A STUDY OF SOME ASPECTS OF THE IMMUNOREGULATION OF GASTRO-INTESTINAL TRICHOSTRONGYLID NEMATODES IN THE SHEEP

DALE. S. SEATON BSc. (Hons)

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF SCIENCE, UNIVERSITY OF EDINBURGH.
OCTOBER, 1988

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 7.9)

Name of Candidate DALE SEATON

Address Moredun Research Institute, 408 Gilmerton Road, Edinburgh EH17 7JH

Degree Doctor of Philosophy *Date* October, 1988

Title of Thesis A Study of some aspects of the immunoregulation of gastro-intestinal trichostrongylid nematodes in the sheep.

No. of words in the main text of Thesis 30,000

Two approaches to understanding mechanisms of immunity to gastro-intestinal nematodes of sheep were undertaken in this thesis: both involved the use of radioisotopes although in quite different ways.

In the first approach infective larvae of Ostertagia circumcincta and Trichostrongylus vitrinus were metabolically labelled with 75Se so that the fate of newly acquired worms could be determined during the course of a trickle infection in lambs.

75Se larvae were produced by the incorporation of selenomethionine into faecal cultures containing worm eggs at concentrations several fold greater than normal. Although these larvae exhibited a wide range of specific activity, virtually all could be detected by autoradiography. In vivo studies showed that almost all labelled worms retained sufficient radioactivity to be distinguished from normal worms 10 days after infection and that their viability measured in terms of their establishment, rate of growth, and sex ratio were unaffected by the radioisotope.

The development of immunity to incoming larvae was subsequently examined in two separate experiments where lambs were continuously infected with 1000 larvae per day of either O. circumcincta or T. vitrinus. Groups of these lambs, together with controls were challenged with 3 doses of 1000 radiolabelled larvae 4, 8 or 12 weeks after the start of infection and killed 10 or 13 days later. Analysis of the number and lengths of both labelled and unlabelled worms suggested differences in the way the two species were being regulated by the sheep. Thus, while immunity to larval establishment developed more rapidly with T. vitrinus, it appeared that established worms of this species were more persistent than those of O. circumcincta where a short period of population turnover probably occurred. O. circumcincta also seemed to be more prone to inhibited development than T. vitrinus, although by the end of each experiment all lambs were highly immune to incoming larvae and contained very few unlabelled worms.

In both experiments immunity was associated with increased numbers of mast cells and IGLs and in the O. circumcincta experiment with antibody titres. However, the precise role of these factors was not clear.

In the second approach attempts were made to identify antigens on the external surface of O. circumcincta.

Surface proteins on the fourth, fifth and adult stages of this parasite were successfully labelled with 125I although the same technique did not work with infective larvae. These proteins could be selectively removed from the worms by the detergent, cetyl-trimethyl ammonium bromide, (CTAB).

Analysis by a combination of polyacrylamide gel electrophoresis under reducing conditions and autoradiography revealed that each stage possessed different proteins. However, it appeared that these were not antigenic since immunoprecipitation and immunoblotting analysis failed to reveal any recognition by antibody from sheep which were immune to infection.

DEDICATION

This thesis is dedicated to my parents and grandparents for their continued encouragement and support throughout the period of my studies.

AKNOWLEDGEMENTS

I would firstly like to thank Dr. David Smith for his guidance and patience throughout this project and also Dr. Peter Wilson, my university supervisor, for helpful discussions and advice.

I am also indebted to numerous colleagues at Moredun for help with various techniques. Thanks are due to Dr. David Jenkinson and Mr. Steven Maley for help with microscopic autoradiography of worm sections and to Dr. Ken Angus and Miss Glynnis Hutchinson for processing and staining pathological sections.

I am also grateful to Mr. Mike McLauchlan for statistical advice, Mrs. Audrey J. Craik for her assistance with computer graphics and Mr. Brian Easter and Mr. Alan Inglis for photography.

I am indebted to the AFRC for providing me with a grant to carry out this research.

Finally, of the many close friends I have made during my stay at Moredun, I would especially like to thank Frank and Liz Jackson, whose help and kindness to me over the years has been unstinting.

CONTENTS

ABBREVIATIONS

BCA	Bicinchoninic Acid
BWB	Blot Wash Buffer
Ci	Curie
c.p.m.	counts per minute
CTAB	Cetyl Trimethyl Ammonium Bromide
Da	Dalton
DAB	Diaminobenzidene
DNB	Denaturing Buffer
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbant Assay
e.p.g.	eggs per gram
Ig	Immunoglobulin
IGL	Intra-epithelial Globule Leucocyte
Iodogen	1,3,4,6, tetrachloro-3-6-diphenylglycouril
M	Molar
PBS	Phosphate Bufferred Saline
r.p.m.	Revolutions per Minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TCA	Trichloroacetic Acid
Tris	Tris(hydroxymethyl)methylamine
WB	Wash Buffer

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Ovine parasitic gastro-enteritis (PGE), resulting from infection with trichostrongyle worms is a global problem which, despite the advent of a variety of efficient anthelmintics and the recommendation of preventative drenching programmes causes considerable production losses to the sheep-farming industry (Brunsdon, 1980).

In Britain, PGE is predominantly a disease of young stock and usually results from mixed roundworm infections. Parasites found in the abomasum include *Ostertagia circumcincta*, *Trichostrongylus axei* and *Haemonchus contortus* whereas *Trichostrongylus vitrinus*, *Trichostrongylus colubriformis* and *Nematodirus battus* inhabit the small intestine. In addition, non-trichostrongylid nematodes such as *Oesophagostomum venulosum* and *Chabertia ovina* may be found in the large bowel. Numerous surveys have shown that in Britain two species predominate, the most common abomasal parasite being *O.circumcincta* (Morgan, Parnell & Rayski, 1951; Crofton, 1954, 1957; Parnell, Rayski, Dunn & Mackintosh, 1954; Connan, 1968a; Reid & Armour, 1975) and the major intestinal parasite being *T. vitrinus* (Parnell *et al*, 1954; Crofton, 1955; Taylor & Cawthorne, 1972; Reid & Armour, 1975). *N. battus* infections have also been shown to cause production losses in young lambs (Stamp, Dunn & Watt, 1955; Thomas & Stevens, 1956; Boag & Thomas, 1975a).

Since the work contained in this thesis has been primarily concerned with infections of *O.circumcincta* and *T.vitrinus* and since it would be impossible to cover all the

published literature on aspects of ovine nematodiasis, emphasis will be placed on work concerning these two parasites.

PHYLOGENY

The class Nematoda contains over half a million species, the majority of which are free-living worms or parasites of plants. However, a large number of animal parasites also exist, and these are classified into 6 orders. The order Strongylida comprises 3 superfamilies, the Trichostrongyloidea, Strongyloidea and Metastrongyloidea containing some of the most important parasites of domesticated stock, including those responsible for the most serious gastro-enteric and pulmonary parasitic diseases.

The generic relationship of members of the family Trichostrongyloidae, Leiper, 1917, one of five families in the Trichostrongyloidea, has been extensively researched and reviewed. Using the classification of Gibbons & Khalil (1982) six subfamilies are recognised, of which the Trichostrongylinae and Ostertaginae are of most relevance here. The subfamily Ostertaginae contains many genera, among them *Ostertagia*, Ransom, 1907 which contains two species of particular importance in livestock production namely *O. ostertagi*, a parasite of the abomasum of cattle, and *O.circumcincta*, a parasite of the sheep abomasum. Recent classifications of the Trichostrongylidae (Gibbons & Khalil, 1982)

has recommended that a separate genus, *Teladorsagia*, Andreeva & Satubaldin, 1954 should be established for the sheep parasite *O.circumcincta*. Although this distinction is now

generally accepted by taxonomists it is, as yet, rarely used by other researchers and consequently throughout this work the former classification *O.circumcincta* will be used.

The genus *Trichostrongylus*, Looss, 1905 is the sole representative of the Trichostrongylinae although it contains several species which are parasitic in sheep, including *T.colubriformis*, *T.axei* and *T.vitrinus*.

LIFE-CYCLE AND PATHOGENESIS

Free-Living Phase

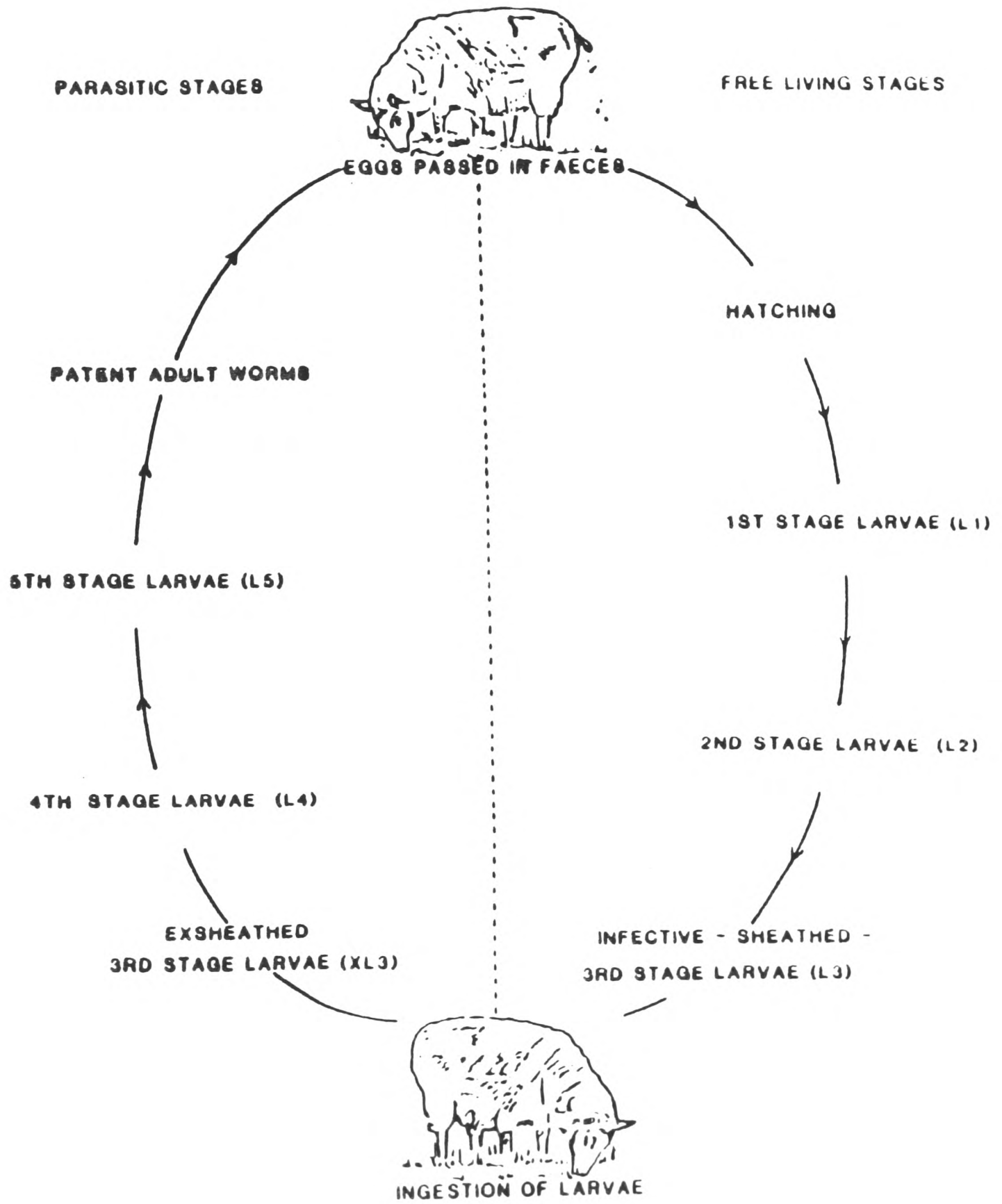
O.circumcincta and *T.vitrinus* share a similar life-cycle, typical to that of the majority of Trichostrongylids, consisting of alternately free-living and parasitic phases (Figure 1.1). Under optimal environmental conditions embryonated eggs passed in the faeces of the host develop into a first stage larva (L1) within 20 to 24 hours. After hatching the L1 feeds and grows until, after a further 12 hours it moults to become a second stage larva (L2). After 20-40 hours feeding the second moult commences. During this moult the L2 cuticle is retained as a protective sheath enclosing the third stage larva (L3). The L3 is the infective stage of the parasite and survives on stored food reserves until it enters a sheep through ingestion with herbage.

Parasitic Phase

(i) *O.circumcincta*

In the rumen the L3, responding to numerous external stimuli, release an exsheathing fluid which facilitates release from the enclosing sheath. On arrival in the abomasum, larvae

Figure 1.1 Life-cycle of *Ostertagia circumcincta* and *Trichostrongylus vitrinus*



penetrate into the gastric glands and approximately 2-4 days later the third moult occurs giving rise to fourth stage larvae (L4). The fourth and final moult is usually completed 6 days later and fifth stage larvae, now effectively immature adults, emerge from the glands onto the surface of the gut where they mature and copulate. The prepatent period is approximately 21 days and after this time eggs are passed out in the host faeces to complete the cycle.

Under certain environmental conditions trichostrongyle L3 on pasture may be 'conditioned' to arrest their development, remaining usually as early L4 in the gastric glands. This phenomenon, known as hypobiosis, is not a permanent condition. Armour, Jarrett & Jennings, (1966) found that although approximately 25% of an *O. ostertagi* infection inhibited as L4, most resumed their development in the glands or had migrated to the mucosal surface 35 days after infection. The factors which predispose larvae to undergo hypobiosis will be discussed in a later section.

Considerable pathological effects are associated with the development of parasitic stages and these have been described by Armour *et al*, (1966). Lesions predominantly develop as a result of the larvae developing in the gastric glands. Parasitized glands enlarge as the worms develop, stretching surrounding glands and stimulating rapid cell division which results in a marked thickening of the mucosa. Furthermore, in parasitized glands differentiated cells are replaced by undifferentiated cells.

These morphological changes in the abomasal mucosa profoundly alter the biochemical conditions that normally exist in the lumen. The loss of functional parietal cells reduces HCl production and as a consequence the abomasal pH increases and any pepsinogen produced is not converted to pepsin. In addition, the breakdown of cellular junctional complexes results in leakage of macromolecules across the abomasal mucosa such that pepsinogen levels in the circulation increase and plasma proteins may be detected in the abomasum. There is a gradual return to a structurally and functionally normal mucosa following the emergence of the parasites from the glands.

(ii) *T. vitrinus*

The development of parasitic stages of *T.vitrinus* and the pathological effects arising from infection have been described by Taylor & Pearson (1979a,b). Ingested L3 exsheath in the abomasum and 2 to 4 days later may be recovered from the small intestine. Here they penetrate into the base of the villi and form tunnels between the basement membrane and villous epithelial cells. Within these sub-epithelial tunnels further development takes place with the final moult to L5 taking place 15 days after initial infection. Adult worms are usually found at least partially in tunnels although the disruption of enterocytes may expose parts of the worm to the intestinal lumen. The prepatent period is similar to *O.circumcincta*.

Trichostrongylus spp. may also arrest their development within the host, although unlike *Ostertagia* they inhibit as exsheathed L3 (Eysker, 1978; Waller, Donald & Dobson, 1981).

As the worms grow, lesions arising from their presence also increase. Villous atrophy occurs, with the development of stunted villi and areas of the mucosa become flattened. The tunnelling of worms causes severe disruption to epithelial cell junctions and this, combined with increased capillary permeability results in a loss of plasma proteins into the intestinal lumen.

The pathology arising from trickle infections of *T.vitrinus* has been examined by Coop, Angus & Sykes (1979) and Jackson, Angus & Coop (1983). In the latter study, after 9 and 14 weeks of daily infection the integrity of the intestinal epithelium had returned to near normal and remaining worms were confined to focal areas known as 'fingerprint lesions', where the mucosa was still completely flattened. These focal lesions were also observed by Coop *et al* (1979) although they were not noted by Taylor & Pearson (1979b). It is probable therefore, that they reflect damage caused by a prolonged continuous assault on the intestinal mucosa. Alternatively, these focal lesions may be remnants of a more extensive villous atrophy in a recovering mucosa (Coop *et al*, 1979).

ECONOMIC CONSEQUENCES OF OVINE GASTRO-INTESTINAL NEMATODIASIS

A. Scale of Problem

The epidemiology of gastro-intestinal nematode infections is such that most grazing ruminants will encounter some level of infection at some stage of their life (Coop, 1982). The effects of this parasitism on any measure of animal productivity will usually be determined by the level of

infection acquired by the host which, in turn, will be affected by a range of factors including climatic conditions, host resistance and pasture stocking rate (Barger, 1982).

Although productivity losses resulting from fatalities and severely affected animals can readily be appreciated, sub-clinical effects are extremely difficult to quantify. Despite this, there is strong experimental evidence to suggest that sub-clinical infections substantially reduce productivity (reviewed by Barger, 1982; Coop, 1982; Holmes, 1986). In Australia it has been estimated that internal parasitism (nematodes, flukes and tapeworms) cost the sheep industry $300 million in lost production in 1985 (Beck, Moir & Meppem, 1985). Precise information detailing the economic losses incurred as a direct result of gastro-intestinal parasitism is not available for British flocks.

B. Manifestations of Gastro-intestinal Nematodiasis on Sheep Production

i) Reductions in Weight Gain

The most obvious effect of nematodiasis on productivity is poor growth rate (Symons & Steel, 1978). Reduction in liveweight gain as high as 79% has been reported in mildly infected grazing lambs (Brunsdon, 1976) although reductions of 20-40% are more usual (reviewed by Barger, 1982). In penned animals Sykes, Coop & Angus (1977) demonstrated a 52% reduction in liveweight gain in lambs trickle infected with *O.circumcincta* and a 50% reduction occurred in lambs infected with *T.colubriformis* (Coop, Sykes & Angus, 1976) although in lambs infected with *T.vitrinus* the body weight of infected animals was

only 8% less than that of controls (Sykes, Coop & Angus, 1979).

ii) Decreased Wool Growth

The adverse effects of parasitism on wool production have been clearly demonstrated. Steel, Symons & Jones (1980) showed that a 53% reduction in wool growth occurred in animals infected with *T.colubriformis* and Symons, Steel & Jones (1981) found a 25% reduction in wool growth of lambs infected with *O.circumcincta* compared to uninfected controls. In addition to reduced fleece weight, production losses may be manifested in a poorer quality of fleece consisting of shorter fibres of reduced diameter (Brunsdon, 1964a; Morris, Anderson & McTaggart, 1977). In Australia it has been estimated that over 60% of all financial losses incurred as a result of internal parasitism are due to reduced wool production (Beck *et al*, 1985).

iii) Reduced Reproductive Performance

Trematode infections have been shown to adversely affect the reproductive performance of ewes by causing lowered lamb birth weights and reduced fertility (Crossland, Johnstone, Beaumont & Bennett, 1977; Johnstone, Coote & Smart, 1979) and reducing the percentage of ewes rearing lambs to weaning (Mackay, 1980). It is probable that nematode infections have similar effects but as yet detailed studies have not been carried out (Holmes, 1986).

C Underlying Mechanisms

The various manifestations of reduced efficiency of production are primarily caused by reduced voluntary food intake combined with impaired digestion, absorption and utilisation of nutrients (Coop, 1982).

i) Reduced Appetite

There are many accounts of reduced appetite and even anorexia caused by nematode infection and these have been reviewed by Symons (1985). In animals given trickle infections with *O.circumcincta*, reductions in food consumption ranging from 6-20% have been recorded (Sykes & Coop, 1977, Coop, Sykes & Angus, 1982; Symons *et al*, 1981). Similarly, hyporexia is a common feature of small intestinal nematode infections of ruminants. In sheep infected with *T.colubriformis* the extent of appetite depression was directly related to the size of the infective dose with 30,000 L3 per week causing a 55% reduction in consumption (Steel *et al*, 1980). Sheep infected with 17,500 *T.colubriformis* L3 per week showed a 15-20% reduction in food consumption (Sykes & Coop, 1976) although no significant reduction could be demonstrated in sheep infected with the same number of *T.vitrinus* L3 (Sykes *et al*, 1979).

The causes of inappetance in infected animals are not known but are likely to be complex (Symons, 1985). It has been suggested that pain may be an important factor (Andrews, 1939; Gibson, 1955) although this is difficult to quantify. Altered gut motility (Gregory, 1985) and changes in the abomasal pH (McLeay, Anderson, Bingley & Titchen, 1973) may also have some importance although this remains to be determined.

ii) Decreased Nutrient Absorption and Utilization

Although depression of appetite is obviously an important factor in the production losses caused by gastro-intestinal nematodiasis, pair-feeding experiments have

demonstrated that it is not the sole cause of poor growth or weight loss (Sykes & Coop, 1977).

The severe damage inflicted on the mucosa by gastro-intestinal nematodes has led some workers to conclude that depressed digestion and impaired absorption of nutrients are major causes of production loss. Evidence to support this view is conflicting and is reviewed by Sykes (1982). Increasingly however, it is believed that the inefficient conversion of available nutrients into energy for growth and production may be of more importance. Sykes & Coop (1976,1977) for example showed that the efficiency of use of metabolisable energy for growth was lowered by 30-40% in animals infected with subclinical infections of either *T.colubriformis* or *O.circumcincta*.

iii) Decreased Mineral Deposition

Numerous studies have clearly demonstrated that parasitised animals have a reduced retention of calcium and phosphorus which results in reduced skeletal growth and mineralisation (Sykes & Coop, 1976, 1977, Sykes *et al*, 1977, 1979). For example, the rate of calcium and phosphorus deposition in lambs infected with *O.circumcincta* has been shown to be only 35% of that in non-infected animals (Sykes *et al*, 1977) and in animals infected with *T.colubriformis* there may be almost complete cessation of skeletal growth and mineralisation (Sykes & Coop, 1976). Since skeletal size determines in part the capacity of the growing animal to accumulate muscle this has obvious long term implications for meat production.

The majority of experiments analysing the effects of parasitism on production have been undertaken with young animals, since in these the effects of infection are more apparent (Holmes, 1986). Comparatively little work has examined the effects of parasitism on older or immune sheep. The limited evidence that is available suggests that the effects of infection on appetite and energy utilisation tend to be similar to those demonstrated in young animals although somewhat less pronounced (Sykes, 1982).

CONTROL MEASURES

Clearly the effects of gastro-intestinal nematodiasis on animal production are considerable. Since complete eradication of helminth populations is not a feasible proposition, methods of controlling the parasites at levels compatible with economic production have been employed. These have been reviewed by Barger (1978), Brunsdon (1980) and Morley & Donald (1980).

Anthelmintics

The most widely used control measure has been the regular treatment of animals with anthelmintic drugs to remove acquired worm burdens (Donald & Waller, 1982). Although such treatments protect the host against serious disease and mortality, animals are often treated only after clinical signs have become apparent and therefore after production losses have already occurred (Donald & Waller, 1982). Moreover, anthelmintic treatment only gives short-term benefits if the animals are returned to contaminated pasture. The effectiveness of

anthelmintic treatment would obviously be enhanced if the likelihood of reinfection of treated animals was reduced (Brunsdon, 1980) and consequently anthelmintic treatment programmes have been integrated with grazing management schemes. These enable the contamination of pastures to be regulated and ensures the most susceptible animals are protected from reinfection. However, on some (e.g. hill) farms pasture management is not possible and on other farms it may place constraints on the best use of the available grazing.

Grazing Management Schemes

The principal aim of grazing management is to prevent contamination of pasture for sufficient time to allow residual contamination to die and thus enable susceptible stock to be re-introduced without risk of infection. To this end a number of different approaches have been used including pasture resting or spelling, rotational grazing and alternate grazing with the same or different species (Morley & Donald, 1980).

The practice of pasture resting by suspending all grazing for a limited period is thought to contribute little to the control of trichostrongylid infections (Brunsdon, 1980; Morley & Donald, 1980). Similarly, rotational grazing schemes have little beneficial effect. The failure of both these enterprises lies mainly in the fact that infective larvae survive long periods on pasture, remaining viable after the relatively short periods of pasture resting that are usually adopted (Barger, 1978). In temperate conditions it would be necessary to withhold stock from pasture for at least 6 months to ensure reduced contamination (Soulsby, 1982) and in most instances this is not an economically valid option.

A more successful approach has been to remove residual larval contamination and prevent new contamination by alternately grazing with animals of the same or different species. Using this system two or more classes of host may graze the same pasture alternately with changeovers only occurring when pastures have become helminthologically safe for the alternate class. The effectiveness of alternately grazing with different host species obviously depends on the degree of cross-transmission of parasites between species but in the most common alternation between cattle and sheep, the majority of parasites have reduced patency in one of the hosts and cross-transmission is therefore of minor importance (Morley & Donald, 1980).

Older animals tend to be more resistant to helminth infection than younger animals and therefore alternately grazing with these two groups may give similarly beneficial results to alternating between different species. This is particularly true of cattle grazing but less so of sheep (Morley & Donald, 1980; Soulsby, 1982). Grazing adult resistant stock alongside younger animals should also dilute pasture contamination to acceptable levels and thus help protect the more susceptible animals. This has been shown to occur in cattle grazing although the equivalent sheep situation remains to be tested (Morley & Donald, 1980).

Anthelmintic Resistance

Although the combined approach of anthelmintic treatment and pasture management has been instrumental in reducing the productivity losses incurred by grazing animals the

emergence of resistance to some of the available broad-spectrum anthelmintic drugs has reduced its effectiveness. Anthelmintic resistance is recognised as a potentially major problem and was believed by Donald & Waller (1982) to be "...the single most important problem confronting the control of helminths in the sheep in the 1980s". A number of reviews deal with the occurrence of anthelmintic resistance in ruminant parasites in Australia (Le Jambre, 1978; Kelly & Hall, 1978; Prichard, Hall, Kelly, Martin & Donald, 1980). Drug resistance is mainly confined to the benzimidazoles group and is most frequently reported where *Haemonchus* spp. are abundant i.e. Australia (Armour & Bogan, 1982). Drug resistance in other species, for example *Trichostrongylus* spp. and *Ostertagia* spp. has mainly been confined to Australia and South America (Barragry, 1984) although there are isolated reports of resistance in Britain (Britt, 1982; Cawthorne & Whitehead, 1983; Cawthorne & Cheong, 1984; Britt & Oakley, 1986).

Vaccination

Although the control of gastro-intestinal nematodiasis is presently achieved through husbandry and chemotherapy the ultimate control measure would be an effective vaccine against these parasites. The fact that continuously infected sheep eventually acquire resistance to re-infection has encouraged efforts in this direction. A number of different methods have been used in attempts to vaccinate against gastro-intestinal parasites (reviewed by Clegg & Smith, 1978; Taylor & Muller, 1980; Lloyd, 1981) but it has yet to be demonstrated that

resistance to infection superior to that which occurs naturally can be achieved by vaccination (Dineen, 1978).

One approach which has had some limited success has been the use of radiation-attenuated L3 vaccines. These vaccines are based on the fact that irradiated larvae are capable of surviving, migrating, and producing functional antigens, but have a reduced pathogenicity (Lloyd, 1981). The only commercial helminth vaccine to be produced so far is of this type, namely "Dictol", which protects cattle against the lungworm *Dictyocaulus viviparus*. Some degree of protection against gastro-intestinal nematodes has also been achieved using this type of vaccine. A number of workers have been able to confer a high degree of protection against *H.contortus* in adult sheep by prior immunization with irradiated *H.contortus* L3 (Jarrett, Jennings, McIntyre, Mulligan & Sharp 1961; Urquhart, Jarrett, Jennings, McIntyre & Mulligan 1966; Smith & Christie, 1979) although attempts to vaccinate young lambs were unsuccessful (Urquhart, Jarrett, Jennings, McIntyre, & Mulligan, 1966). Similarly, Gregg & Dineen (1978) succeeded in immunising adult sheep using irradiated *T. colubriformis* L3 but attempts to immunise 3 month old sheep with the same vaccine were largely unsuccessful (Gregg, Dineen, Rothwell & Kelly, 1978). Some lambs, classed as "high responders" did develop partial protection, however.

Attempts to vaccinate domestic animals with crude somatic extracts of whole worms have not been successful. Rothwell & Love (1974) demonstrated that resistance to *T.colubriformis* could be stimulated in guinea pigs by prior

injection of a soluble protein extracted from 4th stage larvae and adults. In this experiment mature animals responded significantly better to vaccination than immature ones (Rothwell, 1978) and vaccination could be achieved through a number of different routes. However, the efficacy of the extract has not been determined in sheep. Similarly, although excretory/secretory products of fourth stage larvae of *T.colubriformis* were immunogenic in guinea pigs (Rothwell & Love, 1974) successful attempts at immunising sheep with these antigens have not been reported.

In recent years most attempts to control helminth infections through vaccination have aimed at isolating and purifying 'relevant' or protective antigens (Lloyd, 1981). The advent of new immunochemical techniques and also recombinant DNA techniques which enable antigens to be reproduced in sufficient amounts to be used in vaccination trials may bring rewards to this approach although as yet few functional antigens have been characterized. Progress made using these techniques will be discussed in Chapter 6.

Future identification of protective antigens is likely to be an arduous task for at present the target antigens of the host immune response against most parasitic nematodes are not known. It is likely, however, that they will differ between species and may well be specific to different life cycle stages of the parasite. Insight into these problems may be gained through a better understanding of the population dynamics of parasitic nematodes and of the immune mechanisms that operate

against them. With this in mind there has been much work examining these parameters.

REGULATORY MECHANISMS

The factors which regulate the size of parasite populations can be divided into those acting on the free-living worms and those acting on the intra-host population (Barger, 1982). Clearly both these populations are related, the size of one to a large extent determining the size of the other.

Free Living Stages

During the free-living stages of the nematode life-cycle, eggs and larvae are exposed to environmental conditions which can profoundly influence their development and subsequent survival. Of these, temperature and humidity are of particular importance (Levine, 1978; Anderson, Dash, Donald, Southcott & Waller, 1978). The precise effect of temperature and moisture fluctuations may vary, however, according to the particular nematode microhabitat, since soil, herbage and host faeces have very different physical properties (Armour, 1980). Furthermore, in faeces, where development to the L3 usually takes place, additional factors such as consistency and the rate of matter disintegration are also important.

Generally, the infective larva, protected by it's sheath, is the stage most resistant to extremes of temperature and humidity whereas the unembryonated egg and L1 and L2 stages tend to be more susceptible (Levine, 1978). Differences in tolerance exist between different genera. *H.contortus* eggs are intolerant of desiccation (Waller & Donald, 1970) and low

temperatures (Donald, 1968) such that little or no development occurs at temperatures below 9°C. In contrast, *Trichostrongylus* spp. are more able to withstand desiccation (Waller & Donald, 1970) and can develop within a temperature range of 4°C to 27°C (Rose & Small, 1984). Similarly, *Ostertagia* spp. eggs can develop over a wide range of temperatures and are probably at least as tolerant to desiccation as *Trichostrongylus* spp. (Anderson et al, 1978; Callinan, 1978). Nevertheless, the disparity seen between the rate of egg deposition and the rate of infection of grazing animals indicates that there must be enormous destruction of the free-living stages (Gordon, 1973) and indeed Boag and Thomas (1975b) calculated that a mortality rate of over 99% occurred in nematode eggs deposited by sheep.

Parasitic Stages

Acquired Immunity

Under the conditions of continuous infection which normally occur in the field, grazing sheep gradually develop resistance to gastro-intestinal nematodes. This immune response is the major regulatory factor operating against the parasitic stages of gut trichostrongyles and as such may be expressed against newly arriving L3 by prevention of their establishment or against established parasites by eliciting their expulsion or inhibiting their development and egg production. These effects have been reported during infections of both *Ostertagia* spp. and *Trichostrongylus* spp. (Michel, 1963; Chiejina & Sewell, 1974 a,b; Waller & Thomas, 1978 a,b, 1981; Courtney, Parker, McClure & Herd, 1983) but evidence suggests that there are major differences in the sequential development of the

regulatory processes which operate against these two genera (reviewed by Donald & Waller, 1982).

Ostertagia spp.

In the first comprehensive study of the population dynamics of a trichostrongylid infection Michel (1963) described the regulation of *O. ostertagi* in continuously infected calves. In this, and subsequent work, (Michel, 1969, 1970) he concluded that in the first instance parasite numbers were regulated by a density-dependant turnover of the adult population. Michel defined 'turnover' as the constant loss of adult worms and their replacement by fourth stage larvae such that a frequent exchange of the adult worm population occurred. He showed that worms accumulated until numbers stabilized at a level related to the level of larval acquisition. At this point a turnover of the adult population occurred until, with experience of infection, the host built up a resistance to the establishment of incoming larvae (Michel, 1969, 1970). Thereafter there was no recruitment to the adult population and worm burdens eventually declined to very low levels.

Donald & Waller (1982) have reviewed the evidence that *O.circumcincta* infections in sheep are regulated in the same manner as *O. ostertagi* infections in calves. Although the dynamics of the sheep parasite have not been so extensively studied it seems probable that similar regulatory mechanisms do operate. A study using grazing lambs naturally infected with *O.circumcincta* (Waller & Thomas, 1978 a,b) showed that a turnover of the adult population occurred on a monthly basis and that the size of worm burdens was determined by the number of L3

ingested. Similar observations were made by Gibson & Everett (1978) in lambs repeatedly infected with a range of dose rates. Gibson & Whitehead (1981) showed that in lambs infected with 2000 L3/day, 5 days per week, a turnover of the adult population occurred for 10 weeks before resistance to further establishment developed. At this stage few adult worms were recovered and it appeared that worms were being eliminated as late fourth stage larvae. In a similar study, Callinan & Arundel (1982) infected lambs with either 1000 or 10000 *O.circumcincta* L3 twice weekly and found that worm burdens were positively related to the rate of infection and were regulated by turnover. In this instance, however, the authors concluded that resistance to reinfection played little part in the development of immunity.

Trichostrongylus spp.

There is clear evidence that *Trichostrongylus* spp. populations are not regulated in the same manner as *O. circumcincta* populations (reviewed by Donald & Waller, 1982). Many workers (Chiejina & Sewell, 1974 a,b; Waller & Thomas, 1981; Jackson *et al*, 1983; Courtney *et al*, 1983) have shown that there is no turnover of the adult population and worm burdens accumulate until resistance to the establishment of newly ingested L3 develops.

Chiejina & Sewell (1974a) infected 3 week old lambs daily with increasing doses of *T.colubriformis* L3 such that after 10 weeks lambs were receiving 5,000 L3/day. They demonstrated that lambs accumulated worms for the first 12 weeks of infection until a plateau level was reached. At this point

further L3 establishment was prevented although adult worms persisted for a further 8 weeks before they were ultimately expelled. When heavier rates of infection were given to older lambs similar processes occurred but over a shorter time period (Chiejina & Sewell, 1974b). The dynamics of the adult population, summarised by Donald & Waller (1982) as 'accumulation, persistence and expulsion' have also been demonstrated for *T.colubriformis* and *T.axei* (Courtney *et al*, 1983) and *T.axei* and *T.vitrinus* (Waller & Thomas, 1981).

Hypobiosis

The development of nematodes within the host may be temporarily arrested at the L3 or L4 stage,- a phenomenon known as hypobiosis. Despite much work (reviewed by Gordon, 1973; Michel, 1974; Schad, 1977; Gibbs, 1982) the underlying causes of hypobiosis are still not clearly defined. It has been known for some time that nematode larvae arrest their development in response to acquired host immunity (Dineen, Donald, Wagland & Offner, 1965; Soulsby, 1966; Kelly, 1973) but it is also recognised that the hypobiotic state may also be achieved through the influence of environmental factors on the free-living stages. In temperate regions falling temperatures can stimulate the inhibition of development and this can be experimentally mimicked. Thus, Connan (1969) found that large numbers of inhibited *O.circumcincta* larvae amassed in sheep if the infective larvae were previously stored at 4 C. In sub-tropical climates the onset of hot, arid conditions may act as the stimulus and thus hypobiosis can be primarily viewed as an adaptation which permits survival of the worm within the host

when conditions for free-living development are unfavourable (Armour, 1980). Environmental factors other than temperature have also been suggested as being important. Gibbs (1973) demonstrated that inhibition of *H.contortus* could be induced if L3 were subject to a short photoperiod, although Connan (1975) could not confirm this result.

The principal causes of hypobiosis of *Trichostrongylus* spp. are uncertain. Eysker (1978) believed that host immunity was the main cause, but alternatively, Waller *et al*, (1981) thought it unlikely host immunity played an important part. Although hypobiosis may last for prolonged periods, arrested larvae do eventually resume their development. The mechanisms which stimulate this resumption of development are not understood, but may include poor nutrition, concurrent disease and other stress related conditions such as pregnancy and lactation (Soulsby, 1982).

Clearly there are differences in the regulatory processes that operate against *Ostertagia* spp. and *Trichostrongylus* spp. parasitic stages. The development of resistance to both species may, however, be significantly altered by host factors. The reproductive status and age of the host have been shown to be particularly important and evidence has also been presented which suggests that the host genotype may be an influencing factor.

FACTORS AFFECTING HOST IMMUNITY

a) Peri-parturient Relaxation of Immunity.

The fact that pregnancy and lactation can have a profound effect on the immunological responsiveness of the ewe to nematode infections has been demonstrated by numerous workers (Brunsdon, 1964b ; Dunsmore, 1965; Connan, 1968b and O'Sullivan & Donald, 1970). This unresponsiveness may be manifest in a number of ways, including failure to prevent the establishment of newly ingested larvae; inability to expel either established worms or worms that have resumed development after hypobiosis, and failure to suppress the egg production of established female worms (Connan, 1968b, 1976, O'Sullivan & Donald, 1970, 1973). Essentially the differences seen in the immunological responses of lactating and non-lactating animals to nematodes are qualitatively the same as those known to exist between susceptible and resistant non-reproductive sheep (O'Sullivan & Donald, 1970).

A number of theories have been put forward as to the causes of the peri-parturient relaxation in immunity. One suggestion has been that the stress related to pregnancy and parturition may be responsible (Crofton, 1954; Soulsby, 1957). This theory has been discounted, however, since it has been shown that no fall in resistance occurs in ewes which abort (Dunsmore, 1965) and that there is no increase in faecal egg counts of parasitized ewes if their lambs are removed soon after birth (Connan, 1968b). Similarly, O'Sullivan & Donald (1970, 1973) found that any worms acquired by the ewe late in pregnancy were expelled if their lambs were removed.

Crofton (1954) and Spedding (1965) suggested that the onset of lactation was an important factor in the induction of the peri-parturient relaxation of immunity and the results of Connan (1968b) and O'Sullivan & Donald (1970, 1973) confirmed this. However, the loss of immunity in lactating animals is not necessarily absolute. Reid and Armour (1975) showed that although lactating hill sheep were more susceptible to infection with *O.circumcincta* than non-reproductive animals they were still less susceptible than animals with no previous experience of infection. In contrast, Eysker (1981) showed that the numbers of *H.contortus* in lactating ewes approached those observed in previously uninfected animals.

Endocrinological changes have been associated with the peri-parturient relaxation of immunity. Connan (1967) suggested that the lactogenic hormone prolactin was important in immunosuppression of sows and this hormone has been extensively studied in sheep infected with gastro-intestinal trichostrongyles. In general, the levels of susceptibility to infection in the peri-parturient period are mirrored by the levels of prolactin secreted by the ewe (Lloyd, 1983) although during oestrus when a temporary increase in levels of prolactin occurs there is no obvious increase in nematode burdens (Lloyd, 1983) . Nevertheless prolactin and other adrenocorticoid hormones are immunosuppressive *in vitro* (Lloyd, 1983) and if prolactin levels are artificially increased by drug treatment the susceptibility of ewes to infection with gastro-intestinal nematodes increases (Connan, 1974).

b) Immunological Unresponsiveness of the Grazing Lamb

During their first season grazing, lambs gradually develop a protective immune response to infection with gastro-intestinal worms. In most instances this immunity does not reach high levels until the animals are at least 6 months old (reviewed by Lloyd & Soulsby, 1986). Young grazing lambs are therefore at risk from the high levels of pasture larval contamination which result from the peri-parturient relaxation in immunity of the ewe.

The inability of young lambs to mount an effective immune response to helminth infections has been demonstrated by many workers. Manton, Peacock, Poynter, Silverman & Terry, (1962) comparing the response of young and older lambs to challenge with *H.contortus* L3, found that 2-4 month old lambs were not resistant after initial priming with either a single or a series of small daily doses. In contrast, 10-12 month old lambs receiving similar treatments were shown to be almost completely resistant to reinfection. Similar, very pronounced age effects, were observed in experiments with *H.contortus* and *T.colubriformis* when sheep were immunised with irradiated larvae and subsequently challenged (Urquhart *et al*, 1966; Benitez-Usher, Armour, Duncan, Urquhart & Gettinby, 1977; Smith & Angus, 1980).

Chiejina & Sewell (1974a,b) and Waller & Thomas (1981) found that lambs infected with *T.colubriformis* were unable to regulate their worm burdens until at least 5 months of age and Smith, Jackson, Jackson & Williams (1985) showed that 4 1/2 month old lambs were significantly less resistant to challenge

with *O.circumcincta* larvae than 10 month old lambs. This increase in susceptibility was associated with a reduced local immune response to challenge.

The majority of studies investigating the unresponsiveness of young lambs to helminths have been concerned with the apparent failure to develop resistance to re-infection (Soulsby, 1981). Varela-Diaz & Soulsby (1972), for example, showed that although lambs responded to an initial infection with *H.contortus* by producing antibody, no subsequent protection to reinfection could be demonstrated. This situation is in marked contrast to the response to non-helminth antigens. Young lambs can be successfully immunized against bacterial and viral infections (Cole & Morris, 1973) and neonates and even foetal lambs are capable of responding to a range of non-helminth antigens (Silverstein, Uhr, Kraner & Lukes, 1963; Fahey & Morris, 1978). Silverstein, Prendergast & Kraner, (1964) also demonstrated that foetal lambs could reject homografts thus illustrating their capability to mount a cell mediated immune response.

The mechanisms whereby lambs are prevented from developing an effective resistance to helminth infections are not fully understood (reviewed by Soulsby, 1981; Lloyd & Soulsby, 1986). Although the lactating ewe has a lowered immunity to gastro-intestinal nematodes, there is no evidence to relate this to subsequent unresponsiveness in the lamb (Soulsby, 1981). Husband & Lascelles (1975) suggested that an active immune response might be suppressed by passively acquired colostral antibody. However, Dineen, Gregg & Lascelles (1978)

demonstrated that there was no difference in the response of colostrum fed and colostrum deprived lambs following vaccination and concluded that 'feed-back inhibition' did not play an important role. It has become clear, however, that the degree of responsiveness (or unresponsiveness) may to some extent be genetically determined.

c) Genotype

Sheep-breeding experiments undertaken in the 1940's and 1950's demonstrated the heritability of resistance (Warwick, Berry, Turk & Morgan, 1949; Whitlock, 1955; Whitlock & Madsen, 1958) to gut nematodes. Scrivner (1967) showed that resistance to *O.circumcincta* was heritable. Windon, Dineen & Kelly (1980) found that lambs vaccinated with irradiated *T. colubriformis* larvae at 1,2 or 3 months of age could be segregated into 'responders' or 'non-responders' on the basis of their faecal egg counts arising from a subsequent challenge with this same parasite. 'Responder' lambs were shown to respond as well as mature sheep, and their liveweight gains and wool growth were less affected than 'non-responders' . Experiments carried out by Dineen & Windon (1980) and Windon & Dineen (1981) illustrated that this resistance could be selected for. Increased responsiveness, as determined by faecal egg counts and worm burden parameters, could be achieved after only one generation when sires were selected; if both sires and dams were selected the response was more pronounced.

Overall these results confirmed that the response to vaccination was to some extent genetically determined and

suggested that in the long term it might be possible to breed sheep resistant to internal parasites.

MECHANISMS OF IMMUNITY

The various manifestations of the immune response to parasite infection result from many diverse reactions acting both individually and in concert. Despite much work the mechanisms through which immunity is mediated in ruminants remain unclear although a number of observations in sheep and the results of numerous laboratory animal experiments have identified some of the events which play an important role in the overall development of immunity (reviewed by Miller, 1984).

Mucosal Permeability and Immediate Hypersensitivity Responses

During primary infections of *T.colubriformis* and *O.circumcincta*, there is a gradual increase in mucosal permeability, (Smith, Jackson, Jackson & Williams, 1983a) which probably results from parasite-mediated tissue damage (Miller, 1984). In immune animals, however, a more rapid increase in mucosal permeability occurs after challenge. Studies by Smith, Jackson, Jackson, Williams & Miller (1984a) for example, showed that the levels of pepsinogen detected in gastric lymph of primed animals were significantly raised by 24-48 hours after challenge with 50,000 *O.circumcincta* L3 and similarly, when field infected sheep were artificially challenged, there was a rapid loss of plasma proteins into the gut lumen (Yakoob, Holmes & Armour, 1983). The rapidity of these responses probably reflected an immediate hyper-sensitivity reaction to the ingested challenge larvae

(Smith *et al*, 1984a).

During the course of an infection a multiplicity of different inflammatory cells, including mast cells and eosinophils, infiltrate the gastric mucosa and through the release of inflammatory mediators such as histamines and leukotrienes may severely alter its permeability. In ruminants, gastro-intestinal helminth infection is often particularly associated with an increase in the number of mucosal mast cells and globule leucocytes (reviewed by Gregory, 1979). The precise role of these cells is not clear, although recent work suggests that they may be involved in the development of resistance through the release of an inflammatory mediator, designated sheep mast cell proteinase (SMCP). This proteinase, isolated by Huntley, Gibson, Knox & Miller (1986) can be detected in mast cells and globule leucocytes and the appearance of the enzyme in the gastric lymph of sheep infected with *O.circumcincta* coincides with increased mucosal permeability (Smith *et al*, 1984a).

At present the functional activity of SMCP and other inflammatory mediators remains to be characterized. It is, however, likely that they are important components of the immune response since the increased mucosal permeability they promote may facilitate the transfer of serum-derived antibodies and other factors such as complement from the circulation to the mucosal surface (Miller, 1984).

Cellular Responses

Lymphocytes

Smith, Jackson, Jackson & Williams, (1983b) demonstrated that a transient increase in the output of lymphoblasts and IgA-containing cells occurred in the gastric lymph of immune sheep following challenge with 50,000 *O.circumcincta* L3. These workers subsequently demonstrated that partial immunity to this parasite could be transferred between identical sheep with lymphocytes obtained during this response (Smith, Jackson, Jackson, Williams, Willadsen & Fehilly, 1986). Similar results had previously been obtained with *H.contortus* (Smith, Jackson, Jackson, Williams, Willadsen & Fehilly, 1984b). These findings directly demonstrated that lymphocytes could mediate protective immunity but the final effectors have not been defined since an indirect mechanism is thought to be more plausible than a direct cytotoxic effect (Miller, 1984).

Eosinophils

Eosinophil infiltration of the mucosa has been observed in parasitized ruminants (Stewart, 1953; Ritchie, Anderson, Armour, Jarrett, Jennings & Urquhart, 1966; Dobson, 1967) and along with mast cells it is thought that these cells are the major source of leukotrienes generated in the gastric mucosa (Miller, 1987).

Leukotrienes may have some anti-helminth properties (Moqbel, Miller, Wakelin, MacDonald & Kay, 1986) but as yet there is little evidence to suggest that they serve any protective function *in vivo*. Furthermore, Dineen *et al*, (1978) and Dineen & Windon (1980) could not demonstrate any correlation

between numbers of eosinophils and resistance in sheep infected with *T.colubriformis* and the precise role of these cells in the immune response to nematodes therefore remains unclear.

Antibody Responses

The significance of humoral antibodies in the development of acquired immunity has yet to be fully determined (Miller, 1984). It has been demonstrated that the passive transfer of serum from an immune to a susceptible animal can confer resistance to the recipient in laboratory animal model nematode infections (Wakelin, 1975; Love, Ogilvie & McLaren, 1976; Miller, 1980; Dawkins & Grove, 1981; Dobson, 1982) although Rothwell, Adams, Love, Love & McLaren (1980) failed to show any acquired resistance to *T.colubriformis* in guinea pigs. Adams, Merritt & Cripps, (1980) were also unable to demonstrate passive transfer of protection to this parasite and at present there have been no reports of the successful passive transfer of immunity in ruminants.

IgG antibody activity has been detected in both *H.contortus* and *O.circumcincta* infected sheep (Smith, 1977; Smith *et al*, 1983b) although the most important locally produced immunoglobulin isotype in ruminants appears to be IgA (Miller, 1984). Increased amounts of IgA have been observed in abomasal scrapings of sheep immune to *H.contortus* (Smith, 1977) and in the small intestine of *T.colubriformis* infected sheep (Cripps & Rothwell, 1978). Furthermore, analysis of the gastric lymph draining the mucosa of sheep infected with *O.circumcincta* indicated that marked increases in the local production and secretion of IgA antibodies occurred when immune

sheep were challenged (Smith *et al*, 1984a) although peak levels occurred some 4 days after most challenge worms had been eliminated.

The exact role of IgA in the protective response is not known. Smith *et al*, (1985) observed a close inverse correlation between the size of the gastric lymph IgA response of a sheep and the lengths of its worms. These workers speculated that the antibodies might retard the development of *Ostertagia* through interference with the worms ability to feed.

In some nematode infections of rodents, IgE has been associated with increases in mucosal permeability. Gershwin & Dygert (1983) suggested that in cattle an IgE isotype may be involved with changes in permeability, but the full characterization of this isotype was not undertaken, and therefore, at present, the major anaphylactic immunoglobulin in ruminants remains to be determined (Miller, 1984).

Mucus

During gastro-intestinal parasite infections there is a hyperplasia of mucus-secreting goblet cells in the epithelium which results in increased mucus secretion. Hyperplasia occurs in sheep hyperimmunized with *H.contortus* (Christie, Hart, Angus, Devoy & Patterson, 1978) and in sheep infected with *Oes. columbianum* or *T.vitrinus* increased goblet cell numbers are related to the development of resistance (Dobson, 1967; Dobson & Bawden, 1974; Jackson *et al*, 1983)). If mucus production is prevented by corticosteroid treatment, or if the mucus layer is disrupted by intraduodenal administration of mucolytic reagents, the expulsion of *N. brasiliensis* from rats is

blocked (Miller & Nawa, 1979 ; Miller & Huntley, 1982).

Mucus consists in the main of a mixture of glycoproteins, glycolipids and DNA (Forstner, Wesley & Forstner, 1982) but in parasitized animals may also contain a variety of inflammatory mediators derived from the many cells which infiltrate the epithelium during infection (Miller, King, Gibson, Huntley, Newlands & Woodbury, 1986; Douch, Harrison, Elliot, Buchanan & Greer, 1986).

Mucus may also act as a transport medium for specific anti-parasite antibody. Dobson (1966a,b, 1967) recovered high levels of haemagglutinating antibodies in intestinal mucosal washings from sheep infected with *Oes. columbianum* and Douch, Harrison, Buchanan & Greer, 1983, 1986) demonstrated that mucus extracts from immune sheep inhibited larval migration *in vitro* whereas mucus recovered from non-resistant sheep did not. Biochemical analysis of these extracts suggested that the inhibitory effect was due to a slow-reacting substance of anaphylaxis (leukotrienes C4/D4) (Douch *et al*, 1983) but the functional activity of this and other inflammatory mediators as well as antibody in the superficial mucus layer has still to be fully characterized. From available evidence it seems probable that mucus serves an important role as a retainer for numerous anti-parasite substances which are involved in the complex immune response to parasitism.

In summary, it is apparent that a number of factors have been implicated as playing some role in the sheep immune response to gastro-intestinal nematode infection. In immune sheep worm exclusion or expulsion is probably achieved through a

combination of immunologically specific events, such as cellular and antibody responses, and immunologically non-specific components such as the release of inflammatory mediators and the increases in mucus secretion. Much more work is required, however, before the precise mechanisms can be defined.

AIM

The ultimate aim of research into the immunoparasitology of gastro-intestinal nematodiasis of sheep is the development of viable vaccines to these parasites. Although much progress has been made in understanding the intricacies of the host-parasite relationship many aspects still remain to be determined before viable vaccine production can be realised. The quest to discover protective antigens has yet to be successful although the advent of recent immunochemical techniques may provide more information. In Chapter 7 some of these techniques have been used in an attempt to identify 'relevant' antigens of *O. circumcincta* through the characterisation of the surface antigens of four different life-cycle stages of this parasite.

Complementing studies of nematode protective antigens are efforts to elucidate the host immunoregulatory processes operating against the worms. Generally studies of this sort have sought to simulate a natural infection rate through daily dosing with infective larvae. By comparing the size and composition of worm burdens of immune animals and susceptible animals after a challenge infection, information on the immunoregulatory processes can be obtained. The major drawback to this technique, however, is that it is often impossible to

distinguish challenge worms from worms that have established from earlier infections since these latter worms may be retarded in their development. It is thus impossible to follow the fate of a known sub-population of worms in a continuously infected animal. Recently techniques have become available which overcome this problem by enabling infective larvae to be marked. Georgi & Le Jambre (1983) successfully radiolabelled infective *H.contortus* L3 using the radioisotope ^{75}Se in the form of selenomethionine and populations of these worms were identifiable in the worm burdens of continuously infected animals (Barger, Le Jambre, Georgi & Davies, 1985). In Chapter 3 the techniques used for radiolabelling *H.contortus* L3 have been adapted for radiolabelling *O.circumcincta* and *T.vitrinus* L3. Following preliminary experiments these worms were used to examine the population dynamics of these radiolabelled two parasites in continuously infected sheep and these experiments are described in Chapters 4 and 5. It was hoped that through the combination of these different experimental approaches, some insight into the ovine immune response to gastro-intestinal nematodes might be gained.

CHAPTER 2

MATERIALS AND METHODS

SHEEP

The animals used in this work were worm-free Greyface X Suffolk ewe and wether lambs which were born and reared indoors. After weaning the lambs were fed ad lib on a complete ruminant Diet (Ruminant A, Wainmann, Blaxter & Pullar, 1970). All weaned lambs were kept on concrete floors with sawdust bedding which was changed twice weekly.

SOURCE OF PARASITES

The strain of *T.vitrinus* used in experiment was originally isolated from a field infection and had been maintained at the Moredun Institute for 10 years by passage through approximately 30 donor lambs.

The strain of *O.circumcincta* used was isolated 5 years previously from a field infection and had been subsequently maintained by serial passage through worm-free lambs at Moredun.

The strain of *H.contortus* used had been maintained at the Institute for 20 years and was originally supplied by the Central Veterinary Laboratories, Weybridge, Surrey.

FAECAL EGG COUNTS

Rectal faecal samples were weighed and then emulsified in tap water to a concentration equivalent to 10ml of water/gram faeces. A 10ml subsample, removed using a catheter-tipped syringe (4mm diameter), was passed over a 1mm sieve and washed through with an additional 5ml of tap water. The filtrate was poured into a collapsable cellulose acetate tube which was then centrifuged at 1000 r.p.m. for 1-2 minutes. The supernatant

was removed to leave 1ml containing fine faecal debris and eggs. The faecal debris was resuspended in 10-12 ml of saturated salt solution and the tubes were then centrifuged for a further 2 minutes at 1000 r.p.m. The tubes were clamped off using artery forceps just below the meniscus, and the contents of the upper chamber poured into a cuvette. The upper chamber was then rinsed out with saturated salt solution which was also poured into the cuvette. Using an eyepiece graticule, the number of eggs in the sealed cuvette was counted under a microscope (X 40 magnification) to give the total number of eggs per gram faeces. When large numbers of eggs were present, the number seen in two traverses along the cuvette multiplied by a known factor gave the number of eggs per gram faeces (e.p.g.).

NEMATODE EGG EXTRACTION

Large numbers of nematode eggs were extracted from the faeces of donor lambs which had been monospecifically infected. Faeces were weighed and after 1 hour soaking, emulsified in tap water, (10ml / gram faeces). The faecal emulsion was passed over a 1mm sieve and washed with tap water. The retentate was discarded and the filtrate allowed to sediment for one hour before the supernatant was removed with a suction device. The remaining faecal slurry was poured into 100 ml plastic centrifuge bottles and spun for 2 minutes at 1500 r.p.m.. The supernatant was discarded and the pellet of faecal debris containing eggs was resuspended in saturated salt solution, and centrifuged for a further 2 minutes at 1500 r.p.m.. The supernatant which now contained the eggs was poured off over a

38um sieve which was then washed thoroughly with tap water to retain the eggs while removing all traces of salt. Collected eggs were ready for culture.

CULTURE OF INFECTIVE LARVAE

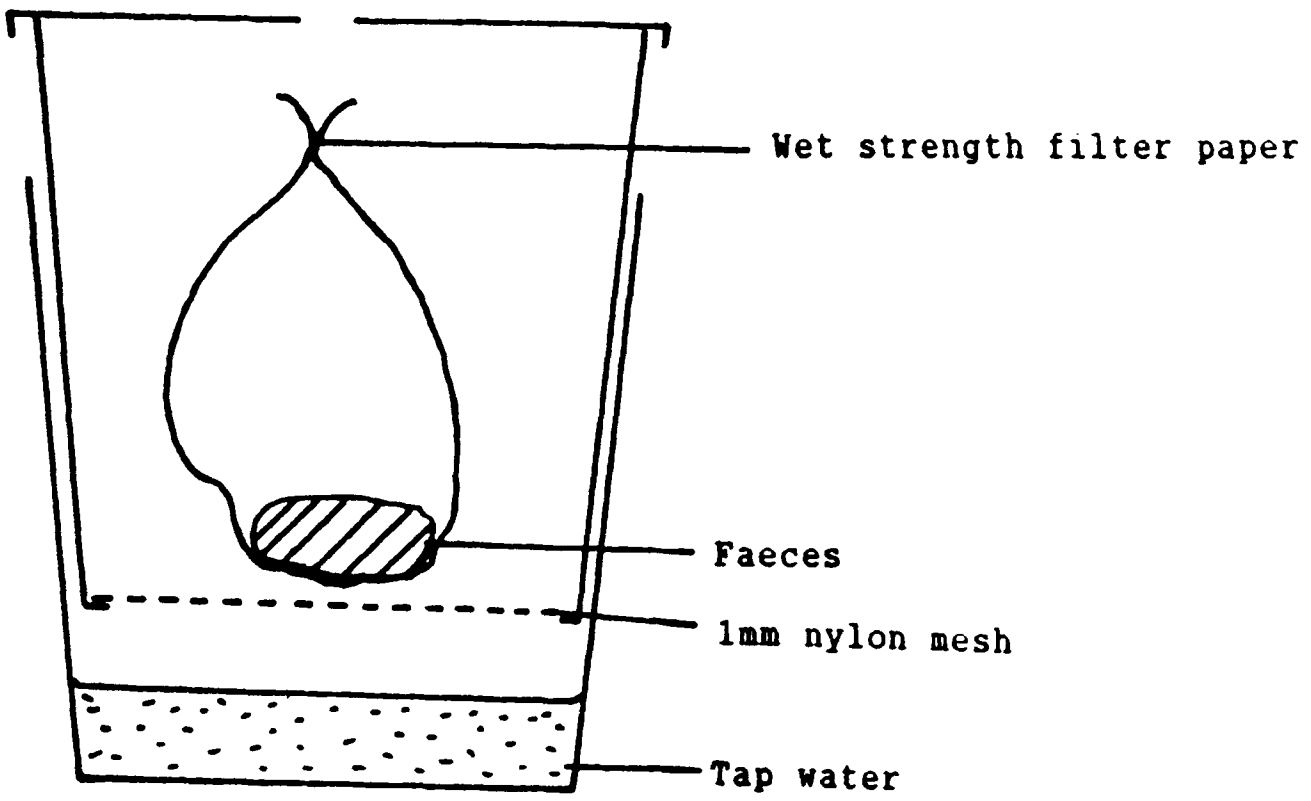
a) Normal Larvae

Faeces collected from monospecifically infected lambs were cultured in 500 ml plastic tubs for 10 days at 22°C. Following this period of incubation the faeces were soaked in warm tap-water for 1 hour and the water was then decanted off and L3 sedimented for 2 hours at 4°C. The volume was reduced and the larvae cleaned by baermanisation, using wet-strength paper (Cleanaroll, Ltd) as the filter medium. Estimations of larval yield were obtained by making a series of dilutions and then counting larvae in an eel worm slide using a stereo microscope (X 60 magnification). Larvae were stored in tap water in 200ml tissue culture flasks (Nunclon) with loosely fitted lids and kept at 4°C until required. Larvae were used within 3 weeks of being harvested.

b) Radiolabelled Larvae

The culture apparatus used for culturing radiolabelled larvae is shown in Fig. 2.1. It consisted of two 250 ml plastic tubs, one placed inside the other, and sealed with a lid. The base of the inner tub was removed and replaced with a plastic grid, situated 3 cm from the bottom of the outer tub which contained 100 ml of water. Radiolabelled L3 were obtained using the following protocol. Approximately 50,000 freshly extracted nematode eggs were thoroughly mixed with 4g of faeces. To this

Figure 2.1 Apparatus used for 'Tub culture' of radiolabelled larvae



mixture, 0.66 mCi of ^{75}Se (L-selenomethionine, ^{75}Se Injection (2-20mCi/mg) B.P. Amersham International Ltd., Amersham, Berks.) was added and mixed thoroughly so that the isotope was evenly distributed throughout the faecal substrate. The resulting mixture was placed inside a bag of coarse mesh filter paper, (Cleanaroll, Ltd) and then placed onto the grid in the inner tub. Tubs were sealed and incubated for 9 days at 22°C, faeces being kept moist by the evaporation of water from the outer tub. After the incubation period, sufficient water was added to submerge the faeces, and thus allow developed L3 to migrate through the filter paper and collect in the outer tub. The following day the radioactive faeces were removed to leave a 'clean' collection of radioactive L3. Collected L3 were washed thoroughly by centrifugation to remove unbound isotope.

DETERMINATION OF LARVAL RADIOACTIVITY

One hundred L3, randomly selected from a population of stored radiolabelled worms were individually removed in 50 µl of water by means of a micro-diluter (Hamilton, micro-lab) and expelled with a further 100 µl of water into a plastic tube (Starsted, Ltd.). Tubes were placed into a Hewlett-Packard gamma-counter and counted for two minutes. The resulting radioactivities were corrected for any soluble isotope in the supernatant, (determined by counting supernatant alone) and for any background counts.

EXSHEATHMENT OF INFECTIVE LARVAE

Infective larvae were washed 3 times in invertebrate Ringers solution and placed in 50 ml of this solution in a 100 ml conical flask. A CO_2/air mixture (500ml CO_2/min. / 1L air/min.) was bubbled through the solution for five minutes after which time the flask was sealed with parafilm and placed in a shaking water bath at 39°C for 25 minutes. Following this incubation the larvae were washed twice using Mapes II medium (pH 2.7), (Mapes, 1969) and incubated for a further 8 hours in the shaking water bath at 39°C. A small number of larvae were removed after the incubation and examined to determine the percentage exsheathment of the population.

RECOVERY OF WORMS FOR QUANTITATIVE PROCEDURES

Sheep were either stunned with a captive bolt pistol and exsanguinated or, if sections of the intestine were required for pathological examination, were killed by an intravenous overdose of sodium pentobarbitone, (Nembutal 1ml/10 kg).

(a) *T. vitrinus*

Immediately after killing the abdomen was opened and the small intestine of the animal was ligated at the pyloric/duodenal and ileo/caecal junctions and then removed. One metre lengths of the gut were opened longitudinally and their contents washed into a 5 litre plastic bucket containing 0.85% saline solution. After 4 hours incubation at 37°C the mucosa was removed by drawing each length of intestine between the index finger and the thumb. The material from this 'saline

digest' was pooled with the collected contents and washings, sedimented and the volume made up to 2 litres with 2% formalin.

b) *O. circumcincta*

Following killing the abomasum was ligated at the omaso-abomasal junction and at the pylorus, removed and opened along its lesser curvature. The contents were collected into a 5 litre container. After thorough washing the tissue was placed into 3 litres of 0.85% saline and incubated for 4 hours at 37°C. After a further wash the material from the saline digest was pooled with the earlier collected washings, sedimented and the volume made up to 2 litres with 2% formalin.

RECOVERY OF LIVE OSTERTAGIA WORMS FOR ANTIGEN PROCEDURES

Immediately after killing the abdomen was opened and the abomasum removed. The contents were discarded and the abomasum was placed in 0.85% saline at 39°C for 4 hours. The mucosal surface was rubbed to remove the mucosa and its parasites and the rubbings were concentrated by sedimentation until the ratio of particulate matter / fluid was approximately 1:3.

A saline solution containing 2.0% molten agar was cooled to 45°C and added to an equal volume of digesta sample, stirring continuously. Sufficient of this mixture was poured into a setting frame to form a layer 1cm thick. This was left to gel for 10-15 minutes at room temperature whereupon the frame base was removed and the agar 'slab' was immersed in 0.85% saline in a Baermann funnel. The slab was left overnight at 37° C and worms, free of digesta were collected from the bottom of the funnel the following day.

ESTIMATION OF WORM BURDENS

Following thorough mixing, 2-10% aliquots were withdrawn from fixed samples using a 50ml catheter tipped syringe and stained using 1-5ml of helminthological iodine for 5 minutes before being washed on a 38µm sieve. The retentate was then searched in 100mm contact plates (Sterilin) using a stereo microscope (x 40 magnification). The number of worms recovered in the aliquot was multiplied by the appropriate factor to give an estimation of the total worm burden.

MEASUREMENT OF WORMS

Worms were picked out and placed in drop of water on a microscope slide and their lengths measured by means of a camera lucida. The magnified image (x 40) of each worm was delineated onto paper and then the length of each drawing was measured with a digitizing apparatus attached to a computer.

PROCESSING OF GUT FOR HISTOLOGY

Small pieces of abomasum or small intestine were fixed flat, serosal side down in a glass Petri dish which was flooded with 10% buffered formol saline solution. Following overnight fixation the pieces were topographically examined and the mucosal condition scored using the same criteria as Coop, Sykes & Angus (1982) for the abomasum and Jackson *et al* (1983) for the small intestine. Following this examination the tissues were processed to paraffin wax, before 5um sections were cut and stained with Toluidene blue using the method of Enerback (1966). For the demonstration of intra-epithelial globule leucocytes

(IGLs) sections were stained with phloxin-tartrazine following the method of Lendrum (1947).

ENUMERATION OF MUCOSAL MAST CELLS AND IGLS

The number of mucosal mast cells and IGLs in stained sections of small intestine or abomasum was determined by counting the number of cells in 20 fields of view (under X40 magnification). To ensure that comparisons between lambs were valid, only crypt cells were counted, as described by Angus & Coop (1984).

STATISTICAL METHODS

In all experiments worm burden data were analysed by t-test on arithmetic values and worm length data by the Mann-Whitney test. Values given in the text are arithmetic means $\pm$ standard error of mean. Correlations between the development of resistance and the number of IGLs, mast cells and also antibody titre were determined by the Spearman rank test.

AUTORADIOGRAPHY

a) Whole Worms

Worms were picked out of fixed samples and placed in drops of 2% formalin on numbered microscope slides. When the drops had evaporated a layer of adhesive tape was placed over the worms to secure them in position. Up to a maximum of 10 slides were attached to a sheet of thin card, which, after being sealed in polythene was placed into a 18cm x 13cm X-ray

cassette (Harmer Ltd.) fitted with an image intensifying screen (Kodak, Ltd). Under a red safe light one sheet of X-ray film (Curix, Agfa-Gevaert) was placed such that it lay between the slides and the intensifying screen and the cassette was closed. After being wrapped in aluminium foil to exclude all light, the cassette was placed at -70°C. Exposure times depended on the amount of radioactivity judged to be present on the parasites, but in most experiments 2 weeks was sufficient. Exposed films were developed using D19 developer (Kodak, Ltd.) and fixed before drying. Images on the developed film could readily be matched up with worms on the slides, and thus radiolabelled parasites could be distinguished from non-labelled worms.

b) Worm Sections

Radiolabelled worms were washed repeatedly before being fixed in 10% formalin, pelleted, and embedded in paraffin wax. Using a rotary microtome, 4-5μm sections were cut and stuck onto microscope slides with a 5% solution of albumin. These sections were dewaxed in xylene substitute and rehydrated through graded alcohols to water before autoradiography. Cleaned slides were immersed in Ilford K2 to give an emulsion layer of 3-4um thickness. When dry the sections were transferred to exposure boxes which were left for 2 months at room temperature before being developed in D19 developer and fixed in 30% hypochlorite. Developed slides were examined with a light microscope (x 100 magnification).

ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

O. circumcincta L3 were exsheathed by CO_2 *in vitro* (see above) and homogenised in ice-cold phosphate buffered saline (PBS) pH 7.2 in a 'teflon-glass' homogeniser (Jencons, Ltd). The homogenate was centrifuged at 29,000g for 1 hour and the supernatant retained as soluble antigen. A protein estimation was made using a bicinchoninic acid (BCA) assay, (Pierce (UK) Ltd., Cambridge, England) and the solution diluted to 5 µg protein/ml in 0.1M carbonate buffer, pH 9.6. MicroELISA plates (M129B, Dynatech Laboratories Ltd, Billinghurst, Sussex), were filled with the antigen solution (150ul/well) and incubated overnight at room temperature. The antigen-coated plates were then emptied and washed 3 times with washing buffer, (WB = PBS containing 0.2% Tween 20) before being stored at 4°C. Sera were diluted 1/400 in WB and duplicates of each sample were tested at 150µl/well. After overnight incubation at room temperature the plates were washed 5 times and an excess biotin-conjugated donkey antibody to sheep $F(ab)_2$ diluted in WB + 10% horse serum was added (150µl/well). One hour later the plates were washed 5 times and 150µl of orthophenylenediamine substrate added. After a 30 minute incubation the reaction was stopped by the addition of 50µl of 2.5M H_2SO_4. Samples were read at 492nm in a multi-channel spectrophotometer. Titrations of both positive and negative control sera were included on each plate. Concentrations of antibody in unknown samples were calculated from standard curves prepared from the positive control and results expressed as a percentage of the positive sample.

RADIOIODINATION OF SURFACE PROTEINS OF *O. CIRCUMCINCTA*

Between 500-1000 freshly harvested worms were used per radioiodination. The worms were washed 3 times in PBS and then 250 to 500 uCi of ^{125}I ($Na^{125}I$, Amersham International Ltd., Amersham, Bucks.) was added to the worms in 0.5 ml of PBS followed by 2 IODO-beads (1,3,4,6,tetrachloro-3-6-diphenylglycouril, IODO-gen, Pierce Chemicals, Rockford, I1, USA). The reaction was allowed to proceed for 15 minutes at 0°C, with periodic shaking. After this time the IODO-beads were removed and the worms were washed 3 times in 10 ml of PBS.

Homogenisation of Iodinated O. circumcincta

All homogenising steps were carried out at 0°C. Radiolabelled worms were washed in 10mM Tris-HCl buffer (pH 8) containing protease inhibitors, 50 ug/ml L-1-tosylamide-2-phenyl-ethylchloromethyl ketone-HCl (TPCK) and 25ug/ml N-tosyl-L-lysine -chloromethyl ketone-HCl (TLCK) resuspended in 0.5 ml of buffer and transferred to a hand-held glass-glass homogeniser. After initial homogenisation 20ul of 0.1mM phenyl-methyl sulphonyl fluoride (PMSF) was added, followed by 120ul of 10% sodium deoxycholate solution. Homogenisation was continued for 5 minutes after which time the solution was spun at 10,000g for 30 minutes in a micro-centrifuge. The supernatant was retained and stored in small aliquots at -20°C.

Detergent Extraction of Iodinated O. circumcincta

Following washing in PBS the radiolabelled worms were transferred to 1ml of 0.25% of cetyl trimethyl ammonium bromide (CTAB) detergent solution containing 10% glucose, 2% Streptomycin/Penicillin and 0.05% Fungizone. The worms were

agitated for 1 hour in this solution at 37°C after which time they were removed and the supernatant (CTAB extract) collected, aliquoted and frozen at - 20°C.

TCA PRECIPITATION OF RADIOLABELLED PROTEINS

The amount of protein associated radiolabel was determined by precipitation by trichloroacetic acid (TCA). To 10 µl of radiolabelled sample, 50 µl of horse serum was added followed by 50 µl of TCA. The resulting precipitate was sedimented by centrifugation at 10,000g for 5 minutes and the supernatant was removed. A further 50µl of TCA was added to resuspend the precipitate and after further centrifugation the supernatant was removed and pooled with the first collected supernatant. The radioactivity of the pooled supernatants and the precipitate were estimated separately in a gamma-counter and thus the percentage of protein associated radiolabel was calculated.

POLY-ACRYLAMIDE GEL ELECTROPHORESIS

Radiolabelled proteins containing between 20,000 and 100,000 TCA precipitable c.p.m. were separated by poly-acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) using a slab gel modification of Laemmli's original procedure (Laemmli, 1970). Samples for analysis were reduced in an aqueous solution of denaturing buffer (63mM Tris (pH 6.8) containing 10% sucrose, 5% SDS, 5% 2-beta-mercaptoethanol and 0.001% Bromophenol Blue) by heating at 100°C for 3 minutes. Reduced samples were loaded into wells

in a 3% acrylamide stacking gel and electrophoresed in the presence of SDS on 12.5% acrylamide resolving gels (16cm x 18cm x 0.75cm) using a Biorad apparatus (Biorad, Model 16CM). Molecular weight markers (BDH Electran standards, BDH Chemicals Ltd., Poole, Dorset, U.K.) covering the range from 12,300 Da - 78,000 Da were always electrophoresed simultaneously. These consisted of Cytochrome c (equine), 12,300 Da; Myoglobulin (equine), 17,200 Da; Chymotrypsinogen A (bovine), 25,700 Da; Ovalbumin (hen egg), 45,000 Da; Albumin (bovine serum), 66,250 Da; Ovotransferrin (hen egg), 78,000 Da.

In some immunoblotting experiments prestained standards covering the range from 14,300 Da to 200,000 Da were also electrophoresed. These consisted of Lysozyme, 14,300 Da; beta-Lactoglobulin, 18,400 kDa; alpha-Chymotrypsinogen, 25,700 kDa; Ovalbumin 43,000 kDa; Bovine serum albumin, 68,000 kDa; Phosphorylase B, 97,400 kDa; Myosin (H-chain), 200,000 kDa. These standards provided instant confirmation that protein transfer had occurred during blotting and provided valuable indication of where to cut the nitrocellulose sheet when individual tracks on the paper were probed separately (see below).

Following electrophoresis gels were fixed and stained with Coomassie Brilliant Blue (0.4g Coomassie Brilliant R250, 5% glacial acetic acid, 40% ethanol, 55% distilled water) for 1 hour and were destained by immersion in a solution of glacial acetic acid, methanol, and distilled water in a volume/volume ratio of 1:3:6 until the desired result was obtained. Gels were dried onto blotting paper using a slab gel drier (Biorad, Model

443) and after being enclosed in polyethene were autoradiographed in 18cm x 13cm X-ray cassettes (Harmer, Ltd.) fitted with an image intensifying screen (Kodak, Ltd) using one sheet of X-ray film (Curix, Agfa-gevaert). The cassettes were wrapped in aluminium foil and placed at -70°C for an exposure time dependent on the amount of radioactivity present on samples.

IMMUNOBLOTTING

Proteins were electrophoresed from the acrylamide gels to nitrocellulose paper (Shleicher & Schull, Dassel, West Germany) using a semi-dry blotting apparatus built at the Moredun Institute based on a design by Kyhse-Anderson (1984). The stacking gel was cut off and the resolving gel was placed on a sheet of nitrocellulose paper soaked in distilled water. The position of the gel was marked on the paper. Two sheets of 3mm chromatography paper soaked in Anode buffer (A) (300mM Tris (pH 10.4) + 20% methanol) were placed on the anode plate of the semi-dry blotter and a further sheet of paper soaked in Anode buffer (B) (25mM Tris (pH 10.4) + 20% methanol) placed on top of these. The nitrocellulose sheet and the gel to be blotted were placed on top of these sheets of chromatography paper and then 3 further sheets of paper soaked in cathode buffer (25mM Tris (pH 9.4) containing 40mM 6-Amino-N-Hexanoic acid + 20% methanol) were placed on top of the gel. The cathode plate was then placed on top of the paper and electrophoresis maintained for 1 hour at a constant current of 150 mA.

After electrophoresis the nitrocellulose paper was incubated overnight at 4°C in Blot Wash Buffer (BWB), (340mM NaCl containing 25mM Disodium EDTA, 0.5% Tween 80 and 10% horse serum) containing 30% pig serum in order to saturate all the protein-binding sites on the paper. The paper was cut into 0.5 cm strips parallel to the direction of electrophoresis and these were then incubated with appropriate dilutions of immune or non-immune lymph samples diluted in BWB containing 10% pig serum for 3 hours at room temperature.

"Immune lymph" consisted of a pool of lymph samples collected from the gastric lymph duct of sheep previously rendered immune to *O.circumcincta* by trickle infection with 2000 *O.circumcincta* L3/day for 9 weeks. Following removal of worm burdens by drenching the sheep were challenged with 50,000 *O.circumcincta* L3. Lymph samples were collected 6 days after challenge at a time when the levels of IgA in the lymph were at their peak (Smith *et al*, 1984a). "Non-immune lymph" consisted of a pool of lymph samples similarly collected from sheep which had no experience of *O.circumcincta* infection.

Following incubation the strips were washed by agitation in 9 changes of BWB over 45 minutes. The strips were then incubated with an excess of peroxidase conjugated pig anti-sheep $F(ab)_2$ for 1 hour before the extensive washing process was repeated. After this time the substrate (100mM Tris (pH 7.4) + 1.1mM 3,3, Diaminobenzidene (DAB)) was added. The reaction was allowed to proceed until sufficient colour was apparent on the strips, after which time the reaction was stopped by the addition of water.

Strips of nitrocellulose that were not probed with lymph samples were stained with Coomassie Blue (4% of Coomassie Brilliant Blue solution described above, 5% glacial acetic acid, 30% methanol and 61% distilled water) for one hour. Strips were then immersed in a destaining solution (5% glacial acetic acid, 30% methanol, and 65% distilled water) for 1 hour. After this time the solution was changed and destaining allowed to continue until the desired effect was obtained.

In some experiments, immunoblotting and autoradiographic techniques were combined to enable a direct comparison between radiolabelled surface proteins and sites of antigen/antibody reaction. In these experiments non-radioactive homogenates produced by grinding worms in PBS were 'spiked' with approximately 200,000 c.p.m. of radiolabelled detergent extracts of the same stage of worm. Following concentration by precipitation with 6 volumes of ethanol overnight at -20°C the combined samples were run on SDS-PAGE and blotted. Strips of the blots were probed with lymph samples and developed with peroxidase-conjugated pig anti-sheep Ig and DAB substrate as described above. Probed strips were then autoradiographed and when the film was developed the radiolabelled (i.e. surface) proteins and proteins recognised by antibodies in the lymph could be directly compared by overlaying the autoradiograph on the blots.

IMMUNOPRECIPITATION

Radiolabelled proteins (between 50,000 - 100,000 c.p.m.) were incubated with immune or non-immune lymph (diluted 1/10 in BWB) for 2 hours at 37°C. Then pig anti-sheep $F(ab)_2$ was added for 1 hour at 37°C to precipitate antibody-antigen complexes. Preliminary experiments showed that 8 volumes of the pig antiserum could precipitate all the immunoglobulin in 1 volume of lymph. Complexes were precipitated by centrifugation at 10,000g for 30 minutes after which time the supernatant was carefully removed and the precipitate washed once in BWB. The radioactivity of precipitate and supernatant was measured in a gamma-counter. The proportion of the total counts which were precipitated was calculated and expressed as a percentage of the number of TCA precipitable counts in the radiolabelled protein solution.

In some experiments samples were pre-absorbed to remove the possibility of any non-specific binding of radiolabelled proteins to non-immune lymph. Samples to be pre-absorbed were incubated with non-immune lymph for 2 hours before being precipitated with pig anti-sheep immunoglobulin in the manner described above. After centrifugation the supernatant was removed and analysed using the standard immunoprecipitation procedure.

CHAPTER 3

THE DEVELOPMENT OF A CULTURE TECHNIQUE FOR RADIOLABELLING *T. VITRINUS* AND *O. CIRCUMCINCTA* L3 AND PRELIMINARY RADIOLABELLING EXPERIMENTS.

INTRODUCTION

This chapter describes the development of an efficient system for culturing *O.circumcincta* and *T.vitrinus* eggs at concentrations several fold higher than those normally found in faeces from sheep infected with these parasites. This was necessary in order to minimise the dilution of isotope used in experiments to metabolically label infective larvae. In initial experiments *H.contortus* eggs were used since infections with this parasite normally produce thousands of eggs/g of faeces. Where possible, similar experiments were undertaken using *O.circumcincta* and/or *T.vitrinus* eggs. Once a satisfactory culture system had been developed experiments were conducted to determine whether the infective larvae of these species could be radiolabelled with ^{75}Se. The viability of radiolabelled worms and the rate of decay of the radiolabel were determined in further experiments carried out *in vitro* and *in vivo*.

A. DEVELOPMENT OF A CULTURE TECHNIQUE

Preliminary attempts to culture *O.circumcincta* and *H.contortus* L3 using the technique described by Georgi & Le Jambre (1983) proved unsuccessful. A modification of the technique whereby eggs were cultured in glass boiling tubes did, however, prove more successful and consequently was used initially.

(1) *Boiling Tube Culture*

(a) Method

Freshly extracted eggs were thoroughly mixed with 3g of faeces from a worm-free sheep and enclosed in a filter paper

bag. The bag was suspended 3cm above 10ml of water in the bottom of a boiling tube which was sealed with a cotton-wool bung. After 9 days culture at 22°C the tube was flooded with water so that the faeces were submerged and the following day the bag of faeces was removed enabling developed L3 to be collected from the remaining water by filtration (Figure 3.1).

(b) Incorporation of Vermiculite

In an attempt to improve the aeration of the eggs in culture, in two experiments vermiculite was incorporated into the faeces substrate. Different substrate mixtures were made consisting of:-

(i) Faeces only

(ii) Faeces: vermiculite, 2:1 ratio

(iii) Faeces: vermiculite, 1:1 ratio

Three gram samples of each of the substrates were mixed with 6000 eggs of either *H.contortus* or *O. circumcincta* and cultured in boiling tubes. Triplicate tubes were prepared for each mixture.

The results of these experiments clearly showed that there was no advantage to be gained from the incorporation of vermiculite into faeces prior to culture. Larval recoveries from the *O.circumcincta* experiment were not significantly increased and in the *H.contortus* experiment the addition of vermiculite actually decreased the recovery of infective larvae (Table 3.1). Following these experiments vermiculite was not used further in the culture procedure.

Figure 3.1. Boiling tube culture technique.

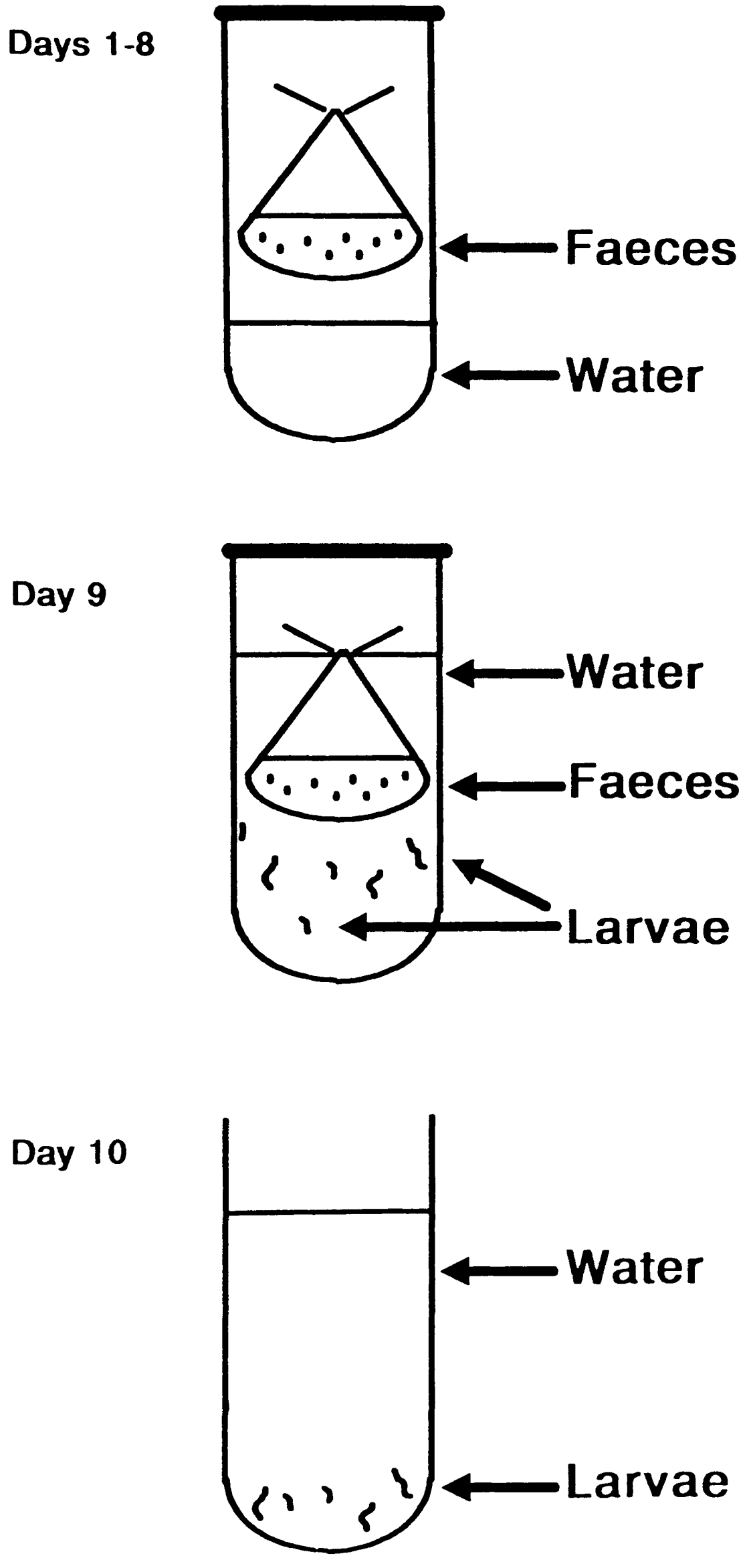
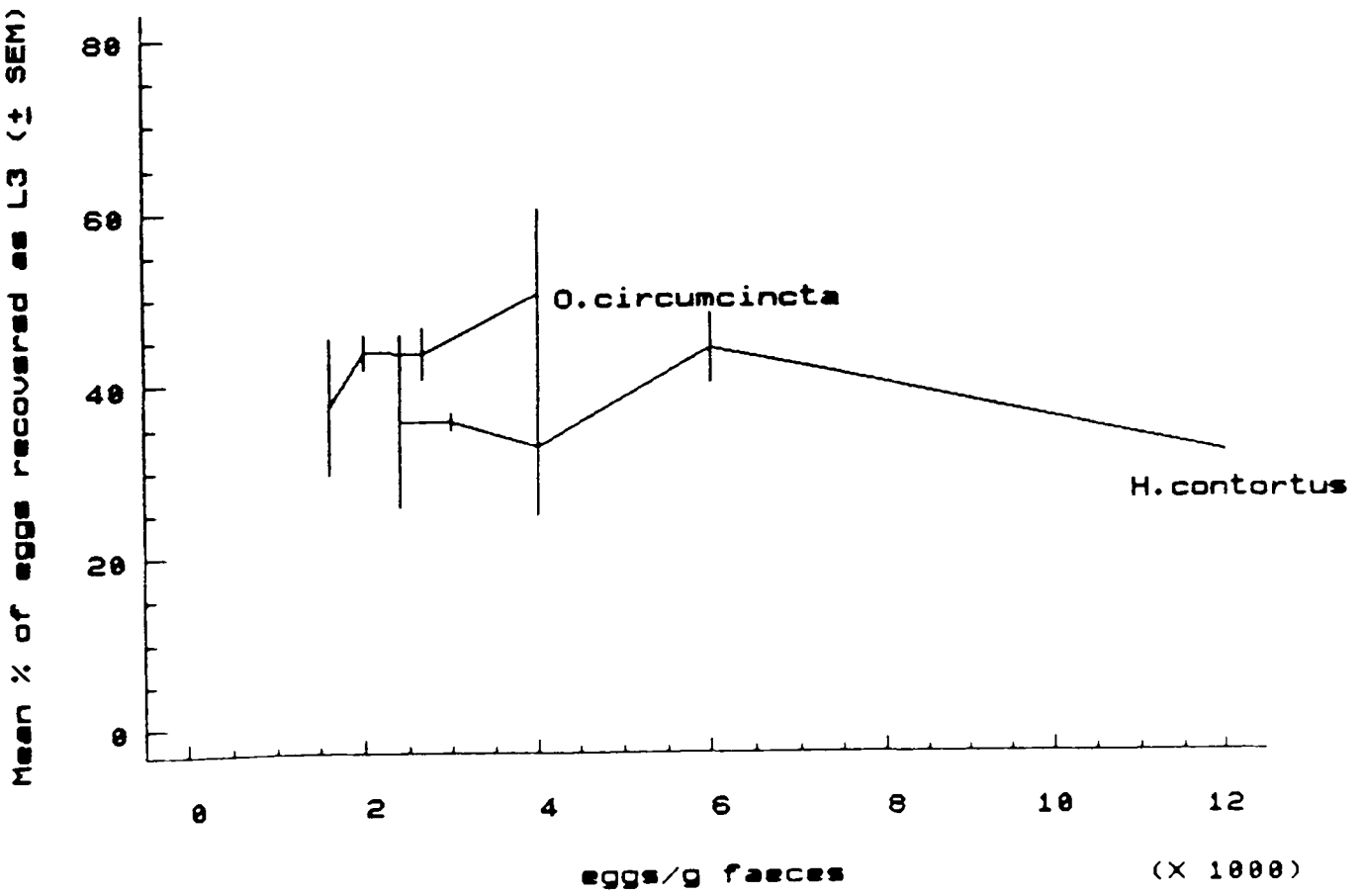


TABLE 3.1 % of *H. contortus* and *O. circumcincta* L3 recovered from eggs cultured in different faeces substrates in boiling tubes.

	H. contortus				*O. circumcinta*			
Substrate	1	2	3	Mean	1	2	3	Mean
Faeces Alone	49.6	47.4	50.7	49.2	35.0	40.6	38.0	37.9
Faeces: vermiculite, 2:1	36.4	41.5	36.1	38.0	30.2	42.9	34.1	35.7
Faeces: vermiculite, 1:1	26.0	35.0	31.4	30.8	36.5	--	--	36.5

Figure 3.2 Mean % recovery of L3 from tube cultures containing different inital concentrations of eggs/g faeces



(c) The Effect of Varying the Concentration of Eggs in Faeces on L3 Recovery

Two separate experiments were undertaken to investigate whether the percentage recovery of *H.contortus* or *O.circumcincta* L3 was affected by the concentration of eggs present in the faecal culture. In each experiment 6000 eggs were cultured on either 4g or 10g of faeces and duplicate cultures were established for each concentration.

In both experiments larval recoveries were not directly related to the concentration of eggs cultured within the range tested: 2400 - 12000 epg for *H.contortus* and 1600 - 4000 epg for *O.circumcincta* (Figure 3.2) even though the latter range is several times higher than that which occurs naturally in *O. circumcincta* infected sheep. This result therefore encouraged further development of the technique.

(2) *Tub Culture*

The apparatus used in the tub culture system was essentially a scaled up version of the tube culture system which enabled larger amounts of faeces to be cultured in one container. Due to the larger surface area this method also enabled the faeces to be kept uniformly moist. Description of the tub culture apparatus can be found in Chapter 2.

(a) The Effect of Varying the Concentration of Eggs in Faeces on L3 Recovery

In a similar experiment to that undertaken with the boiling tube system, the effect of varying the concentration of eggs in the faeces was examined.

O.circumcincta eggs were cultured at concentrations ranging from 500 to 5000 eggs per gram faeces. Duplicate tubs were set up at each concentration. In a separate second experiment concentrations of 4000 epg and 10000 epg were also tested.

The results of these two experiments indicated that even at concentrations as high as 10000 epg, some 50 times higher than that which occurs naturally, over 50% of cultured *O. circumcincta* eggs could be recovered as L3 using the tub culture method (Figure 3.3). This recovery rate was considered efficient enough to warrant proceeding to preliminary radiolabelling experiments. Throughout these experiments the tub culture system was used exclusively.

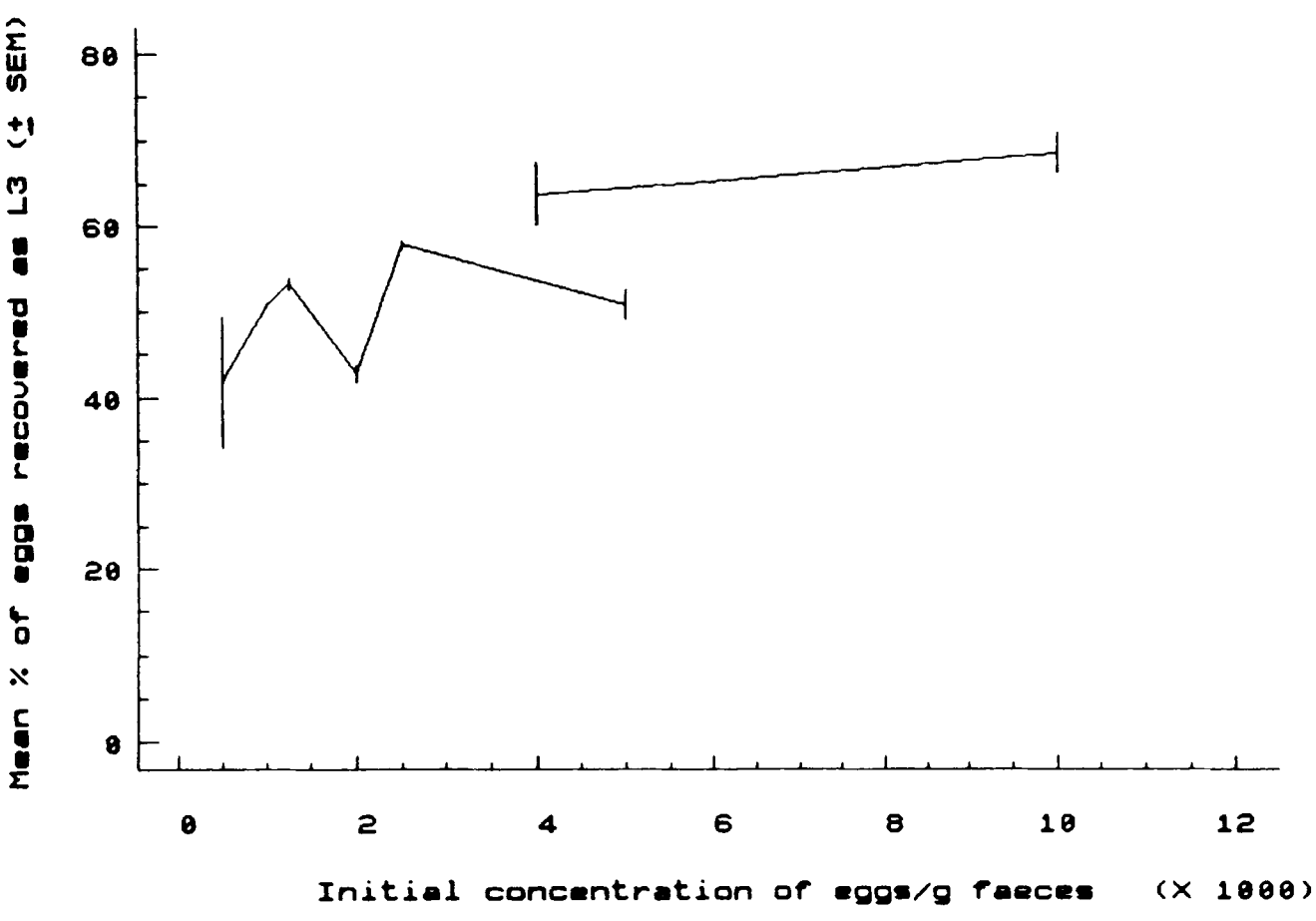
B. PRELIMINARY RADIOLABELLING EXPERIMENT

A preliminary experiment was undertaken to determine whether ^{75}Se-labelled *O.circumcincta* L3 could be produced by the incorporation of selenomethionine into faeces prior to culture.

A total of 300 µCi of ^{75}Se (10mCi/mg) was thoroughly mixed with 4g of faeces containing 7500 eggs/g and cultured in a tub in the manner described in Chapter 2.

One hundred larvae recovered from the culture were individually counted in a gamma-counter to determine the mean and range of larval specific activity. It was apparent that despite thorough mixing of isotope and faeces prior to culture, there was an extensive range in the specific activity of

Figure 3.3 Mean % recovery from tub cultures containing different initial concentrations of eggs/g faeces



recovered L3 (Figure 3.5). The mean specific activity of the L3 was 258 c.p.m. with a range from 81-543 c.p.m.

C. DETECTION OF RADIOLABELLED WORMS BY AUTORADIOGRAPHY

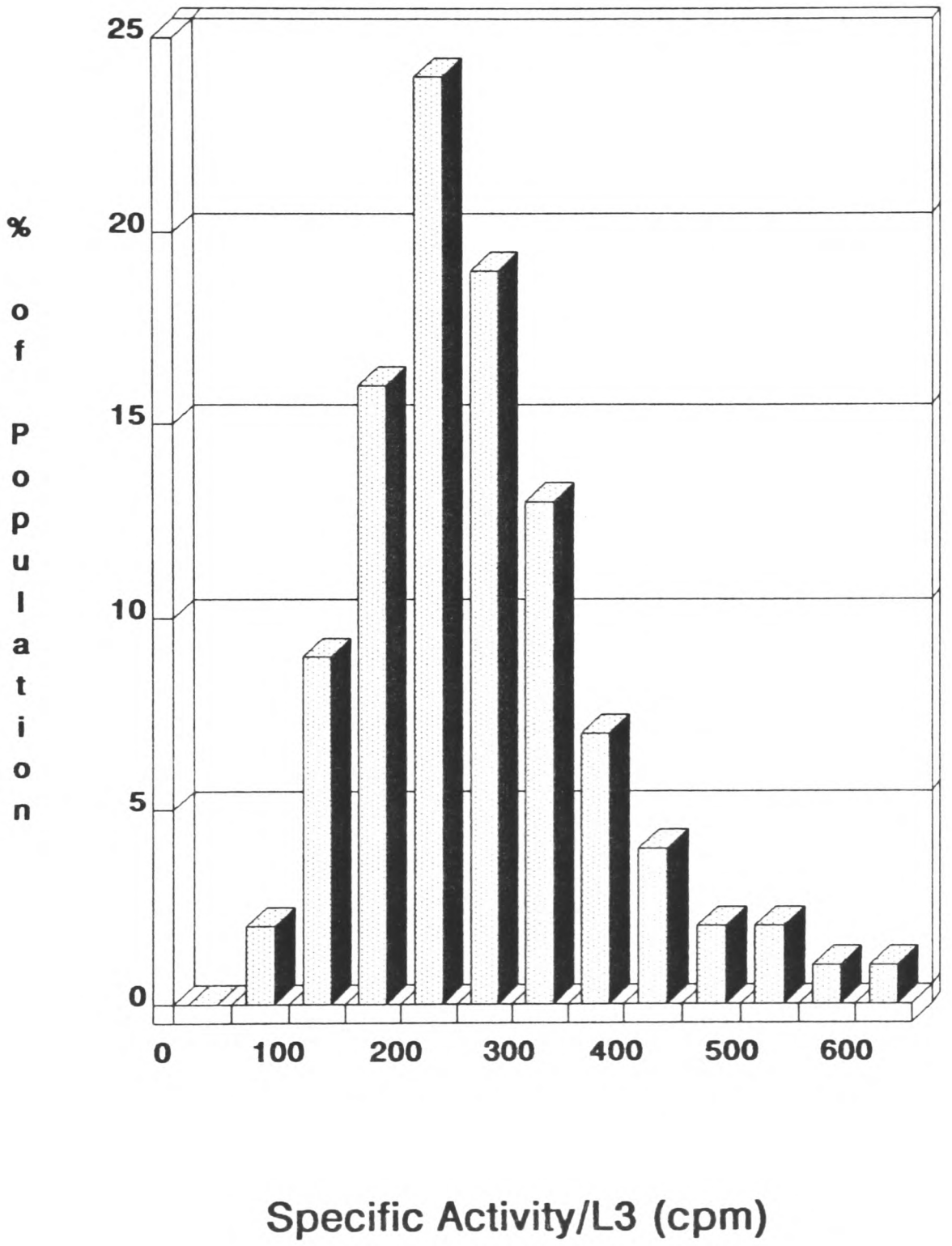
Since the radiolabelling procedure produced a population of larvae with a large range of specific activities. it was important to know whether L3 at the lower end of this range could be detected by autoradiography after a period of infection in the sheep. The following experiment was therefore undertaken using the radiolabelled L3 recovered from the previous experiment.

A single sheep was infected with 8000 radiolabelled *O.circumcincta* L3 and killed 10 days later. Fifty male and 50 female worms removed from the collected worm burden were placed individually on slides and put up to autoradiograph at -70°C (see Chapter 2). The film was developed after 2 weeks exposure.

All of the worms put up to autoradiograph produced an image on the film and 50 of these are shown in Figure 3.4. The autoradiographic images varied in intensity and this was probably related to the fact that the original population of L3 exhibited a range of specific activities (Figure 3.5). However, all of the worms were clearly identifiable when the film was viewed over a light box and thus it was apparent that the autoradiographic technique was sufficiently sensitive to detect 10 day old worms that had developed from L3 with initial specific activities as low as 50-100 cpm.

Figure 3.5

Range of specific activities of radiolabelled *O. circumcincta* L3 recovered in preliminary radiolabelling experiment



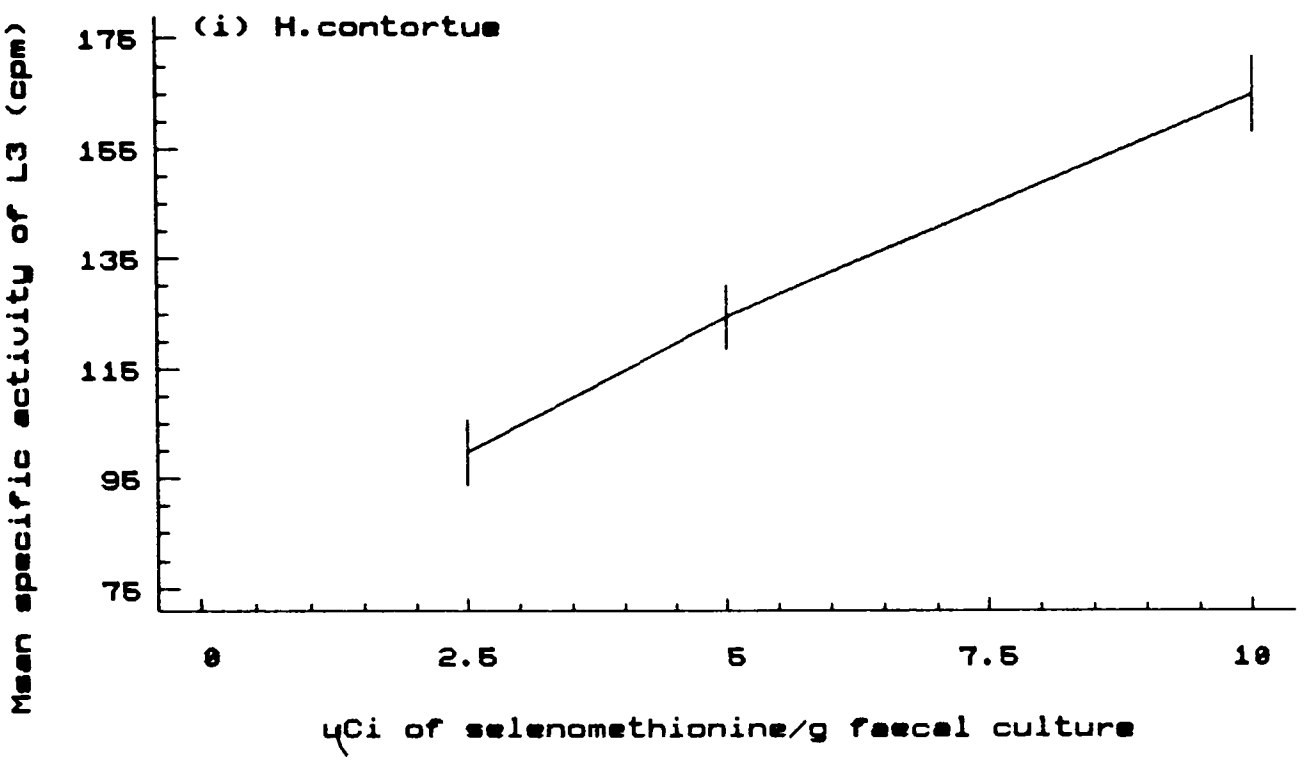
D. RELATIONSHIP BETWEEN CONCENTRATION OF ^{75}SE-METHIONINE IN FAECAL CULTURE AND SPECIFIC ACTIVITY OF DEVELOPED L3

Dose response experiments were undertaken with cultures of *H.contortus* and *O.circumcincta* eggs to examine the relationship between the concentration of radioisotope used in culture and the resulting specific activity of developed L3.

Eight replicate cultures each containing 5000 *H. contortus* epg were prepared and either 0, 2.5, 5 or 10 µCi of ^{75}Se-methionine added per gram faeces. Similarily, twelve replicate cultures each containing 3000 *O.circumcincta* epg were established and either 0, 6.25, 12.5, 25, 50 or 100 µCi of ^{75}Se-methionine was added per gram faeces. The mean specific activity of 50 L3 recovered from each culture was determined by gamma-counting individual worms.

Both experiments demonstrated that the mean specific activity of recovered L3 was directly related to the amount of isotope incorporated in culture (Figure 3.6.(i-ii)). In addition, in the *O.circumcincta* experiment, the range in L3 specific activity became more extensive as the amount of ^{75}Se used in culture was increased (Figure 3.7.(i-v)). For unknown reasons the mean specific activity of larvae recovered from these cultures was much greater than that subsequently achieved in experiments where similar amounts of isotope were used.

Figure 3.6 Relationship between the concentration of selenomethionine added to faecal culture and the specific activity of developed L3



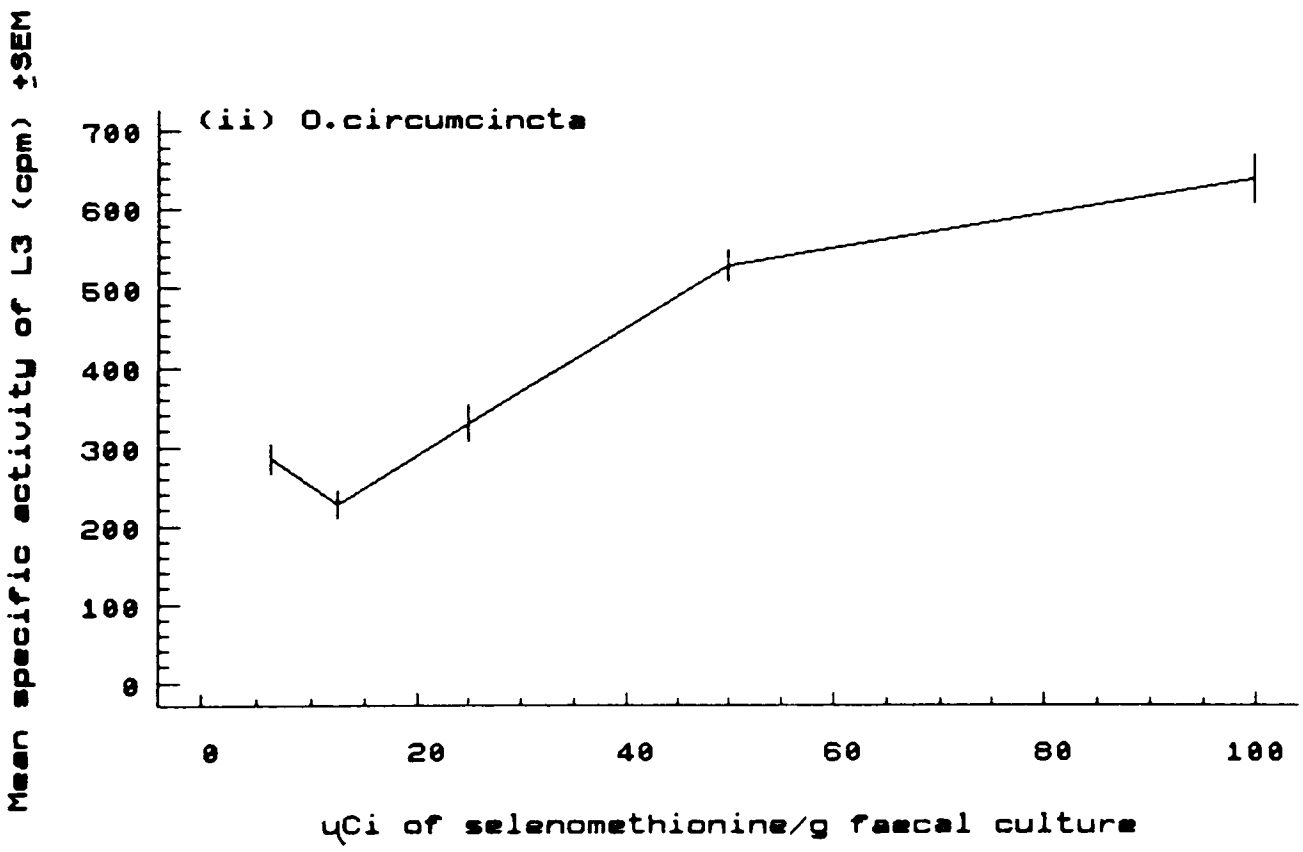
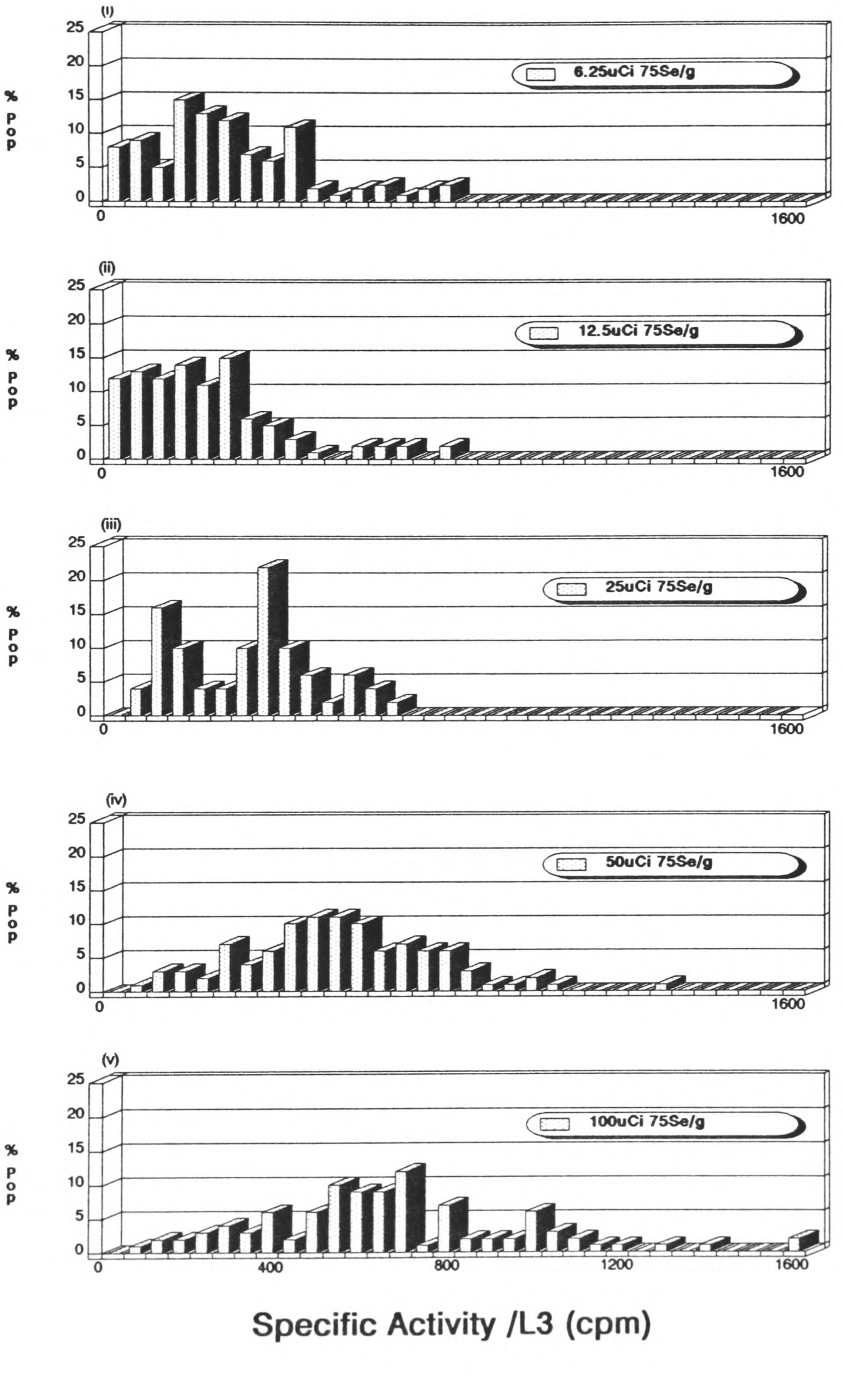


Figure 3.7 (i-v).
Frequency Distribution of *O.circumcincta* L3 specific activities following culture in different selenomethionine concentrations



E. MICROSCOPIC AUTORADIOGRAPHY OF RADIOLABELLED LARVAE

In order to examine the location of isotope in/on radiolabelled larvae, cross-sections of the worms were autoradiographed and viewed by light microscopy using the methods described in Chapter 2.

Cross-sections of radiolabelled *T.vitrinus* larvae following 2 months autoradiographic exposure are shown in Figure 3.8., the darker areas indicating the location of the radioisotope. Significant amounts of isotope were associated with the L3 sheath and could also be located within the somatic tissues of the worm although the extent of incorporation varied between individuals. This variation may account for the range of specific activities routinely observed in a radiolabelled population (Sections B and D).

F. THE EFFECT OF ISOTOPE ON PARASITE VIABILITY

Having established that nematode larvae could be successfully radiolabelled and that radioactive worms could be detected by autoradiography 10 days after infection, it was essential to determine whether their viability was impaired by the incorporation of isotope. Five separate experiments were therefore carried out to determine the effect of radiolabelling on the following parameters:-

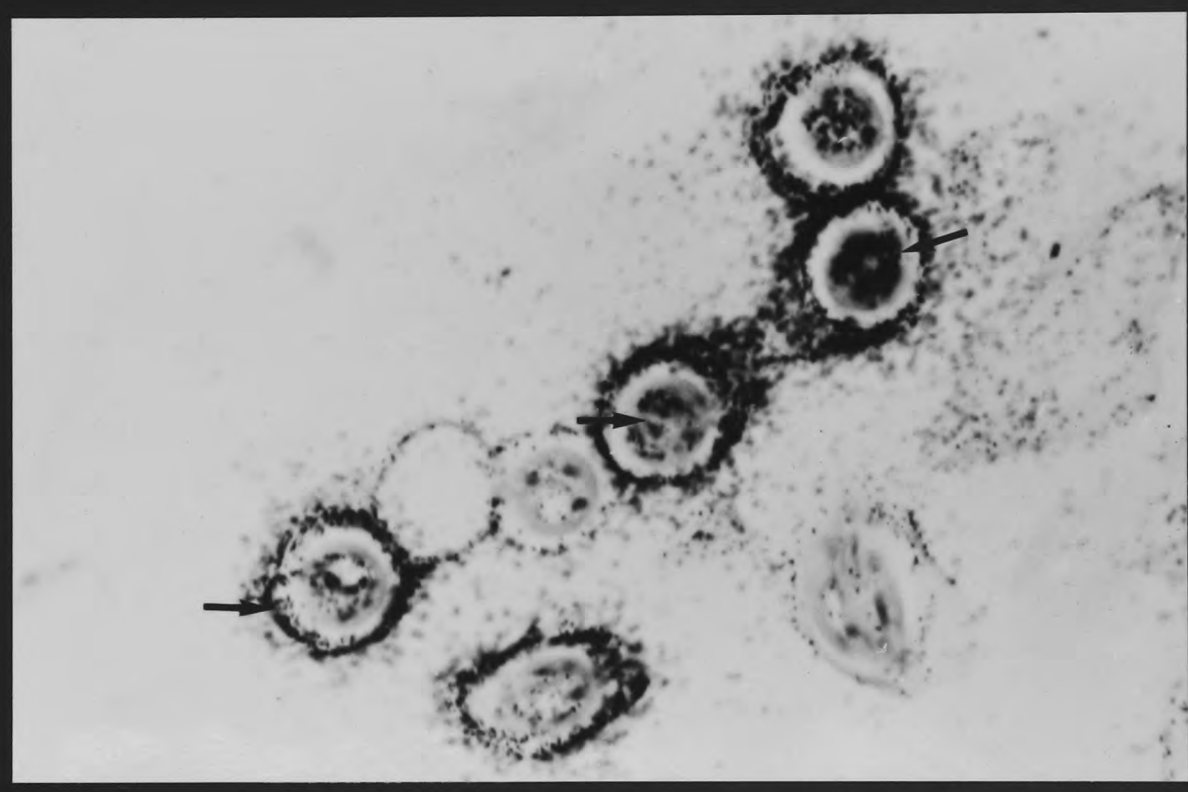
1) the rate of worm establishment.

2) the relative numbers of male and female worms recovered, i.e. the sex ratio.

3) the growth rate of the worms.

Figure 3.8

Cross-sections of radiolabelled *T.vitrinus* L3 following two months autoradiographic exposure. The location of the radioisotope is indicated by the darkened areas (arrowed).



In each experiment a single sheep was infected with equal numbers of radiolabelled and unlabelled larvae, each population having been cultured from the same batch of eggs. A direct comparison of the viability of labelled and unlabelled worms was thus possible within the same sheep. After a period of infection of either 7 or 10 days the animals were killed, their worm burdens determined and random samples of 100 worms were sexed, measured and autoradiographed so that the percentage of radiolabelled worms in the total population could be determined.

In 4 of the 5 experiments almost equal numbers of labelled and unlabelled worms were recovered and it was apparent that labelled and unlabelled worms had grown at similar rates (Table 3.2). In Experiment 2 however, the proportion of radiolabelled worms recovered was lower than expected. This may have been related to the relatively high specific activity of the L3 used in this experiment. However, labelled male worms recovered in this experiment were longer than unlabelled worms although labelled females were markedly shorter than their unlabelled counterparts. There was no major difference in the sex ratios of labelled and unlabelled worms in all five experiments.

G. LOSS OF RADIOACTIVITY DURING STORAGE

The loss of radioactivity from radiolabelled larvae during storage was determined over a 7 week period.

Freshly radiolabelled *T.vitrinus* L3 were washed until radioactivity counts in the supernatant fell to background. Aliquots of 5ml containing approximately 2000 L3 were prepared

TABLE 3.2 The Number, Length and Sex Ratio of Worms Recovered from Sheep Infected with equal Numbers of Labelled and Unlabelled Infective Larvae.

Expt. No.	Species	Infective Dose	Mean specific activity of L_3 (cpm)	Day Killed		% of Population	% Male	Length (mm) ± SEM Male	Length (mm) ± SEM Female
1.	O. circumcincta	8000	253.8	10	Lab	46	51	2.05 ± 0.11	2.43 ± 0.11
					Unlab	54	50	2.17 ± 0.09	2.41 ± 0.11
2.	O. circumcincta	8000	640.2	7	Lab	33	44	3.42 ± 0.24	3.52 ± 0.11
					Unlab	67	50	3.07 ± 0.13	3.91 ± 0.19
3.	T. vitrinus	10000	295.8	10	Lab	52	41	---	---
					Unlab	48	57	---	---
4.	T. vitrinus	8000	192.0	10	Lab	49	46	1.72 ± 0.03	2.03 ± 0.03
					Unlab	51	38	1.67 ± 0.06	2.02 ± 0.03
5.	T. vitrinus	4000	262.8	10	Lab	53	56	1.75 ± 0.03	2.02 ± 0.03
					Unlab	47	59	1.69 ± 0.06	2.08 ± 0.03

and stored at 4°C. The following day and at weekly intervals thereafter one aliquot was removed from storage and the specific activity of 100 individuals was determined.

The decline in mean larval specific activity approximated to the natural decay curve of ^{75}Se (Figure 3.9) with its half-life of 121 days.

H. LOSS OF RADIOACTIVITY FROM INFECTIVE LARVAE DURING EXSHEATHMENT

To determine the loss of larval specific activity following exsheathment 4 experiments were undertaken whereby labelled larvae were stimulated to exsheath *in vitro*.

One hundred L3 were removed from a radiolabelled population and were counted individually to determine their mean specific activity. The remaining worms were stimulated to exsheath and the mean specific activity of 100 of these was also determined. The percentage radioactivity lost on exsheathment was calculated by difference.

On average 38.8% of the larval specific activity was lost when exsheathment occurred (Table 3.3). It is probable that the loss of the sheath itself with its associated radioisotope (Section E) accounted for most of this reduction although isotope located within exsheathing fluids or exposed on the L3 cuticle may have contributed to the loss.

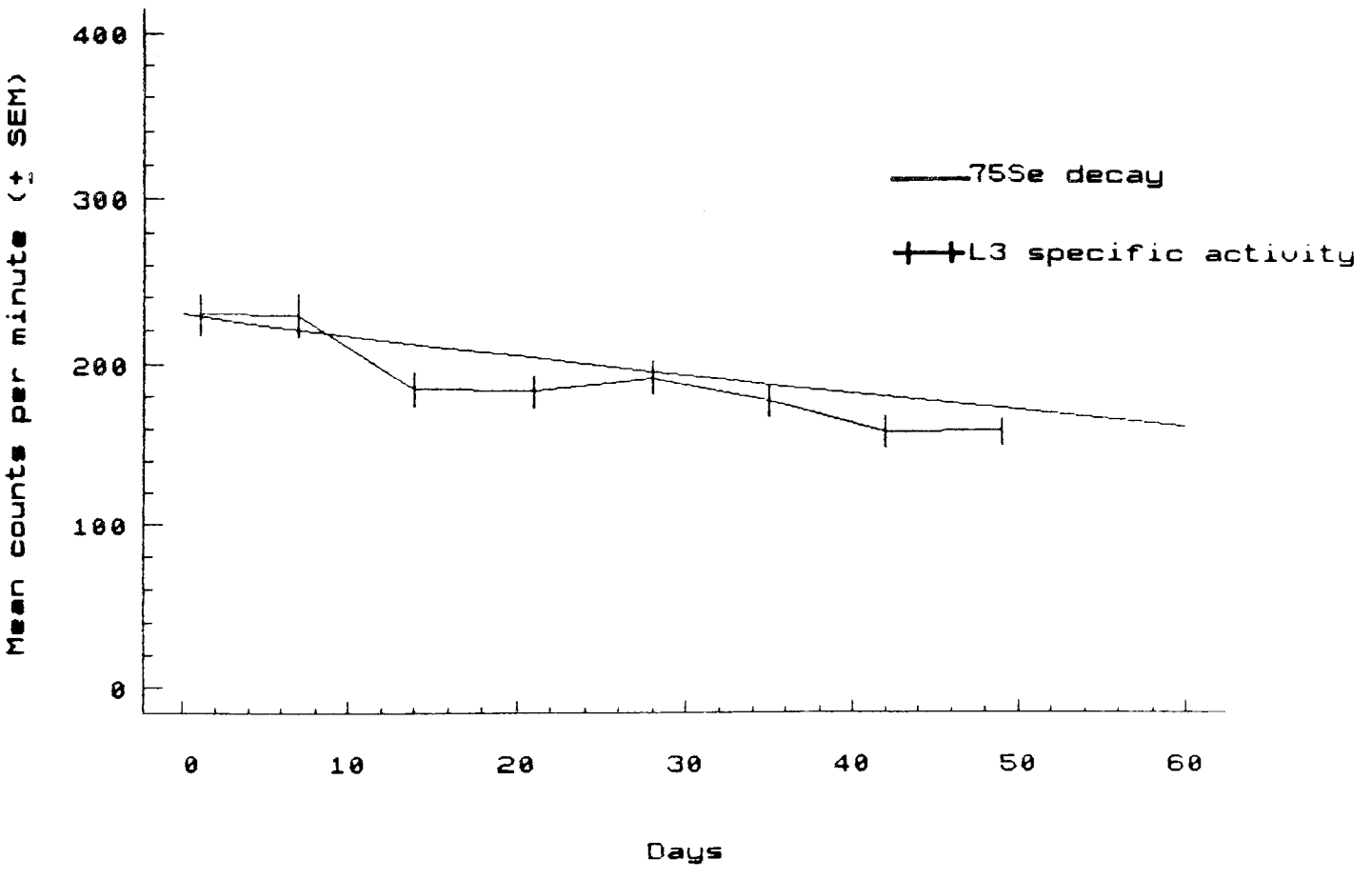
I. LOSS OF RADIOACTIVITY FROM DEVELOPING WORMS IN VIVO

The above experiments showed that approximately 40% of individual worm radioactivity was lost when larvae were

TABLE 3.3 Loss of Radioactivity During Larval Exsheathment

Experiment No.	Species	Mean Sheathed L3 radioactivity (cpm) ± S.E.M	Mean Exsheathed L3 radioactivity (cpm) ± S.E.M.	% Loss
1	T. vitrinus	209.0 ± 10.1	140.3 ± 8.3	32.0
2	T. vitrinus	128.4 ± 8.5	64.5 ± 6.0	49.8
3	O. circumcincta	459.0 ± 16.0	322.0 ± 11.0	29.8
4	O. circumcincta	214.0 ± 12.0	122.5 ± 0.0	42.8

Figure 3.9 Loss of T.vitrinus L3 specific activity during storage compared to the decay curve of 75Se with time



exsheathed *in vitro*. To determine if any further loss of radioactivity occurred as the parasites matured within the host, the following experiment was undertaken.

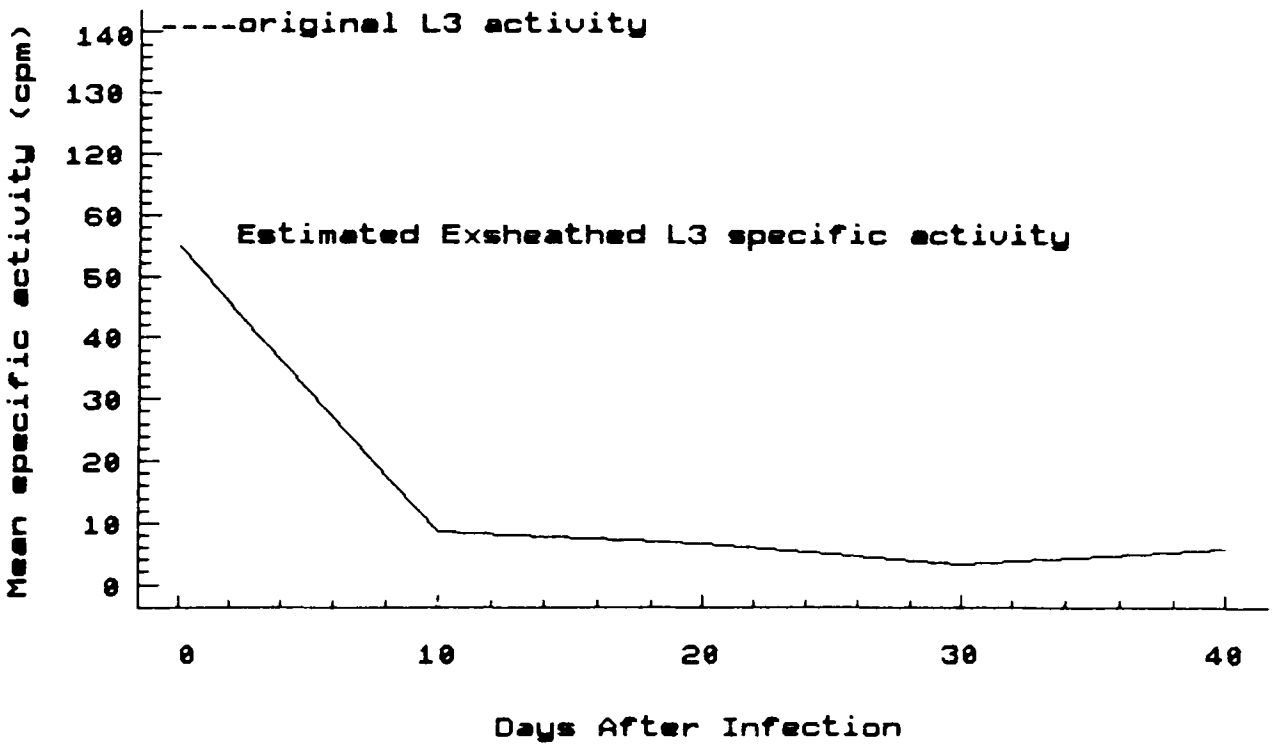
Eight sheep were infected with 800 radiolabelled *T. vitrinus* L3 (mean specific activity = 138.0 cpm) and killed in pairs 10, 20, 30 and 40 days later. The radioactivity of 50 worms recovered from each sheep was determined either by gamma-counting or by autoradiography.

The specific activity of the worms recovered 10 days after infection was very low at a mean of approximately 10 c.p.m. (Figure 3.10). The accuracy of the gamma-counter at these levels is poor but it was nevertheless clear that after day 10 the rate of loss of radioactivity was less. The sensitivity of the autoradiography technique was illustrated by the fact that almost all worms could be detected at day 20 and a mean of 80% of 40 day old worms could be detected after 2 months exposure (Table 3.4), although many images were faint.

TABLE 3.4 Loss of Radioactivity From Developing Worms *In Vivo*

Period of Infection (Days)	% Detected By Autoradiography		
	Sheep 1	Sheep 2	Mean
10	100	100	100
20	100	97	99
30	97	86	92
40	92	68	80

Figure 3.10 Loss of radioactivity on
O.circumcincta worms during in vivo developemnt



DISCUSSION

Numerous problems associated with the tube culture technique including failure to keep faeces uniformly moist were overcome with the development of the tub culture system. This proved to be a satisfactory method of culturing *O. circumcincta* or *T. vitrinus* eggs at densities well above those occurring naturally such that routinely 50-60% of eggs cultured from faeces containing 10,000 epg could be recovered as L3. In addition, faeces retention within the mesh bag and on the grid ensured that the suspension of developed L3 remained essentially debris-free. During radioactive culture, although some soluble isotope passed into the larval suspension, most was retained within the faecal mass and could be disposed of easily.

The amount of ^{75}Se incorporated by *H. contortus* or *O. circumcincta* L3 in any one experiment was directly related to the amount of isotope used in culture, although there was considerable inter-experiment variation. However, a maximum of only 0.6% of the isotope available in culture could be accounted for by the total population of radiolabelled L3 and in these terms the labelling technique could be considered highly inefficient. These results are, however, similar to those reported by Georgi & Le Jambre (1983) who radiolabelled *H. contortus* L3 using a slightly different technique. In both studies, despite the low percentage incorporation of radioisotope, radiolabelled larvae were found to be sufficiently radioactive to be detected after infection in the host.

The means whereby developing larvae incorporate ^{75}Se is unknown although Wilson (1979) has speculated that bacteria naturally present within faeces incorporate the amino acid analogue. He suggested that if these bacteria were ingested by the developing worm, they would be broken down into their constituent amino acids which would be re-metabolised into worm structures. In this connection Georgi & Le Jambre (1983) routinely added bacterial growth medium to cultures to encourage bacterial growth and hence maximise the larval uptake of ^{75}Se. Wilson (1979), however, showed that the addition of *Escherichia coli* directly to culture destroyed the ecological conditions required for larval development, and since satisfactory larval recoveries were obtained in the experiments described here, bacterial nutrient medium was not used.

Populations of radiolabelled worms exhibited a range of specific activities which became more extensive as the amount of isotope used in culture was increased. Similarly, Georgi & Le Jambre (1983) found that a range of larval specific activities occurred even after thorough mixing of the isotope with faecal slurry prior to culture. If the radioisotope was simply added dropwise to the faecal mass an even greater range of larval specific activities resulted. The considerable range of larval activities obtained in the present experiments suggests that some localized areas of high or low isotope concentration may still have existed throughout the cultured faeces even though the isotope was thoroughly mixed with the faeces prior to culture.

During culture the larvae developed and moved around within an environment containing a high concentration of radioisotope. In such a situation it might be expected that a quantity of ^{75}Se would become loosely bound to the outer sheath of the worm. Autoradiography of cross-sections of radiolabelled L3 indicated that significant amounts of radioactivity were associated with the larval sheath and the loss of approximately 40% of larval specific activity during exsheathment tended to confirm this view. However, it is not known if the ^{75}Se lost during exsheathing is attached to the sheath itself or whether a proportion is derived from labelled molecules in the exsheathing fluids.

Microscopic autoradiography of cross-sections of radiolabelled worms revealed that radioisotope was also located within the somatic structures of the worm and the result that worms could be detected up to 40 days after infection confirmed that this was the case. There was rapid loss of radioactivity during the first 10 days of development *in vivo* but slow loss thereafter. This may have corresponded to the period when the worm was undergoing a series of moults to the L5 stage, a time when most active metabolism would be occurring, as found for radiolabelled *H. contortus* by Georgi & Le Jambre (1983). Adult worms recovered after 40 days infection retained only 4% of their original L3 specific activity. This amount was too low for accurate determination by gamma-counting but using the autoradiographic technique 80% of recovered worms could still be detected.

In 4 out of 5 experiments no difference was found between the viability of labelled and unlabelled *O.circumcincta* or *T. vitrinus* worms. Since the proportion of labelled worms detected after a period of infection corresponded to the proportion in the infective dose it could also be concluded that there was no transfer of isotope to unlabelled parasites. In one experiment, however, when *O. circumcincta* L3 with a high initial specific activity of 640 cpm were used, the rate of establishment of labelled worms was significantly lower than that of unlabelled worms. Other measures of viability including the rate of development and the sex ratio of recovered worms were not affected. This latter result was particularly interesting since previous studies have shown that large doses of gamma irradiation of *O.circumcincta* L3 can affect the sex ratio of developed worms (Smith, Jackson & Jackson, 1982) by being detrimental to male worm development. Whether the low establishment in Expt. 2 was attributable to the initial high levels of radioactivity/L3 is open to question especially if one considers that the viability of *H.contortus* L3 was not affected by radioactivity levels more than twice as high as those used in this particular experiment (Georgi and Le Jambre, 1983).

In summary, the results of these experiments showed that large numbers of labelled *O. circumcinta* and *T.vitrinus* L3 could be produced relatively easily. The labelling procedure did not adversely affect their infectivity or growth and yet these parasites could be readily distinguished from normal worms. Thus it was predicted that radiolabelled worms could be used to follow the fate of incoming larvae in repeatedly infected sheep.

CHAPTER 4

THE DEVELOPMENT OF IMMUNITY TO INCOMING LARVAE IN LAMBS EXPOSED TO A TRICKLE INFECTION OF *T. VITRINUS*.

INTRODUCTION

In the previous chapter a technique for radiolabelling nematode L3 with ^{75}Se was successfully developed. Experiments demonstrated that the viability of radiolabelled worms was not affected by the incorporation of isotope and that after infection these worms could be recovered from the sheep and distinguished from normal parasites. In this chapter, radiolabelled *T.vitrinus* L3 were used in an experiment designed to measure the rate of development of immunity to this parasite in lambs continuously infected with 1000 L3 per day.

DESIGN OF THE EXPERIMENT

Twenty-seven 6-month old worm-free Greyface x Suffolk lambs were allocated to 3 equal groups on the basis of their sex and weight and each group was treated as described in Table 4.1. Of the 9 sheep in each group, 5 were infected daily with 1000 *T.vitrinus* L3 for 4 (Group 1), 8 (Group 2) or 12 (Group 3) weeks. The remaining 4 sheep acted as controls and were not infected. Immediately after the allotted infection period, all the sheep within the group were challenged with 1000 radiolabelled *T.vitrinus* L3 for three consecutive days. Ten days after the first challenge dose all 9 sheep in the group were killed.

Faecal egg counts were estimated weekly and worm burdens were estimated from 10% aliquots of the intestinal contents and digests. The worms were picked out, sexed and drawn before being put up to autoradiograph.

TABLE 4.1 Design of the experiment.

Group	Number of Lambs	Treatment	Days of Infection: Challenged (3 x 1000 L3*)	Killed
1.	5	1000 L3/day 4 weeks	29, 30, 31	39
	4	.....		
2.	5	1000 L3/day 8 weeks	57, 58, 59	67
	4	.....		
3.	5	1000 L3/day 12 weeks	85, 86, 87	95
	4	.....		

* Radiolabelled Larvae

The histological condition of the intestinal mucosa was scored and the number of mast cells and intra-epithelial globule leucocytes (IGLs) was determined. Statistical methods are described in Chapter 2.

RESULTS

Faecal Egg Counts

Group mean faecal egg counts increased from Day 17 of the experiment reaching a peak on Day 46. Thereafter, the mean e.p.g. gradually declined until by the end of the experiment the mean count of Group 3 sheep was less than 100 e.p.g. (Figure 4.1).

Total Worm Burdens

Although lambs in Group 2 had been infected with almost twice as many larvae as Group 1 lambs, mean burdens were not significantly different ($p > 0.05$), (Table 4.2.(i-iii)). However, lambs exposed to 12 weeks of continuous infection (Group 3) had significantly lower worm burdens than the other two groups ($p<0.01$) (Table 4.2.(i-iii)). If the mean total worm burden was expressed as a percentage of the number of larvae received (i.e. % establishment), a linear decline was observed between weeks 4 and 12 of the experiment (Figure 4.2).

Radiolabelled Worm Burdens

Approximately 98% of all the worms recovered from the control lambs were radiolabelled, as determined by autoradiography. The mean number of radiolabelled worms recovered from lambs exposed to 4 weeks of previous infection was lower than the mean number obtained in the corresponding

Figure 4.1. Mean egg counts of continuously infected sheep

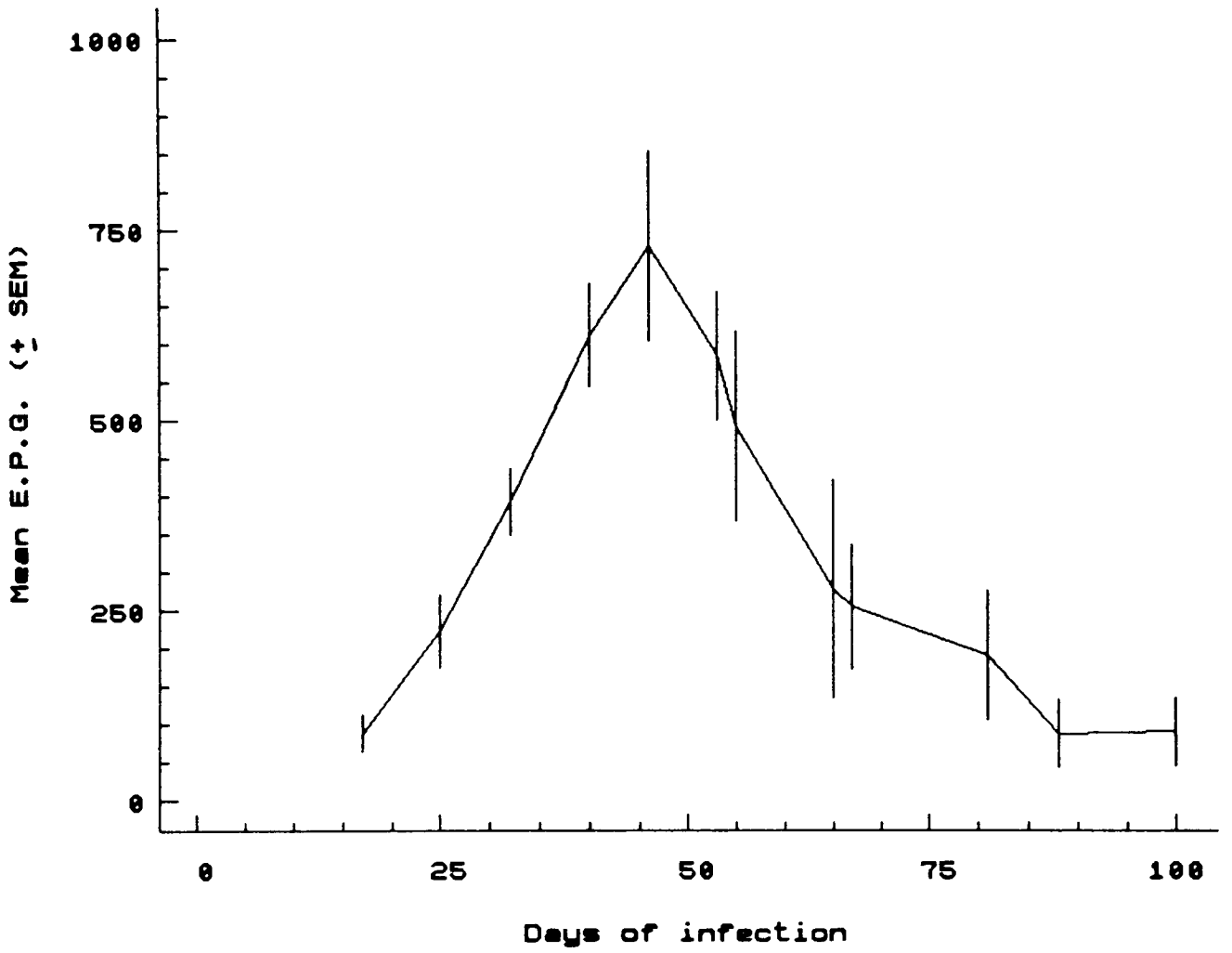


Figure 4.2. Total worm counts (as % of larvae administered) (x--x) and the change in protective immunity (o--o) in sheep continuously infected with T.vitrinus

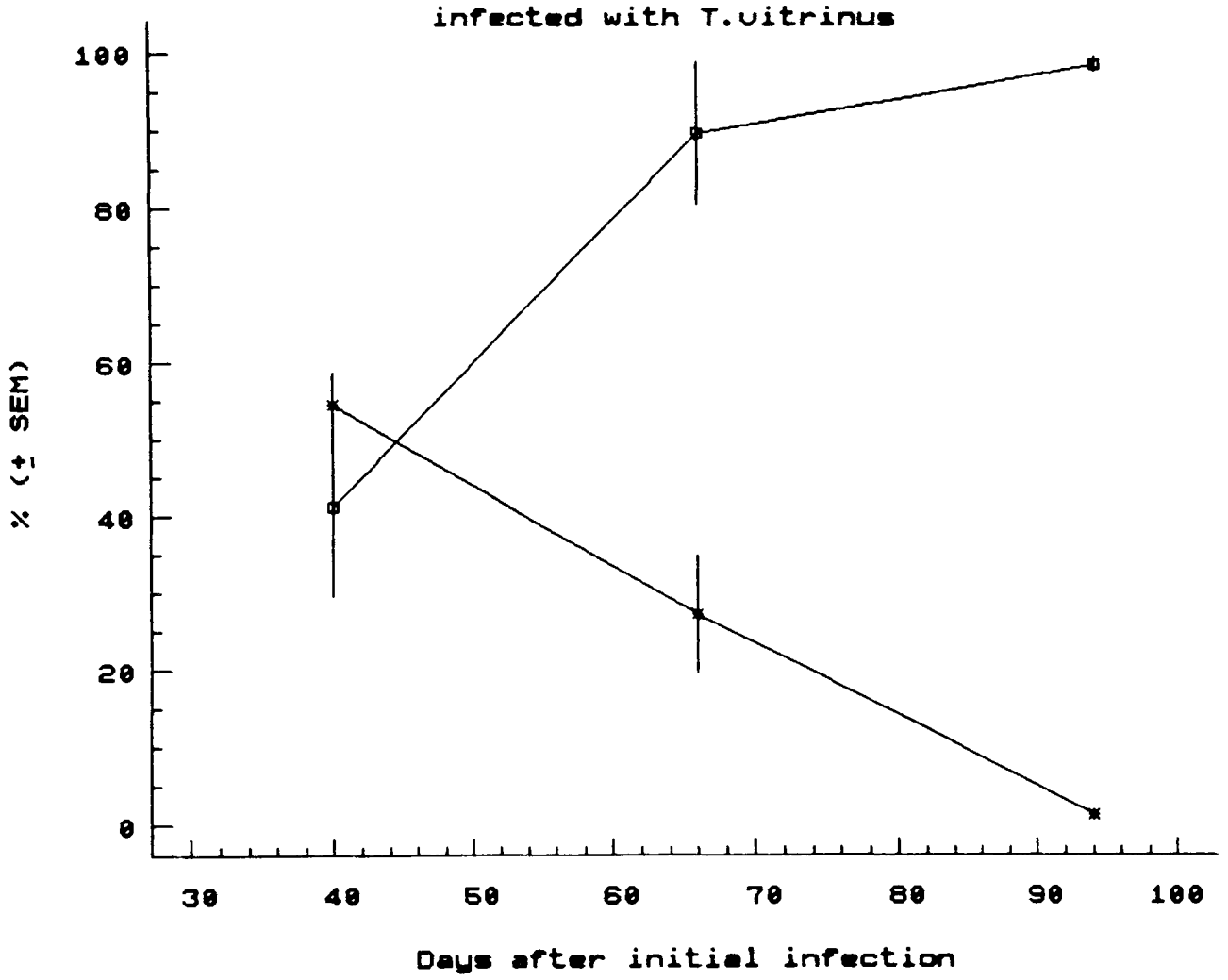


TABLE 4.2 (i)

Numbers of *T. vitrinus* recovered from Group 1 sheep, previously infected for 4 weeks and from control sheep.

Group No.	Sheep No.	Estimated Total Population				%
		Males	Females	Imm*	Total	Est.
1 (Previously Infected)	1.	5000	5700	3500	14500	45.8
	2.	6300	7800	3600	21900	57.1
	3.	7300	9100	4100	20500	66.1
	4.	5400	4400	3800	13600	43.8
	5.	8450	6900	3200	18550	59.9
	Mean	6490	6780	3640	16910	54.5
1 (Controls)	6.	600	650	50	1300	43.3
	7.	750	650	0	1400	46.7
	8.	350	600	0	950	31.7
	9.	300	450	0	750	25.0
	Mean	500	587.5	12.5	1100	36.7

* "Imm" = Sexually Undifferentiated Worms
"Est."=Establishment

TABLE 4.2 (ii)

Numbers of *T.vitrinus* recovered from Group 2 sheep previously infected for 8 weeks and from control sheep.

Group No.	Sheep No.	Estimated Total Population				%
		Males	Females	Imm*	Total	Est.
2 (Previously Infected)	10.	700	1100	80	1880	3.2
	11.	8500	9400	110	18010	30.5
	12.	5850	6700	20	12570	21.3
	13.	8700	8600	190	17490	29.6
	14.	14450	14500	1450	30400	51.5
	Mean	7640	8060	370	16070	27.2
2 (Controls)	15.	350	600	0	950	31.7
	16.	450	900	100	1450	48.3
	17.	350	550	0	900	30.0
	18.	600	500	0	1100	36.7
	Mean	437.5	637.5	25	1100	36.7

* "Imm" = Sexually Undifferentiated Worms
"Est."=Establishment

TABLE 4.2 (iii)

Numbers of *T. vitrinus* recovered from Group 3 sheep, previously infected for 12 weeks and from control sheep.

Group No.	Sheep No.	Estimated Total Population				%
		Males	Females	Imm*	Total	Est.
3 (Previously Infected)	19.	1180	1440	90	2710	3.1
	20.	0	0	30	30	0.0
	21.	10	30	60	100	0.1
	22.	30	30	850	910	1.0
	23.	40	150	1310	1500	1.7
	Mean	252	330	468	1050	1.2
3 (Controls)	24.	230	250	0	640	21.3
	25.	240	250	0	780	26.0
	26.	270	310	0	710	23.7
	27.	300	390	40	730	24.0
	Mean	260	300	10	715	23.8

* "Imm" = Sexually Undifferentiated Worms
"Est."=Establishment

control group (Table 4.3) although this difference was not statistically significant. Most animals challenged after 8 or 12 weeks of daily infection had small numbers of radiolabelled worms, indeed in 3 animals in Group 3 no radiolabelled worms were found. Comparison of the mean number of radiolabelled worms recovered from Groups 2 and 3 with their respective controls showed that these were significantly different ($p<0.001$). By comparing the number of challenge worms established in each infected animal with the mean burden of their respective control group, a "% protection" value was calculated from the formula:-

$$1 - \frac{\text{No. of radiolabelled worms}}{\text{Mean No. of radiolabelled worms in control group}} \times 100\%$$

Group mean protection increased from 41% at day 40 to reach 99% by Day 90 (Fig. 4.2).

Lengths of Radiolabelled Worms

The mean lengths of radiolabelled worms recovered from previously infected and control animals challenged at 4 weeks were similar (Table 4.4). Among the lambs challenged at 8 weeks the parasites recovered from the single sheep that did have a significant labelled population, (No. 14) were stunted, compared to those worms recovered from the controls. The few labelled parasites that were recovered from the remaining sheep challenged at week 8 and those challenged at week 12 were arrested third stage larvae measuring less than 1mm.

TABLE 4.3

Estimated numbers of radiolabelled worms in previously infected and control animals.

Group No.	Sheep No.	Estimated Total	Group No.	Sheep No.	Estimated Total
1 (Previously Infected)	1.	705	1 (Controls)	6.	1300
	2.	428		7.	1372
	3.	1073		8.	950
	4.	653		9.	735
	5.	342			
		----			----
	Mean	640		Mean	1089
		----			----
2 (Previously Infected)	10.	0	2 (Controls)	15.	931
	11.	14		16.	1450
	12.	10		17.	882
	13.	30		18.	1100
	14.	508			
		----			----
	Mean	112		Mean	1091
		----			----
3 (Previously Infected)	19.	0	3 (Controls)	24.	640
	20.	0		25.	780
	21.	0		26.	610
	22.	22		27.	680
	23.	28			
		----			----
	Mean	10		Mean	678
		----			----

TABLE 4.4

Mean lengths of radiolabelled challenge *T. vitrinus*

Group No.	Sheep No.	Mean Length (mm) Male	Female	Imm.	Group No.	Sheep No.	Mean Length (mm) Male	Female
1 (Previously Infected)	1.	1.15	1.52	----	1 (Controls)	6.	1.66	2.07
	2.	1.51	1.79	----		7.	1.59	1.80
	3.	1.66	1.83	----		8.	1.62	1.81
	4.	1.67	1.77	----		9.	1.75	2.02
	5.	1.67	1.69	----				
	Mean	1.53	1.72	----		Mean	1.66	1.93
2 (Previously Infected)	10.	----	----	----	2 (Controls)	15.	1.71	2.17
	11.	1.21*	----	----		16.	1.62	1.86
	12.	----	----	0.64*		17.	1.70	1.95
	13.	----	----	0.66		18.	1.59	1.82
	14.	1.53	1.61	----				
						Mean	1.66	1.95
3 (Previously Infected)	19.	----	----	----	3 (Controls)	24.	1.75	1.96
	20.	----	----	----		25.	1.50	1.89
	21.	----	----	----		26.	1.50	1.82
	22.	----	----	0.64		27.	1.46	1.99
	23.	----	----	0.67				
						Mean	1.55	1.92

* one worm only.

Lengths of Non-labelled Worms.

Comparison of the mean lengths of unlabelled male or female worms recovered from Groups 1 and 2 showed that these were not significantly different (Table 4.5). There was some between sheep variation within groups. Male and female worms recovered from sheep No. 1 and sheep No. 10, for example, were markedly shorter than those from the other sheep in their respective groups. The few adult male and female worms recovered from Group 3 sheep were significantly shorter than those in Groups 1 and 2 ($p<0.001$).

Histopathology

The mean numbers of IGLs and mast cells observed in 20 fields of view for each continuously infected sheep are presented in Table 4.6, together with individual mucosal histopathology scores. Of the lambs exposed to 4 weeks of continuous infection, (Group 1) the mucosa of one animal was extensively flattened whereas in the remainder damage was restricted to 'fingerprint' lesions (Figure 4.3). In Group 2 animals there was severe mucosal damage in the majority of the sheep but by week 14, when lambs exposed to 12 weeks of infection were killed, the mucosa of most had recovered to its normal state. The number of mast cells varied widely between individuals and group means were not significantly different. There was no correlation between the number of mast cells and the number of challenge worms recovered from infected animals. No IGLs were observed in Group 1 sheep or in any of the controls. However, with the exception of one sheep, (No.14), numerous cells were found in sheep from Groups 2 and 3. A

TABLE 4.5

Mean Lengths of Male and Female Worms Recovered from Previously Infected Sheep in Groups 1, 2 and 3.

Group No.	Sheep No.	Length of Males (mm) (± S.E.M.)	Range	Length of Females (mm) (± S.E.M.)	Range
1.	1.	3.83 ± 0.07	2.80-4.40	4.44 ± 0.10	2.95-5.61
	2.	4.19 ± 0.08	3.04-4.96	5.25 ± 0.90	3.37-6.42
	3.	4.27 ± 0.11	2.92-5.20	5.30 ± 0.10	3.02-6.31
	4.	4.34 ± 0.07	2.97-5.28	5.20 ± 0.12	3.16-6.21
	5.	4.51 ± 0.78	2.89-5.34	5.09 ± 0.11	2.94-6.43
		----------		----------	
	Mean	4.23 ± 0.11		5.06 ± 0.16	
2.	10.	3.87 ± 0.70	2.99-5.02	4.31 ± 0.86	2.94-5.31
	11.	4.15 ± 0.52	2.83-4.90	4.97 ± 0.59	4.12-5.96
	12.	4.21 ± 0.50	3.49-5.37	5.08 ± 0.70	3.78-6.08
	13.	4.13 ± 0.45	3.40-4.72	4.73 ± 0.08	2.98-5.74
	14.	4.62 ± 0.08	3.24-6.05	6.18 ± 0.11	3.81-7.33
		----------		----------	
	Mean	4.20 ± 0.12		5.05 ± 0.31	
3.	19.	3.79 ± 0.43	2.86-4.25	4.22 ± 0.08	3.10-5.51
	20.	----	---- ----	----	---- ----
	21.	3.63 ± 0.67	2.96-4.29	3.21 ± 0.03	3.18-3.23
	22.	3.64 ± 0.31	3.33-3.94	3.97	
	23.	3.61 ± 0.24	3.16-4.24	3.80 ± 0.12	3.06-4.52
		----------		----------	
	Mean	3.67 ± 0.41		3.80 ± 0.22	

* One worm only

TABLE 4.6

Numbers of mast cells, IGLs and mucosal histopathology of the the previously infected sheep.

Group No.	Sheep No.	Mean No. Mast Cells ± S.E.M.	Mean No. IGLs ± S.E.M.	Histopathology Score
1.	1.	18.20 ± 1.77	0	F
	2.	5.55 ± 0.64	0	F
	3.	7.65 ± 0.92	0	F+
	4.	8.90 ± 0.91	0	-
	5.	6.20 ± 0.49	0	+++
	Mean	9.30 ± 2.30		
2.	10.	6.50 ± 0.88	6.95 ± 0.55	-
	11.	3.35 ± 0.45	13.95 ± 1.67	F+
	12.	3.55 ± 0.38	4.65 ± 0.82	+++
	13.	0.80 ± 0.22	7.85 ± 0.88	+++
	14.	1.00 ± 0.33	0.00	+++
	Mean	3.04 ± 1.00	6.70 ± 2.30	
3.	19.	1.45 ± 0.39	16.70 ± 1.60	-
	20.	6.10 ± 0.96	16.60 ± 1.06	-
	21.	3.05 ± 0.38	11.65 ± 1.03	+
	22.	5.50 ± 0.98	21.50 ± 1.64	F+
	23.	8.75 ± 0.74	14.70 ± 1.29	-
	Mean	4.97 ± 1.26	16.25 ± 1.60	

+++ = Extensive flattening of the mucosa
++ = Moderate flattening of the mucosa
+ = Focal villous atrophy
- = Normal morphology
F = Fingerprint lesion

Figure 4.3 Examples of fingerprint lesions as seen in the small intestine of Group 1 lambs. Reprinted with permission of the editors of Research in Veterinary Science.

significant negative correlation ($p<0.01$) was found between the number of IGLs and the number of challenge worms recovered from infected animals.

DISCUSSION

After 4 weeks of the experiment, some previously infected animals were already mounting an immune response, which enabled them to regulate incoming or recently established worms. At this stage of the experiment faecal egg counts were still rising and over 16,000 previously acquired worms were present. This suggests that one of the first effects of developing host immunity was to prevent the establishment of incoming larvae or to expel them soon after they did establish. As the duration of infection increased, so did the effectiveness of this response. Thus, after 8 weeks of continuous infection very few radiolabelled worms were recovered from 4 of 5 lambs, although large numbers of mature egg-producing parasites were still present in these animals, and histopathological analysis revealed that substantial areas of the gut were still damaged. One lamb in Group 2 (No. 14) was, however, clearly still susceptible to infection, in that substantial numbers of labelled worms were still establishing. This animal also had the largest adult worm burden of the group, equivalent to 53% of the total non-challenge L3 received. Three of the 5 sheep challenged after 12 weeks of continuous infection appeared to be completely immune to incoming larvae and the few challenge worms that did establish in the remaining 2 lambs were arrested at the third larval stage. Clearly, by this stage of the infection prevention of larval establishment was almost complete.

The 10 day interval between challenge and slaughter employed in the current experiment made it impossible to determine whether labelled larvae failed to establish in the

immune sheep or whether they did establish successfully but were subsequently expelled before slaughter. In a subsequent experiment carried out in the same laboratory (Jackson, personal communication) the interval between challenge and slaughter was reduced to 3 days and very few labelled larvae were recovered from sheep which had been continuously infected for 8 weeks. This indicated that at this stage at least, failure of initial establishment probably accounted for the reduction in labelled larvae seen in the present experiment.

After 4 weeks of infection with 1000 L3/day a mean of 16290 adult worms were recovered, amounting to 58% of the total number of non-challenge larvae received. After 8 weeks of infection a similar number of worms were recovered, equivalent to 28.5% of the total number of L3 administered. It is possible that during the interim period a turnover of the adult population may have occurred and worm numbers thus remained the same as a result of a balance between the number of adult worms lost and the number replaced by incoming larvae. However, analysis of the results suggested that a more likely explanation was that numbers of established worms failed to increase significantly because of the sharp fall in recruitment rate of incoming larvae between 4 and 8 weeks which fell from a mean of 213 L3 per day to only 40 L3 per day over this period.

If it is assumed that this decline is linear, then it is possible to predict the mean total worm burden (N_2) of sheep killed after 8 weeks infection using the equation,

$$N_2 = N_1 + \frac{(r_1 + r_2)}{2} X$$

where N_1 is the observed worm count at 4 weeks, r_1 and r_2 are the recruitment rates at 4 and 8 weeks respectively and X is the number of days of infection. In this particular experiment this gives the following result:- $N_2 = 16910 + (127 \times 31)$, predicting a total worm burden of 20847 worms in sheep killed after 8 weeks infection, a value almost 5000 worms greater than the actual mean worm burden at 8 weeks. This result might be used to argue that some worms were being lost from the established worm population over the 4 week period and thus a very short period of turnover might have occurred. However, in this particular experiment, this may be a misleading interpretation since the mean total of radiolabelled worms in animals killed at 8 weeks is not representative of the majority of animals in the group, being biased by the relatively large numbers of worms in one sheep (No. 14). Further evidence against turnover is provided by analysis of the adult worm lengths. Group mean sizes of adult worms of both sexes recovered after 4 and 8 weeks of infection were not significantly different and frequency distributions of the lengths were very similar. Had a population turnover occurred the mean sizes of male and female worms would be expected to decrease as a result of immunity (see below). Since this clearly was not the case, it seems unlikely turnover occurred and more likely that established worms persisted over this period.

Following the initial establishment of worms and a period when worm burdens remained stable there followed a period when adult worms were expelled from the lambs. Between 8 and 12 weeks the adult population fell from a mean of approximately 16000 to approximately 1000 worms, equivalent to a loss of 535 worms per day. This cannot be considered a period of population turnover since recruitment to the population was to all intents and purposes zero during this period. After 12 weeks of infection all of the lambs had expelled most of their adult worms although inspection of individual adult burdens in Groups 2 and 3 suggests that there was marked individual variation in the rate at which expulsion proceeded. Clearly lamb No. 10 in Group 2 had expelled a large burden by week 8 whereas lamb No. 14 in the same group had expelled few, if any and was still recruiting worms to the population. The few adult worms that were recovered from Group 3 lambs were significantly ($p<0.001$) shorter than those recovered at 8 weeks. The most likely explanation for this result is that these worms established later in the infection and consequently their development was affected by host immunity.

Previous studies have shown that immunity to *Trichostrongylus* spp. is associated with an increase in the number of IGLs in the gastro-intestinal mucosa (Dineen *et al*, 1978, Jackson *et al* , 1983). Similarly an overall positive correlation existed in the present experiment between the development of immunity to incoming larvae and the numbers of IGLs present. The importance of these cells remains unclear, however, since none were observed in intestinal sections taken at 4 weeks, although considerable immunity to incoming worms was apparent at this time.

Mast cells have been associated with the regulation of a number of gastro-intestinal nematode populations in small animals (Russell & Castro, 1979; Lee & Ogilvie, 1981; Miller, Huntley & Wallace, 1981; Miller, Woodbury, Huntley & Newlands, 1983) and in view of this it is interesting that in the present study there was no correlation between the numbers of mast cells and immunity expressed as resistance to new infection. There was also no correlation between mast cell numbers and IGL numbers, a result surprising since Murray, Miller, & Jarrett, (1968) and Huntley, Wallace & Miller (1982) have provided strong evidence that globule leucocytes are in fact discharged mast cells. However, since the animals in this study were killed 7 days after their last challenge, some turnover of the mast cell population may have occurred in the interim period. In addition, by nature of the histological technique used, the functional activity of the cells cannot be measured. In this regard it may be relevant to note that the adult worms recovered from lamb No. 1 were markedly shorter than worms recovered from the rest of the lambs in Group 1 and the mast cell count in this animal was three times as great as the next highest cell count. Furthermore, the one lamb in Group 2 which was most advanced in its immune response in terms of preventing establishment and also in expelling its adult burden had a relatively high mast cell count in relation to the rest of the animals in its group. The role of mast cells and IGLs in the immune response to gastro-intestinal nematodes in sheep clearly needs further investigation.

CHAPTER 5

THE DEVELOPMENT OF IMMUNITY TO INCOMING LARVAE IN LAMBS EXPOSED TO A TRICKLE INFECTION OF *O.CIRCUMCINCTA*.

INTRODUCTION

In the previous chapter it was shown that radiolabelled *T.vitrinus* L3 could be successfully used to study the development of immunity to incoming larvae in continuously infected sheep. It was shown that the first indication of immunity was a partial resistance to the establishment of incoming worms which was apparent after 4 weeks of infection and nearly complete by 8 weeks. Worms that did manage to establish at 8 weeks were inhibited in their development. In this chapter a similar experiment is described whereby the development of immunity to incoming *O.circumcincta* is examined with the use of radiolablled L3. Since the regulatory mechanisms operating against this parasite are generally believed to be different to those operating against *Trichostrongylus* spp (Donald & Waller, 1982) it was considered relevant to compare these two species using a similar experimental approach.

DESIGN OF THE EXPERIMENT

The design of the experiment was similar to that described for the *T.vitrinus* infection experiment. Thirty 6-month old worm-free Greyface x Suffolk lambs were allocated to 3 equal groups on the basis of their sex and weight, and each group was treated as described in Table 5.1. Six sheep in each group were infected daily with 1000 *O.circumcincta* L3 for 4 (Group 1), 8 (Group 2) or 12 (Group 3) weeks. The remaining 4 sheep in each group were not infected and acted as controls. After the designated infection period the previously infected sheep and the controls were challenged on 3 consecutive days

TABLE 5.1 DESIGN OF THE EXPERIMENT

Group No.	Number of Sheep	Treatment	Days of Infection Challenged (3 x 1000 L3*)	Killed
1.	6	1000 L3/day 4 weeks		
	4	---	29, 30, 31	42
2.	6	1000 L3/day 8 weeks		
		---	57, 58, 59	70
3.	6	1000 L3/day 12 weeks		
	4	---	85, 86, 87	98

* Radiolabelled larvae

with 1000 radiolabelled *O.circumcincta* L3. Thirteen days after the first challenge dose all the sheep in the group were killed.

Faecal egg counts from the infected sheep were made weekly and worm burdens were estimated from 10% aliquots of the abomasal contents and digests. The worms were picked out and sexed before being drawn and put up to autoradiograph.

Each sheep was bled weekly and the sera were stored at -20°C. The concentration of antibody directed against *O.circumcincta* L3 antigen was determined for each sample by ELISA. Sera were also used to probe SDS-PAGE blots of exsheathed L3 antigen.

The histological condition of an excised abomasal fold was scored and the numbers of mast cells and IGLs were counted. The statistical methods used to analyse these results are described in Chapter 2.

RESULTS

Faecal Egg Counts

There was large between and within-lamb variation in faecal egg count. The peak mean count was obtained 48 days after the initial infecting dose and numbers remained high until 78 days. Thereafter there was a marked drop in mean e.p.g. which remained below 50 e.p.g. until the end of the experiment (Figure 5.1).

Total Worm Burdens

There were no significant differences between the mean total worm burdens of all three groups of previously infected sheep (Table 5.2.(i-iii)). However, if the total worm counts

Figure 5.1. Mean egg counts of continuously infected sheep

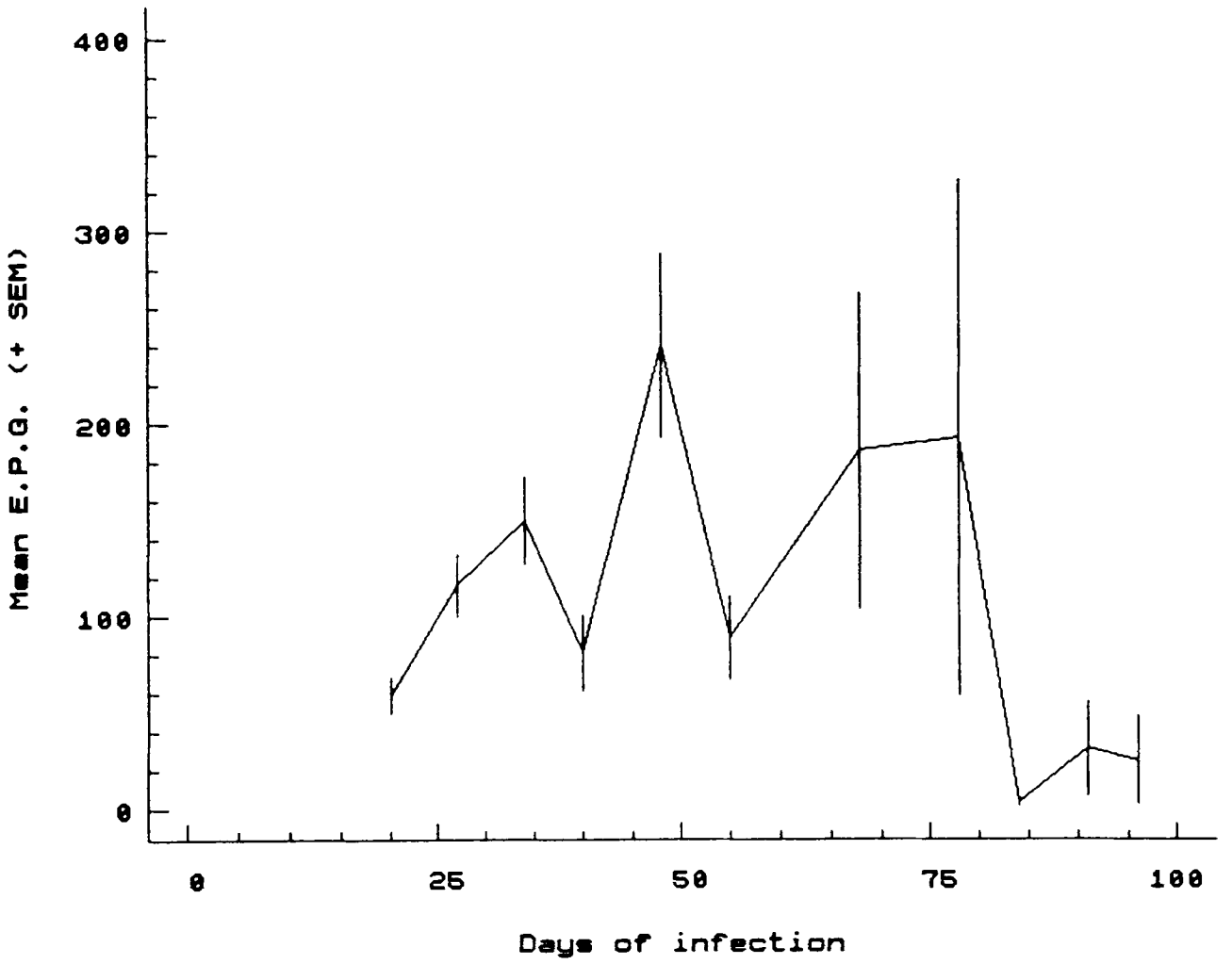


TABLE 5.2(i) Numbers of *O.circumcincta* recovered from Group 1 sheep.

Group No.	Sheep No.	Estimated Total Population Males	Females	Imm*	Total	% Est.
1.(Previously Infected)	1.	6480	5680	1090	13500	42.3
	2.	3400	5120	1280	9800	31.6
	3.	2160	3320	840	6320	20.4
	4.	4280	5120	1400	10800	34.8
	5.	3760	4080	5640	13480	43.5
	6.	3600	4160	4920	12680	40.9
	Mean	3947	4580	2520	11055	35.6
1.(Controls)	7.	640	440	0	1080	36.0
	8.	520	520	0	1040	34.7
	9.	600	720	0	1320	44.0
	10.	720	680	0	1400	46.7
	Mean	620	590	0	1210	40.4

* "Imm" = Sexually Undifferentiated Worms
"Est."= Establishment

TABLE 5.2(ii) Numbers of *O.circumcincta* recovered from Group 2 sheep.

Group No.	Sheep No.	Estimated Total Population Males	Females	Imm*	Total	% Est.
2.(Previously Infected)	11.	5360	7840	1760	14960	25.3
	12.	800	1600	1240	3640	6.2
	13.	8040	10160	4400	22600	38.3
	14.	160	400	2840	3400	5.8
	15.	80	80	2080	2240	3.8
	16.	4520	5000	3800	13320	22.6
	Mean	3160	4300	2027	10027	17.0
2.(Controls)	17.	560	800	0	1360	45.3
	18.	720	840	0	1560	52.0
	19.	520	760	0	1280	42.7
	20.	240	560	0	800	26.7
	Mean	510	740	0	1250	41.7

* "Imm" = Sexually Undifferentiated Worms
"Est."= Establishment

Table 5.2(iii) Numbers of *O.circumcincta* recovered from Group 3 sheep.

Group No.	Sheep No.	Estimated Total Population Males	Females	Imm*	Total	% Est.
3.(Previously Infected)	21.	240	480	0	720	0.8
	22.	0	0	0	0	0.0
	23.	3440	3200	5200	11840	13.6
	24.	1000	2000	4200	7200	8.3
	25.	80	280	680	1040	1.2
	26.	2400	4000	800	7200	8.3
	Mean	1193	1660	1813	4667	5.4
3.(Controls)	27.	440	520	0	960	32.0
	28.	280	320	0	600	20.0
	29.	120	240	0	360	12.0
	30.	400	560	0	960	32.0
	Mean	310	410	0	720	24.0

* "Imm" = Sexually Undifferentiated Worms
"Est."= Establishment

were expressed as a percentage of the number of larvae administered (percentage establishment), a linear decline was observed between weeks 4 and 12 (Figure 5.2).

Radiolabelled Worm Burdens

A mean of over 94% of all the worms recovered from the control lambs were radiolabelled as determined by autoradiography. There was no significant difference between the number of radiolabelled worms recovered from infected and control lambs challenged after 4 weeks (Table 5.3). However, lambs challenged at 8 weeks had significantly fewer radiolabelled worms than controls ($p<0.001$). By 12 weeks, 4 infected sheep were totally resistant to reinfection and the mean numbers of worms recovered from the infected and control groups were significantly different ($p<0.001$).

As described in the previous chapter, a '% protection' value was determined for each continuously infected lamb using the formula;-

$$\% \text{ Protection} = 1 - \frac{\text{No. of Radiolabelled Worms}}{\text{Mean No. of Radiolabelled Worms in Control Group}} \times 100\%$$

There was a sharp increase in group mean '% protection' values between 4 and 8 weeks (Figure 5.2), and this reached 90% by the end of the experiment.

Development of Radiolabelled Worms

No sexually undifferentiated EL4 worms were found in any of the control animals. Radiolabelled male worms recovered from infected animals challenged after 4 weeks were significantly shorter ($p<0.05$) than those recovered from control

Figure 5.2. Total worm counts (as % of larvae administered) (x--x) and the change in protective immunity (o--o) in sheep continuously infected with O.circumcincta

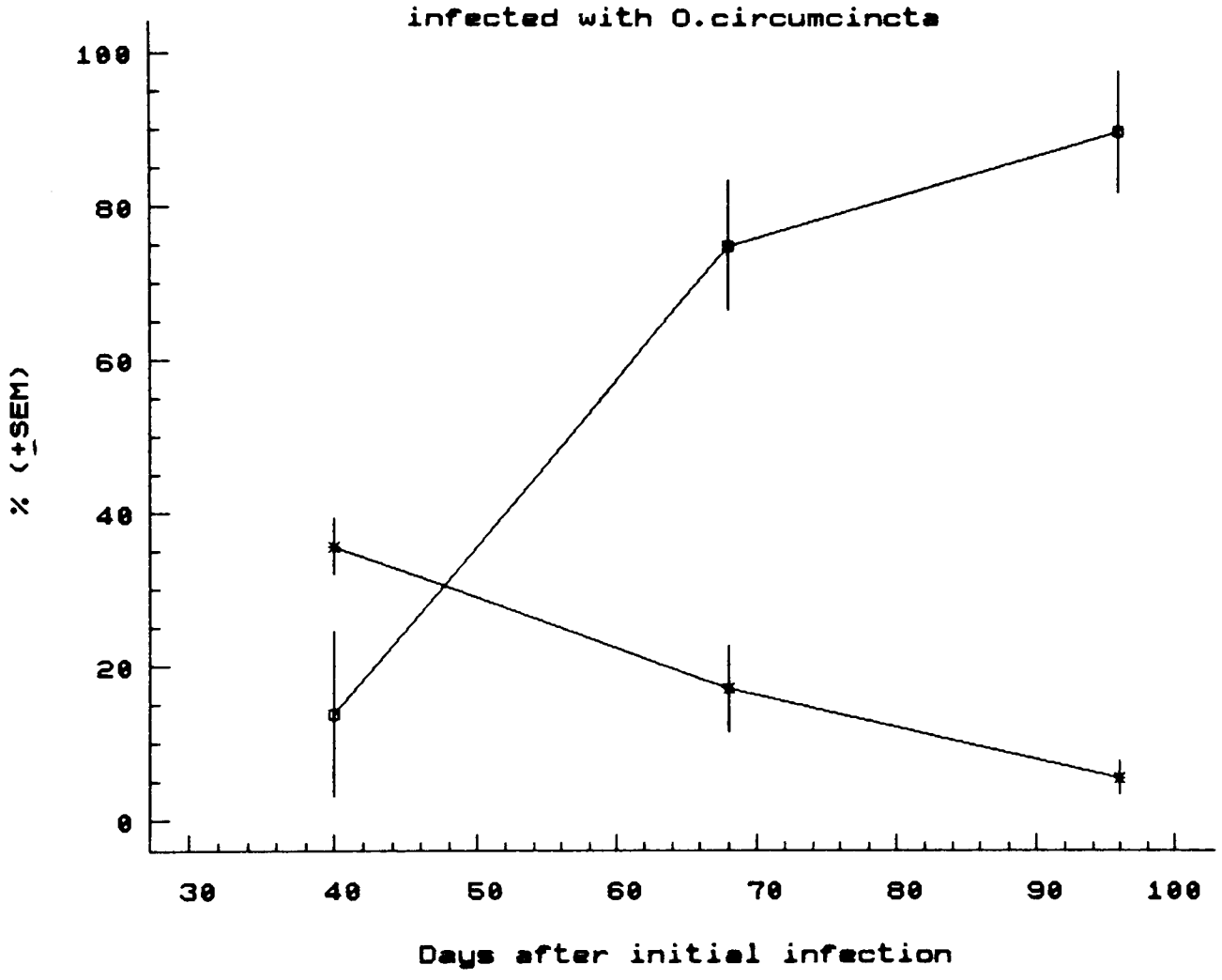


TABLE 5.3

Estimated numbers of radiolabelled challenge L3 in previously infected and control sheep.

Group No.	Sheep No.	Estimated Total	Group No.	Sheep No.	Estimated Total
1. (Previously Infected)	1.	1400	1.(Controls)	7.	1080
	2.	1520		8.	1040
	3.	400		9.	1320
	4.	960		10.	1400
	5.	1640			
	6.	1360			
		——			——
	Mean	1213		Mean	1210
2.(Previously Infected)	11.	360	2.(Controls)	17.	1360
	12.	280		18.	1560
	13.	480		19.	1280
	14.	0		20.	800
	15.	0			
	16.	480			
		——			——
	Mean	267		Mean	1250
3.(Previously Infected)	21.	0	3.(Controls)	27.	960
	22.	0		28.	600
	23.	280		29.	350
	24.	0		30.	960
	25.	0			
	26.	90			
		——			——
	Mean	60		Mean	720

animals (Table 5.4). Radiolabelled female worms recovered from infected animals were more than 1mm shorter than those from the controls but this difference was not statistically significant. Since, with the exception of one lamb (No. 30), 50% or more of the radiolabelled worm population recovered from each continuously infected sheep in Groups 2 and 3 consisted of EL4, measuring less than 2.0 mm the mean lengths of worms recovered from these animals are not shown.

Adult Worm Lengths

The range and mean lengths of 50 male and 50 female adult worms recovered from previously infected animals are shown in Table 5.5. Worms of both sexes recovered from sheep previously infected for 8 weeks (Group 2) were significantly shorter than those recovered from sheep previously infected for 4 weeks (Group 1), females: 6.73 ± 0.11mm vs 7.56 ± 0.11mm, ($p<0.001$): males: 5.25 ± 0.08mm vs 5.56 ± 0.09mm, ($p<0.05$). The number of large worms was also greater in Group 1 than in Group 2 with significantly more female worms greater than 8.00mm ($p< 0.05$) and significantly more male worms larger than 6.00mm ($p< 0.05$). Only three sheep in Group 3 contained sufficient worms for measurement to be made and there was no significant difference in the mean lengths of worms recovered from this group compared to Group 1.

Histopathology

The mean numbers of mast cells counted in previously infected and control animals are presented in Table 5.6 along with IGL counts and individual histopathology scores for previously infected sheep. With one exception (lamb No. 2),

Table 5.4

Lengths of radiolabelled worms recovered from Group 1 Previously Infected and Control Sheep.

Group No.	Sheep No.	Mean Length (mm)		Group No.	Sheep No.	Mean Length (mm)	
		Male	Female			Male	Female
1. (Previously Infected)	1.	4.18	5.75	1. (Con.)	7.	3.89	4.71
	2.	3.33	4.34		8.	3.96	5.33
	3.	2.36	5.92		9.	4.31	5.97
	4.	2.89	3.54		10.	4.74	6.25
	5.	2.39	3.35	Mean (± S.E.M.)		4.23 ± 0.2	5.57 ± 0.3
	6.	2.94	3.59				
Mean (± S.E.M.)		3.02 ± 0.3	4.42 ± 0.5				

TABLE 5.5

Mean Lengths of Male and Female Worms Recovered From Previously Infected Animals in Groups 1, 2 and 3.

Group No.	Sheep No.	Length of Males (mm) (± S.E.M.)	Range	Length of Females (mm) (± S.E.M.)	Range
1.	1.	6.28 ± 0.19	2.9 - 8.7	8.01 ± 0.27	3.4 - 10.9
	2.	5.49 ± 0.22	2.0 - 7.6	7.18 ± 0.23	2.4 - 9.7
	3.	5.08 ± 0.23	1.8 - 7.5	6.78 ± 0.27	2.5 - 9.9
	4.	5.84 ± 0.20	2.7 - 8.2	8.33 ± 0.21	5.2 - 10.7
	5.	5.00 ± 0.27	2.2 - 8.8	7.63 ± 0.34	2.9 - 12.2
	6.	5.57 ± 0.22	2.0 - 8.2	7.47 ± 0.29	2.8 - 10.2
	Mean	5.56 ± 0.09		7.56 ± 0.11	
2.	11.	5.55 ± 0.13	3.4 - 7.7	6.73 ± 0.17	2.8 - 10.5
	12.	4.05 ± 0.29	2.4 - 5.6	5.77 ± 0.34	2.1 - 9.6
	13.	5.24 ± 0.12	2.8 - 6.9	6.92 ± 0.18	2.8 - 9.4
	14.	---- *		----	
	15.	---- *		----	
	16.	5.45 ± 0.10		7.25 ± 0.17	3.4 - 10.1
	Mean	5.25 ± 0.08		6.73 ± 0.11	
3.	21.	---- *		----	
	22.	---- *		----	
	23.	5.74 ± 0.18	2.5 - 7.7	7.46 ± 0.22	2.9 - 12.0
	24.	5.32 ± 0.14	3.0 - 6.6	7.02 ± 0.18	4.4 - 9.8
	25.	---- *		----	
	26.	5.57 ± 0.09	4.0 - 7.9	8.10 ± 0.14	5.4 - 10.2
	Mean	5.57 ± 0.08		7.53 ± 0.12	

* Not Estimated Due to Insufficient Worms For Measurement

TABLE 5.6

Mean Number of Mast Cells for Previously Infected and Control Sheep & Number of IGLs and Histopathology Scores for Continuously Infected Sheep

Group No.	Sheep No.	Mean No. of Mast Cells ± S.E.M.	Mean No. of Globule Leucocytes ± S.E.M.	Histopath. Score	Group No.	Sheep No.	Mean No. of Mast Cells ± S.E.M.
1.(P.I.)	1.	3.0 ± 0.6	0	+++	1. (Con)	7.	5.7 ± 0.6
	2.	3.4 ± 1.1	0	+		8.	2.2 ± 0.5
	3.	11.5 ± 1.1	0	+++		9.	1.9 ± 0.5
	4.	7.3 ± 0.8	0	+++		10.	3.1 ± 0.4
	5.	9.9 ± 1.5	0	+++			---
						Mean	3.2 ± 0.9
	6.	7.1 ± 0.6	0	+++			
		---	---				
	Mean	7.0 ± 1.4	0				
2.(P.I.)	11.	5.9 ± 1.7	7.9 ± 1.7	++	2. (Con)	17.	3.9 ± 0.6
	12.	20.3 ± 1.9	35.2 ± 7.0	+		18.	4.7 ± 0.7
	13.	15.0 ± 1.0	32.8 ± 9.9	+++		19.	2.2 ± 0.5
	14.	13.8 ± 1.0	73.1 ± 11.1	+++		20.	3.1 ± 0.4
	15.	18.2 ± 4.0	3.6 ± 1.1	+++			---
						Mean	3.5 ± 0.5
	16.	8.6 ± 0.8	7.1 ± 2.0	+++			
		---	---				
	Mean	13.6 ± 2.3	26.6 ± 10.9				
3.(P.I.)	21.	19.4 ± 1.4	9.4 ± 2.0	+	3. (Con)	27.	3.9 ± 0.6
	22.	4.0 ± 1.4	15.8 ± 1.0	+		28.	6.8 ± 0.6
	23.	9.5 ± 1.0	0	+++		29.	5.6 ± 0.4
	24.	9.9 ± 1.2	6.3 ± 0.7	+++		30.	2.9 ± 0.4
	25.	8.4 ± 1.0	34.5 ± 0.6	+++			---
						Mean	4.8 ± 0.9
	26.	11.6 ± 0.9	5.4 ± 2.3	++			
		---	---				
	Mean	10.5 ± 2.1	11.9 ± 5.0				

+++ Widespread severe worm damage
++ Moderately severe damage
+ Focal lesions only

P.I. = Previously Infected
Con = Controls

the abomasal mucosa of the sheep challenged after 4 weeks was extensively damaged with widespread loss of specialised secretory cells and copious infiltrates of neutrophils. Numerous larvae were seen in the gastric glands (Figure 5.3). In the lambs challenged after 8 and 12 weeks, individual sheep had evidence of chronic ostertagiasis, including mucosal hypertrophy, loss of differentiation and interstitial infiltrates of mononuclear cells. The numbers of mast cells found in control animals were significantly less ($p< 0.05$) (range = 1.9 - 6.8) than those in each infected group. Although there were significantly more mast cells in Group 2 and 3 lambs compared to Group 1 ($p<0.05$) no correlation was observed between the number of mast cells and the number of challenge worms recovered. No IGLs were observed in any of the control lambs or Group 1 animals. By contrast, numerous IGLs were found in Groups 2 and 3 (Figure 5.4) with the exception of one sheep. A negative correlation ($p<0.01$) was found between the number of IGLs and the number of challenge worms recovered from infected animals.

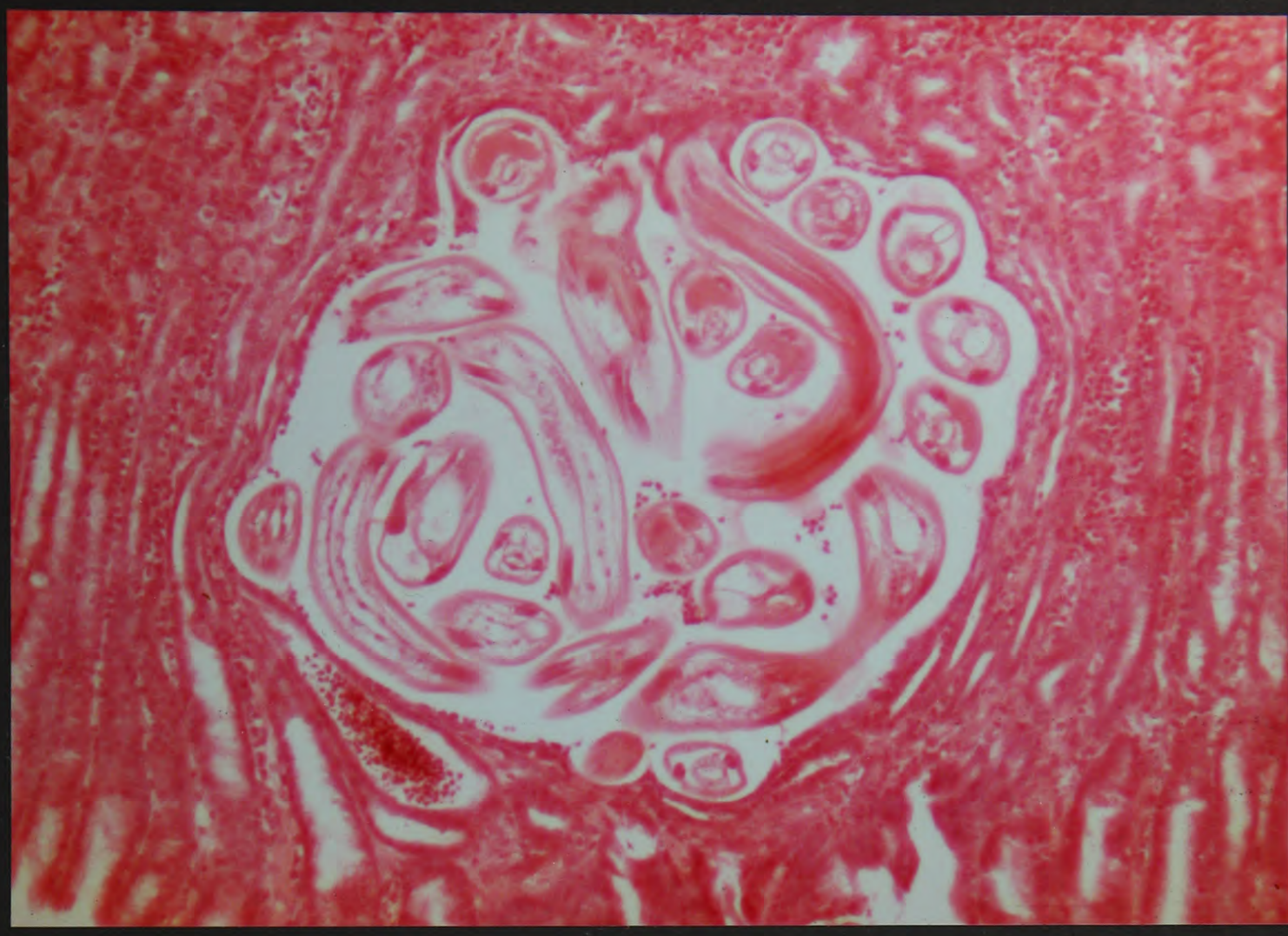
Serology

There was a gradual increase in the mean antibody titres until day 56. This was followed by a more pronounced rise reaching a peak some 10 times above pre-infection values on day 83 (Figure 5.5). There was a significant negative correlation between antibody titre and the number of labelled worms recovered from individual lambs ($p<0.01$).

Sera taken before infection did not react to L3 antigens on a blot. However, at least 9 larval antigens were

Figure 5.3 (above). *O.circumcincta* larvae within a gastric gland of a Group 1 sheep.

Figure 5.4 (below). Intraepithelial globule leucocytes (arrowed) present in the abomasal mucosal of a Group 2 sheep.



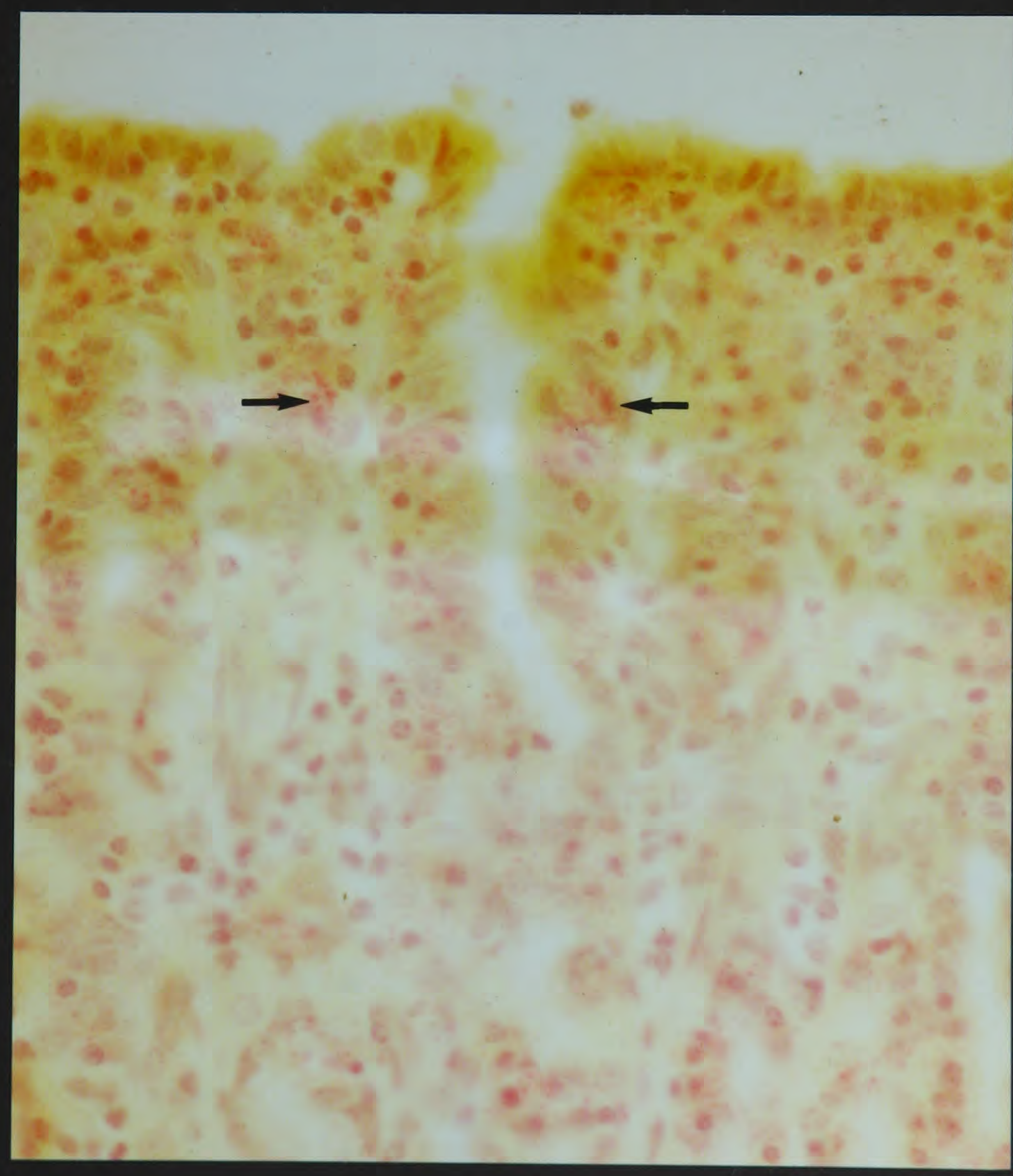
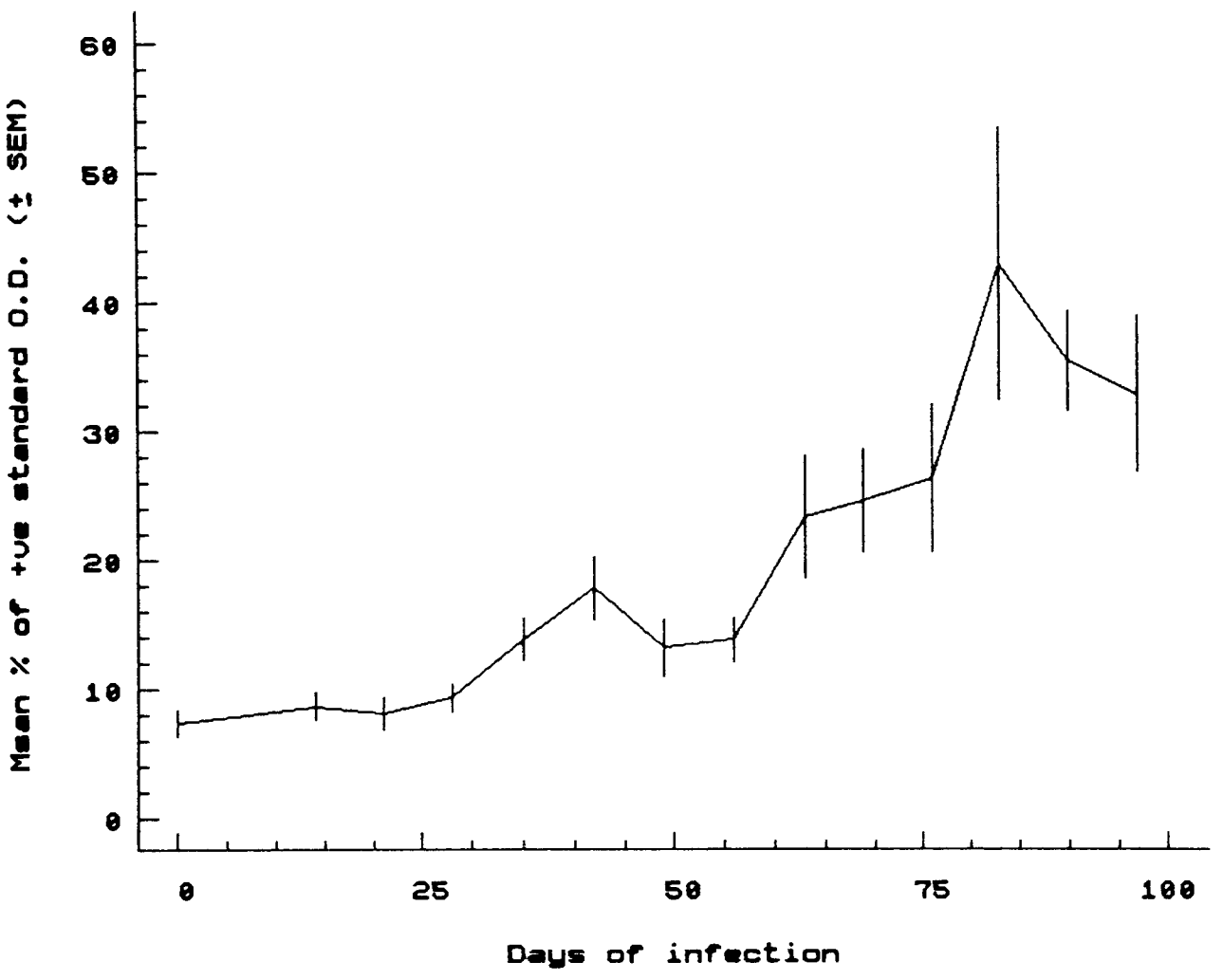


Figure 5.5. Mean serum antibody titres against L3 antigen for continuously infected sheep



recognised by sera from Group 3 animals (Figure 5.6). The intensity of reaction and the number of proteins recognised increased as the experiment progressed, with a smear of proteins between 65 and 80 kDa and two smaller proteins of approximately 58 kDa and 45 kDa being most reactive.

Figure 5.6

Immunoblot of exsheathed *O.circumcincta* L3 proteins probed with sera collected sequentially during infection of Group 3 sheep and peroxidase-conjugated pig anti-sheep $F(ab)_2$.

Track 0 Probed with serum taken prior to infection.

2 Probed with serum taken after 2 weeks of infection.

4 Probed with serum taken after 4 weeks of infection.

6 Probed with serum taken after 6 weeks of infection.

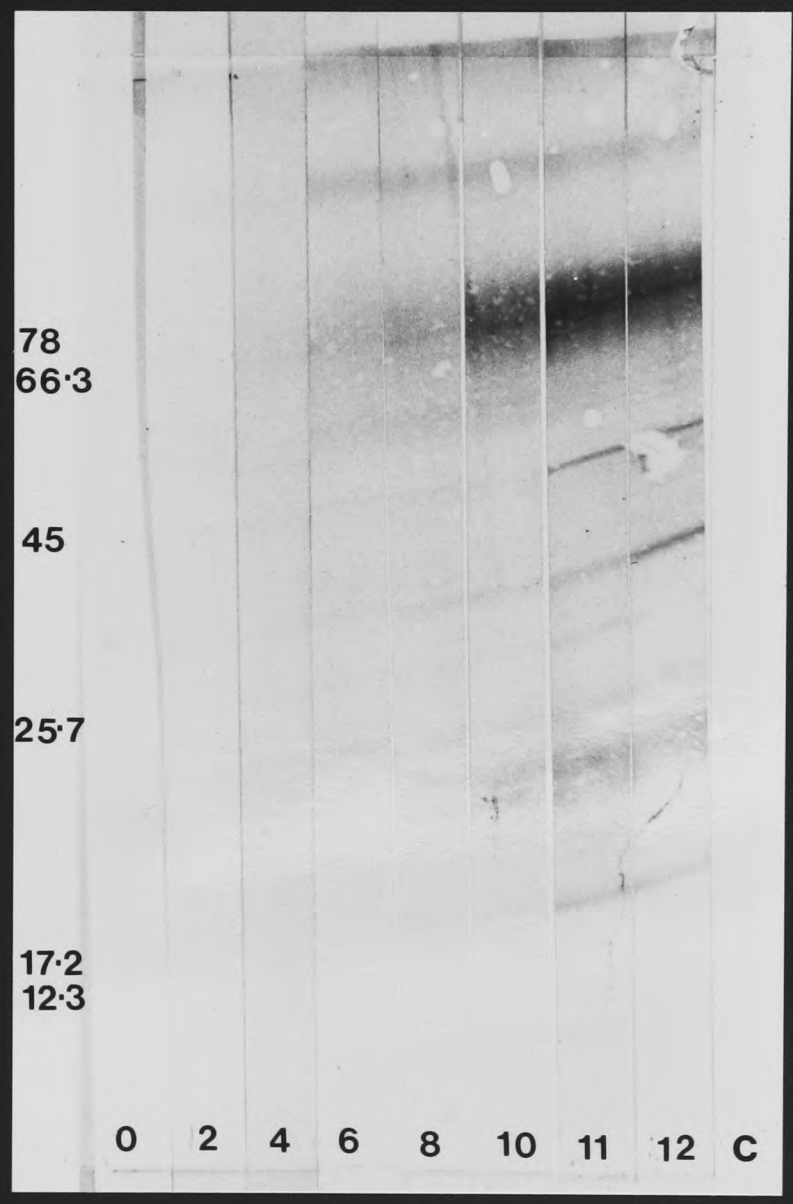
8 Probed with serum taken after 8 weeks of infection.

10 Probed with serum taken after 10 weeks of infection.

11 Probed with serum taken after 11 weeks of infection.

12 Probed with serum taken afetr 12 weeks of infection.

C Control strip, not incubated with serum.


78
66·3
45
25·7
17·2
12·3
0
2
4
6
8
10
11
12
C

DISCUSSION

The number of radiolabelled challenge worms that established in animals infected for 4 weeks and in controls was very similar, and thus it was apparent that there was no appreciable host immune response which prevented incoming larvae from establishing at this time. After 4 weeks of daily infection, a mean of 11,047 worms were recovered, equivalent to 35.3% of the total number of larvae received. After 8 weeks of infection, the mean total burden was similar, amounting to 10,027 worms or only 17% of the larvae received. The rate of recruitment of larvae to the population at 4 weeks can be determined from the number of radiolabelled worms recovered at this time. Thus, since 1213 of the 3000 challenge larvae administered after 4 weeks were recovered, the mean recruitment rate can be calculated as 403 L3 per day. If it is assumed that this recruitment rate is representative of the rate earlier in the infection, then the predicted total worm burden of Group 1 animals can be calculated as (31 x 403) = 12493 worms. Since this figure is close to the observed mean, it would appear that few established worms were lost during the first 6 weeks of infection.

After 8 weeks of previous infection the mean recruitment rate of larvae to the population as determined from the establishment of challenge L3 was calculated as 89 L3 per day. In 2 animals challenged after 8 weeks of infection no radiolabelled worms were recovered, and overall it was clear that between 4 and 8 weeks an immunity to incoming worms developed which resulted in the larval recruitment rate dropping

from 403 to 89 L3 per day. Assuming for a moment that the fall in this recruitment rate was linear, then using the same formula as described in the previous chapter, the predicted total worm count at 8 weeks can be calculated as 11047 + (31 x 246) = 18,673 worms. Since this value exceeds the observed mean worm burden of Group 2 sheep by over 8,600 worms, it can be argued that some established worms were lost during this period. Even if it is assumed that the mean recruitment rate fell abruptly to 89 L3/day immediately after 8 weeks of infection then the predicted total, using these calculations would still exceed the observed count by approximately 4000 worms. Either way the results suggest that established worms were being lost between 4 and 8 weeks of the infection.

Further evidence to support this theory was obtained by analysis of the lengths of non-labelled adult worms. The mean lengths of male and female worms recovered from Group 2 sheep were significantly shorter than those recovered from Group 1 animals. Furthermore, significantly fewer large worms were recovered from animals killed after 8 weeks infection compared to those killed after 4 weeks infection. The only explanation for this difference (assuming that worms do not shrink) is that some worms were lost during this period. It would thus appear that over these 4 weeks of the infection, worm loss and recruitment occurred simultaneously, i.e. a period of population turnover, as originally described by Michel (1963) for *O. ostertagi* in calves, occurred. However, most sheep were totally immune to incoming larvae by 12 weeks and the duration of this period of turnover must, therefore, have been

short in the majority of individuals. It is probable that there was considerable individual variation in the timing of this phenomenon. In sheep Nos. 10 and 11, for example, any period of turnover must have been over by 8 weeks since no larvae were being recruited to the population by this stage.

Although there was no difference in the establishment of challenge worms in infected and control animals at 4 weeks, the challenge worms recovered from the previously infected animals after this time were shorter than those recovered from the controls. By 8 and 12 weeks the majority of the relatively few radiolabelled worms recovered from previously infected animals were early fourth stage larvae. Since no early fourth stage larvae were recovered from any of the control animals, it is assumed that this retardation of development was a consequence of developing immunity.

To summarise the results of this experiment, the first indication of host immunity was a change in the rate of development of newly arrived larvae causing them to be stunted. Between 4 and 8 weeks an immune response against incoming worms developed, such that the number of larvae recruited to the population started to fall. At this time, some established worms were also being expelled, and thus for a short period a turnover of the population occurred. Worms that were lost were replaced by worms which established later in the infection which, because they were subject to an immune response which retarded their development, were shorter than earlier established worms.

The development of protective immunity in this

experiment correlated with the number of IGLs present in the gastric mucosa but no correlation could be demonstrated between immunity and mast cell numbers. However, when serum antibody collected from previously infected sheep was analysed, titres did correlate with the development of protective immunity as determined by the establishment of radiolabelled worms. Immunoblot analysis showed that many antigens present in a somatic extract of exsheathed L3 antigen were recognised by antibodies present in collected sera. The exact role played by these antibodies in the development of protective immunity remains to be defined, however.

CHAPTER 6

NEMATODE SURFACE ANTIGENS

LITERATURE REVIEW

Introduction

The ultimate goal of research into the immunoparasitology of helminth infections of ruminants is the production of effective vaccines against these parasites. Previous attempts to achieve host protection through immunization with crude homogenates of parasites have been unsuccessful (Clegg & Smith, 1978; Lloyd, 1981) suggesting, perhaps, that protective antigens are either not functional in such preparations, are present in amounts too small to be effective or were not administered by the optimum route. To try and overcome these problems the identification and isolation of discrete worm antigens has become a research priority.

Since nematodes are complex, multicellular organisms the identification of specific antigens is not a straightforward task. However, the recent application of techniques which enable individual parasite protein molecules to be radiolabelled with ^{125}I, combined with biochemical, immunological, and recombinant DNA methods has led to renewed optimism that vaccine production may still be a viable, if somewhat long term proposition. At present there are very few reports of antigen characterisation of ruminant nematodes and by necessity therefore, the following discussion is largely restricted to a survey of work undertaken on parasites of man or laboratory animals. The general principles illustrated by this work can be envisaged as being applicable to future work with ruminant parasites.

The somatic antigens of some nematodes have been examined, although most research has concentrated on defining antigens associated with the nematode cuticle (surface antigens) or antigens excreted or secreted by the parasites during *in vitro* culture (ES antigens). Since these molecules are presumably the most accessible to any host immune response it is probable that they will be particularly important.

Cuticle Structure

Despite the fact that the immunogenicity of the nematode cuticle has been recognised for some time (Soulsby & Coombs 1959; Soulsby, 1963) nematode surfaces had not been characterized at the molecular or immunological level until recently. Ultrastructural studies of the cuticle have suggested that it consists of three-layers comprising an outer cortex, a middle matrix layer and an inner basal layer, with the outermost layer of unknown composition and the inner fibrillar layers being similar to collagens (Lumsden, 1975, Lee 1977). Considerable morphological variations in cuticle structure may exist between different nematode species but the basic biochemical composition remains the same. The cuticle has largely been viewed as an acellular exo-skeleton consisting of only inert molecular components even though enzymes and haemoglobin have been shown to be present in the cuticles of some parasitic species (Lee, 1966) and adult stages of *Brugia pahangi* are able to take up nutrients via their surfaces (Chen & Howells, 1979; Howells & Chen, 1981). The true dynamic nature of the nematode cuticle has, however, only come to light through surface protein analyses. Renewed interest in nematode

surface antigens followed the demonstration that anti-surface antibody could mediate leucocyte adherence and cell mediated reactions which killed larval stages of *Trichinella spiralis* and *Nippostrongylus brasiliensis in vitro* (MacKenzie, Preston & Ogilvie, 1978) or resulted in damage to the cuticle of other parasitic nematodes (reviewed by Ogilvie, Philipp, Jungery, Maizels, Worms & Parkhouse, 1980). Following the demonstration of these antibody dependent cellular cytotoxicity (ADCC) reactions, attention has been directed at detailed investigations of the antigenic properties of the nematode surface in order to identify and characterise the particular antigens at which ADCC reactions are aimed.

Radiolabelling of Surface Proteins

Through the application and development of radiolabelling techniques previously used in the analysis of surface components of mammalian cells, detailed information concerning nematode surface proteins has been obtained. In these studies ^{125}I is the radiolabel most commonly used, although a number of different radiolabelling procedures have been utilized (reviewed by Philipp & Rumjaneck, 1984). Further characterization of radiolabelled proteins by poly-acrylamide gel electrophoresis (PAGE) has enabled a 'surface antigenic profiles' to be produced for each stage or species of nematode examined.

Number of Surface Proteins

The surface proteins of *T.spiralis* were the first to be studied in detail through radiolabelling and PAGE techniques (Philipp, Parkhouse & Ogilvie, 1980; Parkhouse, Philipp &

Ogilvie, 1981) although numerous species have since been similarly characterised. These studies have shown that the number of major proteins present on the surface of each nematode is usually 5 or less (Maizels, Philipp & Ogilvie, 1982). This surprisingly small number may reflect a bias towards identifying only those polypeptides containing lysine and tyrosine residues, since only these are amenable to radioiodination with the standard radiolabelling procedures (Maizels *et al* 1982). Polysaccharide or glycolipid antigens present on the cuticular surface would not be radiolabelled using these techniques. It is nevertheless possible that non-protein cuticular components are important in the immunology of some parasitic infections (Selkirk, Denham, Partono, Sutanto & Maizels, 1986).

Stage Specificity of Surface Proteins

Characterization of the surface proteins of 3 parasitic stages of *T.spiralis* following Chloramine-T mediated iodination revealed only 3-4 surface components for each stage (Philipp *et al*, 1980). Comparison of the individual profiles showed that completely different molecules were expressed by each stage. Thus, under reducing conditions, SDS-PAGE analysis resolved bands at 105, 90, 55 and 47 kDa for infective larvae; 64, 58, 34 and 30 kDa for newborn larvae and 40, 33 and 20 kDa for adults. The existence of stage-specific antigens had previously been suggested by the work of MacKenzie *et al*, (1978) who showed that stage-specific antibodies to *T. spiralis* were present in sera taken from infected animals such that absorption of sera with living infective larvae removed antibodies mediating eosinophil adherence to that, but no other stage. Philipp *et al*,

(1980) were, however, the first to directly demonstrate the stage-specificity of surface proteins and thus indicate the dynamic nature of the nematode cuticle. Subsequent work has suggested that the moulting process in itself is not necessarily responsible for all the observed changes in the protein profile between stages. Jungery, Clark & Parkhouse (1983) showed that the profile of newborn larvae of *T.spiralis*_changed 6-8 hours after being shed from the female before any moult occurred, and thus demonstrated that changes in surface proteins could occur between and also within defined developmental stages.

Since these original observations surface protein stage-specificity has been found in many other nematodes, although the extent of specificity varies between different species.

N. brasiliensis surface proteins have been shown to be stage specific (Maizels, Meghji & Ogilvie, 1983a) with different restricted sets being found on L3, L4 and adult stages of the worm. Interestingly in this species a sex-specific surface protein was also identified, with a 90 kDa component only being found on adult male worms. Further evidence of stage-specificity was provided by the demonstration that infections consisting of only one stage elicited antibody which was reactive only to the homologous stage (Maizels *et al*, 1983a). Pritchard, Maizels, Behnke & Appleby (1984) demonstrated that the surface proteins of *Nematospiroides dubius* were also stage specific. One protein identified on L4 6 days after infection could not be detected on adult worms although a further distinct stage-specific molecule was found on the worms by this time.

Filarial worms generally exhibit only partial stage-specificity which is not as absolute as that which exists for *T. spiralis* and *N. brasiliensis* (reviewed by Selkirk *et al*, 1986). Significant differences in the antigenic profiles of larval, adult and microfilarial stages of *B. pahangi* indicated a degree of stage specificity (Maizels, Partono, Oemijati, Denham & Ogilvie, 1983b). Extensive antigenic sharing between different stages also existed, however, since antibody to the surface of every stage could be detected in the serum of animals previously exposed to only one stage. Similarly, Canlass & Piessens (1985) found that monoclonal antibodies were reacted with surface antigens of 3 stages of *B. malayi*, suggesting an extensive distribution of shared determinants.

Cross-reactivity between adult and larval stages of *Litomosoides carinii* has been shown by Philipp, Worms, McLaren, Ogilvie, Parkhouse & Taylor (1984). Of interest in this work, however, was that the principal protein identified on the blood microfilariae was host serum albumin. Albumin has similarly been identified on blood microfilariae of *Wuchereria bancrofti* (Maizels, Philipp, Dasgupta & Partono, 1984) although there is no evidence that such host components play a role in the host-parasite interplay (Philipp & Rumjaneck, 1984).

It has been suggested that the changes in surface proteins which occur during parasite development may correlate with modifications in the parasite's environment (Philipp & Rumjaneck, 1984), and the results of some studies could be interpreted this way. Pritchard *et al* (1984) suggested that differences in surface proteins of different stages might

reflect the contrasting environments occupied by each stage, e.g. larval forms are usually tissue-dwelling whilst adults inhabit the duodenal lumen. On the other hand changes in surface protein composition may be a consequence of an adaptation to immunological rather than environmental constraints (Philipp & Rumjaneck, 1984). For example, since the immune responses to different stages of *T.spiralis* are also stage-specific it could be reasoned that through changing its external antigenic structure, the worm is able to survive the course of a primary infection by keeping ahead of an immune response that is lethal to a secondary infection.

Species Specificity of Surface Proteins

The identification of serologically specific proteins has been an objective of nematode surface antigen research in human parasitology. An obvious requirement for putative immunodiagnostic antigens is that they do not cross-react with sera from patients infected with heterologous parasites and the extent of species specificity is therefore an important consideration for any particular antigen.

In a similar manner to the range in stage-specificities of certain cuticular antigens there is a spectrum of specificities between different species of parasite, some antigens having a class wide distribution whilst others are strictly species restricted (Philipp & Rumjaneck, 1984).

The stage-specific cuticular antigens of *T. spiralis* infective larvae have also been shown to be remarkably species-specific. Parkhouse *et al* (1981) demonstrated that surface antigens did not react with serum antibodies stimulated

by infection with either nematodes of a number of superfamilies or with cestodes or trematodes.

Contrasting with this strict species specificity, immunological cross-reactivity between filarial worms is a common feature (Selkirk *et al*, 1986) and the antigenic similarities between *Brugia* spp. have been particularly well documented. Maizels *et al* (1983b) found that surface antigens of adult *B. timori*, *B. malayi* and *B. pahangi* were all closely homologous as were the surface antigens of infective larvae of all these species and microfilarial antigens of the latter two. The extent of homology was illustrated by the fact that antibody raised against one stage or species reacted with surface antigens from other stages and species, despite the apparent stage specificity revealed by SDS-PAGE analyses. One explanation for this apparent contradiction may have been that although the dominant antigens for each stage were of different molecular weight, there were shared epitopes on surface antigens from each stage (Maizels *et al*, 1983b). Cross-reactivity of surface antigens has been reported for other species. For example, the major surface proteins of microfilariae of *O. gibsoni* cross-reacted with sera taken from patients infected with a variety of filarial and trematode infections (Forsyth, Copeman, Anders & Mitchell, 1981) and adult *O. volvulus* and *O. gibsoni* have been shown to share a 20 kDa surface antigen (Cabrera & Parkhouse, 1986). This antigen had previously been shown to be unreactive with sera from patients infected with *Ascaris*, *Trichuris* or hookworm infected patients although sera from Indian patients infected with *W. bancrofti* significantly cross-reacted. (Philipp, Gomez-Priego, Parkhouse, Davies, Clark, Ogilvie & Beltran-Hernandez, 1984).

The antigenic specificity of *W. bancrofti* itself has been examined by Maizels, Burke, Sutanto, Purnomo & Partono (1986) who found that anti-*B. malayi*, anti-*B. timori* and anti-*Onchocerca* sera all showed some cross-reactivity whereas anti-*Toxocara* serum did not. However, despite this some surface antigens were only recognised by anti-bancroftian sera.

Surface Antigen Turnover

A connection between surface antigens and antigens excreted or secreted *in vitro* was first suggested by the observation of a turnover of the surface antigens of *Ancylostoma caninum* (Vetter & Klaver-Wesseling, 1978). In their experiments exsheathed *A. caninum* infective larvae were first incubated with serum from infected dogs and then with a fluoroscein-labelled anti-dog IgG antibody. At low temperatures the surface of the parasites was shown to be fluorescent, but if the temperature was raised, or if metabolite inhibitors were removed, the fluorescence was gradually released into the surrounding medium. Subsequently Smith, Quin, Kusel & Girdwood, (1981) demonstrated similar anti-metabolite sensitive *in vitro* release of cuticular antigens from *T. canis* and in a number of other species similar processes have been shown to occur (Philipp & Rumjaneck, 1984). Since the cuticle of many nematodes has been demonstrated to be involved in both antigen presentation and secretion, the assignment of antigens into ES and cuticular components may be a somewhat artificial division (Pritchard, 1986).

Surface Analysis of Nematode Parasites of Ruminants

Although radiolabelling techniques have been extensively used to analyse the surface proteins of laboratory animal helminths they have not been used to examine ruminant

nematode parasites. One exception has been the work undertaken with the cattle parasite *O. gibsoni* (e.g. Forsyth, *et al*, 1981) although this parasite has been studied primarily as a model system for the human parasite *O. volvolus.* The extensive literature dealing with antigens of many other parasitic species contrasts with the few reports dealing with ruminant parasites. The limited work that has been published has been restricted to analyses of whole worm homogenates usually without the use of radiolabelling techniques. O'Donnell, Dineen, Rothwell & Marshall (1985) for example, looked for immunogenic antigens in homogenates of *T.colubriformis* L4 by probing PAGE-separated proteins with sera collected from infected sheep or infected guinea pigs. Similarly, Wedrychovicz & Bezubik (1981) and Wedrychovicz (1984) compared the somatic protein profiles of three different stages of *O. circumcincta*. The antigenicity of these proteins was assessed with sera from infected sheep and rabbits by immunoelectrophoresis and results suggested that although a number of proteins were common to all three stages some were restricted to L4 and L5 stages. In this work no attempt was made to radiolabel specific proteins and presumably surface antigens were included in the somatic extracts.

Since it is possible that surface proteins play an important role in stimulating the host immune response to *O. circumcincta*, analysis of the surface proteins of different life-cycle stages of the parasite is desirable. This can be achieved through the application of radiolabelling and PAGE techniques previously described.

CHAPTER 7

CHARACTERISATION AND IMMUNOLOGICAL ANALYSIS OF THE SURFACE PROTEINS OF DIFFERENT LIFE-CYCLE STAGES OF *O.CIRCUMCINCTA*.

INTRODUCTION

Although it has been shown for several nematode infections of laboratory animals and humans that surface proteins of these parasites are antigenic, there have been no equivalent studies for parasitic nematodes of ruminants. Studies of the surface proteins of *O.circumcincta* are described in this chapter. These proteins were defined by labelling live parasites with ^{125}I and subsequently characterised by SDS-PAGE. Surface protein profiles were determined for 3 stages and the antigenicity of these proteins was tested by immunoprecipitation and immunoblotting techniques.

CHARACTERISATION OF *O. CIRCUMCINCTA* SURFACE PROTEINS

Radioiodination of the surface proteins of 4 different life-cycle stages of *O.circumcincta* was attempted using the Iodogen catalyst. The stages examined were exsheathed infective larvae, and worms collected from sheep 5, 10 and 21 days after infection which were classed as fourth stage larvae, early fifth stage larvae and adult worms respectively.

Live parasites were labelled with ^{125}I and then either homogenised or incubated with cetyl-methyl ammonium bromide (CTAB) to obtain a detergent extract. Homogenates and detergent extracts were analysed by SDS-PAGE and autoradiography. Detailed descriptions of these procedures are given in Chapter 2.

Cross-sections of radiolabelled adult worms were autoradiographed to examine the location of the radioisotope on the parasite. The procedure for this analysis was identical to

that for the autoradiography of cross-sections of ^{75}Se-labelled parasites and has been described in Chapter 2.

Results

Labelling with ^{125}I

The surface proteins of L4, EL5 and adult stages were successfully radiolabelled using the Iodogen technique. The amount of protein-associated radioisotope (as determined by TCA precipitability) varied between 36-65 % for the different stages but amounts were similar for detergent extracts and homogenates of the same stage (Table 7.1).

Five attempts to radiolabel the surface proteins of exsheathed infective larvae were unsuccessful. Variation in the number of parasites used from 100-10,000 L3 did not improve the radioiodination and an attempt to radioiodinate CTAB extracted larval proteins using the Chloramine-T catalyst was also unsuccessful.

Location of Radioisotope on Adult Parasites

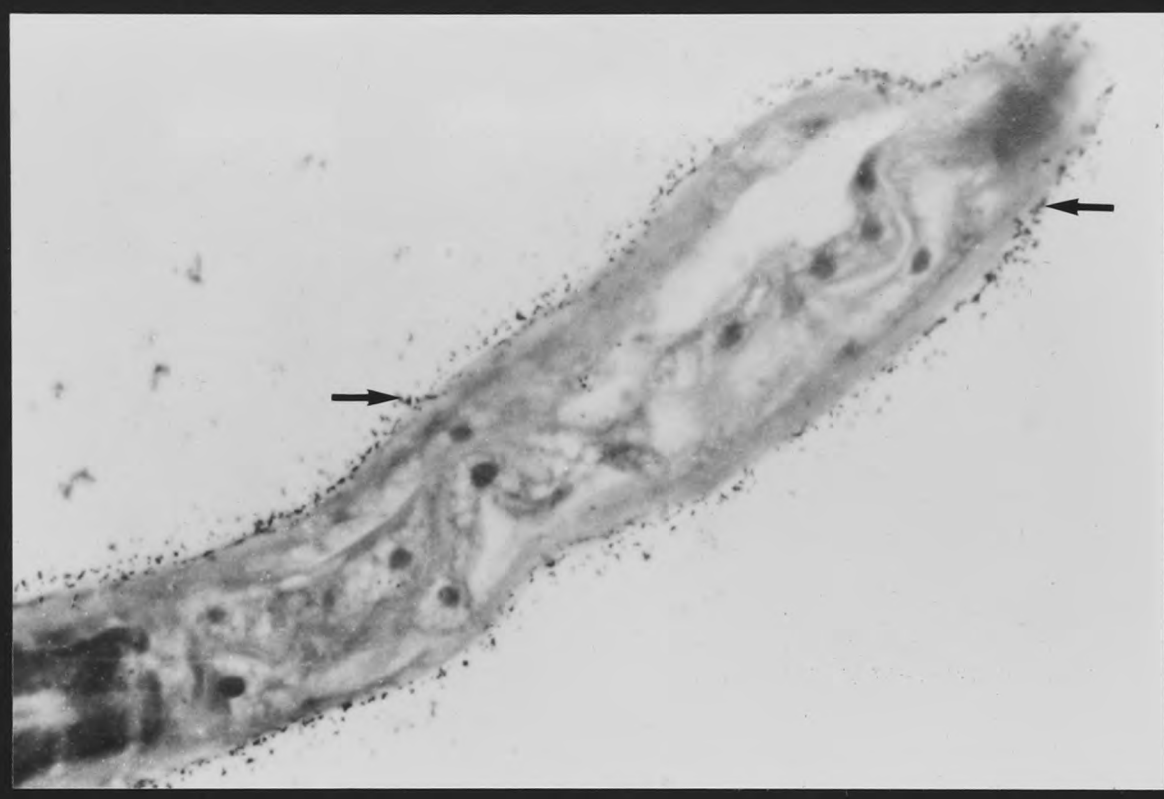
Examination of autoradiographs of cross-sections of radioiodinated adult worms revealed that the isotope was restricted to the surface of the parasite (Figure 7.1). It was clear that the radiolabel had not penetrated into sub-cuticular structures. Unfortunately it was not possible to determine the location of isotope on labelled fourth or early fifth stage parasites. The small size of these worms and the small numbers used in the labelling procedure made it difficult to cut fixed sections.

TABLE 7.1 Radioiodination of Surface Proteins of *O. circumcincta*

Stage of Parasite	No. of Worms Used in Radioiodination	Amount of ^{125}I Used (uCi)	Preparation	% TCA Precipitable
L4	600	300	Homogenate	42.5
			CTAB Extract	36.6
EL5	1000	250	Homogenate	65.0
			CTAB Extract	61.0
Adult	500	250	Homogenate	36.4
			CTAB Extract	42.2

Figure 7.1

Cross-section of radioiodinated adult *O.circumcincta* after 2 months autoradiographic exposure. Radioisotope was restricted to the surface of the parasite (arrowed).



SDS-PAGE and Autoradiographic Analysis

By equalizing the TCA precipitable c.p.m. of samples of CTAB extracts and homogenates a direct comparison between preparations was possible. The profiles of the radiolabelled proteins contained in these preparations were identical for each stage examined (Figure 7.2.(i), 7.3.(i) and 7.4.(i)). In Figure 7.3.(ii) and Figure 7.4.(ii) the Coomassie blue stained gels corresponding to the autoradiographs in Figure 7.3.(i) and Figure 7.4.(i) are shown. These illustrate that although detergent extracts and homogenates expressed identical radiolabelled protein profiles the CTAB extracts did not contain the many somatic and ES proteins which were present in the homogenate preparation. Thus, a relatively 'clean' preparation of surface proteins was obtained using the CTAB extraction method.

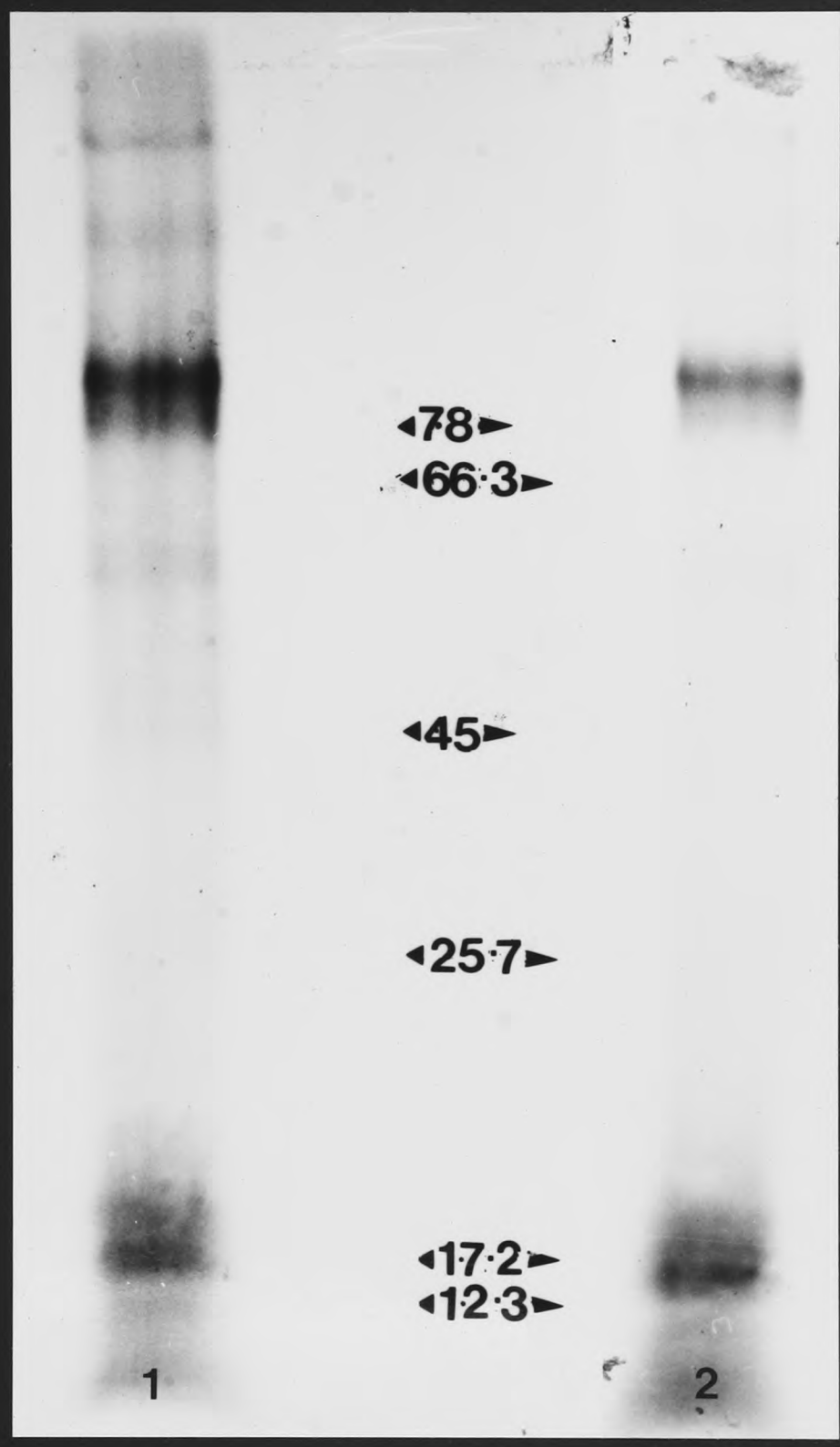
Qualitative and quantitative differences were apparent between the radiolabelled proteins present on each developmental stage. Unfortunately it was not possible to run all 6 surface protein preparations on the same gel and thus make a direct comparison between stages. However, the molecular weights of the labelled proteins were estimated from graphs where the $\log_{10}$ molecular weights of protein standards were plotted against their respective distances migrated. Lines of best fit were drawn with the aid of a computer programmed for regression analysis (Figure 7.5.(i-iii)).

Two heavily labelled proteins of 90 and 16 kDa molecular weight were resolved in the extracts of L4 (Figure 7.2.(i)) whereas extracts of EL5 and adults each produced 4

Figure 7.2.(i) Autoradiograph of polyacrylamide gel of radioiodinated L4 protein preparations.

Track 1 CTAB extract.

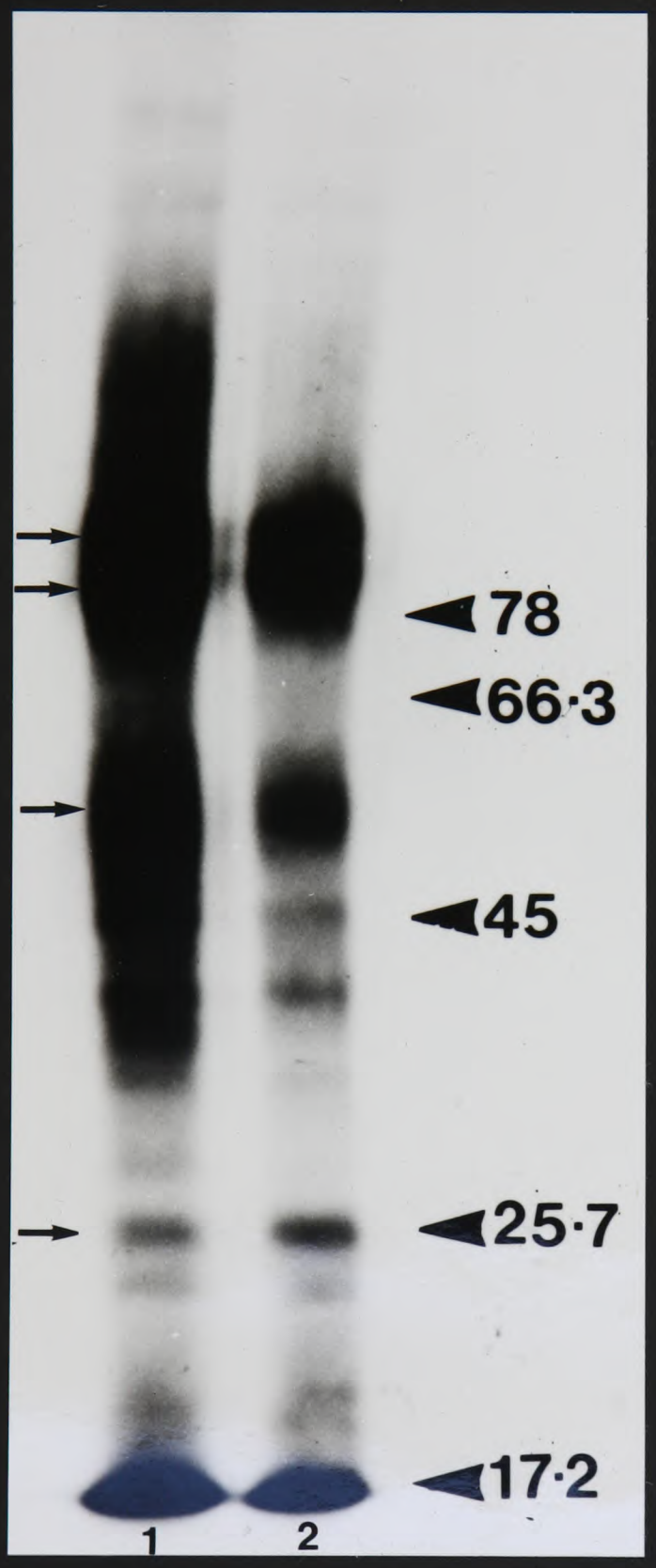
2 Homogenate.


78
66·3
45
25·7
17·2
12·3
1
2

Figures 7.3.(i) and 7.4.(i) Autoradiograph (left) and Coomassie blue stained polyacrylamide gel (right) of radioiodinated EL5 protein preparations.

Track 1 Homogenate.

2 CTAB extract.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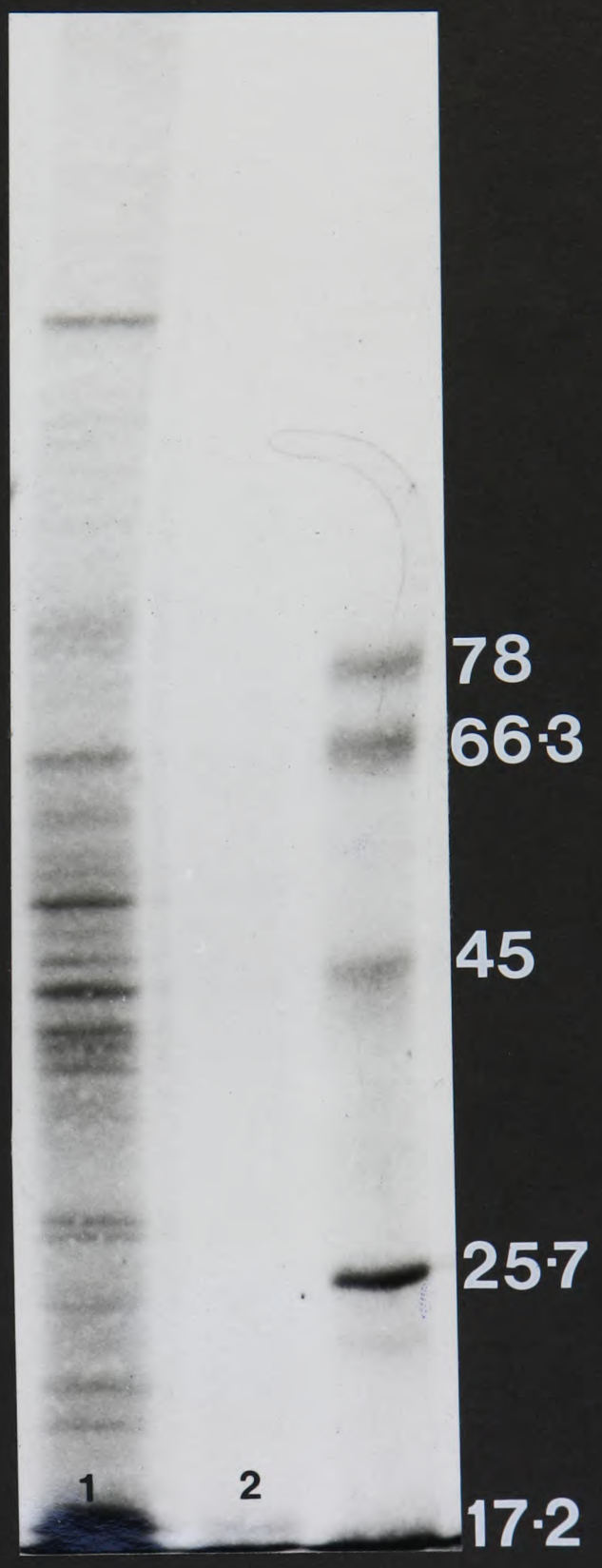

78
66·3
45
25·7
17·2
1
2

Figures 7.4.(i) and 7.4.(ii) Autoradiograph (above) and Coomassie blue stained polyacrylamide gel (below) of radioiodinated adult protein preparations.

Track 1 Approximately 20,000 cpm TCA precipitable cpm of CTAB extract.

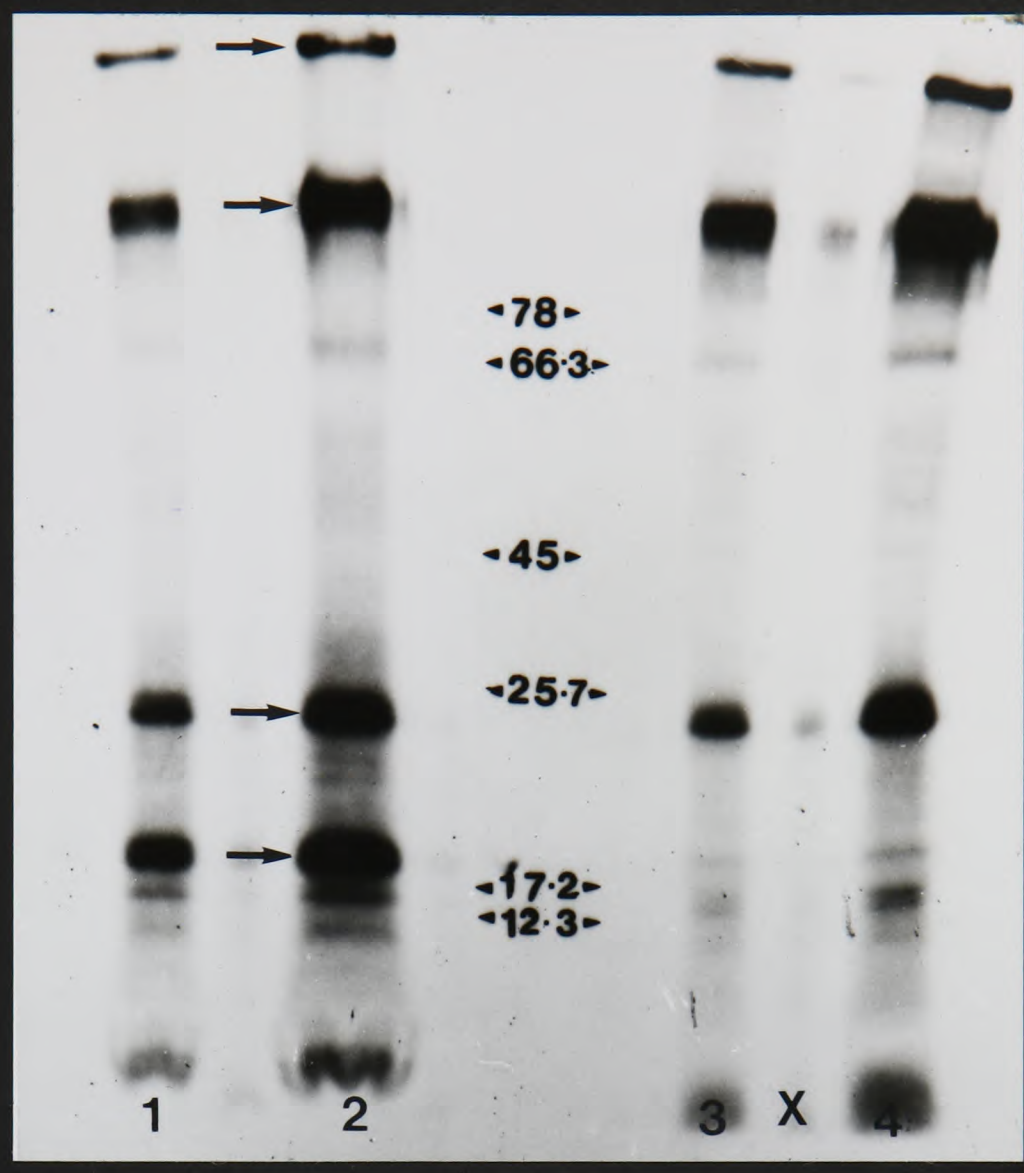
2 Approximately 30,000 cpm TCA precipitable cpm of CTAB extract.

3 Approximately 20,000 cpm TCA precipitable cpm of homogenate.

4 Approximately 30,000 cpm TCA precipitable cpm of homogenate.

X Overflow of sample from track 4.

MW Molecular weight markers.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X
4

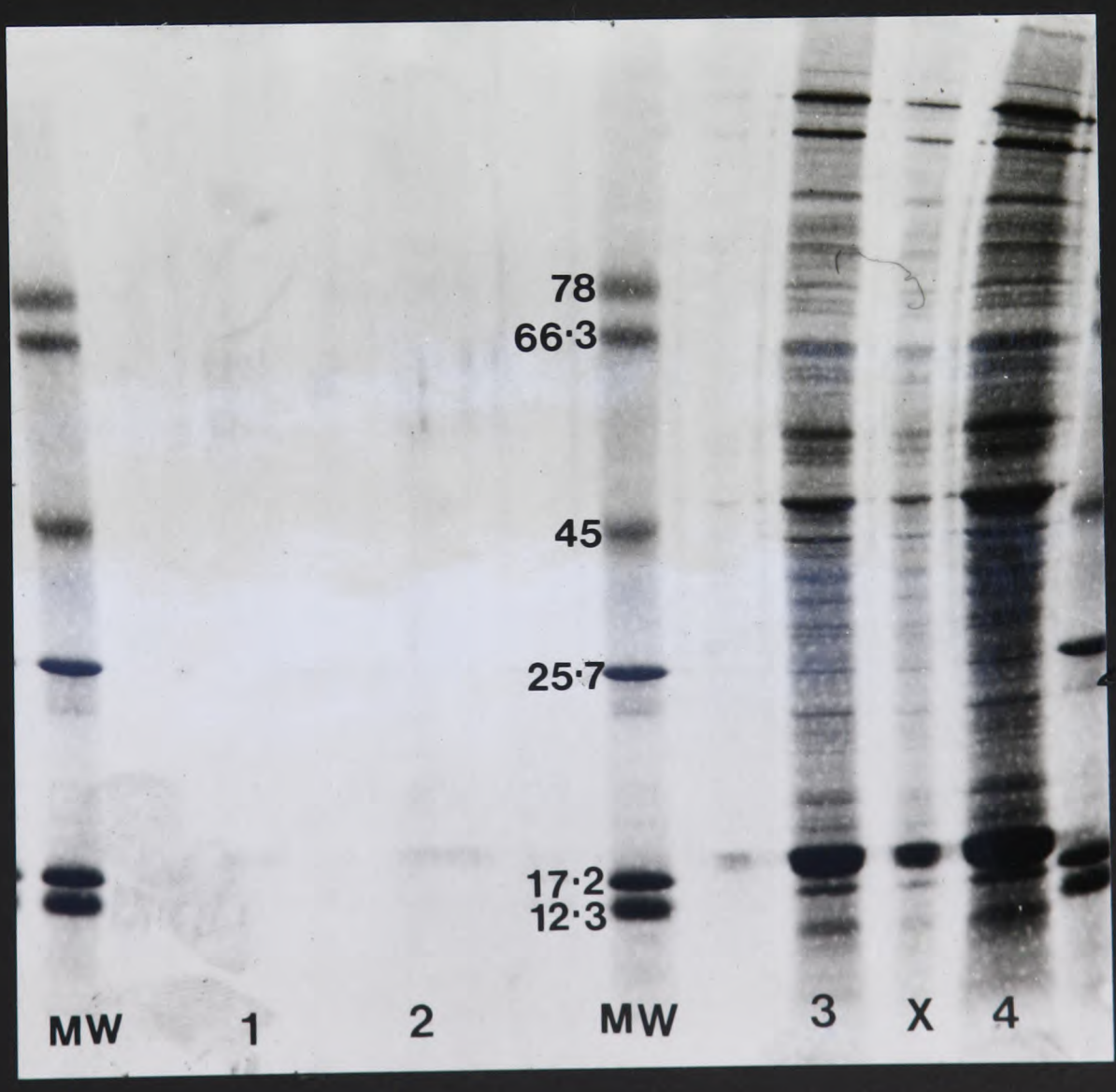
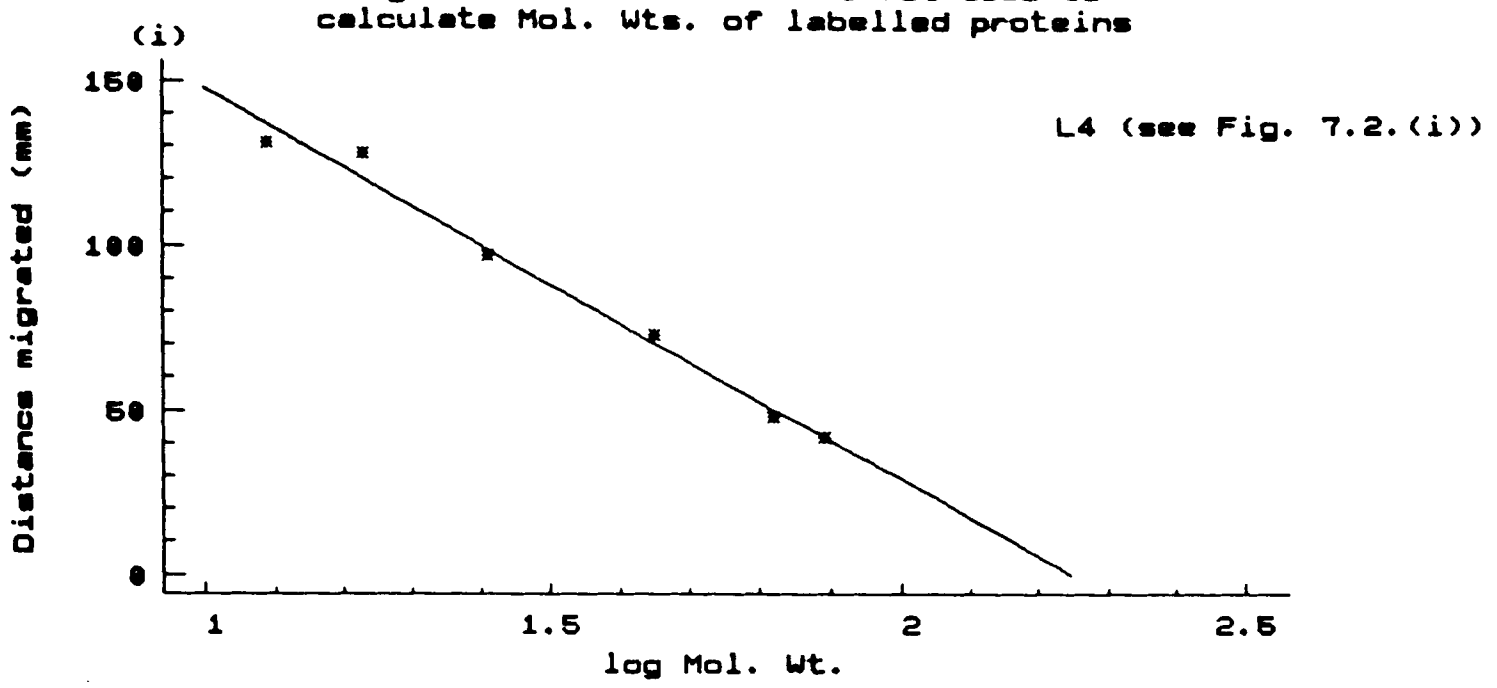
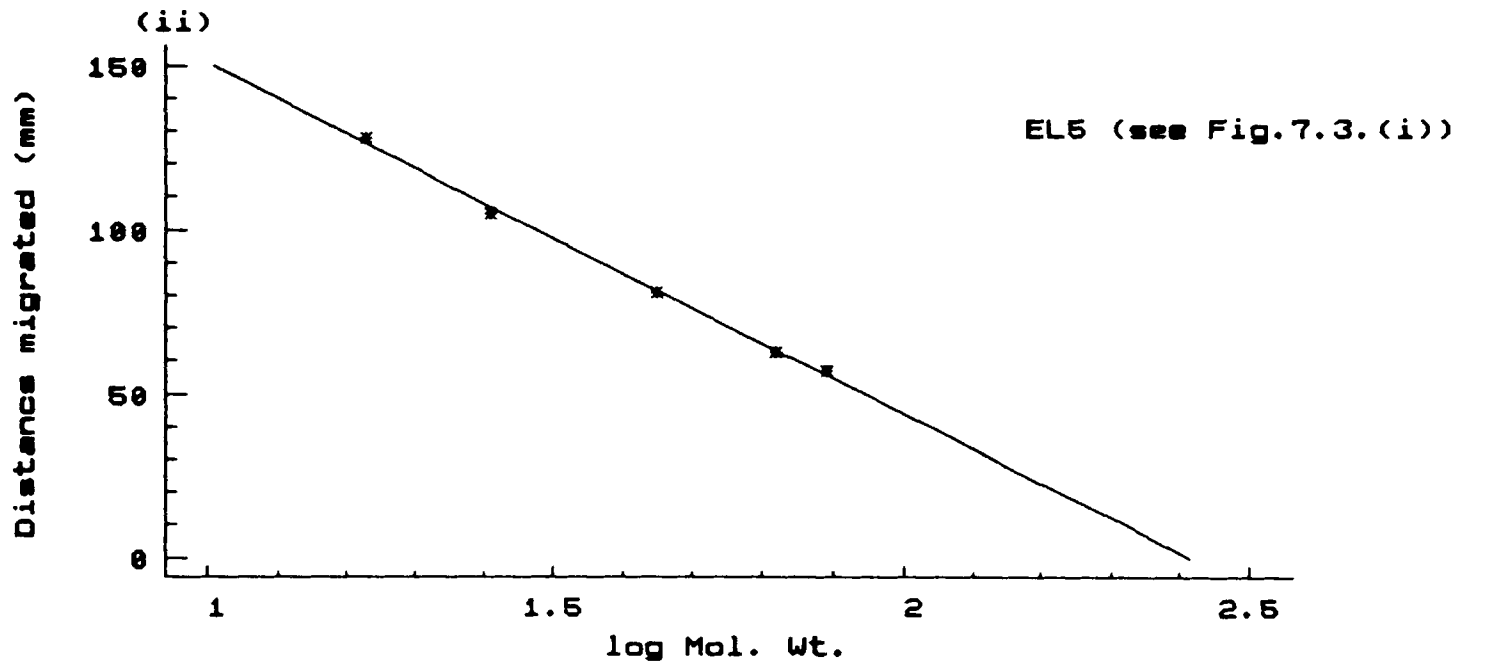

78
66·3
45
25·7
17·2
12·3
MW
1
2
MW
3
X
4

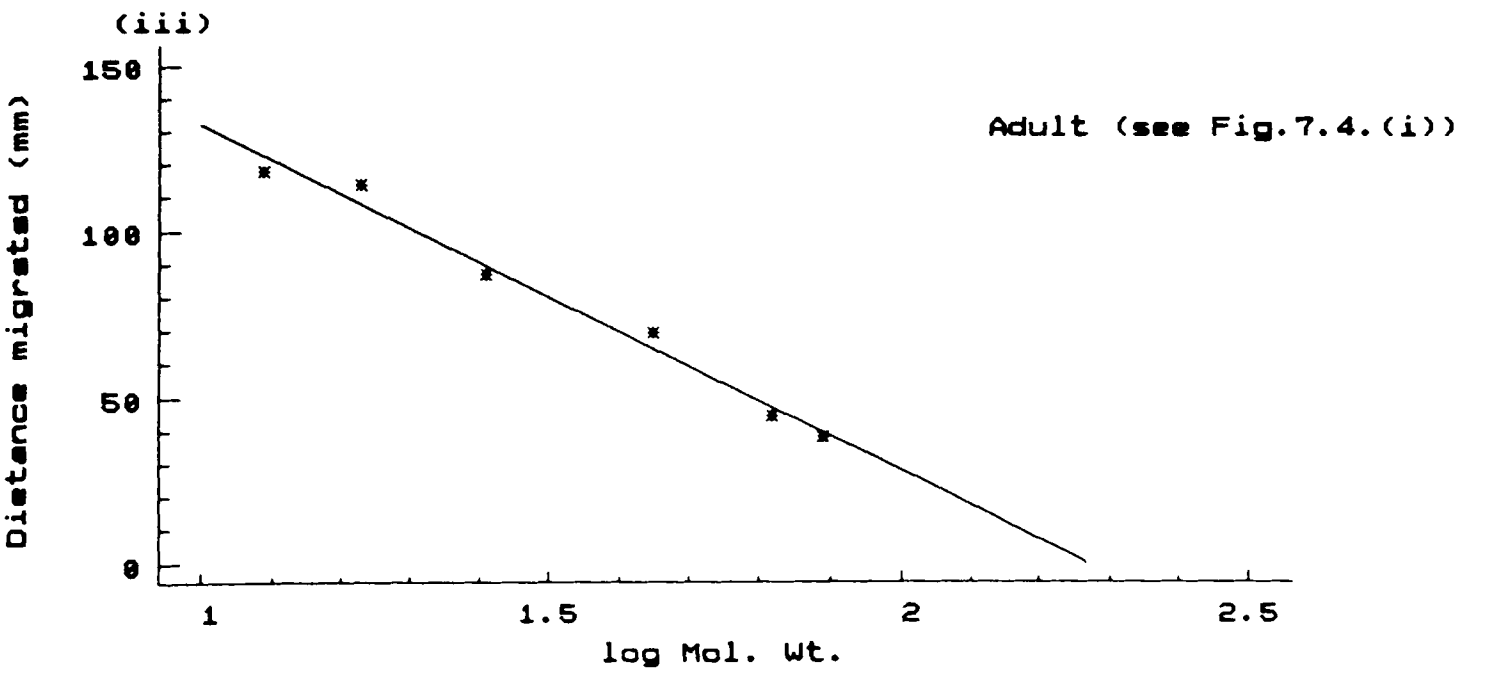
Figure 7.5 Lines of best fit used to calculate Mol. Wts. of labelled proteins



L4 (see Fig. 7.2.(i))



EL5 (see Fig.7.3.(i))



Adult (see Fig.7.4.(i))

heavily labelled bands. These resolved at 87, 82, 54 and 26 kDa (Figure 7.3.(i)) and 177, 109, 24 and 17 kDa (Figure 7.4.(i)) respectively (arrowed). A number of weakly labelled proteins were also present in each sample, especially in the EL5 preparation.

It is considered that the heavily labelled bands resolving at 85 kDa and 87 kDa for L4 and EL5 stages were equivalent proteins, and similarly the major bands at 28 kDa and 24 kDa for EL5 and adult stages respectively were also identical. The major band resolving at 17 kDa on the L4 preparation is probably represented by the 18 kDa band on adult stages. This protein may have been common to all three stages although as the EL5 stage preparation was not fully resolved on this region of the gel this can not be stated with certainty. Despite these similarities, it is obvious that the surface protein profiles for the 3 stages were different, and thus a pronounced stage-specificity in surface proteins was apparent.

IMMUNOLOGICAL ANALYSIS OF RADIOLABELLED SURFACE PROTEINS

Immunoprecipitation

A number of experiments were conducted to examine the antigenicity of surface labelled proteins by immunoprecipitation using lymph collected from sheep immune to *O.circumcincta* or from non-immune sheep. The source of these lymph samples and the immunoprecipitation technique are both described in Chapter 2.

The results of the immunoprecipitation experiments carried out with the radiolabelled preparations are presented in Table 7.2. With the exception of experiments using EL5 samples,

Table 7.2. Results of Immunoprecipitation Studies on Radioiodinated Protein Preparations.

Sample	Procedure	% TCA Precipitable c.p.m. Precipitated by Immune Lymph	% TCA Precipitable c.p.m. Precipitated by Non-immune Lymph
L4 Homogenate	St. Immunoppt.	14.7	14.5
"	"	14.1	16.7
"	"	14.7	12.9
"	Pre-absorbed	4.9	4.9
L4 CTAB Extract	St. Immunoppt.	21.9	16.2
"	"	21.9	18.8
"	"	14.7	10.8
"	Pre-absorbed	6.9	4.7
EL5 Homogenate	St. Immunoppt.	14.4	6.2
"	"	14.3	5.7
EL5 CTAB Extract	St. Immunoppt.	14.2	3.6
"	"	15.2	10.0
Adult Homogenate	St. Immunoppt.	11.3	9.0
"	"	14.0	12.9
"	"	21.9	22.9
"	Preabsorbed	6.2	3.6
Adult CTAB Ext.	St. Immunoppt.	7.8	6.6
"	"	13.0	14.5
"	"	9.6	9.0

St. Immunoppt = Standard Immunoprecipitation Procedure

there was no difference between the number of protein associated c.p.m. precipitated by immune or non-immune lymph. However, in two experiments using the EL5 homogenate preparation the percent c.p.m. precipitated by the immune lymph was over twice that precipitated by the non-immune lymph and a larger difference was found in one of two experiments using the CTAB extract. Only relatively small percentages of the available c.p.m. were precipitated, typically in the range of 10-20%. Similar percentages of c.p.m. were precipitated in homogenate and CTAB preparations providing further evidence of the similarities between the radiolabelled proteins of these two preparations.

Attempts to remove any non-specific binding of labelled protein to non-immune lymph were unsuccessful. Pre-absorbing samples with non-immune lymph served only to reduce the amount of radioactivity precipitated and did not accentuate any differences between the immune and non-immune immunoprecipitates. If the addition of the 2nd precipitating antibody was omitted variable results were obtained which could not readily be interpreted. It is thought these inconsistencies stemmed from the difficulties encountered in separating supernatants from the precipitates, since the latter were difficult to distinguish.

Unfortunately, attempts to analyse immunoprecipitates by SDS-PAGE were unsuccessful. Analysis was hindered by the inability to completely dissolve immunoprecipitates prior to electrophoresis and consequently, autoradiographs failed to reveal any radiolabelled bands.

Immunoblotting

Since attempts to analyse immunoprecipitates by SDS-PAGE were unsuccessful an alternative approach was adopted. Non-radioactive homogenates of L4, EL5 and adult stages were spiked with radiolabelled CTAB extracts of their equivalent stages and run on SDS-PAGE. Blots of these gels were made onto nitrocellulose and strips of this paper were probed with lymph samples and subsequently developed with peroxidase conjugated pig anti-sheep $F(ab)_2$ and substrate. The probed strips were then set up to autoradiograph so that the positions of the radiolabelled surface proteins and the sites of antigen-antibody reaction could be compared (see Chapter 2 for details).

Gels and blots of the adult protein preparation and an autoradiograph of these are shown in Figures 7.6.(i) and 7.6.(ii) respectively. Antibodies present in the immune lymph sample recognised a number of proteins with two bands at 110 kDa and 63 kDa reacting most strongly (arrowed). Some non-specific binding between the proteins and the non-immune lymph sample was apparent above approximately 100 kDa (Figure 7.6.(i), track 4). Considerably less radioactivity was detected on nitrocellulose strips after they had been probed with lymph samples, although comparison of the radioactive profile of the strip which had not been probed (track 5) and that resulting from the dried gel (track 1) indicated that most radioactivity had been transferred during blotting (Figure 7.6.(ii)).

When the autoradiograph was superimposed on the blot, no relationship was observed between the position of the antigen-antibody bands and the radioactive surface proteins.

Figure 7.6.(i) (above).

Polyacrylamide gel and immunoblots of adult homogenate plus radioiodinated adult CTAB extract.

A single 10% gel was loaded with 5 identical tracks containing the antigen mixture. Between each of these tracks, samples of pre-stained molecular weight markers were run. The 4 left-hand tracks of the gel were cut off, stained with Coomassie blue and dried. The remaining tracks of the gel were blotted to nitrocellulose and the 4 right-hand tracks of the paper were stained with Coomassie blue. The remaining antigen tracks were cut out and probed with lymph samples.

Track 1 : Polyacrylamide gel stained with Coomassie blue.

Track 2 : Immunoblot of sample probed with immune gastric lymph and peroxidase conjugated pig anti-sheep $F(ab)_2$.

Track 3 : Immunoblot of sample probed with immune gastric lymph alone.

Track 4 : Immunoblot of sample probed with non-immune gastric lymph and peroxidase conjugated pig anti-sheep $F(ab)_2$.

Track 5 : Blot of sample stained with Coomassie Blue.

MW : Molecular weight markers.

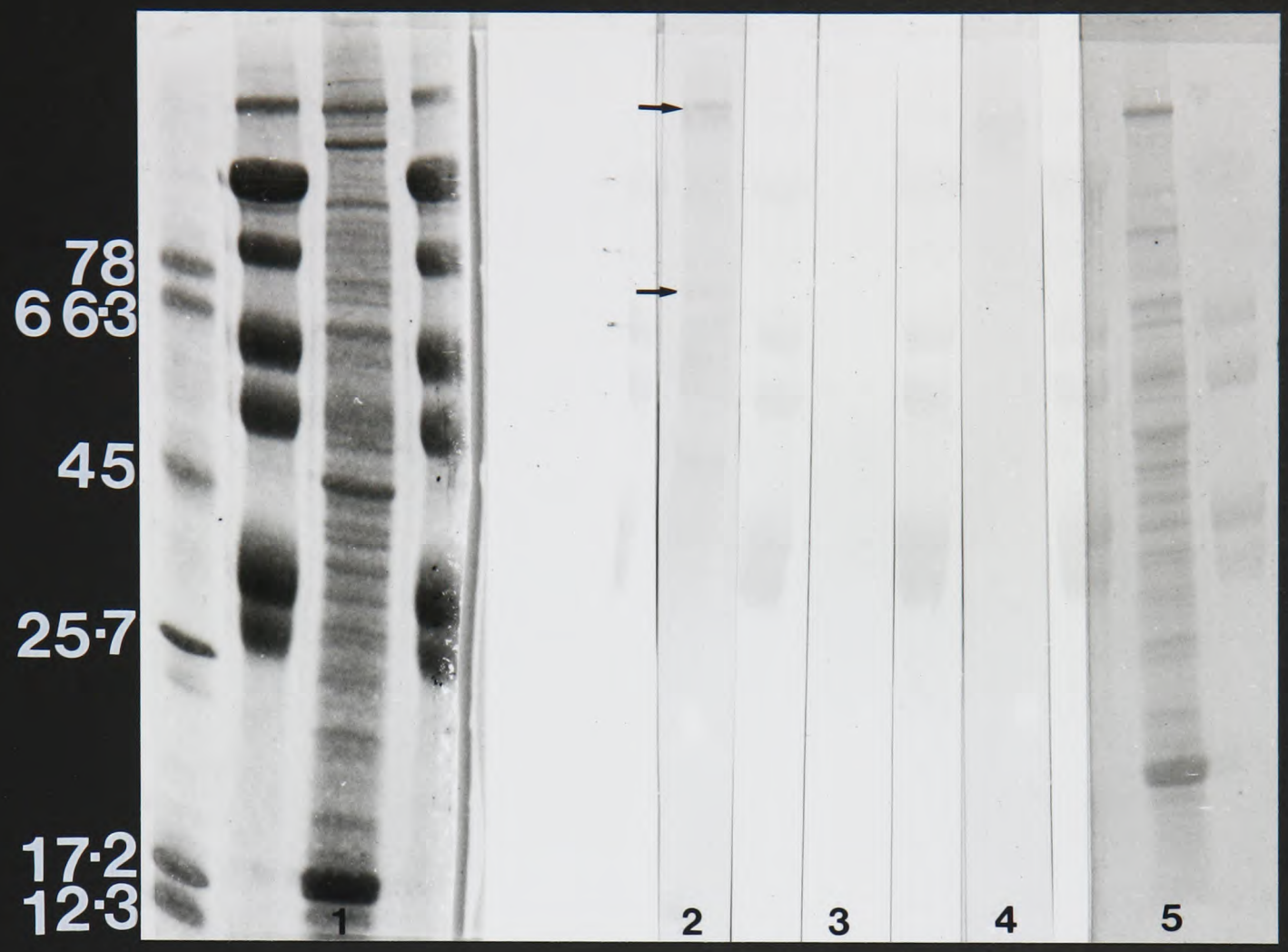
PS : Prestained molecular weight markers.

All of the above sections were stuck down on card and then autoradiographed.

Figure 7.6.(ii) (below).

Autoradiograph of the above gel and immunoblots of adult homogenate plus radioiodinated adult CTAB extract.

Tracks as described for Figure 7.6.(i).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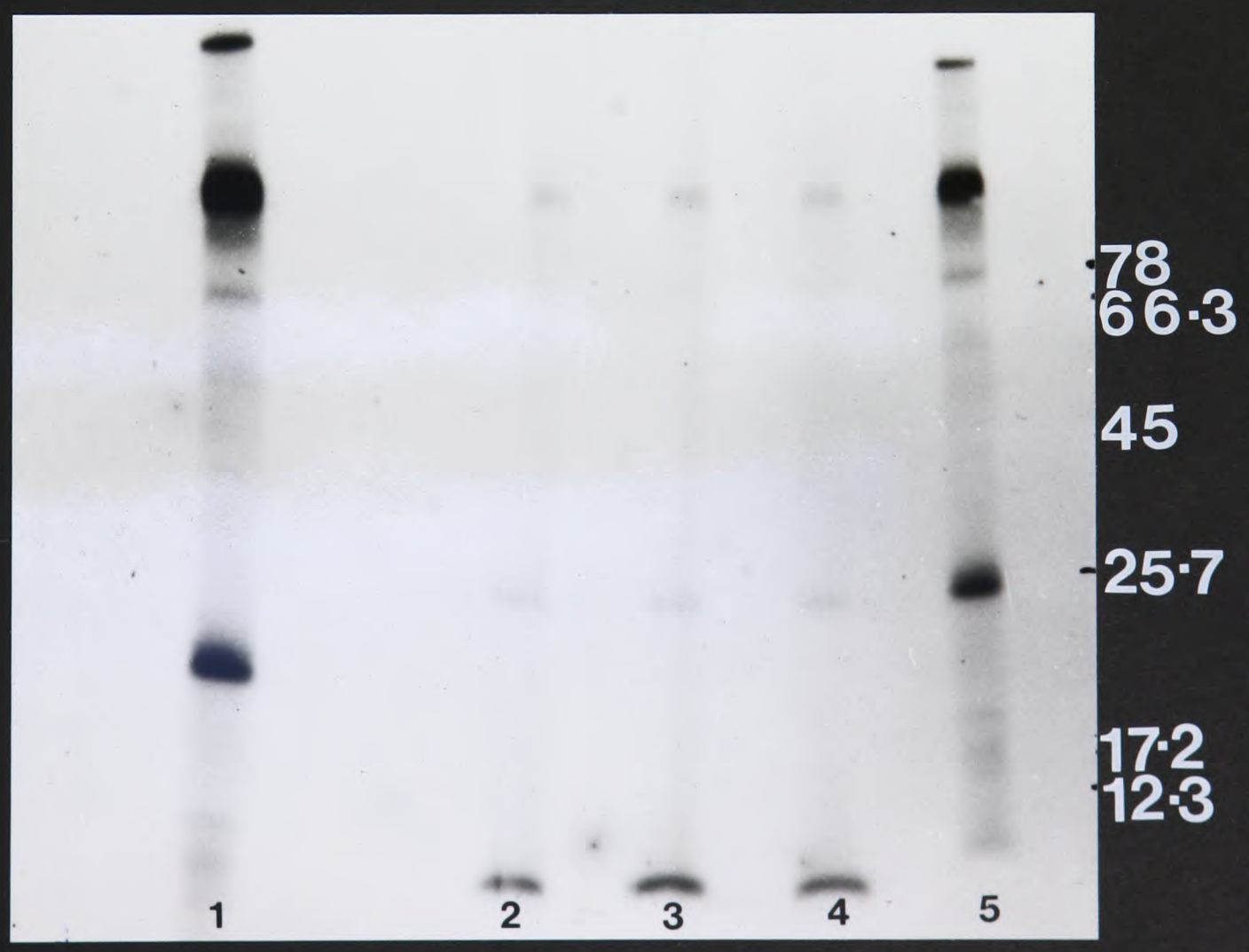
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Similar experiments to the above were carried subsequently carried out with non-radioactive homogenates of EL5 and L4, each spiked with their respective labelled surface proteins. In Figures 7.7.(i) and 7.7.(ii) gels and immunoblots of the EL5 protein preparation and their corresponding autoradiograph are shown. Antibodies present in the immune lymph recognised many proteins, with bands at 60, 58, 47, and 40 kDa reacting particularly strongly (arrowed). Nine other fainter bands could be distinguished on the nitrocellulose probed with immune lymph but no proteins were detected by the non-immune lymph sample. As with the adult protein sample virtually no radioactivity could be detected on strips after they had been probed with lymph samples (Figure 7.7.(ii), tracks 2 & 3). In this particular experiment, the unprobed strip of nitrocellulose paper (Figure 7.7.(i), track 4) was blanked with horse serum by error. Consequently, since the paper was saturated with protein, the transferred EL5 proteins do not show on the Coomassie blue stained strip. This error may also account for the smaller amount of radioactivity detected on this strip compared to the dried gel (Figure 7.7.(ii), tracks 4 and 1 respectively). Despite the large number of proteins recognised by the immune lymph none of these corresponded to radiolabelled proteins.

Gels and immunoblots of the L4 protein preparation and corresponding autoradiograph are shown in Figures 7.8.(i) and 7.8.(ii) respectively. Three proteins of 61, 59 and 50 kDa molecular weight reacted strongly with the immune lymph sample (arrowed) but there was no reaction with non-immune lymph. It is

Figure 7.7.(i) (above).

Polyacrylamide gel and immunoblots of EL5 homogenates plus radioiodinated EL5 CTAB extract.

Track 1 : Polyacrylamide gel stained with Coomassie Blue.

Track 2 : Immunoblot of sample probed with non-immune gastric lymph and peroxidase conjugated pig anti-sheep $F(ab)_2$.

Track 3 : Immunoblot of sample probed with immune gastric lymph and peroxidase conjugated pig anti-sheep $F(ab)_2$.

Track 4 : Blot of sample stained with Coomassie Blue.

MW : Molecular weight markers.

PS : Prestained molecular weight markers.

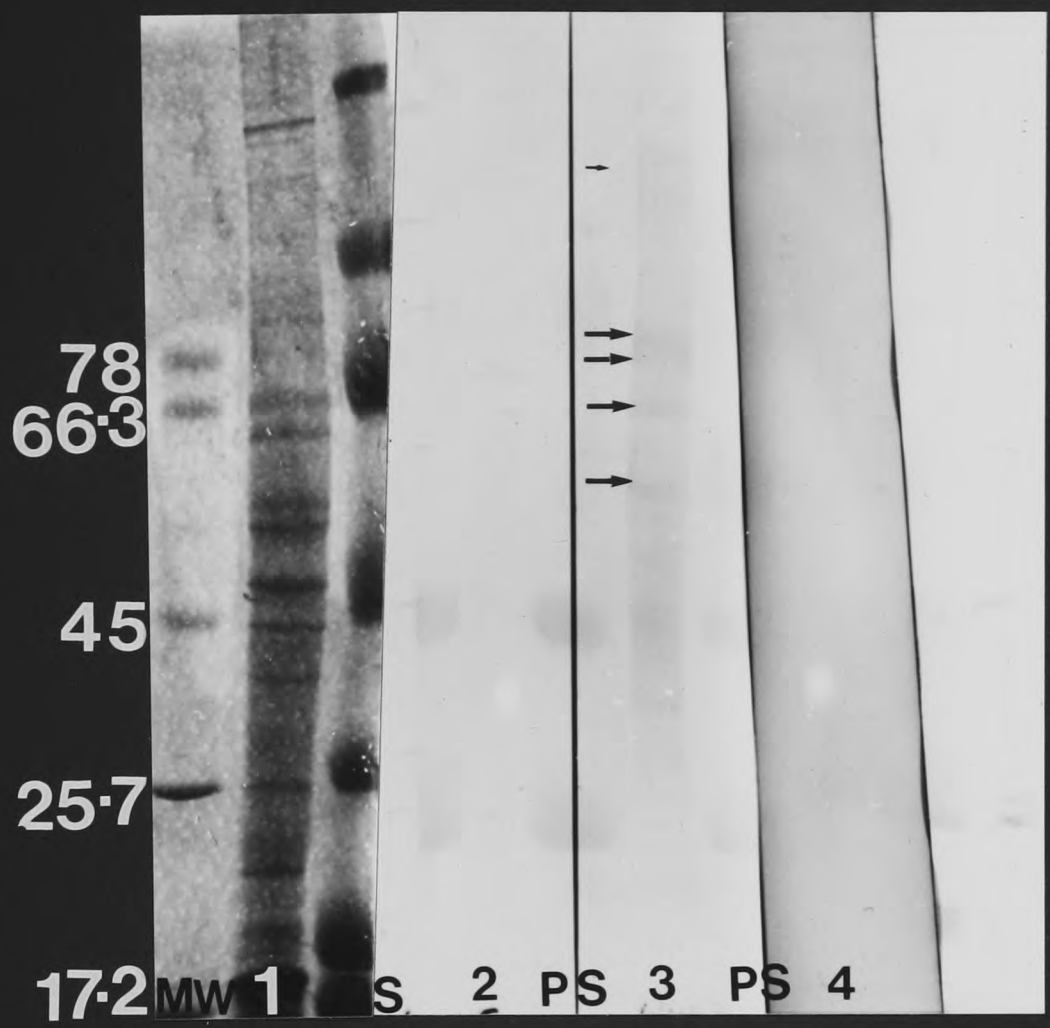
All of the above sections were stuck down on card and then autoradiographed.

Figure 7.7.(ii) (below).

Autoradiograph of the above gel and immunoblots.

Tracks as described above for Figure 7.7.(i).

Artefacts at the top and bottom of the autoradiograph are due to uneven exposure of the film to the developer solution (two films were inadvertently placed in the cassette and both were unwittingly developed at the same time).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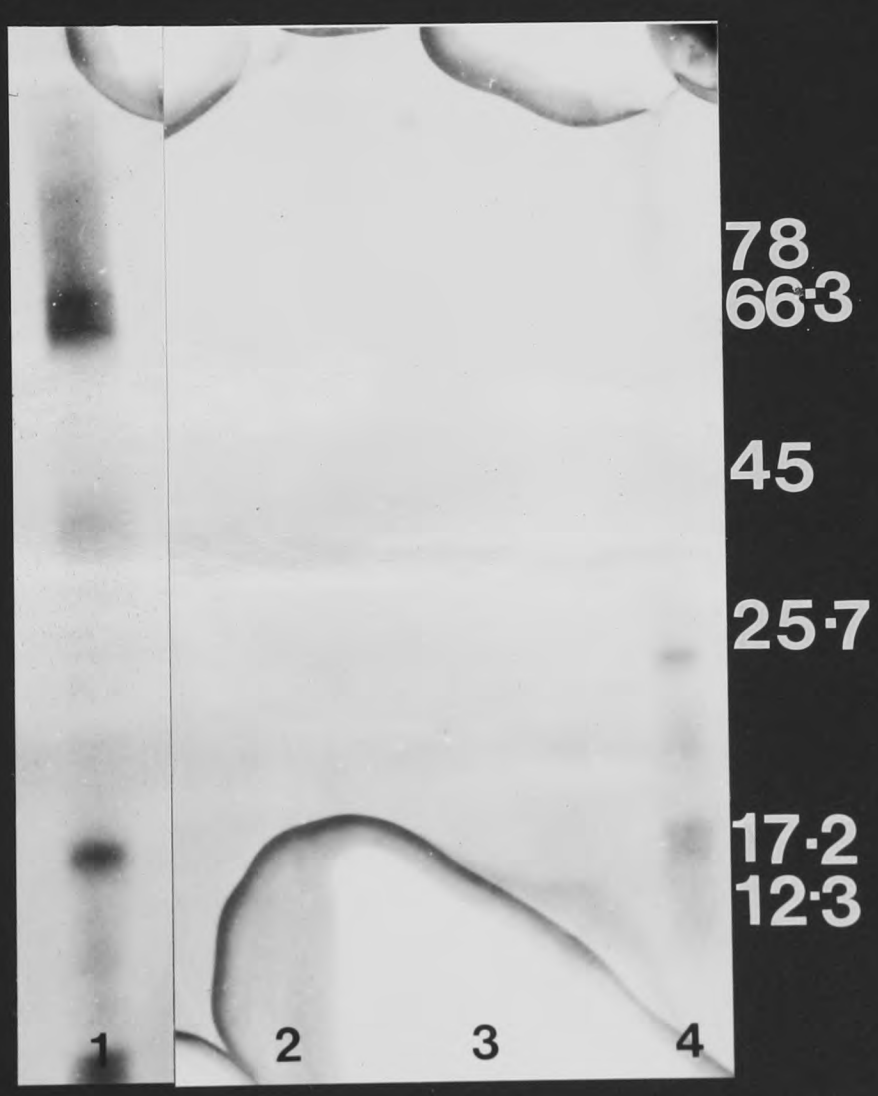
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Figure 7.8.(i) (above).

Polyacrylamide gel and immunoblots of L4 homogenate plus radioiodinated L4 CTAB extract.

Track 1 : Polyacrylamide gel stained with Coomassie Blue.

Track 2 : Immunoblot of sample probed with non-immune gastric lymph and peroxidase conjugated pig anti-sheep $F(ab)_2$.

Track 3 : Immunoblot of sample probed with immune gastric lymph and peroxidase conjugated pig anti-sheep $F(ab)_2$.

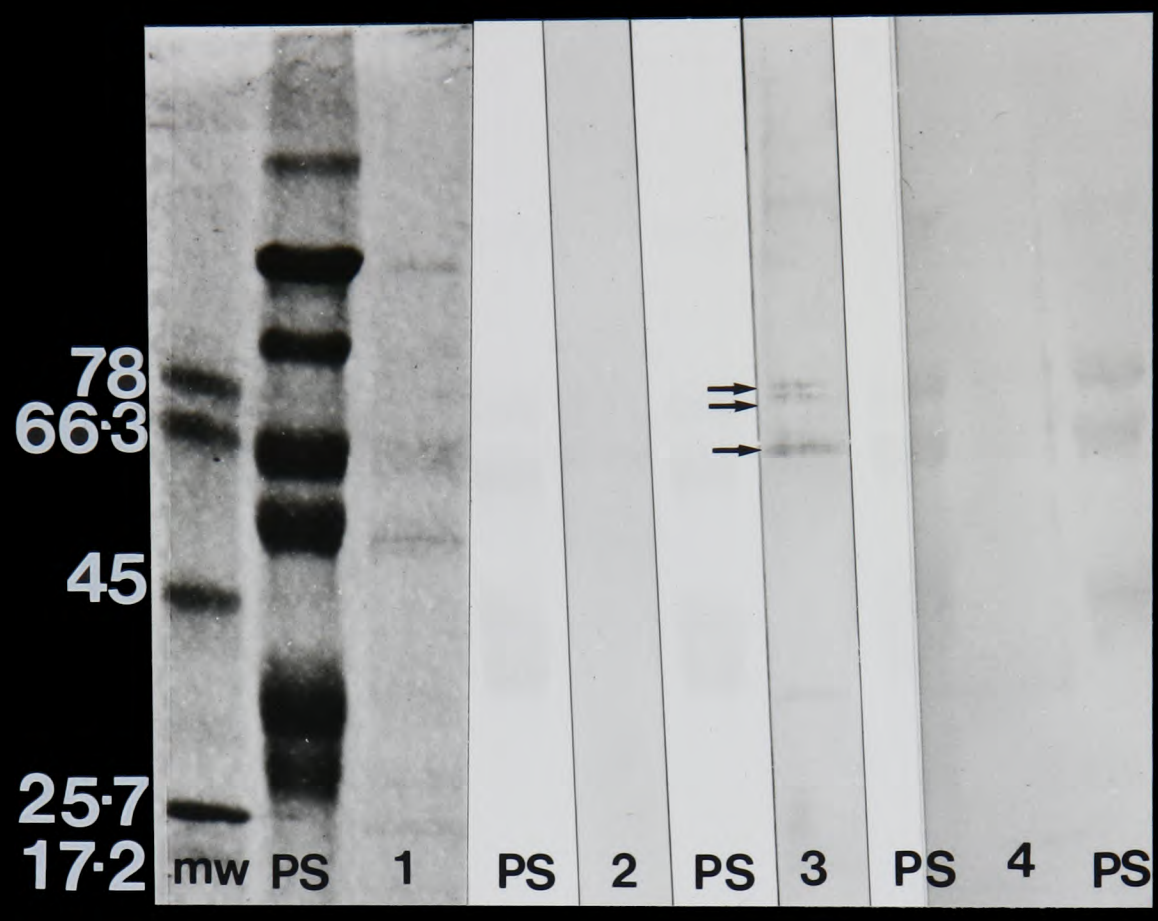
Track 4 : Blot of sample stained with Coomassie Blue.

All of the above sections were stuck down on card and then autoradiographed.

Figure 7.8.(ii) (below).

Autoradiograph of gel and immunoblots of L4 homogenate plus radioiodinated L4 CTAB extract.

Tracks as described above for Figure 7.8.(i).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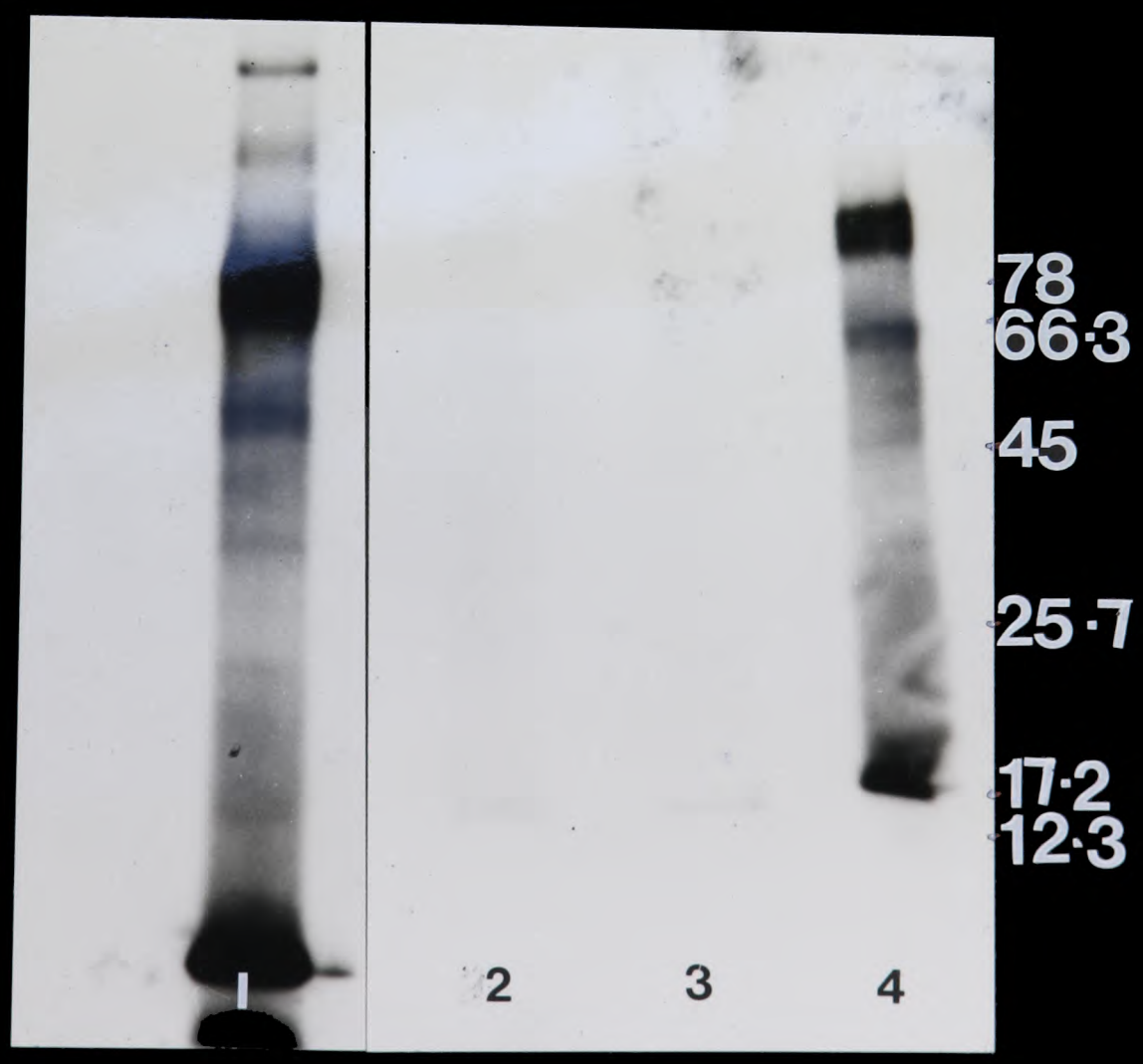
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probable that these proteins are equivalent to those recognised in the EL5 protein preparation. As was found with the two previous samples, little or no radioactivity could be detected on nitrocellulose strips after they had been probed (Figure 7.8.(ii), tracks 2 and 3) and no correspondence was observed between radiolabelled proteins and proteins recognised by antibodies.

In summary, these experiments demonstrated that although a number of proteins were clearly recognised by antibodies present in the immune gastric lymph, none of these could be identified with the radiolabelled surface proteins of each stage of the parasite.

DISCUSSION

The results of experiments described in Chapter 5, suggested that the exsheathed larval stage of *O.circumcincta* may be an important target for the host immune response. It was, therefore, frustrating that attempts to radioiodinate the surface proteins of this stage of the parasite were unsuccessful. The surface proteins of the infective larval stage of other nematode species have been successfully analysed using radioiodination techniques Philipp & Rumjaneck (1984) although Maizels *et al*, (1983a) experienced difficulties radiolabelling *N. brasiliensis* L3. These authors found that reproducible results could not be obtained for this stage of the parasite following Chloramine-T mediated radioiodination and the use of different iodination methods was not beneficial. Maizels *et al*, (1983a) concluded that the fault lay more in a poor extraction of labelled proteins rather than in an initial failure to radiolabel. In the present experiments, although considerable TCA precipitable radioactivity could be detected on exsheathed L3 homogenates or detergent extracts, no protein bands could be demonstrated on SDS-PAGE analysis. Time did not permit the reasons for this failure to be elucidated although a single attempt to iodinate CTAB extracted larval proteins was also unsuccessful.

The use of the Iodogen catalyst resulted in satisfactory iodination of the surface proteins of L4, EL5 and adult stages. Used in the form of coated beads, this catalyst is easier to use than other iodinating reagents but was primarily chosen because of it has been shown by many workers to

exclusively radiolabel proteins associated with the nematode cuticle without facilitating penetration of ^{125}I into sub-cuticular proteins (Forsyth *et al*, 1981; Baschong & Rudin, 1982).

Using microautoradiographic techniques it was demonstrated that following iodination of adult *O.circumcincta* worms, the radiolabel had not penetrated into somatic structures and thus for this stage of the parasite, at least, it could be deduced that radiolabelled proteins were derived exclusively from the surface. Unfortunately, earlier stages of the parasite could not be analysed in this way and so it can only be assumed that similar restrictions applied.

In common with many nematode species the 3 stages of *O.circumcincta* expressed a remarkably restricted set of surface proteins consisting of only 2 to 4 major molecules. Comparison of the SDS-PAGE profiles for the three stages revealed differences which suggested that *O.circumcincta* exhibits stage-specificity in the expression of surface proteins. Some proteins were common to more than one stage, and one 17 kDa molecule may have been common to all three stages.

The cationic detergent, CTAB, has been shown to selectively remove surface proteins from nematodes (Pritchard, Crawford, Duce & Behnke, 1985). Extracts of radioiodinated *O.circumcincta* obtained after 1 hour incubation in this detergent contained radiolabelled proteins identical to those present in homogenates of labelled worms. Furthermore the proportion of labelled proteins (i.e. TCA precipitable counts) in the two types of preparation of each stage were very similar.

Although CTAB extracts contained the same radiolabelled proteins as the homogenates, they lacked the background (E/S and somatic) proteins contained in homogenate preparations, a difference clearly demonstrated by comparing Coomassie stained gels. It was thought that such a relatively 'clean' surface protein preparation would be advantageous in the immunological analysis of these proteins. In the event, less than 25% of the total TCA precipitable radioactivity was immunoprecipitated by host antibodies. In the majority of experiments the amounts precipitated by immune and non-immune lymph were similar, although considerably more TCA precipitable counts in EL5 samples were immunoprecipitated by immune compared to non-immune lymph. This difference was found whether homogenates or detergent extracts of this stage were tested. However, even though EL5 samples contained the greatest number of identifiable surface proteins only 14% of the TCA precipitable c.p.m. were immunoprecipitated by the immune lymph sample and it thus appeared that the majority of the radiolabelled proteins were non-antigenic.

Since analysis of immunoprecipitates by SDS-PAGE was unsuccessful, an immunoblotting technique was used to try and determine which, if any radiolabelled proteins were recognised by sheep antibodies. Unfortunately, this approach also raised problems which had not previously been envisaged. It had been hoped that by overlaying autoradiographs and immunoblots a direct comparison between radiolabelled surface proteins and proteins recognised by host antibodies could be achieved. In the event, the prolonged incubation and washing steps involved in the probing procedure reduced the amount of radioactivity on

immunoblots considerably, making such a direct comparison difficult. It was, however, possible to compare the host-recognised proteins with radiolabelled proteins on unprobed blots and using this method, very little correspondence could be demonstrated.

One explanation for the apparent lack of antibody reactivity against the surface proteins of L4, EL5 and adult stages may have been specificity of the antibody probe used. The lymph samples containing antibody were collected from sheep 6 days after challenge with 50,000 *O.circumcincta* L3 at a time when IgA antibody levels were at their peak. The rapid increase in antibody which occurred to this time after challenge suggests that the response must have been directed at exsheathed L3 or early L4 worms since these were the only stages present to stimulate a response. Nevertheless, a number of non-surface antigens of later developmental stages of the parasite were recognised. Whether any L3 surface antigens would have been recognised by these antibodies can only be a matter for conjecture at the present.

In summary, preliminary experiments have investigated the nature of the surface proteins of different life-cycle stages of *O.circumcincta*. The results of these experiments suggest that L4, EL5 and adult stages of the parasite express different surface proteins, but these are not antigenic to the sheep. Attempts to radiolabel the surface proteins of exsheathed L3 were unsuccessful and it was thus not possible to determine whether these were antigenic. Clearly, further studies are required before definitive statements regarding these proteins can be made.

CHAPTER 8

GENERAL DISCUSSION

The principal objective of the work described in this thesis was to gain further insight into the nature of the ovine immune response to gastro-intestinal nematodes. To this end, two experimental approaches were employed. In the first, a technique was developed to radiolabel infective larvae of *O.circumcincta* and *T.vitrinus* and these larvae were then used to examine the population dynamics of these parasites in continuously infected sheep. In the second approach aspects of the host-parasite molecular interface were examined using both biochemical and immunological techniques to characterise the surface proteins of different life-cycle stages of *O.circumcincta*.

In both experimental approaches the use of radioisotopes was an integral component, although in each case they served very different functions. The use of ^{125}I to specifically radiolabel the surface proteins of parasites is a well-documented technique and results obtained using this method will be discussed later. The application of radioisotopes to non-molecular aspects of parasitology, although less well-documented, is not a new idea either. There are a number of reports of previous attempts to radiolabel parasitic helminths using a variety of different isotopes, including ^{14}C (Ball & Bartlett, 1969), ^{67}Ga (Preston, Macleod, Baker & Jenkins, 1975), ^{32}P (Wilson, 1979) and ^{75}Se (Christensen, 1977; Wilson, 1979). Of these, ^{75}Se, used in the form of selenomethionine, has proved to be particularly successful, enabling Wilson (1979) and Christensen & Nansen (1981) to follow the migration routes of helminth larval stages within the host.

It is only relatively recently that radiolabelling techniques have been applied to aid studies of the population dynamics of gastro-intestinal nematodes of ruminants, even though the desirability of a method of labelling worms is clear. Georgi & Le Jambre (1983) first demonstrated that infective larvae of *H.contortus* could be radiolabelled with ^{75}Se and the culture method used in the present work was a modification of their technique. Using this system up to 60% of eggs added to culture were recovered as radiolabelled L3. Although this recovery rate provided adequate material for the experiments, the extraction of large numbers of nematode eggs from infected faeces prior to culture was laborious and a disadvantage of the method. Techniques which would circumvent this problem, by directly labelling the L3 stage are currently being evaluated in Australia (Windon, personal communication) although as yet no results have been published. Assuming adequate incorporation of isotope can be achieved, such direct labelling techniques would substantially reduce the labour and time required to produce radiolabelled worms.

Populations of radiolabelled *O.circumcincta* and *T.vitrinus* L3 produced in the present work exhibited a wide range of specific activities similar to that described by Georgi & Le Jambre (1983) for radiolabelled *H.contortus*. It is probable that this range resulted from an uneven distribution of isotope in the faecal substrate during culture and thus the development of direct radiolabelling techniques may have the further advantage of reducing this variation. Fortunately, however, the autoradiographic procedure that was employed was

sufficiently sensitive to detect over 90% of labelled worms in the continuous infection experiments.

In a number of other respects the radiolabelled L3 produced in this study were similar to the labelled *H.contortus* produced by Georgi & Le Jambre (1983). The percentage loss of radioactivity from worms during exsheathment *in vitro* and development *in vivo*, for example, was virtually identical to that found by these workers in similar experiments with *H.contortus*. Of more importance, however, was the demonstration that the viability of radiolabelled *T.vitrinus* and *O.circumcincta* L3 was not detrimentally affected by the incorporation of the radioisotope since their establishment, development and sex ratio were comparable to non-labelled worms. Similar results have been demonstrated for *H.contortus* L3 (Georgi & Le Jambre, 1983) and also for radiolabelled schistosome cercariae (Christensen, 1977).

The primary function of radiolabelled worms was to provide a means whereby the fate of a known sub-population of parasites could be followed in animals experiencing a continuous infection. It was, therefore, important to determine that sufficient radioactivity remained incorporated in the worms to enable them to be detected for some time after infection. The manner in which the radiolabel was incorporated into the larvae was not investigated in this work, but it was clear that a significant amount of radioactivity was associated with the surface of the L3 sheath. Clearly this activity would be lost during the early stages of infection as a consequence of larval exsheathment. Experiments also demonstrated that a further

significant loss of radioactivity occurred during the first 10 days of development *in vivo*. However the amount of radiolabel incorporated in the worms remained relatively stable thereafter. It is probable that the high rate of loss initially was the result of the rapid rate of growth that occurs during this period. Worms recovered after 40 days infection retained a mean of 4% of their original activity, a result comparable to the 4.5% found on *H.contortus* worms after 37 days infection (Georgi & Le Jambre, 1983). Since these worms were readily identifiable, by autoradiography the degree of retention of radiolabel was obviously more than adequate to enable these worms to be used in studies of the population dynamics of these parasites.

The immunoregulatory processes operating against populations of *O.circumcincta* and *T.vitrinus* are believed to be different (Donald & Waller, 1982) and it is therefore relevant to directly compare the development of resistance to each of these parasites found in the experiments described in this study.

After 4 weeks of infection different effects of the host immune response on the two species were already apparent. After this period, 4 of the 5 sheep infected with *T.vitrinus* had developed a partial immunity to the establishment of incoming worms. In the *O.circumcincta* experiment, on the other hand only 1 of 6 sheep showed resistance to the establishment of incoming larvae after 4 weeks. However, the radiolabelled male worms recovered from these infected sheep were significantly shorter than those recovered from controls. Female *Ostertagia*

worms were also shorter, although this difference was not statistically significant. It seemed therefore that the first manifestation of host immunity to *O.circumcincta* was a retardation of worm development. However, radiolabelled *T.vitrinus* from previously infected animals were not stunted when compared to worms from their equivalent controls. Thus a second distinction could be made between the effects of the response of the sheep on the two species after 4 weeks.

After 8 weeks infection a strong immunity to incoming *T.vitrinus* L3 had developed, such that only 1 of 5 sheep was still recruiting worms to any degree. At the equivalent stage of the *O.circumcincta* experiment, 2 animals were completely resistant to the establishment of challenge larvae but the remaining 4 animals in the group were still recruiting considerable numbers of worms. In both experiments, the mean number of radiolabelled worms recovered from previously infected animals was significantly less than from the respective control group. The mean '% protection' values were calculated as 89.7% and 75.1% for *T.vitrinus* and *O.circumcincta* infected sheep respectively and thus at this stage of the experiments, a greater degree of resistance, at least in terms of the prevention of larval establishment, was evident against *T.vitrinus* worms as opposed to *O.circumcincta*.

In terms of retardation of worm development, sheep in both experiments were responding similarly by 8 weeks. The worms recovered from the one animal with significant numbers of radiolabelled *T.vitrinus* were stunted compared to controls. The few remaining worms recovered from the remaining infected sheep

were arrested third stage larvae, and therefore, by this stage of the experiment a response which caused inhibition of *T.vitrinus* development was taking place. The equivalent response in *O.circumcincta* infected sheep, which was apparent after 4 weeks, had developed to the extent that the majority of the radiolabelled worms recovered after 8 weeks infection were inhibited at the early fourth stage.

In some respects, therefore, the effects of immune responses on *T.vitrinus* and *O.circumcincta* appeared to be similar after 8 weeks of infection. However, important differences between the 2 species were determined by an analysis of the total worm burdens.

If it is assumed that no regulation of worm numbers occurred between weeks 4 and 8 of the experiment, then, as the period of infection increased a corresponding increase in the total worm burdens would be expected. However, in both experiments, the total worm burdens of previously infected animals at 4 and 8 weeks were very similar and thus it was apparent that the sheep were regulating their worm burdens in some manner. Comparison of the lengths of adult worms at 4 and 8 weeks suggested that the immunoregulatory processes were different in each experiment.

There was no statistical difference between the mean lengths of male or female *T.vitrinus* worms recovered at 4 and 8 weeks, and the size distributions of worm populations were virtually identical. In contrast, adult *O.circumcincta* recovered from infected sheep at 8 weeks were significantly shorter than those recovered at 4 weeks. Since the majority of

these sheep were still recruiting worms at 8 weeks, their predicted total burdens should have been greater than those observed at 4 weeks. Since this was not the case, it is suggested that some established worms were lost during this interval. Support for this hypothesis emerged from analysis of the size distribution of the worms. Significantly fewer large worms were present at 8 weeks than 4 weeks, which, assuming that worms do not shrink, could only mean that some of the largest individuals had been lost.

In essence, the results suggested that adult worms were lost between 4 and 8 weeks and that these lost worms were replaced by newly arriving worms. Since worms recruited later in the infection were subject to an immune response which inhibited their development, the mean length of the adult worms recovered decreased, and the size distribution of worms was also affected. It was therefore clear that a turnover of the population had occurred between 4 and 8 weeks, whereby the numbers of adult worms was kept constant due to a balance in the numbers of worms recruited to the population and the numbers of established worms lost. The onset and duration of the period of turnover varied between individuals. Obviously any population turnover must have ceased by 8 weeks in the two animals which had stopped recruiting larvae to the population by this time.

In contrast to the findings of the *O.circumcincta* experiment, the results suggested that there was no turnover of the *T.vitrinus* adult worm population, and numbers were therefore regulated by a different means. In the *T.vitrinus* experiment only one animal was still obviously recruiting worms to the

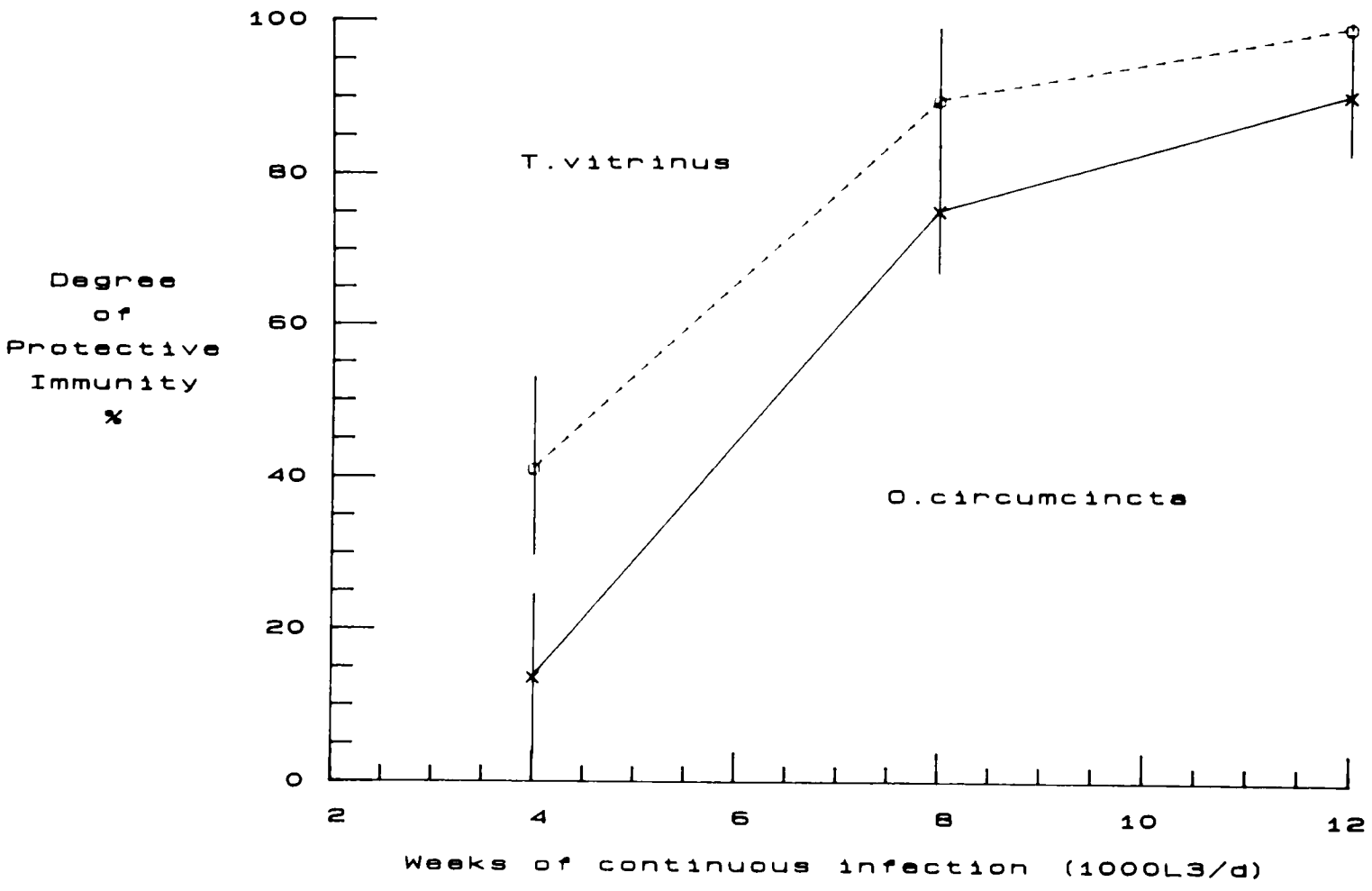
population after 8 weeks. Since the mean total worm burdens remained essentially the same as at 4 weeks it follows that few, if any of the established worms had been expelled, and thus the adult population had remained essentially static. Further evidence to support this hypothesis was provided by analysis of the worm lengths which showed that the mean lengths of worms recovered at 4 and 8 weeks were the same.

After 12 weeks of infection, similar events had taken place in both *O.circumcincta* and *T.vitrinus* infected sheep. By this time 3 of the *T.vitrinus* infected animals were totally resistant to new infection, and in the remaining 2 animals very few radiolabelled larvae were recovered. Similarly 4 of the 6 *O.circumcincta* infected animals were totally immune to radiolabelled larval establishment by this time. Total worm burdens in both groups of animals were very low after 12 weeks infection, amounting to a mean of only 1.2% (*T.vitrinus*) and 5.4% (*O.circumcincta*) of the total larvae received and clearly by this stage of the experiments adult worms had been or were in the process of being expelled. Once again there was variation between individuals in the extent to which this process had progressed.

Fundamental differences were thus found between the regulatory mechanisms operating against populations of *O.circumcincta* and *T.vitrinus*. Resistance to larval establishment developed earlier in the *T.vitrinus* experiment and throughout the infection was maintained at a higher level than that found in the *O.circumcincta* infected sheep (Fig. 8.1). By the end of the experiments a high degree of protection against L3 establishment was found for both species of parasites.

Figure 8.1 The degree of protective immunity directed against incoming *T.vitrinus* and *O.circumcincta* larvae during a continuous infection.

Development of Immunity to Incoming
Larvae in Continuously Infected Lambs



The rate of development of immunity found in the *T.vitrinus* experiment was in broad accord with that found by previous workers who have examined the population dynamics of *Trichostrongylus* spp. in continuously infected lambs (Chiejina & Sewell, 1974b; Waller & Thomas, 1981; Courtney *et al*, 1983; Jackson *et al*, 1983). In each of these studies, as in the present experiment, a resistance to incoming larvae and/or rejection of the developing fourth stages was the primary host response and occurred prior to any rejection of resident adult worms.

In two separate studies Chiejina & Sewell (1974a,b) compared the rate of development of resistance to *T.colubriformis* in lambs of different ages. In the first study, 3 week old lambs subject to an increasing infection rate accumulated worms for 12 weeks before any resistance to larval establishment developed. In 6 month old lambs, however, a strong resistance to re-infection developed after 8 weeks and between 8 and 15 weeks established adult worms were lost. Even in these older lambs, however, the rate of development of resistance to *T.colubriformis* was slower than that found in the present work with *T.vitrinus*. This is consistent with the results of Coop *et al*, (1979) and the recent findings of Eysker (1987) who followed the development of resistance to these two parasites in naturally infected sheep.

Jackson *et al* (1983) examined the pathological changes in the small intestine of lambs continuously infected with *T.vitrinus* and found that in some animals intestinal recovery was apparent by week 9 of the experiment. They estimated that

expulsion of established worms occurred between 10-14 weeks after initial infection, a time somewhat later than that found in the present work. This difference may be related to the different ages of the lambs used in the experiments.

In naturally infected grazing lambs Waller & Thomas (1981) have shown that the regulation of intestinal *Trichostrongylus* spp. follows the same sequence of events as that occurring in artificially infected animals. In their study field-infected animals accumulated worms until they developed a resistance to incoming larvae between 4 and 8 weeks. Established worms persisted until 12 weeks when they were expelled. A similar sequence of events was also described by Eysker (1987) who followed the development of resistance to *T.vitrinus* and *T.colubriformis* in grazing animals by comparing worm numbers recovered from continuously infected and tracer animals. In this study Eysker (1987) also noted large numbers of inhibited third stage larvae in infected lambs. Since tracer animals harboured few inhibited worms, it seemed highly likely that this inhibition was a further consequence of host resistance rather than an environmental conditioning of the larvae.

Turning to the regulation of *O.circumcincta* populations, previous studies have suggested that protective immune mechanisms begin to operate 6 to 10 weeks after the start of a continuous infection (Gibson & Everett, 1978; Jackson & Christie, 1979, 1984; Gibson & Whitehead, 1981; Coop *et al*, 1982). Until recently, however, there have been no studies sufficiently comprehensive to determine whether populations of *O.circumcincta* are regulated by a turnover of adult worms as was originally demonstrated for *O. ostertagi* in calves (Michel, 1963, 1969).

Waller & Thomas (1978a,b) compared the dynamics of *O.circumcincta* in tracer and continuously grazed lambs and found that the worm burdens in both groups corresponded closely to the rise and fall in pasture larval availability. Furthermore female worms recovered from the tracer lambs had prominent vulval flaps, whereas in the grazing lambs worms with poorly developed vulval flaps became increasingly common as the infection period progressed. These results were interpreted as showing that worms were constantly being lost and replaced, and thus a turnover of the population was occurring in the continuously grazing lambs.

Callinan & Arundel (1982) reached similar conclusions after examining the population dynamics of *O.circumcincta* in artificially infected sheep. These workers demonstrated that worm burdens were regulated at a level related to the infection rate, although resistance to worm establishment was not believed to be a contributory factor to this regulation. Instead, they suggested that population turnover was probably the principal regulatory mechanism.

A more comprehensive study of the regulation of *O.circumcincta* in artificially infected animals has recently been undertaken by Hong, Michel & Lancaster (1987) whereby the worm burdens of 18 week old lambs infected daily with 250, 500 or 1000 *O.circumcincta* L3 were monitored over a 20 week period. Results from this work also demonstrated that worm burdens rose to levels which were directly related to the infection rate and were primarily regulated by a loss of adult worms. The kinetics of the worm numbers of lambs receiving 1000 L3/day were similar

to that found in the present work where the same infection rate was used. Thus, mean worm counts at 30 and 60 days (approximately equivalent to the 4 and 8 week groups in Chapter 5) were identical, although by 80 days (equivalent to the 12 week group) worm numbers had begun to decline. As was the case in the present work, Hong *et al*, (1987) found considerable individual variation in worm burdens, such that in some instances they felt justified in excluding some individuals from calculations, classing them as 'abnormal animals'. Nevertheless, the overall pattern of population regulation that emerged from their work was similar to that found in the present study. Thus these authors provided evidence for a population turnover by finding first that the mean population size decreased with time and, second, that female worms with poorly developed vulval flaps became more prevalent as the infection progressed. Since in earlier work (Hong, Michel & Lancaster, 1986) no evidence had been found to suggest that worms shrunk or that vulval flaps regressed during an infection , this result could only mean that some worms had been lost. On the basis of declining worm numbers observed in all three infective groups Hong *et al*, (1987) concluded that resistance to the establishment of incoming larvae developed sometime after 8 weeks. Obviously at this time turnover of the population must have ceased.

It is clear that some insight into the effect of the sheep immune response on trichostrongylid parasites during a continuous infection can be provided by studies of the size and composition of worm burdens. However, as noted by Barger

(1987) the amount of information that can be obtained using such experimental methods is limited. For example, precise details of the development of immunity to larval establishment cannot be obtained since it is impossible to allocate worms recovered at slaughter to a particular infective dose administered during a continuous infection. By using radiolabelled larvae this problem can be overcome since it is possible to follow the fate of a known sub-population of worms within the host. In the present work it was evident that in 6-month old continuously infected lambs, partial resistance to L3 establishment had developed after 4 weeks for *T.vitrinus* and after 8 weeks for *O.circumcincta* times, in each case somewhat earlier than has previously been assumed. In addition, information pertaining to the inhibitory effects of host immunity on worm development have also been demonstrated, which, in a conventional continuous infection study would not have been possible.

Similar insights into the development of immunity to *H.contortus* L3 have been gained by Barger *et al*, (1985) using radiolabelled parasites. These workers were able to demonstrate that immunity to L3 establishment developed after 7 weeks of daily infection and in addition they showed that inhibition of worm development increased as the infection progressed. In a follow up study (Barger & Le Jambre, 1988) the use of radiolabelled worms also enabled the mortality rates of established worms to be determined during a continuous infection.

It would have been of interest to compare the mortality rates of radiolabelled worms in the two species of parasite examined in the present study. It could be envisaged that by following the death rates of radiolabelled adult worms from sheep under continuous infection, confirmation of the regulatory mechanisms operating against each species would be possible. It would be expected that radiolabelled *T.vitrinus* adult worms would persist for a longer period than radiolabelled *O.circumcincta* adults which would be expelled during the turnover process.

The effector mechanisms whereby immunity to *O.circumcincta* and *T.vitrinus* is mediated are poorly understood. In both continuous infection experiments in this study the development of resistance to larval establishment correlated with the number of IGLs found in the gastric mucosa. The importance of these cells in the immune response remains unclear, however, since no IGLs were found in *T.vitrinus*-infected animals at 4 weeks, even though partial immunity to L3 establishment was clearly demonstrable at this time. There was no correlation between resistance and the number of mast cells in the mucosa, nor could any correlation be demonstrated between mast cell and IGL numbers although these cell types are thought to be related (Miller, 1984). Both mucosal mast cells and globule leucocytes have been shown to contain a granule proteinase (designated sheep mast cell proteinase or SMCP by Huntley *et al* (1986) and in recent work, challenge of sheep immune to *H.contortus* or *O.circumcincta* has been associated with increases of this enzyme in serum or

gastric lymph (Huntley, Gibson, Brown, Smith, Jackson & Miller, 1987). It is thought that SMCP is released during immediate hypersensitivity reactions in the gut (Huntley *et al*, 1987) although the functional activities of this enzyme and other inflammatory mediators found in the superficial mucus have not been characterized. Douch *et al* (1983) demonstrated that mucus extracted from sheep resistant to nematode infections could act as a potent inhibitor of larval migration *in vitro* and the results of this work support the theory that the mucus layer serves as a repository for a number of anti-parasitic mediators (Miller, 1987).

The importance of antibody in the sheep immune response to nematodes is unclear. In the present work antibody titres recovered from *O.circumcincta* infected sheep correlated with the overall development of resistance to larval establishment and immunoblotting experiments revealed that a number of exsheathed larval proteins were recognised by host antibodies. Unfortunately it was not possible to determine whether any of these proteins were derived from the surface of the parasite since attempts to radioiodinate exsheathed L3 were unsuccessful. Immunoblots of radioiodinated L4, EL5 and adult preparations suggested that for these stages, at least, surface antigens were not important targets of host antibodies although this result may have been related to the specificity of the antibody probe used. It cannot be assumed, however, that negative results would also have been obtained with the infective larval stage especially since the experiment described in Chapter 5 indicated that this stage of the parasite was a primary target of some aspect of the sheep response.

The identification of protective antigens remains one of the main aims of research into molecular aspects of nematode parasitology and to this end, much work has been devoted to analyses of surface proteins of these parasites. The most commonly used approach, as in the present work, is to specifically label surface proteins with radioactive iodine. However, a different technique has recently been used by Milner, Beall & Orwat, (1987) to compare the proteins of exsheathed L3 and adult stages of *T.colubriformis* and *O.circumcincta*. Biosynthetic radiolabelling with ^{35}S-methionine enabled these workers to identify several hundred different proteins including, it was assumed, any newly synthesised cuticular proteins. Some differences were apparent between the total protein profile of adult and larval stages of *O.circumcincta* although the two stages of *T.colubriformis* exhibited similar profiles. Using serum collected from sheep at intervals after a challenge with 20,000 *O.circumcincta* L3, Milner *et al*, (1987) also determined the development of antibody reactivity against *O.circumcincta* larval antigens by immunoblotting. Although reactivity was detected by 19 days post-infection, this had waned by 81 days and they were unable to determine whether any of the recognised proteins were in fact derived from the surface of the worm.

Analysis of the surface components of nematodes may in the future prove profitable, but as yet there have been no reports of protective immunity being achieved through vaccination with purified surface antigens. Moreover, recent work by Munn, Greenwood & Coadwell (1987) has suggested that

more fruitful results may be gained from an examination of other molecular components of the parasite.

Munn *et al* (1987) demonstrated that some protection to challenge with *H.contortus* could be achieved through vaccination with a worm extract enriched for contortin, a protein present on the luminal surface of the intestine of this species (Munn, 1976, 1977) Young lambs injected with contortin and adjuvant developed a strong humoral antibody response and were less susceptible to haemonchosis when challenged 1 month later. A cell-mediated response could not be detected but the number and weight of worms recovered from vaccinated sheep was markedly reduced compared to control animals.

Munn *et al* (1987) believe that contortin will have value as a component of any future vaccine against *H.contortus* and are currently undertaking improvements in the methods of purification. However, as yet, this antigen has not been shown to provide protection against incoming larvae or early larval stages of the parasite. Since these stages can cause considerable pathological damage to the host much work is clearly still required to identify antigens which may also protect against these stages. Nevertheless, the demonstration that some protection can be achieved through vaccination with an isolated protein is an important discovery and encourages further efforts to determine and isolate protective antigens for other parasites.

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PUBLICATIONS

The following are publications arising from the work described in this thesis;

SEATON, D.S., JACKSON, F., SMITH, W.D. & ANGUS, K.W. (1988). The development of immunity to incoming radiolabelled larvae in lambs continuously infected with *Trichostrongylus vitrinus*. Research in Veterinary Science, (in press).

SEATON, D.S., JACKSON, F., SMITH, W.D. and ANGUS, K.W. (1988). The development of immunity to incoming radiolabelled larvae in lambs continuously infected with *Ostertagia circumcincta*. Research in Veterinary Science, (in press).