ACTION OF THE PESTICIDE DIFLUBENZURON ON *CAENORHABDITIS ELEGANS* (NEMATODA)

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ABSTRACT

The biological effects and mode of action of diflubenzuron, a phenylbenzoylurea pesticide, have been investigated on the free living nematode *Caenorhabditis elegans*.

Population growth of *C. elegans* was found to be significantly retarded by treatment with formulated diflubenzuron whereas treatment with the analytical grade of the compound was found to be ineffective. Nematodes treated with formulated diflubenzuron were able to survive, move, feed, and develop normally but reproduction of adult hermaphrodites was significantly reduced; *C. elegans* treated with 5 and 20ppm diflubenzuron producing 44% and 9 % of the control number of eggs, respectively.

Light microscope and histochemical studies did not detect any effect of diflubenzuron on gonadogenesis in the reproductive tracts. Similarly, embryogenesis of fully formed eggs (with 2 cells or more) from diflubenzuron - treated *C. elegans* was found to be normal and a significant effect on hatching was only found when the egg-producing adults were treated rather than the eggs alone. The uptake and distribution of diflubenzuron in *C. elegans* and its eggs was investigated using ^{14}C-labelled pesticide.

Solubility and sonication tests on unhatched eggs from diflubenzuron-treated adult *C. elegans* suggested changes in the structure of the egg shell and an ultrastructural examination of normal eggs and eggs from diflubenzuron-treated adults showed differences in the structure

of chitin and lipid layers of the egg shell. However, no significant difference in the amount of glucosamine, a chitin precurser, was found in egg shells from control and diflubenzuron-treated samples of eggs.

Throughout this study, an unusually high proportion of unfertilized eggs were produced by diflubenzuron-treated *C. elegans*, and an attempt was made therefore to follow the fertilization process in treated and control nematodes. The Feulgan's stain technique showed that unlike treated nematodes, control nematodes had a greater number of sperm able to encounter mature oocytes; although staining with fluorescent vital dye Nile blue (A) showed that treatment with diflubenzuron did not impede the migration of males sperm within the reproductive tracts of the hermaphrodite. The effects of diflubenzuron on the activity of sperm were also examined *in vitro*.

The results are discussed in relation to the possible mode of uptake and action of diflubenzuron in nematodes and compared to the action of this pesticide in arthropods.

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To the memory

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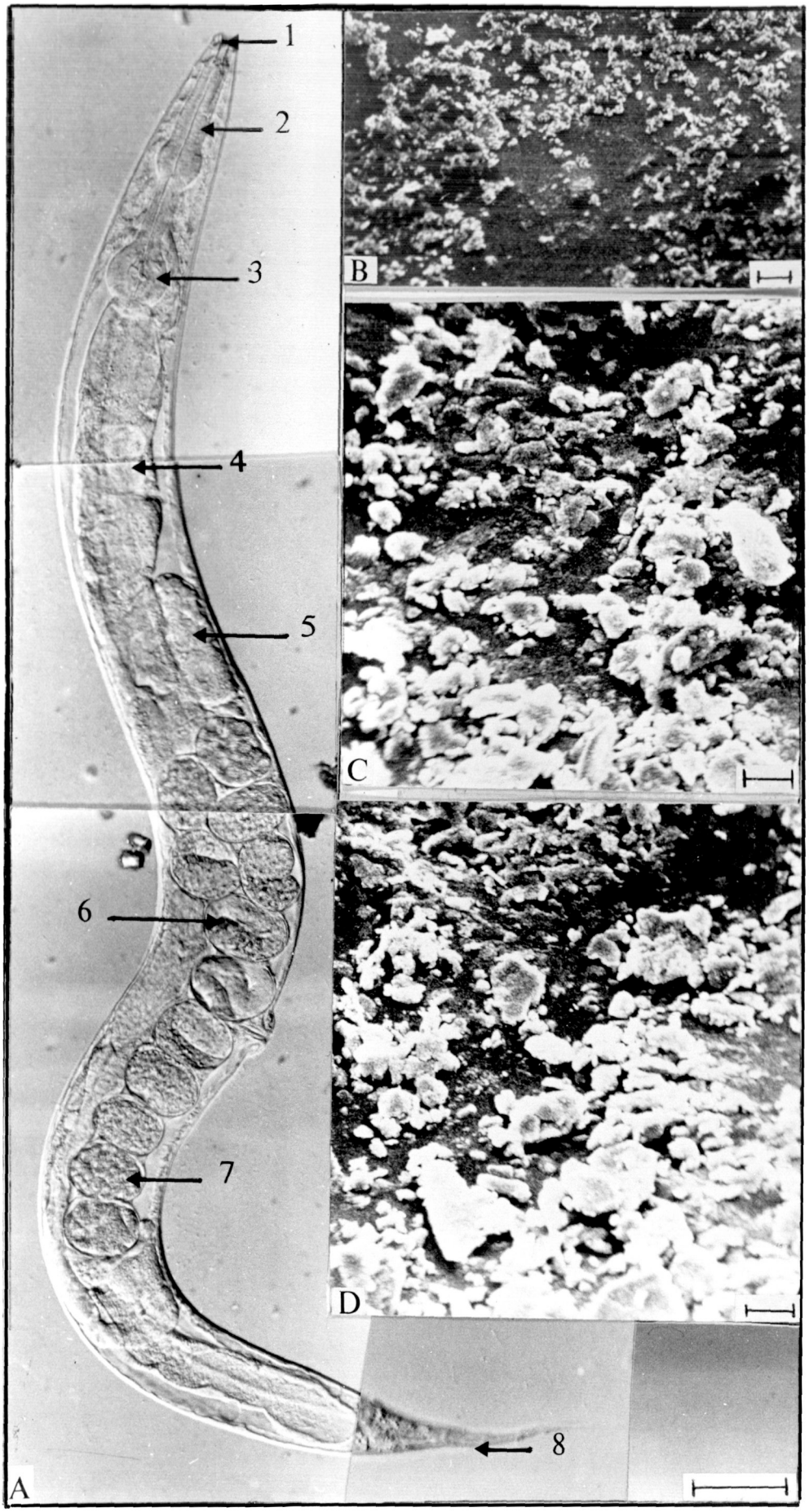
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A) Light micrograph, using Nomarski Interference contrast of an adult hermaphrodite of *Caenorhabditis elegans* (Maupus, 1900); x 300. Scale bar represents 50 µm. (1) Buccal cavity; (2) metacorpus of the pharynx; (3) terminal bulb of the pharynx; (4) intestine; (5) ovary; (6) uterus; (7) an embryonated egg; (8) tail.

B-D) Scanning electron micrographs showing particles of formulated diflubenzuron (25% w.p.). Particles show irregularity in shape and size (B), x 110; Scale bar represents 50 µm.

C) Particles of diflubenzuron formulation A, x 750; Scale bar represents 1.0 µm.

D) Particles of diflubenzuron formulation B, x 750; Scale bar represents 1.0 µm.


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I. GENERAL INTRODUCTION

Drugs and pesticides can be broadly classified by their mode of action into compounds acting on a) energy metabolism, b) synthetic processes and c) membrane function or transport processes (e.g. Corbett, 1974; Van den Bossche, 1976; Gutteridge and Coombs, 1977). The last group can be taken to include neuroactive compounds affecting excitable tissues. Amongst compounds used against animal parasitic helminths are examples of all three types of action (Van den Bossche, 1976; Coles, 1977) but the pesticides currently used against plant parasitic nematodes are either neuroactive (organophosphates and carbamates) or may affect energy metabolism (halogenated hydrocarbon fumigants) (Castro and Thomason, 1977; Wright, 1981).

Compounds affecting synthetic processes in plant parasitic nematodes have been lacking and could represent an important area of nematode biochemistry to be exploited in the search for new nematicides.

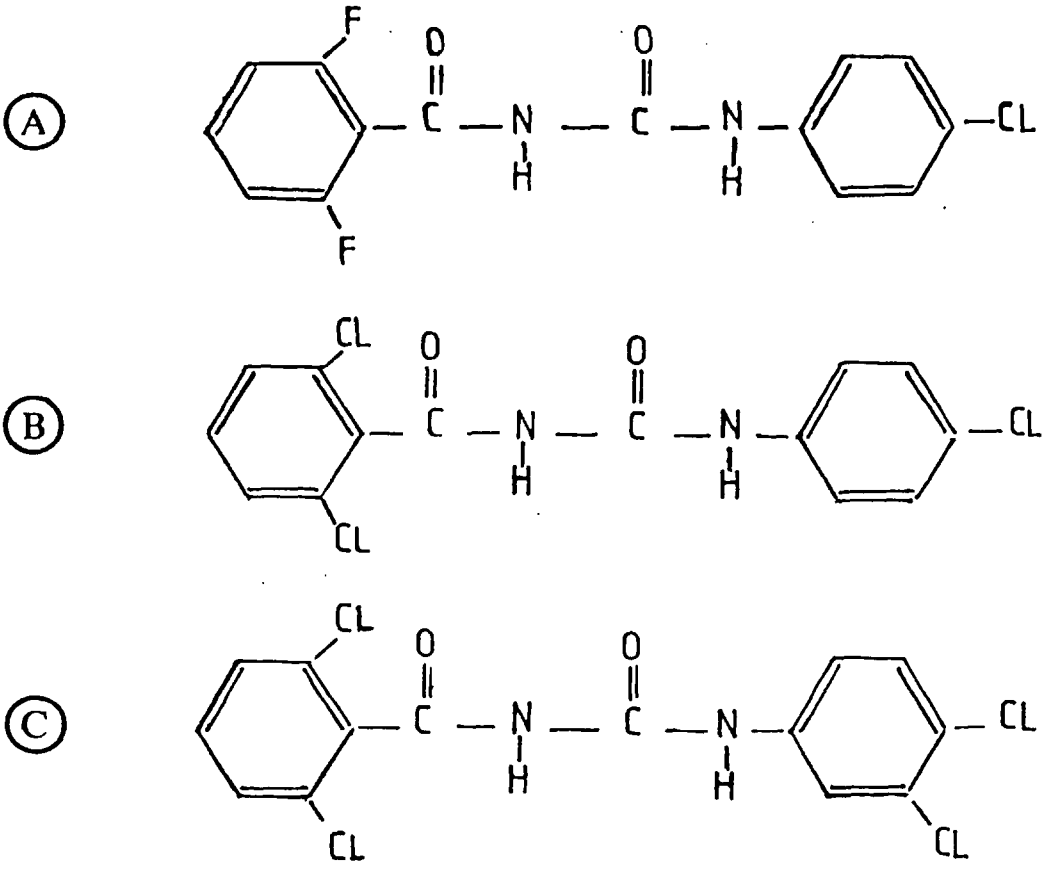
Recently, the insecticide diflubenzuron (Dimilin), known to interfere with chitin synthesis of insects (Wellinga *et al.*, 1973; Post *et al.*, 1974), has been suggested by Veech (1977; 1978a) as a potential nematicide. He found that infusion of diflubenzuron by organic solvents into seeds of cotton plant (*Gossypium hirsutum* L.) significantly reduced the number of egg masses of *Meloidogyne incognita* as well as the number of eggs per egg mass. Veech (1978b) also reported a significant reduction in population growth of the

free living nematodes *Pelodera* sp., *Acrobeloides* sp., and *Panagrellus redivivus* after treatment with 10 µg cm^{-3} diflubenzuron. Ibrahim (1978), similarly demonstrated a significant reduction in population growth and fecundity of *Caenorhabditis elegans* when treated with 5-80 µg active ingredient (a.i.) cm^{-3} of formulated diflubenzuron (wettable powder).

Treatment with diflubenzuron (PH 60-40) and its structural analogues PH 60-38 and DU19,111 (Figure 1) was reported to abolish the larval endocuticle of the insect *Pieris brassicae* (Mulder and Gizswizt, 1973; Post *et al.*, 1974). In insects the larval endocuticle consists mainly of chitin and protein integrated as glycoproteins (Hackman, 1971) and Ker (1977) concluded that as a result of a reduction in chitin content due to treatment with diflubenzuron the insect cuticle becomes structurally weaker. Subsequently, Deul *et al.* (1978) provided evidence that diflubenzuron acts at the final step of chitin synthesis by inhibiting chitin synthetase (Figure 2).

In nematodes, structural chitin only occurs in the median layer of the egg shell (Bird, 1976) and diflubenzuron is therefore believed to interfere with egg shell formation, a process critical to successful reproduction (Veech, 1978b). However, no direct evidence as to the mode of action of diflubenzuron against nematodes has so far been presented.

In the present study, the nematicidal action of diflubenzuron has been examined in some detail using the free-living nematode *Caenorhabditis elegans*.



(A) 1 - [4 - *chlorophenyl*] -3- [2, 6 - *difluorobenzoyl*] *urea*, PH60-40, Diflubenzuron.

(B) 1 - [4 - *chlorophenyl*] -3- [2, 6 - *dichlorobenzoyl*] *urea*, PH60-38.

(C) 1 - [2, 6 - *dichlorobenzoyl*] -3- [3, 4 - *dichlorophenyl*] *urea*, DU-19111

Figure 1. Structural formulae of Diflubenzuron and its analogues. (Ascher and Nemny, 1976).

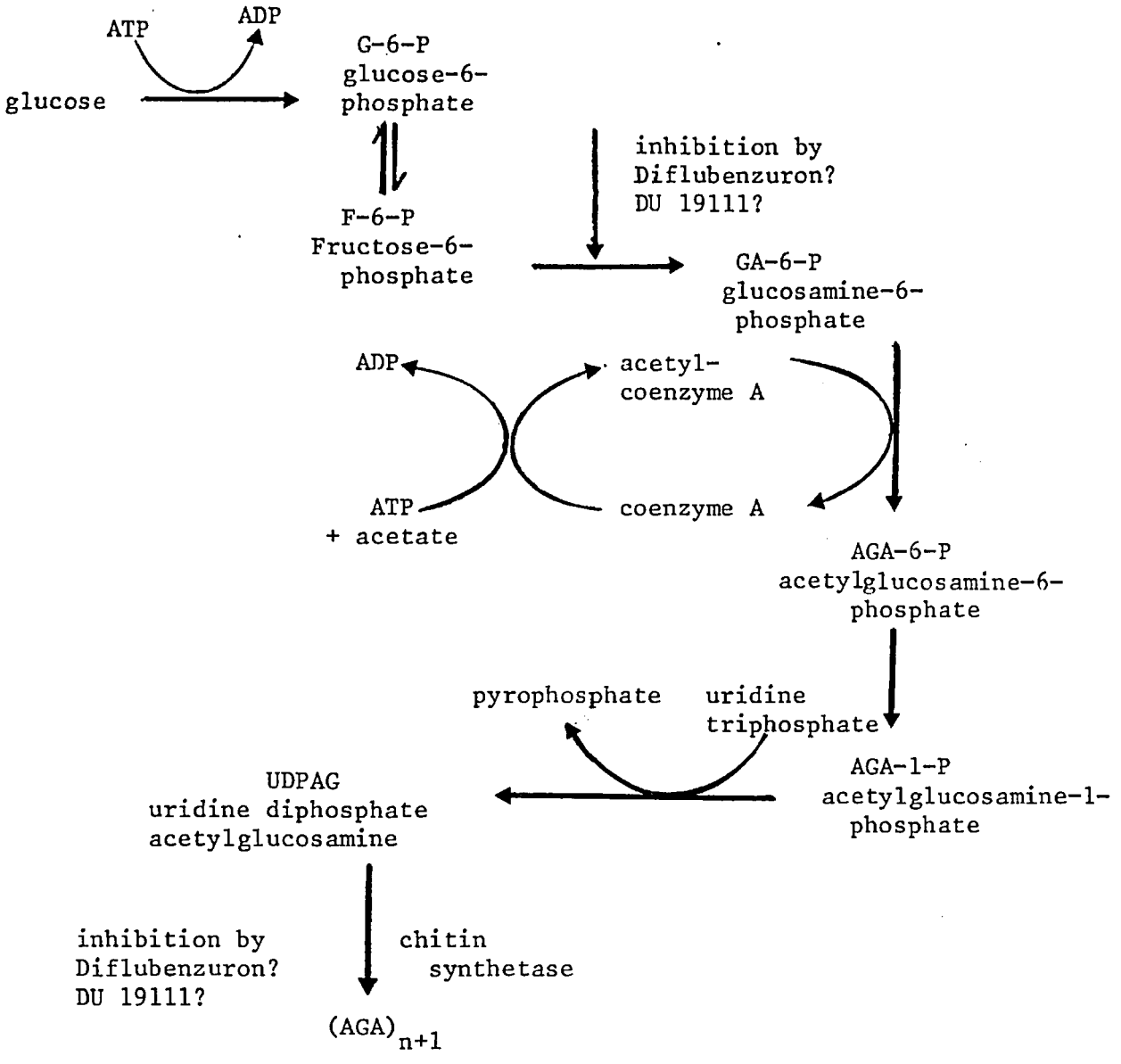


Figure 2. The biochemical pathway of chitin synthesis and the possible target sites of diflubenzuron action (Deul *et al.*, 1978).

II. *CAENORHABDITIS ELEGANS* AS AN EXPERIMENTAL ORGANISM

Caenorhabditis elegans (Dougherty, 1953) was used in the present investigation because of its convenience as an experimental animal and because of the large amount of work carried out on the genetics, development and biochemistry of this species in the last decade (Brenner, 1974; Riddle, 1978; Zuckerman, 1980). *C. elegans* was also suggested by Platzer *et al.* (1977) for use as a test organism for the development of nematicides and anthelmintics. It is a small nematode (the adult is about 1.2mm long and 70 µm in diameter) composed of about 600 somatic cells and with a transparent appearance; factors which make it ideally suited for ultrastructural and developmental studies (Vanderslice and Hirsh, 1976; Sulston and Horvitz, 1977; Riddle, 1978). *C. elegans* can also be readily cultured in a monoxenic state (with one associated organism) on agar with *Escherichia coli* (Brenner, 1974) and has a generation time of 4 days at 16°C and 3.5 days at 20°C (Brenner, 1974; Sulston and Horvitz 1977). The life span of this nematode is about 23 days at 16°C, 14 days at 20°C and 8 days at 25°C (Hirsh *et al.*, 1976).

C. elegans normally reproduces as a self-fertilizing hermaphrodite and thus also has the advantage of a relatively uniform genetical constitution as the animals are driven towards homozygosity (Brenner, 1974).

The reproductive system of a *C. elegans* hermaphrodite has two reflexed gonads, each of which consists of an ovary, oviduct,

spermatheca and a uterus (Hirsh *et al.* 1976). Males occur spontaneously in *C. elegans* cultures at a frequency of about one per 700 worms (Hodgkin, 1974). The females have a chromosomal constitution of 5AA + XX, the males have 5AA + XO with one less chromosome (Brenner, 1974). During larval development the number of non-gonidial nuclei increase from about 550 in the newly hatched J_1 (sexually immature) to about 810 in the mature hermaphrodite (Sulston and Horvitz, 1977). The adult hermaphrodite produces an average of 275 eggs at 25°C (Byerly *et al.*, 1976).

III. ACTION OF DIFLUBENZURON ON *CAENORHABDITIS ELEGANS*

The present study has been divided into five sections. The first section examines the effects of analytical grade diflubenzuron and two of its formulations on the population growth of *C. elegans* The next three sections investigate the toxic effects of diflubenzuron on various biological activities of *C. elegans*, including the processes of egg production and hatching. Ultrastructural and biochemical studies on the action of diflubenzuron on the egg shell of *C. elegans* are described in the final section together with studies on the uptake and distribution of diflubenzuron - ^{14}C.

SECTION I

The Effect of Diflubenzuron On Population Growth Of *C. elegans*

I.A. Introduction

In a preliminary study, Ibrahim (1978) reported that a formulation of diflubenzuron [Dimilin, 25% wettable powder] reduced the population growth of *C. elegans* by more than 60% at concentrations greater than 10 μg a.i.cm^{-3} and totally inhibited reproduction at 80 μg a.i.cm^{-3}. Similarly, Veech (1978b) found that after 10 days treatment with 10 μg cm^{-3} diflubenzuron populations of *Pelodera* sp. and *P. redivivus* were 5 and 50% of the control population, respectively.

In neither of the above studies were the effects of the formulation material incorporated with the active ingredient investigated. The aim of the present work was therefore to examine the nematicidal activity of analytical grade diflubenzuron on *C. elegans* compared to that of two wettable formulations of diflubenzuron and to the formulation material alone.

I.B. Materials and Methods

I.B.1. Culture of *C. elegans*

C. elegens var. Bristol (Strain N_2) was cultured with OP50 a uracil-requiring mutant of *E. coli* on a medium of NG agar (Brenner, 1974).

NG agar consists of :

NaCl	0.3 g
Bactoagar (Difco)	1.7 g
Bactopeptone (Difco)	0.25 g
Distilled water	97.5 cm^3
Cholestrol in ethanol (5mg/cm^{-3})	0.1 cm^3
1M $CaCl_2$	0.1 cm^3
1M $MgSO_4$	0.1 cm^3
1M K phosphate buffer (pH6.0)	2.5 cm^3

added in order and autoclaved.

Sterile Petri dishes (4.5 or 9.0 cm dia.) with NG agar were streaked with *E. coli* using sterile paper strips or flamed platinum wire and similarly inoculated with *C. elegans* after 24 or 48h. Stocks of bacteria and nematodes were maintained at 15 ± 1.0°C and 25 ± 1.0°C. Routine subculturing was carried out every 7-10 days.

I.B.2. The Effect of Analytical Grade Diflubenzuron on Population Growth of *C. elegans*

Stock solutions of diflubenzuron were prepared in acetone or dimethyl sulphoxide due to its low solubility in water (0.2 µg cm^{-3}). The toxicity of these two solvents has been reported to be minimal when applied to axenic cultures of *Aphelenchoides rutgersi* at 0.1 and 0.01% (v/v), respectively (Myers, 1972). These solvents have been also used previously in toxicological studies with diflubenzuron on both insects and nematodes (Mulder and Gizswizt, 1973; Ker, 1977; Veech, 1978a).

Standard solutions of 0.025, 0.05, 0.1, 0.2 and 0.4% (w/v) of

analytical grade diflubenzuron (purity 95-99%, I.C.I. Plant Protection Ltd.,) in acetone or dimethyl sulphoxide (2 cm^3) were added to 98 cm^3 of autoclaved NG agar kept at 45°C and rapidly mixed to make 5, 10, 20, 40 and 80 µg a.i. cm^{-3} solutions of diflubenzuron. Controls of pure NG agar and NG agar with acetone and dimethyl sulphoxide (2% v/v) were also prepared. Pesticide-agar suspensions were then poured into sterile 45 mm diameter plastic Petri dishes and once solidified the agar inoculated with *E. coli* OP50 and incubated at 25°C. After 48h. three second stage juveniles (J_2) of *C. elegans* (Plate I.1) collected from 3-5 day old stock cultures were transferred to each plate. The number of adult and juvenile nematodes on each plate was recorded after 4 and 5 days at 25°C. Each treatment was replicated five times.

I.B.3 The Effect of Formulated Diflubenzuron on Population Growth of *C. elegans*

I.B.3.a Measurement of Particle Size

Two samples (A and B) of a 25% wettable formulation of diflubenzuron were used, one of which (A) had been pulverized (Supplied by I.C.I, Plant Protection Ltd.) to reduce the average particle size.

To measure the distribution of particle sizes in the formulations one or two drops of each formulation in agar (40 µg a.i. cm^{-3}) were placed on a microscope slide and covered with a coverslip (20 x 60 mm). The slides were then examined on an Olympus compound microscope attached to a Fleming Particle Size Analyser, Type 526 (Barnett and Timbrell, 1962; Matthews, 1975) with ten channels (A-J),

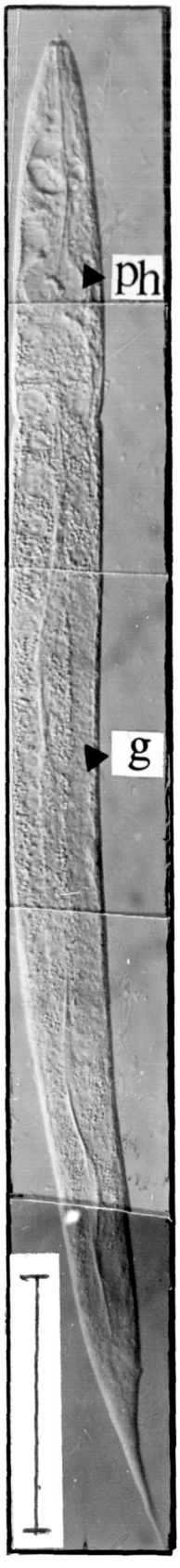


Plate I.1. Second stage juvenile of *C. elegans* (about 600 µm). Scale bar line represents 100 µm. (Ph, pharynx; g, gonad).

each of which was calibrated to a particular shear size. The minimum and maximum particle sizes present were determined, being verified by simple micrometry and these were then classified as channels A and J respectively. The particles on each slide were then counted and recorded in their corresponding channel. About 1,000 particles were measured for each formulation.

I.B.3.b Calculation of the Number Median Diameter (NMD)

The method adopted by Matthews (1975) for measuring droplet size was recommended for diflubenzuron particles (G.A. Matthews, personal communication). The mean diameter within each range (Appendix I.2) was used to calculate the number median diameter (NMD). The number of particles in each class (A-J) was recorded and cumulative percentages plotted on log probability paper against the mean diameter. NMD values were read at the 50 percent intersect and the particle size distribution recorded.

I.B.3.c Results

The number median diameters (NMD) for formulations A and B estimated from Figure I.1., were 1.8 µm and 2.0 µm respectively ($P>0.05$). The distribution of particle sizes in formulations A and B were shown to vary significantly ($P<0.01$, Figure I.2., Appendix I.2).

I.B.3.d The Effect of Diflubenzuron Formulations A and B on Population Growth of *C. elegans*

Samples of formulations A and B were mixed thoroughly with

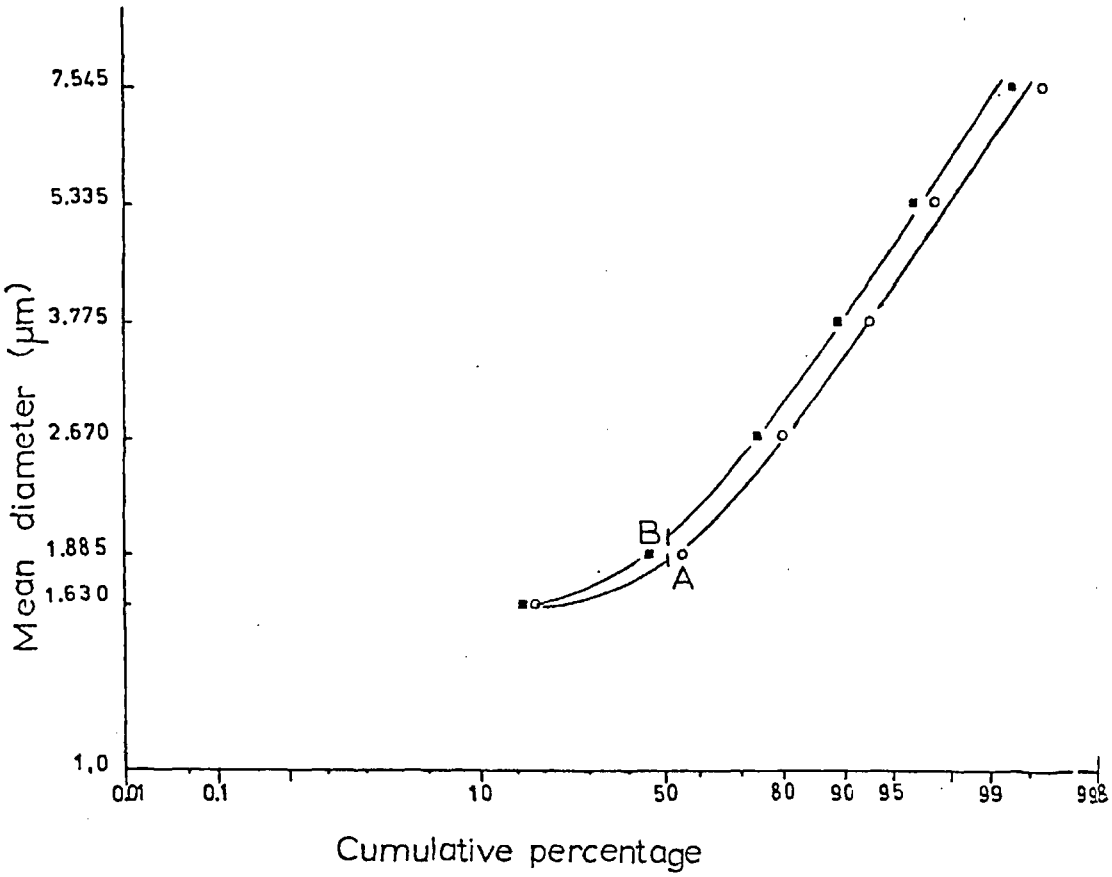


Figure I.1. The number median diameter of particles (NMD) in diflubenzuron from formulations A and B at 50 percent interesect.

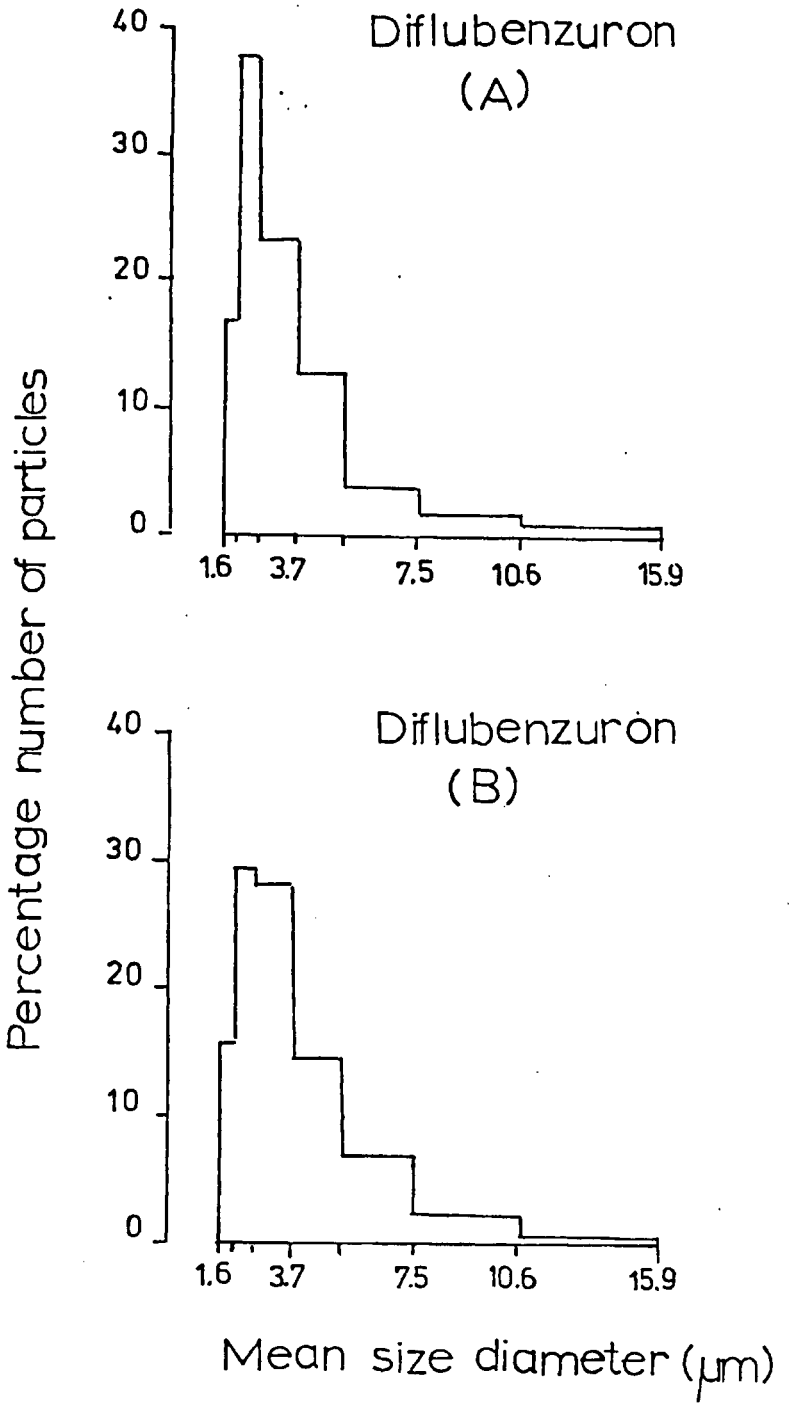


Figure I.2. Distribution of particles in formulations A and B of diflubenzuron, (X^2 = 24.7, P < 0.01).

100 cm^3 of molten autoclaved NG agar at 45°C to give uniform suspensions of 5, 10, 20 and 40 µg a.i. cm^{-3} diflubenzuron. The agar suspensions were then poured into sterile Petri dishes and plates inoculated with *E. coli* OP50 as described earlier. After 48h at 25°C J_2 *C. elegans* were added and the population size recorded after 4 and 5 days. In a separate experiment control agar plates with 0, 15, 30, 60, 120 and 240 µg cm^{-3} of inert carrier for wettable formulations (supplied by I.C.I., Plant Protection Ltd.,) were prepared corresponding to the equivalent amounts of this carrier incorporated with the formulated diflubenzuron tested above. The population growth of *C. elegans* on plates with diflubenzuron carrier was determined after 3, 4 and 5 days.

In both experiments each treatment was replicated five times.

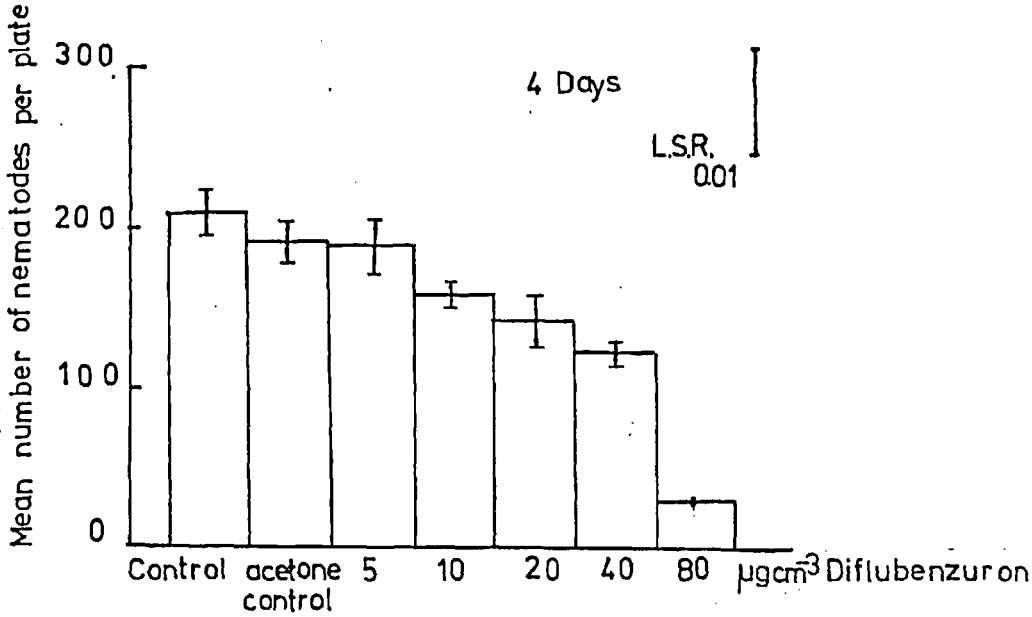
I.C. Results

I.C.1 The Effect of Analytical Grade Diflubenzuron on Population Growth of *C. elegans*

C. elegans populations treated with 0-80 µg diflubenzuron cm^{-3} (in 2% v/v acetone or dimethyl sulphoxide) were compared using Duncan's multiple range test. The data obtained is summarized in Figures I.3 and 4.

There was no significant difference between the mean number of nematodes in the plain agar, or acetone and dimethyl sulphoxide controls ($P>0.05$) after 4 or 5 days treatment (Figure I.3,4). The population growth of *C. elegans* was, however, significantly retarded ($P<0.01$) in plates containing 20-80 µg diflubenzuron cm^{-3} acetone or

Figure I.3. The effect of analytical grade diflubenzuron in acetone on population growth of *C. elegans* after 4 and 5 days treatment.
Bar lines = ± standard error.
$L.S.R._{(0.01)}$ = least significant range, P = 0.01.


Mean number of nematodes per plate
300
200
100
0
4 Days
L.S.R.
0.01
Control
acetone control
5
10
20
40
80
µgcm⁻³ Diflubenzuron

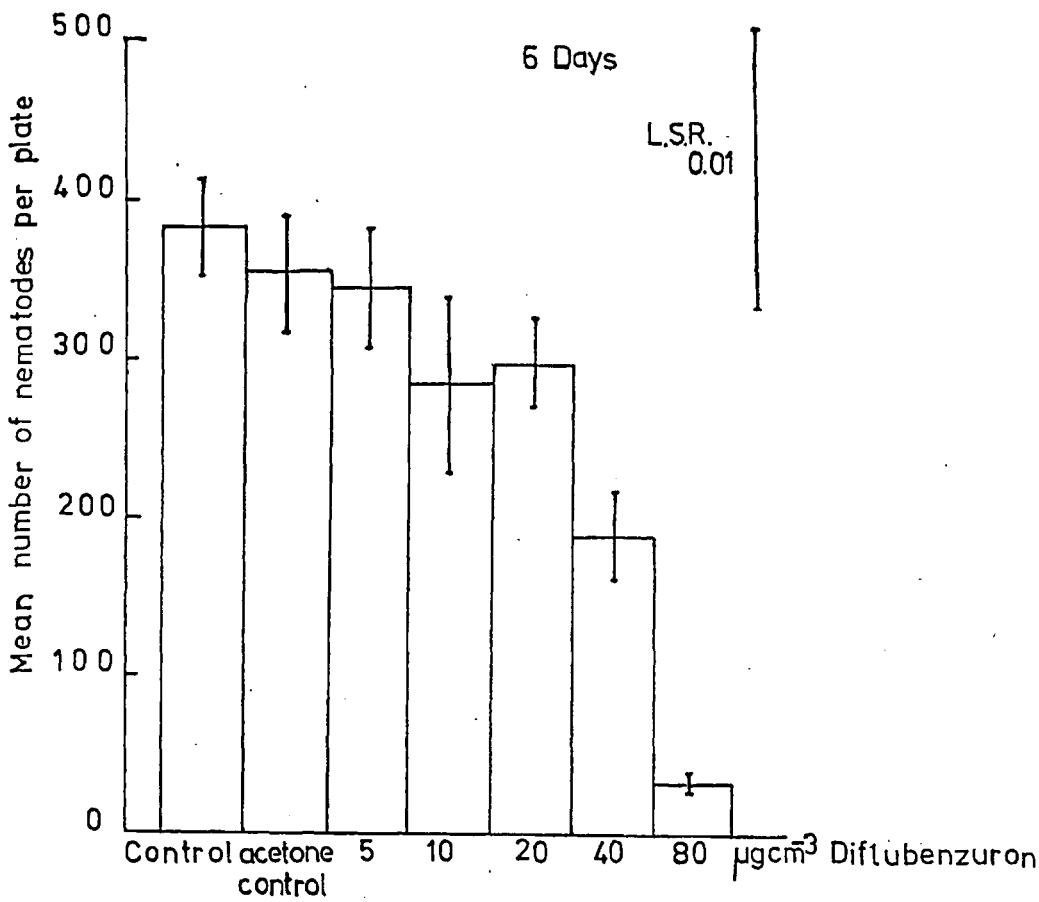
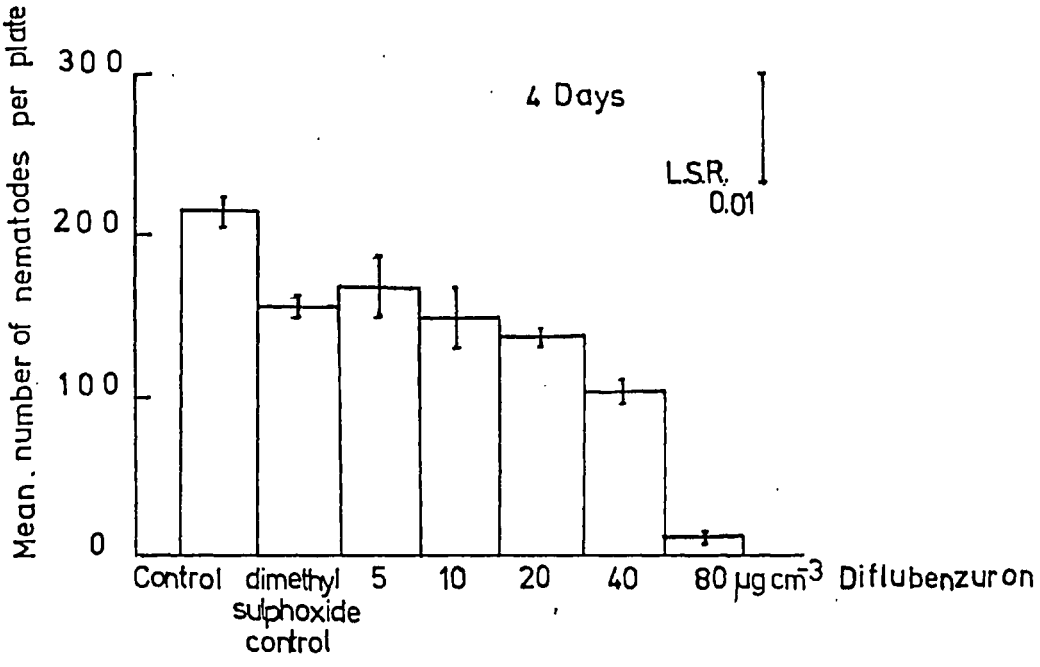
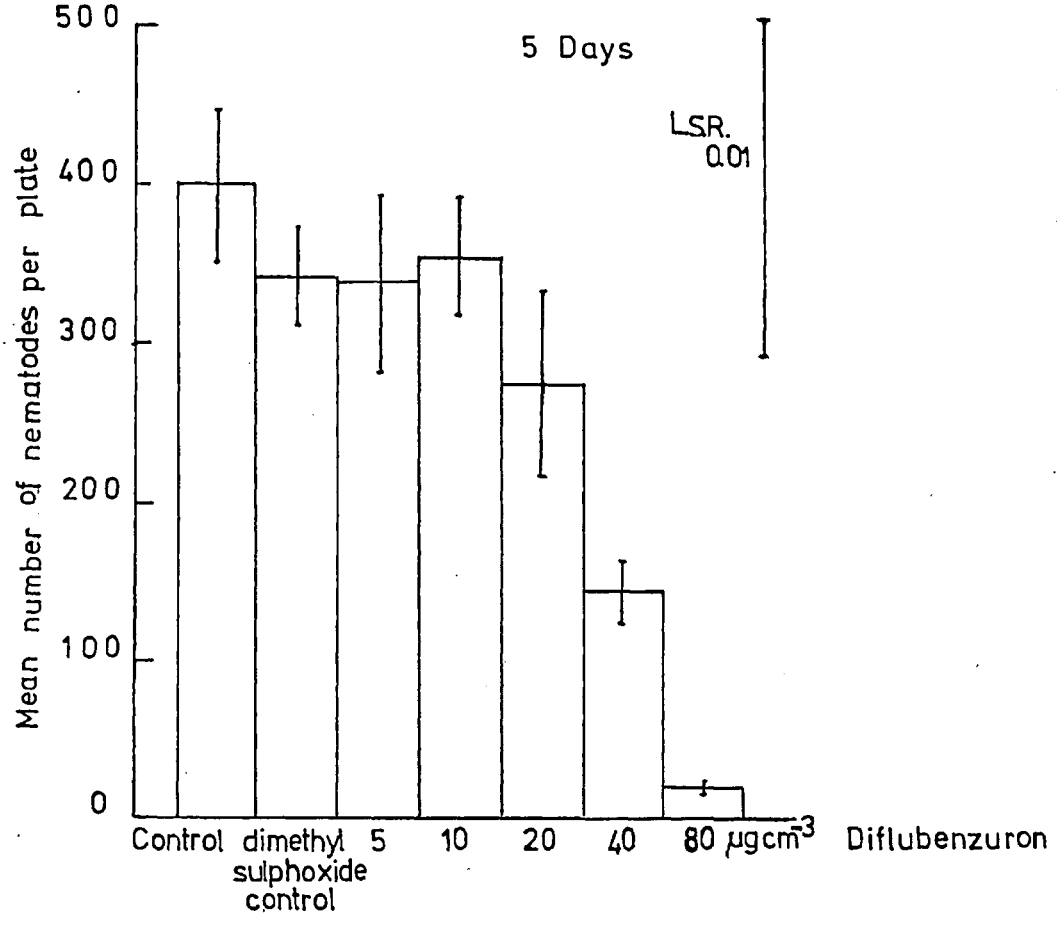

Mean number of nematodes per plate
500
400
300
200
100
0
6 Days
L.S.R.
0.01
Control
acetone control
5
10
20
40
80
µgcm⁻³ Diflubenzuron

Figure I.4. The effect of analytical grade diflubenzuron in dimethyl sulphoxide on population growth of *C. elegans* after 4 and 5 days treatment. Bar lines = ± standard error.

L.S.R. = Least significant range, P = 0.01 .
(0.01)


Mean. number of nematodes per plate
300
200
100
0
4 Days
L.S.R.
0.01
Control
dimethyl sulphoxide control
5
10
20
40
80 µg cm⁻³ Diflubenzuron


Mean number of nematodes per plate
500
400
300
200
100
0
5 Days
L.S.R.
0.01
Control
dimethyl sulphoxide control
5
10
20
40
80 µg cm⁻³ Diflubenzuron

dimethyl sulphoxide after 4 days treatment. After 5 days treatment, a significant reduction in population size was only observed with 40 and 80 µg diflubenzuron cm^{-3} acetone or dimethyl sulphoxide ($P<0.01$; Figure I.3,4). In 80 µg diflubenzuron cm^{-3} acetone, for example, population was reduced by 85% compared to the acetone control.

Percentage changes in population, calculated according to Myers (1972) are plotted in Figure I.5.

I.C.2 The Effect of Diflubenzuron Formulations A and B on Population Growth of *C. elegans*

Population growth of *C. elegans* was not significantly affected compared to control by treatment with 15 - 240 µg cm^{-3} diflubenzuron carrier ($P>0.05$; Figure I.6). However, population growth was significantly reduced in all diflubenzuron treatments ($P<0.01$; Figure I.7) although there was no significant difference between the effects of equivalent concentrations of formulations A and B ($P>0.05$; Figure I.7).

The effects of various treatments of diflubenzuron upon population growth of *C. elegans* are summarised in Table I.1.

The percentage reduction in population growth appeared to be greatest (62 - 99%) in treatments with wettable formulations of diflubenzuron (5-40 µg cm^{-3}). The EC_{50} values for diflubenzuron were estimated from regression equations (Table I.1, Appendix I.7); the EC_{50} for analytical grade of diflubenzuron being more than 40 times the value for the wettable formulation.

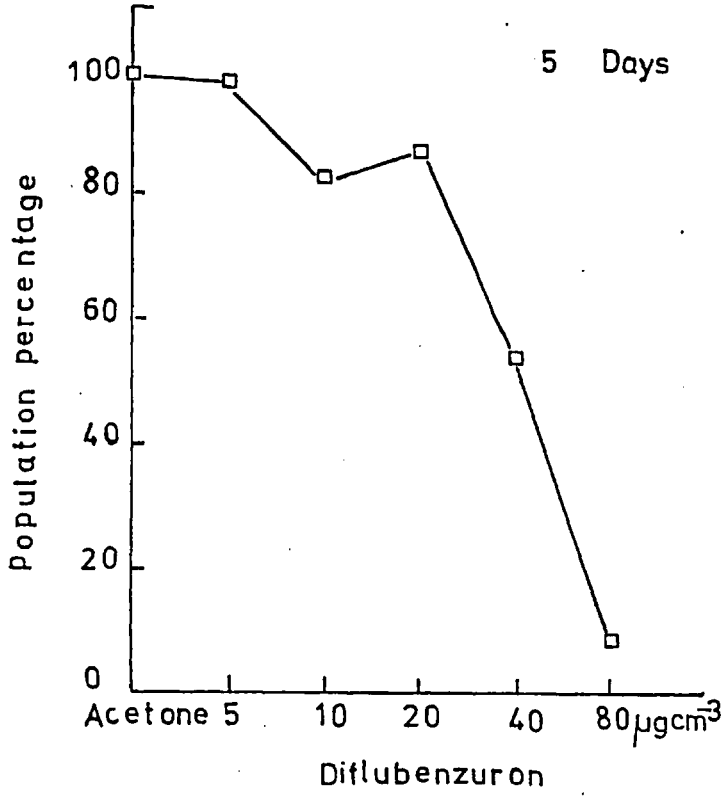
Figure I.5. Population growth of *C. elegans*, expressed as population percentage, after 5 days treatment with analytical grade diflubenzuron in acetone or dimethyl sulphoxide.

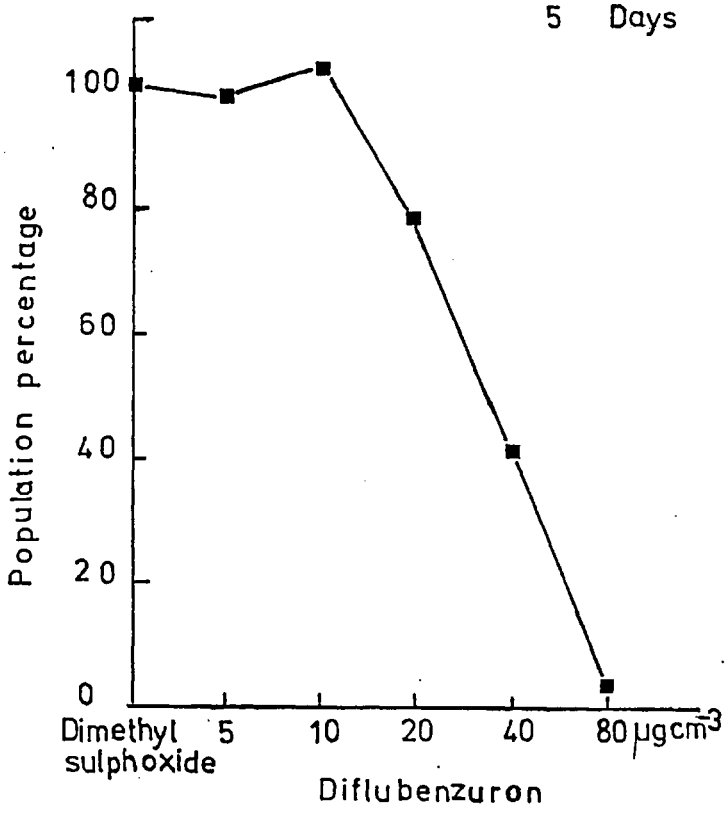
$$\text{Population percentage} = \frac{T - N}{C} \times 100$$

T = total number of living and dead treated nematodes.

N = number of nematodes originally inoculated.

C = net increase of nematode numbers in control treatment.


5 Days
Population percentage
100
80
60
40
20
0
Acetone 5
10
20
40
80 µgcm⁻³
Diflubenzuron


5 Days
Population percentage
100
80
60
40
20
0
Dimethyl sulphoxide
5
10
20
40
80 µgcm⁻³
Diflubenzuron

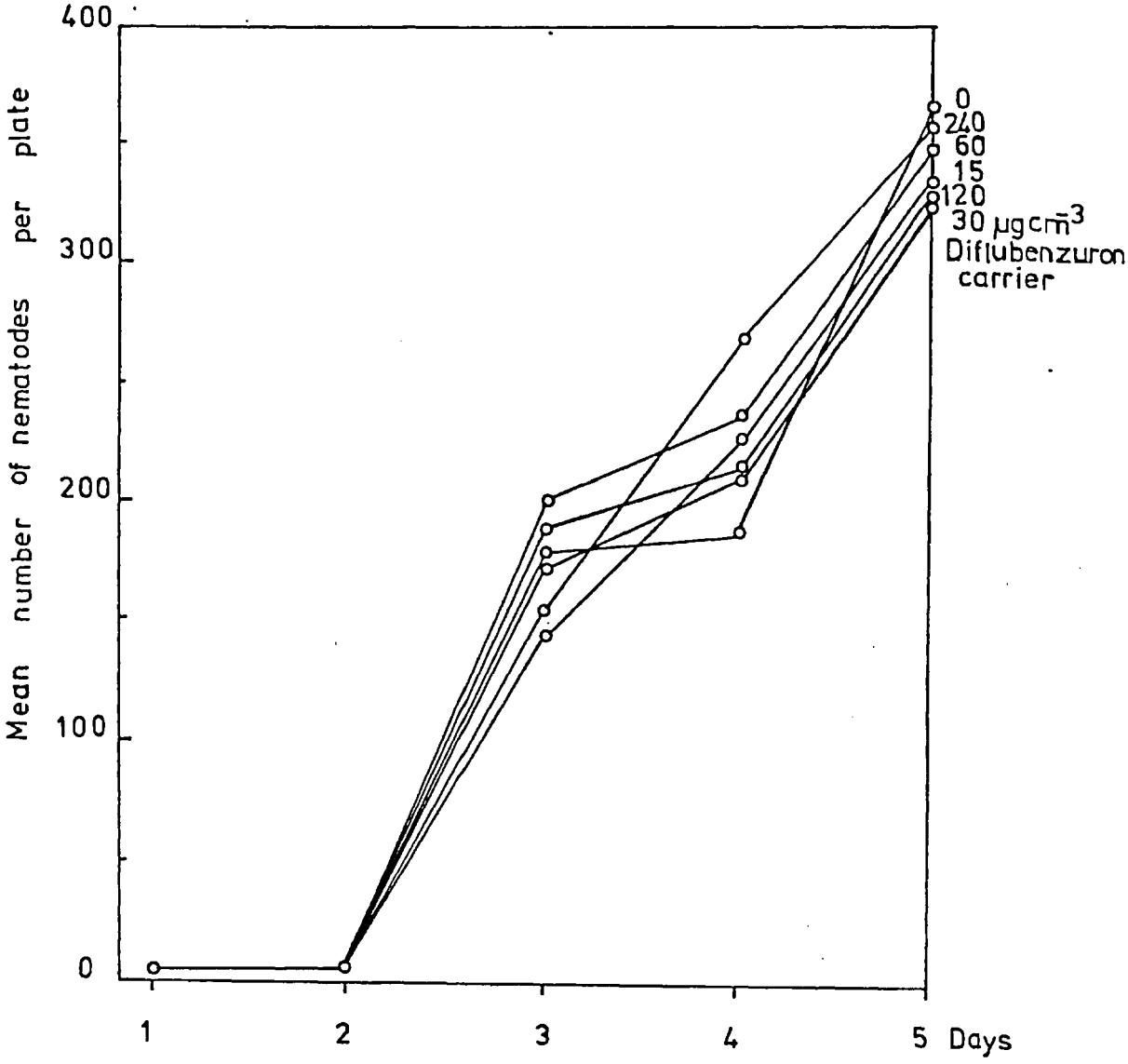
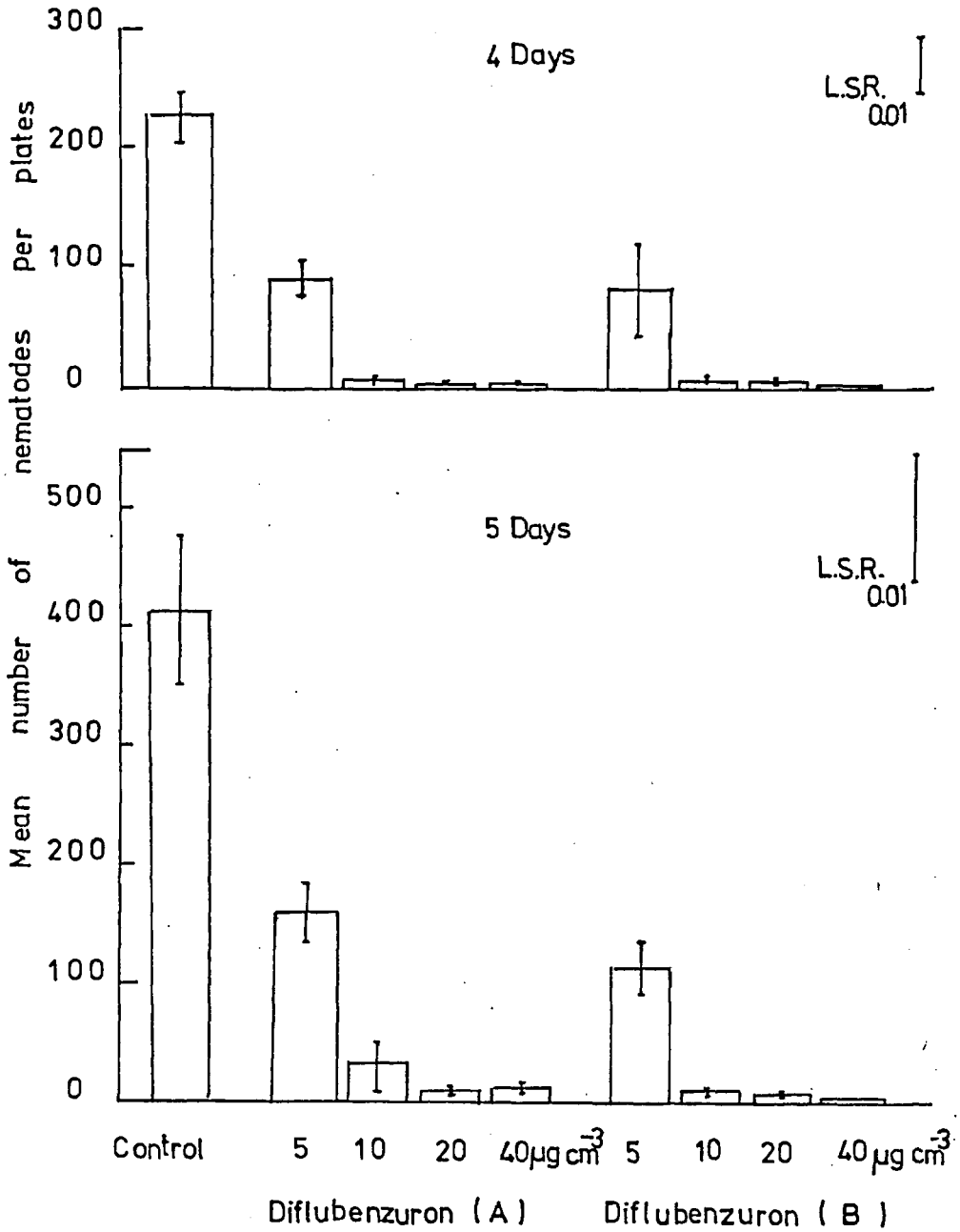


Figure I.6. The effect of diflubenzuron carrier for wettable formulations on population growth of *C. elegans*.

Figure I.7. The effect of diflubenzuron formulations A and B on population growth of *C. elegans* after 4 and 5 days treatment.

Bar lines = ± standard error.

L.S.R. $_{(0.01)}$ = Least significant range, P = 0.01.


4 Days
L.S.R. 0.01
5 Days
L.S.R. 0.01
Mean number of nematodes per plates
300
200
100
0
500
400
300
200
100
0
Control
5
10
20
40µg cm⁻³
5
10
20
40 µg cm⁻³
Diflubenzuron (A)
Diflubenzuron (B)

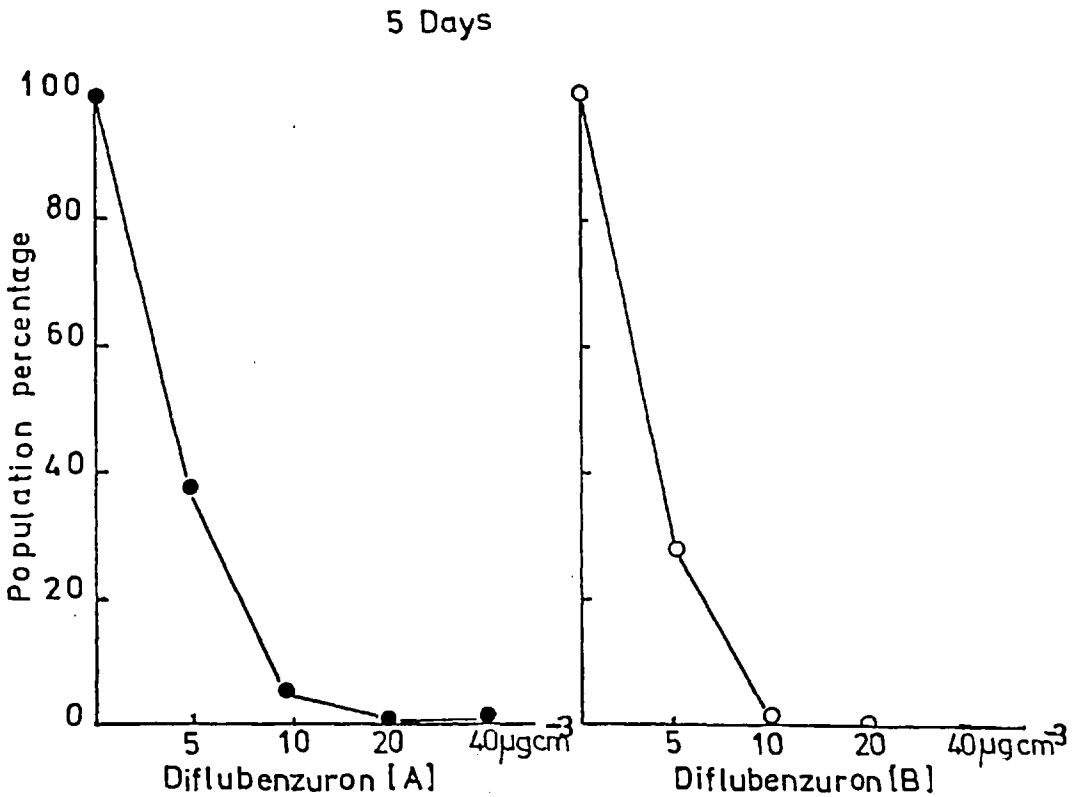


Figure I.8. Population growth of *C. elegans*, expressed as population percentage, after 5 days treatment with diflubenzuron formulations A and B. (see Figure I.5. for calculating population percentage).

Table I.1. Regression Analysis of the Effect of Different Diflubenzuron Treatments on Population Growth of *C. elegans*†

Treatment	Regression equation Y = a+bx	R^2 *	EC_{50} (µg cm^{-3} **)
Diflubenzuron in acetone	Y = -0.61 + 1.4x	0.85	44.6
Diflubenzuron in dimethyl sulphoxide	Y = -1.38 + 1.85x	0.89	46.0
Diflubenzuron formulation (A)	Y = 1.69 + 0.21x	0.69	1.1
Diflubenzuron formulation (B)	Y = 1.8 + 0.14x	0.65	0.2

† Percentages of population reduction were transformed into Log (1 + x) and analysed.

* R^2 = Square of ordinary correlation coefficient, Coefficient of determination.

** EC_{50} = The concentration of chemical used in treatment that results in a 50% reduction of the nematode population compared to the control treatment (Myers, 1972).

SECTION II

Effects of Diflubenzuron on the Biology of *C. elegans*

II.A. Introduction

Pesticides have been reported to affect nematodes in a number of ways. Many organophosphate and carbamate nematicides have been shown to impair movement and feeding of nematodes, thereby affecting development, reproduction, hatching and invasion (Evans, 1973; Nelmes *et al.*, 1973; Mcleod and Khair, 1975; Hague, 1979; Evans and Wright, 1982). On the other hand, benzimidazole anthelmintics do not affect movement directly but have been shown to prevent the growth of *C. elegans* (Platzer *et al.*, 1977) and of adult *Heterodera avenae* and *Globodera rostochiensis* (Cook and York, 1972; Nelmes *et al.*, 1973). Various antimetabolites and growth inhibitors (6- Azauridine, 5- Bromo, 2- Deoxycytidine, Azauracil, Uracil, Uridine) also appear to affect the development of the nematode (Bird and McGuire, 1966; Endo and Schaffer, 1967).

The toxicity of candidate nematicides has been assessed using various techniques. Taylor *et al.* (1957) observed the motility and mortality of nematodes, whereas Keetch (1974) examined nematode posture, feeding activity and dispersal. Other workers have studied the invasion of roots by plant parasitic nematodes and their subsequent development (Cook and York, 1972; Bunt, 1975; Wright and Womack, 1981) while Myers (1972) and Samoiloff (1980) examined nematode reproduction by fecundity experiments and cytological observations on

nematode gonads.

The present study used some of these techniques to investigate the effects of the most active diflubenzuron formulation (B, NMD=2.0 µm) in the population growth experiments (Section I.C.2) on juvenile and adult stages of *C. elegans*.

II.B Materials and Methods

II.B.1 Effect of diflubenzuron on development of *C. elegans*

Eggs of *C. elegans* were collected from 5 day old cultures and batches of 25 - 30 eggs transferred onto NG agar plates containing 0, 5, 10, 20, 40 and 80 µg diflubenzuron cm^{-3}. After 24, 48 or 72h at 25°C nematodes were collected individually using a fine Pasteur pipette with a bulbous head. These nematodes were mounted on thin films of 5% (w/v) agar on microscope slides, narcotized in a drop of 0.25% (v/v) propylene phenoxetol (Ellenby and Smith, 1964) and observed under a light microscope. Body and pharyngeal length and the maximum body width (at the vulval region) were recorded. Five nematodes from each treatment were examined at each time interval.

II.B.2 Effect of diflubenzuron on survival of *C. elegans*

J_2 of *C. elegans* were collected from 3 or 5 day old cultures and batches of 25 - 30 juveniles transferred onto NG agar plates containing 0, 5, 10, 20, 40 and 80 µg diflubenzuron cm^{-3}. The % nematodes surviving was recorded after 48h at 25°C. Each treatment was replicated five times.

II.B.3 Effect of Diflubenzuron on Feeding of *C. elegans*

J_2 *C. elegans* were treated with 0 - 80 µg diflubenzuron cm^{-3} as described above and the rate of feeding of each nematode was determined after 72h at 25°C. Each nematode was transferred into a drop of water on a microscope slide which had been previously coated with a fine layer of 1% (w/v) ion agar smeared with *E. coli* OP50. The rate of pharyngeal pumping was then recorded over a 2 min period. Eight nematodes from each treatment were examined.

Groups of nematodes (25 - 30) were also examined *in situ* on agar plates using an inverted microscope with a X10 objective. The percentage of nematodes exhibiting a pumping motion of the pharynx in 5 sec was determined as described by Byerly *et al.* (1976)

II.B.4 Effect of Diflubenzuron on Movement of *C. elegans*

Individual adult nematodes from diflubenzuron-treated agar plates (0 - 40 µg a.i. cm^{-3}) were transferred onto a thin film of 1.5% (w/v) agarose (0.1 cm^3) on a glass slide. After 30 min tracks produced by the nematodes were photographed and measured using an x-y digitiser (FERRANTI Cetec) linked to a computerised drawing board. This method has previously been found to be more accurate and less time consuming than using calibrated dividers (Turner, 1980). Five nematodes were examined for each treatment.

II.B.5 Effect of Diflubenzuron on Reproduction of *C. elegans*

II.B.5.a Fecundity

Groups of 25 - 30 J_2 of *C. elegans* were inoculated onto NG agar plates containing 0, 5, 10, 20 µg diflubenzuron cm^{-3} as described previously. After 48h at 25°C, juveniles from each treatment were divided into two groups; nematodes in one group were transferred individually to fresh plates containing the same concentration of diflubenzuron, the other group were transferred to clear agar plates. The fecundity of both groups was recorded after a further 48h. For each treatment 24 nematodes were examined.

II.B.5.b Susceptibility of different juvenile stages of *C. elegans* to diflubenzuron

Five J_2 or J_4 *C. elegans* were inoculated onto plates containing 0, 10 or 20 µg diflubenzuron (cm^{-3}). Each treatment was replicated five times. The fecundity of each group of nematodes was observed for 48h after sexual maturation at 25°C.

II.B.5.c Measurement of reproductive tracts

Batches of 20 - 30 J_2 *C. elegans* were treated on NG agar plates containing 0, 10, 20 and 40 µg diflubenzuron cm^{-3}. Ten plates were prepared for each treatment. After 24, 48 and 72h at 25°C, 10 - 15 nematodes were narcotized in 0.25% (v/v) propylene phenoxetol and the reproductive tract of each nematode photographed and measured using a Leitz Dialux microscope equipped with Nomarski (Differential Interference Contrast) optics.

II.B.5.d Histochemical studies on the effect of diflubenzuron on gonadogensis in *C. elegans*

(1) Feulgan's stain

The method of Ward and Miwa (1978) was used in a slightly modified form. Adult nematodes from cultures treated with 0 - 40 µg diflubenzuron cm^{-3} for 72h were collected and transferred into centrifuge tubes (10 cm^3) containing 1 cm^3 of a fixative made of equal parts of 95% (v/v) ethyl alcohol and a saturated aqueous solution of mercuric chloride (Triantaphyllou, 1962). Fixation was carried out for two h at room temperature. The nematodes were then concentrated by low speed centrifugation for 1 - 2 min and this centrifugation process repeated after each of subsequent step. Specimens were first hydrated in 95%, 90%, 70%, 50% (v/v) ethyl alcohol, and distilled water (10 min in each). The samples were hydrolysed in 5N HCL for 30 min at room temperature, stained in Schiff's reagent for two h and rinsed in 10% (w/v) sodium bisulphite (Pantin, 1969). The nematodes were then dehydrated in alcohols and finally transferred carefully into a solid watchglass containing xylene. After clearing nematodes were mounted in Canada balsam and examined by light microscopy. In both treated and untreated hermaphrodites the different phases of gonadial growth and the meiotic maturation divisions of oocytes were observed. The number of oocytes at late diplotene and diakinesis was determined as an indication of the state of oogenesis. Spermatogenesis was assessed by recording the presence of the sperm in the reproductive tract (see Ward and Miwa, 1978; Nelson *et al.*, 1978). About 15 nematodes

from each treatment were stained. In a second experiment, nematodes which had been treated with 80 µg diflubenzuron cm^{-3} were examined.

(2) Fluorescent staining with Hoechst 33258

C. elegans were treated with 0 or 40 µg diflubenzuron cm^{-3} for 72h. Adult nematodes were then stained by a slight modification of the procedure described by Herman *et al.* (1976). Nematodes were gently washed in a watch glass with 1 - 2 cm^3 distilled water and centrifuged as described above. The nematodes were resuspended in a fixing solution made of ethyl alcohol : acetic acid : chloroform (6:3:1 v/v) for 1½ - 2h at room temperature. Specimens were then treated for 10 min in each of the following solutions: 0.15M NaCl, 0.03M KCl, and 0.01 M phosphate buffer (pH 7.0), with centrifugation of the nematodes between each step. The nematodes were finally treated with Hoeschst 33258 (1 µg/cm^3) for 5 min. Stained nematodes were kept in distilled water until examination under a Zeiss fluorescence microscope using excitation filter UGII and barrier filters 47 and 65. The nuclear divisions of oocytes in treated and untreated hermaphrodites were examined and photographed using Ilford HP5 film.

II.C Results

II.C.1 Effect of Diflubenzuron on Development of *C. elegans*

Measurements of *C. elegans* treated with diflubenzuron while at different stages of post-embryonic development are given in Figure II.1. and Appendix II.1.

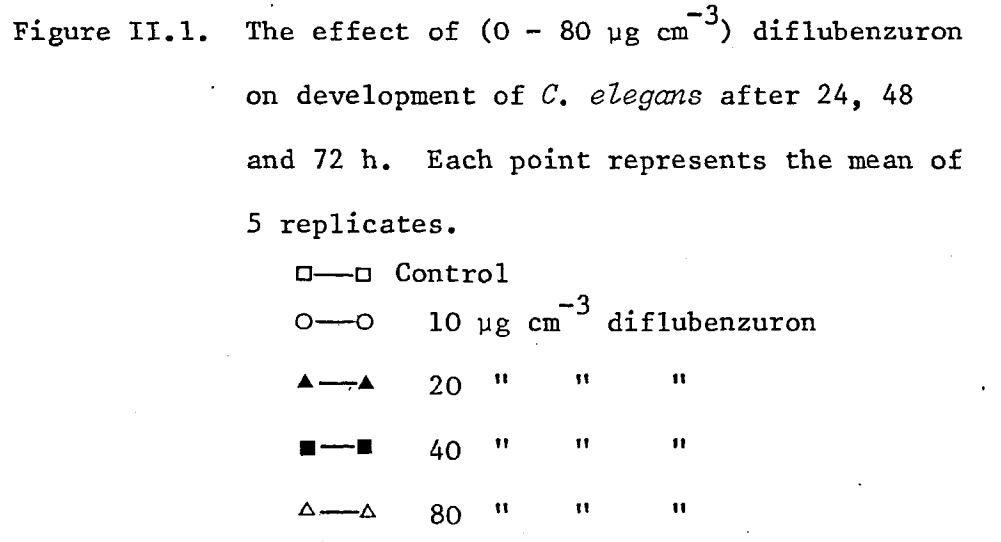
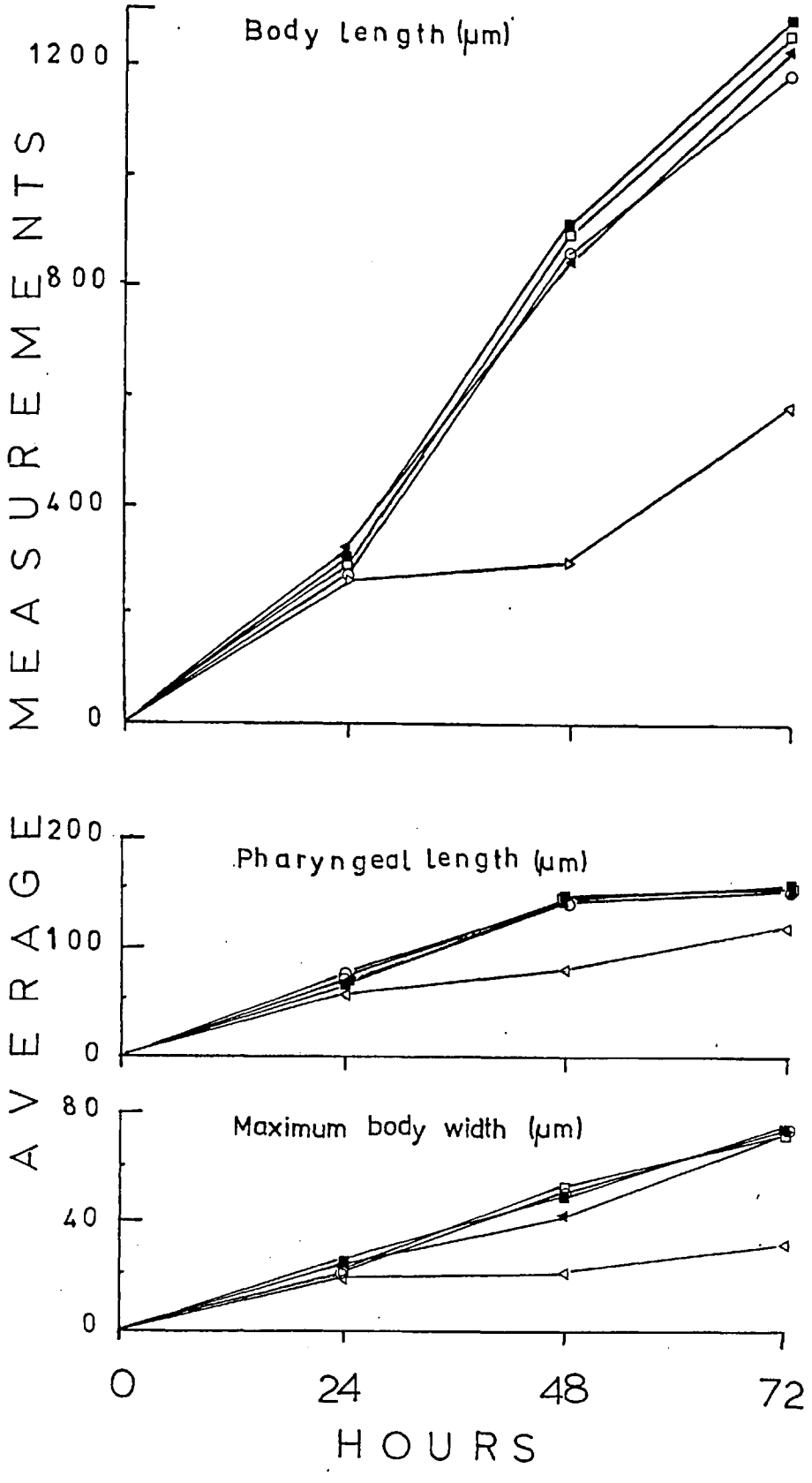


Figure II.1. The effect of (0 - 80 µg cm^{-3}) diflubenzuron on development of *C. elegans* after 24, 48 and 72 h. Each point represents the mean of 5 replicates.


Body length (µm)
1200
800
400
0
MEASUREMENTS
Pharyngeal length (µm)
200
100
0
AVERAGE
Maximum body width (µm)
80
40
0
0
24
48
72
HOURS

In the first 24 h of treatment, *C. elegans* juveniles were able to develop normally in all concentrations of diflubenzuron, but after 48 and 72h treatment nematode development was significantly reduced ($P<0.01$) in 80 μg cm^{-3} diflubenzuron. Measurement of the total body length, pharynx length and vulval width of diflubenzuron-treated (80 μg cm^{-3}) *C. elegans* after 48h represented about 33%, 66% and 43% of the corresponding measurements in control nematodes.

The ratio of the total body length : pharyngeal length appeared to increase as the nematodes developed to maturation in control medium or in 10, 20, 40 μg cm^{-3} diflubenzuron, whereas a significant reduction in this ratio was observed in nematodes treated with 80 μg cm^{-3} diflubenzuron (Table II.1.A). The proportion of body length: maximum body width was unchanged in all treatments of diflubenzuron ($P>0.05$; Table II.1.B).

II.C.2 Effect of Diflubenzuron on Survival of *C. elegans*

Concentrations of diflubenzuron up to 80 μg cm^{-3} had no significant effect on nematode survival ($P>0.05$; Table II.2).

II.C.3 Effect of Diflubenzuron on Feeding of *C. elegans*

Treatment with 10 or 20 μg cm^{-3} diflubenzuron did not significantly affect feeding activity of *C. elegans* ($P>0.05$), whereas 40 and 80 μg cm^{-3} diflubenzuron significantly reduced feeding activity ($P<0.05$ and $P<0.01$, respectively; Figure II.2). However, the percentage of nematodes feeding was not significantly affected by any treatment ($P>0.05$; Table II.3).

Table II.1. The Effect of Diflubenzuron on Development of *C. elegans*

A. Total body length : pharyngeal length

Treatment / Hours	Control	Diflubenzuron ($\mu g\ cm^{-3}$)			
		10	20	40	80
24	3.4	3.3	3.7	3.5	3.2
48	6.5	6.3	6.3	6.5	4.1 *
72	8.1	7.8	8.3	7.8	4.9 *

* Significantly different at $P<0.05$.

B. Total body length : maximum body width

Treatment / Hours	Control	Diflubenzuron ($\mu g\ cm^{-3}$)			
		10	20	40	80
24	13.6	13.6	13.8	13.7	13.4
48	16.9	17.2	21.2	17.7	16.1
72	17.1	15.7	16.9	16.4	17.1

Table II.2 Effect of Diflubenzuron on Survival of *C. elegans*

Treatment	Control	Diflubenzuron (µg cm^{-3})				
		5	10	20	40	80
Mean percentage of survival (n=5)	91.5	86.0	83.3	79.6	82.9	82.5
Range	85-100	76-100	69-93	76-90	64-92	72-89
Means of transformed * values ± S.E.	1.31 ±0.07	1.31 ±0.07	1.28 ±0.09	1.14 ±0.05	1.16 ±0.03	1.16 ±0.03

*Percentages were transformed to angular values (arcsine$\sqrt{p}$)

Table II.3 Effect of Diflubenzuron on Feeding of *C. elegans*

Treatment	Control	Diflubenzuron (µg cm^{-3})			
		10	20	40	80
Mean percentage of nematodes feeding (n=4)	95.3	95.0	90.0	82.8	70.8
Range	88-100	77-100	84-100	71-100	64-76
Means of transformed * values ± S.E.	1.42 ±0.09	1.42 ±0.09	1.29 ±0.7	1.18 ±0.14	1.0 ±0.29

*(arcsine$\sqrt{p}$)

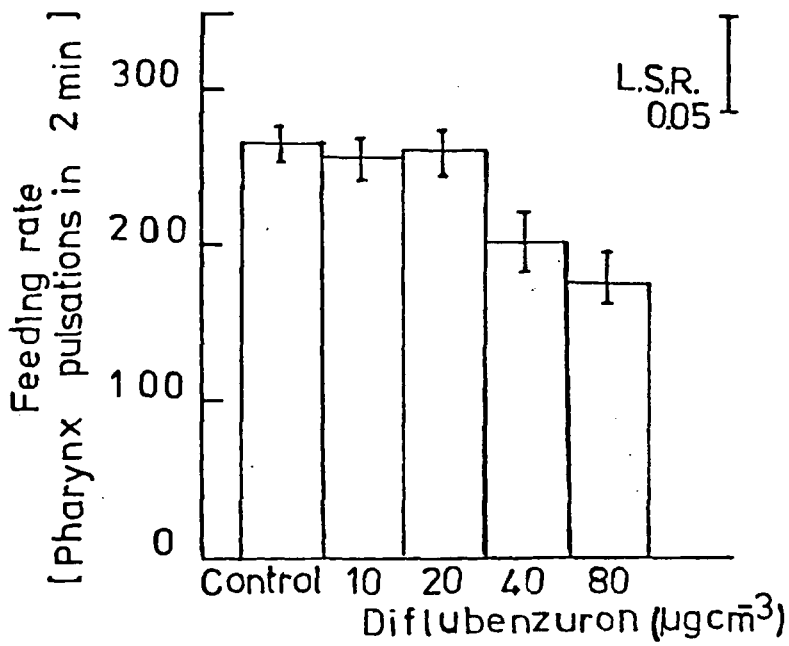


Figure II.2. The effect of diflubenzuron on the feeding rate of *C. elegans*.

Each column represents the mean number of pharyngeal pulsations in 2 min (n = 8).

Bar lines = ± standard error.

L.S.R. (0.05) = Least significant range, P = 0.05.

II.C.4 Effect of Diflubenzuron on Movement of *C. elegans*

Locomotion of *C. elegans* was not affected by 10 - 40 μg cm^{-3} diflubenzuron but was significantly retarded by 80 μg cm^{-3} ($P<0.05$; Figure II.3).

Control nematodes and others treated with 10 - 40 μg cm^{-3} diflubenzuron typically produced sinusoidal tracks in the agarose (Figure II.4). In contrast, tracks made by nematodes treated with 80 μg cm^{-3} diflubenzuron had a flatter shape with fewer sinusoidal waves (Figure II.4.E); the average distance travelled by these nematodes was about 25% of the distance travelled by control nematodes.

II.C.5 Effect of Diflubenzuron on Reproduction of *C. elegans*

II.C.5.a Fecundity of *C. elegans*

After 48h treatment with 20 μg cm^{-3} diflubenzuron the fecundity of *C. elegans* hermaphrodites was found to be significantly reduced ($P<0.01$; Figure II.5), treatment with 5 or 10 μg cm^{-3} diflubenzuron had no significant effect on fecundity ($P>0.05$).

The fecundity of *C. elegans* was significantly affected by all concentrations of diflubenzuron (5 - 20 μg cm^{-3}) when the nematodes were treated for 96h ($P<0.01$; Figure II.5); the number of progeny produced by nematodes on plates containing 5, 10 and 20 μg cm^{-3} diflubenzuron being 44, 15 and 9%, respectively, of the number produced by untreated nematodes (Figure II.5). A total inhibition

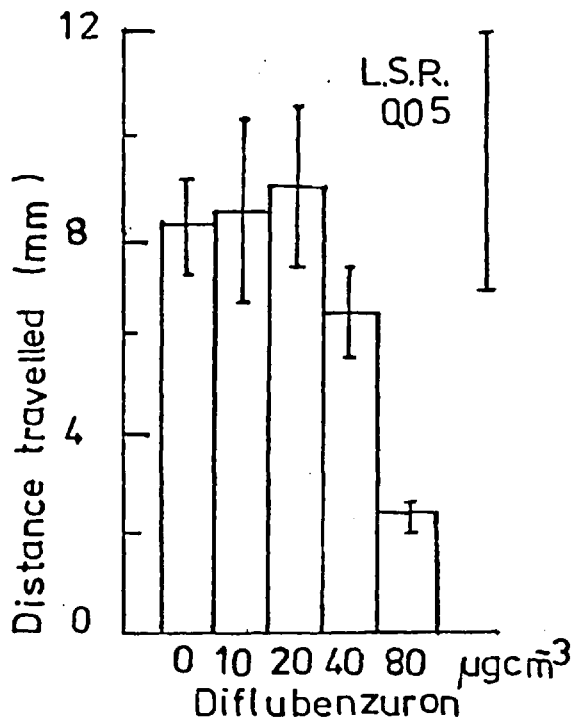


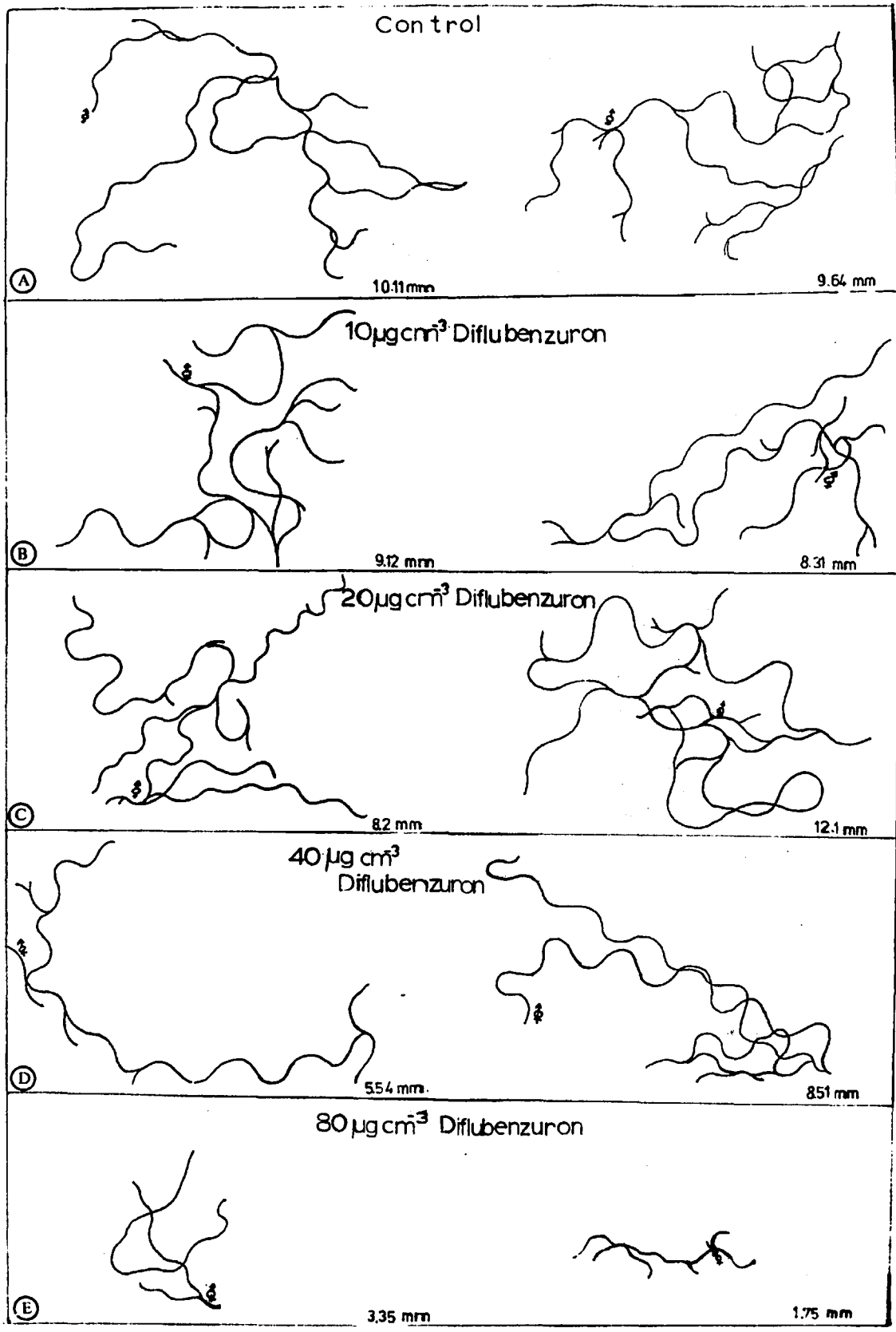
Figure II.3. The effect of diflubenzuron on locomotion of *C. elegans*.

Each column represents the average distance travelled by treated nematodes in 25 - 30 min, (n = 5).

Bar lines = ± standard error.

$L.S.R._{(0.05)}$ = Least significant range, P = 0.05.

Figure II.4. showing representative tracks of control and diflubenzuron-treated *C. elegans* to illustrate the effect of diflubenzuron on the nematode movement.


Control
A
10.11 mm
9.64 mm
10µg cm⁻³ Diflubenzuron
B
9.12 mm
8.31 mm
20µg cm⁻³ Diflubenzuron
C
8.2 mm
12.1 mm
40 µg cm⁻³
Diflubenzuron
D
5.54 mm
8.51 mm
80 µg cm⁻³ Diflubenzuron
E
3.35 mm
1.75 mm

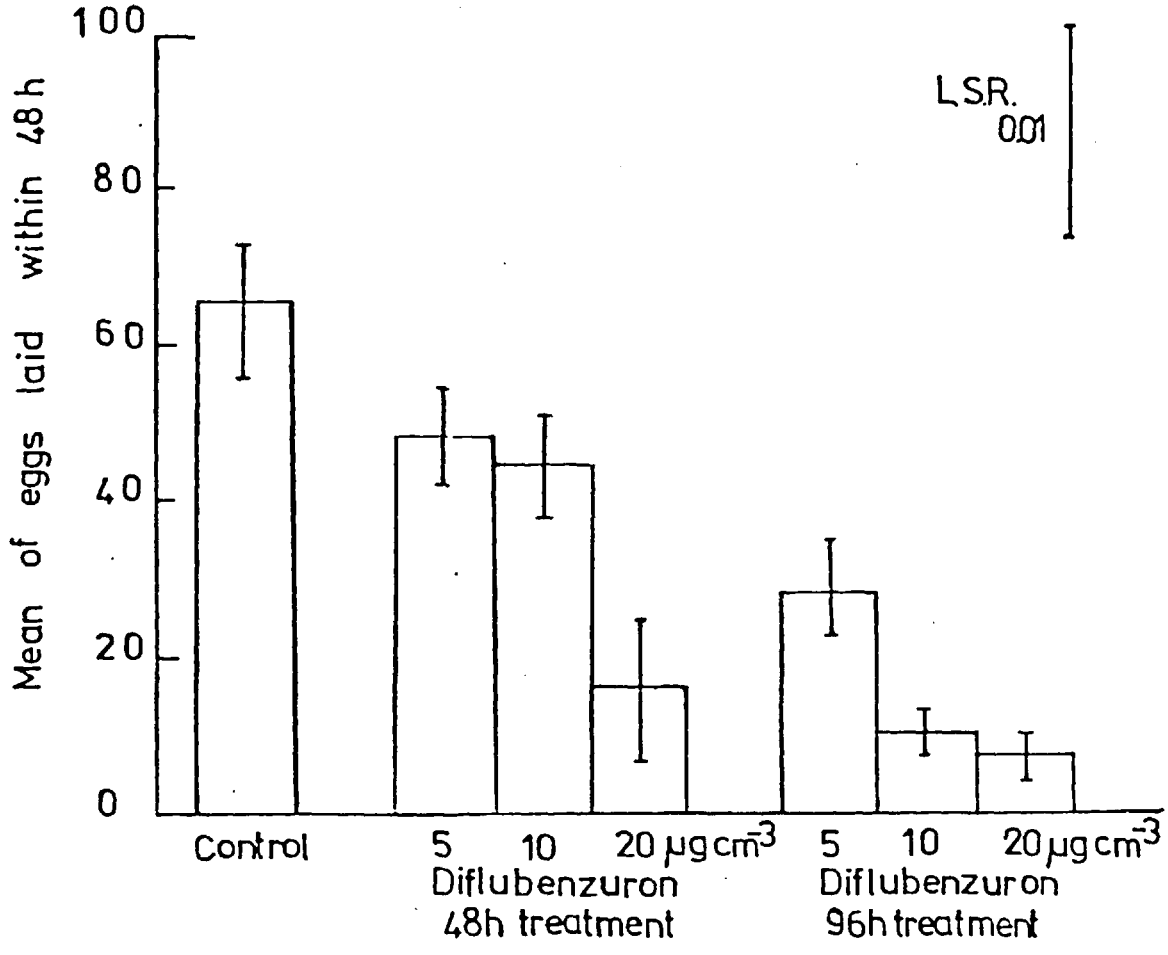


Figure II.5. Effect of diflubenzuron on fecundity of *C. elegans*.

Each column represents the mean of 24 replicates.

Bar lines = ± standard error.

L.S.R.$_{(0.01)}$ = Least significant range at P, 0.01.

of egg production was observed in some individuals treated with 20 μg cm^{-3} diflubenzuron for 48 and 96h (see Appendix II.5).

II.C.5.b Susceptibility of different juvenile stages of *C. elegans* to diflubenzuron

The number of eggs produced by nematodes treated with 10 and 20 μg cm^{-3} diflubenzuron from either the J_2 or the J_4 stage was not significantly different ($P>0.05$) but both were significantly less than in control nematodes ($P<0.01$; Table II.4, Appendix II.6).

Table II.4. Fecundity of *C. elegans* treated with diflubenzuron from different stages during post-embryonic development

Treatment	Mean number of eggs/plate ± S.E.		Mean number of eggs/nematode	
	J_2	J_4	J_2	J_4
Control	267±57.1	-	53	-
Diflubenzuron 10	118±16.5	104±6.3	24	25
(μg cm^{-3}) 20	72±12.2	91±21.8	14	18

J_2 = Nematodes treated from the second juvenile stage.

J_4 = Nematodes treated from the fourth juvenile stage.

II.C.5.c Measurement of reproductive tracts

The reproductive system of *C. elegans* consists of two u- shaped genital tubes with a didelphic form (Bird, 1971). Each tube consists of an ovary which turns 180° to join an oviduct on the ventral side

of the nematode. The oocytes produced in the ovary pass down the oviduct through the spermatheca to be fertilized. The spermatheca is a small flexible tube formed of approximately 24 cells between the oviduct and the uterus. The uteri of *C. elegans* terminate at the vulva in the mid part of the ventral side (Hirsh *et al.*, 1976).

The development of the reproductive system in normal and diflubenzuron-treated *C. elegans* is shown in Figure II.6. After 72h the reproductive system of untreated nematodes is propagated from a group of 10 - 16 cells with length of 47±18 µm to end with mature gonads measuring about 20X the initial length (1006±238 µm).

The reproductive system of *C. elegans* appeared to grow normally when the nematodes were treated in 20 or 40 µg cm^{-3} diflubenzuron (Figure II.6) but growth was significantly suppressed in 80 µg cm^{-3} diflubenzuron ($P<0.01$) where after 72h treatment the gonads were approximately 60% of the length of control gonads (Figure II.6). The development of the reproductive tract appeared to be affected evenly in all parts of the reproductive tract (Appendix II.7).

II.C.5.d. Histochemical studies on the effects of diflubenzuron on gonadogenesis of *C. elegans*

(1) Feulgan's stain

Feulgan stained gonads of *C. elegans* examined in this study appeared similar to those described by Hirsh *et al.* (1976), Ward and Miwa (1978) and Nelson *et al.* (1978).

The oviduct of *C. elegans* hermaphrodites can be distinguished

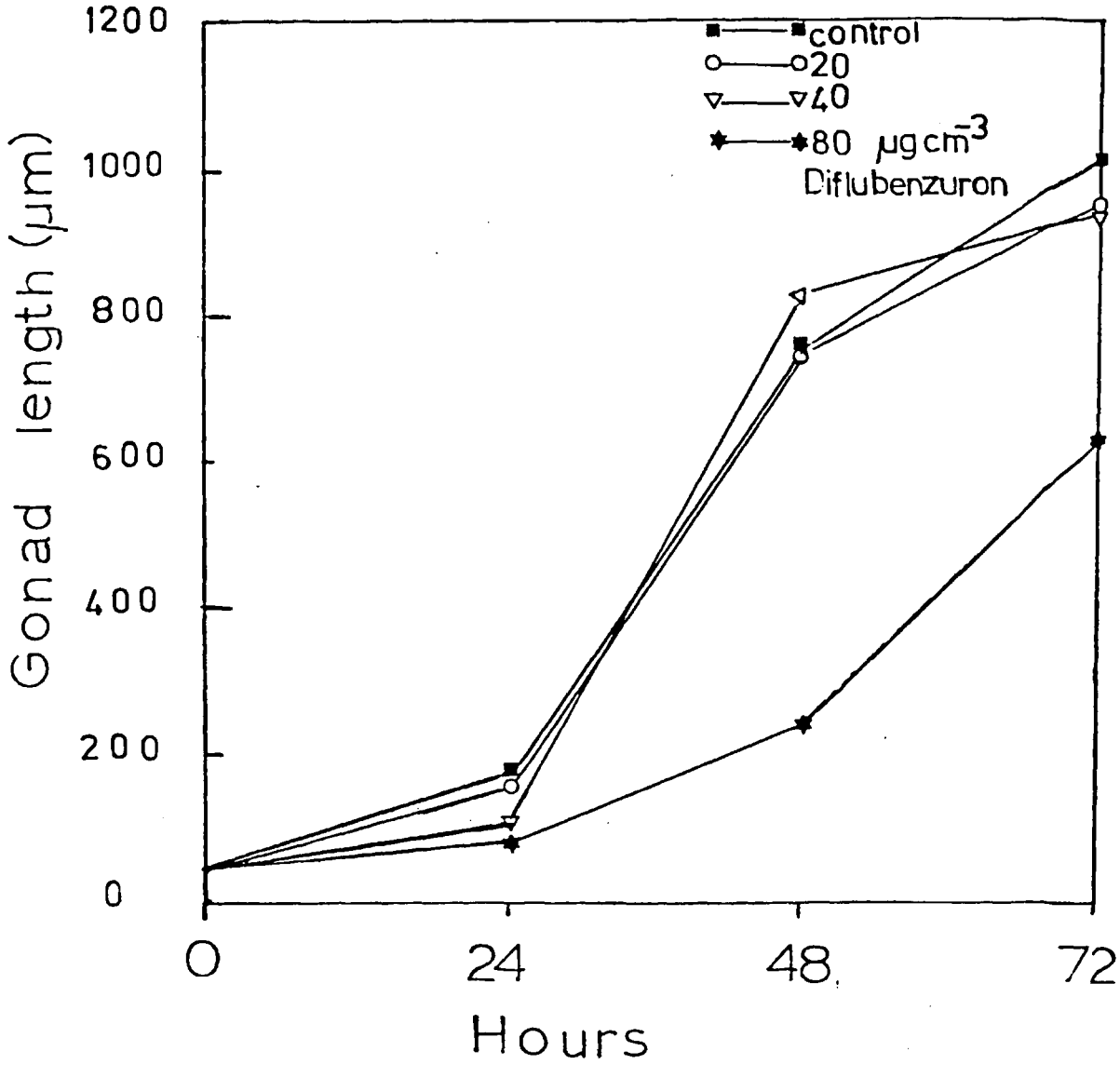


Figure II.6. Effect of diflubenzuron on the growth of *C. elegans* gonads (n = 10).

into a germinal zone and a maturation or growth zone. About 4 - 7 oocytes were visualized across the diameter of the ovary core (about 40 µm) at the germinal zone but the number of oocytes decreased as the oocytes proceeded towards the uterus.

The mitotic oogonial divisions are believed to take place in the germinal zone of the ovaries (see Plate II.1.A,B) and completed at the loop region of the ovotestis (Hirsh *et al.*, 1976). Meiotic division begins in the loop region and continues progressively in the growth zone. The oocytes pass successively through the leptotene, zygotene and pachytene stages of meiotic division culminating in the diplotene and diakinesis stages prior to fertilization (Plates II.1.C and II.2.A).

In the present work observations were conducted mainly on the late meiotic oocytes, where detailed examination was possible (Table II.5). Mature oocytes have much larger nuclei (15 µm dia) than those of oocytes in the germinal and early growth phases (5 µm dia; Plate II.2.A).

A total of 87, 60 and 111 oocytes arrested at late diplotene and diakinesis stages were observed in nematodes treated with 20 and 40 µg cm^{-3} diflubenzuron and control medium, respectively (Table II.5; Appendix II.8).

The total number of arrested meiotic oocytes in *C. elegans* hermaphrodites in 20 and 40 µg cm^{-3} diflubenzuron declined by 22% and 52%, respectively compared to normal nematodes, although these reductions were not significant ($P>0.05$). Similarly, observations

Plate II.1. Feulgan stained ovary of a control untreated *C. elegans*; x 500. The germinal zone of an oviduct (A,B) shows nuclei of oocytes at early (a) and late (b) mitotic stages. The growth zone (C) of *C. elegans* oviduct shows mature oocytes at late meiotic stages (o). Mature sperm (sp) can be seen in *C. elegans* spermatheca as dense spots. (gc, gut cell; vn, ventral nerve cord).

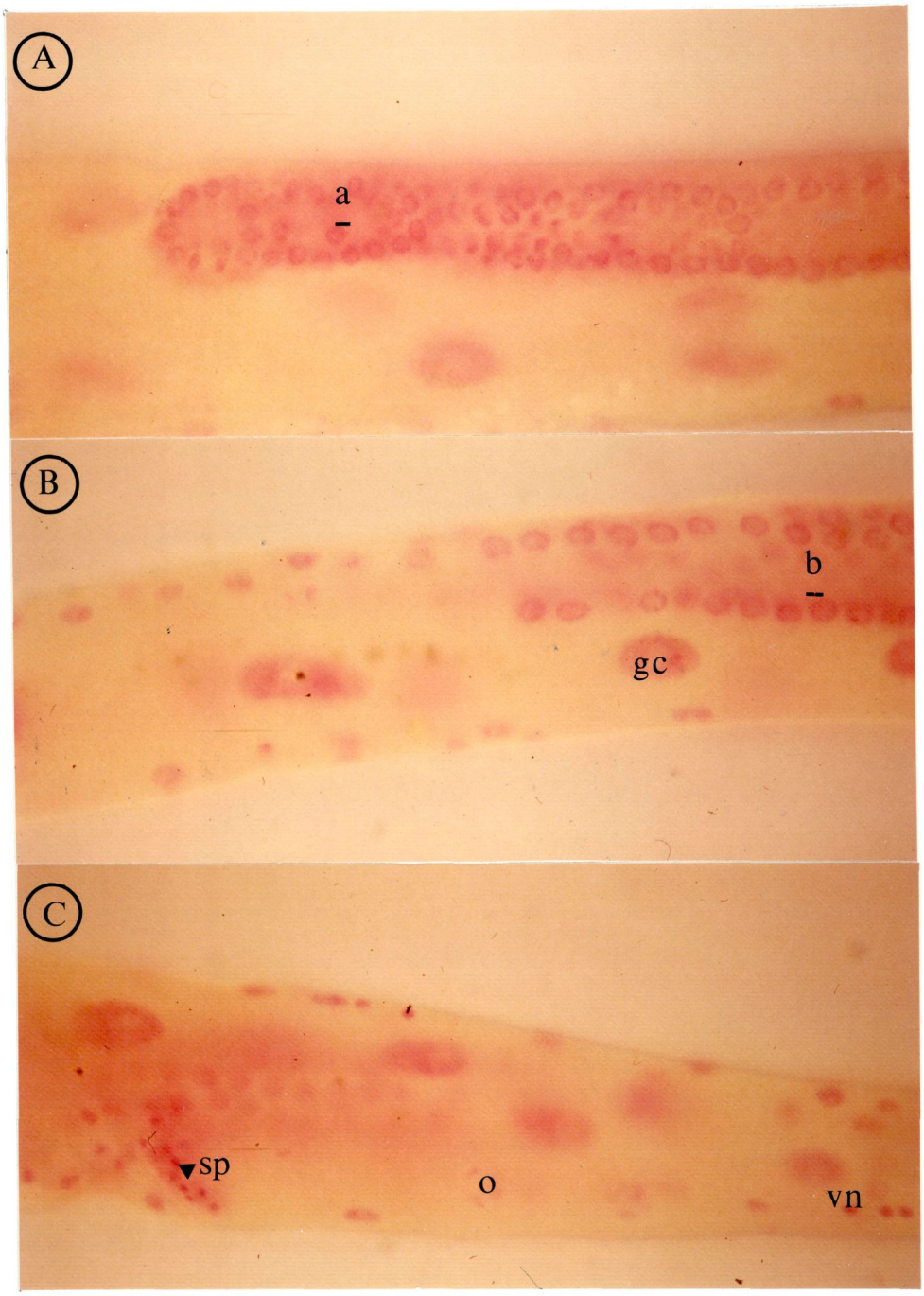

A
a
B
b
gc
C
sp
o
vn

Plate II.2 Feulgan stained gonad of of untreated *C. elegans* showing:

A) Mature oocytes (o) at the diplotene or diakinesis stage; x 1200. Arrows indicate chromosomal structures.

B) Primary spermatocytes of *C. elegans* in the spermatheca; x 500. (gc, gut cell; vn; ventral nerve cord).

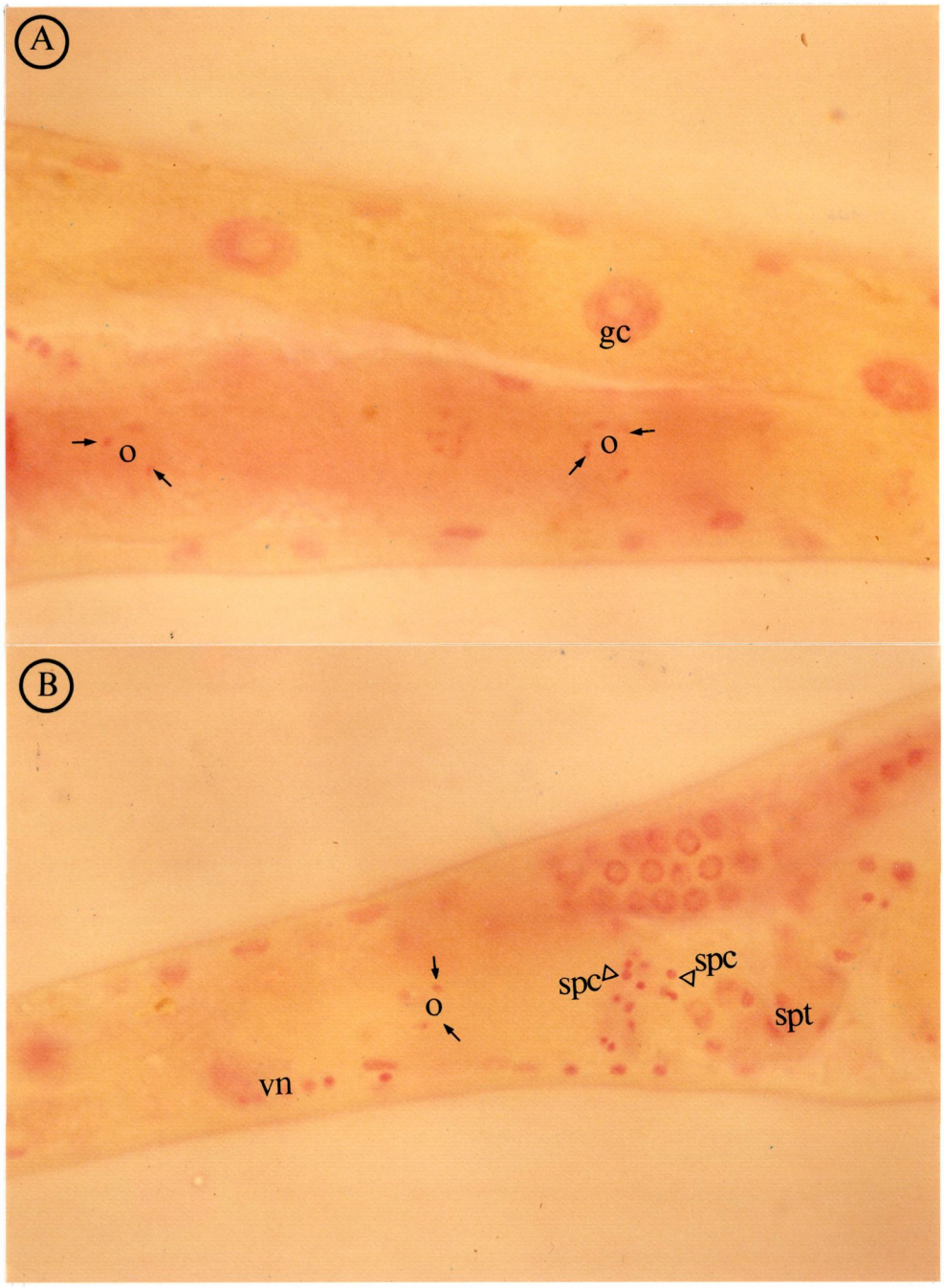

A
gc
o
o
B
o
spc
spc
spt
vn

Table II.5. Effect of Diflubenzuron on Oogenesis of *C. elegans*

Treatment	Mean number of oocytes at diplotene and diakinesis †	Number of replicates	Range	Total number of oocytes anterior + posterior gonads ††	Mean of transformed values, Log (x+1)±S.E.
A					
Control	7.0	15	0-15	56+55 = 111	0.89±0.08
Diflubenzuron ($\mu g\ cm^{-3}$)					
20	6.0	15	0-13	56+31 = 87	0.74±0.09
40	4.0	15	0-7	33+27 = 60	0.65±0.07 L.S.R = 0.27 P>0.05
B					
Control	8.0	13	0-17	58+52 = 110	0.89±0.11
Diflubenzuron 80 $\mu g\ cm^{-3}$	7.0	12	0-16	48+43 = 91	0.84±09 t = 0.14 P>0.05

† The figures represent mean numbers of oocytes in the whole reproductive tract.

†† The sum of both values gives the total number of oocytes examined in each treatment.

on nematodes treated with 80 μg cm^{-3} diflubenzuron indicated that meiotic division continued normally in mature oocytes ($P>0.05$) although this high concentration of diflubenzuron was shown to retard the development of *C. elegans* gonads (Section II.C.5.c).

The observations made on spermatogenesis in diflubenzuron-treated *C. elegans* are summarized in Table II.6. The various stages of sperm development described by Ward and Miwa (1978) were examined in control and diflubenzuron-treated nematodes . Primary spermatocytes were distinguished by their large nuclei with partially condensed chromatin, whereas the nuclei of the spermatids and mature sperm were identified as small dense spots of chromatin (see Plates II.1.C, 2.B).

Production of sperm in *C. elegans* was not found to be significantly affected by diflubenzuron treatment ($P>0.05$; Table II.6, Appendix II.9).

(2) Fluorescent staining with Hoechst 33258

Fluorescence was mainly retained by nuclear material of the oocytes in the ovaries of all control and diflubenzuron-treated *C. elegans*, indicating active nuclear division in these oocytes. In each nematode about 6 - 8 oocytes were found to be at late meiotic stages in which the chromosomes appeared as 3 - 6 dense fluorescent spots (Plate II.3).

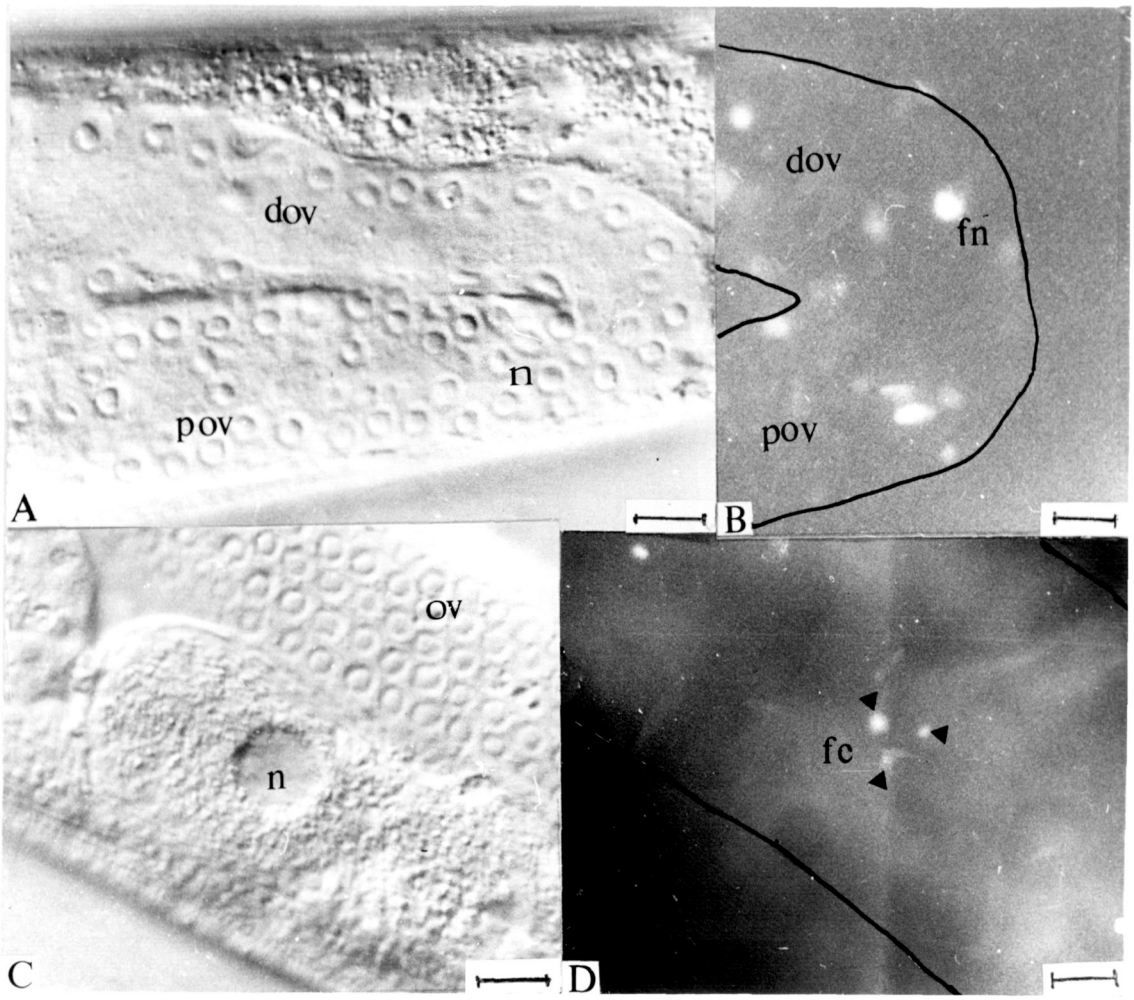
Table II.6. Effect of Diflubenzuron on Spermatogenesis of *C. elegans*

Treatment	Mean number of sperm nuclei at spermatheca region*	Number of replicates	Range	Total number of sperm nuclei in anterior + posterior gonads **	Mean of transformed values, Log (x+1)±S.E.
(A) Control	41	15	0-223	431+279= 710	1.21±0.19
Diflubenzuron ($\mu g\ cm^{-3}$) 20	36	15	0-91	336+210= 546	1.21±0.18
40	30	15	0-98	222+236= 458	0.98±0.22 L.S.R.=0.68 P>0.05
(B) Control	43	12	0-117	289+226= 515	1.42±0.16
Diflubenzuron 80 $\mu g\ cm^{-3}$	36	13	0-134	288+178= 466	1.08±0.23 t=1.20 P>0.05

* The figures represent mean numbers of sperm in the whole reproductive tract.

** The sum of both values gives the total number of sperm nuclei observed in each treatment.

Plate II.3. Light micrographs showing (A) Nomarski Interference contrast of young oocytes in the oviduct of a normal *C. elegans* hermaphrodite, (B) Fluorescent nuclei of oocytes in the proximal or distal ovary arms of *C. elegans* hermaphrodite, stained with Hoechst 33258 and chromosomal material of a mature oocyte (C) in diakinesis, shown as dense fluorescent spots (D); x 1000. Scale bars represent 10 µm. (dov, distal ovary arm; pov, proximal ovary arm; fc, fluorescent chromosomal material; fn, fluorescent nucleus; n, oocyte nucleus; ov, ovary).


dov
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pov
A
dov
fn
pov
B
ov
n
C
fc
D

SECTION III

Effect of Diflubenzuron on Fertilization in *C. elegans*

III.A. Introduction

The presence of an unusually large% of unfertilized eggs of *C. elegans* in culture plates containing diflubenzuron was observed both in the present work (Section I. II) and in earlier studies (Ibrahim, 1978).

C. elegans is a hermaphrodite, able to produce both sperm and oocytes (Maupus, 1900). The process of fertilization in *C. elegans* has been described briefly by Nigon (1949) and Hirsh *et al.* (1976) and in more detail by Ward and Carrel (1979). It was found that towards completion of spermatogenesis sperm accumulated in the spermatheca. In the mean time, oocytes mature through meiosis as they pass down the oviduct, development being arrested at diakinesis I (Hirsh *et al.*, 1976). Oocytes attain maximum size at the end of the oviduct, the nucleolus disappears and the nucleus migrates from the centre of the cell to the end distal from the spermatheca (Ward and Miwa, 1978). Prior to fertilization about 15-20 sperm accumulate on the leading surface of the mature oocyte. The oocyte with its cap of sperm is pushed into the spermatheca by sporadic contractions of the oviduct (Ward and Carrel 1979).

The penetration of *C. elegans* sperm into a mature oocyte is difficult to observe and has been described as an abrupt "plopping"

by Ward and Carrel (1979). Honda (1925) reported that sperm appeared to penetrate anywhere on the anterior third of the oocyte, but Ward and Carrel (1979) suggested there was one site for sperm penetration as was observed previously by Nigon *et al.* (1960). The sperm remains visible inside the oocyte for less than a minute following penetration, then disappears amidst the yolk granules of the oocyte cytoplasm. The oocyte nuclear membrane becomes less distinct and the nucleus undergoes meiotic divisions. Two polar bodies are extruded from the egg cytoplasm and the pronuclei of the oocyte and sperm fuse and cell cleavage commences (Hirsh *et al.*, 1976; Ward and Carrel, 1979).

Fertilized oocytes of *C. elegans* show an increased brownian motion of granules in the oocyte cytoplasm; movement of cytoplasm and the pronuclei having been described by Nigon *et al.* (1960) and Hirsh *et al.* (1976). Recently, Ward and Carrel (1979) found that this activation of the oocyte was due to contact with sperm as it did not occur in oocytes passing through the spermatheca of a sperm deficient mutant hermaphrodite (HC1). They also found that the sperm of the fertilization defective mutant (HC1) activated the oocyte granules but did not penetrate or trigger egg shell formation; the egg shell being formed only if the oocyte is fertilized (Hirsh *et al.*, 1976).

The observed production of increased numbers of unfertilized oocytes in the presence of diflubenzuron suggests that this compound does not act solely by impairing chitin assembly in the developing egg shell, and in the present study the effects of diflubenzuron

on sperm formation, sperm activity and fertilization were investigated.

III.B. Materials and Methods

III.B.1 Preliminary studies

Agar plates containing 0, 5, 10, 20 and 40 µg diflubenzuron cm^{-3} were prepared and inoculated with 5 - 10 J_2 *C. elegans*. Each treatment was replicated five time. After 5 days at 25°C the plates were examined and the number of unfertilized eggs and hatched larvae recorded.

In a similar experiment, *C. elegans* were incubated on agar plates with or without diflubenzuron. After 72h nematodes were transferred individually into drops of distilled water on microscope slides and observed by Nomarski interference contrast optics. The percentage fertilized and unfertilized oocytes in the uterus was recorded. Forty two nematodes from each treatment were examined and representative specimens photographed.

III.B.2 Observations on Fertilization in *C. elegans*

III.B.2.a. Nomarski Interference studies

Control and diflubenzuron-treated (10 and 40 µg cm^{-3}) *C. elegans* (strain N_2) were collected after 60 - 72h and mounted for live observation as described by Sulston and Horvitz (1977). An agar slab about 0.5mm thick was prepared by flattening a drop of 5% (w/v) Difco agar on a microscope slide with a second slide. After the agar had hardened the top slide was carefully removed and a small drop (1 mm^3) of 10% (w/v) polyvinylpyrrolidone (M.W. = 44,000; B.D.H. Ltd, Poole) in S medium (Appendix III.1.A) was placed on

the agar taking care not to break its surface. A single nematode was transferred into the S medium using a fine, blunt Pasteur pippete. A coverslip (12 x 12 mm), thinly coated in the centre with *E. coli*, was lowered onto the agar. The pressure of the coverslip prevents excessive movement of the nematode. Agar protruding beyond the coverslip was removed with a razor blade and the edges of the agar block sealed with silicone or vaseline to prevent dehydration.

The stages prior to fertilization were then observed in each nematode using a Shibaden SV-610 videorecorder and Sony camera attached to a Leitz Dialux microscope with Nomarski Interference optics (NPI x 40/0.65 objective). The uncoordinated, mutant strain of *C. elegans* E369 (Appendix III.1.E) was also examined in control treatments only.

III.B.2.b Fusion of female and male pronuclei

This experiment was to determine whether diflubenzuron prevented fusion between male and female pronuclei in mature oocytes recently encountered by the sperm.

Eggs in spermathecae of control and 10 μg cm^{-3} diflubenzuron-treated *C. elegans* N_2 hermaphrodites were dissected in buffered saline (Ward and Miwa, 1978; Appendix III.1.C) and observed for 20 - 30 min. Ten specimens were examined in each treatment.

III.B.3 Nile blue fluorescence studies

The Nile blue A fluorescence technique was used to study the

movement of male sperm inside the reproductive tracts of *C. elegans* hermaphrodites after mating (Ward and Carrel, 1979). The lipid stain Nile blue A is a fluorescent vital dye taken up readily by tissues of *C. elegans*, including sperm and in the present work a slightly modified staining procedure was used to study the effects of diflubenzuron on the migration of male sperm up the uterus to the spermatheca. A stock solution of Nile blue A (Sigma Ltd. Poole), 5% (w/v) in 95% (v/v) ethyl alcohol was prepared and diluted 8-fold with M-9 buffer (Appendix III.1.B); 6-8 drops of diluted Nile blue A were added to agar plates containing 0 or 40 µg diflubenzuron cm^{-3} and the stain allowed to be absorbed. A number of J_2 *C. elegans* (mutant strain E1467; a strain with a high incidence of males, Appendix III.1.E) were then transferred to the plates. After 48h at 16°C batches of three young males and three young hermaphrodites were transferred to equivalent fresh plates, without stain for 24h. The males and hermaphrodites were then examined individually in a drop of water under a coverslip by incident light fluorescence using a Leitz Dialux 20 microscope with filter block G (BP 350-460;LP 515) Male and hermaphrodite *C. elegans* grown in control medium (without Nile blue A) were examined for autofluorescence.

In a second experiment the concentration of stain was increased to 1:5 in 95% (v/v) ethyl alcohol without appearing to be detrimental to the nematodes. Examination of fluorescent sperm was enhanced by removing the reproductive tracts into a drop of buffered saline. Five nematodes from each treatment were examined and the experiment repeated five times.

III.B.4 Feulgan staining

This technique reveals the presence of sperm pronuclei within the mature oocyte (Triantaphyllou, 1962; Triantaphyllou and Hirschmann, 1962; Hirsh *et al.*, 1976; Ward and Miwa, 1978). *C. elegans* hermaphrodites treated with 0, 20 or 40 µg diflubenzuron cm^{-3} were processed for staining with Feulgan's stain as described previously (Section II.B.5.d). Fifteen nematodes were examined for each treatment and the number of zygotes with visible sperm pronuclei recorded.

III.B.5.a Effect of diflubenzuron on the production of male sperm in *C. elegans*

J_2 *C. elegans* (mutant strain E 1467) were treated with 40 µg cm^{-3} diflubenzuron or control medium at 16°C for 48h. Male nematodes were then transferred onto equivalent fresh plates and examined after a further 24h. Sperm were extruded into a drop of buffered saline (Ward and Miwa, 1978; Appendix III.1.C) by making an incision near the coopulatory bursa. The sperm were counted and their structure examined in buffered saline, Ringer saline (Duggal, 1978; Appendix III.1.D) or S buffer (Sulston and Brenner, 1974; Appendix III.A).

III.B.5.b Effect of diflubenzuron on the activity of male sperm of *C. elegans*

The activity of sperm dissected from diflubenzuron-treated males of *C. elegans* was measured by recording the distance travelled by sperm in 30 min on a thin coating of 0.05% poly-L-lysine (MW. 80,000 Daltons; Sigma Ltd., Poole) on a glass slide. Egg albumin (Wright and Sommerville, 1977) was found to be unsuitable as it did

not provide sufficient attachment for the sperm. Only pseudopodial sperm, as described by Ward and Carrel (1979), were examined and their image was projected on a monitor screen and traced every minute to record the sperm movement.

III.C. Results

III.C.1 Preliminary Studies

An increased production of unfertilized oocytes was observed in *C. elegans* treated in diflubenzuron (Table III.1; Figure III.1). The % of unfertilized oocytes laid in plates with 20 and 40 $\mu g\ cm^{-3}$ diflubenzuron were approximately 3 and 5 times those in the control treatment, respectively ($P<0.01$). Plate III.1. shows the presence of unfertilized oocytes in the uteri of diflubenzuron-treated hermaphrodites. Unfertilized oocytes had an average width and length of 23.6 and 29.5 µm respectively; these oocytes had enlarged nuclei (approximately16.5 µm dia) and presumably they undergo reductive divisions becoming highly polypoid as observed by Ward and Miwa (1978). The cytoplasm of unfertilized oocytes was characterized by a rough and densly granulated appearance (Plate III.1); they also disintegrated when sonicated for 5 sec (amplitude 12 microns) while the fertilized eggs remained intact.

III.C.2 Observations on Fertilization in *C. elegans*

III.C.2.a Nomarski Interference studies

In the ovaries of normal and diflubenzuron-treated *C. elegans* the oocytes attained maximum size (about 29 µm in diameter) and

Table III.1 The effect of diflubenzuron on production of fertilized eggs by *C. elegans*

(A)

Treatment	Control	Diflubenzuron (µg cm^{-3})			
		5	10	20	40
Mean % unfertilized eggs	16.6	20.2	27.9	46.4	74.5
Range	11-26	15-27	22-37	39-53	71-88
Means of transformed values†	0.41	0.46*	0.56**	0.74**	1.06**
(n=5) ±S.E.	±0.04	±0.03	±0.03	±0.03	±0.04

L.S.R. (0.05) = 0.14

† (arcsine $\sqrt{p}$)

* No significant effect (P>0.05).

** Significant effect (P<0.01).

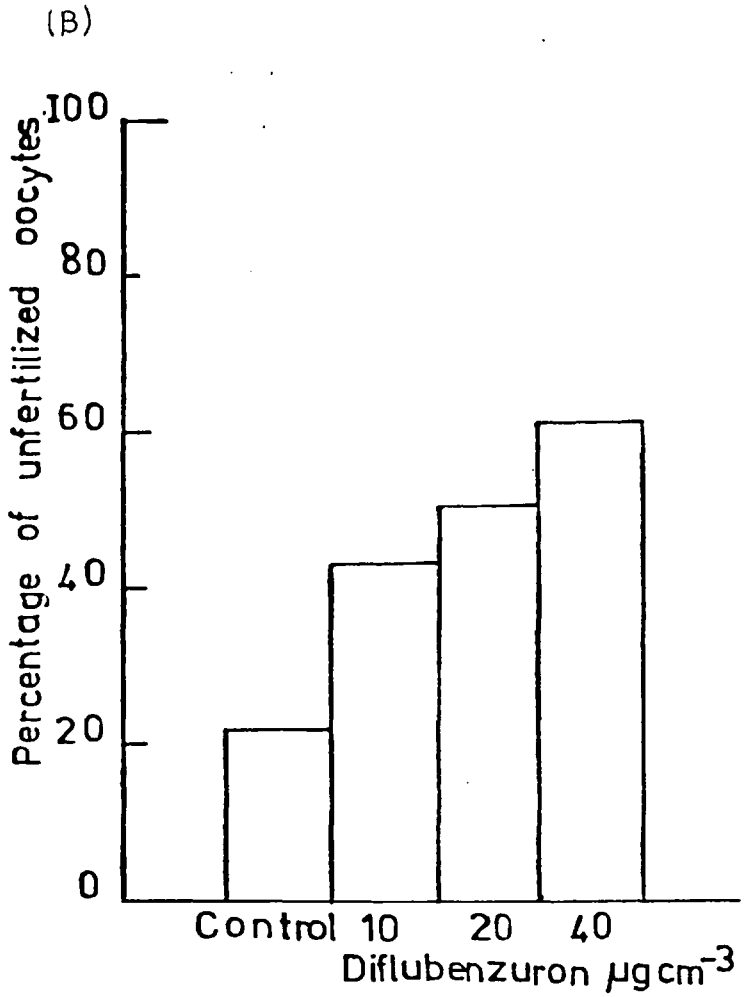


Figure III.1. Effect of diflubenzuron on production of fertilized eggs in *C. elegans* (n = 42).

Plate III.1 Nomarski Interference photomicrographs showing:

A) The uterus (ut) of an untreated *C. elegans* hermaphrodite containing a large number of embryonated eggs (e); x 625. Scale bar represents 10 µm.

B) A number of unfertilized eggs in the uterus of 40 µg cm^{-3} diflubenzuron-treated hermaphrodite; Note the size of proliferating nuclei; x 750. Scale bar represents 10 µm.

C & D) Rough cytoplasmic appearance of unfertilized (uf) eggs in diflubenzuron-treated *C. elegans*; x 750. Scale bars represent 10 µm.

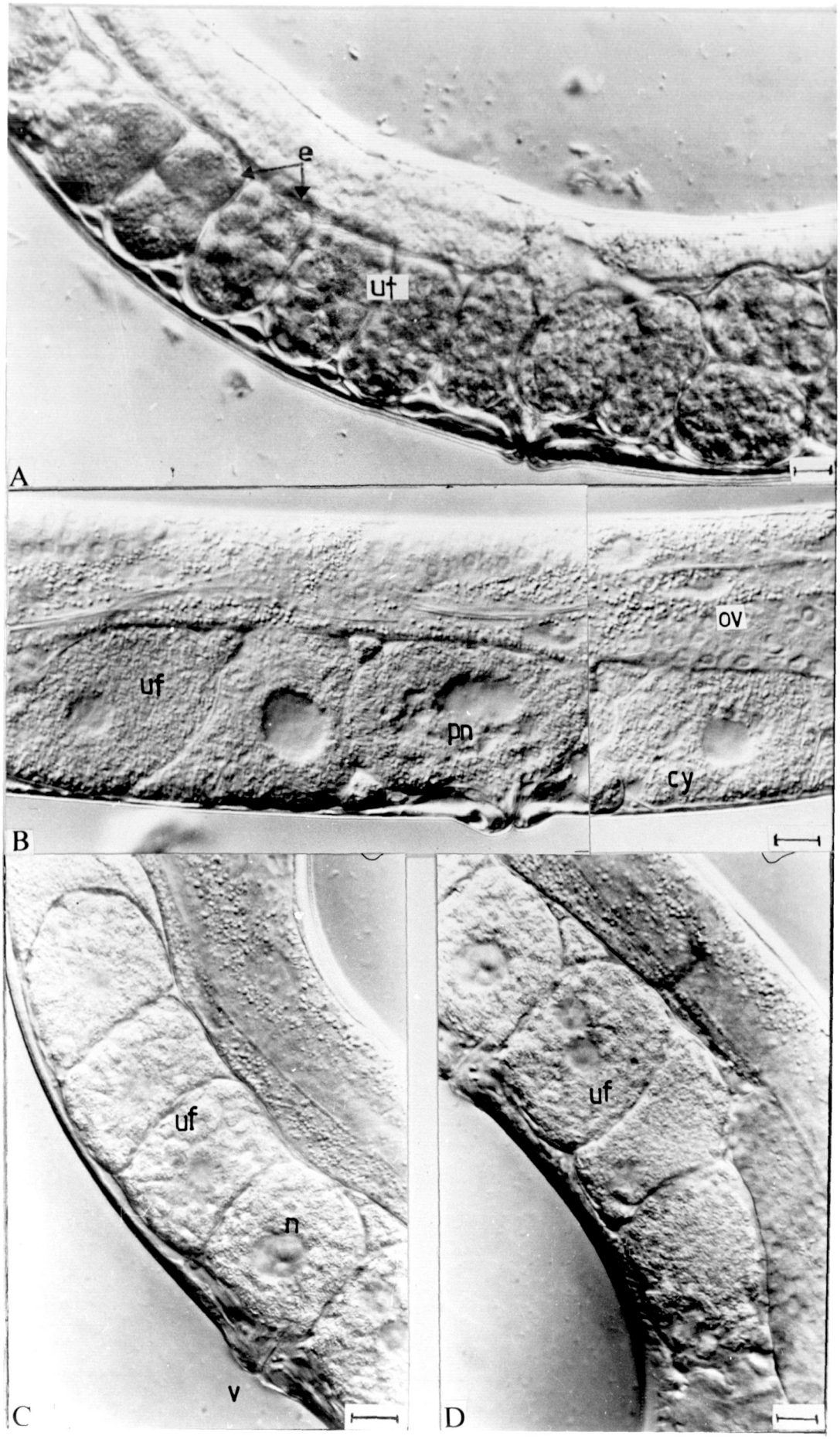

e
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v
C
uf
D

Plate III.2. Nomarski Interference photomicrographs showing:

A) The oviduct structure in a control *C. elegans* hermaphrodite; x 300. Scale bar represents 20 µm.

B) A fully developed oviduct of a 40 µg cm^{-3} diflubenzuron-treated hermaphrodite; x 700

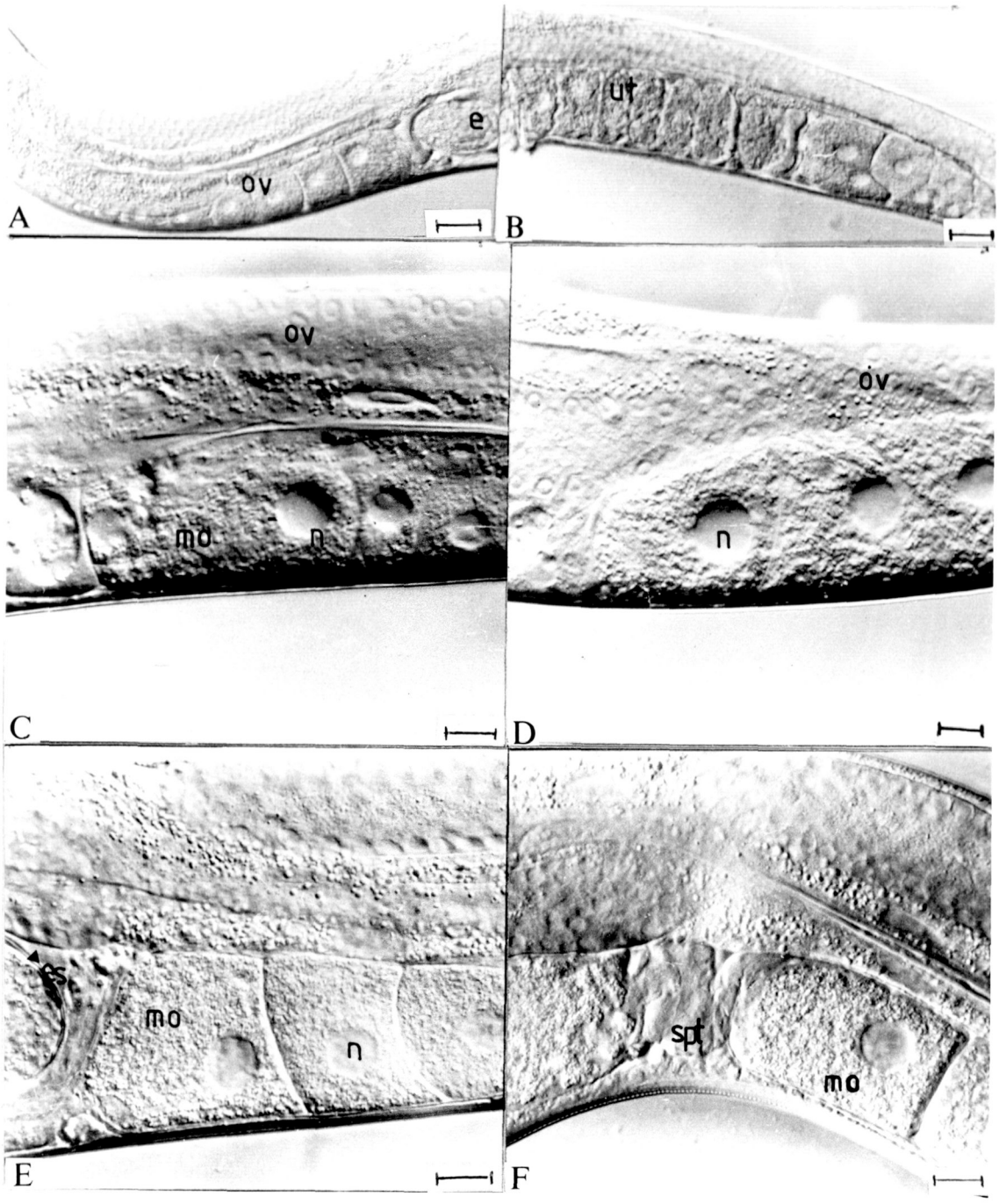
C) Mature oocytes (mo) in the oviduct of an untreated *C. elegans*. Note the gradual increase in size of oocytes; x 700.

D) The normal appearance of oocytes in an oviduct of 40 µg cm^{-3} diflubenzuron-treated hermaphrodite; x 700

E) A fully developed oocyte at the end of an oviduct of a control hermaphrodite of *C. elegans*; 750.

F) The nucleus of a mature oocyte at the end distal from the spermatheca (spt) in a 40 µg cm^{-3} diflubenzuron-treated hermaphrodite; x 750.

Scales bars in B-F represent 10 µm.


A
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spt
mo

reached maturity at the end of each oviduct (Plate III.2). The cytoplasm of mature oocytes showed gentle streaming while the nucleus began to migrate. No significant difference was observed in the time taken for an oocyte nucleus to migrate to the end away from the spermatheca in control or diflubenzuron-treated nematodes ($P>0.05$; Table III.2, Appendix III.3).

Table III.2. Time taken for migration of nuclei in mature oocytes of *C. elegans*

Treatment	Mean time (min) ±S.E.	Range
Control	24.4±4.9	8 - 42
Diflubenzuron ($\mu g\ cm^{-3}$) 10	25.0±2.7	16 - 32
40	24.0+4.0	16 - 47

Polar nuclei were observed in 78%, 84% and 88% of the mature oocytes examined in 40 and 10 $\mu g\ cm^{-3}$ diflubenzuron-treated and control nematodes respectively. The migration of oocyte nuclei was accompanied by contractions of the oviduct walls every 30 - 45 sec in both normal and diflubenzuron-treated nematodes. Both spermatids and fully developed spermatozoa appeared normal in diflubenzuron-treated nematodes (Plate III.3). The distribution of sperm around mature oocytes and in the spermathecae of diflubenzuron-treated and control *C. elegans* is shown in Table III.3 (Appendix III.4).

Plate III.3 Light micrographs, using Nomarski Interference contrast showing:

A) Spermatheca (spt) of a control untreated *C. elegans* hermaphrodite containing about 15 - 20 sperm.

B & C) A number of sperm (sp) in spermathecae of 40 µg cm^{-3} diflubenzuron-treated hermaphrodites. Note the presence of sperm in the vicinity of unfertilized eggs, uf (C).

D) A mature oocyte (mo) of a diflubenzuron-treated *C. elegans* in contact with sperm (about 6).

A - D; x 750. Scale bars represent 10 µm.

E & F) Normal appearance of developing spermatids near to mature oocytes in a control (E) and 40 µg cm^{-3} diflubenzuron-treated hermaphrodite; x 2800 Scale bars represents 2.0 µm.

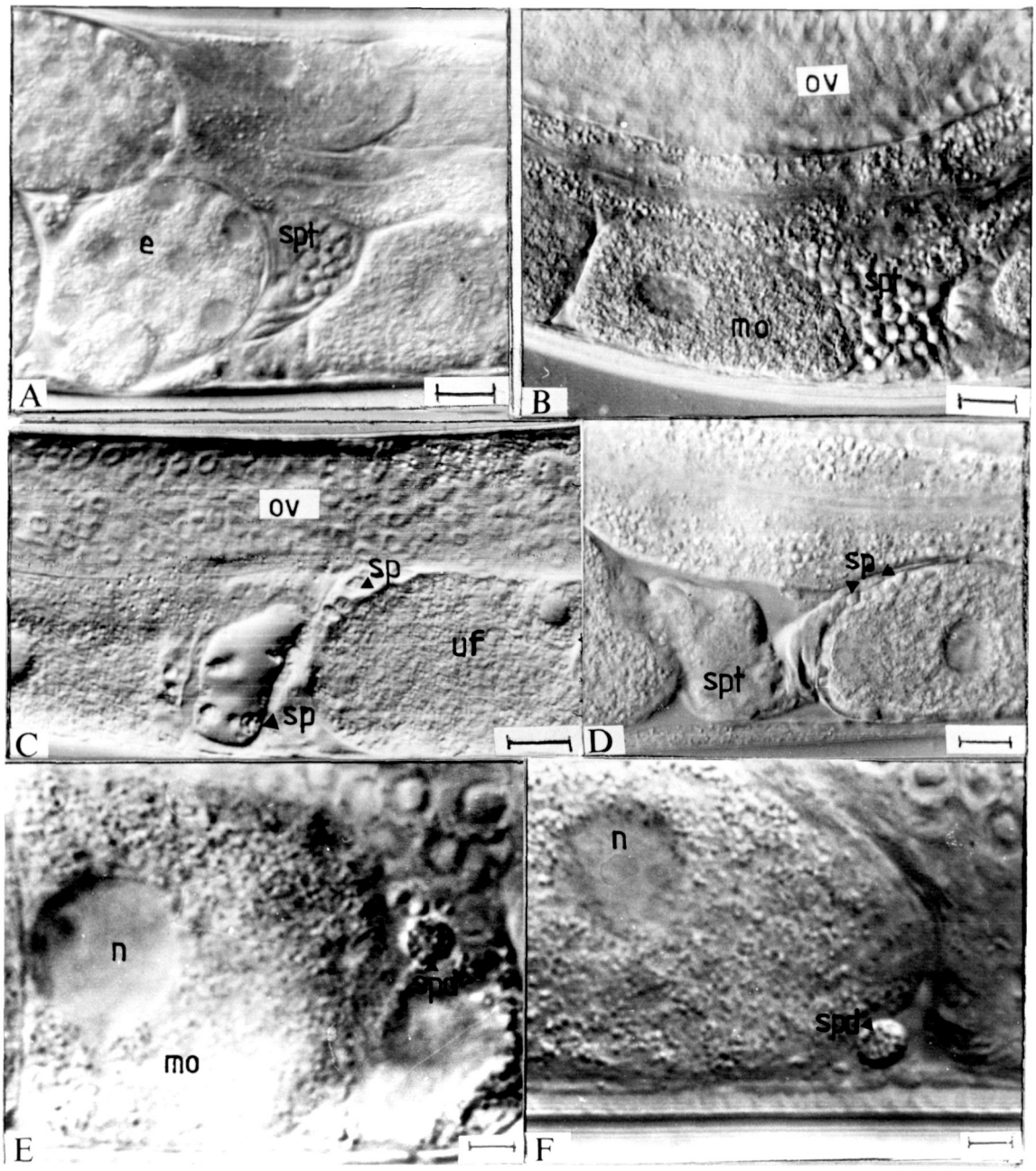

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Table III.3 Effect of diflubenzuron on the distribution of sperm in the oviduct and spermatheca* of *C. elegans*

Treatment	Control	Diflubenzuron (μg cm^{-3})		F ratio
		10	40	
Mean no. of sperm in contact with mature oocytes (n=25)	5.0	5.0	3.0	
Means of transformed values ±S.E.	1.24±0.22	1.31±0.20	0.87±0.19	1.34 P>0.05 N.S.
Mean no. of sperm in spermatheca (n=15)	7.0	6.0	9.0	
Means of transformed values ±S.E.	1.86±0.48	1.47±0.27	1.77±0.34	0.57 P>0.05 N.S.

*Sperm were examined in one gonad for each hermaphrodite.

N.S. No significant effect.

Fifteen nematodes from both the mutant E 369 and the N_2 strain of *C. elegans* were examined in an unsuccessful attempt to follow the penetration of mature oocytes by hermaphrodite sperm.

III.C.2.b Fusion of female and male pronuclei

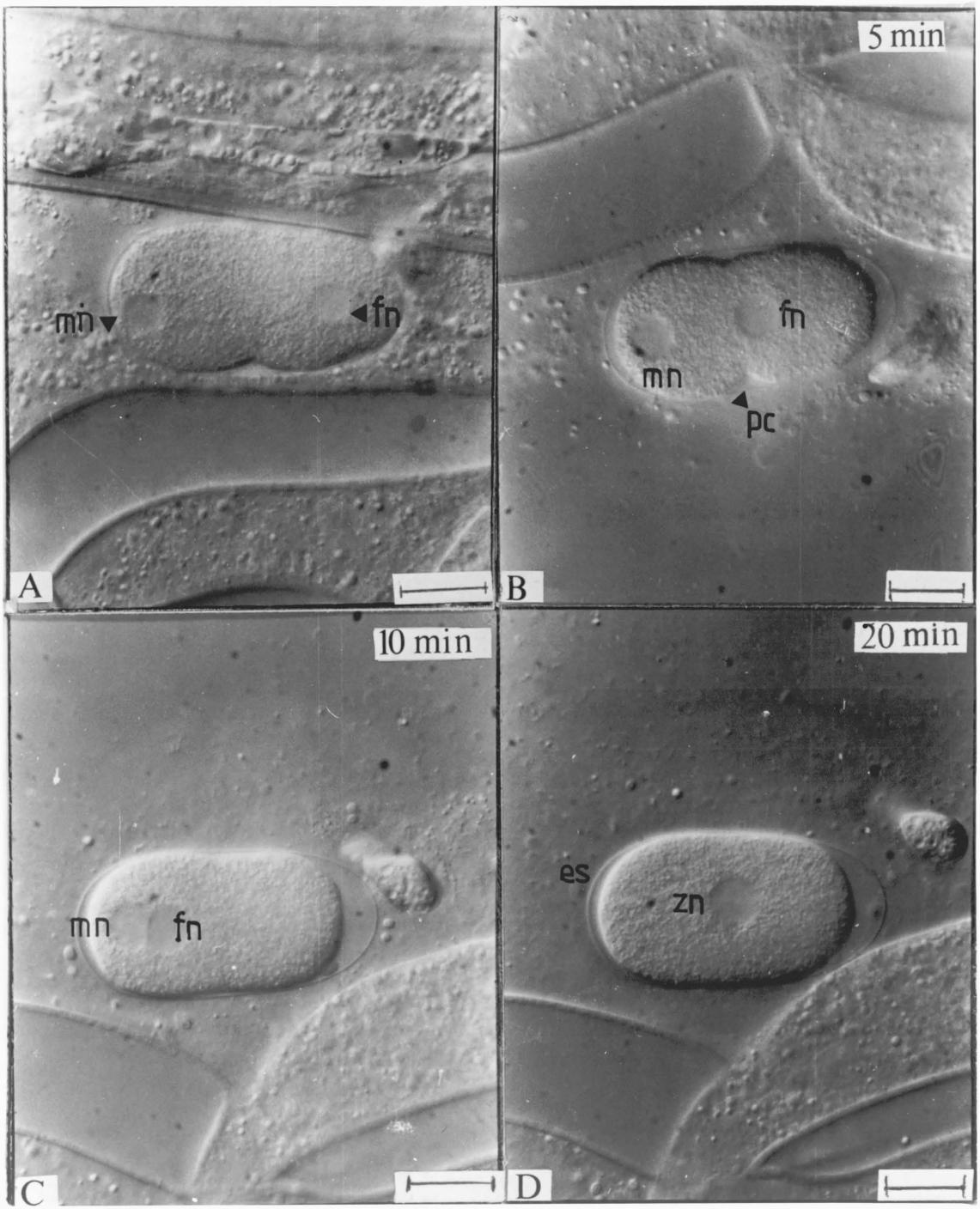
Female and male pronuclei were prominent in the dissected spermathecae of both normal and diflubenzuron-treated *C. elegans* after 2 - 8 min observation. The female pronucleus was eccentrically located on the longitudinal axis as described by Hirsh *et al.* (1978), while the sperm nucleus was at the opposite edge of the egg (Plate III.4, A). According to Schierenberg (1978) eggs at this stage are believed to have been encoutered by sperm 30 min previously.

The changes seen before the fusion of the two pronuclei took place are summarized in Plate III.4. Considerable cytoplasmic and surface membrane activities occurred while the maternal pronucleus migrated towards the paternal nucleus through a pseudocleavage constriction. The apposed pronuclei then moved back, rotated about 90^{o} around the longitudinal axis and finally fused to form the zygote nucleus. There was no significant difference in the time required to complete this process in control (22.6 min) or diflubenzuron-treated nematodes (20.9 min) ($P>0.05$; Appendix III.5).

Deposition of egg shell material was observed in only 3 eggs found in the spermathecae of control nematodes; during this process they attained a highly refractive surface. In other eggs (control and diflubenzuron-treated) only fully developed egg shells were found.

Plate III.4. Fusion of female and male pronuclei in *C. elegans* zygote. The pronuclei appear at the opposite ends of the zygote longitudinal axis [A]. The maternal or female pronucleus (fn) begins to migrate towards the male pronucleus (mn) through a pseudocleavage constriction (pc) [B]. The apposed pronuclei moves towards the mid region of the nematode zygote following the disappearance of the pseudocleavage [C]. Fusion is accomplished within 20 min [D].

X 1000; scale bars represent 12 μm.


mn
fn
A
5 min
fn
mn
pc
B
10 min
mn
fn
C
20 min
es
zn
D

III.C.3 Nile Blue Fluorescence Studies

Observations on normal and diflubenzuron-treated (Him) mutants of *C. elegans* showed similar fluorescence in the spermathecae of both control and treated nematodes ($P>0.05$; Table III.4, Appendix III.6). Few nematodes in either treatment had fluorescent sperm (Table III.4, Appendix III.6); an average of 6 and 5 such sperm being observed in normal and treated nematodes, respectively. Fluorescent sperm were occasionally observed around mature oocytes, presumably pushed back from the dissected spermathecae into the oviducts. The fluorescence of sperm tended to fade after a few minutes under the microscope.

III.C.4 Feulgan Staining

Examination of Feulgan stained eggs (fertilized and unfertilized) in normal or diflubenzuron-treated (20 - 40 $\mu g\ cm^{-3}$) *C. elegans* hermaphrodites showed that a significantly greater number of sperm encountered mature oocytes in the control group ($P<0.01$; Figure III.2). Sperm pronuclei were identified as dense chromatin spots in recently fertilized oocytes (Plate III.5.A), residing at one end in the egg as reported by Hirsh *et al.* (1976). Male pronuclei were observed in only 4.8% and 0.04% of the eggs examined in hermaphrodites treated with 20 and 40 $\mu g\ cm^{-3}$ diflubenzuron, respectively, as compared with 13.2% in control nematodes (Figure III.2; Appendix III.7). However, mature sperm were found in the vicinity of the unfertilized eggs or in the spermathecal region of diflubenzuron-treated nematodes, (Plate III.6) .

Table III.4 Effect of diflubenzuron on migration of sperm from males in the gonads of *C. elegans* hermaphrodites

Treatment	Control	Diflubenzuron ($40\ \mu g\ cm^{-3}$)
% females with fluorescent spermathecae	54.5 (n = 33)	50 (n = 38)
% spermathecae with fluorescent sperm	18.2	23.7
Total number of fluorescent sperm	35	45
% unfertilized oocytes (n = 14)	17.6	86.2

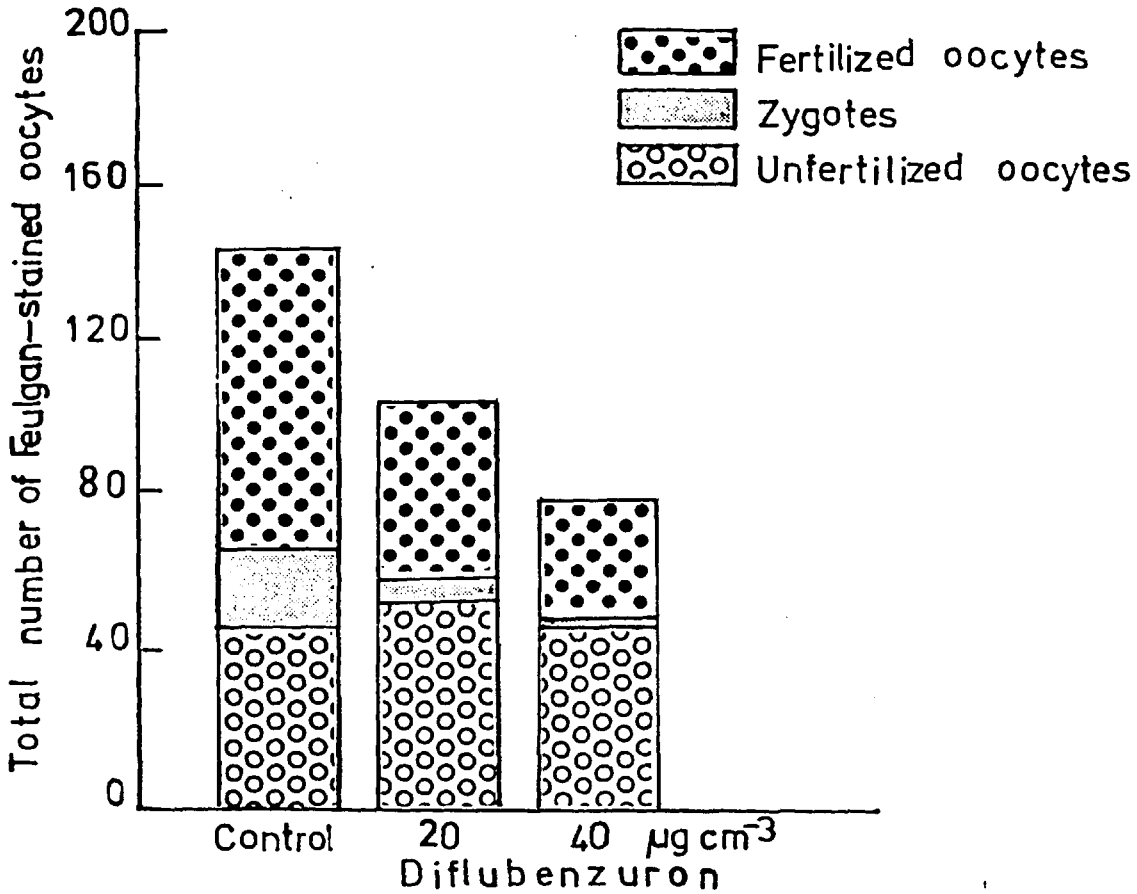
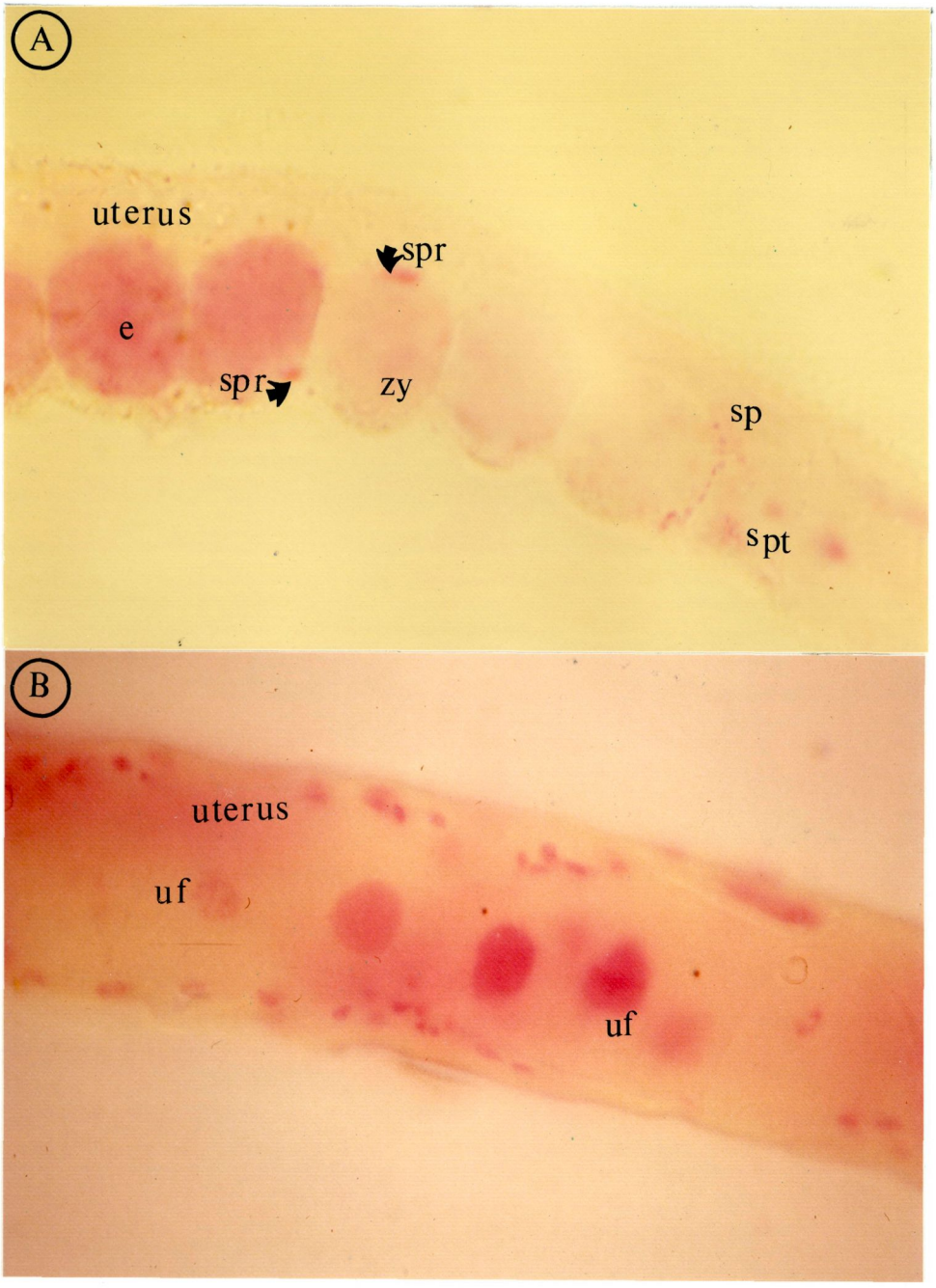


Figure III.2. The number of unfertilized oocytes (not showing sperm pronuclei) compared with the number of zygotes and fertilized oocytes observed in Feulgan stained gonads of control and diflubenzuron-treated *C. elegans*.

Plate III.5 Feulgan stained gonad of:

A) A control untreated *C. elegans* hermaphrodite containing a number of zygotes (zy) and embryonated eggs (e), sperm pronuclei (spr) in nematode zygotes and a file of mature sperm (sp) in the spermathecal region (spt); x 500.

B) A diflubenzuron-treated (40 μg cm^{-3}) hermaphrodite of *C. elegans* showing a number of unfertilized eggs (uf) characterized by large, densly stained nuclei; x 500


A
uterus
spr
e
spr
zy
sp
spt
B
uterus
uf
uf

Unfertilized eggs were characterized by enlarged and densly stained nuclei (Plate III.5.B). The shape of nuclei varied from round to ovoid, exhibiting the chromosomal material in the form of strands and dots (Plate III.6.B).

III.C.5.a Effect of diflubenzuron on the production and structure of male sperm in *C. elegans*

Males of mutant *C. elegans* (E1467) observed in this study seemed to develop normally and production of sperm was unaffected by 40 µg cm^{-3} diflubenzuron (Table III.5, Appendix III.8).

Sperm extruded from normal and diflubenzuron-treated males into buffered saline appeared as irregularly rounded or 3 - 5 angled structures with a maximum diameter of 7 µm (Plate III.7). Sperm nuclei were small spherical bodies found in the central region. Sperm collected in 1:3 diluted earthworm Ringer solution of S buffer acquired a rounded structure with an eccentrical nuclei and densly granulated cytoplasm (Plate III.7). Eventually, most of the sperm ruptured in both earthworm solution and S buffer.

In buffered saline sperm produced 1-7 filaments within 2-5 min measuring 10-17 µm in length and protruding from the pointed ends of the sperm (Plate III.8). These processes were described originally by Ward and Miwa (1978) as a striking morphological specialization and were used in the present study to determine whether diflubenzuron affected the structure of *C. elegans* sperm. In fact no significant change was observed due to diflubenzuron (Table III.5.B, Appendix III.9).

Plate III.6 Feulgan stained gonad of 40 µg cm^{-3} diflubenzuron-treated *C. elegans* hermaphrodites. A(Spermatheca containing fully developed sperm (sp) distinguished as densly stained chromatin spot; x 500.
B) A number of unfertilized eggs (uf) showing proliferating nuclei (n). Note the presence of sperm near to the unfertilized eggs;x1200.(chr, chromosomal material; ov, ovary).

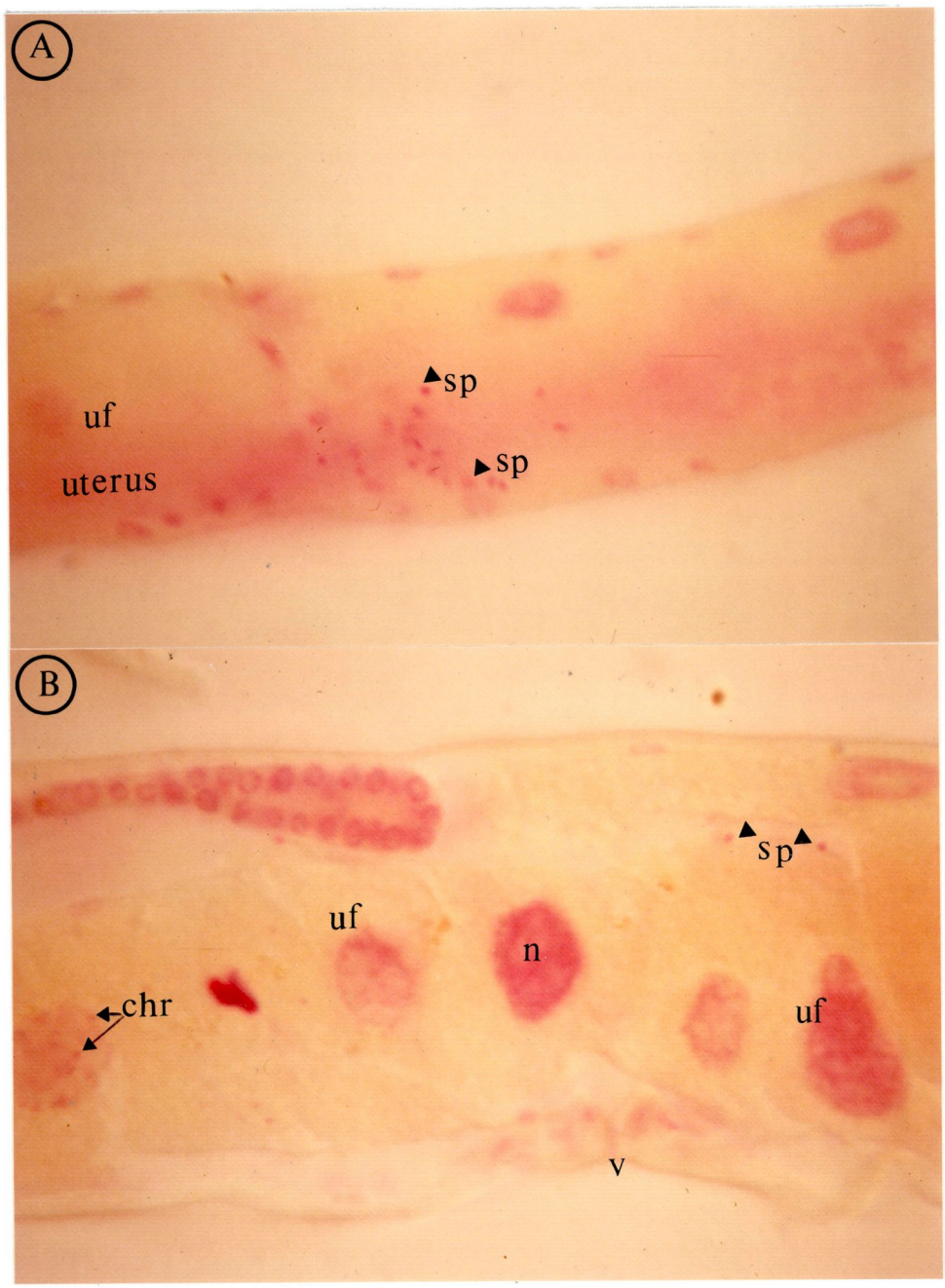

A
sp
uf
uterus
sp
B
sp
uf
n
chr
uf
v

Table III.5 The effect of diflubenzuron on sperm production and sperm structure in males of *C. elegans*

Treatment		Control	Diflubenzuron (40 µg cm^{-3})
(A)	Mean no. of sperm ±S.E. (n = 34)	201±34.5	173±29.6
	Range	34-510	38-485
	t = 1.05 P>0.05		
(B)	% sperm producing filaments (n = 18)	23.8	24.0
	Range	0-82	0-57
	Total no. of sperm examined	1195	1292
	t = 0.43 P>0.05		

Plate III.7. Light micrographs, using Nomarski Interference contrast showing:

A) A large number of sperm extruded from an untreated male of *C. elegans* into buffered saline, x 750. Scale bar represents 5.0 µm.

B) Irregularly rounded and 3-5 angled structure of *C. elegans* sperm in buffered saline; x 1120. Scale bar represents 5.0 µm.

C) Normal structure of sperm from 40 µg cm^{-3} diflubenzuron-treated male of *C. elegans* in buffered saline; x 1120. Scale bar represents 5.0 µm.

D) Rounded structure and granulated cytoplasm of *C. elegans* sperm extruded from untreated males into earthworm Ringer solution; Note eccentric sperm nuclei (n), x 2300. Scale bar represents 5.0 µm.

E) Sperm of control *C. elegans* male in S buffer; x 2300

F) Granulated appearance of sperm collected from 40 µg cm^{-3} diflubenzuron-treated male of *C. elegans* in earthworm Ringer solution; x 2300.

G) *C. elegans* sperm of diflubenzuron-treated male in S buffer; x 2300

Scale bars represents 5.0 µm.

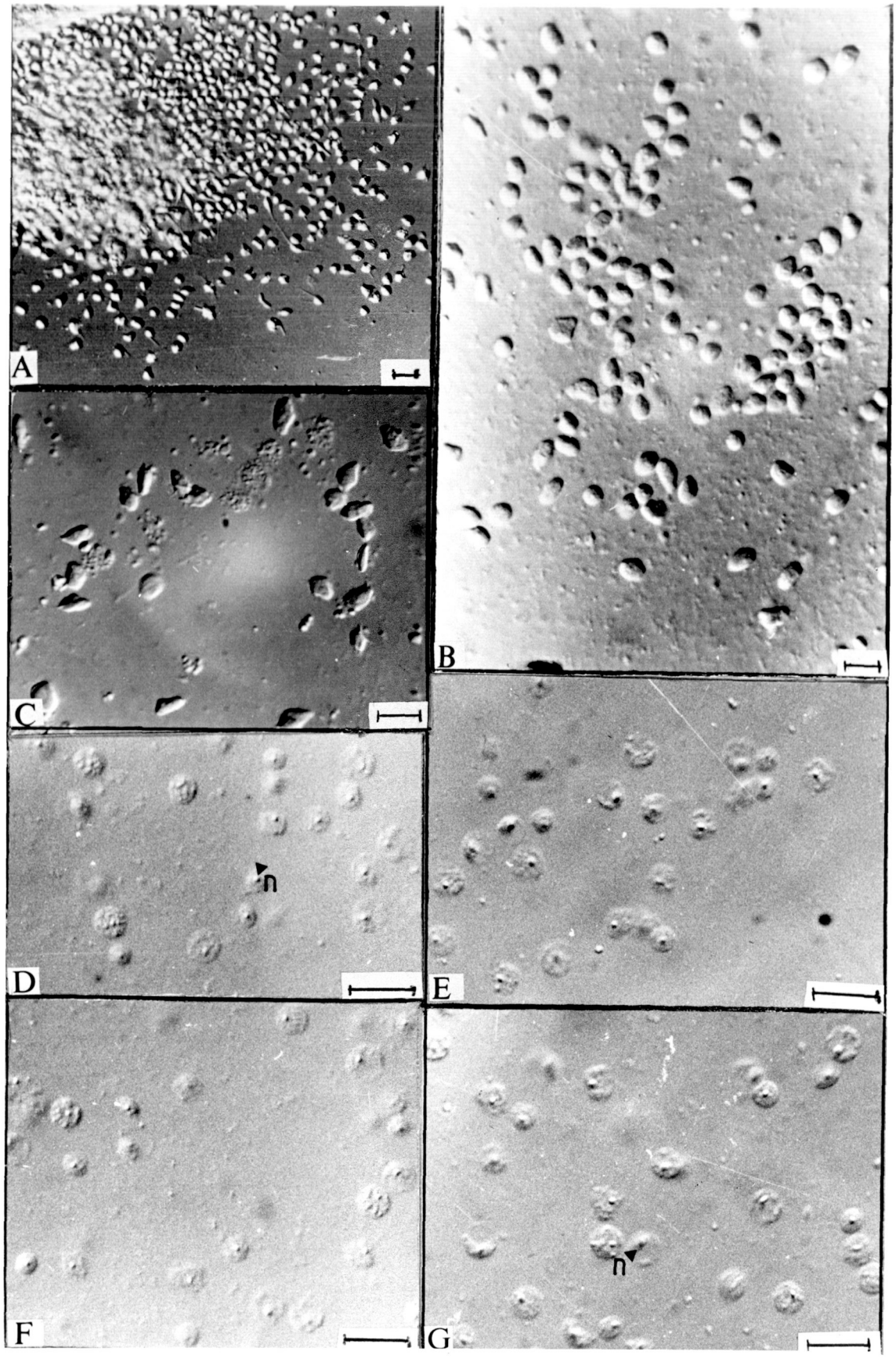
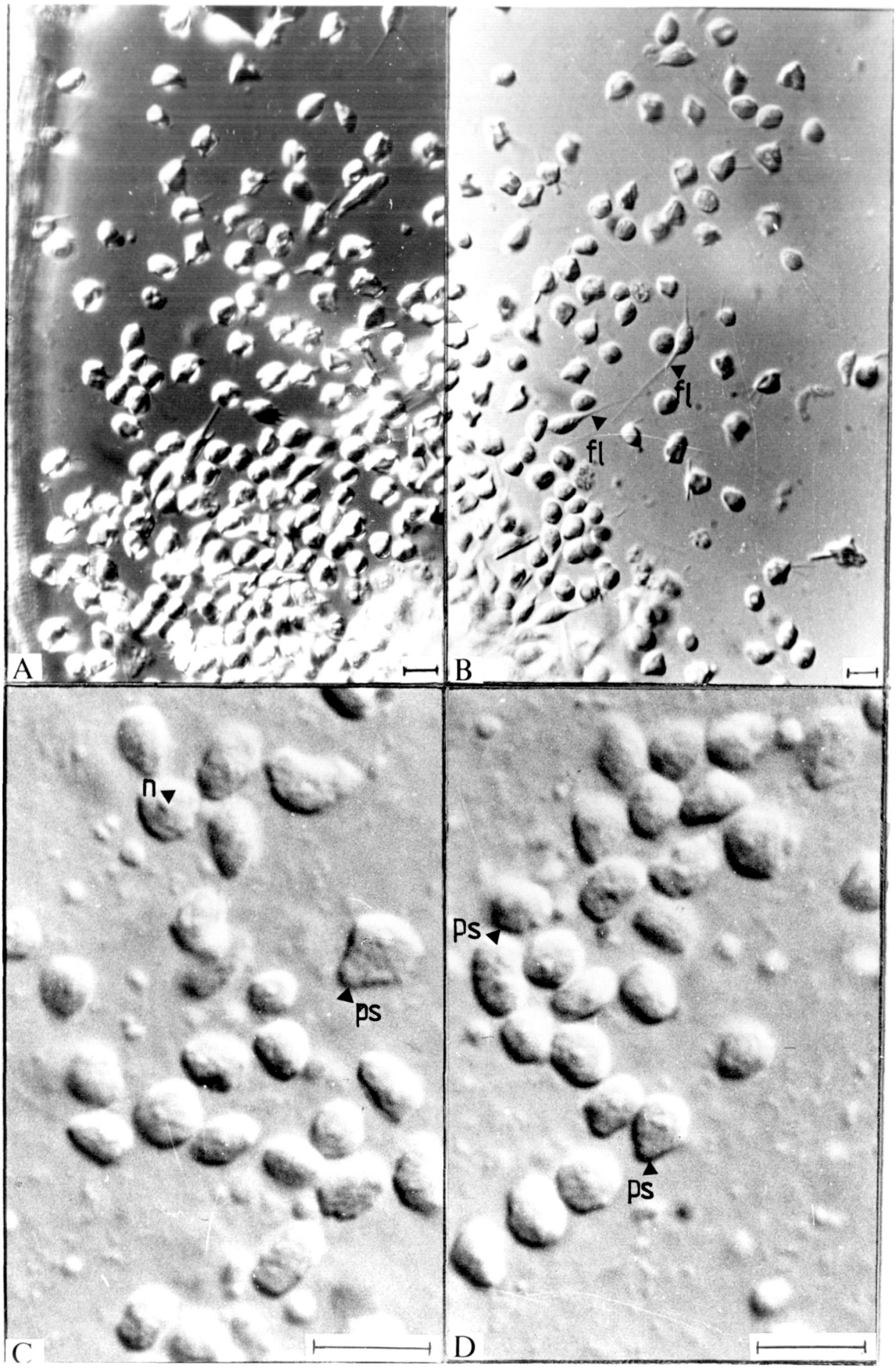

A
B
C
D
n
E
F
G
n

Plate III.8. A & B) Nomarski Interference photomicrographs of *C. elegans* sperm from (A) an untreated and (B) 40 μg cm^{-3} diflubenzuron-treated males, showing numerous filaments (fl) produced in buffered saline; x 1320. Scale bars represent 5.0 μm.

C & D) Pseudopodial sperm (ps) of (C) an untreated and (D) diflubenzuron-treated males of *C. elegans*, extruded into buffered saline on poly-L-lysine surface; x 2480. Scale bar represents 5.0 μm.


A
B
fl
fl
C
n
ps
D
ps
ps

III.C.5.b Effect of diflubenzuron on the activity of male sperm of *C. elegans*

Only 23% and 26% of sperm dissected from diflubenzuron-treated and control males were able to move on polylysine surface; these sperm travelled an average distance of 3.2±1.1 µm and 3.0±1.2 µm respectively in 30 min (Appendix III.10).

SECTION IV

Effect of Diflubenzuron on *C. elegans* Eggs

IV.A. Introduction

Hatching of nematode eggs has been shown to be affected by organophosphate and carbamate nematicides (Wright, 1981), whereas, benzimidazole anthelmintics such as benomyl and thiabendazole do not affect hatching of fully embryonated eggs (McLeod and Khair, 1975).

Amongst potential chitin synthesis inhibitors, the nematicidally-active compound Pentachloronitrobenzene (PCNB) has been reported to cause a significant reduction in hatching of *Aphelenchus avenae* eggs at concentration of 100 µg cm^{-3} (Stokes, 1976). This was believed to be due to the ability of the pesticide to weaken the nematode egg shell, in particular the median chitin layer. Eggs produced by *A. avenae* in the presence of 100 µg cm^{-3} PCNB were much more readily disrupted by ultrasonication than were eggs from untreated nematodes (R. Stokes and D.J. Wright, unpublished). Diflubenzuron may also have an ovicidal effect that could be expressed as an inhibition of embryogenesis or hatching of nematode eggs (Veech, 1978b) and in the present study the effects of diflubenzuron on embryogenesis and hatching of *C. elegans* were investigated. Studies were also made on the effects of diflubenzuron on the structure of *C. elegans* egg shell.

IV.B. Materials and Methods

IV.B.1 Effect of diflubenzuron on embryogenesis *C. elegans*.

Untreated *C. elegans* hermaphrodites were ruptured with a needle and fully formed eggs (at about the 16 cell stage) collected with the aid of a very fine Pasteur pipette fitted with a microsyringe. The eggs were transferred to NG agar plates containing 0, 5, 10, 20 and 40 µg diflubenzuron cm^{-3}. About 20 - 25 eggs were examined for each treatment and the percentage eggs completely embryonated assessed after 12h at 25°C.

In a further experiment, the early stages of embryogenesis were studied as described by Hirsh *et al.* (1976) and Deppe *et al.* (1978). Zygotes were collected from the dissected reproductive tracts of *C. elegans* treated with 0, 10 and 20 µg diflubenzuron cm^{-3}, placed in a drop of S buffer on a glass slide and covered with a coverslip. Embryogenesis was recorded using a video system as described previously (Section III.B.2.a). This study was conducted at room temperature (20° - 25°C) with a heat filter on the microscope light source. The rate of embryogenesis in 20 - 30 eggs from each treatment was examined up to the 16 cell stage.

IV.B.2. Effect of diflubenzuron on the viability and structure of *C. elegans* eggs

IV.B.2.a Collection of *C. elegans* eggs

The following experiments required large numbers of eggs and therefore two collection techniques were investigated.

a.1 Sucrose technique (based on Shaad and Walker, 1975; Patel and McFadden, 1978)

All stages of *C. elegans* were washed from 5 day old plates with 2.0 cm^3 of distilled water using a fine Pasteur pipette and transferred to a centrifuge tube (10 cm^3) with a discontinuous gradient of 30% and 60% (w/w) sucrose (2.0 cm^3 of each). Samples were centrifuged for 5, 10 and 15 min at 383 *g* and eggs in the water layer carefully removed using a pipette. The % eggs recovered at each time was recorded and the process replicated four times.

a.2 Potassium tartrate technique (Clarke *et al.*, 1967)

C. elegans populations were transferred to centrifuge tubes containing a density gradient of 1.0 g cm^{-3} (1.5 cm^3), 0.75 g cm^{-3} (1.0 cm^3), 0.625 g cm^{-3} (1.0 cm^3), 0.5 g cm^{-3}(1.0 cm^3), 0.375 g cm^{-3} (1.0 cm^3) and 0.25 g cm^{-3} (1.0 cm^3) potassium tartrate. Samples were centrifuged at 980 *g* for 1 min and the % eggs in each layer determined. This technique was replicated four times.

a.3 Results

The number of eggs recovered by sucrose density gradient was found to be greatest (94%) after 15 min of centrifugation (Table IV.1) when collected from the top layer of the gradient (Figure IV.1.A).

Similarly, more than 95% eggs were collected by the tartrate gradient (Figure IV.1.B). Both techniques were used in the following studies.

Table IV.1 Sucrose gradient centrifugation of *C. elegans* eggs

Time of centrifugation (min)	No. of eggs + larvae recovered	Mean % of eggs recovered (n = 4)	Mean % of transformed values ± S.E.
5	106+43, 107+67		
	103+22, 106+50	71.5	1.04 ± 0.06
10	209+21, 176+26		
	256+29, 191+10	90.7	1.26 ± 0.03 *
15	137+18, 153+7		
	118+5, 120+11	94.2	1.33 ± 0.03 *

*Significantly greater than after 5 min ($P < 0.01$).

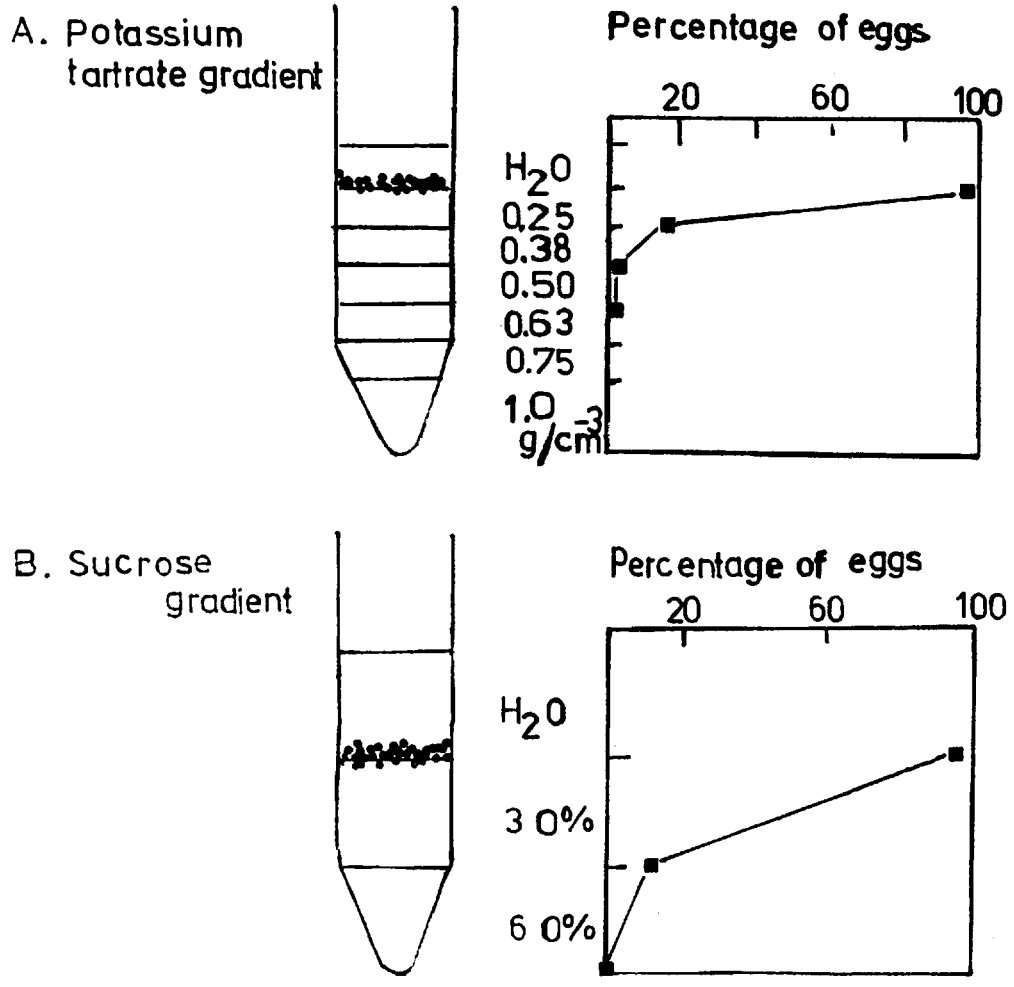


Figure IV.1. Density gradient centrifugation of C.elegans' eggs using Potassium tartrate or sucrose solutions.

IV.B.2.b Effect of diflubenzuron on hatching of *C. elegans* eggs

The following experiments were performed:

1) Eggs collected from normal females and then exposed to diflubenzuron.
2) Eggs collected from, or laid by, diflubenzuron treated hermaphrodites
3) Eggs laid by hermaphrodites treated at different juvenile stages with diflubenzuron.

Experimental procedure

(1) Batches of 50-200 eggs were collected from a 5 day old culture of *C. elegans* using the sucrose gradient centrifugation technique (see IV.B.2.a) and inoculated onto agar plates containing 0, 10, 20, 40 µg diflubenzuron cm^{-3}. The percentage hatch was assessed after 24h at 25°C. Each treatment was replicated four times.

(2) Two batches of *C. elegans* hermaphrodite treated with 0-40 µg cm^{-3} or 0-20 µg cm^{-3} diflubenzuron. Nematodes from the first batch were removed and discarded after 72h at 25°C leaving any eggs remaining on the plate. In the other group, eggs were collected by centrifugation and inoculated onto fresh plates containing 0 or 20 µg diflubenzuron cm^{-3}. The percentage hatch in both groups was recorded after a further 48h.

(3) In this experiment, *C. elegans* were treated with 0-20 µg diflubenzuron cm^{-3} at the J_2 or J_4 stage (Section II.B.5.b)

and the percentage hatching of eggs laid recorded after 48h. Each treatment was replicated five times.

IV.B.2.c Effect of diflubenzuron on the strength and permeability of *C. elegans* egg shells

(1) Solubility test

J_2 *C. elegans* were inoculated onto agar plates containing 0, 10 and 20 µg diflubenzuron cm^{-3}. After 48h at 25°C the nematodes were transferred to equivalent fresh plates without *E. coli* to lay eggs. After 48h the eggs were washed off the plates and collected by sucrose gradient centrifugation in 0.1 cm^3 distilled water. Suspensions of eggs were then pipetted gently into watchglasses containing various test solutions. The following solutions were used: 1M $CaCl_2$; 1N NaOH; 30% (w/v) KOH; 0.1N HCL 50 (v/v) H_2SO_4; Conc. H_2SO_4 sp.gr. 1.84); 5% (w/v) NaOCl; 10% NaOCL (Brown, 1950; Wharton and Jenkins, 1978). The proportion of eggs with intact egg shells was recorded after 24h at 25°C and the degree of "solubility" estimated.

(2) Sonication test

Treatment of *C. elegans* with diflubenzuron was conducted as for the solubility test. Groups of 100 - 200 eggs from each treatment were transferred in 2 cm^3 of distilled water to M.S.E. sonication vials (10 cm^3). The eggs were sonicated for 5, 15 and 30 sec in an MSE 150 watt ultrasonic disintegrater equipped with titanium microprobe (9mm) at an amplitude (peak to peak) of 12 µ (Wright and Awan, 1976). After each period of sonication the eggs were

examined under a binocular microscope to determine the degree of disintegration.

IV.B.3 Light Microscope Observations on the Egg Shell of *C. elegans*

Unhatched eggs laid by *C. elegans* hermaphrodites in 0 or 20 µg diflubenzuron cm^{-3} were collected after 24h by centrifugation and washed gently in distilled water (1 cm^3). A total of 50 eggs from each treatment were examined in a drop of water using Nomarski Differential Interference microscopy. Measurements for the maximum vertical and horizontal diameters of diflubenzuron-treated and control eggs were taken and some eggs were photographed.

IV.C. Results

IV.C.1 Effect of Diflubenzuron on Embryogenesis in *C. elegans*

Embryogenesis of *C. elegans* was not significantly affected by diflubenzuron treatment starting at the 16 cell stage ($P>0.05$; Table IV.2, Appendix IV.1). The early period of *C. elegans* embryonation was separated into the 2, 4, 8 and 16 cell stages (Plate IV.1). The percentage eggs (dissected from nematode gonad) developing up to the 16 cell stage was 83% and 75% in treatments with 10 and 20 µg cm^{-3} diflubenzuron, respectively, and 83% in the control treatment. In both control and diflubenzuron treatments eggs with 2 cells developed to the 16 cell stage within 30 - 40 min. Zygotes were found to develop into 2 cell-eggs after 1 - 4 min and a developmental time of 10 - 16 min was recorded between each of the other stages (Plate IV.1).

Table IV.2 Effect of diflubenzuron on embryogenesis of *C. elegans* from the 16 cell stage to J_1 stage

Treatment	Control	Diflubenzuron (μg cm^{-3})			
		5	10	20	40
Mean % embryogenesis	97.0	91.9	90.0	93.8	89.4
Range	91-100	86-100	83-95	90-96	78-95
Means of transformed† values (n=3)±S.E.	1.39±0.09	1.34±0.12	1.29±0.12	1.32±0.03	1.20±0.08
F ratio = 0.83 ($P > 0.05$)					

† (arcsine $\sqrt{P}$).

IV.C.2 Effect of Diflubenzuron on Hatching of *C. elegans* eggs

Nematode eggs taken from untreated cultures of *C. elegans* hatched normally when subjected to 5 - 40 μg cm^{-3} diflubenzuron (Table IV.3, Appendix IV.2). However, hatching of eggs collected from or laid by diflubenzuron-treated hermaphrodites was significantly affected ($P<0.01$) by concentrations of diflubenzuron greater than 5 μg cm^{-3} (Tables IV.4,5). Hatching was reduced by 82% overall and

Plate IV.1. Early stages of *C. elegans* embryogenesis in control untreated eggs.

A) First cleavage after fertilization.

B) Pregastrula stages with 4 and 8 cells.

C) Formation of 16 cells, about 40 min. after fertilization.

D) A gastrula stage with 30 - 40 cells.

Scale bars represent 5.0 μm.

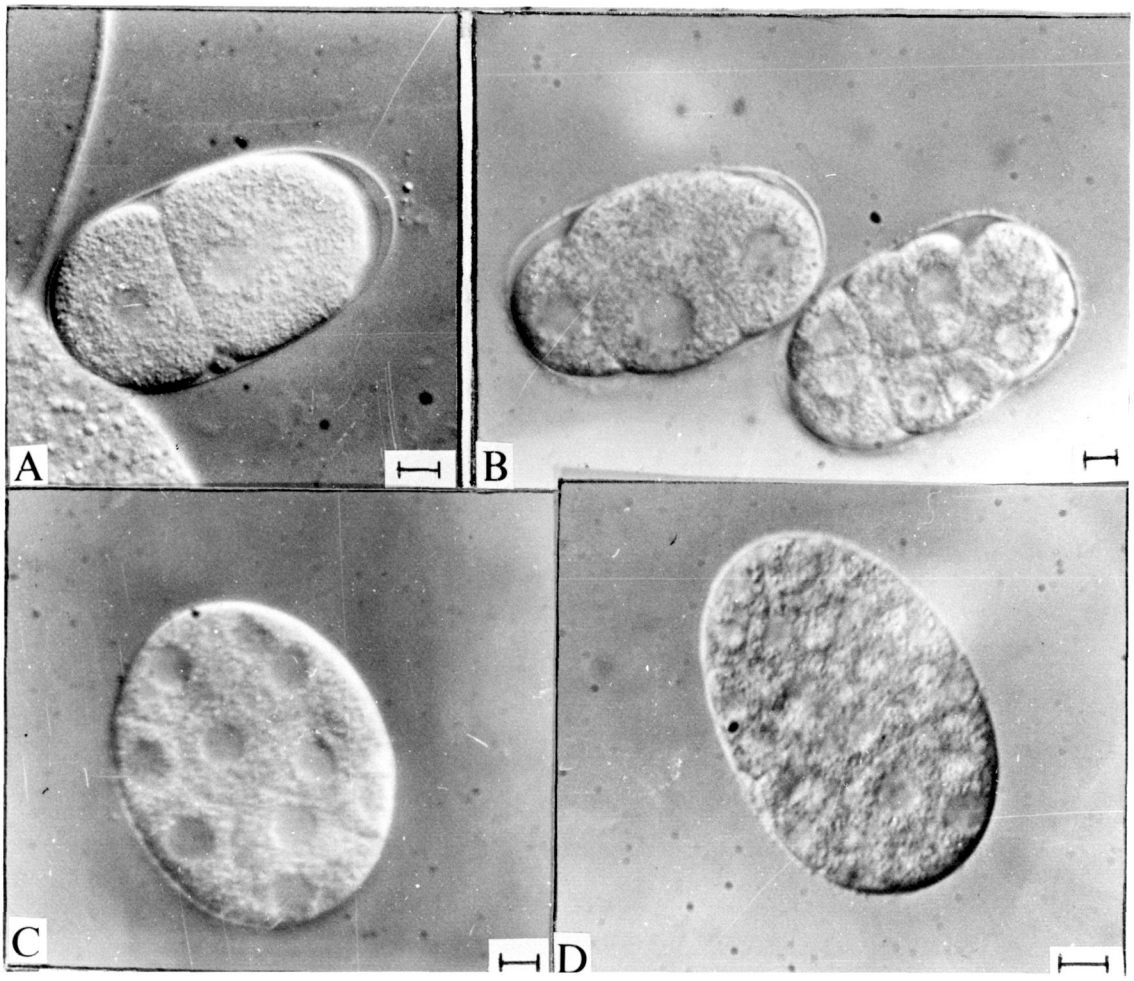

A
B
C
D

Table IV.3 Effect of diflubenzuron on hatching of *C. elegans* eggs (collected from untreated nematodes).

Treatment	Control	Diflubenzuron ($\mu g\ cm^{-3}$)		
		10	20	40
Mean% hatching	80.7	72.9	70.3	75.7
Range	77-89	66-77	67-78	70-82
Means of transformed values * (n=4)±S.E.	1.13±0.35	1.01±0.02*	1.0±0.03*	1.05±0.34*

L.S.R. (0.05) = 0.13

*(arcsine $\sqrt{P}$).

* No significant effect ($P \geq 0.05$)

Table IV. 4 Effect of diflubenzuron on hatching of *C. elegans* eggs (collected after being laid by treated nematodes)

Treatment	Control	Diflubenzuron (µg cm^{-3})		
		5	10	20
Mean % hatching	89.5	69.9	54.3	28.5
Range	77-96	43-88	38-77	19-44
Means of transformed values (n=5±S.E.	1.23±0.05	0.99±0.08*	0.85±0.07**	0.56±0.05**

L.S.R. (0.05) = 0.27

(0.01) = 0.34

EC_{50} = 12.67 (µg cm^{-3})

Y = 2.64 + 2.14X

* No significant effect (P>0.05)

** Significant effect (P<0.01)

Table IV.5 Effect of diflubenzuron on hatching of *C. elegans* eggs laid by treated nematodes)

Treatment	Control	Diflubenzuron ($\mu g\ cm^{-3}$)			
		5	10	20	40
Mean % hatching	85.2	66.5	33.8	18.3	4.3
Range	79-88	49-79	13-44	6-37	0-8
Means of transformed values (n=5)±S.E.	1.19±0.03	0.95±0.07*	0.58±0.06**	0.41±0.07**	0.14±0.07**

L.S.R. (0.05) = 0.25

(0.01) = 0.32

EC_{50} = 8.87 ($\mu g\ cm^{-3}$)

Y = 2.61 + 2.52X

* No significant effect (P>0.05)

** Significant effect (P<0.01).

was completely inhibited in 2/5 of the egg-batches treated with 40 μg cm^{-3} diflubenzuron. Percentage hatching in 10 and 20 μg cm^{-3} diflubenzuron treatments was 35 and 67% less than controls, respectively (Tables IV.4,5).

Hatching was not significantly affected (Table IV.6, Appendix IV.5)in batches of eggs laid by nematodes treated at the J_4 stage with 10 and 40 μg cm^{-3} diflubenzuron.

IV.C.3 The Effect of Diflubenzuron on the strength and permeability of the egg shell of *C. elegans*

Solubility and sonication tests on *C. elegans* eggs revealed distinctive changes in diflubenzuron-treated samples (Table IV.7.A,B). Eggs were weakened by 10% (v/v) NaOCl but the egg structure was unaffected in solutions of $CaCl_2$, NaOH, KOH and HCl. Both normal and diflubenzuron-treated eggs acquired a brown colour accompanied with a strong distension of the egg shells in H_2SO_4 whereas only treated batches attained a colourless appearance in 5% (w/v) NaOCl.

Unlike diflubenzuron-treated eggs, control eggs tolerated sonication for 30 sec (Table IV.7.B). A batch of normal eggs (1800) in distilled water (2 cm^3) did however totally disintegrate after three 15 sec periods of sonication.

IV.C.4 Light Microscope Observation on the Egg Shell of *C. elegans*

Measurements of the diameter of eggs in control and diflubenzuron-

Table IV.6 Effect of diflubenzuron on hatching of *C. elegans* eggs laid by nematodes treated at different juvenile stages (J_2 J_4)

Treatment	Mean % of total hatching	Means of transformed values (n=5)±S.E.
(A) Second juvenile Stage (J_2)		
Control	75.2	1.06±0.05
Diflubenzuron ($\mu g\ cm^{-3}$)		
10	41.6	0.73±0.07**
20	26.2	0.58±0.19**
(B) Fourth juvenile Stage (J_4)		
Diflubenzuron ($\mu g\ cm^{-3}$)		
10	75.2	1.01±0.03*
20	70.3	1.06±0.13*

L.S.R. (0.05) = 0.11

(0.01) = 0.14

* No significant effect (P>0.05)

** Significant effect (P<0.01)

Table IV.7. Effect of diflubenzuron on the strength of *C. elegans* egg shell.

A. Solubility test

Test solution	Treatment		
	Control	Diflubenzuron ($\mu g\ cm^{-3}$)	
		10	20
1M $CaCl_2$	-	-	-
1N NaOH	-	-	-
0.1N NaOH	-	-	-
30% KOH	-	-	-
0.1N HCl	-	-	-
50% H_2SO_4	+	+	+
Conc. H_2SO_4	+	+	+ +
5% NaOCL	-	++	++
10% NaOCL	++	+ + +	+ + +

- No effect observed.

+ Eggs swollen.

++ Eggs with transparent appearance.

+ + <50% of eggs with ruptured or dissolved egg shells.

+ + + >50% of eggs with ruptured or dissolved egg shells.

Table IV.7. (Continued)

B. **Sonication test**

Time of sonication in (sec)	Treatment		
	Control	Diflubenzuron (µg cm^{-3})	
		10	20
5	-	+	+
15	-	++	++
30	-	+++	+++

- No eggs disintegrated.
+ <50% eggs disintegrated
++ >50% eggs disintegrated
+++ All eggs disintegrated.

treated samples showed differences in the structure of treated eggs (Table IV.8, Figure IV.2).

The mean length/mean width ratio of diflubenzuron-treated eggs was found to be 1.39 compared to 1.71 in controls. Treated eggs also had a swollen appearance with a disorganized and retracted cytoplasm indicating changes in the permeability of the egg shell (Plate IV.2).

Table IV.8 Measurements of *C. elegans* eggs

(1) Length (µm)

Treatment	Mean ± S.E.	Replicates	Range	Student's t test
Control	46.3±0.58	48	31.5-55.5	$t = 2.17^{*}$
Diflubenzuron ($20\mu g\ cm^{-3}$)	43.9±0.91	48	31.5-55.5	

(2) Width (µm)

Treatment	Mean ± S.E.	Replicates	Range	Student's t test
Control	27.2±0.26	50	22.5-34.5	$t = 5.75^{**}$
Diflubenzuron ($20\mu g\ cm^{-3}$)	31.5±0.69	48	19.5-43.5	

*Significantly different ($P<0.05$)

**Significantly different ($P<0.01$)

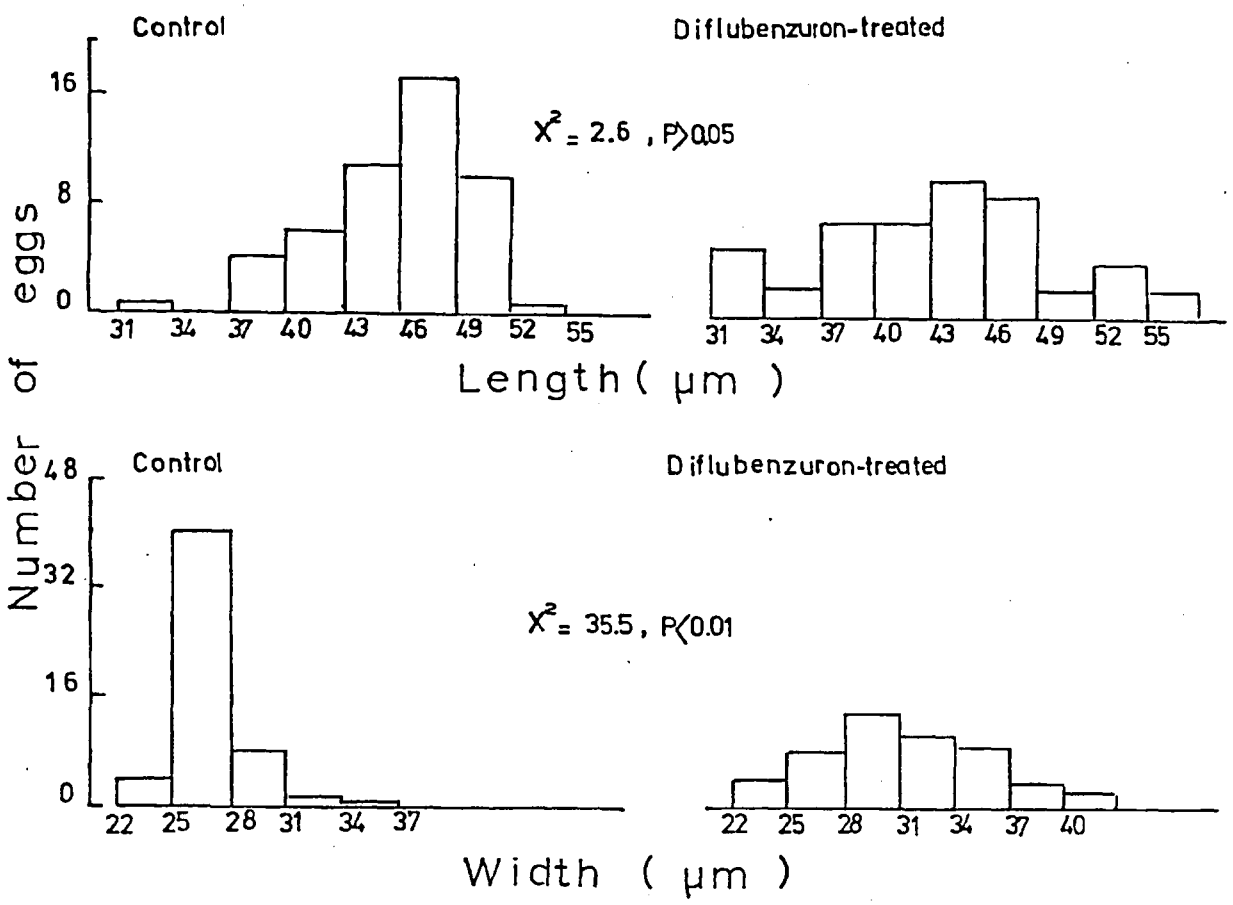


Figure IV.2. Diameters of control and diflubenzuron-treated eggs of *C. elegans*.

Plate IV. 2. Light micrographs showing:

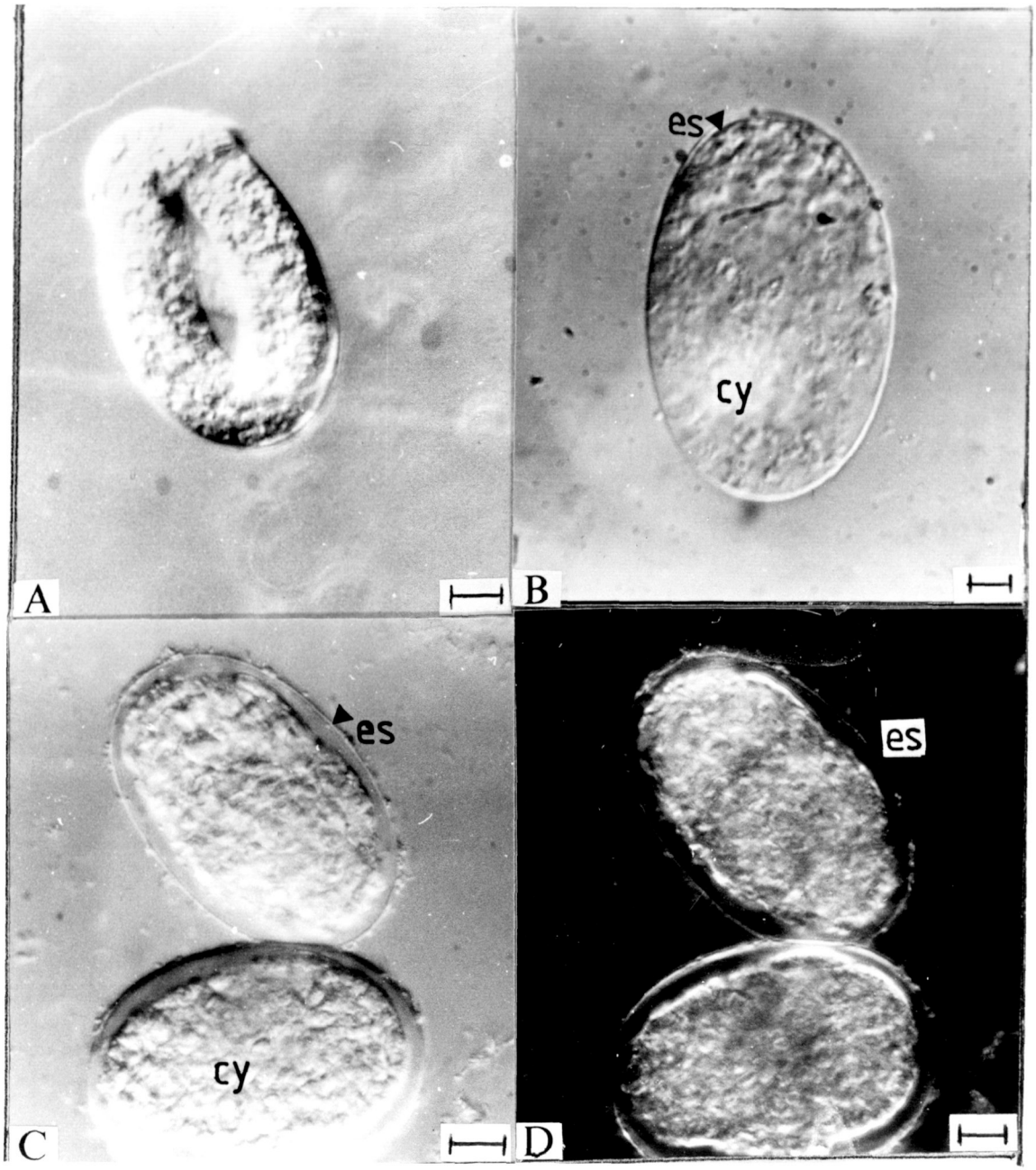
A) The normal structure of an untreated, fully developed egg of *C. elegans* and a highly refractive egg shell; x 1150.

B) Enlarged size of a 10 µg cm^{-3} diflubenzuron-treated egg ; x 1150.

C) Malformed appearance of 10 µg cm^{-3} diflubenzuron-treated eggs of *C. elegans*; x 1150.

D) Dark Nomarski interference contrast of treated eggs shown in (C); x 1150.

Scale bars represents 5.0 µm.


A
B
es
cy
C
es
cy
D
es

SECTION V

Ultrastructural and Biochemical Studies

V.A. Introduction

V.A.1 Ultrastructural Studies

Studies at the light microscope level described in earlier sections revealed some structural and morphological changes in *C. elegans* eggs due to diflubenzuron treatment. In this section investigations were made on the ultrastructural effects of diflubenzuron on *C. elegans* eggs, particularly in the egg shell.

As was mentioned in the general introduction, diflubenzuron is believed to interfere with chitin synthesis in insect larvae (Post, *et al.*, 1974; Deul *et al.*, 1978) and a similar effect in nematodes was anticipated by Veech (1978a, b). In nematodes chitin only occurs in the egg shell (Wharton, 1980); the egg shell generally consisting of three basic layers the outer "vitelline" layer, a chitinous layer and an inner lipid layer (Bird, 1976). In some nematode species there is an additional layer secreted by the uterus and the epithelial cells of the ovary (Yuen; 1971; McLaren, 1973).

The chitinous layer is often the thickest layer in the nematode egg shell and provides structural strength (Wharton, 1980).

In preparing nematode eggs for ultrastructural studies, a number of technical difficulties have been encountered (Morita, 1953; 1956; Bird, 1971). The inner lipid layer of the egg shell apparently dissolves in the solvents commonly used for dehydration prior to

embedding. This problem has now been overcome and the lipid layer can be fixed and rendered relatively insoluble by two successive periods of fixation in primary and secondary fixatives (Foor, 1967; Bird, 1968). An additional problem was that the lipid layer is probably impermeable to most of chemicals used for fixation and embedding. Bird and McClure, (1976) concluded that a suitable method depends on these chemicals penetrating the eggs and he used fixation in gluteraldehyde (3% v/v) followed by cracking of the egg shells by physical pressure.

In the following study satisfactory methods for preparing the eggs and adult females of *C. elegans* were found and the ultrastructure of control and diflubenzuron-treated specimens examined.

V.A.2 Biochemical Studies

In this section, the effect of diflubenzuron on the amount of chitin in egg shell of *C. elegans* was examined.

Several physical and chemical techniques have been used to detect and assay chitin in the nematode egg shell. X-ray diffraction techniques were used to detect chitin by Monné and Hönig (1954), Rudall (1955), Anya (1964) and Clarke *et al.* (1967), while Wharton and Jenkins (1978) used infra-red spectroscopy to demonstrate the presence of chitin in *Trichuris suis*. The Chitosan van Wisselingh colour test for chitin was used by Clarke *et al* (1967), Bird and McClure (1976) and Wharton and Jenkins (1978). Solubility tests for chitin detection of the nematode egg shell have been reported (Monné and Hönig, 1954; Wharton and Jenkins, 1978) and histological

studies made on the egg shells of *Hammerschmidtiella diesingi* and *Aspiculuris tetraptera* (Wharton, 1979b, c). However, the most definitive studies have involved the quantitative analysis of chitin precursors, glucosamine and *N* - acetylglucosamine in chitinase or acid hydrolysates of the nematode egg shell using paper, or gas liquid, or column chromotography (Clarke *et al.*, 1967; Bird and McClure, 1976; Wharton and Jenkins, 1978). Some of the above techniques were used in the present study.

In a final experiment the uptake of ^{14}C-labelled formulated diflubenzuron (25% w.p.) by *E. coli* and the eggs and adult stages of *C. elegans* was determined.

V.B Materials and Methods

V.B.1 Ultrastructural Studies

V.B.1.a. Preparation of *C. elegans* eggs for electron microscopy

Eggs laid on diflubenzuron-treated agar (10 µg cm^{-3}) were collected and treated as described previously (Section IV.B.2); unhatched eggs and hatched juvenile stages being washed carefully from the plates, centrifuged using the sucrose gradient method and collected in distilled water (1.0 cm^3). Eggs from untreated *C. elegans* were collected from dissected hermaphrodites (72h old) or washed from 3-5 day old culture plates and separated as above.

The method described by Wharton (1979a) for the egg shell of *A. tetraptera* was found to be satisfactory for *C. elegans* in a slightly modified form (full details of chemicals used are given in Appendix V.1). Eggs were fixed in 5% (v/v) gluteraldehyde in

sodium cacodylate buffer (pH7.2) for 3h at 5°C, centrifuged, washed in cacodylate buffer and then transferred to 1% (w/v) osmium tetroxide in cacodylate buffer (pH7.2) for 2h at room temperature. Thin layers of 1% (w/v) agar (Difco agar in phosphate buffer, pH7.4) were prepared on clean microscope slides and the fixed eggs positioned in groups on the agar (Ward *et al.*, 1975). A drop of fresh, molten agar was placed on top of each group of eggs and small blocks of agar with eggs dehydrated through a graded series of alcohols (30%, 50%, 70%, 90%, 95% and absolute ethyl alcohol, 15 min in each). The agar blocks were then transferred to propylene oxide (10 min) and continuously rotated overnight in (1:1) propylene oxide and Taab araldite at room temperature (Hayat, 1970). Samples were finally transferred into fresh, well vacuumed araldite, oriented carefully in gelatin capsules and incubated at 60°C for 18-24h.

V.B.1.b. Preparation of adult hermaphrodites of *C. elegans* for electron microscopy

The procedure described by Ward *et al.* (1975) was found to be satisfactory. Nematodes treated on agar containing 0, 20 or 40 µg diflubenzuron cm^{-3} for 60-72h were rinsed off the plates with ice cold 3% (v/v) gluteraldehyde in 0.1M phosphate buffer (pH7.4; Appendix V.1.). Each specimen was cut at the pharynx region to facilitate penetration of the fixatives. After 2h on ice the nematodes were rinsed in phosphate buffer (pH7.4) at 0°C and post-fixed in 0.5% (w/v) osmium tetroxide in phosphate buffer (pH7.4) for 1h at room temperature. Agar blocks with single nematodes were prepared and processed for embedding as described above.

V.B.1.c Sectioning and staining for electron microscopy

Ultrathin sections (500 - 700Å) with a silver or golden interference colour were cut using glass knives on an LKB ultratome 111. Ribbons of transverse sections of adult nematodes or eggs were floated onto 5-10% (v/v) acetone or distilled water and picked up onto 200 µm mesh formvar-coated copper grids (Graticules Ltd., Sovereign Way, Tonbridge, Kent, England). Longitudinal sections of adult worm were collected on 75 µm or 100 µm mesh formvar coated grids. Thick sections (0.5 µm - 1.0 µm) were stained occasionally with 1% (w/v) aqueous toluidine blue in 1% (w/v) borax to locate particular regions of the nematode body.

Ultrathin sections were double stained as described by Hayat (1970) and Glauert (1977). Grids were floated on alcoholic uranyl acetate with the section side down for 10-15 min at room temperature, or for 20-25 min when 0.2% aqueous uranyl acetate was used. The grids were then rinsed with distilled water, dried on filter paper and transferred into Reynold's lead citrate in a CO_2-free atmosphere for 10 min at room temperature. Grids were dried and stored in boxes. Sections were examined in a Phillips EM300 electron microscope operated at accelerating voltage of 60 or 80KV. Micrographs were taken on ILFORD SP353 plates (3¼ x 4").

V.B.2 Biochemical Assay of Chitin in *C. elegans* egg shell

Eggs laid by *C. elegans* treated with 10 µg diflubenzuron cm^{-3} for 72h were collected and centrifuged using a potassium tartrate density gradient (Section IV.B.2.a.). The eggs were carefully washed

in 1% (w/v) NaOCL (McClure *et al.* 1973; Schaad and Walker, 1975; Emmons *et al.* 1979) and sterile distilled water to remove contamination by bacteria. Eggs were then incubated on fresh diflubenzuron-treated plates (10 µg cm^{-3}) at 25°C and collected after a further 24h. Control (untreated) eggs were collected and decontaminated as described above.

Batches of eggs were sonicated for 30 sec (Section IV.B.3.b) to release the egg shells. Attempts to separate the egg shells by density gradient centrifugation were unsuccessful. Instead, whole eggs were used for chitin analysis.

Two methods for chitin analysis were attempted:

Initially a modified Elson-Morgan colorimetric method was used (Clarke *et al.*, 1967). Glucosamine was assayed in hydrolysates eluted with 0.3N HCL from a column of Dowex 50W (8% cross-linked, cation exchange resin). However, poor yields of glucosamine standards were obtained from the column eluates and examination of the absorption spectra (Boas, 1953) detected the presence of chromagens.

Subsequently, glucosamine was assayed as described by Clarke *et al.* (1967), Bird and McClure (1976) with slight modifications. This method is based on the use of ion exchange chromatography for separation of amino sugars and amino acids. Samples of eggs were hydrolysed in 6N HCL at 100°C for 6h and dried at 45°C using a Flash-evaporator. Dry hydrolysates were dissolved in 0.2M citrate buffer, pH2.2 (Table V.1) and aliquots (0.5 - 1.0 cm^3) analysed in a Locarte Amino Acid Analyser (Mark 4). Standard solutions of

glucosamine. HCL (30 or 150 nmole) and amino acids (30 to 60 nmole) were also analysed.

Table V.1. Citrate buffer (pH 2.2)

Na octanoate	0.1 g
Citric acid	21.0 g
NaOH	8.4 cm^3
Conc. HCL	16.0 cm^3
Thiodiglycol	5.0 cm^3
H_2O added to	1.0 litre

Samples were eluted sequentially (Figures V.1-4) from a column of Permutit Zeo Carb 225 resin by a series of buffers of increasing pH and were detected using the ninhydrin reaction (Moore and Stein, 1953; Yemm and Cocking, 1955). The colour complex developed was estimated using a colorimeter at 440 nm for proline and 570 nm for glucosamine and other amino acids (Figures V.I-4). Ten batches of eggs from control and diflubenzuron-treated (10 µg cm^{-3}) samples were examined. The amount of glucosamine in each batch of control and diflubenzuron-treated eggs was determined by calculating the area of glucosamine-peak obtained in each chromatogram, compared with similar peaks made by standard acetylglucosamine (30 or 150 nmole).

V.B.3. ^{14}C-diflubenzuron study

The ^{14}C- radiolabelled diflubenzuron [25% a.i. (w/w) wettable formulation, sp. act. 518 KBq/mg or 643 MBq/mmole] used in this study was provided by Dr. J.A. Veech (USDA, National Cotton Pathology Research Laboratory, College Station, Texas, U.S.A.).

a) Incorporation of ^{14}C-diflubenzuron into *E. coli* (OP50)

NG agar plates containing (40 µg a.i. cm^{-3}) ^{14}C-diflubenzuron

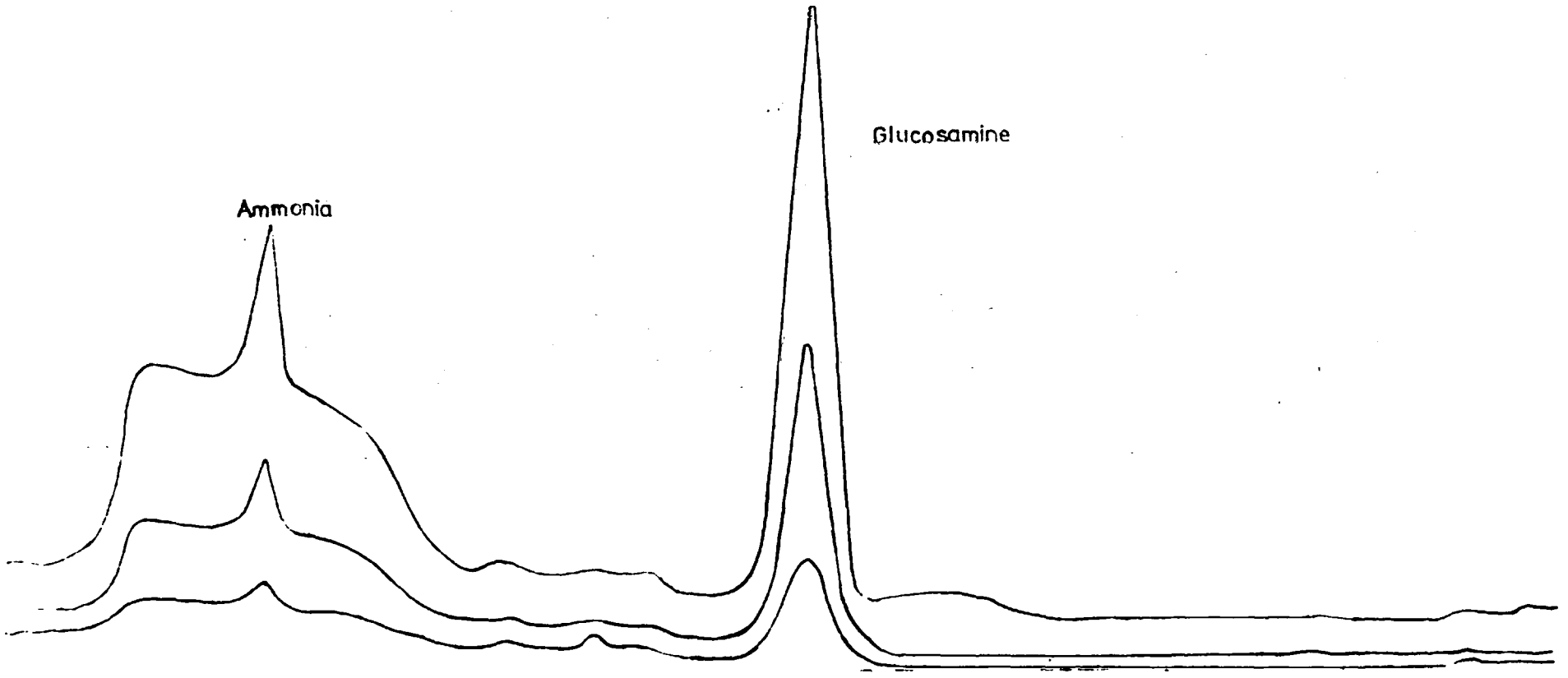


Figure V.1. The peak of standard solution of glucosamine .HCL (150 nmole) detected at 570 nm .

Figure V.2. Representative chromatogram for a mixture of standard solutions of amino acids (60 nmole each, except 30 nmole cystine). The peaks produced by ammonia at 570 nm (upper tracing) and by proline at 440 nm (Lower tracing) were used as reference points.

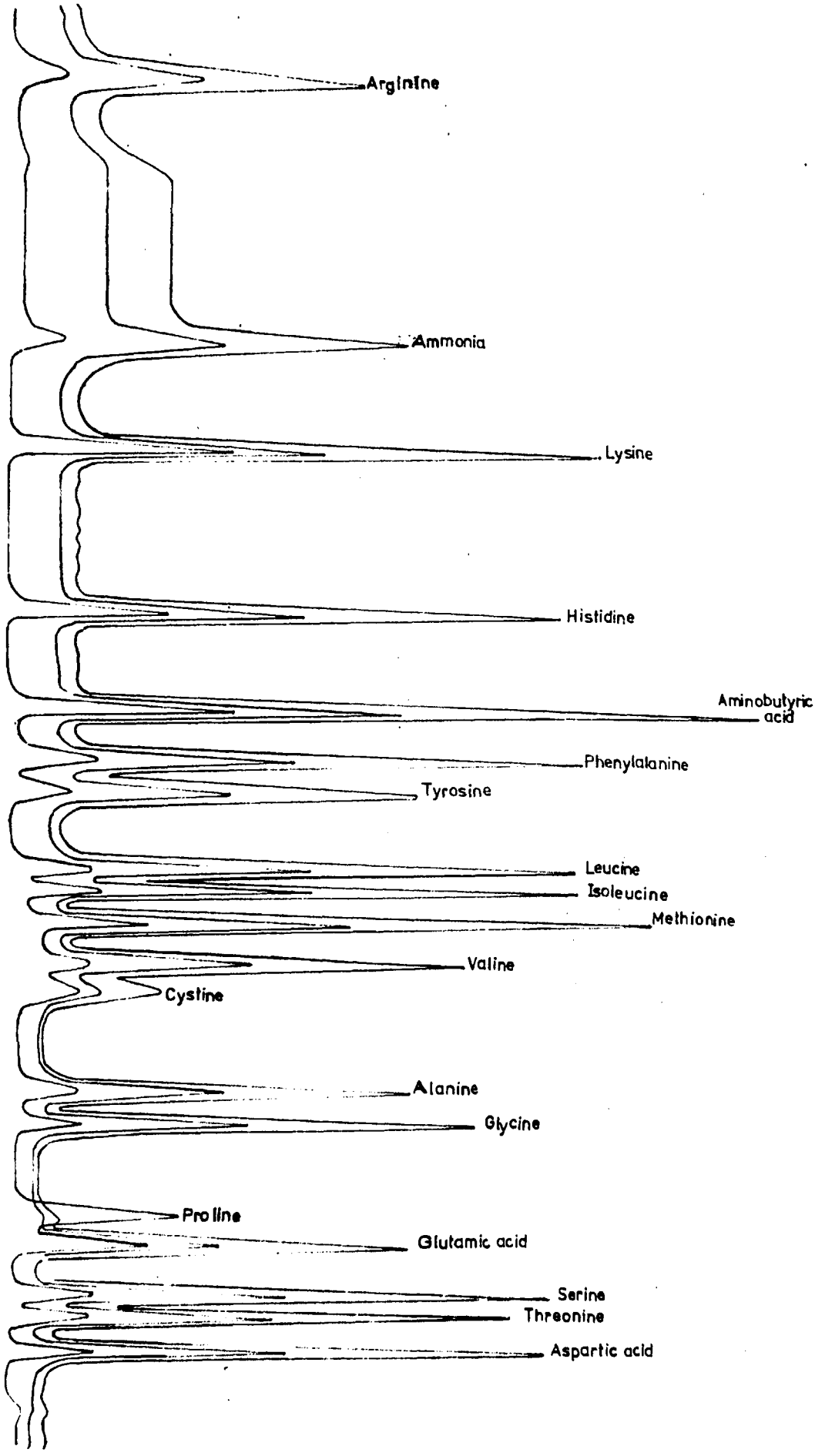

Arginine
Ammonia
Lysine
Histidine
Aminobutyric acid
Phenylalanine
Tyrosine
Leucine
Isoleucine
Methionine
Valine
Cystine
Alanine
Glycine
Proline
Glutamic acid
Serine
Threonine
Aspartic acid

Figure V.3. Representative chromatogram for amino acids and glucosamine obtained from a control sample of *C. elegans* eggs (n = 743).

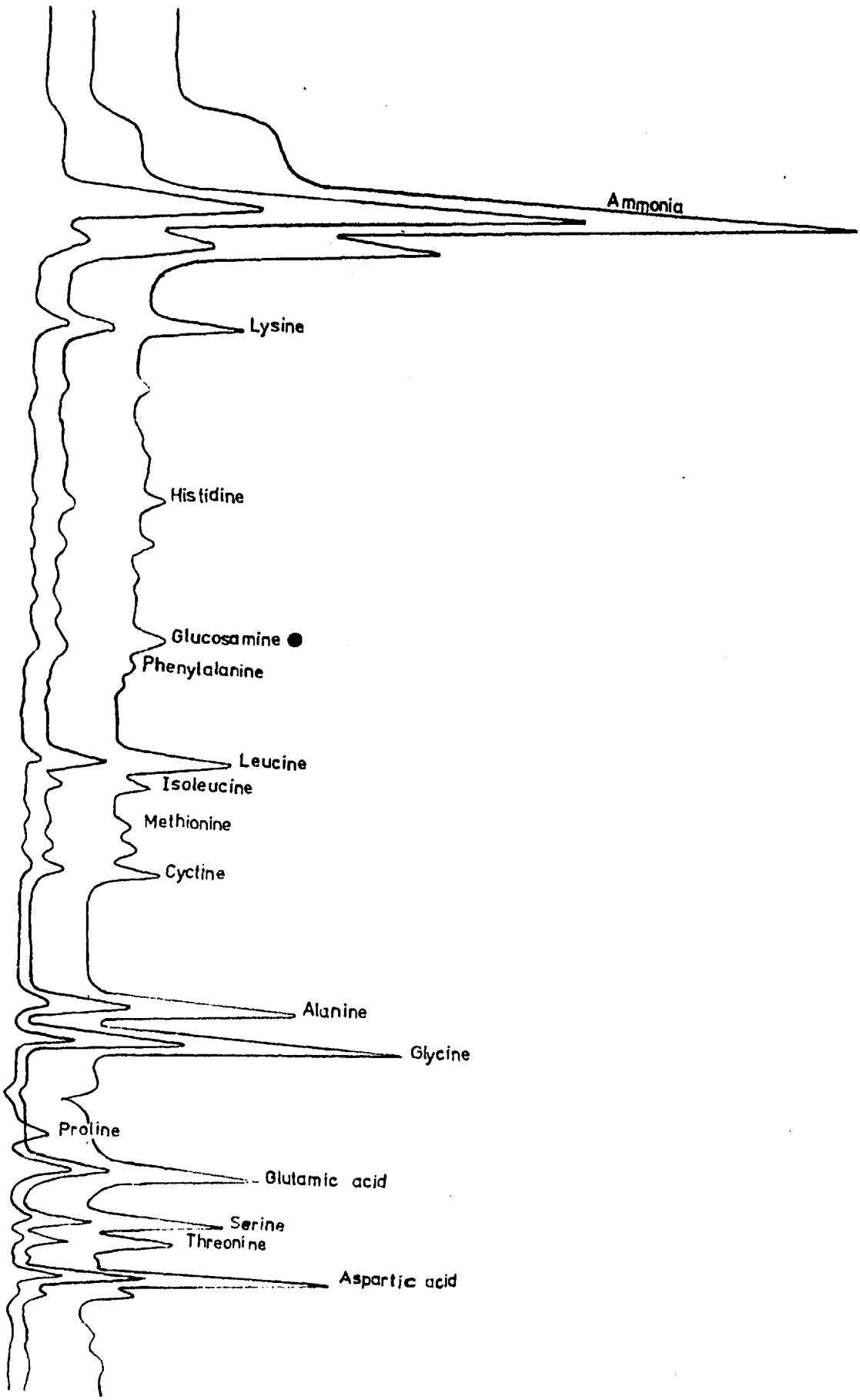
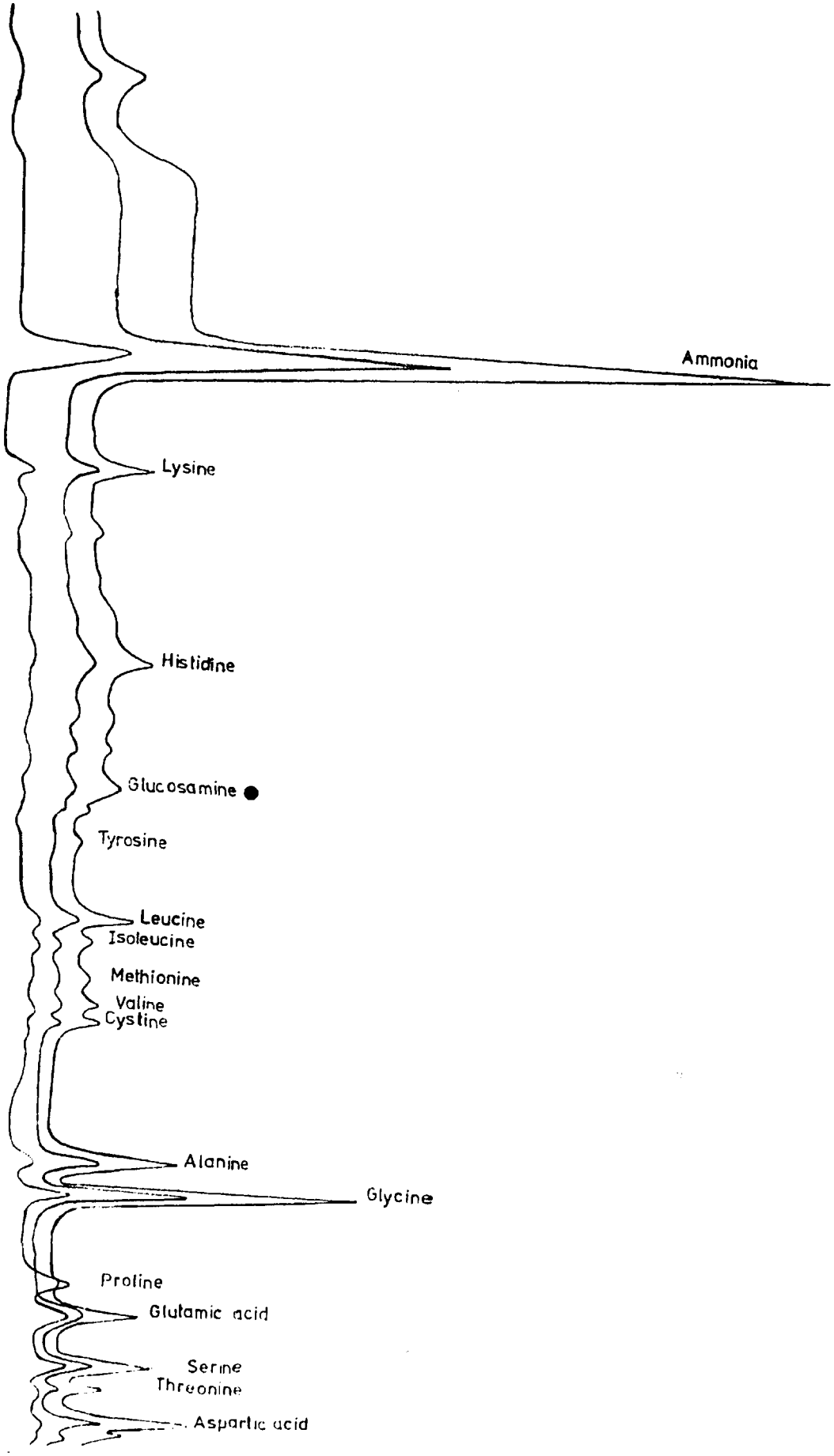

Ammonia
Lysine
Histidine
Glucosamine
Phenylalanine
Leucine
Isoleucine
Methionine
Cyctine
Alanine
Glycine
Proline
Glutamic acid
Serine
Threonine
Aspartic acid

Figure V.4. Representative chromatogram of amino acids and glucosamine obtained from a sample of *C. elegans* eggs (n = 380) treated with 10 μg cm^{-3} diflubenzuron.


Ammonia
Lysine
Histidine
Glucosamine
Tyrosine
Leucine
Isoleucine
Methionine
Valine
Cystine
Alanine
Glycine
Proline
Glutamic acid
Serine
Threonine
Aspartic acid

[Mean radioactivity = 1565 dpm/mg agar] were prepared and streaked with *E. coli* (OP50). After 48 h at 25°C a sample of *E. coli* culture was carefully scraped from the treated plates using a coverslip (1.0 cm dia.), weighed and then placed in a scintillation vial containing 10 cm^3 of Bray's scintillant (Bray, 1960). The vials were counted in a Beckman LS- 250 Liquid Scintillation counter for 50 min or to a 2σ error of 0.5% whichever was the shorter.

b) Incorporation of ^{14}C-diflubenzuron into hermaphrodites and eggs of *C. elegans*

Two groups of 50 - 60 *C. elegans* J_2 were exposed with *E. coli* for 48 h on agar plates containing ^{14}C-diflubenzuron (40 µg a.i. cm^{-3}) at 25°C. The nematodes in one group were washed several times in distilled water to eliminate the bacteria and subsequently transferred to vials with Bray's scintillant and counted as above (a).

Nematodes from the other group were transferred to clean NG agar to lay eggs. After 24 h the eggs laid were washed and collected by sucrose centrifugation (Section IV.B) and radioactivity counted as above. The eggs laid on ^{14}C-diflubenzuron plates by both groups of nematodes were also collected and counted.

A third group of eggs obtained from untreated *C. elegans* were exposed to ^{14}C-diflubenzuron on NG - agar plates for 6 h at 25°C. The eggs were then collected and radioactivity was determined as above.

V.C. Results

V.C.1 Ultrastructural Studies

This section describes the ultrastructure of diflubenzuron-treated eggs and adult *C. elegans* compared to that of control specimens.

V.C.1.a Ultrastructure of the egg shell of *C. elegans*

By analogy with the ultrastructure of the egg shell of other nematode species recently reviewed by Wharton (1980), the three egg shell layers in *C. elegans* were the vitelline layer, the middle chitinous layer and the inner lipid layer (Plates V.1-4). However, the above nomenclature is only used to indicate the general chemical composition of various layers in the nematode egg shell which are in fact believed to have a complex chemical structure.

The vitelline layer of *C. elegans* egg shell appears as a unit membrane showing a slight degree of folding and has a consistent thickness of 6.9Å (Plates V.2-4); this layer shows electron dense staining suggesting that it consists mainly of lipoproteins, similar to the vitelline region in the egg shell of some tylenchid nematodes (Bird and McClure, 1976).

The vitelline layer in the egg shell of *C. elegans* is followed by the median chitinous layer which retains a beam-like structure with an average thickness of 71Å and shows less electron dense staining than the former layer (Plates V.2-4). The chitinous layer of the *C. elegans* egg shell is apparently devoid of microfibrils, which have been found in this region in several nematode species (Neville, 1975; Wharton and Jenkins, 1978; Wharton, 1979e); this layer also appears to condense at the junction with the inner, lipid layer of the egg shell in *C. elegans* Plates V.3, 4. Condensation of chitin was similarly observed in the egg shell of *Ascaris lumbricoides* (Foor, 1967), *Rotylenchulus reniformis* and *M. javanica*

Index of abbreviations used in the ultrastructural studies (Plates V.I-19)

am	amorphous material	luw	lobe of uterine wall
av	autophagic vacuole	mb	membranous body
bc	bacterial cell	mf	myofilament
c	cuticle	mo	mature oocyte
cc	central core	mv	microvilli
chi	chitinous layer	mu	musculature
cj	cellular junction	muf	membrane of unfertilized egg
cm	cellular membrane	mt	microtubule
con	condensation	n	nucleus
cp	cytoplasmic region	ne	neuron
cy	cytoplasm	nt	neural tissue
cz	cytoplasm of zygote	ov	ovary
dg	dense granule	pd	protein droplet
dov	distal ovary arm	ps	pseudocoelom
dp	densely stained patch	pov	proximal ovary
ec	egg cytoplasm	ruf	ruptured unfertilized egg
eps	epithelial surface	sp	sperm
er	endoplasmic reticulum	spt	spermatheca
es	egg shell	slb	sperm lipid body
exd	excretery duct	smv	sperm membranous structures
fm	fragment of membrane	sf	strands of fibrous material
g	gut	Tb	Terminal bar
gc	gut cell	Tw	Terminal web
gl	glycogen	uf	unfertilized egg
gm	granulated material	usp	uterinal space
gcy	granulated cytoplasm	us	uterinal secretion
h	hyaline granule	uv	uterinal vesicle
hy	hypodermis	uw	uterinal wall
inc	intestinal cell	v	vulva
isp	intracytoplasmic space	va	intestinal vacuole
lu	lumen	vb	vesicle body
lb	lamellar body	vl	vitelline layer
lp	lipid layer	vm	vulval muscle
lpb	lipid body	vp	vesicle pore
lp	lipid droplet	yg	yolk granule
lpm	lipoprotein membrane	zes	zygote egg shell
lud	electron lucent droplet		

Plate V.1

A) Light micrograph of an untreated egg of *C. elegans* using Nomarski Interference contrast; X 850. Scale bar represent 10 µm.

B) Electron micrograph (T.S.) of an untreated egg of *C. elegans*; X3908. Scale bar represents 1.0 µm.

C & D) Electron micrographs of regions (1) and (2) of B, respectively; X12,599. Scale bars represent 1.0 µm.

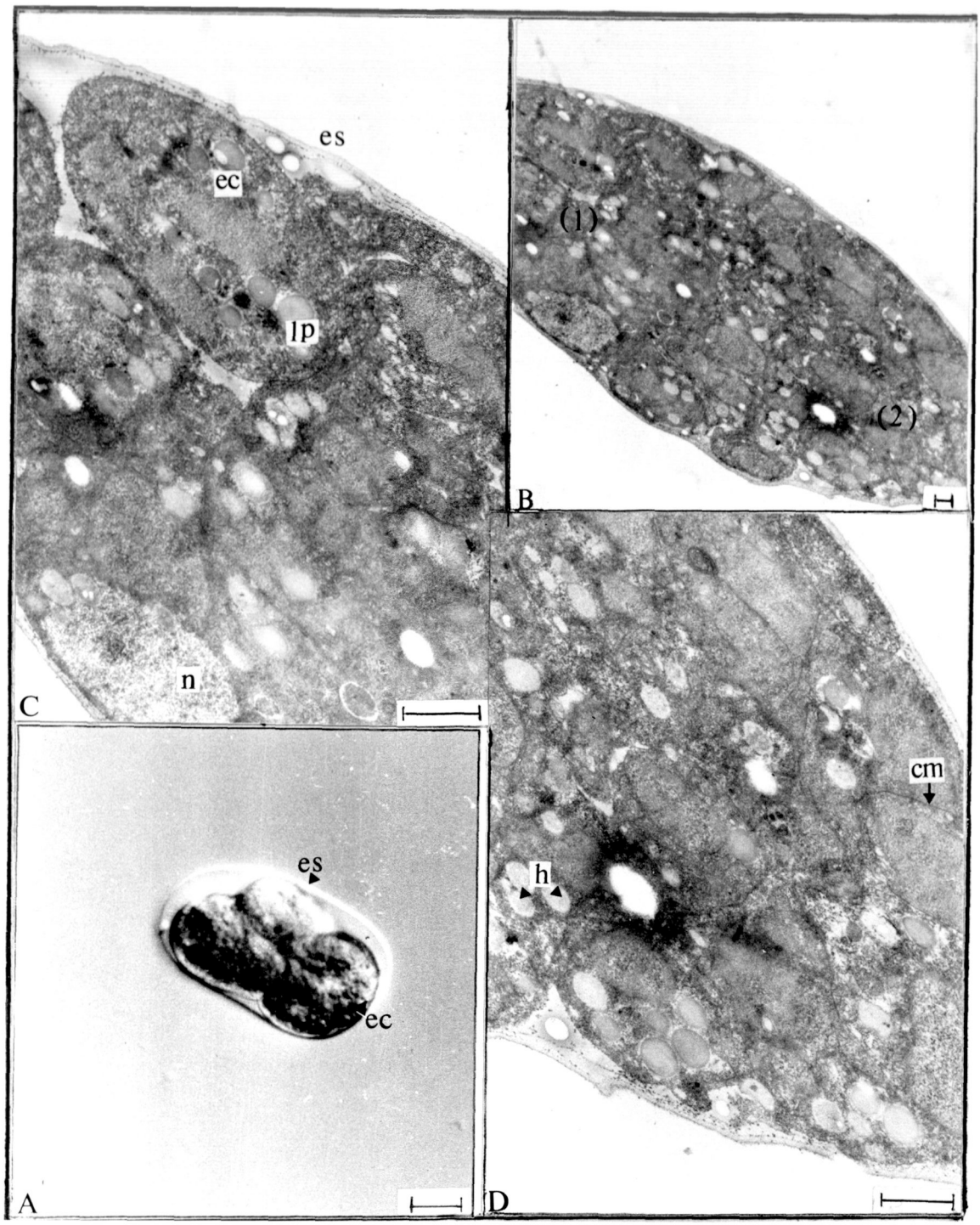

es
ec
lp
n
C
(1)
(2)
B
cm
h
D
es
ec
A

Plate V.2.

A) Electron micrograph (T.S.) through an untreated egg of *C. elegans* showing the egg shell and the developing nematode embryo. The egg shell consists of 3 regions: the vitelline (vl) chitinous (chi) and lipid (lp) layers. The egg cytoplasm appears rich in hyaline (h) and lipid droplets (lp) and glycogen granules (gl). The cytoplasm has contracted away from the egg shell showing the structure of the lipoprotein membrane (lpm) forming the boundary to the lipid layer; X36,252.

B) Electron micrograph (T.S.) of the egg shell and cytoplasm of an untreated *C. elegans* egg, showing the electron dense vitelline layer (vl) and the beam-like structure of the chitinous layer (chi). Note also, the relatively consistent thickness of the lipid layer (lip) and its peripheral membranous structures; X74,315.

C) Electron micrograph (T.S.) of an untreated egg of *C. elegans* showing the 3 distinct layers of the egg shell and the electron dense cytoplasm; X41,022.

Scale bars represent 0.1 μm.

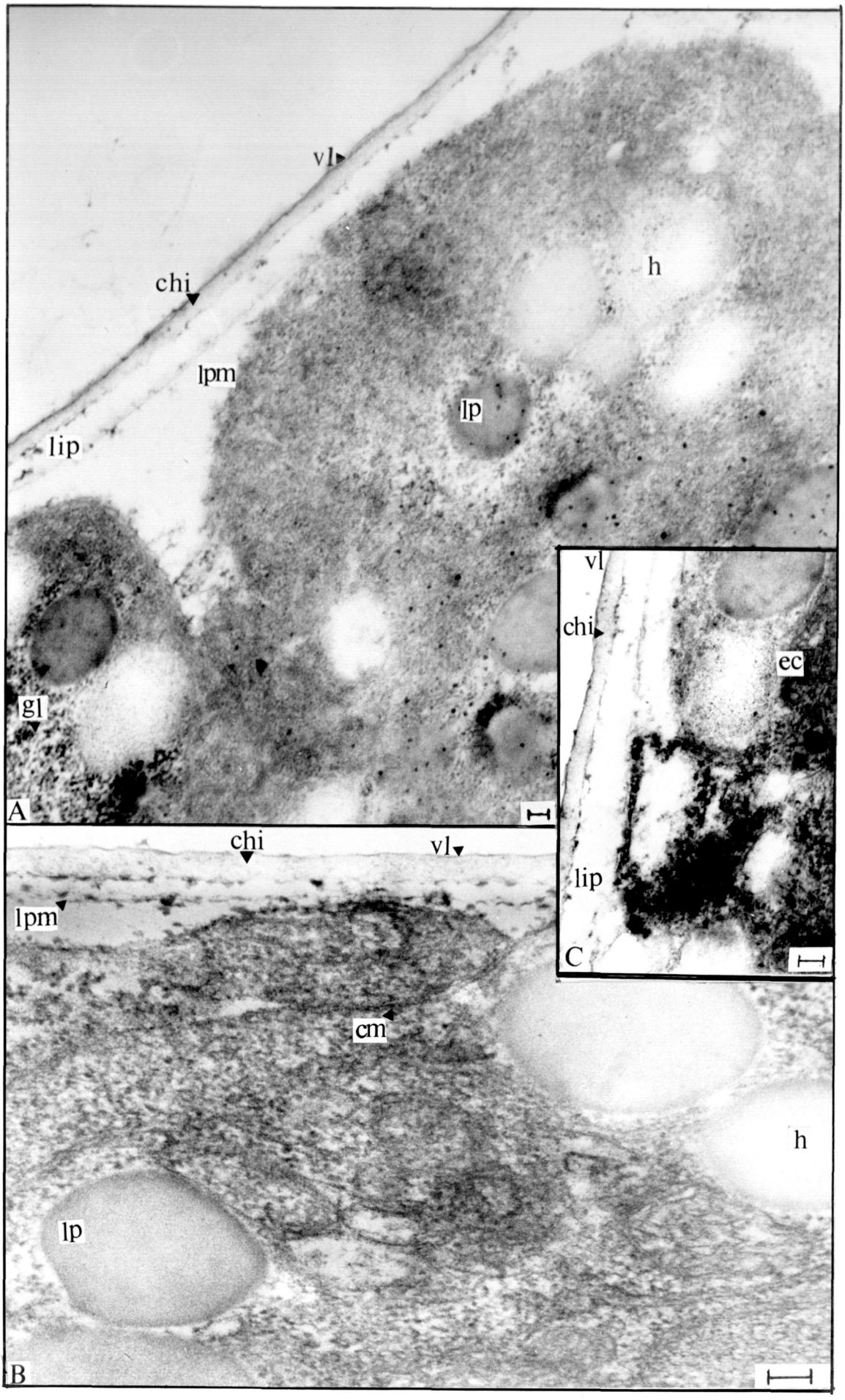

vl
chi
h
lpm
lp
lip
gl
A
vl
chi
ec
lip
C
chi
vl
lpm
cm
h
lp
B

Plate V.3

Electron micrographs (T.S.) of an untreated egg of *C. elegans*,

A) Egg shell with electron dense granules in the lipid layer and lipid body associated with the lipoprotein membrane; 56,263,

B) Densely stained vitelline and chitinous layers; X151,977. Note, condensation of the chitinous layer (con) at the boundary with the lipid layer. Scale bars represent 0.1 µm.

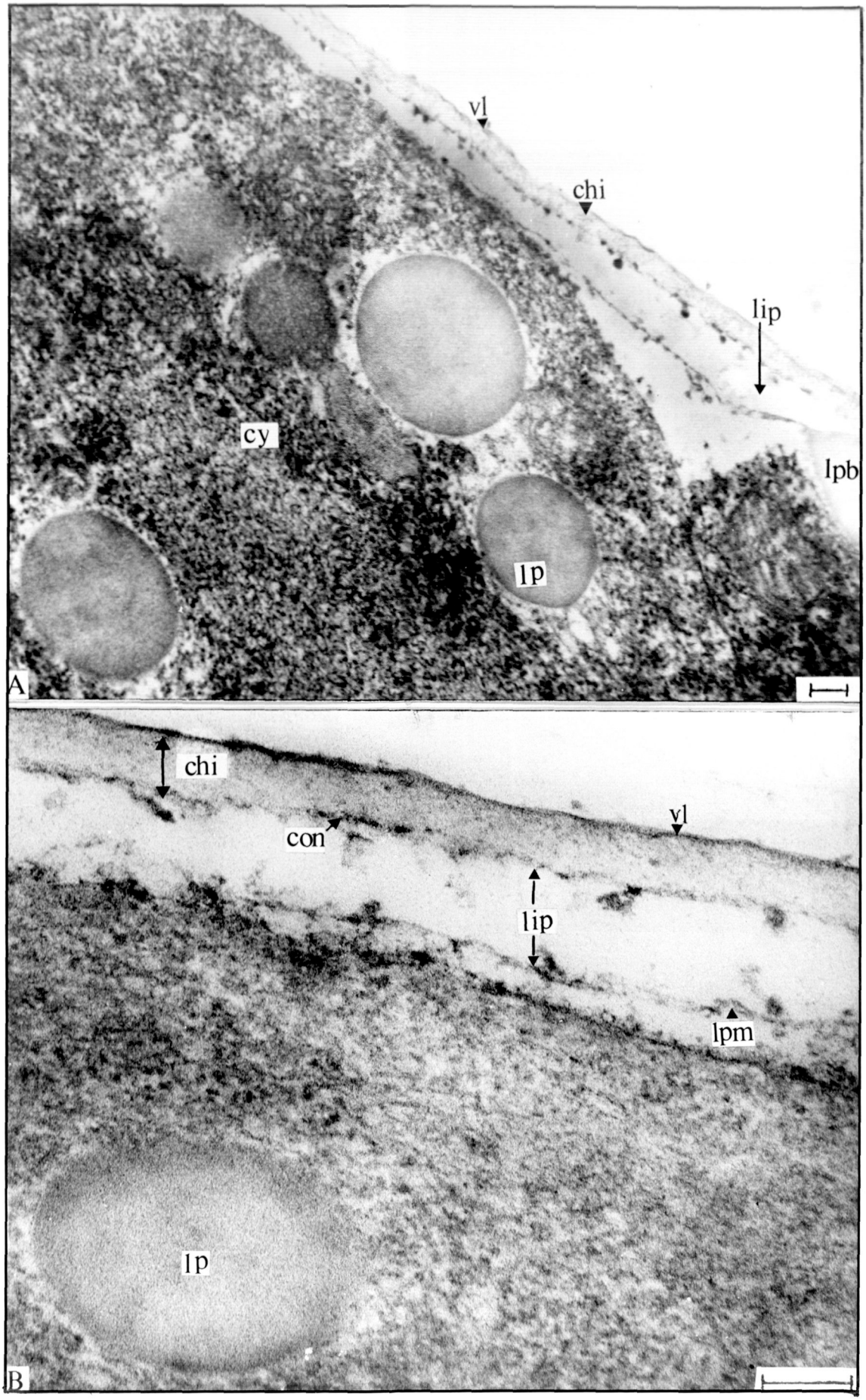

vl
chi
lip
cy
lpb
lp
A
chi
con
vl
lip
lpm
lp
B

Plate V.4

Electron micrographs (T.S.) through untreated eggs of *C. elegans*

A) Vitelline and chitinous layers of the egg shell with a consistent thickness but some variation in thickness in the lipid layer; X38,021.

B) Egg cytoplasm (ec) contracting away from the lipid layer of the egg shell and aggregates of electron dense material at the site of the lipoprotein membrane; X34,026.

C) Chitinous layer of the egg shell showing the greatest thickness found in the present study; X79,607.

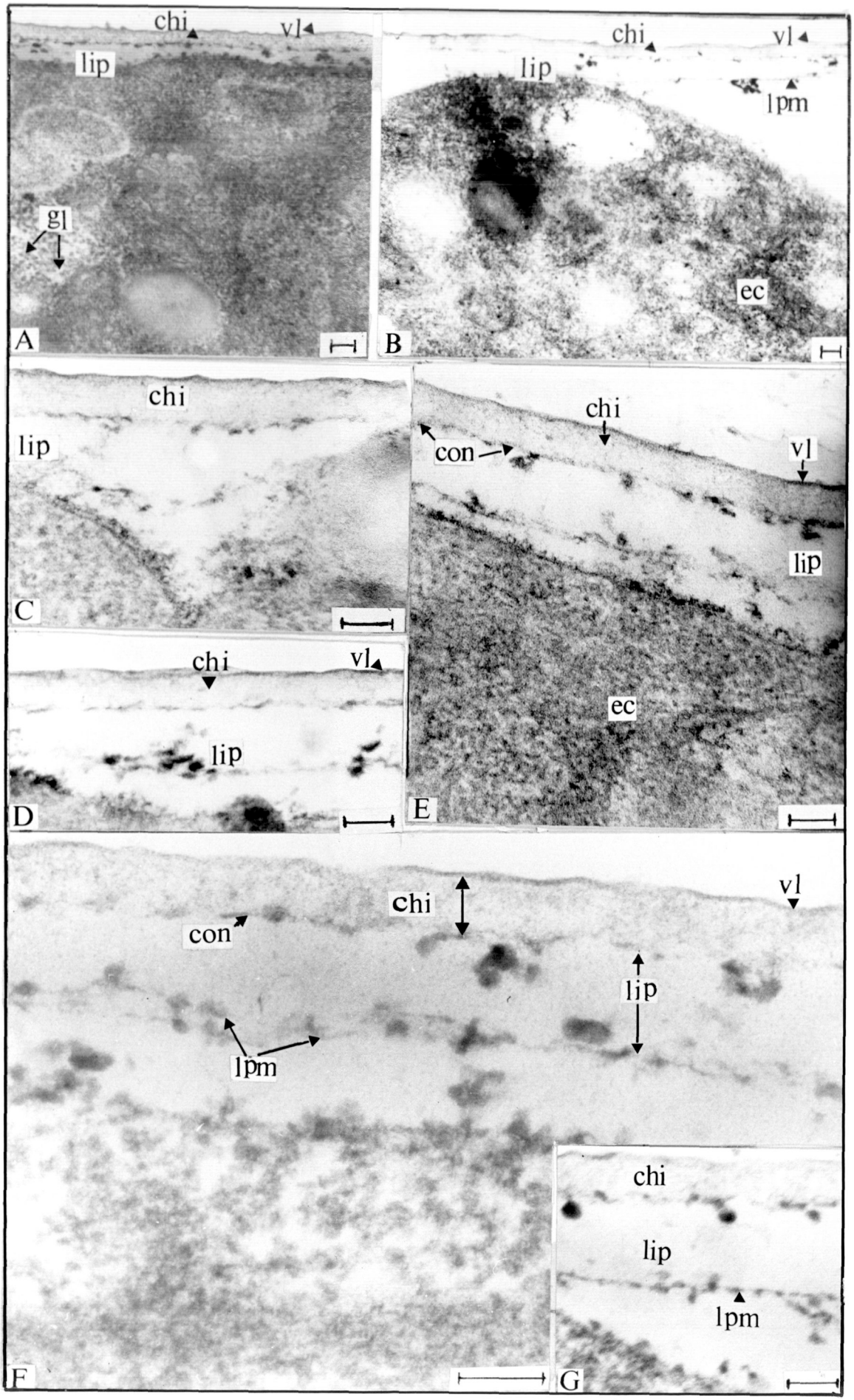
D) Lipid layer of the egg shell containing densely staining material and occasionally attached to the lipoprotein membrane; X79,607.

E) Condensation of the chitinous layer at the boundary with the lipid layer; 79,607.

F) Two lipoprotein membranes at the boundary of the lipid layer of the egg shell; X151,977.

G) Bead-like structure of the lipid membranes of the egg shell; X92,019.

Scale bars represent 0.1 μm.


chi
vl
lip
gl
A
chi
vl
lip
lpm
ec
B
chi
lip
C
chi
con
vl
lip
ec
E
chi
vl
lip
D
chi
vl
con
lip
lpm
F
chi
lip
lpm
G

(Bird and McClure, 1976).

The lipid layer of *C. elegans* , unlike the vitelline and chitinous layers, shows a considerable variation in thickness. A maximum width of 165Å was recorded at the poles of a fully embryonated egg whereas it varied from 40Å to 151Å in other parts of the egg (Plates V.4.A-D). At the boundary between the lipid layer of the egg shell and the cytoplasm of the embryo of *C. elegans* two lipoprotein membranes were discernible (Plate V.4.F). The thickness of each one of these membranes was <30Å and the two membranes were found either superimposed or detached in different sections (Plates V.3.b,4.F). Aggregates of an electron dense material were also observed along these membranes which therefore retained a bead-like structure (Plate V.4.G). A number of such membranes have been described associated with the lipid layer of tylenchid eggs (Bird and McClure, 1976). These membranes are thought to reflect the degree of folding in the lipid layer at a particular stage of its development. However, the lipoprotein membranes have not been observed in the egg shells of nematodes belonging to other orders, although this may be due to the difficulty of preparing thin sections for electron microscopy (Bird and McClure, 1976; Wharton, 1980).

In diflubenzuron-treated eggs (10 µg cm^{-3}), the vitelline layer appeared to be normal whereas the chitinous and lipid layers were considerably affected (Plates V.5-7).

The chitinous layer had a less consistent appearance than normal and therefore did not retain the beam-like appearance found in control

Plate V.5

Diflubenzuron-treated (10 µg cm^{-3}) eggs of *C. elegans:*

A) Light micrograph of an egg using Nomarski Interference contrast; X11,60
Scale bar represents 10 µm.

B) Electron micrograph (T.S.) of an egg showing the granulated appearance of the egg cytoplasm with densely stained patches (dp) and some cellular inclusions. Note malformation in the structure of the egg shell; X34,026. Scale bar represents 0.1 µm.

C) Electron micrograph (T.S.) of an egg showing several changes in the ultrastructure of chitinous layer of the egg shell, particularly at the site of chitin condensation. The lipid layer contains electron dense granules; X151,977. Arrows indicate disintegrated parts of the chitinous layer. Scale bar represents 0.1 µm.

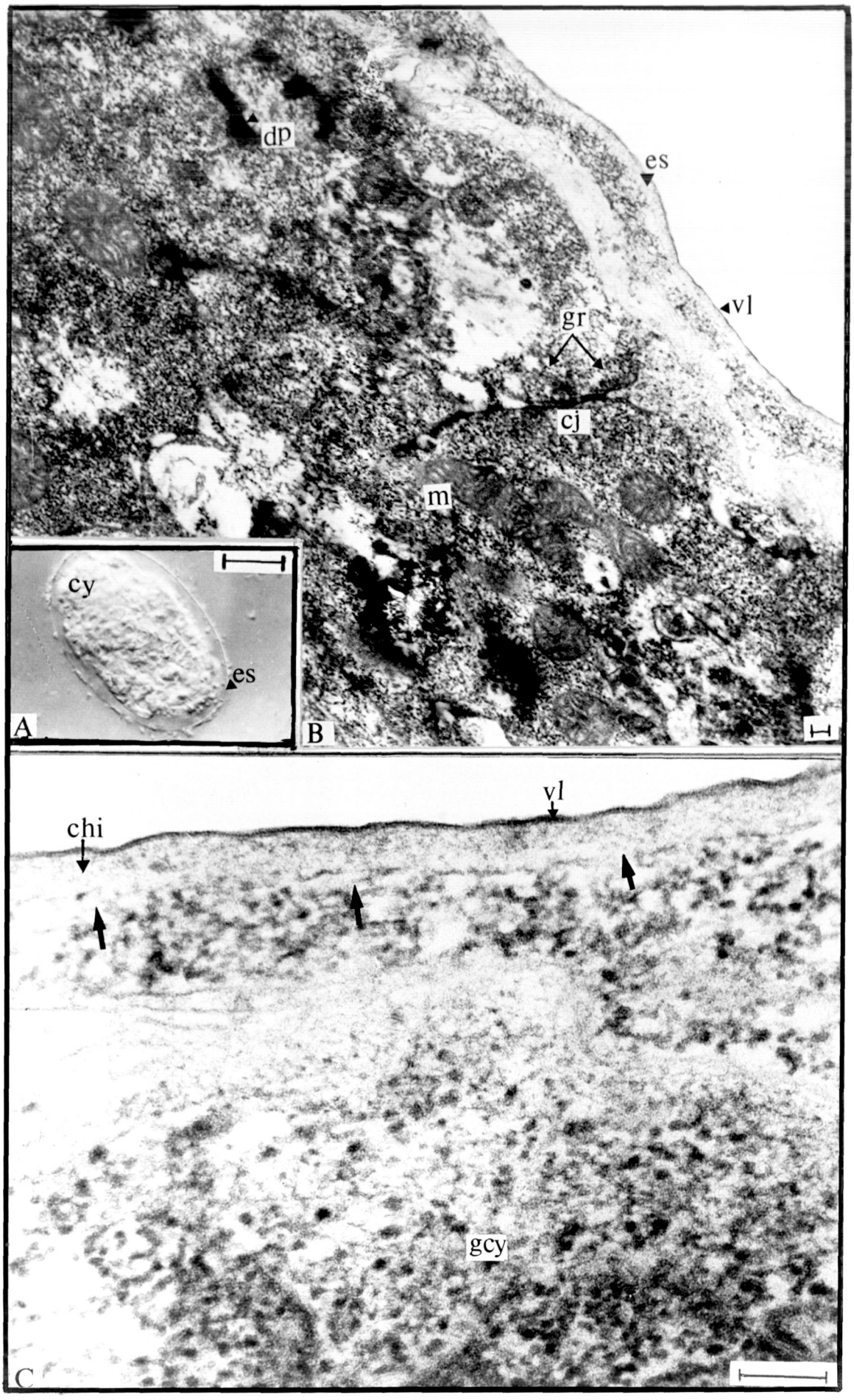

dp
es
vl
gr
cj
m
cy
es
A
B
vl
chi
gcy
C

Plate V.6.

Electron micrographs (T.S.) through diflubenzuron-treated eggs of *C. elegans* showing:

A) Granulated cytoplasm with intracytoplasmic spaces (isp) and dark patches; X 32,214

B) Disrupted configuration of the chitinous layer in the egg shell and fragments of membranous structure (fm); X 86,846.

C) Absence of structural changes in the vitelline layer of the egg shell, but dense granulation in the region of lipid layer; X 86,846.

D) Partially disintegrated chitinous layer, and membranous structures at the site of the egg shell; X 151,977.

Arrows indicate affected regions.

Scale bars represent 0.1 µm.

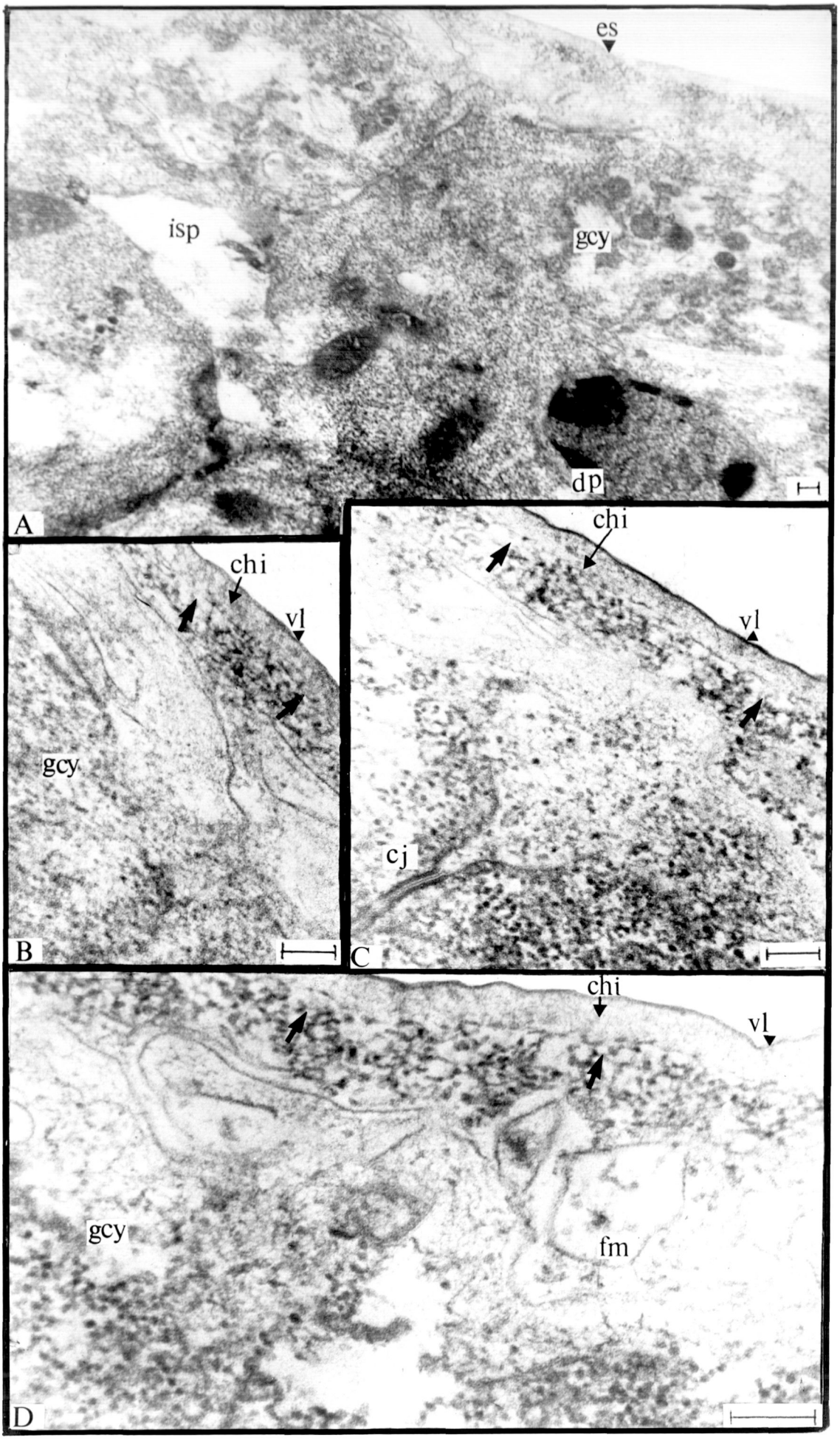

es
isp
gcy
dp
A
chi
vl
gcy
B
chi
vl
cj
C
chi
vl
gcy
fm
D

Plate V.7

Electron micrographs (T.S.) through diflubenzuron-treated eggs showing:

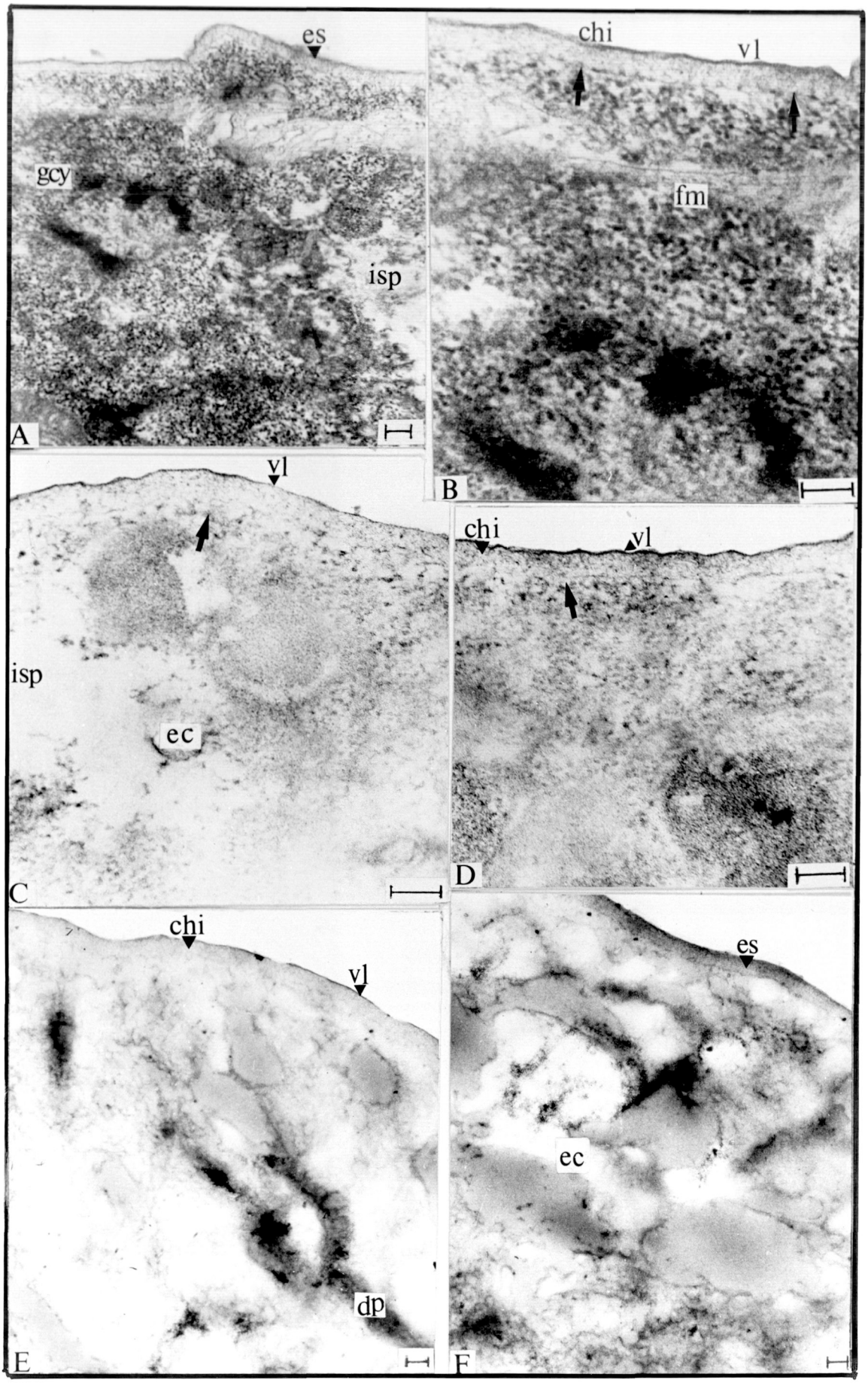
A) Intracytoplasmic space (isp) and granulated cytoplasm (gcy); X 34,565.

B) Egg shell having chitinous layer that does not possess the beam-like structure found in control egg; X 86,846

C & D) Disintegration of the chitinous layer in the egg shell; X 74,315

E & F) Malformation of the egg shell and cytoplasm; X 34,026

Arrows indicate disintegrated parts of the chitinous layer. Scale bars represent 0.1 µm.


es
gcy
isp
A
chi
vl
fm
B
vl
isp
ec
C
chi
vl
D
chi
vl
dp
E
es
ec
F

eggs. This layer seemed to be mostly disintegrated at the margin with the lipid layer, where condensation of chitin occurs in normal eggs (Plates V.5.C, 6.B-D, 7.B-F).

The structure of the inner, lipid layer in treated eggs indicated that production of this layer has been considerably affected by diflubenzuron; the region of lipid layer in diflubenzuron-treated eggs having a granular appearance instead of a normal reticulate organization (Plates V.5, 6, 7).

Discrete membrane-like structures that could be fragments of cellular membranes, were frequently observed at the boundaries between the egg shell and the cellular cytoplasm (Plates V.6.B,D). In comparison to control eggs, the cytoplasm of diflubenzuron-treated eggs was shown to have a malformed structure with dark patches and was also devoid of cellular inclusions (Plates V.6.A, 7.A-F). However, a few treated eggs contained the densely stained hyaline or lipid granules and numerous mitochondria present in untreated eggs (Plates V.5.B).

V.C.1.b Ultrastructural observations on the formation of *C. elegans* egg shell

(1) Sections through the distal ovary arm of *C. elegans* (Plate V.8) show the ultrastructure of mature oocytes prior fertilization and the start of egg shell production. The cytoplasm of these oocytes is rich in yolk granules embedded in strands of compact endoplasmic reticular material; the yolk granules being formed of electron lucent hyaline and lipid droplets. Mature oocytes also

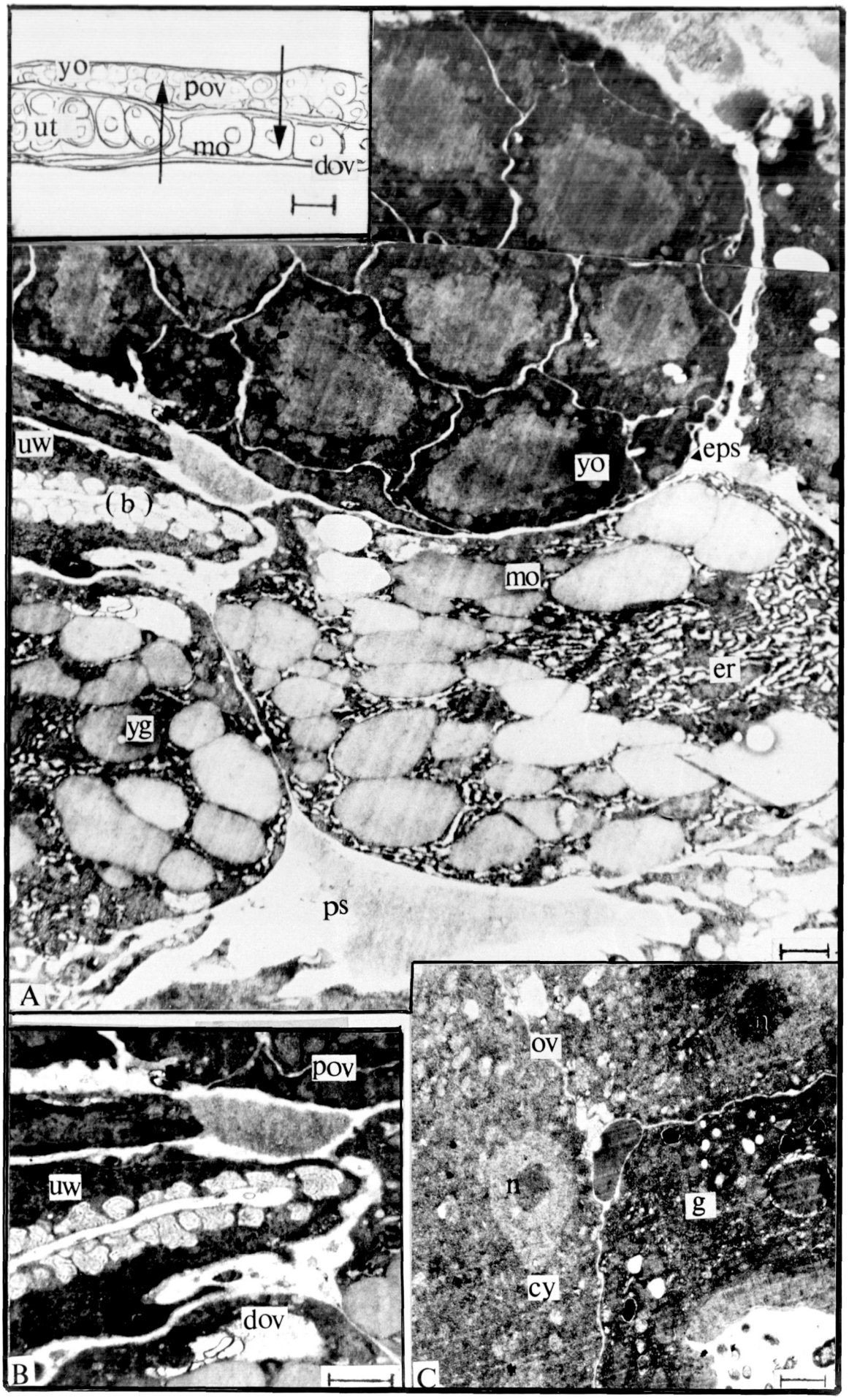
Plate V.8

A) Electron micrograph (L.S.) through the proximal and distal arms of the ovary of an untreated *C. elegans* hermaphrodite showing oocytes at early stages of oogenesis and mature oocytes prior to fertilization. Note extensive network of endoplasmic reticulum and a cytoplasm rich in yolk granules; X 8363.
Inset shows a diagram of the nematode region sectioned (between arrows); X200. Scale bar represents 20 µm.

B) Electron micrograph of the region (b) in (A) showing a part of the uterine wall containing vesicle-like structures; 11,724.

C) Electron micrograph (T.S.) through the ovary of an untreated *C. elegans* hermaphrodite; X 7206.

Scale bars represent 1.0 µm.


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C

appear to have a unit-membrane, mostly intact with the oocyte cytoplasm.

(2) The ultrastructure of *C. elegans* sperm (N_2 strain) in the spermathecal and uterinal regions of the hermaphrodites (Plate V.9) appears to be similar to that of the sperm in *C. elegans* strain HC-D9 described by Nelson *et al.* (1978). However, the sperm were not found to be distinctly differentiated into cytoplasmic and pseudopodial regions as has been observed by Ward and Carrel (1979). The sperm also appear not to have cytoplasmic inclusions apart from numerous membranous organelles, arranged circumferentially or on one site of the sperm (Plates V.9.A,C). These organelles may be associated with microtubular structures and a dense lipid-like body (Plate V.9.B).

(3) Penetration of mature oocytes by the nematode sperm is generally understood to stimulate production of the egg shell (Anya, 1976; Wharton, 1980). Sections through an early stage of *C. elegans* zygotes (Plate V.10.A-D) have shown that the synthesis of the chitinous layer has already been triggered and deposition of an electron dense chitinous material has taken place between the vitelline layer and the cell membrane of the nematode zygote (Plate V.10.A,B). At this stage, production of the chitinous layer is incomplete as there is no indication of chitin condensation at the margin of this layer. Instead, strands of granulated or "fibrous" electron dense material have been observed in the region between the chitinous layer and the egg cytoplasm (Plate V.10.D). The

Plate V.9

Electron micrographs (L.S.) of the sperm (sp) in an untreated *C. elegans* hermaphrodite showing;

A) A number of peripheral vesicular bodies (vb) arranged in one site; X 22,468.

B) Numerous microtubular structures (mt) in conjunction with vesicle bodies around a relatively large lipid body (lb); X 35,948.

C) Membranous structures (sm) in each vesicle and vesicle pores (vp); X47,700.

Scale bars represent 0.2 µm.

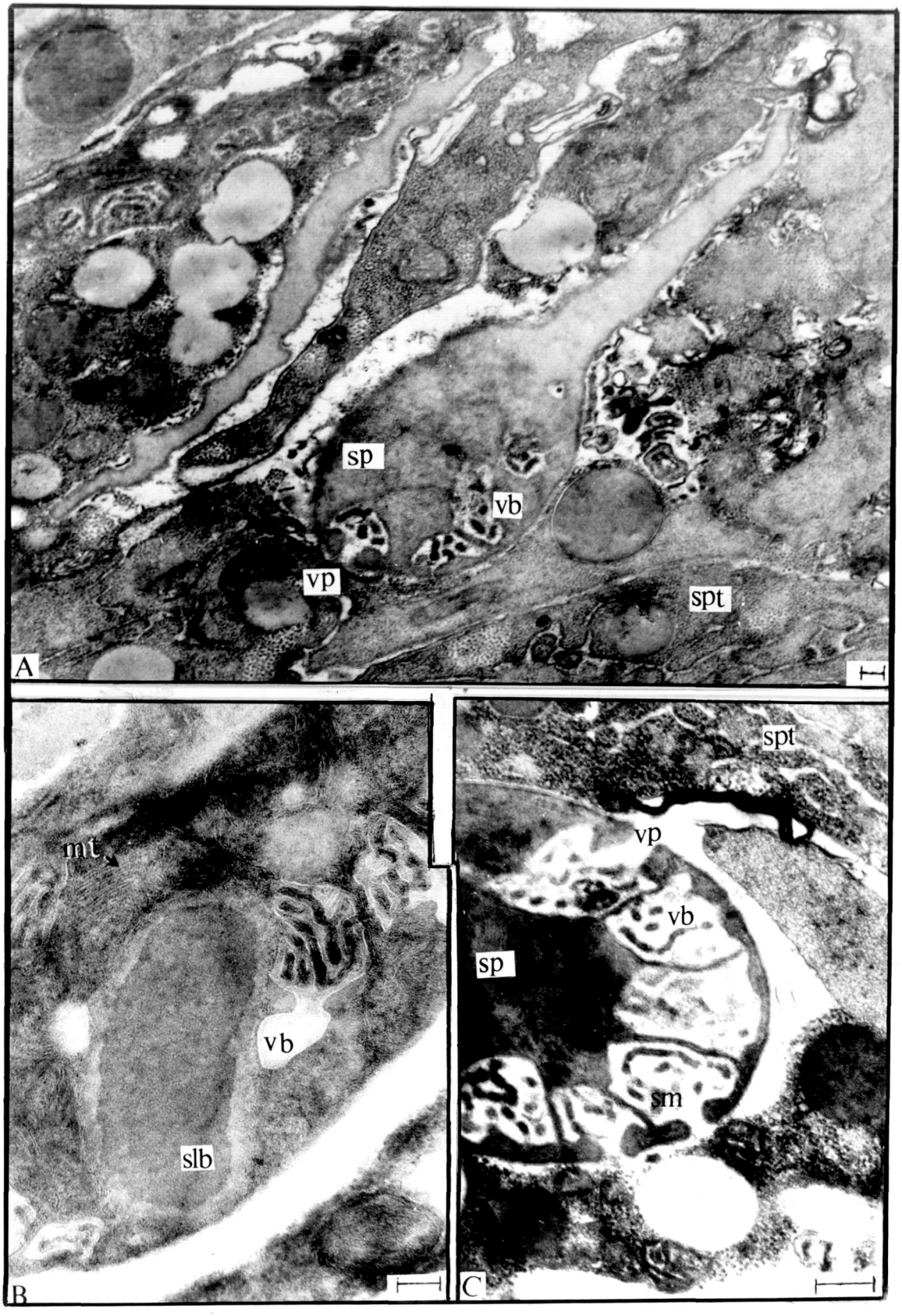
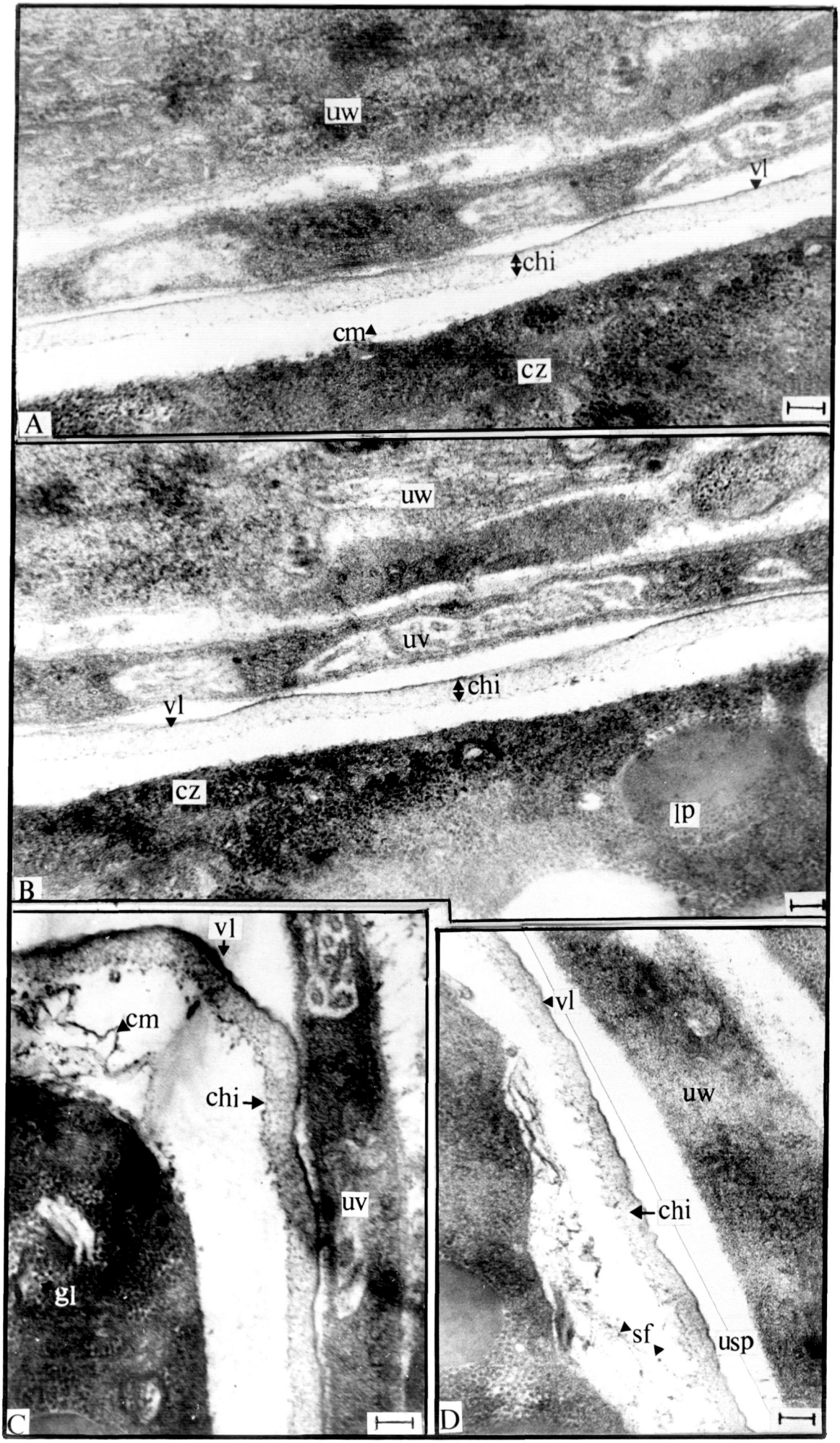

sp
vb
vp
spt
A
mt
vb
slb
B
spt
vp
vb
sp
sm
C

Plate V.10

A-D) Electron micrographs (L.S.) through the egg shell of a zygote in the early stages of formation after fertilization in an untreated *C. elegans* hermaphrodite. The sections show deposition of the chitinous layer between the vitelline layer and cell membrane. At this stage (A-D) condensation of the chitinous layer and formation of the lipid layer have not started. The chitinous layer retains a relatively granulated appearance (C) and shows some electron dense strands (sf) in conjunction with the cell membrane on cytoplasm. Note densely stained cytoplasm containing yolk and glycogen granules (C,D).

A-D; X55,300

Scale bars represent 0.7 µm.


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cm
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thickness of the chitin layer varied from 54Å to 108Å in different specimens, whereas the vitelline layer was <10Å (Plate V.10). Subsequently, the egg shell of the *C. elegans* zygote, in a later stage of development appears to have 3 distinct layers; the vitelline, chitinous and lipid layers with an average thickness of 7Å, 67Å and 75Å, respectively (Plate V.11.A-D). These layers are basically similar to those found in a fully developed egg shell, but at this early stage the lipid layer had a consistent thickness at different sites around the egg shell (Plate V.11.B,c).

(4) Interestingly, longitudinal sections through the uteri of control *C. elegans* revealed the presence of numerous vesicles with microvilli or finger-like structures along the lobulous uterine wall (Plate V.12). The dimensions of these vesicles varied from 180Å to 420Å in length and from 100Å to 300Å in width. They also appeared to be bound by a fine membrane made of 2 or 3 layers (Plate V.12.B).

Since the egg shell of *C. elegans* is considered, to be endogenously produced by the nematode zygote, the function of the uterine vesicles is not thought to be associated with egg shell formation. On the contrary, these structures are suggested to be involved in the production of the extra, uterine layer in the egg shell of the animal parasitic species *A. galli*, *Heterakis gallinarum*, *A. tetraptera* and *H. diesingi* (Simonov and Shigina, 1972; Wharton, 1979 a,c).

(5) The observed production of unfertilized eggs in *C. elegans*

Plate V.11

A) Nomarski Interference contrast of the uterus region of an untreated *C. elegans* hermaphrodite with different stages of fertile eggs. Arrows indicate the region sectioned in B-D below; X 200. Scale bar represents 10 µm.

B) Electron micrograph (T.S.) through a zygote with cytoplasm (cz) mainly consisting of an electron dense reticulate material surrounding a large number of yolk granules; X 6330. Scale bar represents 7.0 µm.

C) Electron micrograph (T.S.) through a zygote in the uterus showing accumulation of cytoplasmic reticulate material at the site of the egg shell; X 19,080. Scale bar represents 0.2 µm.

D) Electron micrograph (T.S.) through a zygote showing a late stage in the formation of the egg shell. The chitinous and lipid layers are fully developed; the vitelline layer is beyond resolution in this section. Note consistent thickness of the lipid layer; X 65,619. Scale bar represents 0.2 µm.

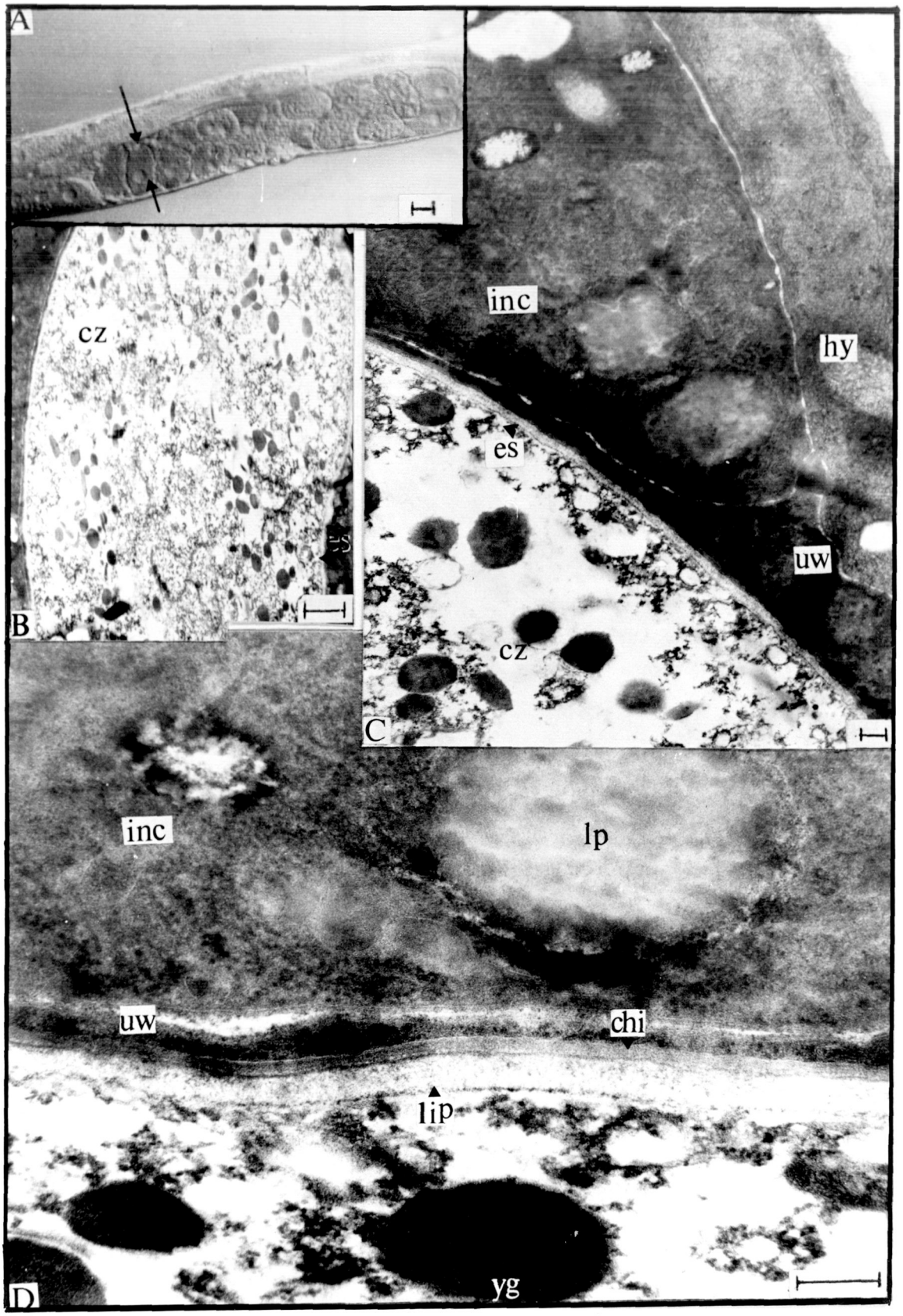

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Plate V.12

Electron micrograph (L.S.) through the uterus of untreated *C. elegans* hermaphrodites showing:

A) Uterine wall (uw); its inner surface with numerous vesicles; X 21,942.

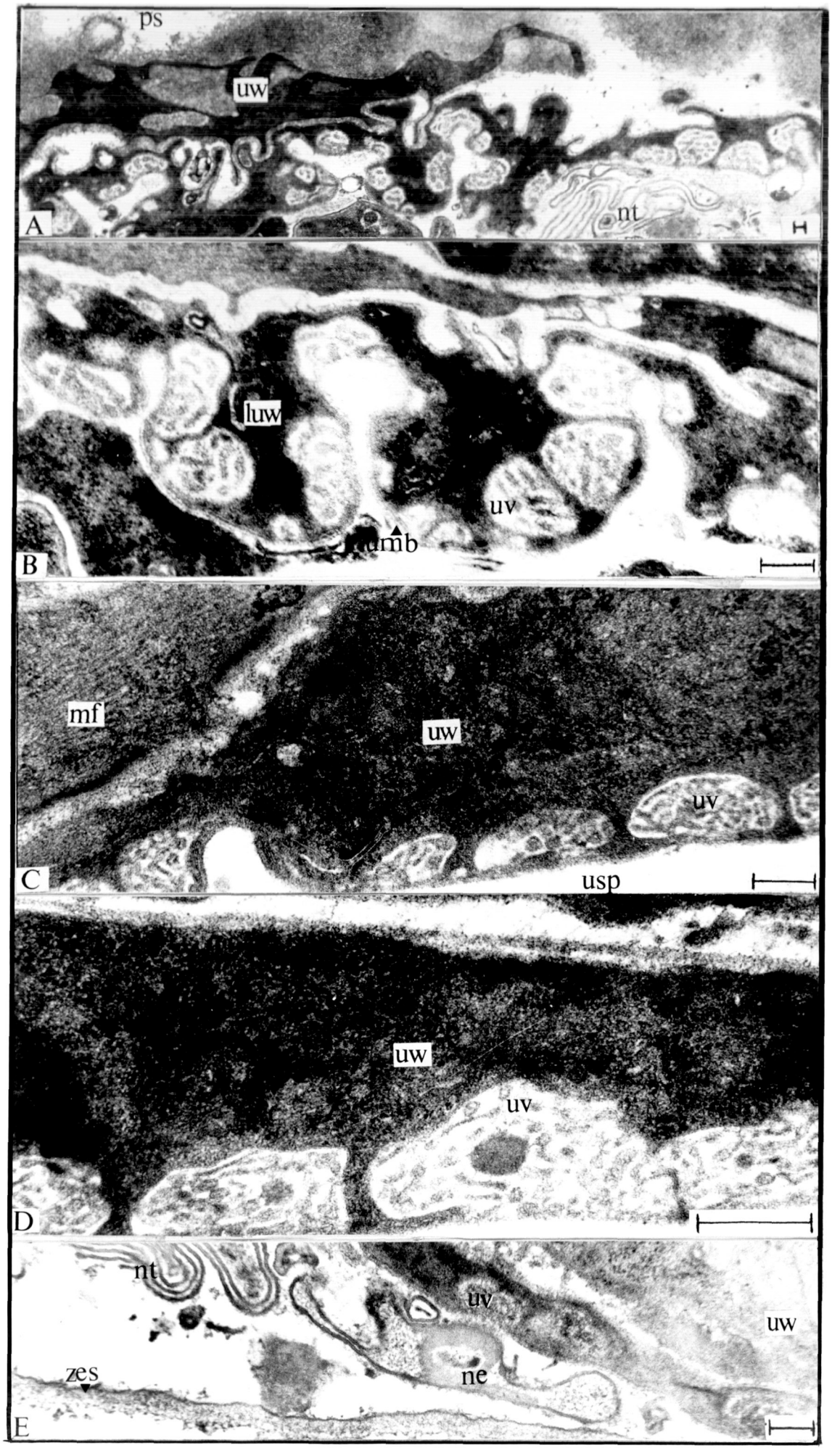
B) Lobular structure of the uterine wall, surrounded by a fine membrane (mb); 47,700.

C) Uterine vesicles (uv) with microvillous structures; X 55,304

D) Electron dense body at the centre of a uterine vesicle; 109,130.

E) Putative neuron (ne) associated with the inner surface of the uterine wall; X 37,976.

Scale bars represent 0.2 µm.


ps
uw
A
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mf
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treated with 40 µg cm^{-3} diflubenzuron (Section III.C) was again evident while examining several longitudinal and transverse sections of the uterus (Plates V.13-15).

Unfertilized eggs appeared to have an electron dense unit-membrane (5.0Å thick) similar to the vitelline layer of a fully formed egg shell (Plates V.2-4). This membrane occasionally detached from the egg cytoplasm forming large spaces, occupied by a dense reticulate material (Plate V.13.C,D); intracytoplasmic spaces were similarly observed (Plates V.13-15). The cytoplasm of these eggs also had numerous lamellar bodies and traces of an endoplasmic reticular system that could be involved in synthetic activity (Plate V. 14.A,B). However, there was no clear evidence for the deposition of an egg shell.

V.C.1.c Effect of diflubenzuron on the ultrastructure of the intestine and body wall of *C. elegans*

(1) The ultrastructure of intestinal cells of *C. elegans* was found to be generally similar to that described in other nematodes (Bird, 1971), Plate V.16.

In control or diflubenzuron-treated (20 and 40 µg cm^{-3}) *C. elegans*, intestinal cells are 2.8 - 3.8 µm wide, with a brush border (0.8 µm) The intestine of *C. elegans* is separated from the pseudocoelom by a basement membrane and each intestinal cell is delimited by a plasma membrane. The mid-region of each cell in treated and control nematodes showed large lipid droplets, glycogen granules, a few mitochondria and an extensive net work of endoplasmic reticulum,

Plate V.13

A) Light micrograph, using Nomarski Interference contrast of the uterus of diflubenzuron-treated (40 µg cm^{-3}) *C. elegans* hermaphrodites showing a number of unfertilized eggs. Note rough structure of the egg cytoplasm and large nuclei; X 520 - Scale bar represents 10 µm.

B) Electron micrograph (L.S.) through the uterus of a diflubenzuron-treated (40 µg cm^{-3}) *C. elegans* showing cytoplasmic material of ruptured unfertilized eggs (ruf); 16,798 - Scale bar represents 0.2 µm.

C) Electron micrograph (L.S.) through an unfertilized egg in the uterus of a diflubenzuron-treated (40 µg cm^{-3}) *C. elegans*. Note structure of egg membrane and intracytaplasmic spaces (isp) at the boundaries of the egg, X 48,320. Scale bar represents 0.2 µm.

D) Electron micrograph (L.S.) through the uterine wall and unfertilized egg of diflubenzuron-treated (40 µg cm^{-3}) *C. elegans*; X 23,280. Scale bar represents 0.2 µm.

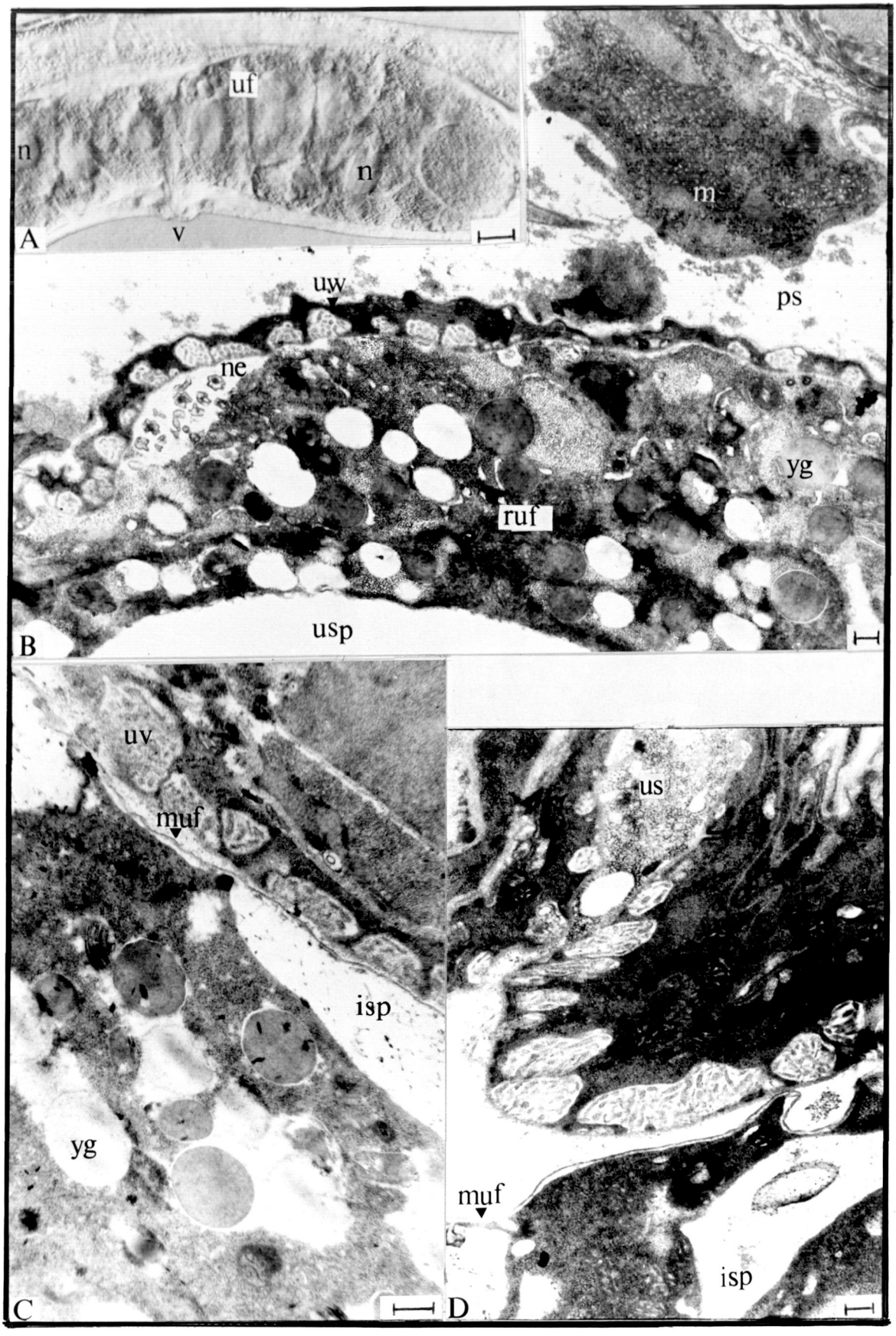

uf
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ruf
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uv
muf
isp
yg
C
us
muf
isp
D

Plate V.14

Electron micrograph (L.S.) through the uterus of diflubenzuron-treated (40 µg cm^{-3}) *C. elegans* showing:

A) Dense granular material (gm) between the uterine wall and membrane of the unfertilized egg, either secreted by the uterus or formed as the cytoplasmic material of unfertilized eggs ruptured; X 43,086.

B) Multilamellar bodies (lb) closely associated with the membrane of the unfertilized egg; X 86,844.

C) Intracytoplasmic space (ISP) at the site of the egg membrane and numerous yolk droplets embedded in the granular cytoplasm; X 43,086.

Scale bars represent 0.2 µm.

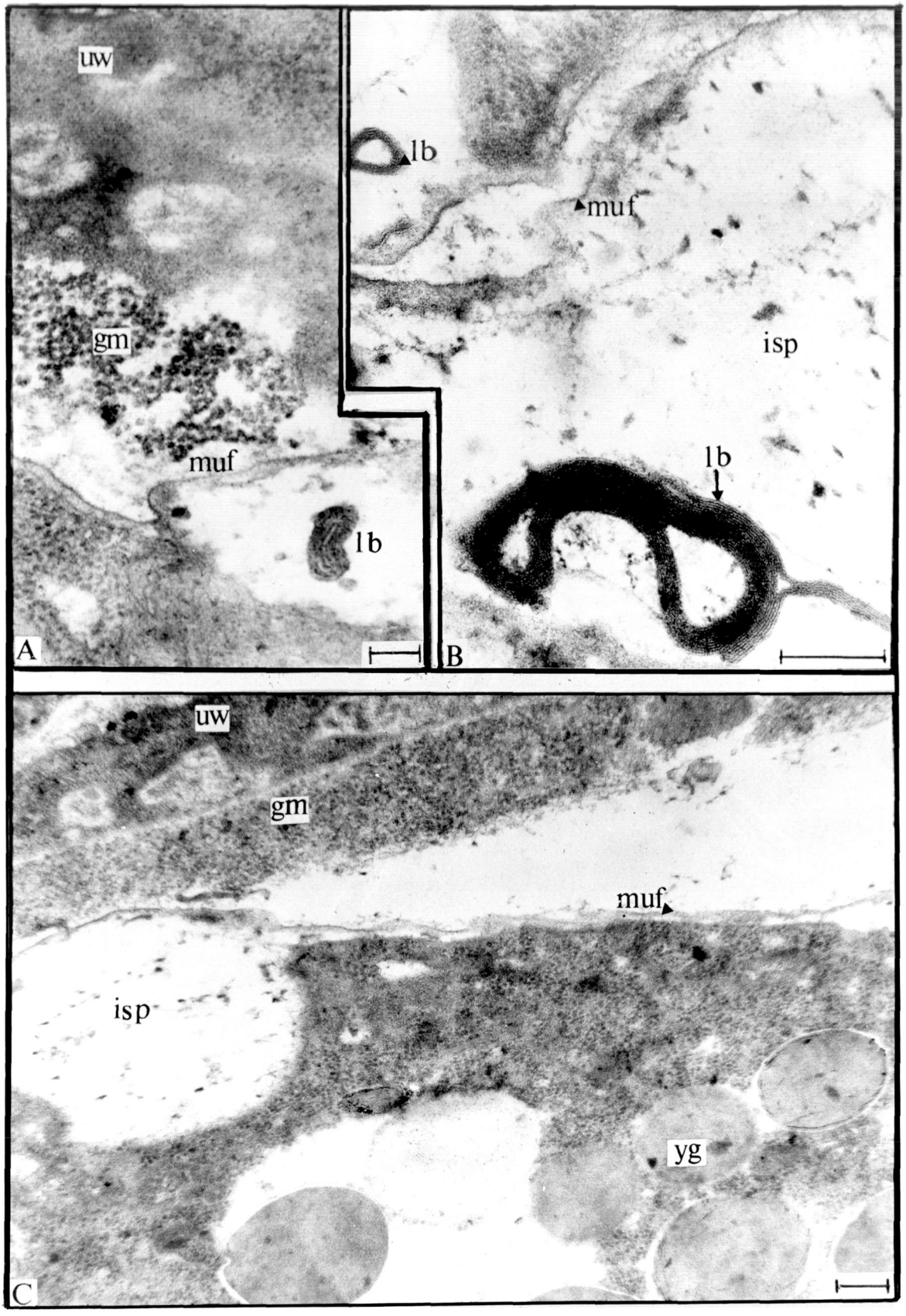

uw
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muf
isp
lb
B
uw
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muf
isp
yg
C

Plate V.15

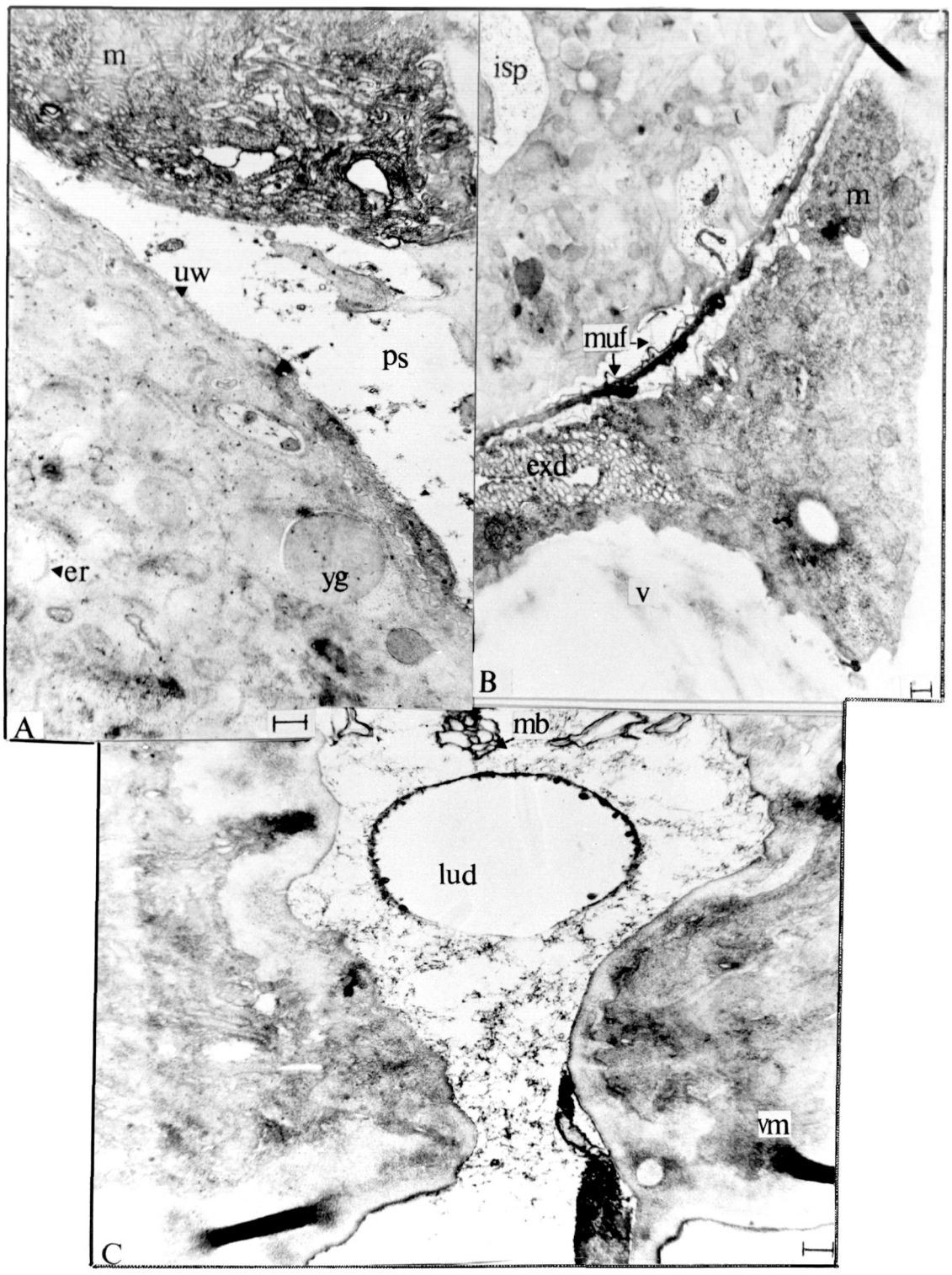
Electron micrographs (T.S.) through the uterus of diflubenzuron-treated (40 µg cm^{-3}) *C. elegans* at the region of the vulva showing:

A) Unfertilized egg with yolk granules and traces of endoplasmic reticulum system (er); X 24,160,

B) Cytoplasm of an unfertilized egg contracting away from the egg membrane; X 9,808.

C) Electron micrograph (L.S.) through the vulva showing "uterinal secretion" at the inner surface of the uterus; the uterinal space also contains membranous bodies (mb) and electron lucent droplets surrounded by electron dense material; X 24,160.

Scale bars represent 0.2 µm.


m
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er
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isp
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muf
exd
v
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mb
lud
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C

particularly in sections in the region of the uterus. The lumen surface of these cells consisted of microvilli, 0.5 µm long (Plate V.16); the microvilli being mostly coated by amorphous material and were more compact in comparison to those in other nematode species (Kessell *et al.*, 1961; Comley, 1980). The cores of the microvilli were joined at their bases by an electron-dense cytoplasmic material to form the terminal web (Plate V .16).

Intestinal cells of diflubenzuron-treated 20-40 µg cm^{-3} *C. elegans* shown to have numerous small vacuoles (about 0.5 µm in diameter) containing several rings of dense particles (Plate V.18). These vacuoles seemed to be associated or coalese with each other. In untreated nematodes such vacuoles were only rarely present, Plate V.17.b.

A few autophagic vacuoles enclosing remains of membranous inclusions were found in diflubenzuron-treated *C. elegans* (Plates V.18) with large dense granules, similar to those found in *C. briggsae* and described as aging granules (Zuckerman, 1976). Autophagic vacuoles were previously observed in the intestinal cells of *A. tetraptera, A. suum* and *Syphacia trachea* after treatment with the anthelmintic Mebendazole (MBZ). These vacuoles were suggested to be formed as a result of an intracellular release of hydrolytic enzymes from secretory granules that accumulate in the cytoplasm of intestine cells (Borgers and Nollins, 1975; Borgers *et al.*, 1975a; Comley, 1980).

In addition, large and small vesicles with multilamellar bodies

Plate V.16

Electron micrographs (T.S.) through the intestinal cells in the mid region of *C. elegans:*

A) Untreated *C. elegans*, showing intestine lumen (lu) with rich microflora and intestine surface with numerous microvilli (mv) connected to the terminal web (tw); X 41,995.

B) *C. elegans* treated with 20 µg cm^{-3} diflubenzuron; X 16,798.

C) *C. elegans* treated with 40 µg cm^{-3} diflubenzuron; X 16,798

D) Terminal bar (tb) closely associated with the terminal web (tw) and surrounded by glycogen granules in an intestinal cell of an untreated *C. elegans*; X 41,995. Scale bar represents 0.2 µm.

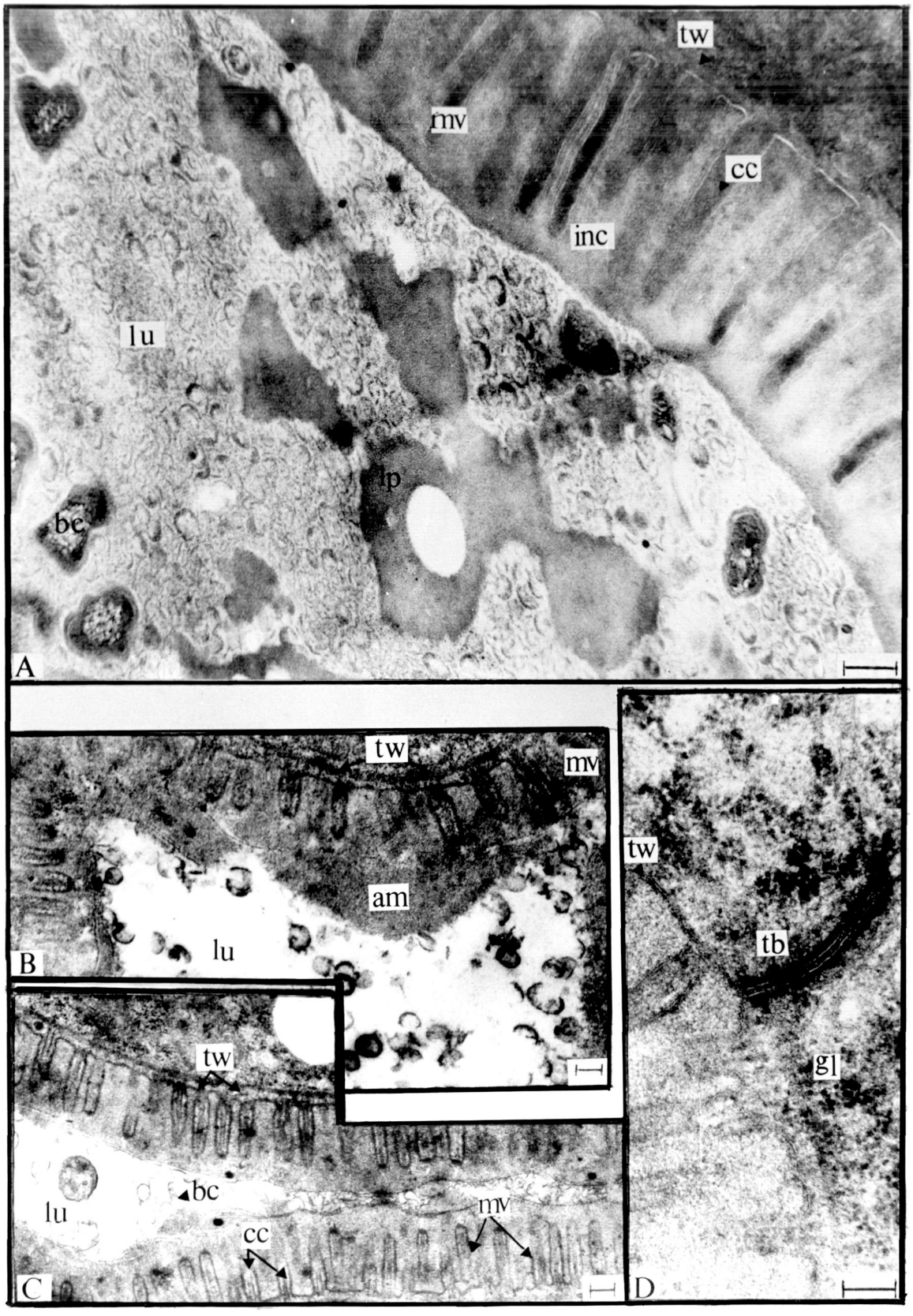

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Plate V.17

Electron micrographs of:

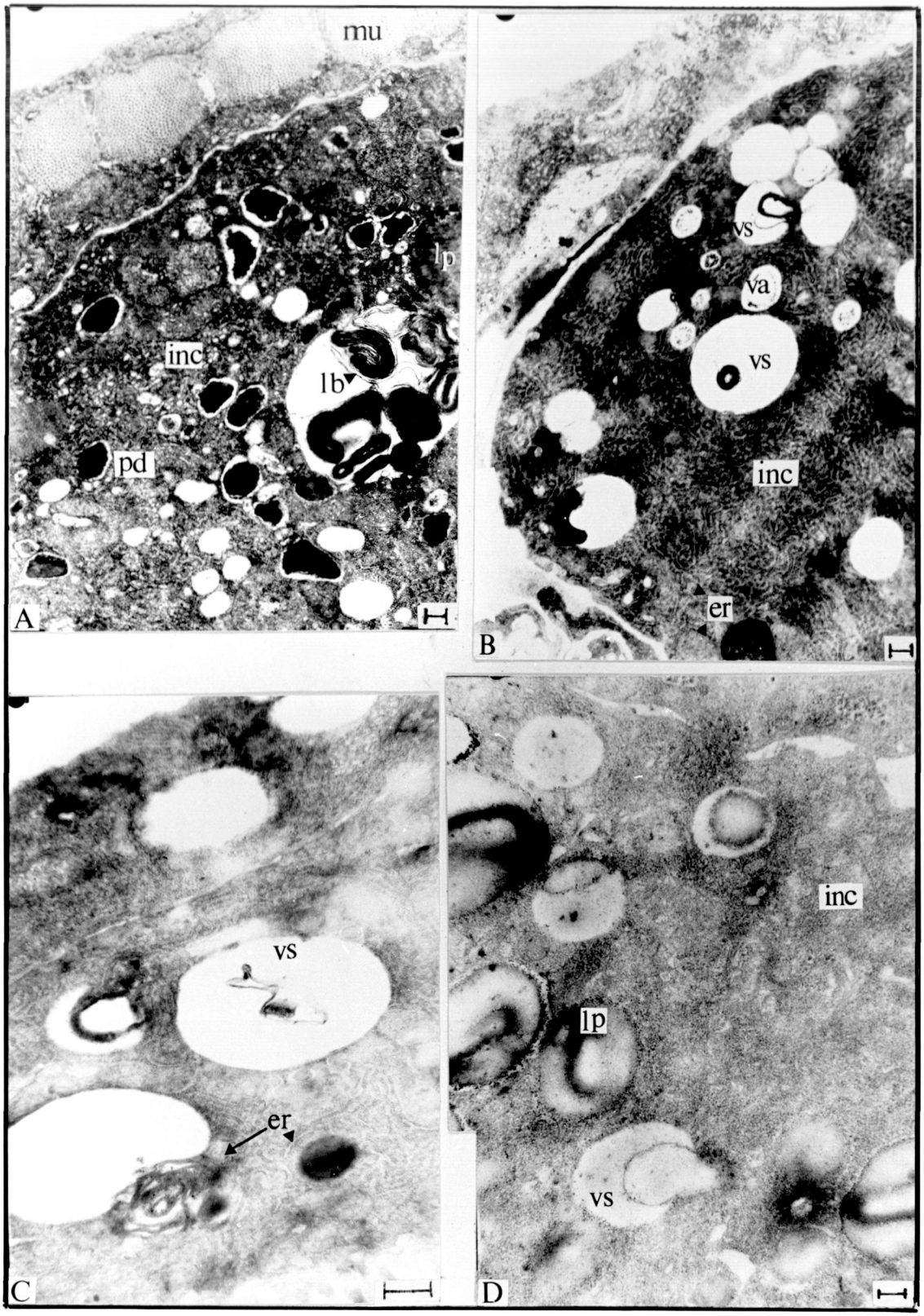
A) T.S. through the body musculature and intestinal cell of an untreated *C. elegans* showing protein or lipid droplets and lamellar bodies; X 15,900.

B) L.S. through the intestine of an untreated *C. elegans* showing active endoplasmic reticulum system, few vacuoles and numerous vesicles (vs) containing lamellar bodies; 17,490.

C) L.S. through an untreated *C. elegans* showing the extension of endoplasmic reticulum system into some vesicles; X 34,565.

D) Intestinal cell in an untreated *C. elegans* showing rich content of lipid droplets; X 22,950.

Scale bar represent 0.2 µm.


mu
lp
inc
lb
pd
A
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va
vs
inc
er
B
vs
er
C
inc
lp
vs
D

were also observed in both diflubenzuron-treated and untreated *C. elegans* (Plates V.17, 18). These structures have been found in a number of nematodes including *A. suum* (Jenkins and Erasmus, 1969) and *Dirofilaria immitis* (Lee and Miller, 1969). They are believed to be involved in destruction and elimination of waste materials. In *C. elegans* the large dense granules and multilamellar vesicles and other intestinal vacuoles appear to represent an important part of the lysosome system found in most animal cells (Dean, 1977).

The intestinal lumen in control and diflubenzuron-treated *C. elegans* was shown to contain the remains of large numbers of digested bacterial cells and large lipid-like droplets (Plate V.16).

(2) The body wall and musculature of *C. elegans* did not show significant ultrastructural changes due to treatment with 20 - 40 $\mu g\ cm^{-3}$ diflubenzuron. The somatic masculature beneath the nematode hypodermis in control or treated *C. elegans* is made of rows of muscle cells between the dorsal, ventral and lateral cords (Plate V.19). Each muscle cell is formed of a contractile and non-contractile region as described in other nematodes (Hirschmann, 1971; Bird, 1971). The contractile portion contained thin and thick myofilaments while the cytoplasmic portion contains numerous mitochondria, glycogen granules and lipid droplets. Glycogen granules were found to be abundantly dispersed around mitochondria, in control and diflubenzuron-treated nematodes.

Plate V.18

Electron micrographs (L.S.) through the intestine of (40 µg cm^{-3}) diflubenzuron-treated *C. elegans* showing:

A) large number of vacuoles (va) either empty or containing electron dense particles; X 11,968

B) Dense granules (dg) varying in size; X 21,340.

C) Dense granules closely associated with an autophagic vacuole (av); X 13,766

D) Active endoplasmic reticulum system (er) of intestinal cells, interrupted with large vacuoles having rings of dense material; X 20,134

Scale bars represent 0.2 µm.

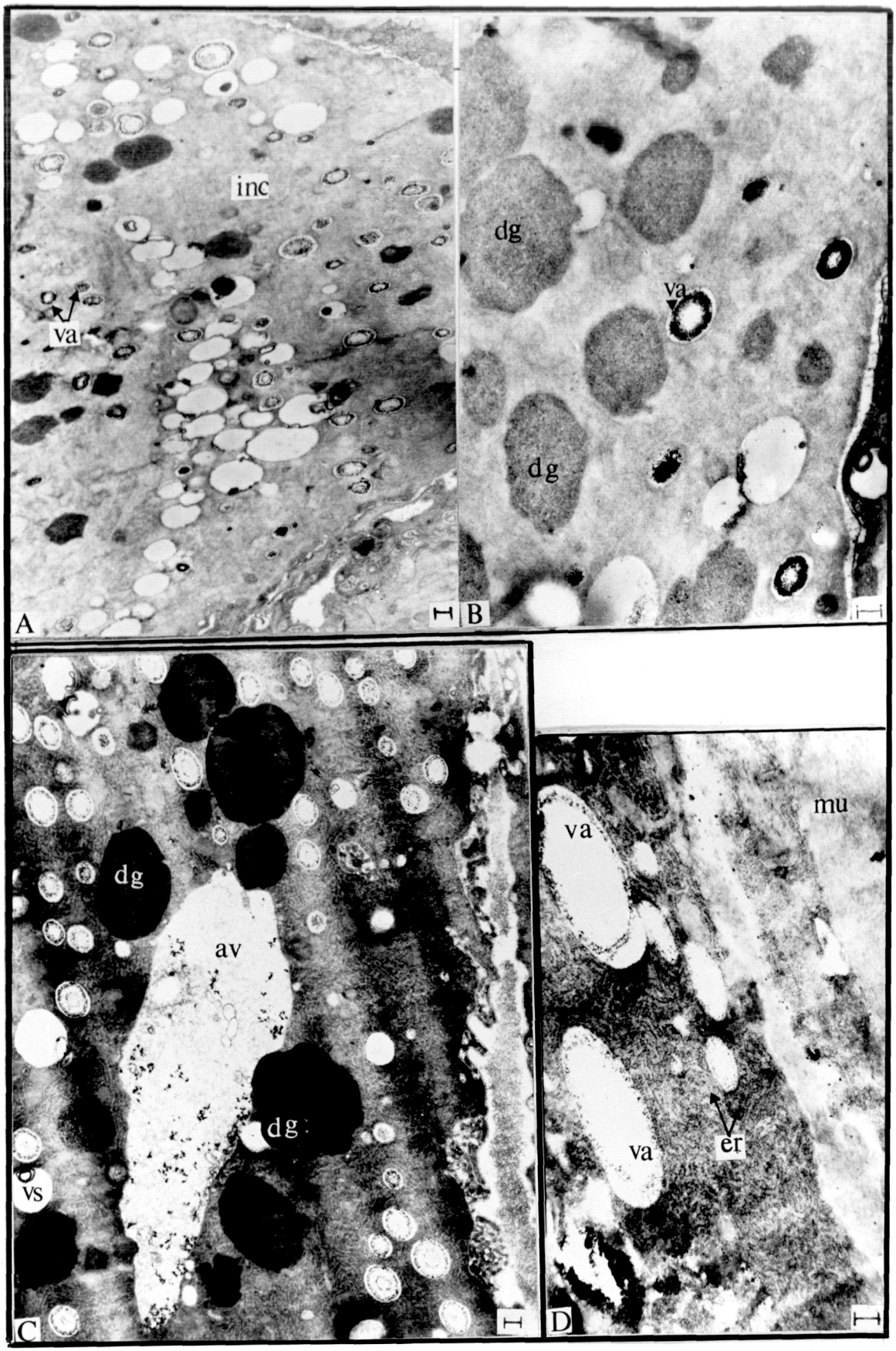

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Plate V.19

Electron micrograph (T.S.) of:

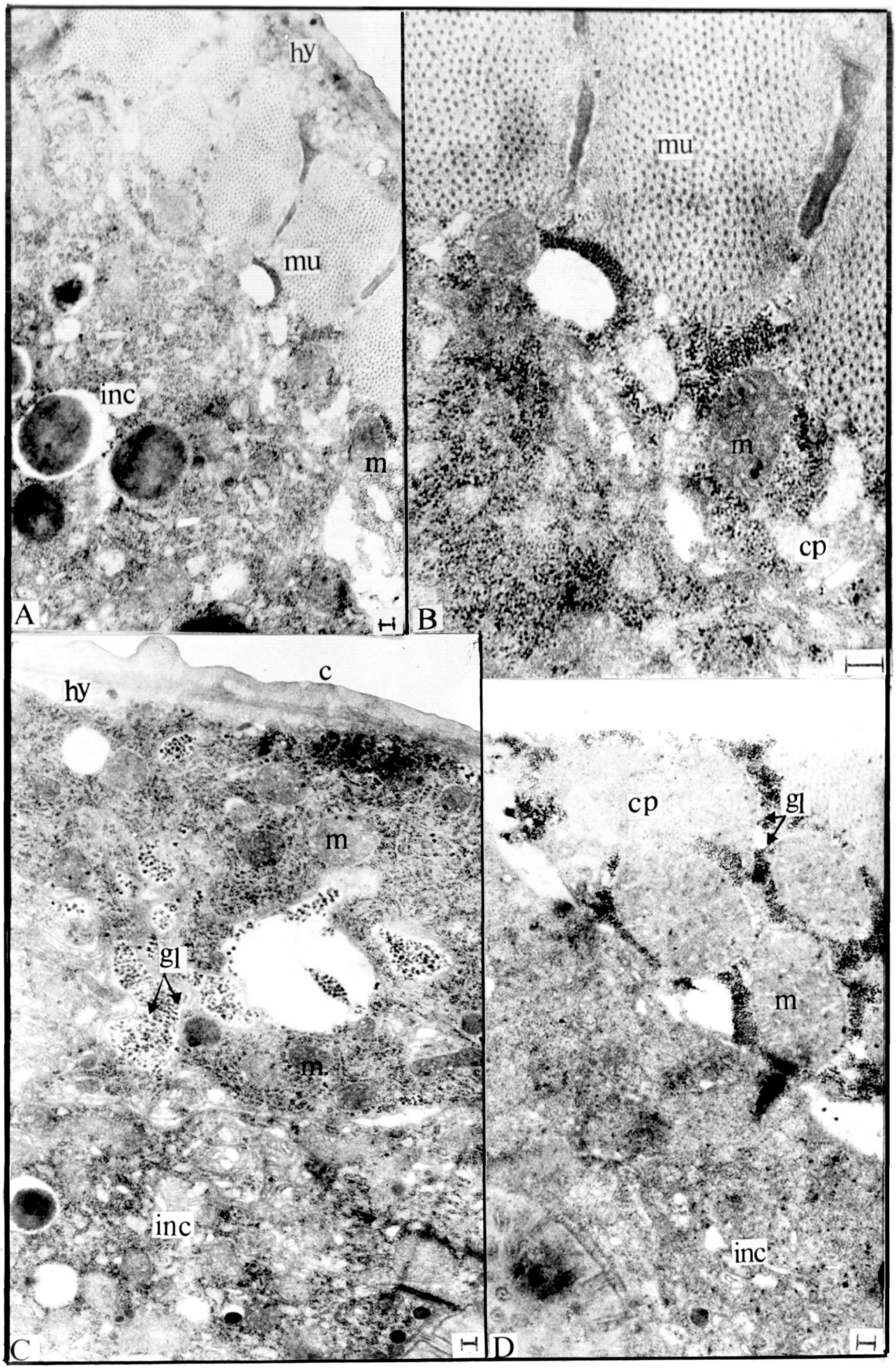
A) Body wall and musculature (mu) of diflubenzuron-treated (20 µg cm^{-3}) *C. elegans*; X 16,798.

B) Cytoplasmic (non-contractile) and contractile regions of the muscles in untreated *C. elegans*; X 55,300.

C) Large numbers of glycogen granules in the cytoplasmic region (cp) of muscles in diflubenzuron-treated (20 µg cm^{-3}) *C. elegans*; 16,798.

D) Numerous mitochondria (m) and cytoplasmic region of muscles in an untreated *C. elegans* 25,440.

Scale bars represent 0.1 µm.


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m
cp
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hy
c
m
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C
cp
gl
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D

V.C.2 Biochemical Assay of Chitin in *C. elegans* egg shell

There was no significant difference in glucosamine content between the egg shells of untreated (control) and diflubenzuron-treated specimens ($P > 0.05$; Figure V.5, Appendix V.2).

V.C.3 ^{14}C-diflubenzuron study

E. coli cultured on NG agar, containing 40 µg a.i. ^{14}C-diflubenzuron cm^{-3} for 48 h acquired a relatively high level of radioactivity, equivalent to about 60% of the level incorporated into the agar (Table V.2, Appendix V.3).

Radioactivity accumulated by *C. elegans* hermaphrodites after treatment with radiolabelled diflubenzuron for 48 h ranged from 8 - 27 dpm per individual in the 5 groups examined. Eggs produced by these diflubenzuron-treated hermaphrodites also contained some radioactivity, the mean amount/egg being approximately 3% of the mean radioactivity/individual hermaphrodite (Table V.2). Radioactivity per egg declined by about 70% on average in eggs laid by diflubenzuron-treated nematodes after transfer to clean agar (Table V.2).

Inconsistent results were obtained when eggs from untreated nematodes were exposed for 6 h to radiolabelled-diflubenzuron (0.05 - 1.46 dpm/egg).

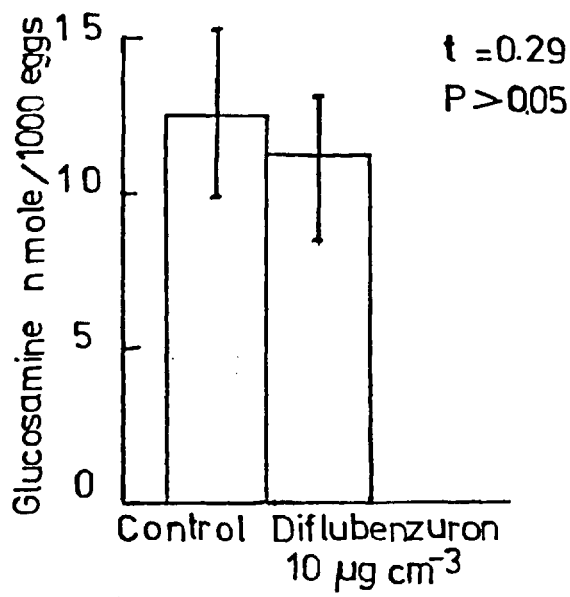


Figure V.5. The effect of diflubenzuron on glucosamine content of *C. elegans* eggs. Each column represents the mean of 10 assays. Bar line = ± S.E.

Table V.2. Incorporation of ^{14}C-diflubenzuron into *C. elegans* hermaphrodites and eggs

Sample	Radioactivity in dpm/unit ± S.E.
E. coli (48 h exposure) (mg)	951 ± 166.9
C. elegans ♀ (individual) (48 h exposure)	15.2 ± 3.3
Eggs laid by ^{14}C-diflubenzuron treated ♀ on treated agar (individual)	0.47 ± 0.07
Eggs laid by ^{14}C-diflubenzuron treated ♀ on plain agar (individual)	0.13 ± 0.03

GENERAL DISCUSSION

The present study has shown that treatment with 10 - 40 μg cm^{-3} diflubenzuron wettable powder formulations reduces population growth of *C. elegans* by more than 90% (Figure I.8); while treatment with similar concentrations of analytical grade diflubenzuron, in acetone or dimethyl sulphoxide were less effective (Figure I.5). The Carrier material incorporated with formulated diflubenzuron proved not to have a detrimental effect on the population growth of *C. elegans* (Figure I.6). These results thus confirm the observations of Ibrahim (1978) and Veech (1978b) on the activity of diflubenzuron on *C. elegans* and some other free living nematodes respectively. A comparison of the population growth of *C. elegans* in various treatments of diflubenzuron showed that the lowest EC_{50} value was obtained with the wettable powder formulation B (<0.5 μg cm^{-3}). In the case of *Pelodera* sp. and *P. redivivus* treated with (25% w.p.) the EC_{50} values for effects on population growth were 4 and 9 μg cm^{-3}, respectively (Veech, 1978b).

The fact that analytical grade diflubenzuron has less effect on *C. elegans*, whether used in acetone or dimethyl sulphoxide, suggests a possible relationship between the nematicidal activity and the physical properties of diflubenzuron, particularly the size and distribution of particles of this chemical in each treatment.

The biological activity of a wide range of pharmaceuticals and pesticides has been reported to be dependent on the particle size of the active compound (Valkerburg, 1973; Maas, 1978). For instance the anthelmintic activity of phenothiazine is known to be inversely

proportional to particle size (Gordon, 1956; Kingsbury, 1958) an effect attributed to increased rates of absorption and decreased rates of excretion of micronized fine particles (<10 μm dia.) of phenothiazine (Farrington *et al.*, 1962). Kelly *et al.* (1975) has also observed the effect of a reduction in particle size on increased anthelmintic efficiency of mebendazole in treatment of *Nippostrongylus brasiliensis*. Moreover, the biological activity of diflubenzuron (PH60 - 40) and its analogue PH60 - 38 was shown to increase as the particle size decreased from 10 μm to 0.5 μm (dia.) when the chemical was applied to *P. brassicae* as a suspension of fine particles sprayed on cabbage leaves (Mulder and Gizswizt, 1973).

In the present study, the nematicidal activity of diflubenzuron formulation B(NMD = 2.0 μm) was in fact slightly greater than that of formulation A although the difference was not significant (Table I.1). This might be due to the difference in the distribution of particle sizes between these two formulations, though the values of their number median diameters (NMD) were similar (Figures I.1, 2).

The decrease in nematode population size by treatment with active chemicals could be the result of increased generation time and lowered fecundity (Somoiloff, 1980) or an increased mortality of the juvenile and adult stages.

In the present investigation, however, survival experiments, (Section II) showed that 10 - 80 μg cm^{-3} diflubenzuron had no lethal effect on the second stage juveniles of *C. elegans*; only 6 - 8% mortality being observed in all treatments. Veech (1978a) similarly

found 93% survival of *M. incognita* second stage juveniles after incubation for 72 h in 1% (w/v) oil based diflubenzuron for 72 h, and that the longevity of *P. redivivus* on diflubenzuron-treated agar (1000 µg a.i. cm^{-3}) was similar to that on the control agar. In addition, there was no significant change in the number of inactive juveniles in aqueous cultures of *Pelodera* sp. or *Acrobeloides* sp. exposed to a 1% (w/v) suspension of diflubenzuron for 7 days (Veech, 1978b).

In the present work, the mobility of *C. elegans* hermaphrodites appeared to be normal after treatment with 10 - 40 µg cm^{-3} diflubenzuron (Figure II.3). Therefore, diflubenzuron seems unlikely to cause a large % mortality of *C. elegans* or retard the nematodes' activity.

Fecundity studies (Section II.C.5), however, showed a reduction in the number of eggs produced by *C. elegans* hermaphrodites treated with diflubenzuron for 96 h; about 56, 85 and 91% reduction in egg production being observed in treatments with 5, 10 and 20 µg a.i. cm^{-3} diflubenzuron, respectively (Figure II.5). This effect of diflubenzuron on *C. elegans* fecundity appeared to be less pronounced when the nematodes were treated for 48h only.

The lowered fecundity of diflubenzuron-treated *C. elegans* could thus account for some of the observed reduction in the nematode population size. Because fecundity, development and feeding activity of nematodes are known to be closely associated (Evans, 1973; Cook, 1977; Samoiloff, 1980), the effects of nematicides on reproduction and development can often be ascribed to inhibition of feeding.

However, in the case of diflubenzuron-treated hermaphrodites of *C. elegans* both development and feeding were found to be normal (Figures II.1, 2, Table II.3).

Reduced fecundity of *C. elegans* could also have been due to either delayed gonad maturation or failure of the nematode gametes to achieve fertilization. No effects of diflubenzuron treatment on mating behaviour were possible since *C. elegans* is a self-fertilizing hermaphrodite.

In fact, the present investigation indicated that diflubenzuron did not generally influence *C. elegans* gonadogenesis (Section II.C.5). Light microscope observations (Figure II.6) showed that the maturation of gonads was only significantly suppressed in nematodes subjected to 80 µg a.i. cm^{-3} after 24, 48 and 72h of treatment, while histochemical results (Tables II.5, 6) showed active oogonial nuclear divisions and normal spermatogenesis in all diflubenzuron-treated (72h) nematodes. These results suggest that *C. elegans* hermaphrodites may tend to recover after the initial 48h of treatment; the rate of gonadial growth in 80 µg cm^{-3} diflubenzuron-treated nematodes being about 90% greater compared to controls (Appendix II.7.B).

On the other hand, observations reported in Section III indicated the possible effects of diflubenzuron on fertilization in *C. elegans*. Nematodes exposed to 20 - 40 µg cm^{-3} diflubenzuron were shown to lay more than 45% unfertilized eggs whereas normal hermaphrodites produced only about 16% unfertilized eggs.

Ward and Carrel (1979) have shown that a shift from production

of fertilized to unfertilized oocytes does not occur abruptly in *C. elegans* but begins about 65h after hatching (at 25°C); only unfertilized oocytes being laid after 100h. They found that *C. elegans* produces excess oocytes and that every available sperm is utilized; only when most of the sperm are exhausted do some oocytes pass through the spermatheca without being fertilized. The results obtained in the present study (Table III.3,5) showed neither sperm production by *C. elegans* males or hermaphrodites, nor the structure of their sperm appeared to be affected by diflubenzuron treatment (10 or 40 µg a.i. cm^{-3}). However, although numerous sperm were found in contact with fully developed oocytes and in the spermathecae of diflubenzuron-treated *C. elegans*, Feulgan staining showed that penetration of oocytes by sperm did not usually take place.

Surface interaction between nematode gametes is considered an important step in fertilization which may involve phagocytic action, liberation of enzymes from the sperm organelles or pseudopodial activity (Anya, 1976). In *C. elegans*, surface contact between gametes was briefly described by Ward and Carrel (1979) as an abrupt ingestion, but pseudopodial sperm were observed in the spermathecae where fertilization takes place. This implies that the pseudopodial activity of sperm may be associated with oocyte penetration.

Pseudopodial activity of sperm before fertilization has been reported in several nematode species including *A. lumbricoides*, *Rhabditis pellio*, *Capillaria hepatica* (Foor, 1970; Beams and Sekhon,

1972; Neill and Wright, 1973). In a detailed study of *P. redivivus* sperm were observed to align in chains after insemination of the female and become amoeboid in uterus with a clear anterior region capable of producing pseudopodia (Duggal, 1978). When the chain of sperm reached the spermatheca pseudopodial activity was only found in the anterior-most sperm adjacent to the descending oocytes. Formation of pseudopodia in *C. elegans* sperm has been shown to be activated by treating nematode spermatids with the ionophore monensin which induced the maturation of spermatids to spermatozoa (Nelson and Ward, 1980).

More recently, Dr. Samuel Ward (personal communication) analysed the pseudopodial activity of *C. elegans* sperm *in vitro* using techniques for labelling cellular membranes and concluded that pseudopodial activity involves the movement of the membrane components of nematode sperm.

An attempt was made in the present study to determine the effects of diflubenzuron on the activity of sperm from *C. elegans* males by recording pseudopodial movement on a positively-charged surface coated with polylysine. However, only limited activity was observed in control sperm suggesting that strong electrostatic forces in polylysine (0.05% w/v) may render the sperm immobile.

In vivo experiments using the fluorescent dye Nile blue were more successful and showed that sperm from 40 µg cm^{-3} diflubenzuron-treated males could actively ascend towards the spermathecae of diflubenzuron-treated hermaphrodites as in control nematodes (Table

III.4). The spermathecae in both control or treated hermaphrodites of *C. elegans* have been shown to acquire Nile blue fluorescence as it diffused from the sperm (Ward and Carrel, 1979). Hence the present observations, imply that sperm of 40 µg cm^{-3} diflubenzuron-treated *C. elegans* males or hermaphrodites were able to retain a normal structure and sufficient level of activity to reach the site of fertilization. Similarly, diflubenzuron appeared not to disrupt the sequence of morphological changes in mature oocytes preparatory to fertilization but it is not known if diflubenzuron would affect oocyte functions involved in fertilization.

While fertilization of *C. elegans* seemed to be somehow blocked by diflubenzuron treatment (10 - 40 µg cm^{-3}), nematode embryogenesis was found to be normal following the fusion of the gamete nuclei in treated hermaphrodites (20 and 40 µg cm^{-3} diflubenzuron). Embryonation from the 16 cell stage to J_1 continued normally in eggs of untreated *C. elegans* after exposing the eggs to 5 - 40 µg cm^{-3} diflubenzuron.

When *C. elegans* eggs were subjected to 10 - 40 µg cm^{-3} diflubenzuron in addition to treatment of the nematodes with diflubenzuron from the J_2 stage, hatching was significantly suppressed (Tables IV.4, 5). On the other hand, hatching appeared to be less affected if the eggs were laid by nematodes treated with diflubenzuron from the last juvenile stage (J_4). From these observations it is believed that the effectiveness of the ovicidal action of diflubenzuron requires early incorporation of the compound into nematode eggs,

probably during egg formation.

Experiments with ^{14}C-diflubenzuron (Section V.B) demonstrated the accumulation of radioactivity in the eggs laid by *C. elegans* exposed for 48h to 40 µg cm^{-3} of the radiolabelled diflubenzuron. While the results of this experiment must be interpreted with caution as the radioactivity was not identified as being due directly to diflubenzuron, it was shown that the amount of radioactivity in the eggs laid apparently decreased when the nematodes were transferred on to plain agar. *C. elegans* hermaphrodites probably acquired much of the radioactivity by ingesting *E. coli* which appeared to accumulate radioactivity to a greater extent than the nematodes. Ultrastructural studies (Section V.C) showed that unlike the anthelmintics mebendazole and thiabendazole used in treatment of *A. tetraptera* (Comley, 1980) diflubenzuron did not appear to reduce the luminal microflora of *C. elegans* nor disturb the integrity of the nematode intestinal cells. This latter observation suggests that normal absorption of the intestinal contents occurs, including presumably diflubenzuron. It seems likely that diflubenzuron enters the nematode egg before or after fertilization, but prior to completion of the egg shell formation. The ovicidal activity of diflubenzuron upon insects has been shown in several reports to be due to inhibition of hatching of embryonated eggs (Abo-Elghar *et al.*, 1976; Bull and Ivie, 1980; Ascher *et al.*, 1980). Diflubenzuron ovicidal effects were shown to be caused both by contact activity on the insect eggs or by treatment of females (Grosscurt and Tipker, 1980). Electron microscopic observations on embryos of the insect *Leptinotarsa decemlineata*

(Colorado potato beetle) following treatment of females with diflubenzuron, showed cuticle malformation indicative of an interruption of chitin synthesis and thus a similar mode of action for the chemical in the larvae and embryos of insects (Grosscurt and Tipker, 1980).

In the present study, the ovicidal activity of diflubenzuron appeared to affect the egg shell of *C. elegans* rather than the embryos directly. Further exposure of eggs from diflubenzuron-treated (10 - 20 $\mu g\ cm^{-3}$) hermaphrodites to diflubenzuron rendered the nematode egg shell vulnerable to strong solvents (NaOCL, Conc. H_2SO_4) and also to ultrasonication (Table IV.7) indicating a considerable change in the permeability and strength of the treated egg shells. The permeability of the egg shell to water was also increased and treated eggs became swollen when immersed in distilled water.

Electron microscopical studies of normal, untreated eggs of *C. elegans* (Section V.C) clearly revealed the 3 distinct layers found in the egg shell of other nematodes (Bird, 1971), namely the vitelline, chitinous and lipid layers. Following treatment of hermaphrodites with 10 $\mu g\ cm^{-3}$ diflubenzuron, the chitinous layer in the egg shell of *C. elegans* was found to be partially disintegrated and demarcation of the inner, lipid layer was not clear. These observations may account for the observed structural weakness of treated egg shell, since the nematode egg shell is thought to acquire its structural strength and permeability properties from the chitinous and lipid layers, respectively. (Bird, 1976; Wharton, 1980).

As in other nematode species (Anya, 1976), the formation of *C. elegans* egg shell is presumed to be activated by fertilization of the mature oocyte. After fertilization, the original oolemma increases in thickness to form the outer vitelline layer of the egg shell, while a new oocyte membrane is produced. The deposition of the middle chitinous layer is thought to be by material that originates in the egg cytoplasm which is then transported through the cell membranes. This phase is followed by formation of the innermost lipid layer and associated lipoprotein membranes. Figure 3 outlines the stages of the egg shell formation in *C. elegans*.

The ultrastructural changes, observed in diflubenzuron-treated egg shells (Plates V.5-7) suggested that synthesis of the chitinous layer was probably interrupted by diflubenzuron after fertilization and production of the vitelline layer. The subsequent formation of the lipid layer is assumed to be inhibited either directly or perhaps more likely, indirectly due to effects on the adjacent chitinous layer.

Disruption of chitin synthesis in insects by phenyl benzoyl ureas was thoroughly investigated by Post *et al.* (1974) and by Deul *et al.* (1978). Using ^{14}C-labelled glucose Post *et al.* (1974) demonstrated blockage of chitin formation by the lack of incorporation of radioactivity from glucose into cuticular chitin through the chitin biosynthetic pathway (Figure 2). Deul *et al.* (1978) found an increase in the level of *N* - acetylglucosamine (AGA) after treating insect larvae with the diflubenzuron analogue, DU 19111. A decrease

Figure 3. Formation of *C. elegans* egg shell

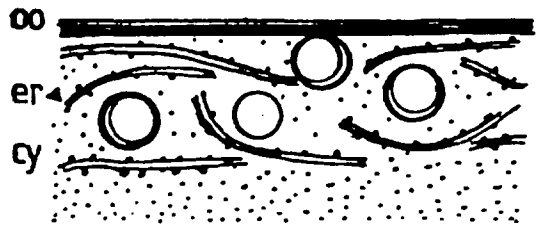
(1) After fertilization, a second oolemma (oo) is formed beneath the original membrane of the mature oocyte and production of the egg shell begins between the two membranes.

(2) The original oolemma thickens to form the vitelline layer (Vl) and the chitinous layer (Chi) is produced between the cell membrane (cm) and vitelline layer.

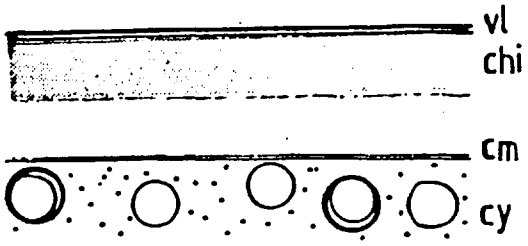
(3) Formation of chitinous layer is succeeded by deposition of lipid layer (lip).

(4) The lipid layer shows two membranes and aggregates of lipoprotein meterial.

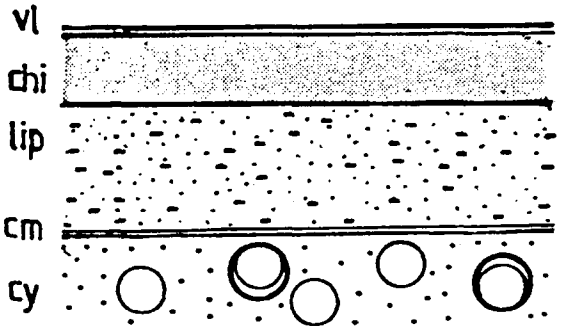
1



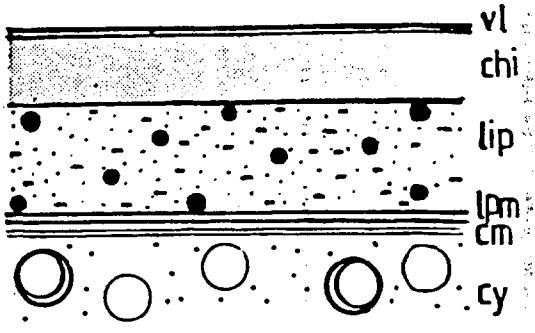
2



3



4



in AGA of the insect larvae would indicate inhibition of an intermediate synthetic step between glucose and AGA but an accumulation of AGA would indicate a blockage of chitin polycondensation (Fig. 2). Subsequently, Deul *et al.* (1978) inferred that DU 19111 could disrupt binding of *N* - acetylglucosamine to the chitin chain, presumably by blocking chitin synthetase, an enzyme necessary for the polycondensation of chitin. Although the accumulation of AGA was not evident after treating the insect larvae with diflubenzuron, Deul *et al.* (1978) believed that the mode of action of both DU 19111 and diflubenzuron was identical due to their structural similarity. In the present work, biochemical assay of the chitin precursor acetylglucosamine failed to show significant differences between control and diflubenzuron-treated eggs of *C. elegans* (Figure V.5). In view of this result together with the ultrastructural observations (Section V.C) and the entomological literature, it seems likely, that treatment with diflubenzuron renders the eggs of *C. elegans* incapable of converting AGA into chitin rather than blocking the intermediate steps between glucose and acetylglucosamine, AGA (Figure 2). However, although diflubenzuron is currently believed to act upon insect cuticle by blocking chitin synthesis, it may also activate the chitinase enzyme which breaks down the endocuticular chitin of the insect (Post *et al.* 1974). A similar mode of action is possible in nematode eggs which are known to have chitinase and lipase enzymes involved in dissociating the nematode egg shell during hatching (Rogers and Sommerville, 1969; Lee and Atkinson, 1976).

CONCLUSIONS

Reductions in population growth of diflubenzuron-treated *C. elegans* were found to be due largely to lowered fecundity and retarded hatching of the nematode.

The observed increase in production of unfertilized eggs in diflubenzuron-treated *C. elegans* was thought to be directly due to pesticide treatment and may account for effects on fecundity since nematode gonadogenesis was not influenced by diflubenzuron. Sperm of diflubenzuron-treated *C. elegans* appeared to show normal activity although further studies are required to determine whether diflubenzuron can affect movement of *C. elegans* sperm *in vivo* in some way. Mature oocytes in the gonads of diflubenzuron-treated hermaphrodites developed normally despite the likely incorporation of diflubenzuron into *C. elegans* oocytes prior to fertilization. Diflubenzuron may disrupt metabolic activity of oocytes or may inhibit the production of chemicals that either attract or activate the sperm before penetrating the oocytes. Alternatively, diflubenzuron could prevent surface interaction between the nematode gametes.

Ultrastructural studies suggested that when *C. elegans* oocytes are fertilized, diflubenzuron incorporated in the eggs interferes with the egg shell formation, in particular the synthesis of the chitinous and lipid layers. The structural weakness of treated *C. elegans* egg shell was also indicated by sonication and solubility tests.

Biochemical studies showed that the amount of the chitin precursor,

acetylglucosamine was similar in diflubenzuron-treated and control eggs. Diflubenzuron was thus thought to interrupt chitin production at the final stages of the synthetic pathway rather than preventing the formation of acetylglucosamine from glucose.

As a result of diflubenzuron's action on the egg shell of *C. elegans* the eggs fail to embryonate or hatch.

In this work, diflubenzuron (25% w.p.) has been shown to possess considerable biological activity that affects target sites in *C. elegans* (fertilization and egg shell formation) which are not exploited by other nematicides or anthelmintics. The production of compounds with such a mode of action would therefore broaden the repertoire of nematicides and anthelmintics available. However, the use of phenylureas or similar acting compounds for the control of plant parasitic nematodes would require the development of more hydrophilic, less persistent compounds and methods of application which could overcome distribution problems in the soil . The importance of proper dispersion of poorly water soluble pesticides in the soil has been shown for example with the nematicide fenamiphos , where good control of *G.rostochiensis* occured only when fenamiphos distributed evenly in the soil (Bromilow and Lord, 1979) .

Appendix I.1.

Physical and Chemical Properties of Diflubenzuron

(Martin and Worthing, 1977).

Trade name	=	Dimilin (Philips Duphar B.V.)
Proposed common name	=	Diflubenzuron
Former code names	=	PH60-40, TH60-40, ENT-29054 OMS 1804, PDD 60-401, DU112307
Chemical name	=	1 - (4 - chlorophenyl) -3- (2,6-diflurobenzoyl)-urea
Emperical formula	=	C_{14} H_9 N_2 O_2 F_2 CL
Molecular weight	=	310.69
Melting point	=	239°C
Solubility		
in water	=	0.2 (μg cm^{-3})
in acetone	=	0.65g/100 cm^3 at 20°C
in polar solvent	=	moderate
in apolar solvent	=	poor

Appendix I.2.

(A) Measurement of Particle Size of Diflubenzuron Formulation A

Channel size	True size (µm) d	Mean size (µm) dm	Number in Class N	% N	Σ % N
A	1.56	0.85	0	0	0
B	1.70	1.63	180	17.54	17.54
C	2.21	1.89	390	38.01	55.5
D	3.12	2.67	246	23.98	79.53
E	4.47	3.78	134	13.06	92.59
F	6.25	5.34	43	4.19	96.78
G	8.84	7.55	24	2.34	99.12
H	12.5	10.67	9.0	0.01	99.13
I	17.68	15.09	0	0	99.13
J	25.0	21.34	0	0	99.13

Total number of particles = 1026

Mean size of particles = 2.24 µm

Number median diameter

(NMD) at 50% = 1.8 µm

Appendix I.2.

(B) Measurement of Particle Size of Diflubenzuron Formulation B

Channel size	True size d	Mean size dm	Number in Class N	% N	Σ % N
A	1.56	0.85	0	0	0
B	1.70	1.63	162	16.02	16.02
C	2.21	1.89	302	29.87	45.89
D	3.12	2.67	292	28.84	74.77
E	4.47	3.78	150	14.83	89.60
F	6.25	5.34	71	7.02	98.68
G	8.84	7.55	28	2.76	99.38
H	12.5	10.67	5	0.49	99.87
I	17.68	15.09	1	0.09	99.98
J	25.0	21.34	0	0	99.98

Total number of particles = 1011

Mean size of particles = 2.8 µm

Number median diameter

(NMD) at 50% = 2.0 µm

Appendix I.3.

The Effect of Diflubenzuron in Acetone on Population Growth of *C. elegans* at 25°C.

(A) Population growth after 4 days

	Controls		Diflubenzuron ($\mu g\ cm^{-3}$)				
	NG agar	Acetone	5	10	20	40	80
	213	156	237	164	112	145	23
	209	205	194	179	168	120	29
	174	189	205	203	144	107	31
	265	214	189	159	187	141	27
	196	221	135	168	124	132	35
Totals	1057	985	960	873	735	645	145
$\bar{X}$	211.4	197	192	174.6	147	129	29
±S.E.	15.0	11.5	16.5	7.8	13.8	6.9	2.0

ANOVA

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	116553	6	19425	29.04	<0.01
Residual	18730	28	669		
Total	135283	34			

Appendix 1.3 (continued)

L.S.R. (0.01) = 62.57

Ranking of means:

Agar control	211.4
Acetone control	197.0
5 µg cm^{-3} Diflubenzuron	192.0
10 " "	174.6
20 " "	147.0
40 " "	129.0
80 " "	29.0

Appendix 1.3 (continued)

(B) Population growth after 5 days

Controls		Diflubenzuron (µg cm^{-3})				
NG agar	Acetone	5	10	20	40	80
336	255	232	418	395	213	40
489	411	309	271	294	206	24
372	293	452	296	307	280	32
428	470	381	312	216	139	28
320	381	366	156	310	117	36
Totals 1945	1810	1740	1453	1522	955	160
$\bar{X}$ 389	362	348	290	304.4	191	32
±S.E. 31.7	39.7	36.7	41.9	28.4	28.9	2.8

ANOVA

Source of Variation	SS	DF	MS = SS/DF	F	P
Treatments	465488	6	77581	14.92	<0.01
Residual	145580	28	5199		
Total	611068	34			

Appendix 1.3 (continued)

L.S.R. (0.07) = 174.85

Ranking of means :

Agar control	389.0
Acetone control	362.0
5 µg cm^{-3} Diflubenzuron	348.0
10 " "	304.4
20 " "	290.6
40 " "	191.0
80 " "	32.0

Appendix I.4

(A) The Effect of Diflubenzuron in Dimethyl Sulphoxide on Population Growth of *C. elegans* at 25°C.

Population Growth after 4 days

Controls		Diflubenzuron (µg cm^{-3})				
NG agar	Dimethyl sulphoxide	5	10	20	40	80
220	184	173	161	115	118	9
256	162	204	185	190	120	23
213	171	153	122	146	137	11
197	139	227	210	157	92	12
229	159	114	107	137	98	25
Totals 1115	815	871	785	745	565	80
$\bar{X}$ 223	163	174.2	157	149	113	16
±S.E. 9.8	7.4	19.7	19.1	6.9	8.1	3.3

ANOVA

Source of Variation	SS	DF	MS = SS/DF	F	P
Treatments	125148	6	20858	25.75	<0.01
Residual	22683	28	810		
Total	147831	34			

Appendix 1.4 (continued)

L.S.R. (0.01) = 68.55

Ranking of means :

Agar control	223.0
5 µg cm^{-3} Diflubenzuron	174.2
Dimethyl sulphoxide control	163.0
10 µg cm^{-3} Diflubenzuron	157.0
20 " "	149.0
40 " "	113.0
80 " "	16.0

Appendix 1.4 (continued)

(B) Population growth after 5 days

Controls		Diflubenzuron (μg cm^{-3})				
NG agar	Dimethyl sulphoxide	5	10	20	40	80
321	413	269	298	242	213	12
514	253	403	427	198	145	15
297	376	364	315	156	125	24
405	390	512	470	339	138	10
538	328	192	295	472	109	30
Totals 2075	1760	1740	1805	1407	730	91
$\bar{X}$ 415	352	348	361	281.4	146	18.2
±S.E. 48.9	28.4	55.1	36.6	56.5	17.8	3.8

ANOVA

Source of Variation	SS	DF	MS = SS/DF	F	P
Treatments	604403	6	100734	12.71	<0.01
Residual	221872	28	7924		
Total	826275	34			

Appendix 1.4 (continued)

L.S.R. (0.01) = 215.36

Ranking of means:

Agar control	415.0
10 µg cm^{-3} Diflubenzuron	361.0
Dimethyl sulphoxide control	352.0
5 µg cm^{-3} Diflubenzuron	348.0
20 " "	281.4
40 " "	146.0
80 " "	18.2

Appendix I.5

The Effect of Diflubenzuron Carrier for Wettable Formulations on Population Growth of *C. elegans*

(A) Population Growth after 3 days

Control	Diflubenzuron Carrier (μg cm^{-3})				
NG agar	15	30	60	120	240
145	127	165	215	124	93
224	142	124	172	219	205
161	253	104	242	117	146
156	178	152	187	153	213
213	293	182	131	257	117
Totals 899	993	727	947	870	774
$\bar{X}$ 179.8	198.6	145.4	189.4	174	154
± S.E. 16.1	32.2	14.1	19	27.5	23.8

ANOVA

Source of Variation	SS	DF	MS = SS/DF	F	P
Treatments	10309	5	2062	0.78	>0.05
Residual	63229	24	2635		
Total	73539	29			

Appendix 1.5 (continued)

(B) Population Growth after 4 days

Control	Diflubenzuron Carrier (µg cm^{-3})				
NG agar	15	30	60	120	240
217	114	152	348	179	250
168	259	210	145	225	313
152	356	165	140	352	350
214	281	192	290	165	217
184	176	411	165	189	189
Totals 935	1186	1130	1085	1110	1325
$\bar{X}$ 187	237.2	226	217.6	222	265
± S.E. 12.7	42.2	47.5	42.7	34.1	30.8

ANOVA

Source of Variation	SS	DF	MS = SS/DF	F	P
Treatments	16269	5	3254	0.48	>0.05
Residual	161780	24	6741		
Total	178049	29			

Appendix 1.5 (continued)

(C) Population Growth after 5 days

Control	Diflubenzuron Carrier (µg cm^{-3})				
NG agar	15	30	60	120	240
299	382	422	397	354	317
.313	479	391	453	290	295
448	257	306	252	426	386
416	371	218	281	384	352
395	283	343	448	246	475
Totals 1871	1772	1681	1831	1700	1825
$\bar{X}$ 374.2	354.4	336	366.2	340	365
± S.E. 29.2	39.6	35.7	42.2	32.4	31.7

ANOVA

Source of Variation	SS	DF	MS = SS/DF	F	P
Treatments	6967	6	1161	0.19	<0.05
Residual	170585	28	6092		
Total	177552	34			

Appendix I.6

The Effect of Diflubenzuron Formulations A and B on Population Growth of *C. elegans*

(A) Population Growth after 4 days

Control	Diflubenzuron formulation A (μg cm^{-3})				Diflubenzuron formulation B (μg cm^{-3})			
NG agar	5	10	20	40	5	10	20	40
269	108	3	5	3	92	3	3	3
245	121	11	3	3	71	13	3	3
171	53	3	3	3	81	11	3	3
218	68	5	3	3	109	6	3	3
262	105	3	3	3	69	5	3	3
Totals 1165	455	25	17	15	422	38	15	15
$\bar{X}$ 233	91	5	3.4	3.0	84.4	7.6	3.0	3.0
±S.E. 17.4	12.9	1.8	0.4	0	7.4	1.9	0	0

ANOVA

Source of Variation	SS	DF	MS = SS/DF	F	P
Treatments	244720	8	30590	100.8	<0.01
Residual	10922	36	303		
Total	255642	44			

Appendix 1.6 (continued)

L.S.R. (0.07) = 42.83

Ranking of means:

Agar control			233.0
5 µg cm^{-3}	Diflubenzuron	A	91.0
" "	"	B	84.4
10 "	"	A	7.6
" "	"	B	5.0
20 "	"	A	3.4
" "	"	B	3.0
40 "	"	A	3.0
" "	"	B	3.0

Appendix 1.6 (continued)

(B) Population Growth after 5 days

Control	Diflubenzuron formulation A (µg cm^{-3})				Diflubenzuron formulation B (µg cm^{-3})			
NG agar	5	10	20	40	5	10	20	40
484	120	13	12	3	117	3	3	3
431	179	60	3	3	180	18	6	3
238	181	33	5	3	95	15	3	3
392	211	29	7	14	88	13	12	3
526	114	4	7	19	113	7	3	5
Totals 2071	805	139	34	42	587	56	27	17
$\bar{X}$ 414.2	161.0	27.8	6.8	8.4	117.4	11.2	5.4	3.4
±S.E. 49.6	18.9	9.6	1.5	3.4	16.9	2.7	1.7	0.4

ANOVA

Source of Variation	SS	DF	MS = SS/DF	F	P
Treatments	744422	8	93053	52.7	<0.01
Residual	64340	36	1787		
Total	808762	44			

Appendix 1.6 (continued)

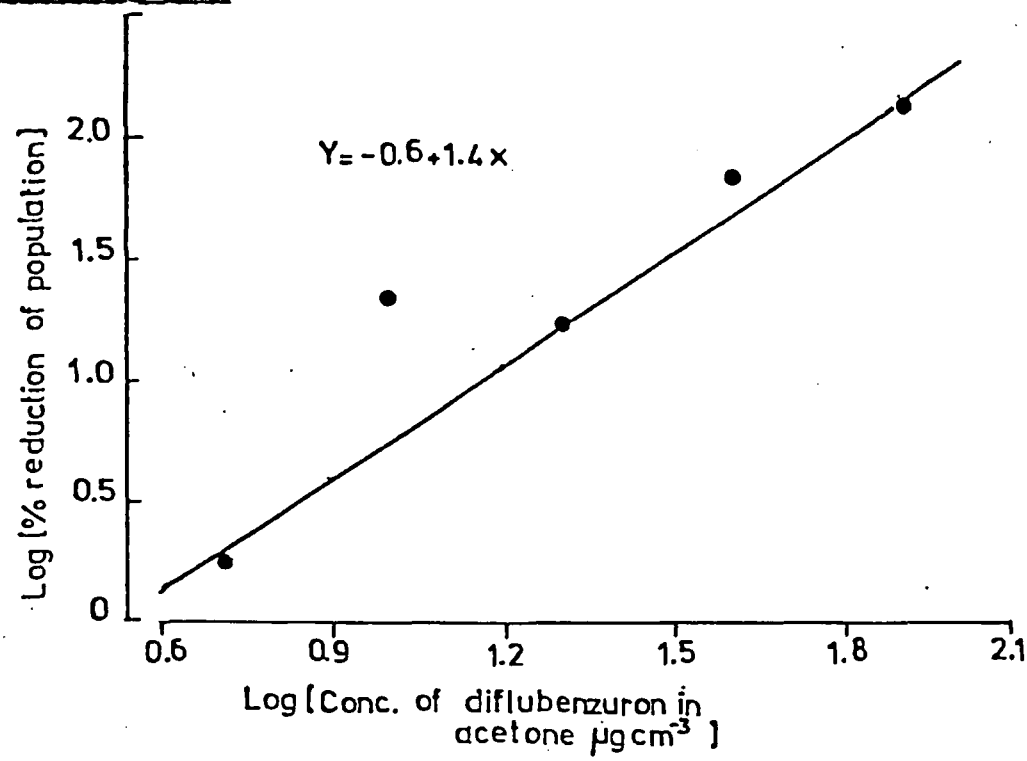
L.S.R. (0.01) = 104

Ranking of means:

Agar control			414.2
5 µg cm^{-3}	Diflubenzuron	A	161.0
"	"	B	117.4
10 "	"	A	27.8
"	"	B	11.2
20 "	"	A	8.4
"	"	B	6.8
40 "	"	A	5.4
"	"	B	3.4

Appendix I.7. (A) Effect of diflubenzuron in acetone on population growth of *C. elegans*

Regression analysis



y = a+bx.

Regression coefficient	S.D. of coefficient	T ratio = coefficient/S.D
a = -0.61	0.47	-1.31
b = 1.40	0.34	4.1

S.D of y about regression line is S = 0.33 (DF = 3)
R^2 = 0.85

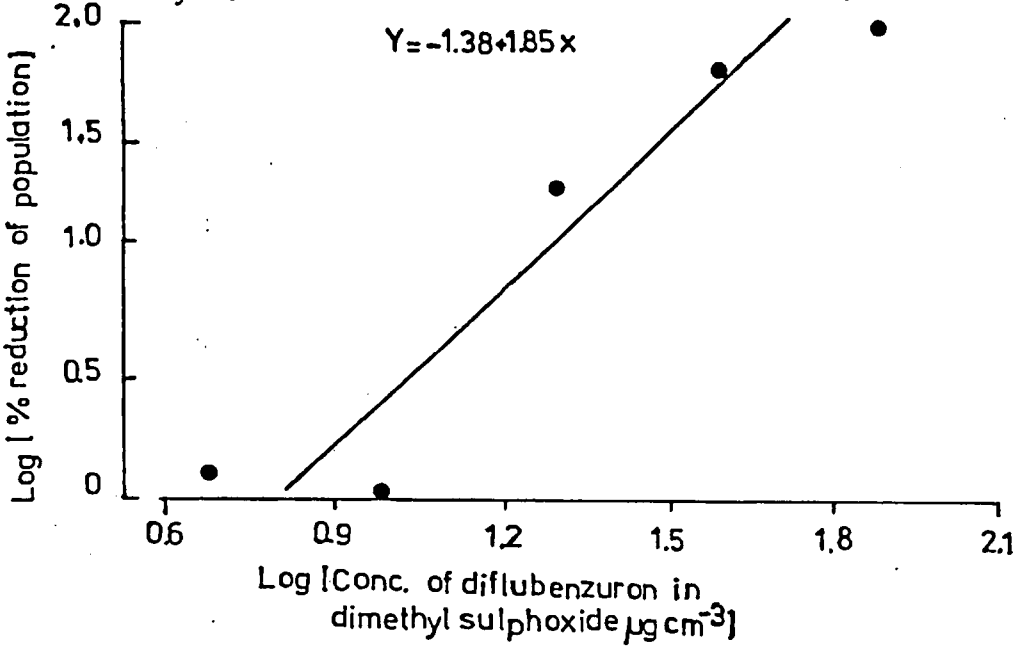
ANOVA

Source of variation	SS	DF	MS = SS/DF
Regression	1.79	1	1.78
Residual	0.32	3	0.71
Total	2.11	4	

Appendix I.7 (continued)

(B) Effect of diflubenzuron in dimethyl sulphoxide on population growth of *C. elegans*

Regression analysis



Regression coefficient	S.D. of coefficient	T ratio = coefficient/S.D.
a = -1.38	0.52	-2.67
b = 1.85	0.38	4.91

S.D. of y about regression line is S = 0.36 (DF = 3)
R^2 = 0.89

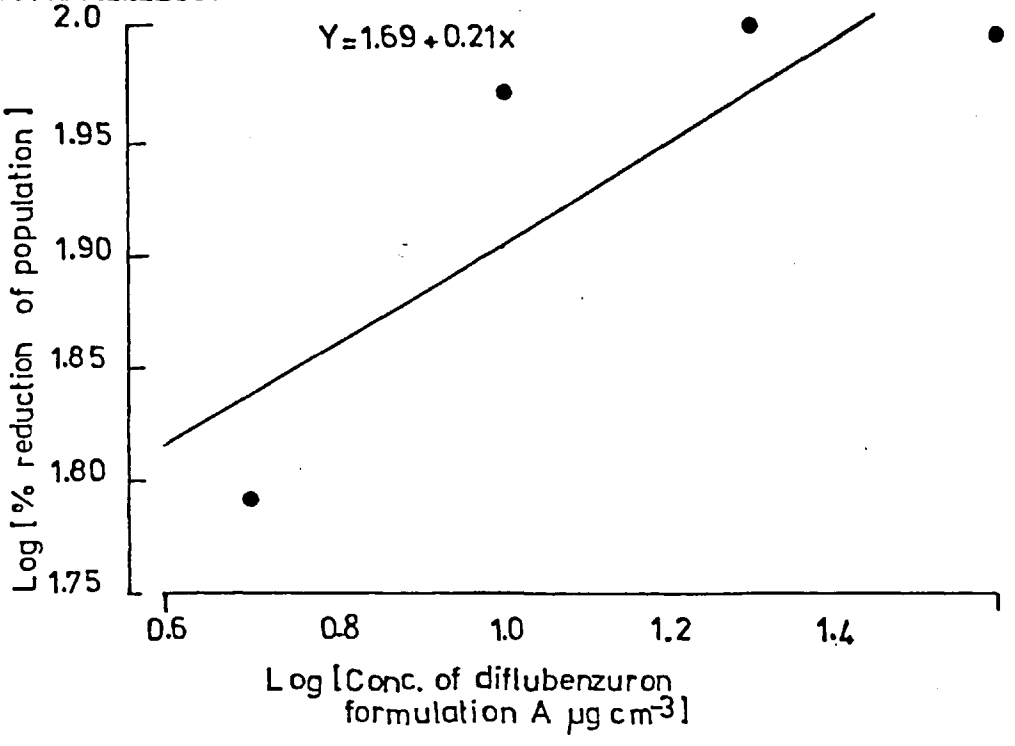
ANOVA

Source of variation	SS	DF	MS = SS/DF
Regression	3.11	1	3.11
Residual	0.39	3	0.13
Total	3.50	4	

Appendix I.7 (Continued)

(C) Effect of diflubenzuron formulation A on population growth of *C. elegans*

Regression analysis



Regression coefficient	S.D. of coefficient	T ratio = coefficient/S.D.
a = 1.69	0.12	13.9
b = 0.21	0.10	2.1

S.D. of Y about regression line is S = 0.68 (DF = 2)
R^2 = 0.69

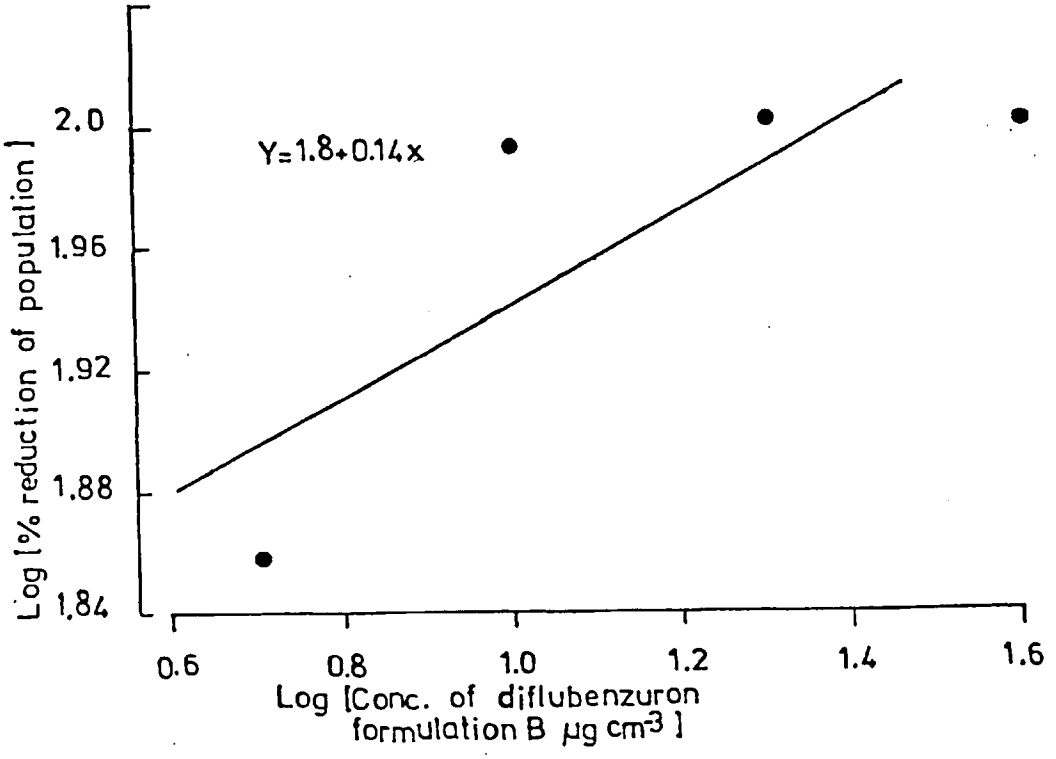
ANOVA

Source of variation	S.D. OF Coefficient	DF	MS = SS/DF
Regression	0.02	1	0.02
Residual	0.01	2	0.01
Total	0.03	3	

Appendix I.7 (Continued)

(D) Effect of diflubenzuron formulation B on population growth of *C. elegans*

Regression analysis



Regression coefficient	S.D. of coefficient	T ratio = coefficient/S.D.
a = 1.8	0.09	19.9
b = 0.14	0.08	1.9

S.D. of Y about regression line is S = 0.06 (DF = 3)
R^2 = 0.65

ANOVA

Source of variation	SS	DF	MS = SS/DF
Regression	0.01	1	0.01
Residual	0.01	2	0.003
Total	0.02	3	

Appendix II.1 Effect of diflubenzuron on development of *C. elegans*

A. Total body length (µm)

Hours	Control	Diflubenzuron (µg cm^{-3})			
		10	20	40	80
	299.9	298.5	463.6	306.1	266.7
	366.7	321.2	278.8	321.2	300.0
24	245.5	280.3	333.3	300.0	278.7
	278.8	312.1	287.9	275.8	266.6
	300.0	266.7	266.7	321.2	242.4
$\bar{X}$	298.2	295.7	326.0	304.8	270.9
±S.E.	±19.9	±10.0	±36.3	±8.4	±9.4
	966.7	719.7	1030.3	975.8	87.9
	848.5	945.5	703.0	797.0	233.3
48	945.5	939.4	757.7	1018.2	442.4
	975.8	863.6	909.1	909.1	421.2
	757.6	845.5	842.8	824.8	300.0
$\bar{X}$	899.0	863	848	905	297
±S.E.	±42.2	±40.8	±57.8	±42.6	±65
	1221.2	1045.5	1239.4	1272.7	500.7
	1175.8	1142.4	1133.3	1242.4	684.8
72	1333.3	1242.4	1330.3	1330.3	430.3
	1272.7	1197.0	1272.7	1330.3	633.3
	1242.4	1272.7	1218.2	1218.2	675.8
$\bar{X}$	1249.1	1180.0	1239.0	1279.0	584,8
±S.E.	±26.4	±40.3	±32.6	±22.8	±51.0

Appendix II.1 (continued)

(Total body length)

24h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	7840	4	1960	1.0	>0.05
Residual	39218	20	1961		
Total	47059	24			

48h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	1365150	4	341287	26.75	<0.01
Residual	255161	20	12758		
Total	1620311	24			

72h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	1725193	4	531298	66.86	<0.01
Residual	129231	20	6462		
Total	1854425	24			

Appendix II.1 (continued)

B. Pharyngeal length (µm)

Hours	Control	Diflubenzuron (µg cm^{-3})			
		10	20	40	80
	87.9	90.9	97.0	87.9	84.8
	93.9	93.9	87.9	90.9	90.9
24	81.8	90.9	87.9	84.8	81.8
	91.9	87.9	87.9	81.8	84.8
	84.8	81.8	81.8	90.9	75.7
$\bar{X}$	88.1	89.0	88.5	87.3	83.6
±S.E.	±2.2	±2.1	±2.4	±1.8	±2.5
	148.5	124.2	151.5	148.5	103.0
	130.3	142.4	133.3	136.4	87.9
48	136.4	154.5	124.2	151.5	103.0
	148.5	130.3	136.4	136.4	90.0
	127.3	136.4	130.3	136.4	75.8
$\bar{X}$	138.2	137.6	135.6	141.8	92.1
±S.E.	±4.5	±5.2	±4.6	±3.4	±5.2
	157.6	151.5	151.5	157.6	115.2
	151.5	151.5	151.5	151.5	127.3
72	157.6	151.5	157.6	157.6	106.1
	151.5	145.5	157.6	157.5	121.2
	151.5	157.6	151.5	151.5	121.2
$\bar{X}$	153.9	151.5	153.9	151.2	118.2
±S.E.	±1.5	±1.9	±1.5	±1.5	±3.6

Appendix II.1 (continued)

ANOVA (Pharyngeal length)

24h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	93.3	4	23.2	0.96	>0.05
Residual	484.6	20	24.2		
Total	577.9	24			

48h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	7581	4	1895	3.48	<0.05
Residual	11430	20	544		
Total	19011	24			

72h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	5062.1	4	1265.5	54.71	<0.01
Residual	462.7	20	23.1		
Total	5524.8	24			

Appendix II.1 (continued)

C. Maximum body width at the vulval region

Hours	Control	Diflubenzuron (µg cm^{-3})			
		10	20	40	80
	21.2	21.2	24.2	21.2	21.2
	24.2	24.2	24.2	24.2	21.2
24	21.2	21.2	24.2	21.2	18.8
	21.2	24.2	24.2	24.2	21.2
	21.2	18.2	21.2	21.2	18.8
$\bar{X}$	21.8	21.8	23.6	22.4	20.1
±S.E.	±2.4	±1.1	±0.6	±0.7	±0.7
	54.5	39.4	60.6	66.7	27.3
	51.5	54.5	36.4	45.5	18.2
48	54.5	51.5	33.3	60.6	27.3
	51.5	51.5	33.3	45.5	24.2
	54.5	54.5	51.5	45.5	18.2
$\bar{X}$	53.3	50.3	43.0	52.7	23.0
±S.E.	±0.7	±2.8	±5.6	±4.6	±2.1
	75.8	75.8	75.8	75.8	21.2
	63.6	75.8	75.8	75.8	42.4
72	75.8	72.8	75.8	75.8	33.3
	75.8	75.8	75.8	75.8	36.4
	75.8	75.8	75.8	75.8	36.4
$\bar{X}$	73.3	75.1	75.8	75.8	33.9
±S.E.	±2.4	±0.6	0	0	±3.5

Appendix II.1. (continued)

ANOVA (Maximum body width)

24h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	32.15	4	8.04	2.67	>0.05
Residual	60.31	20	3.02		
Total	92.46	24			

48h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	3211.8	4	803.0	12.53	<0.01
Residual	1281.7	20	64.1		
Total	4493.5	24			

72h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	6762.3	4	1690.6	91.16	<0.01
Residual	370.9	20	18.5		
Total	7133.2	24			

Appendix II.1. (continued)

(D) Total body length/Length of pharynx

Hours	Control	Diflubenzuron (μg cm^{-3})			
		10	20	40	80
24	3.4	3.3	4.8	3.5	3.1
	3.9	3.4	3.1	3.8	3.3
	2.9	3.1	3.8	3.5	3.4
	3.0	3.6	3.3	3.4	3.1
	3.5	3.3	3.3	3.5	3.2
48	6.5	5.8	6.8	6.6	4.7
	6.5	6.6	5.3	5.8	2.7
	6.9	6.1	6.1	6.7	4.3
	6.6	6.6	6.7	6.7	4.7
	6.0	6.2	6.5	6.5	4.0
72	7.8	7.0	8.4	7.9	4.3
	7.8	7.5	8.2	7.5	5.4
	8.5	8.2	8.4	8.4	4.1
	8.4	8.2	8.4	8.1	5.2
	8.7	8.1	8.0	8.0	5.6

Appendix II.1. (continued)

Total body length/Length of pharynx

ANOVA (Transformed values)

24h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	0.00392	4	0.00098	1.10	>0.01
Residual	0.07789	20	0.00089		
Total	0.02181	24			

48h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	0.05465	4	0.01491	11.0	<0.01
Residual	0.02711	20	0.00136		
Total	0.08676	24			

72h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	0.04939	4	0.01234	36.68	<0.01
Residual	0.00673	20	0.00033		
Total	0.05612	24			

Appendix II.1. (continued)

(E) Total body length/Maximum body width

Hours	Control	Diflubenzuron (μg cm^{-3})			
		10	20	40	80
24	14.1	14.1	19.1	14.4	12.6
	15.1	13.3	11.5	13.3	14.1
	11.6	13.2	13.8	14.1	14.8
	13.1	12.9	11.9	11.4	12.6
	14.1	14.7	12.6	15.1	12.9
48	17.7	18.3	17.0	14.6	17.9
	16.5	17.3	19.3	17.5	12.8
	17.4	18.2	22.7	16.8	16.2
	18.9	16.8	27.3	20.0	17.3
	13.9	15.5	19.6	19.5	16.5
72	16.1	13.9	16.8	16.4	23.6
	18.5	15.1	16.4	15.0	16.1
	17.6	17.1	17.6	17.6	12.9
	16.8	15.8	17.6	16.8	17.4
	16.4	16.8	16.1	16.1	18.6

Appendix II.1. (continued)

Total body/Maximum body width

ANOVA (Transformed values)

24h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	0.000025	4	0.000006	0.02	>0.05
Residual	0.005595	20	0.000280		
Total	0.005620	24			

48h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	0.002849	4	0.00712	2.86	>0.05
Residual	0.004988	20	0.00024		
Total	0.007838	24			

72h

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	0.000476	4	0.000719	0.67	>0.05
Residual	0.003937	20	0.000197		
Total	0.004413	24			

Appendix II.2. Effect of diflubenzuron on survival of *C. elegans*

Treatment	Percentage survival				
	1	2	3	4	5
Control	96 (24/25)	100 (25/25)	89 (25/28)	88 (22/25)	85 (23/27)
Diflubenzuron ($\mu g\ cm^{-3}$) 5	92 (23/25)	76 (23/30)	82 (22/27)	100 (30/30)	96 (24/30)
10	88 (22/25)	76 (19/25)	92 (23/25)	69 (20/29)	93 (26/28)
20	83 (15/25)	80 (20/25)	76 (23/30)	90 (27/30)	89 (24/27)
40	84 (21/25)	64 (18/28)	92 (23/25)	88 (23/26)	88 (22/25)
80	86 (26/30)	72 (18/25)	86 (24/27)	72 (21/29)	89 (24/27)

ANOVA (Transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	0.176	5	0.0351	1.58	>0.05
Residual	0.534	24	0.0223		
Total	0.710	29			

L.S.R. (0.05) = 0.29

Ranking of means: (Transformed values).
1.313, 1.313, 1.282, 1.282, 1.136, 1.156, 1.163. Probits equation: $Y = 3.46 + 0.177x$

Appendix II.3. Effect of diflubenzuron on feeding of *C. elegans*

A. Feeding rate (Number of pharyngeal pulsations in 2 min)

Control	Diflubenzuron (μg cm^{-3})			
	10	20	40	80
254	240	266	190	110
290	273	285	244	214
198	280	230	246	260
266	275	271	180	170
265	219	290	191	195
302	268	170	113	140
240	184	265	156	122
290	290	290	276	180
$\bar{X}$ 263	254	259	200	174
±S.E. ±11.9	± 13	± 13.6	± 19	± 17.8

B. Percentage of nematodes feeding

Control	Diflubenzuron (μg cm^{-3})			
	10	20	40	80
88 (22/25)	84 (21/25)	92 (23/25)	70 (19/25)	64 (16/25
100 (30/30)	100 (30/30)	77 (21/27)	100 (29/29)	73.3(22/30)
100 (30/30)	96 (24/25)	100 (25/25)	71.4(20/28)	76 (19/25)
93.5(25/27)	100 (25/25)	90 (27/30)	84 (21/25)	70 (21/30)
$\bar{X}$ 95	95	90	81	71

Appendix II.3 (continued)

Feeding rate

ANOVA (Transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	52788	4	131977	7.14	<0.01
Residual	64701	35	1849		
Totals	117490	39			

L.S.R. (0.05) = 62.32

Ranking of means,

263, 259, 254, 200, 174.

Percentage of nematodes feeding

ANOVA (Transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	0.4956	4	0.1239	3.25	<0.05
Residual	0.5712	15	0.0381		>0.01
Total	1.0668	19			

L.S.R. (0.05) = 0.425

Ranking of means (Transformed values)

1.141, 1.418, 1.294, 1.182, 1.001.

Appendix II.4. Effect of diflubenzuron on locomotion of *C. elegans*

Distances travelled by *C. elegans* (mm)

	Control	Diflubenzuron ($\mu g\ cm^{-3}$)			
		10	20	40	80
	6.5	9.1	12.1	5.5	1.8
	9.9	6.0	8.2	8.5	3.4
	5.7	8.3	14.4	3.9	1.5
	9.6	16.1	5.7	5.8	2.5
	10.1	5.4	6.7	9.6	2.5
$\bar{X}$	8.4	9.1	9.4	6.7	2.4
±S.E.	±0.9	±1.9	±1.7	±1.0	±0.3

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	164.15	4	41.04	4.87	>0.01
Residual	168.63	20	8.43		
Total	332.78	24			

L.S.R. (0.05) = 5.5

Ranking of means:

9.4, 9.1, 8.4, 6.7, 2.4

Appendix II.5. Effect of diflubenzuron on fecundity of *C. elegans*

4.8 h

Control	Diflubenzuron (µg cm^{-3})		
	5	10	20
40	95	88	8
91	18	4	5
78	6	78	46
25	78	70	6
32	70	32	4
118	74	35	34
119	95	41	0
124	0	26	0
55	84	63	0
23	38	2	0
61	29	96	0
36	3	0	0
23	76	20	24
89	62	26	0
0	84	15	0
51	59	28	0
119	38	72	65
82	72	24	6
46	30	54	57
113	81	70	34
27	20	23	15
36	34	76	45
114	0	111	32
45	12	8	0
Total 1567	1158	1062	381

Appendix II.5 (continued).

(B)

96h

Diflubenzuron ($\mu g\ cm^{-3}$)		
5	10	20
8	6	0
35	6	8
80	47	5
61	6	46
5	23	6
21	13	4
24	0	42
7	0	0
29	4	0
0	0	0
0	16	0
73	18	0
23	4	0
46	22	14
7	6	14
84	2	0
13	19	0
0	16	8
48	7	8
75	11	5
0	0	0
10	0	0
7	8	3
35	12	0
691	246	163

Total

Appendix II.5. (continued)

Treatment	Means ± S.E. (n = 24)	
	48h	96h
Control	-	65.3 ± 8.2
5ppm PH60-40	48.3 ± 6.7	28.8 ± 5.7
10ppm PH60-40	44.3 ± 6.6	10.3 ± 2.2
20ppm PH60-40	15.9 ± 8.3	6.8 ± 2.5

ANOVA

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	69408	6	11568	15.76	<0.01
Residual	118186	161	734		
Total	187594	167			

L.S.R. (0.01) = 26.99

Ranking of means:

65.3, 48.3, 44.3, 28.8, 16.3, 6.8

Appendix II.6 Fecundity of *C. elegans* treated with diflubenzuron at different stages during post-embryonic development.

Control	Diflubenzuron ($\mu g\ cm^{-3}$)			
	10		20	
	J_2	J_4	J_2	J_4
464	110	108	50	29
261	65	93	50	107
233	118	110	113	79
109	133	121	60	162
266	166	86	85	79
Total 1333	592	518	358	456
$\bar{X}$ ±S.E. 267±57.1	118±16.5	104±6.3	72±12.2	91±21.8

ANOVA

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	150897	4	37724	5.76	< 0.01
Residual	131022	20	6551		
Total	281919	24			

L.S.R. (0.05) = 153.47

Ranking of means

286.6, 118.4, 103.6, 91.2, 71.6, 57.32, 23.68, 14.32

Appendix II.7

(A) Effect of diflubenzuron on gonadial growth in *C. elegans*

Treatment	Mean length (µm), n = 10			
	Control	Diflubenzuron (µg cm^{-3})		
		20	40	80
24h Ant. ovary) Uterus) Post. ovary)	173.4	163.7	105.3	82.8
48h				
Ant. ovary	312.6	318	333	109.3
Uterus	129.3	114	149.1	30.6
Post. ovary	311.6	321.5	352.9	105.1
Total	753.5	753.5	835	245
72h				
Ant. ovary	400.5	368.9	363	271.1
Uterus	195	222.5	252	91.9
Post. ovary	411	372.6	337.9	271.1
Total	1006.5	964	952.9	634.1

(B) Size increase of *C. elegans* gonad after 72h treatment

Control	Diflubenzuron (µg cm^{-3})		
	20	40	80
1.33	1.28	1.14	2.59

Size increase = Length after 72h treatment / Length after 48h treatment

(Byerly *et al.*, 1976).

Appendix II.8. Effect of diflubenzuron on Oogenenesis of *C. elegans*

(A)

Number of oocytes at diplotene and diakinesis					
Control		Diflubenzuron ($\mu g\ cm^{-3}$)			
		20		40	
(6+9)	15	(6+7)	13	(3+3)	6
(3+5)	8	(5+4)	9	(1+0)	1
(3+3)	6	(3+3)	6	(2+3)	5
(4+0)	4	(7+0)	7	(3+2)	5
(9+8)	17	(3+3)	6	(4+2)	6
(4+6)	10	(2+2)	4	(4+1)	5
(5+4)	9	(0+0)	0	(4+1)	5
(4+4)	8	(3+0)	3	(0+0)	0
(2+0)	2	(3+0)	3	(2+2)	4
(4+2)	6	(0+0)	0	(0+2)	2
(2+3)	5	(7+5)	12	(4+3)	7
(3+3)	6	(3+0)	3	(1+2)	3
(3+3)	6	(6+1)	7	(1+0)	1
(0+0)	0	(5+4)	9	(2+2)	4
(4+5)	9	(3+2)	5	(2+4)	6
Total	111		87		60
$\bar{X}$	7.0		6.0		4.0

Appendix II.8. (continued)

ANOVA (Transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	0.3094	2	0.1547	1.69	>0.01
Residual	3.8471	42	0.0916		
Total	4.1565				

L.S.R. (0.05) = 0.2688

Ranking of means (transformed values)

0.849, 0.736, 0.647.

(B)

Control		Diflubenzuron (80 µg cm^{-3})	
(5+4)	9	(2+0)	2
(4+4)	8	(3+4)	7
(0+0)	0	(1+5)	6
(6+2)	8	(3+2)	5
(7+4)	11	(12+4)	16
(6+6)	12	(5+6)	11
(3+6)	9	(5+3)	8
(0+0)	0	(4+3)	7
(8+8)	16	(5+8)	13
(7+4)	11	(1+3)	4
(3+0)	3	(7+5)	12
(2+5)	7	(0+0)	0
(7+9)	16	-	-
Total	110		91
$\bar{X}$	8.0		7.0

(t=0.14;P>0.05)

Appendix II.9. Effect of diflubenzuron on Spermatogenesis of *C. elegans*

(A)

Number of sperm nuclei at spermatheca region					
Control		Diflubenzuron (μg cm^{-3})			
		20		40	
(0+0)	0	(15+22)	37	(0+0)	0
(27+0)	27	(27+13)	40	(28+20)	48
(6+0)	6	(43+48)	91	(27+28)	55
(0+0)	0	(29+18)	47	(35+46)	81
(0+0)	0	(5+5)	10	(0+0)	0
(0+4)	4	(42+25)	67	(28+30)	58
(1+25)	26	(12+0)	12	(7+12)	19
(24+4)	28	(0+0)	0	(0+0)	0
(12+20)	32	(12+6)	18	(34+18)	52
(30+42)	72	(0+0)	0	(39+59)	98
(35+48)	83	(9+0)	9	(0+0)	0
(9+9)	18	(38+39)	77	(0+0)	0
(43+30)	73	(71+12)	83	(0+0)	0
(22+6)	28	(33+22)	55	(10+12)	22
(132+91)	223	(0+0)	0	(4+11)	15
Total	710		546		458

Appendix II.9 (continued)

ANOVA (Transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	0.618	2	0.309	0.52	>0.05
Residual	25.211	42	0.600		
Total	25.829	44			

L.S.R. (0.05) = 0.684

Ranking of means (transformed values):

1.244, 1.210, 0.980.

(B)

Control		Diflubenzuron (80 µg cm^{-3})	
(50+12)	62	(7+11)	18
(24+9)	33	(0+5)	5
(48+17)	65	(12+9)	21
(45+72)	117	(72+62)	134
(60+18)	78	(0+0)	0
(9+6)	15	(0+0)	0
(5+11)	16	(0+0)	0
(0+0)	0	(21+12)	33
(7+12)	19	(55+18)	73
(24+47)	71	(0+0)	0
(17+16)	33	(74+38)	112
(0+6)	6	(35+6)	41
-	-	(12+17)	29
515		466	

(t=1.25; P >0.05)

Appendix III.1

Effect of diflubenzuron on fertilization of *C. elegans*

Materials and cultures.

(A) S medium: (Sulston and Brenner, 1974).

1 cm^3 of saturated ethanolic cholestrol was added to 1000 cm^3 of S buffer (0.1M NaCl, 0.05M KH_2PO4. pH6). After autoclaving, the following sterile solutions were added in order,

10 cm^3 M Ktricitrate pH6.

3 cm^3 M $CaCl_2$

3 cm^3 M $MgSO_4$

10 cm^3 trace metal solution (EDTA 5mM, $FeSO_4$ 2.5mM, $MnCl_2$ 1mM, $ZnSO_4$ 1mM and $CuSO_4$0.1mM). Sterilization was performed using Acrodisc (Disposable disc filter) with 0.2 µm pore size, attached to a 10 cm^3 syringe.

(B) Nile blue stain (Ward and Carrel, 1979).

Nile blue A - Nile blue sulfate - Cl Basic blue 12, ClNO. 51180 was purchased from Sigma Ltd. (Poole) and a stock solution, 5% (w/v) in 95% (v/v) ethyl alcohol was diluted 8-fold with M-9 (Brenner, 1974). M-9 buffer was made of 6g Na_2HPO_4; 3g KH_2PO_4; 5gNaCl and 0.25g $MgSO_4$-$7H_2O$ per 1000 cm^3.

(C) Sperm buffered saline (Ward and Miwa, 1978).

33mM KH_2PO_4

63mM Na_2HPO_4

136mM NaCl

1mM $MgCl_2$

(D) Ringer saline solution : (Duggal, 1978)

A stock solution of the earthworm Ringer (Pantin, 1969) was prepared and a test solution was made of 1:3 dilution.

(E) *C. elegans* mutant strains:

1. E369 = unc-51 (e369); (Brenner, 1974)

This strain shows uncoordinated movement and reduced activity.

2. E1467 = him-1 (e1467); (Hodgkin, 1974)

This strain shows a high incidence of males (>0.2%). Male cultures were preserved by adding 6-7 males to a similar number of hermaphrodites on seeded NG plates at 16°C. Routine subculturing was carried out every 10-15 days.

Both mutant strains were obtained from Dr S. Brenner (M.R.C., Hills Road, Cambridge, England).

Appendix III.2 Effect of diflubenzuron on production of fertilized eggs in *C. elegans*

Treatment	Control	Diflubenzuron ($\mu g\ cm^{-3}$)			
		5	10	20	40
Total number of eggs laid + hatched larvae : unfertilized eggs laid	935:122 734:84 957:246 444:51 282:55	873:179 742:201 397:79 751:131 561:82	831:184 465:175 1258:373 703:189 316:76	240:128 176:69 262:117 359:186 270:107	67:49 120:105 43:31 87:67 368:260
Total	3352:558	3324:672	3573:997	1307:607	685:512

ANOVA (transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	1.409	4	0.352	67.05	
Residual	0.105	20	0.005		<0.01
Total	1.514	24			

L.S.R. (0.05) = 0.14

Ranking of means (transformed values) 1.06, 0.74, 0.58, 0.46, 0.41

Appendix III.3.

Time (min) taken for migration of the nuclei in mature oocytes of *C. elegans*

	Control	Diflubenzuron (µg cm^{-3})	
		10	40
	24	32	22
	10	25	47
	8	28	16
	35	24	20
	18	21	19
	34	16	26
	42	20	18
$\bar{X}$ ±S.E.	24.4±4.9	25.0±2.7	24.0±4.0

ANOVA

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	2	2	1	0.01	>0.05
Residual	1879	18	104		
Total	1881	20			

Appendix III.4 Number of sperm in contact with oocytes (O) and in spermathecae (Sp).

	Control O-Sp	Diflubenzuron (μg cm^{-3})	
		10	40
		O-Sp	O-Sp
1	3 - 8	4 - 3	9 - 9
2	0 - 15	8 - 14	0 - 18
3	0 - 0	0 - 3	2 - 12
4	4 - 12	6 - 14	12 - 20
5	6 - 7	6 - 11	3 - 10
6	8 - 4	21 - 4	0 - 11
7	1 - 5	20 - 5	7 - 0
8	0 - 9	9 - 14	0 - 15
9	10 - 6	2 - 7	0 - 0
10	21 - 4	0 - 0	0 - 16
11	2 - 10	0 - 0	1 - 0
12	5 - 7	1 - 4	3 - 9
13	14 - 2	2 - 4	7 - 0
14	4 - 4	0 - 0	0 - 0
15	3 - 5	0 - 0	0 - 18
16	0 ΣX=98	2 ΣX=83	8 ΣX=138
17	0	7	2
18	11	5	3
19	9	0	0
20	14	5	1
21	0	6	0
22	0	1	0
23	0	6	8
24	0	10	1
25	4	0	0
	ΣX=119	ΣX=116	ΣX=67

Appendix III.5 Effect of diflubenzuron on fusion of oocyte and sperm pronuclei in *C. elegans*

Treatments	Time taken for fusion (min) to occur										$\bar{X}$±S.E.
	1	2	3	4	5	6	7	8	9	10	
Control	30	19	24	21	20	30	16	22	20	24	22.6 ±1.4
Diflubenzuron (10 µg cm^{-3})	12	24	21	20	19	21	25	24	21	22	20.9 ±1.1

$t_{0.05} = 0.92$

$P > 0.05$

Appendix III.6 Effect of diflubenzuron on the migration of sperm from males in the gonads of hermaphrodites.

	Spermathecae with fluorescent dye in anterior and posterior gonads		No. of fluorescent sperm	
	Control	Diflubenzuron (40 µg cm^{-3})	Control	Diflubenzuron (40 µg cm^{-3})
1	+,/	+,/	0	1
2	+,/	+,/	5	1
3	+,/	-,-	0	0
4	+,-	-,-	0	0
5	+,-	-,-	4	0
6	+,-	-,-	3	0
7	+,/	+,-	7	8
8	-,-	+,+	0	13
9	+,+	-,+	5	0
10	+,+	-,+	7	3
11	-,/	-,-	0	0
12	+,-	+,-	0	0
13	+,/	-,-	0	0
14	+,/	+,+	0	2
15	+,-	+,/	0	1
16	+,/	+,-	4	0
17	-,-	+,-	0	1
18	-,-	-,-	0	0
19	+,-	-,-	0	0
20	+,-	+,+	0	7
21	-,-	+,+	0	8
22	/,/	+,-	/	0
Totals	18	19	35	45

\+ : Fluorescene present

\- : " not present

/ : Spermatheca not examined

Appendix III.7 Feulgan staining

No. of mature oocytes with visible sperm pronuclei

Treatment		
Control	Diflubenzuron (μg cm^{-3})	
	20	40
2	0	0
0	0	0
2	0	0
0	0	1
0	0	0
1	0	0
2	2	0
2	1	0
0	0	0
2	0	0
3	0	0
2	0	0
0	0	0
0	2	1
3	0	1
Totals 19	5	3

ANOVA (transformed values)

Source of Variation	SS	DF	MS=SS/DF	F
Treatments	2.57	2	1.30	
Residual	8.31	42	0.20	6.5
Total	10.88	44		P<0.01

L.S.R. (0.05) = 0.33 Ranking of means (transformed values)

0.67, 0.19, 0.14

Appendix III.8 Effect of diflubenzuron on production of sperm by *C. elegans* males

Control		Diflubenzuron (40 µg cm^{-3})	
202	101	170	173
210	431	59	102
239	152	91	137
34	198	38	100
107	162	72	170
218	46	264	102
91	220	192	165
154	120	135	128
143	107	140	340
153	423	110	146
81	440	70	141
133	240	157	386
85	249	81	219
304	163	317	121
327	271	281	280
510	92	122	320
279	162	485	68
$\bar{X}$ ± S.E. = 201±20.3		$\bar{X}$ S.E. = 173±17.9	
t = 1.05 P > 0.05			

Appendix III.9 Effect of diflubenzuron on sperm structure in *C. elegans*

% of sperm producing filaments (no of sperm examined)				
Control			Diflubenzuron ($\mu g\ cm^{-3}$)	
1	17.0	(85)	11.2	(89)
2	10.4	(96)	2.5	(81)
3	11.7	(51)	25.5	(102)
4	37.5	(104)	13.7	(73)
5	82.4	(74)	28.6	(14)
6	39.0	(87)	36.5	(41)
7	48.0	(50)	0	(25)
8	20.4	(142)	15.3	(13)
9	50.9	(101)	16.1	(56)
10	18.2	(11)	3.0	(33)
11	4.7	(43)	5.3	(57)
12	11.9	(59)	15.0	(40)
13	21.3	(80)	6.8	(44)
14	45.9	(37)	57.1	(140)
15	0	(40)	73.3	(120)
16	21.6	(37)	30.0	(103)
17	7.1	(56)	9.6	(124)
18	2.4	(42)	5.8	(137)
$t = 0.43$				
$P > 0.05$				

Appendix III.10

Distance travelled by *C. elegans* sperm on polylysine surface (μm)

	Control	Diflubenzuron (40 μg cm^{-3})
	4.5	3.0
	1.5	3.0
	3.0	3.0
	4.5	4.5
	4.5	3.0
	1.5	4.5
	3.0	1.5
	3.0	4.5
	3.0	1.5
	3.0	1.5
$\bar{X}$ ±S.E.	3.2±0.35	3.0±0.38

Appendix IV.1 Effect of diflubenzuron on embryogenesis in *C. elegans*

	Eggs examined : eggs embryonated				
	Control	Diflubenzuron (µg cm^{-3})			
		5	10	20	40
	24:24	20:20	24:20	20:19	23:22
	22:20	22:18	24:23	21:19	23:18
	20:19	20:19	22:20	24:23	20:17
Total	66:64	62:57	70:63	65:61	66:59

ANOVA (on transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	0.060	4	0.075	0.83	>0.05
Residual	0.182	10			
Total	0.242	14			

Appendix IV.2 Effect of diflubenzuron on hatching of *C. elegans* eggs (collected from untreated nematodes)

Eggs inoculated : eggs hatched			
Control	Diflubenzuron ($\mu g\ cm^{-3}$)		
	10	20	40
73:65	223:158	72:48	67:47
110:88	120:88	37:29	44:31
48:40	53:35	174:125	91:75
173:133	217:166	104:76	206:156

ANOVA (on transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	0.046	3	0.015	3.95	>0.05
Residual	0.046	12	0.003		
Total	0.092	15			

L.S.R. (0.05) = 0.13

Ranking of means (transformed values)

1.14, 1.05, 1.01, 1.0.

Appendix IV.3 Effect of diflubenzuron on hatching of *C. elegans* eggs (collected after being laid by treated nematodes)

Eggs inoculated:eggs hatched				
	Control	Diflubenzuron (μg cm^{-3})		
		5	10	20
	254:223	152:65	293:135	109:25
	410:352	248:188	172:65	358:72
	219:210	163:106	138:73	183:81
	512:481	432:307	114:88	219:77
	145:112	164:144	257:168	87:17
Totals	1540:1378	1159:810	974:529	956:272

ANOVA (On transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	1.203	3	0.401	18.32	<0.05
Residual	0.350	16	0.021		
Total	1.553	19			

L.S.R. (0.05) = 0.26

Ranking of means (transformed values)

1.23, 0.99, 0.85, 0.55.

Appendix IV.4 Effect of diflubenzuron on hatching of *C. elegans* eggs (laid by treated nematodes)

	Eggs laid : eggs hatched				
	Control	Diflubenzuron ($\mu g\ cm^{-3}$)			
		5	10	20	40
	123:104	92:45	143:63	78:9	25:2
	87:69	72:56	81:22	112:14	45:2
	50:45	102:81	50:15	45:3	21:0
	112:98	175:125	60:8	96:18	80:4
	48:42	91:47	65:27	89:33	13:0
Total	420:358	532:354	399:135	420:77	184:8

ANOVA (On transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatments	3.48	4	0.869	48.84	<0.01
Residual	0.36	20	0.017		
Total	3.84	24			

L.S.R. (0.05) : 0.25

Ranking of means (transformed values)

1.19, 0.95, 0.58, 0.41, 0.14

Appendix IV.5 Effect of diflubenzuron on hatching of *C. elegans* eggs laid by nematodes treated at different juvenile stages (J_2, J_4)

Treatments	Eggs laid : eggs hatched	Totals
(A) Second juvenile stage		
Control	464:343, 261:186, 266:232, 233:173 109:69	1333:1003
Diflubenzuron ($\mu g\ cm^{-3}$) 10	110:65, 65:36, 118:48, 133:59, 166:38	592:246
20	50:32, 50:20, 113+0, 60:41, 85:8	385:107
(B) Fourth juvenile stage		
Diflubenzuron ($\mu g\ cm^{-3}$) 10	93:65, 109:69, 110:84, 121:104, 96:76	529:398
20	29:19, 107:87, 186:64, 162:153, 79:73.	563:396

ANOVA (on transformed values)

Source of Variation	SS	DF	MS=SS/DF	F	P
Treatment	0.98	4	0.245	4.18	<0.05
Residual	1.17	20	0.058		
Total	2.16	24			

L.S.R. (0.05) = 0.11 Ranking of means: transformed values 1.06, 1.06, 1.01, 0.73, 0.58.

Appendix V.1. Ultrastructural studies

A. Buffers (Lewis and Knight, 1977)

A.1 Sodium Cacodylate (pH 7.2)

81 cm^3 of 0.1N HCl or HNO_3 were added to 50 cm^3 of 0.2 M sodium cacodylate (Hopkin and Williams Ltd., Chadwell Heath, Essex England) and diluted to 200 cm^3 with distilled water.

A.2 Sodium phosphate (pH 7.4)

81 cm^3 of 0.2 M Na_2HPO_4 mixed with 19 cm^3 of 0.2 M NaH_2PO_4.

B. Fixatives (Ward *et al.*, 1975; Wharton, 1979)

B.1 5% v/v) gluteraldehyde (Taab Laboratories, Emmer Green, Reading, Berks, England) in 0.1 M cacodylate buffer.

B.2 3% (v/v) gluteraldehyde in 0.1 M phosphate buffer, pH 7.4.

B.3 1% (w/v) osmium tetroxide (Agar Aids, Stansted, Essex, England) in cacodylate buffer, pH 7.2.

B.4 0.5% (w/v) osmium tetroxide in phosphate buffer, pH 7.4.

C. Embedding medium (Hayat, 1970)

Taab araldite was made of the following recipe;

CY212	24.5 g
DDSA	24.5 g
DBP	0.375 g
BDMA	0.875 g

The resin was mixed and kept for 1 - 2 days at 4°C. Chemicals were purchased from Taab Laboratories (Emmer Green, Reading)

or from Agar Aid Ltd. (Stanstead, Essex).

DDSA - Dodecenyl succinic anhydride

BDMA - Benzyl dimethylamine

DBP - Dibutyl phthalate.

D. Stains (Hayat, 1970; Glauert, 1977)

D.1 Uranyl acetate (Alcoholic); a saturated solution (>8% (w/v)) of uranyl acetate (B.D.H. Chemicals Ltd. Poole, Dorset, England) in 50 (v/v) ethyl alcohol and filtered then stored in a darkened bottle.

D.2 Reynold's lead citrate; two solutions prepared separately of 0.66 g lead nitrate and 0.88 g trisodium citrate were mixed and shaken vigorously; 3.0 cm^3 of IN NaOH was added and the mixture diluted to 25 cm^3 (pH12 ± 0.1). All chemicals were from B.D.H. Chemicals Ltd, Poole.

Appendix V.2 Biochemical assay of chitin in *C. elegans* egg shell

Control treatment										
Replicate	1	2	3	4	5	6	7	8	9	10
Number of eggs	470	1674	714	621	1083	397	748	743	1805	1561
Glucosamine in sample (nmole)	7.3	18.94	4.76	3.35	9.14	13.8	12.0	10.82	6.09	13.53
Glucosamine 1000 eggs (nmole)	15.53	11.31	6.66	5.39	8.44	34.77	16.04	14.56	3.37	8.67
Diflubenzuron (10 µg cm^{-3}) treatment										
Replicate	1	2	3	4	5	6	7	8	9	10
Number of eggs	453	460	767	244	380	670	470	694	582	467
Glucosamine in sample (nmole)	9.0	1.86	1.52	2.03	4.74	6.17	7.85	7.71	7.2	8.8
Glucosamine 1000 eggs (nmole)	19.87	4.04	1.98	8.32	12.47	9.2	16.7	11.11	12.37	18.84

Appendix V.3. ^{14}C-diflubenzuron study

(A) Incorporation of ^{14}C-diflubenzuron into *E. coli* (OP50)

Weight (mg)	CPM	Background	% Efficiency	dPM	dPM/mg
1) 3.71	2081	122	87.9	2229	606
2) 3.40	4380	127	91.2	4663	1371
3) 2.30	2895	136	90.3	3055	1338
4) 6.66	4707	131	90.8	5040	757
5) 2.64	1778	126	91.6	1803	683

(B) Incorporation of ^{14}C diflubenzuron into *C. elegans* hermaphrodites

No. of nematodes	CPM	Background	% Efficiency	dPM	dPM per individual
1) 22	664	129	91.7	583	27
2) 37	724	158	90.3	627	17
3) 45	678	136	90.3	600	13
4) 35	396	134	90.5	290	8
5) 45	553	120	91.4	474	11

$$dPM = CPM - Background \times \frac{100}{\% \text{ Efficiency}}$$

Appendix V.3 (Continued)

(C) Incorporation of ^{14}C-diflubenzuron into eggs laid by treated *C. elegans* ♀ (48h)

(1) Eggs laid on ^{14}C-diflubenzuron-agar

Number of eggs	CPM	Background	% Efficiency	dPM	dPM/egg
1) 350	324	130	88.7	219	0.62
2) 643	338	129	90.6	231	0.36
3) 895	472	126	90.5	382	0.43

(2) Eggs laid on plain agar

Number of eggs	CPM	Background	% Efficiency	dPM	dPM/egg
1) 241	165	132	90.3	37	0.15
2) 350	171	151	90.0	22	0.06
3) 385	181	121	90.7	66	0.77

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