THE MOLECULAR DIVERSITY OF STRAINS OF *OSTERTAGIA OSTERTAGI*

by

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Like - but oh, how different!

William Wordsworth
(1770-1850)

Dedicated to the memory

of

ANNEMARIEKE DUNCAN-LANGEVELD

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DECLARATION

I declare that the work presented in this thesis has been carried out by me. The surface lipid studies were carried out in conjunction with Miss Lorna Proudfoot, Department of Biochemistry, University of Glasgow. Furthermore, some of the studies using biotin-streptavidin affinity have already been prepared and accepted for publication:

Keith, K.A., Duncan, M.C., Murray, M., Bairden, K. and Tait, A. Stage specific cuticular proteins of *Ostertagia circumcincta* and *Ostertagia ostertagi*. International Journal for Parasitology.

Signed .....

M.C. Duncan

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SUMMARY

Increasing evidence for the occurrence of intraspecific variation of nematodes prompted this study of *Ostertagia ostertagi*, a parasite of the bovine abomasum which causes gastroenteritis. Parasites recovered from calves with ostertagiosis have shown differences in their morphology, pattern of development and drug susceptibility. These factors led to experimental work to assess the degree of variation detectable at a molecular and structural level. The techniques applied examined the genetic products of different isolates of *O.ostertagi* and also compared them to other trichostrongyle species. In addition, the stages of the parasite life cycle were followed.

Attempts were made to maintain the parasite outwith the natural bovine host, in laboratory mice and *in vitro* culture. In both cases little success was realised and comparison of samples under these conditions could not be achieved.

Light and scanning electron microscopy showed that the appearance of the seemingly simple nematode altered significantly throughout the life cycle, although not in a strict step-like fashion. A changing appearance was also reflected in the examination of the total and surface peptide profiles of different biotinylated parasite stages by polyacrylamide gel electrophoresis.

Isolates of *O.ostertagi* and different species of trichostrongyle were compared by their charge with the isoenzyme techniques: starch gel electrophoresis and isoelectric focusing, and by their mass with polyacrylamide gel electrophoresis. Analysis of the isoenzyme data indicated variation at both intra- and inter-species levels, although it was greater between the trichostrongyle species. Close similarities were seen between different species and isolates on examination of the peptide profiles indicated by Coomassie blue staining and when biotin-streptavidin affinity labelled a subset of peptides from intact nematodes. There was a slight degree of variation in the homogenate profiles of the different species and isolates examined by biotinylation.

In conclusion, a molecular basis for a low level of intraspecific variation of *O.ostertagi* was recognised. The existence of variation may have important implications in the design of anthelmintic control strategies and the understanding of epidemiology. Furthermore, parasite diversity creates stability in the host-parasite relationship by providing a mixed population of parasites, which may contain certain population subsets which adapt better to changes arising in the environment than other subsets.

ACRONYMS

ALD	-	arrested larval development
APS	-	ammonium persulphate
BSA	-	bovine serum albumin
CO_2	-	carbon dioxide
CTAB	-	cetyltrimethylammonium bromide
CVL	-	Central Veterinary Laboratory, Weybridge
DNA	-	deoxyribonucleic acid
DPI	-	days post infection
EC_{50}	-	the dose estimated to produce the desired effect in 50% of subjects
EDTA	-	ethylenediaminetetra-acetic acid
epg	-	eggs per gramme
ES	-	excretory-secretory products
FITC	-	fluorescein di-isothyocyanate
H and E	-	Meyers Haemotoxylin and Putts Eosin stain
IEF	-	isoelectric focusing
IFAT	-	indirect fluorescent antibody test
L1,L2,..L5	-	larval stages first to fifth
MTT	-	3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NHS-biotin	-	^{12}biotin-N-hydroxysuccinamide
PAGE	-	see SDS-PAGE
PBS	-	phosphate buffered saline
ppm	-	parts per million
psi	-	pounds per square inch
RFLP	-	restriction fragment length polymorphism
RNA	-	ribonucleic acid
SDS-PAGE	-	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	-	scanning electron microscopy
TEMED	-	N,N,N',N'-tetramethylethylenediamine
v/v	-	volume of solute per volume of solvent
w/v	-	weight of solute in volume of solvent

DEFINITIONS

To avoid confusion with the terminology encountered in the text it is important that some background definitions are accepted, since there is a wide variance in usage of certain taxonomic terms. Generally, these have been set along similar lines to those in the World Health Organization's Bulletin "Proposals for the Nomenclature of Salivarian Trypanosomes and for the Maintenance of Reference Collections" Anonymous (1978). Unless stated as otherwise these terms shall refer to pure species samples.

CLONES - Sample organisms derived from a single binary division and consequently generally considered identical.

DEMES - Populations from the same species with varying properties. The varying property may be indicated with a prefix, e.g., zymodemes vary in their enzyme patterns.

LINES - Stocks subjected to altered environments or given different treatments.

POPULATION - The group of parasites present in a single host or culture system at any one time.

PRIMARY ISOLATE - The viable population derived in culture or in an experimental animal after infection with natural material (consequently possibly a mixed group of parasites).

SPECIES - A group of animals with the same major characteristics such that members can interbreed and only varying in minor characteristics.

STOCK - The population derived by passage from the primary isolate and consequently not necessarily homogeneous.

STRAIN - Set of populations from different hosts with similar properties, but varying from other sets of populations.

For the purposes of the recorded study, the samples obtained from different regions were considered to be different isolates with unknown degrees of homology or heterology.

CHAPTER 1

OSTERTAGIA OSTERTAGI:

A PARASITE OF VETERINARY IMPORTANCE

OSTERTAGIA OSTERTAGI: A PARASITE OF VETERINARY IMPORTANCE

1.1 INTRODUCTION

Ostertagia ostertagi is recognised as one of the most pathogenic helminths of veterinary importance in Western Europe and other temperate regions throughout the world. Reports indicate that its distribution closely mirrors that of the major cattle producing areas of the globe (Figure 1.1).

O.ostertagi belongs to the Strongylina suborder of nematodes, in which the males have a copulatory bursa. Further classification places *O.ostertagi* in the superfamily Trichostrongyloidea, because it has a direct life cycle with infection by the third stage larva and since the adult has a small buccal capsule (Table 1.1).

The association between *O.ostertagi* parasites and the abomasal lesions seen in cattle was first made by Ostertag in 1890. Since his observation outbreaks of clinical ostertagiosis have been documented frequently (Stiles, 1900-1901; Ackert and Muldoon, 1920; Bruford and Fincham, 1945; Gracey, 1960). Subsequent to these records, outbreaks have received less publicity and have usually been dealt with by the general practitioner, a fact which may also explain the apparent drop in recognised incidence. The Central Veterinary Office recently recorded the number of outbreaks of the disease from the Veterinary Investigation Centres in England, Scotland and Wales as follows: 1984, 350; 1985, 420; 1986, 155; 1987, 166 and 1988, 110.

With rare exceptions, *O.ostertagi* is restricted to the bovine host, causing a gastritis with varying degrees of severity. The spectrum of disease runs from subclinical cases (with reduced live weight gain) to cases with severe diarrhoea, weight loss and anorexia resulting in dehydration and electrolyte imbalance and eventually death. The range of changes makes it difficult to estimate a cost for lost production, treatment and control. However, Bain and Urquhart (1986) estimated that if parasitic gastroenteritis was left uncontrolled in the United Kingdom, the cost per annum would be £45.7 million. The figure was based on an annual production of 3.2 million calves and the calculated

FIGURE 1.1.

Estimated distribution of bovine ostertagiosis appears to mirror the distribution of the major cattle producing zones of the world.

MAJOR CATTLE REARING ZONES

OSTERTAGIA

TABLE 1.1

Classification of *Ostertagia ostertagi*

Kingdom	-	**Animalia**
Phylum	-	**Nemathelminthes**
Class	-	**Nematoda/Secernentea**
Order	-	**Strongylida**
Suborder	-	**Strongylina**
Superfamily	-	**Trichostrongyloidea**
Family	-	**Trichostrongylidae**
Subfamily	-	**Ostertagiinae**
Genus	-	***Ostertagia***
Species	-	***ostertagi***

(Adapted from Urquhart, Armour, Duncan, Dunn and Jennings, 1987)

costs of lost weight gain and mortality. They suggested 5% of calves would be severely infected with *O.ostertagi*, a quarter of these fatally, 20% moderately infected, 45% sub-clinically infected and 30% free from parasites.

1.2 LIFE CYCLE

The life cycle of *O.ostertagi* is direct, with no intermediate host (Figure 1.2). Embryonated parasite eggs are passed in the faeces of the bovine host, they hatch and develop through two larval stages on the pasture prior to reaching an infective state: the first larval stage (L1) moults its outer sheath to become a second stage larva (L2). Development to the third larval stage (L3), which infects the host, does ~~not~~ include a moult, ~~since~~ but in this case the sheath is retained. The sheath improves larval ability to endure environmental change. The L3s exhibit a high degree of motility and disperse from the faecal pat in moist conditions and are ingested by the host. Development from egg to infective larva may be completed in a minimum of 7 days (Williams, Marbury, Scheide, Luther, Knox, Kimball and Snider, 1984).

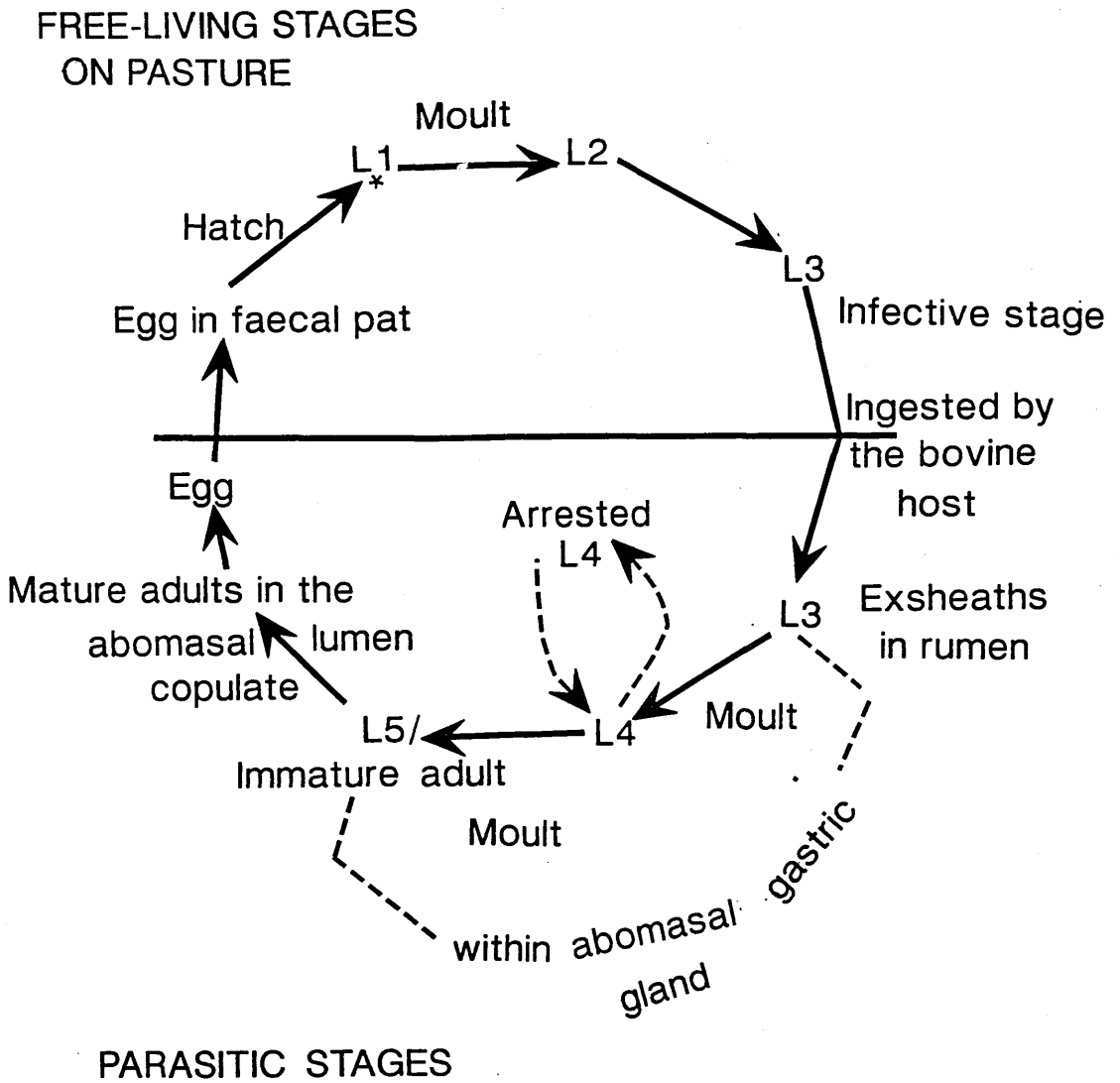
Loss of the protective sheath occurs in the rumen by 2 DPI (days post infection). The larvae then establish in the gastric glands of the abomasum, particularly in the fundic region, although with time the pylorus becomes increasingly affected (Ritchie, Anderson, Armour, Jarrett, Jennings and Urquhart, 1966). The third moult to the fourth stage larva (L4) occurs in the abomasal gastric glands at 3 to 4 DPI. There is no migratory pattern within the host and the parasites moult and develop to the fifth stage larva (L5/immature adult) within the gastric gland. By 12 DPI, 50% of the parasite population is in the immature adult stage (Ritchie *et al.* 1966).

Subsequently, the parasites emerge from the gland and the adults copulate on the mucosal surface. Al Saqur, Armour, Bairden, Dunn, Jennings and Murray (1982a) noted that in *Ostertagia* spp. infections which included *O.leptospicularis* fertilised eggs were present in the adult females prior to emergence from the gastric gland. They suggested that more than one parasite may inhabit a single gland,

FIGURE 1.2

The life cycle of Ostertagia ostertagi.

* L refers to larva



resulting in copulation before emergence. However, this phenomenon may be confined to *O.leptospicularis*, since fertilised eggs have not been found in histological sections from pure *O.ostertagi* infections before ~~larval~~ pre-adult emergence.

The life cycle is completed with the passage of parasite eggs in the faeces. Patency of infection, i.e., period from larval ingestion to egg ~~production~~ output may take a minimum of 17 to 21 days (Anderson, Armour, Eadie, Jarrett, Jennings, Ritchie and Urquhart, 1966). The life span of the adult has a mean of 25 days (Michel, Lancaster and Hong, 1976a) and as early as 28 DPI there is a drop in the number of adult nematodes found by abomasal count at necropsy (Ritchie *et al.* 1966). Murray, Jennings and Armour (1970) found parasite loss was exponential between 17 and 35 DPI. The rate of adult parasite loss is accelerated in diarrhoeic cases and when anthelmintic treatment has been administered.

Parasite development occurs over a varying time scale depending on a possible period of developmental arrest in the host gastric gland, at the early L4 stage, and environmental conditions affecting the progression from egg to infective larva on the pasture, e.g., seasonal variation in temperature, rainfall and humidity. The minimum length of the life cycle is approximately 3 weeks, but where arrest occurs the period may exceed 6 months (Urquhart, Armour, Duncan, Dunn and Jennings, 1987).

The break in parasite maturation is referred to by a number of terms - arrested larval development (ALD), inhibited larval development, retarded larval development and hypobiosis. The phenomenon was first recognised in 1957 by Martin, Thomas and Urquhart. They discovered parasitic gastritis at post-mortem examination of calves in the spring. The condition had previously been encountered only in the autumn at the end of the grazing season, but in this case the calves had had no access to pasture over the winter. The only plausible explanation appeared to be a delay in parasite maturation.

Arrest occurs immediately after the third moult at 3 to 4 DPI, when the early fourth stage larva has established in the gastric gland lumen

(Anderson, Armour, Jennings, Ritchie and Urquhart, 1965a). The larva becomes metabolically inactive: the intestinal lumen closes, partial atrophy of the intestinal mitochondria occurs and neurointestinal activity decreases. In addition, arrested L4s have a clear area caudal to the oesophagus (J.Armour, personal communication). Refractile granules have been observed in the intestinal lumen and possibly these may be equivalent to the increasing number and density of lipid droplets recognised when the nematode *Anguina agrostis* is repeatedly dehydrated (anabiosis) and rehydrated (Preston and Bird, 1987). Often parasites recovered at necropsy from animals infected with an arresting population of *O.ostertagi* form a bimodal distribution. Larvae undergo a rapid growth phase after development to the L4. Arrest occurs prior to the rapid growth phase, so arrested larvae are generally smaller than other parasites present (Armour, 1978).

Resumption in development after arrest often leads to a more synchronous emergence and consequently can result in severe disease. The disease syndrome produced after the larvae have undergone arrest is known as Type II ostertagiosis to differentiate it from the uninterrupted Type I syndrome. The period of arrest itself may be referred to as the Pre-Type II stage and due to the lack of obvious clinical signs usually goes undetected.

The influence of environmental differences on parasite development is well illustrated with an example from New South Wales, Australia. The North Coast has a subtropical climate, while the Northern Tablelands have a colder, drier late autumn and winter. Larval development was assessed following pasture contamination by infected calves in different seasons. On the North Coast there was a rapid rise to peak larval pasture numbers after contamination, followed by a steep fall regardless of season, while in the Northern Tablelands larval development was slower and peak larval counts were achieved in the spring (Smeal, Robinson and Fraser, 1980a).

The parasitic stages of the life cycle could be expected to have a marked effect on the host by their growth and development in the gastric glands. Knowledge of the pathogenesis of ostertagiosis is increasing, but it is apparent that the infection pathway is more

complex than it initially appears; a summary of current information follows.

1.3. PATHOLOGY AND PATHOGENESIS

Murray (1970) reviewed the histological properties of the bovine abomasum. The abomasum is divided into two main areas by a transverse constriction: the fundus and the pylorus. In addition, there is a small cardiac glandular area surrounding the abomasal opening from the omasum. The gastric glands in the fundus are straight tubules opening into the gastric pits, while in the pylorus the tubules are coiled and the pits are deeper. Specialised cells line the gastric gland, in particular the parietal and zymogen cells. Parietal cells produce hydrochloric acid, which converts pepsinogen produced by the zymogen cells to the proteolytic enzyme, pepsin. The intracellular canaliculi and the microvillae of the parietal cells are important for their secretory function.

On infection the exsheathed L3s establish in the gastric glands, where they continue to develop, undergoing a further moult. Prior to emergence, the pathological changes are confined to the parasitised gastric glands and there is little host reaction to the parasite, although from 7 DPI Stringfellow (1974) noticed collagen deposition around the parasitised gland.

The gland distends as the parasite grows from 1.3 to 8mm in length and the gland cells are stretched until the connecting tight junctions may be torn apart (Murray, 1969). To retain epithelial continuity cell multiplication appears to be stimulated (Ritchie *et al.* 1966). Murray (1968) observed the production of incomplete tripartite cell junctions. He noted that 50 to 90% of the zonulae occludentes were partially or completely separated. Electron dense material was seen in dilated spaces between the epithelial cells of parasitised abomasa from 17 DPI (Murray, 1969). From its appearance it was considered that the material was most likely to be protein and these changes in the epithelial tight junctions were taken as evidence of the increasing permeability of the abomasal mucosa to macromolecules.

The specialised parietal cells and zymogen cells are replaced by rapidly dividing non-differentiated cells (Figure 1.3), initially in the parasitised glands, but after emergence the surrounding glands were also affected. Remaining zymogen and parietal cells show evidence of reduced functional activity (Murray *et al.* 1970). In the abomasa of infected calves the zymogen cells contain fewer granules and the parietal cells have reduced numbers of intracellular canaliculi. In addition, local blood vessel dilation and marked cellular infiltration occur in the lamina propria with migration of plasma cells, lymphocytes, eosinophils and mast cells into the area (Figure 1.4) (Anderson, Armour, Jarrett, Jennings, Ritchie and Urquhart, 1967; Murray *et al.* 1970). From 17 DPI mast cell numbers increase and from 35 DPI there is also a marked increase in globular leukocytes (Murray *et al.* 1970).

Maximum visible pathological change due to infection with *O.ostertagi* occurs at parasite emergence from the abomasal gastric glands at approximately 18 DPI, when changes begin to spread to the surrounding tissue. Severity of the lesions is related to the parasite burden and the time span for emergence from the gastric glands (Williams *et al.* 1984). Consequently, while the pre-emergence pathology is similar in both the Type I and Type II case, often the post-emergence pathology found in the Type II disease is more severe, owing to a more synchronous parasite emergence.

By 28 DPI the parasite population begins to decrease, so by 60 to 90 DPI the adult numbers in the lumen are very low. At this stage, the epithelium and then the mucosa have largely returned to the pre-infected state (Ritchie *et al.* 1966). Initially, the undifferentiated cells are replaced by intermediate cells, which develop the normal epithelial pattern with time. At approximately 28 days, the calf's appetite, which is depressed during the period of emergence, begins to return to normal (Murray *et al.* 1970). Armour, Jennings, Kirkpatrick, Malezewski, Murray and Urquhart (1967a) noted that the abomasal tissue apart from the previously parasitised glands returned to normal within 48 hours of removal of adult parasites by thiabendazole treatment.

FIGURE 1.3

Ostertagia ostertagi **larva developing within a bovine abomasal gastric gland. Large numbers of lymphocytes may be seen infiltrating the area. The rapidly dividing, non-differentiating cells lining the gland are illustrated (arrows). Meyers Haemotoxylin and Putts Eosin (H and E). (x 112)**

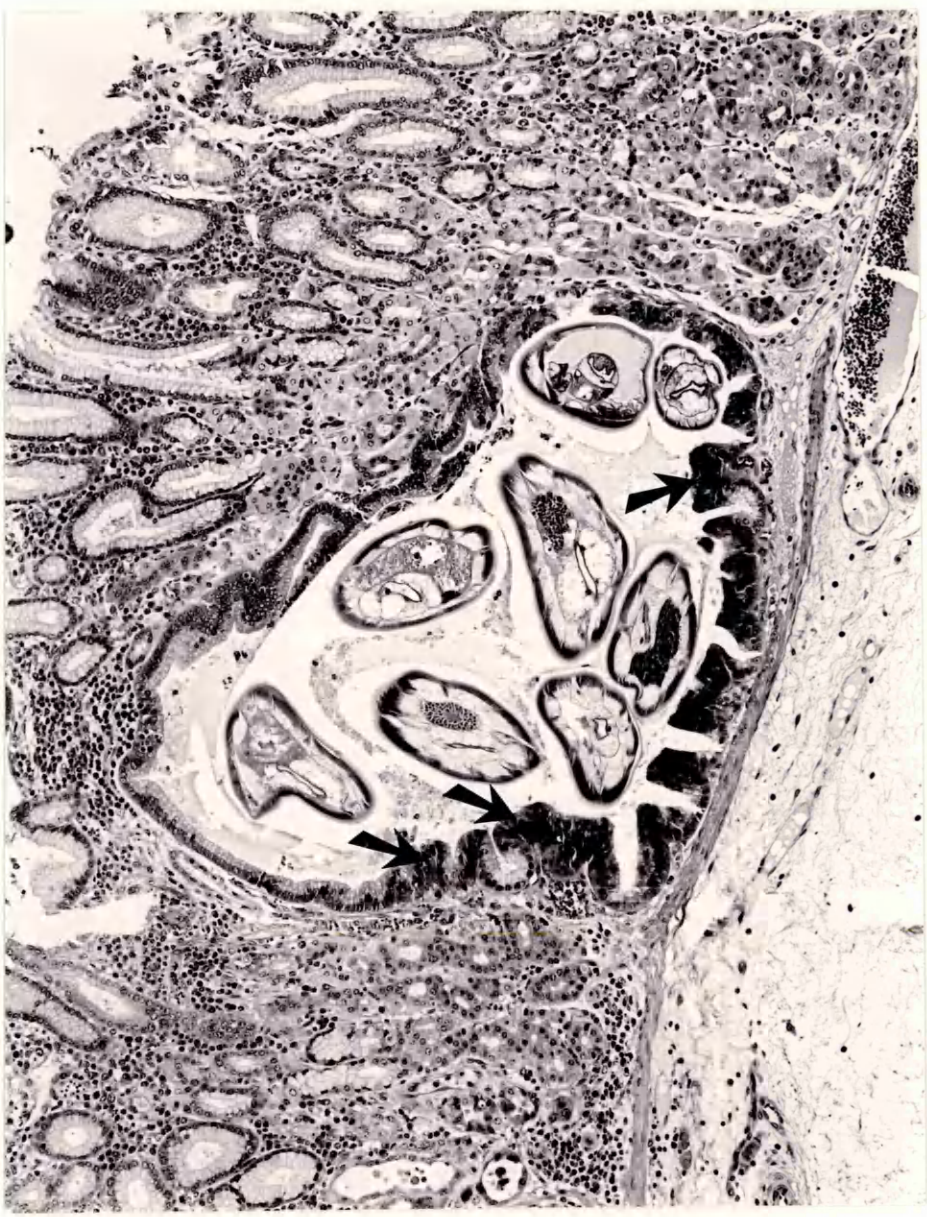
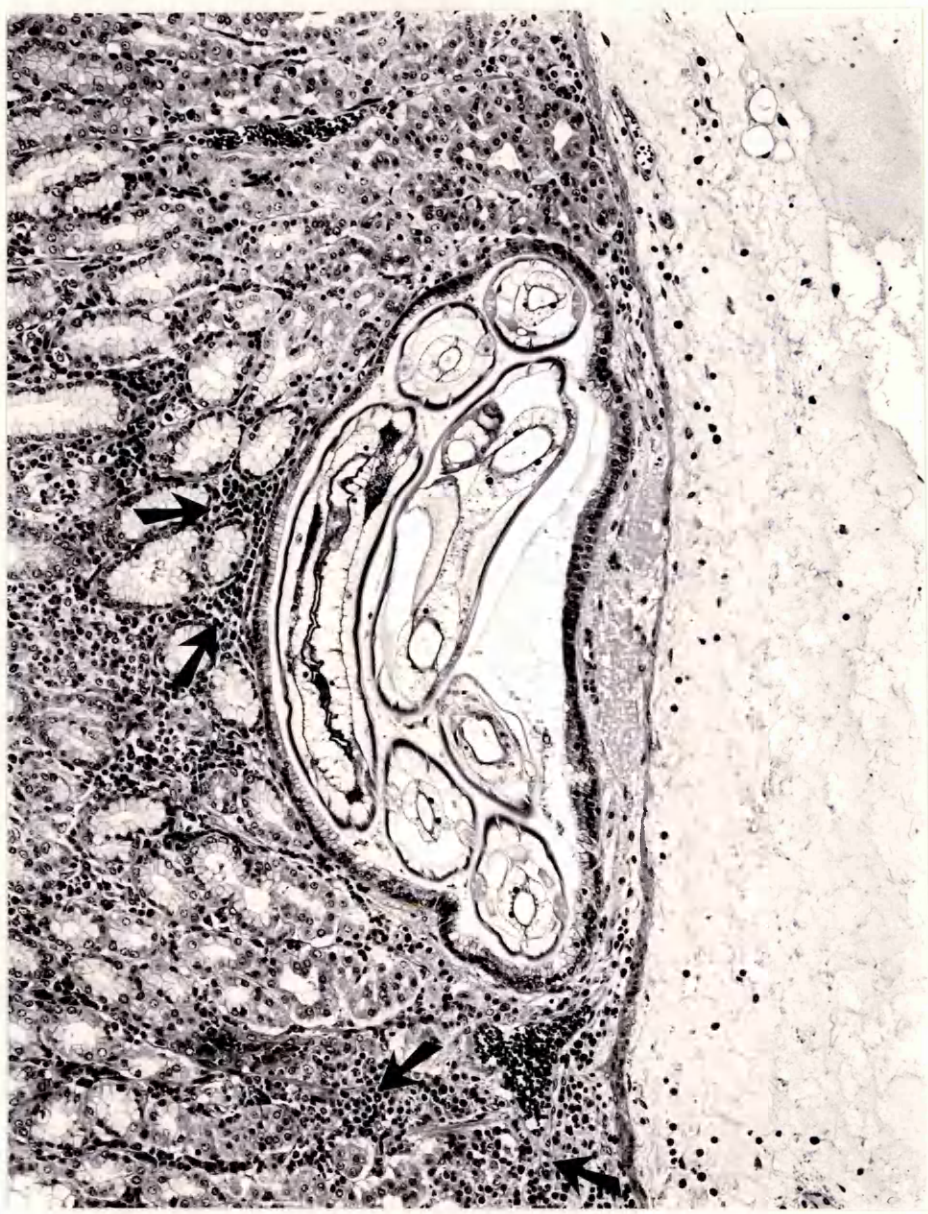


FIGURE 1.4

Longitudinal section through coils of a developing *Ostertagia ostertagi* larva in the gastric gland of an infected bovine abomasum. Inflammatory cells are invading the area in large numbers (arrows). Meyers Haemotoxylin and Putts Eosin (H and E). (x 112)



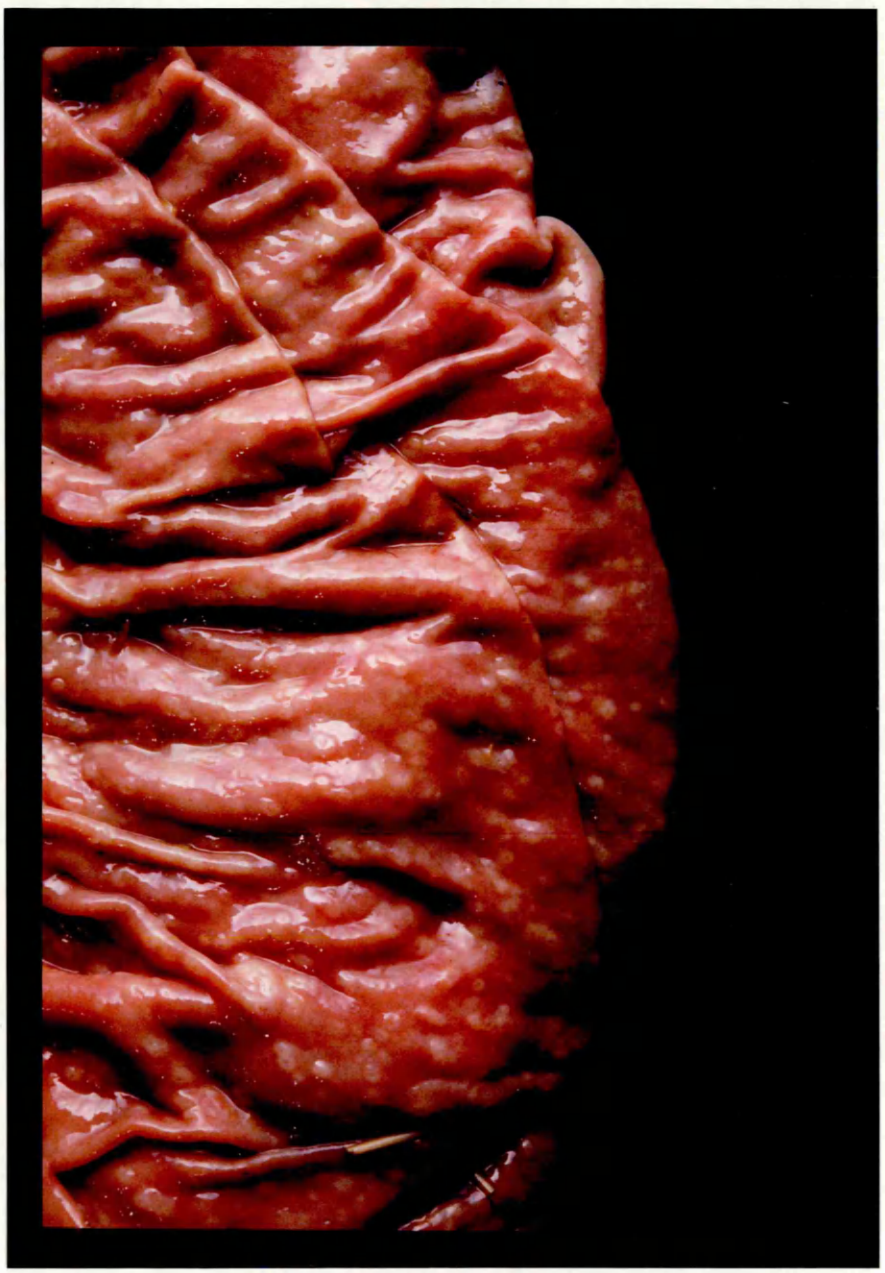
Examination of gross pathology of the abomasum in heavy infections reveals the folds to be markedly oedematous, thickened and hyperaemic. Raised pale nodules 2 to 3 mm in diameter with central orifices are seen throughout the abomasum, but they are particularly numerous in the fundus (Ritchie *et al.* 1966) (Figure 1.5). Coalescence of the lesions occurs in severe infections giving the mucosa the appearance of "morocco leather" or "crazy paving". Superficial cells may slough to give diphtheritic thumbprint lesions. Regional lymph node enlargement occurs as a result of reactive hyperplasia and suppurative lymphadenitis, and may be linked to increased levels of bacteria in the abomasum.

Loss of cell differentiation leads to a lack of acid secretion, so pepsinogen conversion to pepsin is reduced and protein digestion is altered; once the pH rises above 4.5 protein digestion becomes negligible. The normal pH of the abomasum is 2.2 to 3.8. In a heavy infection with greater than 40,000 L3 the pH may rise to 7. McKellar, Duncan, Armour, Lindsay and McWilliam (1987) showed that direct implantation of adult parasites into the abomasum did not itself lead to a rise in pH. They claimed the absence of pH rise on implantation was further evidence for the theory that loss of parietal cell function occurs with the emergence of parasites from the gastric glands (Jennings, Armour, Lawson and Roberts, 1966).

The alteration in protein digestion and absorption of amino acids may in turn affect and reduce the appetite (Holmes, 1986). Decreased appetite is a major sign in both subclinical and clinical cases. Obviously the plane of nutrition will significantly influence the outcome of disease, so higher protein consumption will alleviate the disease problem (Urquhart *et al.* 1987). Actual weight loss is not precisely determinable, since with fluid retention the relative body weight is reduced further than the reduction in live weight gain suggests. By injecting calves with tritiated water and employing the dilution principle, marked reduction in body solids relative to total body weight was confirmed in the Type II infection (Halliday, Dalton, Anderson and Mulligan, 1965). The relative drop in body solids means that even in subclinical infections the condition may be more serious than realised.

FIGURE 1.5

Folds of the fundus of an abomasum infected with *Ostertagia ostertagi*. Pale raised nodules, 2 to 3mm in diameter with umbilicate centres indicate infected gastric glands.



Mulligan, Dalton and Anderson (1963) demonstrated loss of protein in the faeces by injection of radiolabelled polyvinylpyrrolidone into infected calves. In theory the drop in protein digestion may be alleviated by a degree of digestion further down the tract in the ileum. McKellar, Mostofa and Eckersall (1990) found that excretory material from adults and L3s of *O.ostertagi* had a muscarinic effect, which could lead to increased gut motility and so contribute to diarrhoea. Snider (1986) had suggested that the faster passage of food and increased intestinal pH would reduce pancreatic enzyme activity in the intestine, so overall digestion will be impaired. In addition, endogenous protein is lost by a number of routes including increased mucus secretion and urinary loss. Fat and protein deposition are reduced. Production of essential proteins such as the immunoglobulins and albumin must be preferentially maintained.

The aetiology of diarrhoea in ostertagiosis is still not fully understood, but it appears concomitantly with an increase in bacteria in the abomasal fluid. The bacteriostatic effect produced by the acidity of the abomasum for the digestive tract caudal to the rumen is reduced with pH rise. The regulation of the normal gut flora is adversely affected by the loss of bacteriostatic effect of the abomasum and so the population of commensal and opportunist bacteria will rise.

An apparent increase in the selective permeability of the abomasum to macromolecules was mentioned earlier resulting in the loss of plasma protein from the circulation into the surrounding tissue. The animal becomes hypoalbuminaemic and retention of fluid in the surrounding tissue causes oedema (Mulligan *et al.* 1963). Electrolytes are also lost: sodium (Na^+), chloride (Cl^-) (Anderson, Armour, Jarrett, Jennings, Ritchie and Urquhart, 1965b). In addition, there is high potassium loss due to the massive sloughing of epithelial cells (Holmes, 1986).

While plasma proteins are lost from the circulation with increased abomasal permeability, the unconverted pepsinogen is believed to leak back in the opposite direction resulting in elevation of plasma pepsinogen concentrations. Horse radish peroxidase has a similar molecular weight to pepsinogen. However, Stringfellow and Madden

(1979) found the mucosal permeability to horse radish peroxidase was the same in infected and uninfected control calves, which suggested the abomasal permeability was highly selective. When a hypersensitivity reaction is induced in rats immunised against the intestinal nematode, *Nippostrongylus brasiliensis*, the self-cure reaction is simulated. There may be an increase in gut permeability to macromolecules and the rapid worm expulsion may be due to increased antibody transfer to the site (Barth, Jarrett and Urquhart, 1966). Sheep immunised with irradiated *Haemonchus contortus* showed higher levels of IgG and IgA in the serum, however, the rise in IgA in the abomasal mucus was significantly greater (Smith and Christie, 1978). Murray (1972) examined the histological appearance of the *N.brasiliensis* infections in the rat and noted increased concentrations of mast cells with discharge of vasoactive compounds. Murray *et al.* (1970) observed similar mast cell infiltration and globular leukocyte formation in the abomasa of calves with ostertagiosis. Consequently, it was suggested that a hypersensitivity type reaction to the parasite might lead to increased plasma pepsinogen concentration (Armour, Bairden, Duncan, Jennings and Parkins, 1979).

With parasite loss and recovery of the abomasum, a reduction in pepsinogen concentration would be expected at 60 to 90 DPI (Michel, Lancaster, Hong and Berrett, 1978a). However, in reality the plasma pepsinogen concentration remains high after 60 DPI. Consequently, new theories for the rise in plasma pepsinogen level have been suggested. Raised plasma pepsinogen levels were found when adult *O.ostertagi* were implanted directly into the abomasum (McKellar, Duncan, Armour and McWilliam, 1986). It appeared from this work that adult parasites may have an effect on the permeability of the abomasal wall, possibly through their excretory-secretory products or by parasite migration between mucosa and lumen. Alternatively or additionally, the adults or their products might increase pepsinogen secretion from the zymogen cells. Work done by McKellar *et al.* (1990) supports the theory that adult secretions lead to an increase in pepsinogen concentration. They compared the effect of carbachol (parasympathomimetic), atropine (parasympatholytic) and excreted products from adult and L3 *O.ostertagi* on isolated preparations of dispersed abomasal glands. The excreted products resulted in the

greatest increase in pepsinogen concentration, followed by carbachol, while atropine addition reduced the pepsinogen concentration below the control level situation. Moreover, the presence of adult parasites or L3s caused no further increase in pepsinogen.

McKellar *et al.* (1986) found plasma pepsinogen concentrations were high even when low numbers of adults were implanted, which suggested direct secretion of pepsinogen into the blood stream rather than release on mucosal disruption. Irrespective of the mechanisms involved, increased serum pepsinogen levels are commonly used diagnostically for the differentiation of ostertagiosis in stock under a year of age. Concentrations of 3,000mU tyrosine and above are considered significant (Selman, Armour, Jennings and Reid, 1977).

Selective permeability of the abomasal epithelium to other macromolecules may also be affected. The gastrointestinal tract is an important site for immunoglobulin synthesis, so the increased permeability may be instrumental in the local movement of antibody to target areas occupied by parasites (Murray, 1969).

McKellar *et al.* (1987) looked at local and systemic responses to adult parasite implantation. The results suggested that gastrin was released once a threshold level of adult parasites had been reached. An increase in gastrin secretion leads in turn to stimulation of the zymogen and parietal cells, i.e., increasing pepsinogen release and raising acid concentration to try to restore abomasal pH, and alters gastric motility (Titchen and Anderson, 1977; Schillhorn van Veen, 1986). Recently work by Fox, Gerrelli, Shivalkar and Jacobs (1989) has suggested a link between raised blood gastrin levels and reduction in appetite. By injecting calves with a gastric acid secretion inhibitor, they induced an increased blood gastrin level, a depressed appetite and increased plasma pepsinogen concentration. The increase in plasma pepsinogen was not as great as that seen in cases of ostertagiosis suggesting that hypergastrinaemia is not the sole cause for the elevation.

The pathogenic pathways indicated in this section provide an adequate, though still incomplete, explanation of the clinical signs encountered in ostertagiosis. The key factors are loss of specialised cell

differentiation, resulting in loss of enzyme conversion and increased abomasal pH, which in turn cause a disturbance in the normal gut flora and alter protein digestion. The clinical effects of the pathological changes are discussed in the following section.

1.4 CLINICAL SIGNS

A wide spectrum of effects, both sub-clinical and clinical, may result from infection with *O.ostertagi*. Although sub-clinical infection may not be detected, it may cause significant production losses: a lack of live weight gain and a decrease in carcase quality (Entrocasso, Parkins, Armour, Bairden and McWilliam, 1986). In Wisconsin, United States of America, Todd, Bliss and Meyers (1975) found gastrointestinal nematodes were universally prevalent in lactating cows at sub-clinical levels. The cows were found to have low faecal egg counts (chiefly *O.ostertagi*) and dosing with anthelmintics increased milk yield.

In clinical cases rather than a lack of weight gain there is frank weight loss as a result of reduced appetite and profuse watery diarrhoea. The calf's abdomen becomes tucked up, implying pain and faecal soiling is conspicuous around the tail head. The coat takes on a lustreless appearance and the eyes become sunken (Ackert and Muldoon, 1920).

Cases of Type I ostertagiosis result from larvae ingested 3 to 4 weeks previously and are most commonly seen in calves in their first season at grass. Consequently, the diarrhoea is typically bright green and persistent. Anaemia is not found in Type I infections, although when diarrhoea is severe packed cell volume percentage may increase. Armour (1970) stated that clinical signs usually became evident when burdens of 40,000 L3 or more had accumulated at grazing. In Type I outbreaks, disease morbidity is high and 75% of stock will frequently be affected. Providing treatment is instigated within 2 to 3 days of the start of clinical signs the percentage mortality will be low.

The period of larval arrest, when the host is said to be in the Pre-Type II stage, usually goes unnoticed in ostertagiosis. Consequently, the Type II disease is only recognised in older stock after their first season at grass, once larval maturation has resumed (Selman, Reid, Armour

and Jennings, 1976). The diarrhoea is profuse, but intermittent. Anorexia, thirst and hypoalbuminaemia are more marked in Type II ostertagiosis. Halliday *et al.* (1965) showed there was a percentage decrease in body solids relative to total body weight in Type II disease, which was not seen in the Type I infection. There is a decrease in total serum protein, with a drop in the albumin:globulin ratio. Submandibular and brisket oedema may be present. Anderson *et al.* (1965b) observed that a moderate anaemia may be seen in Type II disease; the packed cell volume, haemoglobin concentration and red cell count may be decreased. The appearance of the red blood cells indicates the anaemia is normocytic and normochromic in form.

The morbidity in herds affected by the Type II disease in an outbreak is lower than for Type I ostertagiosis, but the prognosis for infected animals is poorer since the condition has usually advanced further by the time the disease is diagnosed. Clinical Type II disease occurs when large numbers of larvae resume development simultaneously. In some stock Type II larval maturation may be too asynchronous to cause clinical signs (Williams *et al.*, 1984), but the faecal egg output will still contribute to pasture contamination. Michel *et al.* (1976a) suggested that a failure in host resistance could lead to the acceleration of signs in Type II infections. They found the smallest or youngest calves in a group developed Type II signs earlier, i.e., in January rather than March in the United Kingdom.

1.5 DIAGNOSIS

The seasonal occurrence of clinical signs is highly suggestive of ostertagiosis, i.e., reduced appetite, weight loss and profuse diarrhoea from July to September for Type I disease and March to May for Type II disease in the United Kingdom. The whole herd should be examined, particularly in suspected outbreaks of Type II disease, since the time of parasite development may vary with different individuals. A reliable grazing history may be helpful, i.e., records of previous outbreaks, grazing rotations, etc.

Faecal egg counts of greater than 1,000 epg are noted in Type I disease. Generally higher counts are found in more severely infected

individuals. Unfortunately parasitological examination of faeces is not as helpful in Type II cases, since the egg count does not rise until pathological changes are well-established and the rise in egg count itself may be highly variable (Martin *et al.*, 1957; Armour, 1970).

Plasma pepsinogen concentrations may be a useful indication of infection in calves in their first grazing season. Selman *et al.* (1977) suggested plasma concentrations of greater than or equal to 3,000 mU tyrosine were a significant indication of ostertagiosis. However, Hilderson, Berghen, Vercruysse, Dorny and Braem (1989) proposed that many factors could result in levels of greater than 3,000 mU in clinically normal calves:

1) testing irregularities
2) a naturally higher circulating level of pepsinogen in older helminth naive calves
3) a possible hypersensitivity response to reinfection with low parasite numbers
4) apparent ability of calves in good nutritional condition to withstand high parasite burdens.

They proposed that a new threshold for the identification of clinical disease be set at 5,000 mU tyrosine and above, while other evidence of infection would be necessary to confirm a diagnosis at lower pepsinogen concentrations.

Pepsinogen concentrations are less significant in the diagnosis of Type II disease, since they may be highly variable and seldom rise as markedly as in Type I disease (Clements, Hamilton and Redahan, 1977) and as the review by Hilderson *et al.* (1989) indicated plasma pepsinogen concentrations in older calves are generally higher. In addition, Michel *et al.* (1978a) noted that peak pepsinogen concentrations occur after calves have started to recover and gain weight again. The drop in plasma pepsinogen concentration with loss of parasites was very slow, which they felt reduced the suitability of the pepsinogen test as a good method of diagnosis of the Type II disease, since it gives a poor indication of the parasite burden at the time of blood sampling. Furthermore, they found the peak pepsinogen concentration was the same and the pepsinogen profile was similar

regardless of the size of infectious dose and the proportion of the dose developing or arresting.

It has been suggested that increased gastrin levels may be used as a diagnostic tool for ostertagiosis. A threshold burden of parasites must be present before gastrin secretion occurs (McKellar *et al.* 1987). However, unlike plasma pepsinogen concentration, circulating gastrin concentrations drop rapidly on recovery from infection and may consequently be of greater use in diagnosis of Type II infections, although the disease is in an advanced stage before recognition is possible. Gastrin secretion is influenced by roughage and calcium intake (Schillhorn van Veen, 1986), so care in the interpretation of results would be required.

It is clear that it is harder to reach a diagnosis before the disease is advanced in Type II disease, since the pepsinogen concentration, gastrin level and faecal egg count are variably affected and the changes are not apparent until the more synchronous parasite emergence has occurred. Often the post-mortem examination of a suspected Type II case is still the best method of arriving at a quick conclusion, i.e., when weight loss, moderate anaemia and anorexia due to no other obvious cause have been demonstrated.

That a wide spectrum of pathological changes and clinical signs may arise from infections with *O.ostertagi* has been emphasised in the preceding sections and these in their turn appear to be influenced by a complex of inter-related factors. The factors affecting *O.ostertagi* infections may be broadly divided into parasite, host and environmental effects. The experimental work in this study is directed at improving knowledge of the parasite effects on infection, but it is important that these are considered in the light of other available epidemiological information since the factors form an intricate network.

1.6 EPIDEMIOLOGY

Background knowledge indicates that many factors influence the progression and outcome of ostertagiosis. Examination shows these

factors are closely inter-related, but here, for the sake of discussion, they have been roughly subdivided into three categories:

1) the environment,
2) the host,
3) the parasite.

1.6.1 The environment

Seasonal changes in temperature and rainfall, the local climate, have an obvious effect on the development of the free-living stages of *O.ostertagi* and thus, the level of challenge on the pasture. In addition, the climate appears to influence the channeling of the nematode through either a direct or interrupted/arrested form of the parasitic life cycle. Perpetuation of the disease from one year to the next may rely on the parasite ability to withstand harsh climatic extremes on the pasture or by arresting in the host. Periods of inhibition tend to coincide with seasons of extreme environmental hardship for free-living forms, suggesting hypobiosis may prove to be a most useful adaptive technique for nematode parasites (Armour and Bruce, 1974).

1.6.1.1 Development of the free-living stages

Development of the egg to the infective larva on the pasture commences at a mean day-night temperature of greater than 10°C, so in the United Kingdom the cycle begins around April with infectious larvae on the pasture approximately 9 weeks later. As the mean temperature rises so the time taken to complete the step is reduced until it may be completed in 1 week, while as autumn approaches the time period increases again. Consequently, in the United Kingdom, Armour, Jennings and Urquhart (1969) suggested that overwintered eggs probably do not reach the infective stage before May, so infective larvae present on the pasture in early spring must have overwintered there. Only low levels of infection are usually acquired in the spring, these tend not to result in disease or stimulate an effective immunity. These infections are important since they lead to an amplification of pasture contamination resulting in clinical cases later in the season. From June, pasture larval contamination has usually risen, so sufficient infective larvae will be ingested to cause disease.

Both the egg and the infective larva exhibit an ability to survive climatic extremes. In exceptional cases pasture has remained contaminated over successive winters without further grazing by infected stock, e.g., for 4 years in Kentucky as noted by Drudge, Leland, Wyant and Rust (1958). However, pasture contamination generally drops rapidly from 2 months after carrier animals have been removed (Goldberg and Rubin, 1956). In the United Kingdom, overwintered larvae on the pasture have little ability to survive beyond the following June. Consequently, if contaminated pasture is not grazed until late in the season larval numbers will be reduced and pasture growth will dilute their effect further; these facts have been incorporated into some pasture management schemes.

Nematode larvae may be able to overwinter in the soil, as work on *Dictyocaulus viviparus* has suggested (Duncan, Armour, Bairden, Urquhart and Jorgensen, 1979). It would be interesting to speculate whether *O.ostertagi* could survive in a similar manner. The method of larval migration into the soil is uncertain, although it has been suggested that earthworms or beetles may be involved. Gronvold (1979) compared migration of *O.ostertagi* from faecal pats with and without earthworms present. Fifteen times more larvae were found in the top 1cm of soil 50 days after faecal deposition when earthworms were present. Faeces passed by earthworms and their intestinal contents were positive for live *O.ostertagi* larvae. He proposed that earthworms could act as mechanical vectors for *O.ostertagi* and when these vectors are ingested by birds or moles farm to farm transmission might occur. However, he also suggested that the action of earthworms and birds breaking up the faecal pat could increase its desiccation and so decrease the number of viable larvae remaining (Gronvold, 1989).

Cattle tend to discard grass growing in close proximity to faecal pats (Leaver, 1970), so movement away from the pat is more likely to result in ingestion by the host. Anderson (1986) claimed that oxygen deficiency and temperature fluctuations markedly affect migration from the pat. However, moisture is the main requirement for larval migration, thus, in dry summers major infection does not occur until

relatively late, i.e., in the autumn (Armour, 1970). The late infection leads to an increase in the proportion of larvae arresting at the early fourth stage in the abomasal glands. Gronvold (1989) found the effect of rain splash increased dispersion of larvae from the pat; larvae were most easily displaced from pats that had been previously watered.

The effect of climate on the free-living stages has demanded the development of specific survival mechanisms. Climate may influence the level of pasture contamination and thus uptake by the host, but more surprisingly the external environment appears to have a degree of power over the development of parasitic stages.

1.6.1.2 Arrested larval development

The cause of arrested larval development is not fully understood, but there are seasonal differences in its pattern of appearance with locality, implying a close link with the environment. In the United Kingdom, although arrest occurs throughout the year, with 3 to 21 % of the burden affected even in the spring, an increasing proportion of the worm burden inhibits from September onwards (Armour *et al*. 1969). By October 60% of the parasite population is in the Pre-Type II state (Armour *et al*. 1969). The numbers of late L4 and L5 recovered at necropsy remain low until late February, since the majority of larvae are in the arrested early L4 stage. The reversible state of suspended/retarded animation is often likened to the diapause phase of insects. Resumption of development results in the Type II syndrome between March and May with a peak adult population in April (Michel *et al*. 1976a).

In the Southern hemisphere inhibition occurs most often in the spring with increasing temperature and dry weather, e.g., in South Brazil (Suarez, 1985) and Argentina (Pinheiro, Echevarria and Branco, 1978), suggesting that a different stimulus to inhibit may be involved (Armour, 1978). In Northern Nigeria, Hart (1964) found egg counts for *H.contortus* and *Trichostrongylus axei* rose towards the end of the dry season, which runs from October to April/May, since arrested parasites began to resume development. Minimal levels of infection were obtained in the dry season and larval arrest allowed continuation

of the life cycle by avoiding exposure to the hot desiccating pasture conditions.

Arrest occurs slightly later in the autumn-winter season in New Zealand than in the United Kingdom and the Type II syndrome is rarely encountered unless cattle are subjected to additional stress (Brunsdon, 1972). The peak arresting burden is only 8 to 10% of the total burden and Bisset and Marshall (1987) suggested that larvae from New Zealand may have a reduced ability to arrest compared to British larvae or that the phenomenon is not recognised since the resumption in activity is too gradual to produce clinical signs, although the mild climate may be a further factor.

Climate appears to be an important factor in the triggering of arrest, since by cold conditioning larvae for 6 to 8 weeks at 0 to 4°C arrest may be induced in the United Kingdom in summer. Although prolonged conditioning, i.e., for 19 weeks leads to a reduction in the proportion arresting and after 33 weeks selective mortality of types with a propensity to arrest has been found, since no larval inhibition resulted with infections (Armour and Bruce, 1974). Armour *et al.* (1969) showed that approximately 70% of the burden would arrest to produce Type II infections regardless of the ingested dose. However, in Australia, Smeal and Donald (1982a) found that storing L3s up to 10 weeks at 4°C caused only 10% of the population to arrest, i.e., there appeared to be a relatively poorer response to cold conditioning from the Australian larvae. The natural peak inhibition time in Australia is the spring relative to the late autumn for British larvae.

Variation in pattern of arrest does not appear to be influenced by local climate alone. Transfer experiments have shown that an additional explanation is that variation in arrest is caused by genetic differences in the parasite, which in their turn may be affected by the climate and host management factors; these factors will be discussed in the appropriate sections.

1.6.2 The host

The host may contribute to the effect of development and outcome of ostertagiosis by a number of different ways. Hosts may be considered at a number of grouping levels; individuals are known to respond differently to nematode infection, as may particular breeds; herds under different management systems vary in their tendency to infection and their susceptibility to larval arrest. Host immune status and age, sex and its hormonal influences, nutritional status and the effects of intercurrent disease may also play a directional role in the outcome of infection either individually or in association with each other.

1.6.2.1 Level

Individuals within herds may develop ostertagiosis to varying degrees as the clinical signs and pathology will demonstrate; this appears to be due to differences in susceptibility and larval uptake. Parasite uptake may be affected by grazing pattern. Stear, Hetzel, Brown, Gershwin, MacKinnon and Nicholas (1990) showed calves with certain bovine major histocompatibility antigens, CA36 and W7, had lower faecal nematode egg counts, when grazed on pasture with a mixed endemic infection. Faecal worm egg count showed higher heritability than milk production in dairy calves and growth of beef calves.

No information is currently available on the effect of host breed on outcome of infection with *O.ostertagi*, although host breed is known to alter susceptibility to infection with nematodes. Stewart, Miller and Douglas (1937) examined susceptibility of five different sheep breeds to *O.circumcincta*. The least susceptible was the Romney Marsh while the Hampshire was very susceptible. They also noted, however, that there was a large variation in susceptibility of individuals within a breed to the parasite. In 1964, Scrivner compared *Ostertagia* spp. infections in sheep over three successive lamb crops with Targhee, Suffolk, Rambouillet and Hampshire sheep. Targhee sheep were found to have a greater resistance to ostertagiosis by assessment of faecal egg count. The difference in infection levels was attributed to breed genetic differences. Preston and Allonby (1979) compared six sheep breeds for their susceptibility to *H.contortus*. The Red Maasai was

found to be the least susceptible breed and susceptibility increased as follows: Blackhead Persian, Merino, Dorper, Corriedale and Hampshire. Self-cure occurred most frequently in the Red Maasai and in addition their strongyle egg count was low, anaemia seldom occurred and acute clinical cases were rare. They were found to have higher levels of anti-IgA specific to the parasite in the abomasal mucosa than Merinos.

In the United Kingdom, dairy calves suffer from ostertagiosis more commonly than beef calves due to the different management practices. Dairy calves are either born in the autumn and housed over the winter or they are spring-born. They are taken from their dams soon after birth and reared on artificial milk before rapid weaning. They often graze on one pasture until July/August, when they are moved to aftermath grazing only to return later to the first pasture, however, the pasture larval counts will have peaked by this time due to amplification in calves earlier in the season.

The situation is different in beef production systems, where the calves suckle their dams for a longer period. Springborn calves do not usually ingest sufficient herbage to develop disease until the level of overwintered larval contamination has dropped. Additionally, they graze alongside adults with acquired immunity, which contribute only very low parasite egg numbers to the pasture. Autumn born calves are more likely to develop ostertagiosis since the timing of weaning coincides better with pasture contamination, but whether a Type I or Type II infection occurs depends on the management system.

Borgsteede and Eysker (1987) proposed that a seasonal variation in host resistance to infection might have an effect on ability of the parasite to arrest, i.e., environment may affect host and thus arrest. Host management system also appears to have a major effect on susceptibility of parasites to arrest. In Australia, larvae in beef calves have shown a greater propensity to arrest than those in dairy calves. Smeal and Donald (1982b) observed that beef calves are born in one season and graze alongside their dams until weaning, while dairy calves are born throughout the year and are reared away from their dams. Contamination on the beef pasture is low, because the adults are

immune; therefore, larval arrest is required to maintain the parasite life cycle. Pasture contamination under the dairy system is high and sufficient parasites will survive to maintain the infective cycle. They concluded that the production system could inflict selection pressure on the parasite affecting its requirement to arrest.

1.6.2.2 Immune status and age

Immunity may be considered to consist of innate and adaptive components. Development of immunity to ostertagiosis is acquired and age at infection appears to have a significant effect, an observation which should be considered when making decisions about stock management. In areas of the United Kingdom where *O.ostertagi* is endemic, immunity usually develops during the first grazing season, since infection is encountered at low levels initially, although greater numbers of infective larvae begin to develop on the pasture with higher temperature and with increased grass intake on weaning. The development of immunity may explain the end of grazing season drop in parasite burden recognised in September by Anderson, Armour, Jennings, Ritchie and Urquhart (1969). Disease is seen particularly when the balance is upset in favour of the parasite, e.g., early good weather results in high numbers of infective larvae on the pasture earlier in the season. Infection may be seen in older stock, which have previously had no contact with the parasite when they are moved into an endemic area.

Michel, Lancaster and Hong (1973a) demonstrated the acquisition of immunity to *O.ostertagi* by giving daily doses of 1,000 L3 to calves for varying time periods (30, 80, 150 and 250 days). Later, a challenge dose of 30,000 L3 was administered and the level of established parasites assessed on necropsy. Lower nematode burdens established in the calves that had received larval doses over increasing time periods. With the longest immunising schedule - 250 daily doses - the burden recovered was only a twentieth of that found in susceptible controls.

There is no evidence that older animals naturally have higher immunities to ostertagiosis, although they may be better able to

neutralise the effects of infection and develop immunity faster. Smith (1970) compared gastrointestinal infections in 3-month-old and 1-year-old calves when one of the following species was administered: *O.ostertagi*, *Cooperia oncophora* and *Nematodirus helvetianus*. He found parasite establishment in both age groups, but the faecal egg counts were higher in the younger calves. The difference between the egg counts was lowest in the case of *O.ostertagi* infected groups. Furthermore, Michel, Lancaster and Hong (1979) noted that resistance to infection was acquired faster by a 20-month-old heifer compared to a 19-week-old calf, though there appeared to be no significant difference in the peak faecal egg count or plasma pepsinogen concentration.

When only a low level of immunity is acquired in the first grazing season, immunity may wane while the calves are housed over the winter, so they are susceptible to infection in their second grazing season. However, immunity is usually rapidly re-established in this situation and clinical signs are transient if present.

Development of immunity may be strongly influenced by the use of prophylactic anthelmintic drug treatments. Armour (1989) noted that rigorous treatment with highly efficacious anthelmintics in the first grazing season might prevent build-up of immunity and lead to infection resulting in disease later in life. Less efficient anthelmintics allowed better development of protective immunity. Petrie, Armour and Stevenson (1984) reported an outbreak of Type II ostertagiosis in dairy cows. Anthelmintic control had been rigorously enforced in their first grazing season, but in their second grazing season they were moved onto a heavily contaminated pasture. The target milk yields were not realised and increasing deficiency of production correlated with raised plasma pepsinogen concentrations. Cases of severe Type II disease occurred at calving the following spring.

Klesius (1986) claimed that our understanding of immunity to *O.ostertagi* is poor, but that immunity develops slowly and appears to be incomplete. He proposed that *O.ostertagi* may impair the antibody and cell mediated immune responses and so survive better in the young host with an incompletely developed immune system. The host

immune status is influenced by many factors: exposure, time, age, endocrine secretions and prophylaxis. Consequently, the effect of immune status on the outcome of disease may itself be affected by many variables.

1.6.2.3 Sex

Little information is available on the effect of host sex on susceptibility to parasite infection in the veterinary field, let alone *O.ostertagi* itself. However, information available for human and laboratory animal infections suggest that host sex may bias susceptibility to infection through variation in immunity (Alexander and Stimson, 1988) and behaviour characteristics (Bundy, 1988). Alexander and Stimson (1988) pointed out that the endocrine system is involved in regulating immunity and could give rise to a different response to parasitism depending on host sex. In particular, they mentioned a lower cell mediated immune response and higher immunoglobulin levels in females compared to males, while generally the incidence of helminth disease is higher in males. Murray, Jarrett and Jennings (1971) found expulsion of *Nippostrongylus brasiliensis* from rats occurred earlier and more rapidly in female rats than male rats. There appeared to be an earlier rise in mast cell activity associated with expulsion in females. Waddell, Jarrett and Murray (1971) found that castration of infected males accelerated expulsion of the parasite and concluded that hormonal effects were involved in rate of parasite expulsion. Stear *et al.* (1990) found male calves grazing pasture contaminated with mixed gastrointestinal nematode species had significantly higher faecal egg counts.

Lowering of immunity to nematodes frequently occurs in the periparturient season. Lactating dairy cows frequently harbour a low parasite burden (Todd *et al.* 1975; Bairden and Armour, 1981) and anthelmintic treatment has been shown to improve productivity with varying success. The reduction in milk yield has been partially attributed to a hypersensitivity reaction with abomasal oedema and increased permeability of the abomasum. There appears to be a reduction in lymphocyte-mediated response to nematodes peripartum and it has been suggested that increased prolactin levels at the time might play a role. In sheep

injected with a prolactin antagonist no alteration in the pattern of increasing egg count peripartum was noted, implying that prolactin is not the sole cause of the phenomenon (Jeffcoate, Fishwick, Bairden, Armour and Holmes, 1990).

Consequently, host sex should not be discounted as a possible influencing factor to the development of disease, although its effects are poorly understood.

1.6.2.4 Nutritional status

Protein digestion is reduced in the parasitised abomasum, so it is essential to ensure high quality diet is available if a calf's condition is not to deteriorate too severely. Unfortunately, frequently a reduction in appetite is seen in clinical and sub-clinical cases of bovine ostertagiosis, due to the change in protein digestion and also in association with increased gastrin levels. However, it is possible for calves in good nutritional condition to carry nematode burdens subclinically at levels normally associated with disease. In some cases these calves may be recognised only by their unexpectedly high pepsinogen levels on testing (Hilderson *et al.* 1989). In sheep infected with *H.contortus* on a low plane of nutrition higher faecal egg counts were detected than in sheep on a high plane of nutrition (Preston and Allonby, 1978).

1.6.2.5 Intercurrent disease

Little specific information is available about the occurrence of other diseases simultaneously with ostertagiosis. A recognised complex is seen when the liver fluke, *Fasciola hepatica*, is also present, causing a severe haemorrhagic anaemia.

In reality, gastrointestinal parasite populations are seldom purely a single species. Superimposition of different species may have a profound effect on the establishment of individual species. Comparison of infectivity and pathology with *Ostertagia* spp. *in sensu lato* showed that when *O.leptospicularis* was included, severity was potentiated (Al Saqur *et al.* 1982a; Al Saqur, Armour, Bairden, Dunn,

Jennings and Murray, 1982b). Infection may cause immuno-suppression, so the host may become more susceptible to further parasitism. Conversely, simultaneous infection with different parasite species may result in competition for available resources. Frankena (1987) examining interaction of infections between *C.oncophora* and *O.ostertagi*, found the combination led to no "epidemiological effect", i.e., no change in burden, faecal egg count, fecundity or expulsion time. Although previously Kloosterman, Albers and Van den Brink (1984) had shown a negative interaction between sequential infections with *O.ostertagi* and *C.oncophora*, in either order. Priming by the first species led to a reduction in vulval size, worm length, number of ova and burden when the other species was first encountered. They suggested an immune response was the likely cause, probably a shared antigen, since concurrent infection did not produce the same effect. Consequently, the presence of one parasite species may enhance or reduce the establishment of *O.ostertagi*, so affecting the outcome of disease.

Obviously it can be seen from the environmental and host factors discussed that the disease situation can assume an infinite number of permutations. The parasite factors which affect the development and outcome of disease are those of biotic potential, drug sensitivity, antigenicity and virulence. These factors also form the basis for many of the instances of suspected intraspecific variation and are consequently covered in full in the following chapter.

1.7 CONTROL

Possible eradication of parasite disease has been considered by Spedding (1969). In theory the concept was ideal, but should infection reappear the levels of immunity would be very low, so he proposed that keeping disease at a low level would be more desirable. In practice, the aim is to permit sufficient exposure to induce immunity, but without producing evidence of disease.

1.7.1 Traditional methods of control-anthelmintics and pasture management

Currently, parasitic gastroenteritis of ruminants is controlled by strategic prophylactic anthelmintic dosing and pasture management. Ninety-two per cent of farmers questioned in a survey conducted in South-West Scotland carried out grazing management, complemented with anthelmintic control in some cases. The average number of treatments for beef calves was 1.5 per annum and that for dairy calves two treatments per annum (Gettinby, Armour, Bairden and Plenderleith, 1987).

A vast array of anthelmintic drugs and regimens are available to farmers, e.g., avermectins (subcutaneous injection, pour-on), benzimidazoles (drench, intra-ruminal bolus, feedblock), levamisole (drench) and morantel/pyrantel tartrate (intra-ruminal bolus). The preparations vary in their efficacy, duration of action, mode of administration, withdrawal times prior to slaughter and cost. The final choice depends on what suits an individual farmer taking into account husbandry and the typical parasitic problems of the area. To avoid the rapid build up of resistance the same anthelmintic should not be used continuously. Anthelmintic schemes will aid the reduction in pasture contamination and should be enforced with a knowledge of the local disease patterns.

Leaver (1970) compared the weight gain of calves on contaminated pasture when grazed in rotation with heifers either by random pasture use or by grazing the calves ahead of the heifers under a leader-follower system. Half the calves from each group were drenched weekly from late summer onwards. The most significant improvement to weight gain appeared to come from enforcing the leader-follower method of pasture management.

Using the local epidemiological features of the infection, the optimal pasture management system can be employed to reduce pasture contamination. Pasture may be rested beyond the start of a grazing season until the viable overwintered portion of the larval population has decreased, or the land may be used for alternative crops or hosts, i.e., hosts which do not allow *O.ostertagi* to establish. Naturally, the

availability of safe pasture will vary with individual resources. Pasture management and anthelmintic dosing are effective, but they are vulnerable to the vagaries of the weather and human error.

1.7.2 Recent advances and future approaches in control of helminth disease

Currently with increasing awareness of drug related problems, there has been a move to find other methods of helminth parasite control. Many nematode parasites have recently been shown to be developing increasing drug resistance (Chapter 2). Public fears over the long term effect of certain anthelmintics (avermectin) on the environment have been aroused. Drug residues in bovine faeces may effect the ecosystem, since the dung beetles which aid faecal pat breakdown can be destroyed by the insecticidal effect. Further research is required into drug residues, since many factors are involved in the anthelmintic chemical effect, e.g., drug delivery system (the slow release forms having a more lasting effect on the environment) and decomposition, which varies with geographical location and season (Wall and Strong, 1987; Campbell, 1988; Wall and Strong, 1988).

1.7.2.1 The use of host factors to reduce pasture contamination

In theory where no anthelmintic control is employed those hosts adversely affected by the parasite will naturally be selected against (Keymer and Read, 1990). There is no recorded information concerning bovine host resistance to *O.ostertagi*. However, there is increasing proof to show that variation in resistance to nematode infection occurs. With respect to cattle, the trypanotolerant N'dama and West African shorthorn breeds appear to be more resistant to helminthosis as cited by Murray, Morrison and Whitelaw (1982). Certain bovine major histocompatibility antigens have been associated with lower nematode egg counts in areas of endemic *Bunostomum phlebotomum*, *Oesophagostomum radiatum*, *T.axei*, *H.placei* and *Cooperia* spp. (Stear *et al*. 1990).

Albers, Gray, Piper, Barker, Le Jambre and Barger (1987) looked at the genetics of resistance and resilience to *H.contortus* infection in young merino sheep. They found faecal egg count and haematocrit

were heritable traits indicating a degree of resistance, while resilience to infection as measured by depression of productivity was not a significantly heritable trait. Gray (1987) reviewed a study of resistance to haemonchosis looking at different breeds and heritability of traits. Red Maasai sheep were found to have higher ~~circulating~~ mucosal IgA and they were better able to resist establishment of the nematode relative to Merinos. However, the gain in resistance occurred at the expense of meat and wool production. Further work with selective breeding of merinos has suggested genetic correlations between resistance to haemonchosis and production traits which may be used in the future for selection of breeding stock (Gray, 1987). A particular ram, the Golden Ram of Armidale was reputed to carry a gene for major resistance to *H.contortus*, which led to a significant reduction in egg count. However, comprehensive studies on the genetics of resistance have not yet been completed.

It is of interest that a fresh isolate of *H.contortus* showed no apparent alteration in infectivity, when it was subjected to selection pressure. Albers and Burgess (1988) passaged the isolate over nine generations in susceptible (immunosuppressed) sheep and six generations in resistant (repeatedly infected) sheep. The resulting parasite progeny when compared by infection of susceptible sheep showed no significant differences.

These reports suggest a possible future application for the selection of sires to try to improve host resistance without simultaneous adverse effect on the production traits or parasite adaptation. Gray (1987) reported that common genes regulate the response to infection in both high experimental infections or in natural infections. Two main advantages of using resistant animals are the lowering of pasture contamination levels and a decrease in drug requirements.

1.7.2.2 The use of predacious fungus to reduce pasture contamination

To try to decrease pasture contamination, workers are examining the effect of increasing levels of fungi that attack the free-living larvae in the faecal pat (Gronvold, Nansen, Henriksen, Thylin and Wolstrup, 1988). Certain fungi have been found to produce sticky networks for

trapping larvae (predacious fungi), others directly infect larvae with spores (endoparasites) and another group penetrate eggs or larvae with vegetative hyphae (Gronvold, 1989). Direct addition of the predacious fungus to the pasture was not found to aid control. Hashmi and Connan (1989) suggested that improving the pasture environment for fungal growth might be more successful. They found regular feeding of the fungus to cattle led to no reduction in pasture larval levels and produced only low fungal concentration in faeces.

1.7.2.3 Vaccination

Vaccination would appear to be an ideal method of control of ostertagiosis, but unfortunately despite major efforts no effective vaccines have been produced. Armour (1970) suggested that the difficulty in inducing immunity to gastrointestinal nematodes could be linked to the slow development of immunity in natural infection. Generally, there is a poor immune response to multicellular organisms in the digestive tract (Pritchard, 1986). Pritchard (1986) pointed out that frequently infection with nematodes is caused by more than one species and suggested this could be a further reason for difficulties in vaccine production.

A successful vaccine stimulates the immune response to prevent the course of a disease, without producing clinical signs. There are many variables in the approach to vaccination, particularly with respect to dose, frequency and route of immunisation and selection of a suitable immunogen with or without the addition of an adjuvant. The major problem has been the identification and selection of a suitable immunogen. Murray, Robinson, Grierson and Crawford (1979) summarised work done in the immunization of rats against *N.brasiliensis*. They suggested the use of adjuvants to stimulate the appropriate inflammatory cells and multiple vaccine doses to aid generation of the immune response. In addition, the route of vaccine administration and adjuvant concentration were found to affect protection, as measured by a drop in egg count and an increase in intestinal mast cells. Certain stages of the life cycle may appear more immunogenic, since variation in antigenic properties have been recognised and so the optimum stage should be ascertained for vaccine

production (Philipp, Parkhouse and Ogilvie, 1980; Kennedy and Qureshi, 1986; Abraham, Grieve and Mika-Grieve, 1988; Cox, Sharmansky and Boisvenue, 1989). Once a vaccine has been shown to induce immunity, it is important to assess its longevity of efficacy (Miller, 1986).

Live attenuated nematodes

The only successful vaccine commercially available directed at parasitic nematodes consists of an irradiated L3 suspension of *D.viviparus* given orally. Bain and Urquhart (1988) found the vaccine was also effective by subcutaneous injection with the same dosing regime, giving greater assurance of correct administration. Consequently, it appears that it was not essential for parasites to pass through the intestinal wali and mesenteric lymph nodes to stimulate immunity against parasitic bronchitis. A similar vaccine for the sheep lungworm, *D.filaria* is available in areas where the parasite is a problem. In the past, an attenuated vaccine was available against the canine hookworm, *Ancylostoma caninum* and it was used to great effect, but was later removed from the market for commercial and licensing reasons.

Whole nematodes and their extracts

Clegg and Smith (1978) discussed the failure or marginal success of whole nematode extracts in generation of immunity. Different routes of administration and varying immunisation schemes using live *O.ostertagi* L3 have not succeeded in producing protection. Michel *et al.* (1973a) reduced establishment of a challenge dose of L3 after periods of low daily infections. Williams, Roberts and Todd (1974) gave intravenous, subcutaneous and intraperitoneal infections of L3s. No protective immunity was induced. Solely by the intravenous route a patent infection arose and reaction in pulmonary tissue was evident. Although when Rose (1976,1978) performed similar experiments in sheep results were more promising. *In vitro* grown *O.circumcincta* L3, L4 and L5 were given subcutaneously/intramuscularly and their metabolites were given orally/intramuscularly. After oral challenging a marginal ~~protection~~ immune reaction was recognised by a decrease in faecal egg count, parasite burden and worm dimensions with all these protocols. When

administration of low larval doses (25,000 to 50,000 L3) were followed by a low challenge dose (1,000 to 25,000 L3), 36-56% fewer parasites established compared to the numbers in susceptible calves (Herlich, 1976). The nematodes found in the "immunised" calves were smaller and less fecund. However, pathogenic doses produced clinical signs of similar severity in the "immunised" and susceptible calves. Herlich and Douvres (1979) gave calves a series of intraperitoneal doses of *in vitro* grown L3, the recovered culture medium or exsheathed later larval stages. No significant immunity was raised in these tests.

Excretory-secretory products

Attempts to use excretory-secretory products (ES products), i.e., material shed from the nematode surface or excreted metabolic products, have not resulted in any successful commercial vaccines, although they have been observed to generate strong protection, e.g., against *N.brasiliensis*, *Toxocara canis* and *Trichinella spiralis* in mice, *H.contortus* in lambs and *Trichostrongylus colubriformis* in guinea pigs (Maizels and Selkirk, 1988). Previously in work cited by Clegg and Smith (1978), antibody was observed to precipitate around the mouth parts of *N.brasiliensis*, when serum from rats previously immunised against the nematode was added to culture medium, which was thought to imply the precipitation of antibodies with antigens produced or present in the region. Culture fluid from *O.ostertagi* produced no immune protection when tested by Herlich and Douvres (1979), although concentrated metabolites from *in vitro* produced *O.circumcincta* reduced burdens and faecal egg counts, when 3-month-old lambs were immunised (Rose, 1978). Lyophilised material induced no protection. ES from *O.radiatum* L3/L4 induced primed lymphocyte proliferation (Gasbarre, Romanowski and Douvres, 1985). A possible problem of the work done to date on immunisation with ES products has been the use of *in vitro* produced material, while there is still inadequate information regarding possible differences between *in vitro* and *in vivo* produced material.

Lightowlers and Rickard (1988) suggested that vaccine release close to the site of action might be necessary for immune response production against ES antigens. Their work suggested that an ES immunogen

would have to be in close contact with the gastrointestinal tract on administration to raise sufficient immunity, which could present a practical problem.

Internal parasite components

Clegg and Smith (1978) referred to experiments to immunise pigs and guinea pigs against *Ascaris suum*. The malic dehydrogenase of the parasite was found to be a different isoenzyme from that in the host. The enzyme isolated from the nematode successfully produced protection in guinea pigs, but only partial immunity in pigs. Contortin, a component of the intestinal lumen of the L4 and particularly adult of *H.contortus* was used as an immunogen for sheep. The intestinal lumen is bathed in host blood during its digestion, so contortin is readily exposed to the immune system. The contortin preparation was found to give good levels of circulating antibody and protection against infection in the majority of sheep (Munn, Greenwood and Coadwell, 1987).

Heterologous vaccines

Clegg and Smith (1978) mention unsuccessful attempts to produce immunity by heterologous species vaccines by making use of the degree of cross-reaction between species. The example quoted was for work done with *Caenorhabditis briggsae* and *H.contortus*.

Although no successful vaccines have been produced against gastrointestinal parasites, in the wake of advances in other microbiological areas and improved knowledge of parasite biology and immunology, fresh approaches to the problem are constantly being revealed. Maizels and Selkirk (1988) proposed that some form of biological interaction is required between host and parasite for induction of immunity, which may explain why greater success has been attained with living irradiated parasites or soluble products, compared to dead organisms or somatic extracts.

Once antigens with protective abilities have been identified, advancing technology may allow their generation for vaccination purposes. For commercial purposes the facility to produce large amounts of pure

immunogen is important, though the only stage when amplification of the nematode parasite occurs is at egg production. Consequently, facilities for an *in vitro* culture system would be useful. In cases when the culture conditions for a species have been established, they are strictly defined, often unique and require a large labour input for their maintenance. Fortunately with advancements in cloning and vector techniques, alternative sources of purified antigen for vaccination may be available in the future.

In conclusion, novel approaches to anthelmintic control are required, since none of the current methods is without drawbacks. A more complete understanding, not only of the factors that affect host susceptibility, but variation in the biological potential and antigenicity of the parasite are the key to increased knowledge of parasite antigens related to development and differences in immunogenicity, which could lead to strategic advancements in control and classification. From the evidence outlined in the following chapter on intraspecific variation, it is becoming apparent that biological adaptations of strains and species have significant modulating effects which should be considered in conjunction with control schemes. Thus, in this study comparison of different parasite isolates with varying drug susceptibility, biological potential, with respect to arrested larval development, and geographical origin were examined.

CHAPTER 2

INTRASPECIFIC VARIATION OF NEMATODES WITH EMPHASIS ON *OSTERTAGIA OSTERTAGI*

INTRASPECIFIC VARIATION OF NEMATODES WITH EMPHASIS ON *OSTERTAGIA OSTERTAGI*

Intraspecific variations occur when differences are indicated between members of a species, allowing further subdivision and classification. However, intraspecies differences are not so great that sterile hybrids are generated on interbreeding of the group types, as would result from interspecies breeding.

The concept of intraspecific variation in parasites is not new. Strain differences in the parasitic protozoa have been recognised with respect to virulence, immunogenicity and drug susceptibility (Allsopp and Allsopp, 1988; Walliker and Sanderson, 1976; Knowles, Sanderson and Walliker, 1981). Helminth infection may result in a wide range of outcomes, but previously these differences were attributed to variation in the host (nutrition, management, age, sex, breed), and variation in the environment (climate, level of infective challenge), while the parasite was not considered as a source of variance in infections *per se*. Lack of uniformity of experimental design in recorded work has complicated examination of parasite variation.

However, there is now increasing evidence that variation within helminth species contributes to the outcome of infection. Apparent or suspected variation in the following qualities led to the change in thinking:

1) Morphological traits
   - i) Structural polymorphism.
   - ii) Continuous variation.
2) Biotic potential
   - i) Infectivity.
   - ii) Ability to arrest.
   - iii) Fecundity.
3) Antigenicity.
4) Virulence.
5) Susceptibility to anthelmintic drugs.

2.1 MORPHOLOGICAL TRAITS

2.1.1 Structural polymorphism

Structural polymorphism is possibly the most conspicuous of the characteristics to vary within a population, i.e., within a particular host. Lancaster and Hong (1981) cited Ford (1953) who defined polymorphism as "the occurrence together in the same habitat at the same time of two or more discontinuous forms of a species, the rarer (rarest) of which is too frequent to be maintained by recurrent mutation".

A major and a minor form, "morph", of a nematode species are often found together, i.e., one of the morphs is present in a much greater proportion in the population than the other. Morph pairs are found in the prime host, i.e., the host type in which the parasites occur most commonly, but they may be seen also in alternative hosts (Lancaster and Hong, 1981). The Ostertagiinae subfamily exhibits polymorphism (Table 2.1), in addition to sexual dimorphism. In the bovine host, the major morph present is *O.ostertagi* and the minor morph is *O.lyrata*.

Polymorphism of the Ostertagiinae is recognised by the appearance of the accessory reproductive organs of the male on light microscopy; the major morph has a Sjoberg's organ at the genital cone and narrower spicules relative to the minor morph. Sjoberg's organ is defined as the structure surrounding the cloaca of the male's tail, ventral to the dorsal lobe (Gibbons and Khalil, 1983). The variation in appearance of the male Ostertagiinae had led to further classification of the subfamily into many species, but the females could not be differentiated to a similar degree, although the number of females was similar to the total number of all the male "species" types (Lancaster, Hong and Michel, 1983). The difficulty in differentiating the females into *O.ostertagi* and *O.lyrata* suggested that the variation was intra- rather than inter-species.

In bovine ostertagiosis, pure populations of the minor morph, *O.lyrata*, have not been found (Lancaster and Hong, 1981). Selected *O.lyrata* males mated with virgin females yielded a mixed population of males

TABLE 2.1

Major and minor "morphs" of nematodes of the Ostertagiinae subfamily

Host	Major morph	Minor morph
Bovine	*Ostertagia ostertagi*	*Ostertagia lyrata*
Ovine	*Ostertagia circumcincta*	*Ostertagia trifurcta*
		Teladorsagia davtiani
Bovine	*Ostertagia leptospicularis*	*Skrjabinagia kolchida*

in the offspring. With no artificial selection the predominant species appears genetically determined and present in a constant ratio (Lancaster and Hong, 1981). Crossing males of the different morphological forms separately with a pool of females resulted in more than one morph being found in the offspring (Lancaster *et al.*, 1983). By artificial selection the proportion of the minor morph may be increased, i.e., over five generations Lancaster *et al.* (1983) were able to increase the proportion of the minor morph from 2% to 66%. However, the percentage of *O.lyrata* stabilised at 66% and no further increase could be achieved by further selection.

Earlier, Copland (1965) found difficulty in achieving a pure culture of *O.circumcincta* as assessed by appearance of the male accessory reproductive organs, he attributed this to hybridization of species. Daskalov (1975) conducted a series of experiments in which he crossed *O.circumcincta*, *O.trifurcta* and *Teladorsagia* males separately with females from different morphological backgrounds. In the F1 generation males from the other two morphological types arose. Eventually the *O.circumcincta* became dominant in all the mating groups. He concluded that there were no reproductive barriers between the groups, which suggests that the variation among the three parasites of sheep was intra- rather than inter-specific.

Lichtenfels (1983) studied the cuticular ridges or synlophes, by fixing nematodes in 70% alcohol and embedding them in glycerine jelly prior to sectioning. The synlophes allow nematodes to adhere to the gastrointestinal wall. In *Nippostrongylus brasiliensis* the patterns were similar regardless of host source, race and geographical location of origin, i.e., there was no apparent intraspecies variation. Occasionally a slight variation in pattern was noticed along the parasite length, suggesting more comprehensive examination would give more significant information for comparisons. He used the technique to classify species of *Nematodirus*, *Nematodirella* and *Cooperia*; male and female worms of the same species were found to have the same cuticular conformation. Lichtenfels, Pilitt and Lancaster (1988) demonstrated by light and scanning electron microscopy (SEM) that the major and minor morph males, present in the bovine host, (*O.ostertagi* and *O.lyrata*) and the pool of indistinguishable females

had the same arrangement of longitudinal cuticular ridges, oesophagus and bursal rays. The only difference between *O.ostertagi* and *O.lyrata* was the appearance of the spicules and genital cone of the male. They concluded that *O.ostertagi* and *O.lyrata* were not separate species, but represented individuals within one species with a slight variation in structure.

2.1.2 Continuous variation

There is continuous variation in the length and the appearance of the accessory reproductive organs of adult *O.ostertagi*. Michel, Lancaster and Hong (1971) noted that the dimensions of *O.ostertagi* adults ~~decreased~~ altered as an infection progressed, so the length to width ratio changed. The alteration was particularly clear when the burden was low. A decrease in length was most noticeable when low infecting burdens were present, while width decreased more rapidly when the burden was high. Up until 30 DPI parasite size appeared to be related to population density, the parasites were shorter in heavier infections, while after 30 DPI the host immune status seemed to be the prime influencing factor.

Parasite turnover rather than shrinkage was thought to be the most plausible reason for the decrease in parasite length, since there was a concomitant reduction in vulval flap size, alteration in the sex ratio and change in rate of loss when the host was removed from the source of infection. They suggested that a selective loss of large nematodes occurred as the infection progressed (Michel, Lancaster and Hong, 1978b).

Michel, Lancaster and Hong (1976b) noticed variation in the size of the vulval flap of the female *O.ostertagi*. They selected the offspring of adults with differing vulval flap size and used them to dose susceptible and previously infected calves to assess whether decreased vulval size was hereditary or due to host immune status. The progeny of females with well-developed vulval flaps was more likely to have well-developed vulvae itself, but the parasites in previously infected hosts had significantly smaller flaps than those in susceptible calves. They proposed that the reduction in size was due to premature

termination of development rather than selected establishment of certain forms. Vulval flap size appeared to be influenced by heritable parasite factors, but the effect of the host immune status was greater. They suggested host conditions could be affecting the full development of the vulval flap by influencing genetic variation of the helminth.

2.2 BIOTIC POTENTIAL

2.2.1 Infectivity

Infectivity consists of the ability of the parasite to establish in the host and then develop. From the available published data confirmation of a strain effect on infectivity is hard to make, due to the lack of uniformity in experimental design (host age, breed, sex; level of challenge). It is difficult to define parameters for infection, since many factors outwith the parasite may be involved. Variation in strain infectivity in outbreaks may only be estimated in retrospect and is thus subject to unreliability. To prove the existence of variation in infectivity experimentally, samples would have to be assessed from different sources in similar hosts.

Dose rate affects establishment; Ross (1963) reported that in calves given 2,000 L3 of *O.ostertagi* a 60% take in infection resulted, while the take in calves given 100,000 L3 was only 19.6% at slaughter 4 to 6 weeks later. When a very high dose, approximately 800,000 L3 is given a high initial loss of parasites from the abomasum with an earlier increase in duodenal population ~~due to diarrhoea~~ has been found (Anderson *et al.*, 1966, 1967).

The composition of the infecting dose influences the severity of the disease. Infections with gastrointestinal parasites are seldom confined to a single species. Comparison of infectivity and pathology with *Ostertagia* spp. in *sensu lato* showed that when *O.leptospicularis* was present in infection, the severity increased (Al Saqur *et al.*, 1982a and 1982b), i.e., within the genus *Ostertagia* there is variation in infectivity. Al Saqur *et al.* (1982a) noted that when *O.leptospicularis* was introduced to cattle from roe deer there was a rapid increase in the relative ratio of this species to *O.ostertagi*. He suggested this was due

either to a lower susceptibility of the *O.leptospicularis* to the routine anthelmintics used in cattle or due to greater fecundity of the species. He suggested that strain differences were less likely to alter infectivity than the interaction of different species of *Ostertagia*.

Herlich, Gasbarre and Douvres (1984) compared three isolates of *O.ostertagi* by administering them to calves at two different concentrations. The isolates originated from Alabama (passaged 25 years), Weybridge (passaged 20 years) and Louisiana (recent outbreak). The recently isolated strain from Louisiana took longer to develop a patent infection, it produced clinical signs later than the two passaged isolates and a greater proportion of the Louisiana larvae established. *In vitro*, larvae from the Louisiana strain developed at a slower rate than larvae from the other isolates.

From initial work strain variation in infectivity seems likely, but further work is needed to confirm the theory and this will require the infection of large host numbers and the comparison of their effects. Ideally the prime host, bovine, should be infected, but realistically, a suitable model host, if available might give some indication of variation and would allow the comparison of large numbers of animals from similar genetic backgrounds.

2.2.2 Ability to arrest

Arrested Larval Development is the reversible state of suspended animation occurring in the early L4 *O.ostertagi* once the parasite has established in the abomasal gastric gland (Anderson *et al.*, 1965a). Severe clinical signs can develop rapidly if the larvae resume activity synchronously. Modern anthelmintics are effective against the arrested larval forms, however, in the past, the inhibited stage caused major problems, since it was more resistant to anthelmintics, available at that time, an outcome that was attributed to a lower metabolic rate and consequently poor drug uptake.

Intraspecific variation in arrested larval development has been recognised since the 1960s, coinciding with the wider acknowledgement of the Type II condition. Variation occurs in the

propensity and season of arrest and the response to stimuli to enter and exit from the inhibited phase.

Armour, Jennings and Urquhart (1967b) found that a strain of *O.ostertagi* maintained experimentally through a number of passages showed a decreased propensity to arrest compared to a freshly isolated sample. They proposed two possible theories, firstly one strain was naturally more susceptible to arrest, or secondly passaging of the laboratory strain might have eliminated the larvae with a tendency to arrest or favoured a mutant not susceptible to arrest. The second explanation gained increased favour since the larvae were stored at 4°C for lengthy periods prior to calf dosing and cold conditioning appears to trigger larval arrest in the host (Armour, 1978). Consequently, the larvae arising from the eggs collected when patency of infection was first recognised would occur too early to have been derived from previously arrested forms. Sollod (1967) suggested that the field strain could consist of two variants: in one, the larvae overwintered on the pasture and in the other larvae became dormant in the host. Michel (1967) proposed that the higher percentage of arrest in cattle given the field strain was a consequence of the higher infective dose they had received. Further work by Armour, Jennings and Urquhart (1967c) showed that the larval source was more important than the size of infection for the percentage of larvae arresting. Armour and Bruce (1974) suggested that certain strains had an innate tendency for inhibited development. Selective pressures affect the proportion of larvae with the potential for inhibited development. For example, in Australia management systems appear to influence the chances of arrest occurring, so larvae parasitising beef cows are more likely to arrest than larvae in dairy cows (Smeal and Donald, 1982b; Chapter 1, 1.6.2.1). They suggested that without inhibiting *O.ostertagi* would be unable to maintain itself in the beef system, since the calves are born in one season and graze alongside immune dams until weaning; pasture contamination is low due to adult acquired immunity. Earlier, Smeal (1977, cited by Armour, 1978) had suggested that the control of parasitism by using anthelmintics with poor efficacy against arrested forms might affect the prevalence of arrest.

A further example of differences in ability to arrest between isolates from a similar geographical region was recorded by Borgsteede and Eysker (1987). Two Dutch isolates of *O.ostertagi*, from Lelystad and Utrecht, were examined in permanently grazing calves and tracer calves. The results from the permanent grazers showed that both isolates had a propensity to arrest, while data from tracer calves indicated that autumn environmental stimuli influenced arrest of the Utrecht strain, while the Lelystad strain, whether in Lelystad or in Utrecht was not influenced by the same trigger. The results gave further support to the idea that the difference in propensity to arrest appears to be strongly influenced by the parasite strain.

As reviewed earlier (Chapter 1, 1.6.1.2), the season of peak arrest varies and tends to coincide with the periods of greatest climatic extreme to which the free-living phase is subjected. In the United Kingdom, Armour and Bruce (1974) proposed that without the ability to arrest larvae developing on the pasture late in the season would not survive in any great number through periods of environmental hardship; arrest increases as autumn progresses and in this way larvae overwinter in the host. While in the Southern Hemisphere Suarez (1985) in Brazil and Pinheiro *et al.* (1978) in Argentina noted inhibition increased as the climate became warmer and drier, so arresting enabled the parasites to avoid the drought conditions on the pasture.

The trigger stimulating arrest is still poorly understood, but it appears highly complex and varies with different environmental situations. Anderson *et al.* (1965b) postulated that arrested larval development was a function of physiological change to larvae developing on the pasture. Other possible stimuli proposed by Armour and Bruce (1974) were endocrine changes in the host, an intrinsic inherited property of the larvae or an effect of larval ageing. They proposed that with the right stimulus a facultative gene might induce inhibition.

Ross (1963) experimentally infected calves with one or two doses of *O.ostertagi* at varying concentrations, a greater percentage of larvae arrested once a second dose was given. Armour *et al.* (1969) showed that inhibition was not purely a function of the dose, since the

percentage arresting was approximately 70% regardless of the length of time spent grazing. This finding led to the idea that acquisition of immunity by the host was involved in development of arrest (Michel *et al.*, 1973a; Michel, Lancaster and Hong, 1973b) and the resumption of development or at least cessation of inhibition with host immunosuppression supports this. Michel *et al.* (1979) commented on the correlation of increasing host age and previous exposure with higher propensity of larvae to arrest.

In the United Kingdom, an increasing amount of the worm burden inhibits from September onwards (Anderson *et al.*, 1965b) although arrest occurs throughout the year with 3-21% of the burden affected even in the spring. Cold conditioning larvae for 6 to 8 weeks in the United Kingdom led to 60 to 70% of larvae arresting (Armour and Bruce, 1974). Similar cold-conditioning experiments in Australia resulted in only 10% of the population arresting (Smeal and Donald, 1982a). It was suggested that different strains of parasite responding to different triggers to arrest might be involved. Brunsdon (1972) suggested that parasite strain differences could be responsible for the lower proportion of larvae arresting in New Zealand relative to the United Kingdom.

Different patterns and percentages of arrest were observed by Smeal, Fraser and Robinson (1980b) in the distinct climatic regions of the Northern Tablelands, the Northern Coast and the Central Coast of New South Wales, Australia. In a transfer experiment, infective material from the three sites was used to infect groups of cattle in each of the three areas. The pattern of inhibition remained similar to those at the site of origin over the first season and did not alter with the change in climate (Smeal and Donald, 1981).

In the United States of America, the proportion of inhibited larvae in a population from Ohio in the North, was lower in the spring but higher in the autumn than larvae from Louisiana in the South. When larvae were transferred from one site to the other, the pattern of inhibition remained the same as that at the site of origin (Frank, Herd, Marbury and Williams, 1986). The pattern of arrest began to change to the pattern in the new region with time, but the Northern isolate adapted

more slowly than the Southern isolate. Differences in the pattern of arrest were attributed to larval genetic differences (Frank, Herd, Marbury, Williams and Willis, 1988).

Coles (1988) proposed that the percentage of larval arrest in the free-living nematode, *Caenorhabditis elegans*, was genetically determined. Starvation and overcrowding acting via pheromone concentration appear to trigger "entry" to the "dauer" form (Riddle, 1988), a stage of reduced metabolic activity, by acting on a number of identified genes. "Exit" from the dauer form also appears to be related to pheromone concentration. Mutation in a number of identified genes resulted in the inability to arrest or occurrence of arrest independent of environmental changes (Riddle, 1988).

If the stimulus to arrest appears to be incompletely understood, that bringing about resumption in development is even more of a grey area. In the United Kingdom, after approximately 19 weeks of arrest there is a marked increase in mobility of the larvae and an increasing proportion of the immobile larvae are dead rather than arrested (Armour and Bruce, 1974). The time period is equivalent to the natural winter length.

However, not all workers have noticed a synchronous resumption of development. Michel (1970) proposed that a small number of larvae resumed development daily, but the number increased from March onwards and also when there was a breakdown in host immunity with stress, disease or parturition. The adult burden at any one time depends on the adult lifespan and the number resuming activity, i.e., rate of recruitment. Prichard, Donald and Hennessy (1974) found treatment with corticosteroids did not stimulate maturation, but it did end the period of arrest. They proposed that the use of corticosteroids could increase the efficacy of anthelmintics. However, these results were not confirmed by Armour (1978), who superimposed normal and X-irradiated larvae on calves with an arrested population of larvae. Twenty-one days after the superimposed infection, a similar proportion of arrested larvae were found in these calves and those given no extra infection. Adult *O.ostertagi* were found in the challenged calves and these calves showed a rise in plasma pepsinogen concentration, but

reinfection had not induced further larval arrest. Several immuno-suppressant drugs and hormones were tested for their affect on arrested larvae. Only betamethasone, a cortisone, caused a significant resumption in activity, but this was just in 15% of the arrested population.

Several factors appear to contribute to strains arresting or resuming larval development and varying the proportion of larvae affected. Arrested development patterns in parasitic nematodes appear to vary as a result of larval genetic differences, which are in turn affected by selection pressures arising from the environment and management system (Smeal and Donald, 1980b and 1982b; Armour and Duncan, 1987). A better understanding of this strain variation could lead to the development of improved strategies for parasite control.

2.2.3 Fecundity

Fecundity is defined as the ability to produce offspring. There is a strong correlation between intensity of infection and parasite fecundity in helminth disease in man. Assessment of parasite fecundity requires a knowledge of faecal egg count and parasite burden (Keymer and Slater, 1987). Keymer and Slater (1987) warned of the difficulties in assessing helminth fecundity. Variation in parasite fecundity between hosts with low burdens may cover a wide range. Small parasite numbers may produce egg counts of equivalent magnitude to large parasite populations in different hosts. Parasite populations are generally overdispersed, i.e., certain hosts have very high parasite numbers, while the majority have low burdens. Consequently, overdispersion may affect assessment of fecundity, since few hosts with high burdens will be analysed.

The fecundity of *O.ostertagi* has been shown to vary, but the factors causing variation are poorly understood. Michel (1969) found an increase in faecal egg output from deliberately infected calves treated with corticosteroids relative to controls. Host immunity, innate or acquired may affect the number of eggs produced by the parasite population. Smith (1970) found a smaller population of parasites, with fewer eggs in the reproductive tract of the females in yearling

compared to 3-month-old calves following deliberate infections with similar larval doses. Herlich (1976) observed that the parasites of previously infected calves contained fewer eggs than parasites from helminth naive animals. Michel (1978c) noted that the fecundity of adults derived from previously arrested larvae could vary with host parturition and lactation.

2.3 ANTIGENICITY

Antigenicity is known to vary within protozoan species (Allsopp and Allsopp, 1988), but little is known about variability of this parameter in nematodes, let alone *O.ostertagi*. Variable antigenicity implies that different proteins may be important in the pathogenesis of disease or that a particular antigen will have a variable influence on the progression and outcome of disease.

Indications that little or no variation occurs in *O.ostertagi* antigenicity are suggested by work in other species. There is little variation in antigenicity of *O.circumcincta* (W.D.Smith, personal communication). A vaccine containing live irradiated *Dictyocaulus viviparus* larvae continues to be used with efficacy against Parasitic Bronchitis in calves. The vaccine, a larval cocktail, was introduced in 1959 and though the vaccine currently used is derived from the original isolate it continues to induce protective immunity (Bain and Urquhart, 1988). Intraspecific variation of antigenicity of nematodes appears to be low, although little information is available on the subject. However, variation in nematode antigenicity may have important consequences for the development of control strategies and merits further investigation.

2.4 VIRULENCE

Little assessment has been made of intraspecific variation resulting in differences in virulence. Comparison of the outcome of infection and the apparent severity of lesions suggested that strains of *O.ostertagi* isolated in Glasgow, Scotland appeared more pathogenic than those from Wageningen, the Netherlands. The reverse pattern of virulence was thought to occur with *C.oncophora*. Transfer experiments were

carried out and the effects with indigenous and imported larvae compared. Following infection with the Glasgow isolate a greater drop in calf appetite and a different distribution of lesions occurred; the mucosa and submucosa of the fundus were more congested and oedematous. Overall, the Glasgow isolate appeared more pathogenic. The Wageningen isolate induced a greater cell response with polymorphs and lymphocytes infiltrating the abomasal mucosa. The isolate seemed to be more virulent under British than Dutch conditions, but this could be the result of host effects (K.Bairden, personal communication). However, the Two-Sample Mann-Whitney Test showed the differences were not significant (G.Gettinby, personal communication).

Further experimental work is required before any conclusions can be made on intraspecies differences in virulence. To achieve conclusive results large numbers of isolates will have to be compared under similar circumstances, i.e., identical storage conditions prior to infection of similar hosts, and to provide suitable experimental numbers the availability of a laboratory animal host would be an advantage.

2.5 DRUG TOLERANCE / RESISTANCE

When a parasite is said to be tolerant to a certain drug, the implication is that though the population may not have been exposed to that drug previously it is not completely susceptible on initial challenge. Conversely, with drug resistance a decreased susceptibility to a drug develops over successive parasite generations (Rowlands, 1989). The majority of work reported uses these terms synonymously. Drug tolerance and the development of resistance may themselves be considered as signs of variance or its development with time respectively. Fortunately, the strains with a predominantly drug tolerant profile often demonstrate lower pathogenicity. Thus, Kerboeuf, Hubert and Mallet (1989) found there was an inverse relationship between the size of worm burden and the lethal $dose_{50}$ for *Haemonchus contortus*.

Generally, tolerance to drugs may be suggested when treatment results in a low level of parasite expulsion relative to that expected with the agent, only a slight drop in faecal egg count and pathological changes. Breakdown of drug efficacy may be due to a number of different factors, including incorrect drug dosage, incomplete herd treatment allowing a build-up of infective stages on pasture, and unsuitable herd management and husbandry leading to stock contact with contaminated animals or ground.

Tests for resistance to anthelmintics include:

1) the egg hatch assay (eggs from benzimidazole resistant nematodes are resistant to ovicidal action of the drug) (Coles and Simpkin, 1977),
2) *in vitro* larval paralysis test (Geerts, Brandt, Kumar and Biesemans, 1987),
3) low drop in faecal egg count following treatment,
4) tubulin-binding assay [benzimidazoles inhibit the polymerization of nematode tubulin into microtubules and resistant parasites bind less drug (Lacey and Snowdon, 1988)],
5) biochemical tests (increased acetylcholinesterase activity in benzimidazole resistant strains of *H.contortus*, *O.circumcincta* and *Trichostrongylus colubriformis* (Sutherland, Lee and Lewis, 1987).

Anthelmintic resistance has only been reported recently in cattle, although it has been recognised in sheep for a much longer period (Coles and Simpkin, 1977). The apparent lack of drug resistance in bovine parasites was attributed to the lower frequency of anthelmintic dosing in cattle, decreased parasite selection pressure because of differences in host metabolism and parasite environment (Coles, 1986). In addition, a succession of drugs have been used to treat and prevent *O.ostertagi* infection in cattle and although the action of many of these drugs is still poorly understood the changing of drugs employed decreases the chances of development of resistance.

The first reported incident of drug resistance with *O.ostertagi* occurred in 1987 when Geerts *et al.* found a relatively poor drop in *O.ostertagi*

faecal egg count when cattle were treated with levamisole. Borgsteede (1988) recorded an incident where a field strain of *O.ostertagi* was found to be more tolerant than a laboratory strain to morantel tartrate from a sustained release intraruminal bolus; there was a relatively poor fall in faecal egg count and only a slight drop in plasma pepsinogen concentration.

Drug efficacy and dose rate affect the force of the selective pressures inflicted on the parasite leading to adaptation. Herd (1984) proposed that continual release devices for anthelmintics might encourage the development of drug resistance, unless the drug was dispensed at a high constant level with an abrupt termination of release. Similarly, frequent treatment of nematodes with the same anthelmintic might encourage resistance to develop, particularly if inadequate quantities of drug are used. The development of side resistance, i.e., resistance to related drugs of similar action and cross resistance, which is the development of resistance to unrelated drugs, have been recognised and their unfortunate results predicted (Taylor and Hunt, 1989).

Attempts have been made to try to assess the rate of loss of resistance to one drug during treatment with an alternative anthelmintic, i.e., counterselection of the first drug with a second. Martin, Anderson, Brown and Miller (1988) conducted a field trial in sheep over six parasite generations with two thiabendazole resistant *Ostertagia* strains. One strain was treated with levamisole, this led to a small drop in thiabendazole resistance, but the treated strain was still more resistant to thiabendazole than susceptible strains. They concluded that levamisole treatment would not reduce thiabendazole resistance sufficiently to allow its re-introduction for control.

Resistance is thought to be a heritable property of the parasite, so when circumstances are more favourable to the resistant forms, i.e., with drug pressure, they will increase in proportion in the population. It has been suggested that by aiming to control the infection by treating the parasitic stages the more resistant types are the most likely to survive. Consequently, prevention of the development of resistance is desirable, e.g., by the use of drugs in rotation at effective doses, since the problem of removing resistant parasites is so much greater. Van Wyk

have read this, but it was post the thesis submission →

Genetic basis in parasite proven now.
β-Tubulin mutation
RFLP change in H.contortus. (Roos et al)
Possibility of specific DNA probes

and Van Schalkwyk (1990) tried to replace benzimidazole resistant free-living stages of *H.contortus* on the pasture with anthelmintic-susceptible ones. In seasons when there was low pasture contamination some success was achieved, however, further work is required to assess reversion to resistance.

A knowledge of strain variation with respect to anthelmintic efficacy is important for the selection of suitable drug regimens. The rate of development of resistance to a particular drug will vary, but where low levels of drug are administered over long periods of time the opportunity would appear more likely to arise. The experimental work mentioned indicates a slow alteration in the drug resistance characteristic, when selective pressures were applied.

In conclusion, it is now clear that biological variation does exist within the species *O.ostertagi* as assessed by structural polymorphism, arrested larval development and drug resistance, while little is known about differences in antigenicity and virulence. Knowledge of intraspecific variation aids the assimilation of a plan of evolutionary development and information on pattern of parasite spread. Any differences revealed could be used to predict the suitability of control regimes from knowledge of parallel situations. The development of vaccines could also be influenced, since the development of a vaccine with potential efficacy for only a small subset of the population could be noticed and avoided. The major aim of this thesis is to look at the molecular basis of intraspecific variation in the nematode *O.ostertagi* using experimental techniques to try to detect differences between isolates by identifying differences in protein content.

CHAPTER 3

METHODS OF DETECTION OF PARASITE VARIATION

METHODS OF DETECTION OF PARASITE VARIATION

Traditionally, morphological differences have been used to identify species (morphospecies) and developmental stages, requiring the construction of complex keys of classification and the development of parasitological expertise. Many researchers are attempting to supplement the traditional methods of identification with newly developed genetic techniques. These techniques have shown that the routine examination of appearance for differentiation only detects a subset of the possible markers of variation and additional structural and functional differences may occur within a species which may be used to determine intraspecific variation.

The techniques available for differentiation fall into two categories: those that look at the gene itself and those that assess differences in gene products. To gain the maximum information both types of study should be done, since often the techniques are complementary. Many methods and their derivatives have been used to investigate variation, but only those most frequently employed are discussed here.

The DNA forming an organism's genetic code dictates the pattern of messenger RNA produced on transcription. The messenger RNA is read in blocks of three nucleotide bases or codons. Transfer RNA with the corresponding triplet of bases, anti-codons, pair up with the messenger RNA at ribosomes. Each anti-codon has an amino acid bound to it, so the amino acids form peptides in a particular order, which peel away from the RNA. In this way, the amino acid sequence is dependent on the original DNA base order. While each triplet group of nucleotide bases is linked to a certain amino acid, one amino acid can be coded for by more than one triplet group of nucleotide bases, i.e., there is a degree of code degeneracy. In addition, some RNA codons, nonsense triplets, have no known corresponding amino acid. Genetic changes may arise by point mutations at the codon, chromosomal aberrations or alteration in the chromosome number. When a heterogeneous population is subjected to certain conditions particular individuals may be favoured and eventually the population will alter accordingly.

The trichostrongylids are sexually dimorphic and carry XX or XO sex chromosomes (Triantaphyllou, 1983). Herman, Madl and Kari (1979) recorded a method of visualising nematode chromosomes by freezing the parasites between a coverslip and a slide with dry ice and shearing them by flicking the coverslip away. The nematodes were fixed and stained with the fluorescent dye Hoechst 33258. By this method, adult *Ostertagia circumcincta* were found to be diploid and six chromosome pairs were seen (K.Keith, unpublished work).

3.1 DIFFERENTIATION BY EXAMINATION OF GENETIC PRODUCTS

Many approaches to the separation of gene products may be employed. Analysis of differences in size, structure, charge, activity and amino acid sequence are all used. Often as a preliminary step, organisms are broken down into more manageable fragments, e.g., by homogenisation or sonication, followed by a separation process such as centrifugation or density differentiation.

3.1.1 Isoenzyme analysis

Isoenzymes may be assessed by starch gel electrophoresis and isoelectric focusing (IEF). Proteins are applied to a matrix in an electric field and migrate at different rates depending on their net charge and mass. This is the concept employed in starch gel electrophoresis when molecules of low mass will travel further in a given time. In IEF, a pH gradient is produced by addition of ampholines to the matrix and the peptides migrate to their isoelectric point, where they have no net charge. Coomassie blue staining of parasite samples on IEF gels is possible, but complex peptide profiles are often produced which are unsuitable for taxon differentiation, as Isaac-Renton, Byrne and Prameya (1988) found when trying to distinguish strains of *Giardia duodenalis*. Consequently, individual enzyme migration patterns are visualised by providing appropriate substrates and linking them to stain reactions. Examination of a number of different enzymes allows a measure of relatedness to be made between different samples, when more closely related organisms will have more similar enzyme profiles. Leon, Monteoliva and Sanchez-Moreno (1988) showed *Toxascaris leonina*, *Toxocara cati* and *Ascaris suum* had different malate

dehydrogenase patterns on assessment, and within a species males and females also varied.

3.1.2 Sodium dodecyl sulphate-polyacylamide gel electrophoresis (SDS-PAGE)

With SDS-PAGE, protein migration in an electric field produces separation by molecular size. SDS is a detergent with a strong negative charge, which binds to the hydrophobic areas of most polypeptides in a constant ratio, conferring its negative charge on the protein. The negative charge causes the protein to open out and to be attracted towards the positive electrode, i.e., migration is largely independent of protein charge and relies mainly on molecular size; the lower the mass, the further the peptide will travel in a given time. The reducing agent, mercaptoethanol, is often added to the sample to break disulphide bonds. The gel pore size may be altered by changing the acrylamide/bis acrylamide concentration, which affects the rate of molecular sieving of protein samples.

The staining techniques commonly used for evaluation of protein pattern after separation are Coomassie blue and silver stain, which detect 0.1 to 0.5 μg and 1 to 10 ng of protein, respectively, and are adequate for the detection of the majority of proteins. The technique cannot differentiate proteins with distinct sequences but similar molecular weights. SDS-PAGE is a standard technique in many laboratories and in addition to the direct staining methods, samples may be linked to labels, which may be visualised in a variety of ways, e.g., enzyme reactions, fluorescence and radiolabelling.

3.1.3 Two-dimensional gel electrophoresis

Proteins with different molecular weight may appear similar on IEF, while others with varying charge appear the same on SDS-PAGE; two-dimensional gel electrophoresis combines the two techniques and allows the simultaneous differentiation of 1,000 to 2,000 proteins compared to the maximum of 50 proteins in single dimension electrophoresis. However, the results can be difficult to interpret and the technique has not yet been widely applied in nematode taxonomy. The technique is divided into two steps: in the first stage protein

migrates to its isoelectric point dependent on its charge in a fine columnar IEF gel, then in the second dimension SDS is added, giving the sample a blanket negative charge and separation occurs by mass with standard polyacrylamide gel electrophoresis (O'Farrell, 1975).

3.1.4 Monoclonal antibodies

By combining the specific antibody producing properties of B lymphocytes and the longevity of myeloma cell lines, large amounts of antibodies may be generated against particular antigens; these are referred to as monoclonal antibodies. The antibody may be linked to a dye, radiolabel or enzyme. Monoclonal antibody may be used to screen an organism for the presence or absence of a particular antigen, protein or carbohydrate e.g., circulating schistosome antigen may be detected for diagnostic purposes (Noqueiro-Queiroz, Lutsch, Capron, Dessaint and Capron, 1986). Choice of a specific antibody- antigen reaction may form the basis for differentiation of different types of individual. Kennedy, Maizels, Meghji, Young, Qureshi and Smith (1987a) identified a surface carbohydrate present on the surface of *T.canis*, but not on *T.cati*.

3.1.5 Amino acid sequencing

Equivalent proteins present in different individuals may be examined, i.e., their amino acid sequence compared. A protein fingerprint may be developed using enzymes and chemical reagents to separate peptide sections, which may themselves be degraded by sequential release of amino acids from the labelled amino terminal, Edman degradation. The free amino groups are identified by chromatography (3.1.6). The overall sequence is found by piecing together information from overlapping sequences. DNA sequencing is much faster, so often a gene will be cloned to find the amino acid sequence, therefore, amino acid sequencing *per se* is of limited use for the detection of intraspecies variation.

3.1.6 Chromatography

Proteins of a variety of molecular weights may be separated by chromatography. The peptide sample is applied to a matrix and a mixture of solvents allowed to permeate the sample from one direction. The peptides will associate with particular solvents from the mixture, depending on their relative solubility and are carried with the fluid until their solvent is adsorbed to the matrix resulting in a peptide pattern. Many permutations of basic column chromatography have been developed for peptide separation, e.g., charge separation by ion exchange chromatography, size differentiation by gel-filtration chromatography and affinity chromatography, in which a column of beads coated with covalently bound substrate or antibody selects certain peptides.

3.2 DIFFERENTIATION BY EXAMINATION OF THE GENE SEQUENCE

Analysis of variation at the DNA level gives a more direct examination of genetic variation, since the subjectivity produced by morphological and biological examination is not introduced. In addition, the DNA sequence is essentially constant throughout the parasite life cycle and unaffected by environmental factors, which may alter expression of phenotypic characters. (Unusually there is a diminution in the chromatin content of *Ascaris* nematodes in the phase immediately after embryonation (Pasternak, 1988).) A number of different sample types may be used to test for nucleic acid variation, e.g., extracted and cloned genomic DNA, ribosomal RNA, mitochondrial and ribosomal DNA. Intergenic coding portions, i.e., spacers or introns are sections of the genome which are not transcribed and may be a source of variation, which cannot be detected by gene product analysis. Intron variation was found in schistosome species (Rollinson, Walker and Simpson, 1986). Consequently, while the nucleic acid sequence may be used to predict protein sequence, the reverse situation does not hold true, i.e., use of protein sequencing to determine nucleic acid sequence is inaccurate.

The gene sequence is composed of sections with unique information and sections with either a very high number or a moderate number of repeated sequences. Generally, the single copy genes are responsible

for structure and are the ones tested in gene product analysis, while spacer sections are highly repetitive and have a regulatory role. McReynolds, DeSimone and Williams (1986) examined the repetitive sequences of filarial nematodes and found on comparison of *Brugia pahangi* and *B.malayi* that in some regions the homology between species was 95%, while in others it was only 65%.

Nucleic acid techniques are being applied increasingly to the study of nematodes after a troublesome introductory period (Dawkins and Spencer, 1989). Extraction of trichostrongyle L3 sample material was obstructed by the robust retained cuticle of the L2. Initially, low amounts of DNA were recovered on exsheathment, as the cuticle tried to re-establish. Large numbers of parasites were required for DNA and RNA extraction on ice. Furthermore, there appeared to be species specific factors affecting the success of extraction (Dawkins and Spencer, 1989). Generally, nematode DNA is now digested after the cuticle has been removed, rather than mechanically homogenised. Alternatively, material may be generated in large amounts by the polymerase chain reaction.

The helminth genome is approximately 10^8 base pairs long; restriction fragment length polymorphism may be used on sections up to 50 kilobases long, while larger fragments can be analysed by pulse field electrophoresis. The most commonly used methods of nucleic acid analysis are restriction fragment length polymorphism, DNA hybridization and sequencing after chemical digestion.

3.2.1 Restriction fragment length polymorphism (RFLP)

DNA is extracted from the parasite and RNA and protein contaminants are removed. Restriction endonucleases are enzymes purified from bacteria, which cleave the nucleic acid sequence at particular sites four to six base pairs long (Simpson, Walker and Terry, 1986). In the bacteria the enzymes break down foreign DNA as it enters the cell. Endonucleases are incubated with the DNA extract and the digested sample subjected to electrophoresis and transferred to membranes for subsequent hybridization (Southern Blot). The gel may be stained with ethidium bromide to show fragments of varying mass. Beh, Foley and

Goodwin (1989) used RFLPs to identify stage and species specific bands in extracts of the trichostrongyles, *Haemonchus contortus*, *O.circumcincta* and *Trichostrongylus colubriformis*. They suggested the future use of RFLPs to distinguish trichostrongyle species from the egg to the L3, which is not possible by traditional methods.

Ribosomal DNA may be used as an alternative substrate for endonuclease action, since it is abundant, highly repetitive and easily isolated. Mitochondrial DNA contains no introns and variation has been found in the repetitive sequences of *Romanomermis culicivorax*, a mosquito nematode (Beck and Hyman, 1988). Messenger RNA fragments may be separated by Northern Blotting in a similar way to the DNA material in Southern blots, however, with respect to trichostrongyle samples only very low levels of transcription occur in the L3 phase and large amounts of parasite are necessary (Dawkins and Spencer, 1989). However, studies have shown a high degree of conservation between the messenger RNA isolated from different unrelated species of nematode (Nilsen, 1989).

3.2.2 DNA hybridization

The double strand configuration of DNA is separated by heating, denaturation, although on cooling the strands reform, renaturation or hybridization. However, pairing errors often occur and a lesser temperature rise will be required to produce strand separation on the next occasion. The most stable duplex that might form would be that of the two complementary strands matched up once more. The more distant the relationship between two strands the lower the stability of the new duplex to temperature increases: with differences in 1% of nucleotides the temperature required to produce duplex dissociation on heating will be decreased by 1°C (Ferguson, 1980). Rate of duplex formation depends on chances of complementary sequences colliding, which may be measured by hybridization to a radiolabelled probe. All the non-hybridized chains are destroyed by a nuclease, leaving a level of radioactivity proportional to the amount of successful hybridization.

The proportion of particular complementary base pairings, e.g., guanosine--cytosine has been found to correlate with the temperature

of degradation and has been used in the comparison of filarial nematode species. Rothstein, Stoller and Rajan (1988) found only 26 to 28% of base pairings of tested filarials were guanosine--cytosine, which prevented their hybridization with organisms with high levels of the base pairing.

Restriction fragment length polymorphism and hybridization may be combined. After separation of the fragments by electrophoresis and blotting, radiolabelled probes may hybridize to sections of nucleic acid of particular sequences allowing their detection. The probes may be employed specifically to a group, so when new individuals are compared they are either similar or dissimilar. Non-specific probes are used to assess individuals for their reaction with a number of endonucleases (Cameron, Levy, Nutman, Vanamala, Narayanan and Rajan, 1988). By varying the number of endonucleases and probes used the extent of testing may be altered. Using these techniques a physical map of the genome of the nematode *Caenorhabditis elegans* is being developed, many research groups are contributing towards the work and the position of over 500 loci are known (Coulson, Sulston, Brenner and Karn, 1986).

Zarlenga and Barta (1990) claimed RFLP and hybridization techniques might best be applied to species with highly repetitive sequences, so the *Trichinella* species might be assessed, while *Ostertagia* and *Haemonchus* were less suitable. At an intraspecies level, RFLPs have been used to differentiate a DNA sequence in forest form *Onchocerca volvulus* not present in the savannah form (Erttmann, Unnasch, Greene, Albiez, Boateng, Denke, Ferraroni, Karam, Schulz-Key and Williams, 1987) and to distinguish three types of *T.spiralis* among six isolates (Klassen, Thiessen and Dick, 1986 as cited by Zarlenga and Barta, 1990). RFLPs may be complementary to the techniques employed in this study for the comparison of isolates of *O.ostertagi*.

3.2.3 Chemical action and DNA sequencing

The sequence of nucleotide bases present in the genome may be found by chemical hydrolysis and high-performance liquid chromatography. A chemical cleaving at each of the four possible nucleotide bases in a

chain is used on samples of extract. The DNA fragment is radio-labelled and a chemical cleaving at a particular nuclear base is applied. The fragments of varying length are separated by high-performance liquid chromatography and visualised by autoradiography. Combining the information collected for chemicals acting at the different nucleotides allows the elucidation of the DNA sequence.

Thus, intraspecific variation may be examined at the gene or at the product level. The techniques are complementary, both have advantages and disadvantages. In this thesis analysis at the product level was carried out, since these techniques give a rapid overview of the degree of variation. Once knowledge of the degree of variation at the product level has been gained more efficient strategies may be designed to detect variation at the DNA level and loci suited to more detailed analysis may be identified.

CHAPTER 4

FUNDAMENTAL TECHNIQUES

CHAPTER 4

FUNDAMENTAL TECHNIQUES

Fundamental techniques for the experimental work are presented in this chapter, while those relevant to specific sections of the study are described in the appropriate chapters. The sources from which the major reagents originated are listed in Appendix 1.

4.1 SAMPLE ACQUISITION

4.1.1 Origin of isolates

A collection of strains of *Ostertagia ostertagi* was built up from reported outbreaks of ostertagiosis and previously isolated laboratory stocks over a wide range of geographical locations throughout the United Kingdom (Glasgow, Stormont and Weybridge), and abroad (Denmark; The Netherlands: Lelystad, Wageningen; United States of America: Alabama, Louisiana, New Jersey). Recorded in Table 4.1 is a list of the isolates obtained and relevant details of their source, propensity to arrest and their susceptibility to drugs. Only larvae from the United Kingdom were passaged and their numbers amplified in calves. Handling restrictions on samples from abroad prevented their inoculation into stock. It was essential that samples were from a single species to avoid confusion with the intraspecies differences to be examined. To increase the depth of analysis, stocks of different trichostrongyle species were also obtained for comparative purposes, their details may be found in Table 4.2.

Two basic methods for obtaining a contaminant free sample were considered for use:

1) starting with a single pair of nematodes and amplifying their offspring *in vitro*,
2) beginning with a sample contaminated with extraneous matter and nematodes of different species to those under analysis, and trying to remove these contaminants.

TABLE 4.1

Details of *Ostertagia ostertagi* isolates collected for comparative analysis

Isolate(s)	Characteristics of isolates
Glasgow	Larval *Ostertagia ostertagi* maintained by several passages, approximately 3% *O.leptospicularis*. Increased tendency to arrest from September onwards, when 70% of the burden may be affected. Adult *O.ostertagi*.
Weybridge	Larval *O.ostertagi* 1) Weybridge original, isolated from cattle at the Central Veterinary Laboratory (CVL), 1957, multiple passages in worm-free calves (every 1 to 2 years). 2) Weybridge-Glasgow, parasites were obtained from Glasgow 1970 and passaged in Weybridge for 16 years.
Stormont	Larval *O.ostertagi*. Isolate obtained from Weybridge in 1984 and passaged for 4 years.
Lelystad	Larval *O.ostertagi*. 1) Morantel Resistant. Isolated from a mixed infection with *C.oncophora*, 1983 from calves passing a small number of larvae despite carrying an intraruminal bolus of morantel. Passaged 18 times under high pressure with morantel (calves treated or carrying a bolus). No information is available with respect to ability to arrest in the field, no strong seasonal inhibition known. 2) Morantel Susceptible. Isolated 1971, passaged approximately 28 times, with suspensions stored for 1 to 24 months at 4°C. Under no drug pressure. Isolate does not show a great propensity to inhibit either in the laboratory or in the field, but arrest has occurred in calves after repeat infections. Susceptible to all available anthelmintics.
Wageningen	Larval *O.ostertagi*. Isolated Friesland 1971. Acquired from Lelystad mid-1970s. Subsequently, passaged with a certain degree of mixing of the recovered stocks and some further supplementation with Lelystad samples. Susceptibility to inhibition unknown. No drug resistance has been encountered.

TABLE 4.1 (continued)

Danish	Larval *O.ostertagi*. Assendrup strain, isolated Zealand 1988 from faecal culture. Maintained by passage. Suspensions stored in "aged" tapwater, which had been subjected to chlorine fumes. Arrested larval development has been induced by cold conditioning of the isolate for 10 weeks. Susceptible to all commonly used anthelmintics.
Louisiana	Larval *O.ostertagi*. Isolated 1987, Bossier Parish, established over two to three passages in parasite-free donors at grass. Periodically checked for sample purity, since tendency for *C.punctata* contamination. Gravid adult *O.ostertagi* females were identified at slaughter, they were macerated and the eggs collected to obtain a pure sample. A marked propensity for arrested development has been noted from January to May (particularly after March), Type II disease seen from mid-July, but especially August to September. Susceptible to major anthelmintics, although some pockets of resistance to Levamisole and Benzimidazole are suspected in the area.
Alabama	Larval *O.ostertagi*. Isolate from a field outbreak in Auburn, 1970. Maintained as a monospecific infection by continual passage in parasite free calves obtained at 1 day old. Larvae were stored at 4°C for 1 month prior to import. Propensity to arrest unknown. No exposure to anthelmintics.
New Jersey	Larval *O.ostertagi*. A pooled sample was obtained from calves from New Jersey, Pennsylvania and Virginia in 1970. Subsequently, the culture had been passaged at least six times. Cold conditioning results in 20 to 30% of the population arresting, which occurred most frequently in the autumn. No epidemiological studies have been done to examine the Type II disease of the isolate.

TABLE 4.2

Details of the trichostrongyle species samples collected for analysis except for those of *Ostertagia ostertagi*, which are entered in Table 4.1.

Isolate	Characteristics of the isolates
Glasgow	Larval *Cooperia oncophora*, *Haemonchus contortus*, *O.circumcincta* and *Trichostrongylus colubriformis*.
Moredun	Adult *H.contortus* and *O.circumcincta*. Obtained from abattoir at slaughter.
Weybridge	Larval *O.circumcincta* 1) Thiabendazole Resistant isolates (by slaughter trials and ED_{50} by *in vitro* egg hatch assay). i) Banbury, isolated Oxfordshire, 1987, cultured from faeces post-treatment with benzimidazole. Stored in liquid nitrogen, occasional passage in susceptible lambs. ED_{50} = 0.505ppm Thiabendazole. ii) Chalk, isolated Bury St.Edmunds, Suffolk, suspected levamisole resistance, but has not been proved, stored in liquid nitrogen. ED_{50} = 0.264ppm Thiabendazole. iii) CVL, isolated 1982. Passaged every year and challenged with Thiabendazole every 2 years. ED_{50} = 0.748ppm Thiabendazole. iv) Norwich, isolated from faeces. Confirmed Thiabendazole resistant by slaughter trials. 2) Thiabendazole Susceptible i) CVL, isolated 1950s, passaged yearly in worm-free lambs. ED_{50} = 0.053ppm Thiabendazole.

The latter method being significantly inferior, since it can only be as precise as the screening method employed. Initially, production of material for isolate comparison by single pair matings and amplification by passage and maintenance *in vitro* was the aim. However, many problems were encountered in the culture procedures and as a result samples from a number of isolates were tested directly.

4.1.2 Maintenance of calves for the passage of *Ostertagia ostertagi*

Helminth naive, male Friesian calves were acquired at 4 to 6 months of age. They were housed individually on straw and fed a diet of *ad libitum* hay and calf rearing pencils (Spillers).

For the purpose of this study single species infections were obtained from laboratory stocks and fresh isolates. The larval concentration in a suspension may be found by counting the numbers present in aliquots of known volume, allowing the estimation of the amount in the total volume. Ten, 10 µl aliquots were dispensed in a fine streak across a slide, which was examined by light microscopy (x4 to x10 objective). The average number of parasites in 10 µl was found and the figure multiplied by 100 to give the number per ml. The size of the administered dose of L3s varied with the reason for infection. For the collection of faeces for nematode egg culture the dose was 50,000 L3. When the aim was to generate L4 and adult parasites, a dose of 100,000 L3 was given.

The larval dose was suspended in approximately 20ml of water which was tipped over the calf's throat. Daily faecal samples were collected for McMaster faecal egg counts from 16 DPI (Chapter 4, 4.2.1). Once the count exceeded 50 epg, collection for faecal culture was considered worthwhile, although if the material was very important collection was carried out at lower levels. Faecal culture techniques for the production of free-living stages are discussed in full in Chapter 5, 5.2.2.1. The calves were slaughtered at 4 DPI when the aim was to collect L4s and 21 DPI when adults were required.

4.2 PARASITOLOGICAL TECHNIQUES

4.2.1 Modified McMaster technique for the assessment of faecal egg count

The McMaster technique uses the difference in density produced in a saturated sodium chloride solution to separate the lighter parasite egg from heavier faecal matter. The procedure is carried out with a known mass of faeces in a known volume of solution, consequently epg may be calculated (Thienpont, Rochette and Vanparijs, 1979, after the method of Gordon and Whitlock, 1939). A drawback to the McMaster technique is the inability to differentiate trichostrongylid eggs to the species level due to the uniformity of morphology. However, from the L3 stage onwards species differentiation is possible for the experienced parasitologist (Keith, 1953).

To minimise larval development prior to egg count assessment, samples were stored at 4°C in a container with minimal sample-air interface. Two g of faeces were weighed and made up to a 60ml suspension with saturated saline. The suspension was agitated; in some cases glass beads were employed to improve faecal breakdown. After initial mixing, the material was passed through a 250 μm sieve to remove large particulate matter. Aliquots were drawn off and pipetted into the counting chamber of a McMaster slide immediately after agitation. The slide was examined under low power (x10 to x40 objective) and all eggs found within the grid were counted. In total, 10 chambers were assessed and the average count per chamber found (X). The volume of the grid area was 0.15ml, so the epg were calculated as follows:

$X \times 200 = epg$ where X is the average number of eggs in a counting chamber

(60ml of saline contains 2g of faeces
0.15ml of saline contains $2/60 \times 0.15g = 0.005g$
if 0.005g of faeces contains X eggs
1g of faeces contains $X/0.005 \times 1 = 200$ X eggs.)

4.2.2 Collection of *Ostertagia ostertagi* eggs

Parasite eggs were collected for the production of clean samples of free-living parasites to be used for analysis of development and the generation of experimental material. The method was adapted from that described by Le Jambre (1976).

The faecal matter was homogenised to a pouring consistency by adding saturated sodium chloride solution. The homogenate was poured into trays through a 250μm sieve to remove large particulate matter. The dimensions of an *O.ostertagi* egg are 74 to 90μm by 38 to 44μm. Due to the lower density of the nematode egg relative to the faecal matter in salt solution, the eggs rise to the fluid surface and can be collected from the base of Petri dishes or plastic sheeting applied to the surface for 5 minutes.

The material adherent to the dish or sheet was flushed into a sieve with a 38μm pore size with distilled water and the eggs were retained. The surface collection was repeated at least three times. The retained eggs were washed into a concentrating flask and placed at 4°C to minimise development. To clean the eggs further, the supernatant was withdrawn and the sedimented eggs and debris transferred to a centrifuge tube. The volume was trebled by adding saturated sodium chloride solution and centrifuged for 5 minutes at 1,400g. The supernatant containing eggs was collected and poured onto a 38μm sieve. The washings were centrifuged in water to pellet the cleaned eggs.

4.2.3 Preparation of free-living larval suspensions. Two methods were employed to remove particulate matter and dead larvae

4.2.3.1

Five ml of 45% Percoll solution was added to a 15ml centrifuge tube. Five ml of contaminated larval suspension, and water as required, was trickled down the side of the tube doubling the fluid volume. The tubes were centrifuged at 1,400g for 20 minutes. The larval pellet was

resuspended in 10ml of phosphate buffered saline (PBS), pH 7.2 and spun for 5 minutes at 1,400g. The rinsing process was repeated twice. The maximum number of larvae per tube for efficient cleaning was 0.5 to 1.0 x 10^6.

4.2.3.2

Five ml of 60% sucrose solution was added to an equal volume of faecal suspension in a 15 ml centrifuge tube. The tube was spun at 1,400g for 5 minutes. A ring of larvae was seen below the liquid surface and the supernatant was drawn off until all the larvae had been recovered. The larvae were then rinsed three times in excess PBS, which also allowed electrolyte re-equilibriation after the osmotic shock encountered by the larvae in the hypertonic sucrose.

4.2.4 Cleansing of pre-parasitic larval suspensions contaminated with other free-living larvae

Non-parasitic free-living larvae were not necessarily removed by the methods described in 4.2.3.1 and 4.2.3.2. With storage of the suspension at 4°C the majority of free living parasites die within 1 to 2 weeks. However, they can be removed as follows:

The larval suspension was gradually warmed to 37°C, over 15 minutes and made up to 60ml with water. Fifteen ml bovine bile, from a helminth free calf, was added to 60ml of 3% agar, heated to 60°C to allow dissolution, cooled to 48°C, added to the suspension and mixed thoroughly. The agar-larval suspension was poured over a gauze cloth set out in a tray. After the agar had set, the gauze was rolled into a cylinder and suspended in warm water in an elongated Baermann-type funnel. The pre-parasitic larvae alone were collected after overnight incubation at room temperature (Jess Jorgensen, 1975).

4.2.5 Exsheathment of third stage larvae

Larvae in suspension were sedimented by centrifugation at 1,400g for 5 minutes and the supernatant drawn off leaving the larvae in 2ml fluid. The volume was doubled by adding 0.025% sodium hypochlorite

solution (Milton sterilising fluid, Procter and Gamble Ltd.), made up in 0.85% physiological saline, at approximately 20°C. After shaking, the tube was centrifuged at 1,400g for 5 minutes. The supernatant was withdrawn leaving 2ml fluid and the hypochlorite treatment repeated. The larvae were rinsed three times in excess cold PBS, pH 7.2, for 5 minutes at 1,400g. At this stage, the sheath should no longer be seen protruding beyond the actual tail tip on examination by light microscopy. Larval exposure to hypochlorite solution did not exceed 12 minutes, since deeper structural damage is believed to occur after this time (F.W.Douvres, personal communication). The effect was examined by scanning electron microscopy (SEM) as recorded in Chapter 5, 5.1.3.4. Coles, Simpkin and Briscoe, (1980) tried exsheathing larvae in a variety of reagents, e.g., sodium tetraborate, carbon dioxide and carbon dioxide with bile, but they found none of the above gave results of similar reliability to sodium hypochlorite.

Experiments were conducted to test the use of homogenisation for exsheathment. Glass-to-glass and glass-to-teflon homogenisers were used on 3,500 L3 in 1ml of water and aliquots examined after different numbers of strokes. In both cases, after two to ten strokes sheaths were seen in the suspension occasionally, but the exsheathed larvae were inconspicuous. On exceeding 25 strokes few live larvae remained in the suspension. In conclusion, homogenisation was not found to be a satisfactory technique for the preparation of exsheathed larvae, so exsheathment by hypochlorite treatment was employed.

4.2.6 Extraction of fourth stage larvae from the abomasal gastric glands

The method used was adapted from the procedure reported by Gasbarre (1987). To extract L4s, calves were slaughtered at 4 DPI and the abomasa isolated. Each abomasum was opened along the greater curvature and the luminal surface contamination was flushed away with a stream of cold water. The abomasum was cut into strips about 4cm wide, which were suspended in an elongated Baermann-type funnel so maximum surface interface was exposed. The funnel was filled with water acidified with dilute hydrochloric acid (pH 2) at 37°C.

The apparatus was incubated at 37°C. Larvae were drawn off at 30 minute intervals through a tap at the funnel base and the fluid topped up with further dilute acid.

4.2.7 Estimation of adult parasite burden from abomasal worm count

At slaughter, the abomasum was ligated at the cardia and at the junction with the small intestines and isolated from the rest of the digestive tract. It was incised along the greater curvature and the contents were emptied into a bucket. The luminal surface is the main site for the adult *Ostertagia* spp., so the abomasal folds were washed with luke warm water over the bucket and any nematodes dislodged from the surface were retained (Ritchie *et al.*, 1966). The bucket contents were made up to 4 litres with water and thoroughly mixed. Two to 3ml of iodine solution was added to stain the parasites. Rapidly before settling occurred, 40ml of liquid was drawn off. Four ml aliquots were dispensed into a Petri dish. The Petri dish had been subdivided by scratching or drawing lines on the base to allow more systematic examination. Hypochlorite solution was added dropwise to decolourise the background, leaving darkly stained parasites and the samples were examined by light microscopy (x10 objective). Total nematode and individual male and female counts were made. The number of parasites in the abomasum is 100X, when X nematodes are present in 40ml of washings, since the abomasal washings were made up to 4 litres with water. Specimens were fixed in 40ml aliquots at a final concentration of 10% formalin if immediate examination was not possible.

4.2.8 Recovery of adult *Ostertagia ostertagi* from the abomasum

The abomasum was isolated at necropsy. The cardia and the pyloric-duodenal junction were tied off to prevent the loss of any of the contents. The contents were collected in a similar manner to those processed for the estimation of adult parasite burden (Chapter 4, 4.2.7). However, the contents and washings were poured onto gauze squares positioned in a sieve over a bucket. The gauze mesh pore size was chosen to retain adults, but not the majority of the small particulate

matter. The corners of the gauze were drawn together to form a pouch which was suspended in warm water at 37°C and incubated for 2 to 3 hours at 37°C. During this time the adults migrated out of the pouch and sedimented at the base of the beaker, where they could be seen as red entangled clumps of nematodes (Henriksen, 1965). The supernatant was removed and the adults drawn up on a pipette tip. The parasites were cleaned as required by flotation in a sucrose solution, i.e., the volume of the parasite suspension was doubled by adding 60% sucrose solution. Adults are more fragile than L3s due to their increased size and they appear to be affected by osmotic concentration differences to a greater extent; this may be due to a difference in composition of the surface layers. Consequently, the adults were left to settle in the fluid density gradient with time (approximately 5 to 10 minutes) rather than centrifuging them and similar time intervals were allowed for three rinsing steps in excess PBS.

4.2.9 Collection of excretory-secretory products from adult *Ostertagia ostertagi* parasites

Adult parasites were isolated by the method described (Chapter 4, 4.2.8) and washed by flotation in 30% sucrose solution (Chapter 4, 4.2.3.2 and 4.2.8). The parasites were extracted gently on a pipette tip and washed four times for 10 minutes in Hank's Balanced Salt Solution. The Hank's Balanced Salt Solution was supplemented: 1% D-glucose, 0.35% sodium hydrogen carbonate, 1mM sodium pyruvate, 0.24% L-glutamine, 0.04% tripeptide, 0.0004% glutathione, 0.48% streptomycin, 4,800 units penicillin/ml, 1% sodium bicarbonate and 2% fungizone.

In a laminar flow hood, the adults were washed three times by sedimentation in a concentrating flask in RPMI 1640 with 0.25% gentamicin sulphate. A sterile plug of cotton wool was teased out to cover 10ml of RPMI 1640 with 0.25% gentamicin sulphate in a sterile 20ml universal at 37°C. The parasites were added to the top of the cotton wool plug and incubated at 37°C. After overnight incubation, clean viable parasites, which had migrated through the plug were seen. The liquid above the plug was drawn off and the plug removed using sterile forceps. The adults in the RPMI with 0.25% gentamicin

sulphate were transferred to a clean tissue culture flask. Leaving the cap of the culture flask only partially tightened, the parasites were gassed for 1 hour with 5% carbon dioxide at 37°C. The flask top was tightened and the parasites incubated. Medium was withdrawn after varying time intervals by sedimenting the nematodes in the flask and withdrawing fluid using sterile techniques.

The medium was passed through a Millex 0.22µm Millipore low protein retention filter and dialysed against PBS pH 7.2 to remove low molecular weight solutes and solvents. A micro-ultrafiltration system (Amicon Model 8MC) with a PM10 low adsorption filter, 25mm Diaflo membrane was used. The medium was concentrated approximately 20 times in total by centrifugal ultrafiltration using Centricon-10 concentrators with YM membranes, which retain particles with a molecular mass of greater than 10 kilodaltons. The final excretory-secretory sample was stored in aliquots at -70°C (Tomlinson, Christie, Fraser, McLaughlin, McIntosh and Kennedy, 1989).

4.3 PROTEIN TECHNIQUES

4.3.1 Protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis forms the basis for many of the comparative techniques detailed in this thesis, including protein visualisation by Coomassie blue and silver staining, and Western Blotting. Furthermore, certain proteins were labelled with biotin and detected by affinity to streptavidin. A brief description of the standard gel casting technique as modified from Laemmli (1970) is given and the solution recipes listed.

A Protean II Gel Electrophoresis System (Biorad) was used. Two glass plates 16 x 20cm and 18.2 x 20cm were washed with alcohol. The plates were sandwiched together in the alignment slot with a 1.5mm spacer between them, so the lower edges were flush and screw-clamps applied. The unit was fitted into the casting slot over a rubber strip to form a seal which was tested by adding water.

A 10% polyacrylamide slab gel was poured and left to polymerize for a minimum of 30 minutes. Ammonium persulphate (APS) initiates polymerization, while N,N,N',N'-tetramethyl ethylenene diamine (TEMED) catalyses the reaction. A small amount of butan-2-ol was layered on the gel to prevent surface irregularities due to bubbles. Once the gel had polymerized the butanol was poured off and the surface was washed with distilled water, excess water was drawn off using a syringe with a guarded tip.

Depending on the number of samples to be run the appropriate teflon comb was inserted, producing ten or 15 tracks and the stacking gel was poured. The difference in pH and reduction in the gel pore size as the sample migrates through the boundary causes proteins to stack and the bands to sharpen. The gel was left to polymerize for a minimum of 30 minutes. The comb was withdrawn and the remaining liquid in the wells was removed by syringe.

A small amount of water was applied to the cooling core at the rubber insertion point, so when two gels or a gel and a stopper plate were fitted, a seal was formed and the outer plate formed the outer wall of the upper tank. The gels were placed in the buffer tank. Running buffer was added to the tank reservoir until the lower edge of the gel had been covered to a depth of approximately 2cm and the upper tank was almost full (this requires approximately 2.5L of buffer). Air bubbles collecting along the base of the plate were removed by pipette to prevent aberrant patterns caused by uneven current flow.

Sample buffer was added to all samples giving them a blue colouration (Bromophenol Blue) which facilitates loading by pipette and indicates the progress of the protein-dye front during electrophoresis, saturated Tris was added to neutralise acidic samples. Standard Molecular Weight Markers were run alongside the samples, so a linear plot of distance migrated (electrophoretic mobility) against known molecular weight may be plotted on semi-logarithmic paper, enabling weights of sample proteins run simultaneously to the standards to be estimated by interpolation: High Molecular Weight Standard Mixture (in daltons) - MW 29,000 carbonic anhydrase from bovine erythrocytes, MW 45,000

egg albumin, MW 66,000 bovine albumin, MW 97,400 phosphorylase B from rabbit muscle, MW 116,000 B galactosidase *Escherichia coli* and MW 205,000 rabbit muscle myosin.

The safety lid and cables were fitted, the current was set to maximum and the voltage at 50 V to run overnight (approximately 18 hours). Examination of the gel after 1 hour should indicate whether electrophoresis is progressing satisfactorily, the dye front should be straight and any leak from the upper buffer tank should be noticed. Depending on the dye front position it was sometimes necessary to increase the voltage (100V) to speed up migration after 18 hours. Once the protein dye front was just short of the plate edge the current was stopped and the gel could be stained or blotted.

10% ACRYLAMIDE GEL : RECIPE 1

GEL BUFFER

(Stored at 4°C.)

181.5 g	trizma base (TRIS) (1.5 M)	) in 750ml of
26.8 g	ethylene diamine tetra-acetic acid (EDTA)	) distilled water adjusted to pH 8.8.
4.0 g	SDS (0.4%) was added and the solution was made up to 1L with distilled water.	

POLYACRYLAMIDE 1% solution.

(Stored at 4°C.)

1.0g polyacrylamide was dissolved in 75ml warmed distilled water and made up to 100ml with water.

ACRYLAMIDE : BIS ACRYLAMIDE 30 : 0.9%

(Stored at 4°C in an opaque bottle.)

30 g acrylamide

0.9 g N, N'-methylenebisacrylamide (bis acrylamide)

were added to 75ml distilled water and made up to a final volume of 100ml.

From these components a gel buffer stock mix sufficient for 10 gels was produced.

GEL BUFFER STOCK MIX

(Stored at 4°C in an opaque bottle.)

75 ml	gel buffer
99 ml	acrylamide : bis acrylamide
66 ml	polyacrylamide
102 ml	distilled water

10% POLYACRYLAMIDE SLAB GEL SUFFICIENT FOR ONE GEL

34 ml	gel buffer stock mix
250 µl	10% APS (0.1g made up fresh in distilled water)
20 µl	TEMED (added just prior to gel pouring)

STACK BUFFER STOCK

(Stored at 4°C.)

12	g	TRIS (0.5M))	in 150ml distilled water
0.523	g	EDTA (8mM))	adjusted to pH 6.8
0.8	g	SDS (0.4%) was added and the solution made up to 200ml with distilled water	

STACK BUFFER STOCK MIX

(Sufficient for 10 gels.) (Stored at 4°C in an opaque bottle.)

30 ml	stack buffer
16 ml	acrylamide : bis acrylamide
12 ml	polyacrylamide
62 ml	distilled water

4% STACK GEL SUFFICIENT FOR ONE GEL

12 ml	stack buffer stock mix
250 µl	10% fresh APS
20 µl	TEMED (added just prior to gel pouring)

SAMPLE BUFFER

(x 4 concentration of final solution at loading)

40 ml	0.5 M TRIS pH 6.8
8 g	SDS
4 ml	0.2 M EDTA pH 7 (requires an alkali environment to dissolve)
40 ml	glycerol
9 ml	10% mercaptoethanol
2 µl	0.4% bromophenol blue in ethanol

ELECTRODE RUNNING BUFFER X5

60	g	TRIS	)	
288	g	glycine	)	Made up to 2 litres with distilled water.
10	g	SDS	)	
7.44	g	EDTA	)	

Approximately 2.5 litres of X 1 solution required to fill the electrode tanks.

10% ACRYLAMIDE GEL : RECIPE 2

The 29kD band was consistently absent from the Molecular Standards track using the 10% Acrylamide Recipe 1, so the following alternative solution was tried and produced the full range of markers (Harlow and Lane, 1988).

1.5M TRIS BUFFER pH 8.8 was made up by dissolving 181.5g of Trizma base in 900ml of distilled water, the pH titrated to 8.8 and the solution made up to a litre.

1M TRIS BUFFER pH 6.8 was made by dissolving 24g Trizma base in 180ml distilled water, the pH titrated to 6.8 and the solution made up to 200ml.

GEL BUFFER STOCK MIX (sufficient for 10 gels)

139	ml	distilled water
116	ml	acrylamide: bis acrylamide 30:0.9% (as for Recipe 1)
88	ml	1.5M Tris buffer pH 8.8
3.5	ml	10% SDS

10% SLAB GEL (sufficient for 1 gel)

35 ml	gel buffer stock mix
350 µl	10% APS
14 µl	TEMED

STACK BUFFER STOCK MIX

82	ml	distilled water
20	ml	acrylamide: bis acrylamide 30:0.9%
15	ml	1M Tris buffer pH 6.8
1.2	ml	10% SDS

4% STACK GEL (sufficient for 1 gel)

12 ml	stack buffer stock mix
120 µl	10% APS
12 µl	TEMED

STAINING OF POLYACRYLAMIDE GELS FOR THE PRESENCE OF PROTEIN

Silver staining can demonstrate protein at the level of 1 to 10ng, but in most cases detection at the level of 0.1 to 0.5µg with Coomassie blue was found to be sufficient and the clarity of staining was more reliable (Harlow and Lane, 1988).

4.3.1.1 Silver stain

1) The gel was immersed in 50% methanol for 1 to 24 hours on a rotating platform at room temperature.

2) Staining solution was made up from the following constituents in strict sequence. (If the solution turned brown ammonia was added dropwise and the solution was shaken.)

0.5	g	silver nitrate ($AgNO_3$)
17.5	ml	distilled water
0.875	ml	ammonia (NH_3)
12.5	ml	0.36% sodium hydroxide (NaOH)
30	ml	methanol

The gel was incubated for 90 minutes in the solution.

3) Several washes in water were carried out over 10 minutes.

4) A developing solution was made up from 0.005g citric acid and 50µl formaldehyde in 100ml distilled water. The gel was reacted in solution for 5 to 10 minutes in the dark. When clear banding was visible the reaction was stopped with a solution of 50% methanol and 18% thiosulphate in distilled water.

4.3.1.2 Coomassie blue stain

The gel was immersed in the following stain solution for a minimum of 1 hour on a rocking platform, followed by several washes in destain solution to reveal the banding pattern.

Coomassie blue stain

3 g	Coomassie Blue R
500 ml	methanol
70 ml	glacial acetic acid
430 ml	distilled water
1,000 ml	

Destain solution

500 ml	distilled water
400 ml	methanol
100 ml	glacial acetic acid
1,000 ml	

Gels were photographed with a Polaroid Cu-5 Hand Camera and Hood on Polaroid 667 high speed black and white (f16, 1/15s). Gels were preserved by drying; they were laid flat on a sheet of plastic film and a sheet of 3MM paper covered the gel.

The "sandwich" was inverted and dried in a vacuum created by a Biorad Mode 483 Slab Dryer at 80°C for 2 hours.

Shrinkage occurs during drying, so measurements for molecular weight assessment should be made prior to this step.

4.3.2 Protein blotting

Protein separation by molecular weight was achieved by standard methods on polyacrylamide gel (10%) (Chapter 4, 4.3.1). To allow specific immunochemical detection with antibody conjugation and for radiolabelling studies it is convenient to transfer the protein profile to a membranous support, i.e., nitrocellulose acetate. Applying an electric current forcibly elutes the protein from the gel and it binds by electrostatic and hydrophobic forces, to the nitrocellulose (Towbin,

Stacklin and Gordon, 1979). Correct assembly of the apparatus is crucial to the transfer (BioRad Trans-Blot Cell); normal transfer polarity runs from cathode to anode.

The gel holder was assembled in a low tank filled with blotting buffer. The blotting buffer comprised of 72.1g glycine, 15.1g Tris and 200ml methanol, made up to 4 litres with distilled water. All pads and paper were saturated in this buffer. The gel holder was loaded from the cathode, a layer of 'Scotchbrite' pad (3M) and then filter paper (3MM Chromatography Whatman) was added. The gel was positioned on top of this. Nitrocellulose (Hybond-C, Amersham) 0.45µm was soaked in buffer and laid over the gel. Nitrocellulose acetate should be manipulated with gloved hands only, since protein residues may be picked up from the skin, so when necessary forceps should be used to manipulate the nitrocellulose by the edges. A further layer of filter paper was added and a test tube rolled over the surface applying even pressure to dispel air bubbles. The other 'Scotchbrite' pad was applied and the gel holder locked, so firm even pressure was applied. A corner was usually snipped off the gel and the nitrocellulose to allow orientation after blotting. Ideally the gel was left to equilibrate for 30 minutes in the buffer prior to application of an electric current. When there was poor contact between the layers a swirling pattern resulted or high background staining.

The holder was inserted into the blotting tank. When only one gel was to be blotted the slot closest to the cathode was used. Approximately 3 litres of blotting buffer filled the tank. The apparatus was set up in a cold room at 4°C and run at 80 volts for 3 hours.
Non-specific adsorption sites on the nitrocellulose were blocked by immersing the gel for 1 to 2 hours in 10mM Tris-hydrochloride pH 7.4, 1% Tween 20 in 0.9% saline on a rotating platform at room temperature (5% skimmed milk may be added to the solution). The acetate could be stained, i.e., for protein by Ponceau Red staining, to identify antigens by a conjugated antibody reaction or the protein could be labelled with biotin prior to electrophoresis and subsequently its affinity to radiolabelled streptavidin shown by autoradiography.

10X Concentration tris saline blocking solution

12.11g tris

90g sodium chloride

Made up to 1 litre with distilled water.

4ml 25% Tween 20 was included in every 100ml of 1x tris saline solution.

4.3.2.1 Ponceau Red Staining

Protein profiles on nitrocellulose acetate may be assessed by staining with a solution of Ponceau S 0.3% w/v in 5% trichloroacetic acid for 3 minutes followed by washing in tepid water.

4.3.3 Sample preparation for sodium dodecyl sulphate - polyacrylamide gel electrophoresis

Enzymes not generally active in nematode tissue are liberated from storage glands and to an extent from lysosomes during the homogenisation process, leading to a degree of self destruction ("autolysis"/autodigestion). Analysis of an autolysed homogenate does not give a true representation of the living parasite proteins. Therefore, protease inhibitors are added to the homogenisation buffer (Table 4.3) to reduce proteolysis. Larvae were transferred to a 0.1ml glass-to-glass homogeniser in a minimum of supernatant and homogenisation buffer was added. Assessment of homogenisation efficacy was made by examination by light microscopy. After homogenisation, sample buffer was added and the samples were boiled for 3 minutes and then microfuged for 10 minutes at 10,000g. The supernatant alone was loaded, so no large particles obstructed the gel slot base.

4.3.4 The assessment of protein concentration in a parasite sample by the Bradford Protein Assay

The assay solution (Bradford, 1976) was prepared by mixing 200mg Coomassie brilliant blue G-250 and 100ml 95% ethanol for 1 hour, then 200ml 85% phosphoric acid was added. The solution was made up to 1 litre with distilled water and after further mixing the solution

TABLE 4.3

Nematode homogenisation buffer with protease inhibitors for sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Component	Amount	Concentration	Final concentration	Specificity of inhibition
Ethylene-di-amine tetra-acetic acid (EDTA)	4.8ml	0.67mg/ml in distilled water	2mM	Metalloenzymes by chelating action
Phenylmethylsulphonyl -fluoride (PMSF)	5µl	0.17mg/ml in acetone	1mM	Thiolproteases (papain) Serine proteases (chymotrypsin, trypsin, thrombin)
Leupeptin	5µl	0.02mg/ml in methanol	5µM	Serine and thioproteases (trypsin, papain, plasmin, cathepsin B)
Antipain	50µl	0.03mg/ml in methanol	5µM	
N-alpha-p-tosyl-L-lysine chloro-methyl ketone (TLCK)	2µl	0.02mg/ml in methanol	25µg	Thiol proteases (bromelain, ficin and papain) Trypsin irreversibly
N-tosyl-L-phenylalanine (TPCK)	1µl	0.05mg/ml in methanol	50µg	Thiol proteases (bromelain, ficin and papain) Chymotrypsin irreversibly
1,10 phenanthroline	4µl	3.96mg/ml in methanol	2mM	

was filtered. Bovine serum albumin (BSA) standards were made in distilled water at 5, 10, 25, 50 and 100µg/100µl in duplicate. By this method, 100µl of dilute BSA was equivalent to 100µg protein.

The sample under assay was prepared as follows: 100,000 L3s were homogenised in 1 ml of distilled water. Samples were homogenised in water, since the homogenising buffers routinely used contain reagents which interfere with the colorimeter reading. Samples of 100µl of the supernatant and 100µl of the pellet resuspended in 1 ml of distilled water were prepared for comparison.

To each supernatant/pellet and each BSA standard was added 5ml of assay solution. The tubes were vortexed and incubated for at least 20 minutes and not more than 2 hours. After a further burst of vortexing the absorbance was found using a colorimeter set at 595nm (Perkin-Elmer 550A/Cecil digital grating spectrophotometer CE383). The average absorbance values for the protein standards read at 595 nm were plotted against protein concentration and formed a sigmoid curve; samples with higher protein concentration stained more strongly and absorbed more light. The protein concentration of the unknown samples was estimated from the absorbance values on the curve.

CHAPTER 5

THE LIFE CYCLE OF *OSTERTAGIA OSTERTAGI*: PICTORIAL EXAMINATION AND *IN VITRO* CULTURE

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The contents of this chapter are considered in two sections. The first section deals with the changing appearance of *Ostertagia ostertagi* during development and should be considered alongside the associated events described in the life cycle section (Chapter 1, 1.2).

Adequate sample definition is an essential prerequisite for studies where specialised techniques are to be used for the detection of changes within a species or with parasite stage by morphology, protein profile, isoenzyme analysis, surface examination, etc. It is essential to assure that the examination pertains to a single species. Consequently, an ability to recognise the developmental stages of the life cycle by light and scanning electron microscopy (SEM) is important to prevent stage changes confusing possible isolate variation, i.e., a baseline for variation must be established. A pictorial guide to the stages of the life cycle by light microscopy and SEM was considered to be a useful reference point on which to build further knowledge. A major component of the entire study is concerned with the nematode surface and so it seemed particularly appropriate that a SEM examination was made.

The second section is devoted to the techniques for culture of the parasite *in vitro*. Free-living stages are routinely grown in many laboratories, but few workers have successfully maintained the parasitic stages in culture. The importance of *in vitro* culture lies in the generation of samples uncontaminated by other species from material derived from particular isolates.

5.1 A PICTORIAL ANALYSIS OF THE STAGES OF THE LIFE CYCLE OF *OSTERTAGIA OSTERTAGI*

5.1.1 Introduction

With respect to light microscopy, Soulsby (1965) gives a general description of the development of the gastrointestinal nematodes of ruminants and Gibbons and Khalil (1982) have compiled an excellent

illustrated key to the trichostrongyle genera. Threlkeld (1946) gives a brief account of the life history of *O.ostertagi* with some diagrams and photographs. Douvres (1956) and Rose (1969) give full accounts (including diagrams) of the morphogenesis of the parasitic stages of *O.ostertagi*. These latter descriptions and diagrams give the impression of a clear step by step transition from one stage to the next. One of the aims of this study was to assess the clarity of these stage demarcations and to produce a photographic record of development.

Surface topography of nematodes can be well illustrated by scanning electron microscopy. The image is produced by secondary electron production at the sample surface, when a focused electron beam is directed there. The resulting current varies with the sample surface contour. The message is amplified and the voltage variation produced causes a pattern in a cathode ray tube. Increased numbers of secondary electrons are generated at an oblique surface. The part of the sample nearest the incident beam appears brightest. The reader is referred to Hayat (1978) for a full account of the principles and techniques of SEM. Previously SEM studies of *O.ostertagi* have been restricted to infectious larvae and adult stages (Lichtenfels *et al.*, 1988), while no attention has been given to other stages. However, SEM has already been used to demonstrate and confirm information in a way not attainable by light microscopy, e.g., when it was used to aid the differentiation of the Ostertagiinae by examination of the pattern of synlophes, surface ridges, of the adult, giving further support to the theory that major and minor morphs of the trichostrongyle species exist (Lichtenfels *et al.*, 1988) (Chapter 2).

5.1.2 Materials and Methods

5.1.2.1 Parasite source

Early *O.ostertagi* eggs were isolated after maceration of adult females or examined *in utero*. Generally, *O.ostertagi* eggs were collected from calf faeces by flotation in saturated salt solution (Chapter 4, 4.2.2). Faecal cultures were set up and L1s, L2s and L3s were recovered by baermannisation of samples after varying incubation

times (Chapter 5, 5.2.2.1 and 5.2.2.2). The L3 stage alone was subjected to analysis for intraspecies comparison, since maintenance of imported larvae for further development was not permitted. Parasitic larval stages were collected by baermannisation of portions of parasitised abomasum at 4 DPI (Chapter 4, 4.2.6). The abomasal contents and surface washings of calves slaughtered at 21 DPI were baermannised to amass adult parasites (Chapter 4, 4.2.8). Observations of *in vitro* produced "parasite" stages were also made, i.e., from post-exsheathment of the L3 onwards (Chapter 5, 5.2.2.4).

5.1.2.2 Light microscopy

Parasites, suspended in a droplet of water, were examined by direct light microscopy using a Leitz Orthoplan microscope. No stains were employed. The changing morphology was recorded on Fujichrome 50/100 film using an Orthomat camera. Highly motile larvae were fixed in 10% formalin. The magnifications quoted in the figure legends refer to a composite figure consisting of the magnifications produced by the objective lenses (x4, x10, x25, x40), the camera tube factor (x1.5, x2, x2.5) and a factor of enlargement on transfer of the image from the negative to the print.

5.1.2.3 Scanning electron microscopy

L1, L2, L3 and L4 samples were washed by flotation in 30% sucrose and centrifugation at 2,000g, while adults due to their increased size and relative fragility were not centrifuged, but left to settle by relative density with time. Three washes in phosphate buffered saline (PBS), pH 7.2, followed. Gentle washing prevents sample damage, but removes the surface debris, which can result in artefacts seen on final examination of the prepared material.

Parasites were fixed rapidly before degeneration could occur in an isotonic fixative to avoid osmotic shock. Fixative was applied for longer to thicker samples to allow full penetration. Fixation was carried out for 3 hours in 0.1M phosphate buffer pH 7.2 with 2.5% glutaraldehyde and the sample hardened and became brittle. Three washes in 0.1M phosphate buffer followed and the samples were

suspended in 0.1M phosphate buffer until further processing. If the technique was interrupted for greater than 1 week after washing, the samples were given three washes in 0.1M phosphate buffer with 2% zinc prior to the next step.

Most of the supernatant was withdrawn and the sample volume doubled with osmium tetroxide, to give a final concentration of 0.1%. Osmium tetroxide is highly toxic and was added to the sample in the fume cupboard. Heavy metal staining with uranium and osmium improves electron conductivity by increasing electrical emission. The samples blackened. After incubation for 1 hour, the samples were washed three times in distilled water, sedimenting for 10 minutes between washes. At this stage, the samples were too fragile to centrifuge. The sedimented parasites were put into 0.5% uranyl acetate and incubated for 1 hour in the dark, since uranyl acetate is light reactive. Three washes in PBS ensued.

A ring specimen holder was assembled from a series of nylon and metal filters. The sample was laid centrally on a Nucleopore filter: a 5.0μm filter was used for L3, L4 and adult parasites and a 0.2μm filter for L1s and L2s. Water is not miscible with the common transition fluids used in the critical point drier, in this case carbon dioxide (CO_2). Consequently, a transfer fluid miscible with both water and CO_2 must be substituted for the water, i.e., acetone. A series of acetone solutions of increasing concentration were each applied to the specimen carrier for a 10 minute period, i.e., 30%, 50%, 70%, 90%, 100% acetone and 100% anhydrous acetone.

The samples were rapidly loaded into the critical point dryer before the 100% anhydrous acetone was depleted. The critical point dryer had been checked for leaks prior to loading. The CO_2 inlet was opened and the chamber rapidly filled with liquid CO_2. The vent valve was released and the substitution fluid - the acetone - was flushed out, but the liquid level was maintained to prevent turbulence damaging the sample during the procedure. Flushing was repeated four times at 15 minute intervals to remove the acetone. The larger the sample the greater the number of flushes required and the longer the drying time necessary.

Once flushing had been completed, the chamber was filled with liquid CO_2 to just below the pressure gauge inlet and the vent valve was shut. Hairdryers were used to raise the chamber temperature to approximately 40°C for 15 minutes, giving a pressure of 1,400 pounds per square inch (psi), i.e., above the critical point for CO_2 (1,150 psi, at 32°C). Normally on evaporation surface tension causes cell distortion as the surface of the liquid recedes, however, at the critical point liquid and vapour phases are indistinguishable and surface tension is not a problem. Critical point drying was achieved by heating liquid and gas in a sealed container, which caused the liquid density to fall due to evaporation, while the vapour density and pressure increased. The meniscus diffused and disappeared, so CO_2 gas alone remained in the chamber. After 5 minutes at 1,400 psi, the gaseous CO_2 was slowly vented over 10 minutes to avoid condensation due to local cooling. A temperature of greater than 36°C was maintained during the venting process, which was carried out slowly, these precautions prevented local condensation. Finally, heating and gas supplies were cut off and the specimens were withdrawn from the chamber.

The specimen holder was carefully opened and the filter removed. Sample adherent to the filter was mounted directly onto an aluminium stub coated with a piece of double-sided adhesive tape, so the sample filter was slightly smaller than the stub surface. The edges of the filter were sealed with colloidal silver paint, which maintains the electrical conductivity from specimen to stub. Loose sample was gently shaken onto a piece of double-sided tape applied to a stub in a similar manner and edged with silver paint. At this stage, the sample could be stored for a few hours in a desiccator. However, if the delay in sputter coating is prolonged a decrease in surface receptiveness may occur, necessitating a second coating.

The stubs were inserted at the anode and sealed into the Modified Polaron SEM Coating Unit E 5,000. The chamber was evacuated to 0.08 torr by pumping, flushed with argon to remove residual gas and the pressure was increased to not greater than 0.2 torr. Evacuation and flushing was repeated twice and then the pressure set to HT (high tension). During sputter coating, a heavy metal target material is

bombarded with energetic particles in a similar manner to particles in a gaseous discharge tube (Cowell, 1987). Particles are ejected from the target and condense on the sample, which is held at a positive potential. The potential difference (voltage) of the coating unit, from gold cathode (target) to the sample at the anode, was raised to 0.75 kV and argon leaked in until the ammeter gave a reading of 25 mA. The settings were maintained for 8 minutes. The argon was shut off, the potential difference was removed and the pump was turned off. Air was allowed to leak into the chamber using the thumb-leak valve.

The film of gold applied is usually 200-500 Å thick. Film thickness may be calculated by:

a =	approximately	mA	x	kV	x	t	x	k
thickness in angstroms		current (25)		voltage (0.75)		time (2 min)		constant dependent on gas: Argon = 5 Nitrogen/air = 2

Coating the sample with highly conductive material prevents build up of charge from the electron beam. Excess charging distorts the image and damages the specimen by heating. When the coat is too thin charge distortion and damage may occur, while too thick a coat obscures surface detail.

The nitrogen cylinder was turned on and air admitted into the sample chamber of the electron microscope. The chamber was opened, the stage withdrawn and samples were inserted into a rotating carrier using an Allen key. The stage was returned. The door was shut, the nitrogen supply stopped and the chamber evacuated. The electron beam was applied and the image produced on a monitoring screen could be altered by adjusting stage position, magnification and contrast. Surface may be magnified up to 20,000 times.

To record the image on film (Ilford FP4) a section of the frame was chosen, the focus perfected and the full frame picture reselected. A signal profile for the image indicated whether sufficient reflected beam was being received and the contrast was adjusted accordingly.

Once optimal conditions had been achieved the settings were frozen and a single scan exposure made.

5.1.3 Results :

Morphological changes recognised during development

5.1.3.1 Egg

On examination by light microscopy the eggs of *O.ostertagi* were oval and measured 74 to 90 µm by 38 to 44 µm. The cell mass was surrounded by a wide fluid cavity and enclosed by a bilayered boundary. Fertilised eggs were recognised by the presence of centrosomes. Cell division progressed rapidly until a mass of blastomeres were present, the morula, which was the earliest stage found in faecal samples (Figure 5.1). Once 12 to 16 blastomeres/cells were present the embryo was considered to be blastular, fluid gathered centrally and the blastula was formed. Later, the cell mass became indented on one side, which was sometimes visualised. Division was uneven with variation in developing cell sizes. After several divisions the shape of the cell mass began to change; from an oval it elongated and the posterior curved ventrally and anteriorly (Figure 5.2). Two rows of cells formed the intestine, which had a zig-zag appearance immediately before hatching.

5.1.3.2 L1

The L1 was cylindrical and measured 360 to 460 µm long by 20.4 µm wide. Light microscopy showed a twisting intestine, which eventually slanted towards the anus (Figure 5.3). The intestinal cells became increasingly more granular with time (Figure 5.4). The oesophagus was rhabditiform, i.e., the muscle had a slight anterior and posterior swelling. The larvae were active and slithered in a slow flowing fashion. The lateral body margins were parallel for the majority of the larval length. Relative to later stages the width to length ratio appeared high.

On SEM (Figure 5.5) the cuticle was finely striated transversely. Two prominent ridges, alae, ran down the lateral margins, these

FIGURE 5.1:

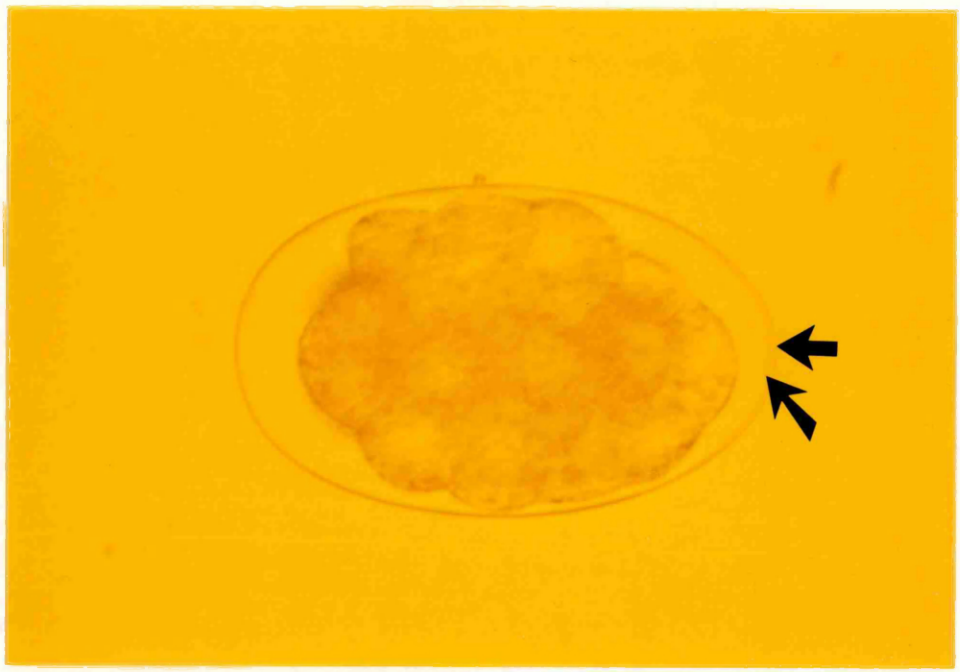
Ostertagia ostertagi **egg in morula stage**
x 210

The bilayered nature of the egg surface may be distinguished at one pole (arrows). Thirteen overlapping granular cells may be distinguished in the view taken at this plane and further cells could be seen by altering the focus. A fluid cavity surrounds the blastular cell mass.

FIGURE 5.2

Ostertagia ostertagi **egg**
x 210

The egg presented in this figure has developed further than the one depicted in Figure 5.1. The number of cells has increased and the cell mass has begun to elongate. Both terminal poles of the body mass have started to curve towards the centre on one side.



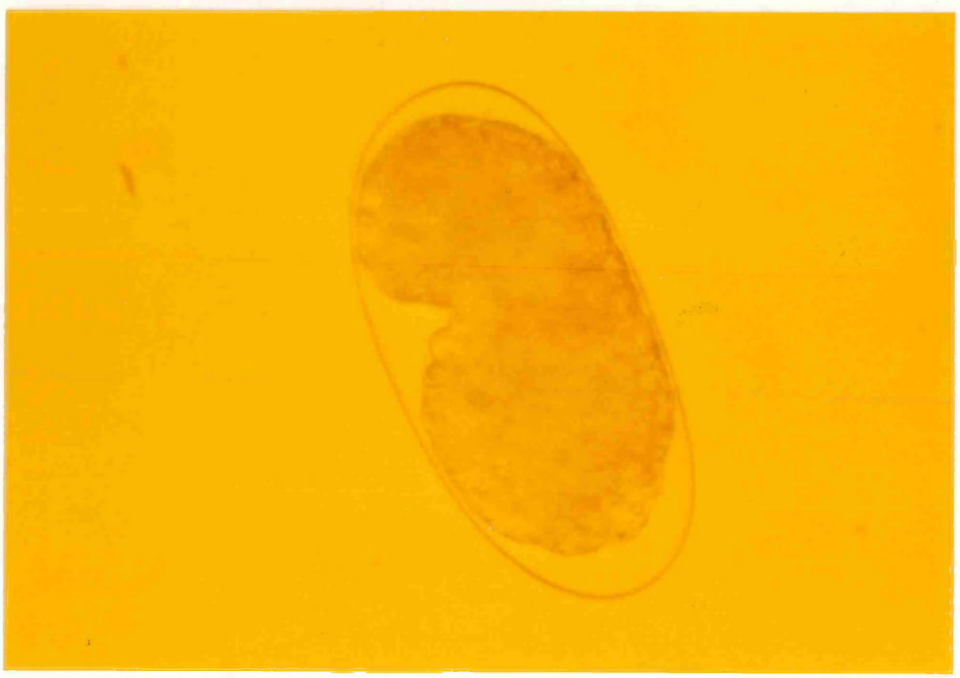


FIGURE 5.3

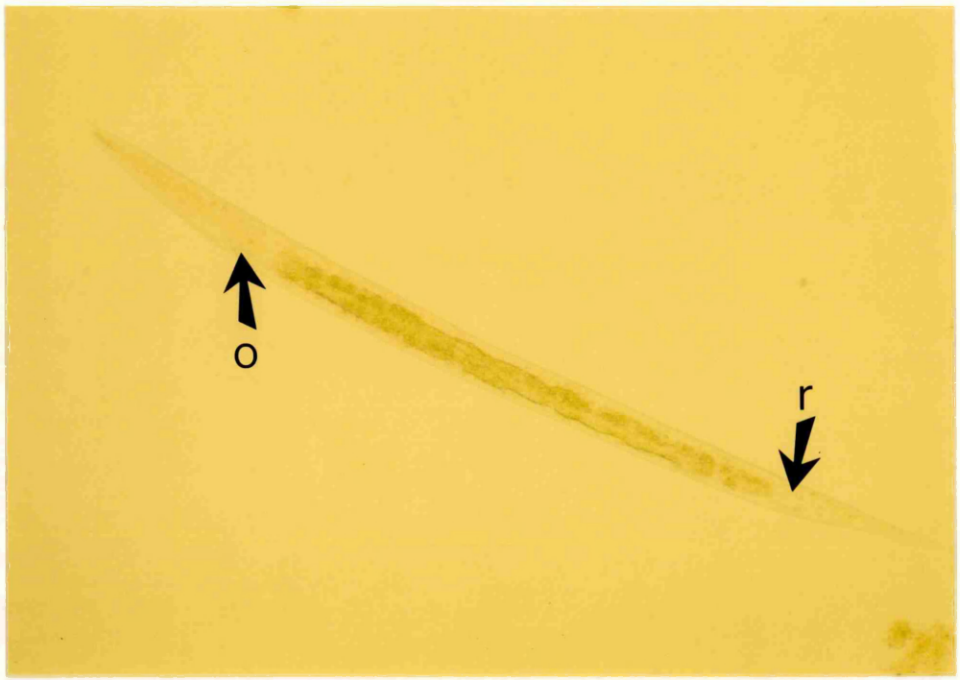
L1 stage *Ostertagia ostertagi*
x 130

The intestinal spiral can be seen clearly, but the caudal muscular bulb of the posterior oesophagus (o) and the caudal slanting of the rectum (r) may only be distinguished with care. The parallel nature of the lateral body margins for the majority of the body length is well illustrated.

FIGURE 5.4

Twisting intestine of the L1 stage *Ostertagia ostertagi*
x 210

The appearance of the intestinal cells became increasingly granular with time.


o
r

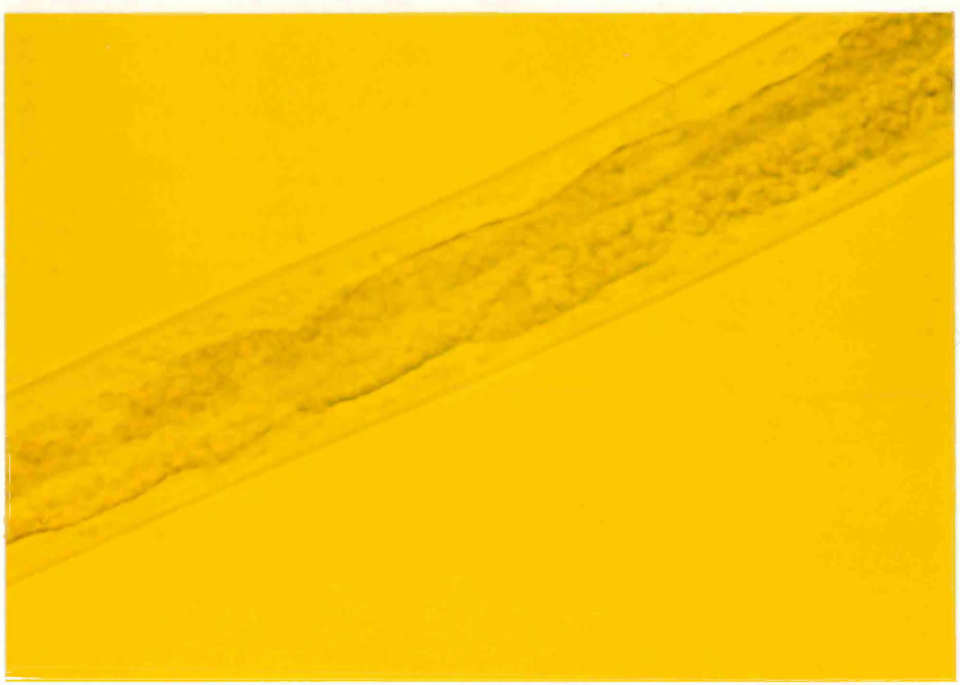
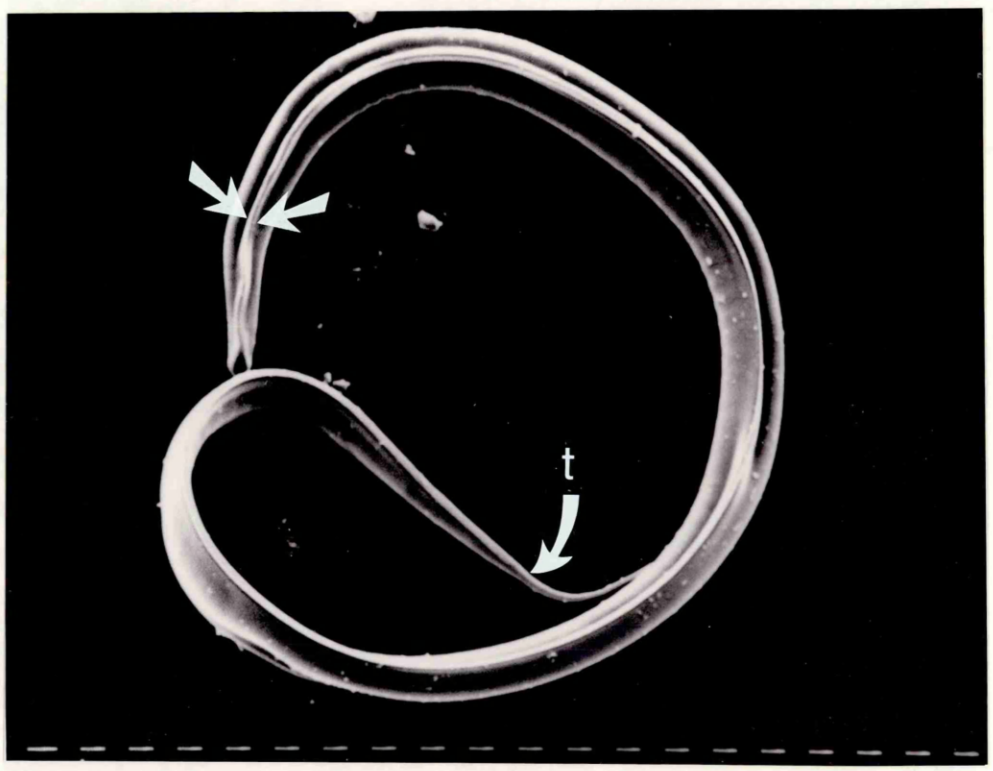


FIGURE 5.5

Scanning electron micrograph of L1 stage *Ostertagia ostertagi* x 400. Scale bar 1 µm.

The prominent lateral alae appear to consist of two ridges (arrows), which give the body a flattened appearance. The tail (t) is steeply tapering, while the anterior is comparatively blunt.


t

seemed to consist of two closely applied elevated sections and the body appeared flattened between them. The anterior cap was blunt compared to the spikey tail.

Later on light microscopy, the outer cuticular surface became thickened, until another layer could be distinguished below. An indented ring clearly demarcated a cone at the most anterior portion of the nematode cuticle. The ring appeared to puncture and the cone "unzipped", so the tip hinged back and the L2 slowly worked out of the cast cuticle to complete ecdysis. Larval activity was decreased immediately prior to the first moult.

5.1.3.3 L2

On light microscopy the larval appearance changed markedly with the transition from L1 to L2. The intestinal lumen straightened and increased in diameter (Figure 5.6). The intestinal cells were flat and indistinct. The oesophagus remained rhabditiform.

However, by scanning examination the image of the L2 had not altered markedly from the L1, although the L2 was longer, approximately 500 µm. The transverse striations were clearer in the L2 and there appeared to be further very fine longitudinal ridges paralleling the lateral alae (Figure 5.7). A ring of projections, sensory structures, surrounded the stoma.

5.1.3.4 L3

The progression to the L3 was preceded by an interval of reduced activity; an inner and an outer layer of cuticle were recognised, eventually separating so the sharp point of the L3 tail was seen well within the remaining L2 sheath causing the sheath to kink (Figure 5.8). With time the gap between the cuticle layers increased until continuity was only retained at the mouthparts.

Differentiation of *O.ostertagi* L3s from those of the other trichostrongyles was difficult. The best parameter was found to be sheath length:total larval length. The sheath was found to contribute

FIGURE 5.6

L2 stage of *Ostertagia ostertagi*
x 140

In the early L2 the intestinal lumen (il) no longer spirals as in the L1, but has become straight and wide. The oesophagus is muscular and rhabditiform (arrows).

FIGURE 5.7

Scanning electron micrograph of the anterior of L2 stage *Ostertagia ostertagi* x 1,600. Scale bar 10 μm

Projections suggestive of sensory structures are demonstrated apparently surrounding the stoma (s). Fine striations are present and the more prominent lateral alae can be seen running down the lateral margins. Fine cuticular ridges (cr) parallel the alae on either side. The corrugated appearance of the alae suggests a slight osmotic imbalance at fixation.

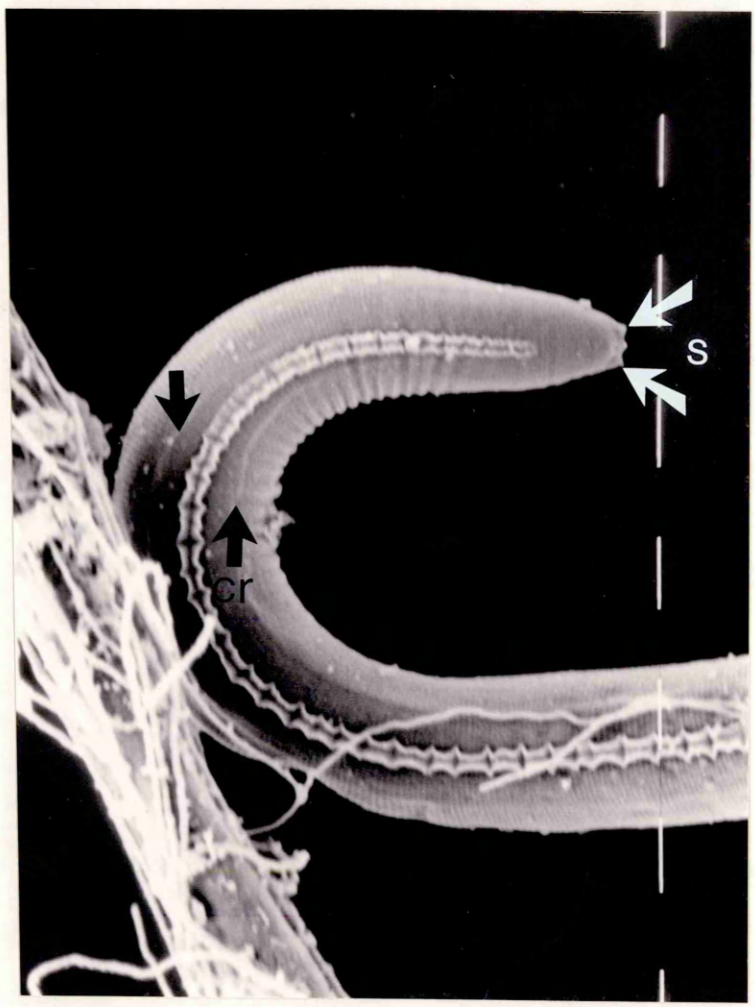

S
cr

FIGURE 5.8

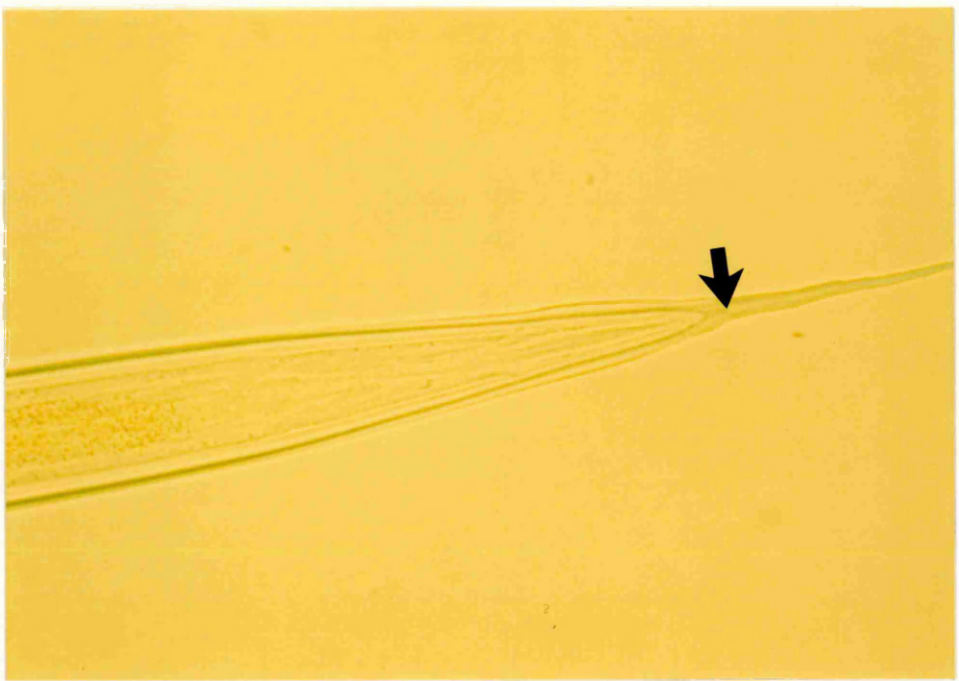
The caudal section of L3 stage *Ostertagia ostertagi*
x 210

By altering the fine focus of the microscope the true L3 tail may be discerned within the retained L2 sheath. The true L3 tail tip is indicated by the arrow.

FIGURE 5.9

Anterior of an *Ostertagia ostertagi* L3
210

The rhabditiform muscular oesophagus of the L1 and L2 stages has been replaced by a wispy filariform oesophagus in the L3. The L3 stage does not ingest nutrients and hence there is no need for a muscular highly developed oesophagus.



approximately a sixth of the total sheath length of 825 to 925 µm. Isolates of the L3 stage obtained from different geographical sources were uniform in appearance. The L3s were highly motile, necessitating fixation for close examination. They moved by a series of rapid sinusoidal undulations, with the body axis perpendicular to the general direction of movement.

The oesophagus was thready (filariform) and mid-way down its length encircled by a wispy nerve ring (Figure 5.9). The globular buccal capsule was not always visible and the stoma appeared closed on light microscopy. Occasionally the excretory pore could be seen ventrally at the level of the posterior oesophagus. The flat intestinal cells filled out with granules. Temporarily 16 discrete cells could be distinguished (Figures 5.10 and 5.11), but with time the differentiation of the intestinal cells was lost until they were obscured by vacuoles and dark granules (Figure 5.12). Caudally the intestine was associated with the sexual primordia.

Despite the closed appearance of the stoma on light microscopy, on SEM a clover-leaf-shaped depression bordered by three lips was apparent (Figure 5.13). In turn, the stoma was ringed by a slight circular depression and surrounded by six triangular projections, cephalic papillae, sensory structures. The transverse annulations and longitudinal ridges were more clearly demarcated than in the previous stages and arose caudal to the ring of projections (Figures 5.13 and 5.14). In the cephalic region a semicircular depression was seen on both lateral aspects between the second and third annulations, sensory amphids (Figure 5.13).

Artificial exsheathing of the L3 is routinely induced by application of sodium hypochlorite solution (Chapter 4, 4.2.5). However, it was suspected that exsheathment may damage the larva (F.W.Douvres and M.W. Kennedy, personal communications). Consequently, samples of L3 exsheathed in sodium hypochlorite were assessed alongside the sheathed L3 to determine whether surface damage had occurred. Hypochlorite exsheathment of the L3s resulted in a bleb-like anterior on light microscopy. On SEM, the exsheathed L3 (Figure 5.15) annulations were irregular and the alar folds did not run as far

FIGURE 5.10

Early L3 stage *Ostertagia ostertagi*
x 50

Temporarily, 16 intestinal cells were clearly distinguished.

FIGURE 5.11

The intestinal cells of early L3 stage *Ostertagia ostertagi*
x 210

Once the intestinal lumen has straightened the cells become temporarily more discrete. However, cell definition is lost with time as the cells become more granular. The larva on the left has clear cell margins and is consequently a less mature individual.

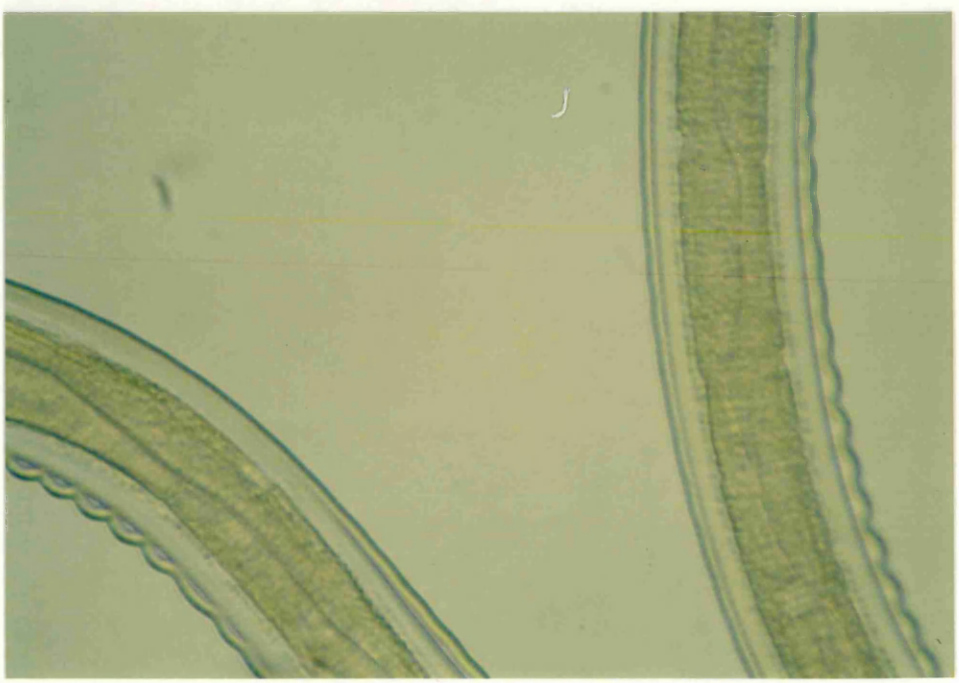


FIGURE 5.12

The intestine of an *Ostertagia ostertagi* L3
x 210

The discrete cell appearance of the early L3 has been lost, as dark vacuoles and granules develop in the nematode digestive system.

FIGURE 5.13

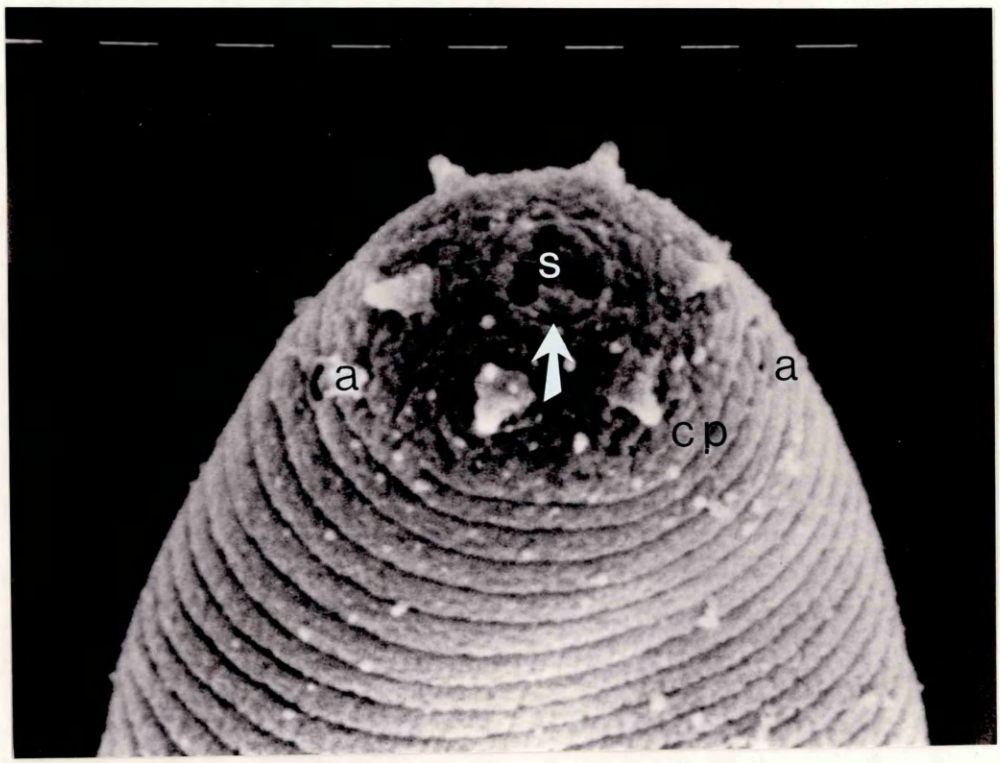
Scanning electron micrograph of the stoma of an L3 *Ostertagia ostertagi* x 12,800. Scale bar 1 μm

The clover-leaf triradiate depression of the stoma (s), may be clearly recognised and it is surrounded by a fine circular depression (arrow). Six triangular projections formed a corona of cephalic papillae (cp) at the anterior margin. The transverse striations arise caudal to the papillae and on the lateral aspects semicircular indentations, sensory amphids (a), may be distinguished between the second and third annulations.

FIGURE 5.14

Scanning electron micrograph of an L3 stage *Ostertagia ostertagi* x 800. Scale bar 10μm

The lateral alae appear to consist of two closely applied ridges. Faint surface annulations can be discerned. The point at which the true tail finishes and only the sheath projects causes a slight kink in the body margins (arrow).


s
a
a
cp

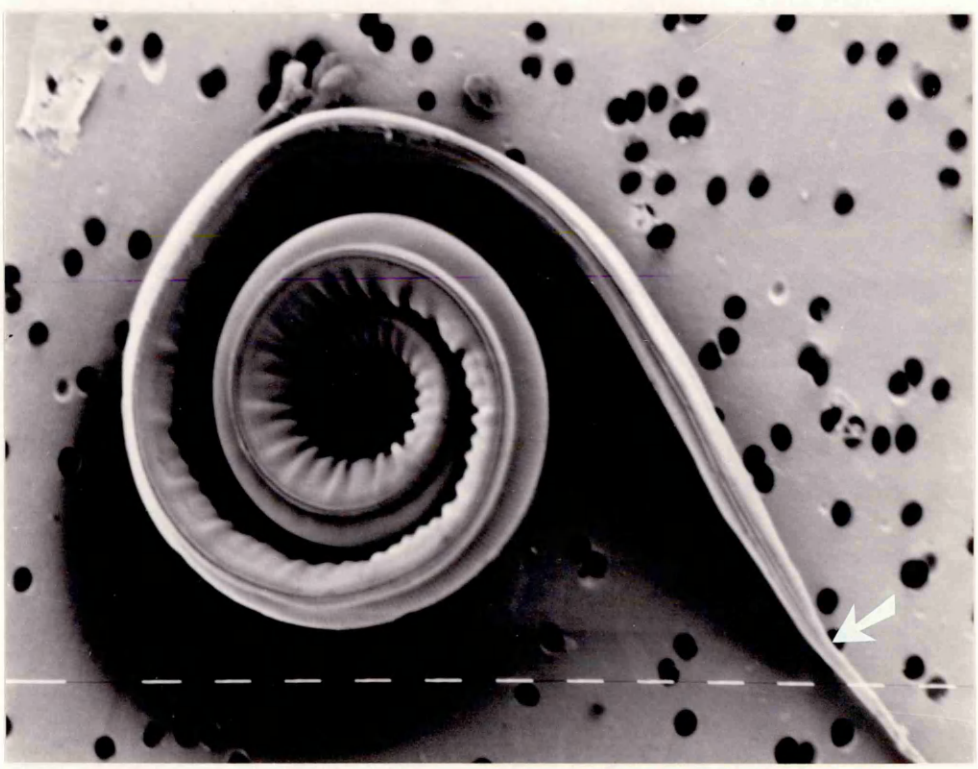


FIGURE 5.15

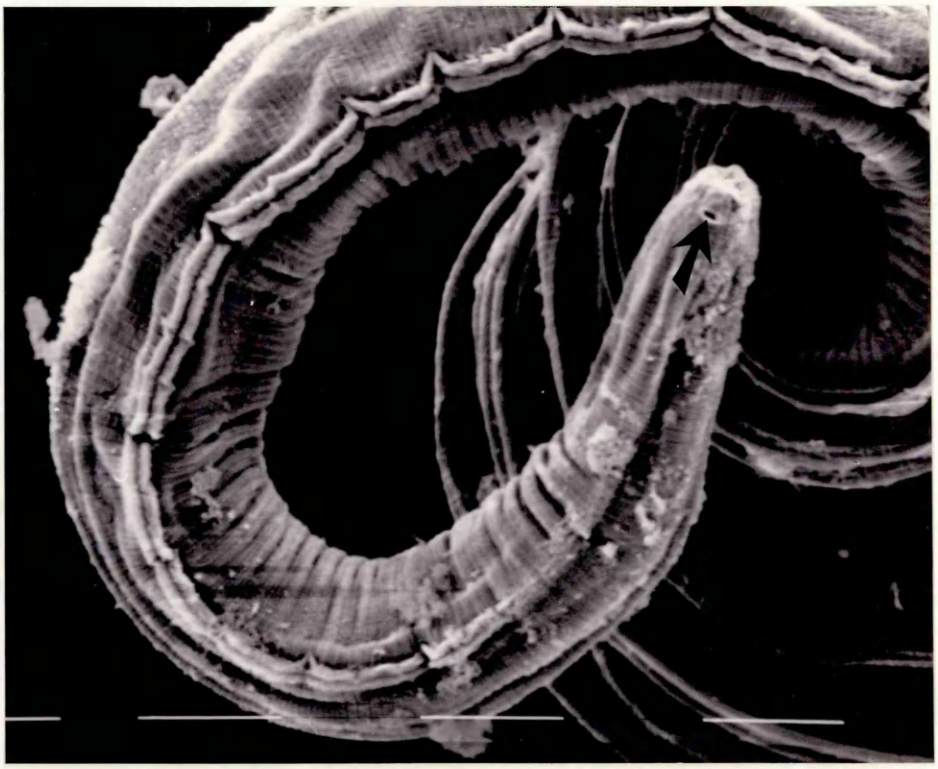
Scanning electron micrograph of L3 stage *Ostertagia ostertagi* after treatment with sodium hypochlorite solution for exsheathment.
x 3,200. Scale bar 10μm

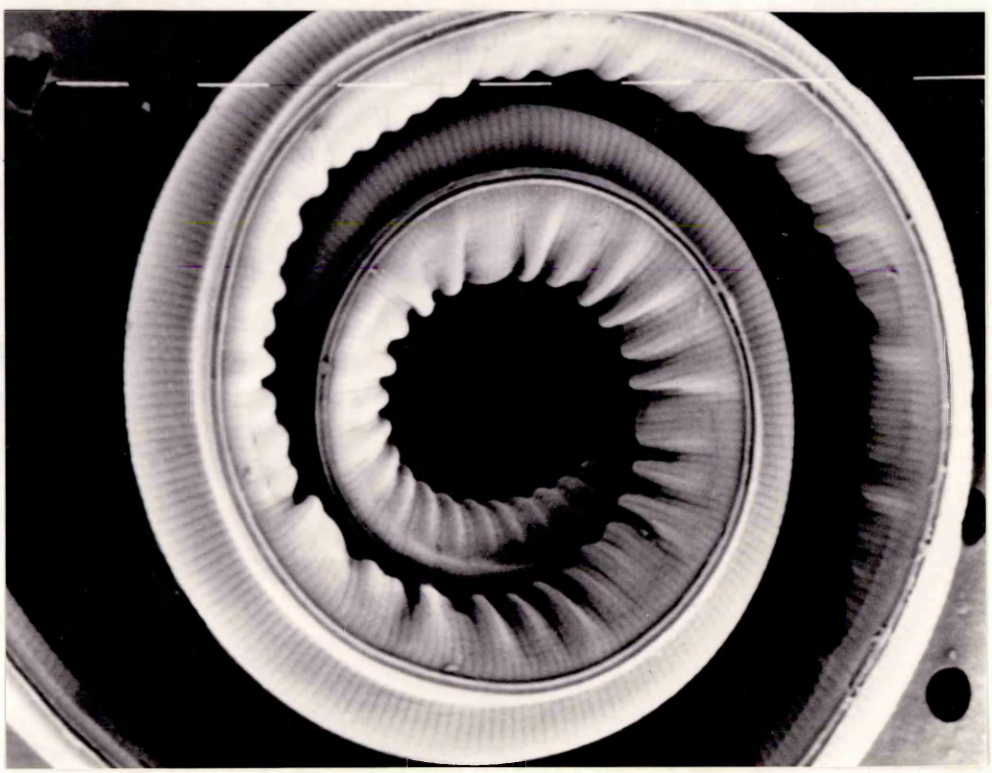
Apart from initial exsheathing and rinsing, the L3 shown was washed and fixed simultaneously with sheathed larvae shown in Figures 5.14 and 5.16. The surface of the exsheathed L3 appears crumpled and irregular, as if fluid has been lost and the larva is in osmotic shock. The surface is covered with irregular tags of material, suggesting some of the retained L2 remains after hypochlorite treatment.

FIGURE 5.16

Scanning electron micrograph showing surface detail of an L3 *Ostertagia ostertagi*
x 1,600. Scale bar 10μm

The sheathed L3 has a smooth surface with only fine annulations, unlike the segmented appearance of the exsheathed parasite with tags of debris attached to it (see Figure 5.15).





anteriorly as those of the sheathed L3s (Figure 5.16). The surface after exsheathment was wrinkled and appeared to have ragged tags of debris stuck to it. The stomal area of the exsheathed L3 was not clearly delineated and the amphids in the cephalic region appeared to be gaping open.

After 2 to 3 days in *in vitro* culture, exsheathed L3s began to develop air pockets below the outer surface, particularly at the level of the excretory glands, anus and tail tip, as seen on light microscopy. Fluid blebs gave the anterior a squared off appearance. The intestinal lumen began to open inwards from the anterior and posterior extremities and the intestinal cells became transparent.

5.1.3.5 L4

After moulting the anterior of the L4 was markedly truncated compared to that of the L3. The buccal capsule became more muscular. The oesophagus enlarged markedly and grew more muscular, resuming a rhabditiform appearance (Figure 5.17). The intestinal lumen widened and the granular material within took on a beaded appearance (Figure 5.18).

Major changes in the reproductive tract occurred. The outline of the male altered: the sheath was stretched around the expanding bursa with the tail tip projecting beyond. A dark elongated mass represented the primitive developing spicule tissue. The sexual primordia of the female developed at a relatively more anterior site in the body. The tapering tail of the female was retained, but approximately mid-way down the body length the cuticle of the developing ovejector area became apparent.

The surface of the L4 had fine annulations by SEM, which arose from a more caudal level compared to the L2 (Figure 5.19). The longitudinal alar ridging had been lost and only a very slight bilateral depression ran down the lateral body margins. The surface had a wrinkled or pitted appearance.

FIGURE 5.17

The anterior portion of an L4 stage *Ostertagia ostertagi* x 165

The L4 has a rhabditiform oesophagus, i.e., double-bulbed (the posterior bulb is indicated - pb) with a nerve ring (nr) present at the constriction.

FIGURE 5.18

Intestinal portion of an L4 stage *Ostertagia ostertagi* x 210

The intestinal lumen of the L4 assumed a beaded configuration and its contents were granular.

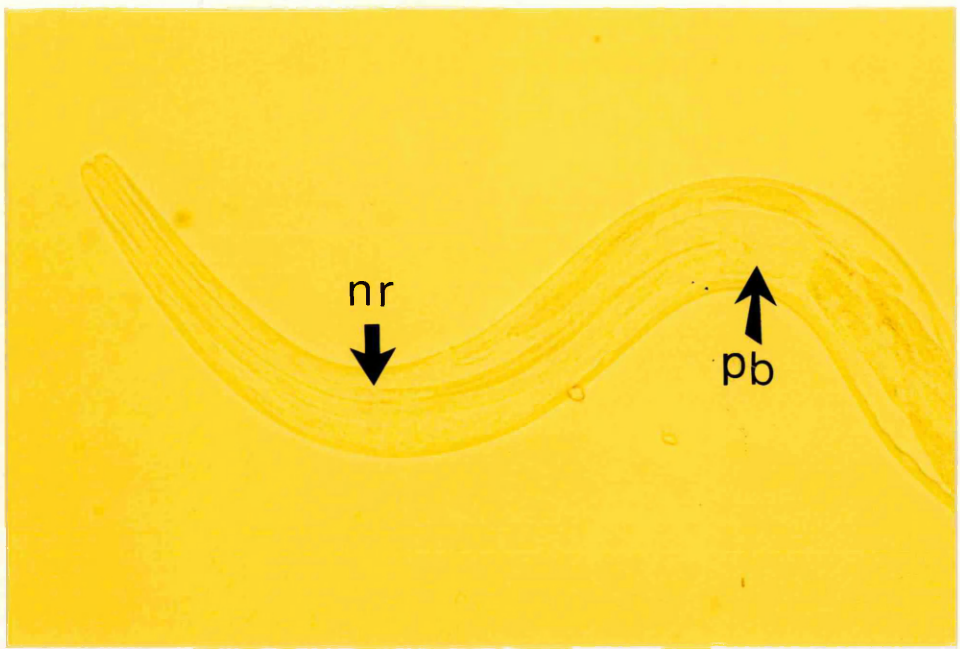
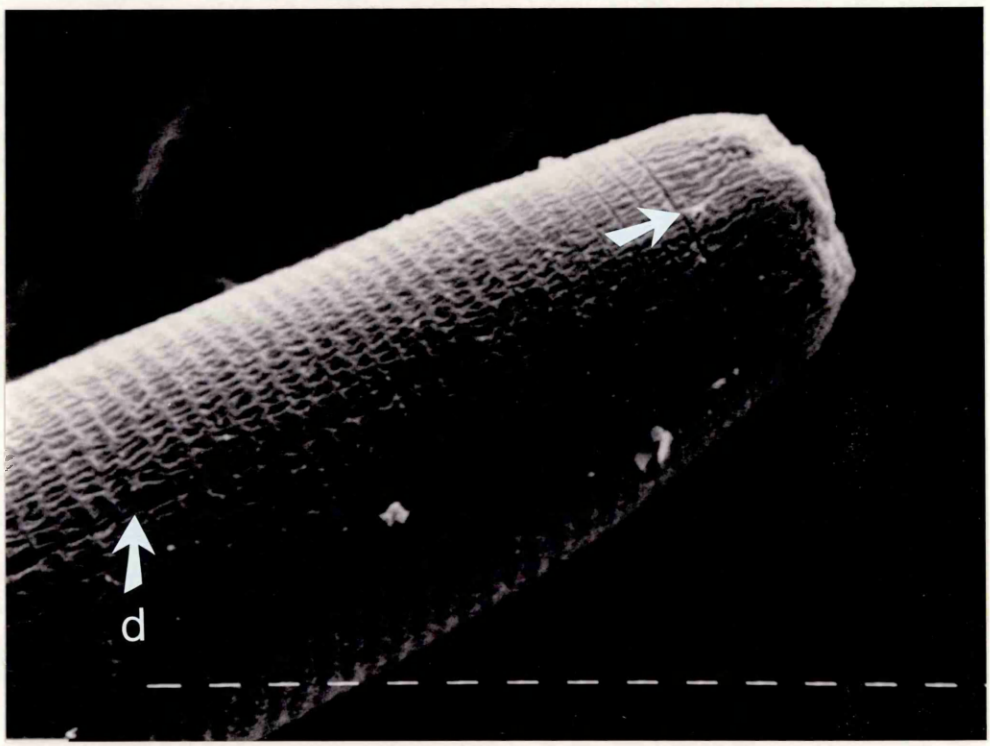

nr
pb

FIGURE 5.19

Scanning electron micrograph of the anterior of an L4 stage *Ostertagia ostertagi* x 6,400. Scale bar 1μm

The surface of the L4 showed regular annulations, which arose from a more caudal level than those in the L3 (arrow). No alae were seen at the lateral margins, instead a depression ran bilaterally and longitudinally (d). The surface has a pitted appearance, which may be due to an osmotic imbalance produced at fixation. However, the sample was prepared simultaneously with the L3s seen in Figures 5.14 and 5.16, suggesting the change is a true reflection of the surface nature.


d

5.1.3.6 Adult

Once the L5 has emerged from the gastric gland and reached sexual maturity it is considered to be an adult. Male *O.ostertagi* may be distinguished from females with the naked eye due to their smaller body length, 6.5 to 7.5 mm compared to 8.3 to 9.2 mm and their bulbous bursal area. The adults are red-brown. *O.ostertagi* were with rare exception the only nematodes found in bovine abomasa at slaughter at Glasgow University Veterinary School.

On light microscopy a short muscular buccal capsule was visible, running into a muscular long oesophagus (Figure 5.20), which could be subdivided into corpus, isthmus and bulb. The male had spicules (approximately 0.22mm long), which divided caudally into two or three and the bursal rays were visible as two lateral lobes, a small dorsal lobe and a ventral lobe surrounding the genital cone (Figure 5.21).

The oviducts and uteri were seen clearly diverging from the vulval flap region (Figure 5.22). One oviduct looped caudally into the tail region and then cranially, so the ovary terminated at a similar level to the opposite ovary (Figure 5.23), which had taken a directly cranial course. The ovaries were tubular and contained several developing cells.

On SEM, the adult surface appeared smoother than in the larval stages and the annulations were very fine and seen most clearly at the extremities. The anterior terminal was cap-like. Cervical papillae projected caudally from the cervical region bilaterally like stumpy conical flaps (Figure 5.24). The longitudinal cuticular ridges or synlophes, 25 to 30 in number, originated in the cephalic region and ran down the tail in the female and just short of the bursa in the male. Terminally some synlophes became conjoined. The synlophes ran continuously onto the female vulva (Figure 5.25), which was smooth and leaf-like against the body and delicately ridged transversely. The annulations were only clearly distinguished at the posterior extremity: in the female they continued down the tapering tail, which terminated in a knob-like tip (Figure 5.26). In the male, the bursal sac was

FIGURE 5.20

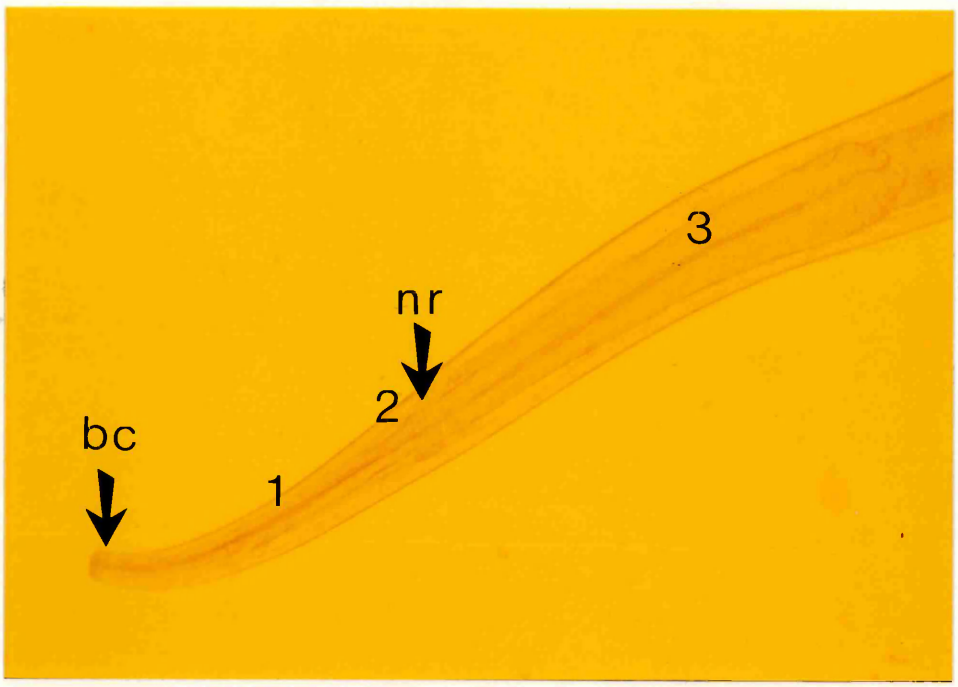
The oesophagus of an adult *Ostertagia ostertagi* x 90

The adult *O.ostertagi* has a muscular rhabditiform oesophagus, which consists of three parts: 1, corpus; 2, isthmus; 3, bulb. The buccal capsule (bc) is cranial to the oesophagus and the nerve ring (nr) may be seen, as in the L4, encircling the oesophagus at the isthmus.

FIGURE 5.21

The accessory sexual organs of an adult male *Ostertagia ostertagi* x 50

The genital cone (gc) is surrounded by the bursal rays (br). The spicules (s), which dilate the vulva of the female are clearly demarcated due to their high chitin content.


3
nr
2
bc
1

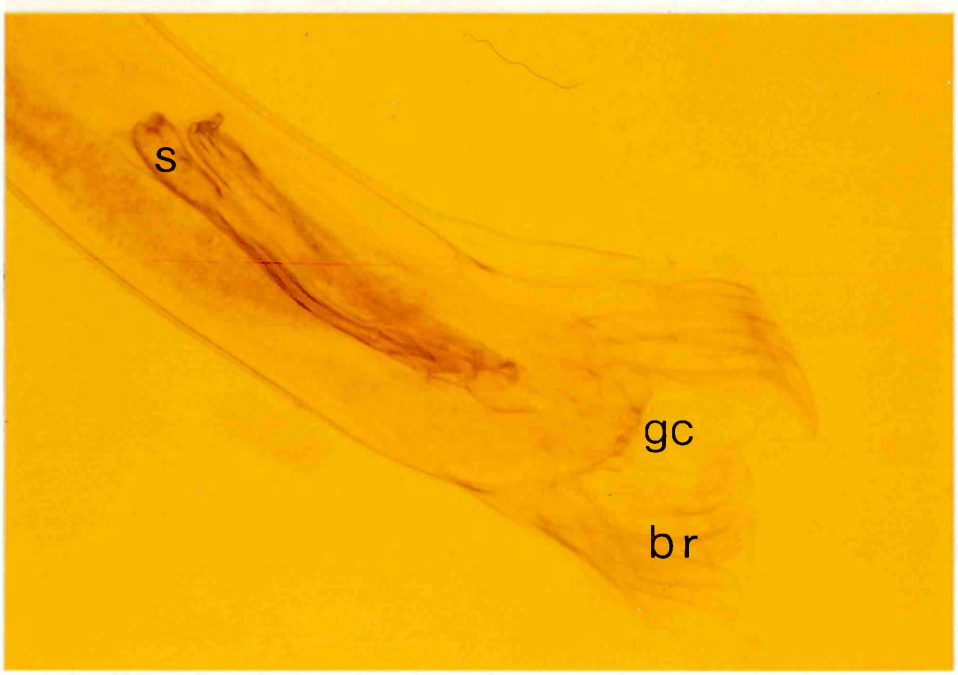

s
gc
br

FIGURE 5.22

The vulval flap of an adult female *Ostertagia ostertagi* x 175

The oviducts (o) diverge beneath the vulval flap (vf). The spiral muscles (sm) of the ovejector may be seen lying between the oviducts and these aid egg ejection.

FIGURE 5.23

The ovaries of an adult female *Ostertagia ostertagi* x 130

The oviducts diverge beneath the vulva, the duct running caudally loops anteriorly, so both ovaries may be seen in parallel (arrows) containing eggs at different stages of development.

FIGURE 5.24

Scanning electron micrograph of the anterior of an adult *Ostertagia ostertagi* x 800. Scale bar 10μm

The sample illustrated was damaged during mounting, however, many important features are still clear. The pattern of the longitudinal ridges, synlophes, indicated by the arrows may be used for identification purposes; they arise at different distances from the anterior cap (a, arrows). A caudally directed cervical papilla (p) was seen on both lateral aspects and further synlophes originated caudal to them (b, arrows).


a
p
b

FIGURE 5.25

Scanning electron micrograph of the vulval flap of an adult female *Ostertagia ostertagi* x 800. Scale bar 10μm

The synlophes or surface ridges may be seen continuing onto the leaf-like vulval flap, pressed against the body wall. In addition, fine striations may be found traversing the flap.

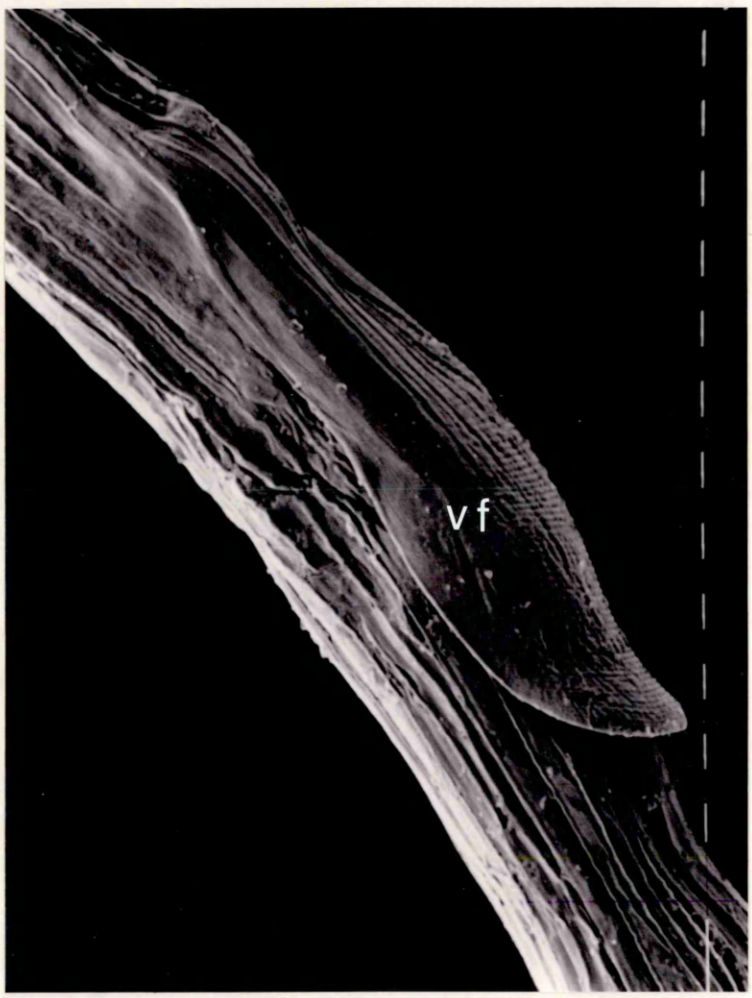

vf

FIGURE 5.26

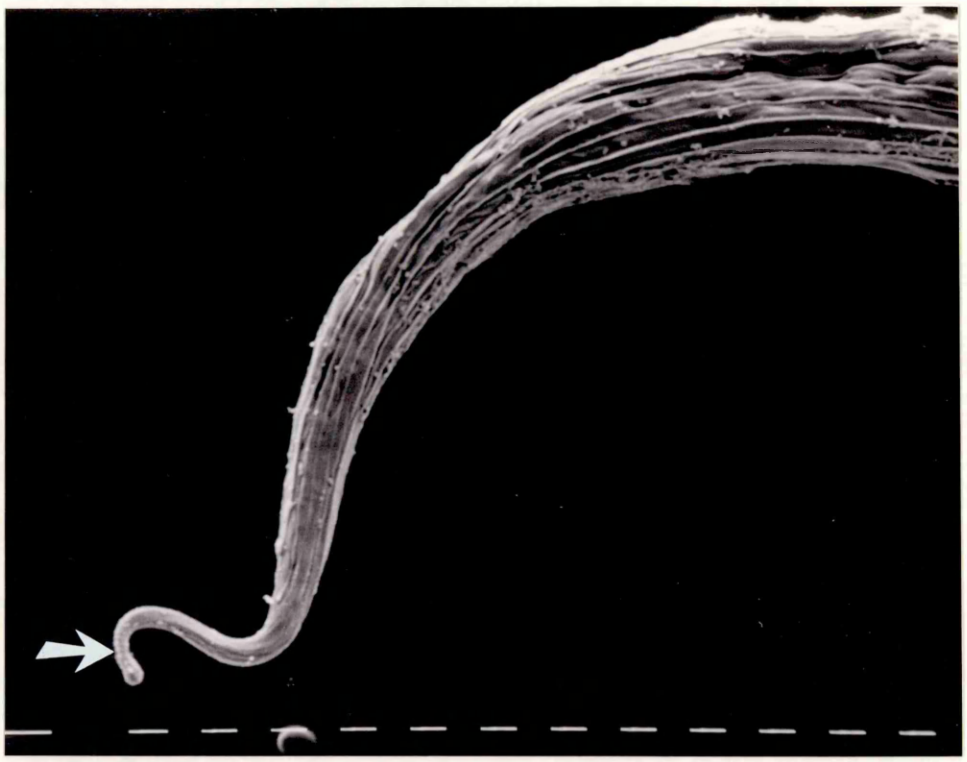
Scanning electron micrograph of the tail of an adult female *Ostertagia ostertagi* x 800. Scale bar 10μm

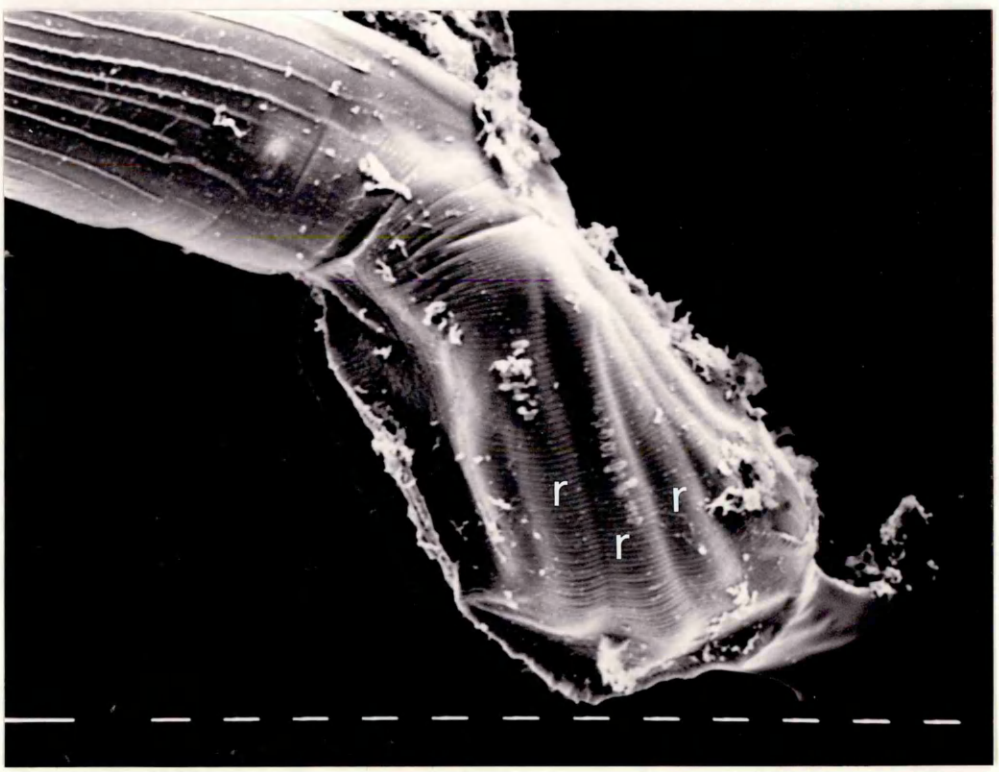
The synlophes or surface ridges on the surface of the adult female parasite fade out immediately proximal to the bulbous tail tip (arrow).

FIGURE 5.27

Scanning electron micrograph of the bursal sac of an adult male *Ostertagia ostertagi*, lateral aspect x 800. Scale bar 10μm

The synlophes or longitudinal cuticular ridges of the male terminate at varying levels cranial to the bursa. The bursal sac has fine transverse striations, which give the bursal rays it emcompasses a scalloped appearance.




r
r
r

faintly striated transversely and stretched over the bursal rays (Figure 5.27). In a few preparations the bursal rays and the gubernaculum were seen protruding through the bursal opening.

5.1.4 Discussion

The changes in morphological appearance of *O.ostertagi* are summarised in Table 5.1. Using light microscopy, trichostrongyle species differentiation from the L3 stage onwards was possible with experience. Keith (1953) demonstrated trichostrongyle species differences at the L3 stage and his criteria were adopted, i.e., sheath length:total body length, appearance of the buccal and sheath regions.

With respect to the earlier stages, *O.ostertagi* has a typical strongyle egg (Soulsby, 1965), which cannot be distinguished simply to the species level by morphology, faecal culture and the examination of L3s are conventionally required. The individual variation in the rate and optimal conditions of development may give some indication of the species involved. Recently, hatching times have been shown to be species specific and equatorial and pole-to-pole dimensions of eggs derived from different nematode species to fall within characteristic ranges, which might allow earlier classification when sufficient sample numbers are available and the source of the sample is a single species (F.Jackson, personal communication)(Christie and Jackson, 1982).

O.ostertagi parasites were easily recognised at necropsy, since they are the most common nematodes of the bovine abomasum. Occasionally *Trichostrongylus axei* was isolated, but it was readily recognised on the basis of its reduced length (5mm) and the appearance of the accessory sexual organs; the female has no vulval flap and the male has thick unbranched spicules of unequal length. *Haemonchus placei* may also rarely be found in the bovine abomasum, but it may be discounted also due to size disparity; it is 20 to 30 mm long. The female has a vulval flap and the ovaries spiral to give the classic barber's pole configuration. The male has barbed spicules. The key devised by Gibbons and Khalil (1982) gives a full account of the distinguishing features of the adult trichostrongyles and proved useful when differentiation was in doubt. Differences

TABLE 5.1.

Summary of the developmental features of the life cycle stages of Ostertagia ostertagi. The main features are underlined.

Stage	Dimensions	Comments
Egg	74 to 90 µm x 38 to 44 µm.	Oval, bilayered boundary, dividing cell mass, which eventually elongates to become vermiform.

Stage	Length	Oesophagus	Intestine	Surface	Comments
L1	360 to 460µm	rhabditiform	twisting	annulations, alae	
L2	500µm	rhabditiform	straight, increased diameter cells indistinct	annulations, alae	
L3	825 to 925 µm	filariform	temporarily 16 intestinal cells, differentiation then lost	annlations, alae, cephalic sensillae, sensory amphids	pointed tail, kink due to sheath
L4	1,200 to 3,200 µm	rhabditiform	beaded	annulations, longitudinal depressions	sexual dimorphism (sheath stretched around the bursa of the male)
L5/ Immature adult	6,500 to 7,500 µm	rhabditiform		synlophes, cervical papillae, fine striations	female-amphidelphic, vulval flap male-bursal sac

between the length of the species of *Ostertagia* have been recognised by light microscopy (Bisset, Kleinjan and Vlassoff, 1984).

Intraspecies variation in morphology has been recognised previously for the Ostertagiinae:

1) the appearance of the male accessory organs led to the further division of the species into two separate species in the past, *O.ostertagi* and *O.lyrata*, although it is now thought that they may be no more than major and minor morphs respectively (Lichtenfels *et al.*, 1988) (Chapter 2, 2.1).

2) the size of the female vulva seems to be influenced by host immune status and parasite heredity (Michel *et al.*, 1976b) (Chapter 2, 2.1).

Female *O.ostertagi* are amphidelphic, due to the central location of the reproductive tract. Douvres (1956) noted spines protecting the vulva; these were not demonstrated by either scanning or light microscopy of the adult females of the Glasgow isolate, in which the surface was smooth. The presence or absence of spines may be a further example of intraspecies variation. Future work to look for intraspecific variation of isolates in the adult stage might prove more rewarding than examination of L3s, since by their more complex design there are more possible sites at which visible variation might occur. However, the import regulations prevented production of parasitic stages by passage of material from foreign sources. No morphological variation was seen between the stocks of L3s obtained from different sources when they were examined by light microscopy.

Identification of developmental stage by morphology was relatively easy for the pre-parasitic stages from the descriptions of Soulsby (1965), although the changing granular appearance of the intestine of some L2 and L3 samples made their differentiation particularly difficult. In contrast, changes in the characteristics of the parasitic stages were often found to be gradual and occurred at disparate rates within the nematode, making precise labelling of stages complicated. The studies of Douvres (1956) and Rose (1969) form an excellent background to the morphological changes, but it was felt that they tended to try to simplify development too rigidly into steps.

Many of the internal organs discussed by Douvres (1956) and Rose (1969) were difficult to visualise or inapparent. Possibly the clearing and staining with alcoholic eosin-Y or cotton blue lactophenol used by these workers are important for the definition of structures, e.g., the excretory cells at the level of the oesophagus and the sexual primordia, which in the L1 are located anterior and ventral to the anus. Application of stains to the light microscope preparations was considered to be undesirable, since further techniques were to be applied to parasites once precise differentiation had been made and no information was available concerning the effect of stains on the reagents involved in these techniques. Consequently, an ability to differentiate the stages without the use of stains had been the aim.

Copulation was not observed on examination by light microscopy due to the clumping behaviour of the nematodes. It is thought that the bursal rays dilate the genital opening and the gubernaculum guides the spicules into place (Georgi, 1985). Non-flagellate wedge-shaped sperm penetrate the eggshell in the oviduct. Once the sperm has entered there is a change in electrical potential along the vitelline membrane and granular material is released into the perivitelline space. No further sperm may enter after the change in the shell.

The boundary layers of the egg are formed in the oviduct by vacuole transport to the surface. The layers are produced endogenously, which accounts for the uniformity of dimensions of the egg during development. Progress of the egg beyond the morula stage was found to be uneven; the cells became committed to particular stem cell pathways, which divided at different rates, i.e., determinate cleavage. The development of the nematode post-fertilisation was found to be rapid and these stages were hard to distinguish. To solve the problem of examination of these ephemeral stages, Cole and Schierenberg (1986) used a laser beam to puncture the eggshell of *Caenorhabditis elegans* allowing fixative to enter and stop development. These specialised techniques were not available and assessment of the early egg stages was felt to be beyond the required scope of the study, particularly when there is no evidence for interspecies variation in these processes.

No assessment of the egg was made by SEM, due to the difficulties of handling such small samples and also since little variation was assumed to occur. However, in retrospect SEM would appear to lend itself to the examination of the early stages of development in the female reproductive tract, when the surface appears to alter suddenly. By serial examination it might be possible to follow chitin deposition and the condensation of lipid vacuoles at the surface, which arise within the egg or to assess whether a visible difference in the surface occurs on fertilisation.

After hatching the organ primordia cell clusters formed recognisable organs in the L1 as described by Georgi (1985). Samoiloff (1973) discovered by applying a focused laser beam to particular areas of *Pangrellus silusiae* he could affect the morphology, e.g., destruction of the nerve ring prevented gonad development, while laser action on the hindgut affected growth. From his work it has been postulated that postembryonic development is under the control of specific regions of the nematode.

Initially, the L1s were very active, feeding on bacteria, but with time they became more lethargic prior to moulting. The L2 also feeds on bacteria and builds up reserves of fat for the L3 stage, when it does not feed. No loss of the L2 sheath occurs at the transition to the L3 stage, although the larva does undergo a period of lethargy. It could be argued that the stage traditionally accepted to be the L3 should really be considered as an L2 stage on account of the lack of second moult and that the L3 stage is the very short-lived stage seen in the host after loss of the retained sheath.

SEM was found to be an excellent technique for the examination of surface topography; the image acquired is that of a three-dimensional structure recorded in two dimensions. No assessment was made of interspecies variation by SEM, since species differences are readily recognised by light microscopy.

The ability of SEM to identify variation is obviously restricted to a small subset of possible markers, i.e., those visualised on the surface. Alteration of sample orientation after fixing or mounting is not

possible which limits views of the sample and is often a disadvantage. It is important that samples should be treated similarly with respect to washing and fixing to minimise artefactual changes. During the serial dehydration it is common for samples to shrink by up to 10%. The shrinkage problem should not be compounded by loss of osmotic balance through inadequate rinsing after sample cleaning in hypertonic solutions, i.e., 30% sucrose. The wrinkled appearance of the L4 could be due to osmotic imbalance, although these larvae were prepared simultaneously to the sheathed L3s illustrated. Aldehydes in the fixative solution will remove surface lipid and since there is a significant proportion of lipid in the nematode epicuticle inclusion of calcium chloride at dehydration to prevent lipid stripping might have improved results. Uneven charge on specimens may lead to beam deflection and reduced resolution. In some cases build up of charge led to thermal damage of samples in the microscope sample chamber, the samples initially turned white and then disappeared leaving a blackened background area.

Fukada, Aji and Tongu (1988) were able to distinguish six Anisakidae larval types from fragments isolated from a patient by the differences in the ratio of distance between transverse striation and the width. Weerasooriya, Fujino, Ishii and Kagei (1986) differentiated four species of Anisakidae by appearance of the lip bulges, external papillary structures, teeth and cuticular ridges. There appears to be little requirement to identify *Ostertagia* spp. from fragments, except possibly to differentiate species on expulsion post treatment, although SEM has been used to further study the relationship between the Ostertagiinae nematode morphs, *O.ostertagi* and *O.lyrata*, giving further evidence that they are subdivisions of the same species (Lichtenfels *et al.*, 1988).

Isolates of *O.ostertagi* were not compared by SEM, which in retrospect was unfortunate, since Eisenback and Hirschmann (1979) found differences in the cephalic structures between populations of one "race" of *Meloidogyne hapla*, a plant nematode. Assuring that samples are fixed in the right position for examination of the cephalic region was found to be difficult, but might be possible by using hairs as props for the parasites (Eisenback and Hirschmann, 1979). The

work recorded here indicates the stomal region of the L3 *O.ostertagi* is complex and could be the site of much variation, which might be studied by SEM.

The L3 undergoes little exchange with the environment, so it was surprising to find such a complex appearance to the anterior i.e., the presence of sensory organs: semicircular amphids (neural) and cephalic papillae. The optional dauer larval stage of the free-living nematode *C.elegans* does not feed, despite showing active movement, so it has some similarities to the L3 of *O.ostertagi*. The appearance of the cephalic region of the dauer form varies from that of the developing larva; the buccal cavity is shut and the lips reduced in size (Riddle, 1988). The stoma of the free-living L3 of *O.ostertagi* appeared open compared to the dauer *C.elegans* illustrated in Riddle's paper.

Comparison of different stages of the life cycle using SEM, clearly illustrated differences, particularly with respect to annulations, alae, sensory and reproductive structures. In many cases, these structures had been inapparent on light microscopy, e.g., by light microscopy the L3 has a simple structure, but on SEM sensory structures were revealed. There appeared to be a general increase in surface complexity with the progression from free-living to parasitic stages.

In addition, SEM gave an insight into the effect of hypochlorite exsheathment on the nematode surface. Tags of the L2 sheath appeared to remain adherent to the surface of the L3, particularly around the stoma. Tension at the amphid on cuticular shedding may have caused the gaping appearance of the orifice in the larvae. The exsheathment results should be considered with the proviso that a satisfactory control is difficult to examine, since this would mean the collection of samples from slaughtered animals, at a stage when larvae are thought to undergo rapid change. It is possible that natural exsheathment might produce a similar appearance to that after hypochlorite treatment or that rapid alteration of the surface could occur in the gastrointestinal tract; further studies are necessary before assumptions can be made in this respect. Assessment of exsheathment by CO_2 action after the method of Boisvenue, Brandt,

Galloway and Hendrix (1983) for *H.contortus* might provide a revealing comparison. However, Lichtenfels, Gamble and Purnell (1990) demonstrated the open appearance of the amphids of *H.contortus* after CO_2 exsheathment by SEM.

The use of SEM for parasitology, particularly with respect to *O.ostertagi*, has not reached its full potential. Further research aimed at assessing differences in structure between isolates and development of the egg could be fruitful. SEM also has a role in pathology studies, Nicholls, Lee and Sharpe (1985) studied haemonchosis by serial biopsy using abomasal cannulae, which allowed repeated work on one host to assess the development of infection. Knowledge of the pathogenesis of ostertagiosis is rapidly advancing, but little information is available about parasite behaviour within the abomasum or the process of emergence from the gastric gland. Cannulation of the abomasum and the use of SEM might provide useful information about nutritional and reproductive habits of the parasite.

In conclusion, light microscopy and SEM are complementary techniques, and were used to gain insight into morphological differences between species, isolates and stages. While light microscopy is essential for visualisation of internal structures, it is unable to show surface changes with the clarity of SEM. Depending on the requirements for and level of differentiation one of the two techniques will prove the more favourable in most situations. It is important that parasite stages can be distinguished, so that comments on stage comparison using specialised techniques may be made in the confidence of adequate sample definition. The key features found to aid stage differentiation were summarised from light microscopy and SEM studies.

5.2 PARASITE CULTURE TECHNIQUES

5.2.1 Introduction

The overall aim of this thesis was to examine intraspecies variation of *O.ostertagi* from different isolates and stages of the life cycle.

Therefore the ability to collect samples of sufficient size from different sources and grow them to suitable stages for comparative analysis was considered to be highly desirable. The purity of comparative samples is crucial to avoid incorrect conclusions from samples of mixed species or contaminated with debris.

Successful *in vitro* cultivation would permit:

1) access to species pure parasite populations at different stages of the life cycle.
2) the maintenance of strains without having to rely on host stock, accommodation and their associated expenses, or producing severe contamination problems.
3) the application of experimental pressures on parasite populations and the study of their effects, e.g., anthelmintic action (Rapson, Jenkins and Topley, 1985), growth regulatory factors (Douvres, Thompson and Robbins, 1980), etc.
4) isolation of male and female pairs before sexual maturity has been reached to enable amplification of parasites from highly defined backgrounds.

The life cycle of *O.ostertagi* can be considered to consist of a free-living phase and a parasitic phase. Culture of the free-living stages is routinely carried out in many laboratories, while relatively few workers have successfully maintained the parasitic phases *in vitro* to complete the cycle.

The present study was undertaken in an attempt to develop standardised *in vitro* methodology that would support the development of the entire life cycle from egg to mature adult to egg.

5.2.1.1 Free-living stages

Development times for certain nematode species have been recorded (Soulsby, 1965); there are marked species differences in the optimum environmental conditions for development (Urquhart *et al.*, 1987) suggesting that these time intervals may not be freely applied throughout the class Nematoda. Consequently, a study of the development of *O.ostertagi* under faecal culture conditions was

considered appropriate to elucidate optimum recovery times for the different stages and thus, produce material for comparative analysis. In addition to the standard faecal culture technique, development of larvae on agar plates was evaluated for the possible advantages of increased sample cleanliness and to test the feasibility of the method for use in conjunction with culture techniques for the parasitic stages.

5.2.1.2 Parasitic stages

Since the 1950s serious attempts have been made to maintain parasitic nematodes by *in vitro* culture. Work on *in vitro* culture systems has centred on the simulation of the natural parasite environment. The alternative approach is to design a chemically defined medium to fit known metabolic requirements, which requires a precise knowledge of the metabolism of individual species to be assimilated and applied (Bryant and Flockhart, 1986).

Previously described culture conditions are highly species specific (Taylor and Baker, 1987), reflecting the spectrum of host and site conditions. To further complicate the picture, different stages of the life cycle may have different metabolic needs, for example, at the transition from free-living to parasitic nematode. Parasitic nematodes ~~have lost~~ do not have the ability to synthesise *de novo* large molecules such as purines, cholesterol, essential amino acids and vitamins, so these must be supplied by the host or culture medium, hence the need for liver extract and serum to provide haemin and sterol in the majority of protocols.

Weinstein and Jones (1959) surface sterilised infective larvae of *Nippostrongylus brasiliensis* and *Necator americanus*. The larvae were maintained in a medium consisting of chicken embryos homogenised in Earle's Balanced Salt Solution, serum, caseinate and antibiotics. The streptomycin and penicillin were added to remove bacteria released from the nematode intestines on exsheathment. Five hundred to 1,000 larvae were inoculated into 2ml of medium per tube, gassed for 30 seconds with 5% CO_2 and maintained at 37.5°C in a roller drum. The larvae were transferred to fresh medium three times weekly. The viable yield was found to vary with larval concentration

in the suspension, type and concentration of serum, addition of vitamin and liver supplements. *In vitro* culture proceeded to the production of fertile eggs, when sperm could be seen in the seminal receptacle.

Leland (1963) maintained *O.ostertagi* to egg-laying/spermatogenesis in a similar medium for 48 days. The medium comprised of chicken embryo extract, Earle's Balanced Salt Solution, calf serum, sodium caseinate, vitamins, porcine liver extract, antibiotic and indicator. Rose (1973) looked at the development of gastrointestinal nematodes, including *O.ostertagi*, adapting the previous method by substituting equine serum for calf serum and lactalbumin hydrolysate for sodium caseinate. Increased numbers of developing parasites were produced when higher concentrations of larvae were used, but when over 2,000 L3 were suspended in 4ml medium there was no further increase. Mature *O.circumcincta* developed by 27 days culture. He tested the theory that aeration of larval suspensions prior to inoculation improved rate of development and found no basis for the idea.

Douvres and Malakatis (1977) developed a two-step culture technique for *O.ostertagi*. In the first step they devised an artificial rumen fluid for exsheathment. The fluid contained reducing agents thought to enhance exsheathment and further development, while in the second step a complex medium resulted in the production of egg-laying adults by 28 days. Their medium using rabbit embryo extract, calf serum, a nucleic acid mix, a fatty acid mix, Kreb's cycle intermediates, vitamins and sugars has been used for a range of nematodes by adding particular supplements for different species. The medium was supplemented with 5% pepsin for *O.ostertagi*. The cultures were gassed with a filtered supply of 85% nitrogen, 10% CO_2 and 5% oxygen at inoculation and at each transfer of medium.

5.2.2 Materials and methods

5.2.2.1 Preparation of faecal egg cultures for the collection of free-living larval stages

Faeces for culture purposes may be collected by a number of different methods, which result in varying degrees of contamination (F.W.Douvres, personal communication); rectal sampling is the optimum, followed by use of harnesses and finally specimens gathered from the ground. Rectal sampling was very time consuming, so often harnesses were fitted to male calves with sample bags being changed at frequent intervals. When animals were uncooperative or when insufficient sample had been collected by other means, faeces were collected from the floor. Free-living nematodes or insect ova are frequent contaminants in samples collected from the ground or when collecting bags are not changed often enough. Samples from different sources were cultured separately to reduce contamination risks. Collection was usually instigated once the faecal egg count exceeded 50 epg, unless the material was going to be in very short supply, when samples were collected at lower counts.

Faeces was mixed with water and insulating material, vermiculite, (Micafil) and moulded into cylinders. These were sealed in disposable plastic containers (Mono-containers, Ltd.) alternatively jam jars or steel bins were used, and incubated at 19 to 22°C for 14 days to allow development from the egg to the L3 (Ministry of Agriculture, Fisheries and Food, 1978). Autoclaved moss and peat have been used as alternatives to insulating material.

At the end of the incubation period, water at 30 to 37°C was added to each container and these were left for 2 to 3 hours to encourage larval migration from the faeces. When a large amount of fungal growth was present on the cultures masks were worn at this stage.

The pooled fluid was strained and poured onto a filter paper (Whatman 113, 18.5cm) under vacuum, so the liquid was removed, but the larvae were retained; application of vacuum for too long can ultimately damage the larvae. A milk filter (Maxa Regal, Blow,

A.McCaskie Ltd., Stirling) was applied above the sample and the paper sandwich inverted so the milk filter was below. Due to the mesh size the infective larvae were able to pass through the milk filter. A Baermann funnel was filled with water at 37°C and the filters were laid on the water surface. Larvae were attracted out of the filter and sedimented in the tubing at the base of the funnel.

From 3 hours onwards, samples were drawn off by opening the tap at the base of the Baermann funnel. The larvae were concentrated by sedimentation and stored at 4°C in a refrigerator.

5.2.2.2 Experiment to assess optimal collection times for free-living larval stages

Thirty faecal cultures were set up in two groups of 15 cultures 12 hours apart by the method described in section 5.2.2.1. In this way, 12 hourly comparative samples could be assessed by simultaneous baermannisation of samples from both groups. The egg count of the material used for culture was 50 epg, i.e., relatively low.

The suspensions run off the Baermann apparatus were sedimented by centrifugation, 5 minutes at 1,000g, and the supernatant was withdrawn. The pellet was assessed for larval presence and where applicable the stage of larva involved.

5.2.2.3 Culture of pre-parasitic stages in a contaminant-free environment

Eggs were collected and cleaned by density differentiation as described in Chapter 4, 4.2.2. Alternatively the eggs, suspended in saturated sodium chloride solution were poured into centrifuge tubes. The surface of the salt solution was level with the tube rim, so on application of a coverslip a positive meniscus was produced. The tubes were centrifuged at 1,000g for 2 minutes. The eggs rose and adhered to the coverslip from where they were rinsed with distilled water. Two further 5 minute washes in distilled water, 1,000g, were necessary to restore an osmotic equilibrium.

A 2% agar solution, made by gentle heating, was poured into Petri dishes to a depth of 0.5cm. A 1.01% yeast extract solution in 0.85% saline was autoclaved for 30 minutes. Nutrient medium was prepared by making a 10% Earle's Balanced Salt Solution (filter sterilized) in the yeast extract solution. The pH of the medium was adjusted to neutrality with sodium hydrogen carbonate.

One ml egg larval suspension and 1ml nutrient medium were added to the Petri dishes. The dishes were incubated at 27°C. To maintain the relative humidity beakers of water were also placed in the incubator. The Petri dishes were examined at intervals for larval development.

5.2.2.4 *In vitro* culture of parasitic stages of *Ostertagia ostertagi*

The medium preparation and inoculation were carried out as described by Leland (1963). These procedures and larval transfers were carried out in a laminar air flow hood, using standard sterile culture techniques and sterile equipment. All the components of the medium were obtained in sterile form or filter sterilised (0.2µm Flowpore D, Flow Laboratories, Sartorius). Components of the medium are listed in Table 5.2.

A stock solution of antibiotics in Earle's Balanced Salt Solution was made for use in larval washes in addition to the culture medium at a concentration of 5ml of antibiotic mixture in a litre of Earle's Balanced Salt Solution, i.e., 200,000 penicillin G, 50,000 units mycostatin and 200mg dihydrostreptomycin. Antibiotics may be included in *in vitro* culture medium, but axenic cultures are maintained without their addition. The pH of the mixture was adjusted to 7.2 to 7.3 with sterile sodium bicarbonate/carbon dioxide at 37.5 to 38.5°C. Two ml aliquots of medium were stored at -20°C. A 2% sodium caseinate solution was made up in Earle's Balanced Salt solution, the pH adjusted to 7.2 at 37.5°C and stored at -20°C. The 2% liver extract was also made up in Earle's Balanced Salt Solution filtered through a 0.2µm Flowpore D and stored. The vitamin mixture was made up on the day the entire culture medium was assembled. A stock solution of the entire medium was produced and stored in 2ml aliquots at -20°C.

TABLE 5.2

Medium for the *in vitro* culture of *Ostertagia ostertagi*

17 ml	chicken embryo extract	
5 ml	bovine serum (collected from a helminth-free calf)	
5 ml	cystine-fortified sodium caseinate	
1.7 ml	2% liver extract	
1.7 ml	vitamins in Eagle's Medium at	
	0.1mg/ml	biotin
	0.1mg/ml	choline
	0.1mg/ml	folic acid
	0.1mg/ml	nicotinamide
	0.1mg/ml	pyridoxal
	0.01mg/ml	riboflavin
0.3 ml	antibiotic mixture	
	(in 5ml of Earle's Balanced Salt Solution:	
	200,000 units	penicillin G
	50,000 units	mycostatin
	200 mg	dihydrostreptomycin)
3 ml	Earle's Balanced Salt Solution	

(From the technique described by Leland (1963).)

Larvae obtained by faecal culture (5.2.2.1) were exsheathed chemically with hypochlorite using the method described in Chapter 4 (4.2.5) to give an inoculant of 500 to 1,000 larvae per 2ml of medium. Five washes of the larvae after exsheathment were made in 0.85% physiological saline followed by five washes in Earle's Balanced Salt Solution with antibiotics. The supernatant was removed leaving the larvae in the smallest possible volume which still allowed their easy transfer to flat-sided culture tubes containing medium, but avoided major medium dilution. The tubes were sealed and inserted into a roller drum (Gallenkampf) turning at 12 revolutions per hour and incubated at 37°C in 5% CO_2.

The tubes were checked daily and the caps loosened for an hour to allow gas exchange. With time the pink-red colouration of the phenol indicator began to turn yellow due to the uptake of nutrients and dispersion of waste products. Three times weekly the larvae were transferred to fresh medium: the samples were spun at 1,000g, the supernatant withdrawn, the larvae washed in antibiotic and Earle's Balanced Salt Solution at 37°C, the supernatant withdrawn and the larvae added to fresh medium at 37°C in a new flask. Twice weekly the culture tubes were checked under an inverted microscope and the larvae assessed for viability and stage. Viability was judged by examination of individuals over a 10 second interval for motility. The culture technique was assessed in quadruplicate.

The study was repeated exchanging abomasal fluid for Earle's Balanced Salt Solution. Abomasal fluid, pH 2.25, was collected from a calf at routine slaughter. The fluid was spun at 3,000g for 15 minutes, the supernatant filtered through 0.2µm Flowpore D and stored at -20°C in 2ml aliquots.

5.2.3 Results

5.2.3.1 Preparation of faecal egg cultures for the collection of free-living larval stages of O. ostertagi

Faecal egg culture consistently produced L3s. Depending on the method of sample collection, varying degrees of contamination with non-parasitic larvae were

revealed, i.e., in descending order of purity: rectal, harness and calf surroundings.

5.2.3.2 Experiment to assess optimal collection times for free-living larval stages of O.ostertagi

The full results of the assessment of the optimal collection times for free-living stages from faecal cultures are recorded in Table 5.3. L1 were present in suspension from 24 hours faecal incubation to 312 hours with an optimum count at 228 hours. L2 were found from 180 to 360 hours, with a marked peak at 228 hours. L3 were present from 324 hours.

5.2.3.3 Culture of pre-parasitic stages in a contaminant-free environment

The use of agar plates for the culture of the pre-parasitic stages of *O.ostertagi* was very time consuming to establish. The developing larvae showed a propensity to burrow into the agar, necessitating an involved recovery by rinsing the plates with further nutrient medium. The washed material was sedimented in centrifuge tubes at 1,000g for 5 minutes. However, the larvae recovered from the agar plates were free from contaminating debris when compared with larvae taken directly from faecal cultures.

5.2.3.4 *In vitro* culture of parasitic stages of *Ostertagia ostertagi*

Culture of the parasitic stages of *O.ostertagi* resulted in a rapid drop in larval motility from the time of medium inoculation (Table 5.4). Three days post inoculation 55 to 73% of larvae were motile and this level was retained for a further 4 days, but a marked drop in viability had occurred by 11 days (9 to 34%). The level continued to drop until 19 days post inoculation when 0 to 2% of larvae were motile. Cast cuticles were observed as evidence of the third moult between 3 and 5 days after medium inoculation. However, no development was seen beyond the early fourth stage using the criteria outlined in the pictorial analysis section. A number of larvae were lost at each transfer to fresh medium. The larvae appeared to adhere to the sides

TABLE 5.3

Larval stage of *Ostertagia ostertagi* at recovery from faecal egg culture after increasing culture times

HOUR	L1		L2	L3
12	-		-	-
24	1		-	-
36	3		-	-
48	4		-	-
60	3		-	-
72	7		-	-
84	6		-	-
96	6		-	-
108	7		-	-
120	5		-	-
132	-		-	-
144	4		-	-
156	7		-	-
168	12		-	-
180	-		3	-
192	10		23	-
204	76	-8*-	611	-
216	26		622	-
228	752		5,163	-
240	87		613	-
252	210		578	-
264	48		871	-
276	10		129	-
288	364		15	-
300	44		405	-
312	300		334	-
324	-		1	88
336	-		-	408
348	-		-	337
360	-		-	292

*The 8 larvae at 204 hours between the L1 and L2 columns were of an appearance intermediate between these stages.

TABLE 5.4

Percentage larval viability with time in culture as assessed by mobility

Sample	Days post culture inoculation						
	0	3	7	11	14	19	22
1	100	75	70	9	5	1	0
2	100	55	55	17	2	1	0
3	100	73	73	31	8	2	0
4	100	65	62	34	1	0	0

of the culture vessel and became trapped in plugs of precipitating medium. Addition of abomasal fluid had no significant effect on the results and the loss of larval motility occurred at a similar rate to that in the trials using Earle's Balanced Salt Solution described above.

5.2.4 Discussion

The routine methods of faecal culture are simple to perform, but in line with the findings of other workers the parasitic stages of the life cycle were harder to maintain. A problem common to the recovery of all stages of the life cycle was the apparent overlap in the rates at which individual nematodes developed. The asynchronous development of recognised stages would require further separation to collect material for comparison of stages: the identification of individuals and their separation into aliquots would be the optimal method of differentiation, although serial sedimentation could be used to roughly separate stages relying on the increase in size with progressive moults. The changes in appearance encountered on morphological examination suggest that the gradual change of features is an ongoing process and possibly the assigning of stages has tended to compartmentalize the organism too severely. Recent work on nematode surfaces has shown that there is a constant surface turnover in addition to the moulting steps, which gives further support to the idea of a low level of continual developmental change (Maizels and Selkirk, 1988).

With respect to the culture of the free-living stages, no difficulty was encountered in either faecal or agar culture techniques in reaching the infective larval. By faecal culture, pure samples of one stage may only be collected on or before 168 hours culture time for L1 or after 336 hours for L3. However, Douvres (personal communication) recovered L3s from faecal culture at 240 hours. The optimal time for collection of L2 would appear to be 228 hours. However, this will not be a pure L2 sample, so further purification would have to be employed as required. To separate out L2s, individual identification would be necessary or with a lesser degree of accuracy, serial sedimentation of different stages relying on the large increase in size with progressive moults.

Comparison of the agar culture to the faecal culture method for collection of free-living larvae, showed the technique to be very time consuming in both the collection of eggs for culturing and recovery of the developed larvae from the plates. The larvae recovered from the plates were free from debris, but this is more likely to be a comment on the cleanliness of the sample originally used to inoculate the plates. The ease of production of faecal culture larvae and the simple available techniques for removing contaminants make faecal culture a more acceptable technique, although agar culture would be useful in the case of pure egg material collected from macerated adult females.

A certain degree of parasitic larval development occurred in culture, but maintenance to the adult stage at 48 days was not achieved as previously reported for the medium (Leland, 1963), i.e., due to larval mortality and decreased rate of development. A visit to the United States Department of Agriculture, Beltsville, Maryland was made. On discussion, possible causes for the poor *in vitro* culture results were suggested:

1) Inadequate aeration of the culture. At Beltsville, surface gassing of the medium at inoculation and each subsequent transfer was carried out with 85% nitrogen, 10% CO_2 and 5% oxygen passed through a filter to provide an aerobic environment.
2) Loss of larvae at transfer. Exchange of medium instead of larval transfer to new medium would reduce larval losses at transfer.
3) Bacterial contamination. Assessment of aliquots of medium for bacterial contamination should be made at medium changes.
4) Alteration to the medium. Due to the variability of embryo extracts from commercial sources fresh extracts were recommended and rabbit embryos were said to be preferable to those of chickens.

Further assessment of *in vitro* culture was planned to implement these suggestions. In addition, to try to identify the stage obstructing continued development later parasite stages were to be inoculated into the medium and their relative development assessed. The importance of larval contact with the abomasum during development is unknown, so the inclusion of cell feeder layers, e.g., parietal and zymogen cells, was thought to be another approach to achieving culture success.

With a change in emphasis of the study these approaches were not tested to assess whether development *in vitro* improved.

However, differences between *in vitro* and *in vivo* developed *O.ostertagi* larvae occur, suggesting samples derived from culture may not be the best for comparative purposes. Weinstein and Jones (1959) noted a comparative reduction in length and rate of development in *in vitro* cultured parasites. Douvres and Malakatis (1977) recognised a relative decrease in the egg size of cultured females. Abraham, Grieve and Oaks (1990) noted that the cuticle of the L4 of *Dirofilaria immitis* developed *in vitro* was thinner than that of *in vivo* parasites. Furthermore, important changes induced by culture conditions may not be immediately apparent as Mirelman (1987) noted for *Entamoeba histolytica*. He found certain isoenzyme types occurred when the organism was stressed by changes in the medium. If alterations between stages are to be examined, samples obtained at different stages of infection might be better recovered at slaughter rather than from culture to rule out possible introduced pressures.

In summary, culture of the free-living stages of the life cycle of *O.ostertagi* were readily achieved, while little development of the parasitic stages beyond the early L4 occurred. Development of all stages within a population was extremely asynchronous, which necessitated sample purification prior to further experimental work. For the purposes of the remaining studies recorded in this thesis, samples were compared after stated serial incubation times, since stage purification was difficult to achieve with sufficient accuracy. The *in vitro* culture of parasitic stages appeared to be a time consuming operation demanding precise attention to the parasite's optimum living requirements. The difficulties faced with the culture system initially prevented its use for the generation of large amounts of experimental material for comparative studies, furthermore, they excluded the use of the technique with the application of selective pressures to the parasite in an attempt to induce variation, e.g., cold conditioning to try to produce arrested larval development *in vitro*.

CHAPTER 6

I. EVALUATION OF THE USE OF THE MOUSE AS A LABORATORY ANIMAL MODEL FOR INFECTION WITH THE NEMATODE *OSTERTAGIA OSTERTAGI*

II. CRYOPRESERVATION OF INFECTIVE STAGE *OSTERTAGIA OSTERTAGI*

CHAPTER 6

Chapter 6 has been divided into two related sections. In the first, the mouse is evaluated as a laboratory animal host and an infectivity model for infections with *Ostertagia ostertagi*. The second section examines the use of cryopreservation for storage of *O.ostertagi* L3s.

Cryopreserved larvae were used to infect mice as a preliminary test to assess the effect of cryopreservation on larval infectivity, thus, linking the sections.

I. EVALUATION OF THE USE OF THE MOUSE AS A LABORATORY ANIMAL MODEL FOR INFECTION WITH THE NEMATODE *OSTERTAGIA OSTERTAGI*

6.1 INTRODUCTION

Passage of *O.ostertagi* in cattle is the normal method of parasite maintenance for experimental purposes. The production of large amounts of experimental material by passage in alternative hosts or by *in vitro* culture has been unsuccessful so far.

The mouse is the most commonly used experimental model for helminth disease; it is particularly useful for the study of host genetics. The availability of large numbers of animals of identical genetic background allows more efficient comparison of variables and/or experimental design. In addition, the immunological status of the mouse can be manipulated more easily than that of large animals, e.g., by irradiation. Cross-contamination with other nematode species is easier to prevent in laboratory animals. Studies in the natural bovine host are expensive in terms of stock, accommodation, labour and feed, and require large numbers of parasites. Many studies of *O.ostertagi* have used small bovine populations and widely varying protocols, while a good experimental model would allow the comparison of suitable sample populations of hosts under different protocols. Thus, the aim of this chapter was to study the potential of the laboratory mouse as a model for *O.ostertagi*.

With increasing evidence for strain variation, the use of an experimental model would allow the examination of several hosts for the comparison of the effects of infection with different isolates and methods of sample preparation, i.e., cryopreserved, refrigerated and fresh. Currently, examination of larvae for motility is the only criterion commonly used to estimate likely levels of establishment subsequent to infection. A reduction in larval establishment is thought to occur when cryopreserved material is used, despite seemingly good motility (M.Taylor, personal communication). Assessment relies largely on visual examination and is subject to environmental changes such as temperature. However, long-term storage of infective larvae in a suspension at 0 to 4°C results in senescence and larval death with time, so the ability to store material without change for long periods would be useful. Neither the correlation between infectivity and motility is known, nor the effect of cryopreservation on this correlation. Ability to passage material in a model host would allow interbreeding of parasite populations from different source types to examine evolutionary changes using reasonable host numbers. Selection pressures could be applied to large host populations and the results might contribute to our understanding of parasite evolution and genetics.

Additionally workers have used models:

1) To improve knowledge of host-parasite interactions by pathological examination of host response to infection.
2) As pilot studies for vaccination schemes, drug efficiency and toxicity trials.

Heterologous species infections have been reported for *O.ostertagi* e.g., a Type II infection recorded in the American Bison included *O.ostertagi* among several nematodes involved (Wade, Haschek and Georgi, 1979). Angora goats grazed on pasture contaminated with *O.ostertagi* and *O.circumcincta* developed ostertagiosis with equal proportions of both species. Later, when the goats were challenged with *O.ostertagi*, they developed infections that remained patent for 5 weeks (Le Jambre, 1978). Williams (1986 and 1987) tested the suitability of the goat as a model of infection for bovine ostertagiosis. He found that infective larvae originating from goats established in

greater numbers in goats compared to larvae of calf origin. However, prolonged passage of parasites in goats did not lead to stabilization of an isolate more adapted to goats than calves. Compared to infection in the bovine host, the pre-patent period of *O.ostertagi* in the goat was frequently extended beyond the usual 18 to 21 days and sometimes patency was never reached. In addition, a lower parasite burden established and the clinical signs were milder and more transient.

Lambs infected with *O.ostertagi* showed very varied signs, though generally their live weight gains were decreased (Coop, Smith, Angus, Graham, Wright and Jackson, 1985). The faecal egg counts were lower in infected lambs compared to infected calves and the nematodes recovered were less well-developed. Refractile crystals were seen in the nematode gut of lamb parasites, which the authors proposed could be due to degenerative changes.

Although, *O.ostertagi* can establish in other ruminants, successful infection of laboratory animals for the production of large amounts of sample material has not been achieved. Snider, Williams, Romaire and Besch (1985) cited Aljeboori (1965), who isolated a single *O.ostertagi* adult from a rabbit stomach 28 days post infection (DPI). Subsequently, they repeated the experiment and found only slight parasite development beyond the early fourth stage larva at 42 DPI. They believed that if infections could have been maintained for longer than 42 days, adults would have been recovered. H.Herlich (personal communication) infected guinea pigs and hamsters with *O.ostertagi*, but found no development beyond the moult to the L4. He related that certain nematode species showed a great ability to develop in a wide range of hosts, while others were highly restricted.

Development of *O.circumcincta* beyond the fourth and fifth larval stages was noted in rabbits and guinea pigs, but no sexually mature adults were recovered, nor did they result when later stages were implanted into guinea pigs or when larvae were transplanted into sheep after initial infection in the rabbit (Zebrowska-Plata, 1980). Corticosteroid treatment did not improve the proportion of nematodes establishing in the guinea pig.

Many parasitic nematodes have now been passaged in model hosts. Using the rabbit as a host, *Cooperia curticei* or *C.punctata* reached patency at around 18 days and a few *Haemonchus contortus*, *Trichostrongylus axei* and *O.circumcincta* have also developed to the adult stage (Wood and Hansen, 1960). Patent infections of *A.suum* have been achieved in the rabbit (Berger, Wood and Willey, 1961). Recently mature ova were seen in the female reproductive tract of *O.circumcincta* infecting the pyloric region of the stomach of experimentally infected Mongolian gerbils (normally the fundic region of the natural sheep host is affected) (Court, Lees, Coop, Angus and Beesley, 1988). No record of attempts to establish *O.ostertagi* in the mouse could be found, therefore the mouse was assessed as a possible host for production of parasite material and for comparison of infections.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Single sex groups of male or female Balb/c or B10HTT mice were selected at 8- to 10-weeks-old. Five to seven mice were housed together in conventional laboratory cages on wood shavings and fed concentrate pellets - SDS expanded rat and mouse no.3 *ad libitum*.

6.2.2 Mouse irradiation

Mice were given a sublethal dose of gamma irradiation: 76 seconds from a ^{60}Cobalt source emitting gamma rays, a 'hard' electromagnetic ionizing radiation at 5.25 Grays/minute (525 rads/minute), i.e., approximately 6.65 Grays (665 rads). The dose causes inhibition of mitosis and lymphocyte disintegration. Maximum immunosuppression occurs at 24 to 48 hours post irradiation, although with time the immune system regenerates. Consequently, the optimum time for infection is 2 days post irradiation, but in these experiments mice were infected after 5 days.

6.2.3 Larval preparation and exsheathment

Larvae were exsheathed in sodium hypochlorite solution and rinsed several times in excess phosphate buffered saline (PBS), pH 7.2 (Chapter 4, 4.2.5). Larvae from Glasgow stock suspensions were used for all the experimental model work.

6.2.4 Larval administration

Varying concentrations of infective *O.ostertagi* larvae were washed three times in distilled water. The larvae were resuspended in a 0.05% agarose solution, since agarose allows a more even suspension of larvae than water due to its higher viscosity. Larval dose concentrations were assessed by averaging the number in ten, 10µl aliquots and calculating the number in the dose volume. Mice were restrained between thumb and forefinger, gathering the excess skin around the neck. They were held upright and steadied by grasping the tail. A 3cm straight bulb-ended catheter (International Market Supply) fitted to a 1ml syringe was eased between the commisures of the mouse's lips and fed down the oesophagus to a depth of approximately 2cm with gentle rotation. The syringe was gradually depressed and withdrawn simultaneously, so depositing a dose of approximately 0.3ml within stomach and oesophagus. In all cases control mice were marked and kept alongside those given larvae. These mice were given an equivalent volume of distilled water and assessed in an identical manner to the infected animals. The day of infection was designated 0 DPI.

6.2.5 Techniques for the assessment of infection

Post-mortem examinations were carried out at certain intervals post infection. The digestive tracts were isolated and prepared for either histological or parasitological analysis. Different mice were used for the two forms of analysis, since the flushing required for parasitological examination may damage the mucosal surface altering the histological pattern.

6.2.5.1 Histology

The stomach was incised along the greater curvature and was immediately fixed in 10% formalin. It was post-fixed in corrosive formol (10% formalin with saturated mercuric chloride). Samples were trimmed, dehydrated, cleared and embedded in paraffin wax. Sections 2μm thick were cut with a Leitz rotary microtome and mounted on glass slides, then stained with Meyers Haemotoxylin and Putts Eosin (H and E) (Cook, 1974). Subsequently, three sections were examined from different sites of the stomach fundus.

6.2.5.2 Parasitology

The digestive tract was opened along its length. The stomach was separated from the intestines and subsequently the two parts were treated individually. Both stomach and intestines were flushed thoroughly with distilled water. The washed tract was examined for gross pathological changes. The material flushed from both areas was collected on a filter paper (Whatman 113) and subjected separately to overnight baermannisation (Chapter 5, 5.2.2.1). Parasites were collected from the Baermann apparatus and concentrated by centrifugation (1,000g). In addition, the filters were examined under a binocular microscope (Wild M7 Heerbrugg) at low power, x 100, for the presence of non-viable or trapped parasites.

6.3 EXPERIMENTAL DESIGNS AND RESULTS

6.3.1 Experiment 1

The Balb/c mouse was evaluated as a laboratory animal host for *O.ostertagi* by administering varying doses of sheathed L3 (the infective stage). Three groups of six male Balb/c mice were used. Five mice received larvae in the doses outlined below, while the sixth mouse acted as a control.

Group 1 - approximately 1,500 L3 in 0.3ml suspension
Group 2 - approximately 300 L3 in 0.3ml suspension
Group 3 - approximately 65 L3 in 0.3ml suspension

A mouse from each group was killed on 4,7,10,15 and 20 DPI and a control mouse was killed on days 4,7 and 10.

In this experiment gross pathology and parasitology examinations alone were made at necropsy. Prior to baermannisation, the contents were aliquotted into Petri dishes and examined under the light microscope. The process was very time consuming and did not aid or improve larval detection, so subsequently the procedure was discarded.

Results:

No gross abnormalities were found on post-mortem examination of the digestive tract. Only one *O.ostertagi* larva was found in a Group 1 mouse at 4 DPI. The sheath had been lost and the blunt anterior appearance was that of an L4. No *O.ostertagi* stages were seen in mice killed at 7,10,15 and 20 DPI. Intestinal contents yielded mouse pinworms (*Syphaciinae* spp.). At doses of 1,500; 300 and 65 L3, long-term establishment of *O.ostertagi* did not occur.

6.3.2 Experiment 2

The Balb/c mouse was evaluated as a laboratory animal host for *O.ostertagi* by administration of a dose of exsheathed L3. Differences in the physiology and structure of the murine and the bovine digestive tracts could have inhibited larval exsheathment and establishment. Consequently, exsheathed larvae were administered and higher doses were given. The experiment was conducted in duplicate. Two groups of ten male Balb/c mice were treated identically. One group contained mice for parasitological investigation and the other group was subjected to pathological examination. Eight mice from each group were given 5,000 exsheathed L3 and the remaining two mice were given distilled water alone and acted as controls. Two infected mice from each group were subjected to parasitological and pathological examinations, respectively, at serial kills 3,7,10 and 20 DPI. A control mouse was euthanised on each of the necropsy days.

Results:

Results were summarised in Table 6.1. The stomachs of the infected mice were ulcerated with petechiae, and gut adhesions were present 7 DPI. Gross pathological changes were not seen at 3,10 or 20 DPI or in the controls. No histological changes were found. High numbers of larvae were apparent in the washings from the digestive tract at 3 DPI, with counts exceeding 1,000 mixed L3s and L4s. The majority had the filariform oesophagus and the pointed anterior of the L3 stage. Some larvae had developed beyond the L3; the excretory pore was conspicuous, the anterior had a truncated appearance and a rhabditiform oesophagus was seen. No parasites were recovered from mice after 3 DPI.

6.3.3. Experiment 3

The Balb/c mouse was evaluated as a laboratory animal host for *O.ostertagi* by administration of sheathed and exsheathed L3 to irradiated mice. Following infection of mice with exsheathed larvae there appeared to be some parasite establishment, consequently, the effect of immunosuppression on the number of parasites establishing was assessed.

The experiment was conducted on 40 irradiated male Balb/c mice, housed in groups of five. Larvae were administered to four mice from each group. Half the groups received sheathed larvae and the rest were given exsheathed larvae. The remaining mice were given agarose alone and acted as controls. The administered doses were as follows:

1) 3,000 sheathed L3

or 2) 5,000 exsheathed L3

An infected mouse from each group was killed on 3,7,10 and 20 DPI. Two mice from each treatment type were subjected to parasitological examination and two to pathological examination at each kill, i.e., the experiment was conducted in duplicate. The remaining mouse from each group acted as an uninfected control and one was euthanised on each of the above days.

TABLE 6.1

Pathological and parasitological findings in mice infected with exsheathed L3 of *Ostertagia ostertagi*

	DAYS POST INFECTION n=2*				
	3	7	10	20	Controls
Gross Findings	NA	stomach ulcers, petechiae	NA	NA	NA
Histology	NA	NA	NA	NA	NA
Pooled baermann larval counts	>1,000	0	0	0	0
Larvae recovered from filters	0	0	0	0	0

* n = 2 mice were examined on each occasion

NA no abnormal changes were detected

Results:

Results are summarised in Table 6.2. In most of the mice given sheathed and exsheathed larvae, hyperaemia and petechiae of the gastric rugae were seen at 3,7 and 10 DPI. Figure 6.1 illustrates the type of petechial stomach lesions seen in an infected mouse, when compared with a control animal. However, neither type of infection demonstrated changes at 20 DPI. Histology sections demonstrated larvae in the gastric glands at 3 DPI with infections of sheathed or exsheathed parasites and in one of the four mice at 7 DPI, a mouse given exsheathed larvae (Figure 6.2). Few changes were seen on histological examination of the fundus. There was some dilation of the glands and loss of cell differentiation as seen in Figure 6.3. The contents of the flushings up to 10 DPI were high in L4s, which were identified by the anal openings and musculature, the prominence of the excretory pore and an overall increase in size relative to the L3. Filters from the Baermann apparatus showed no larvae on examination. No changes were detected in control mice.

6.3.4 Experiment 4

The aim of this study was to further assess the effect of mouse irradiation on improving establishment of exsheathed L3 of *O.ostertagi* in the Balb/c mouse model. The experimental design is summarised in Table 6.3. Seven male and six female mice were given 5,500 and 6,500 exsheathed larvae, respectively. Seven irradiated male mice and eight irradiated female mice were also given 5,500 and 6,500 exsheathed larvae, respectively. All mice were euthanised at 4 DPI.

Results:

Results are summarised in Table 6.4. Larvae were seen in all the histological sections of irradiated female mice infected with *O.ostertagi* and two of the three males. Only one non-irradiated mouse, a female, demonstrated larval establishment. Lower numbers of larvae were seen in gut flushings from non-irradiated mice

TABLE 6.2

Comparison of infection of irradiated mice with sheathed or exsheathed L3 of Ostertagia ostertagi serially killed post infection.

	Sheathed L3				Exsheathed L3			
Days post infection	3	7	10	20	3	7	10	20
Feature								
Gross pathology								
Mouse 1	Light hyperaemia stomach mucosa	NA	NA	NA	Moderate hyperaemia stomach mucosa	Moderate ulceration stomach fundus	NA	NA
Mouse 2	Light hyperaemia and some stomach petechiae	NA	Moderate ulceration stomach mucosa	NA	Light hyperaemia stomach mucosa	Severe stomach ulceration and petechiation	Moderate ulceration stomach	NA
Histology								
Mouse 1	NA	NA	NA	NA	Larvae in gastric glands	NA	NA	NA
Mouse 2	Larvae in gastric glands	NA	NA	NA	Larvae in gastric glands	Larvae in cystic gastric glands	NA	NA
Total larvae recovered by baermannisation	100s	>40	>40	0	100s	100s	>50	0

NA – no abnormal changes detected.

FIGURE 6.1

The stomach of an irradiated mouse 3 days after infection with sheathed L3 of *Ostertagia ostertagi* on the right appears hyperaemic and has petechial haemorrhages when compared to the stomach of the irradiated control mouse on the left.

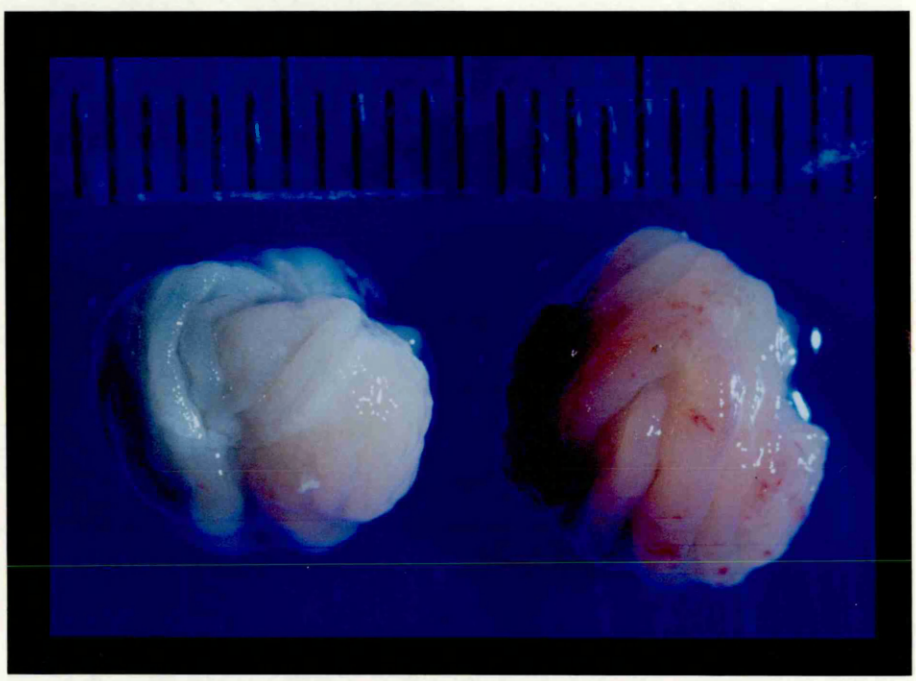


FIGURE 6.2

Transverse sections of *Ostertagia ostertagi* larvae may be seen on histological examination of the stomach fundus 3 days post infection with exsheathed parasites (arrows). There is some hyperplasia of the cells lining the gastric gland and these are poorly differentiated. Meyers Haemotoxylin and Putts Eosin (H and E). (x 112)

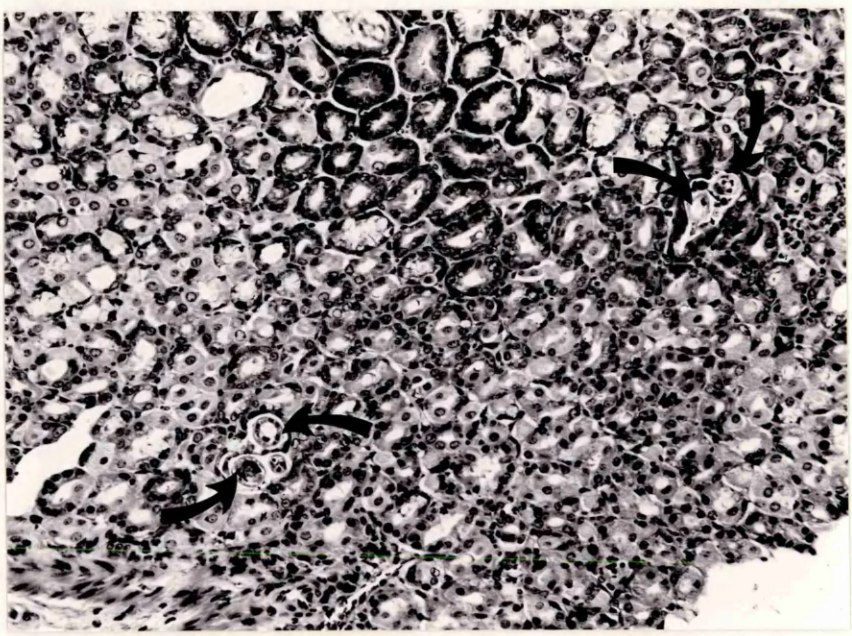


FIGURE 6.3

Fragments of an *Ostertagia ostertagi* larva (arrow) in the gastric gland of the stomach fundus of an irradiated mouse 3 days post infection with exsheathed L3s. Infected glands appeared cystic and showed loss of differentiation of the lining cells as illustrated in this figure. Meyers Haemotoxylin and Putts Eosin (H and E). (x 112)

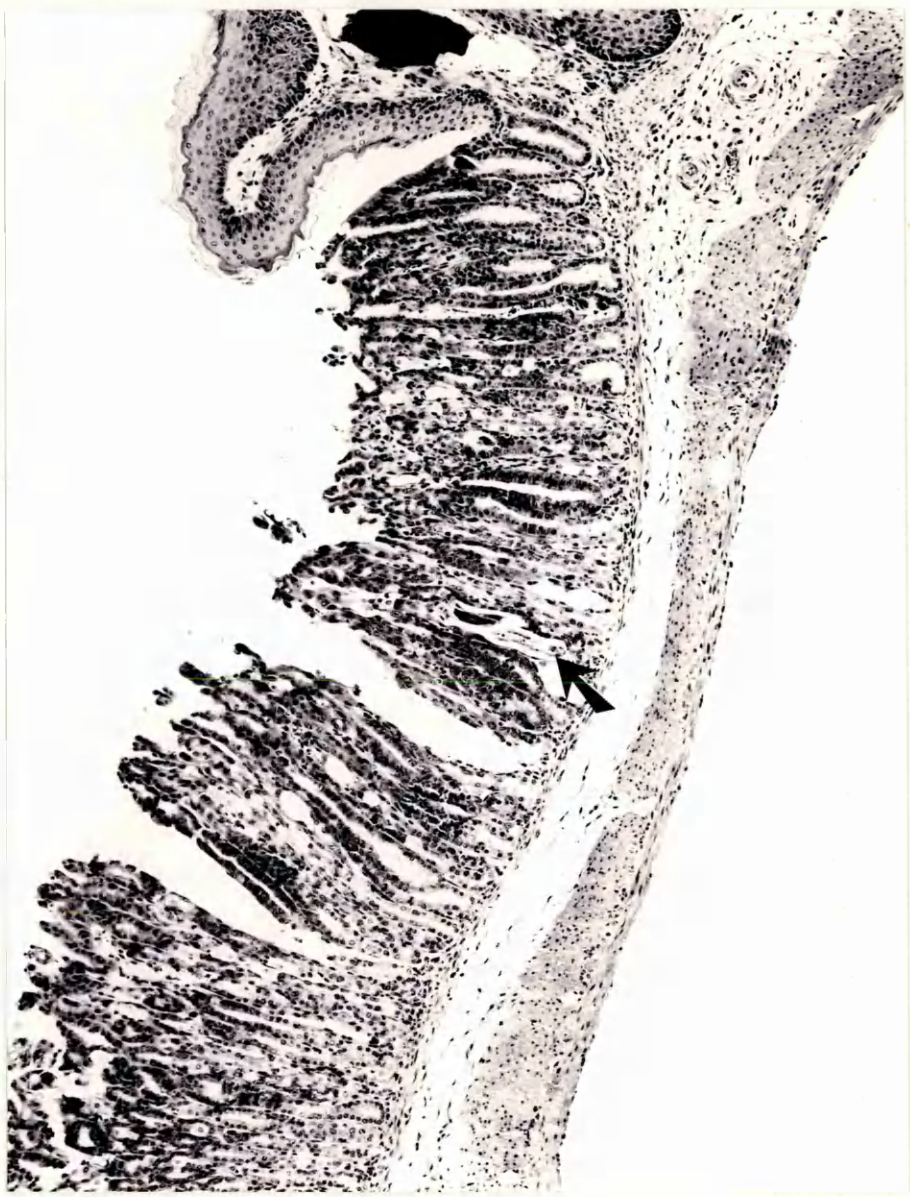


TABLE 6.3

Comparison of the effect of irradiation of groups of male and female Balb/c mice on the establishment of infections with L3 of *Ostertagia ostertagi*

GROUP	STATUS	METHOD OF EXAMINATION
Group I	7 Non-irradiated male mice	-> 4 parasitology examination
	5,500 exsheathed L3	-> 3 histology examination
Group II	7 Irradiated male mice	-> 4 parasitology examination
	5,500 exsheathed L3	-> 3 histology examination
Group III	6 Non-irradiated female mice	-> 4 parasitology examination
	6,500 exsheathed L3	-> 2 histology examination
Group IV	8 Irradiated female mice	-> 5 parasitology examination
	6,500 exsheathed L3	-> 3 histology examination

All mice were euthanised at 4 DPI

TABLE 6.4

Comparison of the effect of irradiation on the establishment of exsheathed L3 of *Ostertagia ostertagi* in groups of male and female Balb/c mice

GROUP	HISTOLOGICAL CHANGES	STOMACH LARVAL COUNTS	INTESTINAL LARVAL COUNTS
I. Male	0/3	0	0
Non-irradiated		0	0
		2	0
		2	2
II. Male	2/3	13	17
Irradiated		4	7
		33	21
		42	8
III. Female	1/2	3	0
Non-irradiated		5	4
		0	1
		2	5
IV. Female	3/3	79	18
Irradiated		49	13
		89	11
		81	8
		83	4

All mice were euthanised at 4 DPI

compared to irradiated mice. Higher numbers of larvae were present in the stomach than in the intestines.

Data were analysed using the Minitab statistical package. The stomach and intestinal counts were transformed to base e logarithmic values and two-way analysis of variance was employed to look at the influence of host sex, irradiation treatment and the interaction of their effects on larval establishment (full details may be found in Appendix 2). Irradiation of the mouse had a highly significant positive effect ($p<0.01$) on larval establishment, while host sex had a smaller effect ($p<0.05$), but still led to an increase in larval establishment in the stomach. There was no evidence for significant interaction between irradiation treatment and host sex. Similar analysis of intestinal larval counts indicated larval establishment was significantly increased by irradiating mice ($p<0.01$), but neither host sex, nor the interaction of irradiation treatment and host sex had a significant effect on larval establishment.

6.3.5 Experiment 5

The results of the previous experiments had shown establishment of larvae at 4 DPI in irradiated Balb/c mice. Experiment 5 was conducted to determine the time at which the larvae were lost. Two groups of five irradiated female B105HTT mice were given 5,000 freshly exsheathed L3s. A further group of six uninfected mice were kept as controls. The mice from one group were euthanised 4 DPI and the remaining group was necropsied 6 DPI. B105HTT mice were used due to the lack of availability of Balb/c mice at the time of experimentation. Samples from two control and two infected mice were examined by histology and from one control mouse and three infected mice by parasitology at both 4 and 6 DPI.

Results:

Only 7,8 and 33 larvae were found on parasitological examination of the stomach washings of the mice given 5,000 freshly exsheathed larvae and euthanised 6 DPI, compared to 42, 75 and 79 larvae at 4 DPI. No larvae were found in the intestinal washings or in the

histology samples at 6 DPI, but 4,8 and 18 larvae were found at 4 DPI. Furthermore, larvae were present in the histology samples taken from those mice necropsied 4 DPI. A marked decrease in larval numbers occurred between 4 and 6 DPI. There was no evidence of parasitological or pathological changes in the control mice.

6.3.6. Experiment 6

Thus, an experiment was carried out to evaluate levels of establishment of larvae cryopreserved and stored in liquid nitrogen for 1 week prior to infection. Details of the experimental design are found in Table 6.5

Three groups of five to seven B105HTT mice were irradiated. Balb/c mice were not available and the results of the previous experiment (6.3.5) indicated establishment was similar in B105HTT and Balb/c mice. Four days later 5,000 exsheathed cryopreserved larvae were administered to six female mice, Group 1 and a dose of 5,000 fresh exsheathed L3 was given to a group of seven females, Group II. The remaining five male mice, Group III received agarose alone and acted as experimental controls.

Results:

The results are summarised in Table 6.6. Larvae were present in the stomachs of all mice infected with fresh or cryopreserved parasites. More larvae were found in mice given freshly exsheathed sample (range 13 to 85) than in mice given cryopreserved larvae (range 1 to 31), but the distributions overlapped. A few larvae were present in the intestine of all mice given freshly exsheathed larvae (range 4 to 11), but in only half of those mice given cryopreserved larvae (range 0 to 27). Histology showed larvae in all infected mice at 4 DPI. Analysis of the effect of larval preparation, cryopreserved or directly exsheathed L3, on establishment of larvae was carried out by Two sample t-test. Larval counts were transformed to base e logarithms for the Minitab statistics package (see Appendix 3). The test assumes the samples have similar distributions and assesses whether this is indeed true. There was no significant difference between the larval

TABLE 6.5

Evaluation of the effect of cryopreservation of exsheathed L3 of *Ostertagia ostertagi* on their ability to establish in the mouse

GROUP	INFECTION STATUS	METHOD OF EXAMINATION
Group I	6 irradiated female mice 5,000 exsheathed cryopreserved L3	-> 4 parasitology examination -> 2 histology examination
Group II	7 irradiated female mice 5,000 exsheathed fresh L3	-> 5 parasitology examination -> 2 histology examination
Group III	5 irradiated male mice dilute agarose alone	-> 1 parasitology examination -> 1 histology examination

All mice were euthanised at 4 DPI

TABLE 6.6

Comparison of establishment of exsheathed L3 of *Ostertagia ostertagi* with or without a period of cryopreservation in liquid nitrogen

GROUP	HISTOLOGICAL CHANGES	STOMACH LARVAL COUNTS	INTESTINAL LARVAL COUNTS
I. Female irradiated mice. Cryopreserved L3.	2/2	20	27
		1	0
		31	0
		13	2
II. Female irradiated mice. Fresh L3.	2/2	28	5
		85	11
		13	4
		13	11
		47	5
III. Male irradiated mice. Controls. No L3s.	0/1	0	0

All mice were euthanised 4 DPI

numbers found in mice given cryopreserved or refrigerated larvae. No larvae were isolated from the control mice.

6.4 DISCUSSION

The mouse was shown to be an unsuitable model for generating passage material of *O.ostertagi*, as no success was achieved in trying to complete the life cycle. The average dose administered to a 75 to 100kg calf is 50,000 to 200,000 L3. An adult mouse weighs 0.02 to 0.04kg, so proportionately the dose would be approximately 14 to 80 L3. Due to physiological and structural differences between murine and bovine digestive tracts, decreased larval establishment was expected, so in addition to a dose within the interpolated range higher concentrations were also given.

Increased establishment of the parasite *O.ostertagi* was found in the laboratory mouse with:

1) Increasing dose
2) Larval exsheathment
3) Mouse irradiation
4) Infection of the female rather than the male mouse.

A marked drop in the number of larvae recovered at necropsy was found between 4 and 6 DPI. Larvae stored by cryopreservation appeared to establish at a reduced level relative to freshly exsheathed larvae, although the differences were not statistically significant. Further assessment of the effects of cryopreservation as a method of larval storage are required to assess the more long-term effects on infection, e.g., whether larval arrest occurs in a similar fashion to that observed after long term storage at 0 to 4°C. Establishment of the parasite for a longer time period would be a prerequisite for a study of this nature.

Comparison of strains of *O.ostertagi* for infectivity and pathogenicity using a laboratory model had also been an original aim from this work. Generally, the low recovery rates and the inconsistency of results from mice of the same strain and batch suggest the level of

experimental error in interpretation of comparative infectivity experiments for *O.ostertagi* is unacceptably high.

A problem frequently encountered in the use of laboratory models is the inadequate mimicking of the conditions the parasite inhabits in the natural host. Even in cases when establishment in laboratory animals appears successful, extreme care must be exercised in extrapolating information and the interpreting of results. Frequently in the model host the pre-patent period is longer, the burden established comparatively lower and clinical signs milder, as has been observed in infection of heterologous hosts, e.g., *O.ostertagi* infection in the goat (Williams, 1986 and 1987). Douvres and Tromba (1971) compared *Ascaris suum* development in rabbits, guinea pigs, mice and swine. They found that larval stage identified on the basis of size, location and length of infection varied among hosts. Consequently, material generated by passage in laboratory hosts may not really reflect the character of the initial inoculum.

Helminths arising from different geographical locations or from different host species may differ in pathogenicity and even morphology, e.g., *Schistosoma intercalatum* strains with different intermediate host specificity were experimentally hybridized *in vivo* in mice and varied in their growth rate, male to female ratio, egg morphology, prepatent period and distribution of eggs between tissue and faeces (Fransden, 1978). Isolates of *Trichinella spiralis* from the Arctic and from temperate zones showed different infectivities using the mouse as a common laboratory host (Read and Schiller, 1969).

Only Balb/c and B10HTT mice were used to evaluate the mouse as a host for *O.ostertagi*. However, in-bred strains of mice vary widely in their susceptibility to nematode infections (Lee and Wakelin, 1982) and other mouse strains might give different results and this approach may be worthy of extended experimentation. In addition, Lichtenfels (1971) found *Nippostrongylus brasiliensis* strains from rats and hamsters behaved differently when used to infect the other host species, e.g., the rat adapted strain persisted longer in the intestine than the hamster strain when both were used to infect hamsters and fewer eggs were seen in the female nematode adapted to the rat.

Other laboratory animal hosts could be evaluated, particularly the rabbit since a single adult *O.ostertagi* has been recovered from that host on a previous occasion (Aljeboori, 1965; Snider *et al.*, 1985). Possibly the rabbit is better adapted to be a model host for the parasite and further work might test this theory. Later stages of *O.ostertagi* might be implanted into laboratory animals to identify the stage at which larvae are rejected as Zebrowska-Plata (1980) did in assessing infection of laboratory animals with *O.circumcincta*.

Infection in a model host was felt most likely to succeed in young animals with a compromised immune system. Bovine ostertagiosis is most commonly seen in young animals, since immunity is acquired; young animals are more severely affected and take longer to develop immunity than older animals (Smith, 1970; Armour, 1978). Animals in a general state of debility are more susceptible to disease, so when concomitant disease is seen the clinical signs seen are often worse.

A complex of interacting host factors affect host susceptibility to parasite infection, for example appetite may influence helminth uptake (Anderson *et al.*, 1969) and the repellent nature of the acid digestive tract to parasite larvae may affect establishment (Stringfellow, 1981). Stress is another factor, which may be involved in parasite establishment. It is known that alterations in corticosteroid levels in cattle infected with *O.ostertagi* lead to increased nematode egg output (Michel, 1969). In addition, susceptibility to infection may be influenced by a multitude of factors that we are simply unaware of, e.g., host sex, other genetically determined parameters (Wakelin 1986; Gray 1987), management systems, etc. Higher numbers of larvae were recovered from irradiated female mice than from males. There is no reliable evidence for host sex affecting the outcome of infections with ostertagiosis in ruminants, although Stear *et al.* (1990) found male calves grazing pastures contaminated with mixed nematode infections had higher faecal egg counts.

In conclusion, no larval development was found beyond the moult to the L4 stage in Balb/c or B105HTT mice. Herlich (personal communication) also found no development beyond the L4 stage in guinea pigs and hamsters. Furthermore, the mouse was found to be a

poor indicator for comparative infectivity, since parasites exhibited unpredictability in establishment when similar infective doses were given, and overall only low numbers of parasites established for a short time. Although initial work has shown that the laboratory mouse, or more precisely Balb/c and B105HTT mice are unsuitable for passage or infectivity studies for *O.ostertagi*, there may well be other possible approaches by which the use of laboratory animal model work could be considered.

II. CRYOPRESERVATION OF INFECTIVE STAGE *OSTERTAGIA OSTERTAGI*

6.5 Introduction

Cryopreservation is a method for long-term storage of parasite samples, which has been used with notable success in haemoparasites and is being increasingly employed in helminth studies, as reviewed by James (1985). Vitrification occurs during cryopreservation, i.e., solidification of a liquid without crystal formation. Providing cell damage is minimised cryopreservation may be used to store organisms at low temperatures until they are required, when they are thawed.

The use of cryopreservation was assessed for possible maintenance of banks of isolates of *O.ostertagi*. Rew and Campbell (1983) found only a 33% loss in survival when *H.contortus* were stored for 10 years by cryopreservation. Ramp, Eckert and Gottstein (1987) cryopreserved L2 of *Toxocara canis* and found that the excretory-secretory antigens produced when these larvae were thawed were indistinguishable from freshly produced material.

Currently, suspensions of L3s are routinely kept for periods at 0 to 4°C, but it is known that storage for 6 to 8 weeks may lead to arrested larval development in the host (Armour and Bruce, 1974). After 19 weeks storage at 4°C the chances of arrest occurring diminish; it appears that inhibition prone larvae are selected out of the population (Armour, 1978). In addition, it has been noted that although larvae are motile in suspensions, after prolonged storage material appears to

be lost from the parasite, since the suspending fluid becomes cloudy and occasionally stationary larvae are seen. When these changes were seen the larvae were baermannised and resuspended in fresh water. However, suspending fluid tested by the Bradford Assay (Chapter 4, 4.3.4) after storage for 1 month had a protein content of 6.2µg/ml. Aliquots of the fluid plated out on MacConkey agar bacteriology plates showed no bacterial growth after 5 days when incubated either aerobically or anaerobically, suggesting the protein was of nematode origin rather than bacterial contamination. As a result, storage at 4°C would not appear to be particularly suitable in the long-term for the parasite and seems to cause selection of subgroups of the population.

The alternative approach to sample maintenance is continual passage of parasite material in calves, a costly procedure, particularly if material is not in constant demand. To date, there has been no success in the completion of the parasite life cycle for generation of sufficient sample material for experimental work either by laboratory models or *in vitro* culture. Continual passage in itself, whether in the definitive host, a laboratory model or *in vitro* may result in sample contamination and population changes and may be a source of further unexpected intraspecific variation.

The rate of cooling is important for the successful recovery of viable organisms. Individual cells have their own optimal rates. With high rates of cooling, intracellular ice may form and insufficient shrinkage occurs before the cell contents freeze. At low rates, the solute concentration becomes relatively high; the freezing point is depressed and water may be drawn out of the cells causing shrinkage. The probability that the suspending medium freezes before the parasites is high due to its comparatively greater volume. In both cases, additional cell damage may occur due to lipid aggregation on solidification. Often the cooling process is divided into two stages: a gradual temperature reduction followed by a rapid drop; some workers use programmable freezers.

In a multicellular organism, as in the case of nematodes, the situation is further complicated, since the constituent cells react individually on cooling. Uga, Araki, Mastsumara and Iwamura (1983) examined the eggs of *Angiostrongylus cantonensis* and found that as eggs developed and the number of cells increased their overall size was reduced and their ability to withstand cryopreservation improved. Their findings suggested that smaller cells are better able to withstand cryopreservation.

The L2 sheath appears to cause a particular problem with cryopreservation by acting as a barrier to water movement, so exsheathing the L3 allows some dehydration pre-cooling, which appears to enhance cryopreservation (James, 1985). Seeding the medium with ice is a technique used by some workers, since it seems to prevent the abrupt damaging increase in temperature with liberation of latent heat of fusion just before the freezing point is reached (Uga *et al.*, 1983).

Cryoprotectants added to the suspension may improve the percentage of live parasites at recovery by a number of methods, e.g., lowering the temperature of ice nucleation thereby reducing ice crystal formation; altering surface kinetics; improving membrane surface resistance to damage especially at thawing; removing intracellular fluid, so reducing the chances of intracellular ice formation. Cryoprotectant action alters with temperature, since the surface is less permeable at low temperatures, consequently, when two-stage cooling is used the concentration of cryoprotectant is often increased at the change to accelerated cooling, i.e., when the surface is less permeable (Ham and Bianco, 1981). However, Coles, Simpkin and Briscoe (1980) reported little improvement in recovery when cryoprotectants were used in the cryopreservation of ruminant nematodes.

Generally the faster the rate of thawing the less chance of small ice crystals seeding and enlarging rapidly with increasing temperature. Conversely, dilution shock may occur on rapid thawing due to cryoprotectant dilution.

With respect to *O.ostertagi* itself, Van Wyk, Gerber and Van Aardt (1977) stored larvae in the gas phase over liquid nitrogen using physiological saline as the suspending medium. They found exsheathed larvae survived a mean of 26 weeks in storage, though few sheathed larvae were recovered at thawing after similar treatment.

The current study was carried out in an attempt to develop a repeatable cryopreservation technique for *O.ostertagi*. Viability or survival after thawing is usually assessed by motility of the parasite *in vitro*. However, in reality only retained ability to infect a host can confirm complete viability, since undetectable damage to the surface or to enzyme systems may have occurred. Thus, in addition to studying motility after cryopreservation, preliminary work was carried out to assess the effect of cryopreservation on infection in model hosts (Chapter 6, 6.3.6). Once the technique was established, it was intended that a bank of different isolates be assembled.

6.6 MATERIALS AND METHODS

6.6.1 Experiment to assess different cryopreservation regimens for *Ostertagia ostertagi*, particularly the influence of exsheathment

Different cryopreservation regimens for the L3 of *O.ostertagi* were evaluated, including the additional influence of larval exsheathment. Details of the experimental design are shown in Table 6.7.

Larvae were exsheathed in sodium hypochlorite solution by the method described in Chapter 4, 4.2.5. Five, 4,000 larvae aliquots were prepared for each of the six regimens and put into 2ml sterile cryotubes (Nunc, Intermed). Prior to storage the L3s were found to have 100% motility on examination by light microscopy. Ten batches of 100 larvae were observed for each regimen. Larvae were considered to be motile when movement was recognised during examination of the microscope field for a 10 second interval. Generally, non-viable larvae in addition to being motionless tend to straighten out, whilst viable larvae are curled up.

TABLE 6.7

Experimental design for comparison of different cryopreservation methods relative to refrigeration as a method of storage of the L3 of *Ostertagia ostertagi*

Group	Preparation
I	Sheathed L3 maintained by refrigeration, 0 to 4°C.
II	Single stage cooling Sheathed L3 directly immersed in liquid nitrogen, -196°C.
III	Two-stage cooling Sheathed L3 held in vapour over liquid nitrogen for 2 hours prior to plunging into liquid nitrogen.
IV	Exsheathed L3 maintained by refrigeration.
V	Exsheathed L3 single stage cooling.
VI	Exsheathed L3 two-stage cooling.

For cryopreservation, the samples were inserted into a carrier cylinder and placed either in the vapour over the liquid nitrogen by wedging the carrier in the neck of the dewar flask (storage cryogenic dewar flask, Taylor Wharton, LD series) or plunged directly into the liquid. After 1 week, the samples were removed from the carriers, immediately submerged in water at 37°C to thaw, left to equilibriate for 30 minutes at room temperature and finally aliquots were assessed for motility.

Results:

Full results are recorded in Table 6.8. Optimal motility was found in the refrigerated samples with average motilities as follows, sheathed 90% and exsheathed 81%, while the cryopreserved exsheathed larvae subjected to two-stage cooling came next at 75%. Percentage larval motilities were markedly lower under the other cryopreservation regimens decreasing in the following order: exsheathed-single stage cooling (32%), sheathed single stage cooling (6%) and finally sheathed two-stage cooling (1%).

6.6.2 Experiment to assess the effect of the cryoprotectants, methanol and glycerol, on recovery levels after cryopreservation

Cryoprotectants, methanol and glycerol, were added to the suspension medium and the motility of exsheathed larvae re-assessed after cryopreservation and thawing. The final concentration of cryoprotectant added was 20%, which appeared to be the commonly accepted experimental level (Ham and Bianco, 1981). The experimental design is shown in Table 6.9. Five 4,000 larvae aliquots were examined for each storage regimen.

Results:

Results are shown in Table 6.10. Two-stage cooling of larvae in water gave higher average percentage motility compared to single stage cooling, 55% and 26% respectively, as in 6.6.1, although the percentage motilities were lower. The relative percentage motility

TABLE 6.8

Comparison of *Ostertagia ostertagi* larval motility after storage for 1 week under a number of preparative regimens

Group and Preparation		Percentage motility	Average
I	sheathed refrigerated	93, 88, 70, 99, 98.	90%
II	sheathed single stage cooling	2, 11, 8, 9, 2.	6%
III	sheathed two-stage cooling	3, 0, 0, 1, 1.	1%
IV	exsheathed refrigerated	83, 84, 94, 74, 70	81%
V	exsheathed single stage cooling	26, 36, 29, 30, 38.	32%
VI	exsheathed two-stage cooling	83, 75, 76, 65, 74.	75%

TABLE 6.9

Cryopreservation protocol for the comparison of the effect of cryoprotectant addition on *Ostertagia ostertagi* larval recovery

Group	Preparation
I	single stage cooling - distilled water
II	two-stage cooling - distilled water
III	single stage cooling - 20% methanol
IV	two-stage cooling - 20% methanol
V	single stage cooling - 20% glycerol
VI	two-stage cooling - 20% glycerol

TABLE 6.10

The effect of methanol and glycerol as cryoprotectants on the motility of exsheathed L3 of *Ostertagia ostertagi* after cryopreservation

Group and Preparation		Percentage motility	Average
I	single stage cooling no cryoprotectant	36, 10, 40, 24, 20	26%
II	two-stage cooling no cryoprotectant	57, 53, 44, 65, 57	55%
III	single stage cooling 20% methanol	0, 0, 0, 0, 0	0%
IV	two-stage cooling 20% methanol	0, 0, 0, 0, 0	0%
V	single stage cooling 20% glycerol	3, 0, 4, 1, 1	2%
VI	two-stage cooling 20% glycerol	3, 5, 3, 0, 0	2%

after two-stage and single stage cooling with glycerol was negligible (2%) and no motile larvae were seen when methanol was added to the suspension as a cryoprotectant.

6.7 DISCUSSION

Refrigeration was the optimal regimen for storage of sheathed larvae for 1 week. With cryopreservation, higher levels of viable parasites were recovered after larvae were exsheathed when judged by motility. More motile larvae remained after two-stage cooling than single-stage cooling. Sheathed L3s showed high levels of mortality on cryopreservation and were more adversely affected by two-stage cooling. The addition of the cryoprotectants methanol and glycerol at 20% of the suspension concentration appeared to reduce viable numbers on recovery.

Cryopreservation of larvae compared to refrigeration for 1 week led to reduced motility. The preliminary studies comparing infection of mice with previously cryopreserved or fresh larvae showed no significant variation in the larval numbers recovered. By standardising methods of sample storage, i.e., by cryopreservation, it was hoped that material could be maintained until all samples required for comparison were available and problems of sample ageing were ruled out. However, as discussed later, samples stored by cryopreservation for isoenzyme analysis appeared to show poorer enzyme activity than freshly homogenised material (Chapter 7). The effect of ageing on nematode enzymes was highlighted by Rothstein (1980), although, in general, little is known about the ageing process itself. Further work is required to ascertain the long-term effects of cryopreservation before the technique can be used with the confidence that errors are not being introduced into experiments, leading to unknown variations in results.

CHAPTER 7

ISOENZYME ANALYSIS OF TRICHOSTRONGYLE SPECIES AND *OSTERTAGIA OSTERTAGI* ISOLATES

7.1 INTRODUCTION

7.1.1 Definition

Different forms of enzymes catalysing the same chemical reaction are known as isoenzymes; they vary in structure so either their kinetic properties and/or their charge are different. Enzymes are proteins and consequently, when the ratio of their net ionic charge to mass/ molecular size varies, they migrate at different rates in an electric field and so they may be separated.

7.1.2 Protein variation implies genetic variation

Since each polypeptide of an enzyme/isoenzyme is coded for by a single gene sequence, variation in the electrophoretic mobility of the same enzyme in two different individuals implies that the polypeptide structure is different, which in most instances arises as a result of a base change in the structural gene. The analysis of the electrophoretic mobility of an isolate for a range of enzymes provides a genetic profile, which may be used to identify strains within a species (Nomura, 1984). Varying presentations, sample track patterns, for a particular enzyme may arise from multiple genetic loci or allele involvement and through post-translational changes.

A zymodeme is a population within a species sharing the same unique combination of enzyme patterns, i.e., zyomodeme profile. The profile gives an indication of presence or absence of an enzyme and the number and configuration of its banding pattern relative to the sample loading position (Bullini, 1982). More closely related individuals have more similar zymodeme profiles and are separated by a shorter "genetic distance". This can be estimated by:

$$\text{The coefficient of similarity} = \frac{\text{Number of common mobility bands}}{\text{Maximum number of bands of an individual}}$$

The trichostrongyles are diploid organisms, so two alleles coding for a simple monomeric enzyme may give rise to a single band in a homozygous individual or to two bands in a heterozygous individual

(Figure 7.1). The pattern is complicated further when the ~~tertiary~~ quaternary structure of the protein consists of more than one chain. An enzyme may vary in its tertiary structure depending on the organism under examination, i.e., the enzyme may be dimeric in one species and monomeric in another, etc.

When the amino acid sequence is sufficiently altered changes in charge, and in a few cases, mass of the protein occur, although alteration in the amino acid sequence does not always result in a change in the charge of the protein. The charged amino acid groups are listed in Table 7.1. Only 25 to 30% of sequence changes may be detected by isoenzyme analysis (Tait, 1982) due to redundancy of the genetic code, lack of charge alteration, etc. Experimentally post translational changes may cause alterations in charge, i.e., by amidation, glycosylation and oxidation (Tait, 1984) and should be minimized by standardisation of techniques, e.g., buffer ionic composition and concentration, matrix composition, voltage, etc.

7.1.3 The use of isoenzyme analysis

Results of sample comparison may produce information on evolutionary pathways and geographical spread of organisms, e.g., *Paragonimus* fluke (Agatsuma and Habe, 1985a and b). Mydinski and Dick (1985) found polymorphism in eight out of 11 enzyme systems in *Trichinella*, which allowed them to divide seven isolates into three groups. Genetic exchange may be detected and taxa defined (Andrews, Adams, Boreham, Mayrhofer and Meloni, 1989), e.g., the differentiation of intraspecific variation from species complexes with two or more morphologically identical cryptic species.

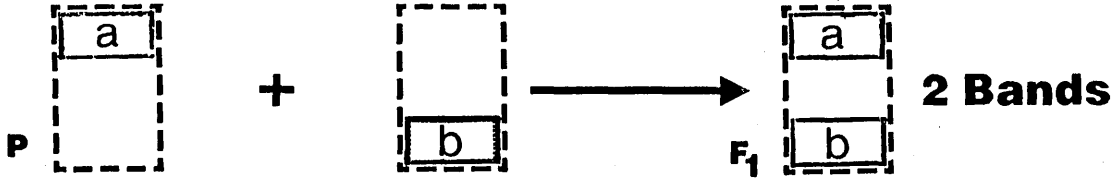
Knowledge of isoenzyme patterns may give useful epidemiological information, e.g., identification of zoonotic trypanosome subspecies (Tait, Barry, Wink, Sanderson and Crowe, 1985) and drug tolerant strains of nematode (Sutherland *et al.*, 1988). Sutherland *et al.* (1988) showed differences in the esterase patterns of benzimidazole resistant and susceptible strains of *Haemonchus contortus*, *Ostertagia circumcincta* and *Trichostrongylus colubriformis*. The resistant nematodes exhibited greater acetylcholinesterase activity and an

FIGURE 7.1

Enzyme patterns depend on the number of protein chains.

DIPLOID - Genetic information from both parents

MONOMERIC ENZYME -



DIMERIC ENZYME -

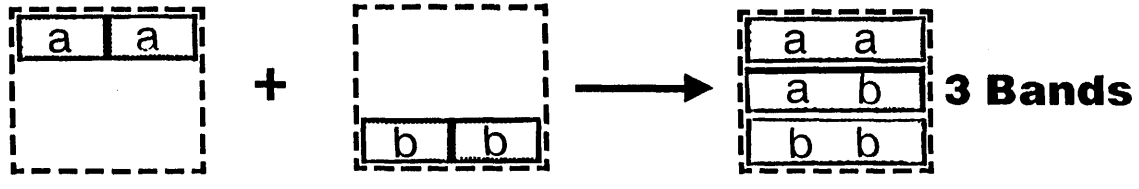


TABLE 7.1

The charged amino acid groups

Positively charged	Negatively charged
Lysine	Free carboxyl groups:
Arginine	Aspartic
Imidazolyls of Histidine	Glutamic

increased number of stained bands for this enzyme. Bryant and Flockhart (1986) commented that aerobic metabolism was more highly developed in drug resistant helminths. Nakamura, Konishi, Kawguchi and Hayashi (1988) found that by quantitative analysis of isoenzymes of *Eimeria tenella* they could examine the effect of different drugs on the coccidia. The action of many anthelmintics is poorly understood, but evidence is emerging that some effect the parasite metabolism. Sharma, Singh and Saxena (1989) found that levamisole inhibited malate dehydrogenase, aldolase and fumarate reductase.

The objective of this study was to ascertain whether isoenzyme analysis could be used to demonstrate differences in isolates of *O.ostertagi* from distinct geographical regions, which appear morphologically identical. Insufficient information was available to estimate the level of variation within a nematode species and other trichostrongyle species were examined to indicate the extent of the inter-species variation relative to these. It was necessary to establish a screen of at least 15 enzyme substrate stain systems. Nei (1978) stated that the number of individuals that must be assessed for the comparison of enzyme heterozygosity may be low provided a large number of loci are examined and the average heterozygosity is low or the genetic distance is great. Enzyme polymorphism may vary, possibly reflecting the compatability or dispensability of resulting alterations on physiological function (Ferguson, 1980). Bullini (1982) suggested a large number of enzymes should be examined for reliable evaluation of differences, i.e., multilocus genetic analysis. He chose to compare 20 enzymes or more, representing different metabolic classes and degrees of variability. In this study, five trichostrongyle species and 11 isolates of *O.ostertagi* were examined by starch gel electrophoresis and isoelectric focusing using 18 basic enzyme substrate systems (plus further non-specific esterases and peptidases).

7.2 MATERIALS AND METHODS

7.2.1 Sample preparation

L3s were readily available in high numbers from a variety of sources, and consequently they were the stage favoured for comparative

purposes. One hundred thousand L3 were homogenised in 100µl of homogenisation buffer. The volume of homogenisation buffer added was equivalent to approximately double the sample volume. Initially, a trypanosome homogenisation buffer used routinely for trypanosome analysis was employed; 50 ml was composed of 0.5% Triton X-100, 10mM Tris-hydrochloric acid pH 7.5, 1mM EDTA and 0.0154% dithiothreitol in distilled water. However, the homogenisation buffer for SDS-PAGE of nematode samples was used latterly (Chapter 4, Table 4.3), since it allowed the preparation of samples for a variety of techniques simultaneously, but contained chelating agents which were occasionally altered on IEF. Homogenates were centrifuged at 10,000g for 5 minutes and the supernatants retained for loading gels. In addition, presence of protease inhibitors in the buffer would reduce degeneration of proteins arising from homogenisation.

In the preliminary stages of the work adult parasite extracts were loaded at 20 adults/sample. Due to the cost of sample production and particularly the problem of obtaining fresh adult material or prepared homogenates from other strain sources, adults were felt to be an inappropriate preparation for comparative assay. The data presented suggests that the patterns produced by the adults and L3s varied in a quantitative manner alone. Furthermore, attempts to produce a larval extract by three repeated freeze-thaw cycles (-17°C) followed by microfuging for 10 minutes at 10,000g were unsuccessful, since the larvae did not appear to have been affected by the processing on examination by light microscopy.

7.2.2 Horizontal starch gel electrophoresis

Many excellent full accounts of isoenzyme techniques are available, e.g., Smith William (1968) and Brewer (1970). The following is a description in brief of the methodology used in the present study.

7.2.2.1 Gel preparation

A perspex template 22 x 15 x 0.6cm^3 and a glass plate were sealed together with silicone grease to form a gel mould. Hydrolysed starch (20g) was heated over a Bunsen flame with 250ml of gel buffer (Table 7.2) in a 1 litre side-arm flask of toughened glass. The solution was

TABLE 7.2

Gel and tank buffers for starch gel electrophoresis

TRIS CITRATE BUFFER pH 7.0

Tank: 0.9L distilled water was added to 16.35g Trizma and 9.04g citric acid and the pH titrated to 7.0 with concentrated hydrochloric acid. The solution was made up to 1L.

Gel: Distilled water was added to 16.7ml of the tank buffer to bring the volume up to 250ml, maintaining the pH at 7.0

TRIS CITRATE BUFFER pH 8.6

Tank: 0.9L distilled water was added to 40.0g Trizma and 8.71g citric acid and the pH titrated to 8.6 with concentrated hydrochloric acid. The solution was made up to 1L.

Gel: Distilled water was added to 20ml of the tank buffer to bring the volume up to 250ml, maintaining the pH at 8.6

TRIS PHOSPHATE BUFFER pH 7.0

Tank: 0.9L distilled water was added to 10.89g Trizma and 17.16g sodium dihydrogen phosphate and the pH titrated to 7.0 with concentrated hydrochloric acid. The solution was made up to 1L.

Gel: Distilled water was added to 12.5ml of the tank buffer to bring the volume up to 250ml, maintaining the pH at 7.0.

TRIS POTASSIUM PHOSPHATE BUFFER pH 9.3

Tank: 0.9L distilled water was added to 21.80g Trizma and 2.72g potassium dihydrogen phosphate and the pH titrated to 9.3 with concentrated hydrochloric acid. The solution was made up to 1L.

Gel: Distilled water was added to 25ml of the tank buffer to bring the volume up to 250ml, maintaining the pH at 9.3.

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swirled throughout heating to avoid temperature gradients, which might result in glass breakage. Once the starch was fully dissolved the solution thickened and cleared, momentarily clouded and thickened further. Once the starch began to boil, the flask was rapidly removed from the heat. Excess boiling will reduce the buffer volume and should be avoided. The solution was degassed by vacuum pump, which causes the starch to boil briefly under reduced pressure, air bubbles are removed and the granule size of the matrix becomes uniform for protein migration. The gel was poured into the mould, so the surface was just proud to the perspex.

The gel was left to set on a level surface for 1 hour at room temperature followed by 1 hour at 0 to 4°C, when slots were cut in a horizontal line across the gel - 1cm wide and 0.4 to 0.5cm deep. Sample to sample cross contamination was prevented by leaving adequate inter-slot distance and assuring the sample did not contact the glass base plate with too deep a slot. Proteins generally carry a negative charge, so by placing the slots eccentrically the area for separation towards the anode was increased.

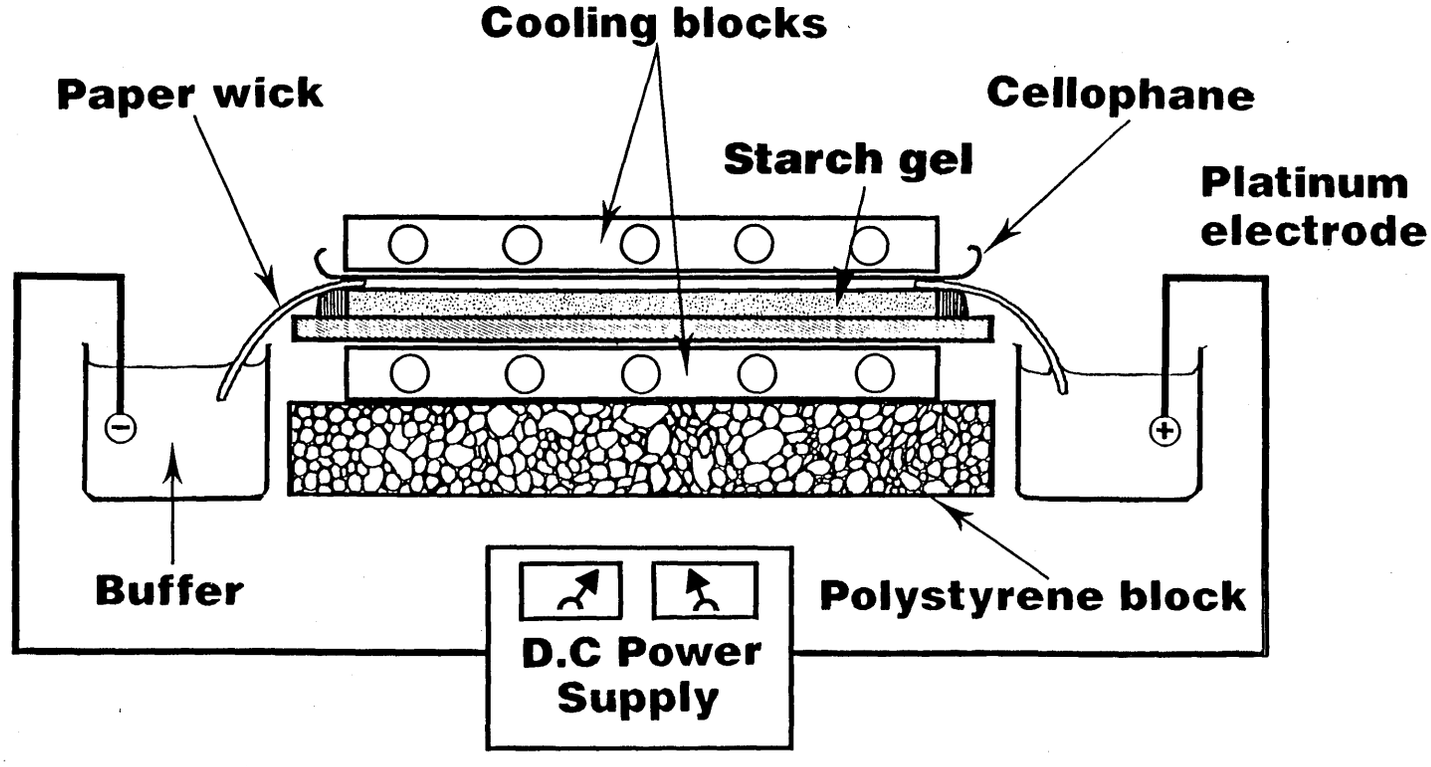
The sample carrier filter was a piece of 3MM Chr paper (Whatman) approximately 0.6 x 0.3cm^2 and it was immersed for 10 minutes in 20ul larval sample, i.e., 20,000L3. Generally, 20µl of solubilised sample produced adequate enzyme activity for interpretation after staining. Smaller amounts of the sample were tested by loading nitrocellulose acetate papers. The filters impregnated with sample were inserted into the slots using forceps.

7.2.2.2 Electrophoresis

The apparatus used is illustrated in Figure 7.2. The gel in its mould was laid between brass cooling plates. Cooled water (Grant FC25 flow cooler) was pumped (Grant FH15 heater) through a series of copper tubes within the plates. Without cooling the heat produced during the experiment resulted in enzyme denaturation and band distortion, particularly if the temperature exceeded 30 to 35°C. The equipment was set up to precool at least 2 hours in advance of the gel run.

FIGURE 7.2

Apparatus for horizontal starch gel electrophoresis.



Five sheets of 1Chr paper (Whatman) were bridged between the buffer tank and the gel surface. The buffer tanks were filled (Table 7.2). In a continuous buffer system the salts in the tank are identical to those in the gel, but in a discontinuous system they are different; only continuous buffer systems were used in this study.

A sheet of plastic was applied to the gel surface to minimise buffer evaporation and to provide electrical insulation. A direct current power supply was applied for 3 hours at 250 to 300 volts, i.e., 2 to 3V/cm (0.07 to 0.08A). In an effort to maximise experimental standardisation, conditions were kept as similar as possible between experimental runs, e.g., time for electrophoresis, cooling and sample concentration. Matrix pore size and protein size are similar, consequently, when a potential difference is applied across the gel molecular sieving occurs, although with starch gel electrophoresis and isoelectric focusing the net protein charge has a greater effect on mobility than mass. The concentration of ions in the gel tank is approximately ten times greater than in the gel. Lower gel buffer concentrations increase the speed of migration, since the sample carries more current and the buffer proportionately less, consequently buffer dilution leads to increased band width.

After completion of the run, the power was switched off and the gel removed from the cooling plates. The sample carrier filters were withdrawn. The mould was lifted away from the gel and replaced by parallel perspex struts relatively thinner than the gel, which enabled a uniform horizontal section of the gel to be cut to give a staining surface. The original mould was replaced and the substrate stain applied.

7.2.3 Isoelectric focusing

A sheet of gelbond (FMC Bioproducts) was laid with the hydrophilic side uppermost on a film of water on a glass plate; the water provides uniform heat dissipation. A template 18.2 x 11.5cm^2 was placed on the gelbond and the layers clamped together and placed level in an incubator at 37°C, so the apparatus was warmed before the gel was poured.

Agarose (0.3g) and sorbitol (3.6g) were placed in a flask with 27ml distilled water and heated in a beaker of boiling water until they had dissolved. A total volume of 0.76ml of ampholine was added in the ratio required to produce the desired pH gradient. The mixture was gently swirled to avoid excess aeration and rapidly poured into the mould to give an even surface. The gel set rapidly since it was only 0.5mm thick, but it was left for 30 minutes at 37°C followed by 30 minutes at 0 to 4°C.

The gel was transferred on the gelbond backing to the cooling plate of the electrofocusing apparatus (LKB Bromma 2117 Multiphor), again over a film of water. Cold water had already been run through the apparatus for 1 hour. Electrofocusing electrode strips (LKB) were impregnated with 0.05M sulphuric acid for the anode and 1M sodium hydroxide for the cathode. Excess water from the edge of the gel bond and reagents from the electrode strips were blotted away. The electrodes were trimmed so they were 3 to 4mm shorter than the gels.

Samples were prepared in a similar fashion to those for starch gel electrophoresis, but they were blotted and applied to the agarose surface. By experience the optimal loading position (high, mid and low relative to the electrodes) and ampholyte ratio were found for samples with a particular substrate stain system. In addition to the samples under test, standard markers could also be run allowing improved comparison of experimental runs (IEF Calibration kit, Pharmacia). Once even electrode contact had been established the power was applied at 6.5 Watts, 20 mA and a maximum voltage of 1 kV (LKB Biochrom 2103 Power Supply). After 30 minutes the pH gradient had established and the sample carrier filters were removed. Excess liquid was blotted away.

With time the current dropped to 4 to 5 mA and the voltage rose from 500 V to 1 kV. At this stage the proteins should have reached and focused at their isoelectric points, but prior to staining this could not be visualised. Consequently, sample carrier filters impregnated with blood were applied adjacent to both electrodes, which resulted in a single fine band when the sample had focused.

The power supply was switched off, the electrodes removed and the gel surface gently blotted. The mould and glass plate were replaced. The gel was separated into sections for staining with particular substrate systems and the cathode region was sealed off with 2% agar. Unless the cathode region is isolated from the rest of the gel, the alkali destabilises and reduces the dye, tetrazolium-phenazine methosulphate, to produce insoluble blue formazan, regardless of the presence of the enzyme under test. The substrate stains were added in a similar fashion to those in starch gel electrophoresis, although relatively smaller volumes of the stain were required.

7.2.4 Substrate staining

The staining protocols employed were adapted from Harris and Hopkinson (1976) and are entered in Table 7.3 with their corresponding buffers in Table 7.4. Reduction of the tetrazolium dye methyl thiazoyl tetrazolium, with the intermediary catalyst phenazine methosulphate results in the formation of formazan, which is blue-black and insoluble. The reagents are light sensitive and the gel should be incubated in the dark. Dehydrogenases are detected with the addition of the coenzymes, nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate or dehydrogenases may be added as linking enzymes allowing the identification of further enzymes. Alternatively, "positive" fluorescent stains produce fluorescent staining in the presence of certain enzymes and "negative" fluorescent staining produces a non-fluorescent area in a fluorescent field; ultraviolet light (366nm) was required for visualisation. Stains were frequently applied in an agar overlay, a 2% agar solution was prepared by gentle heating. The agar was maintained at 55°C in a water bath until required, when the rest of the stain was warmed slightly and mixed in. Results were recorded diagrammatically and by photography (Polaroid Cu -5 Hand camera and Hood) on Polaroid 667 film with different filters for tetrazolium dyes (f16 1/60 second, f11 1/30 second) and the peptidases and acetylcholinesterase (f45 1/30 second, f32 1/125 second). Gels could be fixed overnight as required in 7% acetic acid, rinsed and dried at 37 to 50°C.

TABLE 7.3

Substrate stain systems for isoenzyme analysis
(The buffers for the substrate stains may be found in Table 7.4)

ALANINE AMINOTRANSFERASE (Glutamate pyruvate transaminase) E.C[1].2.6.1.2

188mg DL-alanine and 50mg alpha-ketoglutarate were made up to 10ml with 0.1M Tris-hydrochloric acid at pH 7.0. The pH was checked and 0.04ml L-lactic dehydrogenase (2,750 units/ml) and 5mg reduced nicotinamide adenine dinucleotide (NADH) were added just before the stain was poured onto a piece of 3MM paper laid on the gel surface. (Reduced nicotinamide adenine dinucleotide (NADH) action is destroyed by acid conditions.) The gel was assessed under ultraviolet light at 366nm and the appearance of non-fluorescent patches in the field noted.

ALKALINE PHOSPHATASE E.C.3.1.3.1

25mg beta-napthyl phosphate (sodium salt) and 60mg magnesium sulphate ($MgSO_4.7H_2O$) were homogenised in 0.06M borate pH 9.7 to give a final volume of 25ml. Just before the stain was required, 25mg Fast Blue RR salt was dissolved in 25ml 0.06M borate pH 9.7 and filtered. The borate solutions were combined and poured over the gel in a shallow tray, immersing it. Red-brown staining was observed in the presence of alkaline phosphatase.

ESTERASES

CARBOXYLIC ESTERS - Umbelliferyl derivatives - E.C.3.1.1.1.

A variety of esterases could be tested for by using different 4-methyl umbelliferyl formulations, i.e., acetate, butyrate, heptanoate and propionate.

2mg 4-Methyl-umbelliferyl derivative were dissolved in a few drops of acetone and 10ml of 0.1M phosphate buffer pH 6.5 added. The solution was made up immediately before staining and applied to 3MM paper laid on the gel. The gel was visualised frequently under ultraviolet light (366nm) for the appearance of fluorescent staining.

CHOLINE ESTER E.C.3.1.1.8.

20mg Fast Red TR Salt was dissolved in 50ml 0.2M phosphate buffer pH 7.1. 10mg Alpha-napthyl acetate was added to 1ml 50% aqueous acetone. The solutions were combined just before the gel was stained. A red-brown colouration indicated cholinesterase activity.

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[1] **Enzyme Commission identification number**

TABLE 7.3 (continued)

ACETYLCHOLINE ESTER

100mg Alpha-napthyl acetate was dissolved in 5ml aqueous acetone and 5ml distilled water added. 5 to 6ml of the solution was added to 100mg Fast Blue RR Salt dissolved in 38ml distilled water and the whole solution filtered and poured over the gel. Brown bands appeared where the enzyme was present.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE E.C.1.1.1.49.

10mg Glucose-6-phosphate, 5mg nicotinamide adenine dinucleotide phosphate, 7.5mg MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and 5mg 0.2M magnesium chloride ($MgCl_2$) were made up to 25ml with 0.2M Tris-hydrochloric acid pH 8 and homogenised. 25ml 2% Molten agar and approximately 0.5mg phenazine methosulphate were mixed into the stain before pouring over the cut gel surface. Once the agar had set, the gel was incubated at 37°C in the dark and frequently withdrawn to check for enzyme action which was shown by a blue-black colour.

GLUCOSE PHOSPHATE ISOMERASE E.C.5.3.1.9.

16mg Fructose-6-phosphate, 4mg nicotinamide adenine dinucleotide phosphate and 4mg MTT were made up to 20ml with 0.03M Tris-hydrochloric acid pH 8. Immediately before staining 20ml molten 2% agar, 0.5mg phenazine methosulphate and 10µl glucose-6-phosphate dehydrogenase (140 units/ml) were mixed in and the stain applied to the cut gel surface. After setting the gel was incubated at 37°C in the dark and checked at intervals for enzyme activity indicated by blue-black colouration.

GLUTAMATE DEHYDROGENASE

0.3mg Sodium glutamate, 5mg nicotinamide adenine dinucleotide and 5mg MTT were made up to 30ml with 0.1M phosphate buffer pH 7.4 and homogenised. Just before staining 30ml 2% agar and 0.5mg phenazine methosulphate were added. Glutamate dehydrogenase action was indicated by a blue-black colouration.

GLUTAMATE-OXALOACETATE TRANSAMINASE E.C.2.6.1.1.

66.7mg L-aspartate and 37mg alpha-ketoglutarate were made up to 10ml with 0.1M Tris-hydrochloric acid pH 8. By titration with potassium hydroxide the pH was maintained at 8 (since the action of reduced nicotinamide adenine dinucleotide is impaired by acid conditions). Immediately preceding staining, 5mg reduced nicotinamide adenine dinucleotide and 10µl malic dehydrogenase (6,000 units/ml)

were added and the solution homogenised. 17.5ml 2% Agar was added and the stain poured onto a piece of 3MM paper applied to the cut gel surface. At regular intervals the gel was examined under ultra-violet light (366nm) for dark bands in a fluorescent field.

ISOCITRATE DEHYDROGENASE E.C.1.1.1.42.

20mg Trisodium isocitrate, 5mg MTT, 5mg nicotinamide adenine dinucleotide phosphate and 3ml 1M magnesium chloride (4.06mg $MgCl_2$ in 20ml distilled water) were made up to 25ml with 0.2M Tris-hydrochloric acid pH 8. The solution was homogenised. 25ml 2% Molten agar and 0.5mg phenazine methosulphate were added and the gel stained directly. A blue-black colouration indicated the presence of the enzyme.

MALATE DEHYDROGENASE E.C.1.1.1.37.

2ml 1M Sodium-1-malate pH 7, 5mg nicotinamide adenine dinucleotide and 5mg MTT were made up to 20ml with 0.1M tris-hydrochloric acid pH 8. The solution was homogenised. Prior to staining 20ml 2% agar and 0.5mg phenazine methosulphate were added and the stain applied to the cut gel surface. Malate dehydrogenase activity was indicated by a blue-black colour.

MALIC ENZYME E.C.1.1.1.40.

0.5ml Sodium-L-malate, 5mg nicotinamide adenine dinucleotide phosphate, 5mg MTT and 0.5ml 1M magnesium chloride (4.06g $MgCl_2$ in 20ml distilled water) were made up to 25ml with 0.1M tris-hydrochloric acid. The solution was homogenised and immediately prior to staining 0.5mg phenazine methosulphate and 25ml 2% agar was mixed in. A blue-black colouration indicated sites of enzyme action.

MANNOSE PHOSPHATE ISOMERASE E.C.5.3.1.8.

8mg Mannose-6-phosphate, 25mg adenosine 5'triphosphate, 3mg nicotinamide adenine dinucleotide, 5mg MTT and 0.2ml 1M magnesium chloride (4.06g $MgCl_2$ in 20ml distilled water) were homogenised with 3ml 0.6M tris-hydrochloric acid and 10ml distilled water. Immediately before staining the following reagents were added 8µl glucose phosphate isomerase, 40µl glucose-6-phosphate dehydrogenase (140 units/ml), 0.5mg phenazine methosulphate and 20ml 2% agar and the stain applied. Mannose phosphate isomerase was indicated by a blue-black colour.

PEPTIDASES E.C.3.4.11/13.

A variety of peptidases could be tested for by altering the oligopeptide substrate. 14mg Peptide, 3.5ml o-dianisidine, 4mg snake venom (0.6 units) and 3mg peroxidase (175 units) were made up to 25ml with 0.05M phosphate-hydrochloric acid buffer pH 7.5. In some cases homogenisation was found to be necessary for dissolution. Before application to the cut gel 0.35ml manganese chloride ($MnCl_2$) and 25ml 2% agar was added. High background colouration occurred rapidly if the 0.1M $MnCl_2$ was added too early. Enzyme action produced red staining.

PHOSPHOGLUCOCOMUTASE E.C.2.7.5.1.

40mg Glucose-1-phosphate, 4mg nicotinamide adenine dinucleotide phosphate, 6mg MTT and 0.5ml 1M magnesium chloride (4.06g $MgCl_2$ in 20ml distilled water) were made up to 20ml with 0.025M tris-hydrochloric acid pH 8. Directly before staining, 0.5mg phenazine methosulphate, 10µl glucose-6-phosphate dehydrogenase (140 units/ml) and 20ml 2% agar were added. The solution was swirled to mix and poured onto the cut gel. Blue-black colouration indicated phosphoglucocomutase action.

6-PHOSPHOGLUCONATE DEHYDROGENASE E.C.1.1.1.44.

20mg 6-Phosphogluconate, 5mg nicotinamide adenine dinucleotide phosphate, 5mg MTT and 1ml 1.0M magnesium chloride (4.06g $MgCl_2$ in 20ml distilled water) were made up to 20ml with 0.25M tris-hydrochloric acid pH 8. 20ml 2% Agar and 0.5mg phenazine methosulphate were added to the solution when the cut gel was prepared, the stain was poured and the gel incubated in the dark at 37°C. The gel was frequently examined for the appearance of blue-black bands indicating enzyme action.

SUPEROXIDE DISMUTASE E.C.1.15.1.1.

To 25ml 0.05M tris-hydrochloric acid pH 8.0 was added 5mg MTT, 0.5mg phenazine methosulphate and 25ml 2% agar. The solution was applied to the gel and allowed to darken in the presence of light and then incubated at 37°C, with time paler/yellow areas appeared due to enzyme action.

TABLE 7.3 (continued)

THREONINE DEHYDROGENASE E.C.1.1.1.129

0.3g L-threonine, 10mg nicotinamide adenine dinucleotide, 5mg MTT and 3ml 1M potassium chloride (KCl) were made up to 20ml with 0.15M Tris-hydrochloric acid pH 8 and homogenised. Before applying the stain, 0.5mg phenazine methosulphate and 20ml 2% agar were mixed in. Enzyme presence was demonstrated by a blue-black staining.

TABLE 7.4

Buffers for enzyme substrate stains

0.06M Borate pH 9.7

0.371g Boric acid was made up to 90ml with distilled water, the pH titrated to 9.7 with sodium hydroxide and the solution made up to 100ml.

0.025M Tris-HCl pH 8.0

0.30g Trizma was made up to 90ml with distilled water, the pH titrated to 8.0 with concentrated hydrochloric acid and the solution made up to 100ml.

0.03M Tris-HCl pH 8.0

0.36g Trizma was made up to 90ml with distilled water, the pH titrated to 8.0 with concentrated hydrochloric acid and the solution made up to 100ml.

0.05M Tris-HCl pH 8.0

0.60g Trizma was made up to 90ml with distilled water, the pH titrated to 8.0 with concentrated hydrochloric acid and the solution made up to 100ml.

0.1M Tris-HCl pH 7.0 (pH 8.0)

1.20g Trizma was made up to 90ml with distilled water, the pH titrated to 7.0 (pH8.0) with concentrated hydrochloric acid and the solution made up to 100ml.

0.15M Tris-HCl pH 8.0

1.80g Trizma was made up to 90ml with distilled water, the pH titrated to 8.0 with concentrated hydrochloric acid and the solution made up to 100ml.

0.2M Tris-HCl pH 8.0

2.40g Trizma was made up to 90ml with distilled water, the pH titrated to 8.0 with concentrated hydrochloric acid and the solution made up to 100ml.

0.25M Tris-HCl pH 8.0

3.0g Trizma was made up to 90ml with distilled water, the pH titrated to 8.0 with concentrated hydrochloric acid and the solution made up to 100ml.

TABLE 7.4 (continued)

0.5M Tris-HCl pH 7.5

6.0g Trizma was made up to 90ml with distilled water, the pH titrated to 7.5 with concentrated hydrochloric acid and the solution made up to 100ml.

0.6M Tris-HCl pH 7.4

7.20g Trizma was made up to 90ml with distilled water, the pH titrated to 7.4 with concentrated hydrochloric acid and the solution made up to 100ml.

0.05M Phosphate Buffer pH 7.5

0.705g Disodium hydrogen phosphate was made up to 90ml with distilled water, the pH titrated to 7.5 with concentrated hydrochloric acid and the solution made up to 100ml.

0.1M Phosphate pH 7.0 (pH 7.4)

1.41g Disodium hydrogen phosphate was made up to 90ml with distilled water, the pH titrated to 7.0 (pH 7.4) with concentrated hydrochloric acid and the solutio made up to 100ml.

0.2M Phosphate Buffer pH 7.1

2.82g Disodium hydrogen phosphate was made up to 90ml with distilled water, the pH titrated to pH 7.1 with concentrated hydrochloric acid and the solution made up to 100ml.

7.3 RESULTS

7.3.1 Alanine aminotransferase

In the case of the alanine aminotransferase substrate system, the results were inconsistent and difficult to interpret due to the rapid loss of fluorescence throughout the gel, i.e., high background reaction or poor initial fluorescence. Generally, results for an enzyme only varied slightly between the buffers tested, but repeatability was poor with variation in the number of bands as well as their position and resolution. For these reasons the system was not favoured for inclusion in the screen, although Tris phosphate buffer pH 7 was found to give the best results for *O.ostertagi* with three anodal bands (2.5, 5.5 and 7.5cm) and staining at the slot, while *O.circumcincta* had a single anodal band at 7cm and *H.contortus* showed slot staining and a cathodal band at 2cm.

7.3.2 Alkaline phosphatase

Neither adult nor L3 stage *O.ostertagi* demonstrated presence of alkaline phosphatase either in the homogenate supernatant or its resuspended pellet with Tris citrate buffer pH 7 or pH 8.6, Tris phosphate buffer pH 7 or Tris potassium phosphate buffer pH 9.3. No enzyme activity was seen with *O.ostertagi*, *O.circumcincta* and *H.contortus*, however, *Cooperia oncophora* showed a single band 1cm anodal to the loading slot. Even when sample concentration was increased by a factor of ten, absence of bands was the finding common to all the *O.ostertagi* isolates examined, e.g., Glasgow, Morantel Resistant Lelystad, Morantel Susceptible Lelystad, Weybridge and Weybridge-Glasgow.

7.3.3 Esterases

Esterases present in nematodes hydrolyse the esters of short chain fatty acids and may be subdivided into non-specific esters, e.g., carboxylic esters and choline esters, e.g., acetylcholinesterase, cholinesterase (Barrett, 1981).

7.3.3.1 Carboxylic esters

Four, 4-methyl umbelliferyl esters, carboxylic ester substrates allowed the testing of four esterases: acetate, butyrate, heptanoate and propionate.

1) Acetate

The supernatant of adult homogenised *O.ostertagi* produced a strong band at 1.0cm anodal to the slot by 15 minutes incubation using Tris citrate buffer pH 7.0 and by 2 hours there were further faint bands at 0.5cm and 3.0cm. Bands with similar migration characteristics were seen with some of the other esterase profiles, i.e., bands were seen at 1.0cm with butyrate, heptanoate and propionate and at 3.0cm with butyrate.

2) Butyrate

Results for butyrate esterase were seldom consistent and the bands rarely discrete, however, bands were occasionally seen with adult *O.ostertagi* homogenates at 1.0 and 3.0cm (Tris citrate buffer pH 7).

3) Heptanoate

Anodal bands were seen with all four buffers, Tris phosphate buffer pH 7, Tris potassium phosphate buffer pH 9.3 and Tris citrate buffers pH 7 or pH 8.6 for adult *O.ostertagi*. The common band position anodally at 1.0cm with Tris citrate buffer pH 7.0 was consistent, but slow to develop. Tris potassium phosphate buffer pH 9.3 gave rapid staining and good separation with anodal protein movement to 7.0cm.

4) Propionate

The Tris citrate buffer system pH 8.6 was found to give the clearest results with multiple bands: 2.0, 4.0 and 8.0cm anodal to the slot for *O.ostertagi* L3 from Glasgow and at 2.0 and 4.0cm for Morantel Resistant Lelystad, while *H.contortus* L3 produced a single band at 7.0cm. Adult *O.ostertagi* exhibited banding at the common esterase position of 1.0cm with Tris citrate buffer pH 7.0.

7.3.3.2 Choline esters

Assay for cholinesterase in adult and L3 stages of *O.ostertagi* and *O.circumcincta*, and L3 of *C.oncophora* was made on two occasions employing all four standard buffer systems. Apart from a small amount of stain at the slot, on one occasion with Tris phosphate buffer pH 7 a smudgy anodal triple band pattern was seen with adult *O.ostertagi* (1.8, 2.2 and 3.0cm) and *C.oncophora* had two bands (2.0 and 3.5cm).

7.3.3.3 Acetylcholine esters (Figure 7.3)

Initially acetylcholinesterase was tested for by starch gel electrophoresis, but the sole result was smearing arising from the slot and rapid development of background colouration unless the gel was totally immersed in stain.

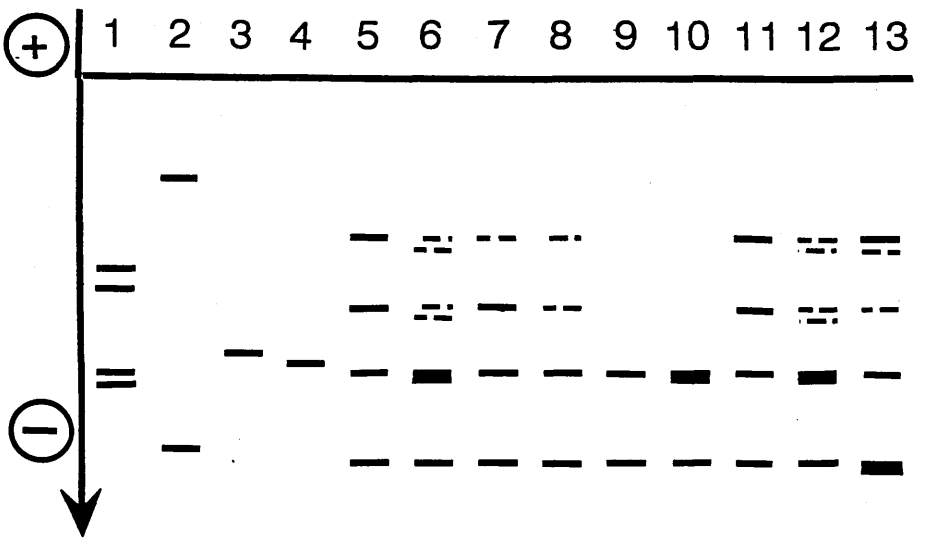
Sutherland (1987) had successfully used the stain for isoelectric focusing, so the samples were re-assessed using isoelectric focusing.

The highest loading position with 0.76ml ampholine pH 3.5 to 10.0 gave the best spread of bands, e.g., for *O.ostertagi* 1.2, 2.0, 2.5, 3.0 and 3.7cm and faintly at 4.4cm cathodal to the loading site. Different species showed significant variation in position and intensity of acetylcholinesterase migration patterns (Figure 7.3).

The results were highly repeatable and strong similarities were seen when isolates were compared. However, differences in the type of banding were observed between the isolates with respect to intensity and nature, i.e., single or doublet (Figure 7.3). On some occasions bands were also seen anodally, these were similar for strains from Glasgow, Morantel Susceptible, Lelystad, Stormont, Weybridge and Weybridge-Glasgow, although extra distinct bands were seen from the Glasgow sample.

FIGURE 7.3

Acetylcholinesterase enzyme patterns by isoelectric focusing: Comparison of trichostrongyle species and isolates of Ostertagia ostertagi.



High load, Ampholines pH 3.5 to 10.

Track	Species
1	Cooperia oncophora
2	Haemonchus contortus
3	Ostertagia circumcincta
4	Trichostrongylus colubriformis
5 to 13	Ostertagia ostertagi

Track	Isolate
5	Alabama
6	Glasgow
7	Resistant Lelystad
8	Lelystad
9	Louisiana
10	New Jersey
11	Wageningen
12	Weybridge
13	Weybridge-Glasgow

7.3.4 Glucose-6-phosphate dehydrogenase (Figure 7.4)

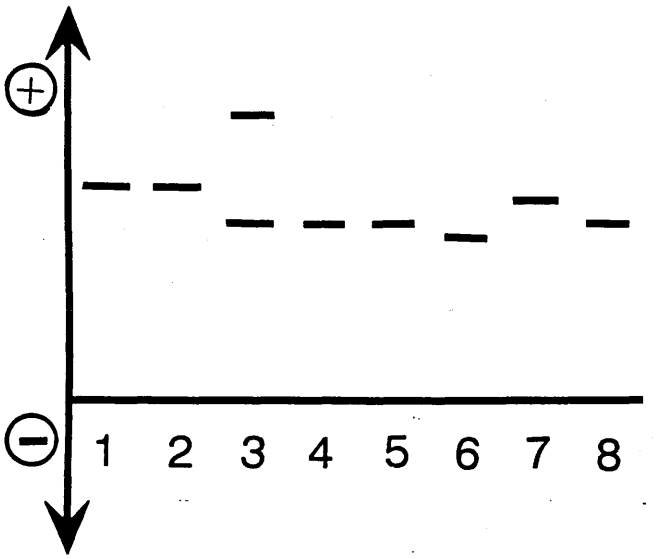
When L3 and adult stages of *O.ostertagi* were tested for glucose-6-phosphate dehydrogenase, optimal resolution was obtained with the Tris phosphate buffer, pH 7; both stages had a band at 3.0cm anodally. *C.oncophora* L3 exhibited two bands at 0.5 and 4.5cm, while *O.circumcincta* L3 showed a very similar pattern to that of *O.ostertagi*. Comparison of different isolates of *O.ostertagi* L3 showed low variation, although enzyme mobility was low and consequently band separation was poor and the staining was badly defined. On a separate occasion, L3 banding for comparison of isolates of *O.ostertagi* was as follows: Alabama, 3.0cm; Denmark, 3.0cm; Glasgow, 2.5 and 4.0cm; Morantel Susceptible Lelystad, 2.5cm; Morantel Resistant Lelystad, 2.5cm; Louisiana, 2.3cm; New Jersey, 2.7cm; Weybridge, 2.5cm (Figure 7.4). The more anodal band found in the Glasgow isolate was inconsistently present. Similar results were achieved on isoelectric focusing, when the optimal conditions were achieved with 0.76ml ampholine pH 3.5 to 10 and the low loading position, though there appeared to be a slight decrease in protein mobility of the *O.circumcincta* enzyme relative to the panel of *O.ostertagi* strains examined and the bands were better focused.

7.3.5 Glucose phosphate isomerase (Figure 7.5 a and b)

Consistent high intensity staining was rapidly obtained when testing for the presence of glucose phosphate isomerase in the trichostrongyles. Gels buffered with Tris citrate pH 7.0 and Tris phosphate pH 7.0 gave the best results. Both adult and L3 samples of *O.ostertagi* (Glasgow stock) gave a common configuration of two anodal bands at 8.0 and 8.5cm with Tris citrate buffer pH 7.0. With Tris phosphate buffer pH 7.0 the glucose phosphate isomerase of adult *O.circumcincta* showed lower glucose phosphate isomerase mobility banding at 1.0 and 1.5cm compared to *O.ostertagi* at 6.0 and 6.5cm. Comparison of *O.ostertagi* isolates on 1 in 20 Tris phosphate gels was found to give diffuse ill-defined bands, so the enzyme was re-assessed on 1 in 10 gels, which would reduce rate of migration and so lead to sharper bands. With the more concentrated gel buffer the mobility of the enzyme in different species was: *C.oncophora* 3.5cm,

FIGURE 7.4

Glucose-6-phosphate dehydrogenase enzyme patterns by starch gel electrophoresis: Comparison of isolates of Ostertagia ostertagi.



Tris phosphate pH 7.0

Track	Isolate
1	Alabama
2	Denmark
3	Glasgow
4	Resistant Lelystad
5	Lelystad
6	Louisiana
7	New Jersey
8	Weybridge

O.circumcincta 4.3cm and *O.ostertagi* 4.0 and 6.0cm (Figure 7.5a). Some variation in band position did occur on comparison of isolates (Figure 7.5a), banding was evident for Morantel Susceptible Lelystad, Morantel Resistant Lelystad, Louisiana and New Jersey at 5.0cm anodally; the Glasgow, Louisiana and Wageningen isolates had bands at 4.0cm and the Glasgow and Wageningen isolates had a further band at 6.0cm. Different single bands of activity were seen at 4.5cm and 4.8cm for the Alabama and Weybridge isolates respectively. Three common band mobilities were observed. Repeated runs indicated that certain isolates had two bands: Glasgow, Louisiana and Wageningen isolates, while others had only one, relatively anodal band: Alabama, Morantel Susceptible Lelystad, Morantel Resistant Lelystad, New Jersey and Weybridge. Figure 7.5b shows a gel stained for glucose phosphate isomerase activity, showing strong similarities between isolates.

Initially, additional smearing was noticed adjacent to the slot, which was thought to be the result of bacterial contaminants released from the nematode gut on homogenisation. To determine whether the levels of bacteria in the gut contents might contribute significantly to the results, homogenates of entire L3 and exsheathed larvae were applied to bacterial culture plates (sheep blood and MacConkey agar). The cultures were incubated aerobically and anaerobically, but no bacterial colonies were observed. Due to their size, bacteria would be more likely to be found in the pellet at homogenisation, but electrophoresis of an extract of the resuspended pellet did not produce the additional bands.

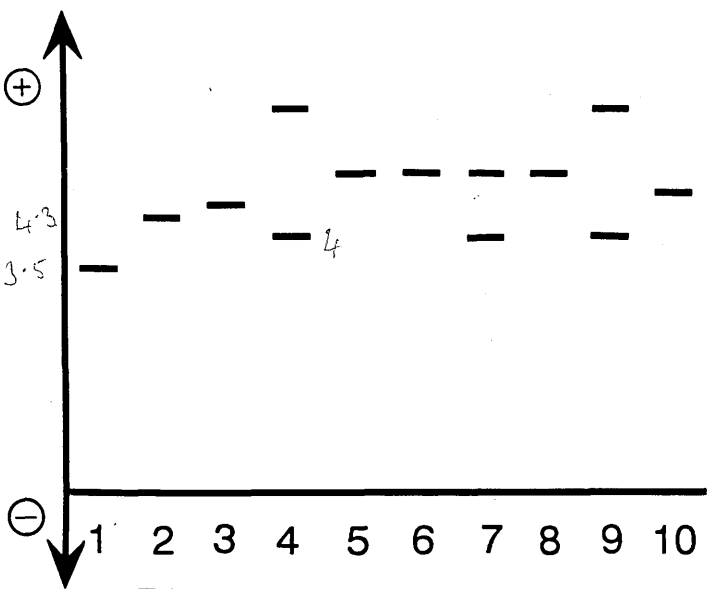
Glucose phosphate isomerase was assayed by isoelectric focusing, with 0.78ml ampholines, pH 3.5 to 10. The low loading position was best, giving a two-banded configuration for the Glasgow L3 stock as in starch gel electrophoresis, but the reproducibility of the results was poor and the activity of the enzyme was lower by this method.

7.3.6 Glutamate dehydrogenase

Glutamate dehydrogenase exhibited two bands of enzyme activity on Tris potassium phosphate pH 9.3 and Tris phosphate pH 7.0 buffers.

FIGURE 7.5 (a)

Glucose phosphate isomerase enzyme patterns by starch gel electrophoresis: Comparison of trichostrongyle species and isolates of Ostertagia ostertagi.

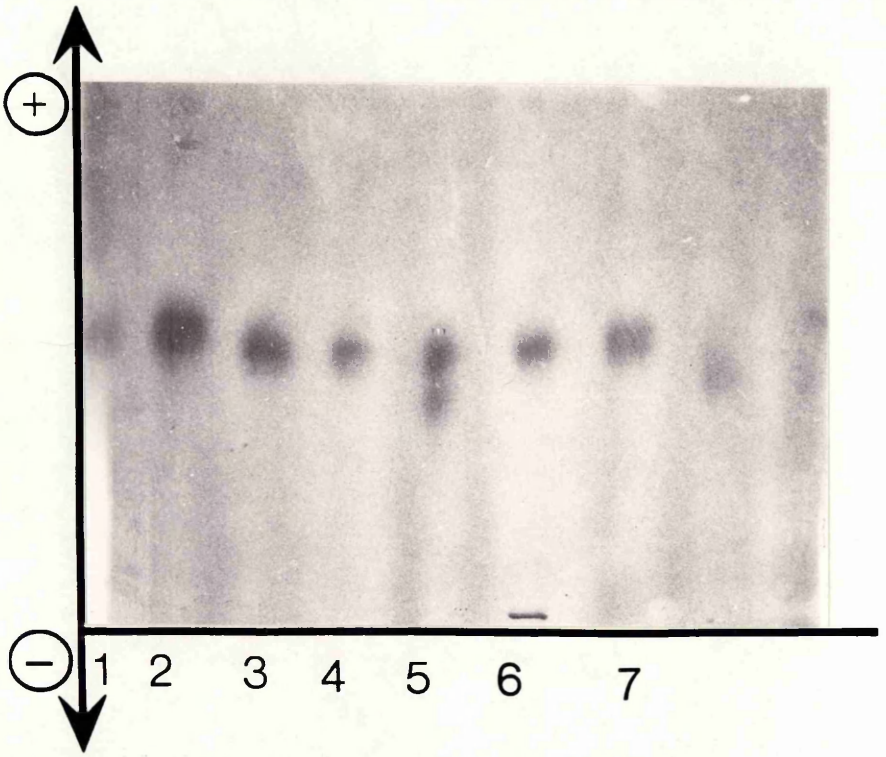


Track	Species
1	Cooperia oncophora
2	Ostertagia circumcincta
3 to 10	Ostertagia ostertagi

Track	Isolate
3	Alabama
4	Glasgow
5	Resistant Lelystad
6	Lelystad
7	Louisiana
8	New Jersey
9	Wageningen
10	Weybridge

FIGURE 7.5 (b)

Glucose phosphate isomerase enzyme patterns by starch gel electrophoresis: Comparison of isolates of Ostertagia ostertagi



Track	Isolate of Ostertagia ostertagi
1	Denmark
2	Glasgow
3	Lelystad Resistant
4	Lelystad Susceptible
5	Louisiana
6	New Jersey
7	Weybridge

The results were clearer with Tris potassium phosphate with anodal bands at 2.5 and 4.5cm for adult *O.ostertagi*, while no activity was detected with L3 samples. A possible reason for this lack of activity could be a lower production of this enzyme in the L3 than the adult, when compared with other enzymes. Later work confirmed the low enzyme activity in the L3; leaving the gel to incubate overnight (rather than the usual limit of 2 hours) revealed a band at 4.5cm in L3 of the Weybridge isolate; *O.circumcincta* L3s had a band at 4.3cm and *C.oncophora* at 4.1cm. The low level of glutamate dehydrogenase activity in L3s meant that staining was not observed for all the isolates of *O.ostertagi* although Wageningen (2cm) and Glasgow (2.25cm) gave results. The inconsistency of enzyme activity was too great to allow comparative work and it was decided to re-assess glutamate dehydrogenase by isoelectric focusing.

Repeated attempts to use the enzyme system with isoelectric focusing encountered similar difficulties, bands were present infrequently. The middle loading position when the ampholines were 0.36ml pH 3.5 to 10 and 0.36ml pH 4 to 6 gave the best results. Focused bands appeared anodally for *O.ostertagi* at 1.5 and 3.0 cm in stocks of adults and L3s from Glasgow and L3s of the Morantel Resistant Lelystad sample.

7.3.7 Glutamate oxaloacetate transaminase

All buffer systems produced bands with adult *O.ostertagi* material, but the highest activity was seen with Tris citrate buffer pH 8.6 with an anodal band at 2cm. The results were repeatable and similar for the L3 stage. L3 of *O.circumcincta* and *C.oncophora* showed greater mobility, 3.5 and 5.5cm, respectively. Results for the enzyme were difficult to interpret since they required visualisation with ultra-violet light and staining spread so rapidly that they required frequent assessment.

7.3.8 Isocitrate dehydrogenase (Figure 7.6)

Isocitrate dehydrogenase consistently gave a well-defined anodal three-banded pattern for the adult and the L3 stage of *O.ostertagi* with all four buffers used, although the best resolution with optimal

separation was obtained with the Tris citrate buffer pH 7.0. The outer bands were of higher activity. In some runs a further band was seen close to the slot cathodally.

Comparing L3 of different species on Tris citrate buffer pH 7.0, the band of lowest mobility of the triple band migrated to 7.5cm for *O.ostertagi*, while with *O.circumcincta* the triple band began at 8.0cm. In contrast, *C.oncophora* produced a six band pattern from 6.0cm, the most anodal bands apparently of similar mobility to those of *O.ostertagi* (Figure 7.6). *H.contortus* was seen to stain as a single band of lower mobility relative to *O.ostertagi*.

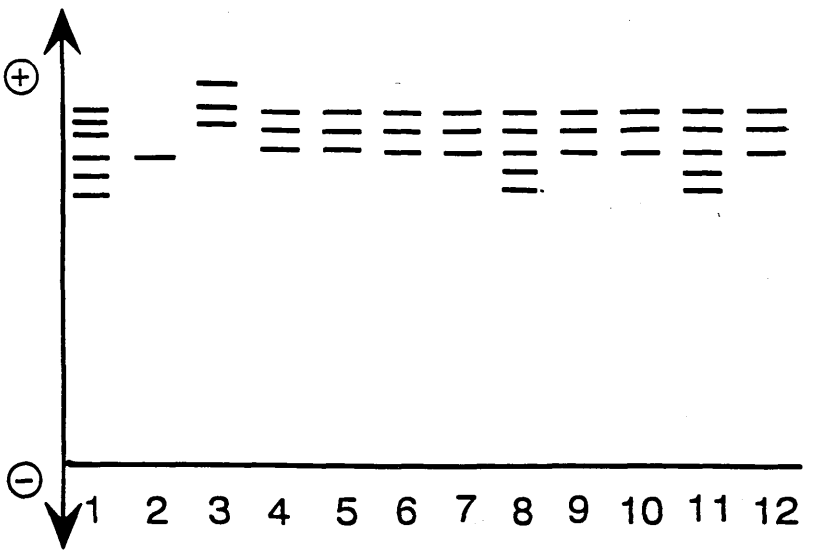
Generally, comparison between isolates demonstrated uniformity, e.g., a three-banded pattern was produced with stocks from Glasgow, Morantel Susceptible Lelystad, Morantel Resistant Lelystad, Stormont, Weybridge and Weybridge-Glasgow, however, Louisiana and Wageningen material had a five-banded pattern with the most anodal bands coincident with those of the triple in the other strains. The results were consistent on repeated experimental runs. On cryopreserving whole L3 or freezing aliquots of homogenate a marked loss of activity was noted relative to freshly prepared homogenates. Isoelectric focusing with 0.76ml ampholines pH 3.5 to 10 gave a triple band pattern at the high loading position, however, the banding was poorly separated from the slot and less discrete than that obtained on starch gel electrophoresis.

7.3.9 Malate dehydrogenase (Figure 7.7)

On Tris citrate buffer pH 7.0 adult *O.ostertagi* gave a single anodal band of low mobility at 1.0cm and a clearer cathodal band at 1.0cm, similar results were found with Tris citrate buffer pH 8.6, Tris phosphate buffer, pH 7.0 and Tris potassium phosphate buffer pH 9.3. Tris phosphate buffer gave the best resolution for species comparison, anodally *O.ostertagi* showed a band of activity at 1.5cm, while the *O.circumcincta* enzyme migrated to 2cm and *H.contortus* enzyme was the most mobile showing a band of activity at 3cm.

FIGURE 7.6

Isocitrate dehydrogenase enzyme patterns by starch gel electrophoresis: Comparison of trichostrongyle species and isolates of Ostertagia ostertagi.



Tris citrate buffer pH 7.0

Track	Species/Isolate
1	Cooperia oncophora
2	Haemonchus contortus
3	Ostertagia circumcincta
4 to 12	Ostertagia ostertagi
4	Alabama
5	Glasgow
6	Resistant Lelystad

Track	Isolate
7	Lelystad
8	Louisiana
9	New Jersey
10	Stormont
11	Wageningen
12	Weybridge

Comparing isolates of *O.ostertagi* of the L3 stage on Tris citrate buffer pH 8.6 indicated a common anodal band at 1.5cm for Glasgow, Weybridge and Weybridge-Glasgow, but the Weybridge and Weybridge-Glasgow isolates had a further band at 6cm. Later experiments frequently showed two bands in the Glasgow strain too. When several isolates were run simultaneously two main bands were identified anodally at 0.3cm in isolates from Alabama, Glasgow, Morantel Susceptible Lelystad, Morantel Resistant Lelystad, Louisiana, New Jersey, Weybridge and Weybridge-Glasgow and at 2.5cm in the Glasgow, Louisiana and Wageningen isolates (Figure 7.7). Alabama alone showed a band of activity at 1.5cm, with a strong anodal smear. This pattern was repeatable and samples of *C.oncophora*, *O.circumcincta* and *T.colubriformis* run alongside showed bands of activity at 1.25, 0.75 and 1.0cm respectively. The poor mobility of the protein under test made identification of minor changes in migration difficult.

Sample storage had a marked effect on enzyme activity: cryopreserved L3 and frozen aliquots of homogenates gave poor staining relative to freshly homogenised material. Certain bands seemed to be more adversely affected, e.g., the cathodal band and the most mobile anodal band.

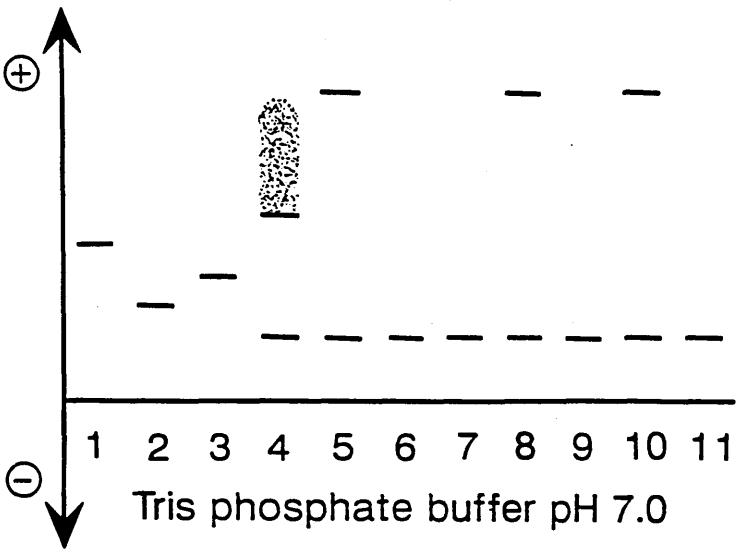
Latterly, isoelectric focusing was undertaken with 0.76ml ampholine pH 3.5 to 10 and staining occurred within 15 minutes. The low load position gave the optimal resolution with small differences in mobility detectable between all species tested: *C.oncophora*, *H.contortus*, *O.circumcincta*, *O.ostertagi* and *T.colubriformis*.

7.3.10 Malic enzyme (Figure 7.8 a,b and c)

Initially attempts were made to assay for malic enzyme by starch gel electrophoresis, but faint bands were seen anodally at 3cm only when adult samples of *O.ostertagi* were run with Tris citrate buffer pH 7.0. Frequently no enzyme activity was observed. Consequently, isoelectric focusing was investigated using 0.76ml ampholines pH 3.5 to 10; the lowest loading position was found to give optimal results, although the staining was still faint. Activity was higher when the

FIGURE 7.7

Malate dehydrogenase enzyme patterns by starch gel electrophoresis: Comparison of certain trichostrongyle species and isolates of Ostertagia ostertagi.



Track	Species
1	Cooperia oncophora
2	Ostertagia circumcincta
3	Trichostrongylus colubriformis
4 to 11	Ostertagia ostertagi

Track	Isolate
4	Alabama
5	Glasgow
6	Resistant Lelystad
7	Lelystad
8	Louisiana
9	New Jersey
10	Wageningen
11	Weybridge

sample concentration was doubled, but the bands were still poorly defined. However, differences between the species tested at the L3 stage were apparent (Figure 7.8a) with a sharp and a broad band of activity present in the majority of the nematodes. Taking the fainter band the mobilities in decreasing order were as follows: *C.oncophora*, *H.contortus*, *T.colubriformis*, *O.circumcincta* and *O.ostertagi*. (Figure 7.8c is the photographic record of the gel, unfortunately the finer bands are barely discernible.) Little variation was noted between the isolates from Glasgow, Morantel Susceptible Lelystad, Stormont and Weybridge-Glasgow (3cm), however, the isolate from Weybridge showed a different mobility (2.7cm) (only the broad band was visualised on this occasion) (Figure 7.8b).

7.3.11 Mannose phosphate isomerase

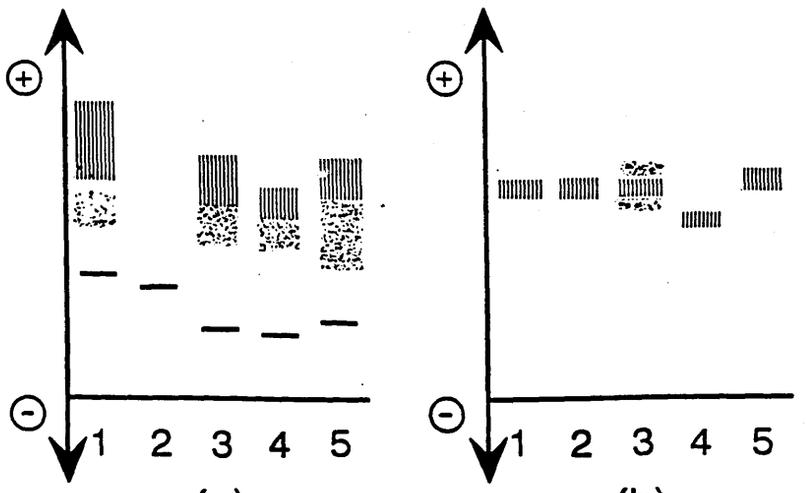
No discrete bands were found on starch gel electrophoresis for mannose phosphate isomerase regardless of the buffer system tried (Tris citrate pH 7.0 or pH 8.6, Tris phosphate pH 7.0, Tris potassium phosphate pH 9.3). Consequently, the enzyme was tested for by isoelectric focusing, which gave optimal results with 0.38ml of each of two ampholines (pH 3.5 to 10 and pH 4 to 6) and the middle loading position. A band of enzyme activity appeared at 2.5cm towards the cathode from the loading position and identical patterns were observed for both the L3 and adult. The band was common to all the isolates examined, i.e., Glasgow, Morantel Susceptible Lelystad, Morantel Resistant Lelystad, Stormont, Weybridge and Weybridge-Glasgow. Despite loading of equivalent larval numbers there appeared to be differences in the relative staining between isolates, although further experimental work would be necessary to confirm quantitative variation in protein.

7.3.12 Peptidases (Figures 7.9 and 7.10)

Poor activity was initially a problem with peptide substrate stains. A slight improvement resulted from doubling the sample concentration, i.e., to 40,000 L3 per track. However, activity was further improved by reducing the pH of the stain buffer from pH 7.5 to pH 6.5, although a further decrease to pH 5.5 did not increase the activity. A marked drop

FIGURE 7.8

Malic enzyme analysis by isoelectric focusing - separate experimental runs for the comparison of a) trichostrongyle species and b) Ostertagia ostertagi isolates.



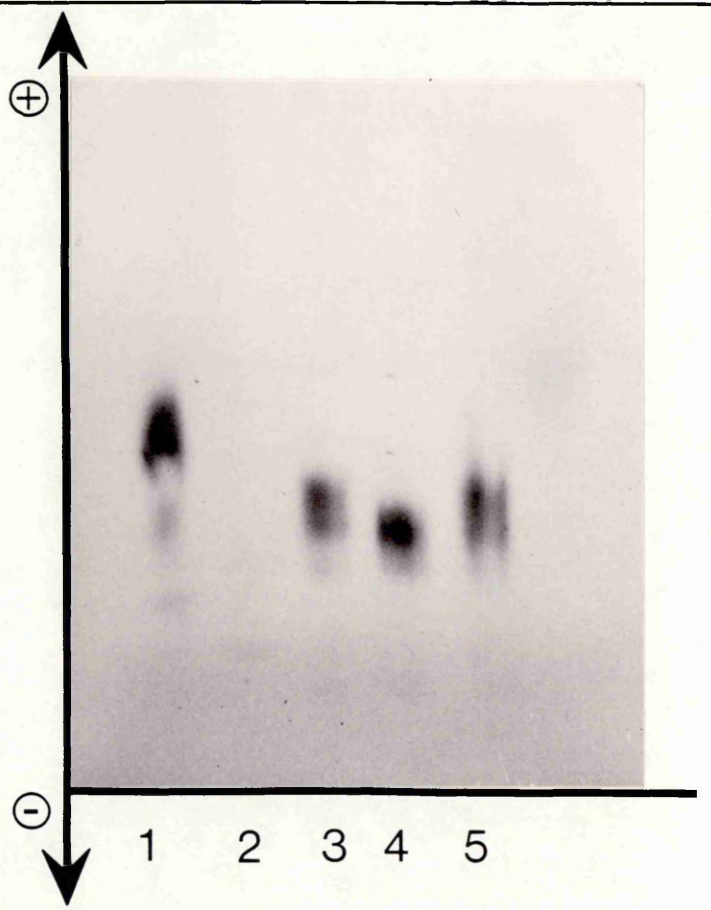
Low load, Ampholines pH 3.5 to 10.

Track	Species
1	Cooperia oncophora
2	Haemonchus contortus
3	Ostertagia circumcincta
4	Ostertagia ostertagi
5	Trichostrongylus colubriformis

Track	Isolate
1	Glasgow
2	Resistant Lelystad
3	Stormont
4	Weybridge
5	Weybridge-Glasgow

FIGURE 7.8 (c)

Malic enzyme analysis
by isoelectric focusing:
Comparison of trichostrongyle species.



Low load, Ampholines pH 3.5 to 10.
Polaroid film (f11, 1/30 second)

Track	Species
1	Cooperia oncophora
2	Haemonchus contortus
3	Ostertagia circumcincta
4	Ostertagia ostertagi
5	Trichostrongylus colubriformis

in enzyme activity was apparent when samples were cryopreserved or stored as frozen aliquots rather than freshly homogenised.

Many of the peptidases appeared to show similar activity with different peptide substrates, e.g., the following substrates showed the same band of activity at 3.5cm with Tris citrate buffer pH 8.6: L-leucyl-tyrosine, L-leucyl-L-leucine, L-leucyl-L-leucyl-L-leucine and L-alanyl-L-tyrosine. A band of activity was observed at 2cm with L-leucyl-tyrosine, L-leucyl-L-leucine, L-leucyl-L-leucyl-L-leucine, glycyl-L-leucine, L-valyl-L-leucine, glycyl-L-tyrosine and L-alanyl-L-tyrosine. The substrate tri-tyrosine showed a band of activity at 3.2cm, while the substrate L-valyl-L-alanine, showed a band of activity at 4cm. Similar patterns were seen in the adult and L3 stages, although the relative activity was generally higher in the adult.

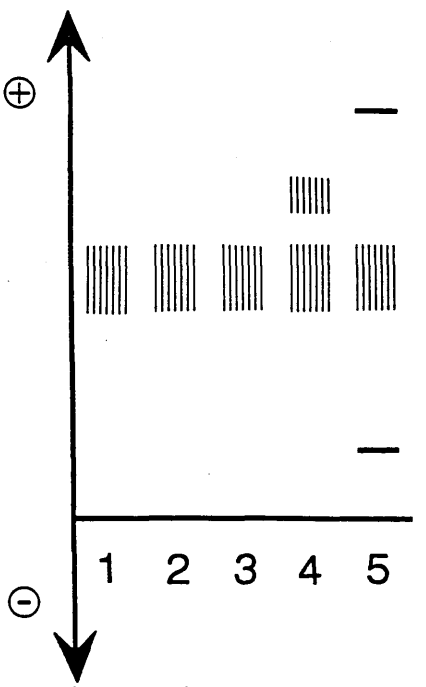
Using L-leucyl-L-leucine as a substrate similar bands were seen with extracts of *O.ostertagi* and *H.contortus*, while *O.circumcincta* had a band of lower mobility. Isolates of *O.ostertagi* frequently exhibited an invariant band, but variation occurred in the presence or absence of additional bands, e.g., L-alanyl-L-tyrosine (Figure 7.9), and glycyl-L-leucine (Figure 7.10). When L-alanyl-L-tyrosine was used as a substrate stain, activity was observed at 3 to 4cm with Tris potassium phosphate buffer with stocks of isolates from Glasgow, Weybridge-Glasgow, Weybridge, Morantel Susceptible Lelystad and Morantel Resistant Lelystad. Weybridge-Glasgow had further bands at 1 and 6cm. A band at 4.5 to 5cm was seen with the Weybridge strain. With glycyl-L-leucine, Alabama, Morantel Susceptible Lelystad, Morantel Resistant Lelystad, Wageningen and Weybridge samples showed bands at 0.5cm and 2 to 2.2cm. The Glasgow stock had multiple bands at 0.7, 2.5, 3.3 and 4.5cm in addition to the band at 2.0cm. The Alabama isolate had further bands at 1.0 and 1.7cm.

7.3.13 Phosphoglucocomutase (Figure 7.11)

Initially, phosphoglucocomutase activity was examined by starch gel electrophoresis. Work to identify the optimal electrophoretic conditions indicated that Tris phosphate buffer pH 7 or Tris citrate buffer pH 7 were the best buffer systems. Although there was some

FIGURE 7.9

Peptidase - L-alanyl-L-tyrosine enzyme patterns by starch gel electrophoresis: Comparison of isolates of Ostertagia ostertagi.

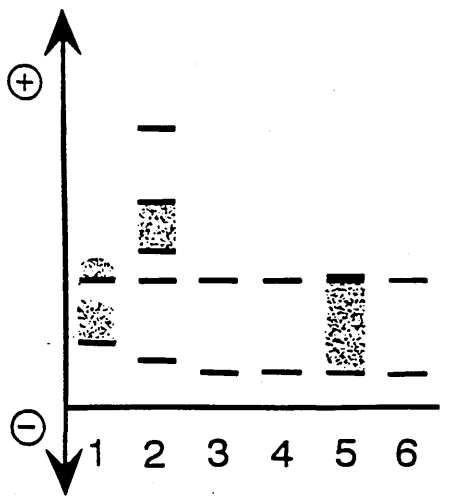


Tris potassium phosphate buffer pH 9.3

Track	Isolate
1	Glasgow
2	Resistant Lelystad
3	Lelystad
4	Weybridge
5	Weybridge-Glasgow

FIGURE 7.10

Peptidase - Glycyl-L-leucine
enzyme patterns by starch gel electrophoresis:
Comparison of isolates of Ostertagia ostertagi.



Tris citrate buffer pH 8.6

Track	Isolate
1	Alabama
2	Glasgow
3	Resistant Lelystad
4	Lelystad
5	Wageningen
6	Weybridge

variability in whether activity was detected, the banding patterns were consistent between runs. Two bands were seen anodally at approximately 3.5 and 5cm (Glasgow, Morantel Susceptible Lelystad, Morantel Resistant Lelystad and Stormont). However, depending on the isolate under examination the more actively staining band varied in mobility, i.e., high mobility band in the Stormont isolate, lower mobility in the Glasgow isolate. The Weybridge sample showed the high mobility band alone, while the material from Alabama showed three bands. Cryopreserving and frozen sample aliquots gave significantly poorer results compared to freshly prepared material.

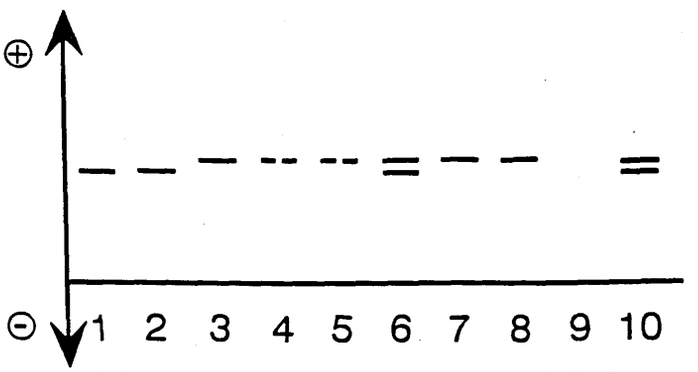
Latterly samples were examined by isoelectric focusing (pH 3.5 to 10 - Figure 7.11). The mid-load position gave the best resolution. A band of activity at 2cm towards the anode was detected with strains from Alabama, Glasgow, Morantel Susceptible Lelystad, Morantel Resistant Lelystad, Louisiana, New Jersey and Weybridge, while the Morantel Resistant Lelystad and Weybridge strains showed a second band at 1.8cm. *C.oncophora* and *T.colubriformis* also showed a band of activity at 1.8cm.

7.3.14 6-Phosphogluconate dehydrogenase

With starch gel electrophoresis, a band of activity, which had migrated little distance from the slot was observed, especially with the L3 samples. Smeary staining was most obvious from the low loading position with isoelectric focusing (0.36ml ampholine pH 3.5 to 10 and 0.36ml ampholine pH 4 to 6). Slight differences in the mobility of the enzyme were seen on isolate comparison with decreasing mobility as follows: Glasgow, Morantel Susceptible Lelystad and Weybridge-Glasgow and finally Morantel Resistant Lelystad. The protease inhibitors in the homogenisation buffer tended to cause electroendosmosis. In electroendosmosis, the presence of ionised groups affect the supporting matrix leading to the migration of buffer ions and the relative development of charge between water molecules; large amounts of liquid collected on the gel surface leading to eventual short circuit and loss of current.

FIGURE 7.11

Phosphoglucocomutase enzyme patterns by isoelectric focusing: Comparison of trichostrongyle species and isolates of Ostertagia ostertagi.



Mid-load, Ampholines pH 3.5 to 10.

Track	Species/Isolate
1	Cooperia oncophora
2	Trichostrongylus colubriformis
3 to 10	Ostertagia ostertagi
3	Alabama
4	Glasgow
5	Resistant Lelystad
6	Lelystad
7	Louisiana
8	New Jersey
9	Wageningen
10	Weybridge

7.3.15 Superoxide dismutase

Diffuse non-specific loss of staining of gels was found regardless of stage, species and strain of parasite, preventing satisfactory interpretation of the gels.

7.3.16 Threonine dehydrogenase

The assay of threonine dehydrogenase by starch gel electrophoresis gave neither good resolution nor sufficient levels of activity, however, isoelectric focusing produced better results in both these respects. Initial work with isoelectric focusing suggested 0.76ml ampholines pH 3.5 to 10 and a mid-loading position gave the optimal enzyme activity. Staining was seen cathodally at 0.8, 1.0 and 1.2cm for samples of Morantel Susceptible Lelystad, Morantel Resistant Lelystad and Glasgow. The bands were faint, despite doubling homogenate concentration, prefocusing the gel, altering the stain buffer pH and changing the salt concentration (potassium chloride) on separate occasions. Overall, the results were also poor with isoelectric focusing and differences between isolates were not detected.

7.4 SUMMARISED RESULTS

The results of the interspecies comparison were analysed using the "BIGSIM" similarity programme with the "GH" adaptation of "SIM" written by Richard Cibulskis in "BASIC" and cluster analysis "4M" written by Chip Buccholtz in "PASCAL". The isoenzyme profile data were recorded in binary form to denote the presence or absence of particular bands (Figure 7.12). Enzymes not tested for in individuals were discarded by the programme. Similarity was calculated for pairs of individuals and a particular enzyme using Jaccard's coefficient a/(a+b+c), where

- a = number of bands common to two individuals
- b = number of bands in individual one, but not in individual two
- c = number of bands in individual two, but not in individual one.

FIGURE 7.12

Binary presentation of isoenzyme data for five trichostrongyle species.

Cooperia oncophora	.01000000.100.11111000000.1111000000000.1.100.0000.10000000.1010000.000000.000000.0110.1000.0000.0000.
Haemonchus contortus	.00110000.000.10000110000.0000110000000.0.000.0010.11111001.0000000.000011.100000.0000.0000.0000.0001.
Ostertagia circumcincta	.00001100.000.01110001000.0000010000000.0.010.0100.01100010.0111000.000100.010000.1000.0100.0000.0000.
Ostertagia ostertagi	.00000111.011.01000000111.1100000111111.0.001.1101.01100100.0000000.110000.001111.1001.0011.0111.1110.
Trichostrongylus colubriformis	.10000000.100.00100000000.0000001000000.0.000.0000.00000000.0000000.000000.000000.0000.0000.1000.0000.

Bands of enzyme activity arising with a particular substrate stain are grouped together, the presence of a particular band is denoted by 1 and its absence by 0. *T.colubriformis* was not included in the cluster analysis data.

A similarity matrix was produced from the information, in which like individuals had a value of one and completely different individuals rated 0 (Figure 7.13): the closest similarity was between *O.ostertagi* and *O.circumcincta* at 0.173 and the least similarity between *O.ostertagi* and *H.contortus* at 0.036. *T.colubriformis* results were discarded since the low number of observations biased the data using cluster analysis, a dendrogram may be developed to give a simplified overview of the degree of variation; the most closely related individuals are picked out and their combined similarity is related to the other individuals, the pattern is built up by adding the most similar individuals consecutively to the group (Figure 7.14).

The binary representation of the comparative data for isolates of *O.ostertagi* is shown in Figure 7.15, while the similarity matrix and the dendrogram are found in Figures 7.16 and 7.17, respectively. The most closely related isolates are those of the Morantel Susceptible Lelystad and Morantel Resistant Lelystad with a similarity coefficient of 0.848 and the least similar, Louisiana and Weybridge-Glasgow at 0.264. The Stormont isolate had originated from Weybridge 4 years before this analysis and the Weybridge-Glasgow isolate had originated from Glasgow 18 years previously, in both cases the isolates still appeared closely related to the material from their source.

7.5 DISCUSSION

It should be stressed that it was not the intention of this study to examine the biochemistry of the parasite, merely to use isoenzymes as markers of variation. Consequently, information about the biochemical function of the enzymes has not been included and the reader is referred to Barrett (1981) for further details.

7.5.1 Alanine aminotransferase

On examination, most helminths have been found to contain alanine aminotransferase (Barrett, 1981). Transaminases catalyse the transfer of an alpha-amino group on an alpha-amino acid to an alpha-keto acid. Alpha-keto acids may be oxidatively deaminated, which is important for the metabolism of excess amino acids for fuel. Although

FIGURE 7.13

Similarity matrix comparing trichostrongyle species byisoenzyme analysis

Cooperia oncophora	1.000				
Haemonchus contortus	0.077	1.000			
Ostertagia circumcincta	0.094	0.112	1.000		
Ostertagia ostertagi	0.041	0.036	0.173	1.000	
(*Trichostrongylus colubriformis*	0.300	0.000	0.083	0.000	1.000)

Examination of the *T.colubriformis* data for cluster analysis was not undertaken due to the comparatively lower level of information available for the species.

FIGURE 7.14

Dendrogram indicating similarity between trichostrongyle species when they were compared by isoenzyme analysis.

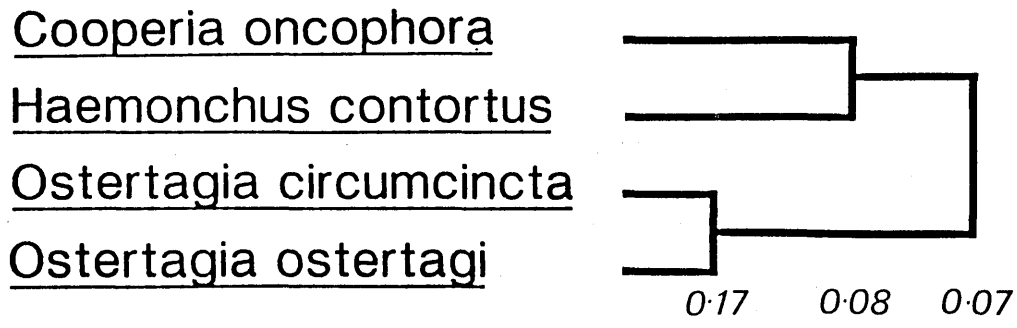


FIGURE 7.15

Binary presentation of isoenzyme data for ten isolates of *Ostertagia ostertagi*.

Alabama	**.0100.0.000.000.0000.000000.11000.00.11100000.000.1101.0.100.010001.111.11110000.**
Glasgow	**.1010.0.111.111.1110.111100.11100.11.11100000.000.1111.1.111.111110.111.11111111.**
Lelystad R	**.1000.0.110.110.0100.111000.01110.11.11100000.111.1100.1.101.110000.101.11110000.**
Lelystad S	**.1000.0.000.000.0100.000000.01110.00.00011100.111.1100.1.110.110000.101.11110000.**
Louisiana	**.0001.0.000.000.0000.000000.11100.00.11100011.000.0001.0.110.000000.101.11000000.**
New Jersey	**.1000.0.000.000.0000.000000.01100.00.11100000.000.1000.0.110.000000.101.11000000.**
Stormont	**.1000.0.000.100.0110.110000.11100.11.11100000.000.1100.1.000.100000.101.00000000.**
Wageningen	**.1000.0.000.000.0000.000000.11100.10.11100011.000.1101.0.000.110000.000.11110000.**
Weybridge	**.1000.0.000.010.0101.111010.11100.11.11100000.100.1110.1.000.110000.101.11111100.**
Weybridge-Glasgow	**.1000.0.000.100.1110.111001.10001.01.11100000.000.1110.1.101.010000.000.11111000.**

Bands of enzyme activity arising with a particular substrate stain are grouped together; the presence of a particular band is denoted by 1 and its absence by 0.

Lelystad R - Morantel Resistant
Lelystad S - Morantel Susceptible

FIGURE 7.16

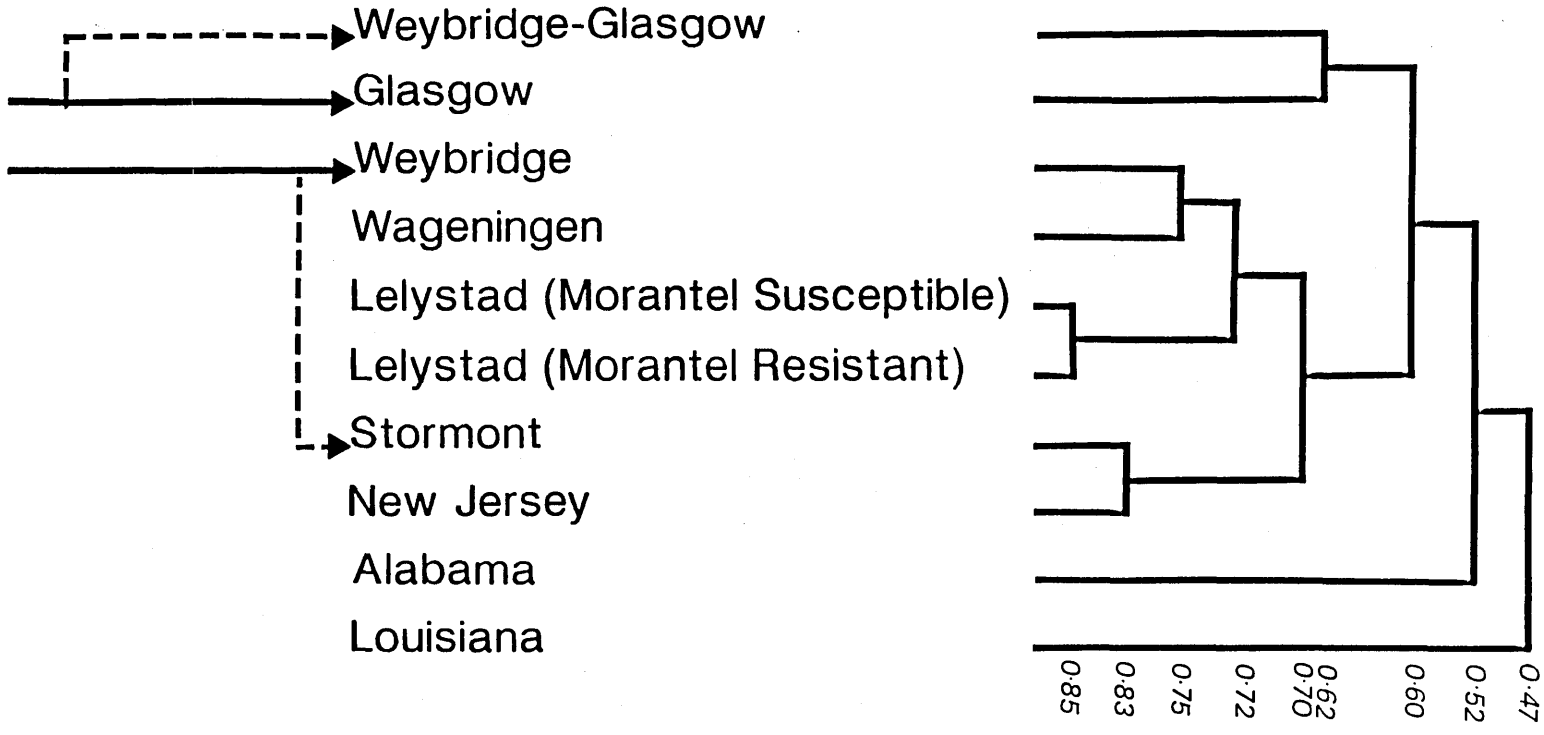
Similarity matrix comparing isolates of *Ostertagia ostertagi* by isoenzyme analysis

Alabama	1.000								
Glasgow	0.552	1.000							
Lelystad R	0.552	0.654	1.000						
Lelystad S	0.427	0.507	0.848	1.000					
Louisiana	0.467	0.490	0.419	0.429	1.000				
New Jersey	0.476	0.571	0.714	0.667	0.610	1.000			
Stormont	0.500	0.670	0.788	0.688	0.520	0.833	1.000		
Wageningen	0.600	0.607	0.752	0.694	0.567	0.620	0.711	1.000	
Weybridge	0.548	0.688	0.763	0.667	0.489	0.722	0.727	0.752	1.000
Weybridge-Glasgow	0.519	0.619	0.671	0.515	0.264	0.511	0.658	0.521	0.630	1.000

Lelystad R - Morantel Resistant
Lelystad S - Morantel Susceptible

FIGURE 7.17

Dendrogram indicating similarity between isolates of Ostertagia ostertagi when they were compared by isoenzyme analysis.
Known links between isolates are shown on the left.



significant differences in species banding patterns were found, the poor repeatability prevented the results of this enzyme system from being used for analysis.

7.5.2 Alkaline phosphatase

Alkaline phosphatase is an enzyme commonly found in the gut and tegument of nematodes; its function is unclear, although the enzyme is often involved in membrane transport mechanisms (Barrett, 1981). It is unlikely that *O.ostertagi* is devoid of the enzyme, instead the enzyme is probably not present in sufficient amounts for visualisation of activity or the surface associated nature of the enzyme prevented solubilisation of the protein when the supernatant or pellet samples were assayed. Alkaline phosphatase was found in *C.oncophora* despite the difficulty encountered in homogenisation of this species due to its coiling behaviour, suggesting the enzyme is probably present in *O.ostertagi*, but is for some reason not revealed. → why not a straight chemical assay

7.5.3. Esterases

Esterases are found throughout the nematode body in varying concentrations (Barrett, 1981). The esterases were considered in three separate groups as follows:

7.5.3.1 Carboxylic esters

Many problems were encountered with the staining system for these esterases. Often rapid uniform staining of the gel occurred and non-specific hydrolysis of the methylumbelliferyl component was suspected. When the technique was repeated with freshly obtained stain constituents, the same non-specific staining occurred. Consequently, it was particularly important that rapid and frequent examinations of gels stained for carboxylic esters were made. Precipitation of certain staining constituents frequently took place prior to pouring, which could be minimised by stain homogenisation. Judging by the common bands present in the profiles, the esterases did not appear to show a high level of substrate specificity, which obviated the inclusion of all four carboxylic esterases in the screen for variation.

The esterases were found to be unsuitable for comparative studies due to the generally poor repeatability of results and difficulty in their interpretation. However, species differences and presence or absence of individual bands in isolates of *O.ostertagi* were observed.

7.5.3.2 Choline esters

The cholinesterase system was tried twice with four buffer systems and enzyme activity on staining rarely resulted in sharp banding patterns. It was decided to discard the system since band activity was seen in the adults, but not in the L3 stage of *O.ostertagi* and increased sample amounts were required. A more selective substrate system was chosen for acetylcholinesterase as a consequence of these results (Sutherland, 1987).

7.5.3.3 Acetylcholine esters

The nematode digestive system is particularly high in acetylcholinesterase and the enzyme is often found in ES products. Acetylcholinesterase is thought to be beneficial to the nematode, since it decreases host peristalsis and may aid anchorage in the gut. Acetylcholinesterase may also reduce the host immune response (Barrett, 1981). Acetylcholinesterase was found to show significant species variation in enzyme migration. Relative intensity of the isoenzyme bands appeared to vary with respect to different isolates.

7.5.4 Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase is important in the pentose phosphate pathway. The enzyme is found in many helminths; it catalyses the step: glucose-6-phosphate --> 6-phosphogluconate, allowing the transfer from the glycolytic to the pentose phosphate pathway. Glucose-6-phosphate dehydrogenase was found to give clear and repeatable results with trichostrongyle material and was considered suitable to screen for comparison of species and isolates. In this respect the enzyme showed differences between species, and only a low level of variation was revealed between the isolates assayed.

7.5.5 Glucose phosphate isomerase

Glucose phosphate isomerase is important in glycolysis, it catalyses the glucose-6-phosphate--> fructose-6-phosphate step in the conversion of glucose to pyruvate with adenosine tri-phosphate production. Glucose phosphate isomerase could be included in a screen for comparison of trichostrongyles, giving repeatable results for all the samples assessed.

Isoenzyme inter- and intra-specific variation was recognised. Single bands were seen in the majority of the species and isolate samples, although two bands were seen with some of the samples. Interspecies differences were recognised, while intraspecies comparison showed common bands, suggesting little enzyme variation at the species level. The two bands of enzyme activity observed for some isolates may be due to heterozygosity of a monomeric enzyme in individuals or the existence of two genotypes of nematode with different monomer isoenzymes within the isolate. The ability to assess enzyme electrophoretic patterns of individual nematodes would help to differentiate which explanation is correct, but unfortunately the sensitivity of the techniques was too low to demonstrate activity with individual nematodes.

7.5.6 Glutamate dehydrogenase

Glutamate dehydrogenase catalyses the oxidative deamination of glutamate amino acid groups by transamination and is consequently important for the excretion of ammonia as a waste product (Barrett, 1981). Satisfactory results were not obtained for glutamate dehydrogenase with infrequent demonstration of enzyme activity from the L3. Lengthening the incubation period and use of adult samples generated greater enzyme activity, suggesting that increased protein loading might be required to improve results. Further study is necessary before glutamate dehydrogenase can be employed in a screen to compare species and isolates of trichostrongyle.

7.5.7 Glutamate oxaloacetate transaminase

Due to difficulty in interpretation of results glutamate oxaloacetate transaminase was not felt to be suitable for accurate isolate comparison, although it did show adequate interspecies variation.

7.5.8 Isocitrate dehydrogenase

Isocitrate dehydrogenase catalyses the isocitrate-oxalosuccinate conversion step in the tricarboxylic acid cycle, but it may also be an important intermediate in other biochemical pathways. Furthermore, it may act as a carrier between the mitochondria and the cytoplasm (Barrett, 1981). A triple banded pattern is indicative of a heterozygote in the case of a dimeric enzyme. The central band (heterodimer) is present at twice the amount of either outer band due to random association of the two allelic variant polypeptides. However, in the results presented here for isocitrate dehydrogenase the two outer bands were more active than the middle band suggesting that either three homozygous enzyme types were present within the population, a complex interaction of subunits was present or a mixture of homozygous and heterozygous individuals within an isolate. The occasional many banded profiles on isolate comparison could be explained similarly, except that the existence of two genotypes must be involved. The pattern of species similarity implies the enzyme is conserved between species. The faint cathodal band found in some experimental runs may be due to contamination (see results for glucose phosphate isomerase, 7.3.5) alternatively there could be two enzyme forms found within an individual - a mitochondrial and a cytoplasmic form. Two enzyme forms of isocitrate dehydrogenase have been discovered in certain nematodes (Barrett, 1981).

7.5.9 Malate dehydrogenase

Malate dehydrogenase reduces oxaloacetate to malate; the process has been shown to be the main source of energy in helminths, consequently the enzyme is an important regulator of energy metabolism (Leon, *et al.*, 1988). These workers found differences in the malate dehydrogenase patterns for adults of different ascarid species and in addition between the males and females. Starch gel electrophoresis

clearly indicated isoenzyme differences between the trichostrongyle species examined and a lower level of variation between isolates of *O.ostertagi*. Two main forms of malate dehydrogenase were found in isolates, together or individually, implying that a mixed population occurred in some cases. The Morantel Susceptible Lelystad and the Morantel Resistant Lelystad isolates showed the same single band, although the other Dutch isolate (Wageningen) produced the two band pattern, possibly this could be the result of a degree of geographical isolation. Isoelectric focusing also clearly illustrated species variation, but was only used latterly and although band separation was poor, the clarity of the results suggest that the technique might contribute further to isolate analysis.

7.5.10 Malic enzyme

Malic enzyme is found in the mitochondria of nematodes and is important in carbohydrate metabolism for the conversion of malate to pyruvate in the glycolytic pathway (Barrett, 1981). Species differentiation was apparent with malic enzyme, though different isolates generally appeared similar.

7.5.11 Mannose phosphate isomerase

Different isolates and stages of *O.ostertagi* tested showed similar mannose phosphate isomerase patterns.

7.5.12 Peptidases

Many of the peptidases showed identical banding patterns with different substrates, suggesting the enzymes are not particularly specific. Although the banding pattern was similar this does not prove that these proteins are the same, it is still possible that the proteins are different but inseparable by isoenzyme analysis.

There were obvious differences in the intensity of staining between the adult and L3, which was probably a result of varying concentrations of the enzyme with stage. Similarity between isolates was high with the presence of common bands, although variation was noted between

additional bands. Some comparisons could also be made between the species examined.

There appeared to be some alteration of the parasite with change in environment, e.g., the Weybridge-Glasgow isolate showed different isoenzyme patterns from the Glasgow sample from which it originated and the Weybridge isolate, suggesting a subset of the parasite population when passaged in isolation had altered in character, possibly certain nematode individuals had been more successful under the new conditions and had been preferentially selected.

7.5.13 Phosphoglucocomutase

Strong inter- and intra-species similarities were seen in the presence of phosphoglucocomutase. Isoelectric focusing gave poor separation of bands from the loading point, which could be the reason for the lower number of bands seen with the technique relative to starch gel electrophoresis.

7.5.14 6-Phosphogluconate dehydrogenase

Without improved levels of activity, 6-phosphogluconate dehydrogenase would be an unacceptable substrate stain for comparison of nematode isolates and species.

7.5.15 Superoxide dismutase

Superoxide dismutase protects cells from potential oxygen poisoning from the superoxide radicals produced in aerobic metabolic processes. The enzyme inhibits the reduction of the p-nitro terazolium dye (Sanchez-Moreno, Leon, Garcia-Ruiz and Monteoliva, 1987). The patterns seen on examination for superoxide dismutase were too diffuse to be used for comparative work.

7.5.16 Threonine dehydrogenase

Threonine dehydrogenase activity was low for screening purposes, but adequately showed isolate similarities.

7.6 GENERAL DISCUSSION

The results of the experiments indicated close similarity in isoenzyme patterns between isolates of *O.ostertagi*, occasionally variation was encountered, but usually in the form of one or a few isolates exhibiting a difference from the majority. However, no obvious link between the individuals showing enzyme variation and the characteristics of arrested larval development and drug susceptibility could be drawn. A lower level of similarity was recognised between nematode species examined by their isoenzymes, although some similarities of pattern were noted. Similar isoenzyme forms seemed to occur with the different stages, but their relative expression altered. Table 7.5 lists the enzyme techniques which were assessed and summarises their suitability for inclusion in a screen for detection of inter- and intra-species variation of trichostrongyles.

Inter-species variation in isoenzyme pattern was demonstrated by starch gel electrophoresis and isoelectric focusing, with distinct patterns for alanine aminotransferase, esterases (carboxylic and acetylcholine), glucose-6-phosphate dehydrogenase, glucose phosphate isomerase, glutamate oxaloacetate transaminase, malate dehydrogenase and malic enzyme. Using other substrate systems there was a degree of overlap between the staining patterns seen in different species, e.g., isocitrate dehydrogenase and phosphoglucocomutase. Ferguson (1980) emphasised that similarity of enzymes between species indicates the early development of these proteins in an evolutionary scale.

However, variation within the species *O.ostertagi* was not so marked with many shared bands and few apparent differences. Intraspecies variation was frequently restricted to relative band intensity, e.g., esterases (carboxylic and acetylcholine), glucose phosphate isomerase and malate dehydrogenase, while in other cases significant differences

TABLE 7.5

Enzymes assayed for screening of trichostrongyle species and isolates of *Ostertagia ostertagi* for variation are listed with a summary of their efficacy for this purpose.

Enzyme	Comments
Alanine aminotransferase	Species differences. Inconsistent, difficult to interpret Not suitable for screen.
Alkaline phosphatase	Lack of staining at high sample concentrations. Not suitable for screen.
Esterases	
1) Carboxylic esters	Species differences with propionate ester. Little specificity of enzyme patterns within carboxylic group. Inconsistent results, hard to interpret due to non-specific staining. Not suitable for screen.
2) Choline ester	Species differences, low activity and definition of staining profile. Not suitable for screen.
3) Acetylcholine esters	Species pattern differences. Isolates varied in the presence or absence of individual bands and their relative intensity. Consistent results. Suitable for screen.
Glucose-6-phosphate dehydrogenase	Species differences. Isolates showed shared and varying staining patterns. Moderately consistent. Suitable for screen.
Glucose phosphate isomerase	Species differences. Isolates common and varying staining patterns. Suitable for screen.
Glutamate dehydrogenase	Species differences. Inconsistent results prevent adequate comparison of isolates. High sample concentration required. Not suitable for screen.

TABLE 7.5 (continued)

Glutamate oxaloacetate transaminase	Species differences. Rapid non-specific staining presented difficulties with interpretation. Not suitable for screen.
Isocitrate dehydrogenase	Species variation, but some overlap of banding patterns. Many shared bands on intraspecies comparison. Consistent clear results. Suitable for screen.
Malate dehydrogenase	Species differences. Strong similarities between isolates, though some variance in the number of bands demonstrated. Suitable for screen.
Malic enzyme	Species differences. High similarity between isolates. High sample concentration required, banding lacked definition. Suitable for screen.
Mannose phosphate isomerase	Isolates varied in relative enzyme activity alone. Suitable for screen.
Peptidases	Little inter- or intra-species variation. Low specificity of enzymes, so selected substrate types were chosen. L-alanyl-L-tyrosine, glycyl-L-leucine and L-leucyl-L-leucine are suitable for screen.
Phosphoglucocomutase	Shared banding patterns between species and isolates, some variation in intensity of activity. Suitable for screen.
6-Phosphogluconate dehydrogenase	Slight differences in enzyme mobility patterns on isolate comparison. Poor mobility. Suitable for screen.
Superoxide dismutase	Diffuse, non-specific staining activity. Not suitable for screen.
Threonine dehydrogenase	Isolates strong similarities. Poor band activity. Suitable for screen.

could not be detected, e.g., malic enzyme, mannose phosphate isomerase and threonine dehydrogenase.

The difference in the scale of the similarity dendrograms indicates simply the greater similarity between isolates than species. Similarity levels of 0.7 and above on a dendrogram are accepted as indicating high similarity (G.Hide, personal communication). There appear to be a cluster of closely related samples: Morantel Susceptible Lelystad, Morantel Resistant Lelystad, New Jersey, Stormont, Wageningen and Weybridge. However, while Glasgow and Weybridge-Glasgow were more similar to each other they were less related to this group. Louisiana and Alabama showed fewer similarities to the rest of the isolates.

High numbers of parasites were required to produce enzyme activity, on average 20,000 L3 per track, so individual nematodes could not be assessed. Consequently, multiple banding could have arisen due to polymorphism or to multiple isoenzyme types in each individual. Comparative work to assess individual variation would be more successful in a nematode of larger size such as *H.contortus*, in which a single adult was found to give a readable pattern on starch gel electrophoresis, unlike *O.ostertagi* in which insufficient protein was present. It had been planned to amplify the offspring of a single *O.ostertagi* mating by *in vitro* culture or implantation of a pair of adults at the start of patency directly into the abomasum of a helminth naive calf. Faecal eggs would be collected for culture of larvae *in vitro* to build up sufficient sample material with similar genetic background by backcrossing. However, little sample material has been generated by these means and the amounts of protein required for the isoenzyme experiments made the techniques impractical. Mirelman (1987) found changes in the zymodemes of *Entamoeba histolytica* could be induced by alterations to the culture conditions, e.g., constituents of the medium and the temperature, so it is possible that culture conditions may alter the isoenzyme profile. The effect of an individual host on its nematode population could similarly alter conditions, i.e., diet, season, management, so it may be inadequate to consider the nematodes of a particular species within a herd as a common pool with little apparent gene flow. In the future the use of gene libraries and cloning

techniques may be employed to generate sufficient sample, once suitable loci for examination have been identified.

Standardisation of samples from different stages for comparison was felt unlikely to produce meaningful data, since it is difficult to produce suitable standards for sample amount due to the disparity in stage size. For many enzyme systems both adult and larval stages were found to show the same banding pattern, although some stages exhibited different relative amounts of the enzymes, e.g., glutamate dehydrogenase was present in very low amounts in the L3 compared to the adult, whereas other enzymes tested at the same concentration showed similar activity. It has been suggested that different stages of the parasite may possess significantly different proportions of enzyme depending on the degree of activity and the form of metabolism (D.Jones, personal communication). O'Riordan and Burnell (1989) demonstrated relative predominance of different metabolic processes in the dauer and adult stages of the free-living nematode *Caernorhabditis elegans*. Barrett (1981) claimed the distribution of the enzyme within a nematode could alter with stage and that simple switching on and off of genes coding for enzymes was unlikely to occur. Leslie, Cain, Meffe and Vrijenhoek (1982) suggested enzyme polymorphism would be a suitable method for matching adult and developing stages when epidemiology makes the life cycle difficult to follow. Andrews, Beveridge, Adams and Baverstock (1988) illustrated that in *Echinocephalus overstreeti* 34 enzyme loci were expressed in both adults and larvae.

Comparison by parasite number would result in the loading of significantly different protein amounts - the L3 being 0.85 mm x 0.025mm and the adult 9.69 mm x 0.11mm . Loading standardised levels of protein also causes problems, since the relative distribution of proteins in the sample alters with size; the earlier stages have a relatively greater surface area to volume ratio and a concomitant increase in structural proteins. However, the main difficulty of stage comparison would be acquisition of sufficient sample, because large sample numbers are required for each stage and other than the L3 these are not readily obtained. When different stages of the parasite are examined further controls are required to verify enzymes are of parasite rather than host

origin, i.e., the samples must be adequately cleaned. Only certain sample concentrations were employed, but it is feasible that increased sample concentration or alteration of other experimental parameters might improve demonstration of enzyme activity, however, the confines of the study did not allow full investigation of stage variation.

To draw quantitative conclusions from the results would require further standardisation of the sample. The method of sample loading for starch gel electrophoresis makes direct comparison difficult, since care must be taken to insert samples to the same depth, so electrodecantation, i.e., sinking of protein in the gel does not affect the staining appearance. When a single band alone stained for an enzyme and differences in activity were observed, sample ageing may have reduced the activity, however, when more than one band is present the relative depth of staining of bands from an isolate may help to identify the more likely cause of variance: sample senescence or isolate variation.

It would be interesting to assess whether alterations in the optimal stain and gel buffer pH change relative to the stage of parasite under examination, i.e., when the vast changes in environment occurring during the life cycle are considered. Optimum enzyme activity occurs at a particular pH, ideally the buffer pH should be similar to this. Sibley, Lawson and Weidner (1986) found the optimal pH ranges for the enzymes superoxide dismutase and superoxide catalase of *Toxoplasma gondii* were very narrow. The band patterns for *O.ostertagi* themselves seem to occur at the same level regardless of stage, so the isoenzyme appears to be the same, but possibly more effective under different conditions, e.g., pH.

Sample storage caused further complications, since it was noticed that preparations stored by freezing at -20°C or -70°C showed reduced activity relative to freshly prepared material. In addition, enzyme activity often appeared at a site more distant from the slot (usually anodally) suggesting some sample degradation. Certain enzymes were affected more significantly, e.g., glucose phosphate isomerase, phosphoglucocomutase and isocitrate dehydrogenase. Due to the

unpredictable loss of activity with storage in homogenate form, the majority of samples were prepared on the day they were required.

In both starch gel electrophoresis and isoelectric focusing, the poor resolution of bands of activity led to an inability to differentiate isoenzymes of similar mobility. The shorter separation distances achieved by isoelectric focusing made differentiation harder, although experimental runs were more comparable. Slight changes in buffer strength and pH and other running conditions may improve the staining clarity, but "fine tuning" of the system requires much work. Staining the standard isoelectric focusing markers was time consuming and since experimentally the objective was to look for isoenzyme differences between isolates, the exact isoelectric point of enzymes was unimportant and so the markers were not frequently used.

In total 11 isolates of *O.ostertagi* were subjected to isoenzyme analysis. There was little variation in enzyme profile between isolates of *O.ostertagi* and the recognised variations did not correlate with the geographical range of sources or their known differences in drug tolerance and propensity to arrest. From work in ascaridoids, Nadler (1986) theorised that the internal environment selects for endoparasite monomorphology. Recent work has suggested that parasites evolve at a similar rate or at a slightly greater rate than their hosts (Fahrenholz rule) when the parasites are highly host specific, so allowing them to adapt to host changes as they arise (Hafner and Nadler, 1988). Enzyme polymorphism may compensate for alterations in the environment and may confer a genetic advantage on a certain group of parasites. Genes may evolve at different rates, which will depend on their metabolic role and selection pressure. Bryant and Flockhart (1986) had suggested that an individual might have multiple enzyme forms to withstand fluctuations in the environmental conditions. Generally, low numbers of different bands were noted for each of the enzymes for an isolate or species, i.e., one to three bands, which may be a reflection on the stability of the host population. Development of amino acid sequence changes depends on the level of selection pressure. Enzyme polymorphism may act as a method to compensate metabolically for alterations in the environment, so conferring a genetic advantage on a certain group of parasites.

In conclusion, the search for intraspecific variation by isoenzyme analysis in *O.ostertagi* suggested a very low level of variation. When variation was present the changes in relative activity or pattern of the bands were infrequent and apparently unrelated, i.e., a sample might vary with respect to one enzyme, but followed the general pattern for the remainder of the enzymes tested. Species differences were more conspicuous, but certain enzyme patterns were similar. Comparison of dendrograms for inter- and intra- species variation showed that similarity clustering was closer for isolates of *O.ostertagi* than that between species of trichostrongyle. In the future the isoenzyme techniques could be further extended to search for variation between stages using quantitative methods, to allow a better understanding of parasite development. The results indicated that similar isoenzymes were present in varying stages of the parasite life cycle, but their relative importance appeared to alter.

CHAPTER 8

SEROLOGICAL RESPONSES TO *OSTERTAGIA OSTERTAGI*

8.1 INTRODUCTION

Host defence mechanisms directed against invading organisms and the damage they cause may be broadly divided into innate and adaptive immune systems (Roitt, Brostoff and Male, 1985). Adaptive immunity is based around the lymphoid system, which recognises infectious agents once they have been encountered a first time, allowing fast mobilisation of defence mechanisms on subsequent occasions. The humoral response is more important than cell mediated immunity for the removal of extracellular parasites. In addition, to the secretion of antibodies into the general circulation, local secretion of antibody at mucosal surfaces is particularly significant in gastrointestinal parasitism, e.g., local IgA levels are more markedly raised than systemic levels in *Haemonchus contortus* infections in sheep (Smith and Christie, 1978). *Ostertagia ostertagi* elicits an immune response, in which circulating levels of IgG_1, IgA and IgM are raised (Jensen and Nansen, 1978); IgA levels are especially high in the abomasal mucosa.

Other defensive mechanisms include the production of chemotactic factors and the activation of the complement system. Mast cells and eosinophils invade the abomasal mucosa in particularly high numbers in *O.ostertagi* infections. The effect of these cells is not fully understood and theories are frequently changing. Murray *et al.* (1970) noted mast cell infiltration into the infected abomasal mucosa and the formation of globular leukocytes, which Armour *et al.* (1979) suggested produces a hypersensitivity reaction with the eventual release of vasoactive compounds. Klesius, Snider, Horton and Crowder (1989) suggested that eosinophil accumulation in response to the infection appeared to contribute more to the pathogenicity of the disease than to nematode expulsion.

Immunity to bovine ostertagiosis is acquired and resistance to infection is influenced by many host and environmental factors, including age, breed, individual, management system, nutritional status, intercurrent disease and prophylactic treatment (Chapter 1, 1.6.1 and 1.6.2). For

example, immunity develops more rapidly in older stock (Smith, 1970), although age immunity *per se* does not occur and mature animals may be affected on moving into an area of endemic *O.ostertagi*, although most stock develop protective immunity as they grow. Furthermore, the management system may influence the host age and level of pasture contamination at initial infection; rigorous treatment with prophylactic anthelmintics may prevent sufficient larval establishment to allow the stimulation of an immune response (Armour, 1989).

In their turn, parasites have developed a number of methods of evading host immune systems. The antigen repertoire presented to the host may alter through successive parasite moults and furthermore, there appears to be a constant loss of surface moieties within a stage (Maizels, Philipp and Ogilvie, 1982), i.e., antigenic turnover occurs. Certain parasites disguise themselves with host antigens, e.g., host macromolecules, particularly histocompatability antigens may fuse to the surface of schistosomes (Trager, 1986). In some cases, parasites may survive at burdens too low to stimulate a host immune response. Direct parasite action on the immune system may reduce the host response directed against them, e.g., Cross and Klesius (1989) found *O.ostertagi* inhibited lymphocyte proliferation in Balb/c mice. Consequently, they suggested incomplete protection of cattle after repeated infection could be due to immunomodulation by *O.ostertagi*. Under certain circumstances the immune system may appear to tolerate the presence of parasites, e.g., during the arrested larval development of L4 *O.ostertagi*. The greater the number and variety of parasite antigens the more difficulty the host usually exhibits in neutralising their action (Wakelin, 1984).

The aim of this section was to examine the antigenicity of *O.ostertagi* by comparing the antibody response generated in cattle infected *per os* and rabbits immunised by injection. Antigen profiles of different parasite stages were obtained by Western Blotting and examined to assess whether the recognised pattern altered with development, since changes might aid evasion of the immune response. Trichostrongyle species were compared by Western Blotting for antigenic cross-reactivity. Interaction between *Cooperia oncophora* and *O.ostertagi*

infections have been noticed, i.e., priming with one species leads to a reduction in the usual vulval size, worm length, number of ova per female and the burden when challenged with the other species (Kloosterman, *et al.* 1984). Inter-species cross-reactivity is important, since with a selected antigen of sufficient immunogenicity it might allow the development of subunit vaccines to a group of parasites simultaneously. Consequently, antigen profiles for different species of trichostrongyle were compared. Previously, attempts to induce immunity to a particular parasite species by immunising with closely related species has had only limited success, e.g., Dineen, Gregg, Windon, Donald and Kelly (1977). They tested the protection induced with irradiated *Trichostrongylus colubriformis* in sheep towards other members of the genera and other trichostrongyle species and found efficacy decreased rapidly with decreased parasite relatedness. However, the poor success of these vaccines may just have been a reflection of the low concentration of the cross-reacting antigen in the preparation or the poor immunogenicity of cross-reacting antigens.

Similarly, within species antigen variation was tested by comparison of isolates of *O.ostertagi*. Variation in antigen response with isolate might have serious implications to the development of control measures and should differences be found they might indicate the extent of vaccine and drug testing required for new products. In addition, surface antigens and their turnover were assessed under a number of conditions using the indirect fluorescent antibody test (IFAT).

8.2 WESTERN BLOTTING

8.2.1 Introduction

The ability of antibodies in a serum sample to recognise parasite antigens may be assessed by Western Blotting. Antigens are separated by polyacrylamide electrophoresis, blotted onto nitrocellulose acetate and incubated with antibody. B cell receptor sites of the first antibody bind the epitopes of the antigen. Unbound antibody is washed away and a second antibody with specificity against the first antibody species is applied. Conjugation of the second antibody to an enzymatic colour

reaction allows visualisation of those antigens recognised by the test sera. The technique may detect as little as 1 to 10 ng of protein, while overloading will occur at greater than 150 µg, leading to band distortion (Harlow and Lane, 1988).

8.2.2 Materials and methods

8.2.2.1 Animals

Lop rabbits were housed singly and maintained on standard rabbit diet:

Rabbit 1 - One hundred thousand sheathed *O.ostertagi* L3 were homogenised in 1ml sterile water and emulsified in 2ml Freund's complete adjuvant. The resulting 2ml of emulsion was divided into four doses and injected intramuscularly into both hind legs and at two subcutaneous sites on the back of the rabbit. Similar booster doses were administered in Incomplete Freund's adjuvant 2 and 4 weeks later.

Rabbit 2 - One hundred thousand exsheathed *O.ostertagi* L3 were homogenised and administered by an identical protocol to that devised for Rabbit 1. The larvae were exsheathed in hypochlorite solution and rinsed three times in excess phosphate buffered saline PBS (pH 7.2) (Chapter 4, 4.2.5).

Blood samples were collected pre-immunisation by aural venepuncture. Post-immunisation blood samples were taken at weekly intervals from 42 days until 72 days after the first larval dose. Unless stated to the contrary, the sera used for blotting and IFAT examination was that taken from the bleed at 72 days post first immunisation.

Stock calf serum samples were collected pre- and post-oral infection of five passage calves each with multiple doses of 100,000 L3. Ten post infection serum samples were collected at weekly intervals from 7 days after infection.

8.2.2.2 Western Blotting

Nematode homogenates (Chapter 4, 4.3.3) were separated by SDS-PAGE, 10% polyacrylamide gels (Chapter 4, 4.3.1) and electroblotted onto nitrocellulose acetate (Chapter 4, 4.3.2). The nitrocellulose was blocked in 10mM Tris-hydrochloride pH 7.4 with 1% Tween 20 in 0.9% saline for 1 hour and then washed in Tris-saline minus Tween. A dilution of the test sera, i.e., first antibody was made up in Tris-saline: 100µl serum in 20ml Tris-saline (1:200). The nitrocellulose was incubated in the solution for 1 to 2 hours on a rotating platform at room temperature (20°C).

Unbound antibody was removed by five to six washes in 100ml Tris-saline on a moving platform at room temperature over 30 minutes. The second antibody conjugated to horse radish peroxidase was diluted with Tris-saline (1:200); commercially prepared donkey anti-rabbit IgG or goat anti-Bovine IgG was used depending on the species from which the first antibody was derived. The nitrocellulose was incubated for 1 to 2 hours at room temperature, followed by a further five to six washes in Tris-saline over 30 minutes. A developer solution, consisting of 0.02% 4-chloro-1-napthol (w/v) and 20% methanol (v/v) in Tris-saline (pH 7.2), was prepared. Once the developer had been added, 100µl 40% hydrogen peroxide was added to initiate the reaction with horse radish peroxidase, producing a purple-black colouration where second antibody was bound to the nitrocellulose. Staining was normally visible by 15 minutes. The colour reaction was halted by immersing the nitrocellulose in deionised water before background staining obscured the banding pattern. The intensity of the staining was reduced on exposure to daylight, consequently results were recorded immediately, although the staining step could be repeated as required.

8.2.3 Results

Experiment 1:

The aim of the first experiment was to identify antigens specific to the retained L2 sheath of the L3 of *O.ostertagi*, by examining the antigenic

patterns produced by sheathed or exsheathed larvae for differences when incubated with sera from rabbits immunised with homogenates of sheathed or exsheathed L3 of *O.ostertagi*.

Homogenates of sheathed and exsheathed larvae were prepared (Chapter 4, 4.2.5, 4.3.4) and loaded at 1,000 and 2,000 L3 per track as duplicate samples on different halves of a 10% polyacrylamide gel. After electroblotting, the blot was halved and the pieces reacted separately with sera from rabbits immunised with exsheathed or sheathed larval homogenates.

Results:

Serum from the rabbit immunised with the exsheathed larvae produced a more intense staining than serum from the rabbit immunised with sheathed larvae with both larval preparations (Figure 8.1). Several bands were common to both types of rabbit serum: 170 and 160 (doublets), 102, 75, 54 and 50 kilodaltons. Serum from the rabbit immunised with sheathed larval homogenates reacted with bands at 85 and 64 kilodaltons in the sample of sheathed larvae, but these bands were not seen with the sample of exsheathed larvae.

Experiment 2:

Serially collected sera samples from a rabbit immunised with exsheathed homogenates of *O.ostertagi* were tested against sheathed L3 homogenate to examine the development of antibody response with time. The experiment was repeated using sera from the rabbit immunised with sheathed larvae and sera from orally infected calves.

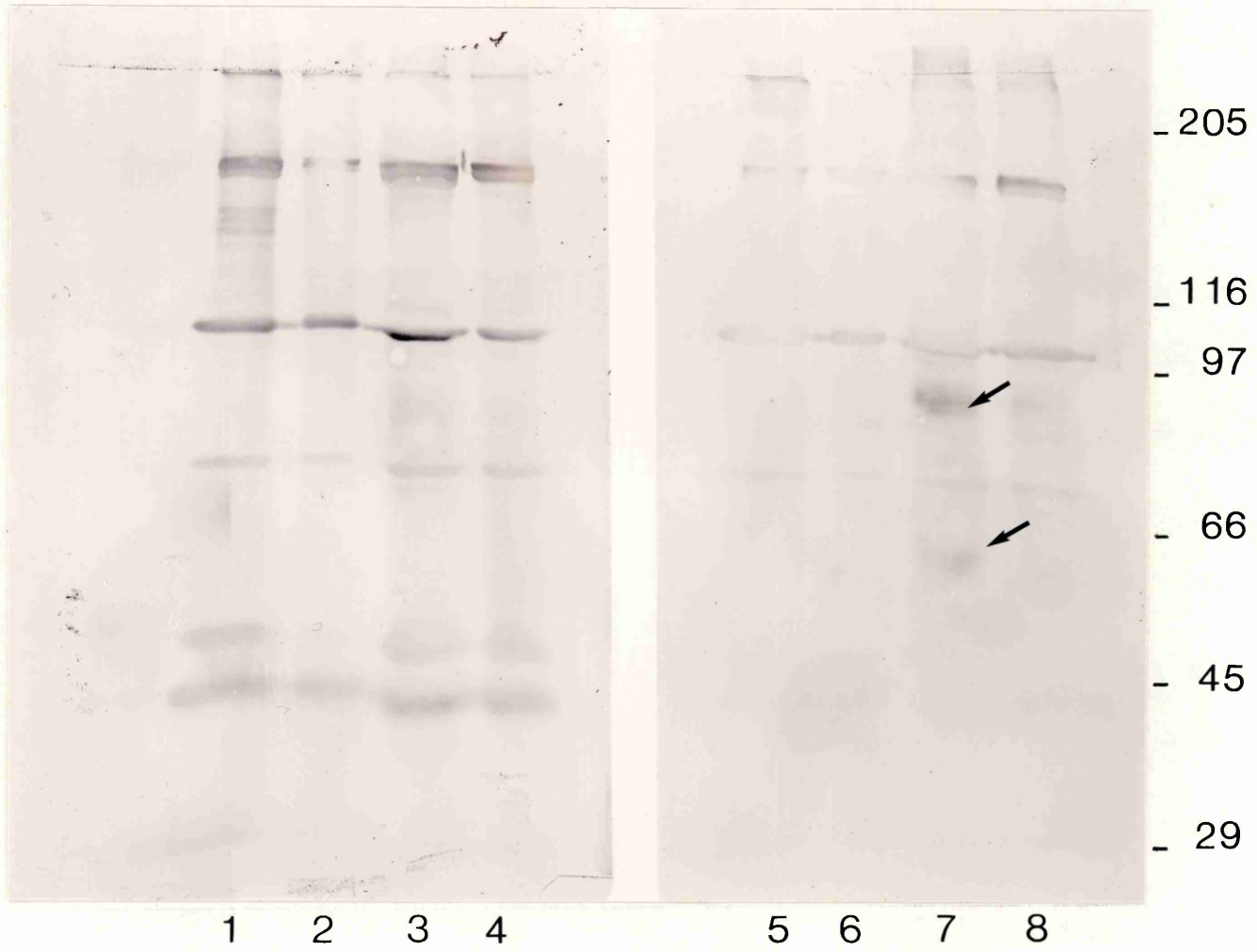
To allow the comparison of large numbers of serum samples against an equivalent antigen two options were available. A different stacking comb was used with a small slot for a molecular mass standard and a wide slot for the sample. The volume of the large slot was estimated to be approximately ten times that of the standard slot size; 30,000 L3 were homogenised in 350µl protease inhibitors (Chapter 4, 4.3.4) and 90µl sample buffer was added. Either the membrane was cut into strips and immersed in different serum samples or a PR200/Decca Probe

FIGURE 8.1

Western Blot. Antigen profiles of sheathed and exsheathed L3 of *Ostertagia ostertagi* probed with sera from rabbits immunised with either sheathed or exsheathed larvae. Molecular mass standards are in kilodaltons.

Lane 1	**-**	**2,000 exsheathed L3s,**	**probed with sera from a rabbit immunised with exsheathed L3s.**
Lane 2	**-**	**1,000 exsheathed L3s,**	**probed with sera from a rabbit immunised with exsheathed L3s.**
Lane 3	**-**	**2,000 sheathed L3s,**	**probed with sera from a rabbit immunised with exsheathed L3s.**
Lane 4	**-**	**1,000 sheathed L3s,**	**probed with sera from a rabbit immunised with exsheathed L3s.**
Lane 5	**-**	**2,000 exsheathed L3s,**	**probed with sera from a rabbit immunised with sheathed L3s.**
Lane 6	**-**	**1,000 exsheathed L3s,**	**probed with sera from a rabbit immunised with sheathed L3s.**
Lane 7	**-**	**2,000 sheathed L3s,**	**probed with sera from a rabbit immunised with sheathed L3s.**
Lane 8	**-**	**1,000 sheathed L3s,**	**probed with sera from a rabbit immunised with sheathed L3s.**

The bands at 64 and 85 kilodaltons in the samples of sheathed larvae (lanes 7 and 8) were only recognised by sera from the rabbits immunised with sheathed larvae, suggesting these antigens may be associated with the retained sheath on the L3.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(Hoeffer Scientific Instruments) was applied, which divided the blot into separate serum reaction chambers by a system of seals. The problem of adequate realignment of the strips for comparison made the second method preferable. It was imperative that the blot surface, which had contacted the gel was uppermost in the probe for detection of reaction. The serum was diluted 1:200 as before, but only 1 to 2ml were required to fill each chamber of the probe apparatus.

Results:

The number and intensity of antigen bands from the sheathed larval homogenate recognised by the rabbits immunised against exsheathed or sheathed larvae increased from 7 to 72 days after the first immunisation (Figure 8.2, Table 8.1). Three antigens were detected by sera 7 days post immunisation (190, 105 and 12 kilodaltons); by 29 days the majority of antigens recognised throughout the remaining sampling time were present. No activity was visible with pre-immunisation serum. Antibody reactions occurred with antigens of similar relative mass when larval homogenates were tested with bovine serum, but the staining was very weak and occasionally banding was seen with pre-immunisation serum.

Experiment 3:

Different stages of *O.ostertagi* were compared on Western Blots prepared with serum from a rabbit immunised with sheathed larvae.

Pre-parasitic larvae taken from 2, 3, 6, 8 and 14 day faecal cultures (Chapter 5, 5.2.2.1 and 5.2.2.2) and L4s from abomasal extraction (Chapter 4, 4.2.6) were loaded at a level of 2,000 to 3,000 larvae per track. Adults from the abomasum (Chapter 4, 4.2.8) were loaded at ten adults per track. Following electrophoresis and electroblotting, blots were reacted with sera from a rabbit immunised with exsheathed larvae.

Results:

On the blot prepared with early faecal culture samples, there was aberrant reverse straining, i.e., white bands were seen on a dark

FIGURE 8.2

Western Blot. Sheathed larval homogenate of *Ostertagia ostertagi* L3 was probed with sera from a rabbit bled at increasing intervals after immunisation with exsheathed larvae. The development of an antibody response was assessed. Molecular mass standards were in kilodaltons.

Lane 1, 7 days;	**Lane 2, 15 days;**	**Lane 3, 29 days;**
Lane 4, 40 days;	**Lane 5, 45 days;**	**Lane 6, 52 days;**
Lane 7, 58 days;	**Lane 8, 65 days;**	**Lane 9, 72 days.**

Increased numbers of bands were detected with time after immunisation; in addition, staining intensity increased and a band at 105 kilodaltons was particularly strong.

"Narrow" tracks are caused by inadequate sealing of the probe apparatus.

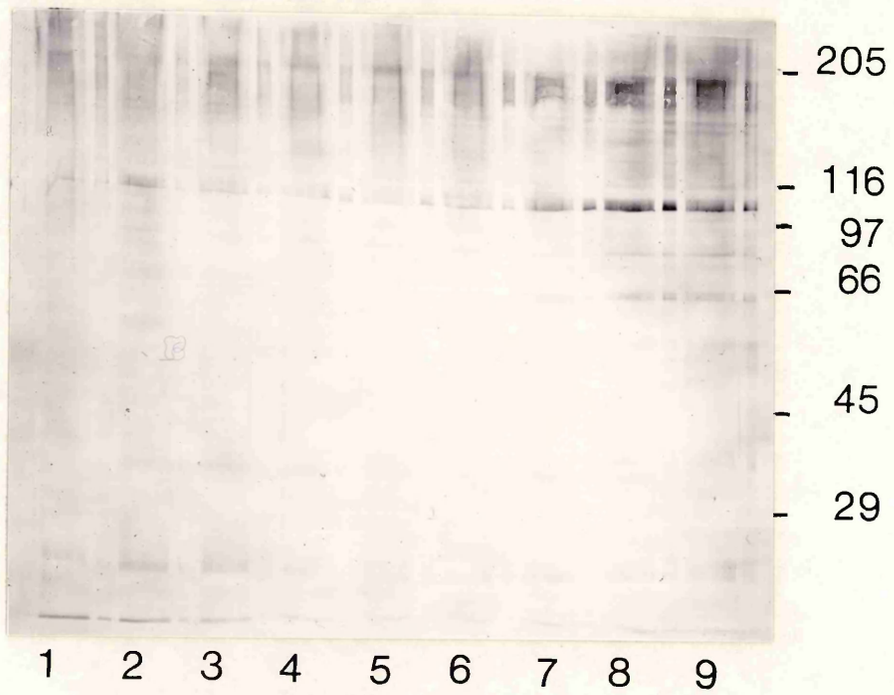
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TABLE 8.1

The development of the immune response towards sheathed larval homogenates of *Ostertagia ostertagi* in a rabbit immunised with exsheathed larvae when assessed by Western Blotting.

Molecular mass in kilodaltons	Days post immunisation								
	7	15	29	40	45	52	58	65	72
190	+	+	+	+	+	+	+	+	+
160			+	+	+	+	+	+	+
155			+	+	+	+	+	+	+
150								+	+
105*	+	+	+	+	+	+	+	+	+
80		+	+	+	+	+	+	+	+
60							+	+	+
28									+
15		+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+

* Staining of this band was particularly intense

ground. No reaction was seen against the 2 or 3 day culture samples (mainly L1s), while bands at 150, 107, 80 and 66 kilodaltons were found at 6 and 8 days (mainly L2s). L3s had additional bands at 190, 15 and 12 kilodaltons, while L4s showed further bands again at 150, 37 and 24 kilodaltons. No satisfactory blots were obtained with adult parasite antigen.

Experiment 4:
Five different species of trichostrongyle nematode were compared by Western Blotting against serum from a rabbit immunised with sheathed *O.ostertagi* homogenates.

Homogenates of *C.oncophora*, *H.contortus*, *O.circumcincta*, *O.ostertagi* and *T.colubriformis* were loaded on a polyacrylamide gel at 1,000 and 2,000 larvae per track. The membrane was incubated with serum from a rabbit immunised with sheathed homogenised L3 of *O.ostertagi*. The experiment was repeated with serum from a calf orally infected with *O.ostertagi*.

Results:

No significant difference was noted in band activity at the two loading levels. The basic patterns were similar for all five trichostrongyle species examined. They all had bands at 100, 64, 36, 31, 21 and 19 kilodaltons. The only differences were in the size of the doublet antigens. The position was 170 and 165 kilodaltons in *C.oncophora*, *H.contortus*, *O.ostertagi* and *T.colubriformis*, but 170 and 175 kilodaltons with *O.circumcincta*. Bovine serum produced very faint reactions with the doublets at 160 to 175 and the band at 64 kilodaltons alone.

Experiment 5:

Different isolates of sheathed *O.ostertagi* L3 were loaded at 2,000 L3 per track and assessed by Western Blotting against serum from a rabbit immunised with homogenates of sheathed L3.

Homogenates from Alabama, Denmark, Glasgow, Lelystad (Morantel Resistant and Susceptible), Louisiana, New Jersey and Weybridge were prepared and loaded at a concentration of 2,000 L3 per track. The resulting blot was reacted with serum from the rabbit immunised with sheathed L3 homogenate, subsequently the experiment was repeated using the serum from the rabbit immunised with exsheathed L3s and the calf serum.

Results:

Serum from the rabbit immunised with sheathed larval extracts gave similar results with all the isolates of *O.ostertagi* examined (Figure 8.3). Bands were present at 170, 160, 107, 78, 48 and 42 kilodaltons and weaker bands were noticed at 150, 96, 65, 62, 58 and 55 kilodaltons. Faint additional bands were seen in the isolates from Denmark and Weybridge at 130 and 113 kilodaltons, Weybridge at 110 kilodaltons and at 80 kilodaltons for Glasgow and Weybridge. The same antigen profile was detected with the bovine serum, but the bands were fainter.

Experiment 6:

The cationic detergent cetyl-trimethyl ammonium bromide (CTAB) was applied to L3 *O.ostertagi* to examine the result of detergent action on the antigenic profile of sheathed and exsheathed larvae as detected by Western Blotting.

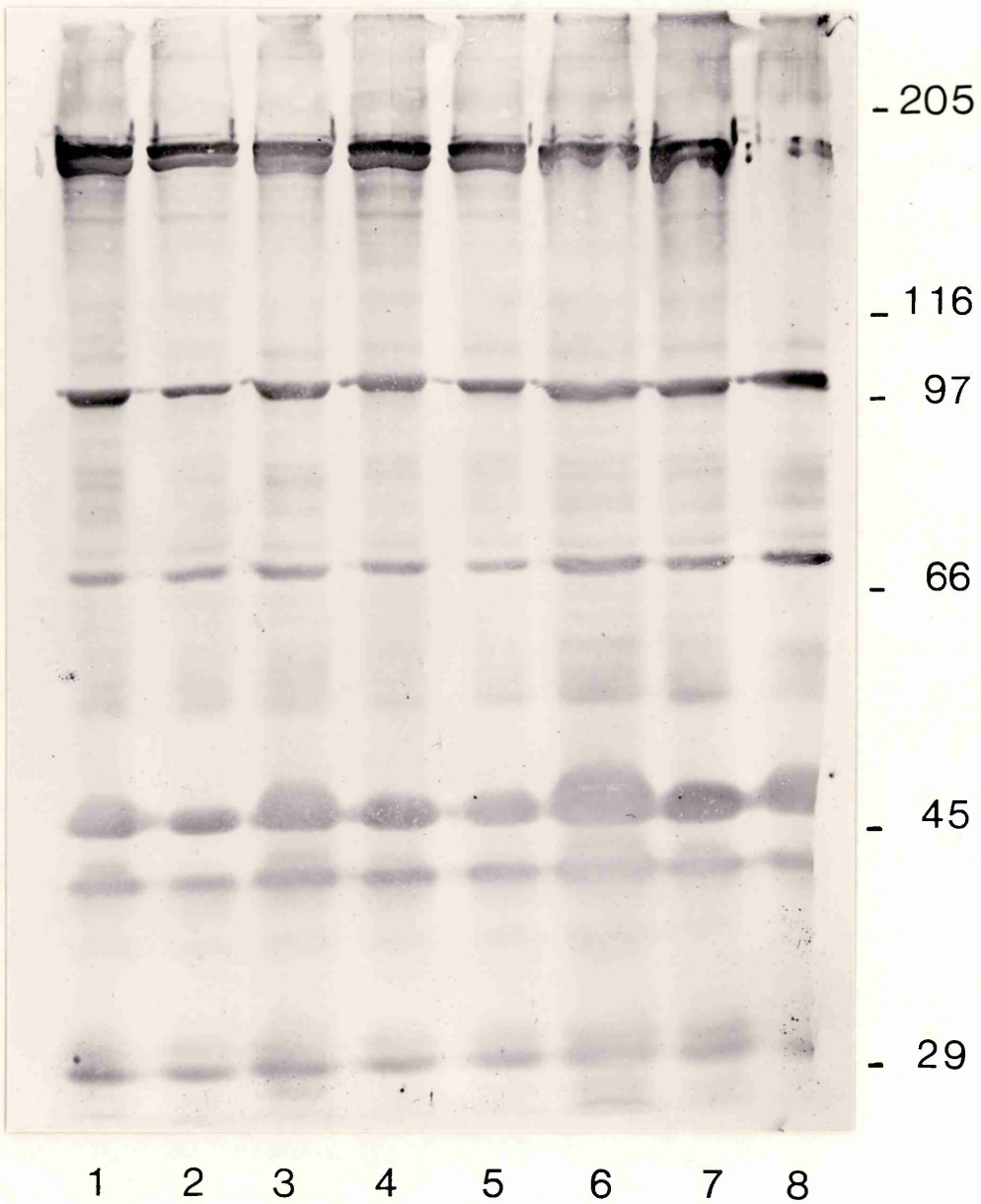
Duplicate samples of 3,000 sheathed and exsheathed L3 of *O.ostertagi* were pelleted in eppendorfs. The supernatant was withdrawn and 1.5ml 0.25% CTAB was added to one sample each of sheathed and exsheathed larvae and these were incubated for 16 hours at 37°C, while the other samples were incubated in distilled water. The CTAB and any stripped material was removed in three rinses in excess PBS (pH 7.2), 10,000g. All four samples were homogenised in 60µl of homogenising buffer with protease inhibitors. Forty µl of sample buffer was added to each of the supernatants retained after centrifugation at 10,000g for 2 minutes. After boiling for 2 minutes and centrifugation at 10,000g for a further 5 minutes the samples were

FIGURE 8.3

Western Blot. Different isolates of *Ostertagia ostertagi* L3 were probed with sera from a rabbit immunised with sheathed L3 of the Glasgow parasite isolate. Molecular mass standards in kilodaltons.

Lane 1, Weybridge;
Lane 2, New Jersey;
Lane 3, Louisiana;
Lane 4, Lelystad (Morantel Susceptible);
Lane 5, Lelystad (Morantel Resistant);
Lane 6, Glasgow;
Lane 7, Denmark;
Lane 8, Alabama.

The strong similarity of the antigen profiles of the different isolates of *Ostertagia ostertagi* is illustrated.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loaded at 1,000 and 2,000 L3 per track. After PAGE and electroblotting the samples were incubated with sera from a rabbit immunised with exsheathed larvae.

Results:

Similar antigen profiles were detected for both the exsheathed and sheathed larvae, with particularly strong bands at 165, 108, 78, 48 and 45 kilodaltons (Figure 8.4, Table 8.2). A clear increase in band activity and resolution was noticed with CTAB treatment of both sheathed and exsheathed larvae, especially at 78 kilodaltons for sheathed larvae. The low molecular mass antigens were poorly transferred at blotting.

8.2.4 Discussion

In all the experiments, bovine serum gave less intense staining than rabbit serum; susceptible host range and route of immunisation may both be factors, but it is also possible that the variation in intensity is a reflection of binding of the second antibody rather than the first antibody. Serum from the rabbit immunised with exsheathed *O.ostertagi* L3s gave more intense staining than the serum from the rabbit immunised with sheathed larvae. Large fragments of sheath tended to remain in the homogenised suspension regardless of the degree of homogenisation, which might have important effects on the antigen pattern achieved by SDS-PAGE. The fragments might have remained with the larval pellet after centrifugation and were therefore not included in the electrophoresed sample and were absent from the antigen profile or their presence in the sample could alter the mobility of the remaining proteins in the sample.

The presence of bands at 85 and 64 kilodaltons in samples of sheathed larvae, which were only detected when antisera to sheathed larvae was applied, implies that these proteins were lost by exsheathment and may be associated with the retained L2 sheath itself. Unfortunately, bands were not detected at similar masses when the experiment was repeated with bovine serum samples; there may be many explanations for the difference: the antigen might not be particularly immunogenic to calves, there may be a difference in the binding nature at either the first

FIGURE 8.4

Western Blot. The effect of detergent, cetyltrimethylammonium bromide (CTAB) action on sheathed and exsheathed L3 of *Ostertagia ostertagi* was examined by probing treated larvae with sera from a rabbit immunised with exsheathed larvae. Molecular mass standards in kilodaltons.

Lane 1, 2,000	**exsheathed L3s, CTAB treated;**
Lane 2, 1,000	**exsheathed L3s, CTAB treated;**
Lane 3, 2,000	**exsheathed L3s, no CTAB;**
Lane 4, blank;	
Lane 5, 2,000	**sheathed L3s, CTAB treated;**
Lane 6, 1,000	**sheathed L3s, CTAB treated;**
Lane 7, 2,000	**sheathed L3s, no CTAB;**
Lane 8, 1,000	**sheathed L3s, no CTAB.**

Treatment of both sheathed and exsheathed larvae with CTAB led to an increase in the intensity of the stained antigen profiles.

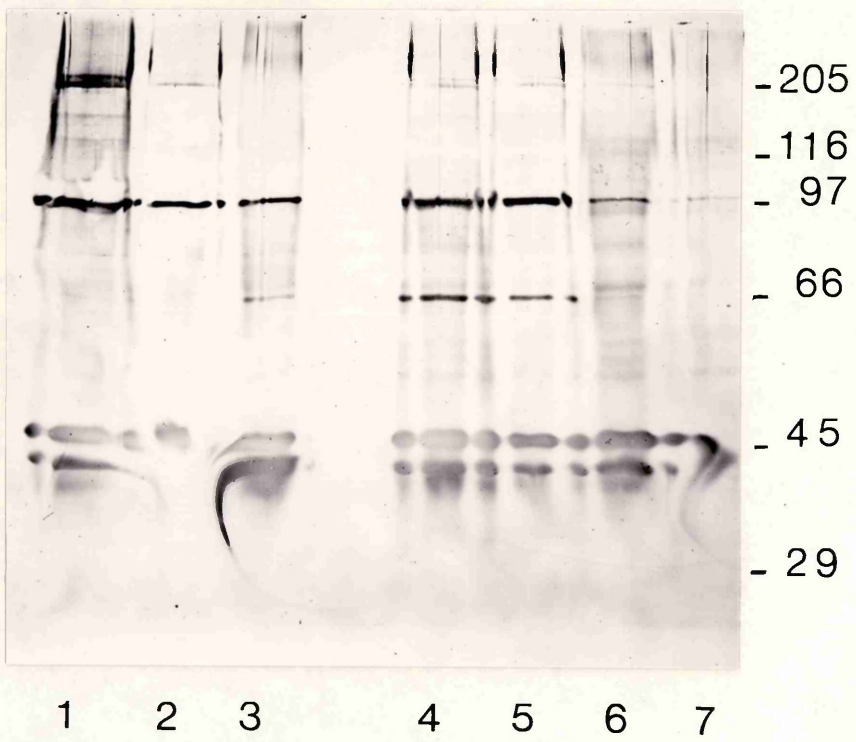
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TABLE 8.2

The effect of detergent treatment on the antigen profile of exsheathed and sheathed larvae recognised by sera from a rabbit immunised with exsheathed larvae

Molecular mass in kilodaltons	Larval preparation			
	Sheathed	Sheathed +CTAB	Exsheathed	Exsheathed +CTAB
165	+	+ (d)	+	+ (d)
150		+		+
135	+	+	+	+
128	+	+	+	+
108	+	+	+	+
105	+			
94	+	+	+	+
82	+	+	+	+
78	+	+	+	
67	+	+		
63	+	+		
60	+	+		
48	+	+	+	+
45	+	+	+	+

(d) denotes doublet banding

or second antibody level, the *in vitro* response might not be a true representation of the effect *in vivo*.

The comparison of L3, L4 and adult *O.ostertagi* by Western Blotting demonstrated that several antigens were common to all three stages. There was a general increase in the number of the bands in the antigen profile in later parasite stages. However, serum from rabbits immunised with different stages might identify additional antigens, so before concluding that the antigen pattern varies little between stages sera collected from animals of the same species immunised with other stages should be assessed.

The antigen profiles of the five trichostrongyle species appeared alike by Western Blotting, implying that rabbits immunised against *O.ostertagi* L3, could recognise similar antigens in other species. The cross-reactivity between trichostrongyle species might explain the occasional presence of antibodies in pre-immunisation calf serum. It seems possible that some of the calves may have been vaccinated orally against *Dictyocaulus viviparus* and this might result in the recognition of shared antigens. Serum collected from non-vaccinated calves pre-immunisation appeared to be a reliable negative control. However, the possible running of homogenates of *D.viviparus* on blots and checking for cross-reactivity would give a more reliable explanation for these unexpected findings.

The comparison of isolates of *O.ostertagi* showed that there were very strong similarities in their antigenic profiles by Western Blotting. The only differences in pattern were very faint and could be due to low levels of antigen rather than a true difference in the antigen profile. Some protein denaturation occurs during sample preparation, electrophoresis and blotting. Consequently, the antigens recognised by blotting may be a subset of the full antigen repertoire. Furthermore, locally produced antibody might have given different results to serum antibody, but measurement of local antibody responses were outwith the scope of this work.

In conclusion, later stages of *O.ostertagi* gave more complex antigen profiles than earlier stages; this was due to an increase in the number

of antigens detected rather than a complete change in the profile. The antigen profiles of the different isolates of *O.ostertagi* were similar as were the profiles of the different trichostrongyle species examined.

8.3 INDIRECT FLUORESCENT ANTIBODY TESTING (IFAT)

8.3.1 Introduction

Indirect fluorescent antibody testing allows visualisation of antibody bound to a parasite. Fluorescent staining is produced by the addition of a second antibody conjugated to a fluorescent dye, e.g., fluorescein di-isothyocyanate or rhodamine, which binds to the first antibody. The distribution of staining may indicate sites of particular immunogenicity. Additionally, surface turnover may be assessed by comparison of staining over time; the effect of temperature and metabolic inhibitors on turnover may also be tested.

8.3.2 Materials and methods

The basic technique is outlined below and further details of individual experimental runs follow. Aliquots of 500 larvae were prepared since they gave a visible pellet and so facilitated supernatant removal. They were washed three times in PBS, pH 7.2, with centrifugation at 2,000g for 5 minutes and finally suspended in 50µl PBS in 1.5ml eppendorfs.

The serum samples were those used in the Western Blotting (Chapter 8, 8.2.2.1), the rabbit post-immunisation sera were collected at the 72 day bleed. Pre-immunisation serum samples acted as the negative controls; two dilutions were used: 1:10, 1:20. Four serial dilutions of the post-immunisation serum were made: 1:10, 1:20, 1:40, 1:80.

Two hundred µl aliquots of serum were incubated with 500 larvae in 50ul PBS for 30 minutes on ice at 4°C and mixed occasionally. The larvae were given five washes in 1.5ml PBS at 2,000g in a MSE Microcentaur centrifuge to remove unbound antibody.

After the final wash the larvae were left in approximately 30µl PBS and incubated for 30 minutes at 4°C in the dark with 30µl FITC

antibody raised against the species responsible for the first level of antibody binding. Unbound FITC-antibody was removed at the end of incubation in five further washes with PBS. The samples were stored in the dark until they could be examined.

Silicone grease was used to demarcate a square on a slide and the sample was pipetted into this area and a coverslip put into place. The slides were examined using a 580 FITC filter (fluorescein emits energy of 554 to 583nm). The larvae were assessed for motility and the intensity and distribution of fluorescence.

8.3.3 Results

Experiment 1:

Sheathed *O.ostertagi* larvae were incubated with sera from the rabbit immunised with homogenates of sheathed *O.ostertagi* larvae at 4°C and exsheathed larvae were reacted with rabbit serum from the animal given exsheathed larvae. An immune response was tested for with the second antibody anti-rabbit IgG (Goat) 1:10 in PBS. The experiment was repeated using calf serum and anti-bovine IgG (donkey).

Results:

The results are shown in Table 8.3. Both the exsheathed and sheathed larvae were recognised by the respective sera from the homologous inoculations. The reaction was stronger for the sheathed larvae, i.e., fluorescence was noted at all dilutions of serum and the intensity was greater and more uniform. No fluorescence was noted when larvae were reacted with pre-immunisation serum. The larval samples in the lowest dilution of post immunisation sera were stored overnight in the dark and reassessed, i.e., those samples with the highest percentage of fluorescing larvae and the greatest intensity of staining. The exsheathed larval sample no longer fluoresced after 16 hours, while the sheathed larvae still had a low level of fluorescence. The results with bovine serum showed very faint surface fluorescence at the lowest dilution of post infection serum, but the intensity was too low for assessment of turnover or for comparative studies.

TABLE 8.3

Examination of the immune response directed against sheathed or exsheathed larvae when reacted at 4°C with serum from rabbits immunised with the equivalent larval antigen as an homogenate.

Tube Number	Larval Type	Serum Type and Dilution	Number Fluorescing and Relevant Comments
1	Ex#	Pre-Ex* 1:10	0:20 100% motile.
2	Ex	Pre-Ex 1:20	0:20 100% motile
3	Ex	Post-Ex 1:10	20:20 100% motile. More difficult to focus precisely on the larval edge relative to sheathed larvae, suggesting a rougher surface.
4	Ex	Post-Ex 1:20	12:20 100% motile. Variable staining of larvae within the batch.
5	Ex	Post-Ex 1:40	2:20 100% motile. Patchy fluorescence
6	Ex	Post-Ex 1:80	2:20 100% motile.
7	Ex	Post-Ex 1:10	Standard FITC 0:20 100% motile.
8	Ex	-	0:20 100% motile.
9	Sh	Pre-Sh 1:10	0:20 100% motile.
10	Sh	Pre-Sh 1:20	0:20 100% motile.
11	Sh	Post-Sh 1:10	20:20 100% motile. Blue-green staining was very intense and clearly demarcated the parasite surface. A fine rim of more yellow- green autofluorescence was recognised within. The tail tip was distinct.
12	Sh	Post-Sh 1:20	20:20 100% motile.
13	Sh	Post-Sh 1:40	20:20 100% motile.
14	Sh	Post-Sh 1:80	20:20 100% motile.
15	Sh	Post-Sh 1:10	Standard FITC 20:20 100% motile.
16	Sh	-	0:20 100% motile.

- Larval type: Ex-exsheathed, Sh-sheathed.
* - Serum type: Pre-Ex/Sh - Pre-immunisation with exsheathed/sheathed larvae, Post-Ex/Sh - Post-immunisation with exsheathed/sheathed larvae.

Experiment 2:

Exsheathed *O.ostertagi* larvae were reacted with sera from the rabbit immunised with homogenates of sheathed *O.ostertagi*, while sheathed larvae were reacted with serum from the rabbit given exsheathed larval homogenates at 4°C.

Results:

Results are entered in Table 8.4. Staining was less intense with all the reciprocal larvae-sera reactions. Sheathed larvae produced a stronger staining reaction than the exsheathed larvae, although there was a loss of uniformity of staining overall and a reduction in intensity with increased serum dilution. Exsheathed larvae were only recognised at the lowest dilution with serum from a rabbit immunised with sheathed larvae.

Experiment 3:

The protocol was essentially the same as in Experiment 1, but the eppendorfs were incubated at 37°C. The effect of increasing the incubation temperature on pattern and intensity of staining was assessed to gain information about changes in metabolism at higher temperatures, which may alter rate of surface turnover and specificity of binding to epitopes.

Results:

Results are given in Table 8.5. Increasing the incubation temperature from 4°C to 37°C led to a reduction in staining intensity of both sheathed and exsheathed larvae. Stain intensity decreased with reduction in serum concentration, especially with the sheathed larvae. In addition, the staining of the sheathed larvae became less uniform with reduction in serum concentration and individual staining varied more.

TABLE 8.4

Examination of the immune response directed against sheathed or exsheathed larvae when reacted at 4°C with serum from the rabbit immunised against the opposite form of homogenised larval antigen.

Tube Number	Larval Type	Serum Type and Dilution	Number Fluorescing and Relevant Comments
1	Ex#	Pre-Sh* 1:10	0:20 100% motile.
2	Ex	Pre-Sh 1:20	0:20 100% motile.
3	Ex	Post-Sh 1:10	16:20 100% motile. Patchy fluorescence, anterior staining strongest.
4	Ex	Post-Sh 1:20	0:20 100% motile.
5	Ex	Post-Sh 1:40	0:20 100% motile.
6	Ex	Post-Sh 1:80	0:20 100% motile.
7	Ex	Post-Sh 1:10	Standard FITC 0:20 100% motile.
8	Sh	Pre-Ex 1:10	0:20 100% motile.
9	Sh	Pre-Ex 1:20	0:20 100% motile.
10	Sh	Post-Ex 1:20	20:20 100% motile. Fluorescence was markedly reduced and patchy relative to the same sample reacted with serum from the rabbit immunised with sheathed larvae.
11	Sh	Post-Ex 1:20	20:20 100% motile.
12	Sh	Post-Ex 1:40	20:20 100% motile.
13	Sh	Post-Ex 1:80	20:20 100% motile. There was a reduction in staining intensity of individual larvae with increased serum dilution.
14	Sh	Post-Ex 1:10	Standard FITC. 0:20 100% motile.

- Larval type: Ex-exsheathed, Sh -sheathed.
* - Serum type: Pre-Ex/Sh-Preimmunisation with exsheathed/sheathed larvae. Post-Ex/Sh-Post-immunisation with exsheathed/sheathed larvae.

TABLE 8.5

Examination of the immune response directed against exsheathed or sheathed larvae when reacted at 37°C with serum from rabbits immunised with the equivalent larval antigen.

Tube Number	Larval Type	Serum Type and Dilution	Number Fluorescing and Relevant Comments
1	Ex#	Pre-Ex* 1:10	0.20 100% motile.
2	Ex	Pre-Ex 1:20	0:20 100% motile.
3	Ex	Post-Ex 1:10	20:20 100% motile. Blotchy staining.
4	Ex	Post-Ex 1:20	20:20 100% motile.
5	Ex	Post-Ex 1:40	4:20 100% motile.
6	Ex	Post-Ex 1:80	3:20 100% motile.
7	Ex	-	0:20 100% motile.
8	Sh	Pre-Sh 1:10	0:20 100% motile.
9	Sh	Pre-Sh 1:20	0:20 100% motile.
10	Sh	Post-Sh 1:10	20:20 100% motile. Marked decrease in staining intensity relative to incubation at 4°C.
11	Sh	Post-Sh 1:20	20:20 100% motile.
12	Sh	Post-Sh 1:40	20:20 100% motile.
13	Sh	Post-Sh 1:80	19:20 100% motile.
14	Sh	-	0:20 100% motile

- Larval type - Sh-sheathed, Ex-exsheathed.
* - Serum type - Pre-Sh/Ex- Preimmunisation with sheathed/exsheathed larvae. Post-Sh/Ex- Post-immunisation with sheathed/exsheathed larvae.

Experiment 4:

The protocol was essentially the same as Experiment 1, but carried out in quadruplicate. Only the 1:10 pre-immunisation dilution was used and 1:10 and 1:20 post-immunisation dilutions. Fluorescence was compared after the four groups of samples had been treated individually as follows:

1) examination on day 1, i.e., as Experiment 1.
2) examination on day 2, i.e., 24 hours after labelling and storage in the dark at 4°C.
3) examination after storage for 24 hours at 4°C, washing five times in PBS to remove unbound conjugated stain moieties and storage for a further 60 hours at 4°C.
4) examination after storage at room temperature for 24 hours (18°C), washing five times in PBS and storage for a further 60 hours at 4°C.

All samples were stored in the dark. Fresh larval samples were prepared for comparison with groups 2, 3 and 4.

Results are shown in Table 8.6. Motility was retained over 84 hours. Over 84 hours fluorescence became reduced and the decrease in intensity of fluorescence was more rapid with the exsheathed larvae.

8.3.4 Discussion

Initial work with the IFAT using sheathed *O.ostertagi* L3 as the antigen and serum from repeatedly infected calves gave poor results. The work was carried out at room temperature, which in retrospect could have led to loss of fluorescence through surface turnover. Vetter and Klaver-Wesseling (1978) studying *Ancylostoma caninum* found incubation over liquid nitrogen or the addition of the metabolic inhibitor, azide allowed IgG from immunised dogs to bind to the infective larval surface. Previously, at room temperature they had been unable to demonstrate indirect fluorescent antibody testing. They suggested this was evidence of cuticle metabolic activity.

TABLE 8.6

Comparison of indirect antibody staining of sheathed and exsheathed *O.ostertagi* reacted with the equivalent larval antigen after varying storage regimes

Serum type		Fluorescence Group 1	2	24 hours Fresh	3	4	84 hours Fresh
Sheathed larvae							
Pre-Sh#	1:10	0:20	0:20	0:20	0:20	0:20	0:20
Post-Sh	1:10	20:20	20:20	20:20	19:20	19:20	20:20
Post-Sh	1:20	20:20	19:20	17:20	20:20	20:20	20:20
Post-Ex	1:10	20:20	20:20	20:20	20:20	20:20	20:20
Exsheathed larvae							
Pre-Ex	1:10	0:20	0:20	0:20	0:20	0:20	0:20
Post-Ex	1:10	20:20	0:20	12:20	0:20	0:20	0:20
Post-Ex	1:20	11:20	0:20	6:20	0:20	0:20	0:20
Post-Sh	1:10	13:20	0:20	10:20	0:20	0:20	19:20

- Serum type: Pre-Sh/Ex - Preimmunisation with sheathed/exsheathed larvae, Post-Sh/Ex - Post-immunisation with sheathed/exsheathed larvae.

Group 1 - examination of freshly labelled larvae.
Group 2 - examination of larvae 24 hours after labelling.
Group 3 - examination of labelled larvae stored for 24 hours at 4°C, washed five times and stored for a further 60 hours at 4°C.
Group 4 - examination of labelled larvae stored for 24 hours at 18°C, washed five times and stored for a further 60 hours at 4°C.

Furthermore, in the early experimental work the controls with pre-immunisation serum appeared to fluoresce in a similar fashion to the post-immunisation samples. However, the intensity was low and the colour was more yellow than that expected with fluorescein staining. Much of the staining in these samples appeared to be internal. Once the correct viewing conditions were employed it became clear that the nematodes were autofluorescing and only a very faint blue-green fluorescein glow was visible at the surface indicating low binding of the bovine antibody.

Fixation of the sample in 10% formalin solution before examination led to larval death and had a marked effect on the surface structure. The process prevented examination of surface turnover and was consequently discarded. Evan's Blue at a concentration of 0.1% quenched autofluorescence, so surface staining alone was shown, but again resulted in larval death and was also discontinued.

Subsequently, much of the work was conducted using serum generated in rabbits, since the positive staining reaction was stronger. The improved staining with the rabbit serum relative to the bovine serum may be due to a greater antibody response with a different route of immunisation or may be a reflection of the more efficient response mounted by the rabbit to *O.ostertagi* antigens or could be a difference in binding at the second antibody level, as mentioned previously.

Exsheathed *O.ostertagi* larvae were recognised by serum from the rabbit immunised with homogenates of exsheathed larvae. The same effect was seen when sheathed larvae were reacted with serum from the rabbit immunised with homogenates of sheathed larvae, but the staining was more intense and uniform with the sheathed antibody-antigen reaction.

The experiments with the reciprocal larval and serum types also led to antibody binding. The serum generated when homogenates of sheathed larvae acted as the immunogen would be expected to react with the surface of the exsheathed larva, but it is more surprising that the serum from the rabbit immunised with the exsheathed larvae could recognise the sheathed larval surface, albeit with less intensity than the exsheathed larva. The ability of antibody raised against exsheathed

larvae to recognise the sheathed larvae could be due to the occurrence of common epitopes on the retained L2 sheath and the true L3 surface or the inclusion of a small amount of sheath material in the immunogen, which is plausible considering the appearance of the exsheathed larvae on SEM (Chapter 5, 5.1.3.4).

Fluorescent staining was less intense when samples were incubated at 37°C instead of 4°C. The results of this study suggest surface turnover occurs at a greater rate at 37°C than 4°C, particularly with sheathed rather than exsheathed L3s. Surface turnover took place at 4°C also, but was more obvious in exsheathed than sheathed larvae.

In conclusion, for IFAT the long-term storage of trichostrongyle larvae should be avoided, but when this is unavoidable the material should be refrigerated. The finding may have wider application, it may help to explain the poor results achieved by biotinylating the surface of the sheathed L3 to examine the protein profile. During biotinylation the samples are incubated at 20°C for 1 hour, which may result in the partial loss of bound protein (Chapter 9).

CHAPTER 9

THE NEMATODE SURFACE: EXPERIMENTAL STUDIES ON *OSTERTAGIA OSTERTAGI*

THE NEMATODE SURFACE: EXPERIMENTAL STUDIES ON *OSTERTAGIA OSTERTAGI*

9.1 INTRODUCTION

The nematode surface is of particular importance as one of the chief sources of antigen initially encountered by the host, since it is the primary interface between host and parasite (Lumsden, 1975). Surface antigens may form a proportion of the excretory-secretory (ES) products of a nematode, which are often immunogenic, these consist of material shed from the nematode surface or secreted or excreted by the nematode. The general structure of the nematode surface has been reviewed recently by Lumsden (1975), Bird (1980) and Wright (1987). The salient points are summarised below beginning at the outer surface and progressing inwards.

Certain nematodes have a surface coat rich in carbohydrate (the glycocalyx), which is demonstrated with cationised ferritin labelling as a gap between the iron particles and the outer nematode structures; the cationised ferritin binds electrostatically to the negatively charged surface. The components of the glycocalyx are thought to be secreted by the epicuticle.

The epicuticle is usually trilaminate, although more layers are present in certain species, and has a high lipid content. The lipid reduces the surface permeability, so little other than some water and non-polar substances penetrate (Bird, 1980). Scott, Diala, Moraga, Ibrahim, Redding and Tamashiro (1988) suggested glycolipid at the nematode surface may stabilise peptides in the cuticle and shield them from the host and so aid persistence of parasitic infection in immunocompetent hosts. The epicuticle is similar to a cell membrane in some ways and a bacterial envelope in others; it is non-particulate, extracellular and largely impermeable (Bird, 1980). On freeze fracture analysis the epicuticle is seen to consist of interdigitating projections (Wright, 1987). A thick proteinaceous layer may exist between the epicuticle and the underlying cuticle (Kennedy, Foley, Kuo, Kusel and Garland, 1987b).

The cuticle may be subdivided into three zones: an amorphous electron dense external cortical zone, a more fluid median zone with globular bodies and a striated basal zone. Elastin predominates in the external cortex of the cuticle, but is gradually replaced by collagen in the deeper layers. The hypodermal cells underneath the cuticle are high in elastin and produce cuticle between moults (Lumsden, 1975). The depths of the different layers vary across species and with development. In addition, the thickness of the cuticle appears to vary with temperature and humidity of the environment encountered by the majority of that species (Bird, 1980). The free-living nematode *Caenorhabditis elegans*, has a resting form, the dauer, which shares the longevity of the free-living infective L3 and the reduction in activity of the arrested L4 of *Ostertagia ostertagi*, respectively. The dauer cuticle is noticeably thicker than that of the active stages (Bird, 1980). Specialized areas are present in the nematode cuticle, for example, the alae (where the collagen is expanded), annulations (where the cortical and median zones are expanded) and pores (Maizels and Selkirk, 1988). Ortega-Pierres, Clark and Parkhouse (1986) observed specific binding of the lectin Concanavalin A and certain monoclonal antibodies to the cloaca of the male *Trichinella spiralis*.

Nematodes moult four times during the life cycle and the accompanying changes in the surface pattern have been illustrated (Philipp, Taylor, Parkhouse and Ogilvie, 1981), although some antigens may persist from one stage to another (Maizels *et al.*, 1982). Abraham *et al.*, (1988) found differences between the stages of *Dirofilaria immitis* in surface charge (L3- negative, L4- weakly positive/neutral) and lectin binding (Concanavalin A bound to the surface of the L4, but not to the L3). The surface structures appear to be highly dynamic; continuous shedding of cells and production of cuticle between moults have been illustrated by radiolabelling parasites and analysing the loss of label into the culture medium (Maizels *et al.*, 1982; Maizels, de Sauvigny and Ogilvie, 1984; Philipp *et al.*, 1980). Marshall and Howells (1986) radiolabelled the surface of L3, L4 and adult *Brugia pahangi*, implanted them into the peritoneum of jirds and compared the radioactivity of recovered parasites after increasing time. Electrophoresis and autoradiography demonstrated that different proteins were lost depending on the stage and that the rate of surface

turnover altered with stage. Rate of surface turnover has a number of contributing factors, for example it is increased with raised temperature and reduced with metabolic inhibitors (Vetter and Klaver-Wesseling, 1978; Smith, Quinn, Kusel and Girdwood, 1981).

The aim of this chapter was to examine and compare the properties of the surface of *O.ostertagi* during development to try to gain information on the regulation of change. In addition, samples of different trichostrongyle species and isolates of *O.ostertagi* from different sources were examined. It is known that related species of nematode may have varying, but also some common epitopes, e.g., *Toxocara cati* and *T.canis* (Kennedy *et al.* 1987a), so a further goal was to test this finding with respect to different trichostrongyle species and to assess the extent of variation within species by examination of different isolates of *O.ostertagi*. In the past, nematode surfaces have been studied *in situ* and after chemical and physical stripping processes; most of the techniques discussed here examine the surface *in situ*, although some study of chemical stripping was also made.

9.2 SURFACE PROTEIN PROFILES

9.2.1 Introduction

Biotin-avidin affinity was first recognised when a diet of raw egg white fed to rats was found to induce biotin deficiency. The glycoprotein, avidin present in egg white bound to the prosthetic group/vitamin, biotin, so preventing its absorption by the intestine. The dissociation constant for the coupling is greater than that in the antibody-antigen reaction (10^{10} to 10^{-12}) at 10^{-15} M^{-1}, so the avidity of the binding can be used as the basis of sensitive labelling reactions. Experimentally streptavidin is used in preference to avidin, since it has an isoelectric point closer to neutrality and it contains no carbohydrate groups which could produce non-specific binding. Each streptavidin molecule has four binding sites for biotin. Streptavidin is isolated from *Streptomyces avidinii* and for experimental purposes it was obtained pre-labelled with radio-iodine by the chloramine-T method.

^{12}Biotin-N-hydroxysuccinamide (NHS-biotin) binds covalently to free lysine amino acid groups and to a lesser extent to tyrosine groups (Hurley, Finkelstein and Holst, 1985). Similarly biotin-hydrazide binds to free carboxyl/keto groups. Consequently, activated biotin reagents may be used to examine surfaces (Hurley *et al.*, 1985), functional groups, antibodies, lectins or other molecules using avidin conjugated to fluorochromes, radioisotopes or peroxidase. Marshall and Howells (1985) compared the available direct radiolabelling techniques and found different amino acids were bound depending on the method; the iodogen, chloramine-T and lactoperoxidase methods bound to tyrosine, the iodosulphanilic acid technique bound to lysine, histidine and tyrosine and the Bolton-Hunter technique bound epsilon amino acids, including lysine. By indirect radiolabelling, when NHS-biotin binds to peptides and the affinity of ^{125}I-streptavidin is employed to probe for the biotin, the biotinylated samples loaded for electrophoresis were not radioactive and thus the handling of radioactive material was much reduced over direct radiolabelling methods. Peptide patterns obtained by electrophoresis do not appear to be substantially altered by sample biotinylation. Furthermore, biotinylated samples may be prepared and stored for later analysis, without the loss of activity which would occur with the equivalent radiolabelled sample at a rate dependent on the isotope half life.

Previously NHS-biotin was used to label intact and disrupted bovine leukocytes (Hurley *et al.* 1985). They found by biotin-labelling intact leukocytes certain peptides were detected, but if the cells were treated with trypsin after biotinylation activity was equivalent to that of the non-treated cell, which suggested the biotin had only labelled the surface. At the start of the study the penetration of biotin at the nematode surface could not be predicted, but the work of Hurley *et al.* (1985) and the known low permeability of the nematode surface (Bird, 1980) suggested investigation would be worthwhile, although the extent of penetration would have to be assessed.

9.2.2 Polyacrylamide gel electrophoresis and biotinylation

Initially, nematode protein profiles were assessed by Coomassie Blue and Silver staining, to obtain a total protein profile for comparison with

biotin-labelled material. The presence of very high numbers of peptide bands produced a complex pattern with nematode homogenates. The purpose of the biotin-streptavidin affinity was to try to identify a subset of nematode polypeptides, perhaps the surface proteins, by labelling intact nematodes rather than total homogenates. The highly restricted permeability of the nematode surface (Bird, 1980) and the peptide profiles of intact labelled leukocytes before and after trypsin treatment (Hurley *et al.*, 1985) suggested that NHS- biotin penetration of *O.ostertagi* would be low. Consequently, labelling of intact parasites might reveal surface peptide profiles of the nematodes.

9.2.2.1 Protein staining of parasite homogenates after polyacrylamide gel electrophoresis:

1. Experiments were conducted to find the optimal *O.ostertagi* sample preparation for separation of polypeptides by polyacrylamide-gel electrophoresis. The experiment was repeated with different parasite stages for comparison of their polypeptide profiles.

Duplicate samples of different numbers of *O.ostertagi* L3, 1,000; 2,000; 5,000 and 10,000, were homogenised by hand (0.1ml glass-to-glass homogeniser, Jencons) in 60µl homogenisation buffer with protease inhibitors (Chapter 4, 4.3.4). The homogenate was spun at 10,000g for 5 minutes (MSE Microcentaur), the supernatant was withdrawn and 15µl of sample buffer was added. The sample was boiled for 2 minutes and centrifuged for a further 5 minutes at 10,000g. A 10% polyacrylamide gel (Chapter 4, 4.3.1) was loaded in duplicate with increasing concentrations of sample to give two identical halves; a standard molecular weight marker was also applied. After electrophoresis, one half of the gel was stained with Silver stain (4.3.1.1) and the other with Coomassie blue (4.3.1.2). Similar analysis was carried out with exsheathed L3s and other parasite stages.

Results:
Similar polypeptide profiles were revealed by Silver and Coomassie blue staining. Although fewer parasites were required for Silver staining (1,000 to 2,000 L3), the Coomassie blue Stain was favoured (5,000 to 10,000 L3), due to its greater simplicity. Large fragments of

the cuticle remained in samples of sheathed L3s and L4 parasites after homogenisation and this appeared to affect the band resolution, which was much improved by using exsheathed larvae. No sheath-specific bands were seen. The optimal loading amounts were similar for all the pre-parasitic larvae, 10,000 L3 was found to give a sample "track" with clearly defined peptide activity, while 5,000 L4s and 20 adults gave sufficient staining activity. The polypeptides identified in the different stages, are shown in Table 9.1. The early pre-parasitic stages had fewer major polypeptides than the L3s and parasitic stages. Figure 9.1 compares L3 and L4 samples after PAGE and Coomassie blue staining. Generally, when a new peptide was first noticed in the profile it remained in later developmental stages. The main differences between stages were in the intensity of bands; the complement of polypeptides barely altered with stage, but their relative importance did. Thus, bands of similar intensity were seen at 90 kilodaltons in the L3 and L4 stages, but the bands at 190, 100, 78 and 52 were stronger in the exsheathed L3 stage.

2. Different trichostrongyle species were examined by polyacrylamide-gel electrophoresis followed by Coomassie blue staining for interspecies protein differences.

Ten thousand sheathed L3s of *Cooperia oncophora*, *Haemonchus contortus* (avermectin susceptible and resistant), *O.circumcincta*, *O.ostertagi* and *Trichostrongylus colubriformis* were prepared as above and loaded on a 10% polyacrylamide gel. After electrophoresis, the gels were stained with Coomassie blue.

Results:
The marked similarity in polypeptide profile between the trichostrongyle species examined was striking (Figure 9.2); Table 9.2 lists the polypeptide bands identified. Additional bands were present in the *H.contortus* samples at 52 to 61 kilodaltons.

3. The polypeptide patterns of isolates of *O.ostertagi* from different geographical sources were examined by polyacrylamide gel electrophoresis and Coomassie blue staining.

TABLE 9.1

Polypeptide profiles of developing *Ostertagia ostertagi* parasites as demonstrated by polyacrylamide gel electrophoresis and direct protein staining with Coomassie blue.

Molecular mass in kilodaltons	L1	L2	L3	ExL3*	L4
235				(+)	
215				(+)	
190		+	+	+++	+
180		+	+	+++	
150			(+)	(+)	
130			(+)	(+)	
110		+	+++	+++	++
105			(+)	++	(+)
100			(+)	+	
90			++	++	++
84					++
78			+++	+++	++
71		+			
68		+	+	+	
56					++
52			++	++	(+)
48		+	+	+	(+)
41	+	+	+++	+++	+++
37		+	++	++	+
31		+	++	+	+
29		+		+	
27		+	+	+	+
25				+	+
24			+	+	+
23				+	
22		+			+
21				+	

*Third stage larvae exsheathed by treatment with sodium hypochlorite solution followed by three rinses in excess PBS.

FIGURE 9.1

SDS-PAGE analysis of the L3 and L4 stages of *Ostertagia ostertagi* stained with Coomassie blue.

Lane 1	**Molecular mass markers in kilodaltons.**
Lane 2	**10,000 L3**
Lane 3	**5,000 L3**
Lane 4	**10,000 L4**
Lane 5	**5,000 L4**

Bands of similar mass were stained in both the L3 and L4 stages, but their relative intensity varied with stage.

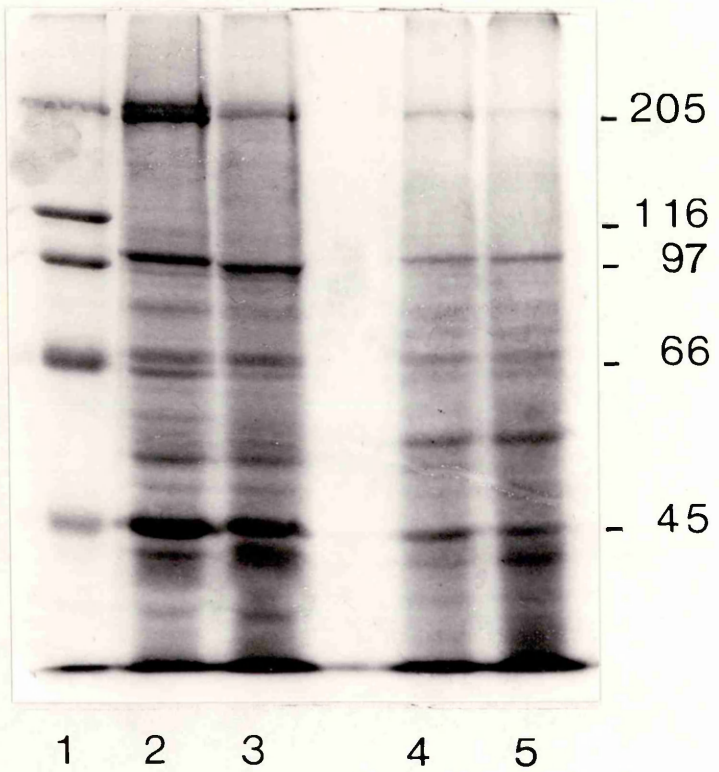

205
116
97
66
45
1
2
3
4
5

FIGURE 9.2

Comparison of six different species of trichostrongyle by SDS-PAGE followed by Coomassie blue staining. Molecular mass standards in kilodaltons.

Lane 1 *Cooperia oncophora*;
Lane 2 *Haemonchus contortus* **(avermectin resistant);**
Lane 3 *Haemonchus contortus* **(avermectin susceptible);**
Lane 4 *Ostertagia circumcincta*;
Lane 5 *Ostertagia ostertagi*;
Lane 6 *Trichostrongylus colubriformis*.

Marked similarities may be seen between the peptide profiles of the different trichostrongyle species examined, although the *Haemonchus contortus* **samples showed additional bands at 52 to 61 kilodaltons (arrows).**

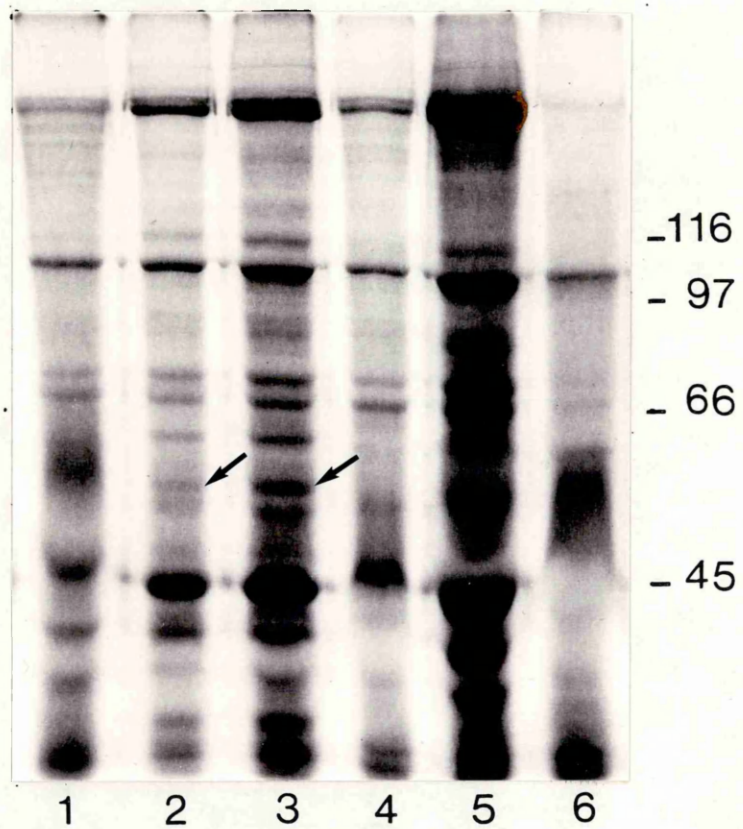

116
97
66
45
1
2
3
4
5
6

TABLE 9.2

Comparison of the major polypeptide patterns exhibited by different trichostrongyle species on polyacrylamide gel electrophoresis followed by Coomassie blue staining.

Molecular mass in kilodaltons	C.o[1]	H.c R[2]	H.c S[3]	O.c[4]	O.o[5]	T.c[6]
190		(+)	(+)		(+)	
175		(+)	(+)		(+)	
160		(+)	(+)	(+)	(+)	
155 (doublet)	++	+++	+++	+++	+++	(+)
145 (doublet)	(+)		(+)		(+)	
140	(+)			(+)	+	
135		+	+	+	+	
130	(+)			(+)		
120	(+)					
110	+	++	++	+	++	
100	++	++	++	++	++	++
84		+	+		+	
80		+	+		+	
74	++	++	++	++	++	(+)
68	++	++	++	++	++	
61		++	++			
54		++	++			
53				+	++	
52		++	++			
46	++	++	++	++	++	
38	++	++	++	++	++	
33	+	+	+	+	+	

1 *Cooperia oncophora* L3
2 *Haemonchus contortus* L3, avermectin resistant
3 *Haemonchus contortus* L3, avermectin susceptible
4 *Ostertagia circumcincta* L3
5 *Ostertagia ostertagi* L3
6 *Trichostrongylus colubriformis* L3

Ten thousand sheathed L3s of isolates from: Alabama, Denmark, Glasgow, Louisiana and Weybridge and 8,000 L3 of Morantel Susceptible Lelystad and Morantel Resistant types were prepared. Samples were loaded onto a 10% polyacrylamide gel and subsequently stained with Coomassie blue.

Results:

No variation in the polypeptides separated by polyacrylamide gel electrophoresis were observed (Figure 9.3). The weaker staining of the Lelystad samples was probably a reflection of the smaller sample size. Bands were present at positions corresponding to 190, 175, 166, 155, 145, 140, 135, 125, 110, 100, 94, 84, 80, 74, 71, 68, 53, 46, 41, 38 and 33 kilodaltons. The major peptides were 155, 110, 100, 74, 68, 53, 46 and 38 kilodaltons.

Discussion:

The optimal concentration of different stages of *O.ostertagi* for protein staining was determined and a table of the major peptides compiled. Proteins of equal mass were found in different stages, but the intensity of the band varied, which might be due to an alteration in expression during development. Poor solubility in the L3 sample of the retained L2 sheath appeared to disturb sample passage during electrophoresis and exsheathment of L3s gave a more distinct profile. The problem of loading equivalent sample amounts when comparing different stages was discussed in Chapter 7: the loading of similar parasite numbers for each stage would result in the relative overloading of adult samples due to the disproportionate increase in size, while loading similar protein amounts would alter the proportion of the protein types represented in the sample, with the increase in surface area to volume ratio in smaller organisms greater amounts of structural proteins would be recorded.

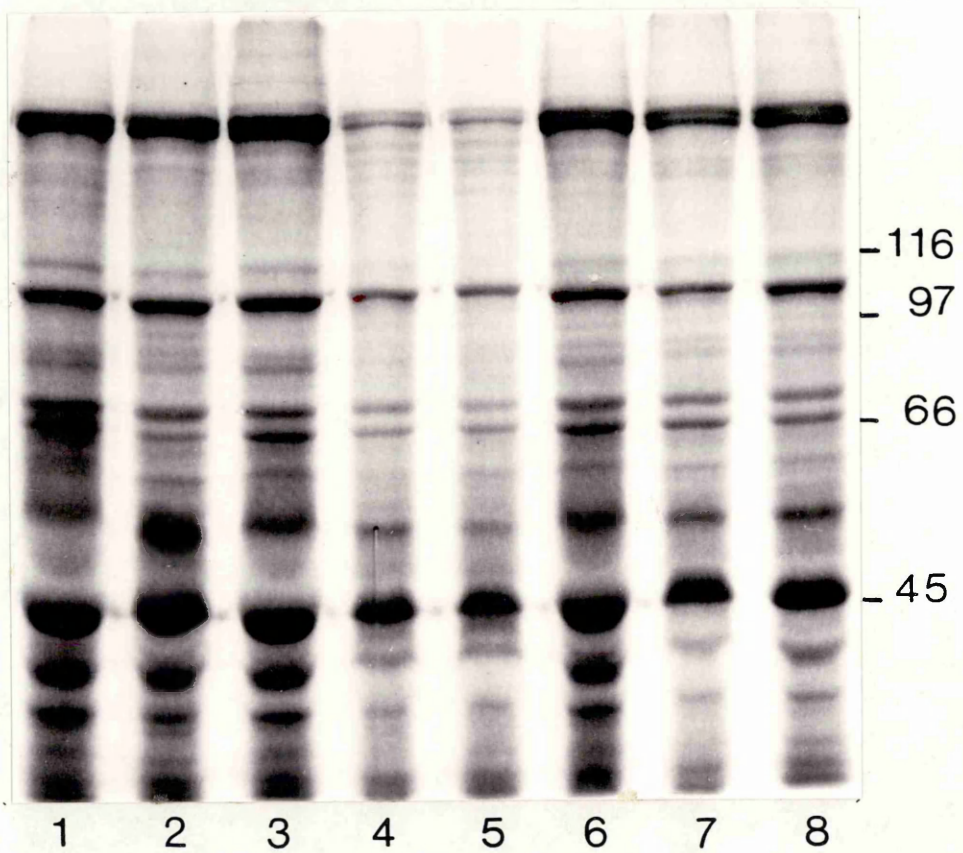
Comparison of trichostrongyle species and isolates of *O.ostertagi* demonstrated the polypeptide profiles were highly conserved, although *H.contortus* appeared to have some extra polypeptides at 52 to 61 kilodaltons, which may be associated with its feeding habits, which are more invasive than those of the other species examined. The lack of labelled peptide in the sample of *T.colubriformis* was probably due to a low sample size.

FIGURE 9.3

Comparison of eight different isolates of *Ostertagia ostertagi* by SDS-PAGE followed by Coomassie blue staining. Molecular mass standards in kilodaltons.

Lane 1, Weybridge;
Lane 2, New Jersey;
Lane 3, Louisiana;
Lane 4, Lelystad (Morantel Susceptible);
Lane 5, Lelystad (Morantel Resistant);
Lane 6, Glasgow;
Lane 7, Denmark;
Lane 8, Alabama.

Close similarity of peptide profile was seen when different isolates of *O.ostertagi* were examined.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From these results it is clear that a large number of polypeptides are present in trichostrongyle homogenates, so variation is difficult to detect, by the use of biotinylation it was hoped to distinguish a subset of peptides for comparison.

9.2.2.2 Polypeptide analysis by biotinylation of parasite samples

1. Experiments were conducted to find the optimal preparation of biotinylated L3s, which might produce a labelled subset of peptides through affinity to radiolabelled streptavidin, following separation by polyacrylamide gel electrophoresis and electroblotting.

Optimal sample amounts were found for biotinylated samples by a trial assessment similar to that used for Coomassie blue staining, but smaller samples were required to produce a visible effect.

Larvae for biotinylation were washed three times in excess phosphate buffered saline, PBS (pH 7.2) by centrifugation at 1,000g. One thousand, 2,000 and 4,000 L3 aliquots were centrifuged in eppendorfs (1.5ml) and the supernatants were discarded.

Preparation of labelled "intact" larvae: The larvae were suspended in 100µl of distilled water, then incubated in the dark at room temperature (20°C) for 1 hour with 3µl of 1% NHS-biotin in dimethyl-formamide, i.e., 30µg NHS-biotin. Unbound biotin was removed by washing the larvae three times in PBS (pH 7.2) at 10,000g for 5 minutes. The supernatant was withdrawn and the larvae homogenised in 50µl of homogenisation buffer with protease inhibitors (Chapter 4, 4.3.4).

Preparation of labelled homogenates: Homogenates of 1,000 to 2,000 larvae or 20 adults were prepared with 50µl of homogenisation buffer and centrifuged at 10,000g for 5 minutes. The supernatants were incubated with 3µl NHS-biotin. The reaction was stopped by adding ethanolamine and Tris pH 7.2 to final concentrations of 10mM and 100mM respectively. SDS-PAGE sample buffer (Chapter 4, 4.3.1) was added and the samples boiled for 2 minutes before loading on to 10% polyacrylamide gels. After electrophoresis, the gels were blotted onto a nitrocellulose acetate membrane (Amersham Hybond C).

Non-specific binding was blocked with 1% Tween 20 in Tris-saline for 1 hour.

The membrane was incubated with 15µl of radiolabelled ^{125}I-streptavidin solution at 100µCi/ml in 30ml Tris-saline, i.e., 0.05 µCi/ml for 1 hour on a rotating platform. With radioisotope disintegration the volume of ^{125}I-streptavidin had to be increased with time (^{125}I has a 60 day half life). The suggested concentration of ^{125}I-streptavidin in the Tris-saline incubation solution is 0.1 to 0.5 µCi/ml. It was important not to add the isotope directly to the blot, since that resulted in indiscriminant binding. Unbound isotope was removed by washing the blot five times in excess Tris saline over 30 minutes. The membrane was heat-sealed into a plastic pouch and placed in a film cassette with a sheet of Hyperfilm-MP autoradiography film (Amersham). Films were developed after varying lengths of time (1 day to 2 weeks) to give the optimal patterns for band clarity on the autoradiograph. Gamma rays produced by ^{125}I-streptavidin disintegration react with the silver bromide in the film emulsion, so portions of film where binding has occurred will be blackened on film development. In this way, the protein binding biotin was indicated by affinity to radiolabelled streptavidin.

Results:

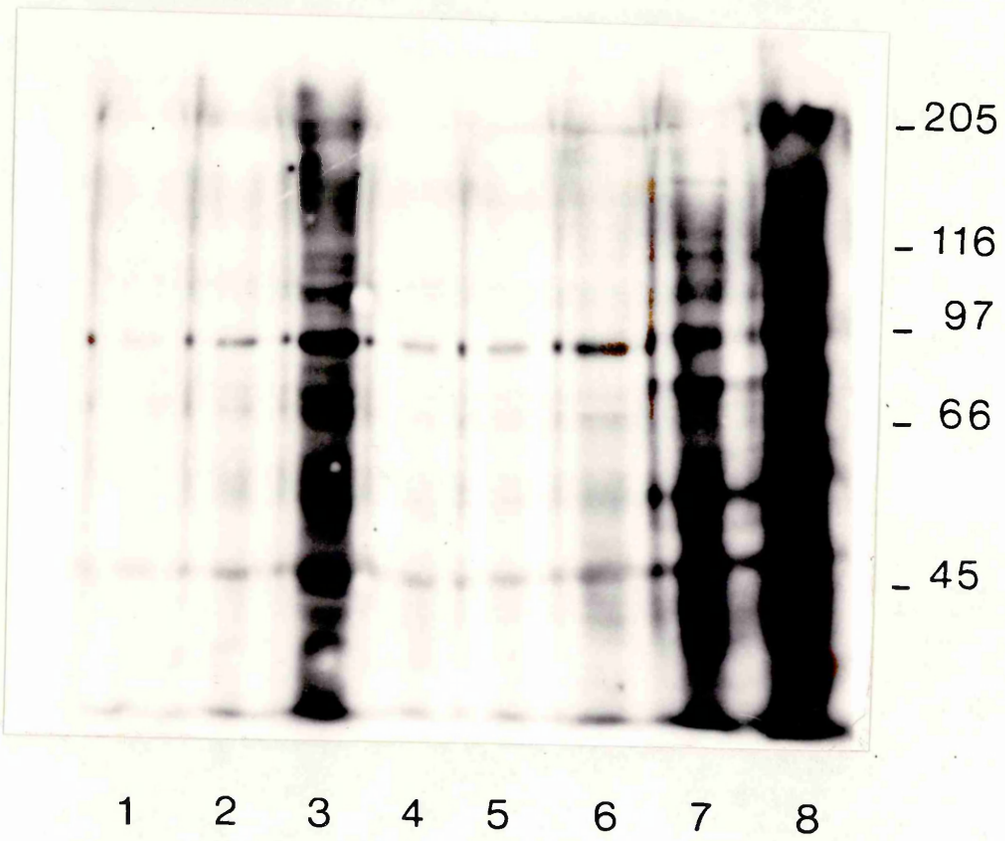
Biotinylation did not appear to affect the motility of intact larvae and the process was repeated with a number of different permutations, Figure 9.4 illustrates some of the findings with L3 preparations. Very low amounts of labelled material were present in tracks when intact sheathed larvae were examined (lanes 4 to 6). More peptides were present in the intact exsheathed sample (lanes 1 to 3). The intact exsheathed sample produced a fainter, but similar pattern to the homogenised samples, although certain bands were comparatively more intense in the exsheathed intact biotinylated specimens in lanes 1 to 3, e.g., 45 and 88 kilodaltons. Samples of 2,000 biotinylated L3 were found to give adequate banding activity with intact exsheathed or homogenised parasites. The background staining could be reduced to leave the bands at 45 and 88 kilodaltons in the exsheathed intact biotinylated samples by stopping further protein biotinylation by

FIGURE 9.4

Analysis of NHS-biotin labelled preparations of L3 of *Ostertagia ostertagi* by SDS-PAGE and electroblotting followed by probing with ^{125}I-streptavidin and subsequent autoradiography. Molecular mass markers are in kilodaltons.

Lane 1	**1,000**	**exsheathed intact biotin-bound L3s**
Lane 2	**2,000**	**exsheathed intact biotin-bound L3s**
Lane 3	**4,000**	**exsheathed intact biotin-bound L3s**
Lane 4	**1,000**	**sheathed intact biotin-bound L3s**
Lane 5	**2,000**	**sheathed intact biotin-bound L3s**
Lane 6	**4,000**	**sheathed intact biotin-bound L3s**
Lane 7	**2,000**	**sheathed homogenate biotin-bound L3s**
Lane 8	**4,000**	**sheathed homogenate biotin-bound L3s**

Bands at 45 and 88 kilodaltons were relatively more intense in the larvae biotinylated intact or as a homogenate.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making the suspension 10mM ethanolamine and 100mM Tris before washing and homogenising the samples.

2. Preparations of different parasite stages were biotinylated and separated by polyacrylamide gel electrophoresis; after electroblotting proteins were indicated by their affinity to radiolabelled streptavidin and their profiles compared.

Two thousand intact and homogenised larvae were biotinylated using the modified method developed as described above. The larvae were taken from faecal cultures at 2, 3, 7, 8 and 14 days, so larvae equivalent to L1, L2 and L3 were collected (Chapter 5, 5.2.2.1 and 5.2.2.2). L4s and adults were collected from abomasa by baermannisation (Chapter 4, 4.2.6 and 4.2.8). Ten adults were found to register sufficient sample activity after biotinylation.

Results:
More polypeptide bands were present in the homogenised than in the intact biotinylated material, although there was a moderate level of background binding of ^{125}I-streptavidin. Comparison of the early faecal culture material indicates alterations in the major proteins detected (Figure 9.5, Table 9.3). Bands at 80 and 44 kilodaltons were maintained throughout the homogenate samples in the early stages and a band at 40 kilodaltons was seen in the intact labelled material (2 to 8 day faecal cultures). Between 3 and 7 days there were clear changes in the major proteins and a great increase in the number of peptides labelling. The homogenate patterns are overexposed, but pattern differences are again obvious. The smudged appearance of the top of the autoradiograph may be due to dirty blotting buffer, poor quality nitrocellulose acetate or poor transfer of peptides of high molecular mass, some of the other gels also show this type of change.

Comparison of the exsheathed L3 and the L4 showed a marked alteration in the major peptide pattern between the stages (Figure 9.6, Table 9.4), unfortunately poor exposure of the L3 samples was found in this autoradiograph, although stage differences could still be detected. Two new intense bands were seen at 56 and 74 kilodaltons in

FIGURE 9.5

Analysis of NHS-biotinylated samples of intact and homogenate larvae of *Ostertagia ostertagi* collected from faecal culture after varying time intervals. Molecular mass markers in kilodaltons.

Lane 1	**intact larvae,**	**2 day faecal culture**
Lane 2	**homogenised larvae,**	**2 day faecal culture**
Lane 3	**intact larvae,**	**3 day faecal culture**
Lane 4	**homogenised larvae,**	**3 day faecal culture**
Lane 5	**intact larvae,**	**7 day faecal culture**
Lane 6	**homogenised larvae,**	**7 day faecal culture**
Lane 7	**intact larvae,**	**8 day faecal culture**
Lane 8	**homogenised larvae,**	**8 day faecal culture**

The major bands detected at 2 and 8 days of faecal culture were different and may correspond with alteration between the L1 and L2 stages.

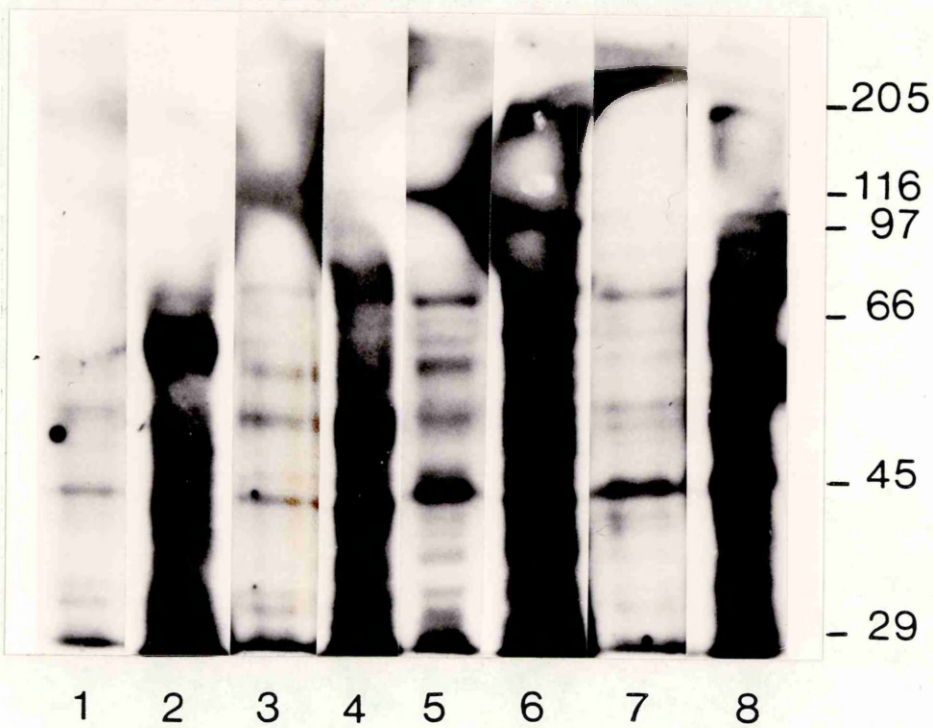
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TABLE 9.3

Comparison of polypeptide profiles of early developmental stages of *Ostertagia ostertagi* collected from faecal cultures, detected after NHS-biotinylation followed by probing with ^{125}I-streptavidin. Polypeptides were separated by polyacrylamide gel electrophoresis and then electroblotted.

Molecular mass in kilodaltons	Time of larval collection from faecal culture							
	2I[1]	2H[2]	3I	3H	7S	7H	8I	8H
215								+
180						+		
175								+
125					+			
120								+
115						+		
110								+
100							+	
95						+		
90		+	+	+		+		
85					+			
83						+		
80				+		+		+
75		+					+	+
72		+		+	+			
68				+	+			
66	+					+		
64			+	+				
62						+		
60				+			+	+
58		+		+		+		
56	+			+				
54		+	+		+		+	
49	+							+
44		+		+		+		+
40	+	+	+	+	+	+	+	+
35		+				+		+
28	+	+	+	+	+	+	+	+

1 Intact labelled samples
2 Homogenate labelled samples
Figure denotes date collected from faecal culture

FIGURE 9.6

Comparison of the L3 and L4 *Ostertagia ostertagi* by NHS-biotinylation of intact and homogenised parasites by SDS-PAGE, electroblotting, followed by probing with ^{125}I-streptavidin and autoradiography. Molecular mass markers in kilodaltons.

Lane 1	**1,000**	**exsheathed L3, intact labelled**	
Lane 2	**2,000**	**exsheathed L3, intact labelled**	
Lane 3	**1,000**	**exsheathed L3, homogenate labelled**	
Lane 4	**2,000**	**exsheathed L3, homogenate labelled**	
Lane 5	**1,000**	**L4**	**intact labelled**
Lane 6	**2,000**	**L4**	**intact labelled**
Lane 7	**1,000**	**L4**	**homogenate labelled**
Lane 8	**2,000**	**L4**	**homogenate labelled**

Two peptides were illustrated with the exsheathed L3 biotinylated intact, compared to four with the intact biotinylated L4.

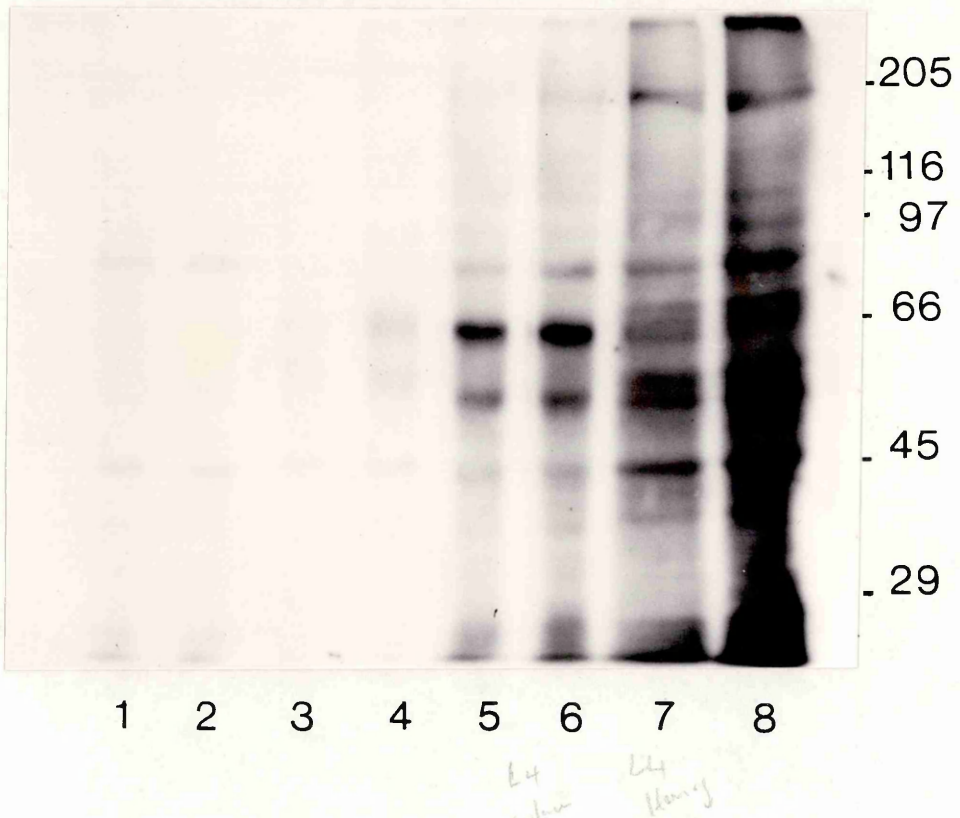
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TABLE 9.4

Comparison of polypeptide labelling with NHS-biotin-^{125}I-streptavidin affinity of exsheathed L3s and L4s, intact and homogenate samples after polyacrylamide gel electrophoresis and electroblotting.

Molecular mass in kilodaltons	Stage and preparation			
	Exsheathed L3s		L4s	
	Intact	Homogenate	Intact	Homogenate
190			+	+
130				+
110				+
96	+			+
94			+	
82		+		+
74			+	+
62		+		
58				+
56			+	+
49				+
43	+	+	+	+
40				+
37			+	+
33			+	+
30				+
24				+
22	+			

the intact labelled L4s, although the two major bands associated with the L3 were still present at 43 and 96 kilodaltons.

Adult *O.ostertagi* were prepared as single sex samples and run alongside L3 aliquots (Figure 9.7, Table 9.5). In addition to the peptide at approximately 45 kilodaltons there was radioactivity at 58, 60 and 94 kilodaltons with intact parasites. On some occasions the female parasite exhibited a further band with the intact sample at 64 kilodaltons.

3. Biotinylated L3s of different trichostrongyle species were separated by polyacrylamide gel electrophoresis and electroblotted, the resulting peptide patterns were visualised by affinity to ^{125}I-streptavidin.

Aliquots of 2,000 L3s of *C.oncophora*, *H.contortus*, *O.circumcincta* and *O.ostertagi* were prepared as described above for biotinylation as intact larvae and homogenates.

Results:
(Figure 9.8 and Table 9.6.) Many polypeptides of similar mass were seen in the homogenates and intact biotinylated L3s of the different trichostrongyle species examined, although some variation was seen in the homogenate patterns. Fewer bands were noticed with the material from intact larvae.

4. The polypeptide profiles of isolates of *O.ostertagi* L3 from different geographical sources were compared by biotinylation-streptavidin affinity. Exsheathed larvae were biotinylated intact and as homogenates before separation by polyacrylamide gel electrophoresis and electroblotting.

Samples of the following larval isolates were compared: Alabama, Denmark, Glasgow, Lelystad (Morantel Resistant and Susceptible), Louisiana, New Jersey, Stormont, Wageningen, Weybridge and Weybridge-Glasgow. For each isolate, 500 L3s were prepared for homogenate analysis and 3,000 L3 surface biotinylated.

FIGURE 9.7

Analysis of NHS-biotinylation of intact and homogenised *Ostertagia ostertagi*, by SDS-PAGE and electroblotting, followed by probing with ^{125}I-streptavidin and autoradiography. Molecular mass markers in kilodaltons.

Lane 1	**Intact**	**adult male**
Lane 2	**Homogenised**	**adult male**
Lane 3	**Intact**	**adult female**
Lane 4	**Homogenised**	**adult female**
Lane 5	**Intact**	**exsheathed L3**
Lane 6	**Homogenised**	**exsheathed L3**

Differences between the L3 and adult stage were clear from examination of the intact biotinylated samples. The female *O.ostertagi* showed an additional band at 64 kilodaltons compared to the male, when biotinylated intact on some occasions.

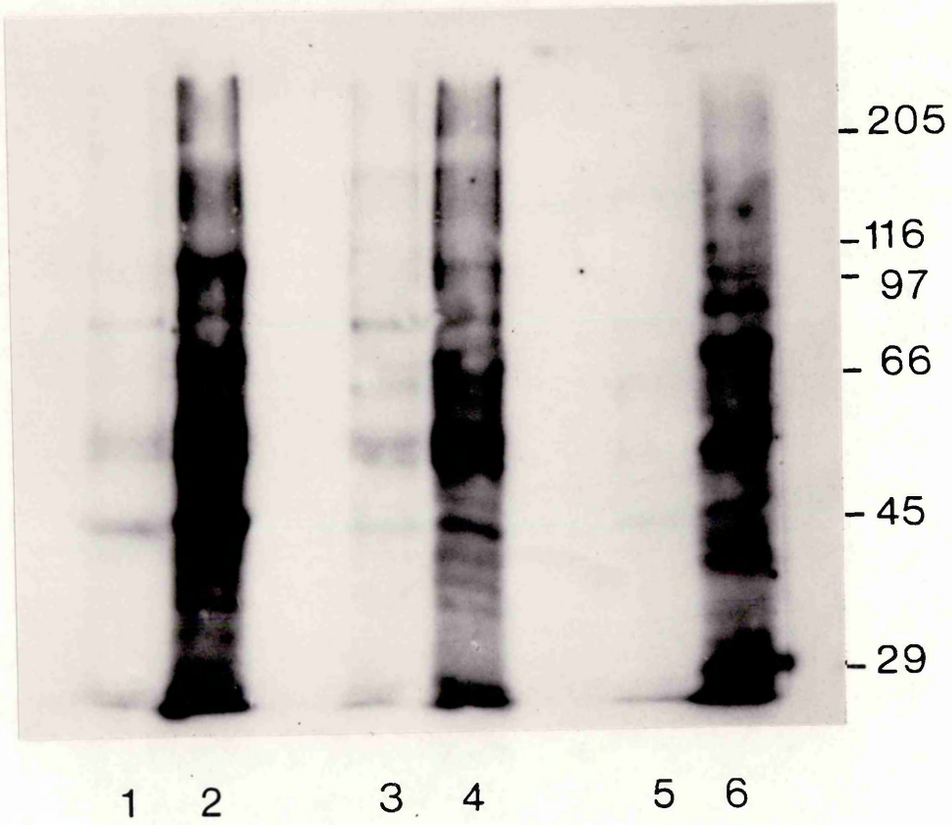
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TABLE 9.5

Comparison of polypeptide labelling with NHS-biotin-^{125}I-streptavidin affinity of male and female adult *Ostertagia ostertagi* and exsheathed L3s after labelling parasites as homogenates and intact.

Molecular mass in kilodaltons	Stage and preparation: Exsheathed L3s I[1]	Exsheathed L3s H[2]	Adult male I	Adult male H	Adult female I	Adult female H
158			+			
118		+				
115				+		
108		+				
97		+		+		+
94			+		+	
84						+
82		+				
79				+		+
74				+		
73	+				+	
64					+	
60			+	+	+	+
58		+	+	+	+	+
56						+
46		+				
45	+		+	+	+	+
39	+			+		+
36				+		+
26		+		+		+

1 intact at biotinylation
2 homogenate at biotinylation

FIGURE 9.8

Four trichostrongyle species were compared by NHS-biotinylation of intact and homogenised exsheathed L3s, after SDS-PAGE, electroblotting, followed by probing with ^{125}I-streptavidin, and autoradiography. Molecular mass markers in kilodaltons.

Lane 1	**2,000**	**intact**	*Cooperia oncophora*
Lane 2	**2,000**	**homogenised**	*Cooperia oncophora*
Lane 3	**2,000**	**intact**	*Haemonchus contortus*
Lane 4	**2,000**	**homogenised**	*Haemonchus contortus*
Lane 5	**2,000**	**intact**	*Ostertagia circumcincta*
Lane 6	**2,000**	**homogenised**	*Ostertagia circumcincta*
Lane 7	**2,000**	**intact**	*Ostertagia ostertagi*
Lane 8	**2,000**	**homogenised**	*Ostertagia ostertagi*

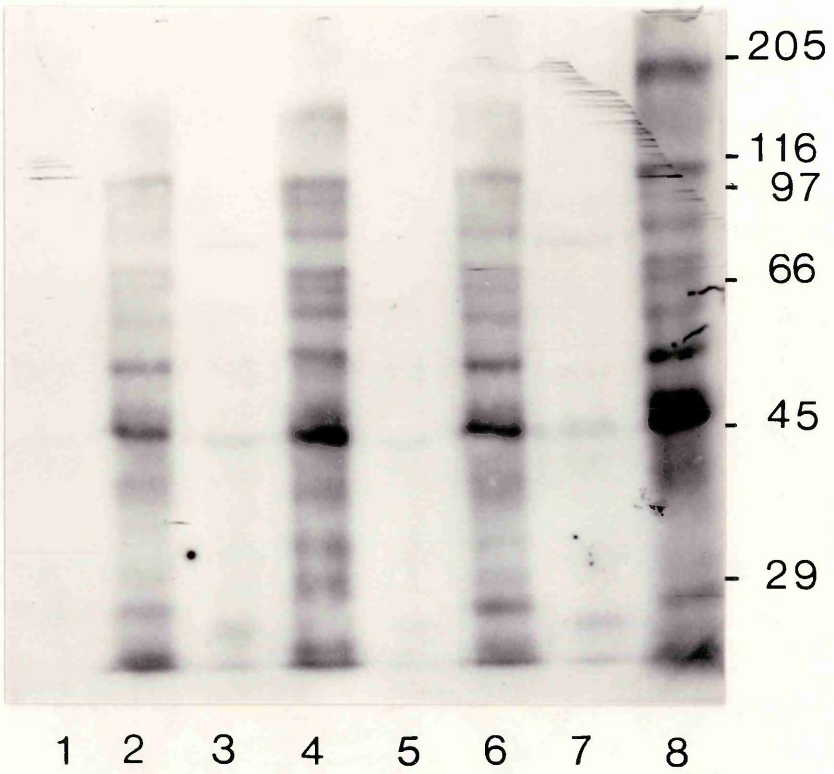
Close similarity of peptide pattern was produced on probing different trichostrongyle species.

FIGURE 9.9

Four different isolates of exsheathed L3s of *Ostertagia ostertagi* were compared by NHS-biotinylation of intact and homogenised exsheathed samples, after SDS-PAGE, electroblotting, followed by probing with ^{125}I-streptavidin and autoradiography. Molecular mass in kilodaltons.

Lane 1	**3,000**	**intact biotinylated exsheathed L3s Alabama**
Lane 2	**500**	**homogenised biotinylated exsheathed L3s Alabama**
Lane 3	**3,000**	**intact biotinylated exsheathed L3s Denmark**
Lane 4	**500**	**homogenised biotinylated exsheathed L3s Denmark**
Lane 5	**3,000**	**intact biotinylated exsheathed L3s Glasgow**
Lane 6	**500**	**homogenised biotinylated exsheathed L3s Glasgow**
Lane 7	**3,000**	**intact biotinylated exsheathed L3s Louisiana**
Lane 8	**500**	**homogenised biotinylated exsheathed L3s Louisiana**

Intact biotinylated samples of different isolates looked similar, but the homogenate samples showed different relative intensities of certain peptides (50 and 68 kilodalton bands - arrowed).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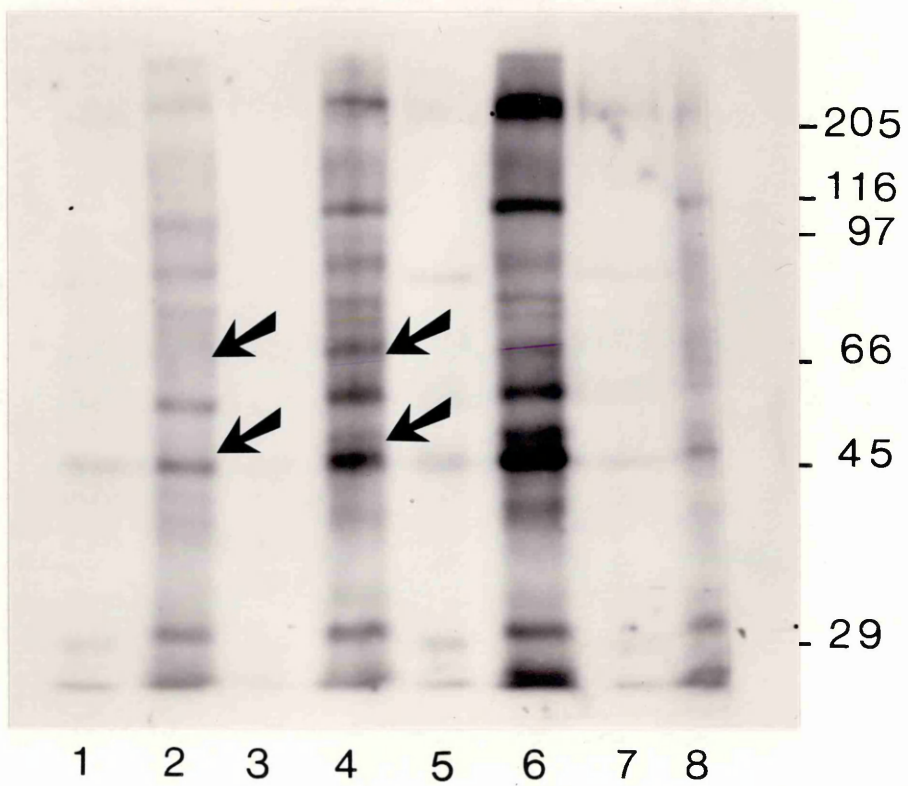
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TABLE 9.6

Comparison of polypeptide profiles of different trichostrongyle species when labelled by NHS-biotin ^{125}I-streptavidin affinity as intact and homogenised larval samples.

Molecular mass in kilodaltons	Intact biotinylation				Homogenate biotinylated			
	C.o[1]	H.c[2]	O.c[3]	O.o[4]	C.o	H.c	O.c	O.o
178								+
151						+	+	
121					+		+	+
119						+		
111						+		
103								+
100					+			
98						+	+	
96				+				
94		+						
91								+
86					+	+	+	+
81							+	
76								+
73					+	+	+	
67								+
64						+		
62				+	+		+	
58								+
52						+		+
50					+	+	+	
46		+	+					
40					+		+	
29						+	+	
26		+	+	+				

1 *Cooperia oncophora*
2 *Haemonchus contortus*
3 *Ostertagia circumcincta*
4 *Ostertagia ostertagi*

Results:

(Figures 9.9, 9.10, 9.11 and Table 9.7.) Unfortunately some aberrant patterns were produced on electroblotting, however, comparison of samples indicates close similarity of the labelled polypeptides, particularly obvious at 45 and 90 kilodaltons in the intact labelled samples. When bound peptides were indicated in larvae biotinylated intact no variation between isolates was seen, although the homogenate bands did appear to vary, the Alabama isolate had no 50 and 68 kilodalton bands which were present in the Denmark, Glasgow and Louisiana samples run simultaneously (Figure 9.9).

Discussion:

Satisfactory binding of ^{125}I-streptavidin was observed on autoradiographs when 2,000 biotinylated exsheathed intact L3s were loaded, but not with a similar amount of sheathed larval material. Examination of the homogenates by light microscopy revealed large fragments of sheath, suggesting that the faint bands with the intact sheathed larvae might be due to poor solubilisation of the biotinylated sample. Reduced biotin uptake by the tough retained sheath could be a further factor.

Samples from intact exsheathed larvae gave a similar range of peptides to the labelled homogenates, although the staining was fainter apart from two bands at 45 and 88 kilodaltons which were relatively more intense than in the homogenate. This result suggested that biotin was penetrating the cuticle or it continued to act during homogenisation. When ethanolamine and Tris (pH 7.2) were added before the three washes in PBS only the bands at 45 and 88 kilodaltons appeared to be biotinylated, suggesting washing in PBS alone did not remove the unbound biotin. Subsequently, exsheathed L3 samples were used for comparative work and the biotinylation step was terminated by addition of ethanolamine and Tris prior to washing in the preparation of intact samples.

Unfortunately with slight variation in protein mobility between experimental runs it is impossible to draw direct comparisons between experimental runs, but many conclusions may be drawn from the qualitative and quantitative differences seen between parasite stages.

FIGURE 9.10

Four different isolates of exsheathed L3s of *Ostertagia ostertagi* were compared by NHS-biotinylation of intact and homogenised exsheathed samples, after SDS-PAGE, electroblotting, followed by probing with ^{125}I-streptavidin and autoradiography. Molecular mass in kilodaltons.

Lane 1	**3,000**	**intact biotinylated exsheathed L3s Lelystad (Morantel Resistant)**
Lane 2	**500**	**homogenised biotinylated exsheathed L3s Lelystad (Morantel Resistant)**
Lane 3	**3,000**	**intact biotinylated exsheathed L3s Lelystad (Morantel Susceptible)**
Lane 4	**500**	**homogenised biotinylated exsheathed L3s Lelystad (Morantel Susceptible)**
Lane 5	**3,000**	**intact biotinylated exsheathed L3s New Jersey**
Lane 6	**500**	**homogenised biotinylated exsheathed L3s New Jersey**
Lane 7	**3,000**	**intact biotinylated exsheathed L3s Stormont**
Lane 8	**500**	**homogenised biotinylated exsheathed L3s Stormont**

Similarity of homogenate and intact biotinylated samples of the different isolates was close.

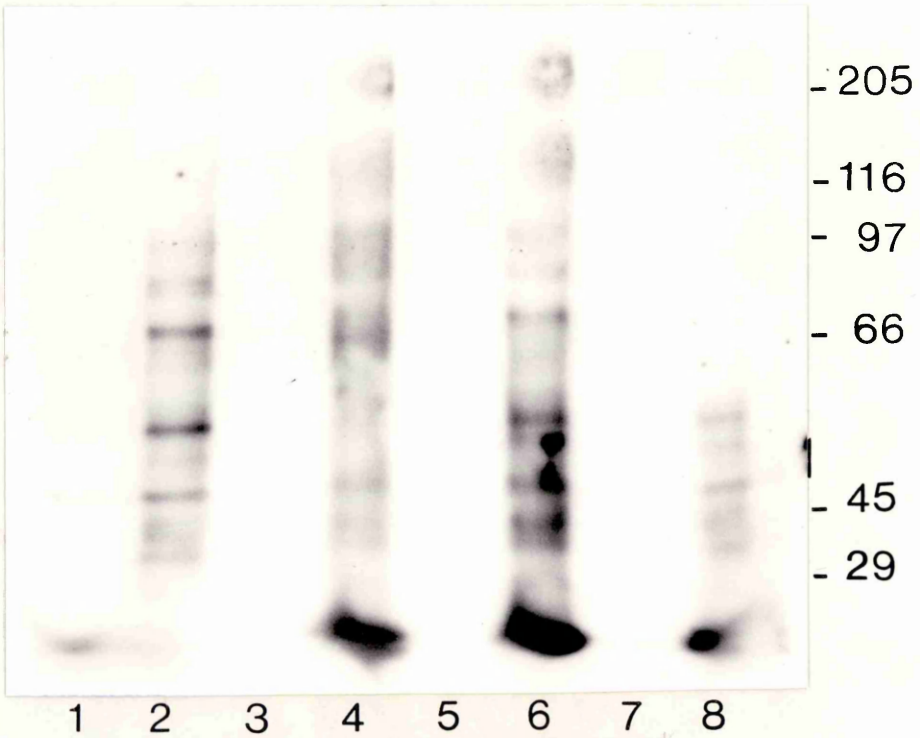

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FIGURE 9.11

Three different isolates of exsheathed L3s of *Ostertagia ostertagi* were compared by NHS-biotinylation of intact and homogenised exsheathed samples, after SDS-PAGE, electroblotting, followed by probing with ^{125}I-streptavidin and autoradiography. Molecular mass in kilodaltons.

Lane 1	**3,000**	**homogenised biotinylated exsheathed L3s Wageningen**
Lane 2	**500**	**homogenised biotinylated exsheathed L3s Weybridge**
Lane 3	**3,000**	**intact biotinylated exsheathed L3s Weybridge**
Lane 4	**500**	**homogenised biotinylated exsheathed L3s Weybridge and Glasgow**
Lane 5	**3,000**	**intact biotinylated exsheathed L3s Weybridge and Glasgow**

Similarity of homogenate and intact biotinylated samples of the different isolates were close.

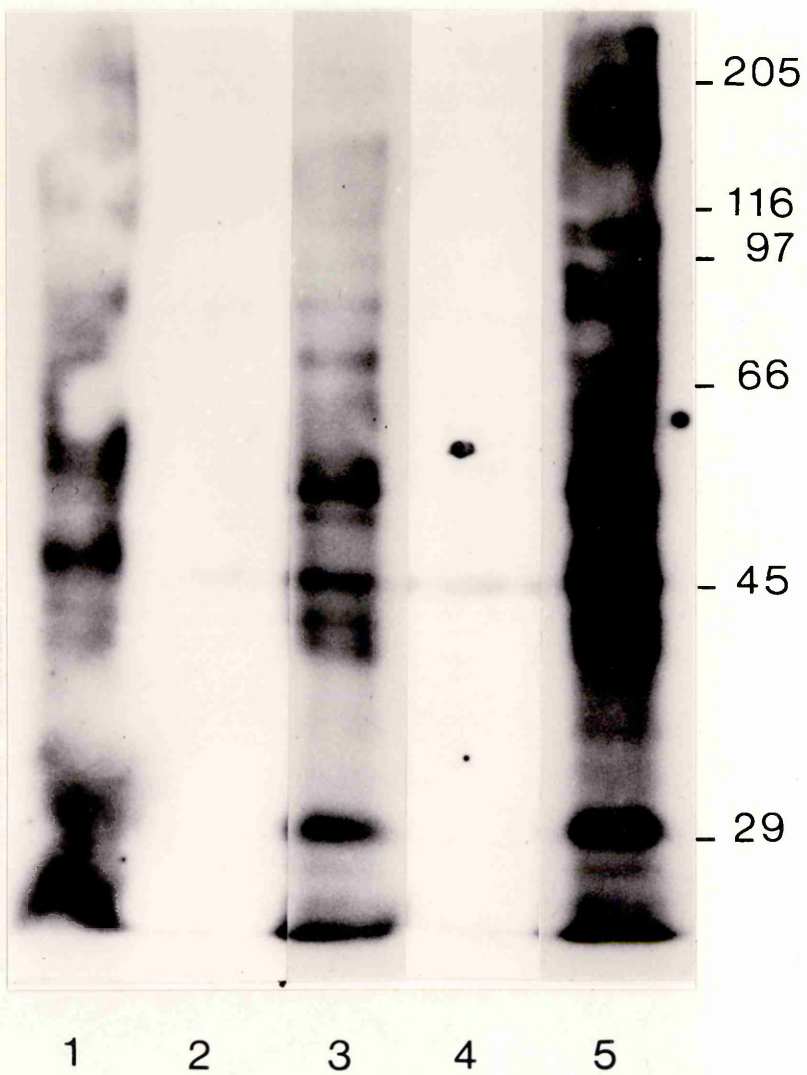
205
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TABLE 9.7

Comparison of polypeptide profiles of different isolates of *Ostertagia ostertagi* L3 stage, when biotinylated as intact and homogenate samples and probed, after polyacrylamide electrophoresis and electroblotting, with ^{125}I-streptavidin.

(a)

Molecular mass in kilodaltons	Isolate labelled intact[1] A	D	G	LR	LS	Lo	NJ	S	Wa	We	WG
174	+										
168			+			+					
151										+	
90	+		+						+	+	+
87						+					
74											+
56						+					
54			+			+					
50										+	
45	+	+	+	+		+	+	+	+	+	+
22	+								+		

(b)

Molecular mass in kilodaltons	Isolate labelled as homogenate A	D	G	LR	LS	Lo	NJ	S	Wa	We	WG
174	+	+	+								
166						+					
144	+										
140		+									
115			+			+					
112	+	+									
104		+		+							
94		+									
92	+		+								
90				+			+				
81		+	+								
78	+										
74	+	+	+	+	+		+				
68		+	+			+					
56	+	+	+								
54				+	+		+				
50		+	+			+	+				
48				+				+			
45	+	+	+	+	+	+	+	+	+	+	+
39	+										
36	+	+	+			+				+	+
34										+	+
26		+	+								
22	+	+	+			+					
18										+	

[1]Isolate abbreviations: A, Alabama; D, Denmark; G, Glasgow; LR, Lelystad Morantel Resistant; LS, Lelystad Morantel Susceptible; Lo, Louisiana; NJ, New Jersey; S, Stormcnt; Wa, Wageningen; We, Weybridge; WG, Weybridge-Glasgow.

Many polypeptides were seen with the homogenised labelled samples, while lower numbers were detected from samples biotinylated before homogenisation, making stage differences generally easier to find with the intact biotinylated samples. The restricted pattern of polypeptides recognised by surface biotinylation suggests only a subset of proteins have taken up biotin and with the impermeability of the cuticle the most likely area to be biotinylated is the surface. Subsequently, work by Alvarez, Henry and Weil (1989) has demonstrated by transmission electron microscopy and the use of streptavidin conjugated to colloidal gold that biotin binding may be restricted to the cuticle of intact *B.pahangi*, although not purely to the surface.

Differences in the polypeptide patterns with stage of intact and homogenised samples were noted in the early larvae particularly between 3 and 7 days of faecal culture. The time interval coinciding with the gradual change in stage from the L1 to the L2 observed in culture. There was a high level of background peptide activity in the intact labelled samples, implying that a number of internal peptides were biotinylated, although the relative intensity of bands suggested that different peptides dominated the pattern relative to those in the homogenate samples. An explanation for biotinylation of internal peptides in these samples might be either further biotinylation was occurring after homogenisation or that the surface of these immature stages is more easily penetrated by the biotin. The intact labelled samples collected on the third day of faecal culture (mainly L1) showed activity at 42, 54, 64 and 72 kilodaltons, though on the seventh day of culture (chiefly L2) only the bands at 42 and 72 kilodaltons remained.

Clear differences were evident particularly in the limited number of peptides noted in the intact labelled sample with the progression from L3 to L4. Usually only two bands were seen in the intact labelled exsheathed L3 at 42 to 45 and 75 to 95 kilodaltons, but two additional bands were seen at 56 and 74 kilodaltons in the L4s. (Greater variation between samples is more likely with polypeptides of higher molecular mass due to the steepness of the gradient on the exponential curve of mass against distance travelled on SDS-PAGE.) Unfortunately L4s were not run simultaneously with the early larvae collected from faecal

cultures, since the L4s looked more like these early larvae than the exsheathed L3s in basic peptide pattern, it would have been useful to have a direct comparison of the intact labelled samples to assess their true level of similarity. The same should be said for comparison with the adult and L4, since one of the additional bands seen in the adult has a very similar mass to one in L4s, i.e., 58 relative to 56 kilodaltons respectively and could be equivalent.

Overall, it appeared that a peptide appeared to be present and surface associated at approximately 45 kilodaltons in all the stages of *O.ostertagi* examined. Other apparently surface associated peptides were present but these altered in their activity with stage, implying alteration in surface structure with stage, which would fit in to the concept of the nematode surface as a dynamic structure. Maizels *et al.* (1982) observed that between stage cross-reactive epitopes aided tolerogenicity of the host to the parasite and so the development of chronic infection. This may indeed be the case sometimes in ostertagiosis since it appears low level infections may persist for long times and there is quite a degree of overlap of similar antigens between stages.

Different trichostrongyle species appear to have peptides of similar magnitude, implying strong conservation of particular proteins or similar proteins. The different distribution of the label suggests that the surface alone was biotinylated in the intact specimens. There appears to be more variation in the peptides of the homogenate material than the intact larvae, which are more likely to be internal peptides if the assumptions from previous experiments are correct. With *O.circumcincta* and *O.ostertagi* the comparison was extended to the L4 and again the similarity in pattern for the intact labelled biotinylated parasites was striking (Keith, Duncan, Murray, Bairden and Tait, in press). Comparison of surface labelled peptides between isolates of *O.ostertagi* also showed close similarities. The results suggest conservation of proteins detected by the biotin-streptavidin technique at both inter- and intra-species levels of classification, although there did appear to be some variation between the total nematode peptide profiles at both the species and isolate level using biotin-streptavidin affinity.

High background reactions could be attributed to over-exposure of film, i.e., too long an incubation, non-specific binding due to inadequate blocking, washing or interaction of protein and biotin. Poor banding also arose due to a number of factors: lack of binding, disintegration of isotope, low protein loading, poor blotting or too short a film exposure. Initially biotinylated standard markers were used, but they developed irregularly on the autoradiographs, consequently the marker tracks were removed before blotting and stained by Coomassie blue and a correction factor employed to accommodate the slight increase in size that occurred with gel expansion on electroblotting. Latterly, the marker track was stained on the blot with Ponceau Red (Chapter 4, 4.3.2.1) before incubation with the radiolabelled streptavidin.

9.2.3 Effect of detergent action on biotinylation

9.2.3.1 Introduction

Detergent stripping of nematode surface structures has enabled workers to study deeper surface strata. Two types of experiment have been done, in one the material stripped by detergent has been examined, while in the other the changed appearance of the surface after the reagent has acted is the focus of attention; in both cases the effect on immunogenicity was assessed. Cetyl-trimethylammonium bromide (CTAB) is a cationic detergent commonly used for the study of nematode surfaces. Cationic detergents are usually employed, since the surface of most nematodes is anionic.

Pritchard, Crawford, Duce and Behnke (1985) used CTAB to strip radiolabelled material from the surface of *Nematospiroides dubius*. On immunoprecipitation with post-infection mouse sera, the shed material appeared to be of similar antigenicity to the whole nematode homogenates. No obvious signs of damage to the surface or deeper structures was found on electron microscopy.

Grencis, Crawford, Pritchard, Behnke and Wakelin (1986) stripped the surface of the muscle stage of *T.spiralis* with detergent and used the extract to immunise mice. On challenge the mice acquired a

significantly reduced muscle burden and the parasites were stunted and less fecund compared to those not previously immunised. They concluded that the mice had developed a degree of protection by immunisation with surface epitopes. Cabrera and Parkhouse (1987) developed an Enzyme Linked Immunosorbent Antibody Test (ELISA) to detergent extracted material from the surface of *Onchocerca volvulus* and *O.gibsoni*, which indicated previously infected individuals.

It has been suggested that *Necator americanus* invokes a poor immune response since surface layers are successively lost during turnover. However, after CTAB action the deeper structures including collagen generated a protective immune response (Pritchard, McKean and Rogan, 1988a and 1988b). They proposed that the nematode might be evading the immune system by concealing epitopes and a vaccine to antigens at a combination of levels in the surface might be more effective.

There are two possible approaches to examination of CTAB action on the surface in conjunction with biotin-streptavidin affinity:

1) Comparison of "surface" labelling of parasites after CTAB action for various times to assess changes to the surface.
2) Examination of material removed by CTAB action.

The second of these approaches was followed, since variation is easier to assess in a developing rather than a reducing assay.

9.2.3.2 Materials and methods. Results.

The method of CTAB action employed by Pritchard *et al.* (1985) was adapted and used; protein staining was assessed before biotinylation studies.

Examination of CTAB action by protein staining:
One ml of 0.25% CTAB was added to 100,000 exsheathed L3s and the suspension was incubated at 37°C for 2 hours. After centrifugation for 1 minute at 10,000g, the supernatant (material stripped by CTAB) was

retained and labelled SN1. The larvae were washed five times in 0.85% sodium chloride. The supernatant was removed and the larvae were homogenised in 100ul homogenisation buffer with protease inhibitors and spun for 5 minutes at 10,000g (Chapter 9, 9.2.2.1). The supernatant, containing material released from the nematode, was collected and labelled SN2. To concentrate the SN1 and SN2 samples, they were precipitated with trichloroacetic acid to a final concentration of 10% and kept on ice for 30 minutes, when they were microfuged at 10,000g for 5 minutes, the supernatants were stored and the precipitated proteins resuspended in four times their volume of sample buffer, saturated Tris was added to increase the pH. Meanwhile the pellet retained from homogenisation was resuspended in 100ul SDS-Tris buffer (0.125M Tris pH 6.8 with 1% SDS), ~~and~~ boiled for 2 minutes and centrifuged for 2 minutes at 10,000g. The supernatant was removed (SN3), it contained substances not released by CTAB or by initial solubilisation. Finally, the pellet was resuspended in 100ul SDS-Tris buffer with 10ul mercaptoethanol; the sample was boiled and centrifuged as before to produce SN4. The harsh extraction steps were included to remove tightly adherent muscle fragments and other remains of the body wall (Cox, Kusch and Edgar, 1981). Sample buffer was added to SN3 and SN4. All the samples were boiled for 2 minutes and loaded on a 10% polyacrylamide gel.

Results:

Coomassie blue showed no polypeptide staining from the CTAB extraction sample (Figure 9.12, lane 2). Very high peptide levels were apparent in the tracks loaded with the supernatant of the larval homogenate (lane 3) and additional peptides appeared to be solubilised with the SDS-Tris buffer and mercaptoethanol treatments as seen in the remaining lanes (4 and 5). The beta-mercaptoethanol extraction solubilises collagen protein in the cuticle.

Examination of CTAB action by biotin labelling:

1) In this experiment the sample was pre-incubated for 1 hour with NHS-biotin. The NHS-biotin reaction was stopped by making the suspension 10mM ethanolamine and 100mM Tris (pH 7.2). The parasites were washed three times in excess 0.85% sodium chloride and spun for 5 minutes at 10,000g. The samples were incubated with

FIGURE 9.12

Exsheathed L3 of *Ostertagia ostertagi* were treated with the detergent, cetyltrimethylammonium bromide (CTAB), and fractions were separated by SDS-PAGE and stained with Coomassie blue.

Lane 1	**Molecular mass standards**
Lane 2	**CTAB stripped material**
Lane 3	**proteins released by homogenisation**
Lane 4	**proteins removed from pellet with SDS-Tris**
Lane 5	**remaining proteins soluble in SDS-Tris buffer and beta mercaptoethanol**

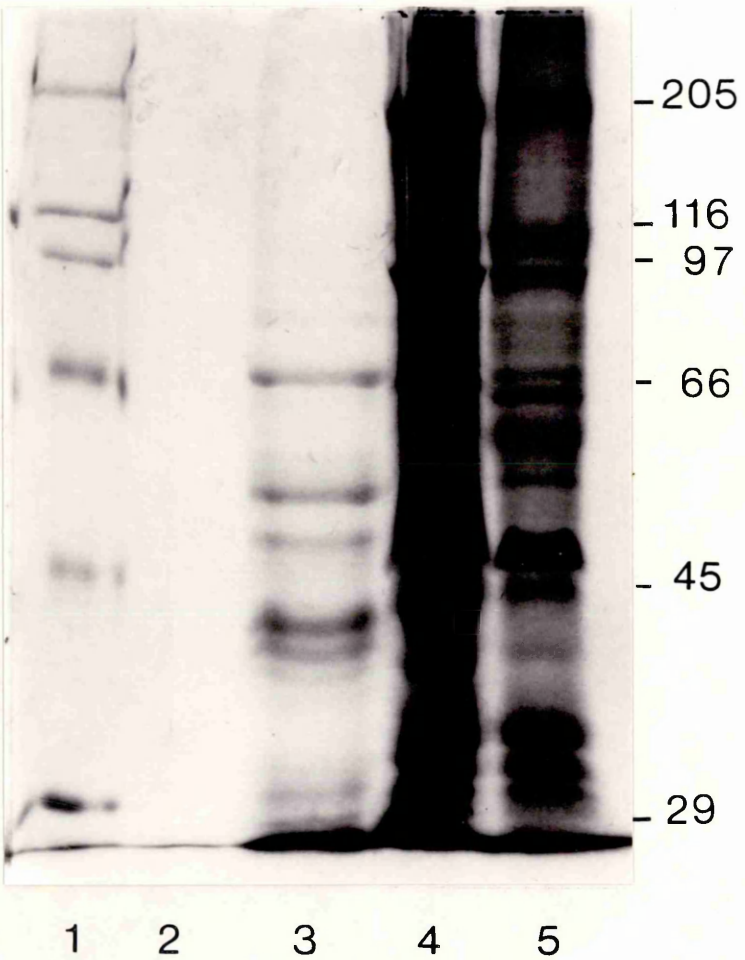
No peptides or insufficient peptide were stripped with detergent to be detected by Coomassie blue staining.

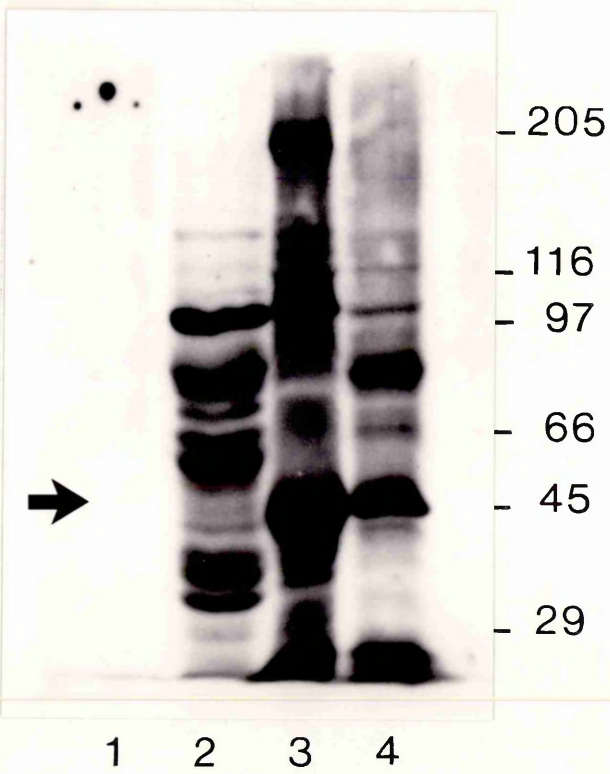
FIGURE 9.13

Exsheathed L3 of *Ostertagia ostertagi* were pre-incubated with NHS-biotin before detergent treatment with cetyltrimethyl-ammonium bromide (CTAB). Fractions were separated by SDS-PAGE, electroblotted, probed with ^{125}I-streptavidin and autoradiographs produced. Molecular masses in kilodaltons.

Lane 1	**CTAB stripped material**
Lane 2	**proteins released by homogenisation**
Lane 3	**proteins removed from pellet with SDS-Tris**
Lane 4	**remaining proteins soluble in SDS-Tris buffer and beta-mercaptoethanol.**

A small amount of peptide of 46 kilodaltons was detected in the CTAB stripped material, although this is poorly indicated in the reproduction (arrow).





why is there so much more activity in these samples compared with those in 9.4 exp

CTAB for 3 hours. Otherwise, the samples were treated as above, but they were loaded onto the gel by syringe. The resulting gel was blotted and reacted with ^{125}I-streptavidin.

Results:
Little sample material was retained in the slot from the CTAB strippings for electrophoresis and this resulted in a band of very low activity at 46 kilodaltons (Figure 9.13, lane 1), unfortunately it is poorly indicated in the photograph. Much higher protein levels and at a greater range of masses were demonstrated in the tracks after homogenisation and solubilisation of samples (Table 9.8).

2) The depth of biotin binding of intact exsheathed larvae was assessed by using fluorescein conjugated streptavidin and examining the effect of the cationic detergent cetyl-methyl ammonium bromide (CTAB).

Ten thousand L3 were exsheathed in sodium hypochlorite solution and rinsed three times in excess PBS, at 10,000g for 2 minutes (Chapter 4, 4.2.5). The larvae were divided equally between eight numbered eppendorfs (1,250 L3s) and subjected to the following treatments:

Tube 1: Thirty ug NHS-biotin was added to the larvae in 100ul of PBS and the tube incubated at room temperature (20°C) in the dark for 1 hour. Larvae were washed three times in excess PBS and the supernatant removed. Excess 0.25% solution of CTAB was added and the larvae incubated 3 hours at 37°C. Three washes in excess PBS followed and fluorescein di-isothyocyanate (FITC)-streptavidin was added to give a concentration of 2ug/ml and incubated for 30 minutes in the dark to retain fluorescein activity. Finally, the sample was washed three times in excess PBS.

Tube 2: The larvae were treated similarly to those in tube 1, but with no FITC-streptavidin incubation.

Tube 3: Larvae were biotinylated and incubated with FITC-streptavidin.

TABLE 9.8

Comparison of polypeptide profiles arising after detergent action on exsheathed L3 of *Ostertagia ostertagi*, which had been previously biotinylated. Peptide separation was achieved by polyacrylamide gel electrophoresis and the electroblot was probed with radiolabelled streptavidin.

Molecular mass in kilodaltons	Larval preparation SN1	SN2	SN3	SN4
205			+	
178			+	
154		+		
142		+	+	+
134			+	
125		+		+
121			+	
112		+		
105			+	+
100		+		
94			+	+
88			+	+
82		+		
75		+	+	
73				+
70		+		+
64		+		
60		+		
50			+	+
46	+	+	+	+
41		+	+	+
37		+	+	+
33		+	+	
29		+	+	+

SN1 CTAB strippings
SN2 Homogenate supernatant
SN3 Solubilised pellet
SN4 Pellet solubilised with mercaptoethanol

Tube 4: Larvae were biotinylated alone.

Tube 5: Larvae were incubated with FITC-streptavidin alone.

Tube 6: Larvae were incubated in CTAB for 3 hours before biotinylation and FITC-streptavidin incubations.

Tube 7: Treatment of larvae was similar to that in tube 6, but no incubation with FITC-streptavidin was given.

Tube 8: The larvae were incubated purely with CTAB.

After the final process for each eppendorf had been completed, the sedimented larvae were applied to a multispot slide coated with poly-tetra-fluoroethylene and dried in a Bunsen flame. The slides were fixed in a solution of 60% ethanol, 30% chloroform and 10% acetic acid for 20 minutes and washed for 2 minutes in a solution of 0.15M sodium chloride, 0.03M potassium chloride and 0.01M phosphate buffer pH 7.2 (Herman, Madl and Kari, 1979). A 1% solution of Evan's Blue in PBS was applied for 5 minutes to quench nematode autofluorescence and the slides were examined by fluorescent microscopy. Results were recorded on Kodak Ektachrome P800/600 Colour Reversal film.

Results:

When FITC-streptavidin had bound to the nematode the surface was green, when no FITC-streptavidin was present the surface was red, intermediate situations were also found. The most strongly fluorescent larvae were those that had been biotinylated before treatment with FITC-streptavidin (Figure 9.14, tube 3). Incubation with CTAB after biotinylation gave a patchy distribution and reduced fluorescence intensity and distribution was patchy (Figure 9.15, tube 1). A further reduction in staining intensity was seen when larvae were biotinylated after CTAB action then treated with FITC-streptavidin, (tube 6). No fluorescence was seen in tubes when no FITC-streptavidin had been added or when no biotin-streptavidin affinity was present (2,4,5,7,8) (Figure 9.16).

FIGURE 9.14

Biotinylated L3s of *Ostertagia ostertagi* after incubation with FITC-streptavidin. The majority of larvae fluoresced throughout their length. (Evan's blue) (x 50)

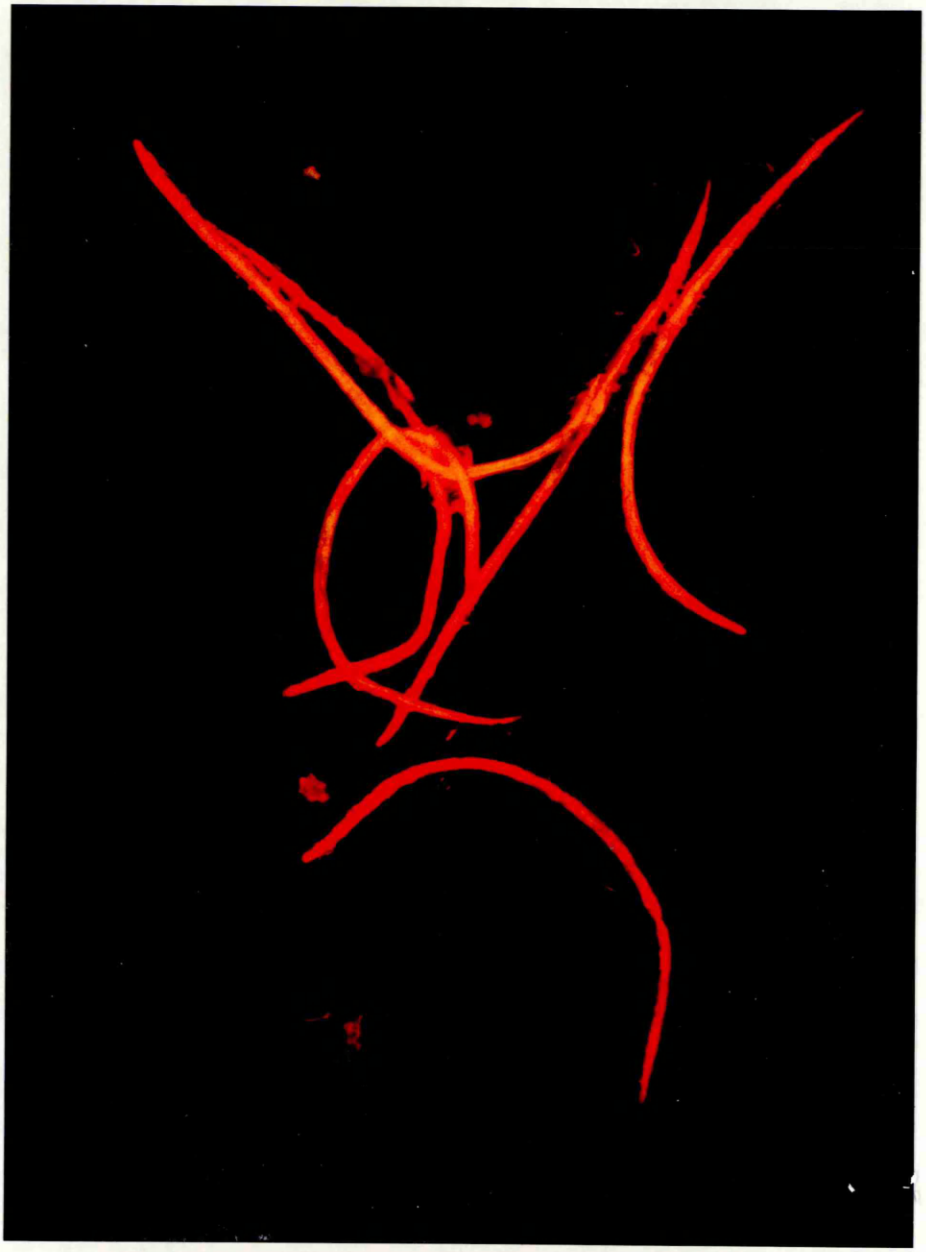


FIGURE 9.15

Biotinylated L3s of *Ostertagia ostertagi* treated with detergent before FITC-streptavidin incubation; some larvae showed patchy fluorescence. (Evan's blue) (x 50)

FIGURE 9.16

L3 not incubated with biotin and/or FITC-streptavidin and consequently showing no fluorescence. (Evan's blue) (x 50)

Discussion:

Little material stripped from the nematode surface was visualised by the biotin-streptavidin affinity reaction, which may be attributed to loading difficulties and a low amount of material being stripped. The CTAB stripped sample was an unexpected consistency and tended to float out of the slot, preventing adequate loading, which was probably due to a high carbohydrate content, since lipid would have been broken down by the SDS present in the sample buffer. When the process was applied to *O.circumcincta* under similar conditions the sample was loaded with no apparent difficulty (Keith *et al.*, in press).

However, the material which was removed by the detergent from *O.ostertagi* was of a mass equivalent to the surface peptide detected previously across the various parasite stages at 46 kilodaltons. In most cases when intact larvae were labelled a restricted group of peptides were indicated, but in this case a large number of bands were demonstrated. Possibly biotin was still acting after homogenisation or the increased size of the larval sample prepared in a similar volume, (100,000 versus 10,000 L3), prevented adequate mixing with the ethanolamine and Tris. When the CTAB extraction process was carried out with biotinylated *O.circumcincta*, the majority of the peptides were seen in the CTAB stripped material, no peptides were present in the homogenate or SDS-Tris extract, but some further bands were detected after beta-mercaptoethanol extraction. Keith *et al.* (in press) concluded that most of the biotin was found in the outer surface layers. By collagenase treatment of the L3 *O.circumcincta*, they found that some biotin penetrated the cuticle surface, since although the majority of biotinylated proteins were not collagens, some collagens were affected.

FITC-streptavidin demonstrated the presence of surface biotinylation of exsheathed L3s and the reduction in fluorescence intensity after detergent action implies that surface biotin was removed. The lower intensity of fluorescence observed when larvae were biotinylated after CTAB action rather than before, implies the surface had been disrupted by the detergent and some of the sites available for biotin binding had been removed.

Marshall and Howells (1985) compared the patterns of staining by the available direct methods of radiolabelling. They discovered with iodogen, lactoperoxidase, chloramine T and iodosulphanilic acid the ^{125}iodine bound to the surface molecules, while iodine penetrated to a considerable depth in the somatic tissue with the Bolton-Hunter technique. Transmission electron microscopy studies with streptavidin conjugated to colloidal gold would be an alternative method of demonstrating depth of penetration with indirect radiolabelling by biotin. Subsequent to the studies outlined in this chapter, work by Alvarez *et al.*, (1989) has demonstrated, by electron microscopy, that sulfo-NHS-biotin binding was restricted to the nematode cuticle of *B.pahangi*, though not purely to its surface and that biotinylated samples produced a similar electrophoretic pattern to iodogen labelled material. Furthermore, Hill, Fetterer and Urban (1990) compared labelling of different stages of *Ascaris suum* with sulfo-NHS-biotin (aqueous soluble) and NHS-biotin (organic soluble). By electroblotting and electron microscopy, they showed no internal peptides were labelled, but sulfo-NHS-biotin penetration appeared to be more restricted than NHS-biotin in later developmental stages, which they proposed indicated a hydrophobic barrier in the cuticle of more developed parasites.

9.2.4 Excretory-secretory products

The term excretory-secretory (ES) products refers to a mixture of substances expelled by the parasite. The material may be shed from the surface or excreted products. In some species these have been analysed in depth, often there is a high enzyme content, e.g., acetylcholinesterase in *Nippostrongylus brasiliensis* (Sanderson and Ogilvie, 1971). The presence of acetylcholinesterase in ES may have a parasympatholytic effect and so reduce gut motility and aids the parasite by producing a holdfast effect. Meghji and Maizels (1986) reported that the ES of *T.canis* are 40% carbohydrate.

Kennedy *et al.* (1987a) commented on the ability of the ES products to induce an antibody response and some ES products have been tested as potential immunogens as reviewed by Maizels and Selkirk (1988) for vaccine production and for the detection of circulating antibody. Rose

(1976) produced some protection in sheep against *O.circumcincta* by immunisation with lyophilised metabolic products. However, Lightowlers and Rickard (1988) suggested by producing ES products nematodes might evade the immune response by shedding immunogenic material.

The aim of this study was to ascertain if the ES material derived from adult parasites was shed from the nematode surface or excreted. Furthermore, a knowledge of any sex linked excretions might be relevant to improving understanding of the parasite behaviour.

Materials and methods:
Adult parasites were maintained in a protein-free medium for 18 hours, 27 hours and 7 days (Chapter 4, 4.2.9). The incubation fluid was withdrawn and the ES protein concentrated by dialysis. Three hundred µg NHS-biotin was added to 1ml of the concentrated ES sample. The solution was incubated for 1 hour and the reaction stopped by adjusting the solution to 10mM ethanolamine and 100mM Tris (pH 7.2). Twenty µl of sample buffer was added to 70µl of ES products. Adult parasites were prepared as single sex, intact and homogenate samples, so the equivalent of 10 adults was loaded per track. After the samples had been boiled for 2 minutes and microfuged at 10,000g for 5 minutes they were loaded onto a 10% polyacrylamide gel. The experiment was repeated loading the ES samples on a 15% polyacrylamide gel to resolve proteins of low molecular mass better.

Results:
On the 10% polyacrylamide gel a single band of activity was seen with the ES products at approximately 29 kilodaltons (Figure 9.17). No peptides similar in mass to the ES products were seen in the intact labelled adults. The ES products in the 15% polyacrylamide-gel showed a group of low molecular mass peptides of 13, 15, 19 and 26 to 32 kilodaltons (Figure 9.18).

Discussion:
A number of peptides of low mass were seen in the ES samples. The peptides differed in mass from all of the peptides demonstrated on the adult surface, suggesting that the products are not simply shed from the

FIGURE 9.17

Analysis of NHS-biotinylated excretory-secretory products of *Ostertagia ostertagi* adults compared with intact and homogenised adult samples. Biotinylated samples were separated by SDS-PAGE, electroblotted, probed with ^{125}I-streptavidin and autoradiographed. Molecular mass standards in kilodaltons.

Lane 1	**excretory-secretory antigens collected at 18 hours**
Lane 2	**excretory-secretory antigens collected at 27 hours**
Lane 3	**excretory-secretory antigens collected at 7 days**
Lane 4	**excretory-secretory antigens collected at 7 days**
Lane 5	**male adults biotinylated intact**
Lane 6	**male adults biotinylated as a homogenate**
Lane 7	**female adults biotinylated intact**
Lane 8	**female adults biotinylated as a homogenate**

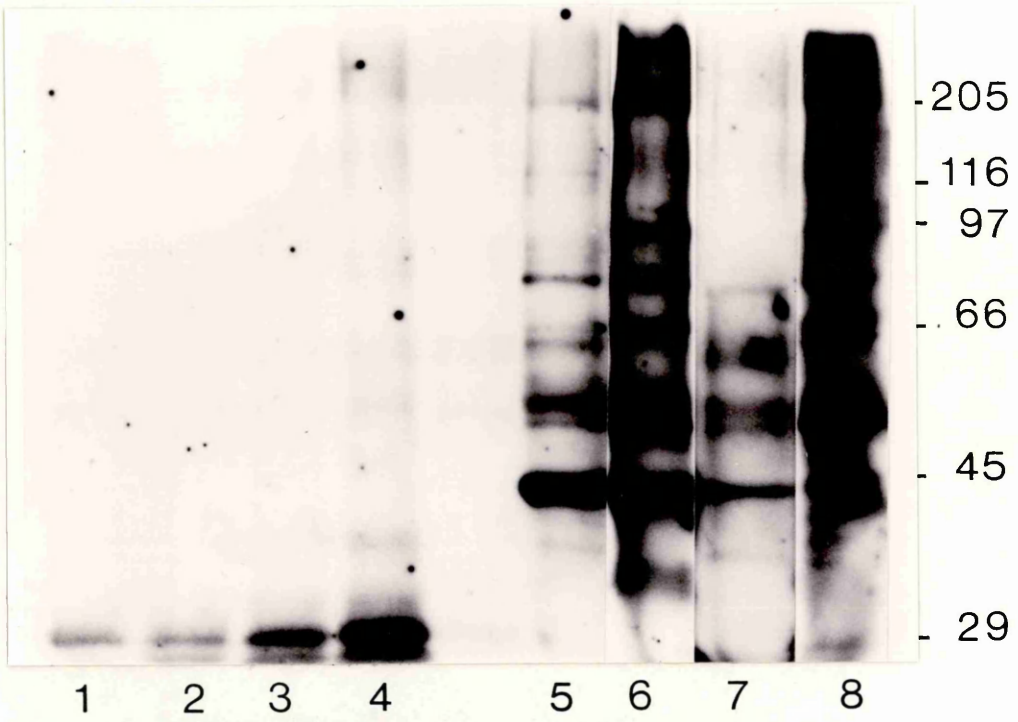
Low molecular mass peptides were detected in the excretory-secretory products.

FIGURE 9.18

Biotinylated excretory-secretory products of adult *Ostertagia ostertagi* were separated on 15% polyacrylamide gels (standard 10% polyacrylamide gel), to better resolve low molecular masses.
After SDS-PAGE and electroblotting, the peptides were probed with ^{125}I-streptavidin and autoradiographs produced.

Lane 1	**excretory-secretory products collected at 18 hours**
Lane 2	**excretory-secretory products collected at 27 hours**
Lane 3	**excretory-secretory products collected at 7 days**
Lane 4	**excretory-secretory products collected at 7 days**

The biotinylated ES products appeared to consist of a number of low molecular peptides or the breakdown products of larger peptides.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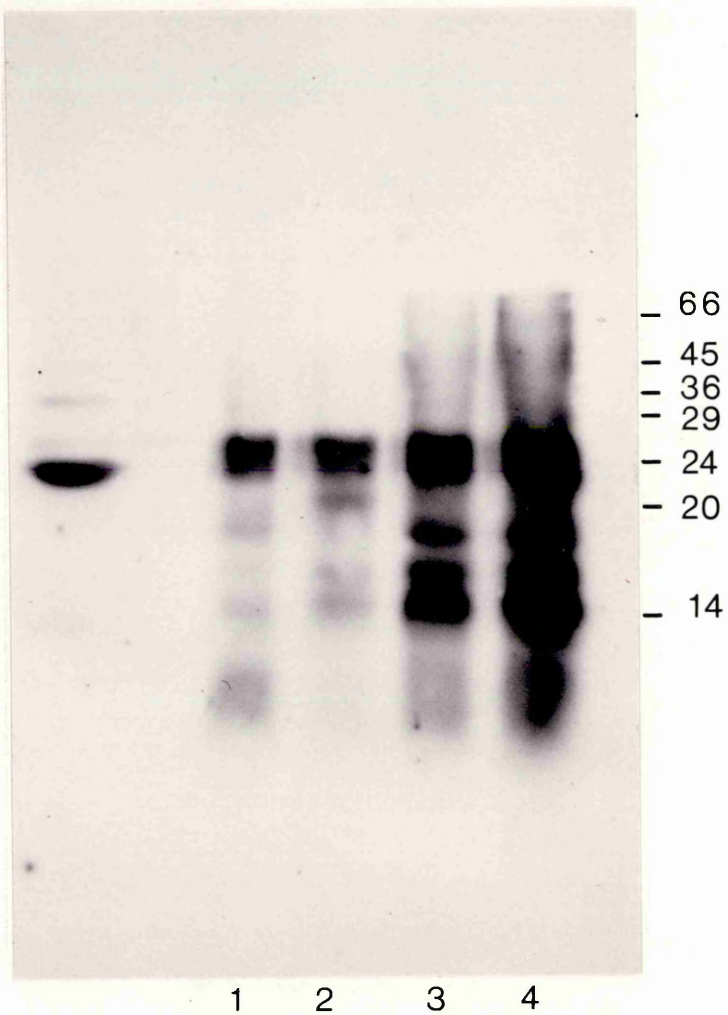
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surface. However, the possibility that these low molecular mass peptides are breakdown products of surface proteins, possibly produced on storage should not be ruled out.

9.3 SURFACE LIPID PROPERTIES

9.3.1 Introduction

Examination of the epicuticle has suggested that nematode surface lipid is heterogeneous and organised into gel and fluid phases called "domains" (Proudfoot, Kusel, Smith, Harnett, Worms and Kennedy, 1990). The lipids present determine the relative rigidity and degree of lipid lateral movement between domains (Proudfoot *et al.* 1990). Furthermore, it has been proposed that the lipid arrangement may alter in response to different stimuli (Kennedy *et al.*, 1987a).

Single or double chain fluorochromes are bound by charge attraction to lipid analogues. Non-polar and anionic analogue probes may be inserted into the surface. The fluorophore moiety is hydrophilic, so the acyl chain tends to penetrate the surface of the nematode leaving the fluorophore exposed at the surface (Foley, MacGregor, Kusel, Garland, Downie and Moore, 1986a). The fluorochromes may be visualised by fluorescence microscopy. Trypan blue is a non-permeant stain, its absorption spectrum overlaps that of the emission spectrum of the fluorescent probes and acts as an energy acceptor (Forster Resonance Energy Transfer). Consequently, if fluorescence is lost when 0.25% Trypan Blue is added to the parasite suspension, the probe could have inserted no deeper than the surface and fluorescence was quenched by the stain.

Lateral diffusion of lipids may be demonstrated by surface labelling with fluorescent lipid analogues and then bleaching the fluorescence from a small area of the surface with an argon or krypton ion laser beam. The rate of return of fluorescence is an indicator of the lipid mobility (Foley, Kusel and Garland, 1986b). Fluorescent probes can be bound to lipids, carbohydrates and proteins and the lateral diffusion of the various surface components may be measured in a similar way,

e.g., fluorescein maleimide binds directly to sulphydryl groups, which allows protein mobility to be examined.

Previously, marked differences between lipid lateral mobility in the trematode, *Schistosoma mansoni* and the nematodes *Toxocara canis* and *T.spiralis* have been noted (Kennedy, *et al.* 1987b). Only one of the probes able to insert into the surface of *S.mansoni* was able to insert into the nematode surface. In addition, fluorescence recovery after photobleaching (FRAP) of lipid was slower and low level in the two nematodes in relation to the trematode, suggesting the domains on the nematode surface are more restrictive than those of the trematode.

The surface of *O.ostertagi* was tested for lipid analogue insertion and lipid diffusion. Different stages of the life cycle were assessed to observe whether variation occurred with development in uptake and diffusion of lipid, which might reflect environmental differences, notably temperature and pH.

9.3.2 Materials and methods

L2s and infective L3 were obtained by faecal culture (Chapter 5, 5.2.2.1 and 5.2.2.2). Third stage larvae were exsheathed by sodium hypochlorite action (Chapter 4, 4.2.5) or with CO_2 while larvae were in a solution of RPMI medium and foetal calf serum. Fourth stage larvae and adults were obtained from abomasal washings (Chapter 4, 4.2.6 and 4.2.8). Parasites were incubated for 10 minutes (37°C) in 1ml of RPMI with 5µl fluorescent probe (10mg/ml in ethanolic solution was added). Three fluorescent probes were used: dioctadecyltetramethyl-indocarbocyanine (Di I_{18})(cationic), 5-N-(octadecanoyl)-amino-fluorescein (C_{18}-Fl)(anionic) and nitrobenz-2-oxa-1,3-diazol stearate (NBD-stearate)(non-polar) - Molecular Probes Inc., Junction City, Oregon, United States of America. The samples were washed four times in RPMI and suspended in 0.25% Trypan Blue. Immediately before analysis, the larvae were paralysed by the addition of levamisole (10µg/ml) or sodium azide (50µg/ml). The fluorescent larvae were examined by ultra violet light at 21°C. Fluorescence was continually monitored by an attenuated focused laser beam. The beam was directed at a spot on the surface with a radius of 1.1µm. Once the

signal was steady the laser power was increased by a power of 10^4 for approximately 0.1 seconds. Approximately half the fluorescent molecules were bleached in this time and the rate of return of fluorescent intensity was assessed.

9.3.3 Results

Only the lipid probe 5-N-(octadecanoyl)-amino-fluorescein (C_{18}-Fl) was taken up at the surface, but NBD-stearate penetrated the surface and dioctadecyltetramethylindocarbocyanine did not insert. High intensity of the fluorophore 5-N-(octadecanoyl)-amino-fluorescein was noted in all the stages studied: L2, L3, L4 and adults of both sexes. L3s exsheathed by hypochlorite action showed labelling, while initially those CO_2 exsheathed larvae did not fluoresce, although uptake of probe rapidly developed with time, i.e., the sheath was lipophilic while the L3 surface was not.

After photobleaching of fluorescent lipid analogues only 27.08% of the fluorescence intensity was recovered in 500 mseconds (Figure 9.19). Lateral mobility of protein was assessed by insertion of fluorescein-maleimide and photobleaching by laser in a similar manner, in this case recovery of fluorescence to 83% of the original level was attained in 500 mseconds (Figure 9.20).

9.3.4 Discussion

O.ostertagi has been found to be similar to other nematode species in having a surface highly restricted to the insertion of lipid analogues (L.Proudfoot, personal communication), only anionic 5-N-(octadecanoyl)-aminofluorescein inserted and the non-polar NBD-stearate penetrated the surface, while cationic dioctadecyltetra-methylindcarbocyanine did not insert.

Despite the apparently dynamic nature of the nematode surface there appeared to be surprisingly little movement of the lipid molecules in *O.ostertagi* (27%). Proudfoot (personal communication) recently found similar recovery from samples of *H.contortus* and *O.circumcincta*. The surface rigidity may be a protective adaptation

FIGURE 9.19

Fluorescence recovery after photobleaching (FRAP): Curve for 5-(N-octadecanoyl) amino fluorescein (C_{18}-Fl) insertion at the surface of Ostertagia ostertagi.

% bleach = 52.8 % recovery = 27.1 beam = 1.1 µm

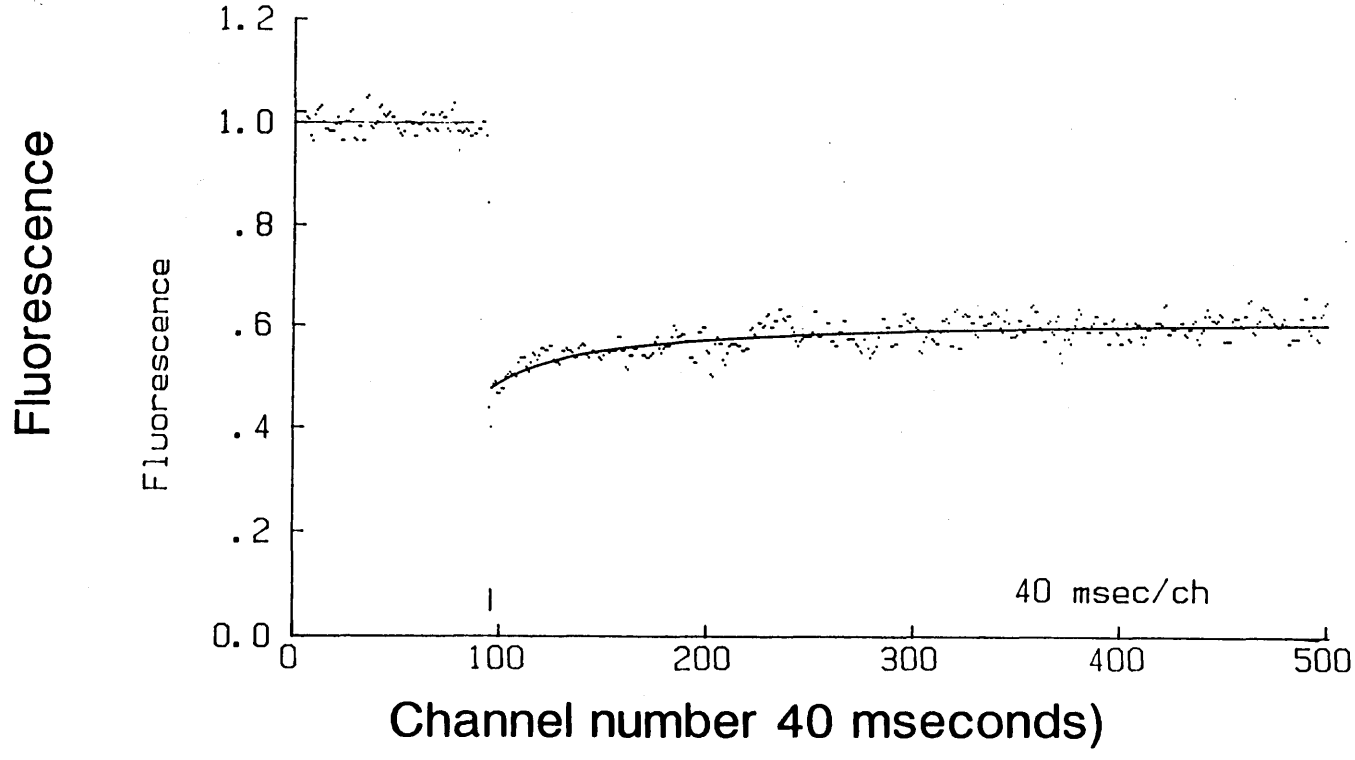
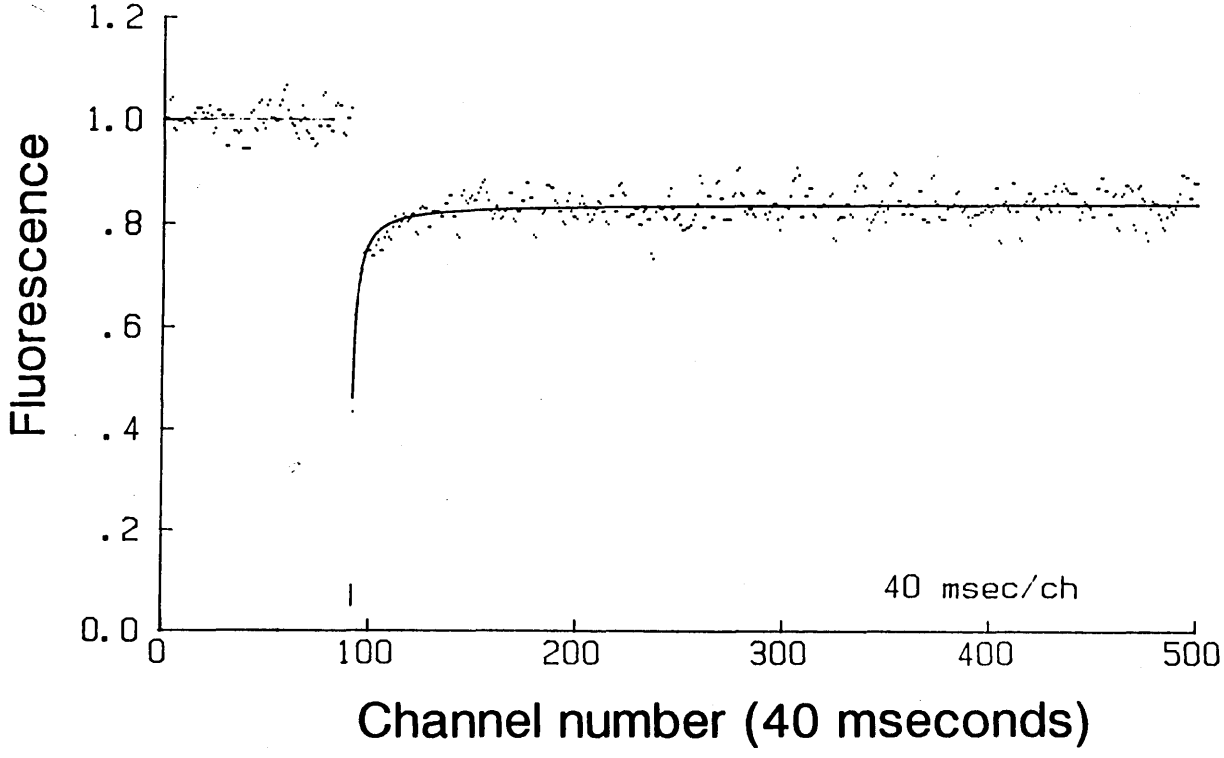


FIGURE 9.20

Fluorescence recovery after photobleaching (FRAP):
Curve for insertion of fluorescein maleimide
at the surface of Ostertagia ostertagi.

% bleach = 91.7 % recovery = 82.3 beam = 1.1 μm



preventing immune attack (Kennedy *et al.* 1987b), so the insertion of complement components would be obstructed and decreasing complement mediated lysis. The only variation in lipid insertion with stage was observed with the non-lipophilic state of the L3 exsheathed in CO_2. The alteration in lipid insertion supports the tentative proposal of Proudfoot *et al.* (1990) that a change in surface lipid composition and/or organisation might initiate parasite changes on entry into the host.

9.4 SURFACE CARBOHYDRATE PROPERTIES BY LECTIN BINDING

9.4.1 Introduction

Lectins are proteins or glycoproteins, frequently multimeric in structure, capable of binding to or agglutinating cells in a similar manner to the antibody-antigen reaction. The linkage formed with the N terminal of a surface sugar residue is reversible and the strength of the interaction is influenced by steric factors. The word lectin is derived from the Latin *legere*, to select, and lectins are highly specific and sensitive markers of carbohydrate groups, although occasionally a sugar residue may react with more than one lectin type. Almond and Parkhouse (1985) used monoclonal antibodies to precipitate four surface proteins from *T.spiralis*, implying a common polypeptide sequence, however, using lectins they were able to differentiate these. Carbohydrates linked to lipids and proteins are present on the nematode surface; they are hydrophilic and so maintain the asymmetrical nature of the surface. The carbohydrates play an important part in cell interactions and may act as receptors to which antibodies may be raised.

Increasing work with lectins labelled by conjugation with fluorochromes, radioisotopes or gold is generating information about nematode surface structures. Differences in the carbohydrate distribution of different stages of the nematode life cycle have been found. Ham, Smail and Groeger (1988) found progressively increased binding of lectins to later stages of *Onchocerca lienalis*. Preston, Jenkins and McLaren (1986) demonstrated altered lectin binding patterns (presence or absence and distribution) with stage in *Trichuris*

muris. These changes were attributed to masking and exposing of binding sites at different stages of development. Little fluorescent labelling occurred immediately after moulting, but increased with time. Cast cuticles were strongly fluorescent.

Preferential binding of lectins to particular areas of the nematode surface has also been demonstrated. Milner and Mack (1988) demonstrated binding of Wheat Germ Agglutinin to the anterior end of *T.colubriformis* particularly the excretory pore. Ortega-Pierres *et al.*, (1986) showed stronger expression of sugar residues with Concanavalin A binding at the eversible cloaca of the female and the copulatory bell of the male *T.spiralis* compared to the rest of the nematode surface. Bone (1986) suggested that carbohydrate distribution might fulfill a pheromone-like role for mating interaction.

Little research has been carried out in nematodes other than the filariids with respect to lectin binding. In this preliminary study progressive stages of the lifecycle of *Ostertagia ostertagi* were examined using five fluorescent lectins: *Arachis hypogaea*, Concanavalin A, *Lens culinaris*, *Ricinus communis* and *Triticum vulgaris* (Table 9.9).

9.4.2 Materials and methods

Aliquots of 150 larvae or 20 adults were washed by sedimentation in 30% sucrose, followed by three 5 minute washes in PBS (pH 7.2) at 2,000g. Pre-parasitic stages were collected by faecal culture and baermannisation after varying time intervals (Chapter 5, 5.2.2.1 and 5.2.2.2). Parasitic stages were collected at slaughter 4 DPI (Chapter 4, 4.2.6) for L4 and 21 DPI (Chapter 4, 4.2.8) for adults. Certain L3 samples were exsheathed using sodium hypochlorite solution as described in Chapter 4, 4.2.5.

The parasites were placed in an eppendorf with 100µl fluorescent lectin and made up to 1ml with PBS, to give a final lectin concentration of 100µg/ml, and incubated for 1 hour at room temperature (20°C). Unbound lectin was removed by three washes in PBS at 10,000g for 5 minutes.

TABLE 9.9

List of lectins used in the examination of the surface of *Ostertagia ostertagi* with respect to carbohydrate configuration.

Lectin	Carbohydrate specificity
Arachina hypogaea (peanut)	D-galactose
Canavalia ensiformis (Concanavalin A from Jack Bean)	N-acetyl-D-glucosamine Methyl-alpha-D-mannopyranoside D-+-glucose D-+-mannose
Lens culinaris (lentil seed)	alpha-D-mannosyl alpha-D-glucosyl
Ricinus communis (castor bean)	D-galactose 1-0-methyl-beta-D-galactopyranoside
Triticum vulgaris	N-acetyl-D-glucosamine

Initially larvae were spotted onto a slide and examined under a FITC-filter, but due to the high degree of parasite motility and the intensity of autofluorescence, assessment of fluorescent staining was obstructed. Subsequently, larvae were spotted onto a multispot poly-tetra-fluoro-ethylene (PTFE) coated slide and the samples were prefixed in a Bunsen flame. The slides were fixed in ethanol:chloroform:acetic acid 6:3:1 for 20 minutes, washed in PBS for 2 minutes and immersed in 1% Evan's Blue to quench the parasite autofluorescence. After further washing in distilled water for 2 minutes the slides were examined for fluorescence.

9.4.3 Results

Labelling of stages of *O.ostertagi* from the L2 to the adult with five fluorescent lectins seldom showed binding and this was of varying affinity. The full results are entered in Table 9.10.

The tail tip of the L2 stage fluoresced weakly with Concanavalin A and *Ricinus communis*. None of the other lectins showed fluorescence with the L2 stage. There was no visible fluorescence with the L3, whether sheathed or exsheathed. The L4 fluoresced strongly throughout its length with *Ricinus communis* and also showed weaker fluorescence with *Triticum vulgaris*. There was also some variation in the staining intensity between individuals. *Arachis hypogaea* binding was specific for the excretory pore of the L4. There was no binding of the fluorescent lectins *Lens culinaris* or Concanavalin A to the L4. Adults stained with three lectins, the intensity was greatest with *Ricinus communis*, then *Triticum vulgaris* and finally Concanavalin A. Lectin binding to adults of both sexes was similar in intensity and present throughout the body length.

9.4.4 Discussion

There were marked differences in lectin binding to *O.ostertagi* and furthermore, binding varied with parasite stage. Only two lectins bound to the pre-parasitic L2 and L3. Generally, greater binding to the parasitic stages was noted. Three lectins bound to the L4 and adult, although the binding of *Arachis hypogaea* to the L4 was restricted to

TABLE 9.10

Results of surface labelling different stages of the life cycle of *Ostertagia ostertagi* with lectins conjugated to fluorescein.

Lectin	Stage				
	L2	L3	Exsheathed L3	L4	Adult
Negative control	A yellow-green autofluorescent rim was seen on the control parasite surface and outlining the intestine, but when Evan's Blue was used the autofluorescence was quenched. In the adult autofluorescence was concentrated at the accessory sexual organs.				
Arachis hypogaea	-	-	-	excretory pore	+[1]
Concanavalin A	tail tip	-	-	-	-
Lens culinaris	-	-	-	-	-
Ricinus communis	tail tip	-	-	+++	+++
Triticum vulgaris	-	-	-	++	++

[1] surface lectin binding has been designated +, ++, and +++ to denote increasing intensity of fluorescence.

the excretory pore. The low level of lectin binding to the L2 and L3 may be advantageous to the parasite. Although the absence of lectin binding in the early stages may mean a lack of the corresponding carbohydrate groups in the assay material, other factors may be responsible, e.g., steric hinderance of receptors or unsuitable concentration of reactants. The optimum situation for the free-living parasite is one of great motility, allowing selection of the best environment until it is ingested by the host, while avoiding nematode-trapping fungi. Lectins are found in all organisms, so it is interesting to speculate over the effect of host and environmental lectins on the parasite. Lectin binding has been shown to be the principle behind the capture technique employed by the nematode trapping fungus *Arthrobotrys oligospora* (Nordbring-Hertz and Mattiason, 1979). Only low levels of parasite surface receptors for lectins are generally found, which may be an advantage to the parasite preventing their impedance. In the parasitic stages increased lectin binding could be an advantage increasing adherence to the host preventing rapid expulsion from the gastrointestinal tract of the host, a holdfast mechanism. A more detailed examination of lectin binding might lead to the design of drugs which are taken up after binding to a lectin moiety.

The L2 showed binding with Concanavalin A and *Ricinus communis* indicative of the presence of N-acetyl-D-glucosamine, methyl-alpha-D-mannopyranoside, D-+-glucose, D-+-mannose, D-galactose and 1-0-methyl-beta-D-galactopyranoside groupings at the tail tip. Considering the L3 retains the L2 sheath, it would appear that some carbohydrate material has been lost from the surface with the development from L2 to L3. The L4, which is closely associated with the gastric glands showed high levels of *Ricinus communis* and *Triticum vulgaris* binding, suggesting the surface is rich in 1-0-methyl-beta-D-galactopyranoside and N-acetyl-D-glucosamine, which was maintained in the adult. D-galactose may be indicated by both *Arachis hypogaea* and *Ricinus communis*, however, since the restricted staining of the excretory pore is present with the former lectin alone the *Ricinus communis* appeared to be indicating 1-0-methyl-beta-D-galacto-pyranoside throughout the surface.

The control samples, to which no lectins had been applied, were seen to autofluoresce faintly as a yellow-green colour at the surface and occasionally along the intestine. The colour produced on autofluorescence was a more yellow-green compared to the blue-green of fluorescein. Particularly strong autofluorescence was noted at the vulval flap of the female and at the bursal sac of the male, but none of the five lectins tested were restricted to these sites to imply the presence of specific carbohydrate. The significance of these findings is not known.

In conclusion, the binding of the five lectins to different stages of the life cycle of *O.ostertagi* was found to be low in the free-living stages, but increased in the parasitic stages. There appeared to be conservation of some surface carbohydrate groupings between stages. A survey with additional fluorescent-labelled lectins would help to give a more complete picture of carbohydrate changes at the parasite surface.

9.5 GENERAL CONCLUSION

Biotin-streptavidin affinity was used to demonstrate the presence of different sets of restricted peptides when different parasite stages were biotinylated intact compared to homogenates; these peptides seemed to be surface associated. By *in vitro* incubation of biotinylated L3s with FITC-streptavidin, reduction in intensity of fluorescence was demonstrated with the addition of CTAB detergent, suggesting biotin had been removed by the detergent. Furthermore, CTAB appeared preferentially to strip one of the peptides labelled on the L3 biotinylated intact, implying the surface had been affected by the detergent. Subsequently, other workers have shown, with transmission electron microscopy, that although biotin may penetrate the nematode surface it remains within the cuticle.

With development the restricted/surface peptides detected with intact biotinylated nematodes appeared to alter, although one peptide at approximately 45 kilodaltons was present in all the stages. There was a general increase in the complexity of the surface peptide profile detected in the later stages. The surface pattern for different

trichostrongyle species and *O.ostertagi* isolates appeared similar, while the homogenate profiles varied slightly in both cases.

Insertion of lipid probes to all the parasitic stages examined was highly restricted. The recovery of fluorescence to an area where a lipid probe was inserted was poor suggesting the surface lipid was limited in its lateral mobility.

Five fluorescent lectins were used to exmine the surface carbohydrate composition of the different stages of *O.ostertagi*. There was a trend towards increased lectin binding in later developmental stages. Lectin binding may affect the interaction of the parasite with its surroundings, and thus, may have important repercussions.

In conclusion, the surface of the parasite *O.ostertagi* appeared to change throughout development with respect to peptides and carbohydrates. However, different species of trichostrongyle and different isolates of *O.ostertagi* demonstrated very similar "surface" peptide profiles, although the total peptide complement did appear to vary.

CHAPTER 10

GENERAL DISCUSSION

GENERAL DISCUSSION

Many inter-related factors may influence the course and outcome of a particular nematode infection; these factors may be broadly divided into three groups: host, environmental and finally parasitic. Parasite variation has only been recognised recently as an influence on disease and the experimental work presented in this thesis was undertaken to assess whether a molecular and structural basis for the variation in a nematode species might be detected.

Ostertagia ostertagi is a nematode found in the bovine abomasum. It was selected for analysis due to its widespread distribution and the economic impact of ostertagiosis on agriculture. The parasite has a simple direct life cycle with free-living and non-migratory parasitic phases. The time scale for the life cycle may vary, for example, the rate of free-living larval development is affected by environmental factors and an optional period of arrested development may occur soon after establishment in the host.

The parasite establishes and develops in the host gastric glands, emerging into the abomasal lumen as an immature adult. Their development affects the highly differentiated cell population of the gastric glands, reducing secretory function and increasing abomasal permeability. Clinical signs vary from lack of weight gain to severe diarrhoea, hypoalbuminaemia and death; it is thought that parasite intraspecies variation might influence the severity of an outbreak.

Pasture management and prophylactic anthelmintics are currently used to control infection, but alternatives are required with the development of drug resistant parasites. Moreover, consumer antipathy to drug treatment of stock due to its association with residues in both bovine by-products and the environment is an increasingly important factor. Ideally, vaccination would be instrumented to prevent parasitic gastroenteritis in the future, but little success has been achieved in the search for suitable immunogens against the nematodes responsible. Examination of a parasite for variation might indicate the usefulness of particular control measure by predicting variations in their efficacy to subsets of the species.

Evidence for intraspecific variation of nematodes is growing; the criteria thought to differ are morphology, biotic potential (including infectivity, arrested larval development and fecundity), antigenicity, virulence and drug susceptibility. Many of these characters have been observed to differ between outbreaks of bovine ostertagiosis, which was a further factor in the selection of *O.ostertagi* for analysis.

Two forms of the male *O.ostertagi* may be found within a population in a host with differing arrangements of their accessory reproductive organs, while the females do not appear to vary. In the past the occurrence of these different "morphs" led to the classification of parasites as either *O.ostertagi* or *O.lyrata*. However, by selective parasite mating it appeared that no reproductive barriers occur between the two types. In addition, the dimensions of *O.ostertagi* may vary in a continuous manner during an infection, initially under the influence of population size and later host immune status.

There is some evidence that individual parasite isolates may have an affect on the infectivity and virulence of infections. However, antigenic intraspecies differences in nematodes were thought to be unlikely based on the continuing efficacy of the lungworm vaccine which is derived from the same source as the material used when the vaccine was introduced over three decades ago.

O.ostertagi may enter a state of arrested larval development, which prolongs its time in the host, allowing avoidance of extremes of the external environment. The proportion of the population affected and the trigger producing arrest has been seen to alter with the location of the outbreak and the management system in which the host is kept. Larval transfer experiments have suggested that the environment may affect the parasite population at a genetic level leading to the selection of nematodes adapted to their new location over many generations, so their development patterns may change, and possibly come to resemble parasites indigenous to the new site.

Reduction in the efficacy of anthelmintics, despite adequate dosing, has been noticed on a number of occasions for many different nematode species, including *O.ostertagi*. Drug resistance is a further

variable criteria and its rate of development depends on the selective pressures applied and the varying effect they have on subsets of the population.

New techniques are being devised to aid the examination of parasite differentiation. These do not purely rely on morphological differences, since other parameters of variation are now accepted. The techniques fall into two main categories, those that scrutinise the gene sequence itself and those that look for changes in the gene products. Both approaches have their advantages and disadvantages. The DNA techniques are more direct and variation in the entire genome rather than the transcribed genome is assessed. Furthermore, an organism's DNA content is generally constant throughout its life cycle while product expression may change. However, to develop a map of the genome would require large amounts of sample and would take a long time to assimilate. Product techniques are cheaper and give a faster overview of variation, but they are not necessarily easier to accomplish. With a background knowledge of variation gained by gene product analysis, suitable loci for further assessment may be suggested to make the work more economical. Consequently, in these initial studies to search for intraspecific variation in *O.ostertagi*, gene product techniques were assayed, i.e., isoenzyme analysis, immunochemical reactions, total and surface proteins.

Isolates of *O.ostertagi* were obtained from several geographical locations in the United Kingdom and abroad for detection of intraspecific variation. A number of different trichostrongyle species were also collected to assess the relative significance of the between isolate variation detected. Basic techniques for collection and preparation of clean samples of different parasite stages were assessed for their application in the detection techniques. Protein separation by molecular mass with polyacrylamide gel electrophoresis and electroblotting was developed as a foundation for techniques with biotinylated samples and for Western Blotting.

Ability to identify parasite samples was a primary requirement of the study, to avoid confusing developmental or species differences with true intraspecific variation, i.e., a baseline for variation was established

by following the development of *O.ostertagi* throughout its life cycle. Morphology was assessed by light and scanning electron microscopy and found to give complementary information suitable for different applications. Although SEM illustrated only the restricted surface sites for variation, the types of characteristic showing intraspecific variation in other species appear to be particularly well-suited to surface examination, e.g., stoma and the accessory reproductive organs. Serial stages of the life cycle were examined for development and key changes identified, although a high degree of overlap between the rate of change of different characteristics was observed.

In vitro culture of the free-living and parasitic stages was assessed with the aim of producing species pure material of more highly defined genetic background than obtained from passage animals. Free-living phases were routinely collected from faecal cultures, but no development of the parasitic stages beyond the early L4 was achieved, so the life cycle could not be completed. Consequently, stocks of different isolates could not be maintained nor selective pressures applied *in vitro*. However, there is some evidence that parasites generated by *in vitro* techniques vary from those developed *in vivo*, so there may be strong reasons for avoiding the use of culture derived material for intraspecies analysis. The simple collection of individual stages of the life cycle was hampered by the asynchronous development of both free-living and parasitic stages within a population, requiring further purification of the sample or collection of material at particular time intervals and examination of sample composition.

Laboratory mice were infected with *O.ostertagi* larvae, firstly to assess their possible use as a model host for generation of material and secondly to test whether the mouse might be suitable for comparative infectivity studies. Initially, an infective larval dose (approximately 65 L3) scaled down from the amount given to passage calves on a weight basis was administered by oral gavage and other groups of mice were given two additional higher doses (approximately 300 and 1,500 L3). At varying times after infection, mice were euthanised and subjected to either pathological or parasitological examination. Only in one of the mice given the highest infection was a single L4 recovered at 4 DPI,

the earliest necropsy time. To assess whether the retention of the L2 cuticle was preventing establishment of the parasite a dose of 5,000 exsheathed L3s was given and higher numbers of larvae were recovered at necropsy. By suppressing the immune system, with a sublethal dose of irradiation and administering 3,000 to 5,000 L3, larvae were seen in histological sections taken 3 DPI with sheathed or exsheathed larvae. Other findings were: a greater establishing burden in the stomach of female mice compared to male mice and a marked drop in parasite numbers between 4 and 6 DPI.

O.ostertagi was not successfully maintained in the laboratory mouse and furthermore, establishment was too short-lived, i.e., few parasites were retained after 7 DPI and varied to such a degree between individual mice that the technique was felt to be unsuitable as a measure of parasite infectivity. Consequently, isolates acquired from different sources were not compared by mouse infection.

Preliminary studies of cryopreservation of *O.ostertagi* were made to evaluate its use for long-term storage of larvae. Sheathed and exsheathed larval aliquots were stored for 1 week by refrigeration or single- or two-stage cooling. Examination for motility showed short-term storage by cryopreservation led to a drop in larval viability, although the exsheathed larvae subjected to two-stage cooling survived in the highest number of these storage methods. Addition of the cryoprotectants, methanol and glycerol, brought no improvement in the number of motile larvae recovered. Although there was some loss of viable parasites maintained in suspension 0 to 4°C with time, larval loss was greater with the cryopreserved material over the same period, so cryopreservation was not recommended for the storage of samples available in only limited quantity. However, when irradiated mice were infected with freshly prepared exsheathed larvae or cryopreserved exsheathed larvae there was no statistically significant difference in level of parasite establishment.

Different isoenzymes may vary in their net charge, which will affect their mobilities in an electric field. The isoenzymes may be visualised by employing a stained substrate system. More closely related individuals will show more similar profiles when a number of enzymes

are tested for. By isoelectric focusing and starch gel electrophoresis the enzyme activities of five different trichostrongyle species and eleven isolates of *O.ostertagi* were compared for similarities.

Initially, the optimal working conditions were found for a single stock of *O.ostertagi* (Glasgow) with respect to enzyme substrate system, buffering and sample concentration, and a screen of thirteen functional tests was established for application to the different samples. Enzyme activity had to be consistent, intense and well-defined for a staining system to be included in the screen.

The degree of variation between different species samples was much greater than that between isolates by examination of similarity and clustering characteristics. In the former the number and position of the bands showed differences, while in the latter the pattern varied less frequently. Occasionally an isolate would differ from the other isolates in the group for a particular enzyme system, but the isolates which varied did not appear to follow a particular pattern. More often the differences between isolates were noted in the nature of the banding activity.

The most closely related species were the two members of the *Ostertagia* genus. The isolates fell into a cluster bearing resemblance to each other and two outlying isolates. Isolates known to be of similar origin appeared to have similar enzyme profiles. Unfortunately, neither starch gel electrophoresis nor isoelectric focusing was sensitive enough to detect enzyme levels in individual nematodes, so the occurrence of multiple banding could not be differentiated into variation of individuals or between individuals in a population. However, the work suggests suitable loci for examination by direct methods when specific material is generated. Furthermore, enzymes suitable for quantitative techniques have been identified by this study, e.g., acetylcholinesterase, glucose phosphate isomerase and malate dehydrogenase; which may have application for analysis of intraspecific and between stage variation or allow the examination of the effect of selective pressures.

Antibody production is particularly important in the defence against extracellular parasites and its local production seems to be highly significant with respect to gastrointestinal parasitism. In addition, circulating IgG, IgM and IgA are raised in infections with *O.ostertagi* and two immunochemical techniques, Western Blotting and the Indirect Fluorescent Antibody Test (IFAT), were employed to assess the systemic response to different antigen preparations and to examine surface turnover.

Western Blots prepared with different parasite stages showed strong similarities in antigen profile, although there was an increase in band number for the later stages of the life cycle. The development with time of the immune response was assessed for rabbits immunised with exsheathed or sheathed L3s by intramuscular and subcutaneous injection and calves given repeated oral infections. The rabbit results showed increased numbers of antigen bands were recognised from one to ten weeks after first immunisation. Antigens indicated at 64 and 85 kilodaltons appeared to be associated with the retained L2 sheath on the L3, since they were only recognised when sheathed L3 samples were incubated with serum from a rabbit immunised with sheathed and not exsheathed L3s. All the different isolates of *O.ostertagi* exhibited similar antigen profiles, but more striking was the uniformity of the patterns of the five trichostrongyle species examined. The findings suggest that if an antigen conferring significant protective immunity could be identified a subunit vaccine might have widespread application among different isolates and species of trichostrongyle other than that from which it was originally derived.

Examination of the nematode surface by antibody binding indicated serum from rabbits immunised with sheathed or exsheathed larvae recognised the homologous antigen more strongly than the heterologous one. Surface turnover was accelerated by increasing the ambient temperature from 4 to 37°C. The loss of bound material was particularly clear in sheathed larvae compared to exsheathed larvae with temperature increase, but long-term storage at 4°C showed greater evidence of turnover in the exsheathed larvae.

With both Western Blotting and IFAT, the results obtained with serum derived from calves infected *per os*, compared to the immunised rabbits was less intense. The reduction in intensity may have been a reflection of greater host susceptibility of the bovine, preventing adequate response to the immunogen, the different route of immunisation (per os versus injected) or a variation in effect at the level of the second antibody binding.

The basic structure of the nematode surface is well known, although between species differences are recognised in the precise composition. Surface structure is of particular interest as the first chief source of parasite antigen the host encounters. Nematodes moult four times during their life cycle and particular changes in the surface structure have been associated with these processes. The surface development of *O.ostertagi* was followed by a number of different techniques, which assessed protein, lipid, and carbohydrate properties. With respect to protein content the L3 stage of different trichostrongyle species and different isolates of *O.ostertagi* were also assessed.

Indirect radiolabelling of intact and homogenised parasites, employing the principle of biotin-streptavidin affinity, indicated restricted uptake of biotin to certain peptides of the intact samples compared to the homogenates. The low permeability of the nematode surface and the previous use of biotin-streptavidin to label the surface of bovine leukocytes suggested that these selected peptides were present on the nematode surface. Later work with FITC-streptavidin lent further support to the idea that biotin does not penetrate sufficiently to bind to internal proteins, since biotinylated intact L3s showed surface fluorescence after incubation with the streptavidin conjugated to fluorochrome. Addition of detergent to the larval samples led to a marked reduction in fluorescence intensity and uniformity of cover was lost, suggesting biotin had been removed. Furthermore, detergent action on biotinylated parasites led to the removal of a peptide of equivalent mass to one of the peptides on the intact labelled nematodes, at 45 kilodaltons, implying surface peptide had been stripped. Subsequently, other workers (Alvarez *et al.*, 1989; Hill *et al.*, 1990) have shown by transmission electron microscopy using colloidal gold

streptavidin that biotin labelled no internal peptides, but remained within the cuticle though not confined to its surface.

Comparison of the different parasite stages by intact versus homogenate labelling indicated a peptide of approximately 45 kilodaltons was common to the surface of all the samples examined. There was a general increase in the number of other surface peptides identified with development, but these varied in mass between stages, indicating the dynamic nature of the surface. In addition, the results from adult parasites indicated possible sex-specific surface peptides.

When the technique was applied to different species and isolate preparations similar masses were found for the biotinylated peptides of the intact samples, while at both levels of classification variation was observed in the homogenate peptides detected. With the interspecies comparison, the *Haemonchus contortus* samples had a number of additional peptides at 52 to 61 kilodaltons, which could be picked up by simple Coomassie blue staining. Preliminary attempts were made to further separate the biotinylated peptides of the homogenate samples from the different species and isolates by two-dimensional gel electrophoresis, but these were unsuccessful due to technical difficulties. Biotinylated adult ES products showed no particular similarity to the banding patterns achieved by labelling of intact adults, implying the peptides were not necessarily of surface origin.

Certain fluorescent lipid analogues could be inserted into the surface of the L2, L3, L4 and adult *O.ostertagi*. The insertion of these analogues allowed lipid lateral diffusion assessment by bleaching fluorescence from an area on the surface with a laser beam and surveying its return to the spot. While, protein was shown to return rapidly by insertion of fluorescein maleimide, which attaches to sulphdryl groups (83% recovery), lipid recovery was only 27%, indicating rigidity of the surface lipid. In this respect *O.ostertagi* follows the pattern previously observed for other nematode species (Proudfoot *et al.*, 1990).

Lectins are glycoproteins which link selectively to the N-terminal of specific sugars and consequently if they are conjugated to a fluorochrome they may be used to examine surface carbohydrate

components. More lectins bound to the parasitic stages of the nematode and the intensity of the staining was also greater than that for the free-living forms. The binding of environmental lectins might alter nematode movement and thus behaviour.

The gene-for-gene hypothesis was first applied to nematode plant parasites and suggests that for each gene conditioning a response to the parasite in the host there is a gene conditioning the pathogenicity of the parasite (Flor, 1956 cited by Barrett, 1981). The gene-for-gene relationship has been claimed to promote the maintenance of genetic diversity (May, 1984). Consequently, it might be expected that, while ruminant hosts have been shown to vary in their susceptibility to gastrointestinal parasitism (Stewart *et al.*, 1937; Scrivner, 1964; Preston and Allonby, 1979; Stear *et al.*, 1990), that the parasite may also show variation in its pathogenic properties. Evidence shows that cases of co-evolution of hosts and parasites occur, so analysis of the developing electrophoretic protein patterns of host and parasite may reveal similar branching patterns (Hafner and Nadler, 1988). Nadler (1987) suggested that the gene-for-gene hypothesis was based on polygenetically determined interactions and the level of variation in the environment correlated with the degree of variability in the host-parasite system. He proposed there would be an increase in the gene flow with increased host mobility, egg dispersal and host migration. Generally, domestic bovine stock is relatively immobile, with animals restricted to a certain location throughout their lives and so parasite egg dispersal tends to occur over only a small area. Thus, the level of *O.ostertagi* variation within an area was expected to be low, but comparison of samples obtained from very different environments were predicted to vary.

The results of the experimental work indicated a low level of intraspecific variation occurs within *O.ostertagi*. Kareiva (1990) suggested a certain degree of variation was necessary to provide stability in the host-parasite system. Furthermore, Bryant and Flockhart (1986) suggested the presence of multiple enzyme forms in an individual would help to accommodate fluctuations in the environment, since different forms might be of greater relative importance under different conditions and allow the parasite to adapt to

changes in the environment. Differences between the surface properties were also noticed with the development of *O.ostertagi*, which may have significant effects on the host ability to recognise the parasite and for the parasite to evade the response. However, there was one peptide of similar mass apparently at the surface of all the developmental stages of *O.ostertagi* examined.

In conclusion, it is important for the development of appropriate control measures that a full appreciation of parasite variation is achieved, so the parasite influence on the course of disease may be better understood. Furthermore, application of the knowledge gained may aid the development of strategic prophylactic measures for the induction of protective immunity and thus, remove the constant pressure to remain one step ahead of the nematode in the development of anthelmintic resistance.

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APPENDICES

APPENDIX 1

PRODUCT SOURCE LIST

PRODUCT	SOURCE
Acrylamide	BDH[1]
Adenosine 5'-triphosphate	Sigma[2]
Agarose (mouse models)	ICN, Immunobiologicals
Agarose (isoelectric focusing)	Pharmacia
Agar Oxoid	
DL-alanine	Sigma
Ammonium persulphate	BDH
Ampholines (pH 4-6,pH 5-7,pH 3.5-10)	LKB
Anti-bovine IgG peroxidase conjugate	Sigma
Antipain	Sigma
Arachis hypogaea - FITC	Sigma
L-aspartic acid	Sigma
D-biotin-N-hydroxysuccinimidester	Boehringer-Mannheim[3]
Butan-2-ol	BDH
Casein (sodium salt)	Sigma
Chick embryo ultrafiltrate	Gibco BRL
Concanavalin A - FITC	Sigma
L-cystine	Sigma
o-dianisidine	Sigma
Dihydrostreptomycin	Sigma
Eagle's medium	Sigma
Earles Balanced Salt Solution	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Boehringer-Mannheim
Fast blue RR salt	Sigma
Fluorescein di-isothyocyanate	
- anti-rabbit IgG (goat) (H and L)	Miles Scientific
Freund's Adjuvant	
Complete - *Mycobacterium tuberculosis*	
Incomplete	Sigma
Fructose-6-phosphate	Sigma
alpha-D-glucose-1-phosphate	Sigma
D-glucose-6-phosphate	Sigma
Glucose-6-phosphate dehydrogenase	Sigma
Glycine	BDH
Horse radish peroxidase conjugated	
anti-rabbit IgG (donkey)	SAPU[4]
DL-isocitric acid (trisodium salt)	Sigma
alpha-ketoglutaric acid	Sigma
Lens culinaris - FITC	Sigma
L-leucylglycylglycine	Sigma
L-leucyl-L-alanine	Sigma
L-leucyl-L-leucine-acetate	Sigma

APPENDIX 1 (continued)

L-leucyl-L-proline	Sigma
Leupeptin	Sigma
Liver extract powder (hog)	Sigma
D-mannose-6-phosphate	Sigma
NN'-methylenebisacrylamide	BDH
4-methylumbelliferyl acetate	Sigma
4-methylumbelliferyl butyrate	Koch-Light
4-methylumbelliferyl heptanoate	Koch-Light
4-methylumbelliferyl propionate	Koch-Light
[3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT)	Sigma
Mycostatin (nystatin)	Sigma
alpha-naphyl acid phosphate	Sigma
beta-napthyl acid phosphate	Sigma
Nicotinamide adenine dinucleotide	Sigma
Nicotinamide adenine dinucleotide phosphate	Sigma
Penicillin G	Sigma
Peroxidase	Sigma
1,10-phenanthroline	Sigma
Phenylmethyl sulphonyl fluoride (PMSF)	Sigma
Poly(acrylamide)	Aldrich Chemical Co.
Ricinus communis - FITC	Sigma
SDS High molecular weight standard mixture	Sigma
Sodium chloride	BDH
Sodium dodecyl sulphate (SDS)	BDH
Sorbitol (sorbite)	BDH
Starch (hydrolysed) (Connaught Laboratories, Canada)	A.R.Horwell
[^{125}I] streptavidin	Amersham
Streptavidin-FITC	Sigma
N,N,N',N'-tetramethylethylenediamine (TEMED)	Sigma
N-alpha-p-tosyl-L-lysine chloromethyl ketone	Sigma
N-tosyl-L-phenylalanine chloromethyl ketone	Sigma
Tris [(hydroxymethyl)methylamine (2-amino-2-(hydroxymethyl)propane-1,3-diol)(tris)]	BDH
Triticum vulgaris - FITC	Sigma
Yeast extract	Difco

1 BDH Ltd., Broom Road, Poole, Dorset, BH12 4NN.
2 Sigma Chemical Co.Ltd., Fancy Road, Poole, Dorset, BH17 7TG.
3 BCL, Boehringer Mannheim, Bell Lane, Lewes, East Sussex, BN7 1LG.
4 SAPU, Scottish Antibody Production Unit, Glasgow and West of Scotland Blood Transfusion Service Law Hospital, Carluke, ML8 5ES.

APPENDIX 2

Stomach and intestinal larval counts of *Ostertagia ostertagi* when administered to male or female, irradiated or non-irradiated mice were analysed for variance. Statistical examination of results of Experiment 4, Chapter 6, Part I.

Stomach Larval Counts

		Radiation Treatment	
		Non-irradiated	Irradiated
Host Sex	Male	0, 0, 2, 2	13, 4, 33, 42
	Female	3, 5, 0, 2	79, 49, 89, 81, 83

Analysis of variance of log transformed data

Source	DF[1]	SS[2]	MS	F[3]
sex	1	4.05	4.05	8.69 significant at p <0.05
irradiation	1	32.91	32.91	70.50 significant at p <0.01
Interaction	1	0.90	0.90	1.92 not significant
Error	13	6.06	0.47	
Total	16			

---

[1] degrees of freedom

[2] sum of squares

[3] sum of squares/degrees of freedom

Appendix 2 continued

Intestinal Larval Counts

		Radiation Treatment	
		Non-irradiated	Irradiated
Host Sex	Male	0, 0, 2, 2	17, 7, 21, 8
	Female	0, 4, 1, 5	18, 13, 11, 8, 4

Source	DF[1]	SS[2]	MS	F[3]	
sex	1	0.09	0.09	0.22	not significant
irradiation	1	11.93	11.93	30.56	significant at $p<0.01$
Interaction	1	0.46	0.46	1.19	not significant
Error	13	5.07	0.39		
Total	16				

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[1] degrees of freedom

[2] sum of squares

[3] sum of squares/degrees of freedom

APPENDIX 3

Statistical examination to evaluate the level of larval establishment after cryopreservation for 1 week. Experiment 6, Chapter 6, Part I.

Stomach larval counts

Cryopreserved larvae	c1 20, 1, 31, 13
Refrigerated larvae	c2 28, 85, 13, 13, 47
c3 = $\log_e$ (c1+1)	
c4 = $\log_e$ (c2+1)	

Two sample c2 Vs c3

	N	Mean	St.Dev.[1]	S.E.Mean[2]
c3	4	2.25	1.54	0.77
c4	5	3.351	0.818	0.37

t = -1.29 df = 4 p = 0.27 not significant

---

1 standard deviation

2 standard error mean

Appendix 3 continued

Intestinal larval counts

Cryopreserved larvae	c5 27, 0, 0, 2
Refrigerated larvae	c6 5, 11, 4, 11, 5

c7 = $\log_e$ (c5+1)

c8 = $\log_e$ (c6+1)

Two sample c7 Vs c8

	N	Mean	St.Dev.[1]	S.E.Mean[2]
c7	4	1.11	1.57	0.79
c8	5	2.033	0.420	0.19

t = 1.15 df = 3 p = 0.34 not significant

---

1 standard deviation

2 standard error mean

Like - but oh, how different!

William Wordsworth
(1770-1850)