CHARACTERISATION OF GLYCOPROTEINS IN POPULATIONS OF *GLOBODERA* AND *MELOIDOGYNE*

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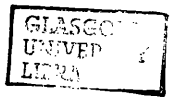

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Abstract

This is a study into the surface glycoproteins and associated terminal sugar groups on the surface of the plant parasitic nematodes in *Globodera* spp., and *Meloidogyne* spp.

The objective was to identify nematode surface glycoproteins and to investigate the possible role in the specific recognition of the suitable host plant by the parasite. In this study different populations of *Meloidogyne* spp (root knot nematode) and *Globodera* spp (potato cyst nematode) were studied using SDS polyacrylamide gel electrophoresis followed by western blotting, and the blots probed for the presence of glycoprotein using digoxygenin conjugated lectins which bind anti-digoxygenin antibody conjugated to alkaline phosphatase.

Different populations of *Meloidogyne* spp (root knot nematode) and *Globodera* spp (potato cyst nematode) were compared using the above method.

All eight populations of *Meloidogyne* tested contained >200 kDa proteins which bound Galanthus nivalis agglutinin (GNA lectin specific for mannose residues) strongly.

The *Globodera rostochiensis* populations R01 and R05-88 could be distinguished by their differential staining of both general glycoprotein stain and peanut agglutinin lectin stain (specific for galactose residues). The *G. pallida* populations Luffness and Halton could not be differentiated from each other by any of the stains.

Also GNA could differentiate five day old nematodes of population AR01 from eight to ten day old populations. The *G. pallida* population Luffness had a 60 kDa protein present in samples of nematode which had been hatched in tapwater only and not potato root diffusate. In addition a 150 kDa protein washed from the potato cyst nematode surface by the detergent CTAB may correspond to a family of proteins of molecular weight between 140 and 150 kDa detected on the glycoprotein stained western blot of the nematodes.

Although more extensive testing is necessary using a wider variety of lectins and populations of nematodes, the differences in glycoprotein profiles of potato cyst nematodes may be useful in identification of nematode populations.

Abbreviations

Potato cyst nematode (Globodera)	PCN
Potato root diffusate	PRD
Canavalia ensiformis lectin	Con A (binds mannose/glucose residues)
Galanthus nivalis lectin	GNA (specifically binds mannose residues)
Sambucus nigris lectin	SNA (specifically binds sialic acid residues)
Peanut lectin	PNA (specifically binds galactose residues)
Mannose	Man
Glucose	Glc
Galactose	Gal
Sialic acid	NeuNAc
N acetyl glucose	GlcNAc
N acetyl galactose	GalNAc
Luffness	Luff
Halton	Hal
Trichloroacetic acid	TCA
Cetyltrimethylammonium bromide	CTAB
Sodium dodecyl sulphate	SDS
Polyacrylamide gel electrophoresis	PAGE
Digoxygenin	DIG

Contents

List of Figures

List of Tables

Chapter 1
Introduction

1.1 Basic introduction to nematodes

1.1.1 General Introduction to nematodes

Nematodes are a ubiquitous group of invertebrates with representatives in almost every kind of environment. Many that live in soil and water are 'free living', some feeding on bacteria, algae or fungi, others preying on small animals including other nematodes. Some feed on higher plants and can damage crops; a few of these can transmit plant viruses.

1.1.2 Nematodes: Basic structure

Nematodes are unsegmented, usually circular in cross-section, and basically bilaterally symmetrical although the anterior region is radially symmetric. The body is usually elongate tapering at each end especially towards the tail. Adults of plant and soil nematodes range from about 0.2mm to just over 1cm (most are in the 0.5 to 1.5mm range).

The basic body structure consists of a flexible body wall (cuticle, hypodermis and somatic muscles) surrounded by a tube-like gut (oesophagus and intestine). The body cavity between the gut and body wall contains pseudocoelomic fluid and sometimes contains fibrous tissue and large cells. The gonads are self-contained tubes and usually lie alongside the intestine in the pseudocoelom. Nematodes do not have a respiratory or circulatory system (Wharton, 1986)

1.1.3 Introduction to *Globodera* spp.

A large part of these experiments were carried out on *Globodera spp.* which are cyst nematodes. These are highly specialised, sessile parasites of important crops, such as *Solanum* species. Adult males are typically elongate, motile nematodes. Pregnant females however, swell into globular or lemon shaped, thick walled organisms, filled with embryonated cysts. The swollen body of a mature female erupts to the root surface while it's anterior end is fastened to a transformed feeding site within the root. Males mate with maturing females soon after they become exposed on the root

Figure 1.1

Photograph showing potato cyst nematodes and cysts

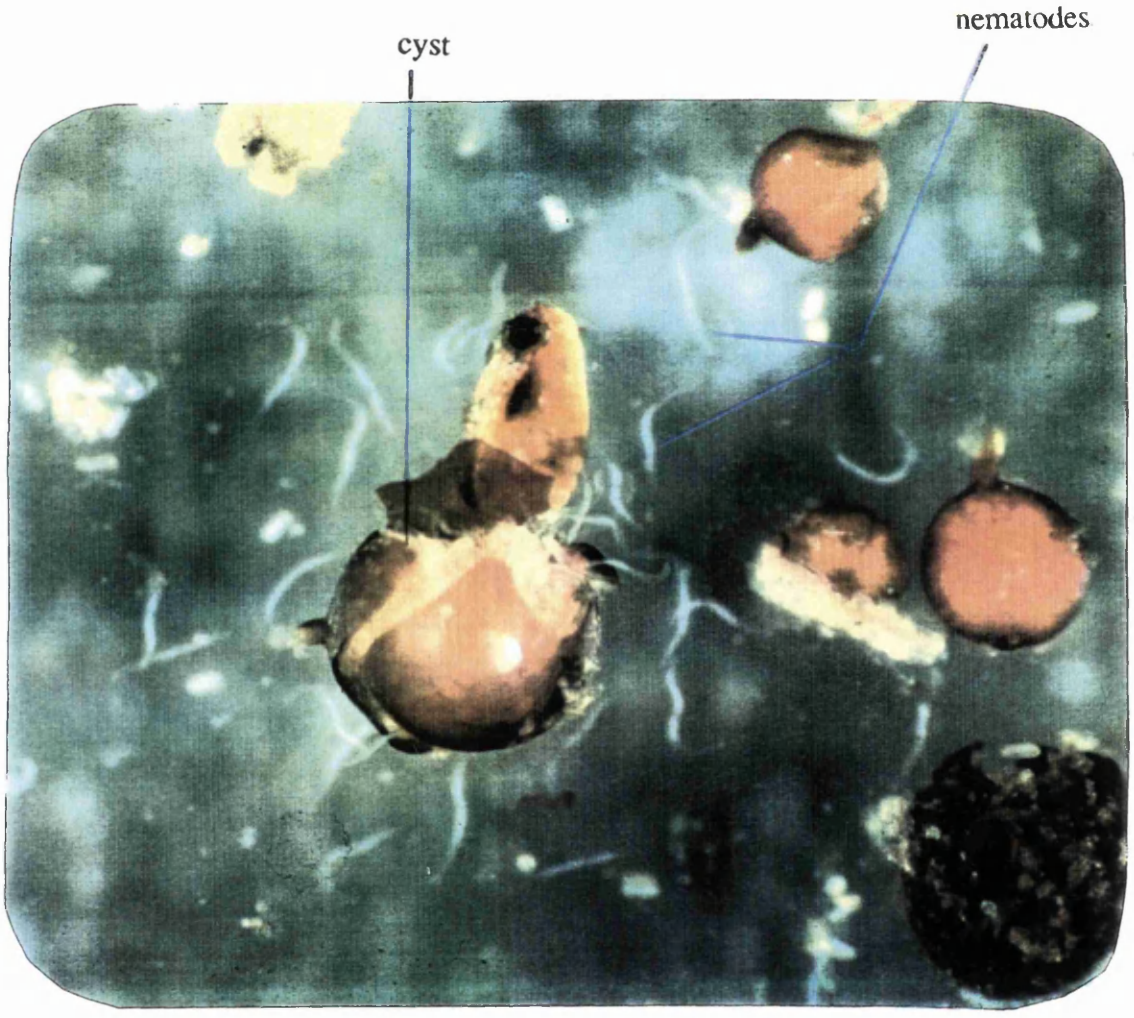
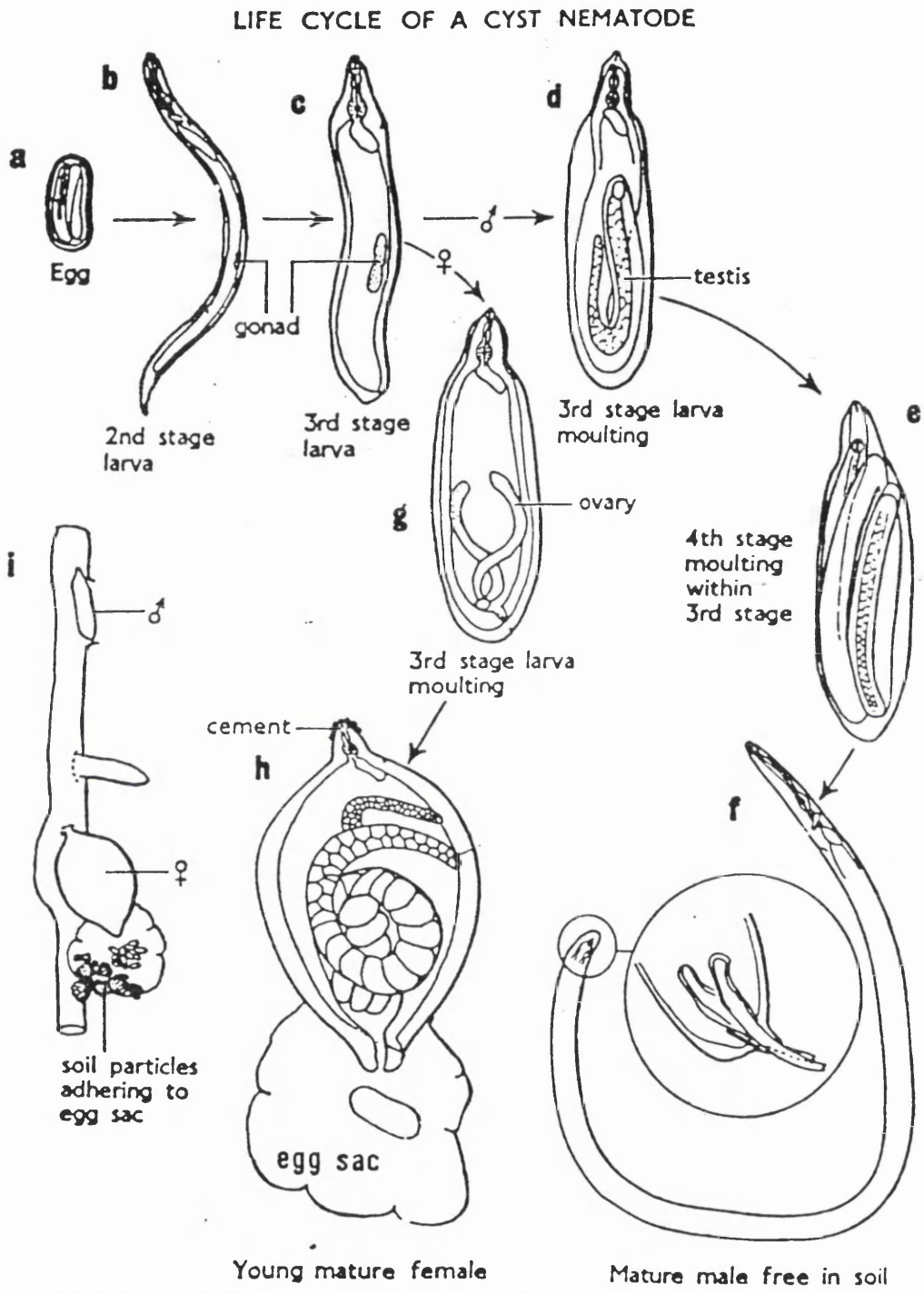


Figure 1.2



Life cycle of a cyst nematode, *Heterodera* sp.

from Jones and Jones,1974

surfaces. Upon death the female's body forms a protective enclosure (cyst) for her progeny. Cysts fall from roots and persist in soil with their infective juveniles that remain viable for many years.

1.1.4 Pathotypes of *Globodera* spp

The various pathotypes of *Globodera* have been differentiated by a scheme devised by Kort *et al*, (1977). *G. rostochiensis* was separated into five pathotypes and *G. pallida* into three. The two species are further subdivided into pathotypes by the reaction of some populations to specific resistance genes possessed by the host plant. Thus, R0-1 and -4 are distinguished from R0-2, 3 and 5 by their almost complete inability to produce females on cultivars with the H-1 gene product (Parrot,1981; Trudgill,1973). The H-1 gene is present in clones of the host species *S. andigena* and the H-2 gene is present on clones of *S. multidissectum.* The distinctions between R0-2, 3 and 5, and between Pa2 and 3 are based on small, quantitative differences in the ability of populations to reproduce on differential hosts with partial or quantitatively inherited resistance (Phillips and Trudgill, 1983).

In order to control more virulent populations of *G. rostochiensis and G. pallida,* potato breeders made crosses between *S. tuberosum* and oligo and polygenic sources of resistance such as *S. kurtzianum, S. vernei* and *S. tuberosum* ssp *andigena.* The resulting progeny of the crosses from the latter two sources were only partially resistant. However Kort *et al* (1977) used certain *S. vernei* clones as pathotype differentials, separating populations into pathotypes depending upon whether the multiplication rate on the differential clone was greater or less than 1 (pf/pi >1 or pf/pi <1) in the pot test. Here Pi is the initial density of cysts in the soil and Pf is the final density of the cysts after the nematodes have been allowed to multiply. The Pf/Pi therefore gives the multiplication rate of PCN in the soil. A similar approach was applied to the assessment of resistance: clones on which the nematode density

Figure 1.3

Geographic origins of nematodes

	Globodera pallida			
Population	Halton	Luffness	Gourdie	Newton
Geographical location	Lincoln	East Lothian	Dundee	Cambridge

	Globodera rostochiensis		
population	AR01	R02	R05-88
Geographic location	United Kingdom	Netherlands	Germany

decreased were classified as resistant whereas those on which the population density increased (albeit only slightly) were classified as susceptible.

Turner *et al* (1983) tested this hypothesis by examining the durability of resistance in *S. vernei* hybrids to *G. pallida*. They found that selection of potato cyst nematodes on these hybrids which were used to differentiate pathotypes by Kort *et al* (1977), resulted in an increase in virulence of these populations on the resistant clones on which they were selected. Subsequent results showed that considerable increases have taken place during selection through only six generations. Populations classified as Pa2 appear to contain a small proportion of virulent individuals. Most European populations of *G. pallida* therefore cannot be pure pathotypes. Instead they appear to be heterogeneous, with virulence genes occurring at different frequencies in different populations. Phillips and Trudgill (1983) suggested that British populations of Pa2/Pa3 form a continuum which seems distinct only when populations at the two extremes are compared. Trudgill (1985) postulated fromdata of Kort *et al* (1977) that the population they classified as R02 was heterogenous and composed of a mixture of virulent and avirulent individuals, with the latter predominating.

This variable or quantitative resistance to *G. pallida* on *S. vernei* and also *S. andigena* hybrids can be explained by assuming that the population level of resistance is determined by the number of individuals in a given nematode population with sufficient virulence genes in their genomes to overcome the complement of resistance genes in the genome of the plant on which the population is tested (Stone, 1985). There has been general agreement that the pathotype scheme as proposed by Kort *et al* (1977) is inadequate and requires modification (Trudgill, 1985; Stone, 1985; Mugniery *et al*, 1989; Anderson and Anderson, 1982). Stone (1985) proposed that only pathotypes defined against identified resistance genes (such as H1 andH2) are scientifically sound and of practical value and that other pathotypes of potato cyst nematodes (R02, R03, R05 and Pa2 and Pa3) should be abandoned.

In this study the various populations of potato cyst nematode (*Globodera* spp) used were as follows. The nematodes from Luffness, although classified as Pa3 under the

Figure 1.4

International scheme for potato cyst nematode pathotype identification

Differential clone	pathotype							
	R01	R02	R03	R04	R05	Pa1	Pa2	Pa3
S. tuberosium ssp *tuberosum*	+	+	+	+	+	+	+	+
S. tuberosium ssp *andigena* H1	-	+	+	-	+	+	+	+
S. kurtzianum 60.21.19	-	-	+	+	+	+	+	+
S. vernei hybrid 58. 1642/19	-	-	-	+	+	+	+	+
S. vernei hybrid 62.33.3	-	-	-	-	+	-	-	+
S. vernie hybrid 65.346/19	-	-	-	-	-	+	+	+
S. multidissectum P55/7 H2	+	+	+	+	+	-	+	+
S. vernei hybrid 69. 1377/94	-	-	-	-	-	-	-	-

In the above scheme devised by Kort *et al* (1977), a population is considered as belonging to a pathotype, if in a standard pot test, the reproductive rate is greater than one (denoted as + in the table) on the appropriate differential clone. An example can be that if a given population of *G. pallida* increases on the *Solanum vernei* clone 62.33.3 then it will be classified as Pa3; if it decreases, it will be classified as Pa2.

pathotype scheme, additionally are able to multiply on the host cultivar known as Morag. The latter behaviour is unique to Luffness since Morag is resistant to all other populations classified as Pa3. This was reported by Phillips *et al* (1992) who studied the genetic variation in British populations of *Globodera* by isozyme and DNA analyses.

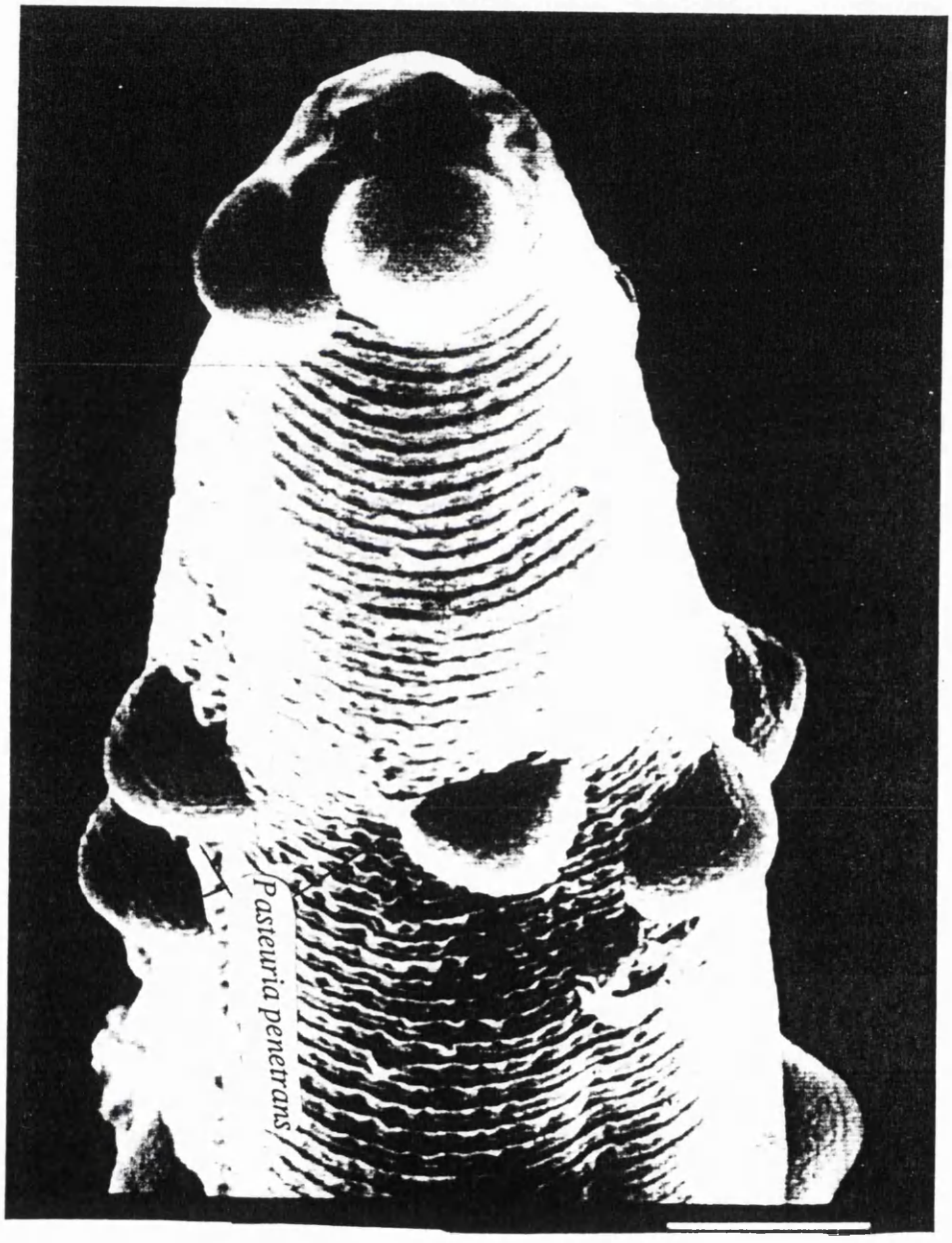
Of the pathotypes of *Globodera rostochiensis* used here the R0-1 was from the U.K. and is totally avirulent on potato genotypes with the H1 gene. The R0-2 was from the Netherlands and the R0-5 is from Germany. Of the *G. pallida* pathotypes, Halton is from Lincolnshire, Luffness from East Lothian, Gourdie from near Dundee and Newton from Cambridgeshire. Under the International pathotype scheme, Halton, which is poorly virulent, would be classed as Pa2; Newton, Luffness and Gourdie which are quite virulent, would be classified as Pa3 as in table 1.5. Fox and Atkinson (1988) used isoelectric focusing to compare pathotyped field populations of PCN and detected differences in non-specific esterase content of the populations. Electrophoretic studies can be very useful in the rapid identification of field populations of plant parasitic nematodes (Evans, 1971).

1.1.5 Introduction to *Meloidogyne* (root-knot nematode)

Meloidogyne are all obligate sedentary parasites of higher plants, generally in the roots but occasionally in stems and leaves, and they usually cause galling of the host tissues. The success of a population of *Meloidogyne* in maintaining itself depends on conditions being favourable during both the free living and parasitic stages of the life cycle. In the free living stage the infective second stage larvae must hatch, migrate through the soil, and find and invade a suitable host plant root, unless they are produced in a large gall or plant organ such as a potato tuber, where the larvae may hatch and migrate to a new feeding site without emerging into the soil. *Meloidogyne* eggs in infective soil are present in egg masses and the moisture tension favouring nematode movement is in the range 0.1-0.25 atm. (Wallace, 1963). On finding a suitable host root (Prot, 1980; Huettel, 1986), the larvae enters and moves through the surface cell layers of the root to

Figure 1.5

Photograph showing docking of *Pasteuria penetrans* on to the surface of *Meloidogyne* spp



Meloidogyne induce multinucleate giant cells without cell wall breakdown whereas *Globodera* spp induce multinucleate syncitia from enlarged cells by cell wall dissolution (Melillo *et al*, 1990; Forrest, 1985). Once inside the plant root the juvenile may develope into a male or a female depending on the conditions within the root (Trudgill, 1967).

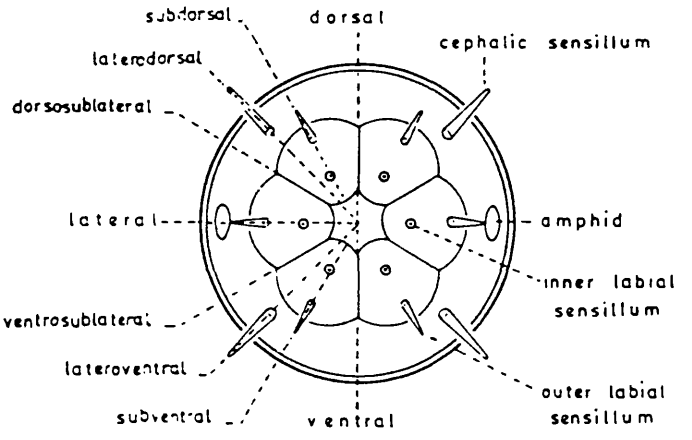
The *Meloidogyne* spp. used in this thesis were raised from single egg-masses individually inoculated into tomato plants of the susceptible cultivar Moneymaker. A range of lines have been raised from field populations collected in geographical locations (West Africa, North and Central America,South East Asia and southern Europe). Single females have been individually identified using their esterase phenotypes as revealed by native 7% PAGE (Fargette, 1987) and corresponding egg-masses individually (from each female) inoculated into tomato plants in order to build up large populations. These females (and lines) belonged to four species; *M. incognita*, *M. arenaria*, *M. javanica* and *M. mayaguensis*. These lines were then submitted to a thorough characterisation. A set of lines was chosen out of these and a more extensive study of their surface composition was aimed at in order to understand any biochemical surface variation in the light of biological characters, such as attachment by the bacterium *Pasteuria penetrans*.

1.2 Nematode sensory apparatus for host finding

One of the developing methods to combat nematode damage to plants is directed at the free living stage of the parasites. This seeks to interfere chemically with the nematode's recognition and penetration of the host plant (Daly, 1984; Dunsenberry, 1983). Closely associated with the nematode host interaction are the the sensory organs or sensilla that regulate nematode movement during host penetration and feeding. The distribution of anterior sensilla on nematodes is given in figure 1.6. The nematode sensillum usually consists of a neural as well as a non neural part. The different nematode sensilla can be grouped according: to body region (such as anterior and

Figure 1.6

Diagram showing positions of sensilla in nematodes



Basic arrangement of the anterior sensilla in nematodes

from Jones, 1959

somatic); to their position (such as peripheral or internal); to the associated organs (such as cuticular or pharyngeal).

The receptors may also be described in relation to their known functions: chemoreceptors, mechanoreceptors, photoreceptors, thermoreceptors and stretch receptors. These may detect changes in the external environment (exteroreceptors) or are sensitive to internal stimuli (proprioreceptors). The basic pattern of an anterior sensillum consists of inner and outer circlets each with six labial sensilla, four cephalic sensilla and amphids. All these sense organs can be termed cuticular sense organs (Wright, 1983).The nematode also has somatic sensilla which either occur as setae or papillae (body pores). They may be numerous and present throughout the body length or in some species they are restricted to body regions (figure 1.3). Setae are bristles on the surface of the nematode and the body pores lead to cuticularly lined sensillar canals that surrounds the receptors. The study of chemosensory responses and chemotaxis-defective mutants combined with detailed anatomical observations (Ward, 1978) has yielded much information on the chemosensitive system of *Caenorhabditis*. The main chemoreceptive organs are the amphids. They possess the highest numbers of receptors that are in contact with the external environment (Aumann, 1993).

1.3 The Lectins

The term lectin may be defined as a carbohydrate binding protein of non immune origin that agglutinates cells or precipitates polysaccharides or glycoconjugates Goldstein *et al*, (1980). As this implies, lectins are multivalent, and so at least possess two sugar binding sites which enable them to cause agglutination. The sugar specificity of lectins is usually defined in terms of the monosaccharides that inhibit the lectin induced agglutination and precipitation reactions. Also lectins, as distinct from antibodies (all of which have a fundamental domain structure) are known to vary greatly in molecular size, amino acid composition, metal requirement and three dimensional structure. Most plant and animal lectins may be classified into a rather limited number of carbohydrate groups. These include the mannose-glucose binding lectins, the N-

acetylgalactosamine/galactose binding lectins, the N-acetyl glucosamine binding lectins,and the L-fucose binding lectins (Lis and Sharon, 1973; Goldstein and Portez, 1986). The lectins also differ markedly with respect to their anomeric specificity. The various lectins used in this study along with their sugar binding specificities are given in figure 1.7.

1.4 Nematode Surface components

The nature of the nematode surface and it's interaction with the host is a fundamental component of the host-parasite relationship (Jansson, 1987; Burrows, 1992; Monsigny *et al*, 1983). Fluorochrome labeled lectins have been exploited in nematology to identify exposed carbohydrate components on the surface of nematodes (Spiegel *et al*, 1982; Zuckerman and Jansson, 1984; Jansson *et al*, 1986; Davis *et al*,1988; Spiegel and Robertson, 1988).

The phenomenon of specificity in plant infections is discussed by Sequeira *et al*, (1978). The authors define specificity in this context as applying to:

(a) Pathogens restricted to a single host species and

(b) Physiologic races of the pathogen which are restricted to a few genotypes of a host species.

Albershiem and Anderson-Prouty, (1975) have advanced the hypothesis that the gene products of the pathogen may be represented by glycosyl transferases, enzymes involved in the synthesis of terminal saccharides on surface glycoproteins. These terminal saccharides are then recognised by lectin receptor molecules; the high specificity of lectins for sugar residues and the very large number of possible configurations of these residues add to the plausibility of this scheme. Both Jansson *et al*, (1986) and McClure and Zuckerman, (1982) agree that Con A binds to *Caenorhabditis elegans* and McClure and Zuckerman further found that Con A bound to the cephalic regions of *M. incognita* although the amphidial exudate was not labelled. Also Zuckerman and Jansson, (1984) and Davis *et al*, (1988) detected

Figure 1.7

Table showing some commonly used lectins and their sugar binding specificities

Lectin	sugar specificity
Canavalia ensiformis (con A)	αMan > αGlc > αGlcNAc
Wheat germ agglutinin (WGA)	(GlcNAc β1-4)3 > (GlcNAc β1-4)2
Galanthus nivalis agglutinin (GNA)	Man α(1-3) Man
	(α1-3 > α1-6 > α1-2)
Sambucus nigris agglutinin (SNA)	NeuNAc α (2-6) Gal/GalNAc
Peanut agglutinin (PNA)	Gal β (1-3) GalNAc

From instruction leaflet in Glycan Differentiation kit (Boehringer Mannheim)

mannose, glucose and sialic acid residues on several nematodes including some *Meloidogyne* species. The blocking of the lectin binding of these sugars modified the chemotactic response. They suggest that a function of these carbohydrates in a matrix is to increase the concentration of the chemotactic factor in the zone of the nematode head, leading to competitive displacement of previously bound molecules and diffusion along the glycocalyx.

In his mini-review, Rusolahti, (1989) has suggested a role for proteoglycans in binding epidermal growth factor. Epidermal growth factor binds to the heparan sulphate chains of a heparan sulphate proteoglycan present in the extracellular matrix. The proteoglycan binding serves to concentrate and localise growth factors, enhancing their availability to growth factor receptors. Experiments done by Robertson *et al*, (1989) indicated the presence of N-acetyl glucosamine, galactose, glucose and mannose residues in association with cephalic chemosensory organs of several nematode species including *Meloidogyne incognita* and *Anguina tritici*. They conclude that these carbohydrates may have a specific role in the recognition of chemotactic factors. The experiments of Jansson *et al*, (1984), Bone and Bottjer, (1985) and Jeyaprakash *et al*, (1985) support this view. Spiegel *et al*, (1982) found sialyl but no galactosyl residues on *Meloidogyne javanica* using FITC conjugates. However McClure and Stynes (1988) found that Con A binds weakly to *Meloidogyne* (*M. hapla, M. arenaria, M. javanica* and *M. incognita*) but that all these races bound UEA (fucose binding), PNA (N-acetyl galactosamine binding), and WGA strongly at the amphidial regions. This effect was blocked by the competing sugar residues in each case. Lectin binding studies by Forrest and Robertson, (1986) have shown that amphidial exudate from potato cyst nematode contain sugars possibly in the form of glycoproteins. It was further found that PCN juveniles which had migrated from the roots of resistant or susceptible potatoes differed in their Con A and WGA binding pattern from freshly hatched nematodes (Forrest *et al*, 1988). They also noted that the labelling of the live nematodes was reduced after treatment with the protease pronase indicating that some of the sugar residues were conjugated to protein. Also Ibrahim, (1991) found that

freshly hatched juveniles of *Meloidogyne javanica* bound Con A more weakly than those emergent from roots. It was suggested that the weak fluoresence by the newly hatched J2 may be associated with lack of contact with the host. The binding of lectins by some nematodes has been found to be age-specific as in Meloidogyne (McClure and Spiegel, 1991) and *C.elegans* (Zuckerman and Kahane, 1983). The free living nematode *C. elegans* is widely accepted as a model for parasitic nematodes since it seems that many of the structural, developmental and dynamic properties of the *C. elegans* cuticle have a direct parallel in parasitic nematodes (Politz and Philipp, 1992).

1.5 Control of nematodes by lectins

Marban-Mendoza *et al* (1987) had success controlling *Meloidogyne* spp. by the application of Con A to the soil arround tomato roots. Also Davis *et al* (1989 a, b) found that treatment of J2 of *M. incognita* with Con A resulted in increased host resistance in soyabean but not with J2 of *M. javanica.* They suggested that some races of *M. incognita* may have an inherent capacity to induce host resistance whereas others cannot promote incompatibility in soyabean no matter what the treatment. Conversely (where incompatibility is a process that can be stimulated) substances such as amphidial exudate may be essential to induce compatibility between host and parasite. It seems that lectins are also used by nematophagous fungi such as *Meria coniospora* and *Pasteuria penetrans* to lock on to the nematode surface (Stirling *et al*, 1986; Jansson *et al*, 1985; Jansson *et al*, 1983; Rosenwig and Ackroyd, 1983; Rosenwig *et al*, 1985; Bird *et al*, 1989; Davies and Danks, 1993). Also the binding of the bacterium *Pasteuria penetrans* to *Meloidogyne* is inhibited if carried out after treatment with 0.1% CTAB (W.Robertson, pers. comm.). However Zuckerman, (1983) proposed that fundamental differences exist between the nematode-plant root and the nematode-predator fungus relationships. In the former, he suggested that the chemotactic factor from the roots bound to the cuticle glycocalyx of the cephalic area of the nematode and then translocated via the sensillum to the sensory membrane receptors, thereby initiating the process which leads to the behavioural response of the nematode. In the latter case,

the specific receptor binds the spores of the nematophagous fungus. This suggests that the nematode has developed mechanisms to direct it to the plant root whereas the nematophagous fungus has used a similar sort of receptor (which is more evenly distributed on the nematode surface) to aid capture of nematodes.

Increasing studies are being conducted co-culturing crop plants with leguminous plants (Marban-Mendoza et al, 1989; Marban-Mendoza et al, 1992) or making use of nematode pathogens (Schuster and Sikora, 1992).

1.6 Objectives

The objectives of the experiments in this thesis are:

(a) To find whether there are glycoproteins on the nematode surface that bind lectins, by the use of SDS PAGE followed by Western blotting of second stage juveniles of potato cyst nematodes (*Globodera* spp.) and root knot nematode (*Meloidogyne* spp).

(b) To attempt to wash off the lectin binding components from the nematode surface by means of detergents, such as CTAB and identify them.

(c) To attempt to distinguish between the different populations of *Globodera rostochiensis* (populations AR01, R05 88 and R02) and *G. pallida* (populations Halton, Luffness and Gourdie) by firstly their general glycoprotein profiles, and secondly their profiles on binding the lectins GNA, SNA and PNA which are specific for mannose, sialic acid and galactose residues respectively.

(d) To attempt to distinguish between nine populations of *Meloidogyne* spp taken from the species *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla* and *M. mayaguensis* by their GNA binding profiles.

Chapter 2
General Materials and Methods

2.1 Globodera: Collection and preparation

2.1.1 Extraction of cysts from soil

250g of dried, sieved soil was weighed out and washed through a Fenwick can. The cysts are collected on a mesh support and left to dry. The cysts can then be separated from other matter by rolling them between two flat sheets of cardboard and are then ready to be counted. The soil was obtained as in Table 1.5

2.1.2 Preparation of potato root diffusate

Sprouted tuber pieces (cv Diseree) were plated in moist sand and grown in the dark at room temperature. On formation of roots, (usually one to one and a half weeks) the roots were washed and then allowed to soak in tapwater in the proportion of 25mls of tapwater to one plant, at 4 ^{0}C for two to two and a half hours (Forrest and Farrer, 1983). The diffusate was then poured through filter paper and then passed through a bacterial filter, aliquoted and stored at 4 ^{0}C for no more than two weeks.

2.1.3 Hatching and Collection of PCN juveniles

Batches of about 300 cysts were put in each of 60 ml canisters with 5 mls of tapwater. The cysts were tumbled for one hour under running water every twenty four hours for a week or until they start to hatch. The diffusate and hatched juveniles were drawn off with a pipette and replaced with fresh diffusate or, when specified, tapwater. The nematodes were then counted and washed as by Forrest *et al* (1986). The nematodes were left to stand at 4 ^{0}C over a period of days when older nematodes were required.

2.2 *Meloidogyne* spp. Collection and Preparation

The populations are renewed by reinfection on to a susceptible variety of tomato plant. The egg-masses are ready to pick off six to seven weeks after infection. Egg-masses from each line can be picked from the root into staining blocks containing a solution of 0.3M sodium chloride which is known to inhibit the hatching of the juveniles. This is so that all the juveniles develope to the point just before hatching. The egg-masses can

be left in the salt solution for a week to ten days to allow enough nematodes sufficient time to develop after which the egg-masses are poured on to a mesh support, and washed with tapwater in a shallow dish of tapwater at 25 ^{0}C for four to five days. This results in a large synchronised hatch and the juveniles were collected at three to four day intervals, counted and prepared for electrophoresis or frozen down for later use.

2.3 SDS PAGE and Western Blotting.

2.3.1 Sample preparation for Electrophoresis

Multiple samples for electrophoresis were prepared by boiling aliquots of approximately two thousand *Globodera* or one thousand *Meloidogyne* in sample-buffer (0.0625M Trizma Base, 2%SDS, 10%glycerol and 5% 2-mercaptoethanol) for about five minutes prior to loading or storing at -20 ^{0}C. Total volume of sample loaded was 20µl. Samples stored at -20 ^{0}C were boiled a second time for five minutes prior to loading. Therefore each well contained protein from two thousand *Globodera* or one thousand *Meloidogyne*.

2.3.2 SDS PAGE

Electrophoresis of the nematode samples was carried out as according to Laemmli, (1970) with the Mini Protean mini gel apparatus (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire) A 10% polyacrylamide gel was used and a constant voltage of 100 volts was applied per gel for one hour. The separated proteins were either stained with Coomassie blue and then destain followed by silver nitrate staining or transferred to nitrocellulose paper by western blotting. Molecular weights were determined by use of molecular weight markers in the range 27 to 180 kDa obtained from Sigma, Pool, Dorset.

2.3.3 Silver staining for proteins resolved by SDS PAGE

To silver stain, the gel was first washed in 50% ethanol three times for 20 minutes each time. This was followed by a pretreatment with 0.2gl^{-1} solution of sodium thiosulphate

($Na_2S_2O_3$) for one minute and rinsed twice with distilled water for 20 seconds each time. The gel was then impregnated with a $2gl^{-1}$ solution of silver nitrate, 0.075% formaldehyde for 20 minutes and again rinsed twice with distilled water for 20 seconds each time. The protein bands were developed by exposure to a solution of $60gl^{-1}$ sodium carbonate, $4mgl^{-1}$ sodium thiosulphate and 0.037% of formaldehyde and development was stopped by immersion of the gel in 50% methanol and 12% acetic acid in distilled water. The gel was dried and stored.

2.3.4 Transfer of proteins from SDS PAGE gels on to nitrocellulose paper by Western blotting.

The transfer of proteins from polyacrylamide gels to nitrocellulose paper was carried out as by Towbin *et al*, (1979) using the mini-protean transblot apparatus (Biorad, Hemel Hempstead, Hertfordshire). A voltage of about 100 volts and 230 mA were applied for one hour in these experiments.

2.3.5- Staining of Western blot

Western blots were stained using a Glycan Detection kit (Boehringer Mannheim Ltd, Lewes, East Sussex) when staining for general glycoprotein. The process of general glycoprotein detection is dependent on vicinal hydroxyl groups in sugars of glycoconjugates being oxidized to aldehyde groups by mild periodate treatment. The spacer-linked steroid hapten, digoxygenin (DIG) is then covalently attached to these aldehydes via a hydrazine group. Digoxygenin labelled glycoconjugates are subsequently detected in an enzyme immunoassay using DIG specific antibody conjugated to alkaline phosphatase.

When staining for particular sugar residues, the Glycan Differentiation kit (Boehringer Mannheim) was used. Protocols for staining were as recommended in the manufacturer's instructions. The specific binding of lectins to carbohydrate moieties is used to identify these structures. The lectins applied are conjugated to the steroid-hapten digoxygenin and detected in an immunoassay as in the detection kit above.

GNA binding was blocked by 200m molar α methyl mannoside and PNA binding blocked by 200m molar N-acetyl D-galactosamine. (Lisa Duncan, pers. comm.).

2.4 Preparation of GNA labelled nematodes.

Freshly hatched juveniles were washed twice in phosphate buffer of pH 6.4 or 7.5 and then incubated in 40µl of total volume of GNA conjugated to digoxygenin (Boehringer Mannheim Ltd, Lewes, East Sussex). The final concentration of digoxygenin was 0.5 mg ml^{-1} in phosphate buffer and the first incubation was for one hour at room temperature. The nematodes then undergo three washes in phosphate buffer at pH 6.4 or 7.5 as before, following which they are incubated in digoxygenin specific antibody conjugated to rhodamine (Boehringer Mannheim) at a final concentration of 50µg ml^{-1} in phosphate buffer at room temperature for one hour. The nematodes are then washed twice and lightly fixed in 1.5% paraformaldehyde for ease of taking photographs under fluorescent light. Controls were carried out with nematodes fixed with no previous treatment of GNA conjugated to digoxygenin and also nematodes which had been incubated in anti-digoxygenylated rhodamine only.

2.5 Preparation of radiolabelled washes of nematodes for electrophoresis and autoradiography

2.5.1 Washing with CTAB

0-4 day old Luffness juveniles hatched in tapwater were collected, and then incubated in 1% CTAB in PBS pH 7 for an hour. Approximately 10,000 nematodes were used. The nematodes are then spun down into a pellet and the CTAB containing supernatant extracted with a pipette. This extract was then iodinated.

2.5.2 Iodination

In this process labelling of protein molecules occurs by electrophilic addition of cationic iodine ^{125}I to tyrosine residues. Iodogen or 1,3,4,6-tetrachloro-3a,6a-diphenilglycoluril (Pierce & Warriner UK Ltd., Chester) is a mild oxidizing agent.

This reagent is practically insoluble in aqueous solution and the iodination proceeds by a solid phase mechanism. Iodogen coated tubes were prepared as described by Johnstone and Thorpe, (1985). The CTAB containing fraction was added to the iodogen tubes. 100μC of $Na^{125}I$ was then dispensed into each tube and incubated at room temperature for 25 to 30 minutes. The labelled solution was passed through a PD-10 Sephadex, GM-25 column (Pharmacia, Milton Keynes, Bucks.) which had previously been washed with PBS and 1% BSA in PBS to block nonspecific sites. The purpose of this step was to enable the excess, unreacted ^{125}I ions to be retained by the column so that the eluted solution should only contain iodine labelled proteins.
Two fractions of eluant from the column (PCN-1 and PCN-2) were tested for amount of free iodine still present. 50μl of the eluants were subjected to Trichloroacetic acid precipitation of bound iodine using 10μl of 1% BSA as carrier protein, and it was found that 90% of the radioactivity in both fractions was due to free iodine. The eluant fraction PCN-1 and PCN-2 were then dialysed with PBS, pH 7.0 at 4°C overnight.

2.5.3 Sample preparation for electrophoresis

The two fractions were further subdivided into 100μl aliquots and these were freeze-dried. The freeze dried samples of PCN-1 were taken up in 80 μl of sample buffer and PCN-2 was taken up in 270 μl of sample buffer. About 5 μl of each sample was loaded so that each track loaded contained approximately 50,000cpm of radioactivity.

2.5.4 Electrophoresis

Electrophoresis was carried out as in section **2.3.2.**

2.5.5 Autoradiography

Following electrophoresis, the gel was stained with Coomassie blue and destained. The gel was then incubated in 1M sodium salicylate for 30 minutes in the dark. The gel was dried down on to filter paper and exposed to Kodak-X-OMAT film at -20°C for four weeks prior to developing film.

Chapter 3
Results

3.1 Introduction

Groups of approximately 2000 nematodes from each of six populations of *Globodera* and approximately 1000 nematodes from each of nine populations of *Meloidogyne* were tested for protein and glycoprotein content by SDS PAGE and western blotting. In all experiments, nematode proteins were prepared by boiling nematodes in sample buffer and samples containing whole nematodes were loaded on to the gel. Further tests were carried out using digoxygenin conjugated lectins to probe for the presence of galactose, sialic acid and mannose residues on nematode glycoproteins by western blotting. Attempts to extract nematode surface proteins by the cationic detergent CTAB are described as well as fluorescent labelling of live nematodes. Figures 2.1 to 3.2 can be consulted together with the corresponding tables 1 to 6.

3.2 *Globodera* spp

3.2.1 Protein profile of *Globodera* spp

From Table 1, Figure 2.1 the protein profiles of all *Globodera* populations are similar to each other and it was not possible to differentiate the populations by one-dimensional SDS PAGE. All populations have a sharp protein band at 112.2 kDa and an intense, broad band of approximate molecular weight 45.4 kDa. Also there are proteins at 38.9, 29.3, 25 and 21 kDa.

3.2.2 Whole *Globodera* tested for general glycoprotein

From Table 2a, figure 2.2a there is a band of molecular weight between 142 and 149 kDa in the *G. pallida* populations of Halton and Luffness (both old and young nematodes), and the *G. rostochiensis* populations of R05 88 and AR01. The *G. pallida* populations of Halton and Luffness (both old and young nematodes) have very similar profiles. However Luffness and Halton differ from *G. rostochiensis* (R05 88 and AR01) since only the latter populations have a band in the region 125 to 128.8 kDa. R05 88 has a broad band at 123 kDa which does not appear in AR01. Luffness

and Halton both have a broad, intense band at 52 to 60 kDa and AR01 has a band of the same breadth and intensity with an Mr of 68.4 kDa. Also there are two intensely staining bands in the R05 88 population only with molecular weights in excess of 200 kDa which appear at the top of the track. All populations have common bands at 105.9-112.2 kDa, 90.8-94.2 and 40.7-42.5 kDa.

Figure 2.2a differs from 2.2b although the staining procedure for glycoprotein was the same in both experiments. It is thought that this may be a result of prolonged storage of the samples used in experiment 2.2a

Figure 2.2b, table 2b shows that all populations of *Globodera* tested for glycoprotein have high molecular weight proteins of about 190 kDa and 177.8 kDa. In addition the *G. pallida* population Luffness where the nematodes where the nematodes were hatched without potato root diffusate (PRD) present have a faint band at 60.5 kDa which is not present in the Luffness nematodes which were hatched in PRD.

All the populations have a broad, intense band between 177.8 kDa and 154.9 kDa which is especially broad in R02. A smeared band of molecular weight 150.7 kDa can be seen in all populations. Figure 2.2c, table 2c shows Halton, Luffness, R02 and R05 88 also tested for general glycoprotein. All populations have three major bands of which the band at 169.8 kDa is very intense. This band is especially broad in the *G. rostochiensis* population R02.

Since the results in Table 2a differed from those of tables 2b and 2c, fig. 2.2c another experiment was carried out to test if the results of Table 2a were as a consequence of proteolysis of the nematodes during storage. Table 2d, figure 2.2d shows PCN again stained for glycoprotein. In this case the nematodes were allowed to stand at room temperature in sample buffer for two and a half hours before carrying out electrophoresis. This was to check whether much protein degradation would take place in this time. The resulting bands show that there has been some protein degradation in this time with one visible lower molecular weight band (66.8 kDa) appearing in all of them. The extra, small band appearing previously in only the water hatched nematodes is not present here and may have been degraded.

3.2.3 Localisation of GNA lectin on the surface of live *Globodera*

The fluorescent studies (Figure 2.6a and c) show that at both pH values GNA has bound to the amphids of potato cyst nematodes.

3.2.4 Whole potato cyst nematodes (*Globodera*) tested for mannose residues

Western blots of eight to ten day old *G. rostochiensis* (population AR01) and *G. pallida* (Luffness population) were probed with *Galanthus nivalis* lectin (GNA) for mannose residues (Figure 2.3a, Table 3) . The GNA seemed to have stained several bands which are common to both species. These included mannose glycoproteins at molecular weights of 123, 39.8, 28.2 22.9 and 21.1 kDa. There was one band at 61.6 kDa, which is present in the AR01 sample but not in the Luffness population. Four day old *G. rostochiensis* (AR01) nematodes (Figure 2.3b, Table 4) were distinguished from those of *G. pallida* (Luffness) by the presence of bands at 55, 29.2, and 26.3 kDa in the *G. rostochiensis*. All bands were blocked by 200mm methyl mannose

3.2.5 *Globodera* tested for sialic acid residues

Western blots of four to six day old *Globodera* (Figure 2.4, Table 5) were probed with *Sambucus nigris* lectin (SNA) which detects sialic acid residues. This indicated that both *G. rostochiensis* and *G. pallida* had one glycoprotein of molecular weight 124.5 which may contain sialic acid residues.

3.2.6 *Globodera* tested for galactose residues

As in Figure2.5, Table 6 only one glycoprotein (molecular weight 95.5 kDa) was present in all the populations, except the eight day old ARO1 population which therefore contains galactose residues

Of the three *G. pallida* populations, Gourdie has a galactose profile consisting of five glycoproteins and was different from both the Halton and Luffness profiles. However Halton and Luffness profiles were similar with one faint band at about 95.5 kDa and

two more intense bands of molecular weights 93 and about 80 kDa. The *G. rostochiensis* populations differed from the *G. pallida* populations of Halton and Luffness by the presence of galactose containing proteins at about 150, 138 and 120-125 kDa. There was a very close match between the AR01 and Gourdie profiles in that they both contained two intensely staining proteins at aproximately 120 and 102-107.8 kDa. Amongst the *G. rostochiensis* populations, AR01 could be distinguished from R05-88 by the lack in R05-88 of the glycoprotein of molecular weight 102 kDa and the presence of a band at 83.2 kDa which was not present in either of the AR01 populations. AR01 had a similar galactose containing glycoprotein profile to that of Gourdie. All bands were blocked by 200mm N-acetyl D-galactosamine

3.3 *Meloidogyne* spp

3.3.1 Protein profiles of nine different populations (lines) of Root knot nematode (*Meloidogyne* spp.)

The protein profiles of the different lines of *Meloidogyne* spp (see Tables 8a, b and Figure 3.1a,b) showed very little difference between the different strains. The lightly silver stained gel (Table 8a) showed major protein bands at 48.4, and 40.1 kDa and lower molecular weight bands at 25 and 22.3 kDa which were present in all the lines. Table 8b shows a gel which was overstained for protein and revealed more bands which stained more faintly including some higher molecular weight bands; the major ones being of molecular weights 141.6 and 112.2 kDa.

3.3.2 Extracts of Root knot nematodes (*Meloidogyne*) tested for mannose-containing glycoproteins

The western blot of *Meloidogyne* populations (referred to as lines or L) probed with GNA for terminally linked mannose residues (Table 9, Figure 3.2) stained proteins of

120 kDa in lines 17, 9, 5, 10 and 32. Lines 17, 5, 10, 32 and 23 had a band at about 50 kDa which varied slightly between the various populations. In addition, lines 17 and 5 seem to have a similar banding pattern as do lines 32 and 9. L 10 has four very faint, high molecular weight bands and L 24 does not seem to contain any glycoproteins with terminally linked mannose residues (at least not of a sufficiently low molecular weight to be resolved here). However the western blot does indicate very intensely staining bands in the region of the stacking gel for all the populations.

3.4 1% CTAB containing washes of *Globodera*

As in Table 7, the CTAB seems to have extracted one or more proteins of molecular weight about 150 kDa from nematodes from population luffness.

Figure 2.1

Nematodes from populations of *Globodera rostochiensis* (populations R05 88 and AR01) and *G. pallida* (populations Halton and Luffness) were separated by SDS PAGE and then Silver stained (as in Materials and Methods section 2.3.3) to stain proteins. Approximately 2000 nematodes were run in each track. Table 1 lists the most prominent bands, which are present in all populations.

Table 1

Protein profile of populations of Globodera

All populations have bands at the following molecular weights (in Daltons)

112,200

45,400

39,800

29,300

25,000

21,000

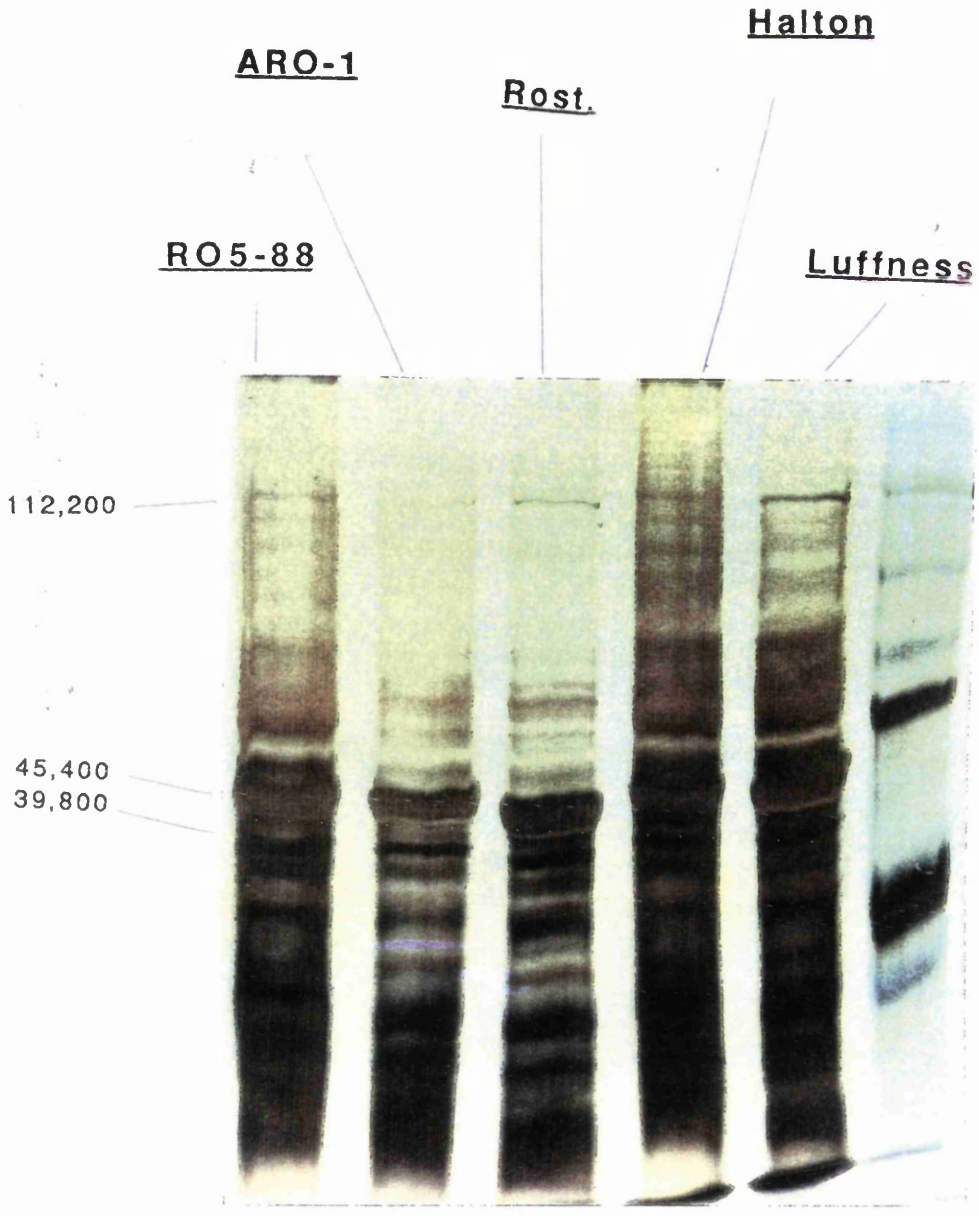

Halton
ARO-1
Rost.
RO5-88
Luffness
112,200
45,400
39,800

Figure 2.2 a

Nematodes from *G. rostochiensis* (populations R05 88 and AR01) and *G. pallida* (Luffness and Halton) were separated by SDS PAGE and then transferred onto nitrocellulose paper. The transferred proteins were tested for general glycoprotein as follows: vicinal hydroxyl groups in glycoconjugates were oxidized to aldehydes by mild periodate treatment. The spacer linked steroid hapten , digoxygenin (DIG) was then covalently attached to these aldehydes via a hydrazine group. Digoxygenin labelled glycoconjugates were detected in an enzyme immunoassay using DIG specific antibody conjugated to alkaline phosphatase. Table 2a is a line drawing of figure 2.2a.

Table 2a

Globodera spp glycoprotein stained

R05 88 (1-3 days old)	AR01 (up to 8 days old)	Luffness (up to 8 days old)	Luffness (1-3 days old)	Halton (1-3 days old)
146,200	149,300	142,900	142,900	146,200
128,800	125,900			
123,000				
112,200	109,200	105,900	105,900	107,600
94,200	90,800	92,900	92,900	92,900
	68,400			
60,300		60,300	60,300-52,500	60,300-52,500
48,400	48,400	55,500		
42,500	40,700	40,700	40,700	40,700

Fig 2.2a

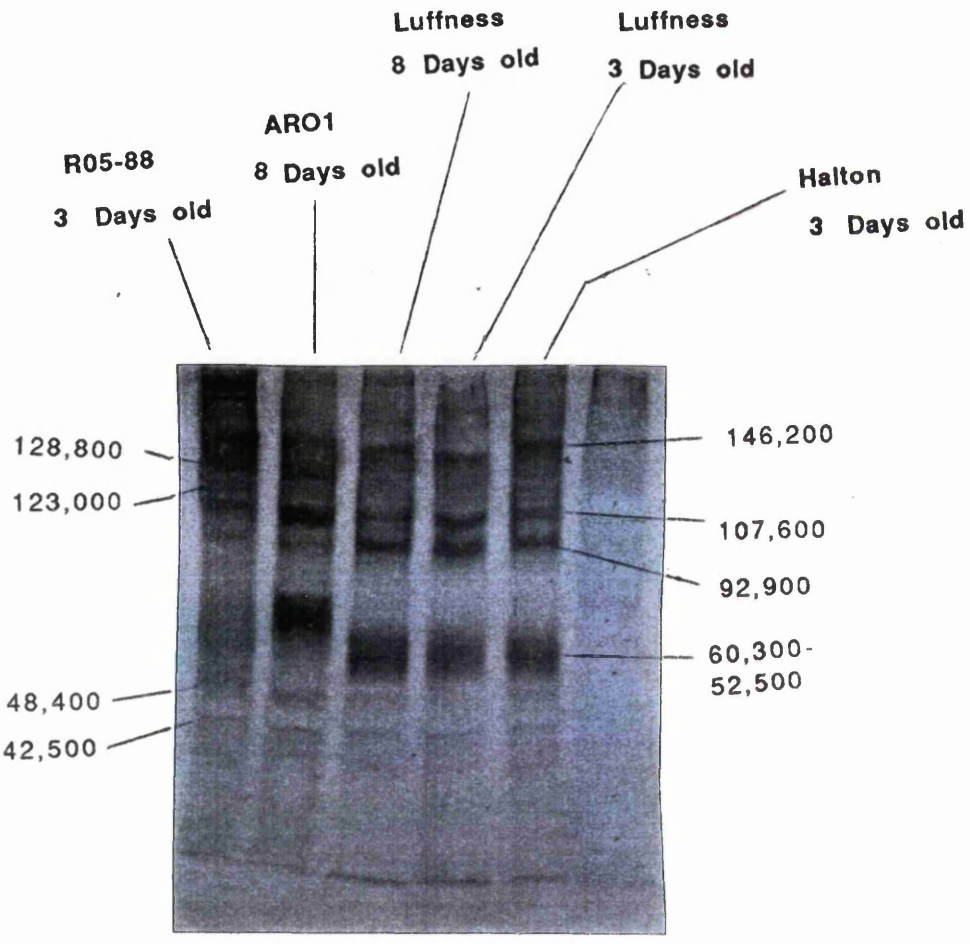

Luffness
8 Days old
Luffness
3 Days old
ARO1
8 Days old
R05-88
3 Days old
Halton
3 Days old
128,800
123,000
48,400
42,500
146,200
107,600
92,900
60,300-
52,500

Figure 2.2 b

Nematodes from *G. rostochiensis* (R02) and *G. pallida* (Halton and Luffness) were treated as in Figure 2.2 a. Table 2.2 b shows a line drawing of figure 2.2 b.

Table 2b

Globodera spp glycoprotein stained

Halton 4 days old +PRD	Luffness 4 days old +PRD	R02 4 days old +PRD	Luffness 2 days old +PRD	Luffness 2 days old -PRD
190,500	190,500	190,500	190,500	190,500
177,800	177,800	177,800	177,800	177,800
		154,900		
150,700-141,300	150,700-141,300	150,700-141,300	150,700-141,300	150,700-141,300
				60,500

Fig 2.2 b

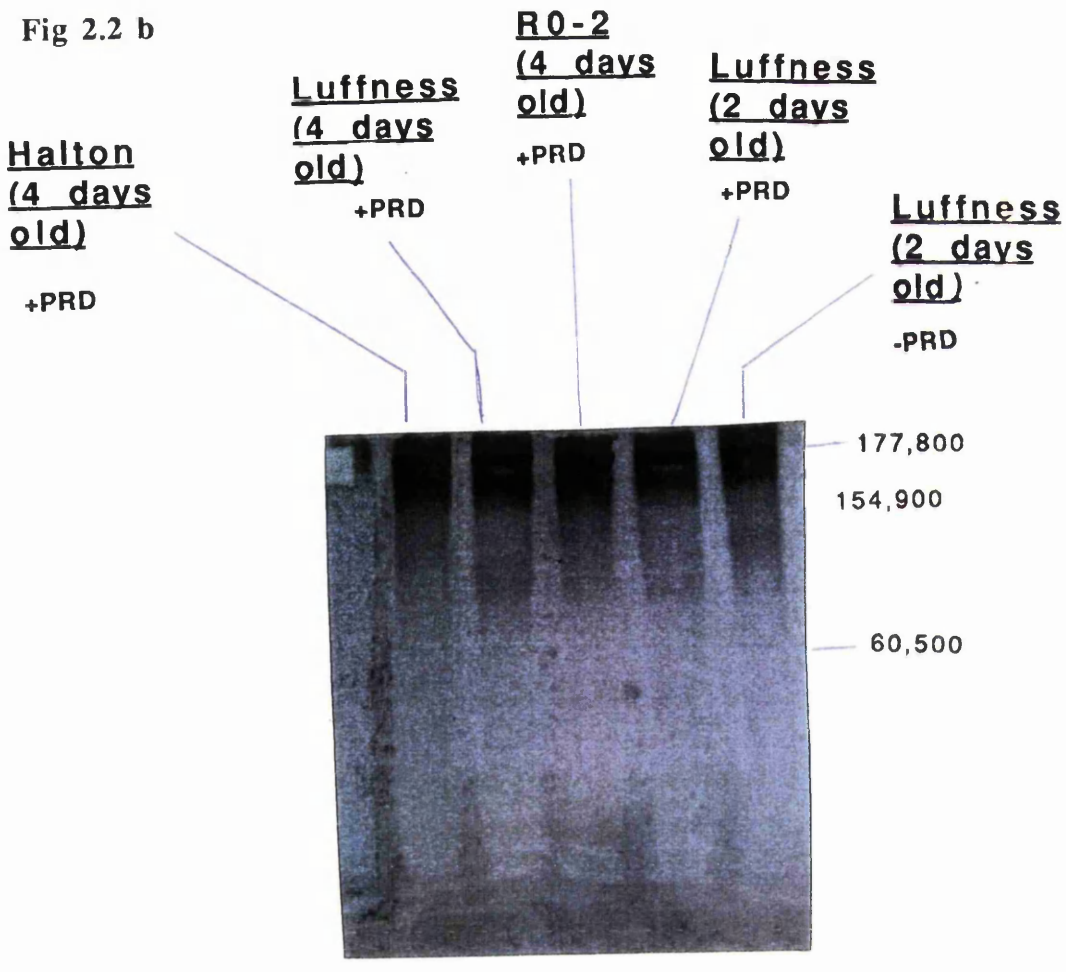

Halton (4 days old) +PRD
Luffness (4 days old) +PRD
R0-2 (4 days old) +PRD
Luffness (2 days old) +PRD
Luffness (2 days old) -PRD
177,800
154,900
60,500

Figure 2.2 c

Nematodes from *G. rostochiensis* (populations R02 and R05 88) and *G. pallida* (populations Halton and Luffness) were treated as in figure 2.2 a to stain for glycoprotein.

Table 2c shows a line drawing of figure 2 c.

Table 2c

Globodera spp probed for glycoprotein

Halton 4 days old +PRD	Luffness 4 days old +PRD	R02 4 days old +PRD	R05 88 4 days old +PRD
184.100	184.100	184.100	179.900
169.800	169.800	175.800-158.500	166.700
144.500-142.900	147.900-142.900	147.900	147.900

Fig 2.2c

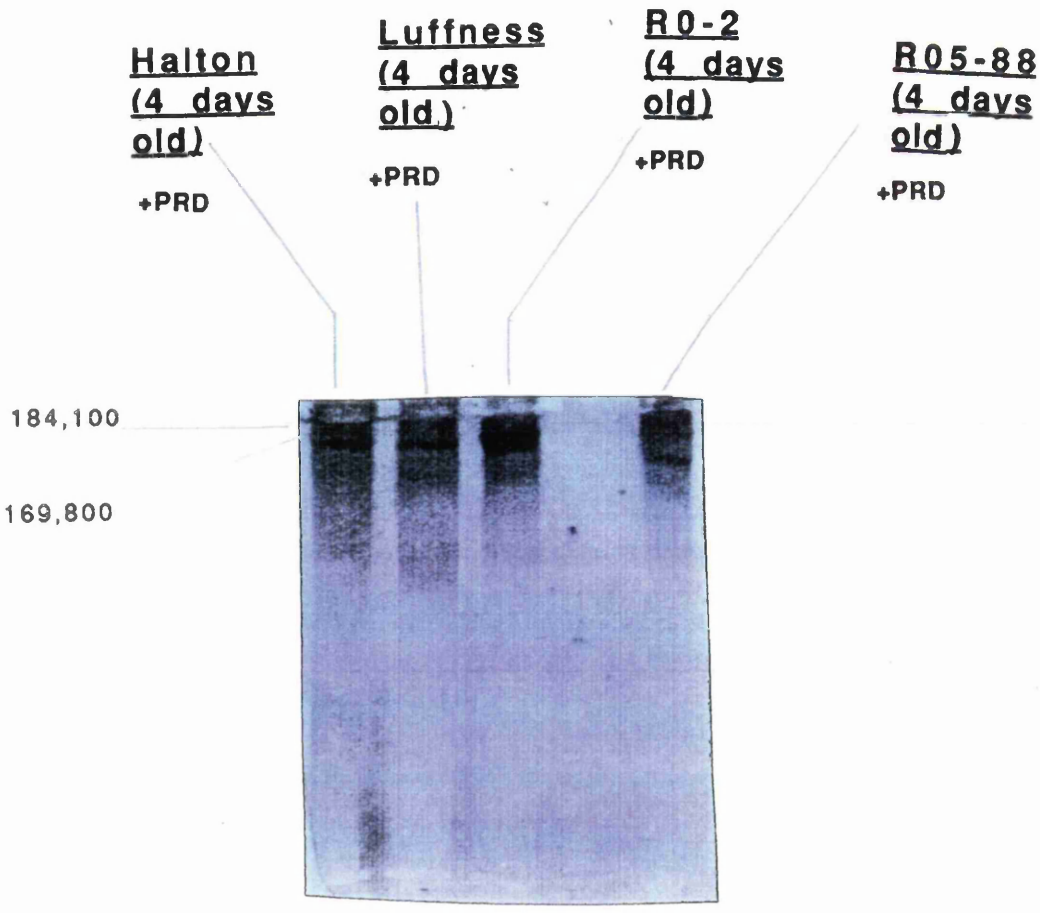

Halton (4 days old) +PRD
Luffness (4 days old) +PRD
R0-2 (4 days old) +PRD
R05-88 (4 days old) +PRD
184,100
169,800

Figure 2.2 d

Nematodes from *G. rostochiensis* (population R02) and *G. pallida* (population Luffness) were treated as in figure 2.2 a after natural degradation at room temperature for two and a half hours. Table 2 d shows a line drawing of figure 2.2 d

Table 2d

Globodera spp probed for glycoprotein

(After natural degradation over 2.5 hours at room temperature)

Luffness 4 days old +PRD	R02 4 days old +PRD	Luffness 2 days old -PRD	Luffness 2 days old +PRD
194,100	194,100	194,100	200,800
169,800 165,200	169,800 165,200	165,200	169,800
		82,200 75,900	82,200
66,800	64,000		

Fig 2.2 d

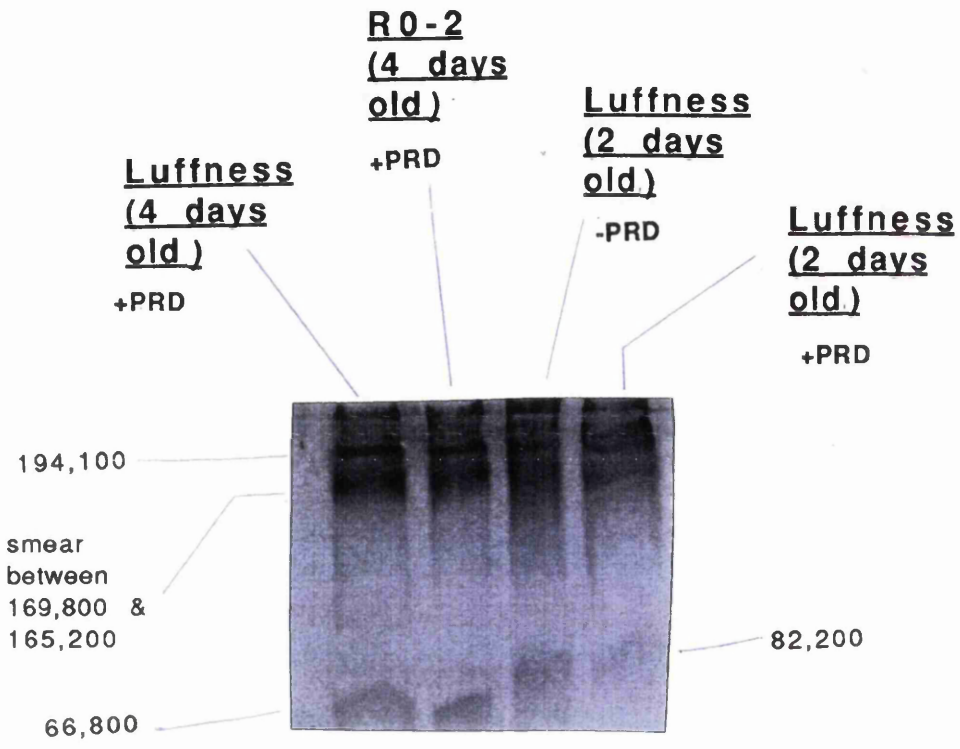

Luffness (4 days old) +PRD
R0-2 (4 days old) +PRD
Luffness (2 days old) -PRD
Luffness (2 days old) +PRD
194,100
smear between 169,800 & 165,200
66,800
82,200

Figure 2.3 a

Eight to ten day old Nematodes from *G. rostochiensis* (population AR01) and *G. pallida* (population Luffness) were separated by SDS PAGE and then transferred on to nitrocellulose paper. The transferred proteins were probed with digoxygenin conjugated to *Galanthus nivalis* lectin (GNA) which binds specifically to terminally linked mannose residues as shown in figure 1.7. The digoxygenin labelled glycoconjugates were detected in an enzyme immunoassay using digoxygenin specific antibody conjugated to alkaline phosphatase. Table 3 shows a line drawing of figure 2.3 a.

Table 3

Globodera spp probed with GNA
(older nematodes)

Luffness 8-10 days old	AR01 8-10 days old
123,000	123,000
	61,600
39,800	39,800
27,200	28,200
22,900	22,900
21,100	21,100

Figure 2.3 a

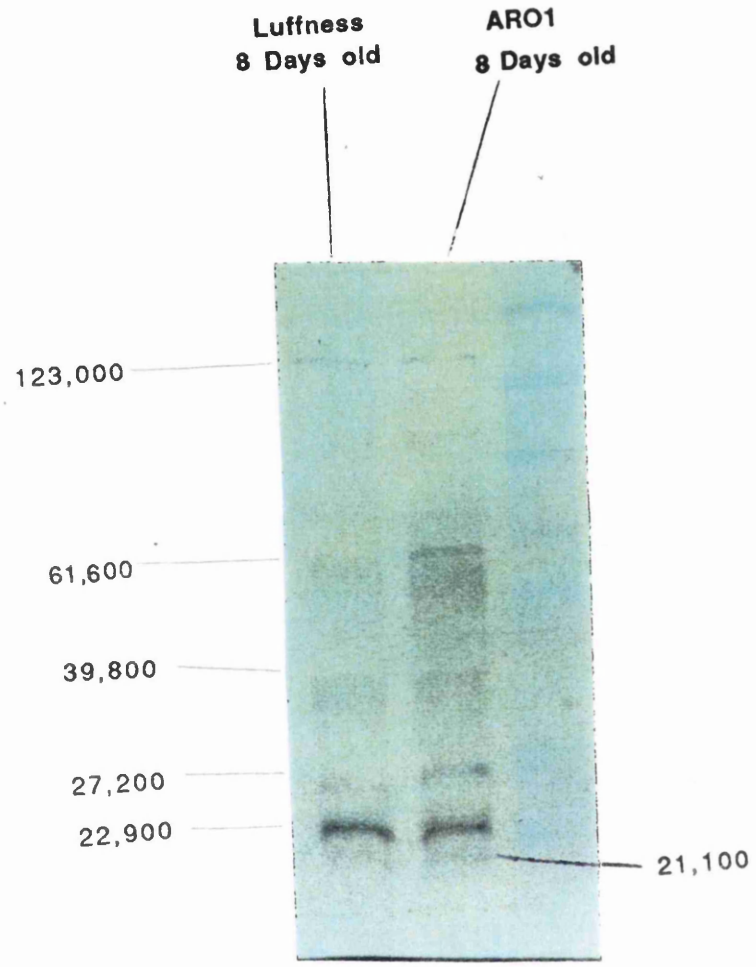

Luffness
8 Days old
ARO1
8 Days old
123,000
61,600
39,800
27,200
22,900
21,100

Figure 2.3 b

Four to five day old nematodes from *G. rostochiensis* (population AR01) and *G. pallida* (population Luffness) were treated as in figure 2.3 a. Table 4 shows a line drawing of figure 2.3 b.

Table 4

Globodera spp probed with GHA
(younger nematodes)

Luffness 4-5 days old	**AR01 4-5 days old**
131,800	131,800
112,200	112,200
86,100	86,100
60,300	58,900-55,600
	41,200
30,200	30,200
	29,200
27,900	27,900
	26,300

Figure 2.3 b

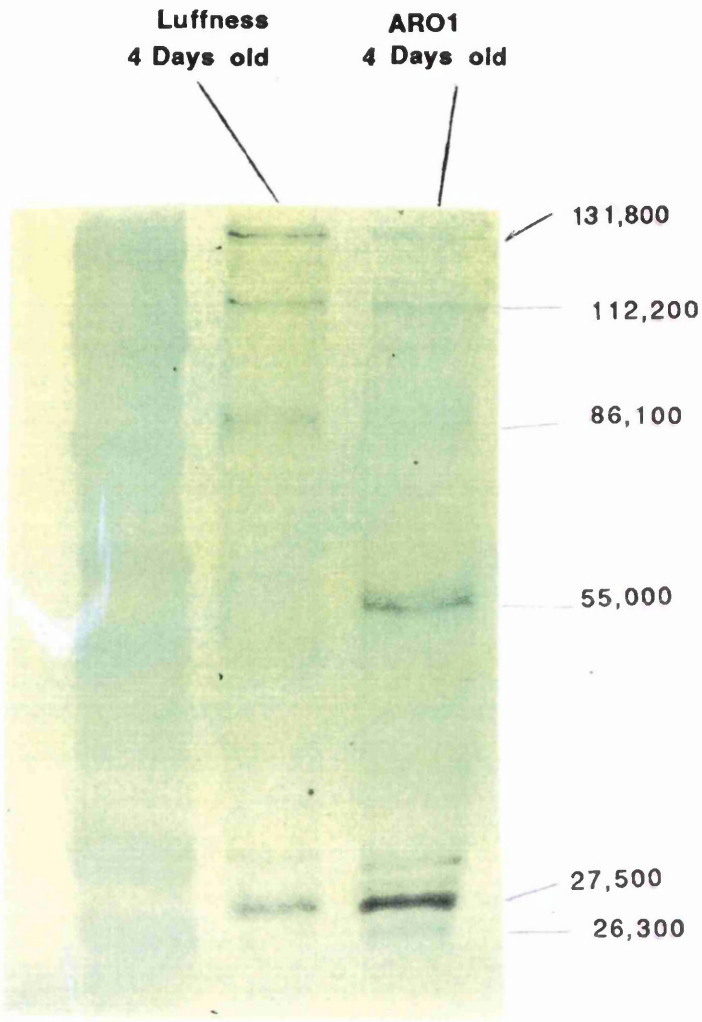

Luffness
4 Days old
ARO1
4 Days old
131,800
112,200
86,100
55,000
27,500
26,300

Figure 2.4

Four to six day old nematodes from *G. rostochiensis* (population AR01) and *G. pallida* (population Luffness) were separated by SDS PAGE and then transferred on to nitrocellulose paper. The transferred proteins were probed with digoxygenin conjugated to *Sambucus nigris* lectin (SNA) which binds specifically to sialic acid residues. The digoxygenin labelled glycoconjugates were detected in an enzyme immunoassay using digoxygenin specific antibody conjugated to alkaline phosphatase. Table 5 shows a line drawing of figure 2.4.

Table 5

Globodera spp probed with SNA

Luffness 4-6 days old	AR01 4-6 days old
124,500	124,500

Figure 2.4

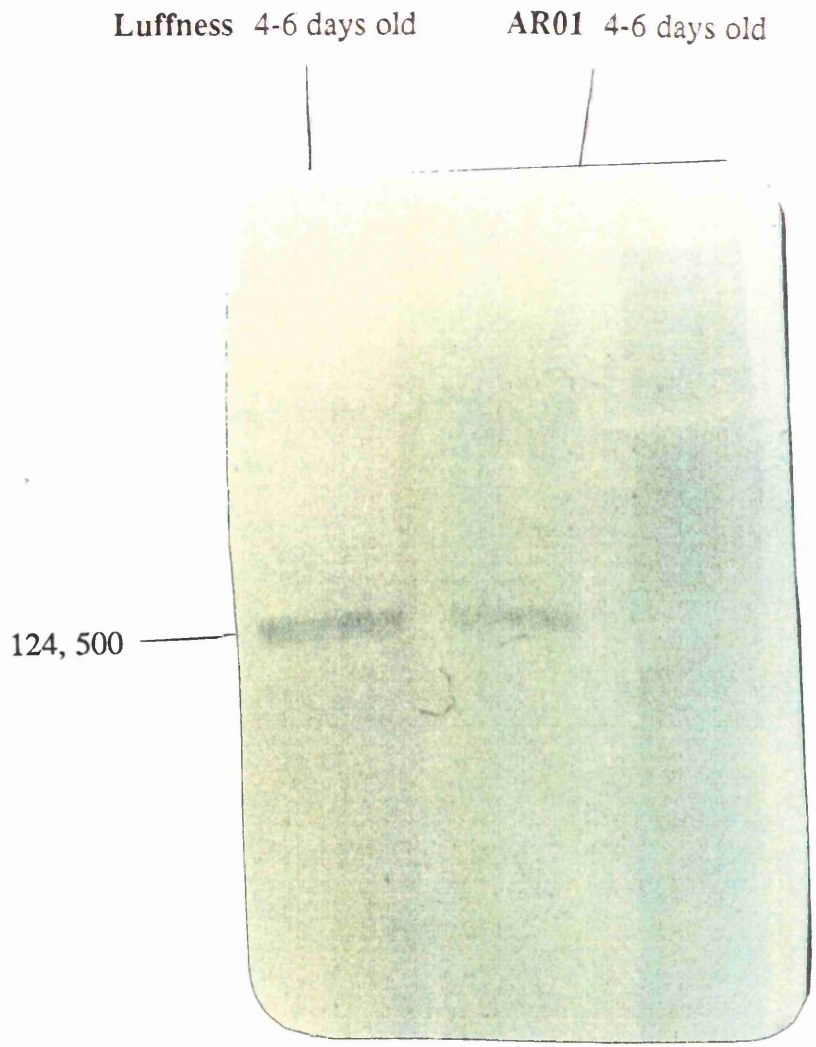


Figure 2.5

Nematodes from *G. rostochiensis* (population AR01 and R05 88) and *G. pallida* (Halton, Luffness and Gourdie) were separated by SDS PAGE and then transferred on to nitrocellulose paper. The transferred proteins were probed with digoxygenin conjugated to peanut agglutinin which binds specifically to galactose residues. The digoxygenin labelled glycoconjugates were detected in an enzyme immunoassay using digoxygenin specific antibody conjugated to alkaline phosphatase. Table 6 shows a line drawing of figure 2.5.

Table 6

Globodera spp probed with PNA

G. pallida			G. rostochiensis		
Gourdie	Halton	Luffness	AR01	R05-88	AR01
			(8-10 Days old) 158,900		
147,200			149,600		153,100
138,000			138,000		138,000
120,000			123,000	125, 900	125, 900
107, 800					102,300
95,500	95,500	95,500		95,500	95,500
	93,000	93,000	91,200		
	79,400	81,300		83,200	

Figure 2.5

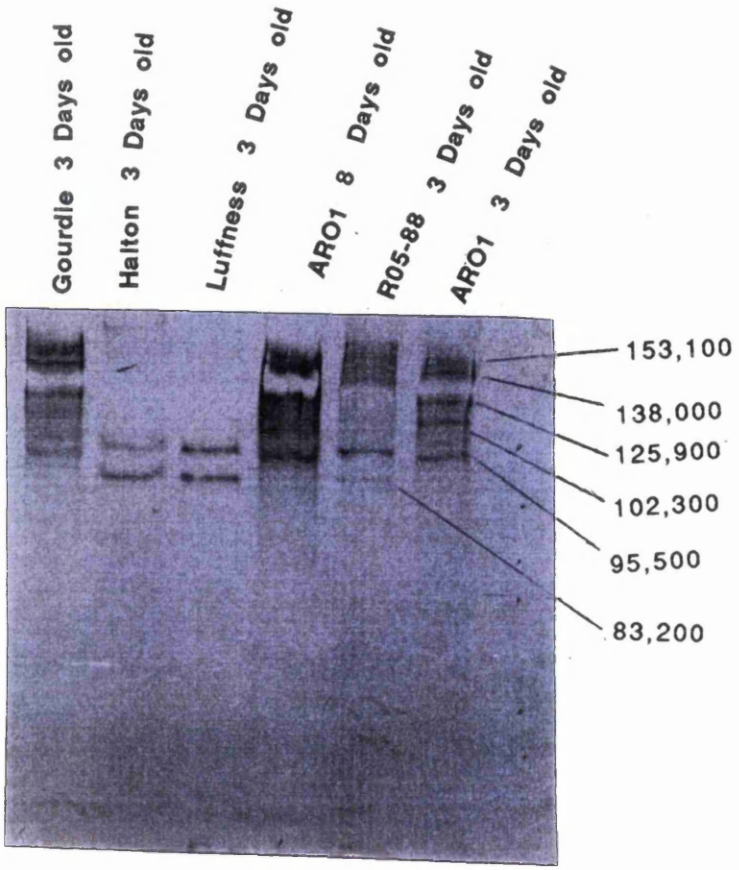

Gourdie 3 Days old
Halton 3 Days old
Luffness 3 Days old
ARO1 8 Days old
R05-88 3 Days old
ARO1 3 Days old
153,100
138,000
125,900
102,300
95,500
83,200

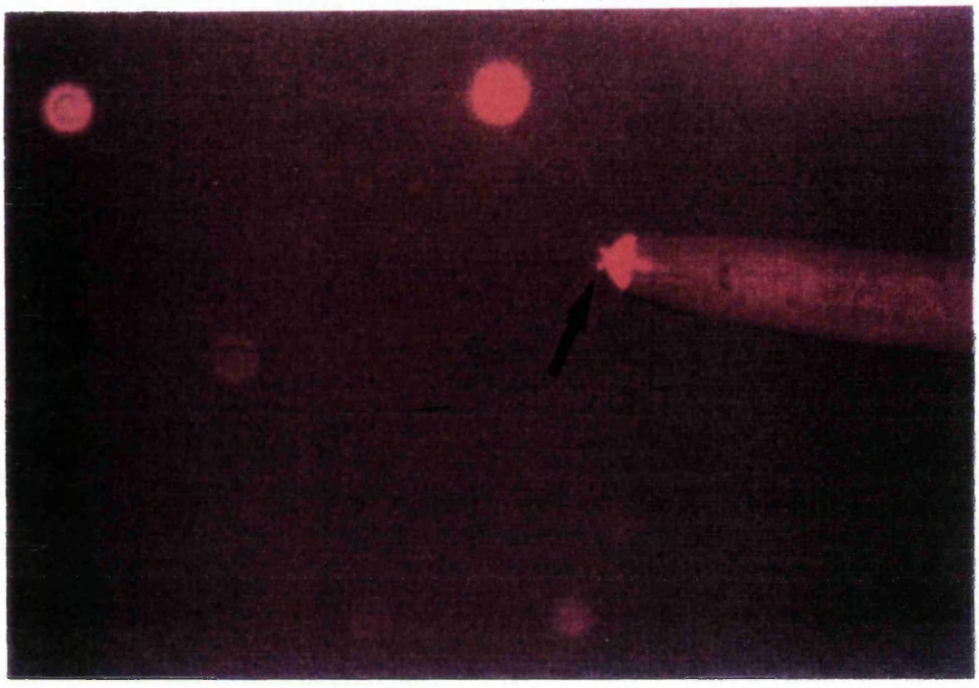
Figure 2.6 a

Nematodes of *G. pallida* (population Halton) were incubated with digoxygenin labelled GNA which is specific for mannose residues, at a pH of 6.4. The digoxygenin glycoconjugates were detected by digoxygenin specific antibody conjugated to rhodamine. The figure shows that amphids of the nematodes have been labelled. Magnification of photograph is times 400

Figure 2.6 b

Nematodes were treated as in figure 2.6 a except that no digoxygenin conjugated GNA was added. Magnification of photograph is times 400

2.6 a



2.6b

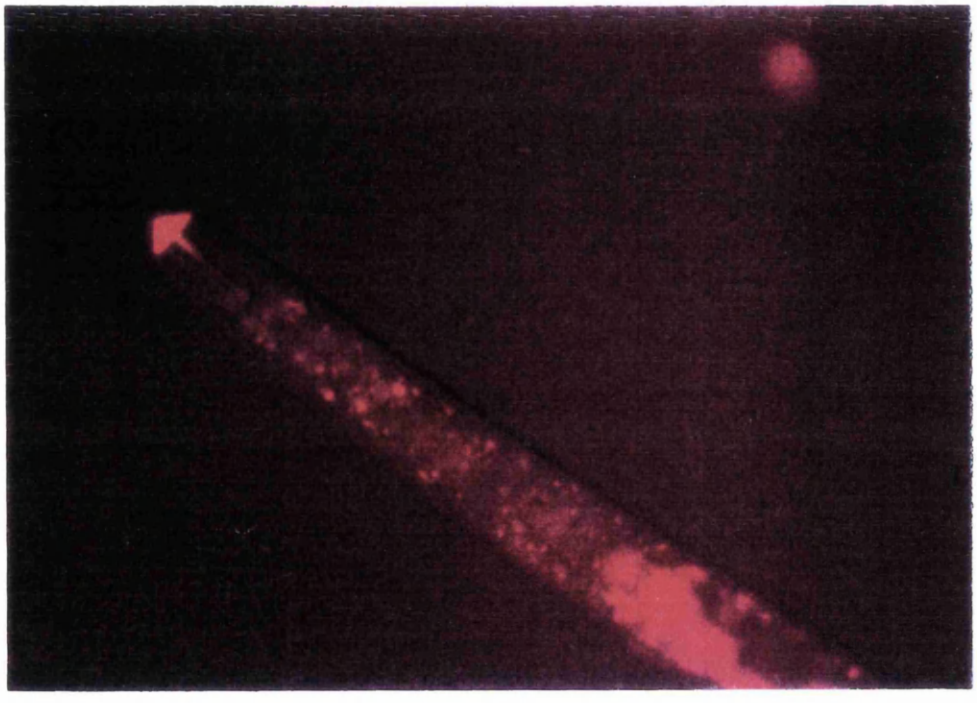


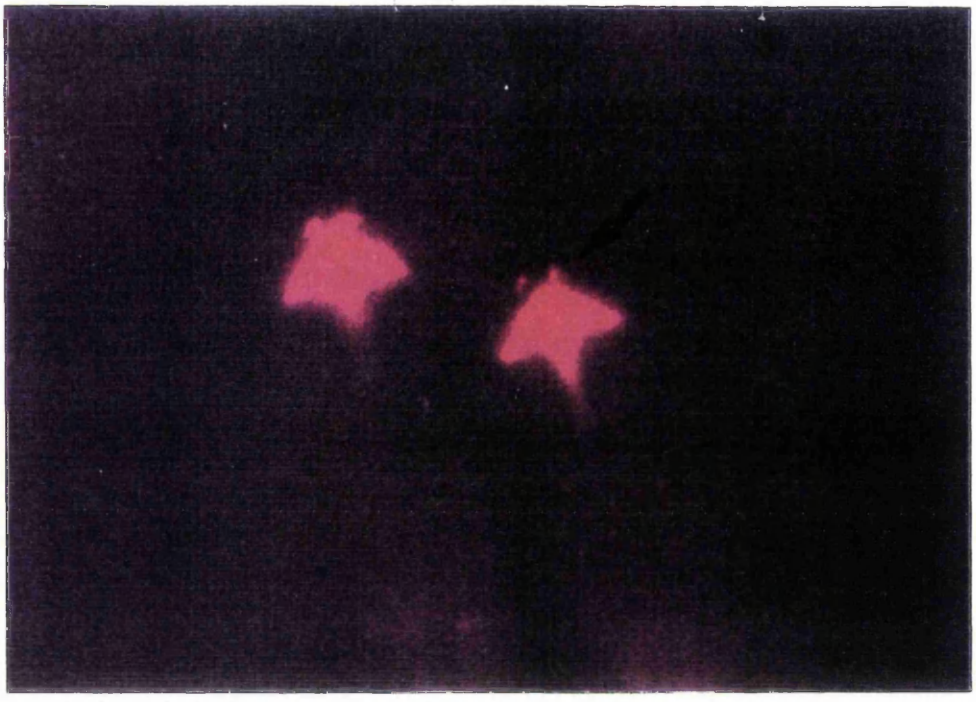
Figure 2.6 c

Nematodes of *G. pallida* (population Halton) were incubated with digoxygenin conjugated GNA which binds specifically to mannose residues, at a pH of 7.5. The digoxygenin labelled glycoconjugates were detected by digoxygenin specific antibody conjugated to rhodamine. The figure shows the amphids of the nematodes have been labelled. Magnification of photograph is times 1000

Figure 2.6 d

Nematodes were treated as in figure 2.6 c except that the incubation step of nematodes with digoxygenin conjugated GNA was missed out. Magnification of photograph is times 400

2.6c



2.6d

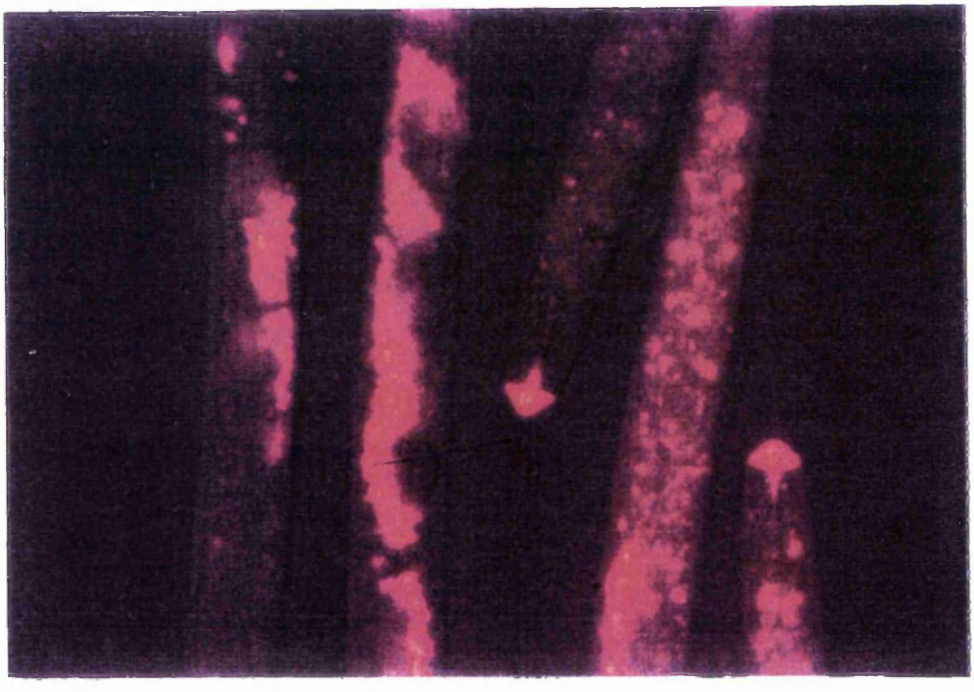


Table 7

Nematodes of *G. pallida* (population Luffness) were washed in 1% of the cationic detergent CTAB for an hour. The nematodes were then spun down and separated from the supernatant. The supernatant (which contained CTAB and proteins washed from nematode surface) was then iodinated and SDS PAGE carried out on the iodinated extract. The resulting gel was autoradiographed and the bands detected are displayed here.

Table 7 bands developed by autoradiography of CTAB washed potato cyst nematodes

sample	band mol. wt.
PCN-1	153,100
PCN-2	149,600

Figure 3.1 a

Nematodes from eight populations of *Meloidogyne* (from *M. incognita*, *M. hapla*, *M. arenaria*, *M. javanica* and *M. mayaguensis*) were separated by SDS PAGE and silver stained for protein (lightly stained). Table 8a lists the prominant protein bands in figure 3.1 a.

Table 8a

Meloidogyne lightly silver stained for protein
3-4 day old nematodes.
All pathotypes contain bands at:

164,100

48,400

40,100

25,000

22,340

Fig 3.1a

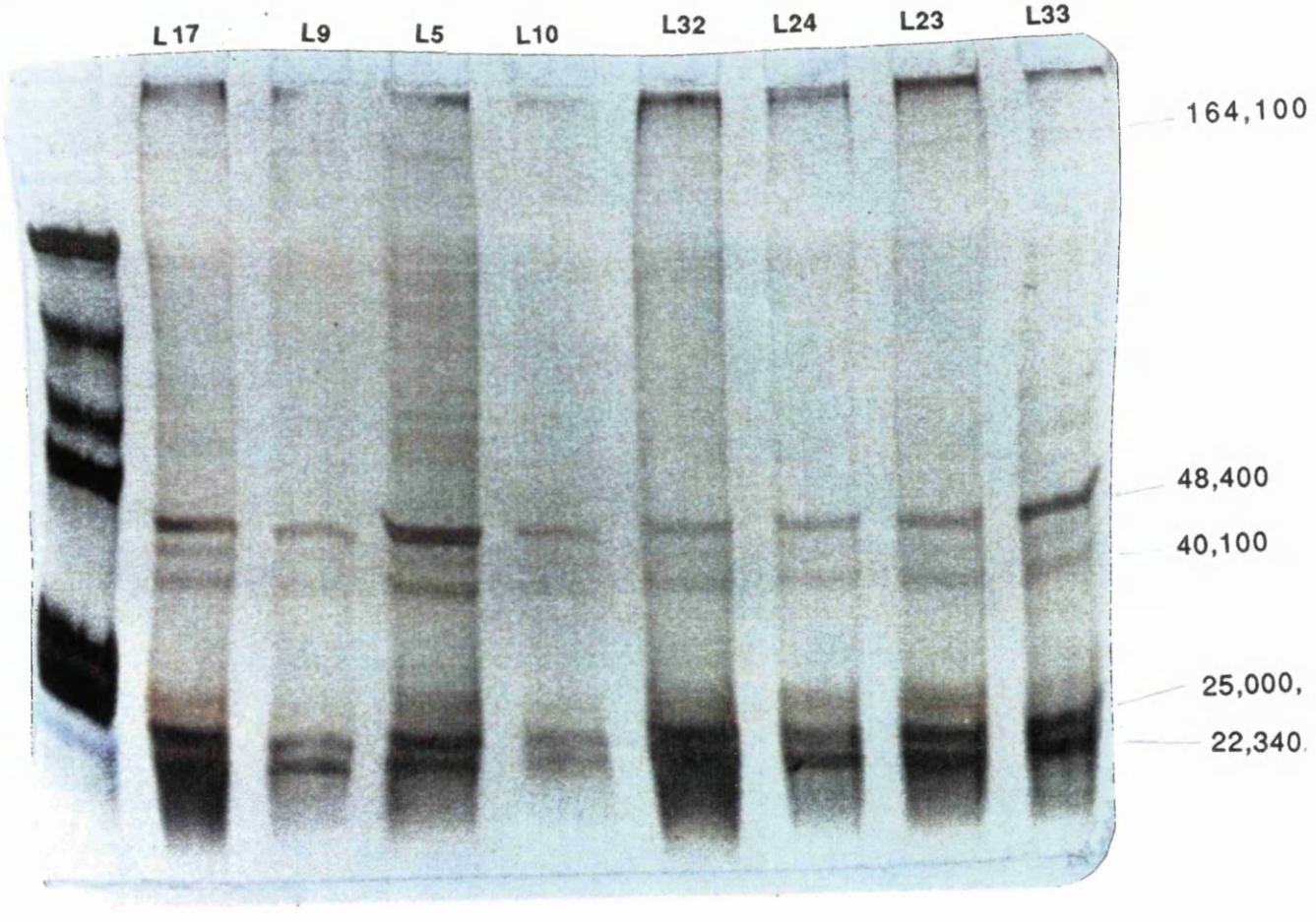


Figure 3.1 b

Nematodes from nine populations of *Meloidogyne* (from *M. incognita*, *M. hapla*, *M. arenaria*, *M. javanica* and *M. mayaguensis*) were separated by SDS PAGE and silver stained for protein (overstained). Table 8b lists the prominant protein bands in figure 3.1 b.

Table 8b

Meloidogyne spp overstained for protein
all lines 3-4 days old

All pathotypes contain bands at:

141,600

112,200

72,400

45,400

43,200

39,100

35,500

22,400

Fig 3.1b

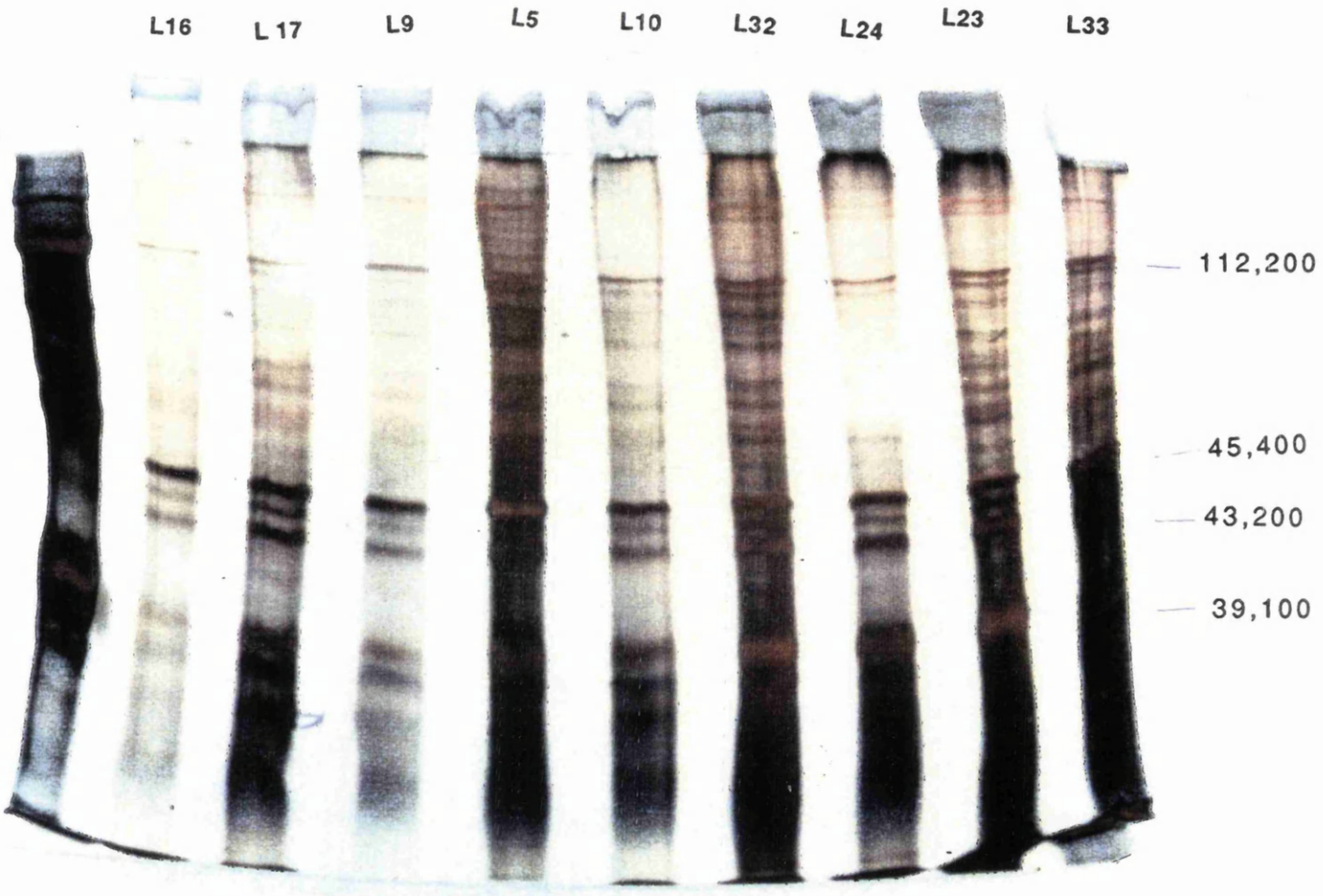


Figure 3.2

Nematodes from eight populations of *Meloidogyne* (from *M. incognita*, *M. hapla*, *M. arenaria*, *M. javanica* and *M. mayaguensis*) were separated by SDS PAGE and transferred on to nitrocellulose paper. The transferred proteins were probed with digoxygenin conjugated to *Galanthus nivalis* lectin (GNA) which binds specifically to terminally linked mannose residues as shown in figure 1.7. The digoxygenin labelled glycoconjugates were detected in an enzyme immunoassay using digoxygenin specific antibody conjugated to alkaline phosphatase. Table 9 shows a line drawing of figure 3.2.

Table 9

Meloidogyne spp probed with GNA

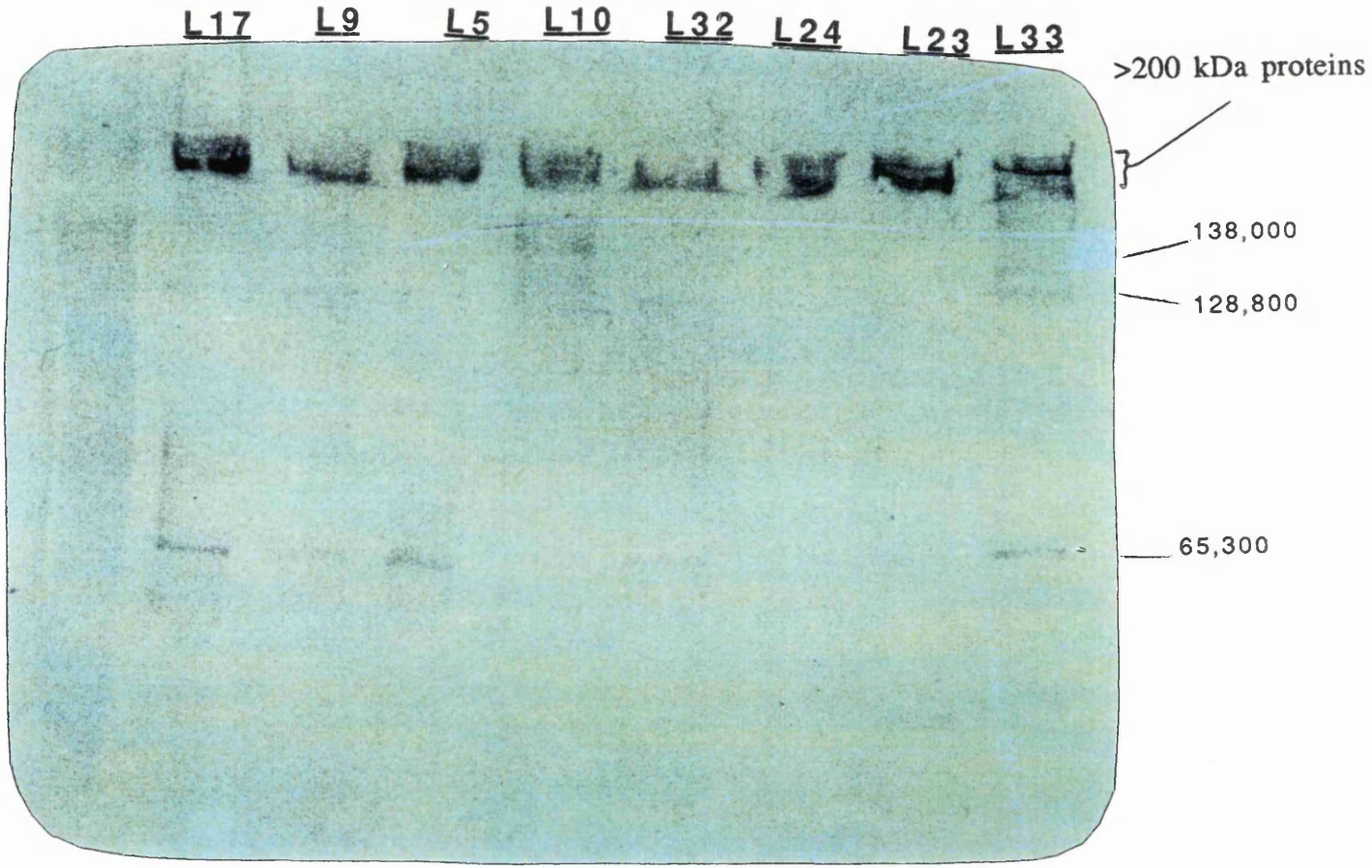
All pathotypes are 3-4 days old

L17	L9	L5	L10	L32	L24	L23	L33
			160,300				
			146,200				138,000
			128,800				128,000
120,200	120,200	120,200	117,500	117,500			
							102,300
						66,800	65,300
51,300	50,100	51,300		50,100		52,500	
		49,000					
43,000							
						30,000	

Table 10 Extent of *Pasteuria* binding to different species of *Meloidogyne* spp.

species	M.javanica		M.incognita			M.arenaria		M.hapla	M. mayaguensis
Lines	23	24	9	16	17	10	32	3 3	5
extent pasteuria binding	High	Low	Low	vhigh	high	Low	medium	not tested	v. Low

Figure 3.2


L17
L9
L5
L10
L32
L24
L23
L33
>200 kDa proteins
138,000
128,800
65,300

Chapter 4
Discussion

4.1 *Globodera* spp

4.1.1 Protein profiles of *Globodera*

From Table 1, Figure 2.1 it can be seen that the protein profiles of the *Globodera* populations are similar. All populations have a sharp band at 112.2 kDa and a dark broad band of molecular weight 43.4 kDa. This is followed by proteins at 38.9, 29.3, 25 and 21 kDa. A separation using two dimensional electrophoresis may show differences between the populations (Mugniery and Bossis, 1993).

4.1.2 *Globodera* probed for presence of general glycoprotein

The results showing pathotypes of *Globodera* probed for general glycoprotein have repeatedly shown the presence of very high molecular weight proteins, which seem to be present in Tables 2b, c and d, but not in Table 2a. Possible reasons for this is that in Tables 2b, c and d the nematodes may have had many proteins forming parts of multiprotein complexes or anchored to the cytoskeleton. It may be that these proteins were not dissociated and that the extra time in storage spent by the samples in Table 2a released more proteins by the actions of the nematode's internal proteases. Referring to figure 1.4 of the *G. rostochiensis* populations, R01 is least virulent and R05 most virulent. The most virulent *Globodera* pathotype is Pa3. Application of biotinylated lectin probes as used by Niedz *et al* (1991) is another sensitive technique in the detection of glycoproteins.

4.1.3 Analysis of Mannose containing glycoproteins from *Globodera*

As shown in figure 1.7 GNA recognises terminally linked mannose residues in western blots and the eight to ten day old *Globodera* populations seem to have five bands in common. However from these results (Tables 3 and 4) it can be seen that nematodes from these two populations do bind GNA differently since only AR01 possesses the band at 61.6 kDa. All these bands were blocked by the sugar methyl

mannose. Forrest *et al*, 1988 showed that mannose residues were present on one day old juveniles of *G. rostochiensis* . The bands labelled with GNA on the western blots are thought to be due to amphidial glycoproteins since mannose has been shown to be present on the amphids by the fluorescent studies on live nematodes using GNA. Comparison of the four day old AR01 with their eight day old counterparts showed the latter had fewer labelled bands. A band in the 55 to 62 kDa range was common to both age groups of nematodes as were bands at 40 and 28 to 29 kDa. There were two bands of lower molecular weight present in both age groups. Three bands with molecular weights of 131.8, 112.2 and 86.1 kDa were present in the younger AR01 whereas the older nematodes only had the band at 123 kDa. The Luffness population also lacked the higher molecular weight bands that were detected in the younger age group. The distribution of glycosylated proteins may vary with age of nematode since it has been found that the nematode's ability to resist nematocides increases with age (W. Robertson, pers. comm.).

4.1.4 Binding of fluorescently labelled GNA to live *Globodera*

The fluorescent studies (Figure 2.6) were carried out using GNA to label live nematodes of *Globodera pallida* and *Globodera rostochiensis* at the pH values of 6.4 and 7.5. According to these results there is no difference in the amount of fluoresence, and therefore GNA attachment between the different nematode species at the pH values of 6.4 and 7.5. Experiments were also done incubating the nematodes in CTAB and then exposing to GNA-DIG rhodamine but these showed that the visible GNA labelling the amphids was still present after washing with CTAB. Previous work has suggested that Con-A binding to potato cyst nematodes varies with pH, most binding occuring at a pH of about 5.5 (Laura Maculloch, Ph.D. thesis, S.C.R.I.). This pH effect may be explained by the fact that Con-A exists as a tetramer at pH 7 and is mostly in dimeric form at pH 5 (Goldstein and Portez, 1986). There may be steric hindrance at pH 7 of the tetramer binding to a

glycoprotein on the nematode surface, and at pH 5 the Con-A molecules in dimeric form are free from this hindrance.

Since the lectin molecule is too large to pass through the nematode cuticle, it can be concluded that one or more of the glycoproteins labelled in the western blots are from the surface. Also, initial experiments at S.C.R.I. using non-lethal washes with 0.1% CTAB of potato cyst nematodes, after which the washes were electrophoresed and silver stained, have detected at least one type of protein (W. Robertson, pers. comm.). The iodination technique for labelling the surface has not been used here, since it has been shown that it's labelling of proteins is not restricted to the surface as ultrastructural studies after radioiodination and found all levels of the cuticle labelled in the animal parasite *Brugia malayi.* (Alverez *et al*, 1989).

4.1.5 *Globodera* probed with SNA

Table 5 shows that potato cyst nematodes (*Globodera* spp.) probed with SNA (which is claimed to recognise sialic acid residues) has stained only one glycoprotein, in both species, of molecular weight 124.5 kDa. Previous studies have used wheat germ agglutinin (Bavananlan and Katlic, 1979; Peters *et al*, 1979; Monsigny *et al*, 1980; Schaver, 1982) and Limax flavus agglutinin (LFA) for this purpose (Robertson et al, 1989) and also by periodate oxidation followed by galactose oxidation treatment (Spiegel *et al*, 1982). However, Bacic *et al* (1990) after extensive tests involving gas chromatography-mass spectrometry and fluorometric analysis could not find any trace of sialic acid residues in the nematode species *Panagrellus redivivus* and *Caenorhabditis elegans.* There is a possibility that the SNA lectin has bound to phosphocholine instead of sialic acid. Testing of the Globodera species with anti-phosphocholine antibodies for the presence of phosphocholine indicated bands at 15 to 20 kDa which may possibly correspond to the 124.5 kDa band detected by the SNA lectin (Lisa Duncan, pers comm).

4.1.6 *Globodera* probed with Peanut agglutinin (PNA)

From table 6 it can be seen that pathotypes of *Globodera* probed with PNA for terminal galactose linked (with a β-1-3 linkage) to N-acetyl galactosamine, vary in the types of glycoproteins that contain this sugar. Although Halton and Luffness are very similar, Gourdie is widely different with respect to PNA binding. A lot of variation occurs within the *G. rostochiensis* and also with the *G. pallida* species although the Luffness and Halton pathotypes show very similar patterns. This is a very interesting result since it seems to show proteins that are common to AR01 (*G. rostochiensis*) and Gourdie (*G. pallida,* pathotype Pa 3) but not to Halton and Luffness (both *G. pallida* pathotypes Pa2 and Pa3 respectively). It may be a consequence of the fact that the international system for classifying the pathotypes of *Globodera* is of an arbritrary nature. This suggests that although any two populations of cyst nematode may carry different virulence genes, their glycoprotein content may not necessarily be different. Conversly, even if two populations of *Globodera* have the same virulence, they may not be similar with respect to glycoprotein profiles.

4.1.7 Iodination of CTAB washings of *Globodera*

1% CTAB washes of nematodes were radio-iodinated in order to attempt labelling and detection of any small amounts of protein that can be washed off by the CTAB (Table 7). This protein may correspond to the protein at a molecular weight between 140 and 150 kDa detected in the glycoprotein blot (Table 2a, Figure 2.2a). The CTAB seems to strip off some proteins used by *Pasteuria penetrans* to attach to the nematode surface. This is supported by the observation that the bacterium no longer binds after treatment of nematodes with the detergent (W. Robertson,pers. comm.). Pritchard and Crawford (1985) also used CTAB to strip antigens from the nematode cuticle. Further experiments can be carried out using larger numbers of nematodes (20,000-30,000). Any differences detected in protein profiles would most probably be due to differences of nematode surface proteins or protein from

exudates from the nematodes present in the various samples. Ultrastructural studies of the PCN carried out after treatment with CTAB would show if any layers of the cuticle had been dissolved. Reddigari *et al* (1986) have used SDS and mercaptoethanol and Davis and Kaplan (1992) have used SDS and CTAB to solubilise proteins from *Meloidogyne*.

However studies by Blaxter *et al* (1992) and Page *et al* (1992) show that in *C. elegans* and also the animal parasite, *Toxocara canis* there is an electron dense coat distinct from the epicuticle. Their conclusions were that in many cases it is the surface coat (which is rich in carbohydrates and is removed by CTAB) rather than the cuticle that displays dynamic properties thought to be involved in the immune evasion by parasites.

It was also investigated whether the presence of potato root diffusate on hatching cysts had any effect on the production of the resulting nematode's surface glycoproteins. The western blot analysis and probing for glycoprotein of the samples of two day old Luffness samples hatched either in root diffusate or tap water showed the presence of an extra glycoprotein of molecular weight 60.5 kDa in the water hatched nematodes only.

Many of the results with western blots show the presence of very high molecular weight material, too high for their molecular weights to be resolved because they remained in the stacking gel. These may be lipoproteins. Further experiments exposing the nematodes to protease before preparation for electrophoresis might yield interesting results. In theory, this would cleave proteins exposed on the nematode surface which could then be separated. One attempt was made at treatment with V8 protease of the nematodes after which the wash containing the V8 protease peptide fragments was subjected to electrophoresis and silver stained for protein, although no bands apart from those due to protease were detected. Another future possibility would be to incubate the nematodes in collagenase (since collagen is a major structural component of the nematode surface) and carry out electrophoresis. This may release proteins anchored on the surface. Glycosidases

such as mannosidase may also be tried, to check if treatment with glycosidases (which cleave the sugar groups from proteins) will abolish some of the bands present in the western blots probed with the various lectins; thus validating the specificity of the detection procedure. However, this last experiment may be practically difficult since, in one trial carried out, the nematodes were very incompatible with the pH and temperature that the mannosidase needed for optimal activity. There is also the possibility that under less than optimal conditions, a large part of the mannosidase may itself autolyse or be degraded by nematode proteases. However it may be possible to carry this experiment out with adjustments to the incubation procedure.

4.2 *Meloidogyne* spp

4.2.1 *Meloidogyne* tested for protein

As in Table 8a and 8b, all the populations of Meloidogyne tested have very similar protein profiles and it was not possible from this result to distinguish them from one another.

4.2.2*Meloidogyne* spp probed with GNA

The western blot of proteins from of *Meloidogyne* probed (Table 9)with GNA shows some differences between the different populations (referred to as lines) tested. *Pasteuria*, which is a nematophagous bacterium, attacks both *Globodera* (potato cyst nematodes) and *Meloidogyne* (root knot nematodes). Rosenweig and Ackroyd (1983) and Rosenweig *et al* (1985) suggested that lectins may be involved in the attachment of nematophagus fungi on the nematode surface. The attachment of the bacterium to the particular nematode surface is therefore an important initial step for predation by the parasite.

From Table 10 populations 17 and 9 belong to *M. incognita* population (or line) 5 belongs to *M. mayaguensis* and lines 10 and 32 are M. arenaria. Lines 23 and 24 belong to *M. javanica* and line 33 is from *M. hapla.* Of these populations, lines

23, 16 and 17 are known to bind large numbers of *P. penetrans* while lines 24, 9, 10 and 5 bind much smaller numbers of the bacterium. Comparing the levels of *Pasteuria* attachment to the numbers of GNA binding glycoproteins in the western blots did not indicate any correlation. However the populations could be distinguished from one another by their GNA binding pattern. This was with the exception that lines 17 and 5 could not be distinguished and neither could lines 9 and 32. An experiment needs to be carried out to see if these bands are blocked by methyl mannose. The above experiment also needs to be repeated with a lower polyacrylamide gel concentration or perhaps a gradient gel, since there was very strong binding of GNA by material which remained in the stacking gel. Davis and Kaplan (1992) carried western blot experiments of CTAB soluble and SDS soluble proteins from four populations of *Meloidogyne* and probed for the presence of mannose (using Con A lectin), sialic acid (using *Limulus polyphemus* agglutinin or LPA) and fucose (using *Lotus tetragonolobus* agglutinin or LTA). They detected proteins of molecular weight 200 kDa or larger. Results on all the populations of *Meloidogyne* tested agree with this finding since material > 200 kDa was intensely stained with GNA. Kaplan and Gottwald detected 12-16 kDa sialic acid containing protein on another tropical phytoparasitic nematode, *Radopolus citrophilus*. Also Spiegel *et al* (1982) detected sialic acid (using WGA) on *M. javanica* and McClure and Zuckerman (1982) detected mannose/glucose residues (using Con A) on *M. incognita* using lectins conjugated to fluorescent molecules. Stewart *et al* (1993) detected a glycoprotein specific to the amphids of Meloidogyne and Hussey *et al* (1990) localised a secretory protein in the oesophageal glands of *M. javanica*. The functions of these proteins are unknown.

4.3 Discussion of lectin binding proteins in *Globodera*

4.3.1 Comparison of lectin binding within *Globodera*

Generally, from Tables 11 to 14 the results of probing nematodes with individual lectins seem to pick out some of the bands also detected by the general glycoprotein

stain. It is therefore more likely that these glycoproteins do contain the particular terminal sugar detected by the lectin. There are also some bands which are in the blots probed with individual lectins but not on the blot stained for general glycoprotein. This may be due to the individual lectins being more sensitive or binding nonspecifically, as would be the case if the bands are not diminished or eliminated by competition experiments with the lectin and the appropriate free sugar.

4.3.1.1 Comparison within *G.pallida*

From Table 12 of eight day old Luffness, it can be seen that there is a band at 123 to 124 kDa which is recognised by both the mannose specific (figure 2.3a) and sialic acid specific stains. The general glycoprotein stain and the mannose stain have both picked out a band at about 40 kDa. Apart from this there are five glycoproteins that recognise sugar groups other than mannose, galactose or sialic acid.

From Table 11 of four day old Luffness, a band at 93 kDa which is detected by both glycoprotein and galactose stains is seen to be present. Another protein at about 60 kDa is indicated in both glycoprotein and mannose stained blots. In addition there are three glycoproteins which contain sugar groups other than the ones tested.

From Table 13 of four day old Halton populations, a protein at 93 kDa has been detected by both general glycoprotein and galactose specific stains. There are four glycoproteins which seem to contain sugars other than galactose.

4.3.1.2 Comparison within *G.rostochiensis*

As Table 14 of three to four day old R05-88 shows, there are proteins of molecular weight 128.8 kDa and between 94 and 95 kDa which are galactose containing glycoproteins. The band at 146.2 kDa may also be represented within the series of

faint, smeared bands between 158.9 and 128 kDa. Five other glycoproteins do not contain galactose.

It can be seen from Table 15 of eight day old AR01 nematodes that there is a protein at 149 kDa recognised by galactose and glycoprotein stains. All the stains have detected a protein at 123 to 125 kDa and so this glycoprotein has mannose, sialic acid and galactose moieties. It may also be possible that there is more than one protein with this molecular wight. Both glycoprotein and mannose stains have identified a protein at about 40 kDa.

4.3.2 General comparison of *Globodera* pathotypes

From the tests that have been carried out (figure 2.2a, figure 2.5) it is possible to distinguish the *G. rostochiensis* population AR01 (pathotype R01) from R05-88 (pathotype R05) since both their glycoprotein and galactose profiles indicate the presence of a protein at 94 to 95 kD only in the latter.

It has not been possible to distinguish between the Halton (pathotype Pa2) and Luffness (pathotype Pa3) populations, as both their galactose and glycoprotein profiles are similar. However, Gourdie (pathotype Pa3) can be easily distinguished from Luffness (also pathotype Pa3) by the former's possession of galactose glycoproteins at 147.2, 138, 120 and about 107.8 kDa (figure 2.5, table 6).

AR01 can not be differentiated from Gourdie, however, from their galactose profiles only and a general glycoprotein stain, mannose stain or a comparison of different stains may reveal differences between the two populations. R05-88 can be separated from Gourdie by the possession of galactose glycoproteins at 120 and 107.8 kDa only by the latter (figure 2.5, table 6). AR01 is also separable from Halton and Luffness by their differential content of galactose, mannose and general glycoprotein. There is no difference in SNA labelled glycoproteins between AR01 and Luffness (figure 2.4). Also, the eight day old AR01 and Luffness populations can be discriminated from their younger (four to five day old) counterparts by the

presence of a protein at 123 to 125 kD bearing mannose (, sialic acid and galactose only in the older nematodes.

4.4 Conclusions

1) It was possible to differentiate at least some of the populations of *Globodera* and *Meloidogyne* by probing them with lectins on a western blot. **2)** It was also possible to tell older nematodes apart from younger ones by their glycoprotein profiles. **3)** The protein at 150 kDa washed off from the nematode surface by the detergent CTAB may possibly correspond to the family of proteins at 140 to 150 kDa as detected by the glycoprotein stain. However more experiments need to be carried out by extending the range of lectin probes and nematode populations thereby making it possible to identify and differentiate at least some of the populations of *Globodera* and *Meloidogyne*.

Table 12 Composite of Globodera rostochiensis population

Luffness stained with three different glycoprotein stains

(samples about eight days old)

glycoprotein stain	mannose specific stain	sialic acid specific stain (4-6 days old)
142,900		
	123,000	124,500
105,900		
92,900		
60,300		
55,500		
40,700	39,800	
	27,200	
	22,900	
	21,100	

Table 11 Composite of Globodera pallida population

Luffness tested with four different glycoprotein stains

(4 days old)

glycoprotein stain (table 2)	(tables3 &4)	mannose specific stain	galactose specific stain
	190,500		
	177,800		
142,900	150,700-141,300		
		131,800	
		112,200	
105,900			
			95,700
			93,000
92,900		86,100	81,300
60,300-52,500		60,300	
40,700			
		30,200	
		27,900	

Table 13 Composite of Globodera pallida population Halton stained with two different glycoprotein stains

glycoprotein stain (table 2a)	(table 2b and 2c)	galactose specific stain
	190,500	
	177,800	
	150,700-	
146,200	141,300	
107,600		95,500
92,900		93,000
		81,300
60,300-		
52,500		
40,700		

Table 14 Composite of Globodera rostochiensis population R05 88 tested with two different glycoprotein stains

glycoprotein stain	galactose stain
	158,900
146,200	
128,800	128,000
123,000	
112,200	
	95,500
94,200	
	83,200
60,300	
48,400	
42,500	

Table 15 Composite showing Globodera rostochiensis nematodes population AR01 tested by four different glycoprotein stains.

(samples about eight days old)

General glycoprotein stain	mannose-specific stain	sialic acid specific stain (4-6 days old)	galactose specific stain
149,300			149,600
			138,000
125,900	123,000	124,500	123,000
109,800			91,200
68,400			
	61,600		
48,400			
40,700	39,800		
	28,200		
	22,900		
	21,100		

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