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Integumental Chitin Synthase Activity in Cell-free Extracts of Larvae of the Australian Sheep Blowfly, *Lucilia cuprina*, and Two Other Species of Diptera

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Abstract

Chitin synthase activity has been demonstrated in crude homogenates of larval integuments from *L. cuprina* and in similar preparations from *Musca domestica* and *Calliphora erythrocephala*. This is the first report of an insect integumental chitin synthase. This activity brings about the incorporation of radioactivity from UDP-*N*-acetyl-[^{14}C]glucosamine into an ethanol- and alkali-insoluble form. A major part of this labelled product has been characterized as chitin by its insolubility in alkali, resistance to degradation by proteases and its susceptibility to digestion by chitinase and HCl. Most of the radioactivity solubilized during digestion by chitinase co-migrates with *N*-acetylglucosamine, glucosamine and chitobiose during paper chromatography. Some radioactivity also becomes incorporated into non-chitin products in this system. There is substantial evidence that incorporation is not brought about by whole epidermal cells or by microbial contamination in the homogenates. The extent of incorporation obtained with the homogenates is limited by the presence of degradative enzymes which rapidly break down the substrate (UDP-*N*-acetylglucosamine). The incorporation was partially inhibited (50–70%) by both polyoxin-D (apparent K_i 0·04 μM) and diflubenzuron (apparent K_i 5–8 μM). This is the first report of a cell-free chitin-synthesizing system derived from insect tissue which is sensitive to inhibition by diflubenzuron.

Introduction

Chitin is essentially a homopolymer of *N*-acetylglucosamine (with occasional glucosamine residues) joined by β-1,4-glycosidic linkages and is an important structural component of arthropod cuticle and of fungal cell walls. In insects it makes up 20–50% of the procuticle (Andersen 1979). In arthropods chitin can be synthesized *de novo* from glucose (Candy and Kilby 1962) and also from *N*-acetylglucosamine, formed by chitinase digestion of the chitin in the old cuticle during each moult (Gwinn and Stevenson 1973).

The final step of the chitin biosynthesis pathway involves the polymerization of *N*-acetylglucosamine residues from the activated substrate UDP-*N*-acetylglucosamine, and is catalysed by the enzyme chitin synthase (UDP-2-acetoamido-2-deoxy-D-glucose: chitin 4β-acetoamidodeoxy-D-glucosyltransferase, EC 2.4.1.16). This enzyme has been studied in fungi and yeasts (Gooday 1977) and, recently, in cell-free membrane and microsomal preparations of extracts of whole insects (Cohen and Casida 1980*a*; Mayer *et al.* 1980). The tissue source from which these insect activities derive has not been established but Cohen and Casida (1980*a*) suggest that in their preparations from *Tribolium infestans* the enzyme comes from the gut, where it is involved in the synthesis of peritrophic membranes.

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Since chitin is present in fungi and insects but is absent from vertebrates, the synthesis of this polymer is an ideal target for selective pesticide action. One group of fungicides which inhibit chitin deposition are the polyoxins (Misato *et al.* 1979). These compounds are structural analogues of UDP-*N*-acetylglucosamine and have been shown to competitively inhibit fungal chitin synthases (Gooday 1977). The benzoylphenylurea insecticides also inhibit cuticular chitin deposition in a variety of insects (Sowa and Marks 1975; Hajjar and Casida 1978) and appear to act on the final polymerization step of the chitin biosynthetic pathway (Verloop and Ferrell 1977), but it is still unclear whether they directly inhibit chitin synthase. Thus diflubenzuron (one of the benzoylphenylurea insecticides) failed to inhibit chitin synthesis in either of the insect-derived cell-free chitin synthase systems described above (Cohen and Casida 1980*b*; Mayer *et al.* 1980). Similarly, in a microsomal chitin synthase system prepared from extracts of larvae of the brine shrimp (*Artemia salina*), inhibition was obtained only after preincubation of the system with diflubenzuron (Horst 1981). Recently, Leighton *et al.* (1981) proposed that diflubenzuron acts indirectly on chitin synthase, by inhibiting a proteolytic enzyme required for the activation of chitin synthase from a zymogen form.

Since the larvicidal action of diflubenzuron (and the other benzoylphenylureas) results from its inhibitory effects on chitin deposition in the integument (Verloop and Ferrell 1977; Turnbull *et al.* 1980), the development of an *in vitro* assay system for an integumental chitin synthase could be an important advance in the investigation of the mode of action of these compounds. In this paper we describe the characteristics of an integumental chitin synthase activity in crude homogenates prepared from early third-instar larvae of the Australian sheep blowfly *Lucilia cuprina*. Integumental chitin synthase activity was also obtained in larval extracts from the blowfly *Calliphora erythrocephala* and the housefly *Musca domestica*. The inhibitory effects of polyoxin-D and diflubenzuron on these integumental chitin synthases have been examined.

Materials and Methods

Chemicals and Experimental Animals

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. Chitinase was from Calbiochem or Sigma Chemical Co.; trypsin was from Worthington Biochemical Corp. and pepsin was from Merck Co. UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (11·95 MBq/mol) was obtained from the Radiochemical Centre, Amersham, U.K. All other fine chemicals were obtained from Sigma Chemical Co.

L. cuprina larvae were reared on pieces of sheep's liver in 150-ml containers (Turnbull and Howells 1980). *C. erythrocephala* larvae were reared by the same method on prime beef. *M. domestica* larvae were reared on an artificial medium of milk powder as described by Grosscurt and Tipker (1980).

Abbreviations used in this article are: NAG, *N*-acetylglucosamine; NAG-1-P, NAG-6-P, *N*-acetylglucosamine-1-phosphate and 6-phosphate respectively; UDPAG, uridine 5′-diphospho-*N*-acetylglucosamine.

Tissue Extracts

Early third-instar larvae were selected from cultures, their anterior ends were cut off and the viscera and haemolymph were gently squeezed out. The resulting integuments were homogenized at 0–4°C in the tissue culture medium of Mitsuhashi and Maramorosch (Buckley 1969) containing 20% (v/v) foetal calf serum and penicillin and streptomycin at 50 and 80 μg/ml respectively. Usually 90 integuments were homogenized in 0·63 ml medium, with 10 strokes of a teflon–glass homogenizer. For experiments with *C. erythrocephala* and *M. domestica*, a modified homogeniza-

tion medium consisting only of the phosphate buffer, inorganic salts, glucose and antibiotics of the above tissue culture medium was used.

Chitin Synthase Assay

Incubations were carried out in 1·5-ml Eppendorf centrifuge tubes at 30°C, normally for 30 min. The reaction mixture contained 10 μl [^{14}C]UDPAG (9·25 kBq), 5 μl 10 mM UDPAG, 5 μl test compound and 80 μl homogenate. Reactions were terminated with 1 ml ethanol–0·3 M ammonium acetate (2:1 v/v), and the insoluble material was collected by centrifugation. Pellets were resuspended in 1 ml 10% (w/v) NaOH, the tubes sealed and then incubated at 70°C for 16 h. The contents of each tube were spotted onto a glass fibre disc (Whatman GF/E or C) and washed with about 400 ml water using a Millipore filtering apparatus. Discs were dried and the radioactivities measured by liquid scintillation counting. Experiments involving homogenates of *C. erythrocephala* and *M. domestica* were performed in glass centrifuge tubes; all volumes were doubled and the reaction mixtures also contained KCN (20 mM).

Analysis of the Products

Acid digestions

All incubations were carried out in sealed 1·5-ml Eppendorf centrifuge tubes. The ethanol-insoluble material from large-scale (three times normal) incubations were deproteinized by digestion first with trypsin (5 mg/ml, final concentration) for 24 hr and then with pepsin (5 mg/ml, final concentration) for 24 h as described by Turnbull and Howells (1982). The residual ethanol-insoluble material was resuspended in 1 ml concentrated HCl and incubated for 16 h at 80°C. The mixture was then neutralized with NaOH and centrifuged to remove insoluble material. Radioactivity in the supernatant fraction was measured by liquid scintillation counting.

Chitinase digestions

The ethanol-insoluble material from six standard incubations was combined and then digested with trypsin and pepsin as described above. The deproteinized products were resuspended in 0·5 ml of 0·05 M sodium acetate buffer (pH 4·7), 0·5 mg of solid chitinase was added and the mixture was then incubated at 37°C for 48 h. (An additional 0·5 mg of chitinase was added after the first 24 h.) The mixture was then centrifuged and the radioactivities in both the supernatant fraction and in the pellet were measured by liquid scintillation counting. Samples of the supernatant fraction were also analysed by paper chromatography.

Paper Chromatography

Whatman No. 3 and Schleicher and Schüll 2043b papers were used. Samples from standard chitin synthase incubations or from chitinase digestions were deproteinized by the addition of an equal volume of 1 M perchloric acid. Insoluble material was removed by centrifugation and the supernatant fraction neutralized using 10 M KOH. Samples were chilled and the precipitate of potassium perchlorate was removed by centrifugation in the cold. The final supernatant fractions were dried under vacuum, redissolved in 50 μl water and spotted on to a chromatogram. These were run in the descending mode for 16 h (or ascending for 4 h) at room temperature in solvent system 1 (Wood 1968) consisting of butan-1-ol–propan-1-ol–acetone–80% (v/v) formic acid–30% (v/v) trichloroacetic acid (8:4:5:5:3 v/v). Some chromatograms were also run in the ascending mode for 3·5 h at room temperature using solvent system 2 (Post *et al.* 1974) consisting of ethyl acetate–methanol–acetic acid–water (65:20:7·5:10 v/v). Chromatograms were first cut into 3-cm strips and then each strip was cut into 1-cm pieces. The radioactivity in each piece was measured by liquid scintillation counting. Marker sugars (about 0·2 mg) were run on separate chromatograms and their positions located by standard chemical staining techniques (Smith 1960).

Results

Characteristics of the Activity

Homogenates of isolated integuments from third-instar larvae of *L. cuprina* incorporate label from [^{14}C]UDPAG into acid- and ethanol-insoluble material. The levels of incorporation range from 400 to 1500 cpm per standard incubation

(0·1–0·3% of input radioactivity). The level is highly dependent on the developmental stage of the larvae, with the most active preparations being obtained from early third-instar larvae selected immediately after ecdysis. The incorporation was quite reproducible when larvae of precise developmental stage were used. In preliminary experiments in which the integuments were homogenized in several different buffers, the level of activity was low and quite variable from preparation to preparation. These problems were largely overcome by homogenizing in cell culture medium (which may better preserve the structural integrity of intracellular components, such as fragments of outer cell membrane) and this procedure was used for most of the experiments described in this paper. However, it is not essential to use this culture medium since reproducible results were also obtained with preparations made from *C. erythrocephala* and *M. domestica* integuments homogenized in a medium consisting of phosphate buffer, inorganic salts and glucose.

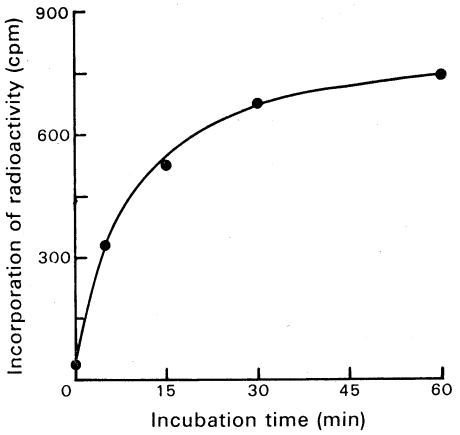


Fig. 1. Incorporation of radioactivity from [^{14}C]UDPAG into alkali-insoluble material by homogenates of larval integuments from *L. cuprina*. The data is from a single experiment and each point is the mean of duplicate determinations. Several such time courses with different preparations of homogenate have been performed and essentially similar curves have been obtained in each case

Incorporation was not linear with time, the graph of the time course being hyperbolic in shape and reaching a maximum after about 60 min (Fig. 1). Standard assays were for 30 min which gives only an approximate measure of initial rate. The hyperbolic shape of the graph suggests that either the substrate concentration is rapidly decreasing during incubation or there is an increase in the level of some inhibitor. From experiments in which the concentration of UDPAG was varied, an apparent K_m of 0·8 mM was estimated. NAG (20 mM), glucosamine (20 mM) or additional Mg^{2+} (10 mM, 10 times the level in the tissue culture medium) had little effect on the level of activity, but UDP (3·8 mM) caused 89% inhibition. KCN (10 mM) stimulated the activity (as much as 100% with some of preparations) and was routinely added in many later experiments.

Analysis of the Products

In zero-time controls, only low background incorporations (5–10% of that of standard incubations) were obtained. Hence non-specific binding of [^{14}C]UDPAG to material from the homogenate is not a significant component of incorporation.

Acid digestions

Although the products were resistant to alkaline hydrolysis (70°C for 16 h in 10% NaOH) they were degraded by strong acid. When ethanol-insoluble labelled

products were first deproteinized by digestions with pepsin and trypsin and then incubated in concentrated HCl for 16 h at 80°C, most of the radioactivity was solubilized, unlike control digestions (water instead of HCl) in which most of the radioactivity remained ethanol-insoluble.

Chitinase digestions

The products were also solubilized by chitinase. The ethanol-insoluble labelled products were deproteinized by digestions with trypsin and pepsin and were then incubated with chitinase for 48 h. About 85% of the original ethanol-insoluble radioactivity remained insoluble after the protease digestions. Following chitinase digestion, the pellets were much smaller and different in appearance compared with control pellets (incubated only in sodium acetate buffer). The chitinase-treated pellets contained only 4–5% of the original precipitable radioactivity; the remainder (about 80% of original) was recovered in the supernatant fraction. However, in the non-chitinase-treated controls, 30–50% of the label was also solubilized. Most of the radioactivity solubilized in both chitinase-treated and control samples co-migrated with NAG and glucosamine when samples were analysed by paper chromatography.

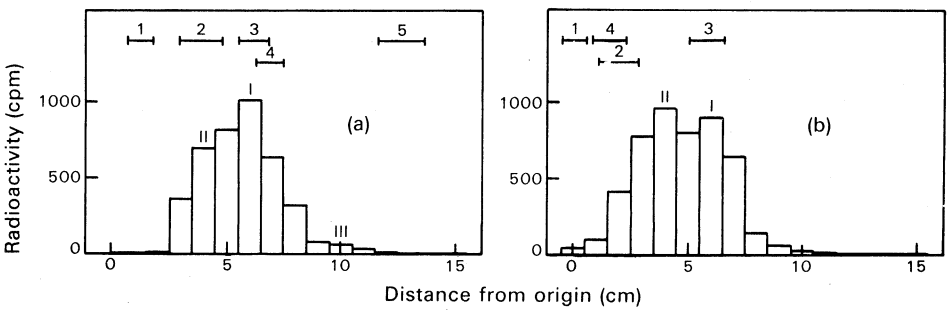


Fig. 2. Chromatogram profiles showing the distribution of ^{14}C-label solubilized from incubation products by chitinase digestion. ^{14}C-labelled products were obtained by using a homogenate of larval integuments from *C. erythrocephala* and were digested with trypsin and pepsin before being incubated with chitinase. Ascending paper chromatograms were run at room temperature in (*a*) solvent system 1 for 4 h, and (*b*) solvent system 2 for 3½ h. After development with the solvent the chromatograms were cut into 1-cm pieces and the level of radioactivity in each piece was determined. The symbols I, II and III show the positions of peaks of radioactivity referred to in the text. The positions of marker compounds are shown above the profiles. 1, UDPAG; 2, chitobiose; 3, NAG; 4, glucosamine; 5, muramic acid.

A chitinase-digestion experiment was also carried out on the products obtained with a preparation from *C. erythrocephala.* (The procedure was as described in Materials and Methods, except that the chitinase digestion was for 30 h with the addition of 1 mg fresh chitinase every 6 h.) The digestion released 96% of the ethanol-insoluble radioactivity into a soluble form, whereas only 4% was solubilized in the non-chitinase-treated control. Samples of the supernatant fractions were analysed by paper chromatography and the profiles of radioactivity obtained on the chromatograms are shown in Fig. 2. In solvent system 1 (Fig. 2*a*) the chitinase-digestible material showed a major peak (I) with a mobility similar to those of the NAG and glucosamine markers. There was a distinct shoulder (II) on the trailing

side of the main peak, which had a mobility similar to that of chitobiose. A third, faster-migrating component (III), which accounts for less than 10% of the total radioactivity, had a mobility different from any of the markers used. Interestingly, the material solubilized in the non-chitinase control ran as a single peak with a mobility very similar to that of peak III. No product with a mobility similar to that of muramic acid was obtained on either chromatogram. In solvent system 2 (Fig. 2*b*) there were two distinct peaks of radioactivity of roughly equal size. One had a mobility very similar to that of the NAG marker, whereas the other co-chromatographed with the glucosamine and/or chitobiose markers.

The digestibility of the radioactive products by chitinase clearly indicated that chitin is a major component formed by these homogenates. However, one (or more) other major ethanol- and alkali-insoluble component seemed to be made, at least by the homogenates prepared from *L. cuprina*. This product, which was initially ethanol-insoluble, was solubilized during prolonged incubation at pH 4·7 (the pH of the non-chitinase control incubation).

Evidence against Whole-cell Contamination of Homogenates

Since labelled chitin is synthesized from [^{14}C]NAG by intact epidermal cells from early third-instar *L. cuprina* larvae (Turnbull and Howells 1982), it was important to consider the possibility that the relatively low incorporations obtained with the homogenates might be due to residual intact cells (particularly since [^{14}C]NAG was shown to be produced during incubation, as a breakdown product of the substrate [^{14}C]UDPAG—see below). Consideration was also given to the problem of microbial contamination, because fungi synthesize chitin and bacteria may convert UDPAG to UDP-*N*-acetylmuramic acid. Both of these nucleotide sugars are used in the synthesis of the β-(1,4)-glycan murein, which is a major component of bacterial cell walls.

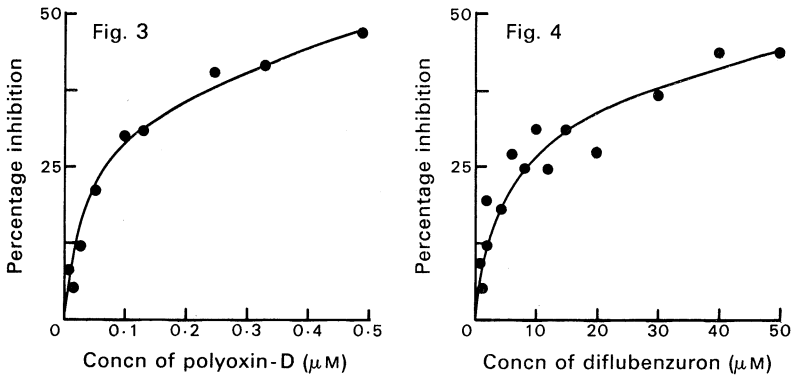
The following observations indicate that the incorporation obtained with the homogenates is not due to the presence of intact epidermal cells. Firstly, examination of stained smears of homogenates using light microscopy showed no intact cells but only pieces of cuticle, cell debris and large numbers of intact nuclei. Secondly, gassing the incubations with nitrogen or the addition of KCN did not reduce incorporation. Both treatments effectively eliminate chitin synthesis by intact epidermal cells (Turnbull and Howells 1982). Thirdly, incubations in which [^{14}C]UDPAG was replaced with either [^{14}C]NAG or [^{14}C]glucosamine (at the same concentration and specific activity as [^{14}C]UDPAG) gave only 11 and 5%, respectively, of the normal level of incorporation. Additionally, the presence of excess unlabelled NAG and/or glucosamine did not reduce the level of incorporation.

The above observations also provide evidence that incorporation was not due to the presence of intact microbial cells. In addition, it would seem highly unlikely that bacterial growth was involved since penicillin and streptomycin were routinely present in all preparations. Two other antibacterial agents, the broad-spectrum antibiotic gentamycin (at an effective antibacterial level of 0·2 mg/ml) and sodium azide (0·4 mg/ml) had no effect on incorporation when added to preparations from *C. erythrocephala*. Finally, no labelled product having a mobility similar to that of muramic acid was seen on any chromatogram.

Effects of Inhibitors of Chitin Synthesis

Polyoxin-D

This structural analogue of UDPAG, which is a competitive inhibitor of the fungal chitin synthases (Gooday 1977), inhibited incorporation by the homogenates when added at low concentrations. However, as shown in Fig. 3, the inhibition was not complete, affecting only about 50% of the total incorporation, even at relatively high concentrations. This suggests that more than one type of labelled product was being formed, the synthesis of one (or more) of these being insensitive to polyoxin-D. This observation in favour of more than one product is in agreement with the results obtained using chitinase digestions. An apparent K_i of 0·04 μM was calculated for the polyoxin-D inhibition (assuming competitive inhibition). This is lower than the K_i values determined for polyoxin-D with the fungal chitin synthases (Gooday 1977).



Figs 3 and 4. Inhibition by polyoxin-D (Fig. 3) and by diflubenzuron (Fig. 4) of chitin synthesis in homogenates of larval integuments from *L. cuprina*. The graphs represent the combined data from five (Fig. 3) and seven (Fig. 4) experiments with different enzyme preparations. Values (means of duplicates) were calculated as percentages of control incorporations (no inhibitor) and each point represents the mean of values from the five (Fig. 3) or seven (Fig. 4) experiments.

Diflubenzuron

This insecticide, which kills larvae of *L. cuprina* (Turnbull and Howells 1980) and inhibits chitin synthesis in intact larval integuments from this insect (Turnbull and Howells 1982), also inhibited incorporation by homogenates of integuments. As with polyoxin-D, inhibition was not complete; although there was variation in the level of inhibition between different homogenates, inhibition was always obtained. For example, the percentage inhibitions obtained with 50 μM diflubenzuron for three different homogenates (mean $\pm$ s.e. of 3–4 determinations) were 49 ± 1, 70 ± 2 and 47 ± 3. Similar results were obtained using homogenates prepared from *M. domestica*. Fig. 4 shows the effect of increasing diflubenzuron concentration on the percentage inhibition of incorporation. Diflubenzuron inhibited to a maximum of about 50% and had an apparent K_i of 5–8 μM. The scatter of the points in Fig. 4 is an indication of the variation in the levels of inhibition between different homogenates and is greater than that seen with polyoxin-D (Fig. 3). Diflubenzuron is extremely insoluble in water and so some of this variability may reflect differences in the concentration

of dissolved diflubenzuron between experiments. In experiments in which both diflubenzuron (50 μM) and polyoxin-D (1 μM) were added, inhibition of incorporation was still incomplete and the level of inhibition obtained depended on the particular homogenate preparation. There seemed to be little, if any, additive effect of these inhibitors.

Substrate Degradation in the Homogenates

The fate of label from [^{14}C]UDPAG during incubations with homogenates of *L. cuprina* larval integuments was analysed by descending paper chromatography. These incubations were carried out in 40 mM Tris buffer (pH 7·5) containing 2 mM EDTA, 2 mM $MgCl_2$, 10 mM glutamine and 16 mM cysteine. Chromatograms of samples from zero-time incubations (developed in solvent system 1) showed the expected single major peak of radioactivity consistent with undegraded UDPAG.

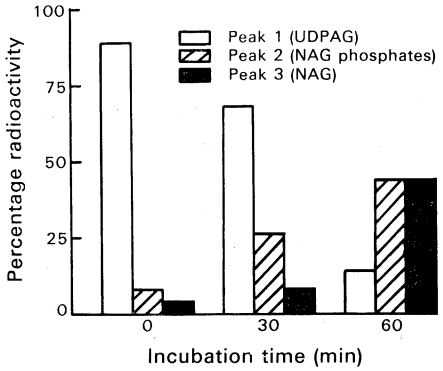


Fig. 5. Degradation of [^{14}C]UDPAG in homogenates of larval integuments from *L. cuprina*. Samples from incubations were deproteinized and the labelled components separated by paper chromatography (solvent system 1, descending mode, 16 h at room temperature). Three distinct peaks of radioactivity were seen on the chromatogram profiles and these had mobilities similar to UDPAG, NAG phosphates and NAG. The histograms show the percentage of total radioactivity in each of the three peaks after 0, 30 and 60 min of incubation.

Samples from incubations of longer duration contained two other labelled components. After incubation for 30 min, the chromatogram showed a major peak consistent with [^{14}C]UDPAG, but a second prominent peak, which co-migrated with NAG-1-P and/or NAG-6-P, was also obvious. A third minor peak, with a mobility similar to that of NAG, could also be distinguished. After incubation for 60 min, the UDPAG peak had almost completely disappeared and the NAG-1-P/NAG-6-P and NAG peaks were prominent and of roughly equal size. Fig. 5 summarizes these results by showing the percentage of total radioactivity in each of the above three peaks after incubation for 0, 30 and 60 min. The UDPAG substrate is apparently degraded rapidly (at a rate of about 60 nmol/h), firstly to NAG phosphates and then to NAG. The rate of degradation of UDPAG at the concentrations routinely used in the chitin synthase assay (0·1–1 mM) is at least two orders of magnitude greater than the rate of *in vitro* chitin synthesis. This probably accounts in large measure for the hyperbolic shape of the time course of chitin synthesis (Fig. 1).

Discussion

Isolated intact integuments from early third-instar larvae of *L. cuprina* incorporate [^{14}C]NAG or [^{14}C]glucosamine into chitin when incubated in insect tissue culture medium (Turnbull and Howells 1982) and, as demonstrated in this article, homogenates of these integuments form chitin from [^{14}C]UDPAG. Thus some of the chitin synthase activity present in the intact integuments is preserved in active form in the homogenates. The rate of chitin formation per integument achieved by the homogenates is about one order of magnitude lower than that taking place in the intact epidermal tissue. This relatively low retention of activity in the homogenates probably reflects, to some extent, a partial inactivation of the membrane-bound chitin synthase enzyme during cell disruption. However, the possible release either of chitin synthase inhibitors or of enzymes which compete for the substrate could contribute to the relatively low level of incorporation. The problems we have experienced in measuring chitin synthase activity reliably are consistent with those experienced by other workers who have attempted to characterize this enzyme from insect tissue. The rate of incorporation obtained in our experiments (equivalent to approximately 200 pmol UDPAG utilized per hour per standard incubation) is similar to those obtained in other *in vitro* chitin synthase systems prepared from insects (Cohen and Casida 1981*a*; Mayer *et al.* 1980).

A major part of the incorporation obtained with these integumental homogenates represented chitin synthesis as judged by the usual criteria; viz. the product was insoluble in warm alkali, was resistant to protease digestion but was solubilized by concentrated HCl and by digestion with chitinase. In the latter case labelled material that co-chromatographed with NAG, chitobiose and glucosamine was obtained. This incorporation is also sensitive to inhibition by polyoxin-D. However, some incorporation always occurred into products that were initially ethanol- and alkali-insoluble, but were solubilized to varying extents during protease digestion and also during prolonged incubation at pH 4·7. The formation of these components appears to be insensitive to inhibition by polyoxin-D and may represent mucopolysaccharide or glycoprotein synthesis. A similar polyoxin-insensitive, diflubenzuron-insensitive activity, resulting in the formation of ethanol-insoluble, chitinase-resistant products, was found in particulate preparations of whole *L. cuprina* larvae and pupae (data not presented). This activity, which probably comes mainly from tissues other than the integument, brings about relatively rapid rates of [^{14}C]UDPAG utilization. We have found that it is virtually impossible to measure chitin synthase activity in homogenates of whole insects due to the high background incorporation into non-chitin material. The low level of polyoxin-insensitive, diflubenzuron-insensitive activity in the integumental homogenates may be due to slight contamination of the integument preparations with viscera and/or haemolymph. No attempt was made to wash the integuments prior to the preparation of the homogenates.

The data presented in this article suggest that several enzyme systems that can act on UDPAG are active in the crude homogenates of larval integuments. The hyperbolic shape of the time course of incorporation is consistent with product inhibition (perhaps by UDP) and/or with a rapid decrease of substrate concentration during incubation. Studies on the rate of degradation of UDPAG indicate that the latter possibility is the more likely explanation. NAG phosphates appear more

rapidly than NAG, suggesting that initially the UDPAG is cleaved into NAG-1-P and UMP. NAG appears later and probably results from the dephosphorylation of NAG-1-P. Consequently, it seems unlikely that there is a significant release of UDP. Fractionating the crude homogenate to enrich for chitin synthase activity may overcome this problem of rapid substrate degradation. Preliminary experiments using homogenates of larval integuments from *C. erythrocephala* indicate that chitin synthase activity is associated mainly with a particulate fraction which sediments at 1000 *g*.

Probably the most notable feature of our results is that incorporation was sensitive to inhibition by diflubenzuron. This is the first report of a cell-free chitin synthesis system from insect tissue in which this compound has had an inhibitory effect. However, an interpretation of this inhibition in terms of the mode of action of diflubenzuron is complicated by the fact that chitin is not the only labelled product formed in our system. There seem to be at least three different interpretations of our data which are consistent with published proposals regarding the inhibitory action of diflubenzuron on chitin synthesis. The first possibility is that diflubenzuron directly inhibits chitin synthase, having a similar mode of action as polyoxin-D. This proposed action of diflubenzuron has been suggested by a number of workers (see Verloop and Ferrell 1977). Consistent with this explanation is the finding that the combined effect of the two inhibitors is little different from the effect of either acting alone. However, this interpretation seems unlikely because diflubenzuron does not inhibit the chitin synthase activities found in preparations from other insects (Cohen and Casida 1980*b*; Mayer *et al.* 1980).

A second possibility is that the polyoxin-D-insensitive incorporation, which occurs in our system, represents not the formation of mucopolysaccharides and glycoprotein *per se*, but of glycolipid or glycoprotein intermediates required for chitin synthesis, and that the formation of these is sensitive to diflubenzuron. The existence of intermediates of this type in insect chitin synthesis has been proposed by Quesada Allué *et al.* (1976) and Marks and Sowa (1976). The involvement of such intermediates is well established in prokaryotic exopolysaccharide formation (Sutherland and Norval 1970) and in mammalian glycoprotein synthesis (Staneloni and Leloir 1979), and an analogy between glycoproteins and the chitin-protein complexes of insect cuticle has been suggested (Hackman 1976). Thus diflubenzuron might act by reducing the level of intermediate or primer available to the chitin synthase enzyme. If this explanation of the action of diflubenzuron is correct, it implies that the polyoxin-D-insensitive activity present in the homogenates of larval integuments is not equivalent to the polyoxin-D and diflubenzuron-insensitive activity present in the particulate preparations from whole larvae. While this is possible it seems unlikely.

A third possibility is that most of the chitin synthase enzyme present in the crude homogenates is initially present in an inactive form, is rapidly activated once incubation begins, and that diflubenzuron inhibits this activation. This interpretation would be consistent with the proposal of Leighton *et al.* (1981), who suggested that diflubenzuron may inhibit a protease-induced activation of the zymogen form of chitin synthase. It would be consistent also with the results of Horst (1981), who found that microsomal preparations from larvae of the brine shrimp (*Artemia salina*) show reduced capacity for chitin synthesis following pre-incubation with diflubenzuron. The essence of this proposal is that polyoxin-D and diflubenzuron both inhibit the final step of chitin synthesis, the former by directly inhibiting the activity of chitin

synthase and the latter by preventing its conversion to an active form. Consequently, their inhibitory effects are unlikely to be additive. The nature of the polyoxin-D-insensitive reaction need not be considered in evaluating this proposal, since it would presumably be diflubenzuron-insensitive also. This third interpretation seems to accord best with our experimental observations.

The data presented in this paper demonstrate that we have obtained an *in vitro* assay system for the integumental chitin synthase of dipteran larvae. This system should provide a starting point for the preparation of a purer chitin synthase which could be used for detailed studies of the properties of such enzymes. Although our studies have not yet resolved questions about the biochemical mode of action of diflubenzuron, the unique property of this integumental chitin synthase system (in comparison with others prepared from insect tissue) is that it has retained a sensitivity to inhibition by diflubenzuron, thus providing a basis for further studies of this problem. Once the homogenates have been fractionated to separate the polyoxin-D-sensitive and -insensitive activities, it will be possible to design experiments to test the various possible actions of diflubenzuron presented above.

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