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Effects of Several Larvicidal Compounds on Chitin Biosynthesis by Isolated Larval Integuments of the Sheep Blowfly *Lucilia cuprina*

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Abstract

Isolated whole integuments from *L. cuprina* larvae rapidly incorporate radioactivity from both *N*-acetyl[1-^{14}C]glucosamine and [1-^{14}C]glucosamine into alkali-insoluble material, a reaction which does not require preincubation of the tissue with β-ecdysone. The labelled product was degraded to *N*-acetylglucosamine during digestion with chitinase, establishing that it consists mainly of chitin. Incorporation was inhibited by polyoxin-D (I_{50}, 6×10^{-7} M) and diflubenzuron (I_{50}, 7×10^{-7}M) but was not inhibited to any marked extent by isoprothiolane, Vetrazin or α-methyl-DOPA. The effectiveness of diflubenzuron as an inhibitor of chitin synthesis in this system (I_{50}, 7×10^{-7} M) correlates well with its potency as a larvicide (LD_{50}, $2{\cdot}1\times10^{-6}$ M), providing additional support for the proposal that this compound kills larvae by interfering with chitin deposition in the cuticle. Polyoxin-D was much more effective as an inhibitor of chitin synthesis (I_{50}, 6×10^{-7} M) than as a larvicide (LD_{50}, $2{\cdot}0\times10^{-5}$ M). It was established that the final intermediate of chitin biosynthesis (UDP-*N*-acetylglucosamine) was formed in the isolated integuments in the presence of diflubenzuron and polyoxin-D. It seems likely therefore that both compounds interfere with the final polymerization step of the chitin biosynthesis pathway.

Introduction

Chitin is essentially a homopolymer of *N*-acetylglucosamine (with occasional glucosamine residues) joined by β-1,4-glycosidic linkages. It is an important structural component of arthropod cuticle and of fungal cell walls. In insects it makes up 20–50% of the procuticle and is deposited as microfibrils originating from the tips of the microvilli of the epidermal cells (Andersen 1979). Pathways for its biosynthesis in arthropods, both from glucose and from *N*-acetylglucosamine (which is formed by chitinase digestion of the chitin in the old cuticle during the moult), via the enzyme *N*-acetyl-D-glucosamine kinase, have been proposed (Gwinn and Stevenson 1973). These pathways are shown in Fig. 1.

The final step of chitin biosynthesis is the polymerization of *N*-acetylglucosamine from the activated substrate UDP-*N*-acetylglucosamine, by the enzyme chitin synthase (EC 2.4.1.16). This enzyme has been well studied in fungi and yeasts (Gooday 1977) but it is only very recently that reliable assay procedures have been reported for it from insect tissues (Cohen and Casida 1980*a*; Mayer *et al.* 1980). It would seem that this final step is a complex one, perhaps involving a glycolipid intermediate (Quesada Allué *et al.* 1976) to allow substrate access to the membrane-bound enzyme (Marks and Sowa 1976). The great potential of chitin synthesis inhibitors as specific fungicides (Misato *et al.* 1979) and insecticides (Verloop and Ferrell 1977) has indicated the need for a better understanding of chitin biosynthesis.

Insect organ culture techniques have provided a useful means for studying aspects of development, physiology and biochemistry *in vitro* (review by Marks 1980). A number of systems have been developed to examine cuticle deposition specifically and the effects of hormones and metabolic inhibitors on this process (Marks and Sowa 1976). The nature and amount of cuticle produced *in vitro* depends on the species of the donor, the stage of development and the specific tissue explanted (Marks and Sowa 1976). The induction of cuticle deposition *in vitro* is also dependent on the presence of and time of exposure to the moulting hormone (β-ecdysone) supplied either intrinsically or extrinsically. Hormone-stimulated epidermal tissue cultures from the rice stem borer (*Chilo suppressalis*) have been used for examining the effects of various chitin synthesis inhibitors on cuticle deposition (Misato *et al.* 1979). The rate of formation of cultured, chitin-containing peritrophic membranes of the blowfly *Calliphora erythrocephala* has also been used to examine the effects of potential chitin synthesis inhibitors (Becker 1978, 1980).

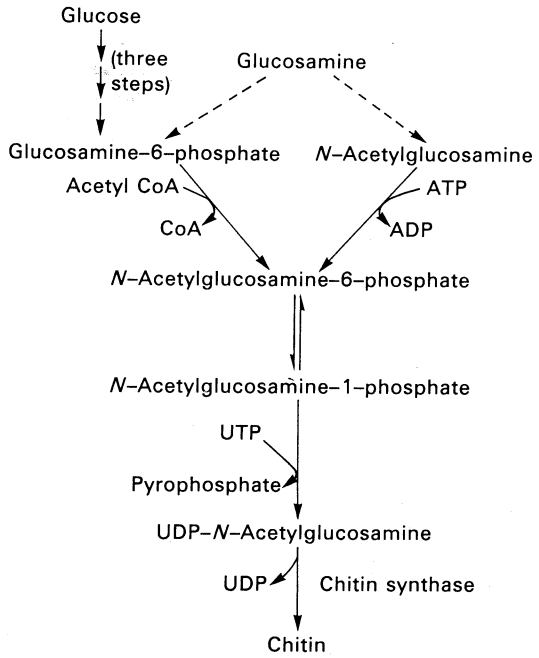


Fig. 1. Postulated pathways for chitin biosynthesis in arthropods (from Gwinn and Stevenson 1973). The broken arrows indicate two possible entry points for glucosamine.

Several biochemical studies of carbohydrate metabolism and chitin biosynthesis have been carried out using cultured insect tissues. Fristorm (1968) obtained ^{14}C-labelling of intermediates of hexosamine metabolism (but not of chitin) in cultured imaginal discs from *Drosophila melanogaster*. Oberlander and Leach (1975) also studied the uptake and incorporation of [^{3}H]glucosamine into cuticle under the influence of α- and β-ecdysone in this culture system. Sowa and Marks (1975) used cultured regenerating cockroach legs to study β-ecdysone-stimulated chitin synthesis from *N*-[1-^{14}C]acetylglucosamine. This incorporation was prevented by chitin synthesis inhibitors. More recently, several other convenient insect culture systems

have been described. Vardanis (1976, 1979) has examined aspects of chitin biosynthesis using cultured adult integument tissue from the grasshopper (*Melanoplus sanguinipes*). Hajjar and Casida (1978), using cultured abdomens from adult milkweed bugs (*Oncopeltus fasciatus*), have measured the relative potencies of a range of benzoylphenylurea insecticides on the rate of *N*-[1-^{14}C]acetylglucosamine incorporation into chitin. In both these systems chitin biosynthesis occurs without pretreatment of the tissues with exogenous β-ecdysone.

In this paper we describe the development and characterization of a relatively simple, reliable, and exogenous hormone-independent system for assaying chitin formation using larval integuments of *Lucilia cuprina*. The potencies of several compounds as inhibitors of chitin biosynthesis have been examined using this system. The toxicities of these compounds against larvae of *L. cuprina* have also been determined, so that comparisons can be made between their potencies as inhibitors of chitin synthesis and their larvicidal activities.

Materials and Methods

Chemicals

Diflubenzuron [1-(4-chlorophenyl)-3′-(2,6-difluorobenzoyl)urea; PH 60–40] was a gift from Philips-Duphar, Amsterdam, the Netherlands; polyoxin-D was a gift from Kaken Chemical Co., Tokyo, Japan; isoprothiolane (diisopropyl 1,3-dithiolan-2-ylidenemalonate) was a gift from Nihon Nohyaky Co. Ltd, Tokyo, Japan; Vetrazin (2-cyclopropylamino-4,5-diamino-*S*-triazine) was a gift from Ciba-Geigy, Australia; commercial chitinase was obtained from Calbiochem, trypsin-TPCK from Worthington Biochemical Corp., New Jersey, U.S.A.; and pepsin from Merck Co., Germany. *N*-Acetyl-D-[1-^{14}C]glucosamine (57·9 mCi/mmol) and D-[1-^{14}C]glucosamine hydrochloride (61 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England.

The sugars used as markers in chromatography were obtained from the Sigma Chemical Co. except for *N*-acetylglucosamine 6-phosphate, which was prepared by a microscale adaption of the method of Distler *et al.* (1958). All other chemicals were of laboratory grade.

The tissue culture medium used routinely was that of Mitsuhashi and Maramorosch and contained 20% (w/v) foetal calf serum, penicillin and streptomycin; it was prepared as described by Buckley (1969).

The abbreviations used in this paper are: NAG, *N*-acetylglucosamine; Gm, glucosamine; NAG-1-P, NAG-6-P, *N*-acetylglucosamine-1- or 6-phosphate; Gm-6-P, glucosamine-6-phosphate; UDPAG, uridine-5′-diphospho-*N*-acetylglucosamine; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

Larval Cultures and Feeding Trials

L. cuprina larvae were reared on pieces of sheep's liver in 150-ml glass jars as described by Turnbull and Howells (1980). Feeding trials were normally conducted as described by Turnbull and Howells (1980) but smaller-scale trials with second-instar larvae were undertaken with polyoxin-D and isoprothiolane (because of their limited availability). In these trials samples of 2–4 ml of blended liver containing the required concentration of the chemical were used with 15–20 early second-instar larvae (about 18 h after hatching). Percentage mortalities were calculated by counting the number of larvae which formed normal pupae.

Chitin Synthesis by Larval Integuments

Standard assay procedure

Early third-instar larvae (usually 5–15 mg liveweight) were selected from small-scale cultures, surface-sterilized in 70% (v/v) ethanol, and their integuments obtained by cutting off the anterior end and gently squeezing out the haemolymph and viscera. When such integuments were examined by light microscopy it was found that the epidermal cell layer and cuticle were essentially intact. The integuments were bisected longitudinally and placed immediately into a drop of oxygenated

culture medium. Initially incubations were performed in 1·5 ml Eppendorf tubes but for later experiments Spinco L-50 centrifuge tubes, cut down to a height of about 1 cm and sealed with a rubber stopper, were used because they gave more consistent results (probably due to the greater surface area). The incubation mixtures consisted of 70 μl medium (previously oxygenated), 5 μl 1 mM ^{14}C-labelled NAG (0·25 μCi) or 5 μl 4 mM ^{14}C-labelled Gm (1·0 μCi) and 5 μl of any test compounds. Incubations were started by the addition of 16 pieces of integument to each tube which was then flushed with oxygen. Incubations were at 30°C normally for 2 h. Subsequently the integuments were washed five times with water, and then deproteinized in 1 ml 10% (w/v) NaOH at 70°C for 16 h. The deproteinized husks were washed five times with water, once with 95% (v/v) ethanol and then homogenized in 1 ml ethanol–0·3 M ammonium acetate (2:1 v/v). The insoluble material was collected on a glass-fibre disc (Whatman GF/E) which was washed with about 100 ml water, dried, and the radioactivity determined by liquid scintillation counting in a Beckman-330 counter.

Chitinase digestion of the product

In order to establish the nature of the insoluble radioactive product, integuments from standard incubations were subjected to protease and chitinase digestions. The method was an adaptation of that described by Porter and Jaworski (1965). Integuments from each of six standard incubations with ^{14}C-labelled NAG were washed five times with water and homogenized in 1 ml water. The insoluble material was obtained by centrifugation (Eppendorf centrifuge) and then washed several times with water. The pellets were resuspended in 0·2 ml 0·01 M HCl containing 1 mg pepsin and then incubated for 24 h at 30°C. After digestion the insoluble material was again pelleted, washed several times with water and resuspended in 0·2 ml 0·06 M TES buffer (pH 7·3). Trypsin (1 mg) was added and the tubes were incubated for 36 h at 30°C. The insoluble material from all six incubations was combined and homogenized in 0·8 ml 0·05 M sodium acetate buffer (pH 4·7). Two 0·3-ml samples of the homogenate were taken and to one was added 1 mg chitinase while the other served as a no-chitinase control. They were incubated at 37°C for 48 h. The insoluble material was collected by centrifugation and the radioactivity in the pellets and in the supernatants of the two samples determined. A 0·25-ml sample of each supernatant was deproteinized with 50 μl 70% (w/v) perchloric acid, neutralized with 10 M KOH and chromatographed as described below in order to determine the nature of the solubilized radioactivity. In addition samples of the insoluble material were taken after the initial incubation and following the protease digestions and treated with 10% (w/v) NaOH in order to determine the levels of alkali-insoluble radioactivity present at each stage of the procedure. In all cases radioactivity was determined by liquid scintillation counting. Samples of insoluble material for counting were collected on glass-fibre discs as described in the previous section.

Chromatographic separation of intermediates of chitin synthesis

The formation of intermediates of the pathways of chitin synthesis during incubations with ^{14}C-labelled NAG and ^{14}C-labelled GM was monitored using paper chromatography. The procedure was adapted from that of Post *et al.* (1974). Integuments from one, two or three standard incubations were combined, washed twice with 0·2 ml culture medium and homogenized in 0·4 ml 0·5 M perchloric acid. The precipitated material was removed by centrifugation. The supernatant was extracted with 0·5 ml ether, neutralized with 10 M KOH at 0°C and salt removed by centrifugation. The supernatant was dried under vacuum, redissolved in 50 μl distilled water, spotted on Whatman No. 3 paper which was developed descending for 16 h at room temperature in GW-3 solvent. This solvent system (Wood 1968) consists of butan-1-ol–propan-1-ol–acetone–80% formic acid–30% trichloroacetic acid (8:4:5:5:3 v/v). The chromatograms were cut into 3-cm strips and then into 1-cm pieces and the radioactivity of each piece determined by liquid scintillation counting. The positions of the unlabelled markers on the chromatograms were determined by staining with aniline–diphenylamine reagent (Smith 1960).

Results

Characteristics of Chitin Synthesis by Larval Integuments

The isolated integuments from early third-instar larvae of *L. cuprina* incorporated radioactivity from ^{14}C-labelled NAG and ^{14}C-labelled Gm into acid- and ethanol-

precipitable, alkali-insoluble material. The levels of incorporation obtained over an incubation period of 2 h were $2{\cdot}2 \pm 1{\cdot}0\%$ of the input radioactivity for ^{14}C-labelled NAG and $2{\cdot}0 \pm 1{\cdot}6\%$ for ^{14}C-labelled Gm, which correspond to 10000–30000 cpm in the standard incubation. Some variation in levels of incorporation occurred between batches of larvae but comparable incorporations were obtained when larvae of the same batch were used. The incorporation was time-dependent (Fig. 2), being linear for at least 90 min. In other experiments (not shown) incorporation continued for at least 180 min, although at a diminishing rate over the final 60 min. In order

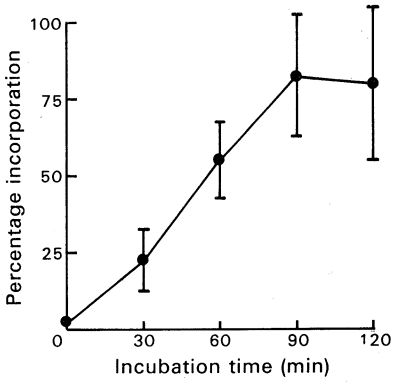


Fig. 2. Incorporation of ^{14}C-labelled NAG into chitin by isolated larval integuments. The data represent the percentages of maximum incorporations for each experiment and 100% incorporations range from 2–5% of input radioactivity. Each point is the mean of five separate determinations and the vertical bars show ± 1 s.d.

to maximize the levels of incorporation we assumed that the time course was linear for 120 min and in all subsequent experiments carried out incubations for this time period. If the rate of incorporation was not constant for the entire 120-min period in some of the experiments in which inhibitors were used, this would probably lead to slight underestimations of levels of inhibition. An almost linear relationship was found between the level of incorporation and the number of pieces of integument per incubation (at least up to 24).

Other properties of this system can be determined from the effects of the treatments listed in Table 1*a*. Incorporations are expressed as percentage of the mean of at least two control incubations with the same batch of larvae, thus allowing for variations in the level of incorporation obtained in different experiments. Incorporation is dependent on the viability and normal respiratory activity of the epidermal cells since replacement of the oxygen atmosphere with nitrogen, or the addition of an inhibitor of oxidative phosphorylation (KCN), caused almost complete inhibition with both substrates. Using ^{14}C-labelled NAG as substrate, the addition of unlabelled NAG substantially reduced incorporation but the addition of similar levels of Gm or Gm-6-P had a much smaller effect (Table 1*a*). Thus, if *N*-acetylations of Gm to NAG or of Gm-6-P to NAG-6-P do occur within the epidermal cells they must occur relatively slowly. The addition of UDPAG had only a small effect in reducing incorporation. Because UDPAG is the final intermediate in the pathway from NAG to chitin, this result suggests that it does not easily penetrate the cell membrane to enter epidermal cells. A similar suggestion was made by Sowa and Marks (1975).

When ^{14}C-labelled Gm was used as substrate the addition of unlabelled Gm reduced incorporation by almost 80% and the additions of NAG or Gm-6-P were both effective in reducing incorporation by more than 50%. This suggests that

alternative pathways probably exist for the incorporation of Gm into chitin: both via *N*-acetylation to NAG and via phosphorylation to Gm-6-P.

Chitinase Digestions

Chitinase digestions of the protease-treated, insoluble labelled material produced in standard incubation with ^{14}C-labelled NAG were carried out in order to establish that the label had been incorporated into chitin. In one experiment labelled alkali-insoluble material (86094 cpm) was used. After the pepsin and trypsin digestions, 70% of this radioactivity remained alkali-insoluble. During the subsequent incubation

Table 1. Effects of (a) metabolic inhibitors and intermediates of chitin synthesis, and (b) potential inhibitors of chitin synthesis on incorporation by isolated larval integuments

Values given are means (±2 s.d. where appropriate). The number of duplicate determinations are given in brackets

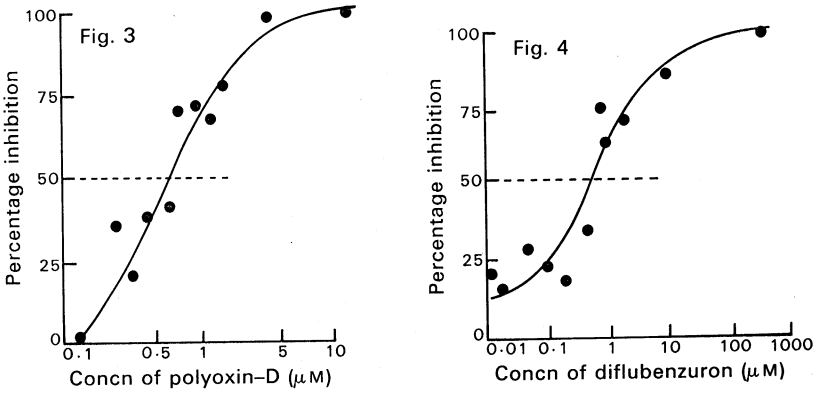
Treatment	Incorporation (as % of control) ^{14}C-labelled NAG as substrate	^{14}C-labelled Gm as substrate
(a) Metabolic inhibitors and intermediates		
Control (with O_2)	100	100
N_2 atmosphere	5±3 (7)	7 (1)
KCN (12·5 mM)	3±1 (3)	6 (1)
NAG (12·5 mM)	13±6 (3)	42 (1)
Gm (12·5 mM)	68 (2)	23 (1)
Gm-6-P (12·5 mM)	91 (2)	43 (1)
UDPAG (1·3 mM)	77 (2)	—
(b) Potential inhibitors		
Diflubenzuron (10 μM)	17±10 (6)	11 (2)
Polyoxin-D (0·13 mM)	4±2 (5)	2 (1)
Isoprothiolane (62 μg/l)	105±17 (4)	95 (2)
Vetrazin (0·39 mM)	107±19 (4)	—

with chitinase 95% of the labelled material was solubilized. In the no-chitinase control incubation only 7% was solubilized. The soluble labelled material obtained after chitinase digestion chromatographed as a single peak coincident with the NAG marker. Results similar to these were obtained in several other chitinase digestion experiments, suggesting strongly that chitin was the major insoluble radioactive product formed by the integuments during incubation with ^{14}C-labelled NAG.

Inhibitor Studies

A number of compounds which have been found either to inhibit chitin synthesis in other systems or to have larvicidal activity against *L. cuprina* (Turnbull and Howells 1980) were tested as inhibitors of chitin synthesis (Table 1*b*). Polyoxin-D, which was established by Hori *et al.* (1974) to be a competitive inhibitor of the fungal chitin synthase enzyme and is an analogue of UDPAG, virtually abolished incorporation when present at 0·13 mM. Similarly, diflubenzuron, which has potent larvicidal activity against *L. cuprina* and which inhibits chitin deposition in other insects

(Verloop and Ferrell 1977), inhibited incorporation by more than 80% when present at only 10 μM. However, isoprothiolane, which is an inhibitor of fungal chitin synthesis via effects on sugar uptake (Misato *et al.* 1979), when present at 62 μg/l (a concentration effective in fungi), had little inhibitory effect. Similarly, α-methyl DOPA and Vetrazin, at levels which kill third-instar *L. cuprina* larvae, had little or no inhibitory effect on chitin synthesis. Since foetal calf serum was present in all incubations, it is possible that the failure of these latter three compounds to cause inhibition might be attributable to binding interactions with the serum proteins.



Figs 3 and 4. Inhibition of chitin synthesis in isolated larval integuments by polyoxin-D (Fig. 3) and by diflubenzuron (Fig. 4). Points (means of duplicates) are expressed as percentages of control incorporations obtained using the same batch of larvae. Inhibitor concentrations are on a logarithmic scale.

In order that a direct comparison of the potencies of polyoxin-D and diflubenzuron as inhibitors of chitin synthesis in this system could be made, the concentrations of each giving 50% inhibition (I_{50}) were determined. The percentage inhibitions obtained with various concentrations of polyoxin-D (Fig. 3) appear to fit a sigmoidal log dose-response curve although the scatter of points is considerable. An apparent I_{50} of 6×10^{-7}M was obtained from the curve of best fit. The data for diflubenzuron (Fig. 4) shows less scatter with an apparent I_{50} of 7×10^{-7}M.

Effects of Inhibitors on Levels of Chitin Intermediates

The effects of polyoxin-D and diflubenzuron on the levels of intermediates of chitin synthesis in the isolated *L. cuprina* larval integuments were studied. Paper chromatography was used to separate the labelled components present in the deproteinized tissue extracts of the cultured integuments. Using ^{14}C-labelled NAG as substrate, in the absence of inhibitors and with GW-3 as the chromatogram solvent system, the profile of radioactivity shown in Fig. 5*a* was obtained. There are three distinct peaks of radioactivity which co-chromatograph with the markers UDPAG (peak 1), NAG-1-P and/or NAG-6-P (peak 2), and NAG (the large peak of residual substrate). In some experiments a shoulder on peak 2 was seen, indicating partial separation of NAG-1-P and NAG-6-P. Chromatography in a second solvent system consisting of pyridine–propan-1-ol–acetic acid–water (8:8:3:4 v/v) described by Post *et al.* (1974), also gave peaks of radioactivity coincident with UDPAG, NAG-6-P,

NAG-1-P and NAG. In addition there was a peak of medium size which co-chromatographed with Gm, suggesting that some deacetylation of NAG occurs in the epidermal cells. This is not detected using the GW-3 solvent system since NAG and Gm do not significantly separate in this system. The profiles obtained after incubations with

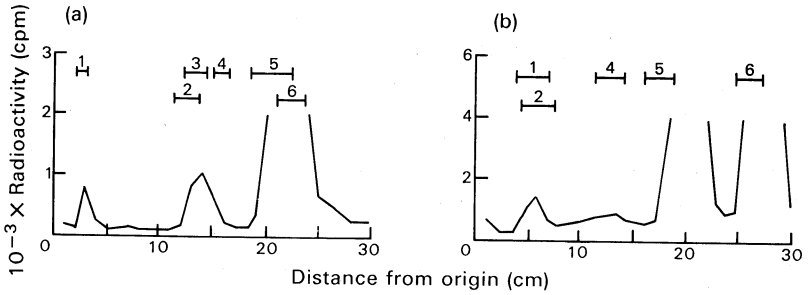


Fig. 5. Chromatogram profiles showing the separation of ^{14}C-labelled intermediates of chitin synthesis. Extracts were prepared from larval integuments following incubation with ^{14}C-labelled substrates. (*a*) ^{14}C-labelled NAG as substrate; chromatogram developed with the GW-3 solvent system. (*b*) ^{14}C-labelled Gm as substrate; chromatogram developed with pyridine–propan-1-ol–acetic acid–water. Decending paper chromatography was used. After development with the solvent, chromatograms were cut into 1 cm pieces and the level of radioactivity in each piece was determined. Positions occupied by marker compounds are shown above the profile: *1*, UDPAG; *2*, Gm-6-P; *3*, NAG-1-P; *4*, NAG-6-P; *5*, NAG; *6*, Gm.

^{14}C-labelled Gm, using GW-3 as the solvent, also showed three distinct peaks and were very similar to that presented in Fig. 5*a*. In the second solvent system the profile shown in Fig. 5*b* was obtained. Peaks of radioactivity corresponding to the markers UDPAG, NAG-6-P, Gm and NAG can be seen. It is clear from these results that

Table 2. Effects of inhibitors on levels of intermediates of chitin synthesis in isolated larval integuments

Values given are means (±1 s.d., where appropriate) of the percentage of ^{14}C-labelled NAG added per incubation incorporated into each component. Data for peaks 1 and 2 were calculated from chromatogram profiles such as that shown in Fig. 5*a*

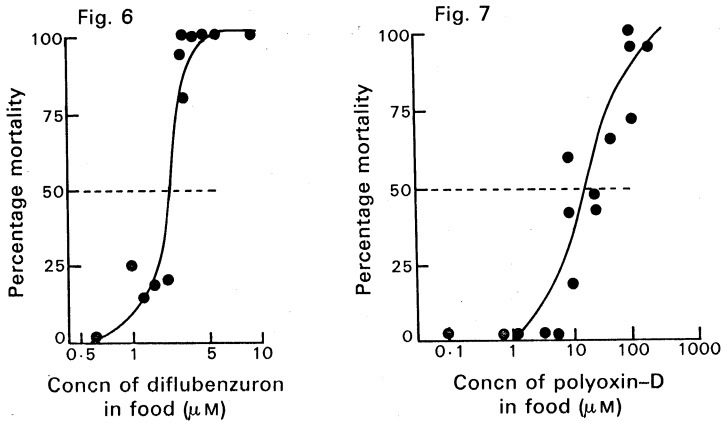
Component	Incorporation (as % of added ^{14}C-labelled NAG)		
	Control[A]	Diflubenzuron (10–25 μM)[A]	Polyoxin-D (0·13 mM)[B]
Chitin	3·0±1·9	0·4±0·3	0·3
Peak 1 (UDPAG)	0·2±0·04	0·2±0·1	0·2
Peak 2 (NAG-1 or 6-P)	0·5±0·3	0·5±0·3	0·3

[A] Means from five determinations.
[B] Means from two determinations.

Gm is converted to NAG, probably by enzymic *N*-acetylation, in the *L. cuprina* epidermal cells. It is possible that Gm may also be phosphorylated to Gm-6-P followed by *N*-acetylation to NAG-6-P. However, this possibility cannot be assessed from these experiments since Gm-6-P is masked by other labelled intermediates in the solvent systems used.

The profiles which were obtained from integuments incubated in the presence of diflubenzuron (10–25 μM) or polyoxin-D (0·13 M), with either ^{14}C-labelled NAG or ^{14}C-labelled Gm as substrate, were exactly the same as those described above. There was no marked increase in the levels of radioactivity in either peak 1 or peak 2 even though incorporation into chitin was reduced by about 90%. Statistical analysis of the data from a number of experiments in which ^{14}C-labelled NAG was used as substrate in the presence of diflubenzuron (Table 2) shows that the percentage of the added radioactivity which appeared in each of the two intermediate peaks are independent of the addition of this inhibitor. Because the final intermediate of the chitin synthesis pathway (UDPAG) is clearly formed in the presence of diflubenzuron, it is unlikely that this inhibitor exerts it effects on chitin synthesis via inhibition of an intermediate step in the pathway. Rather it seems highly likely that diflubenzuron is an inhibitor of the final polymerization step in the *L. cuprina* epidermal cells.

The addition of polyoxin-D, which is known to be an inhibitor of the chitin synthase from fungi and is therefore likely to be an inhibitor of the *L. cuprina* enzyme, failed to cause a build-up in the level of radioactivity in UDPAG (Table 2). This suggests that the chitin synthesis pathway from NAG to UDPAG (Fig. 1) is under metabolic control, perhaps involving feed-back inhibition of NAG-kinase by UDPAG.



Figs 6 and 7. Log dosage–percentage mortality curve for second-instar larvae fed with diflubenzuron (Fig. 6) and with polyoxin-D (Fig. 7).

Larvicidal Activities

An advantage of the chitin synthesis system described here is that it allows direct comparisons to be made between the capacity of a compound to inhibit chitin synthesis and its toxicity against larvae at the same developmental stage. Diflubenzuron is a potent larvicide against *L. cuprina* in feeding trials and Turnbull *et al.* (1980) have presented evidence suggesting that death results from an abnormally formed chitin-containing procuticle in treated larvae. The LD_{50} against first-instar *L. cuprina* larvae was found to be $2{\cdot}1 \times 10^{-6}$M (Turnbull and Howells 1980), with death occurring during the next ecdysis after exposure to this compound. Since the integuments used in the chitin synthesis assay system were from early third-instar larvae, toxicity trials were conducted with second-instar larvae (death therefore resulted from an

abnormally formed third-instar cuticle). The data obtained for diflubenzuron is presented as a log dosage–percentage mortality curve (Fig. 6) from which an LD_{50} value of $2{\cdot}1 \times 10^{-6}$M was obtained. This correlates well with the apparent I_{50} value (7×10^{-7}M) for inhibition of chitin synthesis, providing further evidence that the inhibition of chitin synthesis caused by diflubenzuron is the primary cause of larval death. Polyoxin-D was also shown to be larvicidal in feeding trials (Fig. 7). The LD_{50} ($2{\cdot}0 \times 10^{-5}$M) is substantially higher than the apparent I_{50} (6×10^{-7}M) for inhibition of chitin synthesis, suggesting that lack of penetration through the gut or detoxification within the tissues may limit the effectiveness of this compound *in vivo*. As with diflubenzuron, death of polyoxin-D treated larvae occurs at the next ecdysis following exposure.

The larvicidal activities of two compounds which were ineffective as inhibitors of chitin synthesis were also tested. Isoprothiolane (up to 1 mg/l) was not toxic in feeding trials with second-instar *L. cuprina* larvae. However, Vetrazin was highly effective against larvae at this developmental stage and an LD_{50} value of $1{\cdot}3 \times 10^{-6}$M was obtained.

Discussion

The larval integument system isolated from *L. cuprina* for testing the effects of potential inhibitors and larvicides on chitin biosynthesis has several advantageous features. It requires minimal time for preparation and incorporation occurs spontaneously without a requirement for treatment with exogenous hormones. In addition relatively high rates of incorporation are obtained, obviously reflecting the rapid rates of growth and cuticle deposition which occur in early third-instar larvae (Turnbull and Howells 1980).

There can be little doubt that the insoluble product of this system is [^{14}C]-chitin since it is insoluble in warm alkali, its formation is inhibited by polyoxin-D, and it is degraded to ^{14}C-labelled NAG by digestion with chitinase. It is interesting that this [^{14}C]chitin is almost totally hydrolysed during chitinase digestion. Quesada Allué *et al.* (1976) found that only 30% of the ^{14}C-labelled product formed *in vivo*, following injection of the insect *Triatoma infestans* with ^{14}C-labelled NAG, was chitinase-sensitive. It is possible that the chitin produced in the isolated *L. cuprina* integuments does not become integrated into normal complex chitin/protein cuticular structures, and it is therefore more accessible to the chitinase.

Since the rate of incorporation of ^{14}C-labelled NAG into chitin is substantial in these isolated *L. cuprina* integuments, it seems likely that this compound is an important substrate for chitin synthesis in the intact insect. This would be related to the degradation and re-utilization, during the moult, of chitin from the old cuticle. However, in addition to the re-utilization of old chitin, substantial amounts of totally new chitin must be produced to allow for larval growth. Presumably the pathway for the *de novo* synthesis of this chitin would proceed from glucose (Fig. 1). No definitive conclusions can be made from these studies concerning the route by which Gm enters the chitin biosynthetic pathway. Since both cold NAG and Gm-6-P reduce ^{14}C-labelled Gm incorporation in the isolated integuments, it seems likely that there are alternative routes involving either initial *N*-acetylation or initial phosphorylation.

Diflubenzuron and polyoxin-D were found to inhibit chitin synthesis strongly in isolated *L. cuprina* integuments. The apparent I_{50} for diflubenzuron (7×10^{-7}M) is similar to the value of $5{\cdot}5 \times 10^{-7}$M obtained by Hajjar and Casida (1978) using abdomens of the milkweed bug (*Oncopeltus fasciatus*) but is somewhat higher than the level of $6{\cdot}9 \times 10^{-8}$M calculated by Sowa and Marks (1975) to be the effective concentration of diflubenzuron within the cells of regenerating cockroach leg. Polyoxin-D (apparent I_{50}, 6×10^{-7}M) was considerably more potent in our system than in that used by Hajjar and Casida (1978) (I_{50}, $1{\cdot}2 \times 10^{-5}$M) but an I_{50} value of $7{\cdot}5 \times 10^{-7}$M, similar to ours, was obtained by Sowa and Marks (1975).

The data presented in this paper support the evidence gained in previous *in vivo* studies by Post *et al.* (1974) and Deul *et al.* (1978) that benzoylphenylurea insecticides act by inhibiting some aspect of the final polymerization step of the chitin biosynthetic pathway (see Fig. 1). Whether they act by specifically inhibiting the final enzyme (chitin synthase) is still a matter for speculation. In recent studies with cell-free insect extracts which exhibit chitin synthase activity (Cohen and Casida 1980*b*; Mayer *et al.* 1980) diflubenzuron was found to have no inhibitory effect. Leighton *et al.* (1981) have proposed that diflubenzuron acts indirectly on chitin synthase by inhibiting a proteolytic enzyme required for its activation from the zymogen form.

The similarity between the potency of diflubenzuron as a larvicide (LD_{50}, $2{\cdot}1 \times 10^{-6}$M) and its capacity to inhibit chitin synthesis in isolated *L. cuprina* integuments (apparent I_{50}, 7×10^{-7}M) strongly suggests that its mode of larvicidal action in *L. cuprina* is associated with its ability to inhibit chitin synthesis. This is supported by the observation that the chitin-containing procuticle has an abnormal appearance in electron micrographs of sections of cuticle prepared from diflubenzuron-treated *L. cuprina* larvae (Turnbull *et al.* 1980).

Our finding that polyoxin-D is highly toxic when fed to young *L. cuprina* larvae (LD_{50}, $2{\cdot}0 \times 10^{-5}$M) is the first report that we are aware of concerning the larvicidal action of this compound in a feeding trial. Gijswijt *et al.* (1979) demonstrated that polyoxin-D was toxic when injected into fifth-instar larvae of *Pieris brassicae*, but it was not effective when fed to those larvae (Verloop and Ferrell 1977). However, polyoxin-D is much less potent as a larvicide against *L. cuprina* than is diflubenzuron, even though both compounds are similarly effective as inhibitors of chitin synthesis in isolated larval integuments. This suggests that polyoxin-D is either absorbed less readily in the gut or detoxified to a greater extent within the intact larva compared with diflubenzuron. Poor absorption or ready detoxification probably explain why polyoxin-D has proved to be ineffective as a larvicide in feeding trials with other species of insect.

Vetrazin, a potent larvicide against *L. cuprina*, which is currently marketed in Australia for the control of blowfly-strike in sheep, was not effective as an inhibitor of chitin synthesis in isolated *L. cuprina* integuments. We have been unable to find any evidence that Vetrazin has deleterious effects on the larval moulting process. Its mode of action as a larvicide is still unknown.

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