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STUDIES ON THE ENDOCRINE DEVELOPMENT OF NEMATODES

by

ROGER D.W. DENNIS B.Sc. (Hons.), M.Sc.

Department of Plant Pathology
Waite Agricultural Research Institute
The University of Adelaide
South Australia

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SUMMARY

The results obtained in this work can be interpreted in terms of the following working hypothesis; growth and developmental control mechanisms in nematodes are similar to or the same as those in insects. This implies that the endocrine factors themselves are the same or closely related.

Various physiological and biochemical events of morphogenetic processes in the Nematoda, superficially similar to those in the Insecta, were examined to determine any relationships in the control mechanisms. The nematode, Nematospiroides dubius, reacted in an analogous physiological manner to insect growth hormones at definite stages of the life-cycle. Moulting was stimulated by α -ecdysone and inhibited by synthetic juvenile hormone (SJH), whilst egg-laying by females was unaffected by the former but stimulated by the latter terpenoid. The stimulation by α -ecdysone of the activity of the leucyl naphthylamidase (Induction Ratio 1.709 at 24 hr post-infection) located in the body-wall of Aphelenchus avenae, indicated a possible hormonal correlation between moulting and this enzyme. The purification of the enzyme by $(\text{NH}_4)_2\text{SO}_4$ fractionation, ion-exchange and gel filtration chromatography was unsuccessful due to its instability, with an enzyme purification of 27-fold but an equivalent protein purification of 514-fold. The properties of the crude enzyme extract resembled those of the mammalian (leucyl) naphthylamidases. The enzyme required a thiol group for activity, was inhibited by the sulphydryl reagent β -hydroxymercuribenzoate and puromycin, had a neutral pH optima, and was unstable

following $(\text{NH}_4)_2\text{SO}_4$ and DEAE-cellulose chromatography. Evidence is presented that it may be a Zn-dependent metalloenzyme.

If the mechanism of action of the control of translation of protein synthesis (gene expression) by juvenile hormone was at the level of the polyribosome, then it was not by altered profiles in Panagrellus redivivus. The ribosomes, polyribosomes and polyribosomal RNA's of P. redivivus were considered "normal", following physical, chemical and biochemical comparison with other invertebrates. Also, the cytoplasmic RNA's of A. avenae showed the expected S-values and M.W.'s.

Direct evidence for the presence of morphogenetic hormones of the MH-type (moulting hormone) has been shown by radioimmunoassay for β -ecdysone, and ecdysone-like substances have been shown to occur in the nematodes, P. redivivus and A. avenae. The yield was consistently greater in the latter animal. Indirect evidence from the effect of various Na^+/K^+ ratios, in the absence of Ca^{2+} , on the moulting of fourth-stage larvae of N. dubius also suggests this conclusion. The effect of such hormones on insects is mediated by the alteration of the ionic balance of the nuclear sap, including the above cations (Kroeger, 1963). No β -ecdysone-specific binding proteins were detected in the cytosol fraction of P. redivivus. From equivalence with the properties of the mammalian steroid hormone receptors, this would have given added physiological evidence to the above findings.

STATEMENT

This thesis has not previously been submitted for an academic award at this or any other university, and is the original work of the author, except where due reference is made in the text.

Signed:

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CHAPTER I
GENERAL INTRODUCTION

The basic life-cycle of nematodes is manifested by the free-living forms, where after hatching there are four "larval" (juvenile) stadia, each of which is concluded by a moult, before the formation of the adult (Rogers, 1962). Postembryonic development is characterized by a progressive sequence of changes as the nematodes proceed to the adult stage, e.g. the increase in size and the formation of a functional reproductive system, and without any profound changes in their anatomy or physiology. This is modified in the animal-parasitic forms but not the plant-parasites, where a resting infective stage links the free-living and parasitic-environments encountered during the life-cycle. The minor anatomical but presumed fundamental physiological changes associated with the formation of the infective stage and initial phase of parasitic development (Rogers and Sommerville, 1963), resemble in outline the metamorphosis of many free-living animals, and are associated with the different environments inhabited by the larval (juvenile) and adult forms.

Such anatomical changes that have been studied are associated with the moults that initiate and terminate the infective stage, in particular, of the ensheathed infective larvae. The buccal capsule of the non-feeding third-stage larvae of Haemonchus contortus is re-organised before the third moult (Sommerville, 1964; Mapes, 1969), and the ultrastructure of the cuticle differs from that of preparasitic

or parasitic larval and adult forms in the nematodes, Nippostrongylus brasiliensis (Jamuar, 1966), Nematospiroides dubius (Bonner et al., 1970) and Meloidogyne javanica (Bird and Rogers, 1965; Bird, 1968). This is also seen with the morphological changes that occur in the formation and conclusion of the preadult condition in Paratylenchus projectus and Paratylenchus dianthus (Rhoades and Linford, 1959, 1961).

In its essential outline, growth and development of the free-living nematodes resemble that which occurs in the most primitive insects, the Apterygota (Imms, 1964), i.e. metamorphosis is always of a slight and gradual nature, or more often is absent. The major difference in the pattern of growth is that nematodes increase in size between moults, whilst insects increase in size both during and immediately following the moult (Lee, 1965). The endocrine control mechanism of growth and development in insects, is most perfectly understood in the Holometabola and the Hemimetabola (Highnam and Hill, 1969; Williams, 1970; Gilbert, 1974) with the same mechanism operating for both moulting and metamorphosis. Metamorphosis in insects is closely associated with moulting. There is also evidence that the hormonal regulation of the moulting and of the reproductive cycles in the Ametabola are basically similar (Watson, 1964; Rohdendorf and Watson, 1969).

It is therefore possible to postulate a chain of circumstantial evidence that links the similarity of the physiological and anatomical changes that occur in nematodes during their life-cycle to the known endocrine control of superficially similar processes that occur in insects. This leads to a working hypothesis (which this thesis was

designed to test), that the growth and developmental control mechanisms in the Nematoda are similar to those in the Insecta. This implies that the endocrine factors themselves are the same or are closely related.

Certain aspects of this hypothesis were tested with the view of either substantiating or modifying the ideas put forward. The following questions were asked and tested experimentally. Do nematodes react in a similar physiological manner to the insect growth hormones at definite stages of the life-cycle? Assuming that the RNA species and ribosomes of nematodes are "normal", does moulting hormone (MH) or juvenile hormone (JH) cause the synthesis of a hormone-specific protein(s)? Leucine amino-peptidase (LAP) has been associated with the moulting and exsheathment of nematodes (Rogers, 1965, 1970); how and when does the MH affect this enzyme, if at all? If MH is involved in the postembryonic development of nematodes, can it be detected by direct and/or indirect methods?

In an attempt to answer these questions, nematodes from three different ecological niches were examined; an animal parasite (N. dubius); a plant and fungal feeder (Aphelenchus avenae); and, a bacterial feeder (Panagrellus redivivus).

CHAPTER II

INSECT MORPHOGENETIC HORMONES AND DEVELOPMENTAL MECHANISMS

IN THE NEMATODE, NEMATOSPIROIDES DUBIUS

II.1 INTRODUCTION

Previous studies of the effects of various mammalian and plant hormones on nematodes have mainly revealed indirect effects via the respective tissue treated. Thus males of higher vertebrates are more susceptible than females to helminth infection; whilst gonadectomy of males reduced, and testosterone treatment increased, this susceptibility (Solomon, 1969). However, no sex-linked resistance was detected in mice against the nematode, Nematospiroides dubius (Bryant, 1974). Webster (1967) found indirect stimulation of the multiplication of Aphelenchoides ritzemabosi developing on lucerne callus tissue treated with kinetin, IAA and gibberellic acid (GA_3), as a result of increased feeding sites i.e. cells. Also, the increased reproduction rate of Ditylenchus dipsaci, Pratylenchus penetrans and Pratylenchus zaeae, in response to the application of the plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D), was due to the indirect stimulation of the growth of the alfalfa callus tissue (Krusberg and Blickenstaff, 1964).

At the present time data obtained by in vitro culture have shown juvenile hormone (JH) and mimics to cause inhibition of maturation and of reproduction in Caenorhabditis elegans, Panagrellus redivivus (Dropkin et al., 1971), Caenorhabditis briggsae (Hansen and Buecher, 1970) and Trichinella spiralis (Shanta and Meerovitch, 1970), and specific cell

divisions in the gonadal tissues of the males of Heterodera schachtii (Johnson and Viglierchio, 1970). However, the first two authors showed β -ecdysone to have no effect on growth or development, although Webster and Craig (1969) indicated that the development of a Cephalobus sp. was highly sensitive to a concentration of $1.1 \times 10^{-7}M$. Hitcho and Thorson (1971) extracted a steroid resembling β -ecdysone from Trichinella spiralis that increased moulting and growth of larvae of this nematode; farnesal was also extracted and resulted in increased length of larvae.

The pathway from stimulus to neuroendocrine secretion, to stimulation of the excretory gland to produce and release leucine aminopeptidase (LAP), that results in ecdysis was elucidated by Davey and Kan (1968) for the nematode, Phocanema decipiens. Juvenile hormone produced a moult, due to a stress-type reaction, at a concentration of $10^{-6}M$ (Davey, 1971). Finally, Rogers and Head (1972) have implicated noradrenaline's involvement in the morphogenetic stimulus of Haemonchus contortus, after infection.

The present study is an attempt to elucidate, by more quantitative means, the effects of insect, mammalian and plant hormones on the processes of moulting and egg-laying in the mouse intestinal parasite, Nematospiroides dubius, and to consider the Na^+/K^+ ratio, in context of a "stimulus" for the moulting of nematodes.

II.2 MATERIALS AND METHODS

II.2.A Culture of the nematode (determination of time of the final moult)

The life-cycle studies of Baker (1954) and Bryant (1973) enabled the use of an in vitro incubation technique in order to determine the time of moulting of fourth-stage larvae in the different media. This would enable a standardised regimen to be formulated for the testing of various compounds on the last moult of this nematode.

X About 300 third-stage larvae in 0.1 ml of water were administered by syringe into the mouths of female mice (Sommerville, 1972). At the required time after infection, fourth-stage larvae were removed from the muscle of the small intestine of the mice; all extractions were performed in 0.9% NaCl. The larvae were then cultured in vitro, in 2 ml of the respective media for 48 hr at 39° in the dark (roller tube culture - 12 revolutions per hour). All solutions contained the antibiotics kanamycin (Bristol Laboratories, Australia) and mycostatin (Squibb Ltd, Australia), at the respective concentrations of 5 mg/ml and 100 units/ml. The in vitro incubation media tested were: 199 (C.S.L., Australia), Tyrode's solution (C.S.L., Australia), 0.75% NaCl/0.15% KCl, and 0.9% NaCl. The fourth-stage larvae were isolated at 96, 106, 116, 126 and 136 hr postinfection and cultured in the above media.

In all subsequent experiments, incubations were performed in Tyrode's solution (Fig. 1(d)), of nematodes removed at 106 hr post-infection.

II.2.B Effect of hormones and inhibitors on the last-stage moult

At 106 hr after infection, the larvae were placed in Tyrode's solution, containing the appropriate hormone treatment. The plant growth regulators gibberellic acid GA_3 and kinetin stock solutions were in water. The mammalian sex steroids testosterone and oestradiol- 17β standard solutions were in ethanol, whilst the insect hormones α -ecdysone (Schering AG, Berlin), "dichlorofarnesoate" (Calbiochem, U.S.A. - Law et al., 1966; Vinson and Williams, 1967) and a synthetic juvenile hormone (Ayerst Research Laboratories) with 10% of the biological activity of the pure naturally-occurring hormone, were contained in methanol. These latter compounds were applied to the kimble tubes and the solvent evaporated off under dry N_2 , before addition of the incubating solution. The procedure described in Section II.2,A was then followed. The antibiotics actinomycin D (Merck, Sharp and Dohme) and puromycin di-HCl (Sigma, U.S.A.) were used to examine the physiological effects on the stimulation of moulting by α -ecdysone; each was added separately to a single concentration (2.2×10^{-9} M) of α -ecdysone.

Anti-rabbit immunoglobulin (sheep) labelled with fluorescein (Wellcome Reagents Ltd, England) was incorporated into the incubation medium to determine if the fourth-stage larvae or moulted adults fed within the 48 hr culture period. To this system was added either α -ecdysone (2.2×10^{-9} and 10^{-11}) or synthetic juvenile hormone (3.3×10^{-9} M). After incubation the nematodes were viewed under U/V light microscopy for the detection of green-yellow fluorescence (filters:

N-heat shield; BG 38; two BG 12's; and, K510 suppressor in the eyepiece).

II.2.C Effect of hormones on egg-laying

Females were removed from the lumen of the gut of the mouse at 410 hr postinfection, and the procedure followed as in Section II.2.A.

II.2.D Effect of the Na^+/K^+ ratio on moulting

The worms were removed at 122 hr postinfection and placed in iso-osmotic solutions of: 0.9% NaCl, 0.75% NaCl/0.15% KCl, 0.6% NaCl/0.3% KCl, 0.5% NaCl/0.4% KCl and 0.4% NaCl/0.5% KCl, and the % moult observed after 48 hr at 39⁰. The osmotic pressures were equivalent to the following concentrations: 0.9% NaCl, 317 m-osmol/l; NaCl/KCl ratios, 301 m-osmol/l; Tyrode's solution, 308 m-osmol/l; and, 199, 317 m-osmol/l.

II.2.E Statistical analysis

All results were converted to arc-sine for analysis. They were tested by analysis of variance, except those for the Na^+/K^+ ratios, which were examined by the 't' test. The level of probability used was 1%.

II.3 RESULTS

The criterion of moulting used was the complete separation of the fourth-stage cuticle from the underlying adult cuticle. The adult cuticle was thicker and exhibited developmental polymorphism when compared to the appearance of the larval cuticle, i.e. the adult cuticle

had longitudinal ridges along the length of its surface, as opposed to the horizontal striations of the larva. Males were easily recognised by the copulatory bursa; females were usually tightly coiled, which obscured the vulval opening. This tight coiling was not consistently relaxed by treatment with the anthelmintics piperazine citrate or hexahydrate (12.5 mg/ml) and followed by gentle heating.

II.3.A Time of moulting

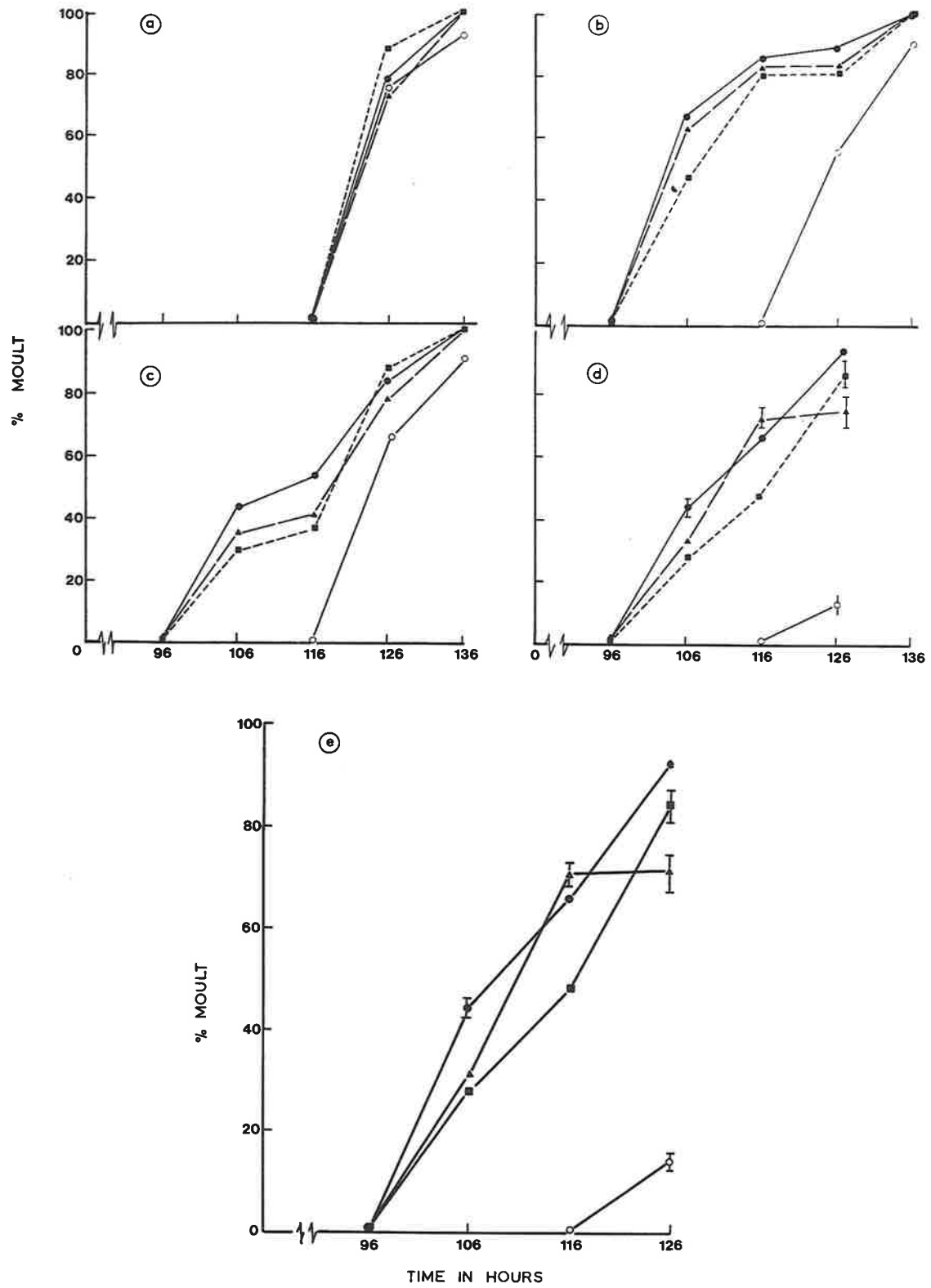
The in vitro rate of development of fourth-stage larvae to adults, i.e. percent moulted, was similar in the 199, Tyrode's and 0.75% NaCl/0.15% KCl media, with 100% attained by 136 hr postinfection. In 0.9% NaCl it was reduced, with 91% adults produced by 136 hr post-infection. A comparison of the development of male and female larvae, revealed a dichotomy in the time at which the majority of adults had appeared. Thus, by 116 hr, 85% of the male larvae were adult in the 199, Tyrode's and 0.75% NaCl/0.15% KCl media. Again, the exception was the 0.9% NaCl solution, where the developmental rate paralleled that of the female larvae in the above media. By 126 hr, 75% of the female larvae had developed to adults. This rate paralleled that which was attained by male larvae in the 199 and Tyrode's media at 106 hr postinfection (Figs. 1(a), 1(b), 1(c)).

II.3.B Hormones and moulting

The percent moult, as against the Tyrode control, was stimulated by α -ecdysone at 2.2×10^{-7} , 10^{-9} and 10^{-11} M. The synthetic juvenile hormone inhibited moulting at 3.3×10^{-7} , 10^{-9} and 10^{-11} M, whilst the

Figure 1. The percent moult of fourth-stage larvae recovered at 10 hr intervals from mice and placed in various media: ●, 199; ▲, Tyrode; ■, 0.75% NaCl/0.15% KCl; ○, 0.9% NaCl. (a) The percent moult of female larvae of population A; (b) The percent moult of male larvae of population A; (c) The combined percent moult of larvae of population A; (d) The combined percent moult of larvae of population B; (e) The combined percent moult of population C. Each point is the mean of three replicates each of about 20 larvae.

FIG.1



'dichlorofarnesoate' produced no significant difference from the control (Fig. 2(a)). Of the plant growth regulators, gibberellic acid at 5.8×10^{-7} , 10^{-8} and 10^{-9} M inhibited moulting, and at 9.3×10^{-7} M kinetin stimulated moulting. The mammalian sex steroids produced significant effects on moulting, oestradiol- 17β inhibited at 3.5×10^{-7} and 10^{-9} M, and testosterone caused the same effect at 3.5×10^{-11} M (Table I). Actinomycin D at 1.6×10^{-5} M and 4.0×10^{-6} M, and puromycin at 5×10^{-4} M and 5×10^{-5} M, caused no significant inhibition of moulting (Table II).

II.3.C Feeding

Neither the fourth-stage larvae nor the moulted adults exhibited autofluorescence. Nematodes in the presence of fluorescent protein (1 mg/ml or 0.5 mg/ml) and the respective insect growth hormone, showed no labelled material in the digestive tract. The only detectable response was from the external surface of the fourth-stage larvae and of the fourth-stage larval cuticles shed from adult nematodes.

II.3.D Egg-laying

The results were assessed as eggs/female/24 hr. In vitro culture of the females removed at 410 hr postinfection, rather than at 250 hr, showed that α -ecdysone and dichlorofarnesoate had no significant effect on egg-laying. The synthetic juvenile hormone stimulated egg-laying at 2.2×10^{-7} and 10^{-9} M (Fig. 2(b)). Gibberellic acid GA_3 and oestradiol- 17β were without effect, although kinetin was again positive at

Figure 2. The effect of insect hormones on (a) the percent moult of fourth-stage larvae, 106 hr post-infection and (b) the number of eggs laid per female per 24 hr, 410 hr post-infection: ●, ecdysone; ▲, dichlorofarnesoate; ■, synthetic juvenile hormone; --- control - Tyrode's solution. Each point for the percent moult is the mean of 3 replicates each of about 20 larvae and for the number of eggs laid is the mean of 4 replicates of 4 females.

FIG. 2

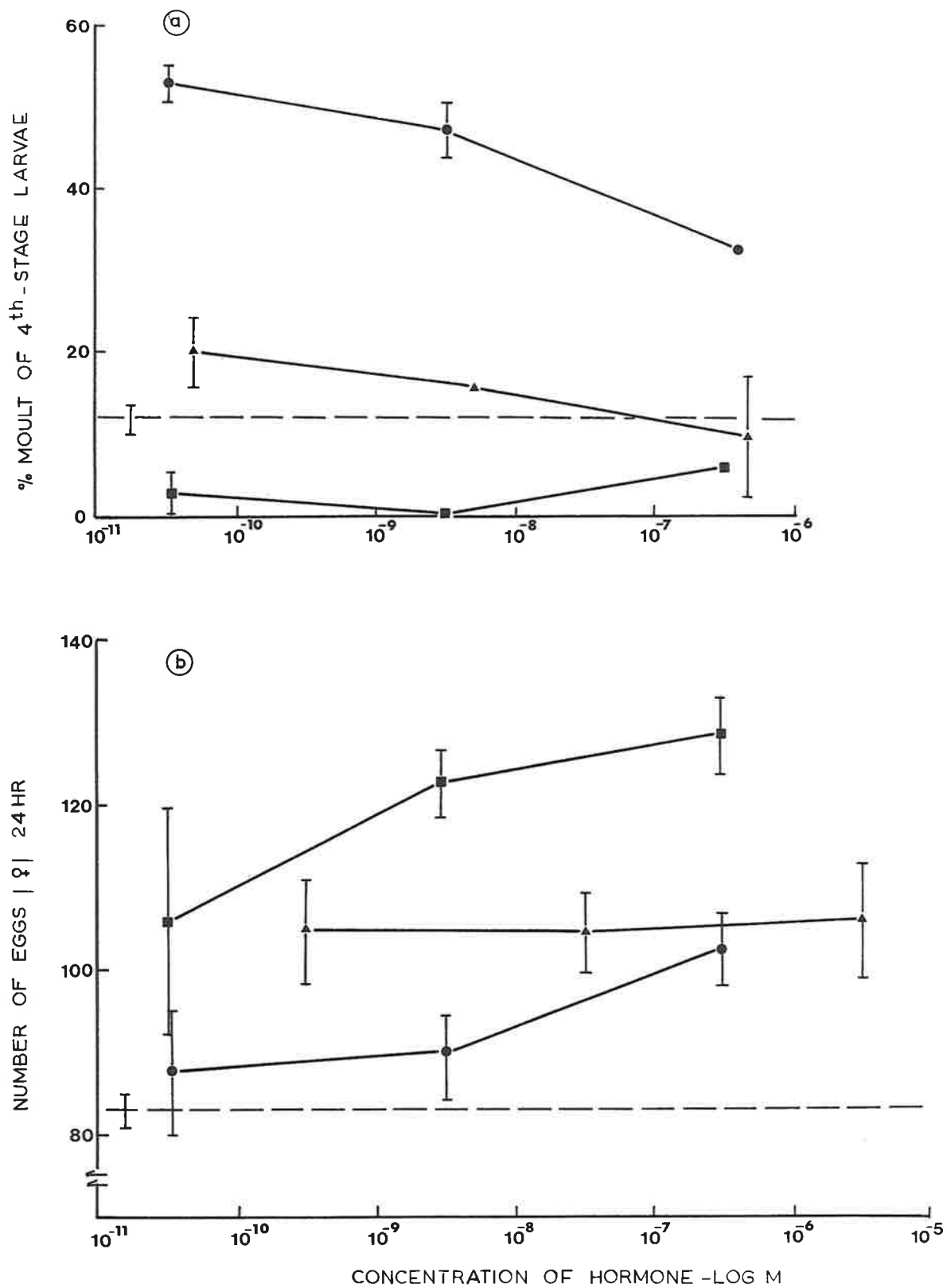


Table 1. The effect of two plant hormones and mammalian sex steroids on the fourth – fifth stage moult of N. dubius.

	Concentration	% Molt	Significance
Gibberellic Acid	$5.8 \times 10^{-8}M$	0.0 ± 0.0	S-ve
	$5.8 \times 10^{-9}M$	14.6 ± 0.16	S-ve
	$5.8 \times 10^{-10}M$	14.3 ± 0.32	S-ve
Kinetin	$9.3 \times 10^{-6}M$	28.5 ± 5.46	NS
	$9.3 \times 10^{-7}M$	33.3 ± 7.1	S+ve
	$9.3 \times 10^{-8}M$	31.4 ± 0.27	NS
Testosterone	$3.5 \times 10^{-7}M$	20.8 ± 0.45	NS
	$3.5 \times 10^{-9}M$	20.7 ± 0.64	NS
	$3.5 \times 10^{-11}M$	14.4 ± 0.53	S-ve
Oestradiol-17 β	$3.7 \times 10^{-7}M$	0.0 ± 0.0	S-ve
	$3.7 \times 10^{-9}M$	13.8 ± 0.34	S-ve
	$3.7 \times 10^{-11}M$	19.6 ± 0.99	NS
Tyrosine Control	-	19.7 ± 1.74	-

Statistical Analysis: Analysis of variance of each hormone against the Tyrosine Control.

F Test $P < 0.01$ Significance tested by L.S.D. at $P < 0.05$

NS Not Significant; S+ve Stimulation; S-ve Inhibition

Values of percent moult are means \pm S.E.M. (Arc-Sine Transformation)

Each figure is the mean of 3 replicates of about 20 larvae each.

Table II. The effect of the antibiotics actinomycin-D and puromycin on α -ecdysone induced moulting of N. dubius fourth-stage larvae^a.

No.	Concentration of α -ecdysone (M)	Concentration of Actinomycin-D	Inhibitor Puromycin	% Moul ^t ^b
1	2.2×10^{-9}	1.6×10^{-5}	-	33.5
2	2.2×10^{-9}	4.0×10^{-6}	-	35.9
3	2.2×10^{-9}	-	5.0×10^{-4}	39.8
4	2.2×10^{-9}	-	5.0×10^{-5}	39.3
5	2.2×10^{-9}	-	-	34.8

a The procedure was as in 'Materials and Methods' Section I. The antibiotic and moulting hormone were added simultaneously.

b The percent result was made on triplicate determinations, with approximately 20 fourth-stage larvae per replicate.

$9.3 \times 10^{-8} \text{M}$. Of significance, was the stimulation by testosterone at all concentrations (Table III).

II.3.E Na^+/K^+ ratio

All concentrations of potassium were significantly higher than 0.9% NaCl (Fig. 3), as no moult occurred in the latter solution. Extrapolation of the points of the curve, showed that 0.67% NaCl/0.23% KCl would give the maximum moulting response. This was estimated to be 54%.

II.4 DISCUSSION

Comparison of the Tyrode controls at 106 hr (Figs. 2(a), (b)) and the timing of the last-stage moult (Fig. 1(d)), indicated that different populations varied considerably in their vitality. This required that each subsequent experiment be related to its own control.

In preliminary experiments, it was necessary to determine the time following infection for larvae to approach the last moult, such that, after in vitro culture for 48 hr at 39° in the dark, a low level of worms would moult to the adult and thus would function as the control. This then allowed the comparison of the control with treatments of various test compounds in a standardised regime. Because of the similarity of the percent moulted at 106 hr in vitro in the 3 media (199; Tyrode's; 0.75% NaCl/0.15% KCl) it was decided to use the simplest medium, i.e. Tyrode's, to avoid any possible complications of the test compound interacting with a component(s) in the complex medium, i.e. 199. In

Figure 3. The effect of varied Na^+/K^+ ratios on the percent moult of fourth-stage larvae, 122 hr post-infection. Each point is the mean of 3 replicates, each of about 20 larvae.

FIG. 3

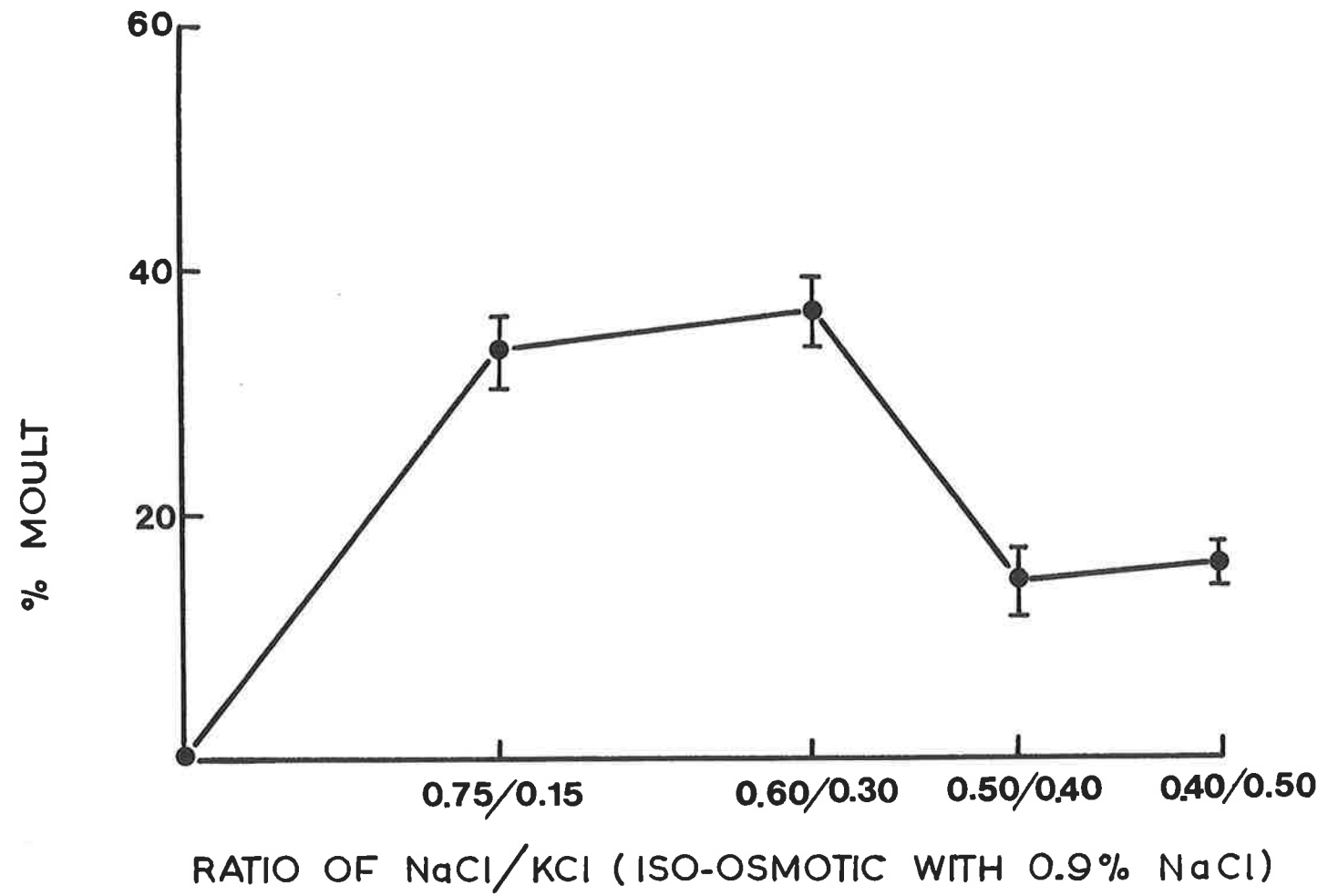


Table III. The effect of two plant hormones and mammalian sex steroids on egg-laying by female N. dubius.

	Concentration	Average number of eggs/female/24 hr	Significance
Gibberellic Acid	$5.8 \times 10^{-7}M$	106.5 ± 7.1	NS
	$5.8 \times 10^{-8}M$	81.49 ± 6.17	NS
	$5.8 \times 10^{-9}M$	64.4 ± 12.7	NS
Kinetin	$9.3 \times 10^{-7}M$	97.93 ± 7.09	NS
	$9.3 \times 10^{-8}M$	126.71 ± 4.53	S+ve
	$9.3 \times 10^{-9}M$	99.4 ± 7.14	NS
Testosterone	$3.5 \times 10^{-7}M$	132.81 ± 7.14	S+ve
	$3.5 \times 10^{-9}M$	134.38 ± 8.97	S+ve
	$3.5 \times 10^{-11}M$	133.94 ± 9.72	S+ve
Oestradiol-17 β	$3.7 \times 10^{-7}M$	111.46 ± 4.55	NS
	$3.7 \times 10^{-9}M$	110.02 ± 8.89	NS
	$3.7 \times 10^{-11}M$	109.33 ± 4.75	NS
Tyrode control	-	83.9 ± 2.23	-

Statistical Analysis: Analysis of variance of each hormone against the Tyrode control.

F Test < 0.01 Significance tested by L.S.D. at $P < 0.05$

NS Not significant; S+ve Stimulated; S-ve Inhibition

Values for eggs are means \pm S.E.M. (Arc-Sine Transformation).

Each figure is the mean of 4 replicates, each of about 4 females.

fact, medium 199 can be deleterious to the limited development of nematodes in vitro, as was found for the third moult of Anisakis sp., obtained from the muscle of the barracouta, Leionura atun. A higher percent moult of these larvae following 6 days incubation at 37° was obtained with the Tyrode's, 0.75% NaCl/0.15% KCl and even 0.9% NaCl solutions. The 0.9% NaCl culture medium was rejected because of the slower rate of moulting and the obvious degeneration and unhealthy appearance of the individuals so incubated.

The majority of males had developed before the appearance of females in this nematode, at least under the present cultural conditions. If this phenomenon is present under in vivo conditions, then presumably it allows for optimal insemination, etc. For the purposes of obtaining a low level of endogenous moulting activity in vitro, nematodes at 106 hr postinfection were chosen for culture. Thus, the moulting assay used was measuring more specifically the number of males moulting in a given time interval, rather than the moulting of both sexes, although females did appear after treatment with α -ecdysone and kinetin. However, it was not expected that a sex-specific response would occur in terms of reaction to α -ecdysone.

α -ecdysone stimulated and synthetic juvenile hormone inhibited moulting at approximately 10^{-11} M. The inhibition of exsheathment of infective third-stage larvae could not be overcome by the same cultural conditions employed for the stimulation of the fourth moult by α -ecdysone. This indicated the absence of a stress-type reaction of α -ecdysone on

this dormant stage and at the concentrations used (2.2×10^{-7} , 10^{-9} and 10^{-11} M). It would be instructive to know the response of infective third-stage larvae, if any, to concentrations of moulting hormone in the region of 10^{-6} - 10^{-7} M. Perhaps, juvenile hormone had a gonadotrophic effect on egg-laying, as the synthetic juvenile hormone stimulated at 10^{-9} M. Such sensitivity would have appeared to preclude a stress reaction response.

The plant growth regulators gibberellic acid GA_3 and kinetin were chosen after preliminary experiments with the nematode, Aphelenchus avenae. Kinetin increased moulting and egg-laying at a concentration of 10^{-7} - 10^{-8} M, which might be considered to be at the borderline of a pharmacological/physiological action. The significance of the observation, that kinetin at these concentrations stimulated the formation of adult females is not known. The increased rate of development of the parthenogenetic, free-living nematode, Cephalobus (Webster and Craig, 1969) by kinetin (4.6×10^{-5} M) may indicate a more general response to pharmacological concentrations of this plant growth regulator. The data presented question the connection between gibberellins and moulting hormone activity in the locust, Schistocerca gregaria (Carlisle et al., 1963), whilst giving additional support of no reciprocal effects (Hendrix and Jones, 1972).

The mammalian sex steroids testosterone and oestradiol- 17β appeared to have no effect on moulting at a physiological concentration, except that the androgen inhibited at 10^{-11} M. Thong and Webster (1971)

observed increases in the length of Cephalobus sp., under the influence of these two hormones, but the rate of development was unaffected.

Within the limitations of this experiment, this would appear to be the case with Nematospiroides dubius. Testosterone also stimulates egg-laying at 10^{-11} M. The growth of N. dubius in vivo was influenced by the host's sex hormones; both indirectly through nutrition, mucopolysaccharide content of the connective tissues, the level of circulating antibodies and the activity of the reticulo-endothelial system, and directly by an effect on the tissues of this nematode (Dobson, 1966). The effect of testosterone on reproduction in vitro, may result from the stimulation of the hypodermis, which shows both neotenuous growth and elevated metabolic activity in H. schachtii female larvae following steroid therapy (Johnson and Viglierchio, 1970). The possibility of a more direct mode of action on the reproductive system, cannot as yet be ruled out.

Oestradiol-17 β stimulated egg-laying at all concentrations tested, but the increases observed were not significant. Although reproduction was temporarily inhibited by α -oestradiol at 40 μ g/ml in the nematode, Caenorhabditis briggsae, testosterone gave a negative result (Rosen et al., 1951).

The study of egg-laying by N. dubius females in vitro, was performed at 410 hr postinfection in preference to 250 hr, because this coincided with the first peak of egg-output in vivo in the mouse (Scott et al., 1959). The time-interval chosen for in vitro incubation was

48 hr, as Nippostrongylus brasiliensis, which has the same output of 1000-1500 fertilised eggs/female/24 hr in vivo, required insemination at least every other day to maintain the normal egg output (Phillipson, 1969).

The simplest explanation of the negative response of actinomycin D (inhibitor of transcription) and puromycin (inhibitor of translation - Rabinowitz and Fisher, 1962), would be that during the period of these experiments the worm did not feed. Thus neither the fourth-stage larvae nor adults formed during the 48 hr incubation period incorporated fluorescent-labelled protein into their digestive tracts. Material did adhere to the cuticle of the fourth-stage larvae but not to that of the adult. The same conclusion of non-feeding was put forward by Despommier and Jackson (1972) for the non-inhibition of development and reproduction by puromycin, in Neoplectana glaseri. The expected inhibition of growth by these two antibiotics was manifested in Panagrellus redivivus, at comparable concentrations to those used in this study (Pasternak and Samoiloff, 1970).

Similar results indicating the necessity of a Na^+/K^+ ratio, rather than either cation on its own, were described by Sommerville (1968) with Haemonchus contortus third-stage larvae. The standard effects such as membrane changes of Na^+ and K^+ ions on cells could include the excitable tissues, such as nerves and muscles. However, Castillo et al. (1964) and Brading and Caldwell (1971) have shown, at least with Ascaris lumbricoides muscle, that the resting membrane potential

is remarkably unaffected by Na^+ , K^+ or Cl^- ions.

Ions (monovalent and divalent cations) have been shown to be of importance in the initial trigger for the initiation of some physiological processes, both directly and indirectly. Barth and Barth (1974) have shown that the normal ionic changes in the development of the gastrula of Rana pipiens, regulate the initiation of induction and differentiation of new cell types. Loss of Na^+ , Ca^{2+} or Mg^{2+} ions from the blastocoel causes presumptive epidermis to form mucus cells, however, if this process is prevented then induction as the neural plate ensues with the formation of pigment and nerve cells. The inhibition of hatching of Trichostrongylus retortaeformis eggs by cations would appear to be indirect. Wilson (1958) suggested that this occurred by prevention of the normal lipid layer breakdown and consequent permeability to water, necessary for hatching.

A plausible alternative was given by Kroeger (1963) and by X Kroeger and Lezzi (1967) for the role of these monovalent cations in insects, whereby changes in the ionic balance of the nuclear sap, including the Na^+/K^+ ratio, resulted in activation in sequence of genes involved in moulting. If the Na^+/K^+ ratio of the nuclear milieu was increased, a juvenile hormone-type response was obtained, whilst reduction of this ratio produced an ecdysone-type response, when considered in terms of polytene chromosome puffing at specific loci (Lezzi and Robert, 1972). This working hypothesis has been further substantiated in the larvae and pupae of Chironomus tentans and C. thummi (Lezzi and Gilbert, 1972), by

the correlation of the coincident puffing patterns following treatment by ecdysone and JH, or K^+ and Na^+ , with the puffing sequences in development and the endogenous hormone titres. The possibility may exist of a similar type mechanism in nematodes, as suggested by the simple expedient of changing the medium ratio of Na^+ and K^+ ions, and observing the percent moult.

The response of N. dubius in vitro to synthetic juvenile hormone and α -ecdysone, is additional circumstantial evidence for the hypothesis, that a comparable type of morphogenetic control exists in the nematodes, as in the insects. Such direct and indirect evidence for the physiological activity of insect growth hormones in nematodes is summarized as follows:

- 1) The stimulation of the last moult of N. dubius by α -ecdysone and inhibition by synthetic juvenile hormone, occurred at physiological concentrations ($\geq 10^{-9}$ M). Also, egg-laying by adult females is promoted at equivalent concentrations of synthetic juvenile hormone.
- 2) The reciprocal effects of active extracts from nematodes and of the pure growth hormones of insects, were identified by bioassay. Hitcho (1970) isolated compounds from the first-stage larvae of Trichinella spiralis, which mimicked the effects of β -ecdysone and synthetic juvenile hormone on in vitro larvae. Extracts of Phocanema depressum caused moulting in insects, whilst α -ecdysone stimulated leucine aminopeptidase (LAP) activity in isolated excretory glands (Rajulu et al., 1972). Similar biological reciprocity was exhibited with extracts of the nematodes, H. contortus and P. redivivus (Rogers, 1973), as well as for

the adults of Ascaris lumbricoides (Horn, 1974).

3) The chemical nature of these active components from nematodes have been examined. Rajulu et al. (1972) from the fourth-stage larvae of Phocanema depressum and Hitcho and Thorson (1971) from the first-stage larvae of T. spiralis, have partially characterised the active principle as α - and β -ecdysone, respectively. β -ecdysone has been identified from the adult of A. lumbricoides (Horn et al., 1974), with an expected low yield of 0.3 μ g/kg wet weight.

4) Although the responses of nematodes to pharmacological quantities of insect growth hormones, analogues and mimics are considered to be non-specific in their effect, such indirect evidence is useful in indicating the possible mode of action and physiological effects involved. Farnesyl methyl ether (FME) induces effects which include, specific gonadal tissue development, an extra moult and hypertrophy of the hypodermis in the male larvae of H. schachtii, possibly by the overstimulation or inactivation of gonadal control mechanisms (Johnson and Viglierchio, 1970). FME inhibits the moult of the fourth-stage larvae of P. decipiens by the premature release of the neurosecretion necessary for ecdysial induction (Davey, 1971). Moulting and development of the first-stage larvae of T. spiralis are similarly inhibited by FME (Shanta and Meerovitch, 1970).

5) Nematodes require a dietary sterol source for normal growth, development and reproduction, as they are incapable of de novo biosynthesis (Cole and Dutky, 1969; Hieb and Rothstein, 1968). The

same is true for insects, where C_{28} and C_{29} phytosterols are dealkylated and reduced to C_{27} sterols, i.e. cholesterol. The maintenance of cellular integrity is their primary function in insect tissues, besides a metabolic role in the synthesis of ecdysones and in reproduction, for the formation of viable eggs and developing embryos (Clayton, 1964; Thompson et al., 1973). This parallelism is thought to indicate the occurrence of similar processes in nematode tissues.

6) The central process of the postembryonic development in insects (Wyatt, 1972), as well as in nematodes, is the moult, whereby the cuticle is periodically shed during the growth and development of the larvae towards the adult. This analogy is more appropriate if the ametabolous development of the Apterygota is considered. The larvae resemble the adult, except in their small size and lack of genitalia, which progressively develop with each moult (Chapman, 1969). This parallelism may again indicate similar or identical mechanisms of morphogenetic control in nematodes, as in the insects.

II.5 CONCLUSION

The only indirect evidence for the physiological activity of insect growth hormones in nematodes, is their insect-type response to such compounds and the probable isolation of β -ecdysone and JH-like substances from their tissues. There is no biochemical evidence available as yet, to indicate the involvement of insect morphogenetic hormones in the growth and development of nematodes. Is there any evidence from structural studies for the presence of nematode growth

hormones, i.e. steroids, terpenoids or polypeptides? The conclusion of Samoiloff (1973), that the post embryonic development of nematodes is under the control of specific regions, can be used to formulate such a search, i.e. growth may be hormonally controlled by the hind-gut region. Two possibilities exist as to the target that the laser microbeam is incapacitating; either cells of the hind-gut region, which may be capable of steroid and terpenoid biosynthesis or neurosecretory cells that may be responsible for the control of aspects of growth, other than ecdysis. Ecdysis is under neurosecretory control by nerve cells anterior to the nerve ring (Davey and Kan, 1967).

The most prominent organelle of steroid-secreting cells is the smooth tubular endoplasmic reticulum (STER) (Christensen, 1965; Locke, 1970). Unfortunately, there are no good electron micrographs of the hind-gut of nematodes to suggest this. The intestinal cells of various nematodes show no evidence of a substantial STER network (Bonner et al., 1971; Jamuar, 1966; Lee, 1969; Lee, 1968). The hind-gut cells of Trichinella spiralis infective larvae show a prominent reticular zone of basal plasma membrane infoldings that might extend to the mitochondria (Bruce, 1966), but less advanced structures are present in the intestinal cells of the above studied nematodes. In the fifth instar of the insect Calpodes ethlius their function is the sequestration of blood proteins, prior to moulting and pupation, by the permanent oenocytes (Locke, 1968). Perhaps a well-developed STER should not be expected, as the degree of development is apparently dependent on the ability of

the tissue to synthesise cholesterol (Christensen, 1965), of which nematodes are incapable. The prothoracic gland of the fifth instar of Calpodes ethlius (Locke, 1970) shows the structure of cells capable of the conversion of cholesterol to ecdysone presumably by the STER-mitochondria-plasma membrane complex.

Presumptive neurosecretory cells have until recently only been detected in the anterior regions of nematodes, in the area of the nerve ring, cephalic sense organs, dorsal and ventral nerve trunks (Davey, 1964, 1971; McLaren, 1972a, b) and hemizonid (Rogers, 1968). Now, neurosecretory-type granules have been detected in the posterior region of the microfilariae of Dirofilaria immitis, in cells associated with the excretory and anal vesicles (Kozek, 1971; O'Leary *et al.*, 1973). Such a neuroendocrine system is the link between environmental cues and developmental and physiological events in the nematode, as suggested by Davey (1972). A second possibility may exist, especially in reactivated larvae in the definitive host, where neurosecretory cells respond to internal signals for this link, e.g. a distended intestine.

Even if no conclusion is at present possible as to the physiological basis of the findings of Samoiloff (1973), the involvement of insect growth hormones in the growth and development of nematodes would appear to be readily testable by the ability of laser-microbeam treated second-stage larvae of Panagrellus silusiae, to develop in the presence of ecdysone and/or to be inhibited by the addition of JH.

The expected bonus of information on the effects of neurosecretion

and growth hormones on the development of the entomophilic nematodes has failed to materialise. The preliminary results of Gordon (1968, 1969, 1970) showed that adults of Hammerschmidtella diesingi in the hindgut of adult females of Blatta orientalis, required a supply of neurosecretory material from the brain of the host for optimum development. The effect was not an indirect one on food uptake or on the composition of various hydrolytic enzymes in the midgut of the host. However, the inverse relationship of adult females of H. diesingi and Leidynema appendiculata following allatectomy and cardiectomy of the adult females of Periplaneta americana was due to reduced nutrition and not altered hormone levels (Hominick and Davey, 1972a, b). The difficulty of the interpretation of real differences remains unresolved, but may be due to differences in the physiology of the host species.

The reproductive system is the only nematode tissue developing by cell division and differentiation during post embryonic growth. Besides moulting, the formation of a functional reproductive system is the major morphogenetic process occurring during postembryonic development. Is it possible to postulate, from a study of the development and function of the reproductive system a co-ordinated form of control involving neuro-endocrine factors?

Structural evidence appears to corroborate this view of development in different nematodes following a precise sequential and temporal pattern. The data supports the suggestion that this pattern is linked to the moulting cycle, however, the definition of moulting

amongst various authors differs and clearly requires standardisation for any comparative findings. The majority of nematodes studied show no division of somatic or germinal nuclei in the genital primordium until the second moult, when nuclear division usually first occurs in the somatic nuclei. Further developmental patterns are of two types; either the absence of divisions of both the somatic and germinal nuclei until the third moult, or the commencing of divisions from the intermoult period of the third-stage larva (Hechler, 1970; Hirschmann, 1962; Roman and Hirschmann, 1969; Yokogawa, 1922). There are three examples, where cell division is confined to the moulting phase with growth by cell enlargement only in the intermoult period; Helicotylenchus dihystrera (Hirschmann and Triantaphyllou, 1967), Helicotylenchus vulgaris (Yuen, 1968) and Aphelenchus avenae (Fisher and Triantaphyllou, personal communication). These authors have shown the restriction of somatic nuclei divisions to the moult phase until the third moult, whilst the male germinal nuclei begin dividing during the third moult and the female germinal nuclei during the fourth stage. Fisher (1970) has established an indirect link between the reception of the stimulus for moulting and gonad development during each moult phase, indicating the possibility of some co-ordinating factor(s) which increase and/or decrease at the time of the moult. The synchronisation of germ cell development in some nematodes is believed due to the rachis (Foor, 1967, 1968), when present.

Biochemical evidence shows that for normal postembryonic development the nematode requires continuous synthesis of DNA, RNA and

protein (Bolla et al., 1972; Pasternak and Samoiloff, 1970) and is not due to pre-programmed stable RNA species. Boroditsky and Samoiloff (1973) conclude that the three phases of gonad development in Panagrellus redivivus, i.e. cell division, cell proliferation and cell differentiation are determined by the pattern of m-RNA synthesis at each moult, which is sufficient for development to the next stage, i.e. next moult. The correlation of specific sequential patterns of development with the temporal macromolecular synthesis of different species of m-RNA and resultant proteins, indicates that the whole process of the formation of the reproductive system is under some type of co-ordinated control. If the hypothesis is correct, this control is hormonal. Davey (1971, 1972) has suggested the possibility of neurosecretory control of development and function of the reproductive system of Phocanema decipiens as such cells show renewed activity 14 days after ecdysis, in vitro.

The functioning of the reproductive system may be under hormonal control, as indicated by the influence of synthetic juvenile hormone on egg-laying. This may follow a similar mechanism of the gonadotrophic activity of juvenile hormone in insects, whereby the synthesis and release of vitellogenin by the fat-body and uptake by the oocytes are increased (Bell and Barth, 1971; Wyss-Huber and Lüscher, 1972).

CHAPTER IIIA

ASPECTS OF THE EXTRACTION OF NUCLEIC ACIDS AND INTACT
POLYRIBOSOMES FROM THE NEMATODES, APHELENCHUS AVENAE
AND PANAGRELLUS REDIVIVUS

IIIA.1 INTRODUCTION

The little that is known of nematode RNA species and polyribosomes suggests that they conform to those of most other animals studied. The two main species of ribosomal RNA in nematodes (Kaulenas and Fairbairn, 1968; Zeelon and Gershon, 1973) and in the cestode Echinococcus granulosus (Agosin *et al.*, 1971) occur at about 18S r-RNA and 28S r-RNA. The ribosomes appear normal, with respect to monosomes, ribosomal subunits, activity in *in vitro* amino-acid incorporating systems and content of RNA (Grummt and Bielka, 1968; Wallach *et al.*, 1973). The ribosomes of Ascaris lumbricoides eggs before embryonation are unusual in that they are in the form of RNase-resistant, non-functional polysomes, which are activated on removal of the protective protein coat by trypsin (Kaulenas and Fairbairn, 1966).

The t-RNA's of Turbatrix aceti have been studied in respect to ageing (Reitz and Sanadi, 1972), showing normal single and isoaccepting species which were charged by specific aminoacylating enzymes. Concomitant with ageing was the detection of altered isoaccepting profiles for arginyl- and tyrosinyl-t-RNA, which were believed to be associated with changes of protein synthesis at the translational level.

Before studying the effects of insect growth hormones on nematodes, it was essential to establish whether the total RNA species, ribosomal

RNA and polysomes were normal, i.e. in terms of S-value, molecular weight and susceptibility to RNase treatment. It was also necessary to devise methods for the routine extraction and purification of these cellular organelles.

This chapter reports the extraction and purification of the polysomes of Panagrellus redivivus and some properties of total RNA from Aphelenchus avenae and the polysomal RNA from P. redivivus.

IIIA.2 MATERIALS AND METHODS

Diethyl pyrocarbonate (DEP) was purchased from Pfizer Chemicals Co. (Australia), Torula Yeast RNA from Signa (U.S.A.), Nonidet P40 from Shell (Australia) and Merthiolate from Elanco Products Co. (U.S.A.).

IIIA.2.A Mass culture of A. avenae

The method used was that of Evans (1970). Cottonwool-sealed conical flasks (500 ml) containing 40 g of wheat seed (cv. Insignia 1971) were autoclaved with 90 ml of rainwater for 20 min at 15 psi, cooled and inoculated with the fungus Rhizoctonia solani, strain 48. After 7 days a sterile inoculum of 500-1,000 nematodes was added to each flask and the flasks incubated in the dark at 25⁰ for 4-6 weeks after which time the nematodes had migrated onto the inner walls. They were harvested, sedimented and decanted to remove the light material and extracted by mistifier. A. avenae was stored at 4⁰, and used within one month.

IIIA.2.B Mass culture of P. redivivus

'Farex' baby powder in large petri dishes was surface-soaked

with distilled water and seeded with a mixture of yeast, bacteria and nematodes from an old culture. The nematodes were harvested from the lid after 3-4 days at 25⁰ in the dark and used within 24 hr.

Before every experiment all nematodes were surface-sterilized on an 8 µm filter with 1 : 10,000 ppm Merthiolate for 30 min, and washed 4-5 times with sterile distilled water.

IIIA.2.C Purification of total nucleic acid of *A. avenae*

The method used was derived from Randles and Coleman (1970). *A. avenae* was pelleted at 3,000 rpm for 10 min, then homogenised for 2-5 min at room temperature in a mixture containing 4 vols 0.05M Tris-HCl buffer (pH 8.0), 4 vols of 90% phenol (500 gm of phenol, 55 ml of water and 0.1% 8-hydroxyquinoline) and 4 vols of 4% SDS. The homogenate was shaken for 45 min at 25⁰ and spun at 10,000 rpm for 10 min to separate the phases. The upper aqueous phase was re-extracted 4 times in 0.5 vols of buffer and 0.5 vols of phenol reagent, followed each time by a spin at 3,000 rpm for 10 min. The final aqueous extract was placed on ice and the nucleic acid material precipitated by 2 vols of 100% ethanol (4⁰). This precipitate was spun down, the ethanol removed and the preparation washed with acetone, dried with ether, the last traces of which were removed in a vacuum desiccator. The preparation was resuspended in 1x TEB buffer and stored at -20⁰ (10x TEB buffer contains Tris 108 g, Na₂EDTA 9.3 g and boric acid 55 g per litre, pH 8.3). The concentration was estimated by spectrophotometer at 260 nm by $O.D. \frac{0.1\%}{1 \text{ cm}} = 25 = 1 \text{ mg/ml}$ (Randles and Coleman, 1970). All precautions were taken to destroy RNase activity on equipment, etc., by autoclaving, alcoholic KOH or 0.1N NaOH.

The extraction method for marker nucleic acids from Nicotiana glutinosa was that of Francki and Jackson (1972), modified to correspond to the technique used on the nematode, A. avenae.

IIIA.2.D Extraction and purification of polysomes of P. redivivus

All procedures were carried out at 0°, unless otherwise stated.

IIIA.2.D.(i) Extraction - A method derived from Randles and Coleman (1972) was used. P. redivivus (450 µl) was homogenised in 2 ml of TMK buffer (0.05M Tris-HCl buffer (pH 7.4), 0.01M MgCl₂ and 0.025M KCl) with 0.005M β-mercaptoethanol (β-MSH) and 20 mg of autoclaved 500-mesh carborundum for 20 seconds at 0°. To this homogenising mixture was added various RNase inhibitors and detergents, to determine their efficacy in the extraction of undegraded polysomes. The homogenate was clarified at 4,000 rpm for 10 min. The extract (75 µl) was centrifuged at 36,000 rpm for 100 min (165,000 g) in an SW41 rotor at 4°, on a linear gradient consisting of a 1 ml cushion of 60% sucrose, a 10 to 40% sucrose gradient and 0.5 ml of 5% sucrose on top, to keep material of low M.W. at the top of the gradient. All gradient solutions were dissolved in 0.01M Tris-HCl buffer (pH 7.3), 0.005M MgCl₂ and 0.005M KCl. The U/V absorbing profile was obtained by passage through an ISCO-fractionator with the detector at 254 nm.

IIIA.2.D.(ii) Purification - The methods of Loeb et al. (1967) and Loeb (1973) were modified for nematode polysome purification. Three ml of pelleted P. redivivus were homogenised for a total of 20 seconds in the following mixture: 3 ml of TMK buffer with 0.005M β-MSH, 30 mg

of autoclaved 500-mesh carborundum, 30 μ l of Triton X-100 and 100 μ g/ml heparin. The homogenate was clarified at 4,000 rpm for 10 min. The supernatant was loaded onto a discontinuous sucrose gradient of 3 ml of 2.0M and 3 ml of 0.5M sucrose, dissolved in 0.01M Tris-HCl (pH 7.3), 0.005M $MgCl_2$ and 0.025M KCl (containing heparin at 100 μ g/ml), and spun for 3 hr at 226,000 g_{max} in a 50 Ti rotor at 4 $^{\circ}$. Pellet resuspension was in 400 μ l of TMK buffer containing 100 μ g/ml heparin without β -MSH and clarified at 4,000 rpm for 10 min. The concentration of ribosomes was calculated on the basis of $O.D. \frac{0.1\%}{1\text{ cm}} = 12.7$ (1 mg/ml) (Randles and Coleman, 1970).

IIIA.2.E Extraction of RNA from polyribosomes

This was done by the pronase-SDS method (Murant et al., 1972). The polysomal RNA was dissolved in 400 μ l of 1x TEB buffer containing 10% sucrose and the concentration determined spectrophotometrically.

IIIA.2.F Sucrose density-gradient centrifugation

IIIA.2.F.(i) A. avenae - Purified nucleic acid (30 μ g) was layered onto a 5-25% linear sucrose gradient in 1x SSC buffer, pH 7.1 (0.015M sodium citrate and 0.15M sodium chloride) with 0.01% SDS and centrifuged for 120 min at 200,000 g_{av} in an SW50 rotor at 25 $^{\circ}$. The U/V absorbing profile was obtained as previously mentioned.

IIIA.2.F.(ii) P. redivivus - RNA (80 μ g) was mixed with 1 ml of 0.005M Tris-HCl buffer, pH 7.3, to reduce the sucrose content to about 2%. It was layered onto a 5-25% linear sucrose gradient in 0.005M Tris-HCl buffer, pH 7.3, and centrifuged, according to the procedure of Rhoads

et al. (1973). The U/V absorbing profile was detected as above.

All centrifugations were performed in a Beckman L2-65 ultracentrifuge.

IIIA.2.G 2.5% polyacrylamide + 0.5% agarose gel electrophoresis (PAGE)

Using the method of Peacock and Dingman (1968) and the apparatus of Reid and Bielecki (1968), the gel was prerun for 1 hr at 20 mA at 5° in running buffer 1x TEB. RNA (20-40 µg) was loaded, containing 45 µl 1x TEB with 10% sucrose for the increased density of the sample. It was run for 10 min at 10 mA, then at 20 mA until the bromophenol blue tracking dye had moved 7-8 cm.

The nucleic acid gels were fixed for 10 min in 5% TCA, briefly washed in distilled water and stained in aqueous 0.02% toluidine blue for 20 min. The gel was destained over a 48 hr period with frequent washes in distilled water.

IIIA.3 RESULTS

IIIA.3.A Problems of polyribosome integrity

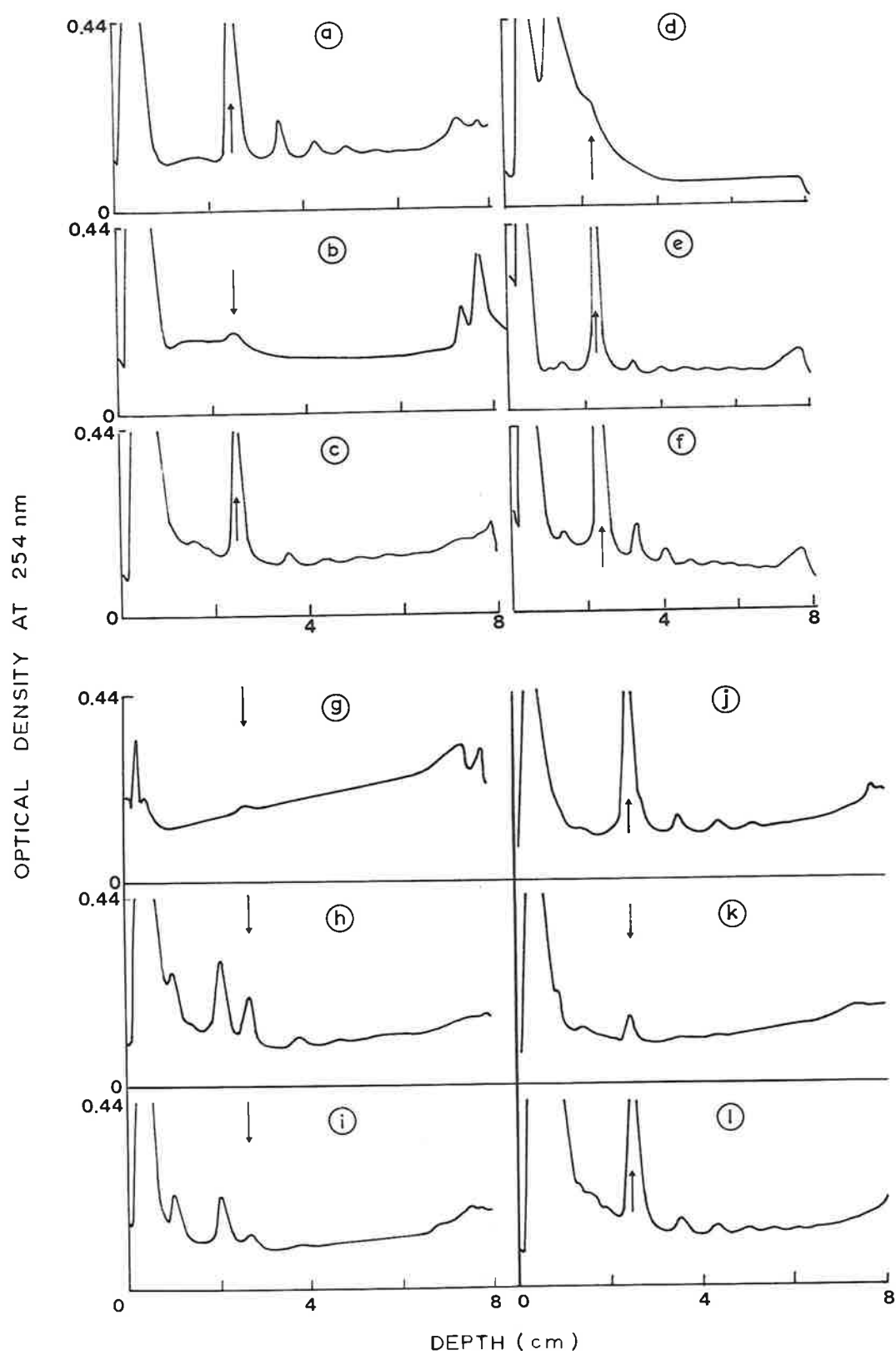
The nonionic detergent Nonidet P40 (Fig. 4(g)) and sodium deoxycholate (DOC; Fig. 4(a)) at 1% final concentration, proved deleterious to the polysomal profiles. The effect of DOC resulted from increased monosome formation, as the polysomal U/V absorbing profile appeared normal. Triton X-100 did not appear to cause any detectable change.

The RNase inhibitors, DEP (2%, 1% and 0.5%) (Fig. 4(h), (i)), bentonite (15 mg/ml) (Fig. 4(k)), t-RNA (0.5 mg/ml) (Fig. 4(d)) and Torula Yeast RNA (5 mg/ml) (Fig. 4(b)) caused the loss of the polysomal

Figure 4. Sucrose density-gradient patterns of polysomes of P. redivivus from a post-mitochondrial supernatant fraction, following treatment with various detergents and RNase inhibitors. The procedure was as in IIIA.2.D.(i), except that Nonidet P40 was centrifuged in a 5 to 25% gradient. The monosome peak is indicated by an arrow.

(a) 1% DOC; (b) 5 mg/ml Torula yeast RNA; (c) 0.75 mg/ml Torula yeast RNA; (d) 0.5 mg/ml t-RNA (unstripped); (e) No heparin added; (f) Heparin to 100 μ g/ml added; (g) 1% Nonidet P40; (h) 0.5% DEP; (i) 1% DEP; (j) 5 mg/ml bentonite; (k) 15 mg/ml bentonite and (l) 2 mg/ml Torula yeast RNA.

FIG. 4



U/V absorbing profile for various reasons. Bentonite was prepared by the centrifugation procedure of Watts and Mathias (1967). However, bentonite (5 mg/ml) (Fig. 4(j)), Torula Yeast RNA (2, 1.0, 0.75 and 0.5 mg/ml) (Fig. 4(c), (1)) and heparin (100 µg/ml) (Fig.(e), (f)) gave equivalent or improved polysomal profiles over the controls (N.B. the amounts shown are the final concentrations of the RNase inhibitors).

Two other variables for polysome extraction were also examined. The time of homogenisation was reduced, following the addition of 500-mesh carborundum for nematode cuticle rupture, from a total of 2 min to 20 seconds. Also, the sucrose density gradient was increased from 5 to 20% to 10 to 40% with a concomitant increase in the time and speed of the centrifugation, to separate the monosome peak from the lighter contaminating material.

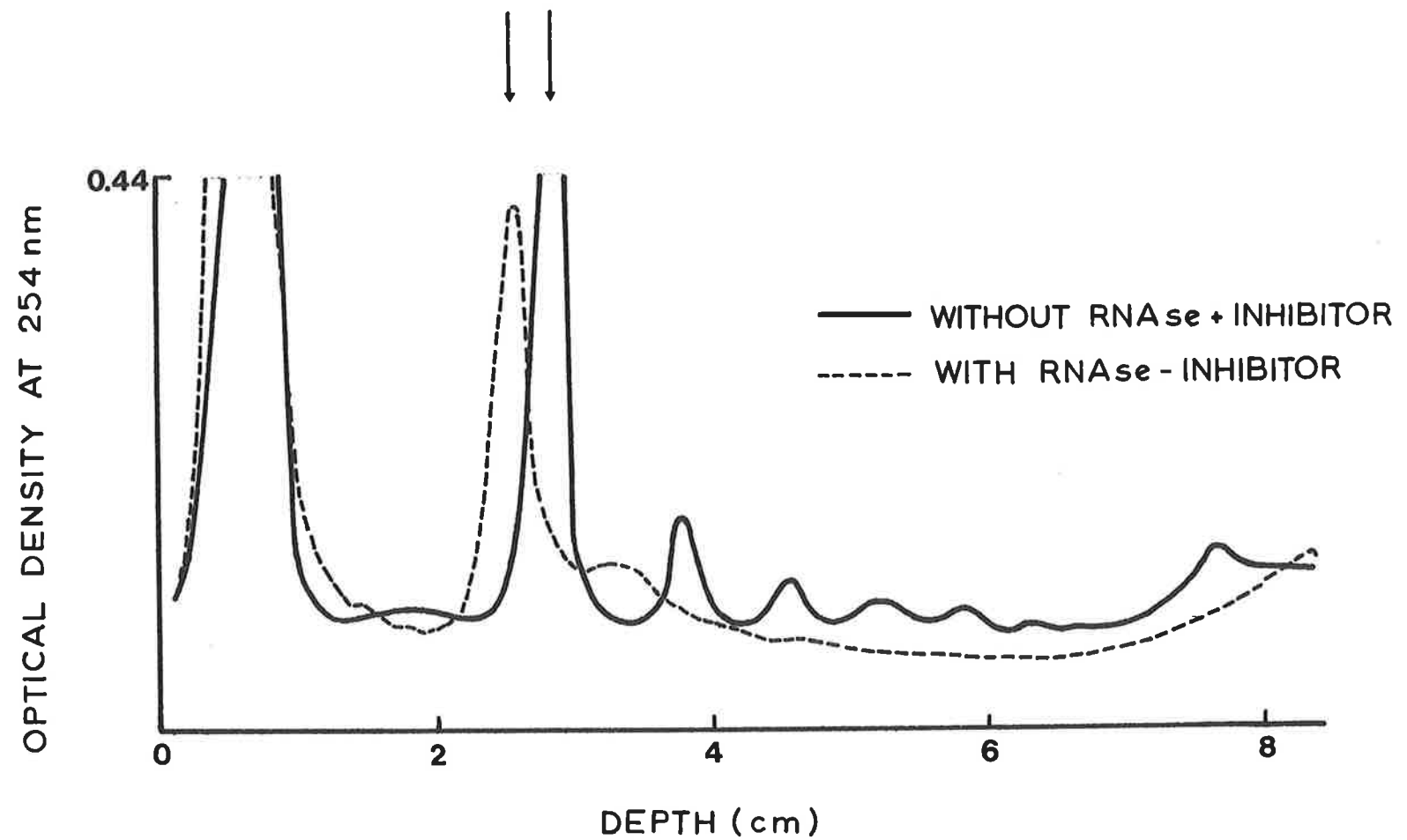
The polysomal profile showed a large monosome peak and, decreasing in order of peak size, 2-6 ribosome polymers (Fig. 5). The main criteria used as evidence of polysomal extraction, were the disappearance of all peaks except the monosome and disome on incubation with 2 µg RNase for 5 min at 5° (Fig. 5). The percentage of monosomes to polysomes was approximately 61% : 39%, as determined by planimetry. The $260_{nm}/280_{nm}$ ratio of purified polysomes was between 1.50 - 1.68,

IIIA.3.B S-values of RNA species by density-gradient centrifugation

The $260_{nm}/280_{nm}$ ratio of both extracts of RNA gave a reading of 2.0, showing the high purity of these preparations. Only two species of RNA were resolved from A. avenae and were approximately 18S r-RNA and

Figure 5. Sucrose density-gradient patterns of polysomes of P. redivivus from a post-mitochondrial supernatant fraction pretreated with and without RNase. The procedure was as in IIIA.2.D.(i). The monosome peak is indicated by an arrow.

FIG. 5



28S r-RNA (Fig. 6(a)); as was shown by direct comparison with the known ribosomal markers of leaf RNA of Nicotiana glutinosa. When incubated with 2 μ g RNase for 30 min at 37⁰, both bands disappeared showing that both species were single-stranded.

Similarly, the 2 ribosomal species of P. redivivus had values approximately of 18S and 28S (Fig. 6(b)). The small U/V absorbing peak had an approximate S-value of 7.0.

IIIA.3.C S-values and M.W.'s estimated by polyacrylamide-agarose gel electrophoresis

The S-values and M.W.'s were determined by a semi-log plot of migration (mm) through the gel against known values for the RNA species of Escherichia coli, bean leaf and Nicotiana glutinosa leaf.

A. avenae - This technique of PAGE yielded 5 species of RNA, which were all single-stranded, as shown by RNase treatment. There was also a single band of highly sheared DNA, possessing an S-value of 30.5. The S-values of the RNA species were 28, 19, 5, 4.7 and 4.3. The corresponding M.W.'s were calculated as 1.2×10^6 d, 7.6×10^5 d, 5.7×10^4 d, 4.1×10^4 d and 2.9×10^4 d (Fig. 7(a)). The 28S and 19S species were ribosomal in origin. The 5S and 4.7S species were also ribosomal in origin and derived from 28S r-RNA. The 4.3S RNA was t-RNA.

P. redivivus - The polyribosomes yielded 8 species of RNA, 4 of which corresponded to the known ones of cytoplasmic RNA. The S-values of these 4 were 28, 18, 4.5 and 4.2. The M.W.s were 1.6×10^6 d, 7.2×10^5 d, 3.6×10^4 d and 2.6×10^4 d (Fig. 7(b)). These 4 species of RNA moved almost equivalent distances to those from A. avenae with the exception of

Figure 6. Sucrose density-gradient centrifugation of total nucleic acid from A. avenae (a) and polysomal RNA from P. redivivus (b). The procedure was as in IIIA.2.F.(i) and (ii), respectively. The polysomal RNA preparation was centrifuged at 40,000 rpm for 6 hr at 4⁰ in an SW41 rotor.

FIG. 6

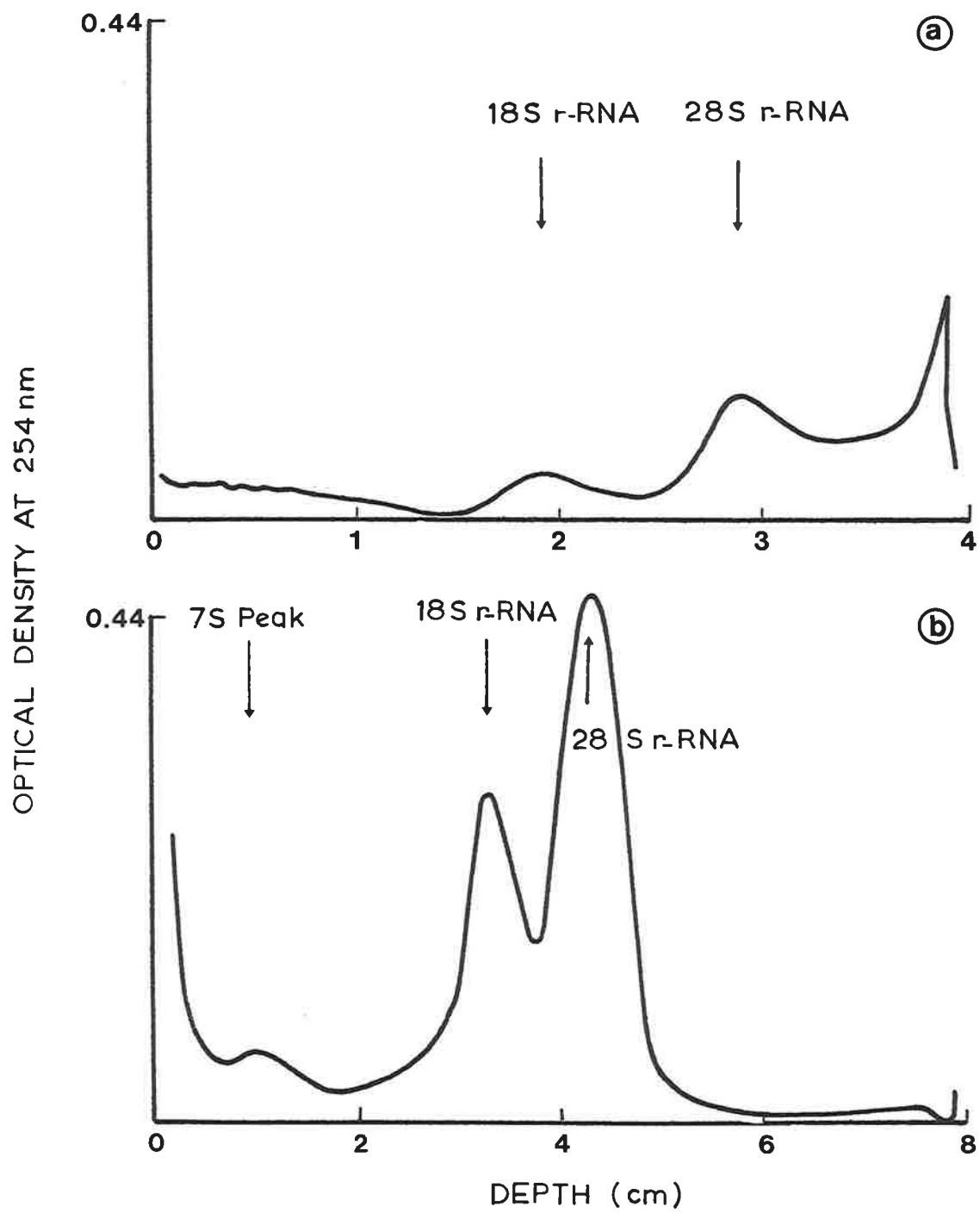
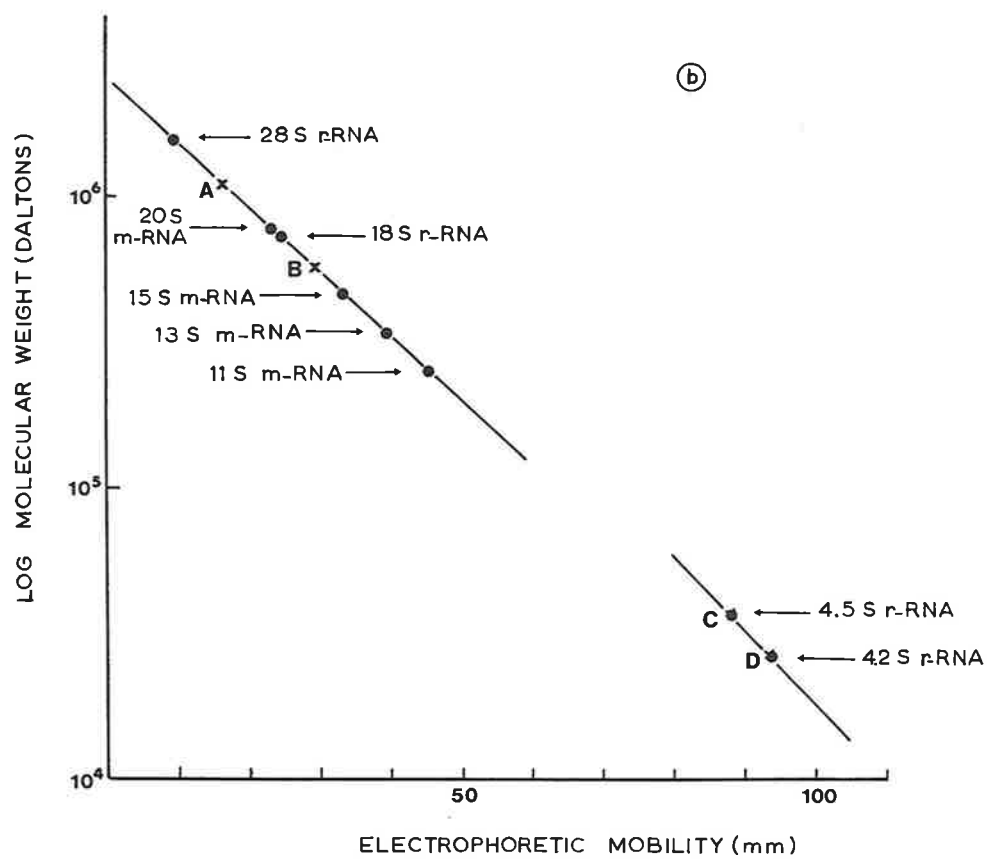
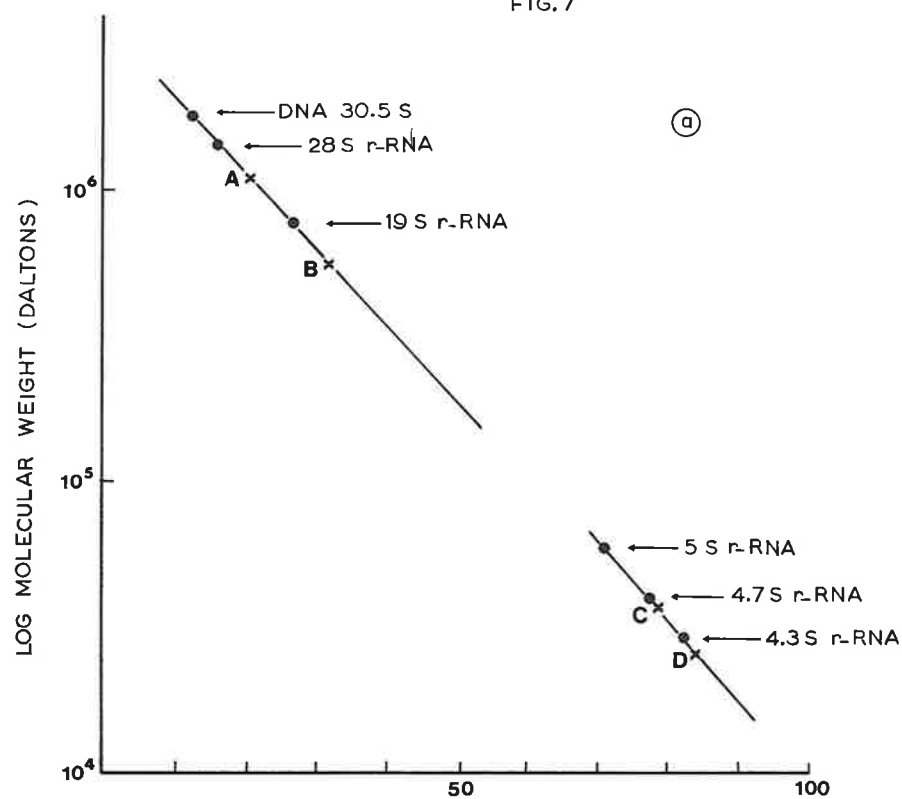


Figure 7. Polyacrylamide agarose gel electrophoresis of the RNA species of A. avenae (o, (a)) and of the polysomal RNA species of P. redivivus (o, (b)), for the estimation of molecular weights, by comparison with known RNA markers of E. coli (x). The procedure was as in IIIA.2.G. The E. coli RNA markers: A, 23.5S r-RNA = 1.1×10^6 d; B, 16.3S r-RNA = 5.6×10^5 d; C, "5"S r-RNA (4.5S) = 3.6×10^4 d; D, "4"S t-RNA (4.2S) = 2.6×10^4 d.

FIG. 7



the slightly heavier 19S r-RNA.

The 4 extra bands had S-values of 20, 15, 13 and 11, whilst the respective M.W.'s were 8.6×10^5 d, 5.2×10^5 d, 3.9×10^5 d and 2.9×10^5 d (Fig. 7(b)).

IIIA.4 DISCUSSION

The RNA species of A. avenae were readily extracted and purified by the phenol-SDS method; and no problems were met with pigments or carbohydrate as had been encountered by Kaulenas and Fairbairn (1966). The parameters of this method had been worked out by Abe et al. (1972(a), (b)), who showed that SDS inhibited RNase activity, whilst solubilising proteins, DNA and chromatin. Also, the pronase-SDS method readily extracted the RNA species present in the polyribosomes of P. redivivus. The RNA species had the normal S-values and M.W. estimates of animal RNA species, e.g. 28S, 18S and 4S of Chironomus tentans (Grossbach and Weinstein, 1968). The 5S r-RNA species from A. avenae was absent from E. coli, bean leaf and P. redivivus, and appeared to be liberated from the 28S r-RNA by heating. Thus, the 5S and 4.7S/4.5S RNA's are of ribosomal origin as shown by Wulff et al. (1972) and Pedersen (1973).

The PAGE technique showed greater resolution and accuracy than did sucrose density-gradient centrifugation, resolving 8 RNA species from P. redivivus as opposed to 2 with the latter technique. This method can resolve RNA species of between 10^4 - 10^8 daltons (Peacock and Dingman, 1967, 1968). The mobility of the respective RNA's is inversely related to the logarithm of the M.W. and the S-value in a linear way. The

separation is mainly on M.W., but configurational changes may cause small changes in mobility. The multiple forms of 23S and 16S ribosomal RNA's found in E. coli (Dahlberg and Peacock, 1971) were not detected in either of the two nematodes investigated. The separation of the r-RNA and t-RNA species of the plant leaf and E. coli preparations, was comparable to the resolution obtained by Loening and Ingle (1967) and Bishop et al. (1967), respectively.

The presence of the 4 extra bands of RNA in the polyribosomes of P. redivivus, and their absence from the total nucleic acid preparation of A. avenae, suggests that they are of messenger-RNA origin. The t-RNA's are attached to the ribosomes, even following purification. As a prerequisite to the identification of non-ribosomal RNA fractions as 'm-RNA', it is essential to study any r-RNA degradation products formed during the cell fractionation procedures. To minimise such breakdowns, it is necessary to add a RNase inhibitor and a mild detergent for membrane dissolution (Dingman et al., 1970; Payne and Loening, 1970; Lonsdale and Boulter, 1974). According to Harris (1974), m-RNA's can be identified by their function and not by their physical or kinetic properties. They are characterised by a heterogeneous distribution, but with a similar sedimentation coefficient to 18S r-RNA, a high degree of secondary structure a chain length longer than the polypeptide for which it codes, and, long sequences of repeated adenylic acid.

The method finally adopted for the purification of the polysomes of P. redivivus closely resembled that of Marmaras and Sekeris (1972) for the epidermis of Calliphora erythrocephala and of Palmiter et al. (1970)

for the chick oviduct. The polyribosomes of P. redivivus showed a relatively low proportion of polysomes to monosomes, i.e. approximately 39% : 61%. Although the small difference between the presence and absence of an RNase inhibitor on the polysome profile, indicated a low level of endogenous RNase activity (cf. ageing plant tissue) (Ramagopal and Hsiao, 1973), it was thought advisable to incorporate an inhibitor as hormones are known, in some instances, to activate such enzymes.

The yield of total nucleic acid was approximately 0.8 mg/g wet weight of A. avenae. The yield of polysomal RNA was approximately 180 µg/ml of P. redivivus, but the yield increased to 276 µg/ml, when this worm was cultured for a further 6 days in nutrient broth containing E. coli strain 086.

Planimetry of the polysome U/V profile gave a reliable measure of the polysome-ribosome distribution under different treatments, as shown by Lecocq et al. (1973). Heparin and Torula Yeast RNA were found to yield, as could be detected from polysomal profiles, largely intact units. DEP was deleterious because of the formation of ribosome subunits and an increase in heavily aggregated material, as was also shown by Anderson and Key (1970). However, it was the favoured RNase inhibitor for plant tissue (Weeks and Marcus, 1969). Bentonite at 15 mg/ml caused the virtual disappearance of the ribosomes. Probably its numerous negative charges chelated Mg^{2+} ions and bound such positively charged proteins as RNase (Watts and Mathias, 1967), as well as, the ribosomes. When used at 5 mg/ml it appeared to have the desired RNase inhibition, without

the disappearance of ribosomes, as suggested by Jernigan et al. (1973). Transfer-RNA gave no protection to the ribosome polymers, because of their complete absence from the U/V absorbing profile. Heparin was used as the routine RNase inhibitor for polysome purification, because it was equally efficient but more convenient to use (Palmiter et al., 1970; Marmaras and Sekeris, 1972; Rhoads et al., 1973).

Nonidet P40 caused complete ribosome loss, whilst DOC removed the membrane-bound ribosomes in the form of monosomes and not as polysomes. This is believed to be due to the release of nuclease, possibly from lysosomes and this destroys the polysome structural integrity. However, it appeared with P. redivivus that in the post-mitochondrial supernatant, i.e. centrifugation of the polysomal homogenate for 15 min at 4,100 g, there was no equivalent to the nuclease inhibitor of rat liver (Howell et al., 1964; Loeb et al., 1967). On the contrary, Triton X-100 yielded only polysomes from the membrane-bound ribosomes and became the preferred detergent.

CHAPTER IIIB

RIBOSOMES AND POLYSOMES OF THE NEMATODE, PANAGRELLUS REDIVIVUS
AND THEIR RESPONSE TO INSECT MORPHOGENETIC HORMONES

IIIB.1 INTRODUCTION

The presence of compounds with insect growth-hormone activity in nematodes, i.e. of the ecdysone (moulting) and the juvenile (inhibitory) type, has not been conclusively demonstrated. Extracts from H. contortus (Rogers, 1973) and T. spiralis (Hitcho, 1970; Hitcho and Thorson, 1971) have been shown to contain active fractions with properties analogous to insect growth hormones. Rajulu et al. (1972) have given evidence for the function of α -ecdysone, as the endogenous neurosecretory hormone involved in the moulting of Phocanema depressum, whilst Thorson et al. (1968) have extracted a compound(s) with juvenile hormone (JH)-like properties from Echinococcus granulosus. Results derived from the addition of insect growth hormones to nematodes are frequently contradictory. β -ecdysone has been shown to elicit no physiological response in Caenorhabditis elegans or P. redivivus (Dropkin et al., 1972), and a further six species of nematode (Hansen and Buecher, 1971). However, Webster and Craig (1969) showed that β -ecdysone increased the rate of development of Cephalobus sp. Juvenile hormone (JH), analogues and mimics have been shown to influence growth and development in several species of nematode (Davey, 1971, 1972; Dropkin et al., 1971; Hansen and Buecher, 1971; Johnson and Viglierchio, 1970; Shanta and Meerovitch, 1970). The moulting of the fourth-stage larvae of N. dubius was stimulated by α -ecdysone and inhibited by synthetic juvenile hormone (SJH)

(Dennis, 1975). The very high sensitivity at low concentration suggested the involvement of these compounds in physiological development.

Information from insects, in particular the effect of hormones on the polyribosomes, may help the interpretation of such processes in the nematodes. After breaking the diapause of the cecropia silkworm, β -ecdysone is the hormone responsible for the early synthesis of all species of RNA and protein (Wyatt and Linzen, 1965; Reddy and Wyatt, 1967; Howells and Wyatt, 1969; Wyatt and Wyatt, 1971) for the stimulation of growth and development. This involves the formation of polyribosomes (Wyatt, 1967) and the induction of the enzyme ornithine decarboxylase (Wyatt et al., 1973). A similar effect on polysomal formation within three hours of the injection of β -ecdysone, has been shown for receptive blowfly larvae (Marmaras and Sekeris, 1972). The little that is known of the response of polysomes to JH, suggests a dissociative effect on these organelles (Cohen and Gilbert, 1973).

The polysomes of nematodes have similar properties to those of other animals (Grummt and Bielka, 1968; Wallach et al., 1973), but the polysomes from the fertilised, unembryonated eggs of A. lumbricoides are remarkably stable to temperature and to low concentrations of RNase, due to their protective protein coat (Kaulenas and Fairbairn, 1966).

This chapter describes some properties of the polysomes of P. redivivus and attempts to detect the appearance of heavy polysomes in response to insect hormones, i.e. the synthesis of hormone-specific proteins. This was in order to obtain more data, as to whether the moulting of nematodes is under hormonal control, by looking at the

possible mechanism of action that is involved.

IIIB.2 MATERIALS AND METHODS

IIIB.2.A Mass culture of *P. redivivus*

The procedure was as in IIIA.2.B.

IIIB.2.B Double-labelling of ribosomal RNA

The method of Loeb (1973) was modified for the culture conditions required in the labelling of *P. redivivus*. Five ml of nutrient broth containing approximately 10^{13} - 10^{15} cells/ml of *Escherichia coli* strain 086, was incubated with 1.5 ml of surface-sterilised nematodes at 25° for 48 hr, with mild shaking. At the same time, to different culture flasks were added either 5 μ Ci of (2 - ^{14}C) uridine (50 mCi/mmol: The Radiochemical Centre, Amersham) or 20 μ Ci of (5 - ^3H) Uridine (29 Ci/mmol: The Radiochemical Centre, Amersham). Synthetic juvenile hormone (Ayerst Research Laboratories - 10% of the biological activity of the pure-naturally occurring hormone) was then added via a flask transfer, in methanol to the ^{14}C -flasks, whilst to the ^3H -control flask solvent only was added, followed by evaporation under dry N_2 . Incubation was continued for the required time interval.

Polysome extraction was carried out as described in IIIA.2.D.

The homogenising mixture contained 2.7 ml of TMK buffer with 0.005M β -MSH (0.05M Tris-HCl, pH 7.3, 0.025M KCl and 0.01M MgCl_2), 100 $\mu\text{g}/\text{ml}$ heparin, 40 mg of autoclaved 500-mesh carborundum, 1% Triton X-100 and 3 ml of control and JH-treated nematodes for co-homogenisation, previously washed four times in sterile distilled water to remove bacteria. The polysome

concentration was estimated spectrophotometrically at $A_{260\text{ nm}}$ by use of the formula for ribosomes of $E_{1\text{ cm}}^{0.1\%} = 12.7$ (1 mg/ml) (Randles and Coleman, 1970).

Polysomes (150 μg) were made to a constant load volume of 750 μl in TMK buffer and centrifuged on a 10 to 40% linear sucrose density-gradient in 0.01M Tris-HCl buffer, pH 7.3, 0.005M MgCl_2 and 0.025M KCl, for 100 min at 160,000 g_{av} in an SW41 rotor at 3° . The U/V absorbing profile and 0.492 ml fractions were obtained by passage through an ISCO-fractionator with the detector at 254 nm. To each vial was added 2.5 ml NCS tissue solubiliser (Amersham/Searle), followed by incubation at 40° for 12 hr in the dark for complete digestion of the water and the organic material. NCS scintillant (15 ml) was added to each vial (7 g PPO, 87.5 mg POPOP to 1 litre toluene), which was then kept in the dark at 5° for 10 hr prior to counting, to reduce any chemiluminescence present. The Packard 3320 scintillation spectrometer was set up for double-label counting; the ^3H -channel at 30 - 175 at 50% efficiency and the ^{14}C -channel at 200 - 1,000 at 14% efficiency, and then each vial counted for 10 min. The percent crossover was determined using standards and the necessary readjustment performed. Because of the slight variation encountered, the percent crossover was determined for each experiment (average 9.7%).

IIIB.2.C Quenching

The possibility existed that the ^{14}C -counts and especially the ^3H -counts would be increasingly quenched with the different percentages of sucrose per vial as derived from fractionation of the gradient. The

'channels ratio method' of Bush (1963), showed that increasing amounts of sucrose from 5% to 40% did not cause quenching. The counting efficiency of the ^3H -channel was 33% and for the ^{14}C -channel it was 72%.

IIIB.2.D Extraction of 70S and 80S ribosomes from *Nicotiana glutinosa* leaves

The method of Randles and Coleman (1970) was used. Leaf tissue (200 mg), 1.2 ml of TMK buffer plus 0.003M β -MSH, 1% Triton X-100 and 12 μg RNase were homogenised for a total of 30 seconds at 0° . The homogenate was clarified at 4,000 rpm for 10 min at 4° .

IIIB.2.E Purification of Tobacco Ringspot Virus from cucumber (TRSV)

The virus was purified from infected cucumber cotyledons, 10 days after inoculation, by the method of Rezaian and Francki (1973). The plants were harvested and the cotyledons weighed, with all further operations being performed at 4° . For every 100 g of plant tissue was added 100 ml of 0.1M phosphate buffer, pH 7.0 and 100 ml of chloroform. This was homogenised for 2 min in a Waring blender, followed by extraction through a double-layer of cheesecloth. The supernatant was spun at 10,000 g for 30 min, and the lower chloroform-plant tissue layer was discarded. The aqueous layer was made 6% to PEG4000 and 1.7% in respect to NaCl and allowed to precipitate the virus and small plant proteins with stirring at 5° for 1.5 hr. The precipitated material was pelleted at 3,500 rpm for 10 min, thence resuspended in 0.1M phosphate buffer, pH 7.0, and clarified by a spin at 10,000 g for 10 min. EDTA was added in the form of a solid to a final concentration of 0.01M and the resultant solution allowed to stand overnight at 5° to dissociate the ribosomes into

subunits. The virus was sedimented by a spin at 160,000 g_{av} for 50 min in a 65 Ti rotor at 3⁰, with resuspension in 800 μ l of 0.01M phosphate buffer, pH 7.0. The viral solution was finally clarified by a spin of 10 min at 10,000 g . The concentration of the virus was estimated by the relationship $E_{1\text{ cm}}^{0.1\%} = 7.0$ (1 mg/ml) at $A_{260\text{ nm}}$.

IIIB.2.F Electron microscopy

The basic technique used for the preservation of polysome structure and of the staining procedure, was that of Lecocq et al. (1971). Serially diluted polysomes, were fixed for 10 min in the following solution: 7% formaldehyde, 50 mM Tris-HCl buffer, pH 7.3, containing 25 mM KCl and 10 mM $MgCl_2$, and on addition of the material was immediately adjusted to pH 6.0 (1M NaOH). Polysomes were stained for 1 min in 2% phosphotungstic acid (PTA) in distilled water at pH 6.8 (negative stain) and the grids then blotted dry. Polysomes were also stained in 1% uranyl acetate (UAc) (positive stain) for 1 hr, followed by four washes in distilled water. All procedures were carried out on carbon-coated formvar treated copper grids. These preparations were examined in a Philips EM100 electron microscope.

IIIB.2.G In vitro amino-acid incorporation

The system used was that developed by Ramagopal and Hsiao (1973). Each tube contained 660 μ l of medium, including 1 μ Ci of ^{14}C -Chlorella protein hydrolysate (The Radiochemical Centre, Amersham) and 150 μ g of protein (supernatant factors). The supernatant factors were prepared by the method of Amaldi et al. (1973), and designated the 'High-Speed

Supernatant' S-105. Six ml of worms were homogenised for 3 min at 4° with 2.5 ml of the TMK buffer with β -MSH (not 1 mM dithiothreitol). All components of the system were stored at -20° , except for pyruvate kinase which was stored at 0° .

Incubations were carried out at 37° for 45 min unless otherwise stated, with preincubation of the medium lacking polysomes for 15 min at 37° . Following incubation in a shaking water bath, the tubes were placed on ice for at least 45 min. To each tube was added 100 μ l of albumin (100 μ g/100 μ l BSA. Fraction V. Sigma Chemical Co., St. Louis) and 0.5 ml of 10% TCA containing 1 mg/ml of casamino acids. The tubes were heated at 85° for 40 min to solubilize the RNA. The hot TCA-precipitable material was filtered through a Gelman type A glass-fibre filter disc of 20 mm diameter and washed five times with five ml of 5% TCA, then dried at 60° for approximately 30 min, placed in a vial, to which was added 10 ml of the NCS scintillant (cooled for 2 hr at 5°). Counting in the ^{14}C -channel was at 14% efficiency, with subtraction of background and appropriate controls for amino-acid incorporation into the hot TCA-precipitable material.

IIIB.2.H Insect growth hormones

Both β -ecdysone (Schwarz/Mann, New York) and synthetic juvenile hormone were dissolved in methanol, stored at 5° and used at a concentration of 1×10^{-5} M.

IIIB.2.I Authoradiography

This was performed on labelled samples of RNA, separated on 2.5% polyacrylamide + 0.5% agarose gels (Peacock and Dingman, 1968), dried for

2 hr by infra-red lamp and allowed to air-dry for a further 24 hr. Kodak X-ray film was placed under pressure in direct contact with the dried gel on plate glass for 22 days in the dark. The film was developed using liquid X-ray developer (Kodak) and fixed with Hypam rapid fixer (Ilford).

IIIB.3 RESULTS

Before the study of the effect of insect growth hormones on the polysomes of P. redivivus could be carried out, it was necessary to characterize some properties of the ribosomes in a general way, to see if they resembled those of other animals.

IIIB.3.A S-value of nematode ribosomes

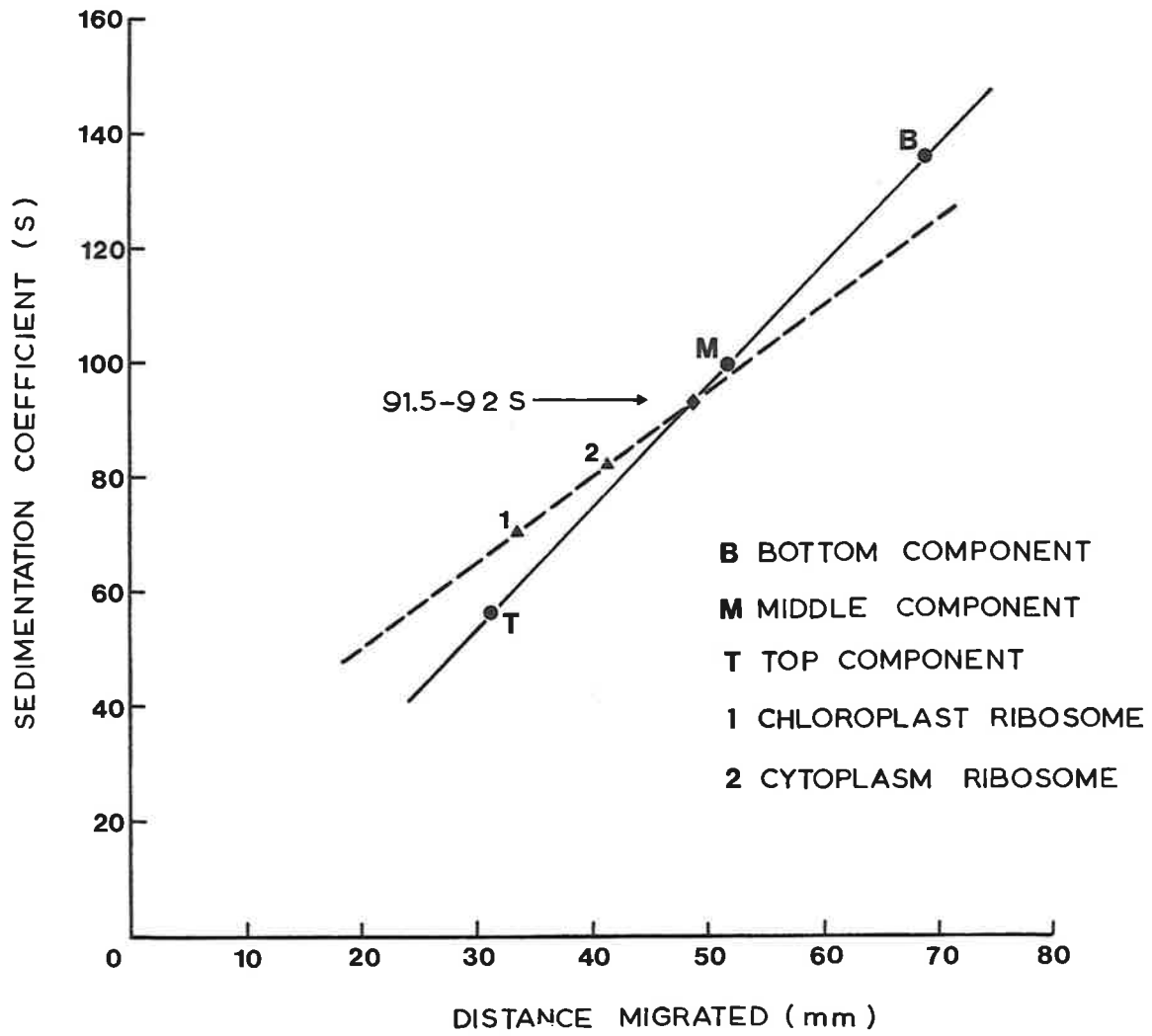
For controls, the 70S and 80S ribosomes of N. glutinosa leaf tissue were used (Boardman et al., 1966). Also for comparison TRSV, a multicomponent virus consisting of top, middle and bottom moieties, with respective S-values of 57, 99 and 136, was used (Randles and Francki, 1965). Using both graphical and direct measurement, a close approximation of 92S was obtained for the ribosomes of P. redivivus (Fig. 8).

IIIB.3.B S-values of polysome units

The S-values of the ribosome polymers were determined by the graphical method of Stutz and Noll (1967). A calibration curve was plotted for the 92S monosome, of distance travelled in the density gradient against the product of the sedimentation coefficient and of the different time intervals of centrifugation, $d \times t$ in St. The S-values

Figure 8. *P. redivivus* ribosome (■) sedimentation coefficient (S-value) as determined from plant ribosome (▲—▲) and TRSV (●—●) markers. 75 μ l of plant and nematode extracts, and 100 μ g TRSV were centrifuged separately or together, as described in IIIB.2.B, for 140 min and analysed by an ISCO fractionator at 254 nm. The results were plotted as distance moved against the known S-value.

FIG. 8



of the unknown ribosome polymers were determined by plotting the distance travelled, and dividing the resultant product by the time of centrifugation. The S-values were estimated as: disome, 128; trisome, 163; tetrasome, 198; pentasome, 240; hexasome, 280.

IIIB.3.C Estimation of ribosomal purity

From the U/V absorbing curve, the $260_{nm}/235_{nm}$ ratio was 1.38 and the $280_{nm}/260_{nm}$ ratio yielded a value of 0.67 - 0.60.

IIIB.3.D Structure

Using the formula of Wettstein et al. (1963) ($\sqrt{S}^3 = kn$, where S = sedimentation coefficient; k = constant related to the polysome mass; n = number of ribosomal units in the polysome), a plot of \sqrt{S}^3 against n for the nematode polysomes yielded a straight-line relationship (Fig. 9(a)).

IIIB.3.E Free polysomes

In order to determine whether this nematode's polysomes were free or membrane-bound, the following procedure was carried out. Nematode polysomes were extracted and centrifuged as in IIIA.2.D, in the absence of heparin, to which was added 1% of Triton X-100 in the homogenisation mixture or in the case of 1% sodium deoxycholate (DOC), to the post-mitochondrial supernatant (more appropriately the post-nuclear fraction). DOC caused no polysome increase, but the monosomes were increased. Triton X-100 increased the polysomes and not the monosomes. The percent increase in the total polysomal area was 9.6% by DOC and 12.4% by Triton X-100 (Table IV).

Figure 9. (a) Relationship between sedimentation rate and particle mass satellite peaks. The numerical S-values were calculated from the 92S ribosome of P. redivivus. The actual S-values were determined according to the method of Stutz and Noll (1967).

(b) The effect on in vitro amino-acid incorporation of the increased concentration of P. redivivus polysomes. Purified polysomes from two different preparations (●, ▲) were used for comparison. The procedure was as that described in IIIB.2.G. Each point is the mean of duplicate determinations.

FIG. 9

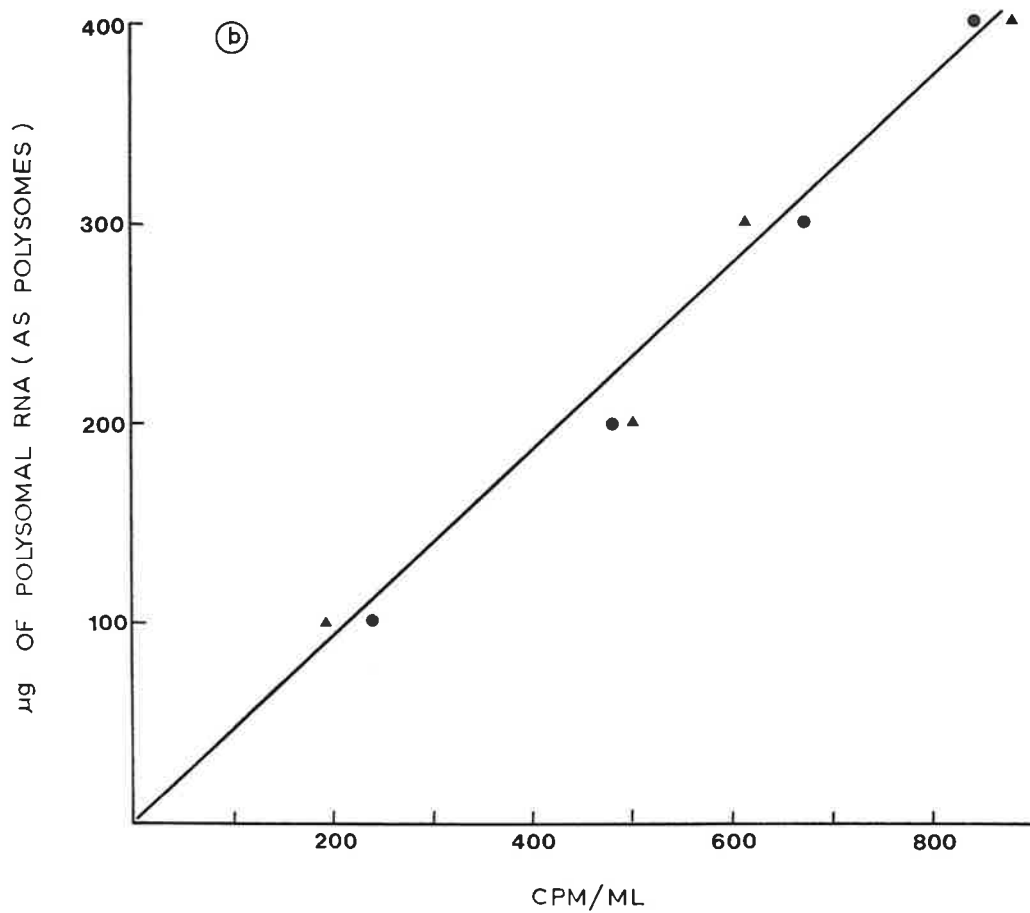
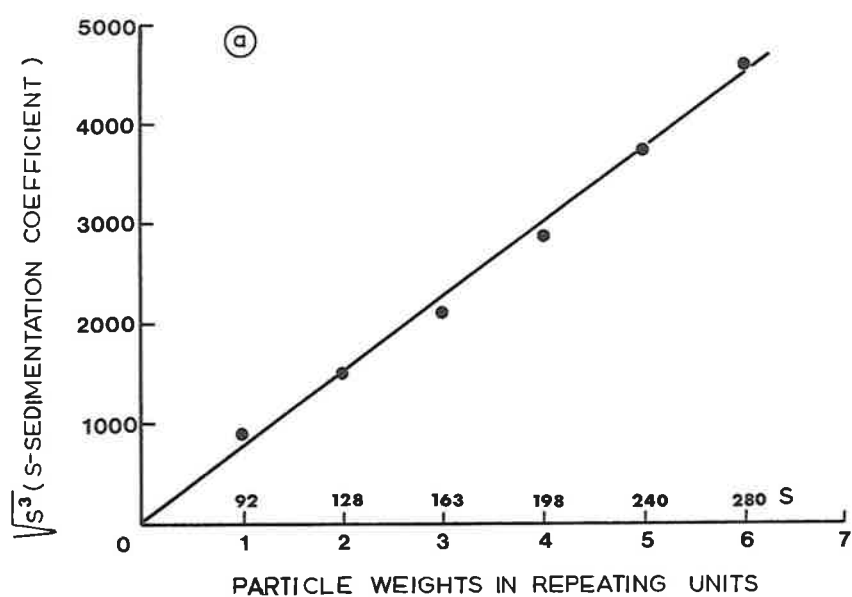


Table IV. The effect of DOC and Triton X-100 on the membrane-bound polysomes of P. redivivus^a.

Treatment	% of each component ^b		Increase in total ribosomal area
	Monosomes	Polysomes	
1. Control	67	33	-
2. Triton X-100	61	39	12,4
3. DOC	68	32	9,6

- a. The centrifugation procedure was as in IIIB.2.B.
- b. The U/V absorbing profile, from analysis by an ISCO-fractionator at 254 nm, was measured by planimetry to determine the area of monosomes, polysomes and total ribosomes. Each figure represents the mean of duplicate determinations.

IIIB.3.F Electron microscopy of polysomes

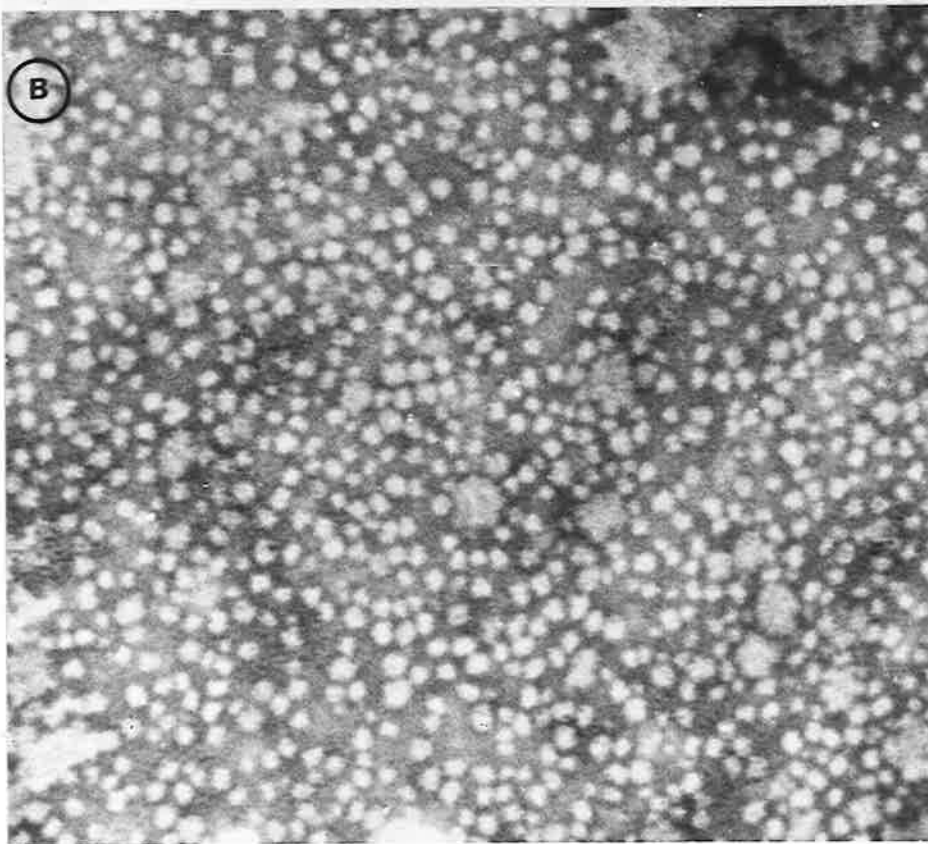
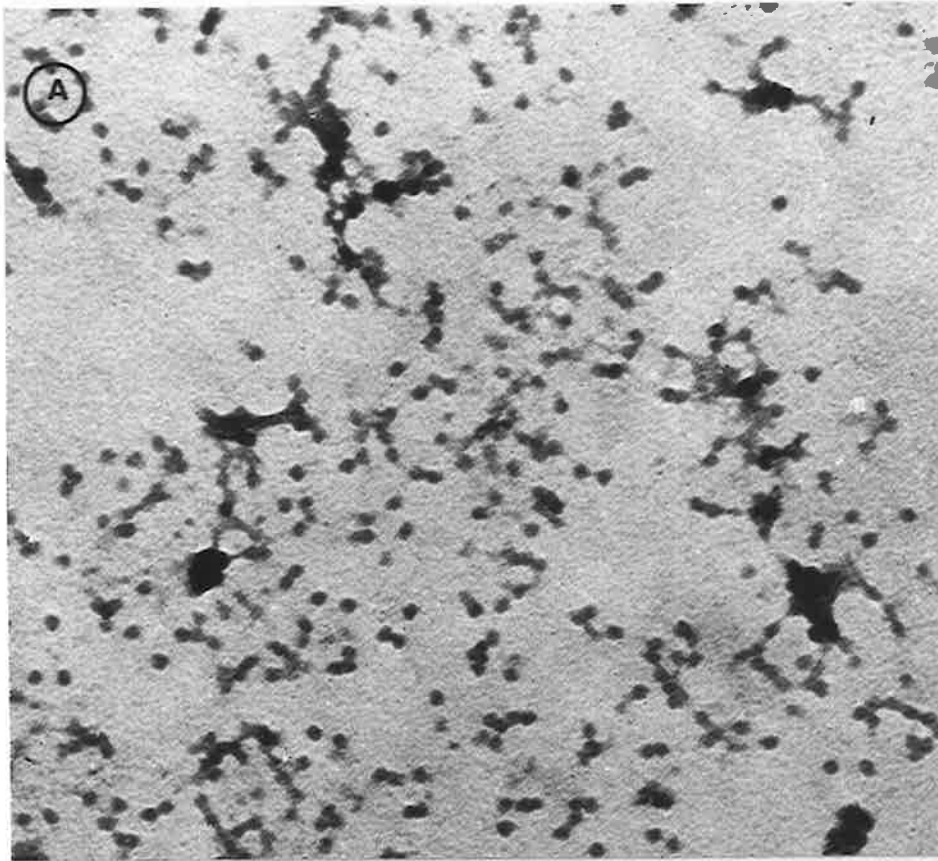
UAc (positive stain) gave better pictures (Plate IA) of isolated ribosomes and what appeared to be clusters and chains of short polysomes. PTA (negative stain) appeared to give an appearance of ribosomes and polysomes clumped together (Plate IB).

IIIB.3.G In vitro amino-acid incorporation by polysomes

The polysomes showed a very low level of amino-acid incorporation into hot TCA-precipitable material. However, they were active, in that a straight-line relationship was evident with a linear increase in the amount of polysomes (Fig. 9(b)). The system had not reached saturation, with respect to the polysomes added, because at 400 μ g the result was

Plate I. Electron micrographs of the polyribosomes and ribosomes of P. redivivus; isolated by sucrose density-gradient centrifugation and stained with (a) uranyl acetate 1% (positive stain) and (b) phosphotungstic acid 2% (negative stain) (x 100,000).

Plate I



still a straight-line relationship.

Incubation with increased amounts of Mg^{2+} from 5, 10, 15 to 20 mM with 300 μ g of polyosomal RNA, showed that 10 mM Mg^{2+} was the optimum concentration in this system.

β -mercaptoethanol (β -MSH) is the usual sulphydryl-donating reagent used in the in vitro medium for incorporation of amino-acids. This compound was compared for its efficacy with another frequently used sulphydryl-compound - dithiothreitol (DTT - Cleland's reagent). The former was used at 5 mM, whilst the latter was added at 10 mM. DTT caused a two-fold increase in incorporation (Table V). β -MSH was inhibitory because of the increased incorporation in its absence (Table V). DTT was used in all subsequent experiments.

Table V. The effect of β -MSH and DTT on the in vitro incorporation of amino-acids in the presence of P. redivivus polysomes^a.

Treatment	¹⁴ C amino-acid incorporation cpm/ml ^b	
	Experiment A	Experiment B
1. β -MSH + (5 mM)	531	421
2. -	759	706
3. DTT + (10 mM)	1043	1032
4. -	661	619

a. The procedure was as in IIIB,2.G.

b. Each figure is the mean of duplicate determinations.

A kinetics experiment was performed to determine the rate at which amino-acid was incorporated into the hot TCA-precipitable material. The amount of polyribosomes was increased to 400 μg and the ^{14}C -amino-acid hydrolysate to 2 μCi . Timed aliquots of 150 μl were taken from the active and the control samples, and treated as previously described. Incorporation was linear for 5 min, after which the rate rapidly decreased (Fig. 10). However, a low rate of incorporation still occurred after 45 min incubation at 37° .

IIIB.3.H Effect of insect growth hormones on nematode polysomes

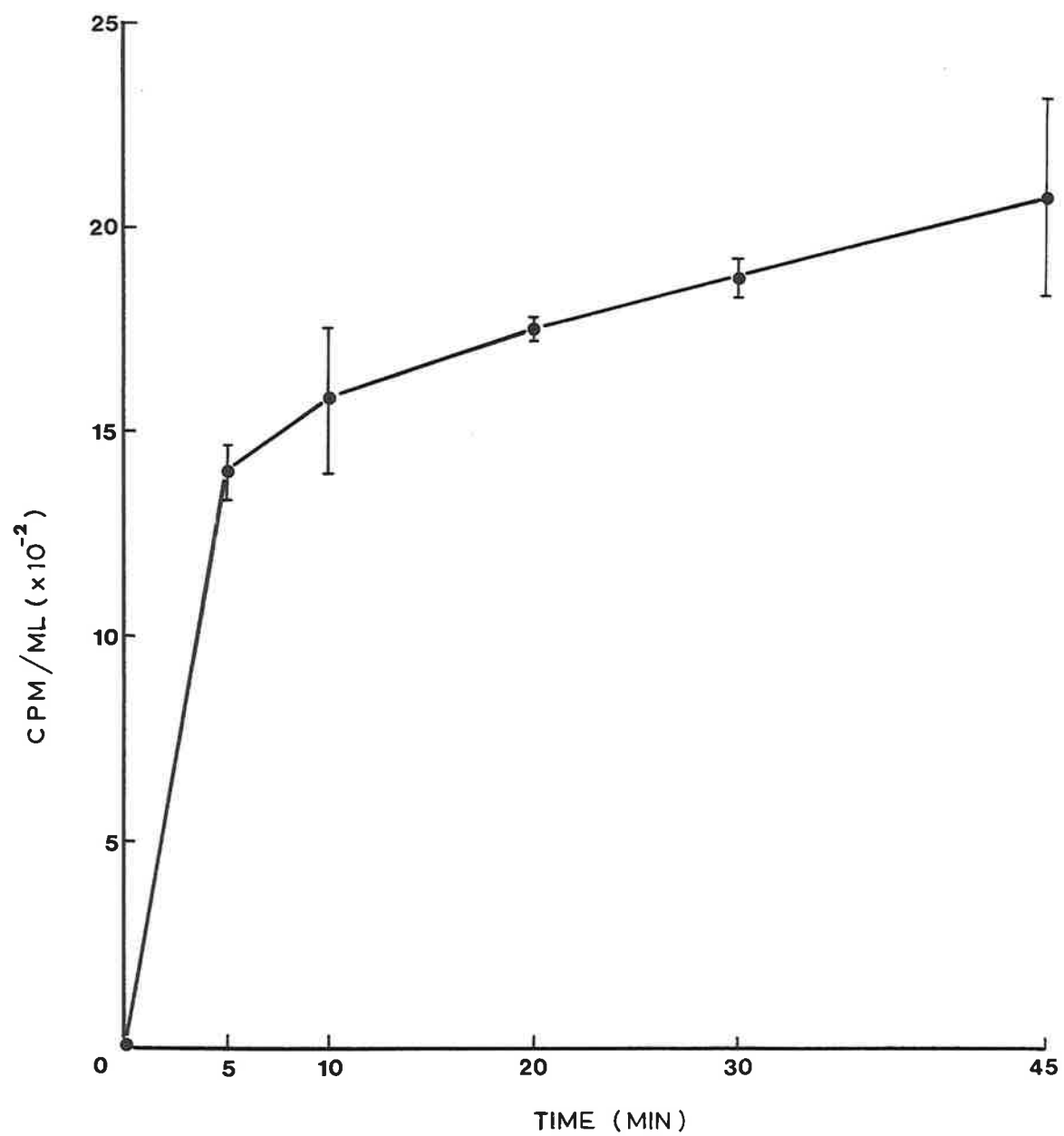
Before it was possible to perform the polysomal double-labelling technique of Loeb (1973), it was necessary to determine the minimum time necessary for label to be predominantly present in r-RNA and to enable these labelled r-RNA's to act as ribosomal markers without interference from m-RNA.

To test for the incorporation of ^{14}C into r-RNA, 2 ml of nematodes and 5 μCi of ^{14}C -uridine were placed in 5 ml of E. coli in nutrient broth, mildly shaken, and examined at intervals of 2, 4 and 6 days. The polysomes were extracted and purified as previously described in IIIA.2.D, and the RNA extracted by the pronase-SDS method of Murant et al. (1972). Labelled ribosomal RNA (20 μg) was counted by the procedure of Siegel (1971) with the scintillation fluid consisting of 1 part Triton X-100, 2 parts of toluene, 0.5% PPO and 0.01% dimethyl POPOP.

Incorporation of label into ribosomal RNA and accumulation of this synthesised material increased with time. The greatest synthesis

Figure 10. Amino-acid incorporation of P. redivivus polysomes in a homologous cell-free system. The procedure was as that described in IIIB.2.G. 150 μ l aliquots were removed at the specified time intervals and processed with hot TCA. Each point is the mean of triplicate determinations. The vertical bars represent the standard error of the mean.

FIG.10



occurred between day 4 and day 6 (Table VI).

Table VI. The incorporation of ^{14}C -uridine into the polysomal RNA of P. redivivus with time^a.

Time of incubation in medium (days)		^{14}C -labelled polysomal RNA ^b	
		Yield (μg RNA/2 ml worms)	Incorporation (cpm/20 μg RNA)
1.	2	378	7,080
2.	4	434	8,139
3.	6	551	14,975

- a. The procedure was as in IIIB,3,H,
 b. The isolation, purification and radioactive determination of polysome RNA was as in IIIB,2,G and IIIB,3,H.

Initially, incorporation of ^{14}C into r-RNA had been tested using 2 ml of nematodes, 3 μCi of ^{14}C -uridine and incubation for the required period of time in 3 ml of the 10% CEE + EMII axenic culture medium of Nicholas (1962). Following 6 days incubation, the rate of incorporation of label into polysomal RNA was 1155 cpm/20 μg polysomal RNA. Because of the low level of incorporation into polysomal RNA, this method was rejected. The cause of the lack of uptake and incorporation of ^{14}C -label into the r-RNA's was probably due to the absence or reduction of feeding in this culture medium (cf, the incorporation rate of ^{14}C -label in the presence of E. coli).

These timed samples were subjected to linear sucrose density-gradient centrifugation, to determine the distribution of label in the ribosomal RNA. RNA (150 μ g) of each sample was diluted with 1 ml of 0.005M Tris-HCl buffer, pH 7.3, to reduce the sucrose content to approximately 2%. It was then loaded onto a 5 to 25% linear gradient in the same buffer and spun at 200,000 g_{av} for 6 hr in an SW41 rotor at 5°. The U/V absorbing profile and 0.492 ml fractions were obtained by passage through the ISCO-fractionator with the detector at 254 nm. The counting procedure was that of Siegel (1971).

By day 2, virtually all radioactivity was present in the 18S and 28S r-RNA, with a small amount in a 7.5S region and another at the bottom of the tube (Fig. 11(a)). At day 2 synthesis was the same in both species of ribosomal RNA, but by day 4 and day 6 the synthesis of 28S r-RNA was greater (Fig. 11(b), (c)).

Confirmation of these results was obtained by autoradiography of 40 μ g samples electrophoresed for 7.9 cm in 2.5% polyacrylamide + 0.5% agarose gels. Counts added for day 2 were 14,160 cpm, for day 4, 16,280 cpm and for day 6, 28,390 cpm. Again, by day 2 virtually all activity was present in the 18S and 28S ribosomal RNA species (Plate II). However, due to the greater resolution obtained, some activity could also be seen in the 4.5S r-RNA and 4.2S t-RNA. Also, slight activity was detected in the 'm-RNA' species running slightly faster than the 18S r-RNA.

Figure 11. The determination of the distribution of ^{14}C -uridine radioactivity in the polysomal RNA of P. redivivus, following incubation for two days (a), four days (b) and six days (c) in culture. Sucrose density-gradient centrifugation was as in III B.3.H.

FIG. 11

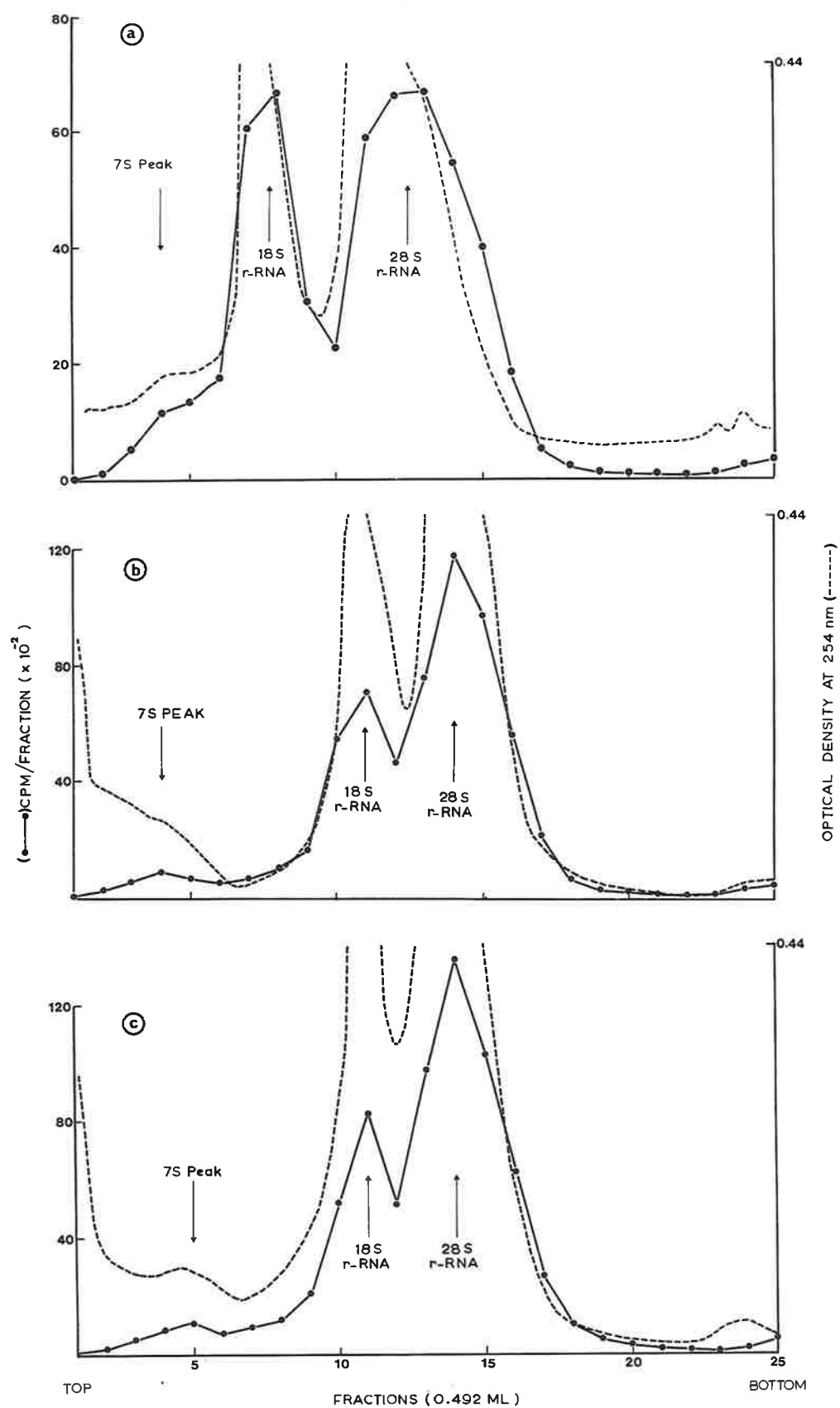
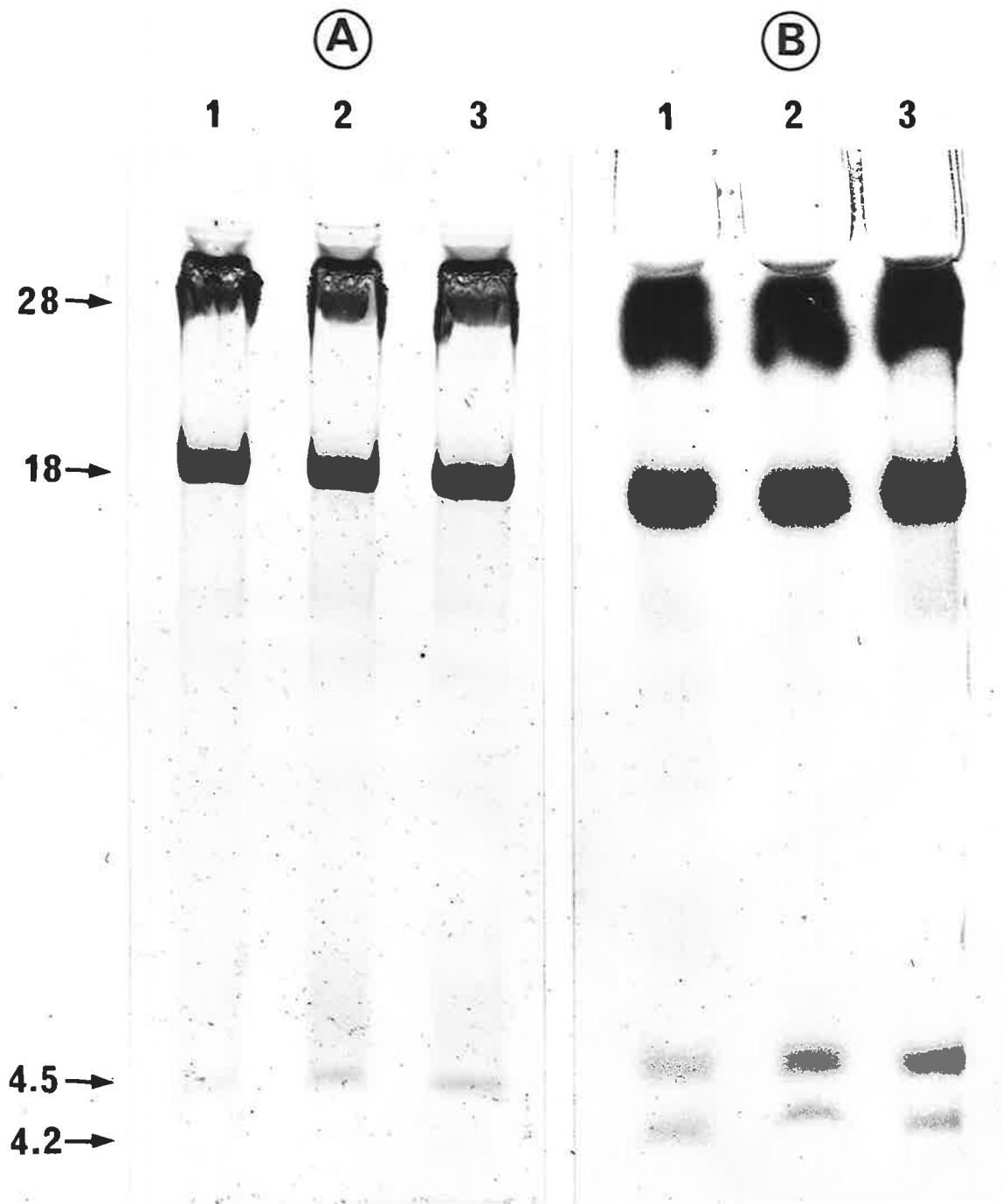


Plate II. PAGE separation and autoradiographic analysis of ^{14}C -labelled polysomal RNA of P. redivivus, following incubation for 2, 4 and 6 days. RNA samples (40 μg) were subjected to electrophoresis in 2.5% polyacrylamide + 0.5% agarose gels at 20 mA/gel slab and run for 7 cm (A - gel stained in 0.02% aqueous toluidine blue; B - subjected to autoradiography, as in IIIB.2.I). The approximate sedimentation coefficients are given. (Sample 1, 2 days incubation; Sample 2, 4 days incubation; Sample 3, 6 days incubation).

Plate II



No response of the polyribosomal U/V absorbing profile was detected at 4.5, 9, 14 or 21 hr of β -ecdysone treatment (Fig. 12). With synthetic juvenile hormone, the time/dose response of the polyribosomes showed no effect at 3.5, 8 or 13 hr post-application, but at 19 hr distinct U/V absorption in the heavy polysome region was detected (Fig. 13).

Therefore, using the double-labelled method for polysomes, extractions, purification, fractionation and radioactivity measurements were performed on 150 μ g of polysomal RNA as polysomes, at 6, 8, 15 and 19 hr post-application of 1×10^{-5} M synthetic JH (Figs. 14, 15, 16, 17). At no time was any difference detected in the polysomal profiles of control and hormone-treated polyribosomes (all results were plotted as percent of the total counts for each vial). In all preparations following the purification of polysomes by sedimentation through a discontinuous sucrose gradient, there was a reduction in the size of the monosome peak, with a relative increase in the disome fraction. This effect was progressively more pronounced with increased time of incubation of the nematodes in culture.

IIIB.4 DISCUSSION

The ribosomes of P. redivivus resemble in a general way, those of other animals. The S-value for the nematode ribosomes was 92, by comparison with two different standards. The S-values of the three components of TRSV have been determined as 57, 99 and 136 by Randles

Figure 12. The response of the polyribosomes of P. redivivus, following incubation of the nematodes in culture with β -ecdysone ($1 \times 10^{-5}M$) for 4.5, 9, 14 and 21 hr. Nematode extracts (100 μ l) were centrifuged as described in IIIB.2.B, and the resultant profile analysed by an ISCO fractionator at 254 nm. The monosome peak is indicated by an arrow.

FIG. 12

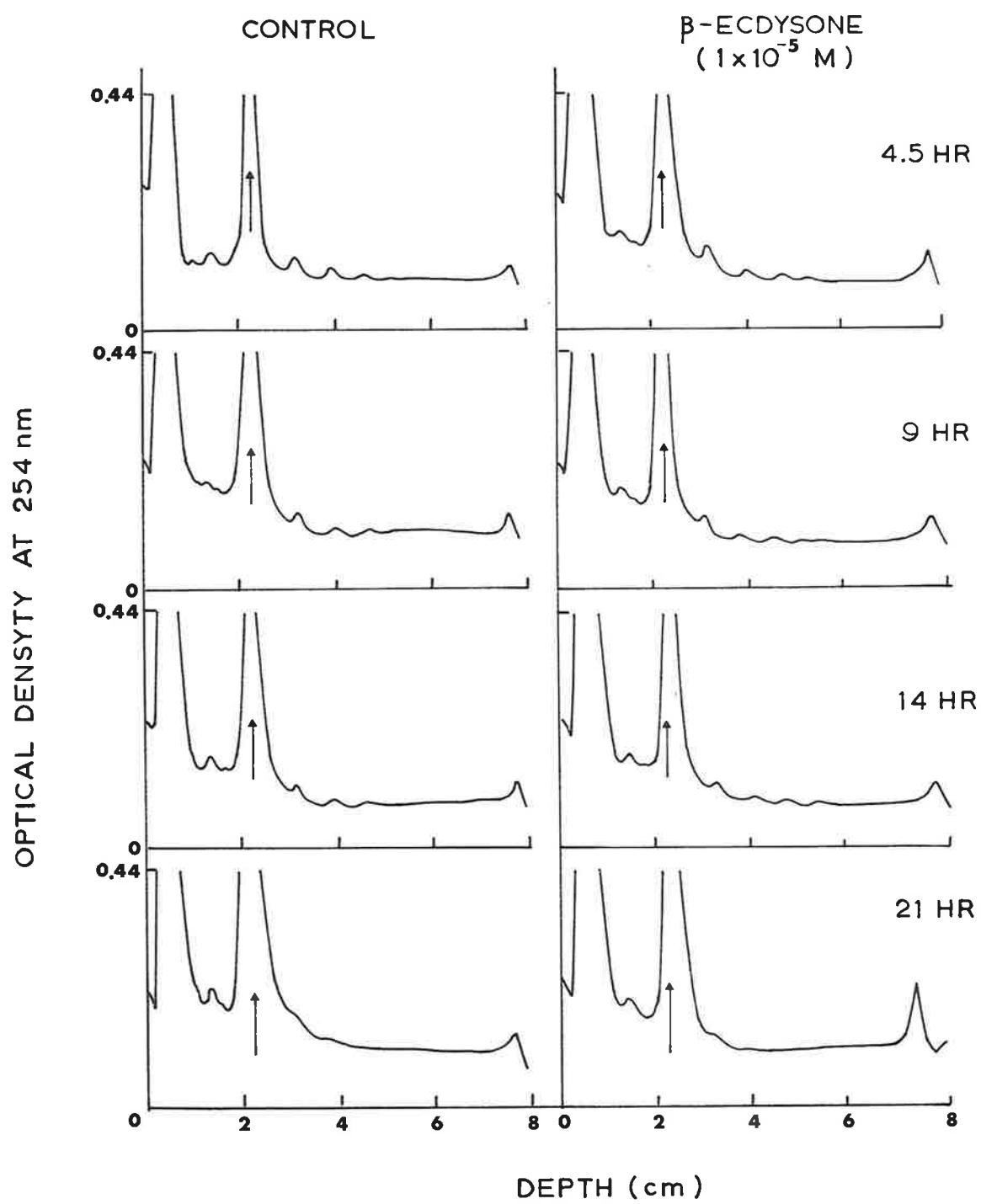


Figure 13. The response of the polyribosomes of P. redivivus, following incubation of the nematodes in culture with synthetic juvenile hormone ($1 \times 10^{-5}M$) for 3.5, 8, 13 and 19 hr. Nematode extracts (100 μ l) were centrifuged and analysed as described in IIIB.2.B. The monosome peak is indicated by an arrow.

FIG. 13

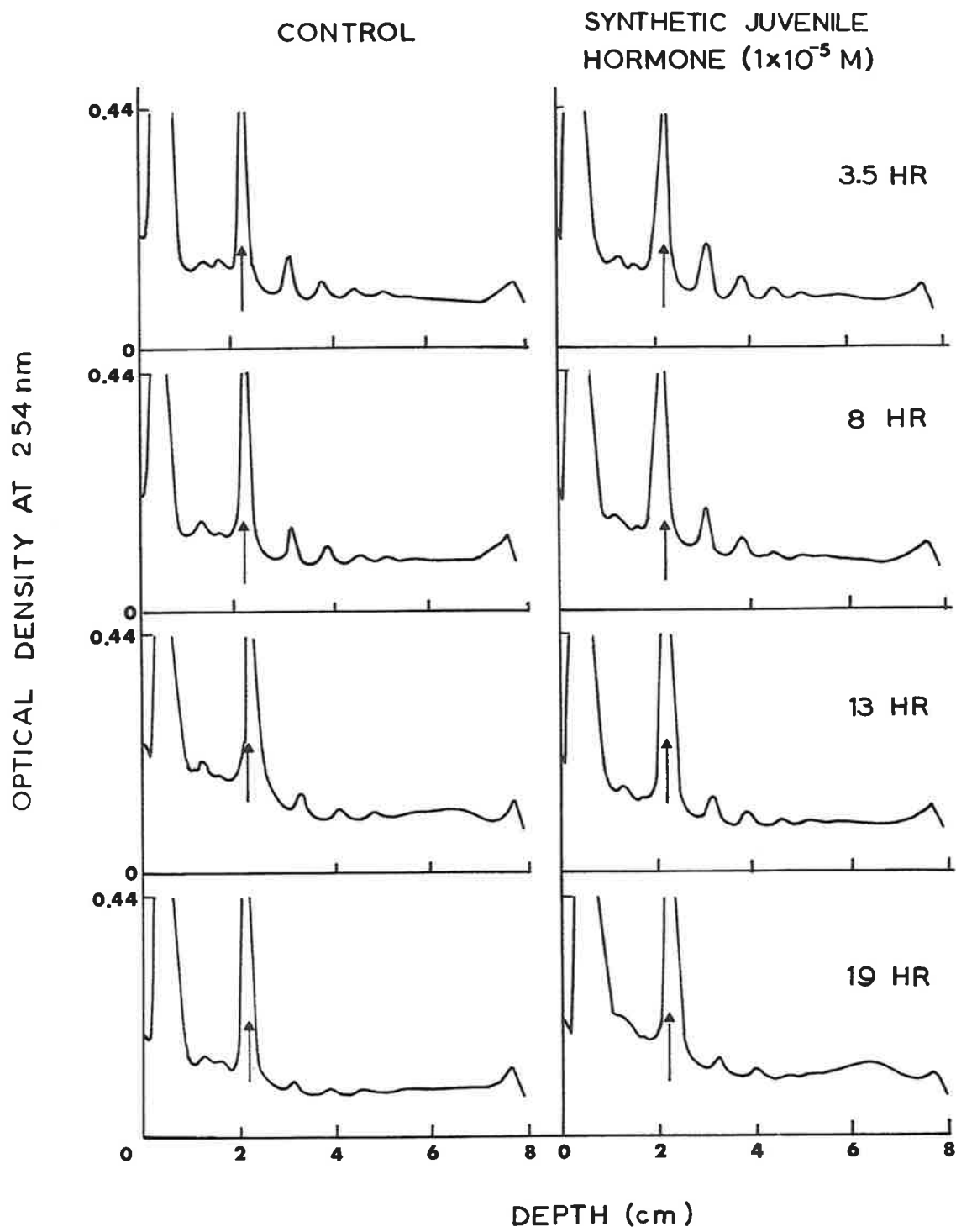


Figure 14. Sucrose density-gradient patterns of purified polysomes of P. redivivus, isolated from a co-homogenate of control and JH-treated nematodes. The hormone treated nematodes were incubated in the presence of $1 \times 10^{-5}M$ synthetic juvenile hormone for 6 hr (a) and 8 hr (b) at 25° . The experimental procedure was as in IIIB.2.B. The monosome peak is indicated by an arrow.

FIG. 14

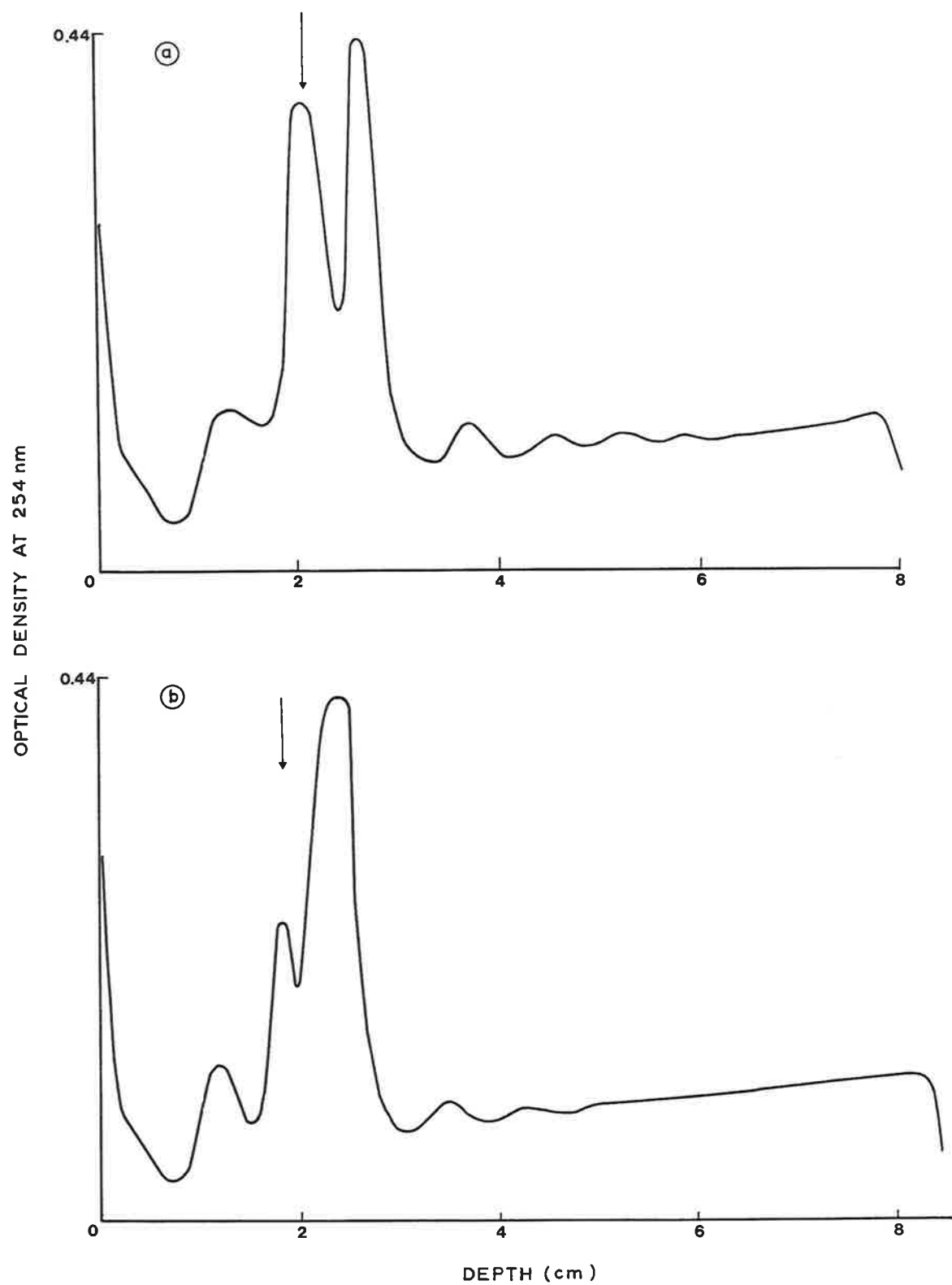


Figure 15. Sucrose density-gradient patterns of purified polysomes of P. redivivus, isolated from a co-homogenate of control and JH-treated nematodes. The hormone treated nematodes were incubated in the presence of 1×10^{-5} M synthetic juvenile hormone for 15 hr (a) and 19 hr (b) at 25° . The experimental procedure was as in IIIB.2.B. The monosome peak is indicated by an arrow.

FIG. 15

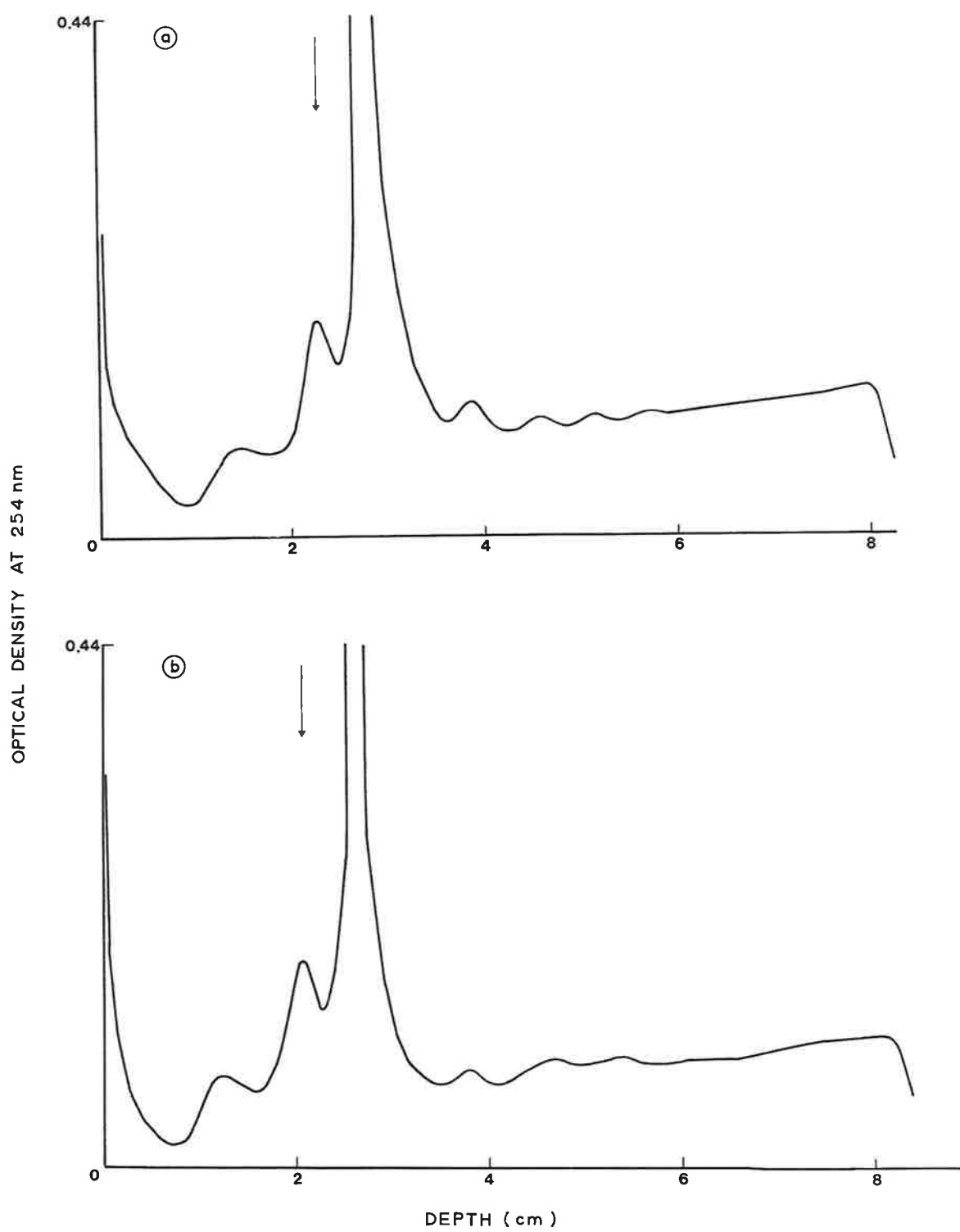


Figure 16. Sucrose density-gradient analysis of the purified polysomes of P. redivivus, isolated from a co-homogenate of control and JH-treated nematodes. The hormone treated nematodes were incubated in the presence of $1 \times 10^{-5}M$ synthetic juvenile hormone for 6 hr (a) and 8 hr (b) at 25° . (The distribution of radioactivity in the gradient was $\bullet\text{---}\bullet$, 3H -labelled ribosomes from control; $\blacktriangle\text{---}\blacktriangle$, ^{14}C -labelled ribosomes from JH-treated animals. The experimental procedure was as in IIIB.2.B.

FIG. 16

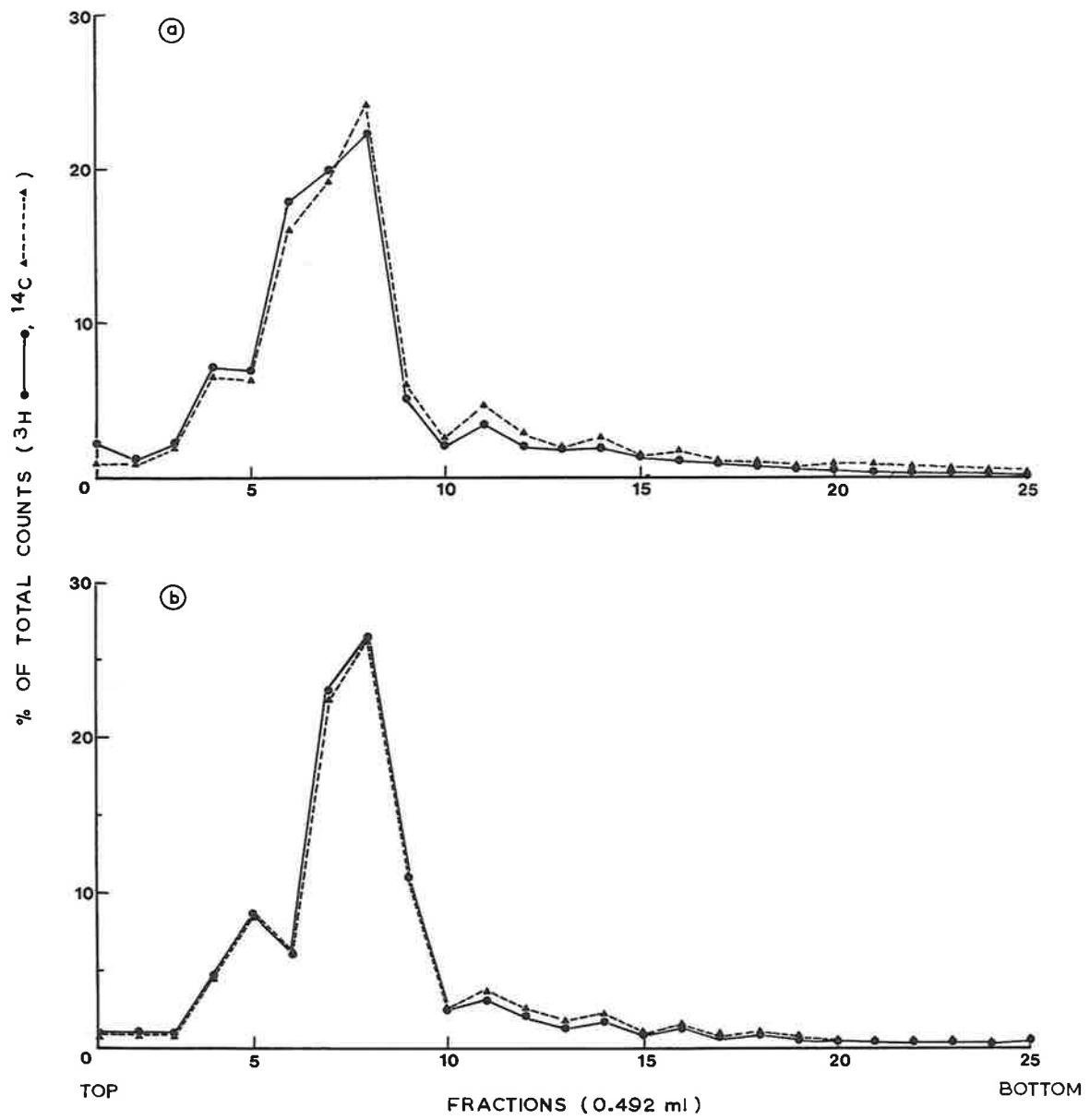
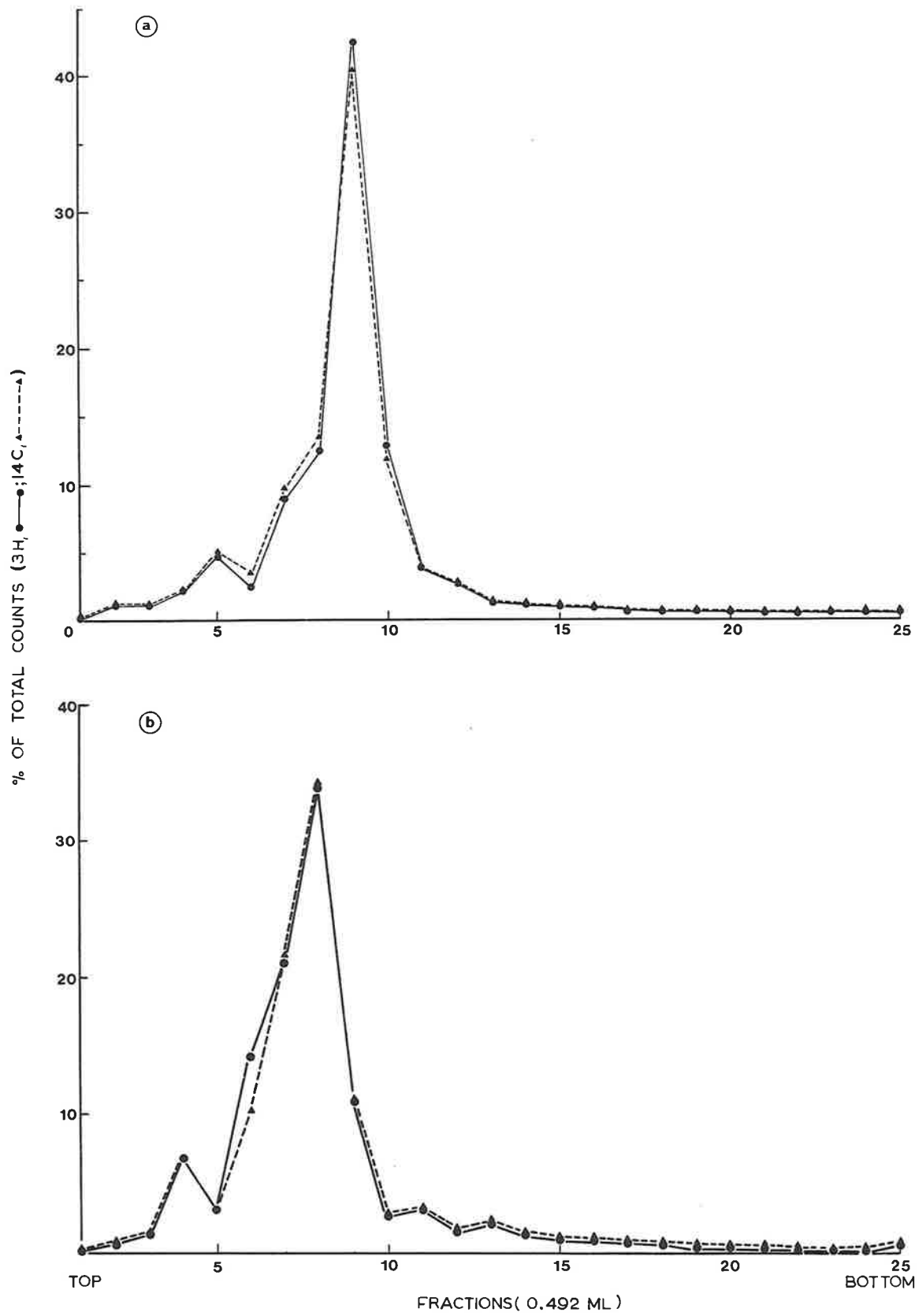


Figure 17. Sucrose density-gradient analysis of the purified polysomes of P. redivivus, isolated from a co-homogenate of control and JH-treated nematodes. The hormone treated nematodes were incubated in the presence of 1×10^{-5} M synthetic juvenile hormone for 15 hr (a) and 19 hr (b) at 25° . (The distribution of radioactivity in the gradient was ●—●, ^3H -labelled ribosomes from control; ▲—▲, ^{14}C -labelled ribosomes from JH-treated animals.) The experimental procedure was as in IIIB.2.B.

FIG. 17



and Francki (1965), or 53, 94 and 128 by Stace-Smith et al. (1965), i.e. a variation of 7%. If this is applied to the nematode S-value, the minimum would be about 85. Although this differed from the estimate of 82S for the ribosomes of Turbatrix aceti (Wallach et al., 1973), the ribosomal subunit equivalent to 64S was consistently detected. These particles may consist of a dimer of two smaller ribosomal subunits, i.e. equivalent to the 37S units.

The value of 1.38 for the $260_{nm}/235_{nm}$ ratio of the ribosomes, agreed with that for the cricket, Acheta domesticus (Kaulenas, 1970). This corresponded to a RNA content of 36%. A $260_{nm}/235_{nm}$ ratio of 1.40 for the ribosomes of 'Jensen sarcoma cells' indicated that 20% extraneous protein was attached (Petermann, 1964). This was low compared to the normally accepted figure of 50% RNA for animal ribosomes (48% RNA in Ascaris lumbricoides (Grummt and Bielka, 1968)). The reason for this low figure may be in the amount of extraneous protein bound under different conditions of pH and ionic strength of the buffer used (Petermann and Pavlovec, 1963; Kaulenas, 1970). This ribosome-attached extraneous protein could also explain the apparently high S-value obtained.

The straight-line relationship for the graph of $\sqrt{S^3}$ against n , suggested that the polysomes were arranged in a clustered structure, not unlike an enlarged ribosome. If the shape was different, then deviation from the straight-line would be expected. This could not be confirmed

by electron microscopy and Lecocq et al. (1971) have shown that the UAc-PTA methods caused an increase in the proportion of light polysomes in the fraction of $n = 2-7$ ribosomes, due to the disaggregation by fixation and staining.

The effect of Triton X-100 on the polysomes of P. redivivus indicated two characteristics of these structures. Firstly, the polyribosomes were mainly free and not membrane-bound, as shown by the small increase in the total polysome area following treatment. Such free polysomes synthesize proteins retained by the cell (Kashiwagi et al., 1971; Uenoyama and Ono, 1972). These free polysomes are believed by Faiferman et al. (1973) to have been derived originally from membrane-bound structures for prior activation of the contained m-RNA. Secondly, the ribosomes bound to the membranes were in the form of polysomes, because Triton X-100 produced an increase only in the area of polysomes, whilst the monosomes were unchanged. Such membrane-bound entities synthesize proteins for secretion and also for the endoplasmic reticulum and derived structures.

The effect of DOC appeared to be more complex. It did not cause any change in the polysome fraction, but there was an increase in the monosome peak. DOC by disruption of lysosomes, causes the release of nucleases that lead to a net increase in monosomes (Howell et al., 1964; Loeb et al., 1967).

The polyribosomes of this nematode are capable of active in vitro

amino-acid incorporation, albeit at a low order of magnitude. The rate of incorporation of labelled amino-acids into hot TCA-precipitable material showed a typical linear curve for the first 5 min, with progressive reduction in the rate with time. This was probably due to the degradation of factors in the S-105 fraction, which are known to be highly labile (Schrader and Huston, 1973). The enzymes of the cell sap were thus stable for 20 min, as they were pre-incubated for 15 min prior to the addition of polysomes. It was assumed that the amino-acid incorporation on purified ribosomes was due solely to chain elongation, as has been demonstrated for other in vitro systems, e.g. Tenebrio molitor (Ilan et al., 1970). Throughout the work on amino-acid incorporation of polysomes, heparin was excluded from the resuspension buffer and the incubation medium, because it was known to inhibit m-RNA translation (Waldman and Goldstein, 1973).

The inactivity of monosomes in in vitro protein synthesis (Noll et al., 1963; Wettstein et al., 1963) may have contributed to the low rate of amino-acid incorporation, due to their high concentration in the polysome extracts. However, Howell et al. (1964) have shown in the rat liver, that membrane-bound ribosomes which sediment with nuclei and mitochondria lose their ability to incorporate amino-acids in vitro, when detached by DOC. The purified ribosomes appeared mainly as ' Mg^{2+} dependent' dimers. This was not accompanied by an increase in the RNase activity in the DOC-treated fractions. A similar

situation may have occurred following the purification of the nematode polysomes from a postnuclear supernatant, i.e. centrifuged at 4100 g for 10 min, where a low rate of amino-acid incorporation in vitro was correlated with a large disome peak. In plant chloroplasts the monosomes are the organelles of amino-acid incorporation (Boardman et al., 1965).

JH caused no increase of heavy polysomes, which would be a prerequisite for the synthesis of a hormone-specific protein(s). The hormone hydrocortisone caused a rapid increase in the polysomal profile of rat liver, without the synthesis of RNA and therefore it was not under transcriptional control (Cammarano et al., 1968; Enwonwu and Munro, 1971; Loeb, 1973).

There are at least five reasons why the JH may not have produced any effect on the polysomes of this nematode:- (a) JH has no physiological effect on this nematode. But, Dropkin et al. (1971) showed that growth and development were inhibited. (b) The pH of the incubation medium was too acidic. JH is very susceptible to acidic conditions, resulting in the rapid loss of biological activity by degradation (Meyer et al., 1970). Throughout the experimental period, the pH remained constant at about 7.2. (c) E. coli may have metabolised the hormone. However, this bacterium does not readily feed on hydrocarbons. (d) The low solubility of the synthetic JH, precluded any biological activity. Kramer et al. (1974) have shown that solutions of 5×10^{-5} M of pure synthetic Hyalophora cecropia juvenile hormone (methyl trans, trans, cis, 3,11-dimethyl-7-ethyl-10,11-epoxytrideca-2,6-dienoate) can be prepared

in 0.02M Tris-HCl, pH 7.3. (e) The JH acts at the level of translation, i.e. modifying protein synthesis and not the polysomal profile. Many hormones stimulate protein synthesis by enhancing the activity of the polysomes in the target tissue, whilst few hormones stimulate the conversion of monosomes to polysomes. Follicle stimulating hormone and luteinising hormone produced an increase in the amino-acid incorporation in vitro of polysomes from the rat testis, without altering the U/V absorbing profile (Abney et al., 1974). This is also the mechanism of action of the plant hormone abscisic acid (ABA) on barley embryos, whereby the unchanged polysomal profile incorporated amino-acids into protein at a rate 40% less than that of the control (Bonnafeous et al., 1973). ABA is a monocyclic sesquiterpenoid (cf. juvenile hormone) (Okhuma et al., 1965) and acts as an inhibitor of the activity of the growth-promoting plant hormones (Addicott and Lyon, 1969; Evins and Varner, 1972; Brown and Sun, 1973), resembling the JH modification of the action of ecdysone in insects (Cohen and Gilbert, 1973).

Such evidence corroborates the work of Ilan et al. (1970) on the mechanism of action of JH on adult cuticular protein synthesis by Tenebrio molitor pupae. The control of m-RNA expression (gene expression) was at the level of translation, by the appearance of a new t-RNA species and activating enzyme, presumably under hormonal influences. The JH mimic acted by the prevention of the appearance of these translational factors; which significantly occurred on either control or

JH-mimic treated polysomes. This indicated further that although JH controlled gene expression at the level of translation, it was independent of the polysomes.

IIIB.5 CONCLUSION

In vitro studies on various insect tissues regarding the action of JH, indicate that it inhibits the stimulation of RNA and protein synthesis by the moulting hormone (Patel and Madhavan, 1969; Congote et al., 1970). Imaginal wing discs show JH inhibition of ecdysone-induced evagination (Chihara et al., 1972) and cuticle deposition (Oberlander and Tomblin, 1972). The mechanism of action of JH on protein synthesis may be by polysome disaggregation (Cohen and Gilbert, 1973). However, the experimental data does not support such a physiological role in the control of morphogenetic events in vivo. The effect on insect cells in vitro is immediate, irreversible and non-specific as elicited by compounds without JH-morphogenetic effects, e.g. methyl epoxyhexadecanoate. Similarly, inhibition can occur in the absence of moulting hormone stimulation, by a combination of specific hormonal effect and non-specific toxic lipoidal effect, as exhibited by a number of unsaturated fatty acid methyl esters, peanut oil and trioctanion (Cohen and Gilbert, 1972). This casts doubt on the physiological validity of some of the data of in vitro JH inhibition.

The results of experiments with P. redivivus showed no evidence of polysome dissociation by SJH, and as it has been shown to be an

irreversible process in insects, would have been expected to be readily detected. However, the significance of such an in vitro result is in doubt because intact organisms were involved in this study. At the present time, there are no reports of the necessary critical experiments to determine the involvement of the moulting hormone(s) and JH in the development and morphogenesis of nematodes. Such experiments are: 1) The effect of JH and moulting hormone(s) together on nematodes, in respect of the inhibition of morphogenetic events, e.g. moulting. 2) The stimulation of RNA and protein synthesis by the moulting hormone(s); and 3) The antagonistic inhibition of the moulting hormone(s) stimulation of RNA and protein synthesis by JH (all experiments performed on age-synchronised cultures). Without such data, it is not possible to hypothesize on the equivalence of JH antagonism of moulting hormone activity in the nematodes. The obvious test system for nematodes is moulting, but Davey and Kan (1967) for Phocanema have shown that this process is not under hormonal control, i.e. neurosecretory cell control, only ecdysis.

Many growth hormones exert their control of gene expression at the level of transcription (m-RNA expression) by stimulation of the rate of RNA synthesis in the target organ, and that this RNA is required for the regulation of growth and of protein synthesis (Tata, 1966; Marmaras and Sekeris, 1972).

This is not the sole form of gene-expression control by such

growth hormones, as it can also occur at the level of translation. Protein synthesis is usually stimulated by the altered activity of the polysomes and by the increase in monomers and polysome aggregates, such that the polyribosomal profile is unaltered (Abney et al., 1974). A few hormones change the polyribosomal profile by the transfer of pre-existing monosomes to polysomes, prior to the synthesis of new ribosomes (Marmaras and Sekeris, 1972; Palmiter et al., 1970).

No alteration of polysomal profiles were detected in the pre-existing ribosomes of P. redivivus, following treatment with β -ecdysone or synthetic JH. Thus, if these hormones are active in the translational control of protein synthesis, it does not occur by the alteration of polysomal profiles. Any hypothesis on the involvement of insect morphogenetic hormones in the physiological development of nematodes must await data from experiments like those mentioned above, which would also determine at which level of gene expression they act.

CHAPTER IV

STUDIES ON THE α -ECDYSONE-STIMULATED ENZYME LEUCYL NAPHTHYLAMIDASE
OF APHELENCHUS AVENAE

IV.1 INTRODUCTION

From studies on the physiological trigger of exsheathment of infective third-stage larvae of Haemonchus contortus and Trichostrongylus axei, Rogers (1960) and Rogers and Sommerville (1960) concluded that the main component of the stimulus for development was the ratio of gaseous CO_2 to undissociated H_2CO_3 in the incubation medium. One of the earliest consequences of renewed development and morphogenesis following activation of the ensheathed infective larvae was the release of an active factor that attacked the second-stage sheath and allowed eventual release of the enclosed third-stage larvae. The properties of this active factor were determined as:- it lost activity following lyophilisation, dialysis and heating; the dialysed component was activated by 0.001M Mn^{2+} or Mg^{2+} , and it was inhibited by Hg^{2+} and Fe^{2+} ions. This suggested that it was a protein, i.e. an enzyme. Rogers (1966) and Rogers and Sommerville (1968) postulated a three-stage process of activation of the infective stage following infection, for the resumption of development. (1) The host stimulus acts on a receptor in the region of the nerve ring-excretory pore, resulting in activation of the neurosecretory system. (2) This causes the release of substances leading to the start of early morphogenesis, which includes the secretion of the exsheathing fluid. (3) This fluid

chemically perforates the sheath, thus releasing the larvae for further parasitic development.

Rogers (1963, 1964, 1965, 1970) identified the active component of the exsheathing fluid as leucine aminopeptidase (LAP), whose properties were similar to those of the classical enzyme from pig kidneys. Thus, following PAGE both biological, chemical and histochemical activities were coincident. It required Mn^{2+} or Mg^{2+} for both activation and stability, but was not a thiol-enzyme due to non-inhibition by di-isopropylfluorophosphate. The pH optimum was 9.5 - 10.0. The role of LAP as the moulting or ecdysial enzyme of nematodes, was also suggested by the studies on Xiphinema index (Roggen et al., 1967) and substantiated by the studies on Phocanema decipiens (Davey and Kan, 1967, 1968). However, this point requires clarification as LAP was not detected in exsheathing fluid of H. contortus (Ozerol and Silverman, 1969, 1972a, b) and H. contortus cayugensis (Slocombe and Whitlock, 1971; Slocombe, 1974). The procedures used would have inactivated or inhibited LAP, but still did not interfere with the formation of the refractile ring.

Although the function of LAP in ecdysis is uncertain, it may be involved in some aspect of the developmental physiology of nematodes. Therefore the enzyme in A. avenae was studied, in an attempt to corroborate data of properties, localisation and response to moulting hormone (α -ecdysone).

IV.2 MATERIALS AND METHODS

IV.2.A Mass culture of *A. avenae*

This was performed as in IIIA.2.A.

IV.2.B Homogenisation procedure

IV.2.B.(i) Preliminary studies. These were performed at 4° in sterile distilled water, unless otherwise stated, at the rate of 100 mg of nematodes (wet weight)/ml, in a powered ground-glass homogenizer for 6 min (N.B. for 1 min intervals interspersed with 2 min of cooling). The homogenate was spun at 20,000 g (13,200 rpm) with the mitochondrial-lysosomal pellet discarded and the supernatant used within 24 hr.

IV.2.B.(ii) Purification studies. Initially, these were performed as above except that an ultrasonic disintegrator (60W M.S.E. England) was used instead of a ground glass homogenizer. This was then changed to extraction in buffer (0.01M Tris-HCl, pH 7.3), containing 6.7×10^{-4} M EDTA. Finally, the procedure adopted was carried out in the same buffer using a French press (2,500 p.s.i.).

IV.2.C Chemical assay

The main chemical assay used was that of Goldbarg and Rutenberg (1958). The substrate 1-leucyl- β -naphthylamide (LNA) was hydrolysed and the released β -naphthylamine was diazotized by NaNO_2 and converted to the Azo dye (blue) by *n*-(1-naphthyl)ethylenediamine dihydrochloride (NED), before measurement of the percentage transmission at 560 nm. One ml of diluted supernatant with 1 ml of LNA (0.2 mg/ml) in 0.1M phosphate

buffer (pH 7.0) was incubated at 37° for the required length of time in a shaking water-bath. The β -naphthylamine released was measured following enzymatic hydrolysis, by stopping the reaction with one ml TCA (40%), removing one ml of solution and adding one ml NaNO_2 (0.1%) for a reaction time of three min. The diazotization reaction was stopped by the addition of one ml ammonium sulphamate (0.5%) for two min, and the Azo dye formed by adding NED (0.5 mg/ml of 95% ethanol) with full development of the colour by 10 min. The amount of absorption was measured using a Beckman 151 spectro-colorimeter.

The calibration curve was linear for 5-80 μg of β -naphthylamine. One unit (LNAU) of enzyme activity was taken as a μg of LNA hydrolysed/min, whilst the specific activity was designated LNAU's/mg protein-N. For enzyme purification the activities measured were changed to ng of substrate hydrolysed, i.e. LNA_mU and LNA_mU's/mg protein-N, respectively. (N.B. Enzymes hydrolysing aminoacyl naphthylamides have been variously described as aminopeptidase A and B, arylamidases, naphthylamidases and aminoacylnaphthylamide aminohydrolases. They will be referred to as naphthylamidases in the following treatise. The connotation (leucyl) naphthylamidase signifies that LNA is one of the substrates hydrolysed by this enzyme.)

The other chemical assay used was that of Rogers (1964). The substrate 1-leucinamide hydrochloride releases NH_3 on hydrolysis, which is measured colorimetrically with Nessler's reagent after Conway micro-distillation. Microtitration cups containing 100 μl 0.01N H_2SO_4 were

placed over wells in microscope slides covered with 54°C M.P. wax and containing 5 µl of nematode supernatant, 5 µl of 0.01M Tris-HCl buffer (pH 7.2) and 1 µl of 0.1M leucinamide hydrochloride. Incubation was for 1 hr at 38°C, and 2 hr at 25°C following the addition of 8 µl of saturated K₂CO₃ to the incubation mixture. Nessler's reagent (50 µl) was added to the microtitration cup, and the resultant mixture measured for absorption at 520 nm, instead of 420 nm (% transmission). The calibration curve was linear for 2-14 µg of NH₃-N. Enzyme specific activity was designated as µg NH₃-N released/min/mg protein-N (S.U.).

IV.2.D Polyacrylamide gel electrophoresis (PAGE) - Protein and enzyme detection

The method of Davis (1964) was used except that 9% gels were prepared, and the apparatus of Reid and Bielecki (1968) was used. The nematode was homogenised as in Section IV.2.B.(i), in the extraction buffer of Staples and Stahmann (1963) (w/v). The load optimum was 100 µg of protein-N for proteins and 200 µg of protein-N for enzyme detection. The electrophoresis was run at RT until the bromophenol blue tracking dye had moved 7-8 cm, i.e. for about 1.5 hr,

IV.2.D.(i) Proteins - The gels were stained in a solution of 7% acetic acid and 25% methanol containing 0.25% Coomassie Brilliant Blue R250 for 15 min, briefly rinsed in distilled water and destained by four washes of the same solvent used for staining, over a period of 30 min (Reid and Bielecki, 1968).

IV.2.D.(ii) Enzymes - All gels were pre-washed for 30 min at 4° in the appropriate enzyme-stain buffer, followed by incubation for the required time interval at 25° in the dark, unless otherwise stated. The gels were fixed by treatment with 7% acetic acid.

IV.2.D.(ii).a Leucyl naphthylamidase - The gels were incubated in a mixture containing LNA (1.37 mM) and Fast Garnet GBC (0.5 mg/ml) in 100 mM Tris-maleate buffer (pH 6.0) for 5 hr. A brick-red colour denoted a positive result (Idahl and Taljedal, 1968).

IV.2.D.(ii).b Non-specific esterases - The gels were incubated in a solution of Fast Blue RR Salt (1 mg/ml) and α -naphthylacetate (0.2 mg/ml) in 0.01M phosphate buffer (pH 6.5) for 22 or 60 min. The appearance of black bands were taken as a positive result (Been and Rasch, 1972).

IV.2.D.(ii).c Acid phosphatase - The gels were incubated in a solution of sodium α -naphthyl phosphate (1 mg/ml) and Fast Blue B salt (1 mg/ml) in 0.1M acetate buffer (pH 5.3) for 60 min at 37°, The appearance of mauve bands was taken as a positive result (Choudhury and Lundy, 1970).

IV.2.D.(ii).d Alkaline phosphatase - The gels were incubated in a solution of sodium α -naphthyl phosphate (25 mM) and Fast Red TR salt (1 mg/ml) in 0.033M Tris-HCl buffer (pH 9.5) for 60 min, to visualize the sites of enzyme activity (Allen and Hyncik, 1963).

IV.2.D.(ii).e Malate/Lactate dehydrogenase - The gels were incubated in a solution containing NAD (1.3 mg/ml), nitro-blue tetrazolium (0.45 mg/ml), either L(+) lactic acid sodium (0.05M) or L(-) malic acid (0.05M) and phenazine methosulphate (0.14 mg/ml) in 0.1M Tris buffer (pH 8.3) for 12 min, to localise the sites of enzyme activity (Gilbert and Goldberg, 1966).

IV.2.E Protein estimation

The method used was that of Jennings (1961), with bovine albumin (Armor Pharmaceutical Co., U.S.A.) as the protein standard and measurement of the optical density of the resultant blue colour at 710 nm.

IV.2.F Incubation with α -ecdysone

Aliquots of the surface-sterilised nematode (0.6 ml) were incubated for various intervals of time at 25⁰ (dark) with mild shaking, in 3.0 ml of Tyrode's solution. α -ecdysone was added at a concentration of 1.8×10^{-5} M, whilst the control contained the equivalent volume of the solvent methanol (N.B. added to incubation bottles and evaporated under a stream of dry N₂, prior to the addition of other components.) The nematodes, after incubation, were treated as in Section IV.2.B.(i). The aliquots of nematodes and Tyrode's solution were increased to 2.0 ml and 10.0 ml, respectively, for the PAGE studies.

IV.2.G Localisation of leucyl naphthylamidase

A mixed population of A. avenae was straightened and fixed by manipulation at 4⁰ in 0.05M phosphate buffer (pH 7.3 or 6.6) containing 4%

glutaraldehyde for 1 hr (Van Gundy, Bird and Wallace, 1967). The nematodes were concentrated by low-speed centrifugation in the respective buffers, and slashed with a razor to produce perforations of the cuticle. Leucyl naphthylamidase was localised either by the method of Nachlas et al. (1960) with the former pH buffer or by its modification due to Cheng and Yee (1968) with the latter pH buffer. The substrates used were LNA and α -leucyl-4-methoxy-2-naphthylamide (LMNA), which yield positive colours of blue-purple and crimson-red, respectively. The perforated nematodes were incubated for various time intervals at 37° in the respective media:-

Components	Volume (ml)	
	Nachlas' Method	Cheng's Method
1. LNA/LMNA (4 mg/ml in distilled water; stored at 40°C)	1.0	-
LNA (8 mg/ml in distilled water; stored at 40°C)	-	1.0
LMNA (8 mg/ml in 0.15 ml methanol/0.85 ml distilled water; stored at 40°C)	-	1.0
2. Sodium chloride (0.85%)	3.5	-
Sodium chloride (0.25%)	-	8.0
3. 0.1M acetate buffer, pH 6.5	5.0	-
0.1M phosphate buffer, pH 6.6	-	10.0
4. Potassium cyanide (2×10^{-2} M)	0.5	1.0
5. Fast Blue B Salt	5 mg	10 mg

Following the prescribed time interval, the nematodes were washed in the respective saline solutions and then treated with 0.1M CuSO_4 for 2 min, ending with two further washes in the respective saline solutions.

They were stored at 4° in the respective saline solution. Specimens were examined in the respective saline solutions at magnifications of 400x and 800x, with comparisons against appropriate timed controls (substrate omitted).

IV.2.H Subcellular localisation of leucyl naphthylamidase

The standard method used for the preliminary fractionation of cellular components was that of Lundgren and Roos (1974). The extraction and resuspending buffer used was 0.01M Tris-HCl (pH 7.3), containing 1×10^{-3} M EDTA and 1×10^{-3} M cysteine. The nuclear pellet obtained by centrifugation at 800 g for 10 min was discarded. The mitochondrial-lysosomal pellet was sedimented by a spin of 26,000 g for 10 min and resuspended in the buffer mentioned. Centrifugation of the resultant supernatant at 102,000 g_{av} for 60 min in an SW50 rotor, produced the pellet of microsomal fraction which was resuspended as before and the supernatant consisting of the soluble fraction. Each fraction was analysed for total and specific activity.

The possibility existed of enzymatic latency in the lysosomes of A. avenae. Therefore, the 800 g supernatant was treated with 1% DOC or 1% Triton X-100 followed by standing for 30 min at 4°, and measurements made to determine the increased enzyme activity, if any.

IV.3 RESULTS

IV.3.A PAGE of nematode proteins and enzymes (soluble)

The resolved bands were characterised by the determination of their Ef values (the ratio of their distance travelled from the origin

to the distance travelled by the tracking dye). The standard 7.5% acrylamide gels resolved 12 protein bands, and this was increased to 16-17 when the gel concentration was changed to 9%. The three major bands according to the intensity of staining had Ef values of 0.52, 0.62 and 0.99 (Fig. 18A). Electrophoresis of the different enzyme activities yielded the following patterns: leucyl naphthylamidase - one band of Ef 0.60 (Fig. 18B); non-specific esterases - eight bands (Fig. 18C); malate dehydrogenase - three bands (Fig. 18D); lactate dehydrogenase - one band (Fig. 18E); and alkaline and acid phosphatase - a single band of the same Ef mobility, i.e. 0.35 (Fig. 18F and G).

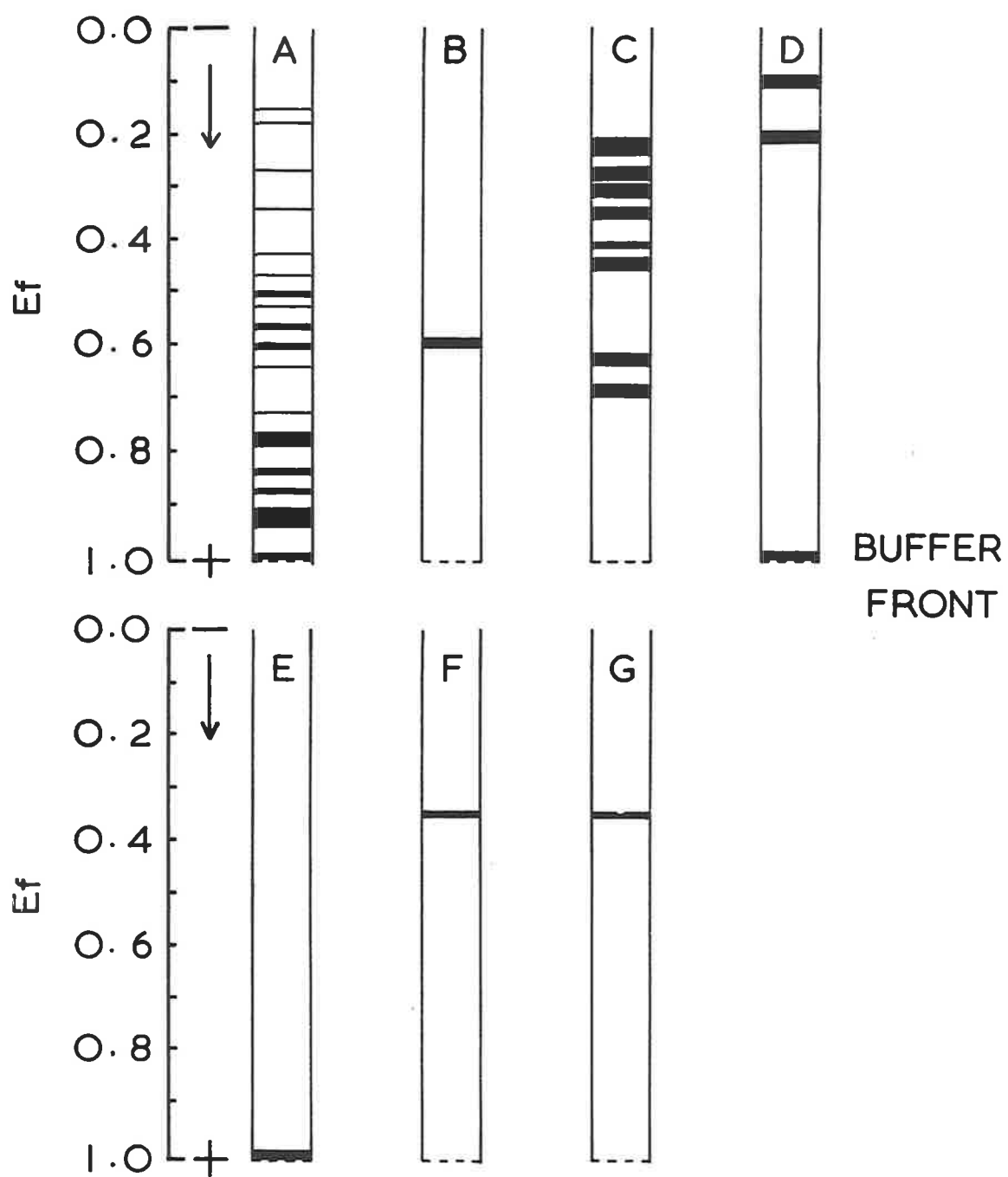
The electrophoresis of nematode extracts following incubation with α -ecdysone (2.2×10^{-8} M) produced no alteration in quantity or quality of protein or enzyme bands. However, after nematodes were incubated with α -ecdysone (1.8×10^{-5} M) for 16 and increasingly after 24 hr, a protein band of Ef 0.55 and the leucyl naphthylamidase of Ef 0.60 showed increased activity, i.e. intensity of staining. It was necessary to quantify this response of leucyl naphthylamidase to ecdysone after 16-24 hr, by chemical assay. The Ef of pig kidney leucine aminopeptidase (Worthington Biochemical Corp., U.S.A.), in the presence of 5×10^{-3} M MgCl_2 , was 0.10 (loading 10 μg).

IV.3.B Chemical assay of leucyl naphthylamidase activity following incubation with α -ecdysone

In response to the presence of α -ecdysone (1.8×10^{-5} M) in the incubation medium, the enzyme showed a small but significant increase in

Figure 18. Electrophoretograms of homogenates of A. avenae, showing bands of protein and enzyme activity (soluble). A, total protein; B, leucyl naphthylamidase; C, non-specific esterases; D, malate dehydrogenase; E, lactate dehydrogenase; F, alkaline phosphatase; G, acid phosphatase. (The E_f is the ratio of the distance travelled by the protein/enzyme band from the origin against that of the tracking dye.)

FIG. 18



activity by 18 hr post-incubation, which rose to a maximum at 24 hr and by 30 hr post-incubation, had declined significantly (Table VII). The leucinamide assay was not continued due to the uncertainty of the identity of the enzyme hydrolysing the substrate; the absence of a zero time control, and, the formation of NH_3 by the supernatant, as well as unexplained variability in the controls.

Table VII. The change in A. avenae leucyl naphthylamidase activity on incubation with α -ecdysone (1.8×10^{-5} M).

Time of Incubation (hr)	N (number of incubation)	Induction Ratio ^a (Treatment : Control)	p ^b
18	4	1.057 ± 0.01435	< 0.05
24	5	1.709 ± 0.2152	< 0.05
30	4	1.313 ± 0.0539	< 0.02

a Values of induction ratio are means \pm S.E.M.

b Significance tested for by the 't' test.

IV.3.C Partial characterisation of leucyl naphthylamidase

IV.3.C.(i) Enzyme kinetics - It was necessary to have linear responses in activity towards increased amounts of enzyme and time of incubation, such that the enzyme maintained zero-order kinetics over the range employed in assays. Also, the pH of enzyme incubation was important in order to determine the optimum conditions for incubation.

IV.3.C.(i).a Rate of substrate hydrolysis as a function of enzyme concentration - Incubation was carried out with varying amounts of

nematode supernatant (equivalent to mg/ml wet weight of nematodes) from 3-100 for 2 hr at 37⁰. The rate of hydrolysis was linear up to 5 mg/ml of nematode tissue homogenate (Fig. 19(a)). In all further experiments 4 mg/ml of nematode tissue homogenate were used, or the amount was increased with a corresponding reduction in incubation time.

IV.3.C.(i).b Rate of substrate hydrolysis as a function of reaction time - Zero-order kinetics was maintained for approximately 2.5 hr and corresponded to 6% hydrolysis of the substrate (Fig. 19(b)).

IV.3.C.(i).c Activity of leucyl naphthylamidase as a function of pH - The optimum pH for substrate hydrolysis was determined in 0.1M phosphate buffer with a pH range from 5.5 to 8.5. The optimum pH for the enzyme was 7.2, following incubation for 2 hr at 37⁰ (Fig. 19(c)).

IV.3.C.(i).d Stability and activity of leucyl naphthylamidase at different pH's - Enzyme activity was stable over the pH range of 6.25 - 8.25 when incubated for 1 hr at 37⁰, prior to assay of the remaining activity with addition of the LNA substrate (Fig. 19(d)). The controls were incubated at 0⁰ for the same time intervals, before addition of the substrate and determination of the LNA-hydrolysing activity. If incubation was extended to 2 hr prior to substrate addition, the maximum percentage of activity remaining was 66% at pH 7.0. The optimum pH for enzyme activity was found to be 5.5. The loss of enzyme activity at pH 4.0 may indicate that this is the isoelectric point of leucyl naphthylamidase (Fig. 19(d)).

Figure 19. (a) The rate of hydrolysis of leucyl- β -naphthylamide by leucyl naphthylamidase as a function of enzyme concentration. Enzyme extracts of various concentrations were incubated for 2 hr at 37⁰, prior to assay.

(b) The rate of hydrolysis of leucyl- β -naphthylamide by leucyl naphthylamidase as a function of time. A standard aliquot of enzyme extract, equivalent to 4 mg/ml wet weight of nematodes, was incubated for various time intervals at 37⁰, prior to assay.

(c) The influence of pH on the activity of leucyl naphthylamidase in 0.1M phosphate buffer (pH 5.5 to 8.5). A standard aliquot of enzyme extract, equivalent to 4 mg/ml wet weight of nematodes, was incubated at various pH values for 2 hr at 37⁰, prior to assay.

(d) The influence of pH on the activity (Δ --- Δ) and stability of leucyl naphthylamidase. A standard aliquot of enzyme extract, equivalent to 10 mg/ml wet weight of nematodes, was incubated at various pH values for 1 hr at 37⁰, prior to assay. Stability was determined by comparison of a preincubation period of 1 hr (\bullet — \bullet) or 2 hr (\times — \times) at 37⁰, with a control at 0⁰ kept for the same interval of time. Acetate buffer (0.05M, pH 3.0 to 6.0) and Tris-HCl buffer (0.05M, pH 6.5 to 9.0) were used.

FIG. 19

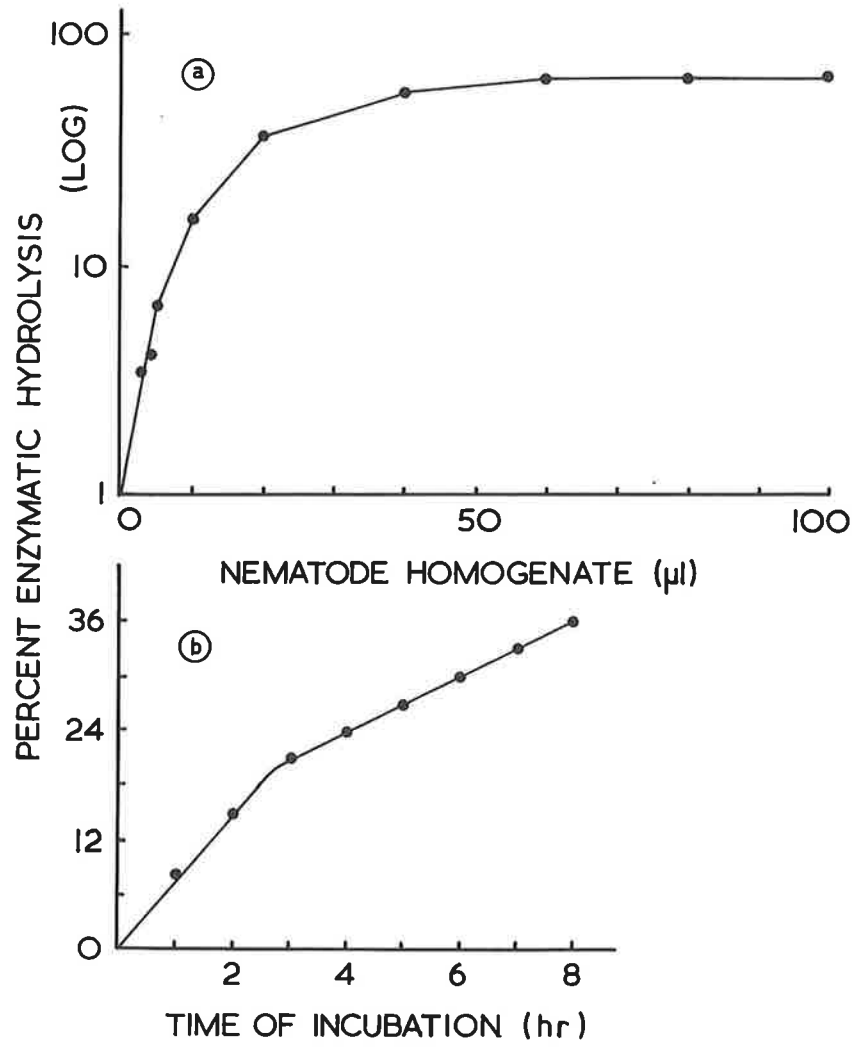
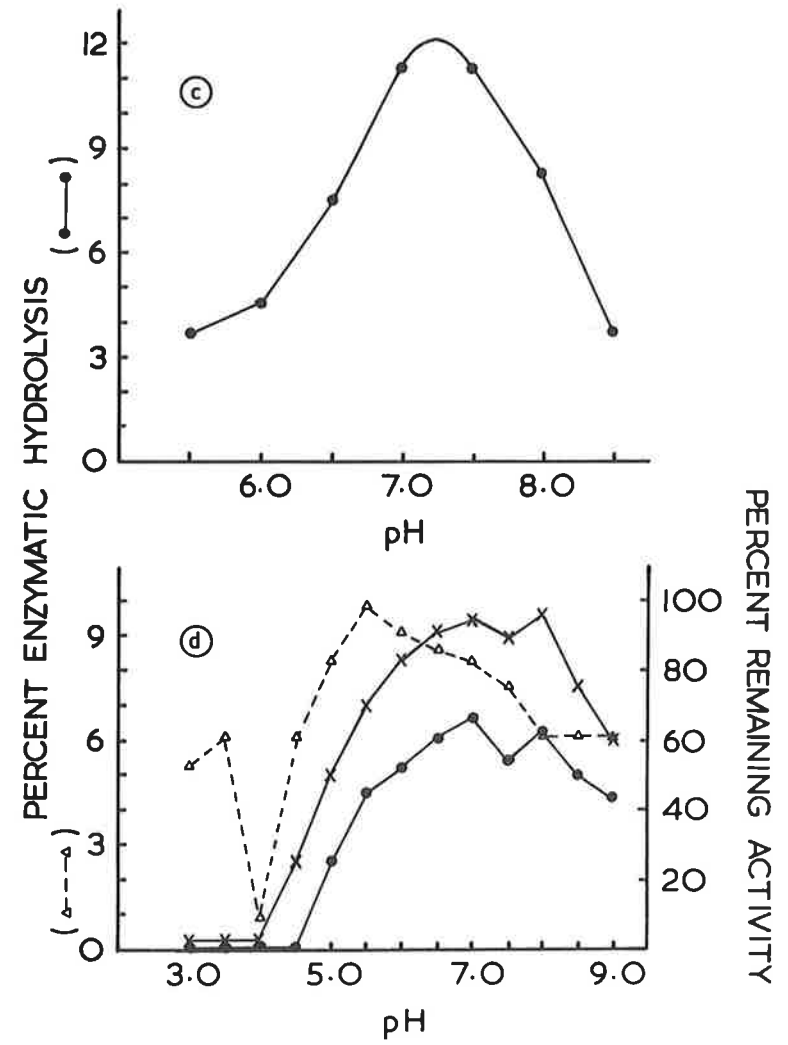


FIG. 19



IV.3.C.(i).e Effect of binding of substrate by nematode protein -

Under the conditions used, i.e. the concentration of LNA and enzyme, this was undetectable.

IV.3.C.(ii) Effect of modifiers - Data obtained by the study of the effect of various modifiers on leucyl naphthylamidase, was necessary to assist in the classification, purification and possible function of the enzyme. The components were added to the homogenate derived from nematode tissue in the extraction buffer 0.01M Tris-HCl, pH 7.2, and the specific activity determined against the relevant control, unless otherwise stated.

IV.3.C.(ii).a Methionine - Enzyme activity was increased by concentrations of 1×10^{-2} and 1×10^{-3} M (Table IX).

IV.3.C.(ii).b Mg²⁺ - Extraction was performed in 0.1M phosphate buffer, pH 7.2, containing 5×10^{-3} M MgCl₂ and with the chemical assay carried out in the presence of 4×10^{-3} M MgCl₂. Enzyme activity was 73.3% that of the control. Incubation of the extract for 1 hr at 37° prior to assay produced no stimulation of enzyme activity, whilst a 2 hr activation period caused the enzyme activity to fall to 53.3% that of the control.

IV.3.C.(ii).c L-alanyl-β-naphthylamide (ANA) - The rate of hydrolysis of ANA was reduced to 30% for the control enzyme (Mg²⁺ absent), and 18.7% for the Mg²⁺-extracted enzyme, when compared to the substrate LNA.

IV.3.C.(ii).d Chelating agents - EDTA, at 4×10^{-3} M and to a greater

extent at 1×10^{-3} M, increased the activity of the enzyme (Table VIII). O-phenanthroline was inhibitory at concentrations of 5×10^{-4} M and above, but stimulatory at lower concentrations (Table VIII). α, α' -dipyridyl inhibited activity at 4×10^{-3} M (Table VIII). The latter two compounds were dissolved in 50% ethanol giving a final concentration in the incubation medium of 5%, followed by enzymatic measurement against appropriate controls. O-phenanthroline was also dissolved by warming the stock solution in a boiling water-bath prior to addition (Prado *et al.*, 1971).

IV.3.C.(ii).e S-S dissociating agents - Of the four reagents tested, cysteine, β -mercaptoethanol (β -MSH) and glutathione were stimulatory to varying degrees (Table IX), although glutathione at 4×10^{-3} M reduced the specific activity to 59% that of the control (Table IX). The greatest stimulation of activity was achieved by cysteine at 1×10^{-3} M (356%). Sodium thioglycollate produced no effect on activity at the concentration applied (4×10^{-3} M),

IV.3.C.(ii).f Sulphydryl reagents - Iodoacetic acid at 4×10^{-3} M and *p*-hydroxymercuribenzoate (Na^+ salt) at 1×10^{-3} M were tried. The former compound had no effect, whilst the latter gave complete inhibition (Table X).

IV.3.C.(ii).g Puromycin - At 5×10^{-4} M puromycin caused marked inhibition of enzyme activity, whilst at lower concentrations the effect was one of slight stimulation (Table X).

Table VIII. The effect of different chelating agents on leucyl naphthylamidase activity.

Substance	Concentration (M)	Naphthylamidase ^a Activity (%)
EDTA	4×10^{-3}	135
	1×10^{-3}	230
α, α' -dipyridyl	4×10^{-3}	20
O-phenanthroline	4×10^{-3}	10
	5×10^{-4}	88
	5×10^{-5}	195
	5×10^{-6}	144

a The enzyme activity of the control, i.e. the nematode tissue extracted in Tris-HCl buffer, pH 7.2, was taken as 100%.

Table IX. The effect of different mild reducing agents on leucyl naphthylamidase activity.

Substance	Concentration (M)	Naphthylamidase ^a Activity (%)
Cysteine	1×10^{-2}	304
	1×10^{-3}	356
Sodium thioglycollate	4×10^{-3}	100
Glutathione	4×10^{-3}	59
	4×10^{-4}	128
	4×10^{-5}	126
L-Methionine	1×10^{-2}	271
	1×10^{-3}	186
β -mercaptoethanol	4×10^{-3}	230

a The enzyme activity of the control, i.e. the nematode tissue extracted in Tris-HCl buffer, pH 7.2, was taken as 100%.

Table X. The effect of different inhibitors on leucyl naphthylamidase activity.

Substance	Concentration (M)	Naphthylamidase ^a activity (%)
Iodoacetic acid	4×10^{-3}	100
p-hydroxymercuribenzoate (Na ⁺ salt)	1×10^{-3}	0
Puromycin	5×10^{-4}	30
	5×10^{-5}	111
	5×10^{-6}	132

a The enzyme activity of the control, i.e. the nematode tissue extracted in Tris-HCl buffer, pH 7.2, was taken as 100%.

IV.3.C.(ii).h Chloride ions - Chloride ions were inhibitory to enzyme activity over the range of 2.5×10^{-1} to 5×10^{-2} M, below which it appeared to be without effect (Fig. 20(a)). The concentration of Cl⁻ ions in the buffer during the chemical assay procedure was 4.5×10^{-3} M.

IV.3.C.(ii).i Divalent and trivalent cations - These were the chloride or sulphate salts of Co²⁺, Hg²⁺, Cu²⁺, Mg²⁺, Pb²⁺, Mn²⁺, Ca²⁺, Zn²⁺, Fe²⁺ and Fe³⁺. All were inhibitory to varying degrees at a final concentration of 1×10^{-3} M, except for the Zn²⁺ ions which manifested a slight increase in activity (Table XI). From 5×10^{-5} M to 1×10^{-3} M there was a small progressive increase in enzyme activity

in the presence of Zn^{2+} ions, with the maximum of 115.1% that of the control.

Table XI. The effect of different divalent and trivalent cations on leucyl naphthylamidase activity.

Substance	Concentration (M)	Naphthylamidase ^a activity (%)
Co^{2+}	1×10^{-3}	70
Hg^{2+}	1×10^{-3}	5
Cu^{2+}	1×10^{-3}	10
Mg^{2+}	1×10^{-3}	90
Pb^{2+}	1×10^{-3}	70
Mn^{2+}	1×10^{-3}	60
Ca^{2+}	1×10^{-3}	70
Zn^{2+}	1×10^{-3}	115
	5×10^{-4}	112
	1×10^{-4}	106
	5×10^{-5}	103
Fe^{2+}	1×10^{-3}	92
	1×10^{-4}	62
(and) Fe^{3+}	1×10^{-3}	76
	1×10^{-4}	62

a The enzyme activity of the control, i.e. the nematode tissue extracted in Tris-HCl buffer, pH 7.2, was taken as 100%.

IV.3.C.(ii).j Enzyme activity in presence of different concentrations of Zn^{2+} and O-phenanthroline - The chelating agent O-phenanthroline preferentially binds Zn^{2+} and with the slight stimulation of activity caused by this divalent cation, it was decided to test Zn^{2+} in the

presence of the chelating agent to determine if this was a Zn-metalloenzyme. Zn^{2+} ions at different concentrations were incubated with the enzyme in three different concentrations of O-phenanthroline. The chelating agent when alone at 5×10^{-5} and 5×10^{-6} M stimulated activity, but in the presence of Zn^{2+} ions produced no stimulation when compared to the control, and inhibition occurred at the highest concentration of the cation used. O-phenanthroline at 5×10^{-4} M was slightly inhibitory, but in the presence of high concentrations of Zn^{2+} ions it was markedly inhibitory. However, at lower Zn^{2+} concentrations stimulation was equally marked, and still increased at a weight ratio of 1:7 and molar ratio of 1:10 of Zn^{2+} to O-phenanthroline (Fig. 20(b)).

IV.3.C.(ii).k Enzyme stability - Prior to the purification of leucyl naphthylamidase it was necessary to find out which components when added to the buffer system, conferred stability on the enzyme and not necessarily activation, for dialysis and diafiltration to be performed. Therefore, various combinations of Zn^{2+} , cysteine and EDTA at final concentrations of 1×10^{-3} M in the homogenate and dialysing buffer were tested to determine the stability of the enzyme following dialysis for 15 hr at 4° .

Enzyme activity prior to dialysis was stimulated in the presence of EDTA, cysteine, and EDTA plus cysteine (Table XII), although the maximum enhancement of 230% that of control occurred with these two components when separate. The combinations of Zn^{2+} + cysteine + EDTA and Zn^{2+} + cysteine proved to be inhibitory (Table XII).

Figure 20. (a) The influence of Cl^- ions on the activity of leucyl naphthylamidase. A standard aliquot of enzyme extract, equivalent to 10 mg wet weight of nematodes/ml 0.01M Tris-HCl, pH 7.2, was incubated with various concentrations of Cl^- ions for 1 hr at 37° , prior to assay. The enzyme activity of the control (---) was measured in the presence of the extracting buffer.

(b) The activity of leucyl naphthylamidase in the presence of Zn^{2+} ions and O-phenanthroline. A standard aliquot of enzyme extract, equivalent to 10 mg wet weight of nematodes/ml 0.01M Tris-HCl, pH 7.2, was incubated with varying concentrations of Zn^{2+} ions and O-phenanthroline at either 5×10^{-4} (●), 5×10^{-5} (x) or 5×10^{-6} M (▲) for 1 hr at 37° , prior to assay. The enzyme activity of the control (---) was measured in the presence of the extracting buffer.

FIG. 20

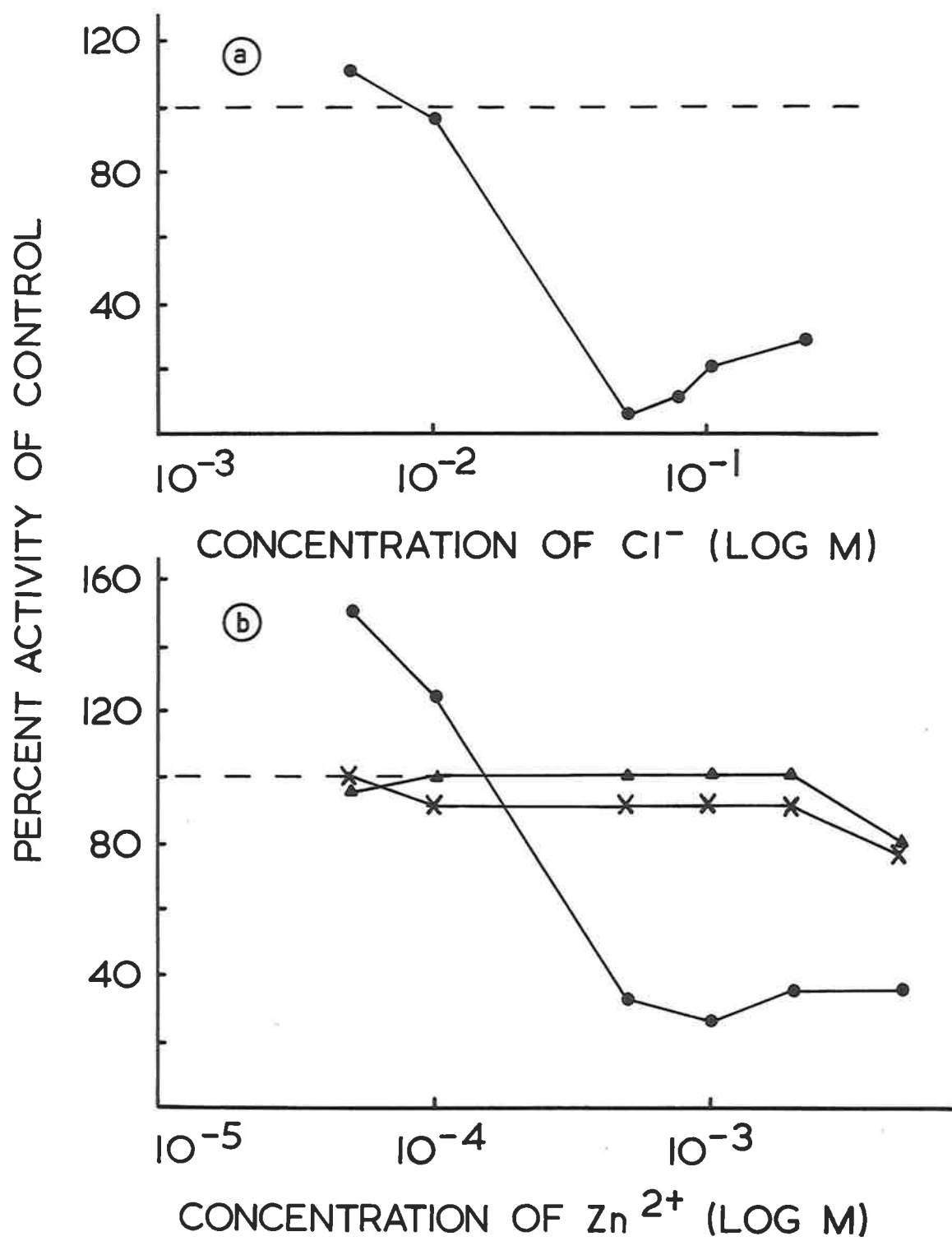


Table XII. The effect of different modifier combinations on the stability of leucyl naphthylamidase activity.

Assay components	Before dialysis Enzyme activity (%)	After dialysis Enzyme activity (%)	After dialysis Activity remaining (%)
Zn ²⁺ Cysteine EDTA Buffer	47	38	31
- Cysteine EDTA Buffer	194	187	37
Zn ²⁺ Cysteine - Buffer	59	100	65
- - - Buffer	100	65	22
- - EDTA Buffer	230	397	67
- Cysteine - Buffer	230	237	40
Control-Non-dialysed Buffer	100	100	39

After 15 hr, the activity of the homogenate left standing at 4⁰ had decreased to 39% of the original activity (control in 0.01M Tris-HCl buffer, pH 7.2), whilst that dialysed had lost 78% of its original activity. All combinations of added components had lost activity over the same time period, the least being with the addition of EDTA to the extracting buffer, i.e. 67% that of control (Table XII).

IV.3.D Enzyme localisation

There are three possible locations of the enzyme in the nematode body (based on the interpretation of the three possible staining patterns), either in the intestine, the body wall or the intestine and the body wall. The method of Nachlas et al. (1960) was negative with all the permutations tried, whilst that of Cheng and Yee (1968) gave a positive result with both substrates used and an optimum incubation time for staining of 20

min. Both substrates gave similar staining patterns, except that LMNA produced a higher incidence of enzyme activity in the pharyngeal bulb. A positive response was seen in the form of granules, vacuoles and background colour. It was present in the body-wall layer (cuticle, hypodermis and somatic muscle) both in front of, surrounding and posterior to the pharyngeal bulb. The pharyngeal bulb itself was positive to the histochemical stain. The reproductive system and intestine did not contain any activity when extruded from the body following cuticle incision. Also, the stain appeared in focus above the intestine, and when the lateral hypodermis/somatic muscle was in focus the colour was stronger than for the intervening intestine.

IV.3.E Subcellular fractionation

Following the differential centrifugation of the nematode homogenate, the specific and total activity of leucyl naphthylamidase was determined for each fraction. The activity recovered was only 65% of the total. The enzyme was contained in the soluble fraction (Table XIII). The treatment of the 800 g supernatant with 1% DOC reduced the specific activity of the enzyme. The total and specific activities were unaltered following treatment with 1% Triton X-100 (Table XIV).

IV.3.F Attempted purification of leucyl naphthylamidase

The procedures of Nakadai et al. (1973a) for the purification of the extracellular enzyme leucine aminopeptidase I of Aspergillus oryzae, were used as the starting point for the purification of the leucyl naphthylamidase of A. avenae. Because the enzyme was first thought to be extracellular, all extractions and manipulations were originally

Table XIII. The subcellular localisation of leucyl naphthylamidase activity.

Fraction	Specific activity LNAU/mg protein- N	Total activity LNAU	% of total activity
800 g supernatant	17.07	23.4	100
26,000 g pellet	5.16	1.5	6.5
102,000 g pellet	1.89	0.27	1.1
102,000 g supernatant	16.41	13.44	57.4

Table XIV. The effect of DOC and Triton X-100 on leucyl naphthylamidase activity.

Substance	Specific activity (%)	Total activity (%)
Triton X-100 (1%)	97.7	94.4
DOC (1%)	49.4	50.0

performed with distilled water (DW), as was done for the extracellular alkaline proteinase of Aspergillus sojae (Hayashi et al., 1967) and the acid carboxypeptidases I-IV of A. oryzae (Nakadai et al., 1972a, b, c, 1973d).

IV.3.F.(i) Problems of leucyl naphthylamidase purification - preliminary studies of fractionation -

IV.3.F.(i).a Amberlite IRC-50 - This weakly acidic, carboxylic-type cationic exchange resin was used to observe if significant fractionation and purification of the leucyl naphthylamidase occurred. Amberlite IRC-50 (2.5 g in 10 ml of 0.05M acetate buffer, pH 4.0) was added to 15 ml of nematode homogenate, the pH adjusted to 4.0 and then stirred for 3 hr at extraction temperature of 4⁰. The non-absorbed material was removed by filtration with 100 ml of 0.01M acetate buffer, pH 4.0, and the acetate fraction adjusted to pH 5.5. The absorbed material was removed by filtration, preceded by stirring with 100 ml of 0.2M phosphate buffer, pH 7.0, for 3 hr and the phosphate fraction adjusted to pH 5.5. The total and specific activities for each fraction were determined (Table XV).

IV.3.F.(i).b Ammonium sulphate precipitation (55-95%) - Solid ammonium sulphate was added to the nematode homogenate to produce a 55% (and a 95%) saturated solution, adjusted to pH 5.5 and stirred for 30 min. The precipitated macromolecular fraction was pelleted by centrifugation at 20,000 g for 20 min. The supernatant was brought

Table XV. The effect of amberlite IRC-50 on the fractionation and the total and specific activities of leucyl naphthylamidase.

Fraction	Total activity (LNAU)	Specific activity (LNAU/mg protein-N)	Increase ^a in specific activity (X)	% of total activity remaining
1. Acetate	180,600	19,860	1.04	16.1
2. Phosphate	0.0	0.0	0	0.0
	<u>180,600</u>			<u>16.1</u>

a Specific activity (1x), is that originally added.

Table XVI. The effect of ammonium sulphate (55-95%) on the fractionation and the total and specific activities of leucyl naphthylamidase.

Fraction	Total activity (LNAU)	Specific activity (LNAU/mg protein-N)	Increase ^a in specific activity (X)	% of total activity remaining
1. 0-55% saturated (NH ₄) ₂ SO ₄	87,300	25,200	1.26	48.3
2. 55-95% saturated (NH ₄) ₂ SO ₄	41,700	56,250	2.14	23.1
	<u>129,000</u>			<u>71.4</u>

a Specific activity (1x), is that originally added.

to 95% saturation and the procedure repeated. The total and specific activities for each fraction were determined following dialysis overnight (O/N) against DW (Table XVI).

IV.3.F.(i).c Rivanol precipitation (6,9-diamino-2-ethoxyacridine lactate) - Rivanol (420 mg) was added to 21 ml of nematode homogenate, the pH adjusted to 6.0 and then stirred for 3 hr at 4⁰. The precipitated macromolecular material was pelleted by centrifugation at 20,000 g for 20 min. The fractions were dialysed overnight at 4⁰, against distilled water. The total and specific activities for each fraction were determined to observe if any significant fractionation and purification of the enzyme had occurred.

Table XVII. The effect of rivanol on the fractionation and the total and specific activities of leucyl naphthylamidase.

Fraction	Total activity LNA _m U	Specific activity (LNA _m U/mg protein-N)	Increase ^a in specific activity (X)	% of total activity remaining
1. Precipitable	4,200	11,880	0.21	10.1
2. Non-precipitable	21,000	6,600	0.12	50.4
	25,200			60.5

a Specific activity (1x), is that originally added.

The extraction of the rivanol-precipitable fraction by precipitation with 67% acetone and resuspension in 0.01M Tris-HCl buffer, pH 7.3, increased the total and specific activity by 48 and 82%,

respectively.

IV.3.F.(i).d Conclusion - Amberlite IRC-50 fractionation indicated the involvement of a cationic moiety in the stability of the enzyme, but the method was discarded because its removal resulted in the reduction of total activity without any increase in the specific activity. Ammonium sulphate fractionation at 55% and 95% saturation was incomplete and the loss of total activity was significant, but the latter fraction showed a measurable increase in specific activity. Rivanol fractionation was almost complete with the enzyme in the non-precipitable fraction, however, significant loss of total and specific activity had occurred. It was discarded because it was shown to be inhibitory, on acetone precipitation of the precipitable fraction.

IV.3.F.(i).e Ammonium sulphate precipitation (55-95%) - The procedure was performed as previously stated in IV.3.F.(i).b, to examine the extent of fractionation and purification of this precipitation step. The total and specific activities of each fraction were determined following the treatment (Table XVIII).

IV.3.F.(i).f Ammonium sulphate precipitation (30-80%) - The procedure was repeated as above, with the altered percent saturations of ammonium sulphate for each fraction, which were adjusted to pH 7.0 prior to being stirred for the 30 min interval. Dialysis was carried out overnight at 4° against 0.01M Tris-HCl buffer, pH 7.3. The extent of fractionation and purification was determined from the total and specific activities of each fraction (Table XIX).

Table XVIII. The effect of ammonium sulphate (55-95%) on the fractionation and the total and specific activities of leucyl naphthylamidase.

Fraction	Total activity (LNA _m U)	Specific activity (LNA _m U/mg protein-N)	Increase ^a in specific activity (X)	% of total activity remaining
1. 0-55% saturated (NH ₄) ₂ SO ₄	24,870	21,780	1.06	9.2
2. 55-95% saturated (NH ₄) ₂ SO ₄	19,170	34,710	1.69	7.1
	<u>44,040</u>			<u>16.3</u>

a Specific activity (1x), is that originally added.

Table XIX. The effect of ammonium sulphate (30-80%) on the fractionation and the total and specific activities of leucyl naphthylamidase

Fraction	Total activity (LNA _m U)	Specific activity (LNA _m U/mg protein-N)	Increase ^a in specific activity (X)	% of total activity remaining
1. 0-30% saturated (NH ₄) ₂ SO ₄	60,900	15,300	0.59	13.8
2. 30-80% saturated (NH ₄) ₂ SO ₄	94,800	34,500	1.33	21.5
	<u>155,700</u>			<u>35.3</u>

a Specific activity (1x), is that originally added.

IV.3.F.(i).g Ammonium sulphate (70-95%) - The procedure in IV.3.F.(i).b was repeated with the altered percent saturations of ammonium sulphate for each fraction. Determination of the total and specific activities of each fraction were used to determine the degree of fractionation and purification achieved.

Table XX. The effect of ammonium sulphate (70-95%) on the fractionation and the total and specific activities of leucyl naphthylamidase.

Fraction	Total activity LNA _m U	Specific activity (LNA _m U/mg protein-N)	Increase ^a in specific activity (X)	% of total activity remaining
1. 0-70% saturated (NH ₄) ₂ SO ₄	98,400	19,200	0.84	63.2
2. 70-95% saturated (NH ₄) ₂ SO ₄	3,870	18,300	0.80	2.5
	102,270			65.7

a Specific activity (1x), is that originally added.

IV.3.F.(i).h Ammonium sulphate precipitation (20-80%) - The procedure was repeated as above with the altered percent saturations of ammonium sulphate for each fraction (IV.3.F.(i).b). The total and specific activities of the enzyme were determined for each fraction to evaluate the extent of fractionation and purification.

Table XXI. The effect of ammonium sulphate (20-80%) on the fractionation and the total and specific activities of leucyl naphthylamidase.

Fraction	Total activity LNA _m U	Specific activity (LNA _m U/mg protein-N)	Increase ^a in specific activity (X)	% of total activity remaining
1. 0-20% saturated (NH ₄) ₂ SO ₄	35,700	9,600	0.50	34.9
2. 20-80% saturated (NH ₄) ₂ SO ₄	27,000	14,700	0.77	26.4
	62,700			61.3

a Specific activity (1x), is that originally added.

IV.3.F.(i).i Ammonium sulphate precipitation (40-95%) - The procedure was repeated as above with the altered percent saturations of ammonium sulphate for each fraction (IV.3.F.(i).b). However, 6.7×10^{-4} M EDTA was added to the extraction and dialysing buffers in an attempt to increase the stability of the enzyme (Rhodes et al., 1966) (Table XXII).

IV.3.F.(i).j The effect of dialysis on enzyme activity - The stability of the enzyme following dialysis was determined by comparison with the total and specific activities of a non-dialysed control. The extraction and dialysing buffer was 0.01M Tris-HCl, pH 7.3, containing 6.7×10^{-4} M EDTA (Table XXIII).

Table XXII. The effect of ammonium sulphate (40-95%) on the fractionation and the total and specific activities of leucyl naphthylamidase,

Fraction	Total activity LNA _m U	Specific activity (LNA _m U/mg protein-N)	Increase ^a in specific activity (X)	% of total activity remaining
1. 0-40% saturated (NH ₄) ₂ SO ₄	3,710	1,374	0.05	2.7
2. 40-95% saturated (NH ₄) ₂ SO ₄	37,837	36,735	1.20	27.9
	41,548			30.6

a Specific activity (1x), is that originally added.

Table XXIII. The effect of dialysis on the stability of leucyl naphthylamidase.

Fraction	Total activity LNA _m U	Specific activity (LNA _m U/mg protein-N)	Increase ^a in specific activity (X)	% of total activity remaining
1. Dialysed (16 hr)	161,100	124,200	0.71	61.2
2. Non-dialysed (16 hr)	235,480	157,170	0.89	89.4

a Specific activity (1x), is that originally added.

IV.3.F.(i).k Conclusion - Dialysis caused the loss of 39% of the total and 30% of the specific activity of the enzyme, whilst on standing at 4° this amounted to 11% of both activities. This partially explains

the loss of 70% of the enzyme activity following $(\text{NH}_4)_2\text{SO}_4$ fractionation and resultant desalting by dialysis, i.e. 35% due to dialysis and 4% due to standing. This reduction of activity was presumed to be due to the removal of a low M.W. factor ($< 5,000$), which was essential for leucyl naphthylamidase stability and activity. In an attempt to avoid this problem, desalting was later performed by gel chromatography with Sephadex G-25. The explanation for the remaining 31% of enzyme activity was originally thought to be due to the length of time of enzymatic dialysis in the presence of $(\text{NH}_4)_2\text{SO}_4$. However, it later became apparent that it was the result of direct inhibition of the enzyme molecule, as it still occurred following desalting by gel chromatography.

Until a measure of stability was achieved with the use of an extracting buffer containing a chelating agent, the precipitation of enzyme activity with ammonium sulphate of different saturations produced a high loss of activity and a variable fractionation. The activity loss was reduced from 84 to 70% and the enzyme almost completely fractionated in the high percent salt solution, when 0.01M Tris-HCl buffer, pH 7.3, and 6.7×10^{-4} M EDTA was incorporated into the system.

The loss of activity was greatest on the first fractionation with ammonium sulphate, i.e. IV.3.F.(i).e, i, with reduction to a constant 35% loss at the third fractionation, i.e. IV.3.F.(i).g, which corresponded to 3.7% of the original enzyme activity. It appeared that this constant loss was due to the process of dialysis. Thus, after

successive ammonium sulphate fractionations, the result is a small amount of enzyme activity of higher stability than the original enzyme.

IV.3.F.(ii) Preliminary studies of purification - The use of the extracting buffer 0.01M Tris-HCl, pH 7.3, containing 6.7×10^{-4} M EDTA, increased the total and specific activities by 28 and 33% respectively. All work was performed at 4° unless otherwise stated.

IV.3.F.(ii).a Crude homogenate - A. avenae nematodes were surface sterilised by 1:10,000 ppm Merthiolate for at least 60 min, and then resuspended in 0.01M Tris-HCl buffer, pH 7.3, containing 6.7×10^{-4} M EDTA at the rate of 1:5 w/v. The nematode suspension was sonicated (60W M.S.E. England) for a total of 25 min. The total and specific activities of the enzyme were determined following centrifugation at 4,000 rpm for 10 min (Table XXIV(A)). Co^{2+} at 6.7×10^{-4} M in the substrate solution for enzyme activation had no effect on enzyme activity (Rhodes et al., 1966).

IV.3.F.(ii).b 20,000 g homogenate - The remaining supernatant was centrifuged at 20,000 g for 15 min to remove the mitochondrial/lysosomal fraction, and total and specific activities of the supernatant determined (Table XXIV(A)). This procedure appeared to remove particulate inhibition of the enzyme, as was shown by the greater increase in the specific activity than the purification of protein.

IV.3.F.(ii).c Activity loss by dialysis - The remaining supernatant was dialysed for 16 hr against the extracting buffer, and the total and

specific activities were re-determined (Table XXIV(A)). Both the total and the specific activity were substantially reduced, without any concomitant rise in the purification of the protein.

IV.3.F.(ii).d Ammonium sulphate fractionation (75-95%) - The macromolecular fractions were precipitated from the remaining supernatant and collected by centrifugation at 20,000 g for 15 min, following 30 min stirring at 75 and 95% saturation with ammonium sulphate consecutively (adjusted pH 7.1). Each fraction was redissolved in the extracting buffer. The fractions were dialysed overnight against the starting buffer, and the total and specific activities determined for each fraction (Table XXIV(A)). Most of the enzyme activity was present in the 75% saturation fraction, although of a lower specific activity than the 95% saturation fraction. This step was also characterised by a significant loss of enzyme activity, and with the increased enzyme activity not equal to the protein purification.

IV.3.F.(ii).e Ammonium sulphate fractionation (40-95%) - The remaining pooled supernatant was treated as previously, with 40 and 95% saturation of the consecutive solutions by ammonium sulphate (Table XXIV(A)). The result was the same as above in terms of the loss of enzyme activity and unequal increase of protein purification, when compared to the specific activity. The 40-95% saturation fraction contained all the remaining enzyme activity.

IV.3.F.(ii).f DEAE-cellulose ion exchange column chromatography -

The anion exchanger (DE 11-Cellulose, Whatman, England) was prepared according to the methodology of the Whatman Manual (1973) and Himmelhoch (1971), and was shown to adsorb nematode homogenate material absorbing at 280 nm, over the pH range 6.4 - 7.4. The column was therefore equilibrated and eluted at pH 7.0. The equilibrated column (1.7 x 15 cm) was loaded with 2.365 mg protein-N and washed with two column volumes of the starting buffer (0.01M phosphate buffer, pH 7.0, containing 6.7×10^{-4} M EDTA) to remove loosely adsorbed material. The column was eluted by a linear gradient of NaCl of 0 - 0.4M in 500 ml of starting buffer, and with 10 ml fractions collected. The enzyme activity and 280 nm absorption was determined for each fraction, as well as the linearity of the gradient (Fig. 21(a)). The active fractions 5-16 were pooled and concentrated by diafiltration to 1.13 ml, by the use of a Model 52 apparatus and UM/10 filter under N_2 of 40 psi (Amicon, U.S.A.). The total and the specific activities for this fraction were determined (Table XXIV(A)). There was a further decrease in total activity, but at the same time a substantial increase in the activity of the enzyme. This was not equal to the degree of protein purification.

IV.3.F.(ii).g Sephadex G-200 gel filtration column chromatography -

The column was prepared and calibrated according to the Pharmacia Instruction Manual (1973) and Rieland (1971) i.e. column volume 158 cc, void volume 66 cc and void volume plus inner volume 148 cc. The

column was equilibrated and eluted with the starting buffer 0.05M acetate, pH 6.0, containing 6.7×10^{-4} M EDTA (loading 0.18 mg protein-N). Fractions of 5 ml were collected and the enzyme activity and 280 nm absorption determined for each one (Fig. 21(b)). The active fractions 19-27 were pooled, diafiltrated to a smaller volume and the total and specific activities determined (Table XXIV(A)). The total activity remaining was 0.3%, whilst the increased enzyme activity was only 14.7x when compared to the protein purification value of 1140x (Table XXIV(B)).

When this procedure was repeated the 'purified' enzyme activity was increased 26.9x (Table XXV(B)). The only alteration was that gel filtration column chromatography was performed using Sephadex G-100 (column volume 302 cc, void volume 110 cc and void volume plus inner volume 295 cc), instead of G-200 and an equilibration and elution buffer of 0.01M Tris-HCl, pH 7.3, containing 6.7×10^{-4} M EDTA.

IV.3.F.(iii) Purification of enzyme - method adopted

IV.3.F.(iii).a Crude homogenate - A. avenae nematodes were surface sterilised with 1:10,000 ppm Merthiolate for at least 60 min, following rehydration of the lyophilised material for approximately 12 hr. The nematodes were suspended in the extracting buffer 0.01M Tris-HCl, pH 7.3, containing 6.7×10^{-4} M EDTA at the rate of 1:5 w/v, before being homogenised twice in a French press at 2,000 - 2,500 psi. The total and specific activities of the extract were estimated, following centrifugation at 4,000 rpm for 10 min (Table XXVI(A)).

Figure 21. (a) Chromatography of the 40-95% $(\text{NH}_4)_2\text{SO}_4$ -precipitable fraction on DEAE-cellulose. The procedure was as in IV.3.F.(ii).f. The parameters measured were: x---x, NaCl concentration; \blacktriangle — \blacktriangle , O.D. at 280 nm; \bullet — \bullet , leucyl naphthylamidase activity on leucyl- β -naphthylamide.

(b) The gel filtration of leucyl naphthylamidase activity through Sephadex G-200. The procedure was as in IV.3.F.(ii).g. The parameters measured were: \blacktriangle — \blacktriangle , O.D. at 280 nm; \bullet — \bullet , leucyl naphthylamidase activity on leucyl- β -naphthylamide.

FIG. 21

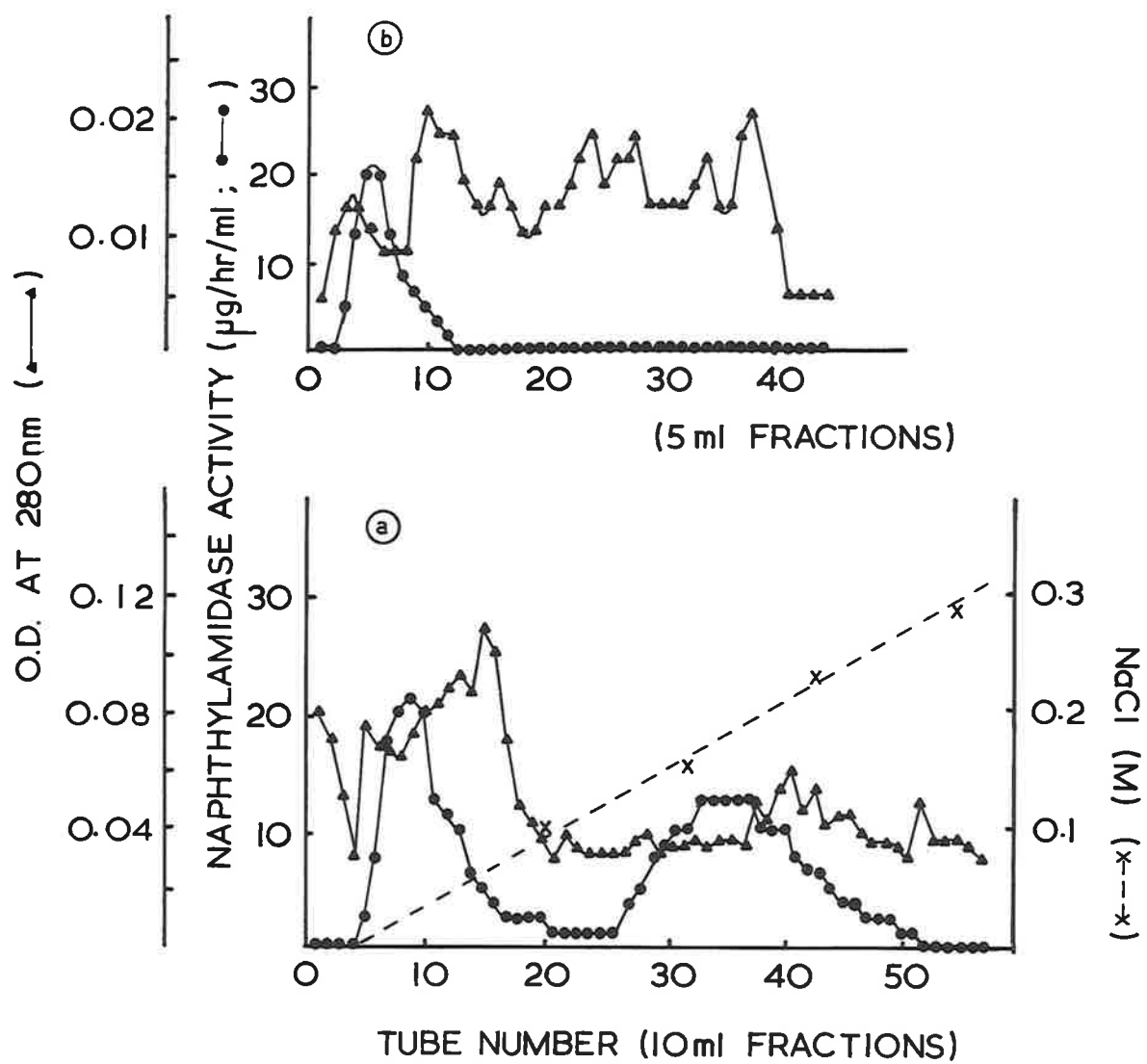


Table XXIV(A). Purification of leucyl naphthylamidase.

Procedure	Total volume ml	Total activity LNAU	Specific activity LNAU/mg protein-N	Yield %
1. Crude extract	38	2.85×10^6	2.53×10^4	100
2. 20,000 g homogenate	36	2.63×10^6	1.76×10^5	92.4
3. Effect of dialysis	34.5	1.61×10^6	1.24×10^5	56.5
4. Fractionation with ammonium sulphate (75-95%)	75% 26	7.02×10^5	7.11×10^4	27
	95% 6	6.9×10^4	1.22×10^5	
5. Fractionation with ammonium sulphate (40-95%)	40% 17	0.0	0.0	0.0
	95% 11	2.31×10^5	9.84×10^4	8.1
6. DEAE-cellulose chromatography	1.13	6.39×10^4	3.53×10^5	2.2
7. Gel filtration on Sephadex G-200	4.2	7.80×10^3	3.72×10^5	0.3

Table XXIV(B). Leucyl naphthylamidase and protein purification of A. avenae extract.

Procedure	Protein-N mg	Increased protein purification (X)	Increased enzyme activity (X)
1. Crude extract	23.94	1	1
2. 20,000 g homogenate	14.94	1.6	6.95
3. Effect of dialysis	12.96	1.8	4.91
4. Fractionation with ammonium sulphate (75-95%)	75% 9.88	2.4	2.8
	95% 0.57	42	4.79
5. Fractionation with ammonium sulphate (40-95%)	40% 5.53	4.3	-
	95% 2.37	10.1	3.88
6. DEAE-cellulose chromatography	0.18	133	13.92
7. Gel filtration on Sephadex G-200	0.021	1140	14.66

Table XXV(A). Purification of leucyl naphthylamidase.

Procedure	Total volume ml	Total activity LNA _m U	Specific activity LNA _m U/mg protein-N	Yield %
1. Crude extract	38	2.85×10^6	1.16×10^4	100
2. 20,000 g homogenate	37.5	2.56×10^6	1.52×10^5	89.7
3. Fractionation with ammonium sulphate (40-95%)	40% - 95% 13.5	- 6.75×10^5	- 1.92×10^5	- 23.7
4. DEAE-cellulose chromatography	2.85	4.68×10^4	6.42×10^4	1.6
5. Gel filtration on Sephadex G-100	1.5	1.77×10^4	3.11×10^5	0.4

Table XXV(B). Leucyl naphthylamidase and protein purification of A. avenae extract.

Procedure	Protein-N mg	Increased protein purification (X)	Increased enzyme activity (X)
1. Crude extract	19.00	1	1
2. 20,000 g homogenate	16.88	1.1	13.1
3. Fractionation with ammonium sulphate (40-95%)	40% - 95% 3.51	- 5.4	- 16.6
4. DEAE-cellulose chromatography	0.73	26	5.5
5. Gel filtration on Sephadex G-100	0.037	514	26.9

IV.3.F.(iii).b 20,000 g homogenate - The supernatant was centrifuged at 20,000 g for 15 min and the mitochondrial-lysosomal pellet discarded. The total and specific activities of the enzyme were assessed (Table XXVI(A)).

IV.3.F.(iii).c Ammonium sulphate fractionation (40-95%) - The macromolecular fractions were precipitated from the supernatant and pelleted by spinning at 20,000 g for 15 min, following 30 min stirring at 40 and 95% saturation with ammonium sulphate, consecutively (adjusted pH 7.1). The 95% saturation fraction was redissolved in the extracting buffer. This was desalted by passage through a pre-equilibrated Sephadex G-25 (fine) column (void volume 55 cc and void volume plus inner volume 110 cc), such that the sample volume was less than 30% of the total column volume. Fractions of 5 ml were collected and the enzyme activity determined for each. The active fractions 3 - 11 (55 - 95 ml of total volume) were pooled and the total and specific activities determined (Table XXVI(A)).

IV.3.F.(iii).d DEAE-cellulose ion exchange column chromatography - The 11 mg of protein-N was charged on a DEAE-cellulose (1.7 x 15 cm) column, equilibrated with the extracting buffer, and washed with two column volumes of extracting buffer to remove loosely adsorbed material. The column was eluted by a linear gradient of 0 - 0.4M NaCl in 600 ml of starting buffer, and 10 ml fractions collected. The enzyme activity and 280 nm absorption was determined for each fraction, as well as the linearity of the gradient (Fig. 22(a)). The active fractions 24 - 37

Table XXVI(A). Purification of leucyl naphthylamidase.

Procedure	Total volume ml	Total activity LNAU	Specific activity LNAU/mg protein-N	Yield %
1. Crude extract	60	1.44×10^6	1.54×10^4	100
2. 20,000 g homogenate	50	1.32×10^6	1.83×10^4	91.4
3. Ammonium sulphate fractionation (40-95%)	40% - 95% 44	- 4.40×10^5	- 4.00×10^4	- 30.6
4. DEAE-cellulose chromatography	6.1	9.63×10^4	5.73×10^4	6.7
5. Gel filtration by Sephadex G-150	1.7	8.5×10^3	4.0×10^4	0.6

Table XXVI(B). Leucyl naphthylamidase and protein purification of A. avenae extract.

Procedure	Protein-N mg	Increased protein purification (X)	Increased enzyme activity (X)
1. Crude homogenate	93.6	1	1
2. 20,000 g homogenate	72	1.3	1.2
3. Ammonium sulphate fractionation (40-95%)	40% - 95% 11	- 8.5	- 2.6
4. DEAE-cellulose chromatography	1.68	55.7	3.7
5. Gel filtration by Sephadex G-150	0.21	446	2.6

(percent transmission < 80%) were pooled and concentrated by diafiltration to 6.1 ml. The total and specific activities were determined for this fraction (Table XXVI(A)).

IV.3.F.(iii).e Sephadex G-150 gel filtration column chromatography -

The 1.68 mg protein-N sample was loaded and eluted through a column of Sephadex G-150 (column volume 319 cc, void volume 115 cc, and void volume plus inner volume 305 cc), pre-equilibrated with the starting buffer (Fig. 22(b)). The 5 ml fractions were collected and the active fractions 25 - 34 (percent transmission < 85%) were concentrated to 1.7 ml by diafiltration. The total and specific activities were determined (Table XXVI(A)).

The above purification procedure was repeated with the results shown in Table XXVII(A), (B).

IV.3.F.(iii).f PAGE of 'purified' enzyme - The 1.3 ml of 'purified' material was concentrated to 105 μ l by the use of a Minicon B15 (Amicon, U.S.A.), mixed with 50 μ l aliquots of 10% sucrose in the running buffer containing 6.7×10^{-4} M EDTA and the samples of 10, 20 and 40 μ g of protein-N electrophoresed for a distance of 8 cm. The control consisted of a nematode homogenate prepared according to IV.2.B.(ii). This showed that the 'purified' material contained 9 detectable protein bands, as compared to the 17 of the control. The E_f values of the protein bands were as follows: 0.03, 0.13 (main), 0.23, 0.34 (main), 0.42, 0.48 (main), 0.52, 0.54 and 0.62.

Figure 22. (a) Chromatography of the 40-95% $(\text{NH}_4)_2\text{SO}_4$ -precipitable fraction on DEAE-cellulose. The procedure was as in IV.3.F.(iii).d. The parameters measured were: x---x, NaCl concentration; \blacktriangle — \blacktriangle , O.D. at 280 nm; \bullet — \bullet , leucyl naphthylamidase activity on leucyl- β -naphthylamide.

(b) The gel filtration of leucyl naphthylamidase activity through Sephadex G-150. The procedure was as in IV.3.F.(iii).e. The parameters measured were: \blacktriangle — \blacktriangle , O.D. at 280 nm; \bullet — \bullet , leucyl naphthylamidase activity on leucyl- β -naphthylamide.

FIG. 22

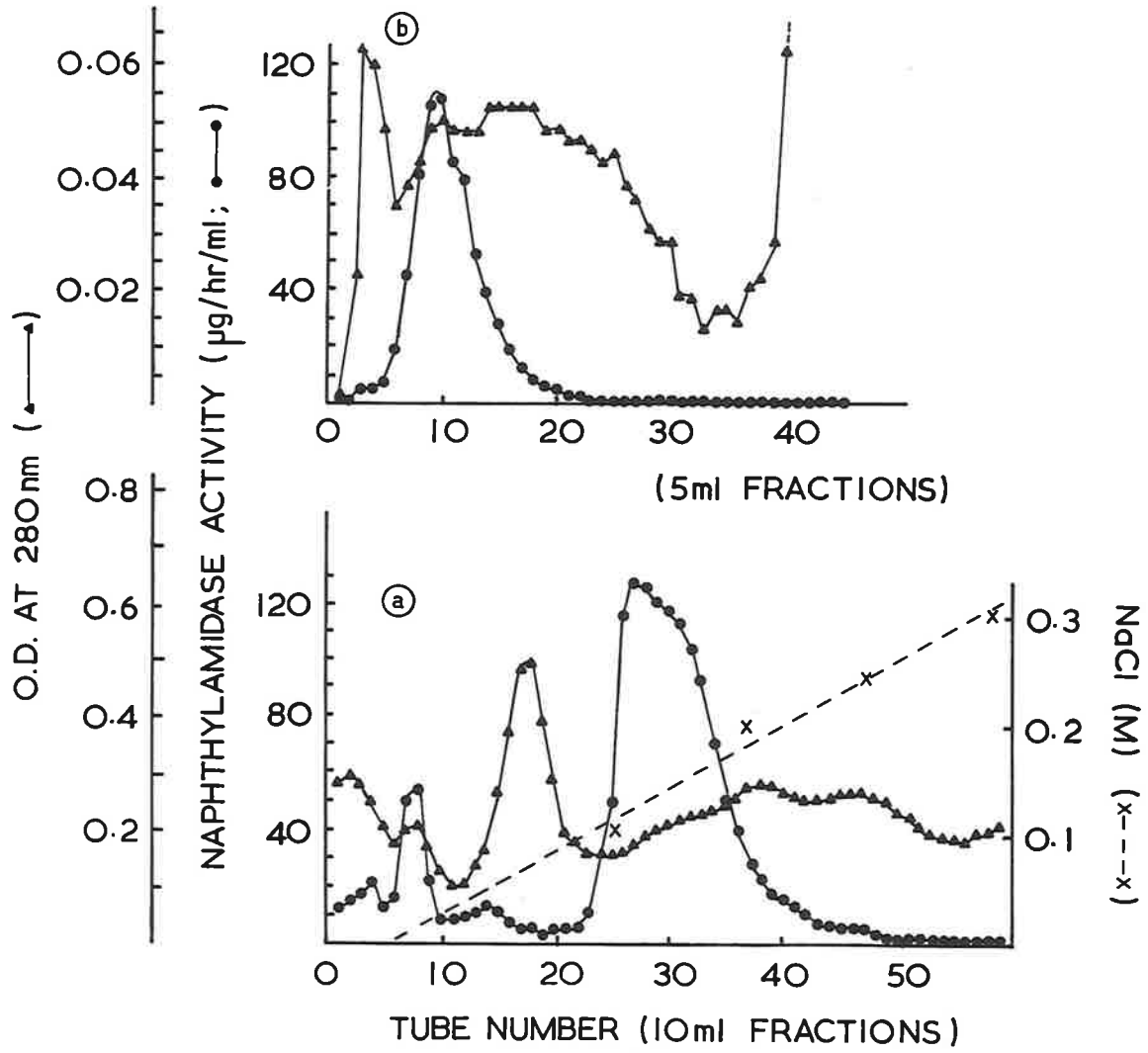


Table XXVII(A). Purification of leucyl naphthylamidase.

Procedure	Total volume ml	Total activity LNA _m U	Specific activity LNA _m U/mg protein-N	Yield %
1. Crude extract	65	2.05×10^6	1.5×10^4	100
2. 20,000 g homogenate				
" supernatant	40	1.36×10^6	3.08×10^4	66.2
" pellet (discard)	35	8.33×10^4	1.45×10^3	4.1
3. Ammonium sulphate fractionation				
40% (discard)	27	3.71×10^4	1.37×10^3	1.8
95%	42	3.78×10^5	3.67×10^4	18.5
4. DEAE-cellulose chromatography	2.84	1.70×10^4	1.96×10^4	0.8
5. Gel filtration by Sephadex G-150		No activity remaining		

Table XXVII(B). Leucyl naphthylamidase and protein purification of A. avenae extract.

Procedure	Protein-N mg	Increased protein purification (X)	Increased enzyme activity (X)
1. Crude extract	136.5	1	1
2. 20,000 g homogenate			
" supernatant	44.0	3.1	2.1
" pellet	57.4	2.4	0.1
3. Ammonium sulphate fractionation			
40%	27.0	5.1	0.1
95%	10.29	13.3	2.45
4. DEAE-cellulose chromatography	0.866	157.6	1.3
5. Gel filtration by Sephadex G-150		No activity remaining	

IV.3.G M.W. estimation of leucyl naphthylamidase

The method used was that of Andrews (1964, 1965). The same Sephadex G-150 column was used as for the attempted purification of leucyl naphthylamidase. It was equilibrated with 0.05M Tris-HCl buffer, pH 7.5, containing 0.1M KCl and calibrated by measurement of the elution volume of various globular protein standards (Horse myoglobin, 16,000d; Bovine serum albumin 70,000d; Yeast alcohol dehydrogenase 150,000d; and, Beef Catalase 250,000d - Sigma, U.S.A.). The protein standards were used at 2 mg loading, except for catalase which required 4 mg and the 'purified' enzyme at 0.021 mg protein-N (Section IV.3.F.(ii).g). Fractions of 3 ml were collected and the protein fractions detected by absorption at 230 nm. The M.W. was estimated by a semi-log plot of elution volume through the column against known values for the globular protein standards. The estimated M.W. of the leucyl naphthylamidase of A. avenae was 130,000 - 140,000d (M.W. \pm 10% - Andrews, 1965). Although all the protein standards used were globular, the horse myoglobin did not behave as though it had a M.W. of 16,000d, but a considerably smaller figure.

IV.4 DISCUSSION

The zymograms of selected enzymes and protein patterns, resembled those obtained by Dickson et al. (1970, 1971) for A. avenae. The single band of leucyl naphthylamidase activity, indicated the presence of a single molecular species, which makes it easier for the interpretation of modifier effects on enzyme activity. α -ecdysone

(1.8×10^{-5} M) appeared to have no quantitative or qualitative effects on any enzyme or protein band, except the increased activity observed, i.e. staining for the leucyl naphthylamidase and the protein band of Ef 0.55. Thus this enzyme was probably being influenced in its activity by the addition of moulting hormone to the nematode. It was therefore necessary to quantify this effect by chemical assay, as well as by the use of simple modifiers to learn something of its properties, as a means to its purification.

Increased enzyme activity was evident 18 hr postincubation with the steroid hormone (induction ratio 1.057), reaching a peak at apparently 24 hr (induction ratio 1.709) and declining more slowly by 30 hr (induction ratio 1.313). It is not known at present if this increase was due to the increased synthesis of the enzyme, decreased degradation of the enzyme or release and activation of a membrane-bound precursor.

There is both direct and indirect evidence of endocrine control of (leucyl) naphthylamidase levels in mammals. Thus, elevated levels occur in the serum during pregnancy (Goldbarg and Rutenberg, 1958; Jones et al., 1972a, b). The hypothalamus of the rat when subjected to treatment with ethinyloestradiol and testosterone showed sex-specific elevation of enzyme level (Kuhl, 1974a, b), whilst (leucyl) naphthylamidases of in vitro grown human cells were altered by physiological concentrations of hydrocortisone, testosterone and oestradiol (Beckman and Lundgren, 1969; Lundgren, 1972). This phenomenon of leucyl naphthylamidase in the nematode A. avenae may resemble the known induction/stimulation of a number of enzymes in insects by the moulting

hormone: α -glutamyl transpeptidase (Bodnaryk et al., 1974); a dipeptidase (Bodnaryk, 1971); a chitinase (Kimura, 1973); dopa-decarboxylase (Sekeris and Lang, 1964; Sekeris and Karlson, 1966); and, ornithine decarboxylase (Wyatt et al., 1973).

The optimum pH for enzyme activity was 7.2, although this was shown to be a composite of pH optimal activity at 5.5 and of optimal stability between pH 6.25 and 8.25.

The nematode enzyme under study had similar general properties to known mammalian naphthylamidases, but not to the aminopeptidases as represented by leucine aminopeptidase (Patterson et al., 1963; Ellis and Perry, 1964, 1966; Smith et al., 1965; Marks et al., 1968; Lundgren, 1971(a); Vanha-Perttula, 1973(a)). However, caution is required in interpretation because of the overlap of properties, within the naphthylamidases e.g. the wide substrate spectrum of most members (Vanha-Perttula, 1973a), as well as with the (leucine) aminopeptidases. Besides, as the physiological substrates for the two groups of enzymes are unknown, the β -naphthylamides (in particular, LNA) are not specific substrates but are hydrolysed by a number of other enzymes, i.e. peptidases and proteinases (Patterson et al., 1963; Idahl and Taljedal, 1968). This does not include the cathepsins B and B₁ from beef spleen as was formerly thought (McDonald et al., 1970). The following properties of the nematode enzyme were found to be common to the (leucyl) naphthylamidases of mammals: a thiol-requiring enzyme, specifically for activation and not stabilisation; inhibition by the sulphydryl reagent *p*-hydroxymercuribenzoate; inhibition by puromycin; a neutral

pH optima; instability following $(\text{NH}_4)_2\text{SO}_4$ and DEAE-cellulose chromatography, as shown by the enzymes of Ascaris suum (Rhodes et al., 1966); and, the enzyme appears to be more labile than leucine aminopeptidase.

Some of the enzymes require a divalent cation for activity (Smith et al., 1965; Ellis and Perry, 1966; Marks et al., 1968; Lundgren, 1971a), whilst others are inhibited by their presence (Patterson et al., 1963). Co^{2+} , Zn^{2+} and Mn^{2+} appear to be the main activators. The enzyme of A. avenae more closely resembled the enzyme II of Vanha-Perttula (1973a), where divalent cations caused moderate inhibition, whilst heavy metals were strongly inhibitory. The exception was the slight stimulation of 15% by the Zn^{2+} ions (1×10^{-3} M). This indicated the possibility that the enzyme might be a Zn-dependent metalloenzyme (Himmelhoch, 1969). This was investigated using the chelating agent O-phenanthroline which forms complexes with group IIB metals, but has little or no affinity for the alkali earth metals. Zn^{2+} at different concentrations was added to the 20,000 g nematode homogenate in the presence of 5×10^{-4} M O-phenanthroline, a concentration that causes 12% inhibition when alone. At a divalent ion concentration of 5×10^{-5} M, the enzyme activity was 50% above that of the control, but at higher amounts there was strong inhibition. The enhanced enzymatic activity at a critical Zn^{2+} concentration in the presence of a preferential chelating agent of group IIB metals suggests this enzyme may require Zn^{2+} for full activity.

The nematode enzyme differed from the mammalian (leucyl) naphthylamidases, by its behaviour towards the chelating agents EDTA, O-phenanthroline and α, α' -dipyridyl. EDTA at a concentration of 1×10^{-3} M enhanced the activity to a maximum of 129% above that of the control. It was also essential for stability, as shown by the reduction to 11% of the loss of activity on standing for 16 hr at 4° , at a concentration of 6.7×10^{-4} M. The loss of activity following dialysis for the same time interval, for the total and specific activities was 39% and 30%, respectively. The increased stability was probably due to the chelation of heavy metal ions and divalent cations.

O-phenanthroline at high concentrations was inhibitory to the activity of the enzyme, perhaps by the mechanism of molecular dissociation as for glutamic dehydrogenase (Yielding and Tomkins, 1962). At lower concentrations it was stimulatory, with a maximum of 94% above the activity of the control (5×10^{-5} M). This response being similar to the enzyme II of rat testis (Vanha-Perttula, 1973a). At lower concentrations it is presumed to act as a typical chelator of heavy metal ions with consequent increased enzyme activity.

The stimulation of activity by the thiol reagents cysteine, β -mercaptoethanol and glutathione, as well as the sulphur containing amino-acid L-methionine, showed that the state of oxidation was important for activity and not stability, because the loss of stability after overnight dialysis in the presence of extracting buffer and

cysteine, was equal to that which occurred in the extracting buffer alone. At present, it is not settled whether this activation is due to the direct reduction of -S-S- bonds to -SH or to the removal of metal inhibitors (West, Todd, Mason and Van Bruggen, 1966). The inhibition of activity in the joint presence of cysteine and Zn^{2+} , indicated an interaction between the amino-acid and divalent cation. This is assumed to be due to mercaptide formation at the -SH grouping, as well as at the $-\text{NH}_2$ sidechain. This results in the loss of efficacy of the two components. A possible conclusion from this is that the direct reduction of the -S-S- enzyme bonds are more important for activity than the mercaptide formation. Because the sulphydryl reagent iodoacetate caused no inhibition, whilst it was complete in the presence of *p*-hydroxymercuribenzoate, it was assumed that the -SH group(s) was masked in the active site (Smith et al., 1965).

As with the uterine (leucyl) naphthylamidases of *A. suum* (Rhodes et al., 1969b) and enzymes III and IV of the rat testis (Vanha-Perttula, 1973), the nematode enzyme was present in the soluble fraction. But the recovery of the nematode enzyme was only 65% of the total activity, due to the instability of this enzyme in its present form and this may have influenced the result. This was contrary to the (leucyl) naphthylamidases of mouse ascites cells (Patterson et al., 1963), rat kidney (Seligman et al., 1970), human liver (Smith et al., 1965) and bovine anterior pituitary gland (Ellis and Perry, 1966), where it was particle bound. Lundgren (1971b) had shown that the isoenzymes of HeLa cells were found in the lysosomal, microsomal and soluble fractions.

The lysosomes of the mouse endocrine pancreas (Idahl and Taljedal, 1968), and of the intestine of A. suum (Rhodes et al., 1966, 1969a) also contained this enzyme as a component. The reason for the non-detection of latency in the 800 g supernatant of A. avenae, could have been due to the inhibition of the particulate enzyme by the non-ionic detergent Triton X-100, as was the case for the isoenzyme of HeLa cells (Lundgren, 1971a). It could also possibly have been due to different properties of the soluble and particulate forms, that caused inactivation of the latter under the experimental procedures used. Thus, the soluble form of leucine aminopeptidase from the pig kidney (Himmelhoch, 1970), has different properties from the particulate form (Pfleiderer, 1970), in terms of their substrate specificity and heavy metal activation.

The properties of the classical leucine aminopeptidase from the pig kidney (Smith and Spackman, 1955; Spackman, Smith and Brown, 1955; Hill et al., 1958; Smith and Hill, 1960), as well as from H. contortus (Rogers, 1965, 1970) and Aspergillus oryzae (Nakadai et al., 1973b, c) differ markedly. Thiol groups are absent because of non-inhibition by sulphhydryl reagents. The pH optimum is 9.1 - 9.3, with Mg^{2+} or Mn^{2+} ion being required for activation and stability. The specific substrate is leucinamide, but hydrolysis of LNA occurs, albeit at a greatly reduced rate, i.e. 10,000 - 20,000x slower (Patterson et al., 1963).

The leucyl naphthylamidase of A. avenae was not activated by Cl^- or Mg^{2+} , as shown by the reduction of activity against that of the control.

The hydrolytic rate for the substrate ANA was only 20% that of the LNA hydrolysed control.

In nematodes the function of enzymes has usually been deduced from localisation studies using histochemical techniques, with assumptions being derived from their location in physiologically specific tissues. Due to the studies of Rogers (1965, 1970) on the correlation of leucine aminopeptidase activity and nematode exsheathment, much work has been focused on the function of this enzyme.

The detection of leucyl naphthylamidases in the excretory canals of adult A. lumbricoides (Lee, 1962) and adult Nippostrongylus brasiliensis (Lee, 1970), have been presumed to play a role in the excretion of metabolised polypeptides and peptides. They have been found in the intestine of developing larvae of Oesophagostomum radiatum (Douvres and Thompson, 1973), adult A. suum (Rhodes et al., 1966, 1969a), larval A. suum (Jenkins and Erasmus, 1971) and are believed to be associated with the digestive metabolism of polypeptides and peptides. Their presence in the subventral excretory gland(s) of adult N. brasiliensis (Lee, 1970) and larvae of Anisakis sp. (Ruitenbergh and Loendersloot, 1971) has suggested a function in the processes of extra-corporeal digestion. Whereas their localisation in the excretory gland(s) of the adult Stephanurus dentatus (Romanowski et al., 1973), fourth-stage larvae of Phocanema decipiens (Davey and Kan, 1967, 1968) and the epidermal cords of larval Xiphinema index (Roggen et al., 1967), has suggested a role in ecdysis for the removal of the old cuticle. This conclusion follows from the work on leucine aminopeptidase and

exsheathment in infective larvae of H. contortus by Rogers (1963, 1964, 1965, 1970). In respect of prescribing a function in moulting for the epidermally located enzyme, few of these histochemical studies are appropriate due to the use of the wrong stage (i.e. the adult), the absence of developing larvae, or the concentration on a particular organ, i.e. the intestine. However, Douvres and Thompson (1973) were unable to detect any cyclic fluctuation of enzyme activity, that could have been correlated with ecdysis.

The detection of the leucyl naphthylamidase of A. avenae in the body-wall suggests its involvement either in the synthesis and secretion of the new cuticle, the maintenance of the growing cuticle (Douvres and Thompson, 1973), or more simply in the tissue metabolism of polypeptides and peptides. Because of the soluble form of the enzyme, it should not be overlooked that this result may be an artefact of localisation, due to the possibility of diffusion (Vanha-Perttula, 1973). The difficulty of determining whether the enzyme was present in the intestine and/or the reproductive system was due to the anatomy of nematodes, i.e. a hollow cylinder of the body wall that surrounded an almost solid cylinder, the intestine. The results of previous studies on a variety of nematode species, indicate that the enzyme is present in the intestine, presumably functioning in digestive metabolism.

Because of the lack of knowledge of a possible metabolic function for the nematode enzyme, it would be instructive to look at what is known for the mammalian forms, although, as yet virtually nothing is known of the natural substrates or physiological functions of this group of exopeptidases (Delange and Smith, 1971).

The naphthylamidases form a distinct class of exopeptidases, which are involved in the hydrolysis of oligopeptides (Lundgren, 1971a). Thus far, they have been shown to function either in a sequence of enzymes for protein degradation, in the regulatory mechanisms of the nervous system or in the regulatory mechanisms of the vascular system.

The proposed function for the nematode enzymes localised in the excretory and digestive systems, as well as the presumed extra-corporeal function of the excretory glands, would come under the category of polypeptide metabolism. This has been suggested for the leucyl naphthylamidases of the rat brain (Marks et al., 1968), the mouse endocrine pancreas (Idahl and Taljedal, 1968) and the dipeptidyl naphthylamidases of the rat testis (Vanha-Perttula, 1973b). The aminopolypeptidase of bovine anterior pituitary tissue also had lysyl (leucyl) naphthylamidase activity, i.e. they were the same enzyme (Ellis and Perry, 1966). Thus, the total hydrolysis of polyalanine (Ala₄, Ala₅) and poly-lysine (Lys₄) was accomplished by the consecutive action of the aminopolypeptidase, an aminotripeptidase and a dipeptidase in the 2.4M (NH₄)₂SO₄ fraction of the tissue homogenate.

There is indirect evidence of the involvement of leucyl naphthylamidase in the regulatory mechanisms of the nervous system. It is suggested that they are involved in the metabolism of biologically active peptides, i.e. polypeptide hormones. Present knowledge of the control of gonadotrophic release by the anterior pituitary gland by the hypothalamus, is that it is mediated by the releasing- and inhibitory releasing-hormones (oligopeptides). Evidence by Kuhl et al. (1974a, b)

supports the idea that naphthylamidases are involved in the formation of physiologically active compounds from inactive precursors, i.e. long-chain peptides or is involved in their degradation. Similarly, Marks et al. (1968) suggest their involvement, either in the release/activation of neurosecretory material, the modification of cerebral proteins to physiologically active proteins or for the inactivation of such compounds.

More direct evidence is available for the involvement of (leucyl) naphthylamidases in the regulatory mechanisms of the vascular system. A (leucyl) naphthylamidase of human liver and plasma (Borges et al., 1974; Guimarães et al., 1973) and horse plasma (Prado et al., 1971) is able to convert kinins that are released from kininogens (serum α_2 -globulin) by kallikreins, to the active polypeptide bradykinin. This is performed by the stepwise and sequential removal of amino acids to the active nonapeptide as shown for kallidin, methionyllysylbradykinin and a tetradecapeptide containing bradykinin. Bradykinin is involved in the inflammation reaction following tissue injury, and it is believed that the inactive enzyme is activated on intracellular release, by the high chloride ion content of the extracellular fluid (Hopsu-Harvu et al., 1966).

The evidence at present available, indicates that the hypodermis is responsible for the synthesis and secretion of the new cuticle in moulting nematodes (Roggen et al., 1967; Kan and Davey, 1968; Bird, 1971), as well as for the maintenance and continued growth of the formed cuticle between moults and particularly following the last moult

(Bird, 1971). It is concluded that the enzyme of A. avenae is involved in a sequence of enzymes for protein degradation, possibly in connection with cuticle physiology. Leucyl naphthylamidase was not detected in the nervous tissue of O. radiatum (Douvres and Thompson, 1973), whilst nematodes do not possess a known form of circulatory system.

The purification of leucyl naphthylamidase was not successful due to the instability of the enzyme under the procedures used. Thus, despite the addition of 6.7×10^{-4} M EDTA to the buffer 0.01M Tris-HCl, pH 7.3, and a consequent rise of the total and specific activities by 28% and 33%, respectively, the enzyme was rapidly inactivated by further purification. The stability of the enzyme activity was increased by this chelating agent, as was shown by a reduction of the activity loss following dialysis from 77% to 33%. The only component untested in this connection was the buffer, perhaps, it caused some measure of enzyme inactivation.

The use of a 20,000 g centrifugation of the crude homogenate removed the effect of an unknown inhibitor(s), as the increased specific activity was approximately 4x greater than the corresponding protein purification. The enzyme became more labile with the extraction and attempted purification from further batches of nematodes. This may have been due to the fact that these nematodes had been lyophilised for storage, and rehydrated 12 hr prior to homogenisation. No activation by the Co^{2+} ion at 6.7×10^{-4} M was evident, as occurred with the various

(leucyl) naphthylamidases of A. suum (Rhodes et al., 1966, 1969a, b). The loss of activity following $(\text{NH}_4)_2\text{SO}_4$ fractionation and dialysis was about 70%, which was the result of an equal proportion of inhibition and inactivation by the above two respective procedures. There is evidence which suggests that the (leucyl) naphthylamidases are labile to $(\text{NH}_4)_2\text{SO}_4$ fractionation with consequent activity loss (Patterson et al., 1963; Rhodes et al., 1966) or that the yield of enzyme is increased if this step is omitted (Marks et al., 1968). It is now apparent that these results are most likely due to indirect inhibition of the enzyme molecule by $(\text{NH}_4)_2\text{SO}_4$. Thus, it is interesting to note that $(\text{NH}_4)_2\text{SO}_4$ is highly inhibitory to the lysyl (leucyl) naphthylamidase of bovine anterior pituitary tissue, i.e. a 78% inhibition at a concentration of 1.6×10^{-1} M (Ellis and Perry, 1966).

This loss of activity also occurred during the desalting process with Sephadex G-25, indicating that some cofactor(s) essential to enzyme stability was lost and had a M.W. considerably less than 5,000d (Craig and King, 1955). This was also accompanied by the loss of enzyme activity due to inhibition by $(\text{NH}_4)_2\text{SO}_4$. This was continued during DEAE-cellulose chromatography again due to the instability of the enzyme. Therefore, once this factor(s) was removed the enzyme was extremely unstable and readily inactivated. The mild reducing agents that were added, were necessary for enzyme activation and not stability.

IV.5 CONCLUSION

The function of the leucyl naphthylamidase of A. avenae is unknown. Its purification is necessary before substrate-specific studies of naphthylamides and more appropriate proteins, polypeptides and peptides can occur.

Thus, the possibility of it being a metalloenzyme has not been resolved. Useful data would be obtained from the addition of various divalent cations following dialysis of the homogenate, as well as the testing of enzyme stability in different buffers and the avoidance of $(\text{NH}_4)_2\text{SO}_4$ in all subsequent purification procedures. These problems need to be resolved with crude preparations, prior to any successful enzyme preparation.

This hydrolytic enzyme with enhanced activity after incubation with moulting hormone (α -ecdysone), presents a problem in possible function(s). A feasible deduction would be that it is involved in some process, where α -ecdysone causes an increase in a physiological and/or biochemical event. Of the indirect evidence available, the suggestion is of a possible role in the phenomenon of moulting-ecdysis. N. dubius incubated with low concentrations of α -ecdysone showed enhanced moulting (Dennis, 1975). This is supported by the moulting activity of extracts from H. contortus (Rogers, 1973) and T. spiralis (Hitcho, 1970; Hitcho and Thorson, 1971) on the nematodes and insects tested. Hitcho and Thorson believe that the active component is β -ecdysone. This steroid-enhanced enzyme is the same as that of the control, i.e. the same E_f value with PAGE, but it may be derived from

a different tissue, e.g. the excretory gland.

An interesting point once the leucyl naphthylamidase has been purified, is whether refractile ring formation would occur with isolated sheaths of H. contortus. If it is positive, then it may indicate a function in cuticle physiology, not necessarily only degradation but also synthesis. The secretion of enzymes from the subventral excretory gland(s) is not unique to the process of exsheathment in infective H. contortus larvae. This has been postulated on structural grounds in the adult Enoplus brevis (Narang, 1970), and adult Stephanurus dentatus (Romanowski et al., 1971, 1973). It has also been shown by the secretion of various proteins from the excretory gland(s), e.g. acetylcholinesterase by a number of fourth-stage larvae and adults of nematodes parasitising the intestinal tracts of certain mammals (Ogilvie et al., 1973), Trichostrongylus colubriformis (Rothwell et al., 1973; Rothwell and Merritt, 1974) and O. radiatum (Bremner et al., 1973). Fourth-stage larvae and adults of S. dentatus secrete trypsin and chymotrypsin inhibitors (Rhoads and Romanowski, 1974).

A common theme of naphthylamidases is their involvement with polypeptides, in the processes of degradation, synthesis and activation. It has been postulated that some of these are 'active' in the physiological sense. There are numerous references in the literature to the presence and function of active polypeptides, e.g. the diuretic hormone of Rhodnius prolixus (Aston and White, 1974), which is neuro-secretory in origin with a M.W. of < 2,000d.

As an adjunct to this, the problem of exsheathment and the

mechanism of action, may be interpreted in terms of a functional polypeptide required in refractile ring formation. Thus, the release of leucine aminopeptidase could be conceived as an intermediate step in the process of formation of an active polypeptide. The tentative conclusions of Ozerol and Silverman (1969, 1970) and Slocombe and Whitlock (1971^b) was that the exsheathing activity resulted from a non-enzymatic substance(s), possibly a polypeptide of little or no tertiary structure. The former authors showed that polypeptides were the main constituent of the metabolites in the exsheathment fluid, which with the contained proteins amounted to 80% of the released material. Such a hypothesis is readily testable by the use of ultrafiltration with membranes of known M.W. retention, as performed by Masiar and Masiar (1974) for the fractionation of isotropically active polypeptides of dog plasma (M.W.'s 1,000 - 10,000d). Is the activity present in a low M.W. fraction? Is the activity present after pronase treatment? Perhaps, a study of the type of amino-acids, peptides and polypeptides released from the sheaths following exsheathing fluid activity, if any, might assist in determining if activity is due to an enzymatic or non-enzymatic substance(s).

CHAPTER V

INVESTIGATIONS ON THE PRESENCE OF ECDYSONE-BINDING PROTEINS
AND ECDYSONE-LIKE MATERIAL IN THE NEMATODES,
PANAGRELLUS REDIVIVUS AND APHELENCHUS AVENAE

V.1 INTRODUCTION

Insect moulting hormone(s) may be detected and quantified by methods of bioassay with a sensitivity of 5×10^{-9} g for α -ecdysone (Kaplanis et al., 1966) or by gas-liquid chromatography (GLC) sensitive to 5×10^{-8} g for α -ecdysone with a flame-ionisation detector (Katz and Lensky, 1970) and to 2×10^{-11} g for β -ecdysone with an electron-capture detector (Borst and O'Connor, 1974). However, both techniques suffer from the necessity of having to purify the extracts prior to examination. This problem is obviated to some extent by the technique of radioimmunoassay (RIA), where crude samples of material may be used (Borst and O'Connor, 1972, 1974), and a sensitivity of 2×10^{-10} g for β -ecdysone-equivalent material may be achieved. The virtually equal cross-reactions of the arthropodan moulting hormones (α -ecdysone, β -ecdysone and inokosterone) for the assay antibody, makes this technique, without prior purification, a method for the measurement of the total ecdysone analogues present. Recently, the sensitivity of the RIA-antibody for β -ecdysone has been increased to 8×10^{-11} g (Lauer et al., 1974(b)), as has the specificity as shown by the reduced cross-reaction with α -ecdysone. This method has been corroborated as a valid procedure, by comparison with the techniques of thin-layer chromatography

(TLC), GLC and high-resolution liquid chromatography (HRLC) in the determination of the titre of β -ecdysone during the metamorphosis of Drosophila melanogaster (Borst et al., 1974) and the secretion of α -ecdysone by the prothoracic glands of Manduca sexta larvae in vitro (King et al., 1974).

The problem of the transport of ecdysones in the haemolymph and tissues of insects has been examined only recently, and as such is still in a state of flux. The data presented by Emmerich (1972), Gorell et al. (1972) and Butterworth and Berendes (1974) support the concept of specific steroid hormone (or metabolite) binding proteins, both extra- and intra-cellular in location. The general properties of these macromolecular fractions (proteins) resembled the specific steroid hormone receptors in the cytosol and nucleus, as detected in the relevant target tissues of various mammals. However, the conclusion of Chino et al. (1970) and Gilbert and Chino (1974) was that at least in the haemolymph, it was transported as the free moiety and not protein-bound.

These two highly sensitive techniques (RIA and detection of receptors) would be useful in both the direct and indirect detection of moulting-hormone(s) in the Nematoda, because of the difficulty of extracting and purifying the minute amounts, if any, of ecdysone-positive material. Both methods were therefore used in an attempt to detect ecdysone-like compounds in the tissues of specific nematodes.

V.2 MATERIALS AND METHODS

Ecdysterone [^3H] (1.0 Ci/mmol) was purchased from New England Nuclear (U.S.A.); testosterone acetate, bovine serum albumin and 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide hydrochloride from the Sigma Chemical Co. (U.S.A.); carboxymethoxylamine hemihydrochloride (α -aminooxyacetic acid hemihydrochloride) from Pfaltz and Bauer (U.S.A.); and N-hydroxysuccinimide from Fluka AG (Switzerland). β -ecdysone was a generous gift from Dr. D.H.S. Horn of C.S.I.R.O. (Australia).

V.2.A Mass culture of *P. redivivus*

This was performed as in IIIA.2.B.

V.2.B Mass culture of *A. avenae*

This was performed as in IIIA.2.A.

V.2.C Methods for the detection of β -ecdysone cytosol-receptors

All procedures were performed at 4 $^{\circ}$, unless otherwise stated.

V.2.C.(i) Sucrose density-gradient centrifugation - The method used was essentially that of Høisaeter (1973). *P. redivivus* at the rate of 0.5 g wet weight/ml of extracting buffer (0.05M Tris-HCl, pH 7.4, 0.0015M EDTA and 0.002M β -MSH) was homogenised with 20 mg of sterile 500-mesh carborundum for a total of 45 sec. Then, spun for 10 min at 4,000 rpm, before loading the remaining supernatant into 0.6 ml cellulose nitrate tubes for centrifugation at 105,000 g_{av} in an SW50 rotor for 60 min. Aliquots (0.5 ml) were incubated for 2 hr with mild shaking in the presence of ^3H - β -ecdysone ($1.2 \times 10^{-8}\text{M}$) and made 10% V/V with respect to

glycerol. This was followed by loading onto linear sucrose density gradients (5-15% or 5-20% in the buffer 0.01M Tris-HCl, pH 7.4, containing 0.0015M EDTA and 0.002M β -MSH) containing glycerol at 10% V/V and centrifuged at 200,000 g_{av} for 17-22 hr in an SW41 rotor. The protein standards that were run concurrently for estimation of the binding protein sedimentation coefficient were ovalbumin (albumin, egg) of 3.6S, alcohol dehydrogenase (Yeast) of 7.6S, and catalase (bovine liver) of 11.4S. The fractionation and U/V absorbing profile (254_{nm}) of the resultant gradients was performed by an ISCO-fractionator. The 0.492 ml fractions were collected directly into scintillation vials, to each of which was added 0.5 ml of distilled water and 10 ml of a Triton X-100/toluene based scintillant (Siegel, 1971), and the vortexed vials counted at 30% efficiency.

V.2.C.(ii) Polyacrylamide gel electrophoresis (PAGE) - This was performed according to the method of Ritzen et al. (1971), whilst the PAGE procedure was as that of Davis (1964). The nematode, P. redivivus, was treated as in the above method up to and including the step of incubation with the moulting hormone, except that the concentration of β -ecdysone used was $1.6 \times 10^{-8}M$. The resultant 0.5 ml aliquots were mixed with 15 μ l of 10% sucrose-bromophenol blue tracking-dye in extracting buffer and loaded onto 9% gels in perspex tubes of 20 cm x 0.6 cm (i.d.). The gels contained glycerol and β -MSH at final concentrations of 10% V/V and 2 mM, respectively, in order to stabilize

the binding protein-steroid complex. The bands were concentrated at 12.5 mA for 30 min and separated at 25 mA, until the buffer front had moved approximately 8 cm. Protein staining was performed according to the method of Reid and Bielecki (1968). The remaining gels were frozen and stored at -20° . Gel fractionation (1.43 mm) was done on a multiple razor-rack, when the gel had thawed slightly to prevent fracturing and the resultant fractions placed in individual scintillation vials. To each was added 3 ml of a solution containing PPO-POPOP scintillation fluid prepared by mixing 220 ml of PPO-POPOP scintillation fluid, 30 ml NCS solubilizer and 25 ml of 8M NH_4OH . The fractions were sealed and digested overnight at room temperature before counting.

V.2.C.(iii) Charcoal/Dextran binding assay - The method used was essentially that of Watanabe et al. (1973). The extracting and charcoal/dextran suspending buffer was the same as that used in Section V.2.C.(i). Homogenisation was carried out as above with the same regimens of centrifugation, to yield 4000 g and 105,000 g supernatant fractions. Aliquots (0.5 ml) were incubated with $2.8 \times 10^{-9}\text{M}$ β -ecdysone for 2 hr, following an initial 10 sec vortex. The charcoal/dextran solution (0.5 ml of Norit A/Dextran T40 or T70) was added for the required period of time, following a 10 sec vortex. This was followed by a spin at 2,000 rpm for 10 min and the removal of a 0.5 ml aliquot. This was placed in a scintillation vial and mixed with 0.5 ml of distilled water and 10 ml of the Triton X-100/toluene based

scintillant, before counting in a Packard Tri-Carb liquid scintillation spectrometer.

V.2.D Protein estimation

All nematode extracts were measured for their protein content, according to the method used in Section IV.2.E.

V.2.E Preparation of β -ecdysone 6-(O-carboxymethyl) oxime (β -ecdysone-6-CMO)

The steroid hapten was prepared by two different methods, that of Fritz et al. (1959) and of Erlanger et al. (1959).

V.2.E.(i) The method of Fritz - A solution of 22 mg (47 μ mol of β -ecdysone containing 16 μ l (100 μ mol) of 2-dimethylaminoethanol in 1.5 ml of an isopropanol-methanol mixture (1 ml : 0.5 ml), was mixed with a 3 ml isopropanol-methanol solution (2.6 ml : 0.9 ml) that contained 23.5 mg (100 μ mol) of α -aminooxyacetic acid hemihydrochloride. This was heated by reflux for 120 min, using a water bath at 70⁰. The resultant product was evaporated to dryness under reduced pressure at 49⁰. This material was taken up in 4 ml of distilled water, the pH adjusted to 8.0 (1M NaOH) and the unreacted steroid extracted three times with an equal volume of water-saturated n-butanol. The phases were separated by a spin at 1,000 rpm for 10 min. The aqueous phase was acidified by 10 drops of 10% HCl to bring the pH to 2.0 and the crude β -ecdysone-6-CMO product extracted three times with an equal volume of water-saturated n-butanol. These were pooled following centrifugation and backwashed

with 2 ml of n-butanol saturated water before the final phase-separation. This was evaporated to dryness under N_2 at 49° , redissolved in ethanol and stored at 5° until required.

The isopropanol used was re-purified in order to remove water and the ketone group content, i.e. acetone, which would combine with α -amino-oxyacetic acid and interfere with the quantitative oximation of the steroid hormone. Similarly, dimethylaminoethanol was also redistilled and stored at 4° , as well as being degassed prior to use, to remove interfering CO_2 .

V.2.E.(ii) The method of Erlanger - Because of the small amount of starting material and the consequent need for a high yield, a second method for hapten formation was tried. A solution of 12.8 mg (26 μ mol) of β -ecdysone in 1 ml of ethanol to which was added 0.3 ml of 2M NaOH, was mixed with 2.75 ml of ethanol containing 12.6 mg (58 μ mol) of α -aminooxyacetic acid hemihydrochloride. This was refluxed for 3 hr at $70^\circ C$ over a boiling water bath. The resultant solution was reduced in volume to approximately 1 ml by evaporation at reduced pressure at 47° , diluted with 4 ml of distilled water and the pH adjusted to 8.0 by 1M NaOH. The unreacted steroid and crude product were extracted, concentrated re-dissolved and stored as in the preceding section.

V.2.F The preparation of testosterone 3-(O-carboxymethyl) oxime
(testosterone-3-CMO)

For the comparative exercise of the determination of the purity, yield and characteristics of the product using a large starting sample, it was decided to synthesise the hapten testosterone-3-CMO under virtually

the same experimental conditions, as for β -ecdysone.

V.2.F.(i) The method of Fritz - A solution of 500 mg (1.5 mmol) of testosterone acetate, 0.66 ml (5 mmol) of 2-dimethylaminoethanol and 1.1 gm (5 mmol) of α -aminooxyacetic acid hemihydrochloride in 45 ml of isopropanol (including a little methanol to solubilize the latter compound), was refluxed for 45 min at 70°. The unreacted steroid was extracted by washing with ethyl acetate. Following acidification, the testosterone-3-CMO product was extracted by two washes of ethyl acetate and this pooled solution was backwashed with distilled water, dried over Na_2SO_4 and evaporated to dryness. The crude product was taken up in ethanol and stored at 5°.

V.2.F.(ii) The method of Erlanger (Erlanger et al., 1957, 1967) - A solution of 572 mg (1.7 mmol) of testosterone acetate and 900 mg (4.2 mmol) of α -aminooxyacetic acid hemihydrochloride in 100 ml of methanol was made alkaline by the addition of 10 ml of 5% NaOH (solution in 50% water : 50% methanol) and treated as in the previous relevant section. The unreacted steroid was removed by extraction with ether. The precipitate formed by acidification of the alkaline aqueous phase was extracted by two ether washes. The pooled ether washes were extracted with distilled water, dried over Na_2SO_4 and then evaporated to dryness. The crude product was taken up in ethanol and stored at 5°.

V.2.G Preparation of β -ecdysone-6-CMO-bovine serum albumin
(BSA) conjugate (antigen)

The method was derived from Lindner et al. (1972). The active ester was formed by dissolving 7 mg (56 μ mol) of N-hydroxysuccinimide in 2 ml of water containing a little dioxane and adding this to a solution of 16.2 mg (28 μ mol) of β -ecdysone-6-CMO in 4 ml of dioxane (pH 5.5). To this was added 11 mg (56 μ mol) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI) in 5 ml of water. The resultant mixture was stirred for 30 min at room temperature. On the premise that 20 hapten residues would be bound per mole of BSA, i.e. the M.W. of the moiety containing one lysine group was 3,500d, 600 mg of protein was dissolved in 0.05M sodium phosphate buffer, pH 7.5. To this was added a few drops of pyridine and the solution warmed to 47⁰, before cooling to room temperature. The two solutions were mixed and stirred, and left for 20 hr at room temperature (pH 7.5). The 27 ml of resultant solution was dialysed against distilled water for 48 hr at room temperature. The remaining insoluble material was removed by centrifugation at 12,000g for 15 min, and the supernatant lyophilised for 48 hr, before storage at -15⁰ in a desiccator.

The hapten testosterone-3-CMO (263 mg) was similarly conjugated to BSA; however, the attempted estimation of the amount of hapten unbound failed because of the residue of pyridine present (Erlanger et al., 1957). This contaminant was not removed by either evaporation under reduced pressure or lyophilisation.

V.2.H Antibody production

The methods of Chase (1967) were used. Two rabbits (16 weeks old) were injected subcutaneously with 3.5 mg of antigen (in equal volume of Freund's complete adjuvant and 0.85% saline). This was repeated 14 days later, except that in this and succeeding subcutaneous injections, the emulsion was formed with Freund's incomplete adjuvant. After a further 14 days, 4 intravenous injections into the ear vein were given at 4 day intervals, with 4 mg of the antigen dissolved in 0.5 ml of 0.85% saline. A booster was given subcutaneously (4 mg of antigen) 6 weeks after the last injection as suggested by Francki and Habili (1972).

The control and immunised rabbits were bled 10 days after the last intravenous and booster injections, with 30-40 ml of blood collected each time. The blood in vaseline-lined beakers was incubated for 1 hr at 37⁰, followed by standing at 4⁰ overnight for clot contraction. The antiserum/serum was collected, spun at 2,000 rpm for 20 min and the supernatant stored at -20⁰ in 3 ml aliquots.

V.2.I Radioimmunoassay

This was performed according to the method of Borst and O'Connor (1972). The required volume of antiserum was made up to 0.5 ml with 0.1M borate buffer, pH 8.4, in glass centrifuge tubes that contained approximately 4,000 cpm of ³H- β -ecdysone, previously added in methanol and dried down under N₂ at 50⁰ to remove the solvent. When the

concentration of antiserum was below 10%, then it was made up to this figure by addition of the required amount of control antiserum. Following incubation for 3 hr at 4⁰ or 1 hr at 37⁰, an equal volume of a 100%-saturated solution of (NH₄)₂SO₄ was added, the mixture stood for 15 min and the precipitate pelleted at 2,000 rpm for 10 min. The precipitate was washed with two volumes of a 50%-saturated solution of (NH₄)₂SO₄ and centrifuged as above. The pellet was redissolved in 1 ml of distilled water, added to a scintillation vial, vortexed with 10 ml of Triton X-100/toluene based scintillant and counted for ³H at 30% efficiency.

V.2.J Extraction of nematode ecdysone-like material

The procedure used was that for the preliminary isolation of an ecdysone-like fraction from the secretion of the prothoracic gland of an insect (King et al., 1974).

The two nematodes used for extraction were A. avenae and P. redivivus. Surface-sterilised nematodes suspended in 0.9% saline were homogenised for a total of 4 min at 4⁰ in the presence of 20 mg of 500-mesh carborundum and spun at 3,000 rpm for 10 min. The supernatant was diluted 2-3 times with 0.9% saline and made 65% with respect to methanol, followed by vortexing and spinning for 10 min at 2,000 rpm. The pellet was re-extracted three times with 65% methanol and the resultant solutions pooled and stored at 4⁰. The extracts were taken to dryness under reduced pressure at 50⁰, and then taken up in distilled water.

This was extracted four times with equal volumes of water-saturated n-butanol, separating the phases by spinning at 1,000 rpm for 15 min. The combined extracts were taken to dryness, re-extracted with four washes of 25% methanol in benzene and again evaporated to dryness. The product was redissolved in 25% methanol in benzene, centrifuged at 2,000 rpm for 10 min and the supernatant stored at 4°.

V.2.K Quenching

The possibility existed that the ^3H -counts of β -ecdysone would be increasingly quenched with the different amounts of nematode homogenate used. Both external and internal standardisation by the 'channels-ratio method' showed that increased amounts of a 4,000 g-supernatant from 0.05 - 0.5 ml (38-380 μg protein-N) did not cause quenching with the scintillant Triton X-100/toluene.

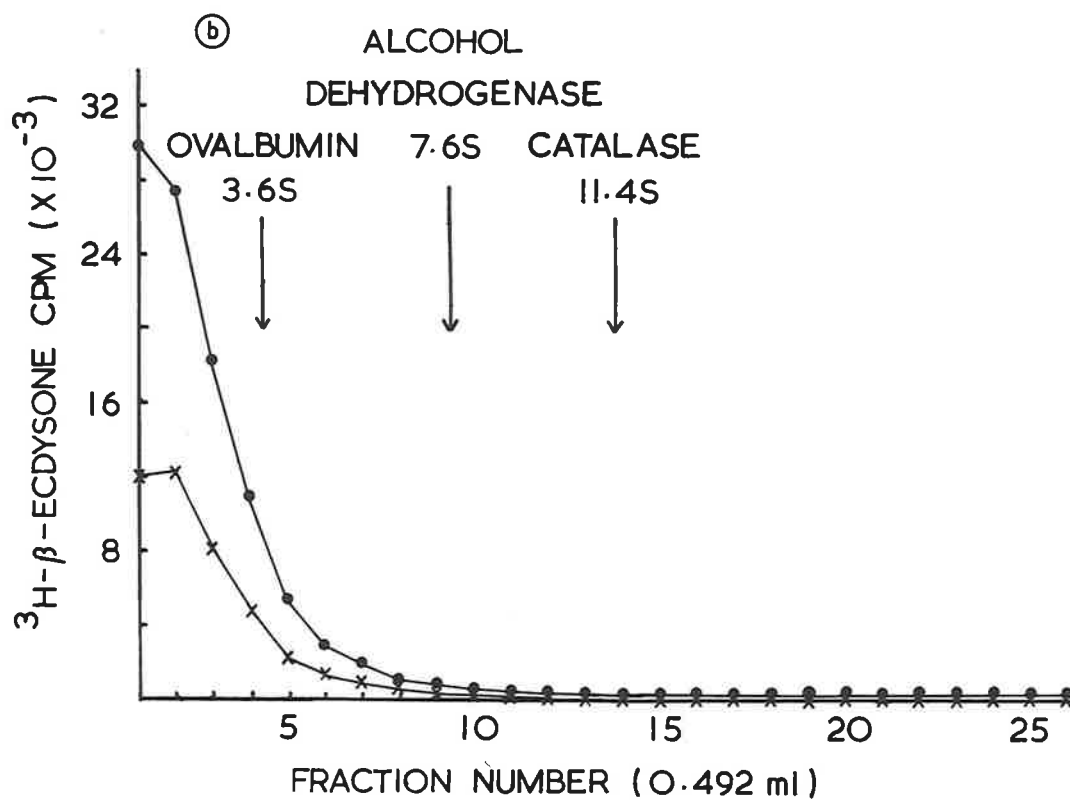
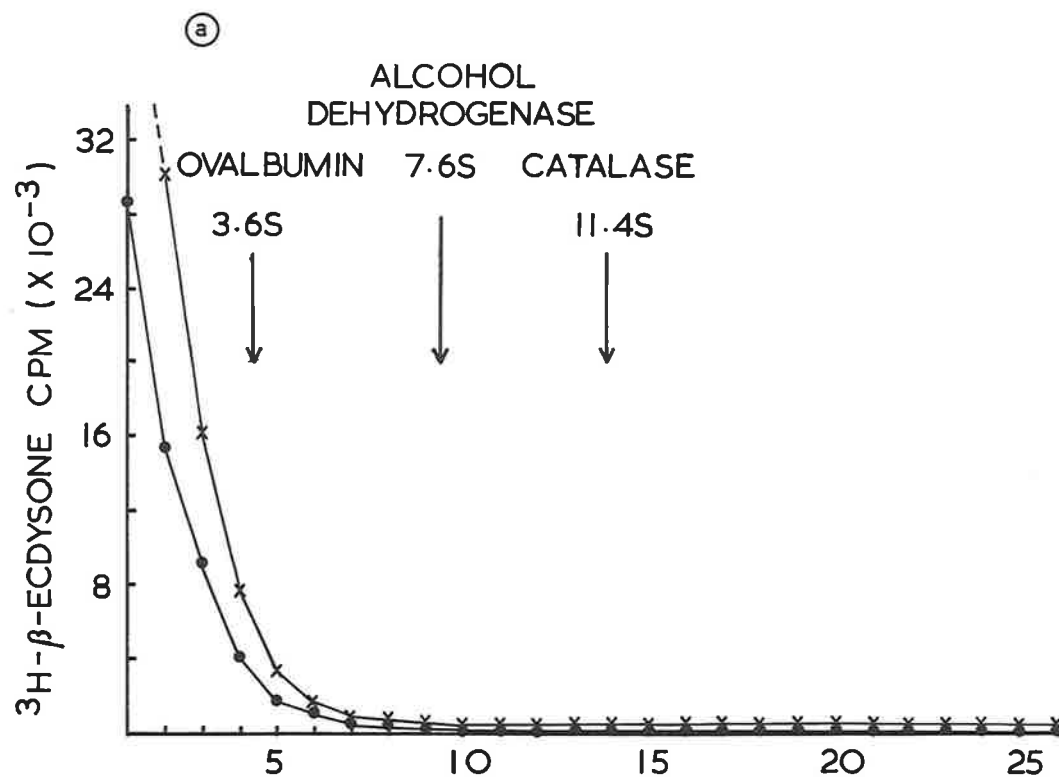
V.3 RESULTS

V.3.A Detection of β -ecdysone cytosol binding proteins

V.3.A.(i) Sucrose density-gradient centrifugation - At no time did the β -ecdysone radioactivity show any association with a macromolecular fraction in the expected 8S range, as for mammalian steroid cytosol-receptors (Fig. 23(a)). Also, no association of the radioactivity peak with a macromolecular fraction was apparent, when the larval forms were enriched from a total of 50% to 87% by a scaled-up version of the glass-bead chromatographic technique of Chow and Pasternak (1969) (Fig. 23(b)).

Figure 23. The incubation of the cytosol fraction (105,000 g-supernatant) with [$^3\text{H}(\text{G})$] β -ecdysone and analysis by sucrose density-gradient centrifugation. The distribution of the radioactivity of the steroid hormone through the gradient was determined for the control (x; incubated in presence of the extracting buffer 0.05M Tris-HCl, pH 7.4, 0.0015M EDTA and 0.002M β -MSH) and the MH-treated supernatant of the nematode (●). The procedure was as described in V.2.C.(i). The protein standards were used as markers for S-value determination, (a) Larvae forming 50% of the total population of the nematode; (b) Larvae enriched to 87% of the total population of the nematode.

FIG. 23



No component of the buffer system appeared to be causing degradation or aggregation of the cytosol proteins. This was examined by the following combinations of the extracting buffer: 0.01M Tris-HCl, pH 7.4, containing 0.0015M EDTA and 0.003M β -MSH (Kato et al., 1974); 0.05M Tris-HCl, pH 7.4, containing 0.0015M EDTA and 0.002M β -MSH (Høisaeter, 1973); 0.01M Tris-HCl, pH 7.5 (Emmerich, 1972); 0.05M Tris-HCl, pH 7.4, containing 0.002M β -MSH; and, 0.01M Tris-HCl, pH 7.4, containing 0.003M MgCl_2 and 0.001M β -MSH. Similarly, 500-mesh carborundum and β -MSH showed no apparent deleterious effects on the cytosol proteins. Both low and high ionic strength buffers were compared, due to the possibility of the former being responsible for aggregation, but they showed no difference in their respective density-gradient profiles of U/V absorbance and of radioactivity. Glycerol was added at the rate of 10% V/V to all incubations and density gradients, as it was shown (Høisaeter, 1973) to stabilize the specific receptor of dihydrotestosterone. Aliquots of 0.5 g wet weight of nematodes/ml of extracting buffer were chosen for homogenisation, as they yielded a greater quantity of measurable protein than either 0.8 or 1.0 g samples (0.5 g wet weight = 690 μg protein-N/ml; 0.8 g wet weight = 560 μg protein-N/ml; and 1.0 g wet weight = 550 μg protein-N/ml).

These results suggested that no β -ecdysone cytosol-receptor was present in the tissues of P. redivivus.

V.3.A.(ii) PAGE - The more sensitive qualitative method of PAGE was used for the attempted detection of ecdysone binding proteins in nematode

tissues. The radioactivity of ^3H - β -ecdysone showed no association with any macromolecular band (i.e. protein), but coincided exactly with moulting hormone electrophoresed in the absence of the 105,000 g-supernatant of the nematode (Fig. 24(a)). The absence of a correlation between a protein band and β -ecdysone suggested that hormone-specific binding proteins in P. redivivus were absent.

V.3.A.(iii) Charcoal-Dextran binding assay - The assay was optimised, as far as possible, prior to the determination of binding of β -ecdysone by the macromolecular fraction, in vitro. The optimum time of free steroid uptake was 20 min, using the 1% Norit A charcoal/0.1% Dextran T40 suspension (Table XXVIII). Non-specific binding of β -ecdysone by BSA (500 μg /0.5 ml buffer) did not occur, as shown by the amount of free steroid hormone that remained unbound in its presence or absence (Table XXVIII).

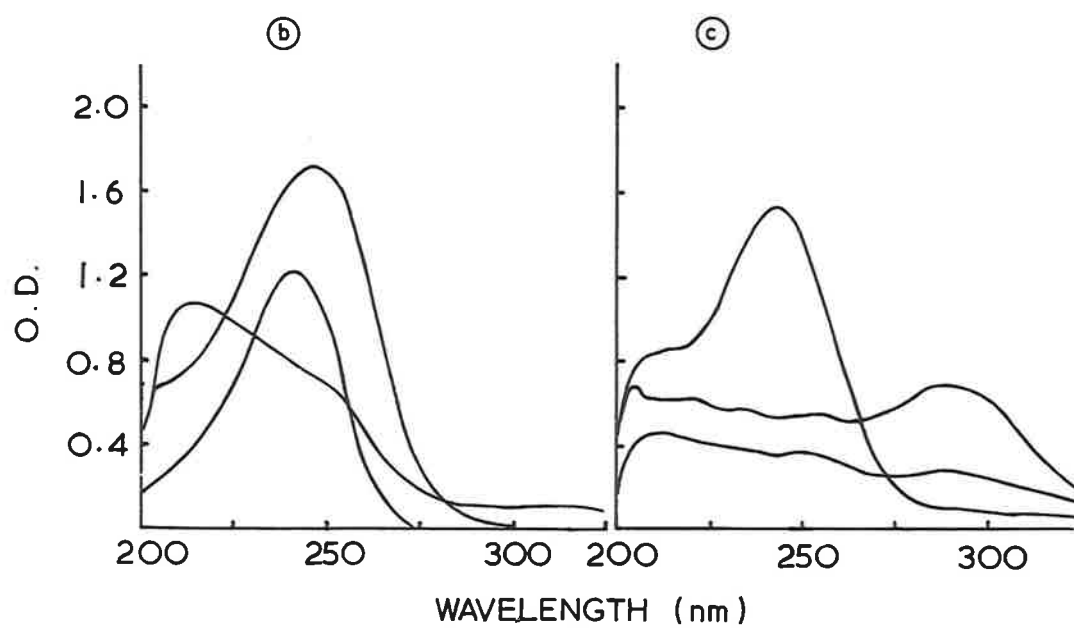
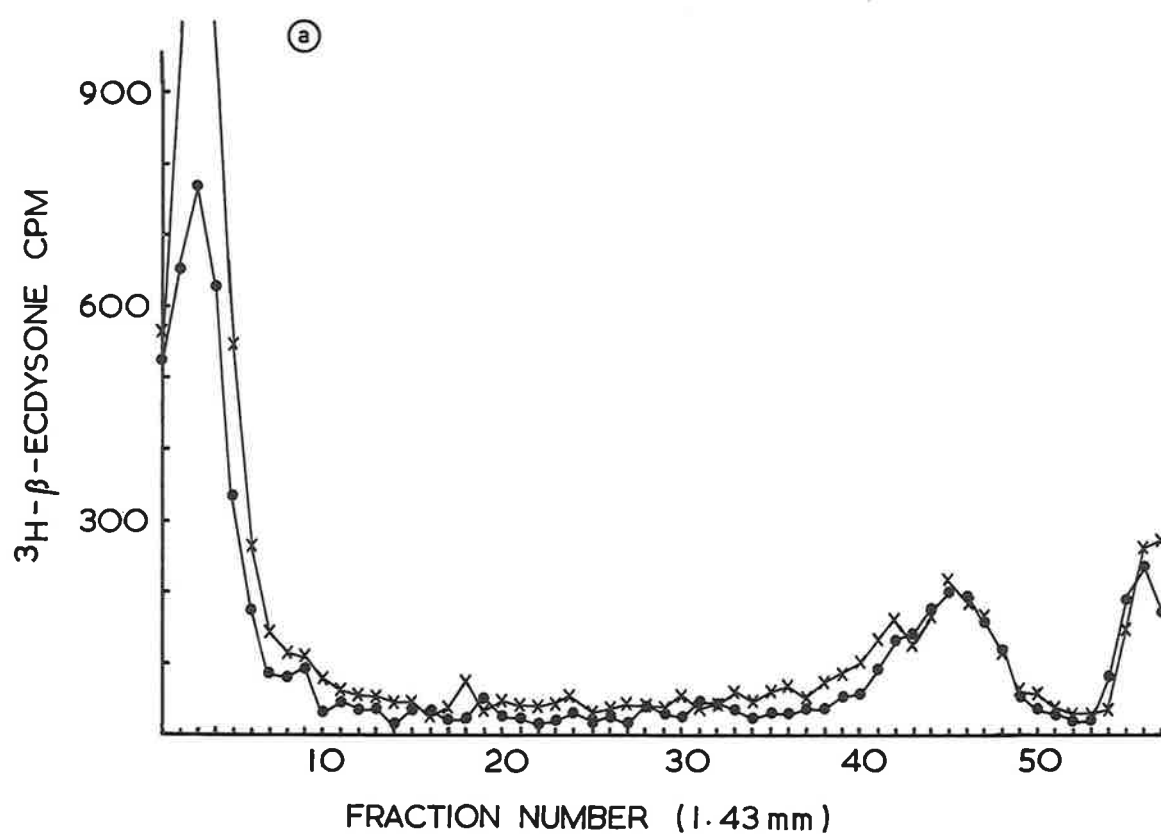
Table XXVIII. The binding efficiency of free ^3H - β -ecdysone by different charcoal/dextran suspensions, in the absence and presence of BSA^a.

Charcoal/Dextran Suspension	Optimum time of uptake (min)	% ^3H -counts remaining
0.5% Norit A/0.05% Dextran T40	60	11.0
1.0% Norit A/0.1% Dextran T40	20	5.2
1.0% Norit A/0.1% Dextran T40 + BSA (500 μg /0.5 ml buffer)	20	5.4
1.0% Norit A/0.1% Dextran T70	30	6.6

a β -ecdysone ($2.8 \times 10^{-9}\text{M}$) was preincubated for 1 hr at 4° in 0.5 ml of extracting buffer (0.05M Tris-HCl, pH 7.4, 0.0015M EDTA and 0.002M β -MSH) then incubated for the times stated above. The procedure was as in V.2.C.(iii).

- Figure 24. (a) The incubation of the cytosol fraction (105,000 g-supernatant) with [$^3\text{H}(\text{G})$] β -ecdysone and analysis by PAGE. The distribution of radioactivity of the steroid hormone through the polyacrylamide gel was determined for the control (x; incubated in the presence of the extracting buffer, 0.05M Tris-HCl, pH 7.4, 0.0015M EDTA and 0.002M β -MSH) and the MH-treated supernatant of the nematode (*). The procedure was as in V.2.C.(ii).
- (b) Ultraviolet spectra of the hapten testosterone-3-CMO in ethanol: testosterone acetate (λ_{max} at 240 nm); testosterone-3-CMO (Fritz product, λ_{max} at 248-249 nm); testosterone-3-CMO (Erlanger product, λ_{max} at 212 nm).
- (c) Ultraviolet spectra of the hapten β -ecdysone-6-CMO in ethanol: β -ecdysone (λ_{max} at 243 nm); β -ecdysone-6-CMO (Fritz product, λ_{max} at 288-290 nm); β -ecdysone-6-CMO (Erlanger product, λ_{max} at 209 nm).

FIG. 24



The charcoal-dextran suspension did not bind BSA (1 mg/ml) to its interstices, as was shown by the constancy of the protein concentration both before and after treatment. Thus, for the control it was 477 ± 14.53 S.E.M. μg protein-N, whilst for 1% Norit A/0.1% Dextran T40 and 0.5% Norit A/0.05% Dextran T40 it was 507 ± 8.82 S.E.M. μg protein-N and 490 ± 10.0 S.E.M. μg protein-N, respectively. The slight discrepancy with the latter two treatments was probably due to the presence of some light-scattering material in the solution. Similarly, no binding of cytosol proteins of the 4,000 g-supernatant of P. redivivus was detected by the charcoal/dextran suspension. In controls of two different populations the protein content of homogenates was 930 and 800 μg protein-N/ml, whilst following treatment it was 980 and 810 μg protein-N/ml, respectively. The charcoal/dextran suspension that was used throughout this study of cytosol macromolecular fraction binding, was 1% Norit A/0.1% Dextran T40.

The assay showed that considerable binding of ^3H - β -ecdysone by the 4,000 g- and 105,000 g-supernatant fractions of P. redivivus was occurring (Table XXIX). This was greater for the former fraction, but when determined on the basis of the specific binding per mg protein-N, it appeared the latter was more active. This preliminary result indicated the possibility of a macromolecular fraction in the cytoplasm, which binds β -ecdysone, i.e. cytosol steroid hormone binding proteins.

Table XXIX. The amount of ^3H - β -ecdysone bound by supernatant fractions of P. redivivus.

Supernatant	% cpm bound	Molarity bound /0.5 ml	Molarity bound/mg protein - N
A _{4,000} g	60.9	1.6×10^{-9}	2.6×10^{-9}
A _{4,000} g	57.5	1.5×10^{-9}	2.4×10^{-9}
A _{105,000} g	50.2	1.3×10^{-9}	3.3×10^{-9}
B _{4,000} g	49.3	1.3×10^{-9}	2.8×10^{-9}
B _{105,000} g	49.2	1.3×10^{-9}	3.7×10^{-9}

The binding of ^3H - β -ecdysone by some soluble protein components of the 4,000 g- and 105,000 g-supernatant, was up to 60% of the steroid hormone added and at a maximum rate of $3.7 \times 10^{-9}\text{M/mg protein-N}$.

One characteristic of mammalian steroid hormone-receptor complexes, is that the hormone is non-covalently bound, i.e. it is removable by treatment with organic solvents. This was investigated with respect to the binding of β -ecdysone to the cytosol macromolecular fraction. This was accomplished by precipitation of the normal 105,000 g-supernatant/ β -ecdysone combination by an equal volume of 10% TCA, placing this on a Gelman type A glass-fibre filter and washing consecutively three times with one ml of 5% TCA; three times with one ml of ether : ethanol (1 : 1); and, two times with one ml of ether. This resulted in the complete removal of all radioactivity from the TCA-precipitate (scintillant fluid: 10 g PPO; 0.25 g dimethyl POPOP per 2.5 l of toluene). There are two possible interpretations of this

result. Either the steroid hormone is non-covalently bound to a specific binding protein, or it is the result of non-specific binding to a protein(s) in the cytosol, e.g. lipoprotein(s). The same result of the complete removal of ^3H - β -ecdysone bound to the cytosol fraction was accomplished, following the addition of 1.5 ml of ethanol to the normal 105,000 g-supernatant/ β -ecdysone combination and a washing procedure on a Gelman filter of three times with one ml of ethanol : water (1 : 1); three times with one ml of ethanol : ether (1 : 1); and, three times with one ml of ether.

The answer to the problem of whether there was specific-binding of β -ecdysone by the cytosol macromolecular fraction, was obtained by determining the distribution of the steroid hormone between the cytosol fraction and supernatant, following precipitation of the cytosol proteins by TCA (Table XXX). The amount of moulting hormone lost by washing the precipitate with 5% TCA closely approximated the amount bound to the cytosol macromolecular fraction (Table XXIX). Due allowance must be made for the different homogenate protein concentrations that were used. In conclusion, it is believed that β -ecdysone was non-specifically bound to a macromolecular component(s) of the cytosol fraction (protein), and the binding was broken following washing with TCA. This was substantiated by the parallel results that were obtained with BSA (1 mg/ml).

V.3.B Characterisation of the hapten testosterone-3-CMO

The products derived from the two methods were compared to gain

more information to optimise the conditions for the formation of the hapten with the polyhydroxy steroid, β -ecdysone.

Table XXX. The amount of ^3H - β -ecdysone bound to supernatant fractions of P. redivivus following washing with 5% TCA.

Treatment	Amount ^3H - β -ecdysone added (cpm)	cpm in supernatant (+ S.E.M.)	cpm in TCA-precipitate (+ S.E.M.)	Molarity of ecdysone removed from TCA-precipitate
TCA-fractionation of 105,000 g-supernatant	10,148	4,222+59.2	12+1.8	1.6×10^{-9}
TCA-fractionation of 105,000 g-supernatant	10,148	3,820+4.2	10+2.3	1.7×10^{-9}
TCA-fractionation of BSA at 1 mg/ml	10,148	5,343+78.6	1.5+0.6	1.3×10^{-9}

V.3.B.(i) Yield - This was 43.6% for the method of Fritz and 5.1% for that of Erlanger.

V.3.B.(ii) U/V spectrum - The Fritz product showed a λ max at 248-249 nm (ethanol) and a shoulder-peak at 208 nm. There was a spectral shift of the molecule from 240 nm (ethanol) for testosterone acetate to the above λ max for testosterone-3-CMO (Fig. 24(b)). The λ max in 0.05M Tris-HCl buffer, pH 8.5, was 252 nm, with a shoulder-peak at 211 nm.

The Erlanger product showed a λ max at 212 nm (ethanol) and a shoulder-peak at approximately 250 nm (Fig. 24(b)). The λ max in 0.05M Tris-HCl buffer, pH 8.5, had shifted to 250 nm, with a peak at 318 nm and a shoulder peak at 211 nm.

Acetone-CMO was also prepared by the method of Fritz and the U/V spectrum used for comparison. It was used as a reference compound because it lacked the U/V absorption characteristics of the steroid moiety. Acetone-CMO (ethanol) showed two definite peaks at 214 and 246 nm, as well as two indefinite shoulder-peaks at 278 and 292 nm. It was assumed that the peak at 214 nm corresponded to the CMO moiety because α -aminoxyacetic acid showed a single absorption peak at 211 nm.

V.3.B.(iii) Molar extinction coefficient (ϵ) - Crystals of the Fritz product and floc particles of the Erlanger product were dried under N_2 in a tared vessel, weighed and a volume of 0.05M Tris-HCl buffer, pH 7.5, added until dissolved. The ϵ -value for the Fritz hapten was estimated spectrophotometrically (15,762). The ϵ -value for the Erlanger hapten was similarly estimated (7,479).

V.3.B.(iv) M.P. of testosterone-3-CMO - Crystals were obtained by dissolving the dried hapten in a minimal quantity of ethyl acetate, followed by the addition of a few drops of methanol. This was continued until small crystals instantaneously formed, after which it was placed at 5°. The Erlanger product did not form crystals under these conditions, but gave the appearance of a heavy floc. The crystals of the Fritz product showed a M.P. of 181-183°, with no apparent decomposition.

Thus, the method of Fritz yielded a product, which was of greater purity and homogeneity than the method of Erlanger. Therefore, the preparation of the hapten β -ecdysone-6-CMO was performed according to this method.

V.3.C The hapten β -ecdysone-6-CMO

Because of the highly polar nature of the β -ecdysone molecule, both ethyl acetate and ether, the normal steroid hormone solvents, were unsuitable for extracting procedures. It was necessary to find a solvent which, although immiscible with water, would extract the steroid hormone and oxime product quantitatively from aqueous solution. This was determined for a number of solvents by the estimation of the partition (Distribution Coefficient K_D) between the solvent and water. The decreasing order of extraction efficiency for β -ecdysone coincided with the decreasing polarity of the solvents tested, i.e. n-butanol > ethyl acetate > chloroform > ether > benzene > toluene (Table XXXI).

Table XXXI. The efficiency of the partition of ^3H - β -ecdysone from aqueous solution by various organic solvents^a.

Partition phases	Organic solvent (cpm/ml)	Water (cpm/ml)	Distribution coefficient K_D	% ^b extracted
Toluene - Water	90	2,380	0.038	3.6
Benzene - Water	90	2,173	0.041	4.0
Ether - Water	114	2,532	0.045	4.3
Chloroform - Water	134	1,648	0.082	7.5
Ethyl acetate - Water	227	2,281	0.099	9.1
n-butanol - Water	1,172	1,226	0.956	48.9

a ^3H - β -ecdysone (4,400 cpm) was partitioned for 30 min between 1 ml each of the equilibrated solvents tested, followed by the counting of 0.5 ml aliquots.

b The counting efficiencies were adjusted because of the different scintillants used for the organic and aqueous solvents.

Therefore, n-butanol was used in all extractions as the water-saturated solvent, because it gave approximately 50% recovery of the steroid from aqueous solution.

The yield of β -ecdysone-6-CMO by the Fritz method was determined by difference, following the estimation of the unreacted steroid. Of two consecutive attempts at quantitative oximation, the yields were 40.3 and 48.7%, with incubation in a water bath at 70° for 45 and 120 min, respectively. The U/V spectrum (ethanol) showed a λ max at 288-290 nm, a peak at 212 nm and a minor peak at 248-250 nm (Fig. 24(c)).

The yield of β -ecdysone-6-CMO by the method of Erlanger was 53.2% (by difference). The U/V spectrum (ethanol) showed a λ max at 209 nm, with peaks at 245 and 288 nm (Fig. 24(c)).

V.3.D Radioimmunoassay (RIA)

The binding activity of the rabbit antisera for the steroid hormone was extremely low, i.e. 4.7% bound with 10% antiserum (Table XXXII). Attempts to increase the binding capacity by lengthening the time of incubation (Table XXXII) or a 2-3-fold increase in the concentration of the immunoglobulin fraction by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Spendlove, 1967) (Table XXXII), were unsuccessful. Increasing the concentration of antiserum by 5-fold did not produce a proportional increase in the binding activity, i.e. 7.7% bound with 50% antiserum (Table XXXII).

The antiserum used for the first RIA was that of the male rabbit, concentrated 4.9-fold by dialysis of the above immunoglobulin fraction against PEG 6000 (polyethylene glycol), i.e. 8.4% of ^3H - β -

Table XXXII. The binding activity for ^3H - β -ecdysone of various titres of rabbit antisera.

Treatment	Concentration of antisera	cpm bound	% counts bound
1) Untreated	10% (male)	222	4.7
	5% (male)	198	4.1
	2% (male)	107	2.2
	1% (male)	115	2.4
	10% (female)	191	4.0
	5% (female)	139	2.9
	2% (female)	79	1.7
	1% (female)	42	0.9
2) Increased % of anti- sera	50% (male)	369	7.7
	50% (female)	284	6.0
3) Increased time of incubation (16 hr, 4 $^{\circ}$)	25% (male)	201	4.2
	10% (male)	143	3.0
	25% (female)	128	2.7
	10% (female)	96	2.0
4) 2-3-fold increase in immunoglobulin fraction by $(\text{NH}_4)_2\text{SO}_4$ precipi- tation	20% (male)	269	5.6
	10% (male)	180	3.8
	20% (female)	199	4.2
	10% (female)	128	2.7

ecdysone bound by the 20% antiserum. The calibration curve was a semi-log plot of increasing amounts of unlabelled β -ecdysone added to the incubation solution, against the corresponding reduction in the labelled hormone bound to the specific antibody, with appropriate controls (Fig. 25(a)). Detectable quantities of ecdysone-like material were found in the extracts of A. avenae and P. redivivus, the concentration being greater in the former nematode (Table XXXIII; 1st assay only).

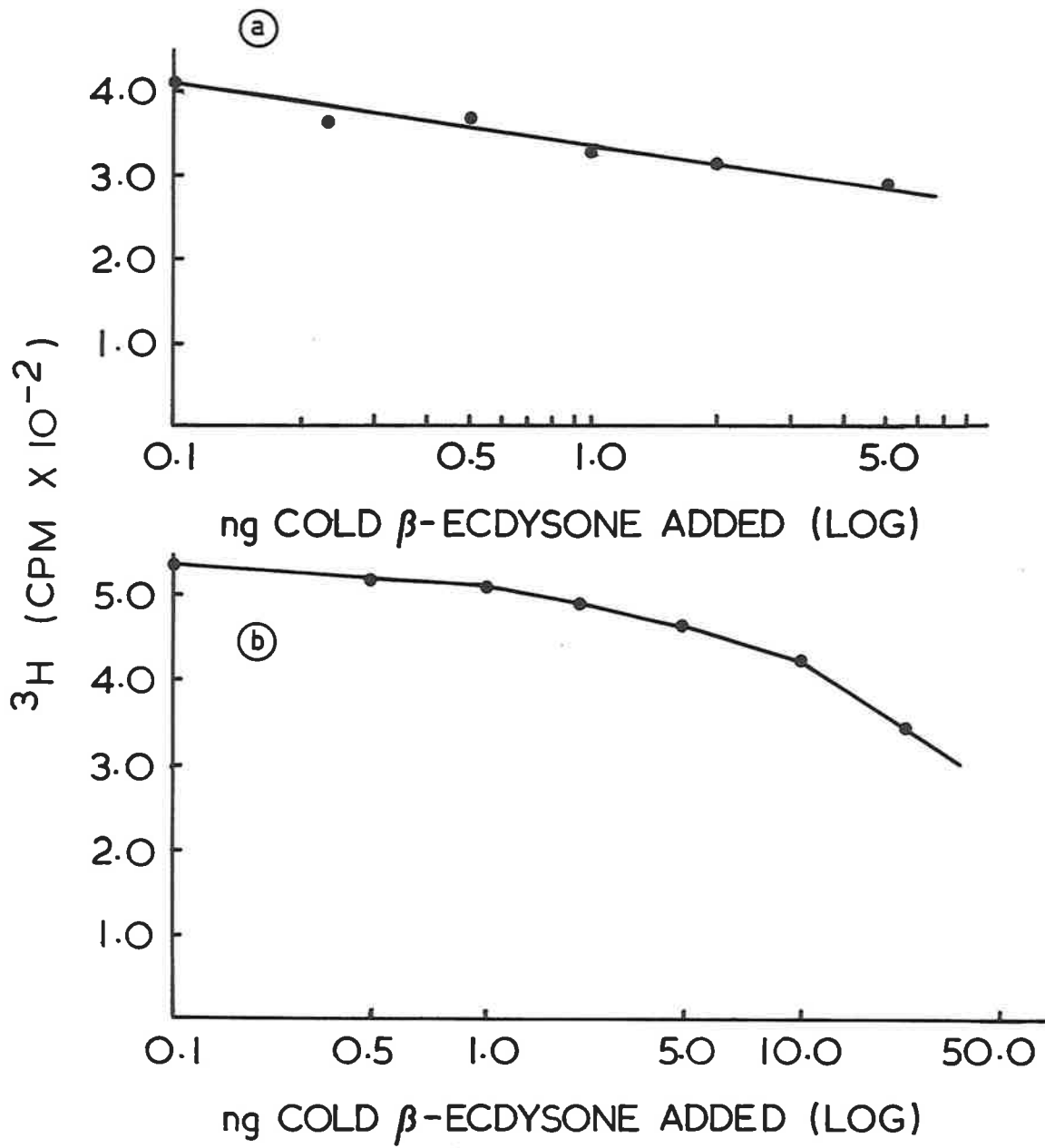
Following a booster injection, the binding capacity of the female rabbit antiserum increased approximately 3-fold, having risen in a 25% antiserum concentration from 5.3 to 16.5% binding of the ^3H - β -ecdysone added. In all succeeding RIA's the female rabbit antiserum at 25% was used. Of three determinations on the two nematode extracts from different populations, measurable quantities of ecdysone-like material were detected (Fig. 25(b), Table XXXIII). Again, a greater amount of ecdysone-positive material was detected in the A. avenae extract.

Table XXXIII. The amounts of ecdysone-like material detected in the extracts of A. avenae and P. redivivus by RIA.

Assay	Amount of nematode extract (μl)	<u>A. avenae</u> ng ecdysone-like material/gm wet weight	<u>P. redivivus</u> ng ecdysone-like material/gm wet weight
1st	50	500	79
2nd	40	266	54.8
3rd	50	114.5	2.4
4th	50	320.6	26.3

Figure 25. The calibration curve for the binding of [$^3\text{H}(\text{G})$] β -ecdysone by 20% (a) and 25% (b) rabbit antiserum, in the presence of increasing amounts of competing unlabelled β -ecdysone. For each assay tube, added labelled β -ecdysone (4000 cpm) and tube contents were incubated for 1 hr at 37° and the procedure of V.2.I followed. Non-specific binding values were subtracted from each determined point on the graph.

FIG. 25



V.4 DISCUSSION AND CONCLUSION

PAGE and sucrose density-gradient centrifugation showed no association of a macromolecular fraction of the cytosol with the steroid hormone, β -ecdysone. All components of the latter technique have been tested for possible interference due to protein aggregation or denaturation. Such macromolecular binding was also absent, following the concentration of the larval stages from 50 to 87%, and larval stages would have been more likely to possess such steroid binding proteins. The preliminary data from the charcoal-dextran binding assay suggested that the cytosol fraction specifically bound up to 60% of the β -ecdysone added, at the maximum rate of 3.7×10^{-9} M/mg protein-N. However, this could be explained in terms of non-specific binding to the cytosol fraction, and hence the hormone was readily removed from the TCA-precipitated protein by three washes with 5% TCA.

The conclusion is that either specific β -ecdysone-binding proteins are absent in P. redivivus, or they are not detectable with the experimental procedures used. As the level of protein extracted was low, it would be useful to increase the concentration of the supernatant protein by polyacrylamide gel or PEG 6000 dialysis, and re-determine the extent of bound β -ecdysone.

The reason for this investigation of the presence of moulting hormone receptors in nematodes was two-fold. Firstly, their occurrence would give indirect evidence to the hypothesis of the involvement of ecdysones in the physiological growth and developmental mechanisms of

nematodes. Perhaps a larger nematode should have been used, such that individual organs and organ systems could have been extracted and tested. This is because the difference between physiological target tissues and non-target tissues is a quantitative one of the amount of specific steroid hormone binding proteins (receptors) present in the cytosol (Jensen and Desombre, 1972; Duval and Funder, 1974).

Thus, specific steroid hormone receptors have been detected in the cytosol of numerous target tissues, which were steroid hormone-dependent tissues, i.e. for growth, development and maintenance. Oestrogen receptors have been detected and characterised from the uterus of the rat (Jensen et al., 1968; Shyamala and Gorski, 1969); the human myometrium (Ratajczak and Hahnel, 1974); and the hypothalamus of the female rat (Kato et al., 1974). Progestin receptors have been described from the uterus of the rabbit and guinea-pig (Philibert and Raynaud, 1974); and the human myometrium and endometrium (Verma and Lauman, 1973). Glucocorticoid receptors have been found in the liver of the rat (Beato et al., 1973; Kalimi et al., 1973; Ribarac-Stepic et al., 1973); the thymus cells of the rat (Bell and Munck, 1973); and the pituitary tumour cells of the mouse (Watanabe et al., 1973). The apparent mineralocorticoid receptor in the liver of the rat, a non-target tissue, was due to a glucocorticoid receptor (Duval and Funder, 1974). Androgen receptors have been isolated from the ventral prostate of the rat (Høisaeter, 1973) and the epididymis of the rat (Ritzen et al., 1971).

Secondly, as a corollary to the detection of specific steroid hormone receptors, is their involvement at some point during the response of the target tissues, in the control of gene expression (Tomkins and

Gelehrter, 1972; Gorski, 1973). Present evidence suggests that the cytosol binding proteins are transported to the nucleus and bound to acceptor sites on the chromatin. This follows the binding of the steroid hormone to the specific receptors in the cytosol and their temperature-dependent transformation (Jensen et al., 1968; Shyamala and Gorski, 1969). This is believed to be the primary event of the target tissue's response to the steroid hormone, as it is not inhibited by actinomycin D, puromycin or cycloheximide (Jensen and Desombre, 1972; Mueller et al., 1972). These inhibitors block all the known early responses of the target tissues to their respective steroid hormone. The result of this nuclear binding is a stimulation of the activity of the soluble and chromatin-bound DNA-dependent RNA polymerases of the nucleus (Desombre et al., 1972; Jensen and Desombre, 1972; Mohla et al., 1972), and in particular, that species of the enzyme that synthesizes DNA-like RNA (Glasser et al., 1972; Doenecke et al., 1973). The specificity of the response of increased RNA synthesis in the target tissue is achieved by the presence of acceptor-sites, for the steroid-receptor complexes, in the nucleus which are unique to the tissue involved (Mohla et al., 1971, 1972). Indirect evidence for the binding of the steroid hormone receptor to the chromatin, is seen by the complex formed between the oestradiol-17 β -receptor and DNA-dependent RNA polymerase I from the oviduct of the quail (Müller et al., 1974).

JH does not occur as a free entity in the insect haemolymph but is transported, like the diglycerides and cholesterol, by specific lipoproteins from the site of release, i.e. the corpora allata, to the

target organ(s) (Whitmore and Gilbert, 1972; Emmerich and Hartmann, 1973; Gilbert and Chino, 1974; Kramer et al., 1974; Krishnakumaran, 1974). This is by virtue of the lipophilic nature of the molecule, i.e. low solubility in an aqueous environment, such as the insect's haemolymph. The mechanism of action of JH (Cecropia JH - C₁₈ and analogues) may parallel that of the steroid hormones, where the primary response of the target tissue is mediated by the binding to specific receptors of high affinity for the hormone, and translocation of the complex to the chromatin of the nucleus. Thus, studies centred on this hypothesis have shown the presence of intracellular binding by membrane-bound ribonucleoprotein moieties (Schmialek, 1973; Ferkovich et al., 1974).

Steroid hormones are not immunogenic, but they may be made antigenic by coupling to a protein, usually BSA, where they are conjugated by amide bonds to the lysine residues present (Erlanger et al., 1957; 60 lysine residues per molecule). Because of the comparatively small amount of β -ecdysone that was available, it was desirable that the method chosen especially for the hapten formation, should yield a product not requiring further purification with the incurrence of further losses. Thus, the formation of testosterone-3-CMO by the method of Fritz, showed most clearly the properties of the hapten as originally characterized by Erlanger et al. (1967), i.e. U/V spectrum, M.P., crystallisation and the molar extinction coefficient (ϵ). The yield of 37.3%, although higher than that of Erlanger's method (5.1%), was considerably less than that

expected. Therefore, as a result it appeared that the Fritz product was of greater purity and homogeneity, and consequently it was used for the formation of the hapten β -ecdysone-6-CMO.

However, for the synthesis of β -ecdysone-6-CMO the decision was less clear-cut, because the yield was slightly greater for the Erlanger method (53%). Also, the observed U/V spectra of both methods did not correspond to the λ max of the hapten formed by Beckers and Emmerich (1973) of 212 nm or that of Borst and O'Connor (1972) of 252 nm. Due to the limited time and amount of material available, it was not possible to purify, estimate the yield or quantify the hapten adequately. The absorption of 288-290 nm may indicate the oxidation of the hydroxyl groups of the molecule. This suggests that any further synthesis of the hapten by refluxing should be performed under N_2 or vacuum, and possibly at a lower temperature and for a longer period of time.

The hapten molecule was conjugated via C-6 of β -ecdysone to BSA, so as to leave the functional groups of ring A and the sidechain, unhindered. This was because from the supposition of Kuss and Goebel (1972) and the findings of Lindner et al. (1972), the antigens of various oestrogens so prepared gave respective antisera of greater specificity. The assumption that one lysine residue of BSA had a M.W. of 3,500d and a maximum of 20 out of the possible 60 residues conjugated to β -ecdysone-6-CMO, appears in retrospect to have been an oversimplification. Thus, it was assumed that from the respective M.W.'s

(β -ecdysone, 480; BSA, 3,500d), 1 part of the hapten would react with 7 parts of BSA. However, this resulted in an excess of BSA over the steroid hapten and probable loss of binding activity. These premises were made in order that the loss, by excess, of the limited amount of β -ecdysone-6-CMO available, was prevented.

Notwithstanding the low order of binding activity of the antiserum it was possible, with appropriate controls (removal of non-specific binding ratio by control antiserum), to detect ecdysone-like material in different populations of A. avenae and P. redivivus. This was consistently higher in A. avenae. Many hidden problems exist, not least of which would be the loss of sensitivity of the antibody following the low binding activity and consequent high concentration of the antiserum used, i.e. 25%. Also, the efficiency of extraction of ecdysone and ecdysone-like material from the nematode homogenates by the procedure used is unknown.

The result of any critical cross-reaction would be to over-estimate the amount of ecdysone-like material present. Therefore, it would be necessary to determine the extent of the cross-reaction with closely and distantly related (structurally) steroids and sterols, that occur in nematodes. This is the main problem with the RIA as a reliable technique, and is usually solved by the selective removal of the competing compounds by TLC and/or column chromatography, prior to the assay of the steroid hormones, such as: oestrogens (Robertson et al., 1973); androgens, oestrogens and progestins (Bremner et al., 1973); glucocorticoides (Farmer et al., 1972; Gross et al., 1972); and,

mineralocorticoids (Waldhausl et al., 1972). The sterol composition of A. lumbricoides (Barrett et al., 1970; Cole and Krusberg, 1967(b)), Turbatrix aceti (Cole and Krusberg, 1968) and Ditylenchus dipsaci and D. triformis (Cole and Krusberg, 1967(a)), show the major sterols so far detected to be cholesterol, 7-dehydrocholesterol and lathosterol. However, because of their distant structural relationship to the polyhydroxy ecdysones, it is unlikely that any cross-reaction would occur. This has been verified by Borst and O'Connor (1972, 1974) for cholesterol. The relatively high amounts of ecdysone-like material detected, especially for A. avenae, may themselves be the result of a cross-reaction with a polyhydroxy molecule closely related to β -ecdysone, e.g. α -ecdysone. It is also conceivable that this molecule is the active principle involved. If this is so, then it would be expected to be present in an approximate ratio of 200 : 1 for α -ecdysone to β -ecdysone, in order to maintain the same level of biological activity, viz. the evagination of the imaginal discs of D. melanogaster (Borst et al., 1974). Perhaps the moulting hormone of insects (β -ecdysone) is characterised by being the most highly hydroxylated and hence biologically active ecdysone, when viewed from an evolutionary standpoint. Comparative information on the moulting hormone(s) of the most primitive insects, i.e. the Ametabola, would be most useful in this respect.

In order to implicate the ecdysone-like material detected, as a factor in the control of growth and development in nematodes, it is necessary to correlate possible changing titres of this moiety with known physiological events. The process of moulting is an obvious

event to examine. During the metamorphosis of Lucilia cuprina (Barritt and Birt, 1970) and Calliphora erythrocephala (Shaaya and Sekeris, 1965), the changing titres of moulting hormone(s) have been partially correlated with the known series of structural and biochemical changes that occur. However, with nematodes the problem is one of finding a species with a suitably synchronised development. This can be circumvented to some extent by in vitro axenic culture, e.g. for the examination of the first, second (Bolla et al., 1972) and fourth moults (Bonner et al., 1971) of the rat parasitic nematode, Nippostrongylus brasiliensis. Another likely physiological process for examination, would be what appears to be facultative dormancy (diapause) in a number of nematode species. RIAs would be used to determine the titre of ecdysone-like material and of JH (Lauer et al., 1974a) both before, during and following this event. A number of nematode species have been shown to exhibit the accepted characteristics of diapause at some stage during their life-cycle; the eggs of Heterodera avenae (Banyer and Fisher, 1971a, b); the eggs of Heterodera rostochiensis (Ellenby and Smith, 1967; Shepherd and Cox, 1967); the fourth-stage larvae of Haemonchus contortus (Blitz and Gibbs, 1971, 1972a, b); and, the probable fourth-stage larvae of Ancylostoma duodenale (Schad et al., 1973). It would be of direct interest to test the two alternative hypotheses of insect diapause for nematodes, namely, whether it is due to a 'hormonal-failure' mechanism (Williams, 1952) or by the maintenance of a high JH titre (Yin and Chippendale, 1973).

CHAPTER VI

GENERAL CONCLUSIONS

The questions that were posed in the Introduction, and experimentally tested, fall into two main categories. First, are morphogenetic hormones of an MH-type and JH-type present in nematodes? The ecdysone RIA has given the first direct indication that ecdysone-like substances are present in the nematodes, P. redivivus and A. avenae. Also, the interpretation given to the effects of various Na^+/K^+ ratios on the moulting of N. dubius, provides indirect evidence for this conclusion. However, no β -ecdysone-specific binding proteins, which would have added physiological evidence to such findings, were detected in the cytosol of P. redivivus.

Second, if the control mechanism of growth and development (morphogenesis) in nematodes is the same as in insects, what possible physiological and biochemical events are involved? Also, what is their mechanism of action? The moulting and egg-laying responses of N. dubius to α -ecdysone and synthetic juvenile hormone indicated two possible processes. In A. avenae after the addition of α -ecdysone, the increased activity of leucyl naphthylamidase, the enzyme thought to be involved with the process of moulting and exsheathment, indicated a possible hormonal correlation between moulting and the postulated moulting enzyme. If the level of regulation of protein synthesis (gene expression) by JH was at the level of translation, then it was not by altered polyribosomal profiles as shown by P. redivivus. The

polyribosomes and polyribosomal RNAs of P. redivivus, were considered to be normal following physical, chemical and biochemical comparison with those of other invertebrates.

At present the working hypothesis still stands, but it requires further examination in the following specific areas to either support or invalidate it. Until more data is forthcoming, the unsaid assumption has been that the different nematodes examined, respond to the application of insect growth hormones by the same physiological and biochemical mechanisms.

An alternative interpretation of the responses of the fourth-stage larvae of N. dubius to hormonal and ionic influences, was that they were due to the activation of the neuroendocrine system. Thus, Maddrell and Gee (1974) have shown that neurohormone release can be mediated by the maintained depolarisation of neurosecretory axons by the elevation of the K-concentration, and that this process is Ca-dependent. The ionic experiments on N. dubius were performed in the absence of calcium. In any investigation of endocrine principles in nematodes, moulting and the development and functioning of the reproductive system, would appear to be the most promising areas for examining hormonal control of morphogenesis. Electron micrographs of hindgut regions of the nematode might confirm or disprove that this area controls growth (Samoiloff, 1973), and if so, might indicate the possible control mechanism, i.e. via steroid hormone-producing cells or neurosecretion.

Prior to any determination of the mechanism of hormonal action on

selective physiological events in nematodes (by JH-type and MH-type compounds), it must be ascertained that JH- and MH-type compounds produce the recognised biochemical effects of growth and development hormones on macromolecular synthesis; the effect of JH and MH together, in respect to the inhibition of morphogenetic events, e.g. moulting; the stimulation of RNA and protein synthesis by MH; and the antagonism of this stimulation by JH. At least the known stability of m-RNA's during differentiation allows other controls of gene expression, besides that of transcription, to occur, i.e. post-transcriptional and translational (Ilan et al., 1970; Ilan et al., 1972; Ilan and Ilan, 1973).

Other enzymes besides the leucyl naphthylamidase of A. avenae should be investigated for induction by JH and MH, followed by a comparative study of their properties and the possibility of cyclic changes associated with development. The LAP of exsheathment and moulting, if examined in respect to the release of amino-acids, polypeptides, etc., from the isolated cuticular sheath, may allow the determination of whether the exsheathing activity is due to an enzymatic or non-enzymatic substance, or whether the activity is due to an intermediate, i.e. a biologically active polypeptide. The location of specific enzymes in cells by immunological methods (Jacobsen and Knox, 1974) would appear to be pertinent to this problem.

Indirect evidence for the involvement of MH in the physiological growth and developmental control mechanisms of nematodes would be gained, if specific hormone receptors were detected. It would be pertinent to

re-determine their presence using β -ecdysone of a higher specific activity, e.g. 50 Ci/mmol, and a nematode supernatant of increased protein concentration, i.e. 2-5 mg protein-N/ml. To substantiate the claim of the presence of ecdysone-like substances in nematodes by RIA, it would be necessary to correlate possible changes of titre during development with known physiological events, e.g. moulting, diapause and the formation and conclusion of the infective resting stage.

There are a number of inherent difficulties in the solution of these problems. Perhaps, the most important is the formation of age-synchronised cultures in vitro, but so far only partial synchrony has been achieved as for Panagrellus silusiae (Samoiloff and Pasternak, 1969). These may be circumvented to some extent by the collection of survival stages which are at a given larval stage in a form of arrested development or cryptobiosis or the collection and keeping of eggs in a solution of sufficient osmotic strength to prevent hatching, but not development, i.e. poised to hatch on the removal of the physical inhibition (Dropkin et al., 1958). Another is the need for highly labelled macromolecular fractions of nematodes, that can be performed by in vitro culture, when development has been ascertained to be the same as that occurring in vivo, e.g. Cooperia punctata (Slonka et al., 1973).

From their need for specific stimuli to overcome physiological blocks in their growth and development, the metazoan parasites are ideal organisms for the study of the hormonal growth of morphogenesis. Smyth

(1969) has brought to the attention of parasitologists and those studying all aspects of growth and development, the advantages as biological models of either nematode, trematode or cestode parasites.

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INSECT MORPHOGENETIC HORMONES AND DEVELOPMENTAL MECHANISMS IN THE NEMATODE, *NEMATOSPIROIDES DUBIUS*

R. D. DENNIS

Department of Plant Pathology, Waite Agricultural Research Institute,
University of Adelaide, Glen Osmond, South Australia 5064

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Abstract—1. The morphogenetic hormones of insects (α -ecdysone at 2.2×10^{-11} M and synthetic juvenile hormone at 3.3×10^{-11} M) stimulated or inhibited respectively the final moult from larva to adult of the nematode, *Nematospiroides dubius*.

2. There was an apparent direct effect of testosterone at 3.7×10^{-11} M on the egg-laying ability of females of this nematode.

3. The plant-growth regulators kinetin and gibberellic acid (GA_3) appeared to have little effect on either moulting or egg-laying.

4. The mechanism of moulting may be determined by ionic balances in certain "critical cells" of the nematode, as has been suggested for the mechanism of ecdysone action in insects.

INTRODUCTION

PREVIOUS studies of the effects of various mammalian and plant hormones on nematodes, have mainly revealed indirect effects via the respective tissue treated. Thus, males of higher vertebrates are more susceptible than females to helminth infection; whilst gonadectomy of males reduced, and testosterone treatment increased, this susceptibility (Solomon, 1969). Webster (1967) found indirect stimulation of multiplication of *Aphelenchoides ritzemabosi* developing on lucerne callus tissue treated with kinetin, IAA and gibberellic acid (GA_3), as a result of increased feeding sites, i.e. cells. Also, the increased reproduction rate of *Ditylenchus dipsaci*, *Pratylenchus penetrans* and *Pratylenchus zaei*, in response to the application of the plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D), was due to indirect stimulation of the growth of alfalfa callus tissue (Krusberg & Blickenstaff, 1964).

At the present time, data obtained by *in vitro* culture, have shown juvenile hormone (JH) and mimics to cause inhibition of maturation and reproduction in: *Caenorhabditis elegans*, *Panagrellus redivivus* (Dropkin *et al.*, 1971), *Caenorhabditis briggsae* (Hansen & Buecher, 1970) and *Trichinella spiralis* (Shanta & Meerovitch, 1970), but, specific cell divisions in the gonadal tissues of males of *Heterodera schachtii* (Johnson & Viglierechio, 1970). However, the first two authors showed β -ecdysone to have no effect on growth or development, although Webster & Craig (1969) indicated that the development of *Cephalobus* was highly sensitive to a concentration of 1.1×10^{-7} M. Hitcho & Thorson (1971) extracted a steroid resembling β -ecdysone from *Trichinella spiralis*, that increased moulting and growth of larvae of this nematode; farnesal was also extracted and resulted in increased length of larvae.

The pathway from stimulus, to neuroendocrine secretion, to stimulation of the excretory gland to produce and release leucine aminopeptidase (LAP), that

results in ecdysis was elucidated by Davey & Kan (1968) for the nematode, *Phocanema decipiens*. Juvenile Hormone produced the same moult, due to a stress-type reaction, at a concentration of 10^{-6} M (Davey, 1971). Finally, Rogers & Head (1972) have implicated noradrenaline's involvement in the morphogenetic stimulus of *Haemonchus contortus*, following infection.

The present study is an attempt to elucidate, by more quantitative means, the effects of insect, mammalian and plant hormones on the processes of moulting and egg-laying in the mouse intestinal parasite, *Nematospiroides dubius* and to consider the Na^+/K^+ ratio, in the context of a "stimulus" for nematode moulting.

MATERIALS AND METHODS

I. Culture of the nematode (determination of time of the final moult)

The life-cycle studies of Baker (1954) and Bryant (1973), enabled the use of an *in vitro* incubation by the technique of Ross (1972), in order to determine the time of moulting of fourth-stage larvae in the different media. This would enable a standardized regimen to be formulated for the testing of various compounds on the last stage moult of this nematode.

About 300 third-stage larvae in 0.1 ml water were administered by syringe into the mouths of female mice (Sommerville, 1972). At the required time after infection, fourth-stage larvae were removed from the circular muscle of the small intestine of the mice; all extractions were performed in 0.9% NaCl. The larvae were then cultured *in vitro*, in 2 ml of the respective media for 48 hr at 39°C in the dark (roller tube culture—12 rev./hr.). All solutions contained the antibiotics kanamycin (Bristol Laboratories, Australia) and nystatin (Squibb Ltd., Australia), at the respective concentrations of 5 mg/100 ml and 100 units/ml.

In all subsequent experiments, incubations were performed in Tyrodé's solution (Fig. 1).

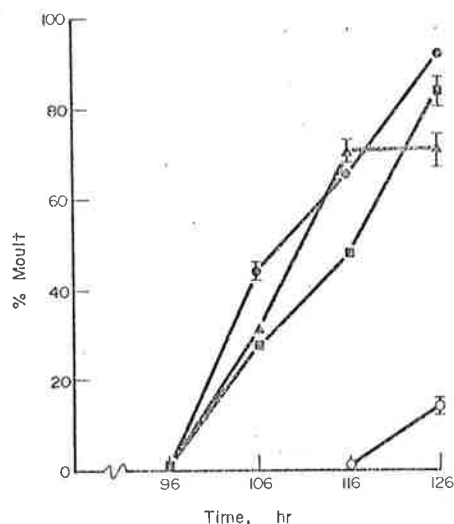


Fig. 1. The % moult of fourth-stage larvae removed at 10 hr intervals from mice and placed in various media: ●—● 199; ▲—▲ Tyrode's; ■—■ 0.75% NaCl/0.15% KCl; ◇—◇ 0.9% NaCl. Each point is the mean of three replicates each of about 20 larvae.

II. Effect of hormones and inhibitors on the last-stage moult

At 106 hrs after infection, the larvae were placed in Tyrode's solution, containing the appropriate hormone treatment. The plant growth regulators gibberellic acid GA_3 and kinetin stock solutions were in water. The mammalian sex steroids testosterone and oestradiol-17 β standard solutions were in ethanol, whilst the insect hormones α -ecdysone (Schering A. G. Berlin), 'Dichlorofarnesoate' (Calbiochem, U.S.A.—Law *et al.*, 1966 and Vinson & Williams, 1967) and a synthetic juvenile hormone (Ayerst Research Laboratories) with 10% of the biological activity of the pure naturally-occurring hormone, were contained in methanol. These latter compounds were applied to the kimble tubes and the solvent evaporated-off under dry N_2 , before addition of the incubating solution. The procedure described in Section I, was then followed. The antibiotics actinomycin D (Merck *et al.*) and puromycin di-HCl (Sigma, U.S.A.) were used to examine their physiological effects on the stimulation of moulting by α -ecdysone; each was added separately to the single concentration (2.2×10^{-9} M) of α -ecdysone.

III. Effect of hormones on egg-laying

Females were removed from the lumen of the gut of the mouse at 410 hr post-infection, and the procedure followed as in Section 1.

IV. Effect of the Na^+/K^+ ratio on moulting

The worms were removed at 122 hr post-infection and placed in iso-osmotic solutions of: 0.9% NaCl, 0.75% NaCl/0.15% KCl, 0.6% NaCl/0.3% KCl, 0.5 NaCl/0.4% KCl and 0.4% NaCl/0.5% KCl, and the % moult observed after 48 hr at 39°C in Tyrode's solution.

V. Statistical Analysis

All results were converted to arc-sine for analysis. All results were tested by analysis of variance, except those for the Na^+/K^+ ratio, which were examined by the *t*-test. The level of probability used was 1%.

RESULTS

The criterion of moulting used was the complete separation of the fourth-stage cuticle from the under-

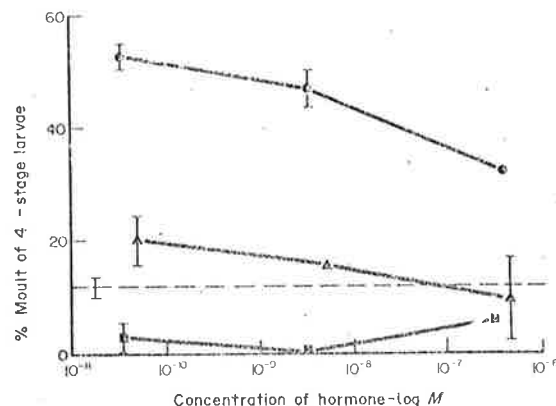


Fig. 2. The effect of insect hormones of the % moult of fourth-stage larvae, 106 hr post-infection: ●—● ecdysone; ▲—▲ dichlorofarnesoate; ■—■ synthetic juvenile hormone; — Control—Tyrode's solution. Each point is the mean of 3 replicates each of about 20 larvae.

lying adult cuticle. The adult cuticle was thicker and exhibited developmental polymorphism when compared to the appearance of the larval cuticle, i.e. the adult cuticle had longitudinal ridges, along the length of its surface, as opposed to the horizontal striations of the larva. Males were easily recognized by the copulatory bursa; females were usually tightly coiled, which obscured the vulval opening.

(a) *Moulting*. The % moult, as against the tyrode control, was stimulated by α -ecdysone at 2.2×10^{-7} , 10^{-9} and 10^{-11} M. The synthetic juvenile hormone inhibited moulting at 3.3×10^{-7} , 10^{-9} and 10^{-11} M, whilst the 'Dichlorofarnesoate' produced no significant difference from the control (Fig. 2). Of the plant growth regulators, gibberellic acid at 5.8×10^{-8} , 10^{-9} and 10^{-10} M inhibited moulting, and at 9.3×10^{-7} M kinetin stimulated moulting. The mammalian sex steroids produced significant effects on moulting, oestradiol-17 β inhibited at 3.7×10^{-7} and 3.7×10^{-9} M, and testosterone caused the same result at 3.5×10^{-11} M (Table 1). Actinomycin-D at 1.6×10^{-5} M and 4.0×10^{-6} M, and puromycin at 5×10^{-4} M and 5×10^{-5} M, caused no significant inhibition of moulting.

TABLE 1. The effect of two plant hormones and mammalian sex steroids on the fourth → fifth stage moult of *N. dubius*.

	Concentration	% moult	Significance
Gibberellic Acid	5.8×10^{-8} M	0.0 ± 0.0	S-ve
	5.8×10^{-9} M	14.6 ± 0.16	S-ve
	5.8×10^{-10} M	14.3 ± 0.32	S-ve
Kinetin	9.3×10^{-6} M	28.5 ± 5.46	NS
	9.3×10^{-7} M	33.3 ± 7.1	S+ve
	9.3×10^{-8} M	31.4 ± 0.27	NS
Testosterone	3.5×10^{-7} M	20.8 ± 0.45	NS
	3.5×10^{-9} M	20.7 ± 0.64	NS
	3.5×10^{-11} M	14.4 ± 0.53	S-ve
Oestradiol-17 β	3.7×10^{-7} M	0.0 ± 0.0	S-ve
	3.7×10^{-9} M	13.8 ± 0.34	S-ve
	3.7×10^{-11} M	19.6 ± 0.99	NS
Tyrode Control		19.7 ± 1.74	

Statistical Analysis: Analysis of variance of each hormone against the Tyrode Control. F Test $P < 0.01$. Significance tested by L.S.D. at $P < 0.05$.

NS Not Significant
S+ve Stimulation
S-ve Inhibition

Values of % moult are means ± S.E.M. (Arc-Sine Transformation). Each figure is the mean of 3 replicates each of about 20 larvae.

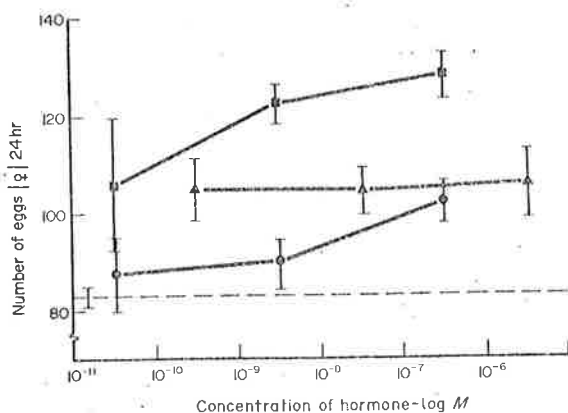


Fig. 3. The effect of insect hormones on the number of eggs per female per 24 hr. at 410 hr post-infection: \circ — \circ α -ecdysone; \triangle — \triangle dichlorofarnesoate; \square — \square synthetic juvenile hormone; ——— Control—Tyrode's Solution. Each point is the mean of 4 replicates each of 4 females.

(b) *Egg-Laying*. The results were assessed as eggs/female per 24 hr. α -ecdysone and "Dichlorofarnesoate" had no significant effect on egg-laying. The synthetic juvenile hormone stimulated egg-laying at 2.2×10^{-7} and 10^{-9} M (Fig. 3). Gibberellic acid GA_3 and oestradiol-17 β were without effect, although kinetin was again positive at 9.3×10^{-7} M. Of significance, was the stimulation by testosterone at all concentrations (Table 2).

(c) Na^+/K^+ ratio. All concentrations of potassium, were significant over 0.9% NaCl (Fig. 4), as no moult occurred in the latter solution. Extrapolation of the points of the curve, showed that 0.67% NaCl/0.23% KCl would give the maximum moulting response.

DISCUSSION

Comparison, of the tyrode controls at 106 hr (Figs. 2 and 3) and the timing of the last-stage moult (Fig. 1), indicated that different populations vary considerably in vitality.

In preliminary experiments, it was necessary to

TABLE 2. The effect of two plant hormones and mammalian sex steroids on egg-laying by female *N. dubius*.

	Concentration	Average No. of eggs/female/24 hrs.	Significance
Gibberellic Acid	5.8×10^{-7} M	106.52 ± 7.1	NS
	5.8×10^{-8} M	81.49 ± 6.17	NS
	5.8×10^{-9} M	64.4 ± 12.7	NS
Kinetin	9.3×10^{-7} M	97.93 ± 7.09	NS
	9.3×10^{-8} M	126.71 ± 4.53	S+ve
	9.3×10^{-9} M	99.4 ± 7.14	NS
Testosterone	3.5×10^{-7} M	132.81 ± 7.14	S+ve
	3.5×10^{-8} M	134.38 ± 8.97	S+ve
	3.5×10^{-11} M	133.94 ± 9.72	S+ve
Oestradiol-17 β	3.7×10^{-7} M	111.46 ± 4.55	NS
	3.7×10^{-9} M	110.02 ± 8.89	NS
	3.7×10^{-11} M	109.33 ± 4.75	NS
Tyrode Control	—	83.9 ± 2.23	

Statistical Analysis: Analysis of Variance of each hormone against the Tyrode Control. F Test < 0.01. Significance tested by L.S.D. at $P < 0.05$.

NS Not Significant
S+ve Stimulation
S-ve Inhibition

Values for eggs are means \pm S.E.M. (Arc-Sine Transformation). Each figure is the mean of 4 replicates each of about 4 females.

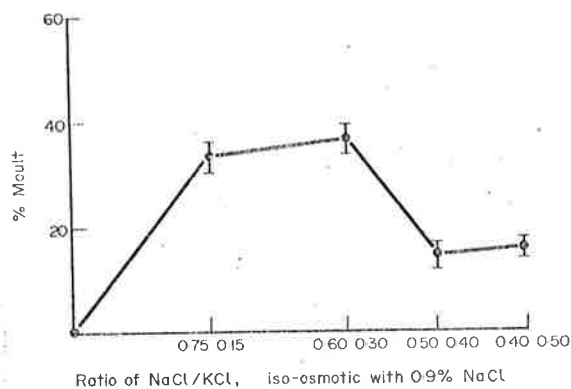


Fig. 4. The effect of varied Na^+/K^+ ratios on the % moult of fourth-stage larvae, 122 hr post-infection. Each point is the mean of 3 replicates, each of about 20 larvae.

determine the time following infection for larvae to approach the last moult, such that, after *in vitro* culture for 48 hr at 39°C in the dark, a low level of worms had moulted to the adult and thus would function as the control. This then allowed the comparison of the control with treatments of various test compounds in a standardized regime. Because of the similarity of the % moulted at 106 hr *in vitro* in the 3 media: 199 (C.S.L., Australia) Tyrode's (C.S.L., Australia) and 0.75%/0.15% KCl, it was decided to use the simplest medium, i.e. Tyrode's, to avoid any possible complication of the test compound interacting with a component(s) in the complex medium, i.e. 199.

The effects of the synthetic juvenile hormone and α -ecdysone, appear to indicate a comparable type of control of morphogenesis in nematodes, as in insects. Thus, α -ecdysone stimulates and synthetic juvenile hormone inhibits moulting at approximately 10^{-11} M. Perhaps, juvenile hormone has a gonadotrophic effect on egg-laying, as the synthetic juvenile hormone stimulates at 10^{-9} M. Such sensitivity would appear to preclude a stress-reaction response.

The plant growth regulators gibberellic acid GA_3 and kinetin were chosen after preliminary experiments with the nematode, *Aphelenchus avenae*. Kinetin increased moulting and egg-laying at a concentration of 10^{-7} – 10^{-8} M, which might be considered to be at the borderline of a pharmacological/physiological action. The data presented appear to refute the connection between gibberellins and moulting hormone activity in the locust *Schistocerca gregaria* (Carlisle *et al.*, 1963), whilst giving additional support of no reciprocal effects (Hendrix & Jones, 1972).

The mammalian sex steroids, testosterone and oestradiol-17 β appear to have no effect on moulting at a physiological concentration, except that the androgen inhibits at 10^{-11} M. However, testosterone stimulates egg-laying at 10^{-11} M. Thong & Webster (1971) observed increases in the length of *Cephalobus* sp., under the influence of these two hormones, but development was unaffected. Within the limitations of this experiment, this would appear to be the case with *Nematospiroides dubius*.

The simplest explanation of the negative response of actinomycin-D (inhibitor of transcription) and puromycin di-HCl (inhibitor of translation—Rabinowitz

& Fisher, 1962), would be that during the period of these experiments, the worm did not feed. Thus, neither the fourth-stage larvae nor adults when incubated with varying concentrations of anti-rabbit immunoglobulin (sheep) labelled with fluorescein (Wellcome Reagent Ltd., England), showed the presence of fluorescence in the intestine (unpublished results). The same conclusion was put forward by Despommier & Jackson (1972) for the non-inhibition of development and reproduction, by puromycin, in *Neoplectana glaseri*. The expected inhibition of growth by these two antibiotics was evident in *Panagrellus redivivus* (Pasternak & Samoiloff, 1970), at comparable concentrations to those used in this study.

Similar results indicating the necessity of a Na^+/K^+ ratio, rather than either cation on its own, were described earlier by Sommerville (1968) with *Haemonchus contortus* third-stage larvae. The standard effects of Na^+ and K^+ ions on cells such as membrane changes, could include the excitable tissues, such as nerves and muscles. However, Castillo *et al.* (1964) and Brading & Caldwell (1971) have shown, at least with *Ascaris lumbricoides* muscle, that the resting membrane potential is remarkably unaffected by Na^+ , K^+ or Cl^- ions.

A plausible alternative was given by Kroeger (1963) and by Kroeger & Lezzi (1966) for insects, whereby changes in the ionic balance of the nuclear sap, including the Na^+/K^+ ratio, resulted in activation in sequence of genes involved in moulting. If the Na^+/K^+ ratio of the nuclear milieu was increased a juvenile hormone-type response was obtained, whilst reduction of this ratio produced an ecdysone-type response, when considered in terms of polytene chromosome puffing at specific loci (Lezzi & Robert, 1972). The possibility may exist of a similar type of mechanism in nematodes, as suggested by the simple expedient of changing the medium ratio of Na^+ and K^+ ions, and observing the % moult.

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Key Word Index—Nematode; *Nematospiroides dubius*; Insect Growth Hormones; Moulting; Egg Laying; Na^+/K^+ Ratio.

ADDITIONAL DATA:V.3.D. Radioimmunoassay (RIA)

The antiserum was finally optimised (FELDMAN & RODBARD, 1971) to 28.4% binding of added ^3H - β -ecdysone, by the preparation of the incubation tubes minus the antiserum/serum 12hr prior to the experiment, and storage at 4°C in the dark. The calibration curve was re-determined (Fig. 26). Cholesterol at the concentrations used did not cross-react with β -ecdysone for binding to the specific antibody (Fig. 26). Measurable quantities of ecdysone-like material were again detected in the extracts of A. avenae, P. redivivus and H. contortus (infective third-stage larvae), the amount being greatest in the former nematode (Table XXXIII(b)).

Table XXXIII(b) The amounts of ecdysone-like material detected in the extracts of A. avenae, P. redivivus and H. contortus by RIA.*

Population [†]	<u>A. avenae</u> ng ecdysone-like material/gm wet weight	<u>P. redivivus</u> ng ecdysone-like material/gm wet weight	<u>H. contortus</u> ng ecdysone-like material/gm wet weight
4	995.6 \pm 28.9	263.2 \pm 12.6	202 \pm 21.9
5	851.3 \pm 18.9	178.1 \pm 7.9	199.8 \pm 24.4
6	930.1 \pm 9.9	270.9 \pm 10.7	212.8 \pm 22.1

*Determined on 50 μl of the nematode extract.

[†]The amounts of ecdysone-like material detected are the means \pm S.E.M. of triplicate determinations.

In an attempt to correlate the levels of ecdysone-like material in extracts of A. avenae and P. redivivus with a meaningful parameter, the proportion of larvae to adults of the different larval stages (populations 4-6) were estimated (Table XXXIV). For P. redivivus, the larval body-length values of

BORODITSKY & SAMOILOFF (1973) were used, and for A. avenae, they were derived from the data of FISHER (1970).

Table XXXIV The population profiles of A. avenae and P. redivivus subsampled prior to RIA analysis.

Population	Adults: Larvae		Ratios ⁺		
			L ₂	L ₃	L ₄
<u>A. avenae</u>	4	0.075 ± 0.01	63	24	13
	5	0.042 ± 0.00 ^{**}	73	20	7
	6	0.047 ± 0.00 ^{**}	60	19	21
<hr/>					
<u>P. redivivus</u>	4	0.835 ± 0.04	65.75 ± 3.06	2.5 ± 0.29	31.75 ± 2.46
	5	1.429 ± 0.07 ^{**}	61.25 ± 2.33 ^{NS}	1.75 ± 0.63 ^{NS}	37.6 ± 2.94 ^{NS}
	6	0.617 ± 0.05 ^{**}	78.75 ± 1.49 ^{**}	2.5 ± 0.29 ^{NS}	18.75 ± 1.44 ^{**}

⁺Where appropriate, the values are the means of 6 replicates ± S.E.M. of 100 nematodes. Significance was determined by the 't' test against population 4 of A. avenae or population 4 of P. redivivus, and designated either,

NS - not significant

* - significant at the 2% level; or

** - significant at the 1% level.

The efficiency of extraction of endogenous ecdysone was estimated by the addition of ³H-β-ecdysone (1 × 10⁵ cpm) to P. redivivus prior to the extraction procedure for ecdysone-like material in this nematode. The value for the recovery of labelled β-ecdysone was 92.1% ± 1.0 (mean ± S.E.M.). For comparison, the extraction efficiency for endogenous cholesterol was estimated (4 × 10⁵ cpm, 4 -¹⁴C-cholesterol) in P. redivivus by the same procedure. The percent recovery was 6.7% ± 0.96 (mean ± S.E.M.).

The specificity of the antibody to β -ecdysone was further examined by the extent to which it was bound to the moulting hormone, when a known amount (10 ng) was added to the extract of P. redivivus, from which the ecdysone-like material was removed by four washes of the aqueous extract with water-saturated n-butanol. The binding of ^3H - β -ecdysone to the antibody was the same as that determined from the calibration curve (Fig. 26), i.e. a reduction of $169 \text{ cpm} \pm 41$ (mean \pm S.E.M.) in the amount of labelled hormone bound to the specific antibody. However, the binding was unexpectedly variable.

V.4. Discussion & Conclusion

Further confirmation of the presence of ecdysone-like material in extracts of the nematodes A. avenae, P. redivivus and H. contortus has been gained by the technique of radioimmunoassay, as well as additional information on the specificity of the antibody for β -ecdysone. There was an absence of cross-reaction with cholesterol, whilst the efficiency of its extraction from nematode homogenates by the procedures used was below 10%. The extraction efficiency for the RIA-positive material approached 100%. This data also showed that on removal of ecdysone-like material from extracts, binding to the moulting hormone was directly proportional to the amount added.

The population profiles of A. avenae and P. redivivus suggest that high larval to adult and fourth-stage larval to second-stage larval ratios result in higher titres of ecdysone-like material, and vice-versa, i.e. more ecdysone-like material is present in moulting larvae than adults and fourth-stage larvae contain a higher titre than second-stage larvae. Concomitant with this interpretation of the correlation between population profiles and the levels of ecdysone-like material detected, an explanation for the higher titre in extracts of A. avenae may have been because of actively developing populations, whilst those of P. redivivus and H. contortus were static. Such results have thus been taken as supporting the hypothesis, that changing titres of ecdysone-like material in nematodes are correlated with moulting and its control.

MODIFICATION:

Chapter IV 'STUDIES ON THE α -ECDYSONE-STIMULATED ENZYME LEUCYL NAPHTHYLAMIDASE OF APHELENCHUS AVENAE', has been changed to that of an appendix; because of its equivocal relevance to the rest of the thesis and the unsuccessful attempt at the purification of the stimulated enzyme leucyl naphthylamidase, whilst the following alterations have been made to it:-

- 1). The correction of Tables XXIV(A), (B) - p. 97.

Table XXIV(A) Purification of leucyl naphthylamidase

Procedure	Total volume	Total activity	Specific activity	Yield
	ml	LNA _{mU}	LNA _{mU} /mg protein-N	%
1. Crude extract	38	2.85×10^6	1.19×10^5	100
2. 20,000g homogenate	36	2.63×10^6	1.76×10^5	92.4
3. Effect of dialysis	34.5	1.61×10^6	1.24×10^5	56.5
4. Fractionation with ammonium sulphate 75%	26	7.02×10^5	7.11×10^4	27
(75-95%) 95%	6	6.9×10^4	1.22×10^5	
5. Fractionation with ammonium sulphate 40%	17	0.0	0.0	0.0
(40-95%) 95%	11	2.31×10^5	9.84×10^4	8.1
6. DEAE-cellulose chromatography	1.13	6.39×10^4	3.53×10^5	2.2
7. Gel filtration on Sephadex G-200	4.2	7.80×10^3	3.72×10^5	0.3

Table XXIV (B) Leucyl naphthylamidase and protein purification of A.avenae extract.

Procedure	Protein-N mg	Increased protein purification (x)	Increased enzyme activity (x)
1. Crude extract	23.94	1	1
2. 20,000 g homogenate	14.94	1.6	1.48
3. Effect of dialysis	12.96	1.8	1.04

4. Fractionation with ammonium sulphate	75%	9.88	2.4	0.60
(75-95%)	95%	0.57	42	1.03
5. Fractionation with ammonium sulphate	40%	5.53	4.3	-
(40-95%)	95%	2.37	10.1	0.83
6. DEAE-cellulose chromatography		0.18	133	2.97
7. Gel filtration on Sephadex G-200		0.021	1140	3.13

2) The correction of Tables XXV(A), (B) - p.98.

Table XXV(A) Purification of leucyl naphthylamidase.

Procedure	Total volume ml	Total activity LNA _m U	Specific activity LNA _m U/mg protein-N	Yield %
1. Crude extract	38	2.85×10^6	1.5×10^5	100
2. 20,000g homogenate	37.5	2.56×10^6	1.52×10^5	89.7
3. Fractionation with ammonium sulphate	40% -	-	-	-
(40-95%)	95% 13.5	6.75×10^5	1.92×10^5	23.7
4. DEAE-cellulose chromatography	2.85	4.68×10^4	6.42×10^4	1.6
5. Gel filtration Sephadex G-100	1.5	1.77×10^4	4.78×10^5	0.6

Table XXV(B) Leucyl naphthylamidase and protein purification of A. avenae extract

Procedure	Protein-N mg	Increased protein purification (x)	Increased enzyme activity (x)
1. Crude extract	19.00	1	1
2. 20,000g homogenate	16.88	1.1	1.01
3. Fractionation with ammonium sulphate	40% -	-	-
(40-95%)	95% 3.51	5.4	1.28
4. DEAE-cellulose chromatography	0.73	26	0.43
5. Gel filtration on Sephadex G-100	0.037	514	3.19

3) IV.3.F.(ii).b - 20,000g homogenate p. 93. The data from the revised Tables XXIV(A), (B) and XXV(A), (B) indicated that no particulate inhibitor(s) was present in the crude extract of A. avenae, because of the lack of increase of specific activity over protein purification following centrifugation.

4) IV.3.F.(ii).g-Sephadex G-200 gel filtration column chromatography p.95-96
The revised estimates of the final activity of the enzyme leucyl naphthylamidase, following the same protocol of purification, were 3.13 x compared to a protein purification of 1140 x (Table XXIV(B)) and 3.19 x compared to a protein purification of 514 x (Table XXV(B)).

IV.4. DISCUSSION p.114 There was no indication of an inhibitor(s) of leucyl naphthylamidase in crude extracts of A. avenae, and therefore the appearance of the enzyme becoming more labile during the extraction and attempted purification from further batches of lyophilised nematodes, was more apparent than real. Further attempts at purification should therefore concentrate on developing a protocol to stabilise the enzyme prior to fractionation, and avoid the procedures used which only exacerbated the lability of this protein.

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- p.23 "Jamuar, 1966b".
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- p.38 "Lecocq et al., 1971".
- p.70 "Van Gundy et al., 1967".
- p.95 "Reiland, 1971".
- p.108 "West et al., 1966".
- p.109 "Spackman et al., 1955".
- p.118 Delete 'b' from "Slocombe and Whitlock, 1971b".
- p.119 Delete 'brackets' from "Lauer et al., 1974(b)".
- p.147 "Robertson et al., 1972".
- p.152 "Jacobsen and Knox, 1973".

p.170 "Horn et al., 1974" should be listed before Howell et al., 1964".

Additional

Data: FELDMAN, H. and RODBARD, D. (1971).

p.(i) Mathematical theory of radioimmunoassay.

In "Principles of Competitive Protein-Binding Assays" (Odell, W.D. and Daughaday, W.H. eds.), pp 158-203. J.B. Lippincott, Philadelphia.

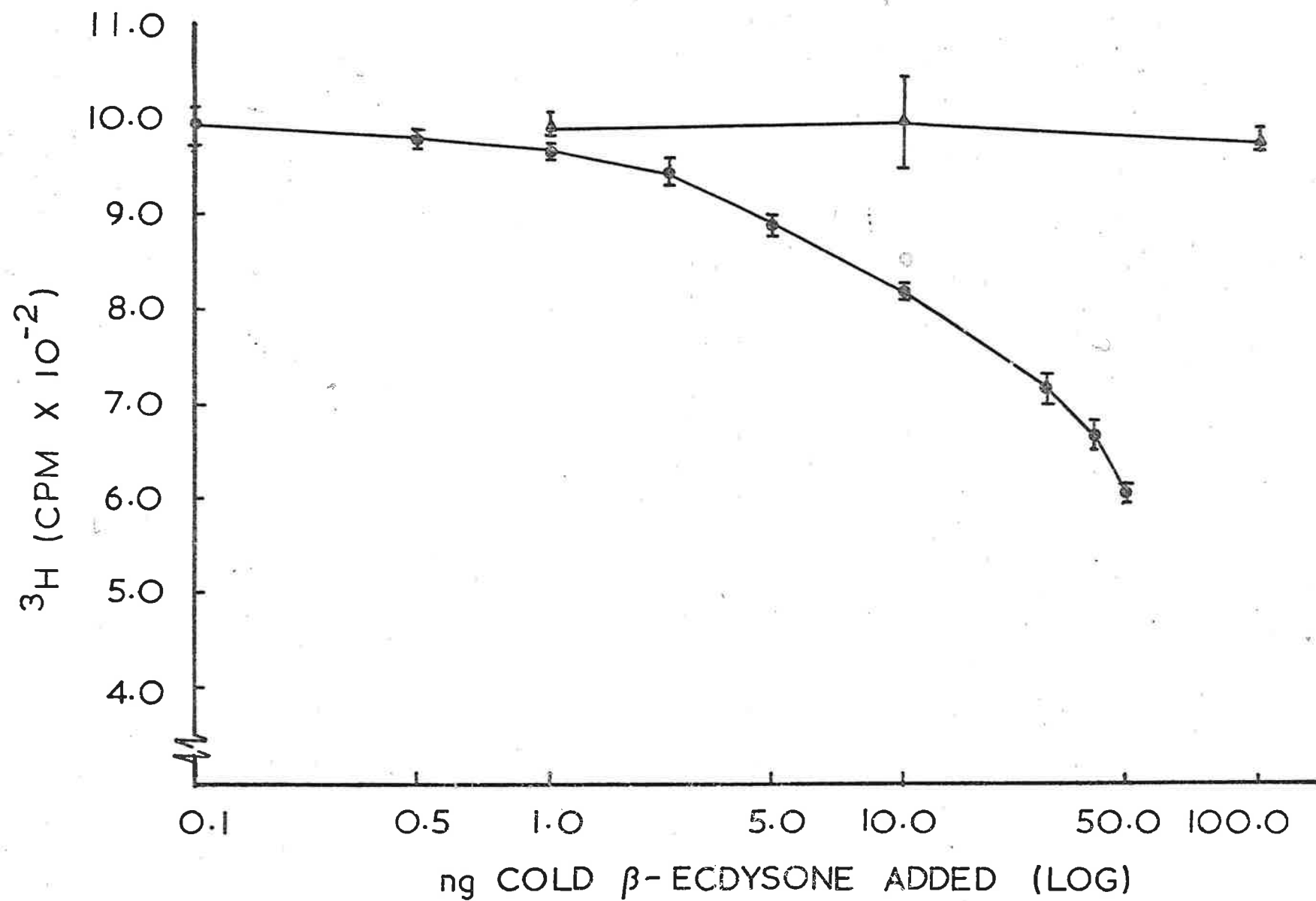


Figure 26. Calibration curve for the binding of ($^3\text{H}(\text{G})$) β -ecdysone by 25% rabbit antiserum, in the presence of increasing amounts of competing unlabelled β -ecdysone (o) or cholesterol (). For each assay tube, added labelled β -ecdysone (3500 cpm) and tube contents were incubated for 3 hr at 4°C and the procedure of Borst & O'Connor (1972, 1974) followed. Nonspecific binding values were subtracted from each point on the graph. Each point is the mean of triplicate determinations and the vertical bars represent the standard error of the mean.