



University  
of Glasgow

Duncan, Lisa Helen (1995) *An investigation of the secretions of the potato cyst nematode globodera pallida.*

PhD thesis

<http://theses.gla.ac.uk/4356/>

Copyright and moral rights for this thesis are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

**AN INVESTIGATION OF THE SECRETIONS  
OF THE POTATO CYST NEMATODE  
*GLOBODERA PALLIDA***

**A Thesis Submitted for the Degree of  
Doctor of Philosophy  
by**

**LISA HELEN DUNCAN, B.SC.  
UNIVERSITY OF GLASGOW,  
DECEMBER 1995**

## ACKNOWLEDGEMENTS

I would like to thank Professor Gordon Lindsay for making available the facilities of the Division of Biochemistry and Molecular Biology, and the Scottish Office Agriculture, Environment and Fisheries Department for funding in part the work described in this thesis.

My first thanks go to my two supervisors John Kusel and Walter Robertson for their help, encouragement and laughs throughout the course of this project. Many thanks are also due to Dr. Les Fixter, for proofreading this thesis, and for his patience with me throughout. I am also grateful to Professor Hillman at the Scottish Crop Research Institute for his helpful discussion.

Another big thank you goes to the members of Lab. C15, both past and present. To Dr. Jay (Praise the Lord!) Modha, for all his "help", literary criticisms and Madonna tapes; to David "Shine" Taylor for keeping Jay under control; to Symone Lima and Pepe Publio for the tales of Brasil, and late nights; to Chris Redman for finally developing a sense of humour (mine's a pint!), and a special thank you to Ms. Clare Roberts for all those "we'll just go for one" on dreary Monday nights - Yes, Yes, Yes!

I would like to take this opportunity to say a special thank you to Joyce Thornhill, for the friendship and support she has given me. And for all the nice pictures of snails!

This is also a chance to say thank you to Ailsa Smith and Lee Robertson at the Scottish Crop Research Institute, for making all my visits there such a good laugh, and for showing me the sights, or should that be pubs, of Dundee.

Thanks also has to go to Kathy Li, my flatmate, for listening to my ranting and raving throughout the writing up of this thesis, and for the constant supply of Hula Hoops and Rocky bars.

Finally, I would like to thank my family for all their love and support, and to say that this thesis is dedicated to them.

## LIST OF ABBREVIATIONS

ABA	abscisic acid
ABP	auxin binding protein
AF18	5-N-(octadecanoyl)-aminofluorescein
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CPA	chlorophenoxyacetic acid
Con A	concanavalin A
Diazo-2AM	1,2-bis-(2-amino-5-di-azoacetylphenoxy) ethane- N, N, N', N' -tetraacetic acid tetrakis methyl ester
DMSO	dimethyl sulphoxide
DMT	5-methoxy-N,N-dimethyl tryptamine
DSA	<i>Datura stramonium</i> agglutinin
ES	excretory / secretory
GA <sub>3</sub>	gibberellin
IAA	indole 3-acetic acid
IP <sub>3</sub>	inositol trisphosphate
MAA	<i>Maackia amurensis</i> agglutinin
NITR.5	tetracarboxylate 2-nitrobenzhydrol
PBS	phosphate buffered saline
PCN	potato cyst nematodes
PMSF	phenylmethanesulphonyl fluoride
PRD	potato root diffusate
SNA	<i>Sambuca nigra</i> agglutinin
TBS	tris buffered saline
TLCK	tosyllysine chloromethyl ketone
TPCK	tosylphenylalanine chloromethyl ketone
WGA	wheat germ agglutinin

## CONTENTS

	PAGE
Title	i
Acknowledgements	ii
Abbreviations	iii
Contents	iv
List of Figures and Tables	x
Summary	xiii
<b>CHAPTER 1. AN INTRODUCTION TO POTATO CYST NEMATODES</b>	
1.1. Potato Cyst Nematodes	1
1.2. Potato Cyst Nematode Classification	1
1.2.1. Species	1
1.2.2. Populations	2
1.3. The Life Cycle of Potato Cyst Nematodes	6
1.3.1. Hatching	8
1.3.2. Events Associated with Hatching	8
1.3.3. Hatching Factors	9
1.4. Host Finding	10
1.5. Anterior Sense Organs	11
1.5.1. Amphidial Secretions	13
1.5.2. The Role of Amphidial Secretions	14
1.6. Formation and Characteristics of the Syncytium	17
1.7. Feeding Behaviour	19
1.8. Nematode Induced Gene Expression in Syncytia	21
1.9. Oesophageal Gland Secretions	25
1.10. Isolation of Oesophageal Gland Secretions	28
1.11. The Excretory-Secretory System	30
1.11.1. Functions of the Excretory-Secretory System	32
1.11.2. The Role of Excretory-Secretory Products	34
1.12. Other Sources of Secretion	35
1.13. Plant Defense Responses	37
1.14. Resistance Reactions Against Potato Cyst Nematodes	37
1.14.1. H1 Resistance	38
1.14.2. Polygenic Resistance	40
1.15. Aims of the Thesis	41

**CHAPTER 2: MATERIALS AND METHODS**

2.1. Parasite Material	42
2.2. Potato Root Diffusate Production	42
2.3. Preparation of Parasite Proteins	43
2.3.1. Amphidial/Excretory Pore Secretions	43
2.3.2. Whole Body Preparations	44
2.3.3. Homogenate Preparations	44
2.3.4. Collection of Excretory-Secretory Products	45
2.3.5. Methoxy Dimethyl Tryptamine Induced Secretions	45
2.4. Anti-Serum Production	46
2.4.1. Luffness Anti-Serum	46
2.4.2. Excretory-Secretory Anti-Serum	46
2.5. Origin of Other Anti-Sera	47
2.5.1. Anti-Serum D16	47
2.5.1.1. Affinity Purified D16	48
2.5.2. Anti-Serum 5559	48
2.6. Protein Determination	49
2.7. Protein Separation	49
2.7.1. Sample Preparation	49
2.7.2. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis	50
2.7.3. Silver Staining	50
2.7.4. Molecular Weight Determination	51
2.8. Blotting Studies	52
2.8.1. Immunoblotting	52
2.8.2. Lectin Blotting	53
2.9. Fluorescence Studies	54
2.9.1. Indirect Immunofluorescent Antibody Test	54
2.9.2. Periodate Treatment	55
2.9.3. Direct Fluorescence Using Fluorochrome-Conjugated Lectins	55
2.10. Induction of Secretions	55
2.10.1. Methoxy dimethyl tryptamine (DMT)	56
2.10.2. Phytohormones	56
2.11. Lipid Probe Binding	57
2.11.1. 5-(N-Octadecyl) Aminofluorescein	57
2.11.2. PHK 26	57
2.11.3. Trypan Blue Quenching of Surface Fluorescence	57

2.11.4. Quantitation of Fluorescence and Statistical Analyses	58
2.12. Caged Compounds	58
2.13. Electron Microscopy Studies	61
2.13.1. Pre-Embedding Surface Labeling of Whole Specimens	61
2.13.1.1. Immunogold Labelling of the Surface	61
2.13.1.2. Fixation	61
2.13.2. Post-Embedding Labeling of Cut Sections	62
2.13.2.1. Fixation	62
2.13.2.2. Immunogold Labeling	62
2.13.3. Sections for Ultrastructural Observations	63
2.13.4. Sectioning	63
2.13.5. Counter Staining	63
2.13.6. Viewing and Photography	63
2.14. Ion Exchange Chromatography Purification of Putative Nematode Auxin Binding Protein	63
2.15. Appendix	65
2.15.1. General Buffers	65
2.15.2. Electrophoresis and Blotting Solutions	65
2.15.3. Silver Staining	66
2.15.4. Electron Microscopy Buffers	66

### **CHAPTER 3: ISOLATION AND CHARACTERISATION OF SECRETIONS FROM *GLOBODERA PALLIDA***

3.1. Introduction	68
3.2. Results	68
3.2.1. Lectin Binding Studies	68
3.2.1.1. Lectin Binding to Whole Parasites	68
3.2.1.2. Lectin Binding to Whole Body Extracts	68
3.2.2. Collection of Secretions	75
3.2.2.1. Excretory-Secretory Products	77
3.2.2.2. Amphidial/Excretory Pore Secretions	77
3.2.3. Examination of Unstained and Stained Amphids using Electron Microscopy	79
3.2.4. Electrophoresis of Collected Secretions	82
3.2.4.1. Excretory-Secretory Products	82
3.2.4.2. Amphidial/Excretory Pore Secretions	82

3.2.5. Indirect Immunofluorescent Antibody Test	82
3.2.5.1. ES Anti-Serum	82
3.2.5.2. Luffness Anti-Serum	85
3.2.6. Periodate Treatment of Nematodes prior to Indirect Immunofluorescence	89
3.2.6.1. Luffness Anti-Serum	89
3.2.7. Immunoblot analysis of Nematode Whole Body Extracts	91
3.2.7.1. ES Anti-Serum	91
3.2.7.2. Luffness Anti-Serum	91
3.2.8. Electron Microscopy Studies	94
3.2.8.1. Pre-Embedding Immunogold Labelling of the Surface	94
3.2.8.2. Post-Embedding Immunogold Labelling of Cut Sections	96
3.3. Discussion	96

#### **CHAPTER 4: INDUCTION OF SECRETIONS IN *GLOBODERA PALLIDA* AND *GLOBODERA ROSTOCHIENSIS***

4.1. Introduction	106
4.2. Results	106
4.2.1. Induction of Secretions by Methoxy Dimethyl Tryptophan	106
4.2.2. Analysis of Induced Secretions	107
4.2.2.1. SDS-PAGE Analysis of Induced Secretions	109
4.2.3. Immunoblot Analysis of Induced Secretions	109
4.2.3.1. ES Anti-Serum	109
4.2.3.2. Luffness Anti-Serum	112
4.2.4. Indirect Immunofluorescence Studies	112
4.2.4.1. Luffness Anti-Serum	112
4.2.4.2. ES Anti-Serum	114
4.2.4.3. Anti-Serum 5559	117
4.2.4.4. Normal Rabbit Serum	117
4.2.5. Periodate Treatment	117
4.2.6. Lectin Binding Studies	119
4.2.7. Electron Microscopy Studies	120
4.2.7.1. Pre-Embedding Immunogold Labelling with Luffness Anti-Serum	120
4.2.8. Induction of Secretions by Phytohormones	124

4.2.9. Indirect Immunofluorescence Studies	126
4.2.9.1. Luffness Anti-Serum	126
4.2.9.2. Other Anti-Sera	126
4.2.10. Lipid Probe Studies	128
4.2.10.1. Insertion of 5-N-(octadecanoyl) Aminofluorescein	128
4.2.10.2. Insertion of PKH 26	128
4.2.11. Effect of Induction of Secretions on Lipid Probe Insertion	130
4.2.11.1. Methoxy dimethyl tryptamine	130
4.2.11.2. Indole 3-Acetic Acid	130
4.2.12. Trypan Blue Quenching of Surface Fluorescence	132
4.2.12.1. 5-N-(Octadecanoyl) Aminofluorescein	132
4.2.12.2. PKH 26	134
4.2.13. Trypan Blue Quenching of Fluorescence Following Induction of Secretions	134
4.3. Discussion	136

## **CHAPTER 5: IDENTIFICATION AND CHARACTERISATION OF A PUTATIVE NEMATODE AUXIN BINDING PROTEIN**

5.1. Introduction	144
5.2. Results	146
5.2.1. Investigations Using Caged Compounds	146
5.2.1.1. Effect of Calcium Depletion	146
5.2.1.2. Addition of Caged Second Messengers	147
5.2.2. Investigations Using D16 Anti-Serum	150
5.2.2.1. Indirect Immunofluorescence Studies	150
5.2.2.2. Post-Embedding Immunogold Labelling	150
5.2.3. Immunoblotting Studies	151
5.2.3.1. D16 Anti-Serum	151
5.2.3.2. Affinity Purified D16 Anti-Serum	153
5.2.4. Ion Exchange Chromatography Purification of Putative Nematode Auxin Binding Protein	153
5.3. Discussion	156

## **CHAPTER 6: FINAL DISCUSSION: HYPOTHESIS AND SYNTHESIS**

6.1. A Hypothesis	160
6.2. Strategies of Bacterial Plant Pathogens	161

6.3. The Role of Phytohormones in Disease	163
6.3.1. Auxin and Cytokinin as Modulators of Disease	164
6.4. Auxin	165
6.4.1. Transduction of the Auxin Signal	165
6.4.2. Auxin Induced Genes	166
6.4.3. Consequences of Altered Auxin Signal Transduction	169
6.5. The Role of Auxin in <i>Globodera</i> Infection: A Synthesis	172
6.6. In Summary	173
6.7. Future Work	173
<b>LITERATURE CITED</b>	<b>176</b>

## LIST OF FIGURES AND TABLES

FIGURE	PAGE
1.1 The life cycle of Potato Cyst Nematodes	7
1.2. Structure of a typical amphid	12
1.3. Organisation of the three oesophageal glands	26
1.4. The ES system of <i>Caenorhabditis elegans</i> .	31
2.1. Structure of caged compounds	60
3.1. Lectin binding to the surface of <i>G. pallida</i> .	69
3.2. Lectin blot of whole body extracts of <i>G. pallida</i> and <i>G. rostochiensis</i> probed with Con A.	71
3.3. Lectin blot of whole body extracts of <i>G. pallida</i> and <i>G. rostochiensis</i> probed with GNA.	72
3.4. Lectin blot of whole body extracts of <i>G. pallida</i> and <i>G. rostochiensis</i> probed with SNA.	73
3.5. Lectin blot of whole body extracts of <i>G. pallida</i> and <i>G. rostochiensis</i> probed with WGA.	74
3.6. Immunoblot analysis of whole body extracts of <i>G. pallida</i> and <i>G. rostochiensis</i> probed with the monoclonal antibody TEPC 15.	76
3.7. Production of stained secretions from the amphidial and excretory pores of <i>G. pallida</i> ..	78
3.8 Transverse section through the amphid of <i>G. pallida</i> .	80
3.9 Transverse section through the amphid of <i>G. pallida</i> which has undergone the staining procedure used to produce secretions.	81
3.10. SDS-PAGE gel of ES secretions from <i>G. pallida</i> .	83
3.11. SDS-PAGE gel of secretions collected from <i>G. pallida</i> Luffness, Derby and Newton populations.	84
3.12 Indirect immunofluorescence of <i>G. pallida</i> Luffness population probed with Luffness anti-serum.	86
3.13. Indirect immunofluorescence of <i>G. pallida</i> Loc population, and <i>G. rostochiensis</i> , Ro3 probed with Luffness anti-serum.	87
3.14 Indirect immunofluorescence of <i>G. pallida</i> Luffness population probed with normal rabbit serum.	88
3.15. Immunoblot analysis of whole body extracts of <i>G. pallida</i> and <i>G. rostochiensis</i> populations using ES anti-serum.	92
3.16. Immunoblot analysis of whole body extracts of <i>G. pallida</i> and <i>G. rostochiensis</i> probed with Luffness anti-serum.	93

3.17.	Longitudinal section through the head of <i>G. pallida</i> Luffness population labelled with Luffness anti-serum.	95
3.18	Electron micrograph showing transverse section through the metacarpal bulb of <i>G. pallida</i> Luffness population probed with Luffness anti-serum.	97
3.19.	Electron micrograph of a longitudinal section through the oesophageal region of <i>G. pallida</i> Luffness population probed with Luffness anti-serum.	98
4.1.	Stained secretions produced from <i>G. pallida</i> following incubation with DMT in the presence of Coomassie Blue R250.	108
4.2.	SDS-PAGE gel of secretions collected following incubation with DMT.	110
4.3.	Immunoblot analysis of DMT-induced secretions probed with ES anti-serum.	111
4.4.	Immunoblot analysis of DMT-induced secretions probed with Luffness anti-serum.	113
4.5.	Binding of Luffness anti-serum to <i>G. pallida</i> Luffness population after incubation with DMT.	115
4.6.	Binding of Luffness anti-serum to <i>G. rostochiensis</i> (Ro3) following incubation with DMT.	116
4.7.	Binding of anti-serum 5558 to <i>G. pallida</i> Luffness population.	118
4.8.	Longitudinal section through the cuticle of <i>G. pallida</i> Luffness population probed with Luffness anti-serum.	121
4.9.	Longitudinal section through the cuticle of <i>G. pallida</i> Luffness population probed with Luffness anti-serum following incubation with DMT.	122
4.10.	Longitudinal section through the cuticle of <i>G. pallida</i> Luffness population probed with Luffness anti-serum following incubation with DMT	123
4.11.	Secretions produced by <i>G. pallida</i> Luffness population following incubation with IAA.	125
4.12.	Binding of Luffness anti-serum to <i>G. pallida</i> population Luffness following incubation with IAA.	127
4.13.	Insertion of lipid probes into the surface of <i>G. pallida</i> .	129
4.14.	Effect of DMT and IAA on the insertion of lipid probes in to the surface of <i>G. pallida</i> .	131
4.15.	Trypan Blue quenching of lipid probes inserted in to	

	the surface of <i>G. pallida</i> .	133
4.16.	Effect of DMT and IAA on Trypan Blue quenching of lipid probes inserted in to the surface of <i>G. pallida</i> .	135
4.17.	Staining of the amphidial canals of <i>G. pallida</i> with lipid probes following surface quenching by Trypan Blue.	137
5.1.	Effect of calcium depletion on AF18 insertion following incubation with DMT.	148
5.2.	Effect of caged second messengers on AF18 insertion into the surface of <i>G. pallida</i> Luffness population.	149
5.3.	Immunoblot analysis of whole body extracts of <i>G. pallida</i> probed with D16 anti-serum and KLH anti-serum.	152
5.4.	Immunoblot analysis of whole body extracts of <i>G. pallida</i> probed with affinity purified D16 anti-serum.	154
5.5.	Elution profile of nematode homogenate obtained using 10-400mM Imidazole-HCL gradient.	155
6.1.	Possible interactions occurring between <i>Globodera</i> and its host during infection.	170

<b>TABLE</b>		<b>PAGE</b>
1.1	Summary of the international pathotype scheme.	3
3.1.	The effect of sugars on the binding of normal rabbit serum to the amphids of <i>G. pallida</i> .	90

## SUMMARY

Initial experiments performed in this study allowed species differentiation of *Globodera pallida* and *G. rostochiensis* following immunoblotting with the lectin, wheat germ agglutinin (WGA), and the monoclonal antibody TEPC 15. Further studies were aimed at the isolation of amphidial secretions. Two methods of collecting secretions were attempted, one involving the modification of a previously described staining method, the other relying on the collection of ES products from living, sterile nematodes. Secretions collected from *G. pallida* using the two different methods were analysed using SDS-PAGE electrophoresis. Secretions were also used for anti-serum production, giving two anti-sera, Luffness anti-serum and ES anti-serum. These were subsequently used for immunoblotting and indirect immunofluorescence studies. Indirect immunofluorescence studies indicated that the two anti-sera recognised different nematode components. This was further confirmed by immunoblotting studies which revealed that Luffness anti-serum recognised a number of nematode proteins, and was capable of differentiating both between and within species of *G. pallida* and *G. rostochiensis*. In contrast, ES anti-serum recognised only two proteins which appeared to be conserved between the two species. Observations also indicated the presence of a nematode lectin component present in amphidial secretions with apparent specificity for N-acetylgalactosamine.

Experiments were also performed to examine different methods of inducing secretions. Previous research (Goverse *et al.*, 1994) has shown that the serotonin agonist 5-methoxy dimethyl tryptamine (DMT) is an effective inducer of nematode oesophageal secretions. Comparison of DMT-induced

secretions with ES secretions using SDS-PAGE electrophoresis revealed that the protein profiles were similar, although some proteins were more abundant following induction with DMT. Treatment of *G. pallida* with DMT followed by indirect immunofluorescence with Luffness anti-serum revealed an increased and altered distribution of antibody binding on the nematode surface. Further studies indicated that this phenomenon was unique to Luffness anti-serum, and was also associated with surface changes as determined by insertion of the lipid probe aminofluorescein 18 (AF18). Later experiments showed that these changes could be mimicked by substituting the phytohormone indole 3-acetic acid (IAA) for DMT, raising the possibility that IAA acts as a host cue to the nematode upon invasion of the plant root. To test this hypothesis nematode homogenates were probed using anti-serum D16, an anti-serum with specificity against the auxin binding domain of the auxin binding protein of maize. Immunoblots reveal the presence of a 45kDa protein which appears to be specific for D16 while initial immunoelectron studies show that D16 appears to localise to structures around the head of the nematode indicating that *Globodera* may possess an auxin receptor. Using these findings it has therefore been possible to propose a hypothesis of possible interactions that may be occurring between *Globodera* and its host.

**CHAPTER 1**  
**AN INTRODUCTION TO POTATO**  
**CYST NEMATODES**

## 1.1. POTATO CYST NEMATODES

The potato cyst nematodes (PCN) comprising the two species *Globodera pallida* and *G. rostochiensis*, are regarded as major pests of the potato *Solanum tuberosum*. It is estimated that total annual potato production approaches 300 million metric tonnes (Horton, 1987). These figures make potato one of the top five food crops in the world giving some indication of the impact of PCN as a commercial pest. Their dissemination probably coincided with the export of potatoes from the South American Andes to Spain in the sixteenth century. Since then they have become of economic importance in most temperate regions of the world.

Symptoms of PCN infection include poor growth, with plants exhibiting signs of water stress and mineral deficiency. Death may also occur. Poor growth can result in reduced yield due to the lack of development of the root system. In instances of severe infection with a large number of PCN, fewer tubers may be harvested than were initially planted.

## 1.2. POTATO CYST NEMATODE CLASSIFICATION

### 1.2.1. SPECIES

The two species of PCN have only been separated fairly recently. The similarities in their morphology and the overlap of morphological characteristics initially led to difficulties in classification. Indeed, the various species of cyst nematodes, including PCN, were thought to be races of *Heterodera schachtii*. Originally reclassified as *Heterodera rostochiensis* the two species of PCN were separated into *H. pallida* and *H. rostochiensis*, partly on the basis of female colouration (Stone, 1973). Finally the subgenus *Globodera* was changed to generic rank to which round cyst nematode species, including PCN, have been assigned.

### 1.2.2. POPULATIONS

It has become clear that much variation exists between populations of PCN in their ability to reproduce on resistant potato plants. In an attempt to identify and name these populations various systems have been adopted. The discovery of populations of PCN which could multiply on otherwise resistant potato cultivars led to the development of two different classification systems based in the UK and the Netherlands which classified the different populations as pathotypes (Kort *et al.*, 1977). However, it was apparent that the two separate systems caused confusion with the result that Kort *et al.* (1977) proposed an international, unifying scheme for the classification of PCN pathotypes. In this scheme pathotypes were defined as variants of a potato cyst nematode species which differ from others by their ability to multiply on particular potato genotypes known as differential hosts.

The scheme relies on the differential reproduction of PCN populations on several clones of potato. Pathotype classification depends on the ability of PCN to reproduce, with the ratio of cysts produced, (Pf), compared to the initial inoculum, (Pi), being used as the defining factor, (Pf/Pi). Using this scheme three pathotypes of *G. pallida* (Pa1, 2 and 3), and five of *G. rostochiensis* (Ro1, 2, 3, 4 and 5) were designated with the understanding that additional pathotypes could be added if required. Table 1.1 summarises the international pathotyping scheme.

For many years now the scheme has formed the basis by which decisions are made in controlling PCN by the use of resistant cultivars. Despite this, the scheme can be regarded as having several drawbacks, not least the laborious, time-consuming methods required. The major criticism of the scheme is that it gives little information on the degree of virulence exhibited



by pathotypes, with pathotypes being classified as virulent or avirulent. For example, *S. tuberosum* spp *tuberosum* is considered to be a universal host for all PCN pathotypes, but Pf/Pi values varying from 66 to 11 are obtained with different PCN populations (where Pf/Pi  $\leq$ 1.0 indicates resistance). This variation in response makes the interpretation of results more problematic. It has been shown that European differentials are unsuitable for many South American populations due to their ability to reproduce well on them. However, using a different set of four differential clones it was possible to distinguish four European pathotypes of *G. rostochiensis* and three of *G. pallida*, as well as three additional South American pathotypes of *G. pallida* (Canto Saenz & Mayer de Scurrah, 1977). Trudgill (1985) notes however, that many of the pathotypes described may be artefactual, resulting from the use of an arbitrary Pf/Pi value ( $>$  or  $<$ 1) resulting in the above mentioned variability in Pf/Pi values. Indeed, a more recent scheme suggested by Nijboer and Parleveit (1990) recognises only three pathotypes of *G. rostochiensis*. These are Ro1 (formerly Ro1 and Ro4), Ro3 (formerly Ro2 and Ro3), and Ro5. Another complicating factor occurs in that populations may arise in which a mixture of pathotypes are present. It should be noted that in this thesis nematodes will be named according to population, with the pathotype following in parentheses where appropriate.

It is clear that control of PCN using resistant cultivars requires the accurate identification of the virulence characteristics of pathotypes. In an attempt to move away from the time-consuming schemes described, many researchers have attempted to differentiate pathotypes using biochemical and molecular techniques. For example, using two-dimensional gel electrophoresis of proteins, Bakker *et al* (1988) showed that *G. pallida* and *G. rostochiensis* exhibit differences in approximately 70% of their polypeptides.

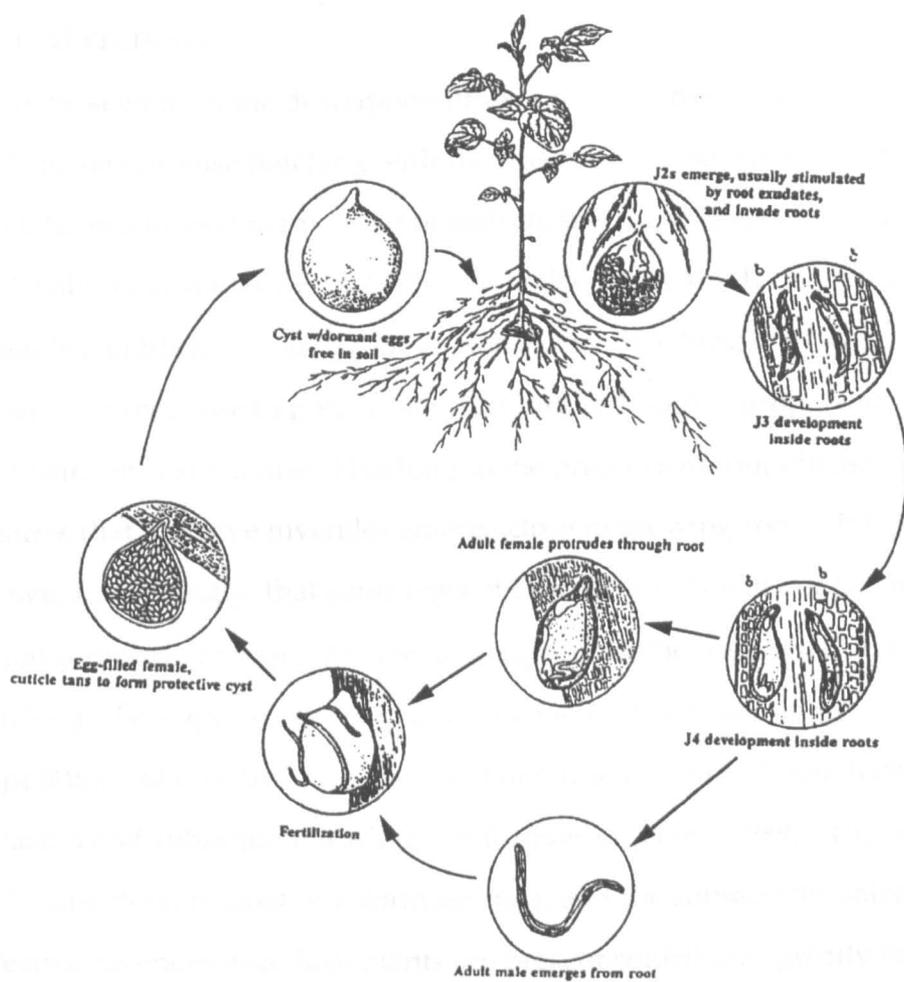
As pointed out by Gheyson and Van Montagu (1995), this is greater than the differences that are apparent between orang-outangs and humans using this assay (Goldman *et al.*, 1987). The two species of PCN have also been distinguished serologically. Schots *et al.* (1989) produced monoclonal antibodies that allowed the identification of a number of species, including *G. pallida* and *G. rostochiensis*, by utilising differences in species-specific egg proteins (Schots *et al.*, 1992). Separation of species has also been achieved at the DNA level using analysis of restriction length fragment polymorphisms. This method uses restriction enzymes to generate fragments of DNA which can then be separated by gel electrophoresis. Differences in DNA sequences alter the restriction sites that are present, and as a result a different banding pattern is thus generated. This technique has been successfully used by De Jong *et al.* (1989) to directly visualise differences between *G. pallida* and *G. rostochiensis*. However, although separation at the species level has been accomplished, reliable differentiation of population and pathotypes still remains problematic, especially for *G. pallida*. Population and pathotype differentiation has been accomplished, at least in part, using random amplification of polymorphic DNA (RAPD) (Folkertsma *et al.*, 1994). This polymerase chain reaction (PCR) based technique uses a random primer to generate reaction products after annealing to fortuitously homologous sequences. Using this technique it was observed that *G. rostochiensis* populations appeared to cluster in groups with similar pathotype classifications. *G. pallida*, however, appeared highly variable and no similarity between pathotype identification and RAPD generated data could be made.

### 1.3. THE LIFE CYCLE OF POTATO CYST NEMATODES

Figure 1 shows the life cycle of PCN. PCN are sedentary endoparasites. They exhibit a complex parasitism which ends when the body wall of the dead female tans to form a protective cyst. The cyst can enclose up to 400 eggs which are highly resistant to unfavourable conditions and can remain viable for up to 20 years. In the absence of a host plant about a third of the eggs will hatch each year. In the presence of a host however, diffusate from the roots stimulate the majority of the eggs to hatch. Second stage juveniles are released, the first moult having occurred within the eggs. These move through the soil towards host roots by following a gradient of compounds leached from the root (Rawsthorne & Brodie, 1986). Penetration of the root occurs just behind the growing point, with the nematode moving intracellularly through the cortex until it reaches the vascular tissue. At this point the nematode selects a single cell upon which to feed. Ultimately this cell will form the syncytium - the feeding site of the nematode. Syncytial formation is an absolute requirement for completion of the life cycle, with the syncytium acting as a transfer cell to provide nutrients for the nematode (Pate & Gunning, 1972; Gunning, 1976).

Once the feeding site is established the nematode becomes sedentary with the loss of locomotory muscles. In this site it passes through three developmental stages to reach adulthood. Sex determination is dependent upon syncytial size with large syncytia producing females which remain sedentary. Smaller syncytia produce vermiform males which emerge from the root. Cyst formation occurs as the developing females increase in size and erupt through the root surface. At this point their spherical bodies are exposed while their heads and necks remain in the root allowing continued feeding and egg production. Mature females release pheromones to attract males so fertilisation can occur with fertilised eggs developing into embryos

**FIGURE 1.1.** The life cycle of potato cyst nematodes. Adapted from Brodie, Evans & Franco, (1993).



which are retained within the female body. Following death of the female, the cuticle hardens to form the distinctive cysts which are left in the soil after harvest to await the arrival of another host plant.

### **1.3.1. HATCHING**

It can be seen from the description of the life cycle that it is essential for PCN to synchronise hatching with the presence of a host plant. In the case of PCN, and indeed many other nematode species, this synchrony is a result of the ability of unhatched infective juveniles to remain dormant until suitable conditions occur. As described earlier, hatching of PCN generally occurs as a response to potato root diffusate, although a proportion of eggs will hatch in water alone. Hatching in the presence of root diffusate thus ensures that infective juveniles emerge close to growing roots. It has been shown, interestingly, that some eggs of *G. rostochiensis* will not hatch on an initial contact with root diffusate, but require another exposure prior to hatching. This appears to be an effective method of maintaining populations of infective juveniles, and of reducing competition during invasion and subsequent feeding site formation (Perry, 1989). The ability of PCN, therefore, to exist in a dormant state, and the subsequent hatching of infective juveniles once host plants are present contributes greatly to their survival and dissemination.

### **1.3.2. EVENTS ASSOCIATED WITH HATCHING**

Upon interaction with root diffusate the shell of PCN eggs undergoes a permeability change. This appears to be due to calcium-dependent changes in the inner lipoprotein membranes of the lipid layer found in the egg (Clarke & Perry, 1985). The change in permeability results in the release of trehalose from the perivitelline fluid of the egg. This loss allows rehydration of the juvenile PCN. Once sufficiently hydrated, the juvenile is

able to move, allowing it to cut the eggshell with its stylet. These changes occur during the first 24 hours following exposure to root diffusate.

Juveniles, however, usually do not emerge from eggs until 3 days after this. Significant changes can be observed in this initial 24 hour period, including increased lipid metabolism (Robinson *et al.*, 1985), and increased oxygen consumption (Atkinson & Ballantyne, 1977).

Thus trehalose appears to be a factor of great importance in the hatching process. It is thought that trehalose may serve several functions. For example, Perry (1989) has suggested that the trehalose content of the perivitelline fluid may stabilise the lipid membranes of the eggshell. It is also thought that trehalose may have cryoprotective properties, thus shielding the juveniles from environmental extremes. Indeed, in support of this it has been demonstrated that chilling the eggs of *Nematodirus battus* results in an increase in the concentration of trehalose (Ash & Atkinson, 1983; Ash & Atkinson, 1986).

### 1.3.3. HATCHING FACTORS

As well as having a role in the permeability changes that occur in the lipid layer, it is thought that root diffusates may also have a direct effect on the juvenile itself. With this in mind, a bimodal action for the root diffusate hatching stimulus was proposed (Perry, 1986). For example, exposure of *G. rostochiensis* to potato root diffusate has a stimulatory effect on the dorsal oesophageal gland with an increase in secretory granules. It is thought that the accumulation of secretory granules may be in preparation for entry into the root. Further, the response appears to be to specific fractions of the diffusate. Partial purification of active fractions in potato root diffusate indicated that some had a stimulatory effect as described, but did not induce hatching (Atkinson *et al.*, 1987). To date few hatching factors have

been identified. Some progress has been made with *H. glycines* using dried and powdered roots of kidney bean. This has resulted in the isolation and characterisation of hatching factors named glycinoclopin A, B and C (Masamune *et al.*, 1982; Fukuzawa *et al.*, 1985a, b). The above section has dealt only briefly with some of the issues concerned with hatching. For an in depth review of the subject Perry (1989) should be consulted.

It can be seen from the description of the life cycle that to reach maturity PCN must first locate and penetrate host roots, and secondly initiate and maintain the syncytium. Despite many advances in the understanding of PCN biology relatively little is known about the process of infection and the mechanisms that are involved in those processes. This thesis will examine the role of various secretions produced by *G. pallida* in the infection process. There are several sources of secretions in nematodes but for the purposes mentioned secretions from the amphids, the oesophageal glands, the excretory / secretory (ES) system and the cuticle will be discussed. The initial part of this introduction will consider host finding and feeding site formation by *Globodera*, and the role that various secretions may have in these processes. The different resistance reactions that may occur in PCN infection will be considered, and the importance of these in the context of, for example, syncytium formation. Finally, the aims of this thesis will be described.

#### **1.4. HOST FINDING**

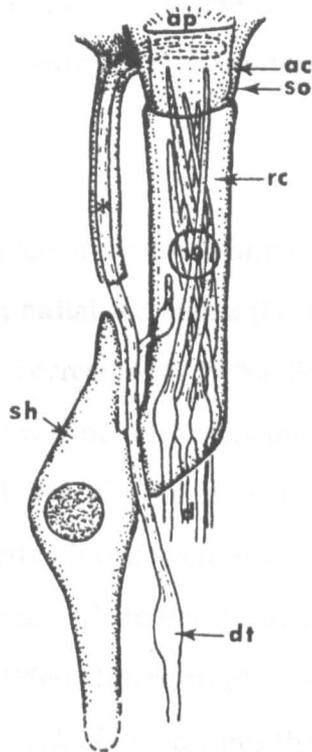
Once hatched, infective second stage juveniles must locate and move towards host roots if they are to complete the life cycle. It is known that several types of gradient including amino acid, sugar, ion and pH gradients exist around the roots (Etherton & Higginbotham, 1960; Miller & Gow, 1989), and as such may be used by nematodes in location of a host plant. It

is perhaps more likely that specific components of root exudate are involved in host finding by the nematode, especially in host-specific interactions such as those seen with PCN. Indeed, this was proposed as early as 1925 by Steiner.

### 1.5. ANTERIOR SENSE ORGANS

In nematodes the anterior sense organs are composed of 12 labial sensillae, 4 cephalic sensillae and 2 amphids (McLaren, 1976; Wright, 1980). The amphids are the largest and most complex of these, and their structure appears to be well conserved within plant-parasitic nematodes. Figure 1.2 shows the structure of a typical amphid. It is generally accepted from ultrastructural and neuroanatomical studies that the amphids are the main chemosensory organs (Ward, 1973; Lewis & Hodgkin, 1975; Bargmann *et al.*, 1990). The use of *Caenorhabditis elegans* as a model has allowed the characterisation and analyses of some chemotactic responses in nematodes and the reader is referred to the following references (Bargmann & Horvitz, 1991; Sengupta *et al.*, 1993; Troemel *et al.*, 1995). The amphids of PCN are situated laterally on either side of the mouth and can be described as a cuticle lined pore that is open to the exterior. Each amphid consists of a glandular sheath cell, a supporting socket cell, and dendritic processes which are located at the base of the pore. The number of dendritic processes varies between species, although seven processes are usually present in the amphid. Other processes may pass through the amphid but form structures at the head of the nematode. It is not known if these structures are associated with amphidial function (Wergin & Endo, 1976; Trett & Perry, 1985a).

**FIGURE 1.2.** Diagram showing the amphid of the plant-parasitic nematode *Xiphinema americanum*. Abbreviations: ap, amphidial pore; ac, amphidial canal; so, socket cell; rc, receptor cavity; sh, sheath cell; d, dendrite; dt, dendrite tip. 19 indicates the number of dendritic processes in the receptor cavity. (Adapted from Wright, 1980).



### 1.5.1. AMPHIDIAL SECRETIONS

Dendritic processes project into the amphidial cavity and are bathed in secretions produced by the amphidial sheath cell. These secretions are highly glycosylated. Initial studies indicating the presence of carbohydrate residues in amphidial secretions came from the observation that the adhesion of some nematophagous fungi appeared to occur exclusively to chemosensory structures. It was shown that this was mediated by a lectin-carbohydrate interaction (Nordbring-Hertz & Mattiasson, 1979; Jansson & Nordbring-Hertz, 1983), with the carbohydrate being located in the amphidial secretions.

Concurrent work using lectin binding studies confirmed the presence of glyco-conjugates in amphidial secretions (Forrest & Robertson, 1986; Aumann & Wyss, 1987; Forrest *et al.*, 1988a; Forrest *et al.*, 1988b; Aumann & Wyss, 1989), although it was not clear whether secretions were composed of glycoprotein, glycolipid or a mixture of the two. The nature of the glyco-conjugate as at least partly glycoprotein was confirmed by investigating the effects of various enzymes and chemicals on lectin binding (McClure & Stynes, 1988; Aumann, 1989). Interestingly, recent work carried out on the closely related *Heterodera schachtii* indicates that its amphidial secretions are composed exclusively of O-glycans (Aumann, 1994). O-glycan linkages are known to be the major constituents of mucus, although they are also found in some cell membrane-associated molecules (Hilkens *et al.*, 1992). This apparently supports previous findings by Aumann (1989) who showed that amphidial secretions were very resistant to proteolytic attack, often a consequence of O-glycosylation due to the relative resistance of the glycosylated regions to protease degradation. It is thought that this resistance is due to the attached carbohydrate residues blocking access to the peptide core as removal of the carbohydrate allows subsequent protease

digestion (Kozarky *et al.*, 1988). Another effect of O-glycosylation may be to extend the functional domain of a molecule out from the cell surface, thus allowing interactions with extracellular molecules (Jentoft, 1990).

### 1.5.2. THE ROLE OF AMPHIDIAL SECRETIONS

In recent years the role of amphidial secretions has been subject to much speculation. As stated earlier, it is generally considered that the amphids fulfil a chemosensory function. Experimental evidence however, has been forthcoming to suggest various, possibly multifunctional, roles for the amphids and their secretions.

The similarity in structure between nematode amphids and insect chemosensilla has led to the suggestion that the secretions may assist in capture and transport of chemotactic stimuli to the dendritic membranes in a similar manner to insects (Zuckerman, 1983). It has been suggested by Vogt *et al.* (1990) that capture, transport and degradation of odorant molecules was mediated by two kinds of protein in insect sense organs, namely odorant binding proteins (OBPs) and odorant degradative enzymes (ODEs). They suggested that odorant molecules bound to OBPs and were thus transported to receptor sites at the dendritic processes. Once released, the odorant molecule was degraded by ODEs to prevent repeated stimulation of the dendrites. Using probes derived from conserved regions of insect OBP it has been possible to identify genes that may encode similar proteins in *Meloidogyne* spp., *Heterodera schachtii* and *G. pallida* (Jones *et al.*, 1992). Also in the context of insect chemosensilla, amphidial secretions have been compared to the secretions which bathe the tarsal receptors of the blowfly *Phormia regina* and are thought to act as an  $\text{Na}^+/\text{K}^+$  reservoir for the maintenance of the receptor membrane potential (Broyles *et al.*, 1976).

Trett & Perry (1985b) have suggested that the secretions may act to maintain electrical contact between the bases and tips of dendritic processes.

It has been postulated that carbohydrate residues may have important functions in transduction of a chemosensory signal (Zuckerman, 1983; Zuckerman & Jansson, 1984). This hypothesis has been tested (Jansson *et al.*, 1984; Jayaprakash *et al.*, 1984), and it was shown that treatment of the free-living nematode *Caenorhabditis elegans* with the enzyme mannosidase or the lectin Concanavalin A (Con A) resulted in altered chemotactic responses towards a food source. Interestingly, normal chemotactic responses were resumed within a few hours which appears to be consistent with receptor renewal (Harford & Ashwell, 1982). Similarly, antibodies directed against a glycoprotein (gp32) found in the amphids of *Meloidogyne* significantly retarded orientation of nematodes to host roots (Stewart *et al.*, 1993b). Localisation studies show that gp32 is associated with the secretory material filling the amphidial canal, as well as the sheath cell. This may implicate gp32 in the primary events of signal reception and/or transduction (Stewart *et al.*, 1993a).

Other evidence for a chemosensory function of amphids have come from studies of nematicide action. Low concentrations of nematicides can impair responses to chemoattractants with no effect on motility (Di Sanzo, 1973). Later studies showed that exposure to the carbamoyloxime nematicide, aldicarb, resulted in the hypertrophy of the internal dendrite terminals within the amphidial sheath cell, a reduction in surface volume of the dendritic processes and the appearance of electron-lucent granules in the cytoplasm of the amphidial sheath cell (Trett & Perry, 1985b). Interestingly, these neuroanatomical effects were restricted to the amphids, and not observed in the sheath cells or dendrites of the labial or cephalic sensilla. It

was therefore suggested that aldicarb may have an effect via disruption of cholinesterase activity that has been reported (Cuany *et al.*, 1984). As stated by Perry (1994) however, this is still speculative.

A further suggestion is that amphidial secretions may be involved in initiation and/or maintenance of the host-parasite relationship. Forrest & Robertson (1986) were first to demonstrate the occurrence of carbohydrate residues on the surface of PCN. Further, the work also indicated that these carbohydrates were confined to small regions of the head, and that they could differ between populations and individuals. These differences may play an important role in determining whether the nematode can establish a successful infection. This hypothesis is further reinforced by the knowledge that glycoproteins rich in mannose and galactose extracted from *Cladosporium fulvum* act as elicitors of phytoalexin production in tomato (de Wit, 1980). Similarly, Huang & Barker (1991) showed that localisation of the phytoalexin glyceollin I occurs close to the head region of *Heterodera glycines* in resistant soybean cultures. Again this suggests that carbohydrate residues located close to the feeding site of the nematode play an important role in determining the host response to nematode infection.

It has been postulated by Endo (1978) that the feeding plug which is secreted by cyst nematodes once the feeding site is established may originate from the amphids. However, later studies suggest that feeding plug material may in fact be secreted through the cuticle (Jones *et al.*, 1993). The role of the feeding plug is itself uncertain. It has been suggested by Endo (1978) that the plug may function as a seal at critical stages in the infection and feeding process. The plug may first function as a seal during initial stylet penetration and probing. Secondly, the plug may seal the cell wall following stylet withdrawal that occurs during the moulting process.

The amphids may have multiple functions which are not necessarily mutually exclusive. It is known that in animal parasitic nematodes such as *Necator americanus* the amphids change upon infection of the host. The associated increase in secretory activity is thought to coincide with the onset of anti-coagulant production (McLaren, 1974; McLaren *et al.*, 1974). A recent study by Jones *et al.* (1994) shows however that the amphids change very little throughout the different stages of *G. rostochiensis*, indicating that they may fulfil the same role throughout the life cycle. Interestingly, although amphidial structure appears to be conserved throughout the life cycle of PCN it has been shown that the secretions themselves do change (Forrest *et al.*, 1988a). Freshly hatched juveniles which had been exposed to potato root diffusate (PRD) produced an abundance of amphidial secretion which filled the amphidial canal and protruded from the pore. The secretion appeared to be composed of dark reticulate osmiophilic material in a light matrix. In contrast to this, developing juveniles established within roots had secretions which had a fibrillar appearance. This may indicate that amphidial secretions have a function in pathogenesis or the establishment of infection.

#### **1.6. FORMATION AND CHARACTERISTICS OF THE SYNCYTIIUM**

Once the nematode has located a host it must enter the root and establish a syncytium. Using the stylet to break through cells the nematode migrates through the root until it reaches the vicinity of the vascular cortex. Here the movement of the stylet changes to gentle thrusts as it selects the single cell from which the syncytium will develop. Once the cell has been selected, the nematode uses its stylet to gently break through the cell wall until it rests between the cell wall and the plasma membrane. Upon selection and penetration of the cell the stylet tip remains in place for several hours although feeding does not occur (Wyss, 1992). Initiation of syncytium

formation is thought to occur upon the introduction of nematode oesophageal secretions to the cell, presumably during this period (Jones & Northcote, 1972). As yet the biochemical mechanisms of syncytial formation are unknown, although the events leading to syncytium formation are presumed to occur in this initial stage.

Ultrastructural studies using the *Arabidopsis thaliana* - *Heterodera schachtii* system have allowed detailed observations to be made of syncytium formation. Generally, the syncytium is composed of numerous expanding cells which have fused together following partial cell wall dissolution. It has been shown that parenchyma cells adjacent to differentiated proto- and metaxylem tracheary elements are generally selected as initial cells by cyst nematodes. Upon interaction with the nematode these cells rapidly enlarge and a single compartment of cytosol is formed containing large amounts of mitochondria, plastids, rough endoplasmic reticulum and dictyosomes. Similarly, an increase in cytoplasmic streaming and nuclear enlargement can be observed within hours of the commencement of feeding (Wyss, 1992). It is possible to observe multivesicular bodies and small vesicles containing fibrillar material in close contact with the plasmalemma, and it has been suggested that these may be involved in the cell wall dissolution that occurs in these areas (Sijmons *et al.*, 1994). Other features associated with syncytium development include stimulation of division of some stelar parenchyma cells, cell wall thickening of phloem elements, and the deposition of osmiophilic material close to the head of the nematode. As development progresses more cells are incorporated, with the syncytium expanding longitudinally towards the base of the root. Expansion generally occurs along the two tracheary elements of the early metaxylem with the syncytium eventually reaching 2-3mm in length. Cell wall protuberances can be seen adjacent to conductive tissues, especially xylem tracheary

elements. These are lined with plasmalemma which enhances short distance solute transport (Jones, 1981), and their structure appears to be typical of transfer cells. The development of these cell wall protuberances is generally taken as an indication of enhanced short distance transport of solutes (Jones & Northcote, 1972). It has been suggested by Bockenhoff & Grundler (1994) that water is likely to become a limiting factor in syncytial development, and that the cell wall ingrowths described may be part of the apoplastic pathway for water and ions. It is perhaps of note that *Meloidogyne incognita* directs the expression of the *TobRB7* gene during infection. The *TobRB7* protein is a water-pumping, membrane-associated protein. It is thought that expression of this protein increases the water content of the giant cells induced by the nematode (Conkling *et al.*, 1990; Opperman *et al.*, 1994). Interestingly, expression of *TobRB7* is not up-regulated in syncytia induced by cyst nematodes.

### 1.7. FEEDING BEHAVIOUR

Once the initial cell has been selected it has been observed that feeding appears to occur in 3 distinct phases;

1. Withdrawal of nutrients from the syncytium by the action of the metacorpul bulb.
2. Withdrawal of the stylet and its reinsertion into the syncytium.
3. Salivation with the consequent production of a feeding tube.

Feeding behaviour follows this pattern throughout the development of the syncytium (Wyss & Zunke, 1986; Wyss, 1992). Syncytial size, and thus presumably the quantity and quality of food available to the developing nematode, is an important factor in sex determination. Generally, females develop on much larger syncytia than males, probably a reflection of the greater nutrient requirement involved in egg production. Based on measurements of syncytium size, pump volume and pump frequency, it is

estimated that adult females of *Heterodera schachtii* can withdraw up to four times the syncytium volume per day (Seymour, 1983; Sijmons *et al.*, 1991; Wyss, 1992). An efficient method of solute transport is thus essential, and is reflected by the large number of cell wall protuberances present in syncytia.

It has been observed that feeding behaviour is associated with the production of the feeding tube which remains within the cell following withdrawal of the stylet. Each new round of feeding results in the production of a new feeding tube. The role of the feeding tube and the mechanism of its formation remain unknown, although the feeding tube is thought to filter cell components as they are ingested by the nematode. Innovative studies by Bockenhoff & Grundler (1994) using micro-injection techniques to introduce fluorescently labelled dextrans into the syncytium of *H. schachtii* have shown that the parasite can ingest only dextrans of less than 20kDa from the syncytium. It is of note that the stylet orifice of *H. schachtii* has a diameter of about 100nm whereas the size limit for uptake of dextrans corresponds to a Stokes radius of 3.2-4.4nm. From these observations it has been postulated that the size limit observed may be due to an additional sieving mechanism, probably provided by the feeding tube.

Similar techniques carried out with the membrane impermeable dye Lucifer Yellow has shown that the syncytium appears to be symplastically isolated from adjacent cells despite the presence of plasmodesmata. After injection the dye remained confined to the syncytium, with no movement even after three weeks (Bockenhoff & Grundler, 1994). Plasmodesmata can be considered as the equivalent of gap junctions in animal systems. They are cytoplasmic junctions whose frequency and transport function is developmentally and environmentally regulated (Robards & Lucas, 1990). Various studies have shown that they allow movement of molecules with a

molecular weight generally less than 1kDa, although under certain conditions such as the presence of azide or anaerobiosis, the size exclusion limit can increase to as high a mass as 7-10kDa (Cleland *et al.*, 1994). The small size of Lucifer Yellow (570Da) usually allows its transport through plasmodesmata, and as such it is commonly used to study intercellular communication between plant cells (Palevitz & Hepler, 1985; Fisher, 1988). The confinement of Lucifer Yellow to the syncytium suggests that the plasmodesmata which are present in small numbers in the syncytium are non-functional. The phenomenon of symplastic isolation reported by Bockenhoff & Grundler (1994) may be a consequence of the difference in pressure that is generated between the syncytium and adjoining cells. Oparka & Prior (1992) have demonstrated that symplastic transport in a higher plant can be blocked by increasing the pressure differential between adjacent cells. Their results showed that an artificially generated pressure gradient of 2000hPa led to a blockage of plasmodesmatal transport. When it is considered that a pressure gradient of approximately 5000hPa exists between syncytia and adjacent cells, then symplastic isolation may be a consequence. Alternatively, closure of plasmadesmatal junctions may be required to generate the pressure recorded in syncytia.

### **1.8. NEMATODE INDUCED GENE EXPRESSION IN SYNCYTIA**

It is clear that feeding site formation results from a complex interaction between nematode and plant. From the very nature of feeding site formation it may be presumed that the changes involved must occur as a result of altered patterns of plant gene expression. One area that is of great interest is the identification of host genes altered in their expression following nematode infection, and that are specific to the feeding site structures. Although this field is relatively recent in plant-nematode

research, encouraging progress appears to have been made (Gheyson & Van Montagu, 1995).

Analysis of plant gene expression patterns at feeding sites was initially problematic due to the limited amount of material available. PCR techniques, however, have overcome this problem, with the result that several genes involved in the feeding site have been identified using a variety of methods. It perhaps should be emphasised at this point that genes of particular interest are those genes induced specifically by the nematode, as opposed to genes induced by a general response to, for example, wounding.

For example, differential screening of a cDNA library produced from tomato galls induced by *Meloidogyne incognita* has allowed the identification of genes that are locally and highly induced in giant cells. One of these genes, *Lemmi 9*, has been shown to have high sequence similarity with the cotton gene *lea4-A*. *Lea4-A* has been characterised as a late-embryogenesis abundant protein (Galau *et al.*, 1993). It is thought that these proteins may have a role in protecting the embryo during desiccation of the seed. A putative role for *Lemmi 9* has thus been suggested by Van der Eycken *et al.* (1995) who postulate that it may act as an osmoprotectant in giant cells. Screening of cDNA libraries for nematode-induced genes has also allowed the identification of the extensin gene as being up-regulated by the nematode (Niebel *et al.*, 1993). Normally the extensin protein is thought to function in the termination of cell expansion, either by cross-linking to itself or to other cell wall components (Carpita & Gibeaut, 1993). In uninfected roots it has been shown that extensin production is induced in actively dividing cells, probably due to the need for new cell wall structural components. Similarly, high extensin expression has been observed in galls

of *M. incognita*. Interestingly, induction appears mainly in cortex and pericycle cells, whereas little induction could be observed in the cell walls of the giant cells (Van der Eycken, 1994). These results have been interpreted as reflecting the requirement of cortex cells to resist increasing pressure created by dividing pericycle cells, whereas the large cell walls need to maintain a loose cell wall structure to enhance solute uptake from the vascular system (Van der Eycken, 1994; Van der Eycken *et al.*, 1995).

A similar approach by Bird and Wilson (1994) has identified several nematode-induced genes. These include genes encoding products with homology to a plasmalemma proton ATPase, the largest subunit of RNA polymerase II, a ubiquitin carrier protein and a *myb*-like transcription factor. The identification of a *myb*-like transcription factor that is up-regulated by *Meloidogyne* spp. may indicate that a cascade mechanism may be responsible for some of the cellular changes seen. The proto-oncogene *c-myb* was the first cellular *myb* gene isolated from animals (Gonda *et al.*, 1985). It was shown to encode a transcriptional regulator essential for the proliferation of haematopoietic cells (Luscher & Eisenman, 1990; Shen-Ong, 1990). *Myb*-like genes have also been isolated from a variety of plants, including *Antirrhinum* (Jackson *et al.*, 1991), *Arabidopsis* (Oppenheimer *et al.*, 1991) and potato (Baranowskij *et al.*, 1994). The function of many of these *myb*-like genes in plants is unknown. However, it has been demonstrated that *myb*-like genes are required for the formation of flavonoids and derived pigments (Cone *et al.*, 1993; Grotewold *et al.*, 1991), as well as being involved in epidermal cell development (Oppenheimer *et al.*, 1991). It appears then, that *myb*-like genes may have important roles in regulating plant metabolism and development. Regulation of such transcription factors by the nematode would thus appear to be very much to its advantage.

Interestingly, recent work has indicated that *myb*-like transcription factors may be involved in gibberellin-regulated gene expression, and Jacobsen and Gubler (1993) have identified three different *myb*-regulated mRNA size classes. Indeed, evidence is available that suggests gibberellin-regulated processes such as  $\alpha$ -amylase induction in barley aleurone cells (Gubler *et al.*, 1995) and chalcone synthase production in *Petunia* (Weiss *et al.*, 1990; Solano *et al.*, 1995) are under the control of gibberellin-regulated *myb*-like genes. This suggests an involvement of upstream interactions, for example, in gibberellin perception or signal transduction in nematode up-regulation of *myb*-like transcription factors.

Gene expression studies have also been performed *in situ* using promoter- $\beta$ -glucuronidase (GUS) fusions. This assay works on the basis that fusing the *gus* gene with the promoter from a gene which is expressed (for example, in feeding sites) will, after addition of a chromogenic GUS substrate, result in a blue colour change that can be easily observed. This method has several advantages in that it allows screening of many plants simultaneously, as well as facilitating comparison of gene induction at different time points. Genes identified by this method include the TobRB7 membrane protein mentioned earlier (Opperman *et al.*, 1994). Further advances using this technique have come from using *Arabidopsis thaliana* as a host plant (Sijmons *et al.*, 1991). This work is further enhanced by the progress that has been made in identification and sequencing of *A. thaliana* genes, specifically those genes involved in cell cycle regulation. Niebel *et al.* (1995) have shown that *Heterodera schachtii* and *Meloidogyne* spp. induce the gene *cdc2a* within one hour of feeding site initiation. Similar induction of *cyc1 At* has also been observed, although expression appears more transient (Niebel *et al.*, 1994). Gheyson and Van Montagu (1995) have argued that although induction of cell cycle genes by *Meloidogyne* spp. might be expected due to the processes

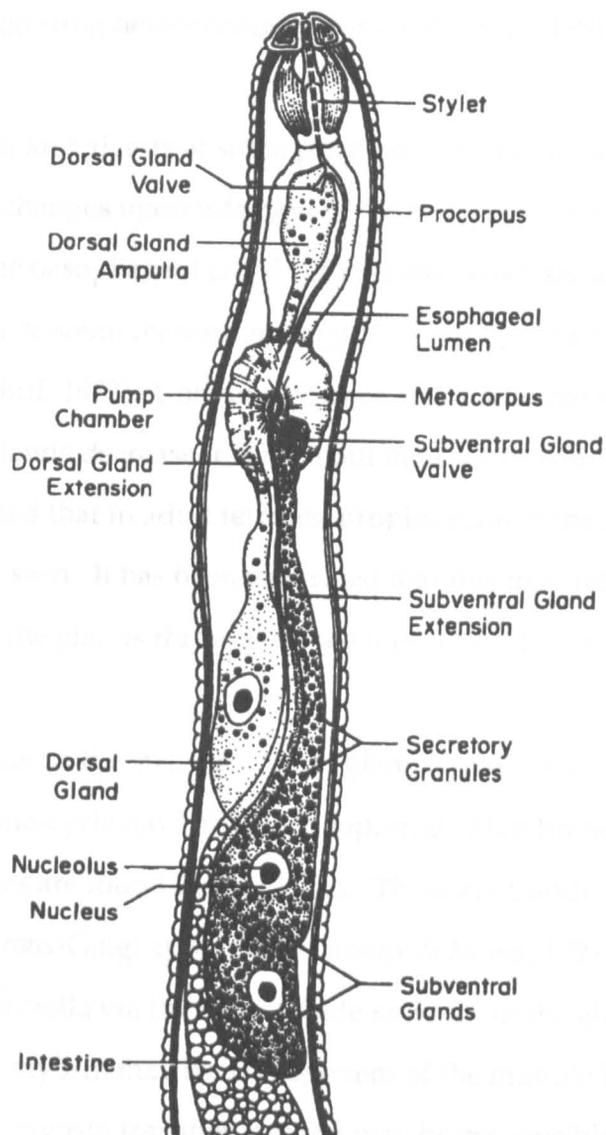
of endomitosis and cell proliferation that are a feature of *Meloidogyne* infection, it is perhaps more surprising that similar results were observed with cyst nematodes. They suggest that *cdc 2a* induction by cyst nematodes probably occurs as a consequence of the endoreduplication that has been observed in syncytia (Endo, 1971), since *cdc 2a* is probably expressed in the S phase of the cell cycle (Hemerly *et al.*, 1993). Similarly, they argue that *cyc1 At* expression may be a consequence of the induction of an abortive mitosis that is blocked in very early phase.

It can thus be seen that feeding site formation appears to be an extremely complex process, involving both temporal and spatial shifts in gene expression. Although some progress has been made in identifying genes involved in the initiation and formation of the feeding site, the triggers for these changes still remains to be made clear. It has long been considered that nematode oesophageal gland secretions have some function in this process. The next section, therefore, will examine attempts that have been made to isolate and characterise such secretions.

### 1.9. OESOPHAGEAL GLAND SECRETIONS

Molecules secreted by the oesophageal glands of nematodes appear to have critical roles in the plant-nematode interaction. The oesophagus of *Globodera* spp. is divided into a non-muscular procorpus, a muscular metacarpus containing a pump chamber, with a posterior glandular region (see Figure 1.3). Three oesophageal glands are present, consisting of one dorsal gland and two subventral glands. Each gland is composed of a single cell. A cytoplasmic extension containing an array of longitudinally orientated microtubules ends in an ampulla that acts as a collecting reservoir. The ampullae are themselves connected to the lumen of the oesophagus via ducts terminated with valves (Endo, 1984). The cells of all

**FIGURE 1.3.** Organisation of the three oesophageal glands. (Adapted from Hussey, 1992).



three glands contain lobed nuclei with a prominent nucleolus, Golgi complexes, rough endoplasmic reticulum, ribosomes and secretory granules, all of which are typically associated with secretory glandular cells. Nerve processes and neurosecretory cells can be found associated with the gland cells, suggesting neurosensory regulation (Endo, 1984).

The oesophagus and glands of some plant-parasitic nematodes undergo morphological changes upon infection of the host plant. It has been reported that the oesophageal glands of infective juveniles of *Meloidogyne incognita* and *M. javanica* increase in length but not volume upon interaction with the host (Bird, 1983). Conversely, once nematodes have become sedentary the glands decrease in length but increase in volume. In the same study it was noted that in adult females atrophication of the subventral glands could be seen. It has been suggested that this may reflect changes in the functions of the glands throughout the life-cycle (Hussey, 1989).

As well as changes in the structure of the glands, changes in their contents throughout the life-cycle have also been reported. Membrane-bound secretory granules are found in the glands. These are budded from the cisternae of the *trans*-Golgi apparatus (Hussey & Mims, 1991) and move forward to the ampulla via the microtubule network in the gland extension. It is thought that myosin may be a component of the granule bilayer, suggesting that a myosin transport system may be responsible for the movement of granules described. In support of this it has been demonstrated that a monoclonal antibody shows cross-reactivity with secretory granules and muscle in *Meloidogyne* spp.

Various changes have been noted in the form of these granules. Bird (1967) described secretory granules in the subventral glands of pre-parasitic *M.*

*javanica* as being large with distinct outer membranes. Parasitic juveniles recovered from roots three days post infection had granules which were smaller with indistinct membranes. Similarly, in the soybean cyst nematode *H. glycines* the secretory granules in the dorsal gland cell of preparasitic juveniles are small and electron dense. These enlarge and become less dense as parasitism commences (Endo, 1987; Endo, 1993). Granules within the subventral glands are also initially large with irregular cores, but become smaller and more electron dense. However, Hussey (1989) suggests that variations in the fixation protocols used in these studies may account for some of the differences seen.

#### **1.10. ISOLATION OF OESOPHAGEAL GLAND SECRETIONS**

Much work has been directed at the isolation and characterisation of these secretory molecules in order to obtain an insight into their nature, and allow speculation regarding their biochemical role. Hussey (1989a) suggested that the position where the gland ducts emptied into the oesophageal lumen could indicate any potential role in pathogenesis, since it can be presumed that only those components secreted through the stylet can have a direct role in the plant-nematode interaction (Goverse *et al.*, 1994). Early attempts at isolation of oesophageal gland proteins were not conclusive (Poehling & Wyss, 1980; Reddigari *et al.*, 1985), and relied on indirect methods, such as differential centrifugation, to collect candidate molecules. Using a more direct approach Veech *et al.* (1987) collected stylet secretions from adult females of *Meloidogyne* species. These secretions were subjected to electrophoresis which revealed at least nine protein bands. Later work looked at novel immunisation procedures as a way of raising monoclonal antibodies against oesophageal gland proteins. Several monoclonal antibodies have now been produced against secretions from various plant-parasitic nematodes, including *Heterodera* and *Meloidogyne* (Atkinson *et al.*,

1988; Atkinson & Harris, 1989; Hussey, 1989b; Hussey *et al.*, 1990; Davis *et al.*, 1992). The production of monoclonal antibodies against oesophageal gland proteins has provided a means to answer various questions. Using them it has been possible to show that stylet secretions are derived from both the dorsal and subventral glands (Davis *et al.*, 1994; Goverse *et al.*, 1994). Similarly, it has been possible to monitor the expression of secretory antigens throughout the life cycle of the parasite. From this, evidence is available indicating that secretions from the oesophageal glands may change throughout the life-cycle of some plant-parasitic nematodes. Parasitic stages of *M. incognita* taken from tomato revealed differential temporal and spatial expression of several oesophageal gland antigens (Davis *et al.*, 1994). For example, a monoclonal antibody specific to subventral gland and stylet secretions of infective juveniles did not bind to the oesophageal glands of early parasitic juveniles or any later parasitic stage. This may indicate a role for secretory components in penetration and invasion of the root. A similar phenomenon has been observed in *H. glycines*, with changes occurring in dorsal and subventral gland antigens during infection of soybean (Atkinson & Harris, 1989). Despite the progress made with monoclonal technology only one secretory molecule, a 212kDa glycoprotein from *Meloidogyne incognita*, has been isolated to date (Hussey *et al.*, 1990).

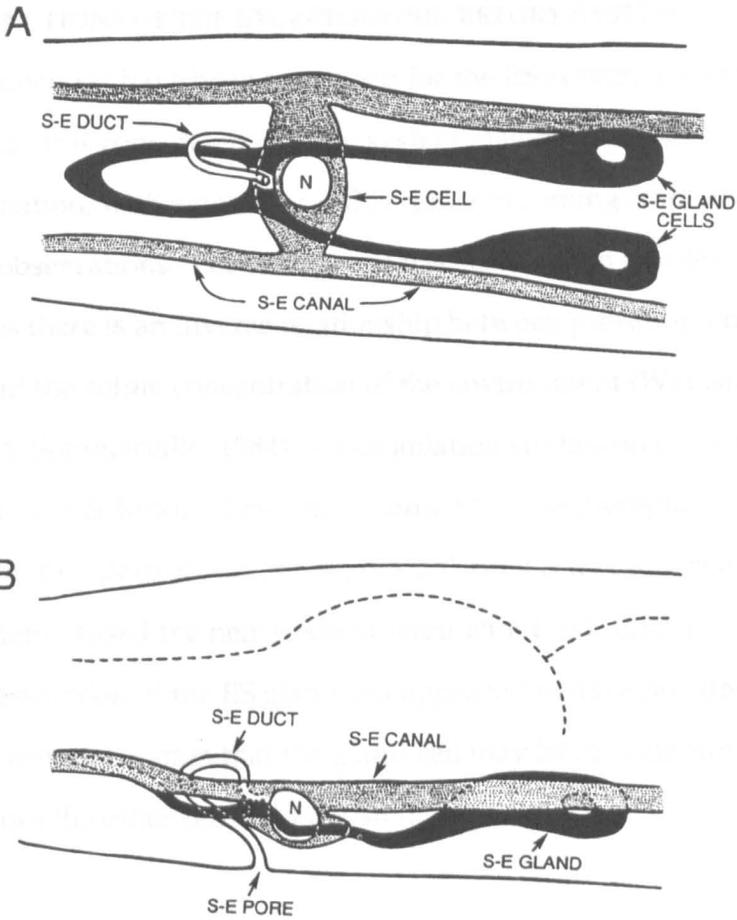
Previous work has shown that several plant-parasitic nematodes exhibit stylet activity, including the production of secretions, when incubated in the presence of dihydroxybenzene derivatives or certain neurotransmitters (McClure & von Mende, 1987). The induction, forward movement and accumulation of granules in the subventral glands is associated with exposure to these compounds. More recently it has been shown that incubation of nematodes with the serotonin agonist, 5-methoxy-N, N-

dimethyl tryptamine (DMT) results in the production of large quantities of stylet secretions (Goverse *et al.*, 1994). This has allowed the collection of such secretions from *H. glycines*, and the production of monoclonal antibodies against the oesophageal glands. Despite these advances the crucial question of what roles these secretions have in the plant-nematode interaction remains unanswered.

### 1.11. THE EXCRETORY-SECRETORY SYSTEM

In nematodes the excretory-secretory (ES) system opens to the environment through the ES pore. In the secernentean nematodes, to which *Globodera* spp. belong, much variation exists in ES system architecture. Generally speaking, ES system structure is based on an H-shaped tubular system consisting of two long canals joined by a transverse canal (see Figure 1.4). These are connected to the ES pore by a median duct. An ES cell may be associated with the transverse canal, and two other ES cells may also be involved. The ES gland cells of those secernentean nematodes examined ultrastructurally have been full of secretory granules (Wharton & Sommerville, 1984; Nelson *et al.*, 1983). Modifications of the structure occurs in different nematodes, sometimes involving the loss of one or more of the components. The origin and role of the ES system has been subject to much speculation (Bird, 1971). It is of interest to note that the structure of the ES system has been shown to change throughout the life-cycle of many nematodes. It has been suggested that such differences may reflect changes in the environment of the nematode as it proceeds through its life cycle. For example, in *Caenorhabditis elegans* all stages except the dauer larva have numerous secretory granules in their ES gland cells. However, upon entering the developmentally arrested state characteristic of the dauer form, the secretory granules are replaced by a loose membranous network. When development recommences following an increase in available food,

**FIGURE 1.4.** Diagram of reconstruction of ES system of *Caenorhabditis elegans*. A) Ventral view, B) Lateral view. (Adapted from Nelson *et al.*, 1983).



secretory granules reappear (Nelson *et al.*, 1983). Changes in structure have also been reported for parasitic nematodes such as *Stephanurus dentatus* (Waddell, 1968) and *Meloidogyne javanica* (Bird, 1971). It therefore appears to be a feature of many nematodes that the function of the ES system in the larval stage is different from that of the adult, with its morphology being dependent upon the physiological state of the environment.

### 1.11.1 FUNCTIONS OF THE EXCRETORY-SECRETORY SYSTEM

Various functions have been suggested for the ES system in secernentean nematodes. It is considered that the system may have a role in osmoregulation, with evidence for this function coming from several different observations. For example, it has been shown that for certain nematodes there is an inverse relationship between pulsation rate of the ES system and the solute concentration of the environment (Weinstein, 1952; Wharton & Sommerville, 1984). Laser ablation studies on *Caenorhabditis elegans* (Nelson & Riddle, 1984) also pointed to an osmoregulatory function. They found that destruction of the pore cell, duct cell or associated ES cells in the system caused the nematode to swell with fluid, finally resulting in death. Destruction of the ES gland cell appeared to have no effect and this has led to the speculation that the gland cell may have some function distinct from the other cells in the system.

It has been suggested that the ES system may have a secretory role. Lee (1970) found that the ES glands of *Nippostrongylus brasiliensis* contained esterase, cholinesterase and aminopeptidase activities. Similar materials have been detected in the ES glands of *C. elegans* (Mounier, 1981; Nelson *et al.*, 1983). This may suggest a role in exodigestive feeding for the secretions. It has also been suggested (Davey & Sommerville, 1982) that such activities may function in the exsheathment process of some nematodes.

Material derived from the ES system may form the surface coat seen in some nematodes. The surface coat was originally described as a fuzzy layer coating the epicuticle (Luft, 1976; Bird & Zuckerman, 1989). This was originally designated the glycocalyx, but is now generally referred to as the surface coat after Wright (1987). Little is known of the composition of the surface coat, although studies indicate that it may be glycoprotein in nature (Premachandran *et al.*, 1988). Similarly, it appears that some components of the surface coat may be lipid in nature. For example, lipase treatment was shown to increase labelling of the lectin wheat germ agglutinin (WGA) to the surface of *Anguina tritici* (Spiegel & Robertson, 1988; Spiegel *et al.*, 1991), the suggestion being that removal of lipid residues allowed better access for lectin binding to occur.

The origin of the surface coat still remains a matter of controversy (Spiegel & McClure, 1995). Some evidence is available, however, that suggests the ES system may be responsible, at least in part, for the surface coat observed in many nematodes. Binding of WGA to ES secretions and the outer surface of *Anguina agrostis* led Bird *et al.* (1988) to speculate that glyco-conjugates leave the ES pore and spread over the nematode surface. Staining of *Meloidogyne* secretions using Coomassie Blue also results in coating of the cuticle in the region of the excretory pore, as well as amphidial and buccal staining (Premachandran *et al.*, 1988). In animal parasitic nematodes, Meghji & Maizels (1986) found that monoclonal antibodies to ES products reacted both with the secretions and the surface coat of *Toxocara canis* although it appears in some cases that components of the surface coat may be secreted through the cuticle (J. Modha, pers. comm.).

### 1.11.2. THE ROLE OF ES PRODUCTS

Work in this area has generally focused on the effects of ES products of animal-parasitic nematodes. Much of the groundwork for collection and characterisation of ES products has been carried out using *Toxocara canis* as a model due to its longevity in culture, coupled with its ability to secrete copious amounts of material (Maizels & Page, 1990). Since then, ES products from many animal-parasitic nematodes have been characterised and various roles suggested for them. These roles include proteolysis of host proteins (Robertson *et al.*, 1987), immunomodulation due to acetylcholinesterase activity (Rathaur *et al.*, 1987; Robertson *et al.*, 1987), eosinophilia (Sugane & Oshima, 1984) and immune evasion due to shedding of surface components (Smith *et al.*, 1981; Maizels *et al.*, 1984). Many other roles have been postulated for ES products, and the ways in which they may affect the infection process. For a comprehensive review of the role of ES products in various animal parasitic nematodes the following references can be consulted (Parsons *et al.*, 1986; Maizels & Selkirk, 1988; Smith, 1991). It should be emphasised however, that ES products of animal-parasitic nematodes do have a definite role in both the infection process, and the outcome of infection.

As yet no ES products from plant parasitic nematodes have been characterised. Indeed, the only suggestion to date which can perhaps be regarded as comparable in terms of evasion of host responses has involved the seed gall nematodes *Anguina agrostis* and *A. tritici*. It was shown that the cuticle surface of these nematodes had binding sites for wheat germ agglutinin (WGA) (Spiegel & Robertson, 1988). A later study showed that WGA itself was present on the surface, and that it appeared to be derived from the wheat plant (Spiegel *et al.*, 1991). From this it has been suggested that the nematode uses host WGA to avoid recognition by the plant.

However, the source of these binding sites remains unclear, and it is not known whether they constitute part of the cuticle, or are present as secretions. Other researchers have suggested a role for the surface coat in plant-nematode interactions. It is thought that carbohydrate moieties that are components of the surface coat may act as specific elicitors during the plant-nematode interaction, and may therefore have a role in determining the outcome of infection (Kaplan & Davis, 1987). Lin & McClure (1993) have shown that second stage juveniles of *Meloidogyne incognita* surface labelled with radioiodine release  $^{125}\text{I}$  into their environment, indicating that the surface coat may be shed. It has been suggested, therefore, that this dynamic property of the surface could allow evasion of recognition phenomena (Spiegel & McClure, 1995). It has also been suggested (Bird *et al.*, 1988) that the surface coat may serve as a lubricant as the nematode moves through soil. Again, as with the amphids and amphidial secretions, the ES system, and its related secretions, may fulfil more than one function that are not necessarily mutually exclusive.

#### 1.12. OTHER SOURCES OF SECRETION

Several species of cyst nematodes have been shown to have secretions on the cuticle surface that apparently originate from within the cuticle upon commencement of feeding behaviour (Brown *et al.*, 1971; Forrest *et al.*, 1989; Aumann *et al.*, 1991). It was originally thought that components of the secretions were produced by fungi that metabolised products excreted by the nematode (Brown *et al.*, 1971). The resulting layer, termed the subcrystalline layer, was thought to act as a barrier to potential pathogens. Several studies (Zunke, 1985; Endo & Wyss, 1992) have shown, however, that nematodes maintained in sterile conditions still exhibit the subcrystalline layer, thus discounting the possibility of a symbiotic interaction. Observations made during a study by Endo & Wyss (1992)

show that cuticular secretions appear to originate in the hypodermis as secretion vesicles, most probably derived from the Golgi system of the hypodermis. This extends the observation that cuticular secretions originate from lacunae in the median layer of *G. rostochiensis* (Forrest *et al.*, 1989). Cuticular secretions thus appear to be products of the hypodermis (Endo & Wyss, 1992).

Cuticular secretions have been shown to be present in all stages of developing females, and up to the J3 stage of males of *H. schachtii* (Zunke, 1985). This may indicate that they are associated with feeding, and may occur as a result of nematode metabolism. Various other functions have been suggested for cuticular secretions. They may act as a protective layer for the cuticle that masks recognition by the host plant (Aumann *et al.*, 1991). Alternatively, it is thought that the secretions may anchor the nematode in place during feeding (Forrest *et al.*, 1989). However, the fact that secretions occur over the entire surface of the cyst nematodes examined (Zunke, 1985; Aumann *et al.*, 1991) perhaps indicates that they have a more general function (Endo & Wyss, 1992).

In summary then, it can be seen that there are various sources of secretions that may have a function in the plant-nematode interaction. It is known that at least some of these secretions contain carbohydrate residues, and as such may be important in acting as elicitors that determine whether or not infection will be successful. The following section will discuss the various resistance reactions that can occur as a result of PCN infection.

### 1.13. PLANT DEFENSE RESPONSES

In previous sections nematode-induced gene expression in relation to syncytial formation has been discussed (see Section 1.8). Infection of a plant by a pathogen, however, also results in the induction of defence-related gene expression that is quite distinct from those events occurring within the syncytium. These responses can be localised to the site of infection, or may involve systemic accumulation of defence compounds in tissues distant from the infection site (Cramer *et al.*, 1993). The events governing plant-defence gene expression and regulation are complex. The subject has been the focus of many reviews, and the reader is referred to Collinge & Slusarenko (1987), Cramer *et al.*, (1993), Kuc, (1995), Baron & Zambryski, (1995) and Smith, (1996). The following section will describe resistance reactions occurring against PCN in relation to syncytial formation.

### 1.14. RESISTANCE REACTIONS AGAINST POTATO CYST NEMATODES

Resistance reactions against infection by PCN vary depending on whether they are due to a major gene mediated response or polygenically inherited resistance. The co-evolution of PCN and their hosts means that the selection of genes conferring resistance in their hosts is likely. Indeed, species of wild and cultivated potato in the Andean region are sources of resistance against PCN. Several species of potato are used in breeding resistant potato cultivars. For example, *Solanum tuberosum* spp. *andigena* (Juz. et Buk.) Hawkes CPC 1673 is the source of the H1 major gene which confers fully effective resistance to pathotypes Ro1 and Ro4 of *G. rostochiensis*. *Solanum vernei* (Bittm. et Wittm.) clones CPC 2487 and 2488 provide polygenically based resistance to *G. rostochiensis* and *G. pallida* (Rice *et al.*, 1985). Resistance reactions can vary according to the source and can involve failure of infective juveniles to remain in roots, an imbalance of the

sex ratio with more males being produced, slowed development or death (Williams, 1956; Trudgill & Parrott, 1969; Turner, 1980; Philips *et al.*, 1982).

#### 1.14.1. H1 RESISTANCE

An ultrastructural study by Rice *et al.* (1985) showed that the pattern of cellular changes in roots of plants carrying the H1 gene can be divided into two phases. The first concerns cells damaged as the nematode moves towards the vascular cortex, and the subsequent degeneration and death of intact cortical cells surrounding the body of the nematode. Once the juvenile has come to rest, various changes can be observed in the undamaged cortical cells that surround the body of the nematode. Cytoplasm in these cells becomes granular, and nuclear enlargement occurs. By 42 hours breakdown of the tonoplast and plasmalemmal disintegration can be seen. Organelles lose their standard shape, and the nuclear envelope appears disrupted. These changes result ultimately in death. The changes do not appear to be caused by physical damage, and are not typical of a hypersensitive response in that they occur in an extended period over 42 hours (Rice *et al.*, 1985). It has been suggested (Rice *et al.*, 1985) that the necrotic cells surrounding the syncytium may contain phenolic substances since they stain intensely with safranin and toluidine blue. The distribution of the response suggests it is elicitor-induced, perhaps indicating the involvement of cuticular components and/or nematode secretions.

Despite the death of these cells, syncytial development is still initiated. Concomitant with syncytial development is the appearance of a necrotic layer of cells at the boundary of the syncytium. Organelles can not be observed in these cells, which ultimately surround the boundary of the syncytium (Rice *et al.*, 1985). Syncytial development initially proceeds as normal, but by 7 days cytoplasmic contents have degenerated despite the

incorporation of surrounding cells, and organelles are no longer present. The cytoplasm is much reduced, giving the syncytium a vacuolated appearance. Similarly, cell wall protuberances associated with solute transfer in a normal syncytium are absent. Rice *et al.* (1987) question whether the initial hypersensitive reaction observed around the body of the nematode is required for the later response that limits feeding site development. They suggest that the timing and distribution of the responses indicate that different processes may be involved, the first being a response to the exterior of the nematode, while the second occurs as a response to some component of juvenile oesophageal secretions. It appears then, that limitation of the syncytium by the boundary of necrotic cells, and the absence of wall protuberances prevents the syncytium from providing adequate nutrients for growth and development of the nematode. Resistance conferred by the H1 gene is particularly effective in that it allows second stage juveniles to invade and partially develop. However, it does not allow complete development of the nematode, but the resulting loss of locomotory muscle that has occurred with partial development renders the nematode unable to leave the root.

It was originally argued that H1 resistance occurs as a result of gene-for-gene interactions (Jones, 1974; Parrott, 1981). Briefly, the gene-for-gene hypothesis proposes that in a resistance reaction, specific compounds produced directly or indirectly by a dominant avirulence gene of a pathogen interact, again directly or indirectly, with the product of a resistance gene from the host to initiate the incompatible reaction (Cramer *et al.*, 1993). In simplest terms, this can be envisaged as avirulence genes encoding products that act as signal molecules which bind to host cell receptors encoded by resistance genes. The first fungal avirulence gene to be characterised was the *avr9* gene from *Cladosporium fulvum*. It encodes a

small peptide which, in the presence of gene products encoded by the resistance gene *cf9* activates host defences (Van den Ackerviken *et al.*, 1992). Evidence also suggests that some avirulence genes encode enzymes involved in the modification of cell surface glycoproteins and glycolipids. A comprehensive review of gene-for-gene interactions can be found in Keen (1990). Evidence for a gene-for-gene interaction between PCN and potato was provided by Janssen *et al.* (1991) who produced pure strains of *G. rostochiensis* that were virulent or avirulent for potatoes carrying the H1 gene. Using these strains it has been possible to show Mendelian proof for the interaction (Janssen *et al.*, 1991; Bakker *et al.*, 1992). This is the only clear example of Mendelian proof for a gene-for-gene interaction between a plant and nematode.

#### 1.14.2. POLYGENIC RESISTANCE

Anatomical changes mediated by polygenically mediated resistance appear to be less drastic. The severe necrosis seen with the H1 response is absent with the implication being that different mechanisms are responsible (Rice *et al.*, 1987). As before the invading juvenile moves through the root to the cortex. Once the nematode has come to rest the cells adjacent to the body undergo a hypersensitive type response, although the nematode is still able to initiate syncytial formation. The syncytium expands by incorporating cells and cytoplasm proliferates in some parts of the syncytium, but does not become dispersed as normal. After about a week, syncytia are highly vacuolated and the cytoplasmic contents appear to have degenerated. Plastids and other organelles can not be distinguished in the cytoplasm. Generally, juveniles fail to develop beyond the second stage and have syncytia that are degenerated, suggesting they are unable to gain sufficient nutrients (Rice *et al.*, 1987). It has been suggested that expression of resistance derived from *S. vernei* may depend upon a balance between

numbers of resistance and virulence genes. This may affect the intensity, extent and timing of the response which may in some cases allow normal development.

With both H1 and polygenic resistance, it can be seen that a disturbance of normal syncytial formation and function occurs. It has been postulated that nematode secretions/excretions may act as elicitors of resistance in plants. However, the paucity of information regarding any secretory products of PCN, or indeed any plant-parasitic nematode, means that there is little evidence to support this theory. It is clear that a better understanding of the events occurring during syncytium formation and development will help elucidate resistance mechanisms against PCN, especially in the case of the H1 major gene mediated response.

#### **1.15. AIMS OF THE THESIS**

Initially, the aim of this thesis was the isolation and characterisation of secretory material, especially amphidial secretions, from *G. pallida* and *G. rostochiensis*. However as work has progressed it has become clear that there are fundamental aspects of the PCN/potato interaction which require clarification. Of these syncytium formation and maintenance, a process central to PCN parasitism is extremely important, being in itself crucial to the understanding of resistance reactions against the parasite. Although isolation of some secretory material has been accomplished, later work in this thesis looks at the induction of secretions in *G. pallida* and how this may affect surface properties. Similarly, host cues to the nematode have been investigated. Finally, a scheme has been proposed hypothesising how PCN may alter physiological conditions in the host thus allowing syncytium formation.

**CHAPTER 2**  
**MATERIALS AND METHODS**

The following chapter describes the protocols and materials used throughout the project. All reagents used in the following protocols were obtained from Sigma, UK unless otherwise stated. An appendix listing all solutions used in the following protocols can be found at the end of the chapter.

## 2.1. PARASITE MATERIAL

All cysts of *Globodera pallida* (E, Luffness, Loc, Newton, Gourdie and Derby populations) and *G. rostochiensis* populations (pathotypes Ro1, Ro3, Ro4 and Ro5) were kindly provided by the Scottish Crop Research Institute, Invergowrie, Dundee.

Hatched second stage juveniles (J2) of *G. pallida* and *G. rostochiensis* populations were obtained as described previously (Forrest & Farrer, 1983). Cysts were presoaked in tap water until hatched nematodes were seen after approximately 6 days. The tap water was then replaced with potato root diffusate (PRD) produced from the susceptible potato cultivar Désirée. Hatched nematodes were either used immediately or harvested by centrifugation using an MSE Microcentaur bench centrifuge and the pellet stored at -20°C.

## 2.2. POTATO ROOT DIFFUSATE PRODUCTION

Potato root diffusate (PRD) was produced using Désirée potatoes, a variety with low resistance to nematode infections. The potatoes were allowed to sprout by leaving them at room temperature for a few days. When sprouts could be seen they were excised from the potato using a scalpel blade and placed in canisters containing moist sand. The canisters were kept in the dark at room temperature and examined every few days until good root growth was observed. When sufficiently

large the roots were removed from the sand, rinsed and placed in 250ml of distilled water at 4°C. Sprouts taken from 6 small potatoes were used for each batch of PRD. After 2 hours the roots were removed and the remaining diffusate filtered first through Whatman Number 1 filter paper to remove grit and other large particles, and then through a bacterial filter (Millipore, pore size 0.2µm) to remove any micro-organisms. Filter sterilised PRD was kept at 4°C and used within one week of production.

## **2.3. PREPARATION OF PARASITE PROTEINS**

### **2.3.1. AMPHIDIAL/EXCRETORY PORE SECRETIONS**

Amphidial/excretory pore secretions were collected using a modification of the staining procedure originally described by Premachandran *et al.* (1988). Various stains at different concentrations were used initially to determine which combination gave optimal results. The combination which appeared to give the best results is described here.

Approximately 50 000 freshly hatched J2 were harvested and washed by centrifugation as described earlier, and the pellet suspended in a final volume of 0.5ml distilled water. An equal volume of 40% methanol containing 0.1% Coomassie Blue R250 was added to the nematode suspension, leaving the nematodes in a final concentration of 0.05% Coomassie Blue R250 in 20% methanol. The solution was placed in a shallow dish and covered with a large coverslip smeared with nail varnish. The dish was then left to incubate overnight allowing the production of stained secretions from the amphidial and excretory pores. Secretions were collected by passing solution containing the nematodes through a modified syringe which had polyester mesh

(Lockertex, Walsall, UK) of pore size 15µm heat welded across the syringe barrel (Forrest, 1987). Nematode bodies were retained in the barrel of the syringe, allowing the secretions to pass through in solution. Secretions were concentrated and washed by centrifugation in 20% methanol with a final wash in distilled water and stored at -20°C prior to use.

### **2.3.2. WHOLE BODY PREPARATIONS**

Nematodes were harvested by centrifugation as before and washed extensively in Phosphate Buffered Saline (PBS, see appendix). The pellet was then resuspended in Sample Buffer (see appendix) made up without bromophenol blue. The volume of Sample Buffer used was adjusted according to the number of nematodes, with 100µl per 50 000 nematodes taken as standard. Samples were then boiled for 10 minutes, allowed to cool, and the resulting supernate collected by passing the samples through a modified syringe (described earlier). After protein determination using the method of Winterborne (1986), a small amount of bromophenol blue was added to the samples to allow visualisation during electrophoresis.

### **2.3.3. HOMOGENATE PREPARATIONS**

Nematodes were washed extensively in 10mM imidazole buffer pH 6.5 before being resuspended in a small volume of the same buffer containing a cocktail of the protease inhibitors PMSF, TLCK and TPCK (see appendix). Samples were sonicated (sonicator type 7532A, Dawe Instruments Limited) at 50W for 4 periods of 15 seconds, with 30 second intervals between sonications to allow cooling. All sonications were carried out on ice. Samples were centrifuged as described following

sonication to pellet insoluble material, and the supernate removed and stored at  $-20^{\circ}\text{C}$  prior to use.

#### **2.3.4. COLLECTION OF EXCRETORY-SECRETORY PRODUCTS**

Approximately 70 000 nematodes were washed extensively, first in PBS then in sterile PBS containing penicillin ( $100\text{IUml}^{-1}$ ) and streptomycin ( $100\text{IUml}^{-1}$ )(Gibco). Using aseptic technique, nematodes in a final volume of 3ml PBS (Pen/Strep) were placed in one well of a sterile 6 well tissue culture plate, and incubated at room temperature for 24 hours. The nematodes were then transferred to sterile eppendorfs, and centrifuged as before. The supernatant was removed and stored at  $-20^{\circ}\text{C}$ , and the pelleted nematodes were resuspended in 3ml PBS (Pen/Strep) as before. This procedure was carried out 3 times over 3 days. To ensure that nematodes remained viable throughout the procedure, and that suspensions remained free of bacterial or fungal contamination, suspensions were regularly examined microscopically. All PBS (Pen/Strep) supernatants collected in this manner were stored at  $-20^{\circ}\text{C}$  prior to freeze drying under vacuum (Lyolab A, Life Science Laboratories) and the freeze-dried material stored at  $-20^{\circ}\text{C}$ .

#### **2.3.5. 5-METHOXY-N, N-DIMETHYL TRYPTAMINE INDUCED SECRETIONS**

All experiments performed using secretions induced by methoxy dimethyl tryptophamine (DMT) were carried out using secretions collected by Lee Robertson, Scottish Crop Research Institute.

Nematodes were washed extensively in sterile distilled water, then incubated in water containing penicillin ( $100\text{Uml}^{-1}$ ), streptomycin ( $100\mu\text{gml}^{-1}$ ) and amphotericin B ( $0.25\mu\text{gml}^{-1}$ ) overnight at  $4^{\circ}\text{C}$ . After overnight incubation nematodes were again washed extensively in

sterile distilled water, then resuspended in Clark and Lubs buffer pH 6.8 (see appendix) containing  $200\mu\text{gml}^{-1}$  DMT and ampicillin ( $75\mu\text{gml}^{-1}$ ). Nematodes were incubated overnight at room temperature then pelleted by centrifugation as before, and the supernate removed into Centriplus concentrators (Amicon) with molecular weight cut off at 10kDa. The supernate was centrifuged (3000g, 8.5 hours) to concentrate secretions, the protein concentration determined and stored at  $-20^{\circ}\text{C}$  prior to use.

## **2.4. ANTI-SERUM PRODUCTION**

### **2.4.1. LUFFNESS ANTI-SERUM**

A mixture of secretions were collected from *G. pallida* population Luffness using the method described in Section 2.3.1 of Materials and Methods and used to immunise a rabbit. Secretions were collected from approximately 50 000 nematodes, washed and resuspended in a final volume of  $30\mu\text{l}$  of sterile distilled water. This was made up to  $500\mu\text{l}$  with sterile PBS and Freund's Complete Adjuvant (FCA) added to give a final volume of 1ml. Subcutaneous injections were administered to a rabbit over multiple sites and the rabbit boosted in a similar manner 6 weeks later except that Freund's Incomplete Adjuvant (FIA) was used. The rabbit was bled 4 weeks after the final injection, the blood allowed to clot, and the antiserum isolated by centrifugation of the blood clot and stored in aliquots at  $-20^{\circ}\text{C}$ .

### **2.4.2. EXCRETORY-SECRETORY ANTI-SERUM**

ES secretions were collected from *G. pallida* population Luffness and freeze-dried as described in Section 2.3.4 of Materials and Methods. Freeze-dried secretions were reconstituted in a total of  $500\mu\text{l}$  sterile PBS and this was made up to 1ml with Freund's Complete Adjuvant (FCA).

Subcutaneous injections were administered to a rabbit over multiple sites and the rabbit boosted in a similar manner 6 weeks later except that Freund's Incomplete Adjuvant (FIA) was used. The rabbit was bled 4 weeks after the final injection and the anti-serum isolated as described earlier.

## **2.5. ORIGIN OF OTHER ANTI-SERA**

### **2.5.1. ANTI-SERUM D16**

All D16 anti-serum was a kind gift of Dr. Richard Napier at Horticulture Research International, Kent.

D16 is an anti-serum raised against the 16 amino acid portion of maize auxin binding protein (ABP1) that has been shown to constitute the auxin binding domain of the protein (Venis *et al.*, 1992). Data have shown that there is almost complete sequence conservation in this region between the different species of plant investigated, and that the region contains the longest stretch of wholly conserved sequence in ABP1. The protocol describing the production of D16 can be found in Venis *et al.*, (1992), but will be described briefly here.

The ABP1 peptide ARG-THR-PRO-ILE-HIS-ARG-HIS-SER-CYS-GLU-GLU-VAL-PHE-THR was synthesised by standard solid phase procedures and azo-coupled to keyhole limpet haemocyanin (KLH) through an added N terminal tyrosine. Anti-serum to the conjugate was raised in rabbits by injecting conjugate (1mg) with FCA, with 3 boosts of conjugate (0.5mg) administered sub-cutaneously at 4 weeks interval. The rabbit was bled 2 weeks after boosting.

### 2.5.1.1. AFFINITY PURIFIED D16

IgG fractions of D16 were prepared using ammonium sulphate precipitation and anion-exchange chromatography (Veris *et al.*, 1992). Affinity purification was achieved by coupling an ABP1 peptide-tuberculin derivative conjugate to React Gel 6X (Pierce & Warriner). This matrix was tumbled overnight at 4°C with the D16 IgG fraction in PBS. Centrifugation (3000g, 2 mins) was used to pellet the gel, which was washed several times in saline. Affinity purified IgG was obtained by desorption with 0.5M acetic acid for 5 mins followed by neutralisation with NH<sub>4</sub>OH.

### 2.5.2. ANTI-SERUM 5559

Anti-serum 5559 was a kind gift of Dr. Jock Forrest of the Scottish Crop Research Institute, Dundee.

Anti-serum 5559 was raised in rabbits in an attempt to obtain anti-serum with specificity against epitopes of amphidial secretions. The immunisation procedure was as follows. The initial immunisation was with J2 of *G. rostochiensis* population (pathotype) Ro1 in the presence of FIA. The rabbit was then boosted another 3 times as before, except nematodes had been adsorbed against anti-serum 4031 prior to immunisation. Anti-serum 4031 was raised in rabbits by immunising with nematodes which had undergone washes in sodium hypochlorite. The rationale behind the immunisation procedure was based on electron microscopy observations that alterations in the structure of amphidial secretions could be seen following hypochlorite treatment (Forrest *et al.*, 1988b), therefore hypochlorite treatment might expose novel epitopes within amphidial secretions.

## **2.6. PROTEIN DETERMINATION**

Two methods of protein determination were used according to the manner of homogenate production. For samples prepared in the presence of Sample Buffer all determinations were carried out according to the method of Winterborne (1986). This method is a dye-binding assay that measures the amount of dye that binds to protein dried onto filter paper.

Briefly, the exact concentration of a  $1\text{mgml}^{-1}$  solution of bovine serum albumin (BSA) was calculated using its absorbance at 280nm. 1, 2, 5 and  $8\mu\text{l}$  of this solution, and up to  $8\mu\text{l}$  of the test solution was then blotted onto 1cm squares of Whatman 3MM paper and allowed to dry. Once dry the paper was stained using 0.45% Commassie Blue R dissolved in 40% ethanol containing 12% glacial acetic acid for 1 hour. Destaining was then performed in 10% (v/v) ethanol, 5% (v/v) acetic acid until a clear background was obtained. The paper was then dried, and the squares cut out and placed in 1ml of desorbing solution, 1M potassium acetate dissolved in 70% (v/v) ethanol, for one hour. The absorbance of the liquid was then read at 590nm. The absorbance value was plotted against the protein content of the standard, and this was used to determine the amount of protein in the test solution.

All other protein determinations were performed according to the method of Bradford (1976), using BSA as a standard.

## **2.7. PROTEIN SEPARATION**

### **2.7.1. SAMPLE PREPARATION**

Following protein determination where appropriate, samples were dissolved in Sample Buffer. Samples were placed in a boiling water bath

for a few minutes, and allowed to cool before being loaded for electrophoresis.

### **2.7.2. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL**

#### **ELECTROPHORESIS**

Electrophoresis was performed on precast 10-20% gradient gels (Bio-Rad, UK) using the Mini-Protean II system (Bio-Rad, UK) according to the method of Laemmli (1970). The pre-cast gels obtained from Bio-Rad are ready for electrophoresis. Prior to electrophoresis, gels are prepared by removing the gel comb so that wells are present, and removing the cellophane strip at the base of the gel to ensure buffer contact. The gels are then clamped in the Mini-Protean II apparatus in preparation for loading of samples. Between 10-20 $\mu$ g of protein per sample was loaded. Electrophoresis was performed at 150V (producing a current of 75mA) for 2 gels. Gels were run until the dye front reached the base of the gel plates. It should be noted that some gels presented in this thesis were run using a Pharmacia Phast Gel system, which is indicated where appropriate.

Once separated, proteins were either silver-stained, or were transferred onto nitrocellulose paper by electroblotting according to the method of Towbin (1979).

### **2.7.3. SILVER STAINING**

Gels were fixed in 40% (v/v) methanol, 10% (v/v) acetic acid for 1 hour then placed in 8.3% (v/v) glutaraldehyde for 30 minutes. Gels were then rinsed in distilled water (4 washes of 30 minutes) and placed in Staining solution (see appendix) for 1 hour. After staining, gels were rinsed in distilled water (3 washes of 15 minutes), then placed in Developer (see

appendix) until bands could be seen. Upon the appearance of bands the reaction was stopped by adding 5ml of 2.3M citric acid per 100ml of developer.

#### 2.7.4. MOLECULAR WEIGHT DETERMINATION

Molecular weights of the separated proteins were calculated using Sigma molecular weight markers (low and high range) for silver-stained gels, and prestained markers (Bio-Rad, UK) for molecular weight determination on nitrocellulose paper. The molecular weight markers used are listed below.

##### BIO-RAD PRESTAINED MARKERS

PROTEIN	MW (KDA)
myosin	204
$\beta$ -galactosidase	121
bovine serum albumin	82
ovalbumin	50.2
carbonic anhydrase	34.2
soybean trypsin inhibitor	27.5
lysozyme	19.4
aprotinin	7.3

##### SIGMA MOLECULAR WEIGHT STANDARDS - LOW

PROTEIN	MW (KDA)
albumin	66
ovalbumin	45
glyceraldehyde-3-phosphate dehydrogenase	36
carbonic anhydrase	29

trypsinogen	24
trypsin inhibitor	20
$\alpha$ -lactalbumin	6.5

#### SIGMA MOLECULAR WEIGHT MARKERS - HIGH

PROTEIN	MW (KDA)
myosin	205
$\beta$ -galactosidase	116
phosphorylase b	97
fructose-6-phosphate kinase	84
albumin	66
glutamic dehydrogenase	55
ovalbumin	45

Relative molecular weights were calculated by plotting a standard graph of log molecular weight of the protein standards against the distance they had travelled from the top of the gel. Molecular weights of unknown proteins were calculated by measuring the distance travelled on the gel and using this to determine the molecular weight from the graph.

## 2.8. BLOTTING STUDIES

### 2.8.1. IMMUNOBLOTTING

For immunoblotting 25 $\mu$ g of BSA-equivalent protein per lane was electrophoresed as described in Materials and Methods and transferred onto nitrocellulose paper (Bio-Rad). Transfer took place at 250mA for 75 minutes using an ice block as coolant. To prevent non-specific binding of antibody, the nitrocellulose paper was blocked with PBS containing 5% (w/v) Marvel and 0.1% (v/v) Tween 20. Thereafter all dilutions and

washings were performed in PBS containing 0.1% Tween 20. Proteins were detected by incubation of the nitrocellulose paper with the primary antibody (dilution varied according to which anti-serum was used) for 2 hours at room temperature. This was followed by horseradish peroxidase conjugated anti-rabbit IgG (1:400) as secondary antibody for 2 hours at room temperature. Detection of bound antibody was carried out using 3, 3-diaminobenzidine (30mg) in 100ml PBS containing 20 $\mu$ l 6% hydrogen peroxide). This solution was kept on the nitrocellulose paper until distinct bands could be seen. The reaction was then stopped by washing in distilled water.

### 2.8.2. LECTIN BLOTTING

The following lectins (specificities shown) were used in the procedure described below.

LECTIN	SPECIFICITY
Concanavalin A (Con A)	$\alpha$ Man > $\alpha$ Glc > $\alpha$ GlcNAc
<i>Galanthus nivalis</i> agglutinin (GNA)	Man $\alpha$ (1-3) Man ( $\alpha$ 1-3 > $\alpha$ 1-6 > $\alpha$ 1-2)
<i>Datura stramonium</i> agglutinin (DSA)	Gal $\beta$ (1-4) GlcNAc GlcNAc-Ser/Thr
<i>Maackia amurensis</i> agglutinin (MAA)	Neu NAc $\alpha$ (2-3) Gal
Wheat germ agglutinin (WGA)	(GlcNAc $\beta$ 1-4) <sub>3</sub> > (GlcNAc $\beta$ 1-4) <sub>2</sub>
<i>Sambuca nigra</i> agglutinin (SNA)	Neu NAC $\alpha$ (2-6) Gal/GalNAC

For immunoblotting 25 $\mu$ g of BSA-equivalent protein per lane was electrophoresed and transferred onto nitrocellulose paper as before. Lectin blotting was performed using the Glycan Differentiation Kit obtained from Boehringer Mannheim. To prevent non-specific binding

the nitrocellulose paper was blocked with the blocking reagent provided in the kit. Blocking was performed for 1 hour at room temperature. Lectin binding was performed by incubating the nitrocellulose with the following lectins, WGA and Con A ( $100\mu\text{gml}^{-1}$ ), MAA ( $50\mu\text{gml}^{-1}$ ) or SNA, DSA and GNA ( $10\mu\text{gml}^{-1}$ ) for 1 hour at room temperature. All lectins used in this procedure were digoxigeninylated, therefore detection of bound lectin was performed using digoxigenin anti-serum conjugated to alkaline phosphatase (1:1000) for 1 hour at room temperature. All washings and dilutions were carried out using Tris Buffered Saline (TBS, see appendix) except for the dilution of the lectins where TBS containing  $1\text{mM MgCl}_2$ ,  $1\text{mM MnCl}_2$  and  $1\text{mM CaCl}_2$  was used. Detection of bound antibody was performed using BCIP/NBT FAST tablets. As a control, all lectin blots were repeated in the presence of 100-200mM of the appropriate competing sugar.

## **2.9. FLUORESCENCE STUDIES**

All fluorescence microscopy was performed on a Leitz Laborlux S microscope (Leica, UK) equipped for quantitative fluorescence and photography.

### **2.9.1. INDIRECT IMMUNOFLUORESCENT ANTIBODY TEST**

Indirect immunofluorescence (IFAT) was carried out on living nematodes or on nematodes fixed in 1% (v/v) formaldehyde for 15 minutes. Nematodes were incubated for one hour with primary antibody (1:50) and for 30 minutes with the secondary antibody. Tetrarhodamine isothiocyanate (TRITC) conjugated goat anti-rabbit IgG was used as the secondary antibody. Nematodes were washed extensively after each antibody incubation, with PBS being used for all washings and dilutions. Preliminary experiments showed that fixation

in 1% formaldehyde for 15 minutes either prior to IFAT, or following IFAT had no effect on fluorescence. Prior to viewing nematodes were thus fixed as described to aid photography.

### **2.9.2. PERIODATE TREATMENT**

Periodate treatment was carried out on fixed nematodes prior to IFAT. Nematodes were fixed in 1% formaldehyde for 15 minutes then washed in 50mM sodium acetate buffer pH 5.8 and incubated with 10mM sodium m-periodate in the same buffer for one hour. Treatment with 50mM sodium acetate pH 5.8 alone was used as the control.

### **2.9.3. DIRECT FLUORESCENCE USING FLUOROCHROME-CONJUGATED LECTINS**

Living nematodes were exposed to fluorescein isothiocyanate (FITC) or TRITC-conjugated lectins. Freshly hatched nematodes were harvested by centrifugation and washed extensively prior to incubation with TRITC-Con A, TRITC-GNA and FITC-WGA at a concentration of  $100\mu\text{gml}^{-1}$  for one hour at room temperature. All washings and dilutions carried out in PBS according to the method of Forrest & Robertson (1986). After exposure to the lectins, nematodes were fixed in 1% (v/v) formaldehyde for 15 minutes prior to viewing.

### **2.10. INDUCTION OF SECRETIONS**

To aid visualisation of any secretions that were induced using the following methods all incubations were performed in 24 well culture plates thus easing microscopical observation. Similarly, all incubations were performed in the presence of 0.025% Coomassie Blue R250 as this allowed direct visualisation of secretions. Secretions were immediately

obvious once produced, appearing as blue-stained particulate material that was often still associated with the nematode.

#### **2.10.1. 5-METHOXY-N, N-DIMETHYL TRYPTAMINE**

DMT was a kind gift from Dr. Richard Hussey, University of Georgia. DMT was prepared as a  $10\text{mgml}^{-1}$  stock dissolved in PBS. To determine the optimum concentration for the induction of secretions nematodes were incubated in the following range of concentrations: 0, 10, 25, 50, 100, 200 and  $400\mu\text{gml}^{-1}$ . All incubations were carried out in the presence of 0.025% Coomassie Blue R250. Optimum concentration for the production of secretions was  $200\mu\text{gml}^{-1}$  therefore all further experiments with DMT were carried out at this concentration.

#### **2.10.2. PHYTOHORMONES**

Stock solutions of the phytohormones indole-3-acetic acid (IAA), gibberellic acid, abscisic acid (ABA) and kinetin were prepared as follows:

IAA: prepared as a  $10\text{mgml}^{-1}$  stock dissolved in 100% ethanol. Stored in aliquots of  $1\text{mgml}^{-1}$ , using PBS as diluent.

Giberellic acid: prepared as a  $10\text{mgml}^{-1}$  stock dissolved in PBS.

Abscisic acid: prepared as a  $10\text{mgml}^{-1}$  stock dissolved in 1M NaOH.

Stored in aliquots of  $1\text{mgml}^{-1}$ , using PBS as a diluent.

Kinetin: prepared as a  $10\text{mgml}^{-1}$  stock dissolved in 1M NaOH. Stored in aliquots of  $1\text{mgml}^{-1}$ , using PBS as a diluent.

Initial incubations were carried out using concentrations of phytohormones recommended for use in plant tissue culture (Sigma). This was in the range of  $0.1\text{-}10\mu\text{gml}^{-1}$ . It was decided, however to increase the concentration range to  $50\mu\text{gml}^{-1}$ . As before, all incubations

were carried out in the presence of 0.025% Coomassie blue R250 as described earlier.

## **2.11. LIPID PROBE BINDING**

### **2.11.1. 5-(N-OCTADECYL) AMINOFLUORESCEIN**

AF18 (Molecular Probes) was prepared as a 10mgml<sup>-1</sup> stock dissolved in 100% ethanol. Nematodes were washed extensively in PBS and then AF18 added to the nematodes to give a final concentration of 10µgml<sup>-1</sup>. Nematodes were incubated for 10 minutes at room temperature. After incubation with AF18 nematodes were again washed extensively, and 20mgml<sup>-1</sup> carbachol added to stop the parasites moving before viewing under the microscope.

### **2.11.2. PKH 26**

The lipid probe PKH 26 was obtained as the PKH 26 Red Fluorescent Cell Linker Kit. All PKH-26 labelling experiments in this project were carried out using distilled water for all washings and dilutions. PKH-26 is provided as a stock solution of 1mM in 100% ethanol. Labelling of the parasite surface was performed by incubating washed nematodes in PKH-26 diluted 1:1000 for 10 minutes at room temperature. Following incubation nematodes were washed again, and 20mgml<sup>-1</sup> carbachol added to stop the parasites moving before viewing under the microscope.

### **2.11.3. TRYPAN BLUE QUENCHING OF SURFACE FLUORESCENCE**

Trypan Blue is a non-permeant molecule which will quench the fluorescence from molecules found within 4nm of the dye by resonance energy transfer (Foley *et al.*, 1986). Fluorescence which is unquenched by addition of Trypan Blue therefore is either due to insertion of the

fluorescent probe into layers further than 4nm from the surface of the parasite, or due to internalisation of the fluorescent probe. Quenching experiments were performed with Trypan Blue to examine the depth of insertion of AF18 and PKH 26. Nematodes were labelled with either AF18 or PKH 26 as described previously, then 0.25% (w/v) Trypan Blue added prior to viewing and quantitation of fluorescence.

#### **2.11.4. QUANTITATION OF FLUORESCENCE AND STATISTICAL ANALYSES**

All measurements were performed on a Leitz Laborlux S microscope (Leica, UK) with 3-1 PLEOMOPAK fluorescence illuminator, under a x40 objective lens. Fluorescence was quantified using a Leitz MPV compact photometer with MPV-COMBI control electronics attached to a PC (Silicon Valley) installed with MPV-STAT software for data and statistical analysis (Leica).

For each different treatment, between 30 and 40 readings were taken from different nematodes, with each experiment being repeated a minimum of three times. Comparisons of the different treatments were done using the Student's t-test, taking  $P < 0.05$  as being significant.

#### **2.12. CAGED COMPOUNDS**

It is recognised that access of bioactive mediators to the interior of the nematode is difficult due to the impermeability of the nematode cuticle. To circumvent this problem, second messengers and chelating agents were introduced to the nematode in the form of caged compounds. Caged compounds are bioactive molecules which have been rendered hydrophobic and inactive by linkage to a photo-sensitive group (McCray & Trentham, 1989). Exposure to ultra-violet light releases the cage,

giving a fully bioactive molecule. Figure 2.1 shows the structure of the caged compounds used in the following experiments.

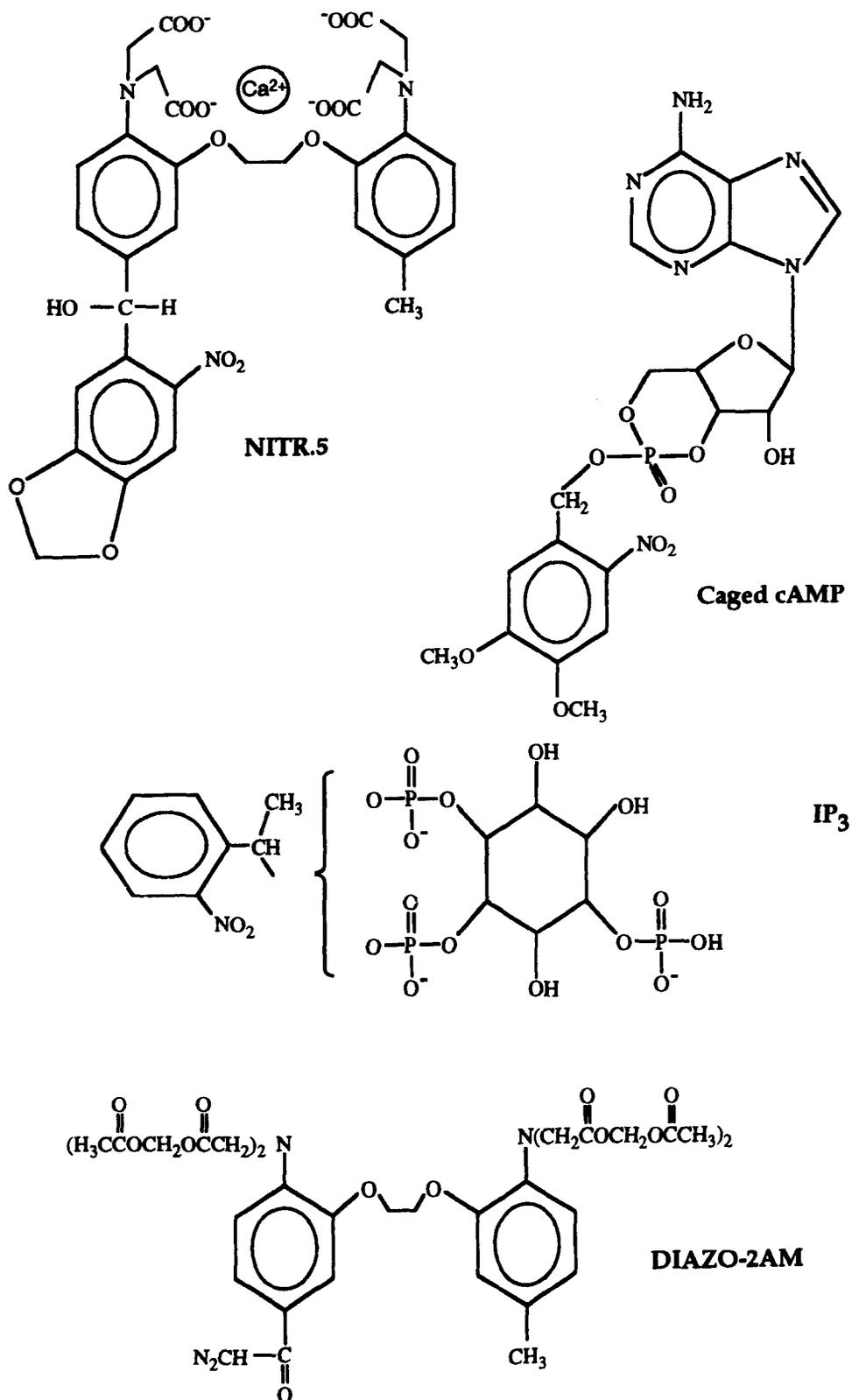
Stock solutions of the following caged compounds were prepared as follows:

caged cAMP	10mM stock. Used at 10 $\mu$ M
tetracarboxylate 2-nitrobenzhydrol (NITR-5)	5M stock. Used at 0.5mM
1,2-bis-(2-amino-5-di-azoacetylphenoxy) ethane-N, N, N', N' -tetraacetic acid tetrakis methyl ester (Diazo-2 AM)	1mM stock. Used at 20 $\mu$ M
inositol trisphosphate (IP <sub>3</sub> )	5mM stock. Used at 10 $\mu$ M

All stocks were dissolved in dimethylsulphoxide, and diluted in PBS for use.

Washed nematodes were incubated with the various caged compounds in a microtitre plate for 1 hour at room temperature. After incubation, nematodes were removed from the plates, washed 3 times by centrifugation as described earlier, then returned in a small volume to the microtitre plate. Activation of the caged second messengers was achieved by exposing the nematodes to an ultra-violet light source for 30 seconds (Leitz UV light source; power - 150mW cm<sup>-2</sup> at 310nm and 13mW cm<sup>-2</sup> at 365nm, measured using a Macam UV 103 digital radiometer). Nematodes were then transferred to eppendorf tubes,

Figure 2.1. The structure of caged compounds



washed again, then labelled with AF18 as described previously and surface fluorescence quantitated.

### **2.13. ELECTRON MICROSCOPY STUDIES**

Slightly different fixation procedures were used depending on whether nematodes were to be used for structural observations and immunogold surface labelling of specimens or for immunogold labelling of cut sections. The protocols used are described below.

#### **2.13.1. PRE-EMBEDDING SURFACE LABELLING OF WHOLE SPECIMENS**

For this procedure living nematodes were first surface labelled with immunogold, then fixed in preparation for sectioning as described in section 2.13.4.

##### **2.13.1.1. IMMUNOGOLD LABELLING OF THE SURFACE**

Living nematodes were surface labelled using the same procedure as described for indirect immunofluorescence studies with all washings and dilutions performed in PBS. Nematodes were incubated with primary antibody (1:50) for 1 hour at room temperature. Immunogold labelling of bound antibody was performed by incubating with goat anti-rabbit IgG conjugated to colloidal gold (1:50, 10nm gold) for 1 hour at room temperature. Nematodes were washed extensively between each step.

##### **2.13.1.2. FIXATION**

In the following protocol all washings and dilutions were carried out using 0.1M phosphate buffer pH 6.8 (see appendix). Following immunogold labelling of the surface as described above samples were fixed for 1 hour in 3% (v/v) glutaraldehyde, rinsed three times, and

placed in 1% (w/v) osmium tetroxide for 30 minutes. Samples were washed again before embedding in 1% (w/v) Water Agar (Oxoid). Blocks containing individual nematodes were cut out and processed through a graded ethanol series, with two final changes in 100% propylene oxide. Finally samples were placed in Emix resin (Agar Scientific) at 35°C for a minimum of 2 hours, before polymerisation at 70°C overnight.

### **2.13.2. POST-EMBEDDING IMMUNOGOLD LABELLING OF CUT SECTIONS**

In this procedure nematodes were fixed first to allow sectioning as described in 2.13.4, and then the sections themselves underwent immunogold labelling.

#### **2.13.2.1. FIXATION**

In the following protocol all washings and dilutions were carried out using 0.1M phosphate buffer pH 7.2 (see appendix). Samples were fixed for 1 hour in 2% (w/v) paraformaldehyde, rinsed three times and embedded in 1% (w/v) Water Agar (Oxoid). Blocks containing individual specimens were cut and dehydrated in a graded ethanol series with two final washes in 100% propylene oxide. Blocks were then placed in LR White resin (Fisons) overnight at room temperature, before polymerisation at 55°C overnight.

#### **2.13.2.2. IMMUNOGOLD LABELLING**

Sections fixed as described in 2.9.2.1 were placed on copper grids and incubated in PBS containing 1mgml<sup>-1</sup> BSA, and 5% goat serum before incubation with primary antibody in PBS containing 0.1% BSA, 0.05% Tween 20, 1% goat serum and 2.08% NaCl for 2 hours at room temperature. Grids were then washed in TBS containing 0.2% (w/v)

BSA then again with TBS containing 1% BSA, before incubation with goat anti-rabbit IgG conjugated to colloidal gold (1:50, 10nm gold) in TBS containing 1% BSA for 2 hours at room temperature. Grids were washed as before, stained and viewed as described below.

### **2.13.3. SECTIONS FOR ULTRASTRUCTURAL OBSERVATIONS**

Specimens which were to be used for ultrastructural examinations only were fixed as described in 2.13.1.2. Following fixation, sections were cut and stained as described in sections 2.13.4 and 2.13.5 below.

### **2.13.4. SECTIONING**

Silver grey sections were cut on a Reichert Ultracut and collected on plastic coated copper grids.

### **2.13.5. COUNTER STAINING**

All sections were counter stained with a saturated solution of uranyl acetate in 50% (v/v) ethanol for 2 minutes followed by staining with saturated lead citrate for 7 minutes.

### **2.13.6. VIEWING AND PHOTOGRAPHY**

Sections were viewed on a JEOL 1200EX electron microscope at 80kV. Photographs of sections were taken using Agfa Scientia EM Film.

## **2.14. ION EXCHANGE CHROMATOGRAPHY PURIFICATION OF PUTATIVE NEMATODE AUXIN BINDING PROTEIN**

A Mono Q anion anion exchange column was attached to FPLC apparatus (Pharmacia) and equilibrated with 10mM imidazole pH 6.5. Approximately 20mg of nematode homogenate, prepared as described in Section 2.3.3, was loaded onto the column and eluted over a 30ml

gradient from 10-400mM imidazole pH 6.5. 1ml fractions were collected. After collection, fractions were precipitated using 10% ice cold trichloroacetic acid (TCA). Remaining TCA was removed by two washes in ice cold acetone and the remaining pellet resuspended in sample buffer prior to SDS-PAGE. Electrophoresis of samples was performed on 10-20% gradient gels as described previously and fractions assayed by immunoblotting with anti-serum D16.

## 2.15. APPENDIX

### 2.15.1. GENERAL BUFFERS

#### PHOSPHATE BUFFERED SALINE (PBS)

sodium chloride	8.5g/L
sodium dihydrogen orthophosphate	0.39g/L
disodium hydrogen orthophosphate	1.07g/L

#### TRIS BUFFERED SALINE

Tris-HCl	6.05g/L
sodium chloride	8.76g/L

pH adjusted to pH7.5 using concentrated HCl.

#### CLARK AND LUBS BUFFER

50ml of 0.1M  $\text{KH}_2\text{PO}_4$  (0.68g).

Add 3.5ml of 0.1M NaOH, then make up to 100ml with distilled water.

#### PROTEASE INHIBITORS

10 mM TPCK

100mM PMSF

20mM TLCK

Dissolved in 100% ethanol. Diluted 1:100 for use.

### 2.15.2. ELECTROPHORESIS AND BLOTTING SOLUTIONS

#### RUNNING BUFFER (10X concentrated)

glycine 144g

Tris base 30g

SDS 2.5g

Made up to 1L with distilled water.

**TRANSFER BUFFER**

Tris base 3.02g

glycine 14.4g

Made up to 1L in 20% (v/v) methanol.

**SAMPLE BUFFER**

0.5M Tris pH 6.8 0.6ml

sodium dodecyl sulphate 0.1g

glycerol 0.25ml

2-mercaptoethanol 50 $\mu$ l

Made up to 1ml with distilled water then a few crystals of bromophenol blue added for colour.

**2.15.3. SILVER STAINING****STAINING SOLUTION**

20% (w/v) silver nitrate 1ml

4% (w/v) sodium hydroxide 5ml

NH<sub>3</sub> 700 $\mu$ l

Make up to 100ml with distilled water

**DEVELOPER**

3% (w/v) sodium carbonate

50ml of 37% formaldehyde per 100ml of developer

**2.15.4. ELECTRON MICROSCOPY BUFFERS****PHOSPHATE BUFFER (0.1M pH 6.8)**Na<sub>2</sub>HPO<sub>4</sub> 0.69gNaH<sub>2</sub>PO<sub>4</sub> 0.61g

Made up to 100ml with distilled water

## PHOSPHATE BUFFER (0.1M pH 7.2)

$\text{Na}_2\text{HPO}_4$  1.02g

$\text{NaH}_2\text{PO}_4$  0.33g

Made up to 100ml with distilled water

**CHAPTER 3**  
**ISOLATION OF SECRETIONS**  
**FROM *GLOBODERA PALLIDA***

### 3.1. INTRODUCTION

It seems clear that secretions produced by PCN will have important functions throughout the life-cycle of the nematode. It appears that many of these secretions are glycosylated, and it has been suggested that differences in glycosylation may determine host responses that occur upon infection. The first series of experiments described in this chapter therefore, were performed to investigate patterns of lectin binding to homogenates of different populations of PCN. Different methods were then used to collect secretions from *Globodera pallida*, with the subsequent production of two anti-sera. The remainder of the work described in this chapter details blotting and indirect immunofluorescence studies performed with the anti-sera.

### 3.2. RESULTS

#### 3.2.1. LECTIN BINDING STUDIES

##### 3.2.1.1. LECTIN BINDING TO WHOLE PARASITES

Washed nematodes were incubated with  $100\mu\text{gml}^{-1}$  of Con A, WGA or GNA as described in the Materials and Methods 2.9.3. The specificity of lectin binding was investigated by pre-incubating each lectin with its appropriate competing sugar for 30 minutes prior to incubation with the parasites. The results show that Con A, GNA and WGA bind specifically to the amphids of *G. pallida*. Figure 3.1 shows the binding of WGA and Con A to the amphids of *G. pallida*. In all cases, binding could be abolished by incubation with the appropriate competing sugar, indicating that a specific carbohydrate-lectin interaction was occurring.

##### 3.2.1.2. LECTIN BINDING TO WHOLE BODY EXTRACTS

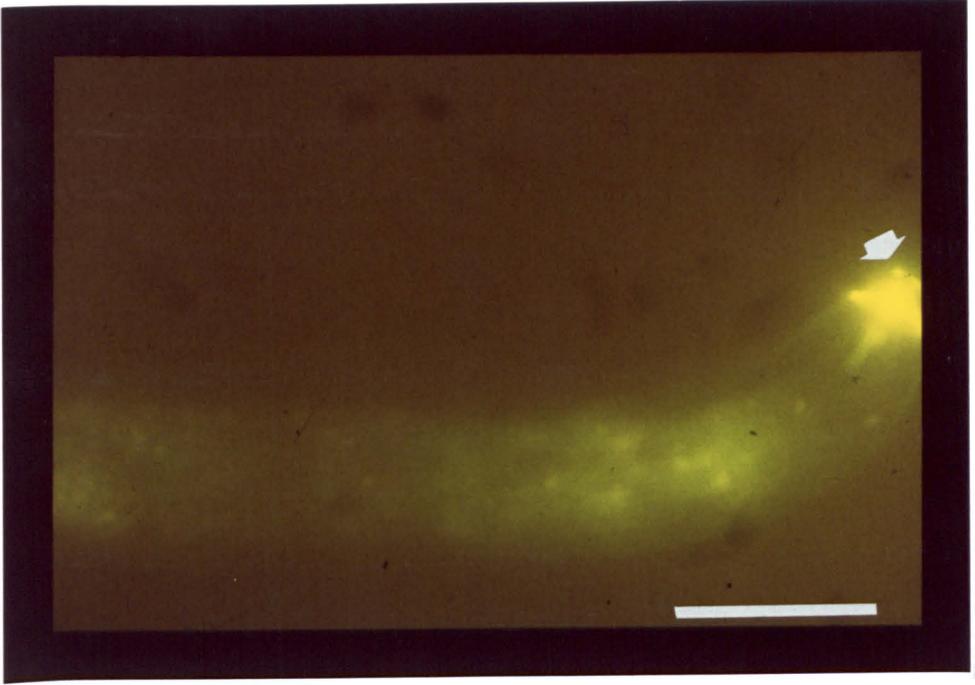
Using lectin binding studies performed on whole nematodes it can be seen that Con A, GNA and WGA bind to the amphids, although differentiation

**FIGURE 3.1.** Lectin binding to the surface of *G. pallida*.

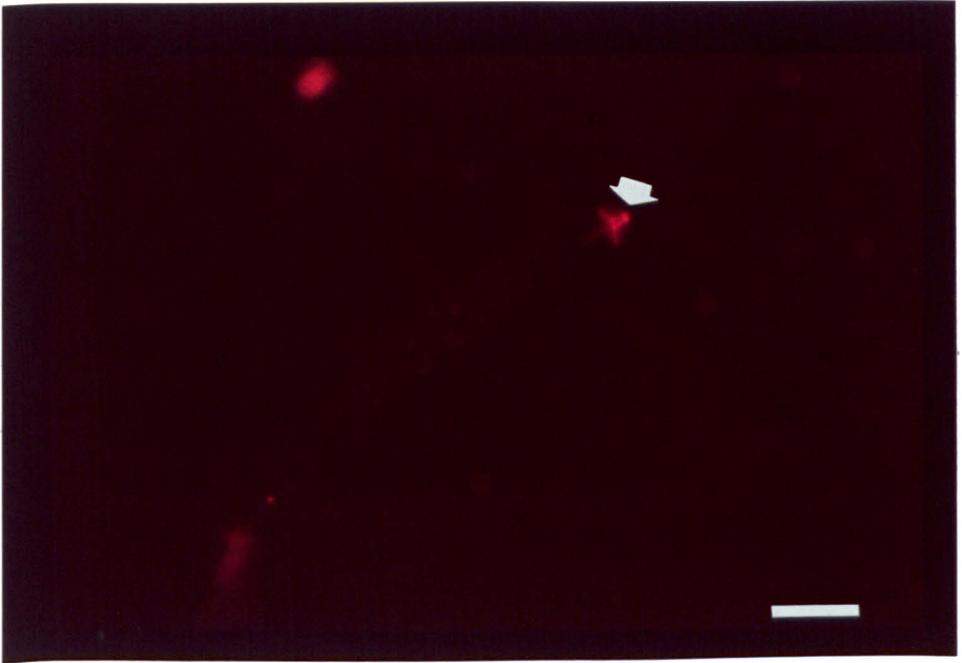
Nematodes were washed extensively prior to incubation with FITC- or TRITC conjugated lectin ( $100\mu\text{gml}^{-1}$ ) for 1 hour as described in Materials and Methods 2.9.3.

- a) Fluorescent micrograph showing binding of FITC-WGA to the amphids of *G. pallida* (arrowed). Bar represents  $30\mu\text{m}$ .
  
- b) Fluorescent micrograph showing binding of TRITC-Con A to the amphids of *G. pallida* (arrowed). Bar represents  $30\mu\text{m}$ .

a



b



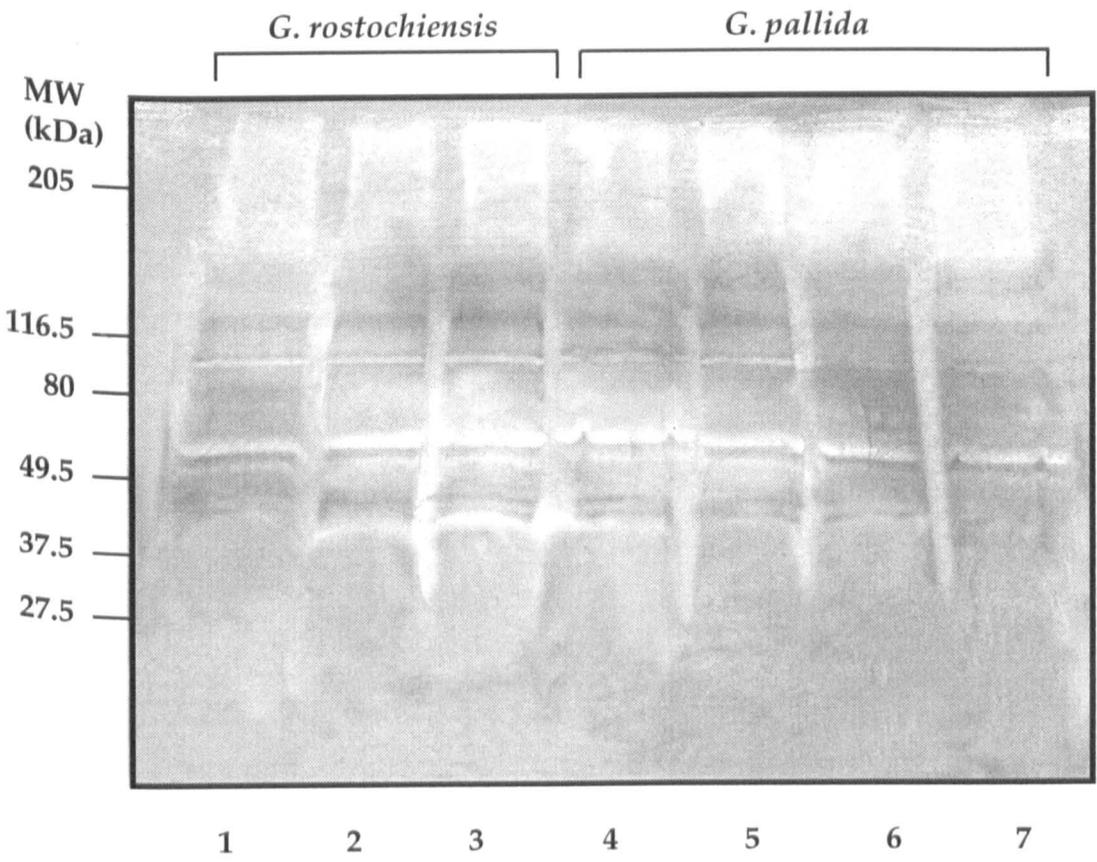
of the populations or pathotypes used was not possible. In an attempt to differentiate species or populations, it was decided to probe blots of nematode homogenates.

Whole body extracts of different populations of *G. pallida* and *G. rostochiensis* were electrophoresed on a 10-20% gradient gel, and the proteins then transferred on to nitrocellulose paper as described in Materials and Methods 2.7.2. The nitrocellulose paper was then probed with the following lectins: Con A, GNA, SNA, MAA, DSA and WGA. Figures 3.2 - 3.5 show the pattern of binding obtained with these lectins. Of these lectins, DSA and MAA (Gal $\beta$ (1-4) GlcNAc and Neu NAc  $\alpha$  (2-3) Gal specific respectively) did not bind to any nematode proteins. With all of the other lectins, however, binding was apparent. Con A and GNA are mannose-specific lectins recognising  $\alpha$  Man and  $\alpha$  Glc or Man  $\alpha$ (1-3) Man respectively. From the blots, (Figures 3.2 and 3.3) it can be seen that both lectins recognise many bands, although the lectins do not appear to be able to differentiate between the populations used. Interestingly, although both lectins are mannose-specific, the banding pattern seen on the blots are not identical, indicating that Con A and GNA recognise different species of mannose containing glycoproteins. In contrast SNA, which is Neu NAc  $\alpha$  (2-6) Gal/GalNAC specific, binds to a single band of molecular weight 128kDa (Figure 3.4). Again, this band does not vary between species or populations. Of the lectins tested, WGA (specificity (GlcNAc  $\beta$  1-4)<sub>3</sub> > (GlcNAc  $\beta$  1-4)<sub>2</sub>) alone was able to distinguish differences between *G. pallida* and *G. rostochiensis*. Figure 3.5 shows a blot probed with WGA. From the blot it can be seen that differences are apparent between *G. pallida* and *G. rostochiensis*, and also between the different population of *G. pallida*. No differences are apparent between the *G. rostochiensis* populations tested, all having 2 bands present of molecular weight 144kDa and 110kDa

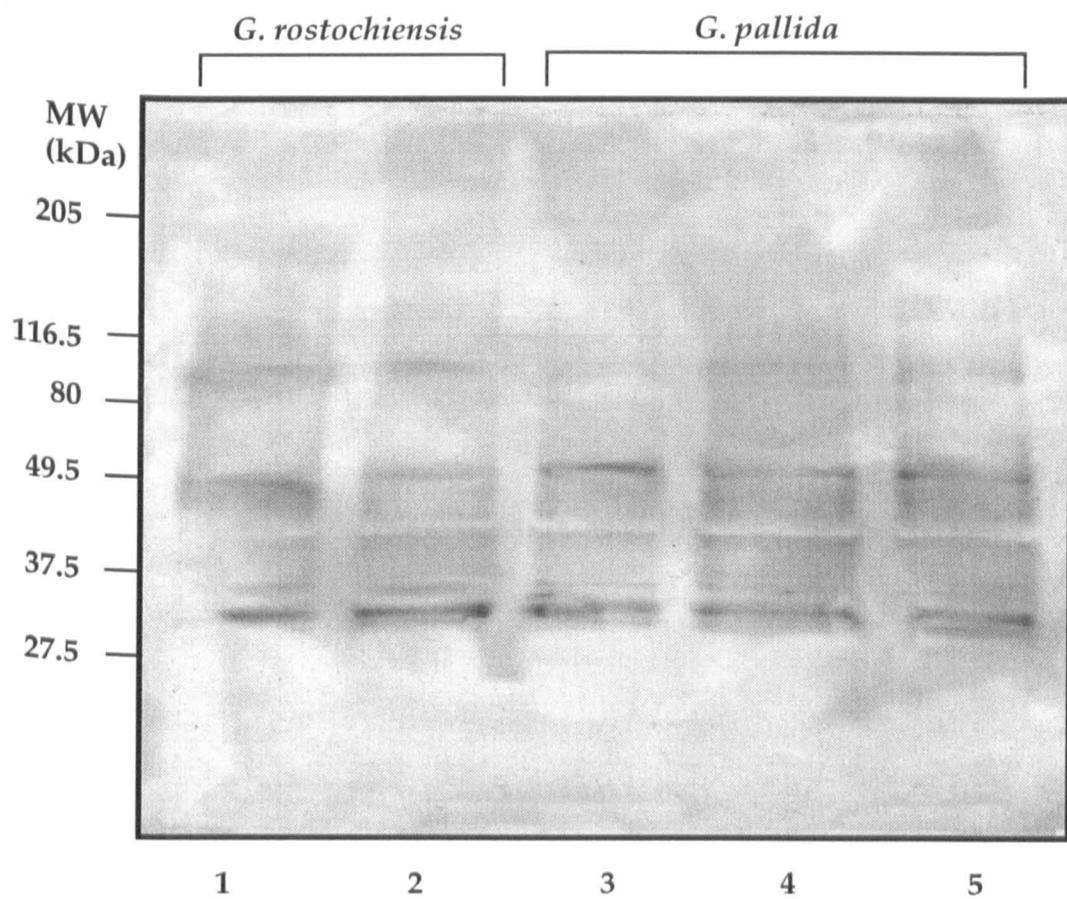
**FIGURE 3.2.** Lectin blot of whole body extracts of *G. pallida* and *G. rostochiensis* probed with Con A.

Nematode whole body extracts (25µg BSA-equivalent protein) were electrophoresed on a 10-20% gradient gel and electroblotted onto nitrocellulose paper as described in Materials and Methods 2.7.2. Blots were probed with Con A (100µgml<sup>-1</sup>) as described in Materials and Methods 2.8.2.

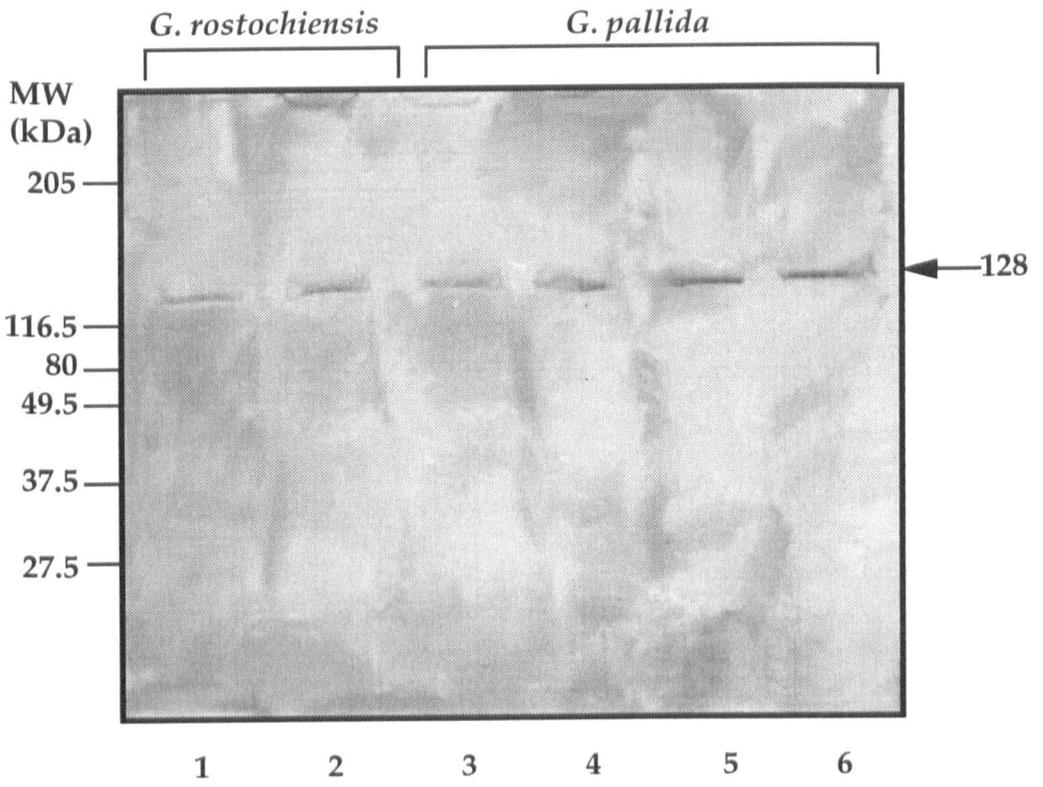
Lanes:            1 - *G. rostochiensis* Ro1  
                      2 - *G. rostochiensis* Ro3  
                      3 - *G. rostochiensis* Ro4  
                      4 - *G. pallida* Luffness population  
                      5 - *G. pallida* Derby population  
                      6 - *G. pallida* Newton population  
                      7 - *G. pallida* Gourdie population







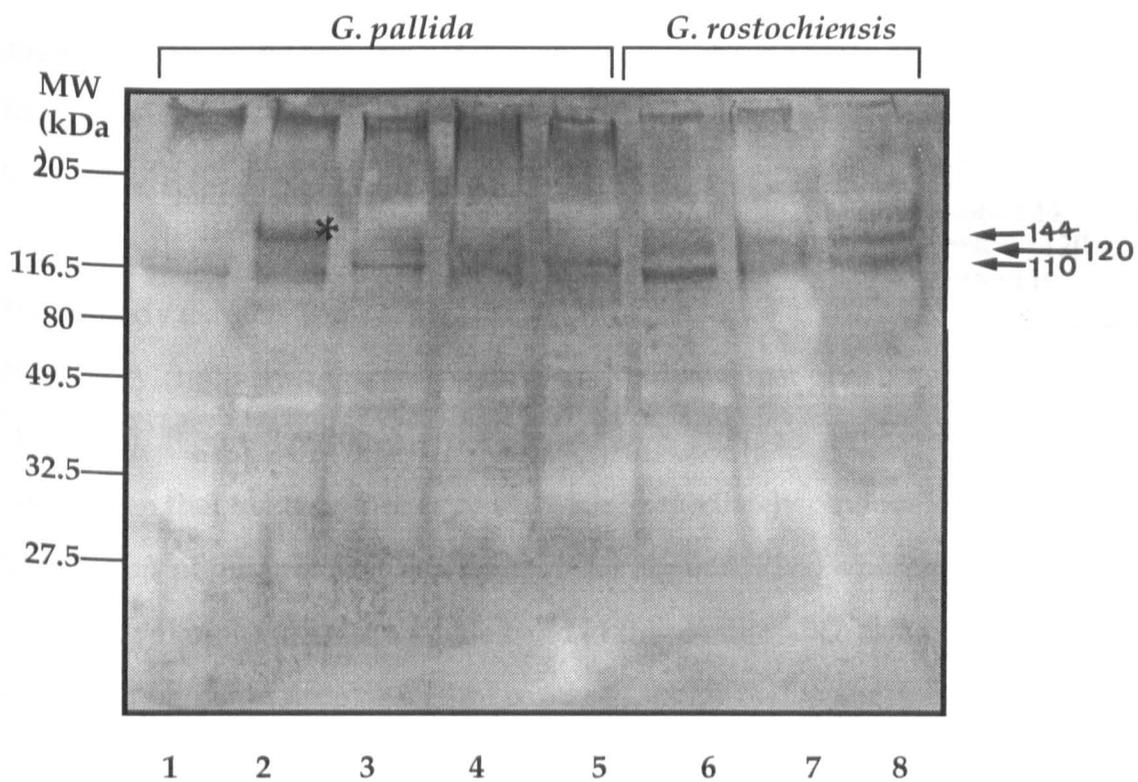




**FIGURE 3.5.** Lectin blot of whole body extracts of *G. pallida* and *G. rostochiensis* probed with WGA.

Nematode whole body extracts (25µg BSA-equivalent protein) were electrophoresed on a 10-20% gradient gel and electroblotted onto nitrocellulose paper as described in Materials and Methods 2.7.2. Blots were probed with WGA (100µgml<sup>-1</sup>) as described in Materials and Methods 2.8.2.

Lanes:            1 - *G. pallida* E  
                      2 - *G. pallida* Luffness  
                      3 - *G. pallida* Loc  
                      4 - *G. pallida* Newton  
                      5 - *G. pallida* Gourdie  
                      6 - *G. rostochiensis* Ro1  
                      7 - *G. rostochiensis* Ro3  
                      8 - *G. rostochiensis* Ro4



respectively. In contrast *G. pallida* Loc, Newton and Gourdie populations show 2 bands at 144kDa and 120kDa. *G. pallida* Luffness and E populations differ again, Luffness exhibiting a major band at 150kDa and a minor band of 110kDa, whereas E exhibits a single band at 120kDa.

To ensure that the presence of bands in any of the blots was not due to non-specific binding, all blots were also performed in the presence of 200mM of the appropriate competing sugar. In all cases, inclusion of competing sugar led to the disappearance or a reduction in intensity of all labelled bands.

Whole body extracts were also probed for the presence of phosphorylcholine using a monoclonal antibody against phosphorylcholine (TEPC 15). Figure 3.6 shows a blot probed with TEPC 15. From the blot it can be seen that the two species of PCN are immediately obvious, *G. rostochiensis* populations having a doublet at about 45kDa, whereas *G. pallida* populations possess a single band at this position. *G. pallida* also displays a higher molecular weight band of 130kDa that is not present in *G. rostochiensis* populations. As a control, mouse ascites fluid alone was used. No bands could be seen, indicating that TEPC 15 recognises proteins containing phosphorylcholine.

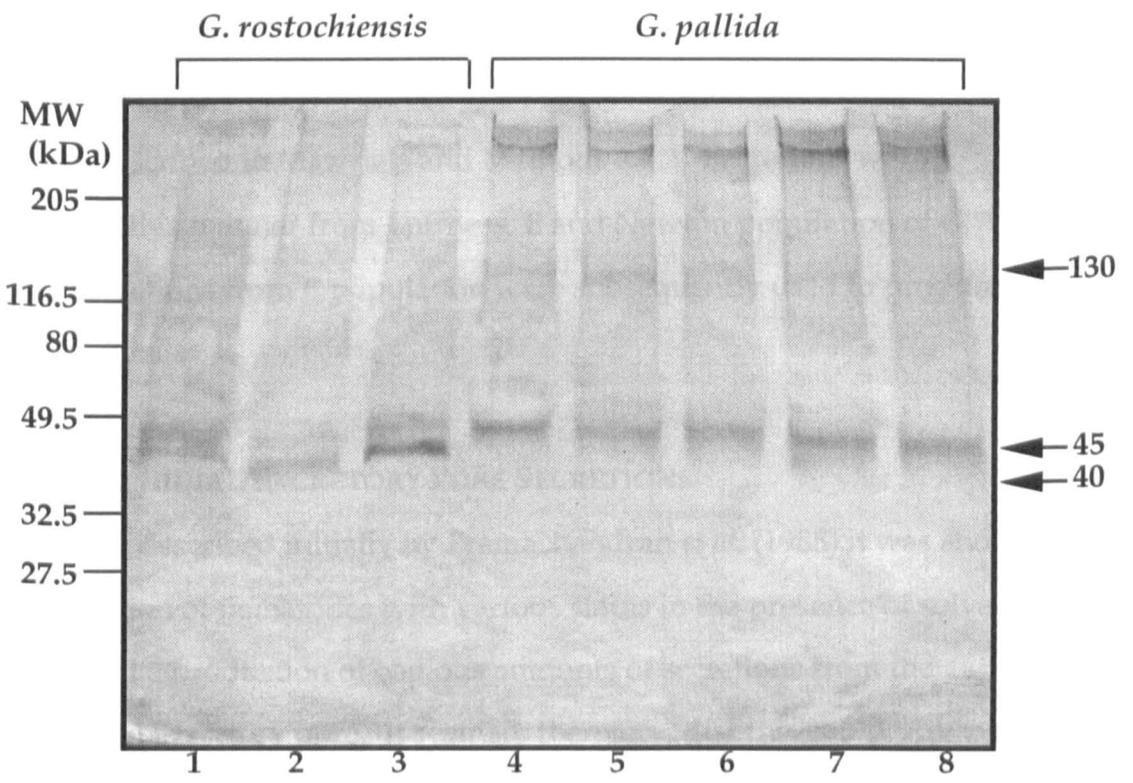
### 3.2.2. COLLECTION OF SECRETIONS

The blotting experiments described in the previous sections indicated that it was possible to differentiate between *G. pallida* and *G. rostochiensis* using various lectins and the TEPC 15 monoclonal antibody. However, using this method it was not possible to determine the origin of such molecules, that is, it remained unclear whether molecules were associated with secretions, or were localised to the interior of the parasite. It was decided, therefore, to collect secretions from PCN to see if differences in lectin binding could be

**FIGURE 3.6.** Immunoblot analysis of whole body extracts of *G. pallida* and *G. rostochiensis* probed with the monoclonal antibody TEPC 15.

Nematode whole body extracts (25µg BSA-equivalent protein) were electrophoresed on a 10-20% gradient gel and electroblotted onto nitrocellulose paper as described in Materials and Methods 2.8.1. TEPC 15 was used at 1:1000.

Lanes:            1 - *G. rostochiensis* Ro1  
                      2 - *G. rostochiensis* Ro3  
                      3 - *G. rostochiensis* Ro4  
                      4 - *G. pallida* E  
                      5 - *G. pallida* Luffness  
                      6 - *G. pallida* Loc  
                      7 - *G. pallida* Newton  
                      8 - *G. pallida* Gourdie



observed in these. Such an approach, however, entailed various problems, not least the large amounts of parasite material that would be required to collect sufficient secretions for analysis. As an alternative to probing secretions collected directly from parasites, it was decided to collect secretions and use them instead for anti-serum production. Two methods were used to collect secretions, with the subsequent production of two polyclonal anti-sera. These are described in the following sections.

### 3.2.2.1. EXCRETORY-SECRETORY PRODUCTS

Excretory-secretory (ES) secretions were collected and used to raise ES anti-serum as described in Materials and Methods 2.4.2. Secretions were collected in this manner from Luffness, E and Newton population of *G. pallida*. Secretions from E population were subsequently used to produce ES anti-serum.

### 3.2.2.2. AMPHIDIAL/EXCRETORY PORE SECRETIONS

In a method described initially by Premachandran *et al.* (1988) it was shown that incubation of nematodes with various stains in the presence of solvents resulted in the production of copious amounts of secretions from the amphids and excretory pore. It seemed, therefore, that this could prove an effective method for collecting a mixture of secretions from *G. pallida*.

Initial experiments were performed to determine which dye and solvent combination gave optimal results. Various stains were used including eosin and toluidine blue. It was eventually decided that 0.025% (w/v) Coomassie Blue R250 dissolved in 20% (v/v) methanol gave the best results. Figure 3.7 shows the production of stained secretions from *G. pallida* following incubation with the combination as described. The production of secretions using this method was also observed using time-lapse photography which showed that the first secretions to appear were

**FIGURE 3.7.** Production of stained secretions from the amphidial and excretory pores of *G. pallida*.

Nematodes were suspended in a solution containing 0.025% (w/v) Coomassie Blue R250 in 20% (v/v) methanol as described in Materials and Methods 2.3.1. An aliquot of this suspension was then placed on a slide, and covered with a coverslip which was sealed with nail varnish. Stained secretions are arrowed. Bar represents 30 $\mu$ m.



produced from the excretory pore after approximately 6 hours incubation, followed by secretions from the head region about 4 hours later. By scaling up this method as described in Materials and Methods 2.3.1, secretions were collected from Luffness, Derby and Newton populations of *G. pallida*. Secretions from *G. pallida* Luffness population were subsequently used to raise Luffness anti-serum.

### 3.2.3. EXAMINATION OF UNSTAINED AND STAINED AMPHIDS USING ELECTRON MICROSCOPY

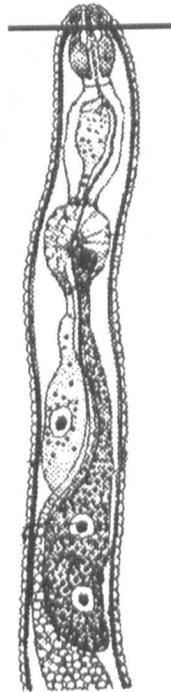
To determine the effect of the staining procedure used to produce secretions on amphidial structure and secretions, sections cut through the amphids were examined using electron microscopy. Samples were fixed, processed and stained for ultrastructural observations as described in Materials and Methods 2.13.3. Figure 3.8 shows an electron micrograph of a transverse section cut through a normal amphid. It shows that in normal amphids, the amphidial secretions (arrowed) appear amorphous, having no regular structure. In contrast, the staining procedure used to produce secretions appears to have had profound effects upon the structure of amphidial secretions that are present in the pore. Figure 3.9 shows an electron micrograph of a transverse section, this time cut through the amphid of a nematode that has undergone the staining procedure. The amphidial canal is again obvious, but the structure of the secretions (arrowed) has changed, becoming much more regular and lamellar in structure. The structure of the secretions appear similar to that observed in liposomes. This may indicate the presence of a lipid or glycolipid component in the secretions.

**FIGURE 3.8.** Transverse section through the amphid of *G. pallida*.

Samples were fixed and processed as described in Materials and Methods 2.13.1.2.

The electron micrograph shows a section cut through the amphid about 5 $\mu$ m back from the tip of the nematode as indicated by the diagram below. Bar represents 200nm.

Abbreviations: ap, amphidial pouch; cs, cephalic structure; as, amphidial secretion



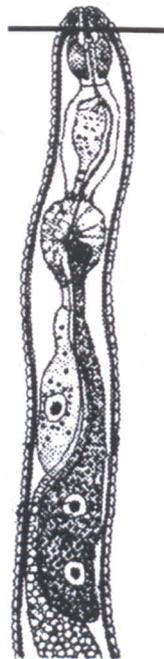


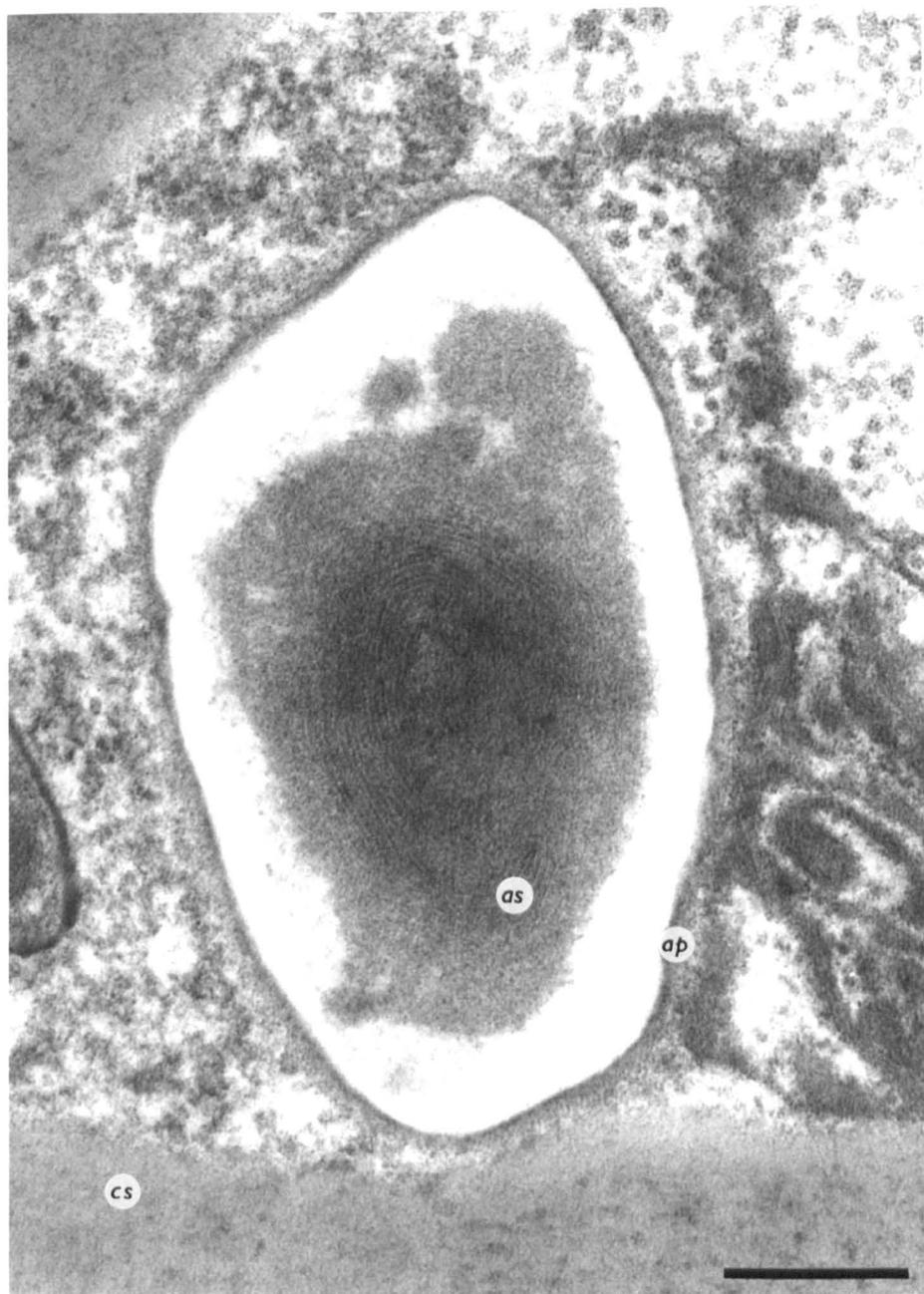
**FIGURE 3.9.** Transverse section through the amphid of *G. pallida* which has undergone the staining procedure used to produce secretions.

Samples were fixed and processed as described in Materials and Methods 2.13.1.2.

The electron micrograph shows a section cut through the amphid about 5 $\mu$ m back from the tip of the nematode as indicated by the diagram below. Bar represents 200nm.

Abbreviations: ap, amphidial pouch; cs, cephalic structure; as, amphidial secretion





### **3.2.4. ELECTROPHORESIS OF COLLECTED SECRETIONS**

#### **3.2.4.1. AMPHIDIAL/EXCRETORY PORE SECRETIONS**

Amphidial/excretory pore secretions were subjected to electrophoresis on a 10-20% gradient gel, and visualised by silver staining. Figure 3.10 shows a photograph of the gel. Electrophoresis reveals a smeared region of approximately 40-60kDa in all populations, which is perhaps indicative of a high carbohydrate content. A band which appears at <20kDa (arrowed) is also present in all populations. It should be noted that due to the method used in obtaining the secretions it was not possible to quantitate the amount of protein loaded onto the gel. Differences, therefore, may be quantitative rather than qualitative.

#### **3.2.4.2. EXCRETORY-SECRETORY PRODUCTS**

ES products collected as described in Materials and Methods 2.3.4 were subjected to electrophoresis on a 10-20% gradient gel and silver stained. SDS-PAGE electrophoresis reveals several bands ranging from about 14-100kDa (Figure 3.11). The most abundant of these proteins has a molecular weight of about 70kDa.

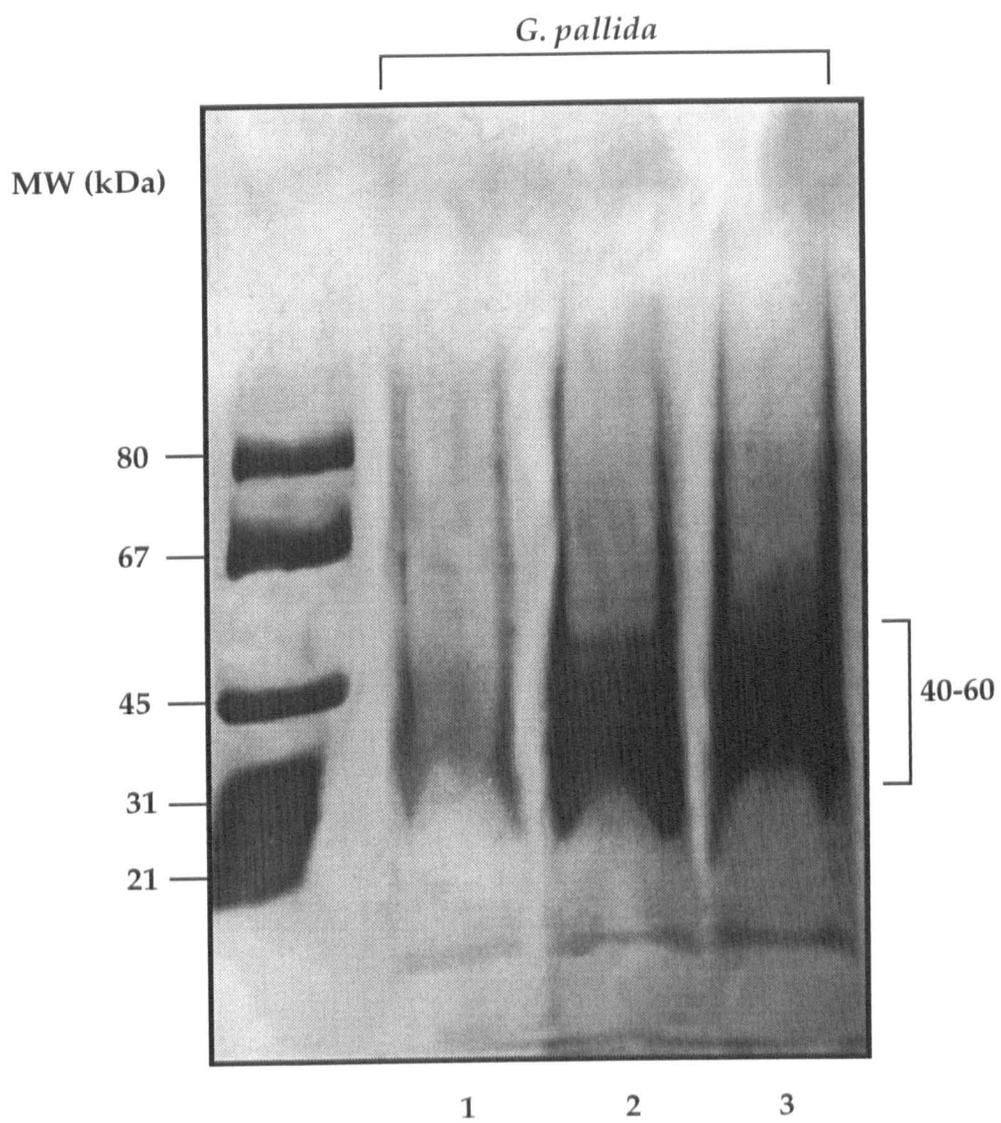
### **3.2.5. INDIRECT IMMUNOFLUORESCENT ANTIBODY TEST**

In order to determine if secretions were surface localised, indirect immunofluorescent antibody test (IFAT) was used. IFAT was performed as described in Materials and Methods 2.9.1. In all of the following experiments, primary antibody was used at a concentration of 1:50.

#### **3.2.5.1. ES ANTI-SERUM**

When indirect immunofluorescence studies were performed with ES anti-serum on whole nematodes, very faint fluorescence could be seen covering the surface of some nematodes. This pattern of antibody binding was only

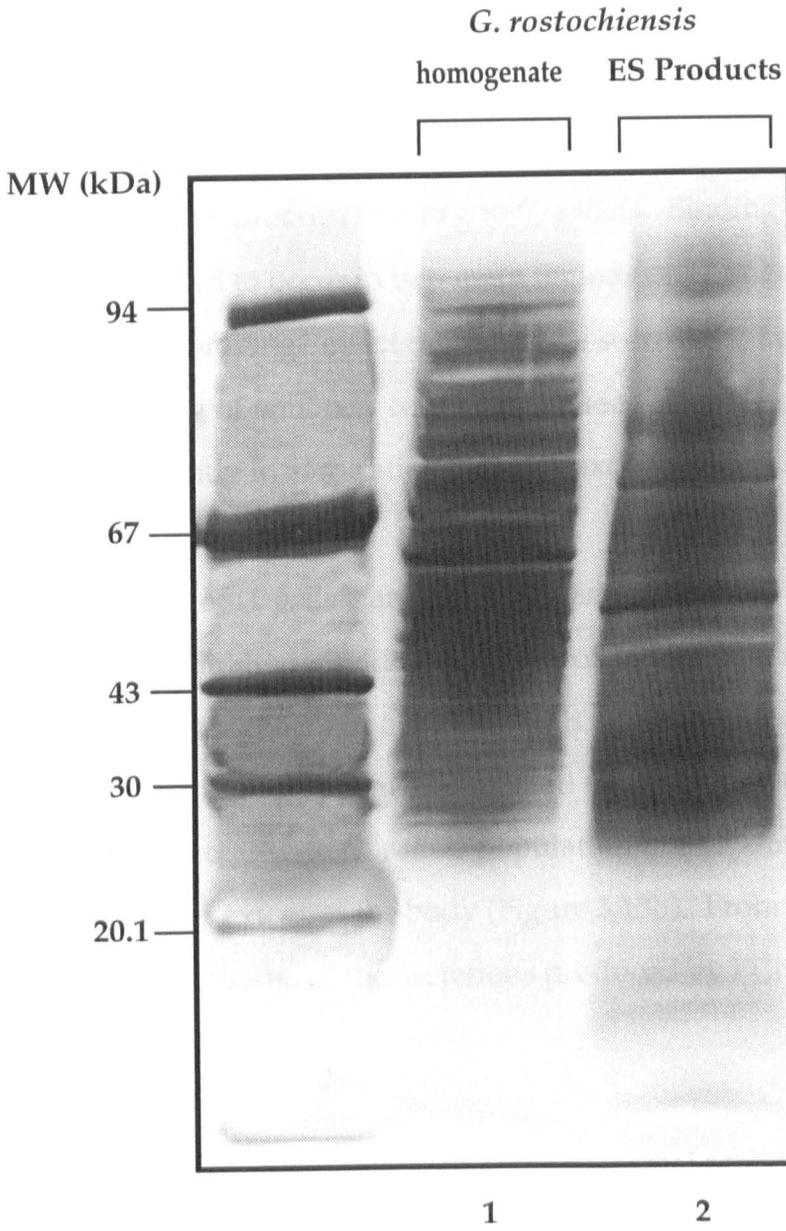




**FIGURE 3.11. SDS-PAGE gel of collected ES secretions.**

Secretions were collected from *G. rostochiensis* using the modified staining procedure described in Materials and Methods 2.3.1. Secretions (15µg BSA-equivalent protein) were electrophoresed on a 10-20% gradient gel and silver stained as described previously.

Lanes:        1 - *G. rostochiensis* Ro1 (homogenate)  
                  2 - *G. rostochiensis* Ro1 (ES products)



observed in the minority of nematodes, most showing no fluorescence. Of the nematodes that bound antibody, surface fluorescence was very faint, and proved impossible to photograph. In an attempt to improve binding, the protocol was repeated on fixed nematodes, and also performed at 4°C. The same results, however, were obtained.

### 3.2.5.2. LUFFNESS ANTI-SERUM

Indirect immunofluorescence was performed on living and fixed nematodes, both giving the same pattern of antibody binding. Figure 3.12 shows the pattern of antibody binding to *G. pallida*. Binding of Luffness anti-serum appeared to occur in two main regions on the nematode. Most obvious was the "capping" effect that could be seen at the head of the nematode. Binding of antibody could also be seen along the body of the nematode, apparently in association with the excretory pore.

Other populations of *G. pallida* and *G. rostochiensis* were also tested to see if any differences in antibody binding could be observed. Interestingly, some differences were apparent. For example, with population (pathotype) Ro3 of *G. rostochiensis* binding appeared restricted to a small region of the head (Figure 3.13a), whereas with *G. pallida* population Loc binding appeared to occur over a greater extent of the body (Figure 3.13b). From these results it appears that at least some of the secretions produced by PCN are surface localised.

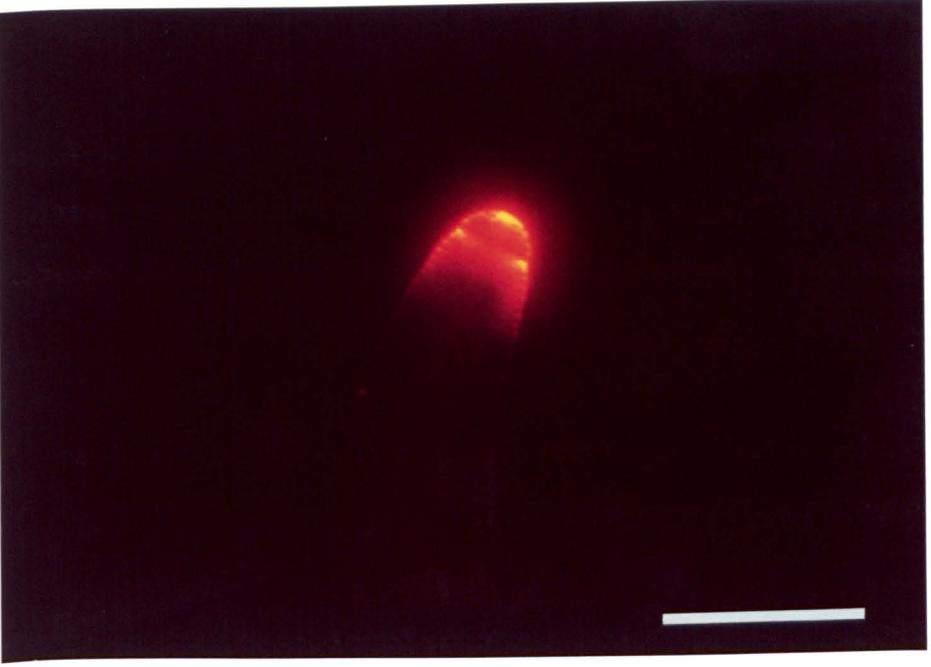
In these indirect immunofluorescence studies, normal rabbit serum (NRS) was used as a control to eliminate the possibility of non-specific binding. Interestingly, when NRS was used very distinct binding could be seen to the amphids (Figure 3.14). Incubation with TRITC-conjugated secondary antibody alone or FITC-BSA alone gave no fluorescence, indicating that a

**FIGURE 3.12.** Indirect immunofluorescence of *G. pallida* Luffness population probed with Luffness anti-serum.

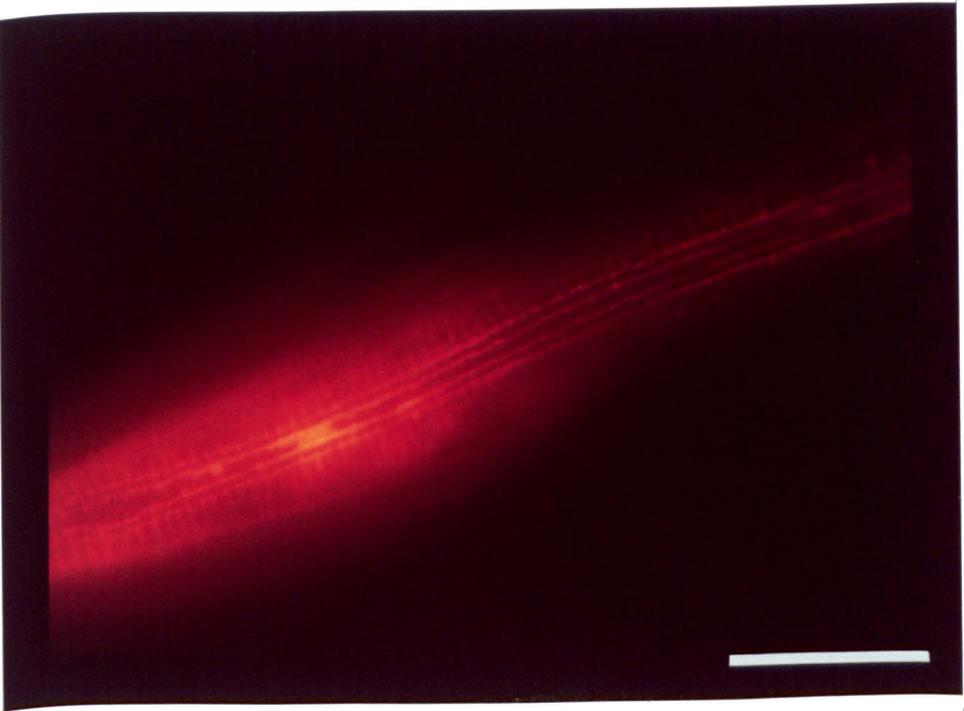
Washed nematodes were incubated with Luffness anti-serum (1:50), followed by goat anti-rabbit IgG-TRITC as described in Materials and Methods 2.9.1. Prior to viewing nematodes were fixed in 1% formaldehyde for 15 minutes.

- a) Binding of Luffness anti-serum to the head of *G. pallida* Luffness population. Bar represents 30 $\mu$ m.
  
- b) Binding of Luffness anti-serum to the body of *G. pallida* Luffness population. Bar represents 30 $\mu$ m.

a



b

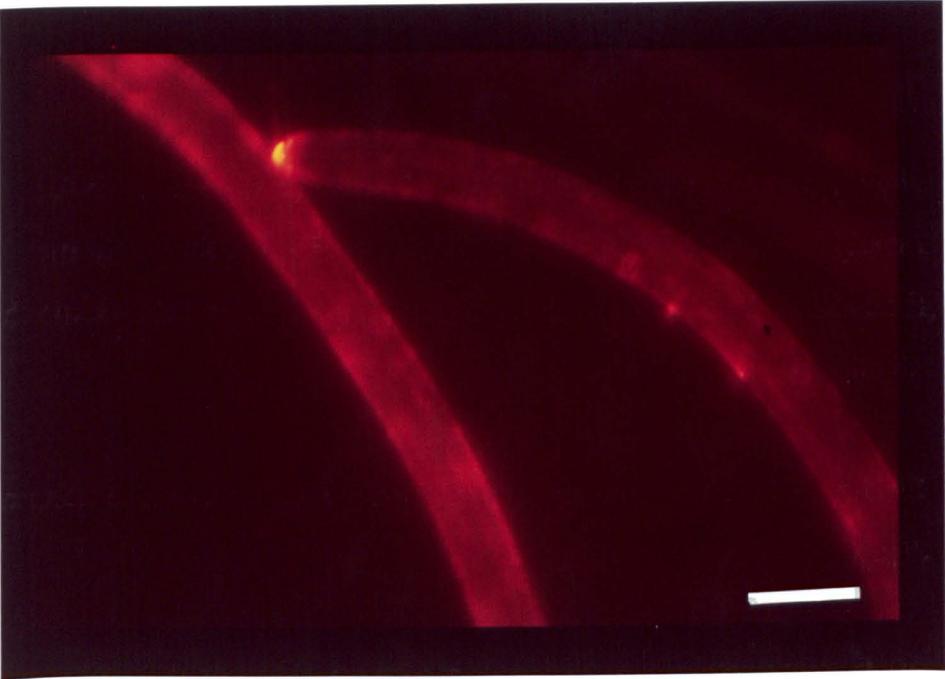


**FIGURE 3.13.** Indirect immunofluorescence of *G. pallida* Loc population, and *G. rostochiensis* Ro3 probed with Luffness anti-serum.

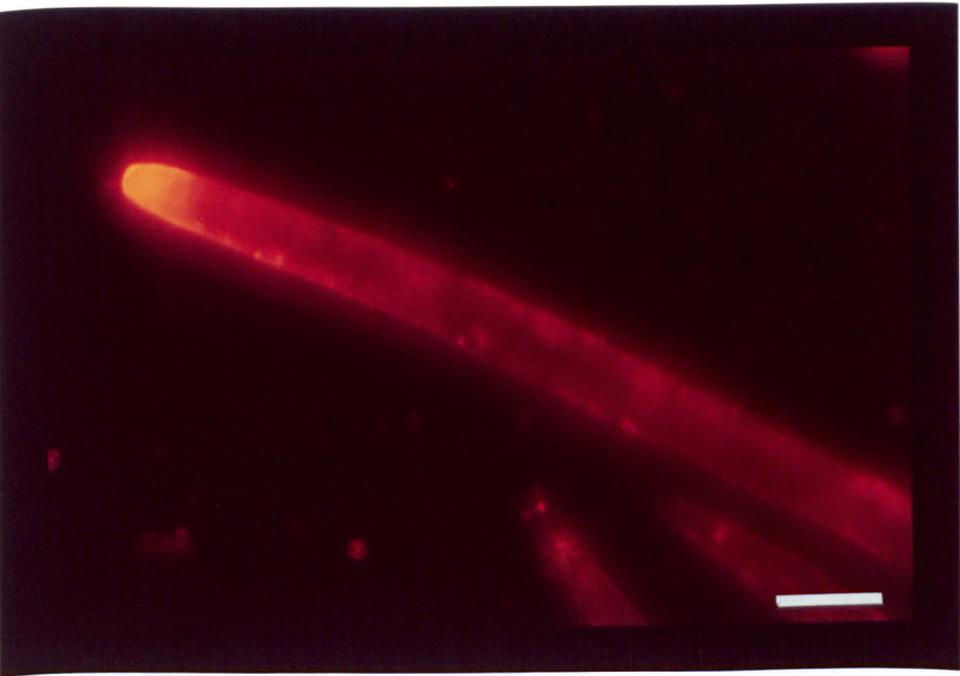
Washed nematodes were incubated with Luffness anti-serum (1:50), followed by goat anti-rabbit IgG-TRITC as described in Materials and Methods 2.9.1. Prior to viewing nematodes were fixed in 1% formaldehyde for 15 minutes.

- a) Binding of Luffness anti-serum to *G. rostochiensis* Ro3. Bar represents 30 $\mu$ m.
  
- b) Binding of Luffness anti-serum to *G. pallida* Loc population. Bar represents 30 $\mu$ m.

a



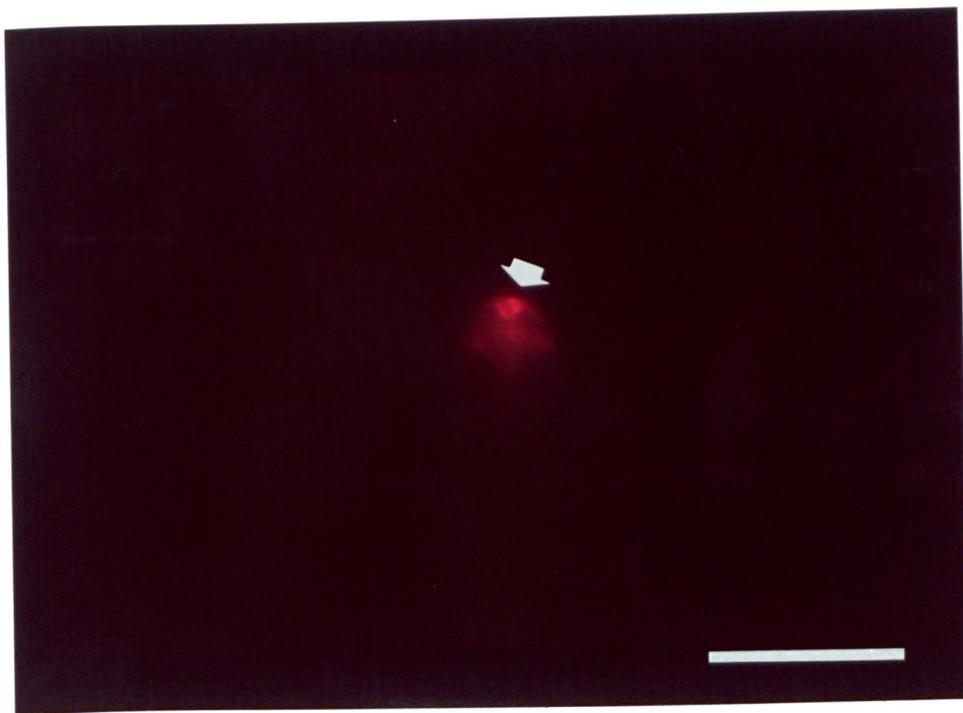
b



**FIGURE 3.14.** Indirect immunofluorescence of *G. pallida* Luffness population probed with normal rabbit serum.

Washed nematodes were incubated with normal rabbit serum (1:50), followed by goat anti-rabbit IgG-TRITC as described in Materials and Methods 2.9.1. Prior to viewing nematodes were fixed in 1% formaldehyde for 15 minutes.

Binding of normal rabbit serum to the amphids of *G. pallida* Luffness population can be seen (arrowed). Bar represents 30 $\mu$ m.



specific component of the NRS was binding to the amphids. To investigate this phenomenon further, indirect immunofluorescence studies using NRS in the presence of competing sugars were carried out. Nematodes were pre-incubated with 200mM of  $\alpha$ -L-fucose, methyl  $\alpha$ -D-mannopyranoside, N-acetyl D-glucosamine or N-acetyl D-galactosamine respectively for 30 minutes prior to addition of NRS. Of all the sugars tested, N-acetyl D-galactosamine alone was able to inhibit binding of NRS to the amphids. Table 3.1 summarises the inhibition of NRS binding by competing sugars.

### **3.2.6. PERIODATE TREATMENT OF NEMATODES PRIOR TO INDIRECT IMMUNOFLUORESCENCE**

Periodate treatment was not performed with ES anti-serum due to the inconsistent results obtained with the anti-serum.

#### **3.2.6.1. LUFFNESS ANTI-SERUM**

Nematodes fixed in 1% (v/v) formaldehyde were exposed to 10mM sodium metaperiodate for one hour prior to indirect immunofluorescence as described in Materials and Methods 2.9.2. After treatment no antibody binding could be observed on the surface of the nematode indicating that the epitopes involved in antibody recognition are predominantly carbohydrate in nature. Interestingly, after periodate treatment, binding could still be observed to the amphids. Again, other populations of *G. pallida* and *G. rostochiensis* were examined. In all cases the same results were obtained.

Periodate treatment was also repeated on nematodes probed with NRS. In these nematodes, binding of NRS to the amphids could still clearly be seen indicating that the amphidial components involved in the interaction are not carbohydrate in nature.

**TABLE 3.1.** The effect of sugars on the binding of normal rabbit serum to the amphids of *Globodera pallida*.

Sugar (200mM)	Binding of NRS
fucose	+
$\alpha$ -methyl mannoside	+
N-acetyl glucosamine	+
N-acetyl galactosamine	-

+ binding of NRS

- no binding of NRS detected

### 3.2.7. IMMUNOBLOT ANALYSIS OF NEMATODE WHOLE BODY

#### EXTRACTS

##### 3.2.7.1. ES ANTI-SERUM

Whole body extracts of *G. pallida* and *G. rostochiensis* were electrophoresed using the Pharmacia Phast gel system, and the proteins then transferred onto nitrocellulose paper as described in Materials and Methods 2.8.1. ES anti-serum was then used to probe the separated proteins (Figure 3.15) From the blot it can be seen that ES anti-serum recognises two proteins of molecular weight around 90 and 30kDa. There appear to be no differences between the two species.

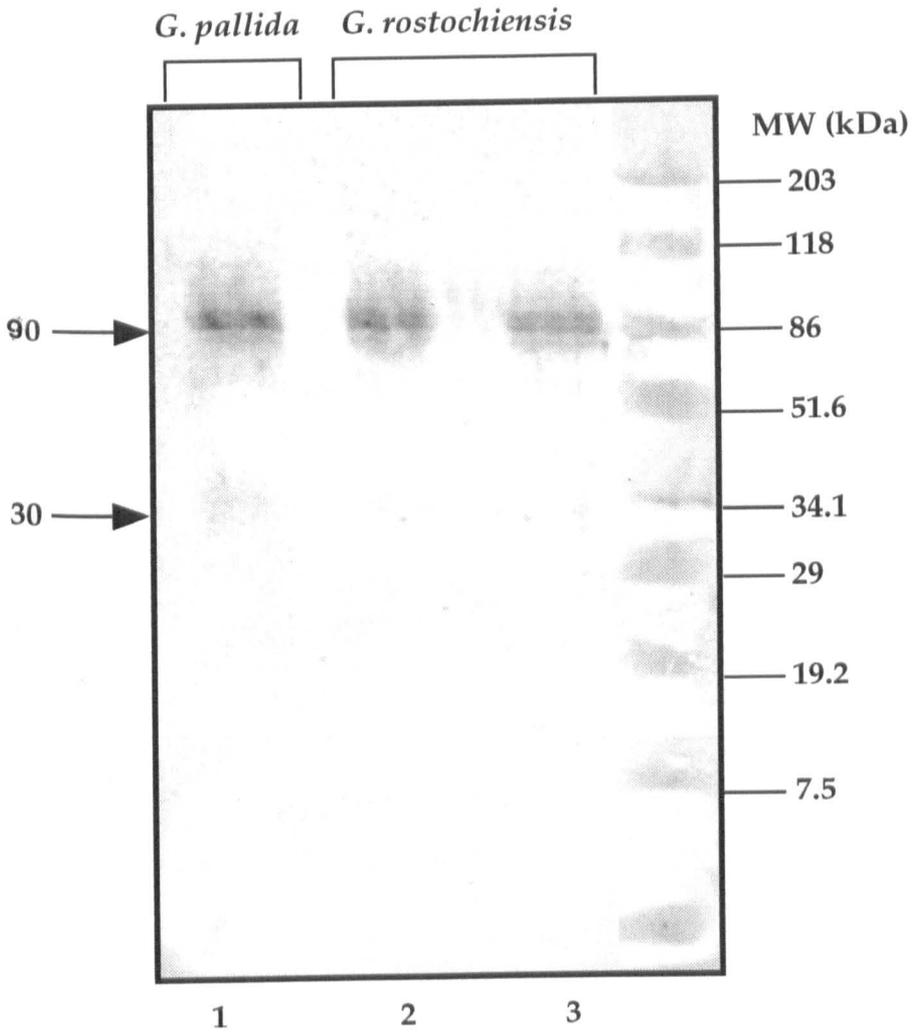
##### 3.2.7.2. LUFFNESS ANTI-SERUM

Whole body extracts of several populations of *G. pallida* and *G. rostochiensis* were electrophoresed on a 10-20% gradient gel, and the proteins then transferred onto nitrocellulose paper. Luffness anti-serum was then used to probe the separated proteins. Figure 3.16 shows the resulting blot. It can be seen that there are clear differences between the two species. For example, in *G. pallida* there is a clear doublet at 30kDa (arrowed) which is not present in *G. rostochiensis*. In contrast, a band of approximately 40kDa (arrowed) which is present in *G. rostochiensis* is absent from *G. pallida*. Interestingly, this 40kDa band appears to differ slightly in molecular weight between the different populations of *G. rostochiensis*. As well as inter-species differences it is also possible to detect intra-species differences. The clearest example of this occurs with population (pathotype) Ro5 where two bands at 65kDa and 80kDa are missing, (asterisks), but are present in all the other *G. rostochiensis* populations. Similarly, population Luffness of *G. pallida* appears to lack a band of 55kDa which is present in the other populations. As well as qualitative differences, some quantitative differences are also apparent. For

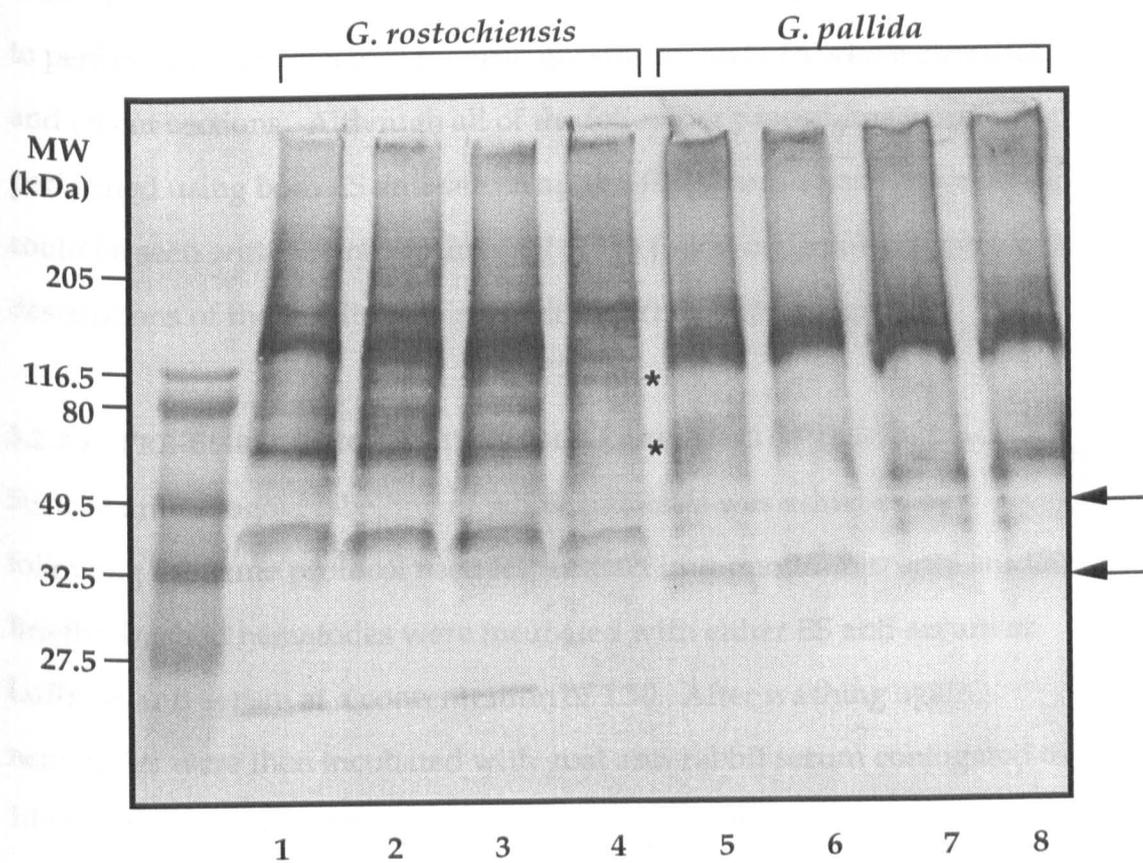
**FIGURE 3.15.** Immunoblot analysis of whole body extracts of *G. pallida* and *G. rostochiensis* using ES anti-serum.

Secretions were electrophoresed on a 12.5% homogenous Phast gel followed by electroblotting onto nitrocellulose. Blots were probed with ES anti-serum (1:500). Approximately 0.125 $\mu$ g BSA-equivalent protein was loaded per lane.

Lanes:        1 - *G. pallida* Luffness population  
                  2 - *G. rostochiensis* Ro1  
                  3 - *G. rostochiensis* Ro1







example in *G. pallida* populations Luffness and E a doublet at 50kDa is reduced in intensity compared to the other populations.

### 3.2.8. ELECTRON MICROSCOPY STUDIES

In order to investigate more fully the possible origins of the secretions to which ES anti-serum and Luffness anti-serum were raised, it was decided to perform immunoelectron microscopy studies on both whole parasites and on cut sections. Although all of the following procedures were performed using both ES anti-serum and Luffness anti-serum, no binding could be seen with ES anti-serum. All of the following results therefore are descriptions of the results obtained with Luffness anti-serum.

#### 3.2.8.1. PRE-EMBEDDING IMMUNOGOLD LABELLING OF THE SURFACE

Surface immunogold labelling of whole parasites was achieved by following the same protocol used for indirect immunofluorescence studies. Briefly, washed nematodes were incubated with either ES anti-serum or Luffness anti-serum at a concentration of 1:50. After washing again, nematodes were then incubated with goat anti-rabbit serum conjugated to 10nm colloidal gold. After labelling nematodes were fixed in 2% paraformaldehyde and processed and stained as described in Materials and Methods section 2.13.1.2.

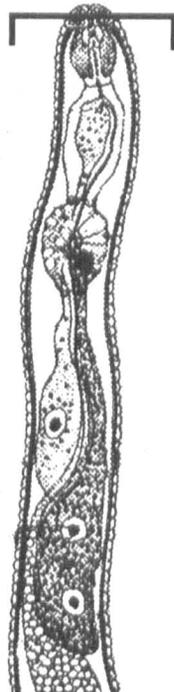
Although many sections were cut and examined, most of these showed a lack of antibody binding which appeared to be in direct conflict with the results obtained using fluorescence microscopy. For example, Figure 3.17 shows a longitudinal section through the head of *G. pallida* Luffness population. Fluorescence microscopy results had indicated that this region would be heavily labelled. However, the electron micrograph shows that the gold particles are very sparsely distributed in this area. One interesting

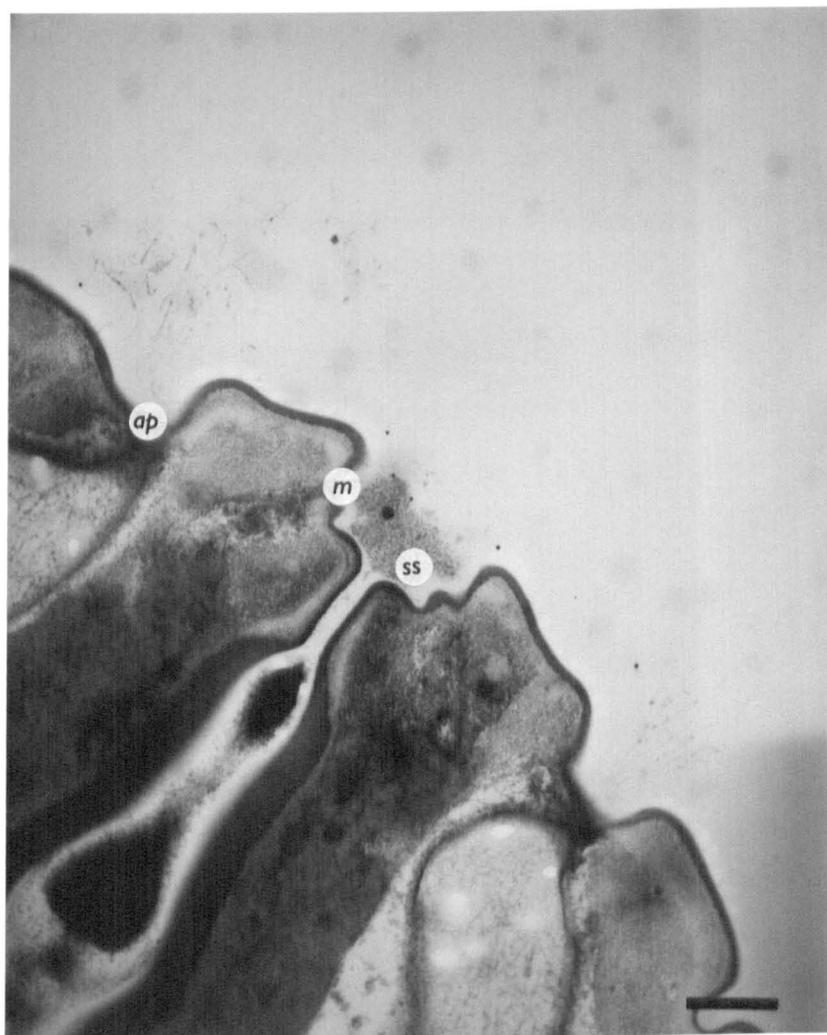
**FIGURE 3.17.** Longitudinal section through the head of *G. pallida* Luffness population labelled with Luffness anti-serum.

Whole nematodes were labelled with Luffness anti-serum (1:50) followed by goat anti-rabbit IgG conjugated to colloidal gold (10nm). Samples were then fixed and sectioned as described in Materials and Methods 2.13.1. Longitudinal sections were cut through the head as indicated in the diagram below.

Bar represents 200nm.

Abbreviations: m, mouth; ss, stylet secretions; ap, amphidial pouch





point noticed however, was that clumps of labelled material could be seen lying away from the surface of the nematode. A possible explanation for this is presented in the discussion.

### 3.2.8.2. POST-EMBEDDING IMMUNOGOLD LABELLING OF CUT SECTIONS

To allow the localisation of secretions within the nematode, sections were cut and used for immunogold labelling. Glutaraldehyde fixed specimens were processed, cut and then immunogold labelled using Luffness anti-serum at a dilution of 1:10 000, as described in Materials and Methods 2.13.2. Sections were cut through the anterior end of the specimen allowing visualisation of structures in the head and oesophageal regions. Figure 3.18 shows a transverse section cut through the metacorpul bulb which is found at the base of the stylet. Antibody can clearly be seen labelling the contents of the metacorpul bulb indicating that at least some of the secretions seen are of oesophageal origin. When sections were cut further back through the nematode to allow visualisation of the oesophageal glands no obvious binding of antibody could be seen to any of the gland structures. Considering the previous result illustrated in Figure 3.18 this might be surprising due to the presence of labelled material in the metacorpul bulb. Instead it appears that this material may in fact be derived from the intestinal contents. Figure 3.19 shows labelling of the contents of the intestinal canal which can be seen lying adjacent to one of the oesophageal glands. The oesophageal gland can be seen to contain secretory granules which are free of label, in contrast with the contents of the intestine which lies adjacent.

## 3.3. DISCUSSION

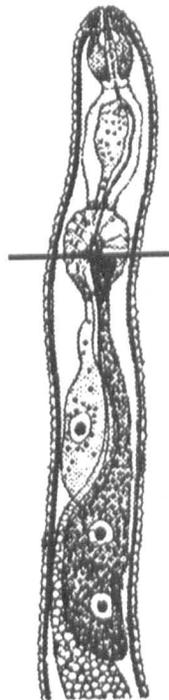
The initial experiments reported in this chapter confirmed previous results that localised lectin binding to the amphids of *G. pallida* and *G. rostochiensis*

**FIGURE 3.18.** Electron micrograph showing transverse section through the metacorporeal bulb of *G. pallida* Luffness population probed with Luffness anti-serum.

Samples were fixed for 1 hour and sectioned as described in Materials and Methods 2.13.2. Cut sections of these samples were then immunogold labelled using Luffness anti-serum (1:10 000) followed by goat anti-rabbit IgG-TRITC conjugated to colloidal gold (10nm) (1:50).

Sections were cut about 100 $\mu$ m back from the tip of the nematode as indicated by the diagram below. The electron micrograph shows labelling of the contents of the metacorporeal bulb (arrowed). Bar represents 200nm.

Abbreviations: rm, radial muscle; pc, pump chamber of the tri-radiate metacorporeal bulb



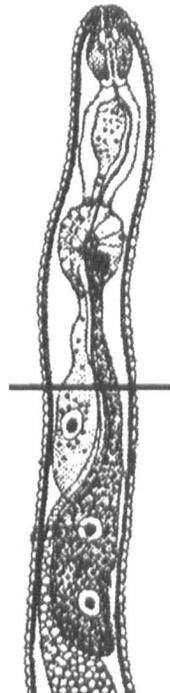


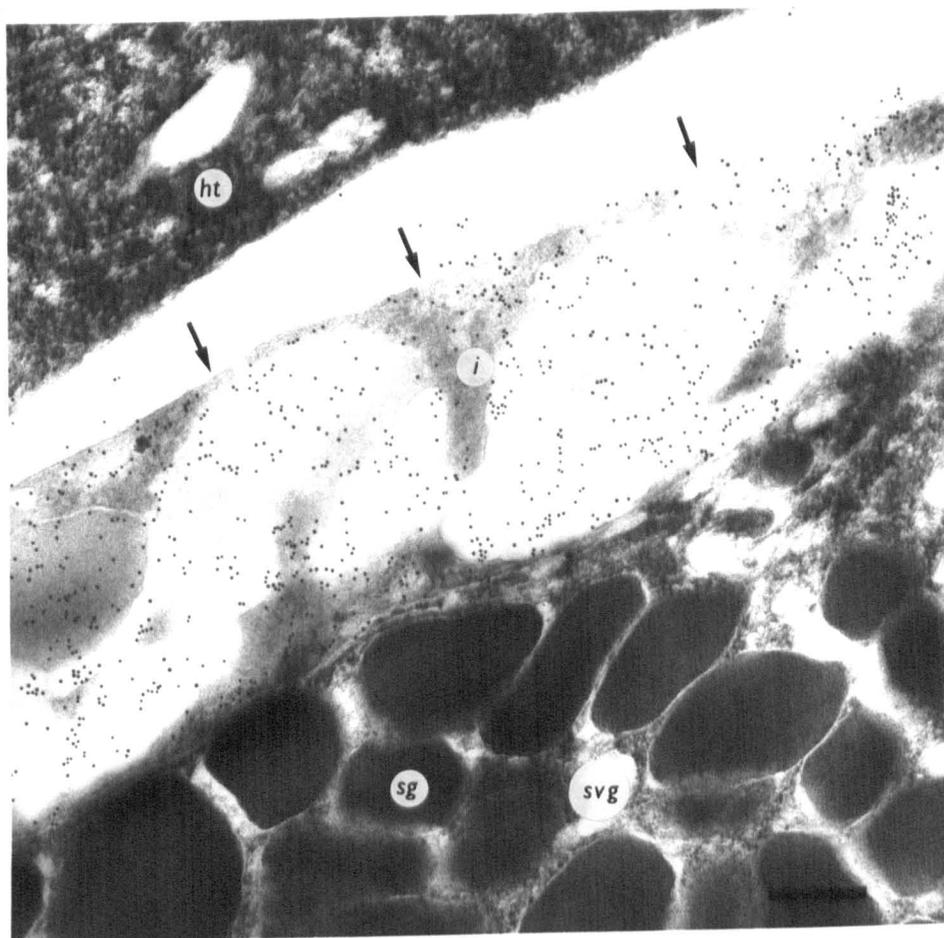
**FIGURE 3.19.** Electron micrograph of a longitudinal section through the oesophageal region of *G. pallida* Luffness population probed with Luffness anti-serum.

Samples were fixed for 1 hour and sectioned as described in Materials and Methods 2.13.2. Cut sections of these samples were then immunogold labelled using Luffness anti-serum (1: 10 000) followed by goat anti-rabbit IgG-TRITC conjugated to colloidal gold (10nm) (1:50).

Sections were cut about 120µm back from the tip of the nematode as indicated by the diagram below. The electron micrograph shows labelling of the intestinal contents (arrowed). Bar represents 200nm.

Abbreviations: svg, subventral gland; sg, secretory granule; i, intestine; ht, hypodermal tissue





(Forrest & Robertson, 1986; Aumann & Wyss, 1987; Aumann, 1994). Results obtained confirmed that binding of the lectins Con A, GNA and WGA occurs specifically to the amphids. In contrast to the findings of Forrest & Robertson (1986) which showed differences in binding of Con A to *Globodera* populations, no differences between the populations tested were found. This can be explained, however, since different populations were used in this study.

Lectin binding was investigated further by using digoxigeninylated lectins to probe blots of whole body extracts of different *Globodera* populations. Other workers have successfully used this technique to differentiate some *Meloidogyne* spp. (Davis & Kaplan, 1992), and the burrowing nematodes *Radopholus citrophilus* and *R. similis* (Kaplan & Gottwald, 1992). Of the panel of lectins tested, Con A, GNA, SNA and WGA all bound specifically to nematode glycoproteins. Of these lectins WGA alone revealed differences both between and within species. Species differentiation was also observed when TEPC 15, a monoclonal antibody against phosphorylcholine, was used to probe blots of whole body extracts. As with WGA, TEPC 15 could differentiate between *G. pallida* and *G. rostochiensis*, although not between populations. It has been argued that differences in glycosylation may determine whether the nematode can establish a successful infection. Bearing in mind the evidence that glycoproteins rich in mannose and galactose can elicit phytoalexin production (de Wit, 1980), then differences such as those highlighted by WGA may act as determinants for the outcome of infection. Similarly, the differences revealed by TEPC 15 indicate that phosphorylcholine moieties are present on different proteins in *G. pallida* and *G. rostochiensis*. Such differences may similarly influence infection events.

The key aim of the work described in this section was to isolate amphidial secretions specifically and in sufficient quantities for further experiments. The first method (Method 1), was based on a staining protocol originally described by Premachandran *et al.* (1988), and allowed the collection of a mixture of amphidial and excretory pore secretions following incubation of the nematodes with Coomassie Blue R250, methanol and solvent. In contrast, the second method, (Method 2), allowed the collection of ES products from living nematodes kept in aseptic conditions.

SDS-PAGE electrophoresis of secretions collected using Methods 1 and 2 revealed quite different protein profiles. For example, amphidial/excretory pore secretions collected using Method 1 appeared on gels as a smear in a region between 40-60kDa. From the results obtained it can be seen that the secretions collected are, at least partly, proteinaceous in nature. This appears to corroborate previous work (Spiegel & Robertson, 1988; Forrest *et al.*, 1988; Aumann, 1989) on the nature of amphidial and excretory pore secretions. This method, however, also resulted in the death of the nematodes, allowing the criticism that secretions collected in this way may differ substantially from those found in living nematodes. The appearance of electrophoresed secretions as a smear may indicate that the secretions are highly glycosylated. Alternatively, since the staining method results in the death of the nematodes, the process may result in the release of degradative enzymes such as proteases which leads to the smeared appearance of the gels. In contrast, electrophoresis of ES products collected using Method 2 revealed about sixteen distinct bands ranging in weight from 14-100kDa.

These results, therefore, indicate that the two methods resulted in the collection of distinct nematode proteins. It is useful to speculate upon the possible role and origin of the secretions collected by the two methods. As

stated before, secretions collected using Method 1 are derived from dead nematodes, and as a result may be artefactual in their content. Despite this, observation of nematodes stained in this manner clearly shows that the majority of secretion visible appears to be derived from the amphidial and excretory pores. Method 2 allowed the collection of ES products from living nematodes. This method did not allow visualisation of secretions, nor was it possible to determine the origin of secretions collected in this manner. These secretions, however, may more truly reflect secretions (and excretions) that are produced by the nematode in the pre-parasitic stage of infection.

Secretions collected using Methods 1 and 2 were also used to raise polyclonal anti-sera in rabbits, resulting in Luffness anti-serum, and ES anti-serum respectively. When these anti-sera were used for indirect immunofluorescence and immunoblotting studies, further differences between the collected secretions were confirmed. Indirect immunofluorescence studies performed with Luffness anti-serum, (raised against secretions collected using Method 1), revealed that antibody binding occurred consistently to the surface of both *G. pallida* and *G. rostochiensis*, in particular as a "cap" around the head region, and on the body in the vicinity of the excretory pore. Interestingly, this characteristic antibody binding can be completely abolished by periodate treatment, indicating a predominance of carbohydrate epitopes. Some variation was observed between different populations tested, with some populations binding more antibody to the surface than others. The characteristic pattern of binding, however, was always present. When ES anti-serum was used for indirect immunofluorescence studies very inconsistent binding was seen. Faint binding of antibody to the surface of a very small number of nematodes in any particular sample was observed. The majority of

nematodes, however, showed no binding. This appears to indicate that the antibody recognises a fragile surface layer of the nematode that is easily lost upon washing or handling of the nematodes.

Indirect immunofluorescence studies using Luffness anti-serum therefore confirmed that some of the secretory material collected using Method 1 was surface associated. Although it is tempting to speculate that the material associated with the body wall posterior to the head originates from the excretory pore, the origin of material located at the head of the nematode is open to question. Material found here may be of amphidial, subventral or dorsal gland in origin. The origin of collected secretions was further investigated using electron microscopy. Pre-embedding immunogold labelling of the surface of whole nematodes using Luffness anti-serum resulted in very sparse surface labelling, although from fluorescence microscopy results intense staining would have been expected. One possible explanation for this anomaly may lie in the glutaraldehyde fixation procedure used. When viewing the specimens it was often possible to see clumps of heavily labelled material lying away from the surface of the nematode. It is possible then that the glutaraldehyde fixation required for this procedure may allow the loss of the labelled layer which can subsequently be seen lying away from the nematode trapped in agar.

To localise secretions to structures within the nematode, post-embedding immunogold labelling of nematode sections was performed. When sections were cut through the base of the metacorporeal bulb, labelling of secretory material contained within the bulb could clearly be seen, raising the possibility that labelled secretions were derived from the oesophageal glands. Further sections indicated however that the oesophageal glands were not labelled, although the contents of the intestine were. An

explanation for this may lie in the fact that the contents of both the ventral and oesophageal glands ultimately end up as part of the intestinal contents. It is possible that movement of material from oesophageal gland to the intestine is accompanied by changes, brought about by example with enzymes, which allows the recognition of this material by Luffness anti-serum. Pre- and post-embedding immunogold labelling of nematodes was also performed using ES anti-serum. However, in all of the samples examined no labelling could be seen on the nematode.

Immunoblotting studies with Luffness anti-serum and ES anti-serum also gave very interesting results. When Luffness anti-sera was used to probe whole body extracts of different populations of *G. pallida* and *G. rostochiensis*, it could be seen that the anti-serum could differentiate between the two species, and also between different populations of *G. rostochiensis*. When it is considered that binding of Luffness anti-serum to the surface of *Globodera* can be abolished by periodate treatment, then it seems likely that the majority of the epitopes recognised by the anti-serum are carbohydrate in nature. The implication of this, therefore, is that differences revealed by blotting may be due to glycosylation differences present between species, and some populations. As stated earlier, this may have important consequences for the outcome of infection.

When ES anti-serum was used to probed blots, two bands were recognised. These bands did not appear to differ between species, or between populations suggesting that the proteins recognised by ES anti-serum fulfil a conserved role in the species. It is perhaps surprising that ES anti-serum appears restricted to these proteins, when electrophoresis of collected ES products reveals many more proteins. It is possible that the proteins

recognised on the blots are simply much more immunogenic than any of the other components.

One interesting phenomenon observed as a result of indirect immunofluorescence studies was the binding of control normal rabbit serum (NRS) to the amphids. Control experiments using FITC-BSA and secondary antibody conjugate eliminated the possibility that this was a non-specific interaction due to, for example, hydrophobic interactions. Further studies, using competing sugars, indicated the involvement of N-acetylgalactosamine residues. The interaction therefore appears to be due to N-acetylgalactosamine residues found on immunoglobulin molecules (Taniguchi *et al.*, 1986) binding to a component of the amphidial secretions. Periodate treatment of nematodes prior to incubation with NRS did not affect binding to the amphids indicating the amphidial component involved is protein in nature. Such results indicate the presence of a nematode lectin in the amphids. The function of such a lectin, however, remains open to speculation.

Further experiments were also performed concerning the adhesion of human red blood cells to the surface of the nematode. Initial experiments appeared to confirm the results obtained with competing sugars since red blood cells of blood group A, B or AB, with galactose or N-acetylgalactosamine as their main determinant, bound beautifully to the head region of the nematode. Red blood cells of blood group O whose main determinant is fucose did not bind to the surface of the nematode. Unfortunately this experiment proved impossible to repeat despite many attempts, leaving erythrocyte binding results invalid.

In summary, the different methods used to collect secretions yielded different and distinct types of protein. No evidence could be obtained however, that any of the methods resulted in the collection of amphid specific material in sufficient quantity for further experimentation. Indirect immunofluorescence studies indicated that the two anti-sera raised using material collected using Methods 1 and 2 recognised different nematode components. This was further confirmed by immunoblotting studies which revealed that Luffness anti-serum recognised a number of nematode proteins, and was capable of differentiating both between and within species of *G. pallida* and *G. rostochiensis*. In contrast, ES anti-serum recognised only two bands which appeared to be conserved between the two species. Observations also indicated the presence of a nematode lectin component present in amphidial secretions with apparent specificity for N-acetylgalactosamine.

**CHAPTER 4**  
**INDUCTION OF SECRETIONS**  
**IN *GLOBODERA PALLIDA* AND**  
***G. ROSTOCHIENSIS***

## 4.1. INTRODUCTION

One of the main problems encountered in attempts to analyse secretions from plant-parasitic nematodes is the small amount of material that can be easily collected. Some progress was made when it was demonstrated that several plant-parasitic nematodes could be induced to produce secretions upon exposure to dihydroxybenzene derivatives, as well as certain neurotransmitters, such as serotonin (McClure & von Mende, 1987). More recently, it has been shown that the serotonin agonist, methoxy dimethyl tryptamine (DMT) could induce the production of large amounts of secretion (Goverse *et al.*, 1994).

This chapter will describe experiments carried out to confirm that DMT could induce secretions in *Globodera pallida* and *Globodera rostochiensis*, and its effect on the distribution of secretions as determined by indirect immunofluorescence and electron immunogold studies. This is followed by a description of various lipid probe studies performed to determine if changes were occurring at the surface of the parasite following the induction of secretions.

## 4.2. RESULTS

### 4.2.1. INDUCTION OF SECRETIONS BY METHOXY DIMETHYL TRYPTAMINE

Initial experiments were performed to confirm that methoxy dimethyl tryptamine (DMT) could indeed induce the production of secretions from *Globodera pallida* and *G. rostochiensis*. Washed nematodes were incubated in different concentrations of DMT ranging from 0-400 $\mu$ gml<sup>-1</sup>. As described in Materials and Methods 2.10.1, all incubations were performed in the presence of 0.025% (w/v) Coomassie Blue R250 to ease the visualisation of secretions.

Generally, after addition of DMT, stained particulate matter could be seen appearing in solution within minutes. This was followed by an accumulation of secretions at the head of the nematode, although it was not possible to distinguish if these were amphidial, oesophageal or a mixture of both (Figure 4.1). Different types of secretion appeared to be produced. For example, sheet-like material could be seen apparently sloughing off from the sides of the nematode. Induction of secretions appeared optimal at  $200\mu\text{gml}^{-1}$  with movement of the stylet also being associated with the production of secretions. The procedure was repeated using different populations of *G. pallida* and *G. rostochiensis*, all giving the same result. Addition of DMT therefore appears to result in a large increase in secretory activity.

Incubation with DMT was also carried out in the absence of Coomassie Blue R250. In the absence of Coomassie Blue R250 it was not possible to see particulate or sheet-like material as described before. It was still possible however, to see unstained secretions collecting at the head of the nematode. Interestingly, when nematodes were incubated with DMT alone they could be seen to clump together. This did not appear to be restricted to any particular region of the nematodes, with adhesion appearing to occur immediately upon contact.

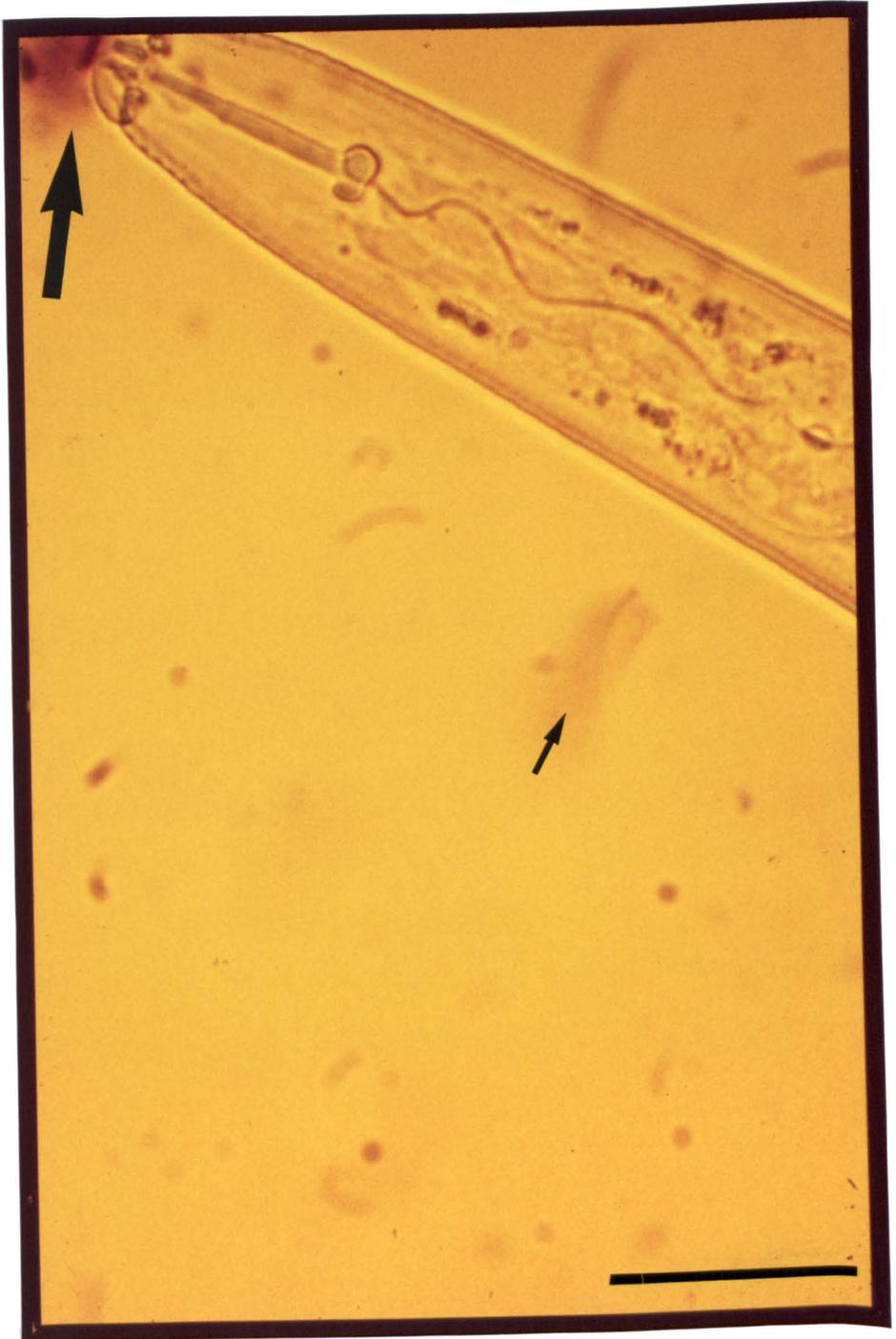
#### 4.2.2. ANALYSIS OF INDUCED SECRETIONS

To compare those secretions induced by DMT with those collected using the methods described in Chapter 3, secretions induced by DMT were collected for electrophoretic and immunoblot analysis. The following work describes experiments performed using DMT-induced secretions collected by Lee Robertson of the Scottish Crop Research Institute, Dundee.

**FIGURE 4.1.** Stained secretions produced from *G. pallida* following incubation with DMT in the presence of Coomassie Blue R250.

Washed nematodes were incubated with  $200\mu\text{gml}^{-1}$  DMT in the presence of 0.025% (w/v) Coomassie Blue R250 as described in Materials and Methods 2.10.1.

Stained secretions can be seen accumulated at the head of the nematode and also free in solution (both arrowed). Bar represents  $30\mu\text{m}$ .



#### 4.2.2.1. SDS-PAGE ANALYSIS OF INDUCED SECRETIONS

Secretions from about 1 million *G. rostochiensis* (Ro1) collected following overnight incubation with 200 $\mu$ gml<sup>-1</sup> DMT as described in Materials and Methods 2.3.5 were electrophoresed on a 12.5% SDS-PAGE homogenous gel using the Pharmacia Phast gel system. Proteins were then visualised by silver staining.

Silver staining reveals at least 16 distinct proteins, ranging in molecular weight from 14-100kDa (Figure 4.2). It is interesting to note that this gel closely resembles that obtained with collected ES (Figure 3.11), although some components appear to be more abundant.

#### 4.2.3. IMMUNOBLOT ANALYSIS OF INDUCED SECRETIONS

DMT-induced secretions from *G. rostochiensis* were collected and electrophoresed as described previously. In the following experiments collected secretions and whole body extracts of nematode proteins were electrophoresed on the same gel to allow a direct comparison of the proteins recognised. In all of the following blots *G. rostochiensis* (Ro1) only were used. The following sections describe the results obtained after blotting with Luffness anti-serum and ES anti-serum.

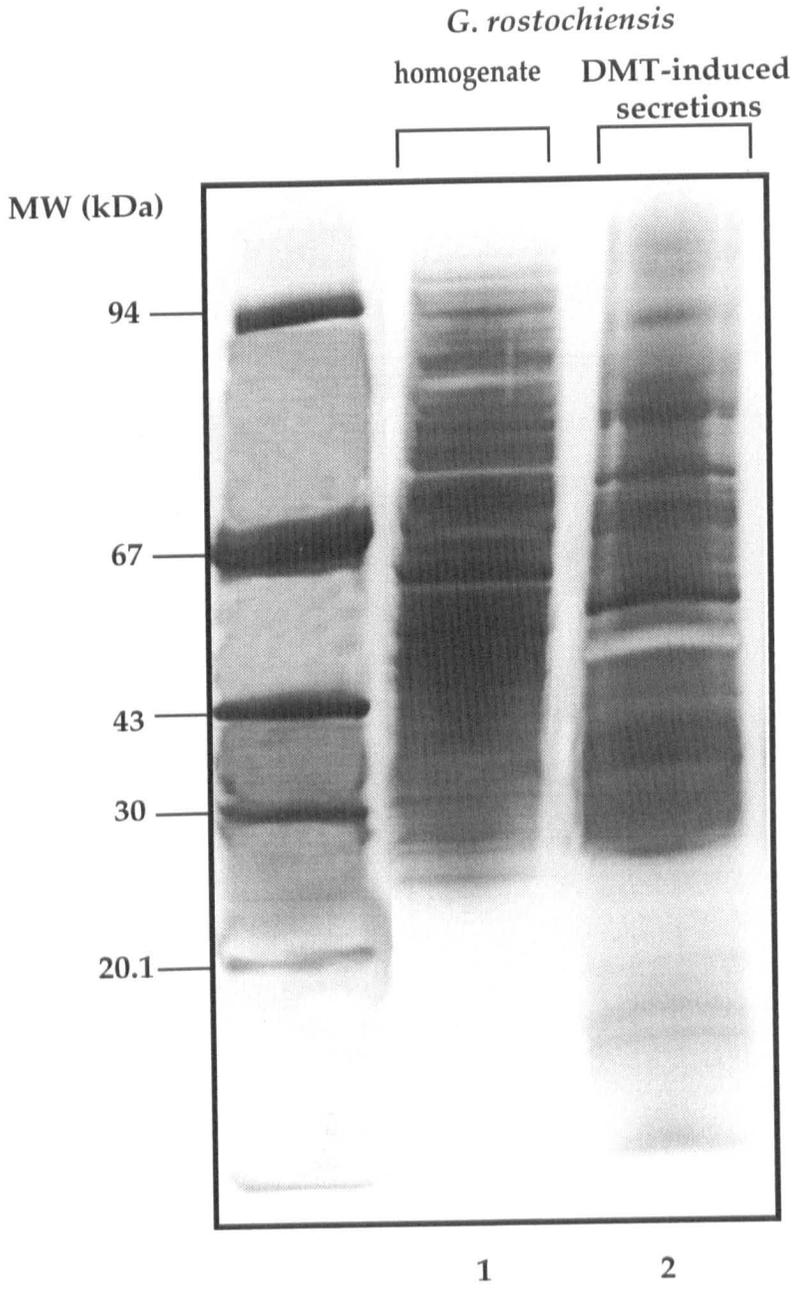
##### 4.2.3.1. ES ANTI-SERUM

When ES anti-serum was used to probe blots of DMT-induced secretions, the anti-serum recognised one band of approximately 90kDa. Figure 4.3 shows an immunoblot of whole body extracts and DMT-induced secretions of *G. rostochiensis* probed with ES anti-serum. Previous studies have shown that ES anti-serum recognised two bands between 90kDa and 30kDa in whole body extracts of nematodes (See Section

**FIGURE 4.2.** SDS-PAGE gel of secretions collected following incubation with DMT.

DMT-induced secretions were collected from *G. rostochiensis* Ro1 as described in Materials and Methods 2.3.5. Collected secretions were electrophoresed on a 10-20% gradient gel and silver stained as described in Materials and Methods 2.7.3. Approximately 15 $\mu$ g BSA-equivalent protein was loaded per lane.

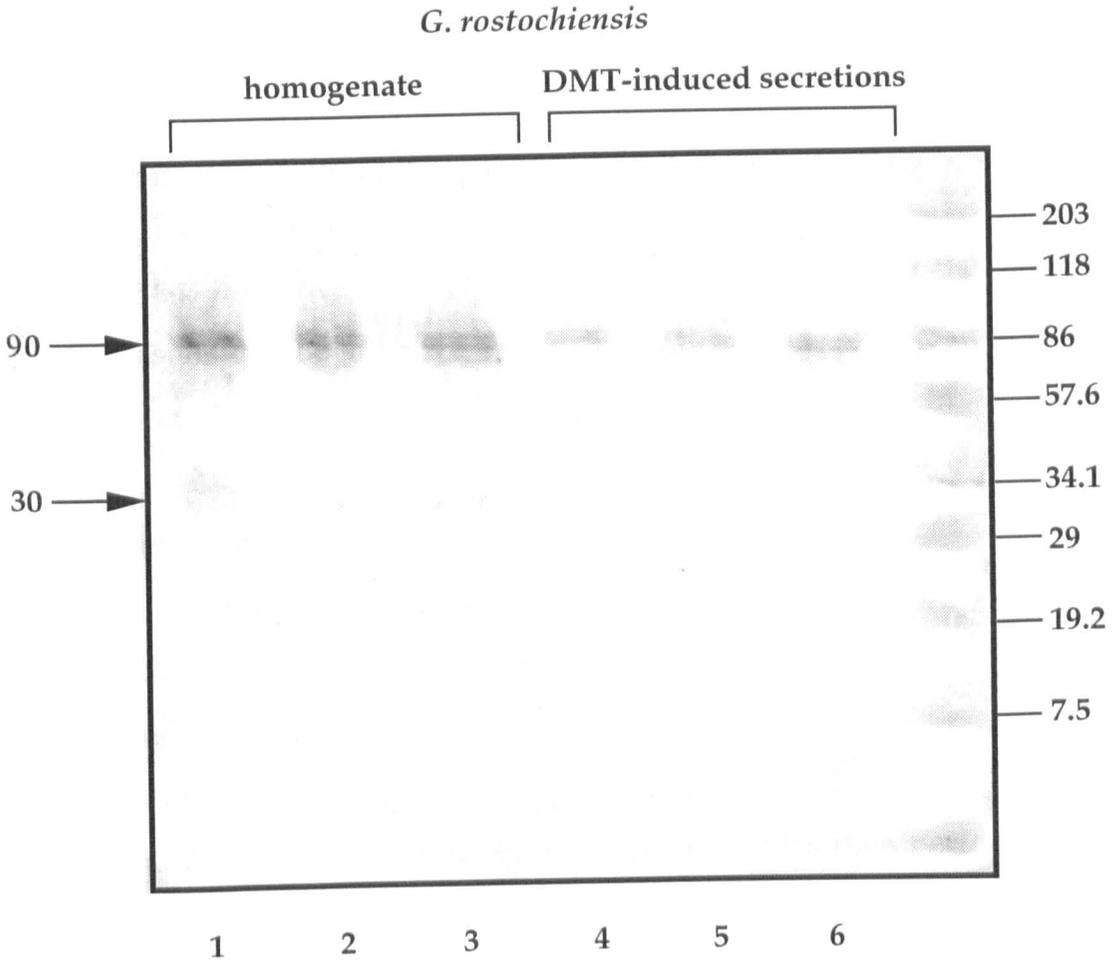
Lanes:           1 - *G. rostochiensis* Ro1 (homogenate)  
                  2 - *G. rostochiensis* Ro1 (DMT-induced secretions)



**FIGURE 4.3.** Immunoblot analysis of DMT-induced secretions probed with ES anti-serum.

DMT-induced secretions were collected as described in Materials and Methods 2.3.5. Secretions were electrophoresed on a 12.5% homogenous Phast gel followed by electroblotting onto nitrocellulose. Blots were probed with ES anti-serum (1:500). Approximately 0.125 $\mu$ g BSA-equivalent protein was loaded per lane.

Lanes:            1 - *G. rostochiensis* Ro1 (homogenate)  
                      2 - *G. rostochiensis* Ro1 (homogenate)  
                      3 - *G. rostochiensis* Ro1 (homogenate)  
                      4 - *G. rostochiensis* Ro1 (DMT-induced secretions)  
                      5 - *G. rostochiensis* Ro1 (DMT-induced secretions)  
                      6 - *G. rostochiensis* Ro1 (DMT-induced secretions)



3.2.7.1). The 90kDa protein therefore appears to be specific to DMT-induced secretions.

#### 4.2.3.2. LUFFNESS ANTI-SERUM

Luffness anti-serum was also used to probe blots of whole body extracts and DMT-induced secretions of *G. rostochiensis*. Surprisingly, Luffness anti-serum did not react with any component of DMT-induced secretions (Figure 4.4). In contrast, as described earlier, several bands were recognised in whole body extract samples.

#### 4.2.4. INDIRECT IMMUNOFLUORESCENCE STUDIES

From the results described above, it can clearly be seen that incubation of *G. pallida* and *G. rostochiensis* with DMT results in an increase in secretory activity. DMT also appeared to be able to cause changes at the surface of the parasite as indicated by the clumping phenomenon observed. It was therefore decided to perform indirect immunofluorescence studies to determine whether changes at the nematode surface could be visualised by antibody binding.

Nematodes were incubated with 200 $\mu$ gml<sup>-1</sup> DMT for 1 hour, then fixed in 1% formaldehyde. Indirect immunofluorescence was then performed using various anti-sera. The protocol was also repeated using living nematodes, and nematodes fixed after indirect immunofluorescence, all combinations giving identical results.

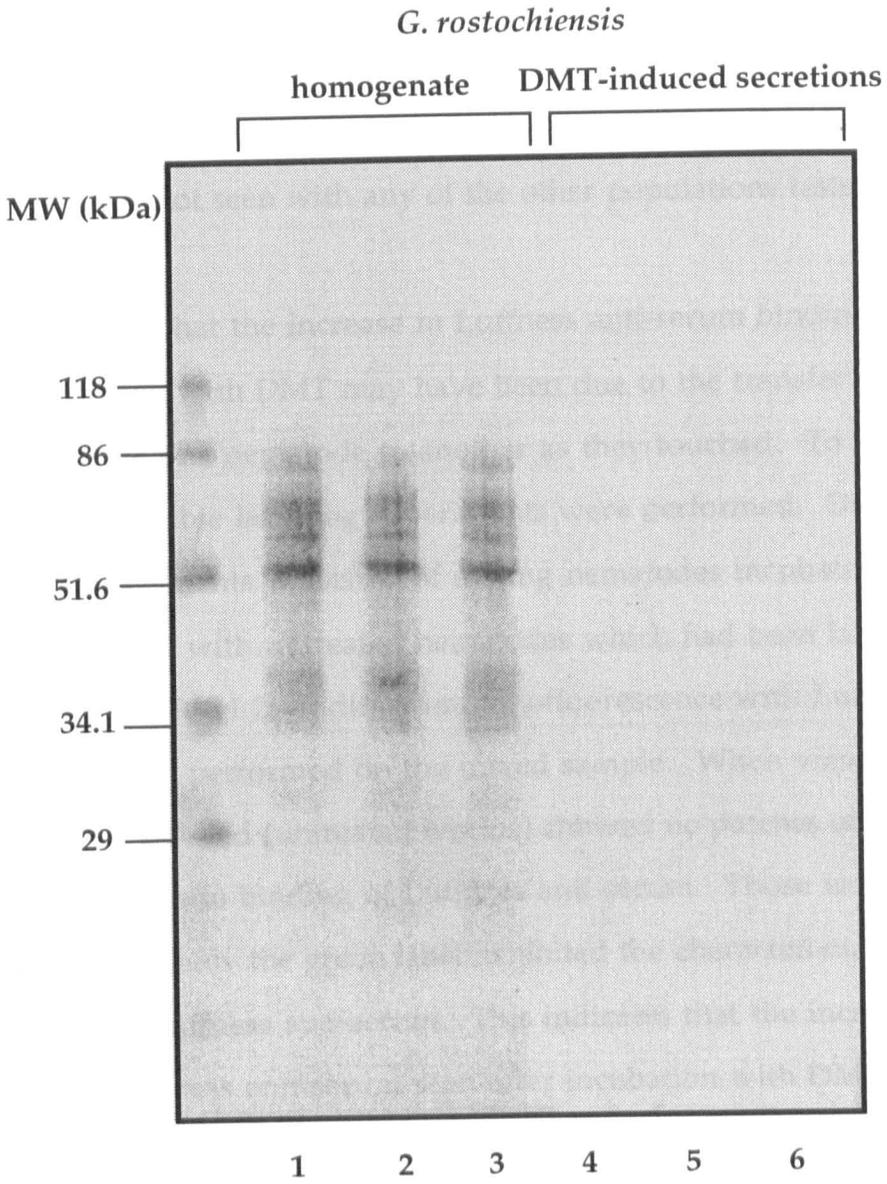
##### 4.2.4.1. LUFFNESS ANTI-SERUM

As described in Chapter 3, binding of Luffness anti-serum to *G. pallida* results in a distinctive pattern of antibody binding to the head and body of the nematode (illustrated in Figure 3.11). Following incubation with DMT, however, this distinctive pattern of antibody binding is

**FIGURE 4.4.** Immunoblot analysis of DMT-induced secretions probed with Luffness anti-serum.

DMT-induced secretions were collected as described in Materials and Methods 2.3.5. Secretions were electrophoresed on a 12.5% homogenous Phast gel followed by electroblotting onto nitrocellulose. Blots were probed with Luffness anti-serum (1:400). Approximately 0.125 $\mu$ g BSA-equivalent protein was loaded per lane.

Lanes:        1 - *G. rostochiensis* Ro1 (homogenate)  
                  2 - *G. rostochiensis* Ro1 (homogenate)  
                  3 - *G. rostochiensis* Ro1 (homogenate)  
                  4 - *G. rostochiensis* Ro1 (DMT-induced secretions)  
                  5 - *G. rostochiensis* Ro1 (DMT-induced secretions)  
                  6 - *G. rostochiensis* Ro1 (DMT-induced secretions)



dramatically altered. DMT appears to cause an increased and altered distribution of antibody binding over the entire surface of the nematode (Figure 4.5). As before, populations from both *G. pallida* and *G. rostochiensis* were tested. In all cases, incubation with DMT caused a marked increase in antibody binding to the surface of the nematode. One interesting observation made, however, was that incubation of *G. rostochiensis* population (pathotype) Ro3 with DMT resulted in shedding of bound antibody from the surface of the nematode (Figure 4.6). This was not seen with any of the other populations tested.

It was thought that the increase in Luffness anti-serum binding seen after incubation with DMT may have been due to the transfer of secretion from one nematode to another as they touched. To investigate this further, double labelling experiments were performed. Double labelling experiments consisted of mixing nematodes incubated with  $200\mu\text{gml}^{-1}$  DMT with untreated nematodes which had been labelled with the lipid probe PKH 2. Indirect immunofluorescence with Luffness anti-serum was then performed on the mixed sample. When viewed, the green PKH 2 labelled (untreated worms) showed no patches of surface fluorescence due to binding of Luffness anti-serum. Those worms which did not show the green label exhibited the characteristic increase in binding of Luffness anti-serum. This indicates that the increase in binding of Luffness anti-serum seen after incubation with DMT is not due to transfer of secretions from one nematode to another.

#### 4.2.4.2. ES ANTI-SERUM

As described previously, binding of anti-ES to untreated nematodes gave variable results, with only some nematodes showing a very faint binding of antibody to the surface. Since DMT results in an obvious

**FIGURE 4.5.** Binding of Luffness anti-serum to *G. pallida* Luffness population after incubation with DMT.

Nematodes were incubated with  $200\mu\text{gml}^{-1}$  DMT for 1 hour prior to indirect immunofluorescence with Luffness anti-serum (1:50) as described in Materials and Methods 2.9.1.

Binding of Luffness anti-serum can be seen all over the surface of *G. pallida*. Bar represents  $30\mu\text{m}$ .

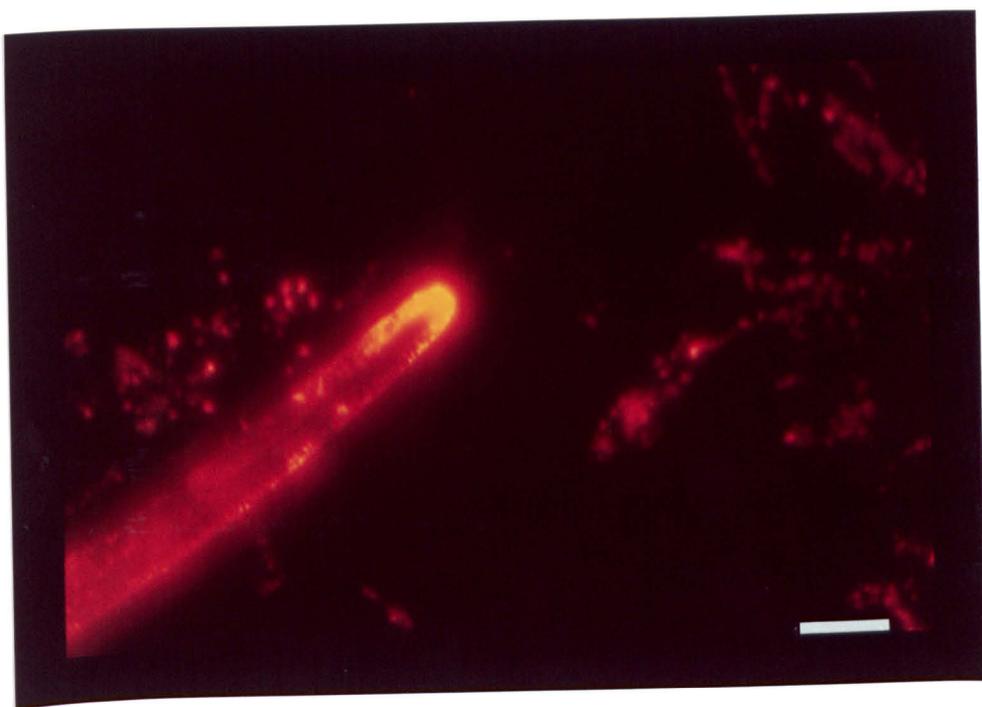


**FIGURE 4.6.** Binding of Luffness anti-serum to *G. rostochiensis* (Ro3) following incubation with DMT.

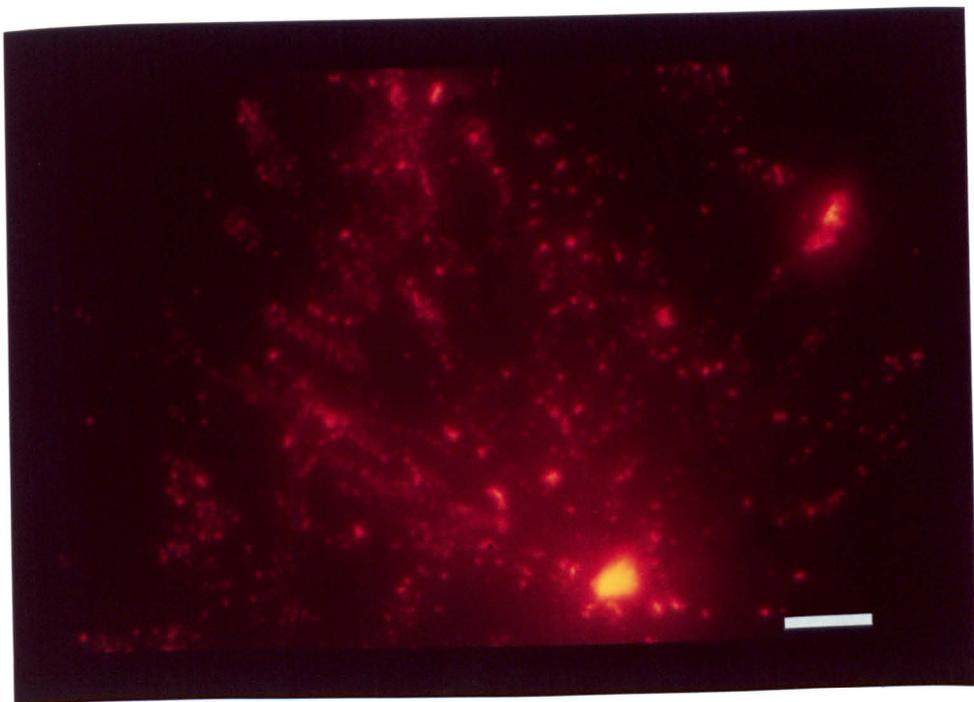
Nematodes were incubated with  $200\mu\text{gml}^{-1}$  DMT for 1 hour prior to indirect immunofluorescence with Luffness anti-serum (1:50) as described in Materials and Methods 2.9.1.

- a) Binding of Luffness anti-serum to *G. rostochiensis* (Ro3). Antibody can be seen bound to the nematode, although shedding from the surface is also apparent. Bar represents  $30\mu\text{m}$ .
  
- b) Binding of Luffness anti-serum to material that has been shed from the surface of *G. rostochiensis* (Ro3). Bar represents  $30\mu\text{m}$ .

a



b



increase in secretion, indirect immunofluorescence with anti-ES was performed on DMT treated worms to determine if antibody binding would increase. No changes, however, in antibody binding could be seen.

#### **4.2.4.3. ANTI-SERUM 5559**

To determine whether the phenomenon of increased antibody binding following incubation with DMT was unique to Luffness anti-serum, the procedure was repeated using anti-serum 5559. Anti-serum 5559 was a kind gift from Dr. Jock Forrest of the Scottish Crop Research Institute, Dundee. It is a polyclonal anti-serum raised in rabbits with specificity against the surface of *G. pallida*. On untreated nematodes, binding of anti-serum 5559 occurs uniformly over the length of the nematode (Figure 4.7a). Incubation of nematodes with DMT does not appear to alter the pattern of antibody binding (Figure 4.7b).

#### **4.2.4.4. NORMAL RABBIT SERUM**

As shown in Figure 3.13, NRS binds specifically to the amphids in an interaction that appears to be mediated by N-acetyl galactosamine. Incubation of nematodes with DMT prior to indirect immunofluorescence with NRS did not result in an increase of antibody binding.

#### **4.2.5. PERIODATE TREATMENT**

Indirect immunofluorescence of nematodes incubated with DMT shows that DMT causes a large increase in secretion, with a concomitant increase in binding of Luffness anti-serum. Previous experiments (described in Section 3.2.6) show that binding of Luffness anti-serum is directed against carbohydrate epitopes, as determined by periodate

**FIGURE 4.7. Binding of anti-serum 5559 to *G. pallida* Luffness population.**

Nematodes were incubated with 200 $\mu$ gml<sup>-1</sup> DMT prior to indirect immunofluorescence with anti-serum 5559 (1:50) as described in Materials and Methods 2.9.1.

- a) Binding of anti-serum 5559 to *G. pallida* Luffness population incubated in PBS alone. Bar represents 30 $\mu$ m.
  
- b) Binding of anti-serum 5559 to *G. pallida* Luffness population following incubation in 200 $\mu$ gml<sup>-1</sup> DMT for 1 hour. Bar represents 30 $\mu$ m.



treatment. Nematodes which had been incubated with DMT were thus exposed to periodate to determine if the secretions induced by DMT exhibited mainly carbohydrate epitopes.

Nematodes incubated in the presence of  $200\mu\text{gml}^{-1}$  DMT for 1 hour were fixed in 1% formaldehyde. Periodate treatment consisted of exposure to 10mM sodium metaperiodate for 1 hour prior to indirect immunofluorescence with Luffness anti-serum. After treatment no antibody binding could be observed on the surface of the nematode, although binding to the amphids could still be observed, indicating that all surface epitopes recognised by Luffness anti-serum are carbohydrate in nature.

#### 4.2.6. LECTIN BINDING STUDIES

To investigate whether exposure to DMT would increase lectin binding over the nematode surface, nematodes were incubated with  $200\mu\text{gml}^{-1}$  DMT for 1 hour then fixed in 1% formaldehyde. Fixed nematodes were then incubated with WGA, Con A, GNA and potato lectin fluorochrome conjugates ( $100\mu\text{gml}^{-1}$ ) as described in Materials and Methods 2.9.3. WGA, Con A and GNA bind specifically to amphidial secretions of PCN (Figure 3.1), whereas potato lectin exhibits no binding. After exposure to DMT, binding of WGA and Con A was identical to that of the PBS controls. Binding of potato lectin however, showed some slight differences. After DMT treatment a small number of nematodes showed very faint binding to the head region, although this was too faint to photograph.

#### 4.2.7. ELECTRON MICROSCOPY STUDIES

From previous results it can clearly be seen that DMT induces secretions in both *G. pallida* and *G. rostochiensis*. In section 4.2.2, it was shown that at least some of the secretions induced by DMT are surface-associated, and are recognised specifically by Luffness anti-serum. It was therefore decided to investigate the effect of DMT at an ultrastructural level, using sections probed with Luffness anti-serum.

##### 4.2.7.1. PRE-EMBEDDING IMMUNOGOLD LABELLING WITH LUFFNESS ANTI-SERUM

Nematodes which had been incubated with DMT for 0, 15 and 60 minutes were surface immunogold labelled using Luffness anti-serum. After labelling, nematodes were fixed in 3% glutaraldehyde, processed and stained as in Materials and Methods 2.13.1. Longitudinal sections were cut through labelled nematodes pre-treated with DMT for 0, 15 and 60 minutes respectively. From the electron micrographs, the architecture of the cuticle layers can clearly be seen, with the outermost layer, the epicuticle, present as a dark electron-dense layer. In nematodes not incubated with DMT, very sparse labelling of the surface and no outer layer of secretion above the epicuticle can be seen (Figure 4.8). After 15 minutes incubation with DMT, an increase in antibody binding was clearly visible, and a broken layer of secretion was seen lying on the epicuticle (Figure 4.9). Finally, after 60 minutes incubation with DMT, a thick layer of very heavily labelled secretion was apparent, lying above the epicuticle (Figure 4.10). These results confirm those results obtained with fluorescence microscopy.

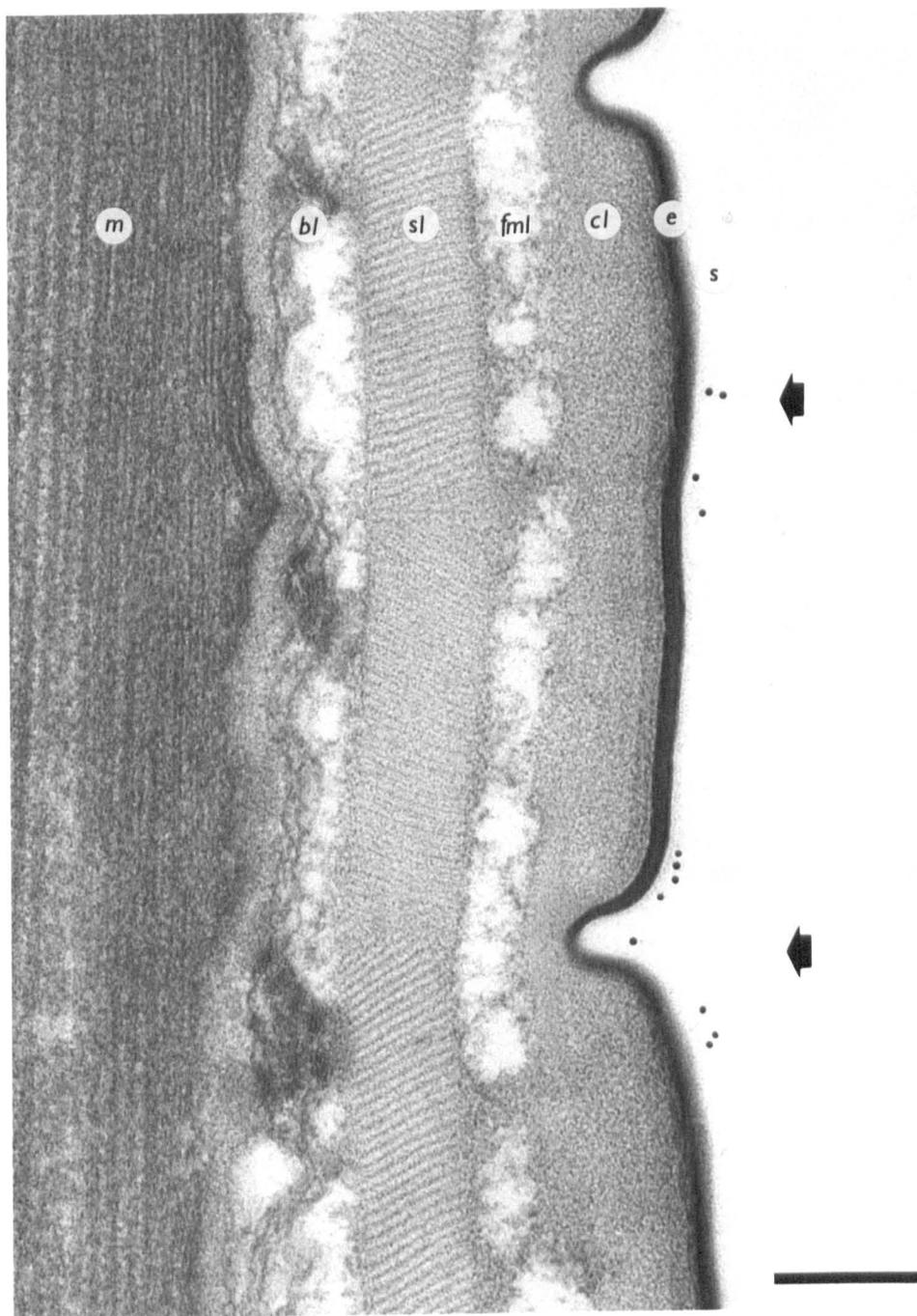
**FIGURE 4.8.** Longitudinal section through the cuticle of *G. pallida* Luffness population probed with Luffness anti-serum.

Washed nematodes were fixed, embedded and sectioned as described in Materials and Methods 2.13.2. Sections were probed with Luffness anti-serum (1:10 000). Longitudinal sections were cut through the cuticle as indicated in the diagram below.

A small area of antibody binding can be seen occurring on the surface of the nematode (arrowed). Bar represents 200nm.

Abbreviations: s, secretion; e, epicuticle; cl, cortical layer; fml, fenestrated median layer; sl, striated layer; bl, basal layer; m, muscle





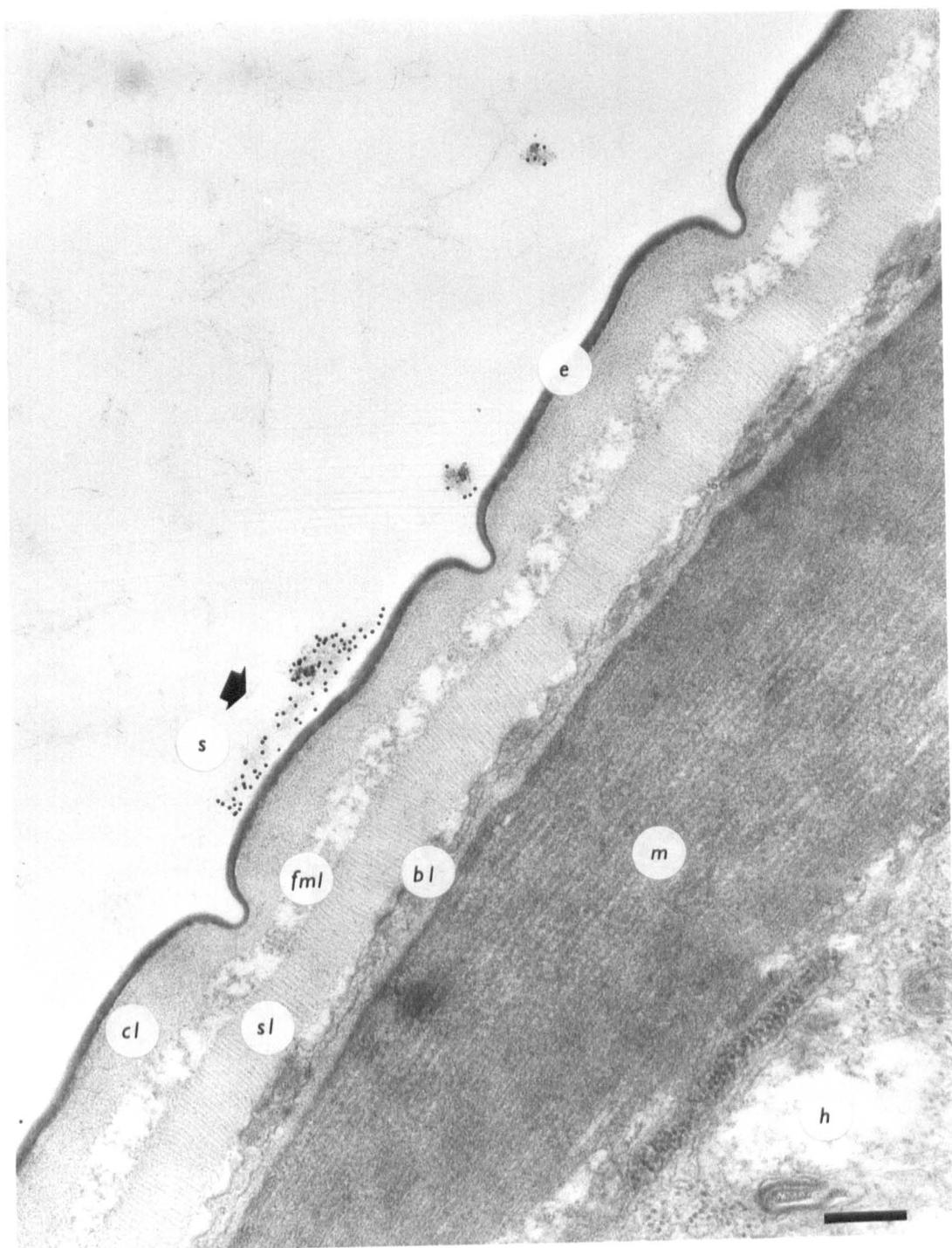
**FIGURE 4.9.** Longitudinal section through the cuticle of *G. pallida* Luffness population probed with Luffness anti-serum following incubation with DMT.

Nematodes were incubated with  $200\mu\text{gml}^{-1}$  DMT for 15 minutes then fixed, embedded and sectioned as described Materials and Methods 2.13.2. Sections were probed with Luffness anti-serum (1:10 000). Longitudinal sections were cut through the cuticle as indicated in the diagram below.

A small area of antibody binding can be seen occurring on the surface of the nematode (arrowed). Bar represents 200nm.

Abbreviations: s, secretion; e, epicuticle; cl, cortical layer; fml, fenestrated median layer; sl, striated layer; bl, basal layer; m, muscle





**FIGURE 4.10.** Longitudinal section through the cuticle of *G. pallida* Luffness population probed with Luffness anti-serum following incubation with DMT.

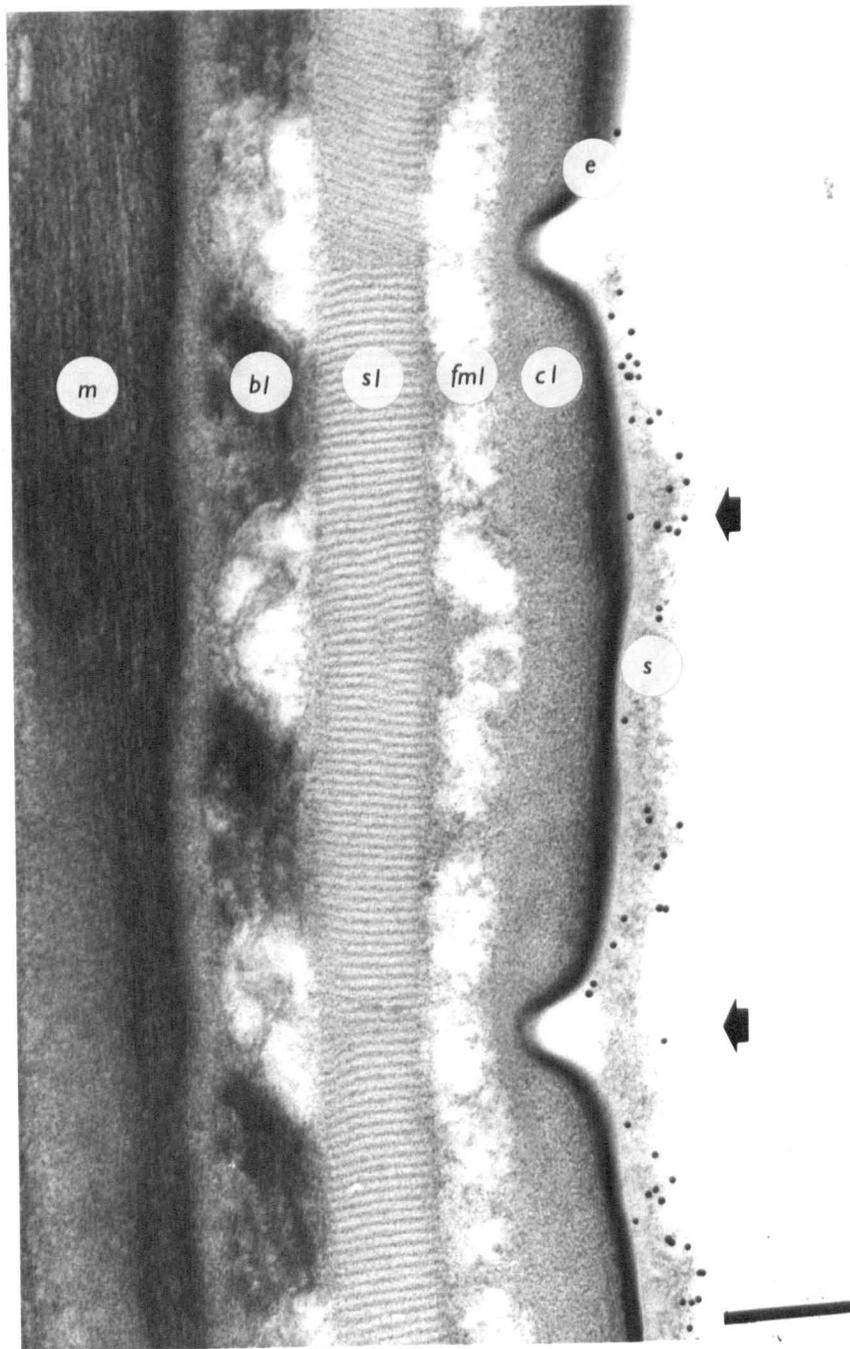
Nematodes were incubated with  $200\mu\text{gml}^{-1}$  DMT for 60 minutes then fixed, embedded and sectioned as described Materials and Methods 2.13.2.

Sections were probed with Luffness anti-serum (1:10 000). Longitudinal sections were cut through the cuticle as indicated in the diagram below.

Antibody binding can be seen occurring on the surface of the nematode (arrowed). Bar represents 200nm.

Abbreviations: s, secretion; e, epicuticle; cl, cortical layer; fml, fenestrated median layer; sl, striated layer; bl, basal layer; m, muscle





#### 4.2.8. INDUCTION OF SECRETIONS BY PHYTOHORMONES

Following the results obtained with DMT it was decided to investigate endogenous plant chemicals as possible agents that induce secretions. The structures of DMT and serotonin are similar to the phytohormone, indole acetic acid. It was therefore decided to test a panel of phytohormones to investigate their effect on the production of secretions. Nematodes were incubated with indole 3-acetic acid (IAA), gibberellin (GA<sub>3</sub>), abscisic acid (ABA) and kinetin as described in Materials and Methods 2.10.2. As before, all incubations were performed in the presence of 0.025% (w/v) Coomassie Blue R250.

Of the phytohormones tested, IAA alone appeared to be able to induce secretions of *G. pallida* and *G. rostochiensis*. Although incubation with IAA as described induced secretions in a similar manner to DMT, the time scale involved was much longer requiring an overnight incubation. Since the concentration of IAA used was relatively low in order to mimic levels *in planta*, it was decided to increase the concentration of IAA. When the concentration of IAA was increased to 25-50 µgml<sup>-1</sup>, secretions appeared within 3 hours. As before, the appearance of secretions was also associated with movement of the stylet.

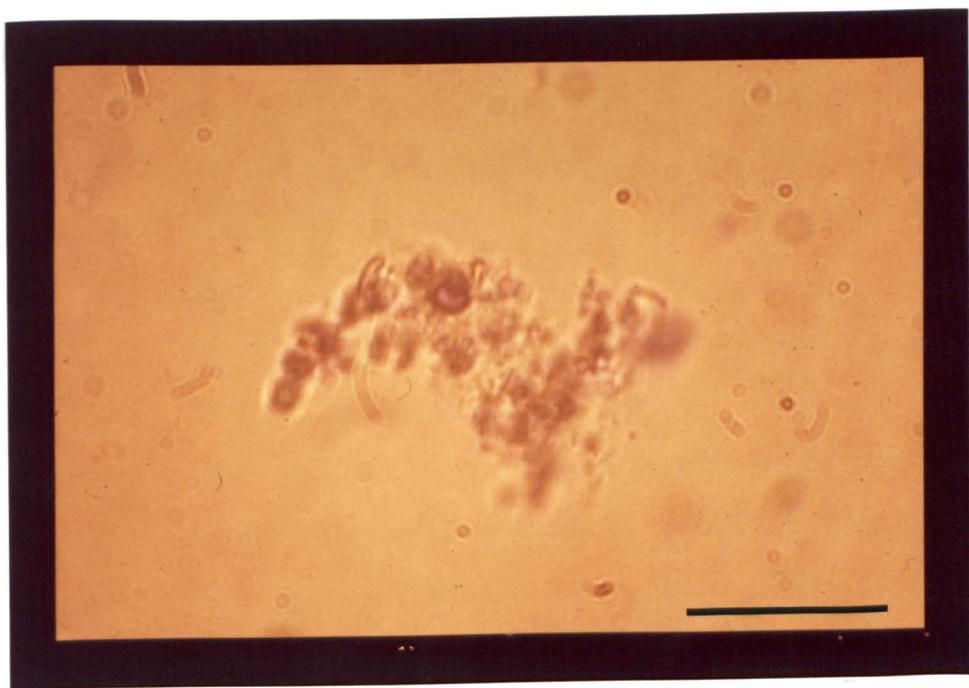
As was observed with DMT-induced secretions, various types of secretion appeared to be induced by IAA. As before, material could be seen apparently sloughing off from the sides of the nematode, as well as accumulating at the head of the nematode (Figure 4.11a). Interestingly, some nematodes produced secretions from the head which formed small regular spheres when free (Figure 4.11b). These maintained their regular form even when pressure was applied to the coverslip.

**FIGURE 4.11.** Secretions produced by *G. pallida* Luffness population following incubation with IAA.

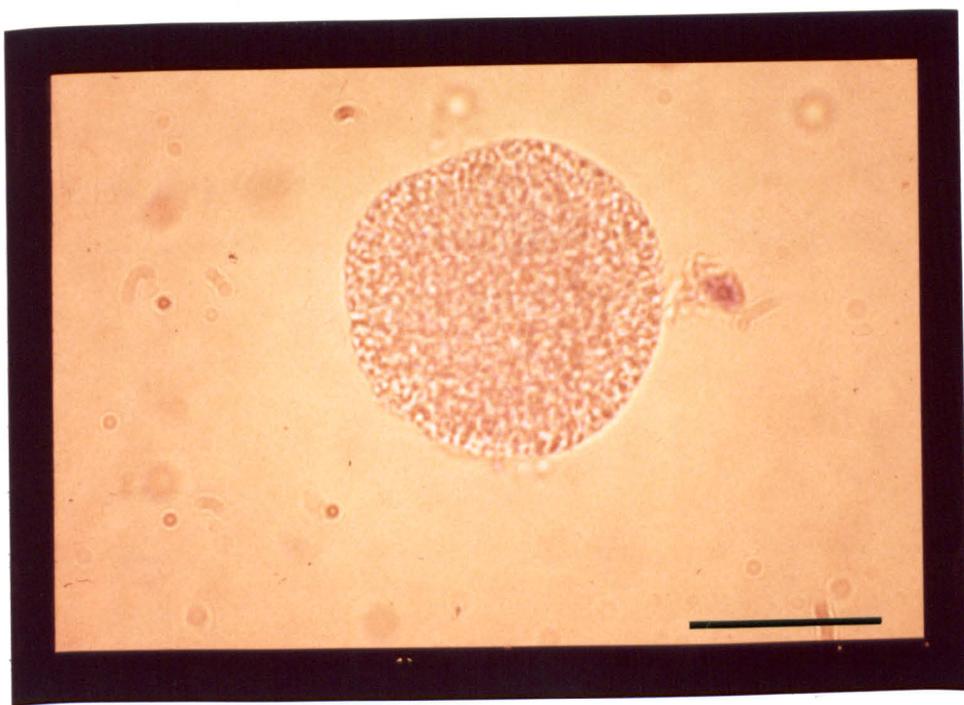
Washed nematodes were incubated with  $25\mu\text{gml}^{-1}$  IAA as described in Materials and Methods 2.10.1.

- a) Irregular form of secretion produced by *G. pallida* upon incubation with IAA. This form of secretion was generally found accumulated at the head of the nematode. Bar represents  $30\mu\text{m}$ .
  
- b) Regular form of secretion produced by *G. pallida* upon incubation with IAA. This form of secretion tended to be found free of the nematode. Bar represents  $30\mu\text{m}$ .

a



b



Secretions with such structures were never observed using DMT. Another dissimilarity to events observed with DMT is that incubation with IAA did not result in the adhesion of nematodes to one another. This appears to indicate that subtle differences may exist between secretions induced by DMT and IAA.

#### **4.2.9. INDIRECT IMMUNOFLUORESCENCE STUDIES**

Indirect immunofluorescence studies were performed using various anti-sera on nematodes incubated with IAA to determine whether IAA acted in the same manner as DMT. Nematodes were incubated in  $25\mu\text{gml}^{-1}$  IAA for 3 hours then fixed in 1% formaldehyde as described before. Indirect immunofluorescence was performed using various anti-sera.

##### **4.2.9.1. LUFFNESS ANTI-SERUM**

Following incubation with IAA, indirect immunofluorescence with Luffness anti-serum revealed an altered and increased distribution of antibody binding over the surface of the nematode (Figure 4.12). The effect seen was similar to that observed using DMT, although not quite as marked. The same pattern of antibody binding was seen in *G. pallida* and *G. rostochiensis*.

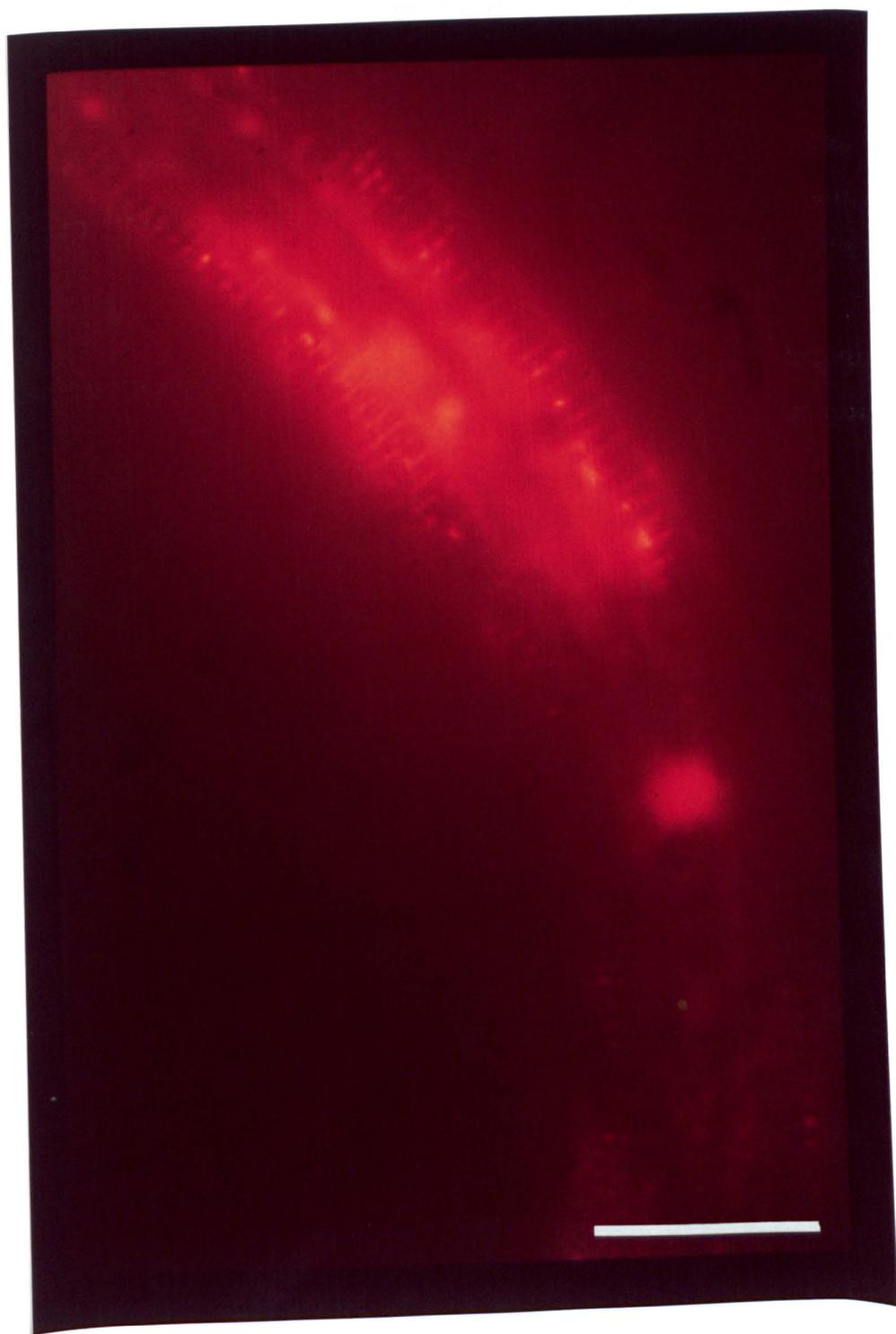
##### **4.2.9.2. OTHER ANTI-SERA**

Indirect immunofluorescence studies were also repeated using ES anti-serum, anti-serum 5559 and NRS. No increase in antibody binding was observed with any of these anti-sera, again indicating that IAA induces secretions recognised exclusively by Luffness anti-serum.

**FIGURE 4.12.** Binding of Luffness anti-serum to *G. pallida* population Luffness following incubation with IAA.

Nematodes were incubated with  $25\mu\text{gml}^{-1}$  IAA prior to indirect immunofluorescence with Luffness anti-serum as described in Materials and Methods 2.9.1.

Binding of antibody can be seen occurring over the body of the nematode. Bar represents  $30\mu\text{m}$ .



#### 4.2.10. LIPID PROBE STUDIES

From the results described above it can be seen that DMT and IAA can induce secretions in both *G. pallida* and *G. rostochiensis*. The induction of secretions associated with the onset of parasitism in PCN may indicate that IAA is acting as a host cue to the nematode. In previous studies by Proudfoot *et al.*, (1993a, b), it was shown that the surface of animal-parasitic nematodes undergoes rapid changes in response to initial contact with host signals. Of these changes, one of the most marked involved an alteration in the lipophilicity of infective larvae, so that insertion of the lipid probe 5-N-(octadecanoyl) aminofluorescein (AF18) increased following exposure to host conditions. This provides a simple marker with which to examine the effect of host signals on the parasite surface. It was therefore decided to examine the effect of DMT and IAA on insertion of AF18 and PKH 26 into the surface of the parasite. Initial studies were therefore performed with AF18 and PKH 26 to determine the pattern of insertion into the surface of the nematode.

##### 4.2.10.1. INSERTION OF 5-N-(OCTADECANOYL) AMINOFLUORESCIN

Washed nematodes were incubated with  $10\mu\text{gml}^{-1}$  AF18 for 10 minutes as described in Materials and Methods 2.11.1 to allow insertion of the probe into the nematode surface. When viewed, it could be seen that incubation with AF18 results in the even insertion of the probe over the whole surface of the nematode (Figure 4.13a).

##### 4.2.10.2. INSERTION OF PKH 26

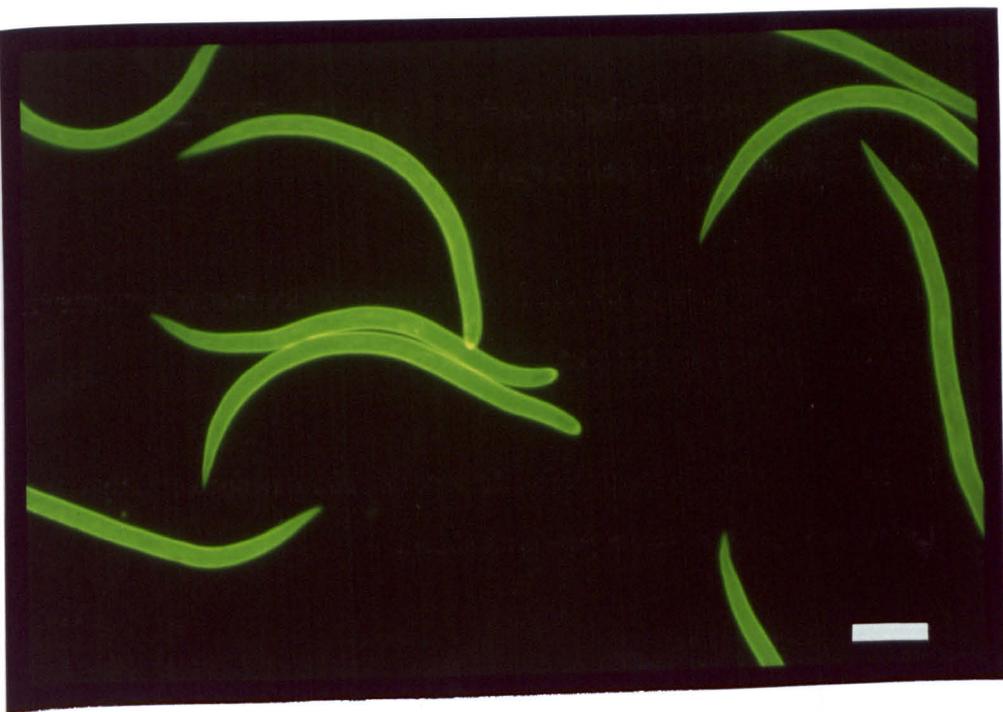
Washed nematodes were incubated with PKH 26 ( $1\mu\text{M}$ ) for 10 minutes as described in Materials and Methods 2.11.2. As with AF18, PKH 26 inserts evenly over the whole surface of the nematode (Figure 4.13b).

**FIGURE 4.13.** Insertion of lipid probes into the surface of *G. pallida*.

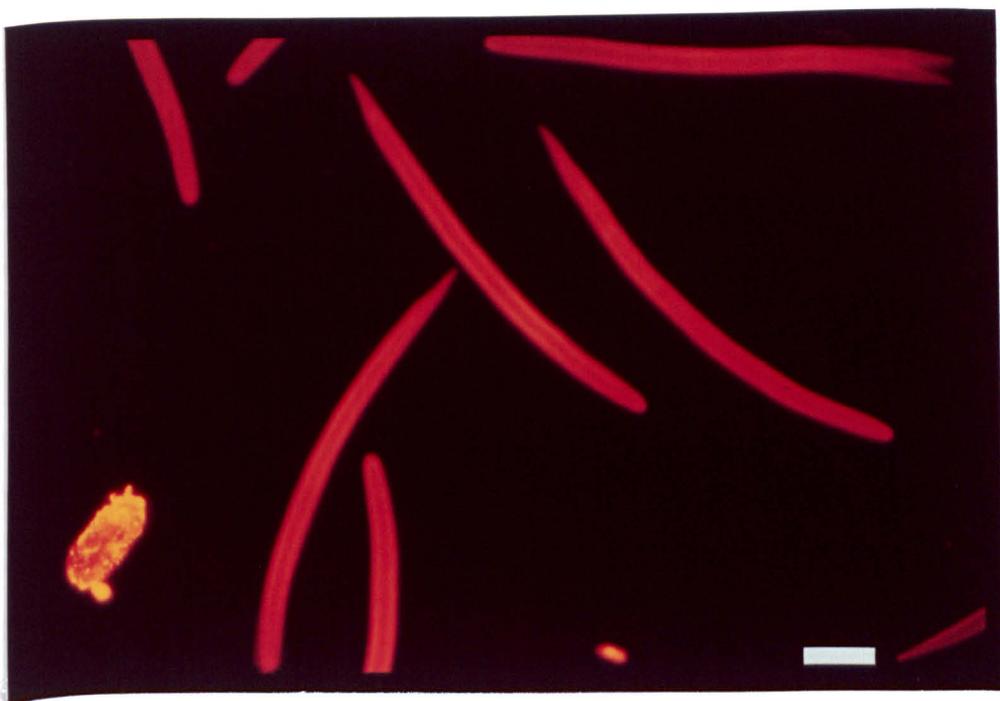
Washed nematodes were incubated with either AF18 or PKH 26 as described in Materials and Methods 2.11.

- a) Insertion of AF18 in to the surface of *G. pallida* occurs evenly over the whole surface. Bar represents 30 $\mu$ m.
  
- b) Insertion of PKH 26 in to the surface of *G. pallida* occurs evenly over the whole surface. Bar represents 30 $\mu$ m.

a



b



From these results it can be seen that both AF18 and PKH 26 insert evenly over the whole surface of the parasite, thus facilitating quantitation of surface fluorescence. Both AF18 and PKH 26, therefore were subsequently used to determine whether DMT and IAA could affect surface lipophilicity.

#### **4.2.11. EFFECT OF INDUCTION OF SECRETIONS ON LIPID PROBE INSERTION**

To determine whether the induction of secretions resulted in alterations in surface lipophilicity, nematodes were incubated with either DMT or IAA prior to labelling with either AF18 or PKH 26.

##### **4.2.11.1. METHOXY DIMETHYL TRYPTAMINE**

Washed nematodes were incubated with  $200\mu\text{gml}^{-1}$  DMT for 1 hour prior to incubation with either AF18 or PKH 26 as described previously. Quantitation of fluorescence was then performed to determine the effect of DMT. When nematodes were probed with AF18 following incubation with DMT, AF18 still inserted evenly over the whole surface of the nematode. When quantitation of fluorescence was performed, however, it revealed that insertion of AF18 had decreased by about 20% as compared to controls (Figure 4.14). As with AF18, when nematodes were probed with PKH 26 after incubation with DMT, PKH 26 still inserted evenly over the surface of the nematode. Quantitation, however, revealed that incubation with DMT reduced PKH 26 insertion by approximately 20% (Figure 4.14).

##### **4.2.11.2. INDOLE 3-ACETIC ACID**

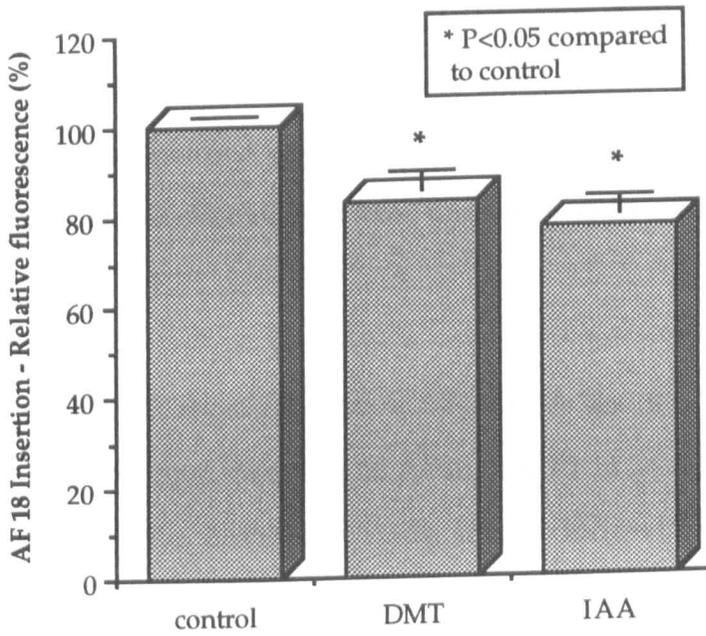
Washed nematodes were incubated with  $25\mu\text{gml}^{-1}$  IAA for 3 hours prior to incubation with either AF18 or PKH 26 as described previously. Quantitation of fluorescence was then performed to determine the effect

**FIGURE 4.14.** Effect of DMT and IAA on the insertion of lipid probes in to the surface of *G. pallida*.

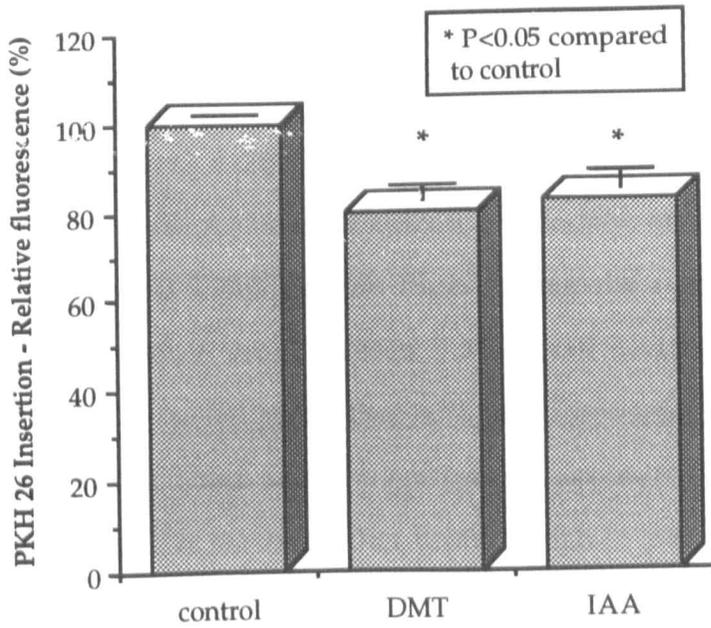
Washed nematodes were incubated with either IAA ( $25\mu\text{gml}^{-1}$ ) for 3 hours or DMT ( $200\mu\text{gml}^{-1}$ ) for 1 hour, then probed with AF18 or PKH 26 as described in Materials and Methods 2.11. Quantitation of surface fluorescence was performed as described previously.

The results are presented as a histogram. It is possible to see that incubation with either DMT or IAA results in a 20% decrease in total fluorescence compared to controls ( $P < 0.05$  when compared to control). This is true of both AF18 and PKH 26 insertion.

## AF 18



## PKH 26



of IAA upon insertion of the probes. When nematodes were probed with AF18 following incubation with IAA, a decrease in AF18 insertion of about 20% was observed. Similarly, PKH 26 insertion also decreased by about 20% after incubation with IAA (Figure 4.14). These results therefore appear to be in contrast with those obtained using animal-parasitic nematodes, where exposure to host cues results in an increase in surface lipophilicity.

#### **4.2.12. TRYPAN BLUE QUENCHING OF SURFACE FLUORESCENCE**

In order to determine whether insertion of AF18 and PKH 26 was confined to the surface layers of the nematode, it was decided to perform Trypan Blue quenching studies. Nematodes were probed with AF18 or PKH 26 as described previously, then Trypan Blue added to give a final concentration of 0.25% (w/v). Quantitation of fluorescence was then performed as before. Trypan Blue quenching studies were also performed on nematodes incubated with DMT and IAA to determine if either of these treatments resulted in internalisation of the lipid probes.

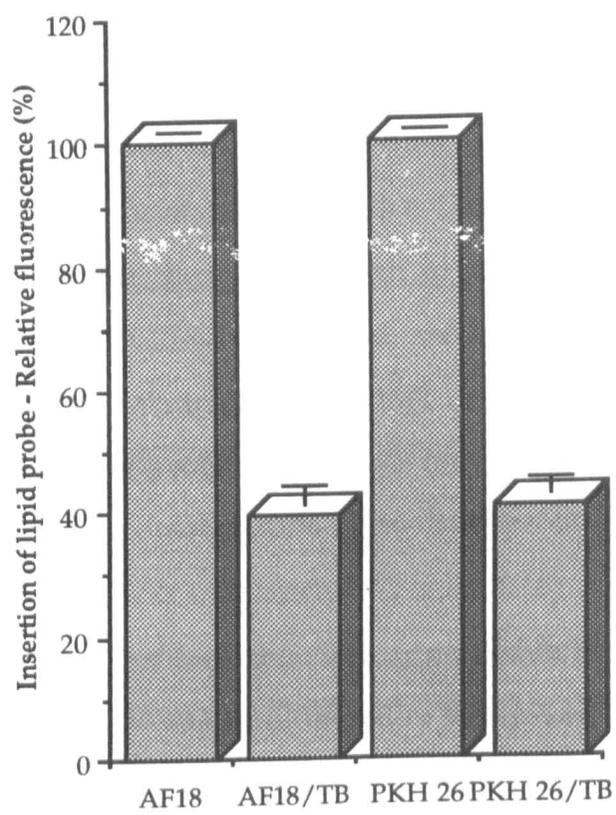
##### **4.2.12.1. 5-N-(OCTADECANOYL) AMINOFLUORESCIN**

Nematodes were labelled with AF18 as described previously, and surface fluorescence quenched using 0.25% Trypan Blue. When the remaining fluorescence was quantitated, it could be seen that overall fluorescence had decreased by around 60% indicating that 40% of fluorescence is due to probe that can not be quenched, and is therefore not accessible to Trypan Blue (Figure 4.15). It is assumed that probe which is inaccessible to Trypan Blue is internalised.

**FIGURE 4.15.** Trypan Blue quenching of lipid probes inserted in to the surface of *G. pallida*.

Washed nematodes were labelled with AF18 or PKH 26 as described in Materials and Methods 2.11 and Trypan Blue added to give a final concentration of 0.25%. Quantitation of fluorescence was performed as described earlier.

The results are presented as a histogram. It can be seen that quenching of surface fluorescence with Trypan Blue results in a decrease in fluorescence of 60% with both AF18 and PKH 26.



#### 4.2.12.2. PKH 26

As with AF18, quantitation of fluorescence following Trypan Blue quenching revealed that fluorescence had decreased by around 60% (Figure 4.15).

#### 4.2.13. TRYPAN BLUE QUENCHING OF FLUORESCENCE FOLLOWING INDUCTION OF SECRETIONS

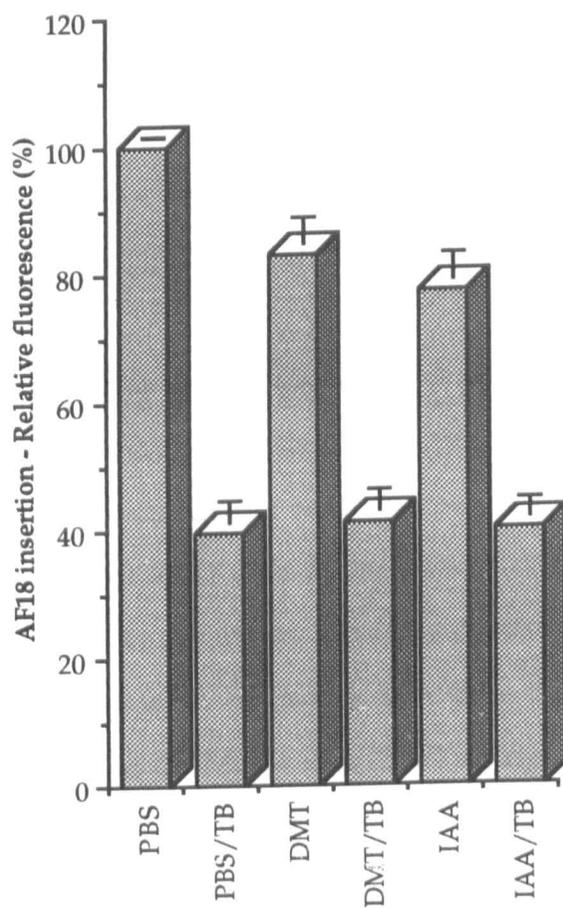
The studies described above indicated that AF18 and PKH 26 were acting in a similar manner. Further studies were thus carried out using AF18 alone. Nematodes were incubated with either DMT ( $200\mu\text{gml}^{-1}$ , 1 hour) or IAA ( $25\mu\text{gml}^{-1}$ , 3 hours), then probed with AF18 as described previously. Trypan Blue (0.25%) was added, and quantitation of surface fluorescence performed. Incubation with DMT or IAA appeared to have no effect on the amount of probe unavailable for quenching. In both cases there was still approximately 40% of the lipid probe (relative to untreated control) which was unquenched (Figure 4.16). Incubation of the nematodes with DMT or IAA therefore appeared to have no effect on the amount of probe internalised, therefore differences observed when fluorescence was quantitated were due to differences occurring in surface fluorescence. Since 60% of fluorescence is surface associated, and incubation with DMT or IAA results in a 20% decrease in total fluorescence, this means that in effect there is a 33% decrease in surface fluorescence (as opposed to total fluorescence) following incubation with DMT or IAA.

Some other interesting observations were made during Trypan Blue quenching experiments. It can be observed that in any sample of freshly harvested nematodes, a very small proportion will exhibit movement of the stylet, although no associated secretions will be produced. In the

**FIGURE 4.16.** Effect of DMT and IAA on Trypan Blue quenching of lipid probes inserted in to the surface of *G. pallida*.

Washed nematodes were incubated with either IAA ( $25\mu\text{gml}^{-1}$ ) for 3 hours or DMT ( $200\mu\text{gml}^{-1}$ ) for 1 hour, then probed with AF18 as described in Materials and Methods 2.11. Trypan Blue quenching of surface fluorescence was carried out by adding Trypan Blue to give a final concentration of 0.25%. Quantitation was then performed as described earlier.

The results are presented as a histogram. It can be seen that incubation with DMT or IAA does not change the amount of probe inaccessible to quenching which remains at 40%.



samples tested those nematodes exhibiting movement of the stylet also showed staining of the amphidial canals and associated cell bodies (Figure 4.17). The number of nematodes staining in this manner did not increase upon incubation with DMT or IAA, although it is of interest to note that it was confined to nematodes which exhibited stylet thrusting. However, although all nematodes stained in this manner exhibited stylet thrusting, not every nematode exhibiting stylet thrusting (many in the case of DMT treatment) showed staining.

### 4.3. DISCUSSION

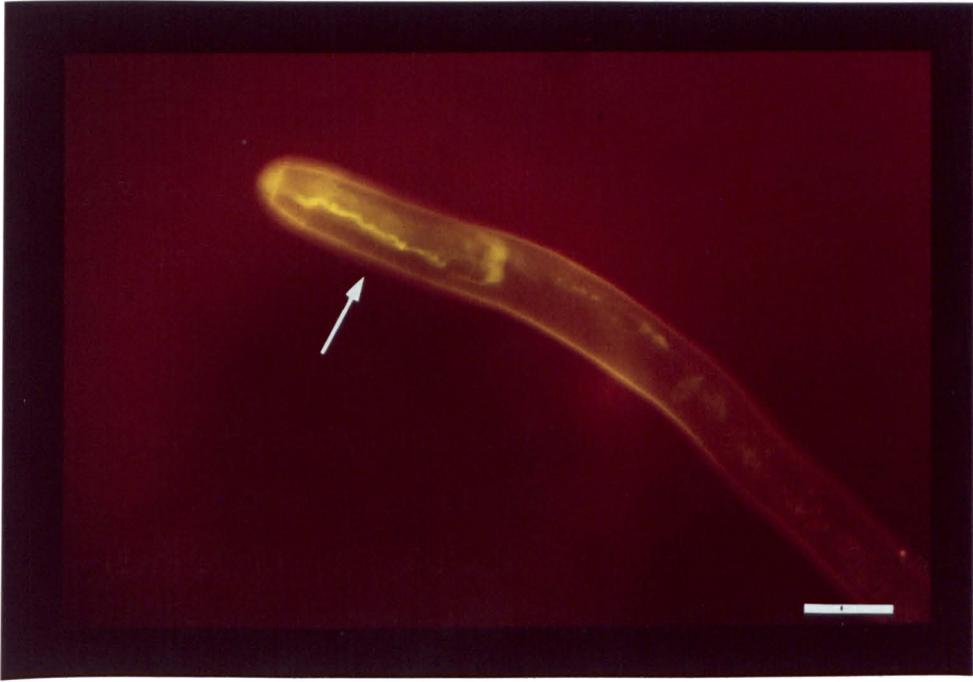
Initially the work in this chapter was started with the aim of finding a more efficient manner of inducing collectable amounts of secretions from *Globodera* spp. Previous reports have successfully described the use of the serotonin agonist DMT as a method for inducing *Heterodera* secretions, and thus initial experiments were performed to see if DMT had a similar effect on *Globodera*. It can be seen from the results described that incubation of *Globodera* with DMT causes a marked change at the surface of the parasite, as well as inducing large amounts of secretion. One of the initial observations made was the phenomenon that following addition of DMT it was possible to see nematodes adhering to one another. At first it was thought that this could be occurring due to transfer of secretions between nematodes. Various double labelling experiments, however, showed that there was no transfer of secretions from one nematode to another, indicating that profound changes were occurring at the surface of the nematode. These secretions therefore may have moved through the cuticle following stimulation of the nematode with DMT.

**FIGURE 4.17.** Staining of the amphidial canals of *G. pallida* with lipid probes following surface quenching by Trypan Blue.

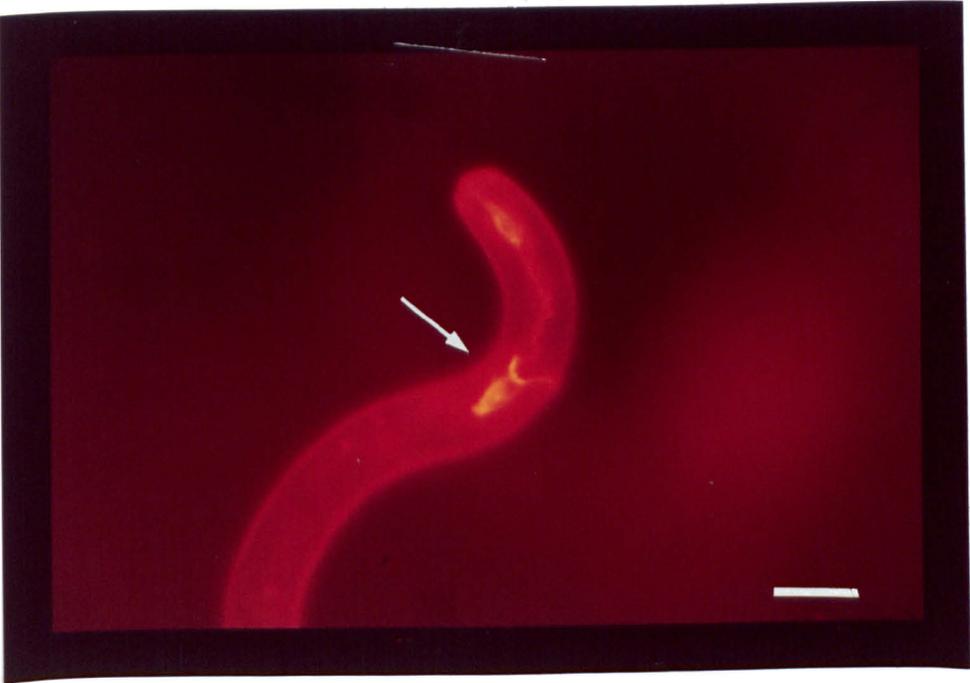
Washed nematodes were labelled with AF18 or PKH 26 as described in Materials and Methods 2.11 and Trypan Blue added to give a final concentration of 0.25%.

- a) AF18 labelling of the amphidial canals of *G. pallida* (labelled) following Trypan Blue quenching. Bar represents 30µm.
- b) PKH 26 labelling of the amphidial canals of *G. pallida* (labelled) following Trypan Blue quenching. Bar represents 30µm.

a



b



SDS-PAGE electrophoresis of DMT-induced secretions also yielded interesting information. As expected, DMT-induced secretions were completely different from those collected using Method 1 described in Chapter 3. More interestingly, when compared to secretions collected using Method 2, the majority of bands appeared to be in common. Several components, however, could be seen in DMT-induced secretions that had appeared to become more abundant.

Following these observations it was decided to investigate the effect of DMT on binding of various anti-sera to the surface of nematodes. With Luffness anti-serum an altered and increased distribution of antibody binding to the nematode surface was immediately obvious following incubation with DMT. When these experiments were repeated using anti-serum 5559 (anti-serum with specificity against the surface of *Globodera*), ES anti-serum and various lectins, no differences could be observed indicating that the phenomenon observed was unique to the Luffness anti-serum. Results described in Chapter 3 indicate that Luffness anti-serum recognises various secretions produced by *Globodera*, some of which are surface associated. The differences described in antibody binding however can not only be attributed to increased secretion from the mouth, amphids and/or excretory pore. When nematodes are incubated with DMT in the presence of 0.025% Coomassie Blue R250 sheetlike secretions could be seen apparently sloughing off from the sides of the nematodes, as well as secretions produced from the head region. It appears then, that at least some of the antibody binding seen may be due to material that has moved through the cuticle. This appears to be corroborated by the double labelling experiments described earlier. Immunogold labelling of cut sections was attempted to determine if this was the case. Unfortunately, the fixation

and embedding process left specimens with the same refractive index as the embedding resin, making it near impossible to orientate and cut the specimen from within the block. Several attempts were made to cut sections processed in this way, all unsuccessfully. However, it is hoped that attempts will be successful in the future. Results obtained from such specimens would allow observations to be made on whether secretions are indeed passing through the cuticle.

Other populations of both *G. pallida* and *G. rostochiensis* were also incubated with DMT. All of the populations tested showed increased secretion as determined by visualisation with Coomassie Blue, and binding of Luffness anti-serum. With population (pathotype) Ro3 however, shedding of bound antibody was observed. This was not seen with any of the other populations tested. Periodate treatment of nematodes incubated with DMT was also carried out to determine whether induced secretions differed from those recognised by Luffness anti-serum on untreated nematodes. Periodate treatment completely abolished antibody binding on nematodes as before, again indicating that the secretions must have a high carbohydrate content. It is interesting to note that despite results which indicate the high carbohydrate content of these associated secretions, there is a remarkable paucity of lectin binding on the surface of *Globodera*.

Secretions induced by DMT were also used for immunoblotting studies. Results obtained with indirect immunofluorescence studies had indicated that Luffness anti-serum appeared to specifically recognise secretions induced by DMT. Surprisingly then, when DMT-induced secretions were immunoblotted with Luffness anti-serum no reactivity could be seen on the blots. In contrast, when blots were probed with ES

anti-serum, one band of 90kDa was recognised. These results have several implications. They suggest that DMT induces two distinct sources of secretions, most obviously those surface-associated secretions recognised by Luffness anti-serum, as well as an increase in soluble secretions that do not associate with the nematode surface and are recognised by ES anti-serum.

As stated earlier, one aim of the work performed in this chapter was that of finding alternative methods of inducing secretions in *Globodera*. Previous work by McClure & von Mende (1987) had demonstrated that some plant compounds such as catechol and caffeic acid could induce secretory activity in various plant-parasitic nematodes. With this in mind it was decided to incubate *Globodera* with a range of different phytohormones.

Of those tested IAA alone was able to induce secretions in the same manner as DMT. At this point it is interesting to note the similarity in structure between DMT, serotonin and IAA. Indeed, this raises interesting questions about the mode of action of DMT and IAA. It seems likely that IAA acts as a signal to the nematode, the implication being that such a response must be receptor mediated. The efficiency of DMT in inducing secretions may be due to its similarity in structure to IAA. Alternatively, DMT may simply be acting as a serotonin agonist resulting in contraction of nematode musculature (especially those muscles controlling the oesophageal gland ducts) with the resulting production of secretions as described. Serotonin receptors have been described in the animal parasitic nematode, *Ascaris suum* (Albrecht & Walter, 1991; Brownlee *et al.*, 1995), and in the free-living nematode *Caenorhabditis elegans* (Johnson *et al.*, 1996). In *A. suum*, the

pharyngeal component of the enteric nervous system exhibits immunoreactivity for serotonin. Addition of serotonin to the nematode resulted in a stimulation of pharyngeal pumping (Brownlee *et al.*, 1995). DMT may therefore have a similar effect in *Globodera*. One obvious way to test this hypothesis would be immunolocalisation studies using anti-sera raised against serotonin. The similarity in structure between the compounds indicates however that a shared receptor may be involved.

From these results the hypothesis can be presented that IAA may act as a cue which prompts the nematode from a pre-parasitic to the parasitic stage of infection. It is reasonable to assume that plant-parasitic nematodes such as *Globodera* must receive host cues as they move from a free-living soil environment to the environs of the plant root. Detailed observations of *Heterodera* following infection show that there are several events associated with infection, including the commencement of stylet thrusting, and the production of oesophageal secretions (Wyss & Zunke, 1986; Wyss, 1992). Exposure to both DMT and IAA results in stylet thrusting and the production of oesophageal secretions. Previous studies by Proudfoot *et al.* (1993a,b) have demonstrated that the surface of animal-parasitic nematodes undergoes rapid changes in response to initial contact with host signals. One of the most marked changes involved an alteration in the lipophilicity of infective larvae so that insertion of the lipid probe 5-N-(octadecanoyl) aminofluorescein (AF18) increased following exposure to host conditions. This provides a simple marker with which to examine the effect of host signals on the parasite surface.

Exposure to DMT or IAA results in events associated with the onset of infection (stylet thrusting, and production of secretions), and as such may be regarded as mimicking host stimuli. Quantitation of AF18 insertion into the surface of *Globodera* following incubation with DMT or IAA therefore seemed an obvious experiment to perform. From previous results obtained with animal-parasitic nematodes it would not have been unreasonable to expect an increase in AF18 insertion following exposure to IAA or DMT. However, results obtained show that the opposite of this is true, and that incubation with IAA or DMT in fact causes a decrease in total AF18 insertion by 20%, although this is a 33% decrease in surface fluorescence as determined by Trypan Blue quenching. It is known that following incubation with DMT or IAA there is a large increase in the amount of highly glycosylated secretions upon the surface of the nematode. It has been shown with other parasites that a high degree of surface glycosylation can prevent effective insertion of lipid probes. Indeed, Foley *et al.* (1988) demonstrated that the lipid probe merocyanine would only insert into larval stages of *Schistosoma mansoni* following transformation of cercaria with the concomitant loss of the cercarial glycocalyx.

Trypan blue quenching studies similarly revealed interesting data about insertion of AF18. From the results it can be seen that approximately 40% of the AF18 is internalised, and that the proportion of internalised AF18 does not change following treatment with DMT or IAA. One other observation made as a result of Trypan blue quenching studies was that a very small proportion of all the samples tested showed staining of the amphidial canals and associated cell bodies. In all cases this was observed in approximately 1% of each sample, irrespective of treatment. In all cases such staining was associated with stylet thrusting. It should

be noted however, that the converse is not true. Since such staining is associated with stylet thrusting (even in untreated samples) this may be a reflection on the ability of such nematodes within a population to respond extremely quickly to host stimuli. To test such a hypothesis would involve isolating those nematodes displaying early stylet thrusting for use in infection bioassays.

The results presented in this chapter suggests the intriguing hypothesis that the phytohormone IAA acts as a host signal to *Globodera*. Data presented in the final results chapter tests this hypothesis by examining *Globodera* for the presence of an auxin binding protein (ABP) using anti-serum raised against the auxin binding domain of maize ABP.

**CHAPTER 5**  
**IDENTIFICATION OF A**  
**PUTATIVE NEMATODE AUXIN**  
**BINDING PROTEIN**

## 5.1. INTRODUCTION

The work described in Chapter 4 presents evidence that the phytohormone indole 3-acetic acid (IAA) acts as a signal to *G. pallida* and *G. rostochiensis* to produce secretions associated with the onset of parasitism. One implication of this, therefore, is that IAA may interact with a specific receptor to give the responses described.

Receptor-mediated events are generally associated with intracellular signalling pathways that occur upon release of second messengers (Berridge, 1993; Foster, 1993; Bygrave & Roberts, 1995). Methods, therefore, of investigating the second messenger systems that may be involved in a known response include the addition of exogenous second messengers, or second messenger agonist or antagonists directly to the system. In nematodes, however, the impermeability of the cuticle poses a barrier to the entry of compounds that may elicit a response. This obstacle has been recently overcome by the use of a novel set of compounds known as photoreactive caged compounds (For a review, see McCray & Trentham, (1989). The term "caged" was originally used to describe photosensitive precursors in which the effector is rendered inert and hydrophobic through a covalent bond to the photosensitive group (Kaplan *et al.*, 1978).

Activation of the caged molecule is achieved by brief exposure to ultra-violet light ( $\lambda = 310\text{-}360\text{nm}$ ) which breaks the photosensitive linkage, releasing the active molecule.

As described in Chapter 4, insertion of the lipid probe AF18 in animal-parasitic nematodes is a convenient marker of induced lipophilicity that occurs following the receipt of host signals. Using AF18 as an insertion marker, Modha *et al.*, (1995), utilised caged second messengers to dissect the pathway involved in the signal transduction mechanism leading to

increased surface lipophilicity of the surface of *Trichinella spiralis*. The initial work described in this chapter, then, describes experiments performed with caged compounds which were used to attempt to identify some of the second messengers activated upon interaction with DMT or IAA. In these experiments, insertion of AF18 as a measure of surface lipophilicity was chosen rather than the induction of secretions since AF18 insertion is easily quantifiable.

In conjunction with the second messenger investigations, various experiments were performed to determine whether *Globodera* possessed proteins containing an IAA (auxin) binding domain. Although the identification of auxin (IAA) binding proteins (ABPs) in plants has been the subject of much discussion, convincing evidence has been presented by Venis and Napier (1995) for the existence of an auxin binding protein (ABP1) from *Zea mays* which fulfils the criteria expected of a genuine receptor. Their work with ABP1 has led to the production of various anti-sera (Napier *et al.*, 1988) which were shown to be specific against conserved regions of ABP1 (Napier & Venis, 1990). Of particular interest is anti-serum D16 which was raised against a sixteen amino acid sequence shown to embrace the auxin binding domain of ABP1 (Venis *et al.*, 1992). Raised against a synthetic oligopeptide embracing the auxin binding region, D16 recognises all maize ABP isoforms, as well as ABP homologues in several other plant species tested. D16 is also able to induce hyperpolarisation of the transmembrane potential of tobacco protoplasts in a manner identical to that of IAA itself (Venis *et al.*, 1992). From this evidence it therefore appears likely that the peptide sequence used for anti-sera production does indeed lie within the auxin binding domain of ABP1, and that D16 interacts specifically with the auxin binding domain of the protein. Subsequent data have shown that there is almost complete sequence conservation in this

region between species tested, and that the region contains the longest stretch of wholly conserved sequence in ABP1.

Such an anti-serum, therefore, is potentially useful in allowing the identification of molecules containing the conserved region associated with auxin binding in other species. The final experiments described in this chapter are performed using D16 anti-serum and affinity purified D16 anti-serum.

## 5.2. RESULTS

### 5.2.1. INVESTIGATION USING CAGED COMPOUNDS

As described in Chapter 4, incubation of *G. pallida* and *G. rostochiensis* with either DMT or IAA results in a consistent, reproducible reduction in AF18 insertion by about 20%. This decrease in AF18 insertion, therefore, appears to represent a reliable marker with which to gauge events occurring as a response to DMT or IAA. It was therefore decided to use caged second messengers to mimic these surface events.

#### 5.2.1.1. EFFECT OF CALCIUM DEPLETION

Initial studies with caged compounds investigated the effects of depleting calcium levels prior to incubation with DMT. In these experiments, DMT alone was used since it brings about a more rapid response at the nematode surface than IAA. In this way, it was hoped to test whether DMT-mediated events were linked to increases in calcium levels within the nematode. Washed nematodes were incubated with Diazo-2 AM (20 $\mu$ M) and the compound photoactivated as described in Materials and Methods 2.12. This was immediately followed by incubation with 200 $\mu$ gml<sup>-1</sup> DMT for 30 minutes. An incubation of 30 minutes was chosen since preliminary experiments showed this was still sufficient to cause a 20% decrease in

AF18 insertion. Following incubation with DMT, nematodes were washed again, probed with  $10\mu\text{gml}^{-1}$  AF18, and surface fluorescence quantitated as before. When quantitation of surface fluorescence was performed, addition of DMT still resulted in a 20% decrease in surface fluorescence (Figure 5.1). This indicates that depletion of endogenous calcium does not appear to affect those changes induced by DMT. DMSO controls were also performed using the final concentration of DMSO that would be present in the sample with DIAZO-2 AM. These showed no alterations in insertion of AF18 when compared to PBS controls ( $P > 0.05$  when compared to control).

#### 5.2.1.2. ADDITION OF CAGED SECOND MESSENGERS

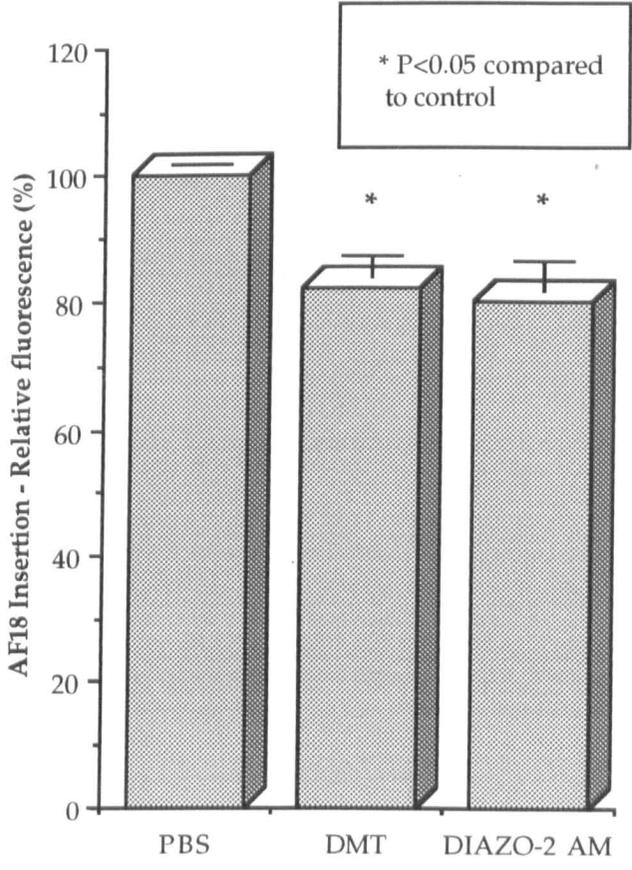
Since depletion of calcium did not appear to affect DMT-mediated changes, it was decided to use caged compounds to introduce a series of second messengers to the nematode. Washed nematodes were incubated with caged cAMP, caged  $\text{IP}_3$ , or NITR.5 (a calcium releasant), and the compounds photoactivated as described previously. Nematodes were then washed and probed with  $10\mu\text{gml}^{-1}$  AF18, and surface fluorescence quantitated as before.

Quantitation of surface fluorescence indicated that the release of caged second messengers had no effect on the insertion of AF18 into the nematode surface. All samples tested showed the same level of surface fluorescence as controls with no significant differences observed,  $P > 0.05$  for all caged second messengers when compared to PBS control (Figure 5.2). DMSO controls were repeated as before. These showed no alterations in insertion of AF18 when compared to PBS controls ( $P > 0.05$  in all cases).

**FIGURE 5.1.** Effect of calcium depletion on AF18 insertion following incubation with DMT.

Washed nematodes were incubated with 20 $\mu$ M Diazo-2 AM (a caged calcium chelator) for 1 hour, and the compound photoactivated as described in Materials and Methods 2.12. Nematodes were then immediately incubated with 200 $\mu$ gml<sup>-1</sup> DMT for 30 minutes. Nematodes were then washed, probed with 10 $\mu$ gml<sup>-1</sup> AF18, and surface fluorescence quantitated as before. Experiments were repeated at least three times using nematodes from the same batch of cysts. In each experiment at least 40 readings from different nematodes were taken for each sample.

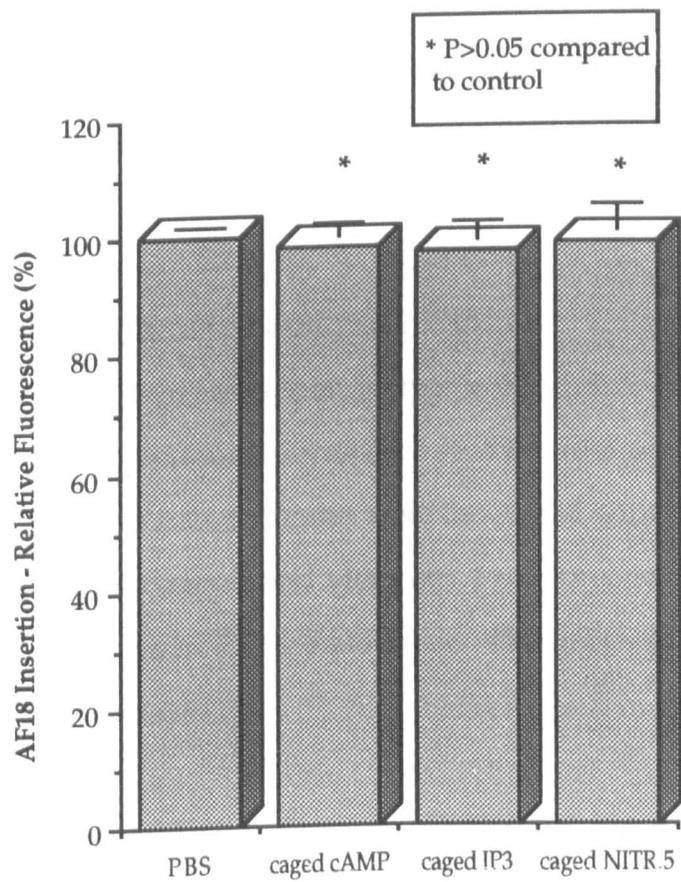
Results are presented as a histogram, showing levels of fluorescence of calcium depleted samples compared to PBS treated controls, and DMT treated controls. In this case, PBS controls were taken as representing 100% relative fluorescence. DMT treated controls show a significant ( $P < 0.005$ ) decrease of 20% in surface fluorescence compared to PBS controls, as do calcium depleted DMT treated samples.



**FIGURE 5.2.** Effect of caged second messengers on AF18 insertion into the surface of *G. pallida* Luffness population.

Washed nematodes were incubated with caged cAMP (10 $\mu$ M), caged IP<sub>3</sub> (10 $\mu$ M) or NITR.5 (0.5mM), and the compounds photoactivated as described in Materials and Methods 2.12. Nematodes were then washed again, probed with 10 $\mu$ gml<sup>-1</sup> AF18, and surface fluorescence quantitated as before. Experiments were repeated at least three times using nematodes from the same batch of cysts. In each experiment at least 40 readings from different nematodes were taken for each sample.

Results are presented as a histogram, showing levels of fluorescence of PBS treated controls compared to samples treated with caged second messenger. PBS controls represent 100% relative fluorescence. None of the second messengers used causes a decrease in AF18 insertion,  $P > 0.05$  when compared to PBS control in all cases.



## **5.2.2. INVESTIGATIONS USING D16 ANTI-SERUM**

Although the various experiments performed with caged second messengers yielded no information about possible receptor signalling mechanisms, it was decided to further investigate the existence of a nematode auxin binding protein using D16 anti-serum. Various studies were thus performed to determine if D16 anti-serum reacted with any nematode proteins. These experiments are described in the following sections.

### **5.2.2.1. INDIRECT IMMUNOFLUORESCENCE STUDIES**

To determine whether D16 anti-serum reacted with surface components of the nematode, indirect immunofluorescence studies were performed on whole nematodes. Washed nematodes were incubated with D16 anti-serum as described in Materials and Methods. Prior to viewing nematodes were fixed for 15 minutes in 1% formaldehyde. This was also repeated on living, and pre-fixed nematodes.

In all cases, no binding of D16 anti-serum could be seen on the surface of the nematode. It has been shown that D16 has auxin agonist activity when added to tobacco protoplasts (Venis *et al.*, 1992). Observations of nematodes treated with D16 anti-serum failed to show the characteristic induction of secretions that occurs upon incubation with IAA, indicating that D16 anti-serum does not appear to act as an agonist in the nematode. This may be explained by the fact that the size of the immunoglobulin molecule would prevent interaction with internal receptors.

### **5.2.2.2. POST-EMBEDDING IMMUNOGOLD LABELLING**

Since indirect immunofluorescence indicated that D16 anti-serum did not bind to the surface of the nematode, electron microscopy was used to see if

the antibody would bind to internal structures. Post-embedding immunogold labelling of sections cut through the anterior region of the nematode was performed as described in Materials and Methods 2.13.2. When sections were viewed no definite results were obtained that localised D16 to a particular structure. However, in some nematodes staining was localised to areas close to the amphids. One problem that may have affected staining was incomplete penetration by the resin resulting in an incompletely fixed section. The result of this was that structures within the nematode appeared misplaced, and this may account for the staining seen. Results obtained however do indicate that D16 may localise to sensory structures at the head of the nematode. Controls performed using KLH anti-serum showed no binding.

### 5.2.3. IMMUNOBLOTTING STUDIES

To determine whether D16 anti-serum showed any cross-reactivity with nematode proteins, it was decided to use the anti-serum to probe whole body extracts of *G. pallida*. Whole body extracts were electrophoresed on a 10-20% gradient gel, and electroblotted as described previously. Blots were probed initially with whole D16 anti-serum. Since keyhole limpet haemocyanin (KLH) was used as a carrier protein in the immunisation procedure, blots were also repeated using anti-serum raised against KLH. This was done to ensure that any bands recognised were not due to the presence of KLH, or KLH-like, epitopes.

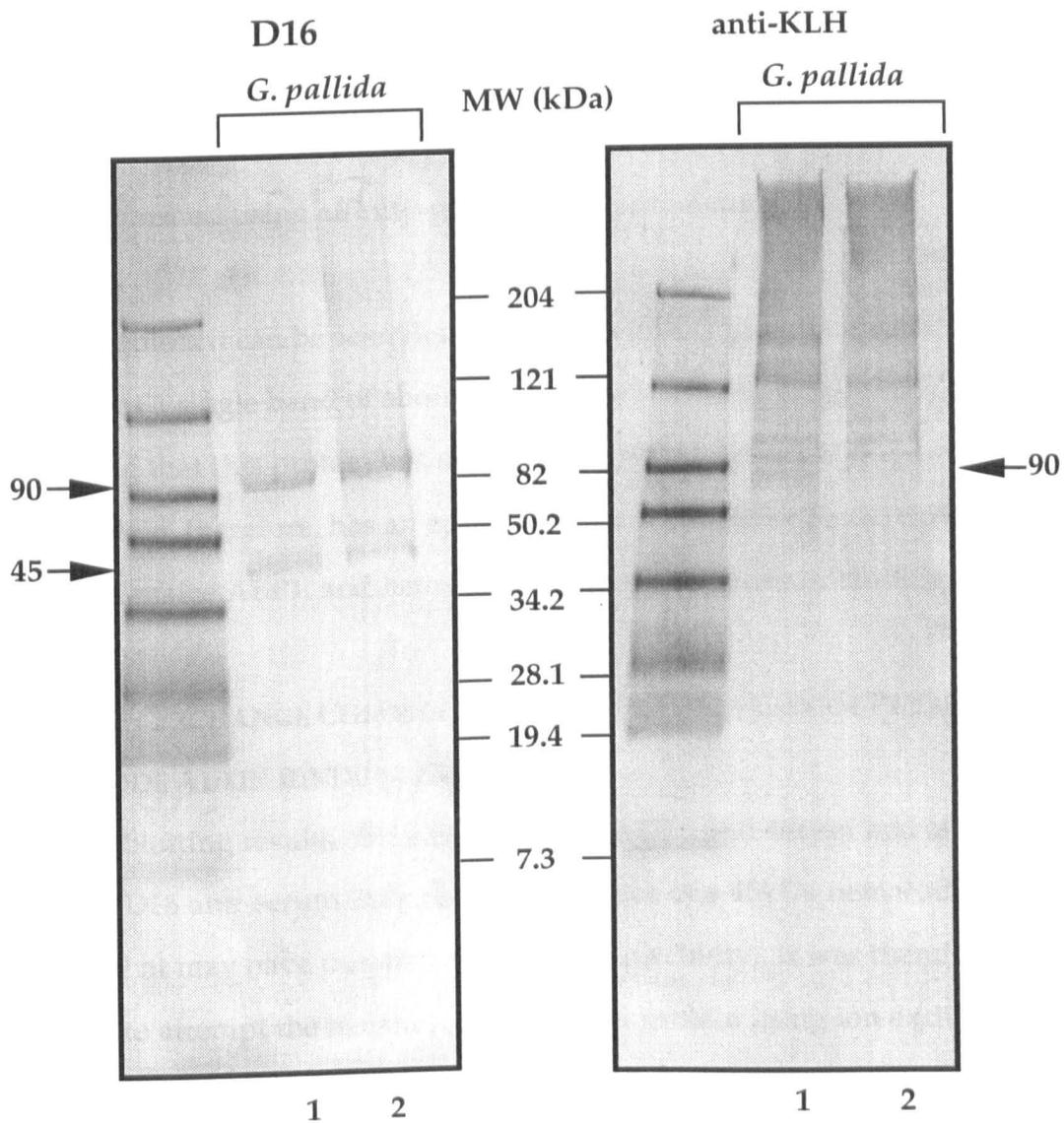
#### 5.2.3.1. D16 ANTI-SERUM

When blots of whole body extracts of *G. pallida* were probed with D16 anti-serum, two bands of molecular weight 45kDa and 90kDa could be observed (Figure 5.3). No other bands appeared to be present. In contrast, when blots were probed with KLH anti-serum, five bands of a higher molecular

**FIGURE 5.3.** Immunoblot analysis of whole body extracts of *G. pallida* probed with D16 anti-serum and KLH anti-serum.

Whole body extracts of *G. pallida* Luffness population (25µg BSA-equivalent protein) were electrophoresed on a 10-20% gradient gel and electroblotted as described in Materials and Methods 2.7. Blots were then probed with D16 anti-serum (1:250), or KLH anti-serum (1:400).

- a) Immunoblot of *G. pallida* whole body extracts probed with D16 anti-serum. All lanes contain whole body extracts of *G. pallida* Luffness population. Two bands of 90kDa and 45kDa (both arrowed) are recognised.
  
- b) Immunoblot of *G. pallida* whole body extracts probed with KLH anti-serum. All lanes contain whole body extracts of *G. pallida* Luffness population. Several high molecular weight bands ranging from 90-150kDa are recognised.



weight ranging from about 90-150kDa could be seen (Figure 5.3). This indicates that the 90kDa band present in both blots is due to cross-reactivity with KLH-like epitopes. The 45kDa band present in the blot probed with D16 anti-serum therefore appears to be specific to the anti-serum.

#### 5.2.3.2. AFFINITY PURIFIED D16 ANTI-SERUM

The results described above indicated that a band of about 45kDa appeared to be recognised specifically by D16 anti-serum, which was not present in blots probed with KLH anti-serum. To confirm that this was the case, blots were performed using affinity-purified D16 anti-serum.

From the blots it can be seen that affinity-purified D16 anti-serum recognises a single band of about 45kDa in whole body extracts of *G. pallida* indicating that this protein has no cross-reactivity with KLH (Figure 5.4). This protein, therefore, has an epitope which is similar to the auxin binding region of maize ABP1, and hence may be involved in auxin binding.

#### 5.2.4. ION EXCHANGE CHROMATOGRAPHY PURIFICATION OF PUTATIVE NEMATODE AUXIN BINDING PROTEIN

Immunoblotting results obtained with whole D16 anti-serum and affinity purified D16 anti-serum indicated the presence of a 45kDa nematode protein that may have putative auxin binding activity. It was therefore decided to attempt the isolation of the 45kDa protein using ion exchange chromatography.

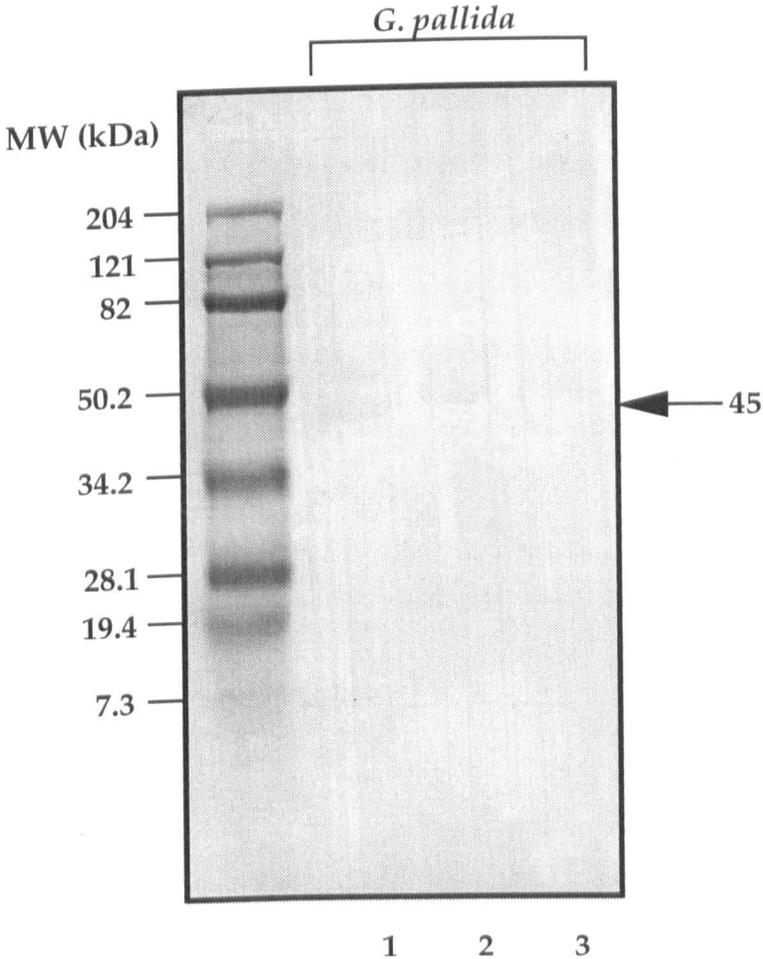
Earlier work performed by Lee Robertson at the Scottish Crop Research Institute, Dundee using isoelectric focusing gels had shown that the pI of the 45kDa protein recognised by D16 anti-serum was around 5.5.

Homogenates of *G. pallida* were thus prepared in 10mM imidazole-HCl

**FIGURE 5.4.** Immunoblot analysis of whole body extracts of *G. pallida* probed with affinity purified D16 anti-serum.

Whole body extracts of *G. pallida* Luffness population (25µg BSA-equivalent protein) were electrophoresed on a 10-20% gradient gel and electroblotted as described in Materials and Methods 2.7. Blots were then probed with affinity purified D16 anti-serum (1:100).

All lanes contain whole body extracts of *G. pallida* Luffness population. From the blot it can be seen that one band of 45kDa (arrowed) is recognised.

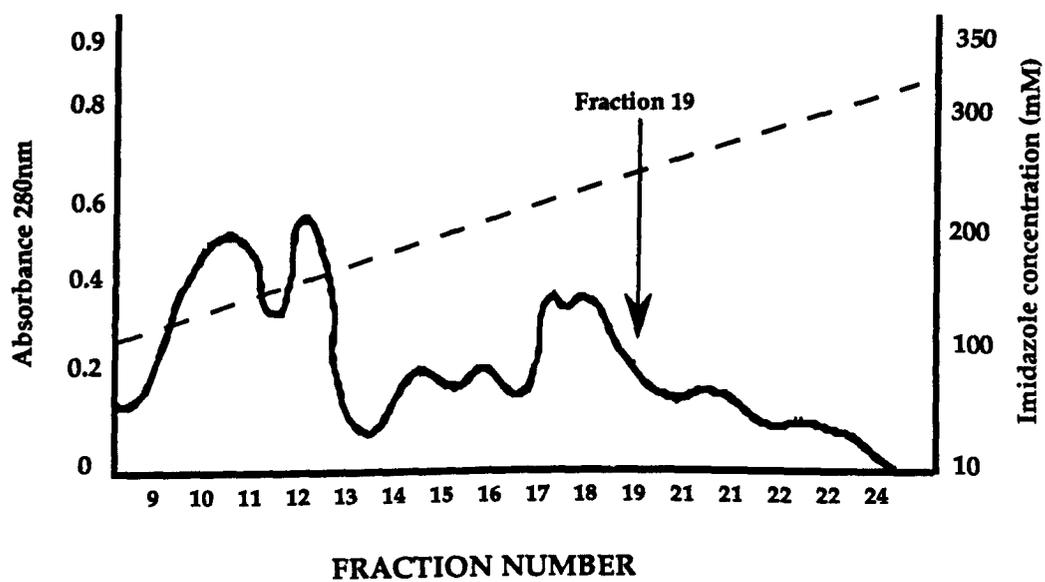


**FIGURE 5.5.** Representation of the elution profile of nematode homogenate obtained using 10-400mM imidazole-HCl gradient.

A Mono Q anion exchange column was attached to FPLC apparatus (Pharmacia) and equilibrated with 10mM imidazole pH 6.5.

Approximately 20mg of nematode homogenate, prepared as described in Section 2.3.3, was loaded onto the column and eluted over a 30ml gradient from 10-400mM imidazole-HCl pH 6.5. 1ml fractions were collected.

Protein concentrations were estimated using absorbance at 290nm. The diagram shows the elution profile from fractions 9 to 22. Fraction 19 is arrowed since this fraction showed reactivity with D16 anti-serum.



buffer pH 6.5. Approximately 20mg of nematode homogenate was loaded onto a Mono Q anion exchange column attached to a FPLC apparatus. Proteins were eluted over a 30ml gradient from 10-400mM imidazole pH 6.5, and 1ml fractions were collected. Figure 5.5 shows the elution pattern obtained. Collected fractions were electrophoresed on a 10-20% gradient gel as described previously, and the proteins electroblotted onto nitrocellulose paper. The blots were then probed with D16 anti-serum. Binding of D16 anti-serum could be seen to correspond to fraction 19 of the preparation. The elution of this fraction corresponded to an imidazole-HCl concentration of approximately 250mM. Unfortunately, a very high background was present on the blot preventing effective scanning and presentation.

### 5.3. DISCUSSION

Experiments performed using caged second messengers in an attempt to understand second messenger signalling events were largely unsuccessful. Initial experiments examined the effect of chelating endogenous calcium prior to incubation with DMT. Chelation of calcium, however, did not affect the insertion of AF18 into the surface of the nematode, indicating that other second messengers are involved. Subsequent studies using other caged second messengers gave no further information on possible mechanisms involved. One explanation may centre on the fact that the changes brought about by DMT involved a relatively small decrease of around 20% in total fluorescence due to AF18 insertion. Changes brought about by individual second messengers could, therefore, give even smaller reductions. Such changes, therefore, might be impossible to detect, at least to a significant statistical level. This is in contrast to the animal-parasitic nematode systems where host cues can lead to an increase in AF18 insertion

by up to 300% (Modha *et al.*, 1995), making changes resulting from single second messengers relatively easy to detect.

The results presented in this final chapter also go some way to indicating the presence of an auxin binding protein in *Globodera pallida*. This appears to correlate with previous data in which IAA was shown to induce secretions, as well as changes in the surface properties of *Globodera pallida*. It is perhaps likely then that the ABP identified may act as an auxin receptor in *Globodera* spp. The work detailed in chapter 5 presents only the data obtained with work carried out on *Globodera pallida*. Concurrent work carried out by Lee Robertson under the supervision of Walter Robertson at the Scottish Crop Research Institute, Dundee however has also shown that the plant-parasitic nematodes *Meloidogyne*, *Xiphinema* and *Longidorus* also possess proteins that are recognised by D16. Although *Meloidogyne* is a sedentary parasite that forms galls, it is of note that *Xiphinema* and *Longidorus* are ectoparasitic. Feeding by *Xiphinema* still requires the formation of galls indicating that a disruption of normal cellular controls has occurred. It is also interesting to speculate upon the location of such an ABP in nematodes. One obvious location for them is in the amphids, possibly at the ciliary membranes of the dendritic processes. Indeed, preliminary results from electron microscopy does localise some antibody binding to the head region of the nematode. In the case of *Longidorus* and *Xiphinema* it is more likely that ABPs would be found in their feeding structures considering their ectoparasitic nature. Further electron microscopy studies are required to clarify this point.

D16 was also used to probe homogenates from the aphid *Myzus persicae*. Again, D16 recognised a band present in the homogenate. At first glance, this may seem rather surprising. However, it has been documented that

certain plant growth substances, including IAA, appear to be responsible for certain stages of growth and development in some aphid species (Bur, 1975). More specifically IAA appeared to cause enhanced wing development and increased wing size. The presence of an auxin binding protein or receptor is therefore not completely unlikely. It is been shown that the gene conferring aphid resistance in tomato is tightly linked to the *Mi* gene conferring *Meloidogyne* resistance (Kaloshian *et al.*, 1995). This may indicate a common mechanism of resistance, and it is tempting to speculate that this may involve disruption of auxin perception by the pathogen.

Control experiments carried out using anti-KLH antibodies were interesting in that they indicated the presence of epitopes showing cross-reactivity with KLH. Similar results have been obtained with other nematodes including *Trichinella spiralis* and it has been suggested that these molecules may have a role as a cuticular structural component (Modha *et al.*, 1994). The presence of such proteins in *G. pallida* is therefore not too unlikely.

The work presented here details a very preliminary characterisation of an auxin binding protein in *G. pallida*. Much work is still required to give more information on these potentially useful proteins, including their localisation, purification and sequence. Work which will be carried on from these initial findings will include further purification of the 45kDa ABP from *Globodera* followed by antibody production and protein sequencing. Also, similar experiments will be performed with *Meloidogyne* homogenates.

The presence of such a receptor in the plant pathogens described above may have interesting applications in the field of transgenic plants. As stated previously, maize ABP has a molecular weight of 23kDa. Results

indicate that *Globodera* possess an ABP with a molecular weight of 45kDa. This may indicate that the nematode protein will possess regions which are not shared by the plant, for example, an additional membrane spanning region. New technology has allowed the expression of antibody fragments, "plantibodies" in plants, and it is possible to envisage methods whereby transgenic plants may be engineered expressing antibody fragments against nematode-specific regions of ABPs as a possible mechanism of resistance.

In the final chapter a hypothesis is proposed in which it is stated that one possible mechanism of feeding site induction and maintenance involves the regulation of plant growth substances, specifically auxin, by the nematode. The chapter begins with a description of auxin signal perception and transduction in the plant, followed by various ways in which the nematode may interfere with these processes.

**CHAPTER 6**  
**FINAL DISCUSSION:**  
**HYPOTHESIS - SYNTHESIS**

## 6.1. A HYPOTHESIS

Infection by *Globodera*, or indeed any nematode which is sedentary for much of its life cycle, requires an intimate and elaborate association with host tissue that allows the nematode to complete its life cycle resident within the host root. This interaction, by its very nature, must be highly complex and dynamic, and will require crosstalk between nematode and plant. Using the results described in the previous chapters, as well as research that has taken place on other plant pathogens the following hypothesis can be presented as follows:

**Phytohormones induce the production of specific secretions in *Globodera rostochiensis* and *Globodera pallida*, and these specific secretions are themselves involved in the alteration of the perception or signal transduction of phytohormones such that the syncytium is initiated and maintained.**

In support of this hypothesis, evidence from research on the involvement of phytohormones in bacterial plant pathogenesis is presented below. This is followed by a brief description of the possible role of auxin and cytokinin in the disease state. The molecular mechanisms of auxin signal transduction and auxin gene induction are then described, as possible mechanisms of nematode intervention. Finally, the possible effect of auxin on nematode gene regulation is discussed.

## 6.2. STRATEGIES OF BACTERIAL PLANT PATHOGENS

In many ways the strategies of sedentary plant-parasitic nematodes resemble those of bacterial plant pathogens such as *Pseudomonas savastanoi*, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. All require an intimate association with the host plant resulting in the formation of specialised structures which will provide the nutrients for the pathogen. For example, many plant pathogenic bacteria form galls or tumours, while sedentary nematodes form syncytia or giant cells. The formation of such feeding structures by the nematode generally involves the redifferentiation of already differentiated cells, although the mechanism of formation of such structures remains unknown. In contrast, it has long been recognised that phytohormones have a major role in the pathogenesis of bacterial plant disease (Smidt & Kosuge, 1978; Garfinkel *et al.*, 1981).

Bacterial plant pathogens appear to have several strategies whereby alterations in phytohormone levels and activities are used to facilitate and maintain infection. One central theme is that the bacteria themselves carry the genes responsible for phytohormone biosynthesis. For example, genes encoding enzymes necessary for auxin and cytokinin biosynthesis have been identified and sequenced from *P. savastanoi*, *A. tumefaciens* and *A. rhizogenes* (Barry *et al.*, 1984; Klee *et al.*, 1984; Akiyoshi *et al.*, 1985; Powell & Morris, 1986). All three bacteria have genes encoding tryptophan monooxygenase and indoleacetamide hydrolase, necessary for auxin biosynthesis. Similarly, *P. savastanoi* and *A. tumefaciens* carry genes encoding isopentenyl transferase, the first enzyme required in cytokinin biosynthesis (Morris, 1986). Many other plant pathogenic bacteria carry genes associated with auxin biosynthesis and the main aim of the examples listed above is to demonstrate that the capacity to synthesise phytohormones is widespread amongst soil and plant-associated bacteria.

To cover in detail other bacteria carrying similar genes is beyond the scope of this discussion although the reader is referred to two reviews which cover the area in great detail (Morris, 1986; Costacurta & Vanderleyden, 1995).

Bacterial plant pathogens possess other strategies for altering phytohormone levels other than *de novo* phytohormone synthesis. Estruch *et al.* (1991a, b) have demonstrated that *A. rhizogenes* carries the genes *rolB* and C (root locus genes). Their products encode for two  $\beta$ -glucosidases that catalyse the *in vitro* hydrolysis of the inactive glucoside conjugates of auxin and cytokinin respectively. Recent evidence, however, indicates that at least for the Rol B protein, this may be incorrect. Experiments by Maurel *et al.* (1994) revealed a strong cooperativity between auxin and *rol B* transformation in increasing auxin sensitivity. This has led to the suggestion that auxin and *rol B* act to alter the mechanism of auxin perception and signal transduction at the plasma membrane. *Agrobacterium* may also regulate auxin/cytokinin levels by the synthesis of antagonists. Korber *et al.* (1991) showed that gene 5 of *Agrobacterium* encodes the production of the auxin antagonist indole-3-lactate (ILA). ILA undergoes autoregulated synthesis, and acts to antagonise auxin by competing for auxin binding sites. The authors postulate that the gene 6b protein may similarly act as a cytokinin antagonist.

Although the above is but a brief description of various strategies used by bacterial pathogens it serves to outline three ways in which phytohormone levels may be modulated by a pathogen, that is, *de novo* synthesis, altered perception and antagonist production. Given that the infection process of sedentary nematodes similarly requires an alteration of host physiology then the involvement of phytohormones also seems likely. One possibility

then, is that sedentary nematodes may possess the ability to synthesise phytohormones in the same manner as their bacterial counterparts. Indeed, this was suggested as early as 1974 (Giebel, 1974). Indirect evidence for the presence of nematode-associated auxin has been presented. For example, indole compounds were identified by paper chromatography in extracts of the galls of various *Meloidogyne* species (Balasubramanian & Rangarwami, 1962; Bird, 1962). To date, however, there is no direct evidence that nematodes themselves synthesise phytohormones.

Due to advances in phytohormone biology and the advent of sophisticated biochemical techniques, the task of ascertaining the presence of nematode-associated phytohormones should be relatively straightforward. Several different approaches exist including immunoassay for phytohormones, and GC-MS analysis of, for example, nematode secretions. At a molecular level it should also be possible to probe the nematode genome for homologues to bacterial genes associated with phytohormone synthesis. This approach would also allow identification of genes with similar functions to *rol B* and gene 5.

### 6.3. THE ROLE OF PHYTOHORMONES IN DISEASE

The ability of phytohormones to modulate disease resistance has been noted since the 1960s. For example, Ingram (1967) showed that plant tissue cultures were able to express race-specific resistance when supplied with combinations of cytokinins and auxins. Interestingly, it was noted that resistance could be suppressed by increasing the ratio of cytokinin to auxin (Helgeson *et al.*, 1976; Haberlach *et al.*, 1978). Similarly, evidence has indicated that auxin can down-regulate genes involved in plant defence responses (Dominov *et al.*, 1992), inhibit the induction of the hypersensitive response (Robinette & Matthyse, 1990) and induce susceptibility to some

pathogens (Simons *et al.*, 1972; Hoffman & Zscheile, 1973). It should be noted that plants may be relatively insensitive to the absolute concentrations of auxin and cytokinin, but may be sensitive to the ratio of their concentrations (Yamada, 1993). Similarly, different tissues may exhibit different sensitivities. Thus even minor changes in phytohormone concentrations may have effect.

### 6.3.1. AUXIN AND CYTOKININ AS MODULATORS OF DISEASE

Since their discovery it has been shown that the phytohormones auxin and cytokinin have fundamental roles in the regulation of plant cell growth (Evans, 1984; Davies, 1988). Indeed, this has led Hobbie *et al.* (1994) to postulate that perhaps all changes in cell division or elongation are mediated by auxin or cytokinin. The very nature of plant growth development as a process that requires the continuous regulation of cell division and elongation goes some way to indicating the diversity of responses that exist to these ubiquitous molecules. Their role as regulatory molecules also implicates them as possible mediators in the disease state caused by those pathogens that require an alteration in host physiology as part of their infection process.

It should be noted that despite the advances in plant physiology, there still remains a basic lack of information about the molecular mechanisms of phytohormone action when compared with the advances made in animal hormone signalling systems. This has meant unfortunately, that attempts to formulate a more detailed hypothesis have been hindered, especially in relation to the involvement of phytohormones. One aspect of phytohormone action is that more than one phytohormone may be involved in any specific process. This is further complicated by the fact that phytohormones can act both synergistically and antagonistically. In this

next section I will attempt to outline the reasons which I consider implicate the phytohormone auxin in the formation of the feeding site induced by *Globodera* spp. Phytohormones such as cytokinin, abscisic acid and gibberellin are not considered here at length due to their poorly understood mode of action. It is likely that these are similarly involved. For example, recent evidence indicated that gibberellin is responsible for regulation of myb-like transcription factors (Weiss *et al.*, 1990; Jacobsen and Gubler, 1993; Gubler *et al.*, 1995; Solano *et al.*, 1995). This suggests an involvement of upstream interactions, for example, in gibberellin perception or signal transduction in nematode up-regulation of myb-like transcription factors.

#### 6.4. AUXIN

Auxin appears to be involved in many aspects of plant growth, including phototropism, elongation growth, lateral branching of roots and shoots and vascular differentiation (Droog *et al.*, 1993), but as stated previously the molecular details remain unknown. Indeed, it has only been relatively recently that auxin binding proteins have been identified (Jones, 1990; Napier & Venis, 1990; Palme, 1993). Controversy still exists over the location and action of such proteins, and their role as potential receptors.

##### 6.4.1. TRANSDUCTION OF THE AUXIN SIGNAL

Structural analyses of auxin binding proteins so far isolated reveal them to be extrinsic proteins with no hydrophobic membrane spanning domain. As such, it has been suggested by Klambt (1991) that a transmembrane docking protein must therefore be involved in transduction of the auxin signal. This has led to the suggestion by Millner (1995) that this putative docking protein may belong to the G-protein coupled receptor superfamily.

Evidence to support this theory has been forthcoming. Unpublished work by Bennet and co-workers has indicated that the *AUX 1* gene encodes a

protein with seven transmembrane domains and a hydrophilic amino terminus. Interestingly, mutations in *AUX 1* cause agravitropism and resistance to auxin. The predicted structure of the *AUX 1* protein is similar to that of the gonadotrophin receptors (McFarland *et al.*, 1989). These receptors are unusual in that their ligands are relatively large glycoproteins of 23-30kDa, as opposed to the much smaller ligands normally bound by G-protein coupled receptors. The *AUX 1* protein therefore appears to fulfil some of the properties expected of a G-protein-linked "docking" protein.

#### 6.4.2. AUXIN INDUCED GENES

One of the major problems involved in the identification of auxin-induced genes has been the inability to discriminate between primary hormonal responses in growth regulation, and those responses that occur as a consequence of primary reactions (Takahashi *et al.*, 1995). Similarly, as auxin is involved in the processes of cell division and differentiation then many auxin induced genes may be part of general cellular pathways of growth control and metabolism. Indeed, these are the very genes that would require altered regulation to induce and maintain the nematode feeding site.

One auxin induced gene that is of particular interest in relation to induction of the nematode feeding site is the *cdc 2* protein kinase gene whose product is a key regulatory component in cell cycle control. It has been demonstrated that a plant homologue of *cdc 2* exists, and that it is localised to meristems and regions of cell proliferation. It has been suggested that the effect of auxin on cell division may be due to changes in *cdc 2* gene expression. Indeed, so rapid is *cdc 2* mRNA induction by auxin, John *et al.*, (1993) speculated that this was a direct response to auxin rather than a secondary one.

Several other gene families are also induced by auxin including SAUR (small auxin upregulated RNA) genes (McClure & Guilfoyle, 1987), genes GH1 and GH3 (Hagen *et al.*, 1984), the pIAA genes (Theologis *et al.*, 1985) and the genes pJCW1 and pJCW2 (Walker & Key, 1982). The function of these genes remains unknown, but interesting observations have been made of their regulation. It has been shown that as well as being induced by auxin, SAUR genes can be induced by protein synthesis inhibitors such as cycloheximide. Induction did not appear to be due to increased transcription (Franco *et al.*, 1990), and thus it was suggested that SAUR gene expression was under the control of a repressor. Interesting clues are also emerging as regards the function of the pIAA genes. Originally isolated from pea (Theologis *et al.*, 1985), these genes are members of a gene family encoding short-lived nuclear proteins. One of their domains is predicted to form an amphipathic  $\beta$  -  $\alpha$  -  $\alpha$  structure similar to the DNA-binding domain found in the Arc family of prokaryotic repressors (Pabo & Sauer, 1990; Abel *et al.*, 1994), suggesting a function in gene regulation. Several other auxin-induced genes contain this putative DNA-binding domain including GH3 (Hagen *et al.*, 1984), and ARG 3 and ARG 4 (Yamamoto, 1994).

Interestingly, cycloheximide induction of pIAA genes has also been reported (Theologis *et al.*, 1985), again suggesting the existence of a controlling repressor protein. With this in mind it has been suggested by Hobbie *et al.* (1994) that pIAA genes may autoregulate their synthesis to higher levels and thus control the expression of genes downstream through direct promoter interactions.

Another gene shown to be responsive to auxin is ARCA (Ishida *et al.*, 1993). ARCA encodes a protein that appears to be related to G $\beta$  subunits due to the presence of characteristic WD40 motifs. Proteins possessing the WD40 motif often have the ability to form complexes with proteins containing a

specific 34 residue motif (tetraricopeptide repeat proteins). These findings have led Ishida *et al.* (1993) to postulate that ARCA may have a role in regulating cell division under the ultimate control of an auxin signal.

One feature of auxin-induced genes is the presence of auxin responsive elements (ARE) located in the promoter region. Ballas *et al.*, (1993) identified such an ARE in the promoter of pIAA genes which was shown to confer responsiveness to auxin. It appears that the element is conserved in several other auxin-inducible genes, and it is thought that some nuclear proteins may bind to the ARE. It has been suggested (Ballas *et al.*, 1993) that in the absence of auxin repressor binding to a promoter bound activator would prevent further interaction with required transcription factors. The presence of auxin would cause a modification of the repressor such that necessary interactions could take place thus allowing the start of transcription.

From the examples given above it can be envisaged that infection by sedentary nematodes leads to an uncoupling of the normal cellular controls normally exerted in the plant cell. Indeed, such an example has already been documented in the case of *Meloidogyne* and the tobacco root-specific gene *TobRB7*. The *TobRB7* gene encodes a protein of 250 amino acids with several membrane spanning regions (Yamamoto *et al.*, 1990). Expression appears to be limited to root meristematic and immature vascular cylinder regions, suggesting a developmental role for the protein. Experiments performed by Yamamoto *et al.*, (1991) showed that sequences controlling root-specific expression were located between -636 and -299 nucleotides from the site of transcription initiation. All gene expression was lost with a promoter deletion to the -299 position (construct  $\Delta$  0.3).

Promoter tagging studies (Opperman *et al.*, 1994) show that *TobRB7* is one of the genes affected by *Meloidogyne* infection, with both a spatial and temporal shift in gene expression occurring. *Meloidogyne* infection thus causes a major alteration in the control of plant gene expression. This is further confirmed by the evidence that *Meloidogyne* infection of tobacco carrying the  $\Delta 0.3$  construct still induces the expression of the *TobRB7* gene. It appears then that the nematode-responsive element of the *TobRB7* promoter is different to the root-specific element, and that nematode infection appears to uncouple control from the root-specific element.

#### 6.4.3. CONSEQUENCES OF ALTERED AUXIN SIGNAL TRANSDUCTION

Although the molecular mechanisms of auxin action are still only beginning to be unravelled it can be seen from the examples above that there are several strategies that could be used by the nematode in the infection process. Already mentioned in this discussion has been the suggestion that sedentary nematodes may synthesise phytohormones, and that this may be responsible, at least partly, for the pathogenesis of disease. Alternatively, the nematode may bypass *de novo* synthesis of phytohormones and instead produce molecules which interfere with normal plant gene regulation. From the examples given above of auxin-induced genes containing an auxin-responsive element under the control of a putative repressor, then the establishment of a nematode infection may result as a consequence of several possibilities. For example, repressor molecules controlling auxin-induced gene expression may be altered or rendered inactive by molecules produced by the nematode. Similarly, nematode molecules may alter bound repressors (in a manner similar to that postulated for auxin) such that transcription can occur. Possible interactions that may be occurring are summarised in Figure 6.1.

**FIGURE 6.1.** Possible interactions occurring between *Globodera* and its host during infection. (Adapted from Millner, 1995).

A schematic representation of *Globodera* within the host root is shown in Figure 6.1a where the nematode can be seen feeding from the syncytium. Figure 6.1b represents possible interactions that may be occurring between nematode products and nuclear events in the auxin signalling pathway that in turn may lead to the initiation of the syncytium.

Evidence to date indicates that initial events in the auxin signalling pathway involve interaction of IAA with auxin binding proteins (ABP). ABPs appear to mediate their effects via a transmembrane docking protein encoded by AUX1. Translated proteins resulting from auxin induction may include general transcriptional repressors or activators (PSIAA4/6), or may affect the timing of cell division (ARC A). Possible interactions that may be occurring between nematode products and components of the signalling pathway are indicated by ●.

Abbreviations: IAA - indole acetic acid; ABP - auxin binding protein; AUX1 - transmembrane docking protein; AUXRE - auxin responsive elements; SAUR - small auxin upregulated RNA; PSIAA4/6 - auxin-induced rapidly turned over proteins PSIAA4 and PSIAA6; ARC A - auxin-induced G $\beta$ -like protein; ● - nematode products.

Figure 6.1a.

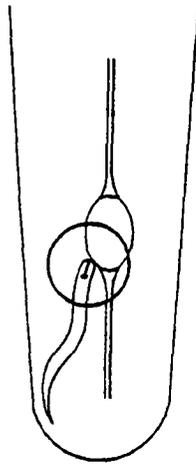
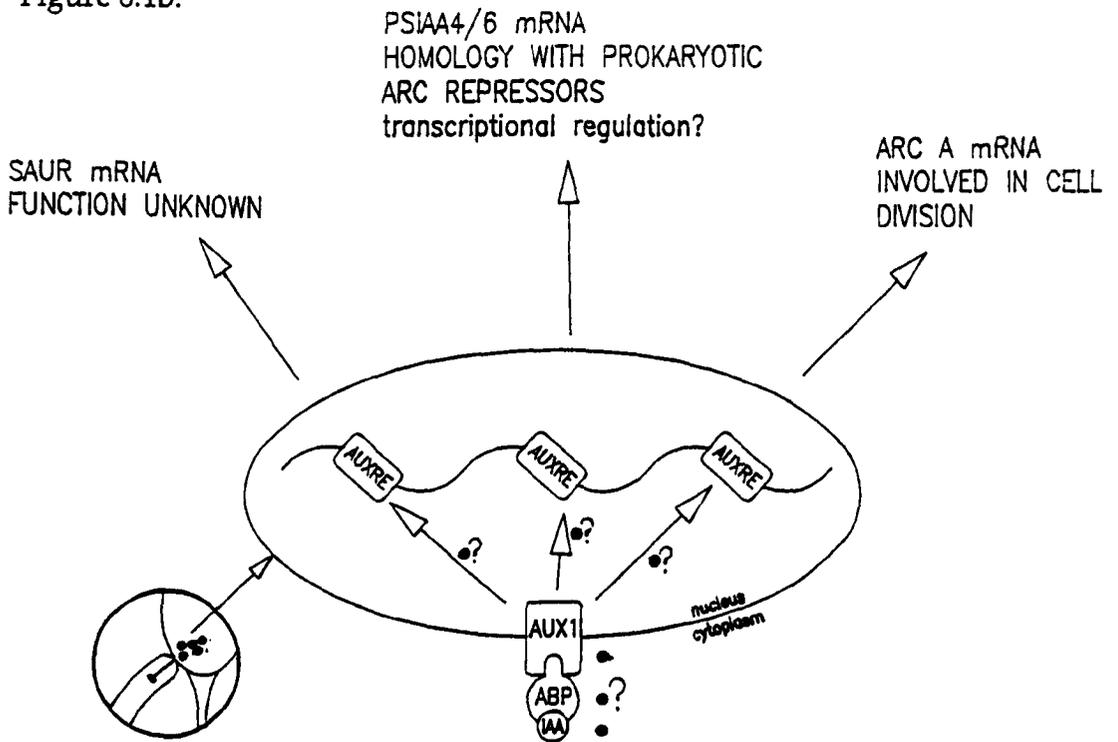


Figure 6.1b.



In this way the vastly complex processes of cell regulation could be altered, theoretically by the production of one protein from the nematode. To extend this hypothesis further, then this may have implications for the basis of H1 resistance. H1 resistance has been shown by several workers to be a gene-for-gene interaction. If infection by the nematode is dependent upon the production of a protein that alters gene expression as described, H1 resistance may be governed by a plant gene product that would in some way negate the effects of the nematode protein.

The events described above concern interactions that would be occurring early on in the transduction of a received (altered) auxin signal. As yet few data exist detailing the roles of auxin-induced genes, or the consequences of their altered expression. Similarly, auxin (or auxin-induced events) may be responsible for the down regulation of certain responses. For example, less well documented responses to auxin include inhibition of plant defence responses (Dominov *et al.*, 1992), and inhibition of the hypersensitive response (Robinette & Matthysse, 1990). The requisitioning of such key responses by the nematode would thus appear to be an extremely effective method in facilitating those changes required for infection.

The production in recent years of plants with mutations specifically affecting phytohormone synthesis and perception should facilitate investigations into feeding site induction by sedentary nematodes. The use of *Arabidopsis* as a model system which can be infected with sedentary nematodes may allow rapid progress in this area, although any advances would have to rely on a better knowledge of auxin signal transduction.

### 6.5. THE ROLE OF AUXIN IN *GLOBODERA* INFECTION: A SYNTHESIS

The plant-nematode relationship is highly complex and elaborate, and requires the perception of signals by both the plant, and the invading nematode. Results described in Chapter 5 detail the identification by immunological techniques, and partial characterisation of a putative auxin binding protein from *G. pallida*. This appears to be the first clear evidence that the nematode could respond to specific signals from its host, and for the presence of specific plant compound receptors in the nematode. This is further supported by recent work performed by Lee Robertson (Scottish Crop Research Institute, Dundee) which indicated that those secretions induced by IAA did indeed differ from ES secretions or DMT-induced secretions. Such a finding may have important implications in, for example the regulation of gene expression by the nematode during the course of infection. It is known that the presence of the nematode is required to maintain the integrity of the feeding site through out the course of infection. Removal of the nematode results in the degradation of the feeding site. This observation indicates therefore that maintenance of the feeding site requires a continuous input from the nematode that would similarly require continuous regulation depending on the state of the feeding site. For example, the integrity of the feeding site may rely on levels of auxin, or the presence of an auxin-induced gene product. It is absolutely essential that the nematode can detect and respond to such host molecules. A novel and interesting approach to such a theory would be to investigate the nematode genome for the presence of auxin responsive elements that have already been identified in plants. It is tempting to speculate that upon entry into the host plant, molecules such as auxin are responsible for regulating the expression of genes required in the infection process. Such an approach may allow interesting insights into new aspects of host-parasite relationships.

## 6.6. IN SUMMARY

From the discussion above it can be seen that there are new and exciting aspects of the plant-nematode relationship that are only just beginning to become obvious. The hypothesis presented in this final chapter states that an altered regulation of phytohormones, specifically auxin, is ultimately responsible for the establishment and maintenance of the feeding site. With the advances being made in auxin research, and indeed phytohormone research in general, evidence to support or contradict this hypothesis should be forthcoming.

## 6.7. FUTURE WORK

From the work described in this thesis, several avenues of further research are apparent. Of these, the most obvious appears to be the further application of the two anti-sera, Luffness anti-serum and ES anti-serum. Interesting results have already been obtained using Luffness anti-serum. The anti-serum shows cross-reactivity with the root-knot nematode *Meloidogyne incognita*, appearing to recognise sensory structures within the nematode, as well as cuticular components (Rosane Curtis, pers. comm). Initial results from immunoblotting studies suggest the possibility of shared antigens and it is hoped to characterise these. Further investigations are planned to determine whether Luffness anti-serum can differentiate species of *Meloidogyne* in the same manner as *Globodera*. It is also hoped to carry out similar investigations using ES anti-serum.

The two anti-sera raised are also useful tools with which to probe a cDNA library, raising the possibility of cloning novel nematode genes. One problem that can be envisaged with Luffness anti-serum is that the anti-serum recognises largely carbohydrate epitopes, making screening of a cDNA library expressed in a bacterial vector problematic. One possible

solution to this problem would be to use an expression library expressed in a eukaryotic vector. ES anti-serum is perhaps a better candidate with which to screen an expression library, recognising only two bands in nematode homogenates, one of which is clearly immunodominant. ES anti-serum is particularly interesting since it has the potential to allow cloning of an ES product from a plant-parasitic nematode. It is also hoped to screen several libraries, including *Globodera* and *Meloidogyne* libraries, with D16 anti-serum in an attempt to clone putative nematode auxin binding proteins. Further work in this area includes the purification and characterisation of the protein.

The methods of secretion collection and induction of secretion described are also worthy of further investigation. One interesting, and important, experiment that should be performed is the comparison of DMT-induced secretions with those secretions induced by IAA. As stated earlier, preliminary work by Lee Robertson indicates that differences are indeed apparent. This may indicate that IAA acts to induce secretions with different functions from those induced by DMT. Such studies may also be of importance as regards *Meloidogyne* secretions. Ongoing work carried out by Lee Robertson also indicates that the phytohormones cytokinin, gibberellin and abscisic acid can induce secretions in *Meloidogyne*, as well as auxin. Comparisons of the secretions induced by different phytohormones may allow insight into the function of different secretions produced by the nematode.

Finally, although the field of plant nematology research has advanced much in recent years, especially in terms of molecular biology, there are many fundamental questions regarding feeding site formation and maintenance that remain unanswered. The techniques described in this

thesis allowing the isolation of nematode oesophageal secretions will hopefully lead to novel investigations of the mode of action of these proteins on the plant cell, and help elucidate the mechanism of formation of the nematode feeding site. The hypothesis presented in this final chapter provides a basic framework with which to test the idea that phytohormones are fundamentally involved in the formation and maintenance of the nematode feeding site. With the advances that have been made in plant molecular biology, especially with the advent of techniques such as promoter tagging, it should be possible to define a role, if any, for phytohormone involvement in sedentary nematode infection.

## LITERATURE CITED

- ABEL, S., OELLER, P.W. & THEOLOGIS, A. (1994) Early auxin-induced genes encode short-lived nuclear proteins. *Proceedings of the National Academy of Sciences USA* **91** 326-330
- AKIYOSHI, D.E., REGIER, D.A., JEN, G. & GORDON, M.P. (1985) Cloning and nucleotide sequence of the *tzs* gene from *Agrobacterium tumefaciens* strain T37. *Nucleic Acids Research* **13** 2773-2778
- ALBRECHT, S. & WALTER, R.D. (1991) Hallucinogenic and neuroleptic drug-interactions with potential neurotransmitter receptors in parasitic nematodes. *Molecular and Biochemical Parasitology* **45** 289-296
- ASH, C.P.J. & ATKINSON, H.J. (1983) Evidence for a temperature-dependent conversion of lipid reserves to carbohydrate in quiescent eggs of the nematode *Nematodirus battus*. *Comparative Biochemistry and Physiology* **76** 603-610
- ASH, C.P.J. & ATKINSON, H.J. (1986) *Nematodirus battus* - development of cold hardiness in dormant eggs. *Experimental Parasitology* **62** 24-28
- ATKINSON, H.J. & BALLANTYNE, A.J. (1977) Changes in the oxygen consumption of cysts of *G. rostochiensis* associated with the hatching of juveniles. *Annals of Applied Biology* **87** 159-166
- ATKINSON, H.J. & HARRIS, P.D. (1989) Changes in nematode antigens recognised by monoclonal antibodies during early infections of soya bean with the cyst nematode *Heterodera glycines*. *Parasitology* **98** 479-487
- ATKINSON, H.J., HARRIS, P.D., HALK, E.J., NOVITSKI, C. & LEIGHTON-SANDS, J. (1988) Monoclonal antibodies to the soybean cyst nematode *Heterodera glycines*. *Annals of Applied Biology* **112** 459-469
- ATKINSON, H.J., TAYLOR, J.D. & FOWLER, M. (1987) Changes in the 2nd stage juveniles of *Globodera rostochiensis* prior to hatching in response to potato root diffusate. *Annals of Applied Biology* **110** 105-114

- AUMANN, J. (1989) Enzymatic effects on lectin binding to *Heterodera schachtii* (Nematoda: *Heteroderidae*) males. *Nematologica* 35 461-488
- AUMANN, J. (1994) The chemical nature of the amphidial and "excretory" system secretions of *Heterodera schachtii* (Nematoda: *Heteroderidae*) males. *Fundamental and Applied Nematology* 17 186-189
- AUMANN, J., ROBERTSON, W.M. & WYSS, U. (1991) Lectin binding to cuticle exudates of sedentary *Heterodera schachtii* (Nematoda: *Heteroderidae*) second stage juveniles. *Revue Nematologie* 14 113-118
- AUMANN, J. & WYSS, U. (1987) Lectin binding sites on mobile stages of *Heterodera schachtii* (Nematoda: *Heteroderidae*). *Nematologica* 33 410-418
- AUMANN, J. & WYSS, U. (1989) Histochemical studies on exudates of *Heterodera schachtii* (Nematoda: *Heteroderidae*) males. *Revue Nematologie* 12 309-315
- BAKKER, J. BOUWMAN-SMITS, L. & GOMMERS, F.J. (1992) Genetic relationships between *Globodera pallida* pathotypes in Europe assessed by using 2-dimensional gel electrophoresis of proteins. *Fundamental and Applied Nematology* 15 481-490
- BAKKER, J., SCHOTS, A., BOUWMAN-SMITS, L. & GOMMERS, F.J. (1988) Species-specific and thermostable proteins from 2nd-stage larvae of *Globodera rostochiensis* and *G. pallida*. *Phytopathology* 78 300-305
- BALASUBRAMANIAN, M. & RANGARWAMI, G. (1962) Presence of indole compounds in nematode galls. *Nature* 194 714-715
- BALLAS, N., WONG, L.-M. & THEOLOGIS, A. (1993) Identification of the auxin-responsive element, *AuxRE*, in the primary indoleacetic acid-inducible gene, *PS-IAA4/5*, of pea (*Pisum sativum* L.). *Journal of Molecular Biology* 233 580-596
- BARANOWSKIJ, N., FROHBERG, C., PRAT, S. & WILLMITZER, L. (1994) A novel DNA-binding protein with homology to myb oncoproteins containing only one repeat can function as a transcriptional activator. *EMBO Journal* 13 5383-5392

- BARGMANN, C.I. & HORVITZ, H.R.** (1991) Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in *Caenorhabditis elegans*. *Neuron* **7** 729-742
- BARGMANN, C.I., THOMAS, J.H. & HORVITZ, H.R.** (1990) Chemosensory cell function in the behaviour and development of *Caenorhabditis elegans*. In: Cold Spring Harbour Symposia on Quantitative Biology, Vol. LV. Cold Spring Harbour Laboratory Press pp529-538
- BARON, C. & ZAMBRYSKI, P.C.** (1995) The plant-response in pathogenesis, symbiosis, and wounding - variations on a common theme. *Annual Review of Genetics* **29** 107-129
- BARRY, G.F., ROGERS, S.G., FRALEY, R.T. & BRAND, L.** (1984) Identification of a cloned cytokinin biosynthetic gene. *Proceedings of the National Academy of Sciences USA* **81** 4776-4779
- BERRIDGE, M.J.** (1993) Inositol trisphosphate and calcium signalling. *Nature* **361** 315-325
- BIRD, A.F.** (1962) The inducement of giant cells by *Meloidogyne javanica*. *Nematologica* **8** 1-10
- BIRD, A.F.** (1967) Changes associated with parasitism in nematodes. I. Morphology and physiology of preparasitic and parasitic larvae of *Meloidogyne javanica*. *Journal of Parasitology* **53** 768-776
- BIRD, A.F.** (1971) Specialised adaptation of nematodes to parasitism. In: Plant Parasitic Nematodes. (Zuckerman, B.M., Mai, F.M. & Rohde, R.A., eds) pp35-49 New York, Academic
- BIRD, A.F.** (1983) Changes in the dimensions of the oesophageal glands in root-knot nematodes during the onset of parasitism. *International Journal for Parasitology* **13** 343-348

- BIRD, A.F., BONIG, I. & BACIC, A.** (1988) A role for the excretory system in secernentean nematodes. *Journal of Nematology* **20** 493-496
- BIRD, A.F. & ZUCKERMAN, B.M.** (1989) Studies on the surface coat (glycocalyx) of the dauer larva of *Anguina agrostis*. *International Journal for Parasitology* **19** 235-240
- BIRD, D.M. & WILSON, M.A.** (1994) DNA sequence and expression analysis of root-knot nematode-elicited giant cell transcripts. *Molecular Plant-Microbe Interactions* **7** 419-424
- BOCKENHOFF, A. & GRUNDLER, F.M.W.** (1994) Studies on the nutrient uptake by the beet cyst nematode *Heterodera schachtii* by *in situ* microinjection of fluorescent probes into the feeding structure in *Arabidopsis thaliana*. *Parasitology* **109** 249-257
- BRADFORD, M.M.** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72** 248-254
- BRODIE, B.B., EVANS, K. & FRANCO, J.** (1993) Nematode parasites of potato. In: *Plant-Parasitic Nematodes in Temperate Agriculture* (Evans, K, Trudgill, D.L. & Webster, J.M., eds). Chapter 3 pp 87-132 Cab International
- BROWN, G., CALLOW, R.K., GREEN, C.D., JONES, F.G.W., RAYNER, J.H., SHEPHERD, A.M. & WILLIAMS, T.D.** (1971) The structure, composition, and origin of the subcrystalline layer in some species of the genus *Heterodera*. *Nematologica* **17** 591-599
- BROWNLEE, D.J.A., HOLDENDY, L., FAIRWEATHER, I. & WALKER, R.J.** (1995) The action of serotonin and the nematode neuropeptide KSA YMRFamide on the pharyngeal muscle of the parasitic nematode, *Ascaris suum*. *Parasitology* **111** 379-384
- BROYLES, J.L., HANSON, F.E. & SHAPIRO, A.M.** (1976) Ion dependence of the tarsal sugar receptor of the blowfly *Phormia regina*. *Journal of Biological Chemistry* **237** 2736-2743

- BUR, M. (1975) Einfluß von phytohormonen auf entwicklung und fortpflanzung von blattläusen (Homoptera; Aphidinea; Aphididae). *Entomol. Gener.* **10** 183-200
- BYGRAVE, F.L. & ROBERTS, H.R. (1995) Regulation of cellular calcium through signalling cross-talk involves an intricate interplay between the actions of receptors, G-proteins, and second messengers. *FASEB Journal* **9** 1297-1303
- CANTO SAENZ, M. & MAYER DE SCURRAH, M. (1977) Races of the potato cyst nematode in the Andean region and a new system of classification. *Nematologica* **23** 340-349
- CARPITA, N.C. & GIBEAUT, D.M. (1993) Structural models of primary cell walls in flowering plants - consistency of molecular structure with the physical properties of the walls during growth. *Plant Journal* **3** 1-30
- CLARKE, A.J. & PERRY, R.N. (1985) Eggshell calcium and the hatching of *Globodera rostochiensis*. *International Journal for Parasitology* **15** 511-516
- CLELAND, R.E., FUJIWARA, T. & LUCAS, W.J. (1994) Plasmodesmal-mediated cell-to-cell transport in wheat roots is modulated by anaerobic stress. *Protoplasma* **178** 81-85
- COLLINGE, D.B. & SLUSARENKO, A.J. (1987) Plant-gene expression in response to pathogens. *Plant Molecular Biology* **9** 389-410
- CONE, K.C., COCCIOLONE, S.M., BURR, F.A. & BURR, B. (1993) Maize anthocyanin regulatory gene *pl* is a duplicate of *c1* that functions in the plant. *Plant Cell* **5** 1795-1805
- CONKLING, M.A., CHENG, C.L., YAMAMOTO, Y.T. & GOODMAN, H.M. (1990) Isolation of transcriptionally regulated root-specific gene from tobacco. *Plant Physiology* **93** 1203-1211
- COSTACURTA, A. & VANDERLEYDEN, J. (1995) Synthesis of phytohormones by plant-associated bacteria. *Critical Reviews in Microbiology* **21** 1-18

- CRAMER, C.L., WEISSENBORM, D., COTTINGHAM, C.K., DENBOW, C.J., EISENBACK, J.D., RADIN, D.N. & YU, X. (1993) Regulation of defence-related gene expression during plant-pathogen interactions. *Journal of Nematology* 25 507-518
- CUANY, A., BERGE, J.-B., BRIDE, J.-M. & HAMADENE-SELLAMI, S. (1984) Inhibition et reactivation des acetylcholinesterases amphidiales de *Meloidogyne javanica* par l'éthoprophos et l'aldicarbe: tentative de corrélation avec une réaction comportementale. *Revue de Nématologie* 7 173-176
- DAVEY, K.G. & SOMMERVILLE, R.I. (1982) Changes in the optical-path difference in the oesophageal region and the excretory cells during exsheathment in *Haemonchus contortus*. *International Journal for Parasitology* 12 503-507
- DAVIS, E.L., ALLEN, R. & HUSSEY, R.S. (1994) Developmental expression of oesophageal gland antigens and their detection in stylet secretions of *Meloidogyne incognita*. *Fundamental and Applied Nematology* 17 255-262
- DAVIS, E.L., ARON, L.M., PRATT, L.M. & HUSSEY, R.S. (1992) Novel immunisation procedures used to develop monoclonal antibodies that bind to specific structures in *Meloidogyne* spp. *Phytopathology* 10 1244-1250
- DAVIS, E.L. & KAPLAN, D.T. (1992) Lectin binding to aqueous-soluble and body wall proteins from infective juveniles of *Meloidogyne* spp. *Fundamental and Applied Nematology* 15 243-250
- DAVIES, P.J. (1988) The plant hormones: Their nature, occurrence, and functions. In: *Plant Hormones and their Role in Plant Growth and Development*. (Davies, P.J., ed). pp1-11. Kluwer Academic Publishers, Dordrecht
- DE JONG, A.J., BAKKER, J., ROOS, M. & GOMMERS, F.J. (1989) Repetitive DNA and hybridisation patterns demonstrate extensive variability between the sibling species *Globodera rostochiensis* and *G. pallida*. *Parasitology* 99 133-138
- DE WIT, P.J.G.M. & ROSEBOOM, P.H.M. (1980) Isolation, partial characterisation and specificity of glycoprotein elicitors from culture filtrates, mycelium and cell walls of *Cladosporium fulvum* (syn. *Fulvia fulva*). *Physiological Plant Pathology* 16 391-408

- DI SANZO, C.P.** (1973) Nematode responses to carbofuran. *Journal of Nematology* **5** 22-27
- DOMINOV, J.A., STENZLER, L., LEE, S., SCHWARZ, J.J., LEISNER, S. & HOWELL, S.H.** (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. *Plant Cell* **4** 451-461
- DROOG, F.N.J., HOOYKAAS, P.J.J., LIBBENGA, K.R. & VAN DER ZAAL, E.J.** (1993) Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione-S-transferases and one member indeed shows *in vitro* GST activity. *Plant Molecular Biology* **21** 965-972
- ENDO, B.Y.** (1971) Synthesis of nucleic acids at infection sites of soybean roots parasitised by *Heterodera glycines*. *Phytopathology* **61** 395-399
- ENDO, B.Y.** (1978) Feeding plug formation in soybean roots infected with the soybean cyst nematode. *Phytopathology* **68** 1022-1031
- ENDO, B.Y.** (1984) Ultrastructure of the oesophagus of larvae of the soybean cyst nematode *Heterodera glycines*. *Proceedings of the Helminthological Society of Washington* **51** 1-24
- ENDO, B.Y.** (1987) Ultrastructure of oesophageal gland secretory granules in juveniles of *Heterodera glycines*. *Journal of Nematology* **19** 469-483
- ENDO, B.Y.** (1993) Ultrastructure of subventral gland secretory granules in parasitic juveniles of the soybean cyst nematode, *Heterodera glycines*. *Journal of the Helminthological Society of Washington* **60** 22-34
- ENDO, B.M. & WYSS, U.** (1992) Ultrastructure of cuticular exudations in parasitic juvenile *Heterodera schachtii*, as related to cuticle structure. *Protoplasma* **166** 67-77
- ESTRUCH, J.J., CHRIQUI, D., GROSSMAN, K., SCHELL, J. & SPENA, A.** (1991a) The plant oncogene *rolC* is responsible for the release of cytokinins from glucoside conjugates. *EMBO Journal* **10** 2889-2895

- ESTRUCH, J.J., SCHELL, J. & SPENA, A. (1991b) The protein encoded by *rolB* plant oncogene hydrolyses indole glucosides. *EMBO Journal* **10** 3125-3280
- ETHERTON, B. & HIGGINBOTHAM, N. (1960) Transmembrane potential measurements of cells of higher plants as related to salt uptake. *Science* **131** 409-414
- EVANS, M.L. (1984) Functions of hormones at the cellular level of organisation. In: Hormonal Regulation of Development II. *Encyclopaedia of Plant Physiology*, **10** pp23-79. (Scott, T.K., ed), Springer-Verlag, Berlin
- FISHER, O.G. (1988) Movement of lucifer yellow in leaves of *Cloves blumei* Benth. *Plant Cell Environment* **11** 639-644
- FOLEY, M., KUSEL, J.R. & GARLAND, P.B. (1988) Changes in the organisation of the surface membrane upon transformation of cercariae to schistosomula of the helminth parasite *Schistosoma mansoni*. *Parasitology* **96** 85-97
- FOLKERTSMA, R.T., VANDERVOORT, J.N.A.M.R., VANGENTPELZER, M.P.E., DEGROOT, K.E., VANDENBOS, W.J.R., SCHOTS, A., BAKKER, J. & GOMMERS, F.J. (1994) Interspecific and intraspecific variation between populations of *Globodera rostochiensis* and *Globodera pallida* revealed by random amplified polymorphic DNA. *Phytopathology* **84** 807-811
- FORREST, J.M.S. (1987) A modified syringe for processing second stage juveniles of *Globodera pallida* for scanning electron microscopy. *Nematologica* **32** 123-124
- FORREST, J.M.S. & FARRER, L.A. (1983) The response of eggs of the white potato cyst nematode *Globodera pallida* to diffusate from potato and mustard roots. *Annals of Applied Biology* **103** 283-289
- FORREST, J.M.S. & ROBERTSON, W.M. (1986) Characterisation and localisation of saccharides on the head region of 4 populations of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. *Journal of Nematology* **18** 23-26
- FORREST, J.M.S., ROBERTSON, W.M. & MILNE, E.W. (1988a) Changes in the structure of amphidial exudate and the nature of lectin labelling on freshly hatched,

invaded and emigrant second stage juveniles of *Globodera rostochiensis*. *Nematologica* **34** 422-431

FORREST, J.M.S., ROBERTSON, W.M. & MILNE, E.W. (1989) Observations on the cuticle surface of second stage juveniles of *Globodera rostochiensis* and *Meloidogyne incognita*. *Revue Nematologie* **12** 337-341

FORREST, J.M.S., SPIEGEL, Y. & ROBERTSON, W.M. (1988b) A possible role for the amphids of the potato cyst nematode *Globodera rostochiensis* in host finding. *Nematologica* **34** 173-181

FOSTER, D.A. (1993) Intracellular signalling mediated by protein-tyrosine kinases - networking through phospholipid metabolism. *Cellular Signalling* **5** 389-399

FRANCO, A.R., GEE, M.A. & GUILFOYLE, T.J. (1990) Induction and superinduction of auxin-responsive mRNAs with auxin and protein synthesis inhibitors. *Journal of Biological Chemistry* **265** 15845-15849

FUKUZAWA, A., FURUSAKI, A., IKURA, M. & MASAMUNE, T. (1985a) Glycinoeclepin-A, a natural hatching stimulus for the soybean cyst nematode *Journal of the Chemical Society-Chemical Communications* **4** 222-224

FUKUZAWA, A., MATSUE, H., IKURA, M. & MASAMUNE, T. (1985b) Glycinoeclepin-B and Glycinoeclepin-C, nortriterpenes related to Glycinoeclepin-A. *Tetrahedron Letters* **26** 5539-5542

GALAU, G.A., WANG, H.Y.C. & HUGHES, D.W. (1993) Cotton *Lea5* and *Lea14* encode atypical late embryogenesis abundant proteins. *Plant Physiology* **101** 695-696

GARFINKEL, D.J., SIMPSON, R.B., REAM, L.W., WHITE, F.F., GORDON, M.P. & NESTER, E.W. (1981) Genetic analysis of crown gall: Fine structure map of the T-DNA by site-directed mutagenesis. *Cell* **27** 143-153

GHEYSON, G. & VAN MONTAGU, M. (1995) Plant/nematode interactions, a molecular biologists approach. *Nematologica* **41** 366-384

- GIEBEL, J.** (1974) Biochemical mechanisms of plant resistance to nematodes: A review. *Journal of Nematology* **6** 175-184
- GOLDMAN, D., GIRI, P.R. & O'BRIEN, S.J.** (1987) A molecular phylogeny of the hominoid primates as indicated by two-dimensional protein electrophoresis. *Proceedings of the National Academy of Sciences USA* **84** 3307-3311
- GONDA, T.J., GOUGH, N.M., DUNN, A.R. & BLAQUIERE, J.** (1985) Nucleotide sequence of cDNA clones of the murine *myb* proto-oncogene. *EMBO Journal* **4** 2003-2008
- GOVERSE, A., DAVIS, E.L. & HUSSEY, R.S.** (1994) Monoclonal antibodies to the oesophageal glands and stylet secretions of *Heterodera glycines*. *Journal of Nematology* **26** 251-259
- GROTEWOLD, E., ATHMA, P. & PETERSON, T.** (1991) Alternatively spliced products of the maize *P* gene encode proteins with homology to the DNA-binding domain of *myb*-like transcription factors. *Proceedings of the National Academy of Sciences USA* **88** 4587-5491
- GUBLER, F., KALLA, R., ROBERTS, J.K. & JACOBSEN, J.V.** (1995) Gibberellin-regulated expression of a *myb* gene in barley aleurone cells - evidence for *myb* transactivation of a high-pI alpha-amylase gene promoter. *Plant Cell* **7** 1879-1891
- GUNNING, B.E.S.** (1976) Introduction to plasmodesmata. In Intercellular Communication in Plants: Studies on Plasmodesmata, pp1-13 (Gunning, B.E.S. & Robards, A.W., eds.) Springer-Verlag
- HABERLACH, G.T., BUDDE, A.D., SEQUEIRA, L. & HELGESON, J.P.** (1978) Modification of disease resistance of tobacco callus tissues by cytokinins. *Plant Physiology* **62** 522-525
- HAGEN, G., KLEINSCHMIDT, A. & GUILFOYLE, T.** (1984) Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. *Planta* **162** 147-153

- HARFORD, J. & ASHWELL, G.** (1982) The hepatic receptor for asialoglycoproteins. In: *The Glycoconjugates* (Horowitz, M.I., ed) vol. 4 pp27-55 Academic Press, New York
- HELGESON, J.P., HABERLACH, G.T. & UPPER, C.D.** (1976) A dominant gene conferring disease resistance to tobacco plants is expressed in tissue cultures. *Phytopathology* **66** 91-96
- HEMERLY, A., FERREIRA, P., ENGLER, J., ENGLER, G., VAN MONTAGU, M. & INZE, D.** (1993) Developmental control of cell division - spatial and temporal regulation of cdc2 expression. *Journal of Cellular Biochemistry* **S17B** 29
- HILKENS, J., LIGTENBERG, M.J.L., VOS, H.L. & LITINOV, S.V.** (1992) Cell membrane-associated mucins and their adhesion modulating property. *TIBS* **17** 359-363
- HOBBIE, L., TIMPTE, C. & ESTELLE, M.** (1994) Molecular genetics of auxin and cytokinin. *Plant Molecular Biology* **26** 1499-1519
- HOFFMAN, S.E. & ZSCHEILE, F.P.** (1973) Leaf bioassay for *Helminthosporium carbonum* toxin - search for phytoalexin. *Phytopathology* **63** 729-734
- HORTON D.** (1987) Potatoes: Production, marketing, and programs for developing countries. Westview Press. Boulder Colorado.
- HUANG, J.S. & BARKER, K.R.** (1991) Glyceollin I in soybean-cyst nematode interactions. 1. Spatial and temporal distribution in roots of resistant and susceptible soybeans. *Plant Physiology* **96** 1302-1307
- HUSSEY, R.S.** (1989a) Disease-inducing secretions of plant-parasitic nematodes. *Annual Review of Phytopathology* **27** 123-141
- HUSSEY, R.S.** (1989b) Monoclonal antibodies to secretory granules in oesophageal glands of *Meloidogyne* species. *Journal of Nematology* **21** 392-398

- HUSSEY, R.S. (1992) Secretions of oesophageal glands of root-knot nematodes. In: *Nematology, from molecule to ecosystem*. (Eds, Gommer, F.J. & Mass, P.W.T.H). pp42-50
- HUSSEY, R.S. & MIMS, C.W. (1991) Ultrastructure of feeding tubes formed in giant-cells induced in plants by the root-knot nematode *Meloidogyne incognita*. *Protoplasma* **162** 99-107
- HUSSEY, R.S., PAGUIO, O.R. & SEABURY, F. (1990) Localisation and purification of a secretory protein from the oesophageal glands of *Meloidogyne incognita* with a monoclonal antibody. *Phytopathology* **80** 709-714
- INGRAM, D.S. (1967) The expression of R-gene resistance to *Phytophthora infestans* in tissue cultures of *Solanum tuberosum*. *Journal of General Microbiology* **49** 99-108
- ISHIDA, S., TAKAHASHI, Y. & NAGATA, T. (1993) Isolation of cDNA of an auxin-regulated gene encoding a G-protein beta-subunit-like protein from tobacco BY2 cells. *Proceedings of the National Academy of Sciences USA* **90** 11152-11156
- JACKSON, D., CULIANEZ-MACIA, F., PRESCOTT, A.G., ROBERTS, K. & MARTIN, C. (1991) Expression patterns of *myb* genes from *Antirrhinum* flowers. *Plant cell* **3** 115-125
- JACOBSEN, J.V. & GUBLER, F. (1993) GARC and DNA-binding proteins: The new wave in GA action research. In: *The Past, Present and Future of Plant Biology*. (Ho, T.-H. D. & Pakrasi, H., eds) pp45-49 Washington University
- JANSSEN, R., BAKKER, J. & GOMMERS, F.J. (1991) Mendelian proof for a gene-for-gene relationship between *Globodera rostochiensis* and the H1 resistance gene from *Solanum tuberosum* spp. *andigena* CPC 1673. *Revue Nematologie* **14** 213-219
- JANSSON, H.-B., JEYAPRAKASH, A., DAMON JR, R.A. & ZUCKERMAN, B.M. (1984) *Caenorhabditis elegans* and *Panagrellus redivivus* : enzyme-mediated modification of chemotaxis. *Experimental Parasitology* **58** 270-277

- JANSSON, H.-B. & NORDBRING-HERTZ, B. (1983) The endoparasitic nematophagous fungus *Meria coniospora* infects nematodes specifically at the chemosensory organs. *Journal of General Microbiology* **129** 1121-1126
- JAYAPRAKASH, A., JANSSON, H.-B., MARBAN-MENDOZA, N. & ZUCKERMAN, B.M. (1984) *Caenorhabditis elegans*: lectin-mediated modification of chemotaxis. *Experimental Parasitology* **59** 90-97
- JENTOFT, N. (1990) Why are proteins O-glycosylated? *TIBS* **15** 291-294
- JOHN, P.C.L., ZHANG, K., DONG, C., DIEDELICH, L. & WIGHTMAN, F. (1993) p34<sup>cdc2</sup> related proteins in control of cell cycle progression, the switch between division and differentiation in tissue development, and stimulation of division by auxin and cytokinin. *Australian Journal of Plant Physiology* **20** 503-526
- JOHNSON, C.D., REINITZ, C.A., SITHIGORNGUL, P. & STRETTON, A.O.W. (1996) Neuronal localisation of serotonin in the nematode *Ascaris suum*. *Journal of Comparative Neurology* **367** 352-360
- JONES, A.M. (1990) Do we have the auxin receptor yet? *Physiologia Plantarum* **80** 154-158
- JONES, F.G.W. (1974) Host-parasite relationships of the potato cyst nematode: A speculation arising from the gene-for-gene hypothesis. *Nematologica* **20** 437-443
- JONES, J.T., PERRY, R.N. & JOHNSTON, M.R.L. (1993) Changes in the ultrastructure of the cuticle of the potato cyst nematode, *Globodera rostochiensis*, during development and infection. *Fundamental and Applied Nematology* **16** 433-445
- JONES, J.T., PERRY, R.N. & JOHNSTON, M.R.L. (1994) Changes in the ultrastructure of the amphids of the potato cyst nematode, *Globodera rostochiensis*, during development and infection. *Fundamental and Applied Nematology* **17** 369-382
- JONES, J.T., PERRY, R.N. & JOHNSTON, M.R.L. & BURROWS, P.R. (1992) Investigations of the secretions of nematode sense organs using molecular biological techniques. *Nematologica* **38** 417

- JONES, M.G.K. (1981) Host cell responses to endoparasitic attack: structure and function of giant cells and syncytia. *Annals of Applied Biology* **97** 353-372
- JONES, M.G.K. & NORTHCOTE, D.H. (1972) Nematode-induced syncytium - a multinucleate transfer cell. *Journal of Cell Science* **10** 789-809
- KALOSHIAN, I., LANGE, W.H. & WILLIAMSON, V.M. (1995) An aphid-resistance locus is tightly linked to the nematode-resistance gene, Mi, in tomato. *Proceedings of the National Academy of Sciences USA* **92** 622-625
- KAPLAN, D.T. & DAVIS, E.L. (1987) Mechanisms of plant incompatibility with nematodes. In: *Vistas on Nematology* (Veech, J.A. & Dickson, D.W., eds). pp267-276 Society of Nematologists
- KAPLAN, D.T. & GOTTWALD, T.R. (1992) Lectin binding to *Radopholus citrophilus* and *R. similis* proteins. *Journal of Nematology* **24** 281-288
- KAPLAN, J.H., FORBRUSH, B. & HOFFMAN, J.H. (1978) Rapid proteolytic release of adenosine 5' triphosphate from a protected analogue; utilisation by the Na:K pump of human red blood cell ghosts. *Biochemistry* **17** 1929-1935
- KEEN, N.T. (1990) Phytoalexins and their elicitors. *ACS Symposium Series* **439** 114-129
- KLAMBT, D. (1991) A view about the function of auxin binding proteins at plasma membranes. *Plant Molecular Biology* **6** 1045-1053
- KLEE, H., MONTOYA, A., HORODYSKI, F., LICHTENSTEIN, C., GARFINKEL, D., FULLER, S., FLORES, C., PESCHON, J., NESTER, E.W. & MILTON, G. (1984) Nucleotide sequence of *tms* genes of pTiA6NC octopine Ti plasmid: two gene products involved in plant tumorigenesis. *Proceedings of the National Academy of Sciences USA* **81** 1728-1791
- KORBER, H., STRIZHOV, N., STAIGER, D., FELDWISCH, J., OLSSON, O., SANDBERG, G., PALME, K., SCHELL, J. & KONCZ, C. (1991) T-DNA gene 5 of

*Agrobacterium tumefaciens* modulates auxin response by autoregulated synthesis of growth hormone antagonist in plants. *EMBO Journal* 10 3983-3991

**KORT, J., ROSS, H., RUMPENHORST, H.J. & STONE, A.R.** (1977) An international scheme for identifying and classifying pathotypes of potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. *Nematologica* 23 333-339

**KOZARSKY, K., KINGSLEY, D. & KRIEGER, M.** (1988) Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors. *Proceedings of the National Academy of Sciences USA* 85 4335-4339

**KUC, J.** (1995) Phytoalexins, stress metabolism, and disease resistance in plants. *Annual Review of Phytopathology* 33 275-297

**LAEMMLI, U.K.** (1970) Cleavage of the structural proteins during assembly of the head of bacteriophage T4. *Nature* 227 680-685

**LEE, D.L.** (1970) Moulting in nematodes: the formation of adult cuticle during final moult in *Nippostrongylus brasiliensis*. *Tissue and Cell* 2 139-153

**LEWIS, J.A. & HODGKIN, J.A.** (1975) Specific neuroanatomical changes in chemosensory mutants of the nematode *Caenorhabditis elegans*. *Journal of Comparative Neurobiology*. 172 489-510

**LIN, H.-J. & MCCLURE, M.A.** (1993) Surface coat proteins from the nematode *Meloidogyne incognita*. Proceedings of the APS/SON Joint Meeting 1993.

**LUFT, J.H.** (1976) The structure and properties of the cell surface coat. *International Review of Cytology* 45 291-382

**LUSCHER, B. & EISENMAN, R.N.** (1990) New light on Myc and Myb. 1. Myc. *Genes and Development* 4 2025-2035

**MAIZELS, R.M., DESAVIGNY, D. & OGILVIE, B.M.** (1984) Characterisation of surface and excretory-secretory antigens of *Toxocara canis* infective larvae. *Parasite Immunology* 1984 6 23-37

**MAIZELS, R.M. & PAGE, A.P.** (1990) Surface associated glycoproteins from *Toxocara canis* larval parasites. *ACTA Tropica* **47** 355-364

**MAIZELS, R.M. & SELKIRK, M.E.** (1988) In *The Biology of Parasitism* (Englund, P.T. & Sher, A., eds) pp285-308, Alan R. Liss

**MASAMUNE, T., ANETAI, M., TAKASUGI, M. & KATSUI, M.** (1982) Isolation of a natural hatching stimulus, Glycinoeclepin-A, for the soybean cyst nematode. *Nature* **297** 495-496

**MAUREL, C., LEBLANC, N., BARBIER-BRYGOO, H., PERROTRECHENMANN, C., BOUVIER-DURAND, M & GUERN, J.** (1994) Alterations of auxin perception in *rolB*-transformed tobacco protoplasts - time-course of *rolB* messenger RNA expression and increase in auxin sensitivity reveal multiple control by auxin. *Plant Physiology* **105** 1209-1215

**MCCLURE, B.A. & GUILFOYLE, T.** (1987) Characterisation of a class of small auxin inducible soybean polyadenylated RNAs. *Plant Molecular Biology* **9** 611-623

**MCCLURE, M.A. & STYNES, B.A.** (1988) Lectin binding sites on the amphidial exudates of *Meloidogyne*. *Journal of Nematology* **20** 321-326

**MCCLURE, M.A. & VON MENDE, N.** (1987) Induced salivation in plant-parasitic nematodes. *Phytopathology* **77** 1463-1469

**MCCRAY, J.A. & TRENTAM, D.R.** (1989) Properties and uses of photoreactive caged compounds. *Annual Review of Biophysics and Biophysical Chemistry* **18** 239-270

**MCFARLAND, K.C., SPRENGEL, L., PHILLIPS, H.S., KOHLER, M., ROSENBLIT, N., NIKOLICS, K., SEGALOFF, D.L. & SEEBURY, P.H.** (1989) Lutotrophin-chorionogonadotrophin receptor: an unusual member of the G-protein coupled receptor family. *Science* **245** 494-499

**MCLAREN, D.J.** (1974) The anterior glands of adult *Necator americanus*. (Nematoda: *Strongyloidea*) - 1. Ultrastructural studies. *International Journal of Parasitology* **4** 25-37

- MCLAREN, D.J.** (1976) Nematode sense organs. In: *Advances in Parasitology* (Davies, B., ed) vol 14 pp195-265 Academic Press, New York
- MCLAREN, D.J., BURT, J.S. & OGILVIE, B.M.** (1974) The anterior glands of adult *Necator americanus* (Nematoda: *Strongyloidea*) II. cytochemical and functional studies. *International Journal of Parasitology* **4** 39-46
- MEGHJI, M. & MAIZELS, R.M.** (1986) Glycoproteins of the parasitic nematode *Toxocara canis*. *Molecular and Biochemical Parasitology* **18** 155-170
- MILLER, A.L. & GOW, N.A.R.** (1989) Correlation between profile of ion current circulation and root development. *Physiologia Plantarum* **75** 102-108
- MILLNER, P.A.** (1995) The auxin signal. *Current Opinions in Cell Biology* **7** 224-231
- MODHA, J., ROBERTSON, W.M., KENNEDY, M.W. & KUSEL, J.R.** (1994) Characterisation of a major surface-associated excretory-secretory antigen of *Trichinella spiralis* larvae with antibodies to keyhole limpet haemocyanin. *Parasitology* **109** 531-538
- MODHA, J., KUSEL, J.R. & KENNEDY, M.W.** (1995) A role for second messengers in the control of activation-associated modification of the surface of *Trichinella spiralis* infective larvae. *Molecular and Biochemical Parasitology* **72** 141-148
- MORRIS, R.O.** (1986) Genes specifying auxin and cytokinin biosynthesis in phytopathogens. *Annual Review of Plant Physiology* **37** 509-538
- MOUNIER, N.** (1981) Location of neurosecretory-like material in *Caenorhabditis elegans*. *Nematologica* **27** 160-166
- NAPIER, R.M. & VENIS, M.A.** (1990) Monoclonal antibodies detect an auxin-induced conformational change in the maize auxin binding protein. *Planta* **182** 313-318

- NAPIER, R.M., VENIS, M.A., BOLTON, M.A., RICHARDSON, L.I. & BUTCHER, G.W.** (1988) Preparation and characterisation of monoclonal and polyclonal antibodies to maize membrane auxin binding protein. *Planta* **176** 519-526
- NELSON, F.K., ALBERT, P.S. & RIDDLE, D.L.** (1983) Fine structure of the *Caenorhabditis elegans* secretory-excretory system. *Journal of Ultrastructure Research* **82** 156-171
- NELSON, F.K. & RIDDLE, D.L.** (1984) Functional study of the *Caenorhabditis elegans* secretory-excretory system using laser microsurgery. *Journal of Experimental Zoology* **231** 45-56
- NIEBEL, A., DE ALMEIDA-ENGLER, J., HEMERLY, A., FERREIRA, P., VAN MONTAGU, M. & GHEYSON, G.** (1995) Induction of *cdc2a* and *cyc1* expression in *Arabidopsis thaliana* during early phases of plant-root-knot and plant-cyst nematode interactions. Proceedings of the 22nd International Nematology Symposium of the European Society of Nematologists 1994. *Nematologica* **41** 325
- NIEBEL, A., ENGLER, J.D., TIRE, C., ENBLER, G., VAN MONTAGU, M. & GHEYSON, G.** (1993) Induction patterns of an extensin gene in tobacco upon nematode infection. *Plant Cell* **5** 1697-1710
- NIEBEL, A., GHEYSON, G. & VAN MONTAGU, M.** (1994) Plant cyst-nematode and root-knot nematode interactions. *Parasitology Today* **10** 424-430
- NIJBOER, H. & PARLEVLIT, J.E.** (1990) Pathotype-specificity in potato cyst nematodes, a reconsideration. *Euphytica* **49** 39-47
- NORDBRING-HERTZ, B. & MATTIASSON, B.** (1979) Action of a nematode-trapping fungus shows lectin-mediated host-microorganism interaction. *Nature* **281** 477-47
- OPARKA, K.J. & PRIOR, D.A.M.** (1992) Direct evidence for pressure-generated closure of plasmodesmata. *Plant Journal* **2** 741-750

- OPPENHEIMER, D.G., HERMAN, P.L., SIVAKUMARAN, S., ESCH, J. & MARKS, M.D. (1991) A *myb* gene required for leaf trichome differentiation in *Arabidopsis* is expressed in stipules. *Cell* **67** 483-493
- OPPERMAN, C.H., TAYLOR, C.G. & CONKLING, M.A. (1994) Root-knot nematode directed expression of a plant root-specific gene. *Science* **263** 221-223
- PABO, C.T. & SAUER, R. (1992) Transcription factors; structural families and principles of DNA recognition. *Annual Review of Biochemistry* **61** 1053-1095
- PALEVITZ, B.A. & HEPLER, P.K. (1985) Changes in coupling of stomatal cells of *Allium* and *Commelina* demonstrate by microinjection of lucifer yellow. *Planta* **164** 473-479
- PALME, K. (1993) From binding proteins to hormone receptors? *Journal of Plant Growth Regulation* **12** 171-178
- PARROTT, D.M. (1981) Evidence for a gene-for-gene relationship between resistance gene H1 from *Solanum tuberosum* spp. *andigena* and a gene in *Globodera rostochiensis* and between H2 from *S. multidissectum* and a gene in *G. pallida*. *Nematologica* **27** 172-179
- PARSONS, J.C., BOWMAN, D.D. & GRIEVE, R.B. (1986) Pathological and haematological responses of cats experimentally infected with *Toxocara canis* larvae. *American Journal of Tropical Medicine and Hygiene* **25** 974-981
- PATE, J.S. & GUNNING, B.E.S. (1972) Transfer cells. *Annual Review of Plant Physiology* **23** 173-196
- PERRY, R.N. (1986) In: Cyst Nematodes. (Lamberti, F. & Taylor, C.E., eds) pp119-131 Plenum Press
- PERRY, R.N. (1989) Dormancy and hatching of nematode eggs. *Parasitology Today* **5** 377-383

- PERRY, R.N. (1994) Studies on nematode sensory perception as a basis for novel control strategies. *Fundamental and Applied Nematology* **17** 199-202
- PHILLIPS, M.S., FORREST, J.M.S. & FARRER, L.A. (1982) Invasion and development of juveniles of *Globodera pallida* in hybrids of *Solanum vernei* X *S. tuberosum*. *Annals of Applied Biology* **100** 337-344
- POEHLING, H.-M., & WYSS, U. (1980) Microanalysis of proteins in the aseptic host-parasite system: *Ficus carica* - *Xiphinema index*. *Nematologica* **26** 230-242
- POWELL, G.K. & MORRIS, R.O. (1986) Nucleotide sequence and expression of a *Pseudomonas savastanoi* cytokinin biosynthetic gene: homology with *Agrobacterium tumefaciens* *tms* and *tzs* loci. *Nucleic Acids Research* **14** 2555-2559
- PREMACHANDRAN, D., VON MENDE, N., HUSSEY, R.S. & MCCLURE, M.A. (1988) A method for staining nematode secretions and structures. *Journal of Nematology* **20** 70-78
- PROUDFOOT, L., KUSEL, J.R., SMITH, H.V., HARNETT, W., WORMS, M.J. & KENNEDY, M.W. (1993a) Rapid changes in the surface of parasitic nematodes during transition from pre-parasitic to post-parasitic forms. *Parasitology* **107** 107-117
- PROUDFOOT, L., KUSEL, J.R., SMITH, H.V. & KENNEDY, M.W. (1993b) External stimuli and intracellular signalling in the modification of the nematode surface during transition to the mammalian host environment. *Parasitology* **107** 559-566
- RATHAUR, S., ROBERTSON, D.B., SELKIRK, M.E. & MAIZELS, R.M. (1987) Secretory acetylcholinesterase from *Brugia malaya*. *Tropical Medicine and Parasitology* **38** 71-76
- RAWSTHORNE, D. & BRODIE, B.B. (1986) Relationship between root growth of potato, root diffusate production, and hatching of *Globodera rostochiensis*. *Journal of Nematology* **18** 379-384

- REDDIGARI, S.R., SUNDERMANN, C.A. & HUSSEY, R.S.** (1985) Isolation of subcellular granules from second stage juveniles of *Meloidogyne incognita*. *Journal of Nematology* **17** 482-488
- RICE, S.L., LEADBEATER, B.S.C. & STONE, A.R.** (1985) Changes in cell structure in roots of resistant potatoes parasitised by potato cyst nematodes. 1. Potatoes with resistance gene H1 derived from *Solanum tuberosum* spp. *andigena*. *Physiological Plant Pathology* **27** 219-234
- RICE, S.L., STONE, A.R. & LEADBEATER, B.S.C.** (1987) Changes in cell structure in roots of resistant potatoes parasitised by potato cyst nematodes. 2. Potatoes with resistance derived from *Solanum vernei*. *Physiological and Molecular Plant Pathology* **31** 1-14
- ROBARDS, A.W. & LUCAS, W.J.** (1990) Plasmodesmata. *Annual Review of Plant Physiology and Plant Molecular Biology* **41** 369-419
- ROBERTSON, B.D., RATHAUR, S., MCKERROW, J.H. & MAIZELS, R.M.** (1987) Functional analyses of the excretory-secretory molecules of *Toxocara canis* infective larvae. *Journal of Cellular Biochemistry* **11A** 172-180
- ROBINETTE, D. & MATTHYSSE, A.G.** (1990) Inhibition by *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* of development of the hypersensitive response elicited by *Pseudomonas syringae* pv. *phaseolicola*. *Journal of Bacteriology* **172** 5742-5749
- ROBINSON, M.P., ATKINSON, H.J. & PERRY, R.N.** (1985) The effect of delayed emergence on infectivity of juveniles of the potato cyst nematode *Globodera rostochiensis*. *Nematologica* **31** 171-178
- SCHOTS, A., GOMMERS, F.J. & EGBERTS, E.** (1992) Quantitative ELISA for the detection of potato cyst nematodes in soil samples. *Fundamental and Applied Nematology* **15** 55-61
- SCHOTS, A., HERMSEN, T., SCHOUTEN, S., GOMMERS, F.J. & EGBERTS, E.** (1989) Serological differentiation of the potato cyst nematodes *Globodera pallida* and

- Globodera rostochiensis*. 2. Preparation and characterisation of species-specific monoclonal antibodies. *Hybridoma* 8 401-413
- SENGUPTA, P., COLBERT, H.A., KIMMEL, B.E., DWYER, N. & BARGMANN, C.I. (1993) The cellular and genetic basis of olfactory responses in *Caenorhabditis elegans*. *CIBA Foundation Symposia* 179 235-250
- SEYMOUR, M.K. (1983) The feeding pump of *Ditylenchus dipsaci* (Nematoda, Tylenchida). *Nematologica* 29 171-189
- SHEN-ONG, G.L.C. (1990) The *Myb* oncogene. *Biochimica et Biophysica ACTA* 1032 39-52
- SIJMONS, P.C., ATKINSON, H.J. & WYSS, U. (1994) Parasitic strategies of root nematodes and associated host cell responses. *Annual Review of Phytopathology* 32 235-259
- SIJMONS, P.C., GRUNDLER, F.M.W., VON MENDE, N., BURROWS, P.R. & WYSS, U. (1991) *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *Plant Journal* 1 245-254
- SIMONS, T.J., ISRAEL, H.W. & ROSS, A.F. (1972) Effect of 2,4-dichlorophenoxyacetic acid on tobacco mosaic virus lesions in tobacco and on the structure of adjacent cells. *Virology* 48 502-525
- SMIDT, M. & KOSUGE, T. (1978) The role of indole-3-acetic acid accumulation by alpha methyl tryptophan-resistant mutants of *Pseudomonas savastanoi* in gall formation on oleanders. *Physiology and Plant Pathology* 13 203-209
- SMITH, C.J. (1996) Accumulation of phytoalexins - defence-mechanism and stimulus-response system. *New Phytologist* 132 1-45
- SMITH, H.V. (1991) In Parasitic Nematodes - Antigens, Membranes and Genes (Kennedy, M.W., ed) pp116-139, Taylor & Francis

- SMITH, H.V., QUINN, R., KUSEL, J.R. & GIRDWOOD, R.W.A. (1981) The effect of temperature and anti-metabolites on antibody binding to the outer surface of second-stage *Toxocara canis* larvae. *Molecular and Biochemical Parasitology* **4** 183-193
- SOLANO, R., NIETO, C. & PAZARES, J. (1995) Myb.PH3 transactivation factor from *Petunia hybrida* induces similar DNA-bending/distortions on its two types of binding sites. *Plant Journal* **8** 673-682
- SPIEGEL, Y. & MCCLURE, M.A. (1995) The surface coat of plant-parasitic nematodes - chemical composition, origin and biological role - a review. *Journal of Nematology* **27** 127-134
- SPIEGEL, Y., MCCLURE, M.A., KAHANE, I., ROBERTSON, W.M. & SOLOMON, R. (1991) Wheat germ agglutinin bound to the outer cuticle of the seed-gall nematodes *Anguina agrostis* and *Anguina tritici*. *Journal of Nematology* **23** 451-456
- SPIEGEL, Y. & ROBERTSON, W.M. (1988) Wheat germ agglutinin binding to the outer cuticle of the plant-parasitic nematode *Anguina tritici*. *Journal of Nematology* **20** 499-501
- STEINER, G. (1925) The problem of host selection and host specialisation of certain plant-infesting nemas and its application in the study of nemic pests. *Phytopathology* **15** 499-534
- STEWART, G.R., PERRY, R.N., ALEXANDER, J. & WRIGHT, D.J. (1993a) A glycoprotein specific to the amphids of *Meloidogyne* species. *Parasitology* **106** 405-412
- STEWART, G.R., PERRY, R.N. & WRIGHT, D.J. (1993b) Studies on the amphid specific glycoprotein gp32 in different life-cycle stages of *Meloidogyne* species. *Parasitology* **107** 573-578
- STONE, A.R. (1973) *Heterodera pallida* n. sp. (Nematoda: Heteroderidae), a second species of potato cyst nematode. *Nematologica* **18** 591-606

- SUGANE, K. & OSHIMA, T. (1984) Induction of peripheral-blood eosinophilia in mice by excretory and secretory antigens of *Toxocara canis* larvae. *Journal of Helminthology* **58** 143-147
- TAKAHASHI, Y., ISHIDA, S. & NAGATA, T.J. (1993) Functions and modulations of auxin-regulated genes. *Plant Research* **106** 357-367
- TANIGUCHI, T., MIZUOCHI, T., BELAE, M., DWEK, R.A., RADEMACHER, T.W. & KOBATA, A. (1986) Structures of the sugar chains of rabbit immunoglobulin G: Occurrence of asparagine-linked sugar chains in the Fab fragment. *Biochemistry* **24** 5551-5557
- THEOLOGIS, A, HUYNH, T.V. & DAVIS, R.W. (1985) Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. *Journal of Molecular Biology* **183** 53-68
- TOWBIN, H., STAHELIM, T. & GORDON, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences USA* **76** 43-50
- TRETT, M.W. & PERRY, R.N. (1985a) Functional and evolutionary implications of the anterior sensory anatomy of species of root-lesion nematode (genus *Pratylenchus*). *Revue Nematologie* **8** 341-355
- TRETT, M.W. & PERRY, R.N. (1985b) Effects of the carbamoyloxime, aldicarb, on the ultrastructure of the root-lesion nematode, *Pratylenchus penetrans* (Nematoda: *Pratylenchidae*). *Nematologica* **31** 321-334
- TROEMEL, E.R., CHOU, J.H., DWYER, N.D., COLBERT, H.A. & BARGMANN, C.I. (1995) Divergent seven transmembrane receptors are candidate chemosensory receptors in *Caenorhabditis elegans*. *Cell* **83** 207-218
- TRUDGILL, D.L. (1985) Potato cyst nematodes: a critical review of the current pathotype scheme. *EPPO Bulletin* **15** 273-279

- TRUDGILL, D.L. & PARROTT, D.M. (1969) The behaviour of nine populations of the potato cyst nematode *Heterodera rostochiensis* towards three resistant potato hybrids. *Nematologica* 15 381-388
- TURNER, S.J. (1980) Resistance in *Solanum vernei* hybrids to potato cyst nematodes. Ph.D Thesis, University of Birmingham, U.K.
- VAN DEN ACKERVENKEN, G.F.J.M., VAN KAN, J.A.L. & DEWIT, P.J.G.M. (1992) Molecular analyses of the avirulence gene *avr9* of the fungal tomato pathogen *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. *Plant Journal* 2 259-266
- VAN DER EYCKEN, W. (1994) Molecular analysis of the compatible plant-nematode interaction: tomato - *Meloidogyne incognita*. Ph.D Thesis, Universteit Gent.
- VAN DER EYCKEN, W., ENGLER DE ALMEIDA, J., VAN MONTAGU, M. & GHEYSON, G. (1995) Molecular study of the compatible interaction: *Meloidogyne incognita* - tomato. Proceedings of the 22nd International Nematology Symposium of the European Society of Nematologists, Gent (Belgium), 1994. *Nematologica* 41 350
- VEECH, J.A., STARR, J.L. & NORDGREN, R.M. (1987) Production and partial characterisation of stylet exudate from adult females of *Meloidogyne incognita*. *Journal of Nematology* 19 463-468
- VENIS, M.A. & NAPIER, R.M. (1995) Auxin receptors and auxin binding proteins. *Critical Reviews in Plant Science* 14 27-47
- VENIS, M.A., NAPIER, R.M., BARBIER-BRYGOO, H., MAUREL, C., PERROT-RECHENMANN, D. & GURN, J. (1992) Antibodies to a peptide from the auxin binding protein have auxin agonist activity. *Proceedings of the National Academy of Sciences USA* 89 7208-7212
- VOGT, R.G., RYBCZYNSKI, R. & LERNER, M.R. (1990) Biochemistry and Molecular Biology of pheromone reception and transduction. *Abstracts of Papers of the American Chemical Society* 199 76

- WADDELL, A.H. (1968) The excretory system of the kidney worm *Stephanurus dentatus* (Nematoda). *Parasitology* **58** 907
- WALKER, J.C. & KEY, J.L. (1982) Isolation of cloned cDNAs to auxin-responsive poly (A)<sup>+</sup> RNAs of elongating soybean hypocotyl. *Proceedings of the National Academy of Sciences USA* **9** 7185-7189
- WARD, S. (1973) Chemotaxis by the nematode *Caenorhabditis elegans*: identification of attractants and analysis of the response by use of mutants. *Proceedings of the National Academy of Sciences USA* **70** 817-821
- WEINSTEIN, P.P. (1952) Regulation of water balance as a function of the excretory system of the filariform larvae of *Nippostrongylus muris* and *Ancylostoma caninum*. *Experimental Parasitology* **1** 363-377
- WEISS, D., VAN TUNEN, A.J., HALEVY, A.H., MOL, J.N.M. & GERATS, A.G.M. (1990) Stamens and gibberellic acid in the regulation of flavonoid gene expression in the corolla of *Petunia hybrida*. *Plant Physiology* **94** 511-515
- WERGIN, W.P. & ENDO, B.Y. (1976) Ultrastructure of a neurosensory organ in a root knot nematode. *Journal of Ultrastructure Research* **56** 258-276
- WHARTON, D.A. & SOMMERVILLE, R.I. (1984) The structure of the excretory system of the infective larva of *Haemonchus contortus*. *International Journal of Parasitology* **14** 591-600
- WILLIAMS, T.D. (1956) The resistance of potatoes to root eelworms. *Nematologica* **1** 88-93
- WINTERBORNE, D.J. (1986) Cell growth determined by a dye-binding protein assay. *Biochemical Society Transactions* **14** 1179
- WRIGHT, K.A. (1980) Nematode sense organs. In: *Nematodes as biological models*, Vol. 2: Ageing and other model systems. New York, Academic Press pp237-297

- WRIGHT, K.A. (1987) The nematode cuticle, its surface and the epidermis, -function, homology, analogy - a current consensus. *Journal of Parasitology* **73** 1077-1083
- WYSS, U. (1992) Observations on the feeding behaviour of *Heterodera schachtii* through out development including events during moulting. *Fundamental and Applied Nematology* **15** 75-89
- WYSS, U. & ZUNKE, U. (1986) Observations on the behaviour of second stage juveniles of *Heterodera schachtii* inside host roots. *Revue Nematologie* **9** 153-165
- YAMADA, T. (1993) The role of auxin in plant-disease development. *Annual Review of Phytopathology* **31** 253-273
- YAMAMOTO, Y.T. (1994) Further characterisation of auxin-regulated mRNAs in hypocotyl sections of mung bean (*Vigna radiata* (L.) Wilczek): Sequence homology to genes for fatty-acid desaturases and atypical late embryogenesis-abundant protein, and the mode of expressions of the mRNAs. *Planta* **192** 359-364
- YAMAMOTO, Y.T., CHENG, C.-L. & CONKLING, M.A. (1990) Root-specific genes from tobacco and *Arabidopsis* homologous to an evolutionarily conserved gene family of membrane channel proteins. *Nucleic Acids Research* **18** 7449
- YAMAMOTO, Y.T., TAYLOR, C., ACEDO, G.N., CHENG, C.-L. & CONKLING, M.A. (1991) Characterisation of *cis*-acting sequences regulating root-specific gene expression in tobacco. *The Plant Cell* **3** 371-382
- ZUCKERMAN, B.M. (1983) Hypotheses and possibilities of intervention in nematode chemoresponses. *Journal of Nematology* **15** 173-182
- ZUCKERMAN, B.M. & JANSSON, H.-B. (1984) Nematode chemotaxis and possible mechanisms of host-prey recognition. *Annual Review of Phytopathology* **22** 95-113
- ZUNKE, U. (1985) The formation of the sub-crystalline layer of *Heterodera schachtii* under aseptic conditions. *Nematologica* **31** 117-120