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THE INHIBITION OF INSECT CHITIN SYNTHESIS BY TUNICAMYCIN

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SUMMARY. The conversion of [^{14}C]-labeled glucosamine to [^{14}C]-labeled insect chitin was inhibited by tunicamycin, a nucleoside antibiotic which specifically blocks the biosynthesis of dolichyl diphosphate N-Acetylglucosamine. A method of short-term culturing is described for the analysis of chitin and glyconjugates biosynthesis by epidermal tissue of Triatoma infestans.

INTRODUCTION. The biosynthesis and sequential deposition of chitin (β -1-4-linked poly-N-Acetylglucosamine) and proteins are major events in the formation of the insect cuticle (1-3). The fact that no insect chitin has been found to be free of aminoacids (3,4) indicates the existence of stable connecting links between chitin and protein (5,6).

Although a fungal chitin synthase has been well characterized (7-10), very little data exist about insect chitin biosynthesis (3,11,12). Most of the knowledge on this area comes from "in vivo" experiments (13,14) and imaginal disks (15-17) or epidermal tissue culture studies (18-20). Since there are some difficulties to obtain a cell-free assay system for measuring insect chitin synthase (3,12), reports on this field are scarce (12,21-24).

We previously reported (23) that newly moulted imagos of the blood sucking bug Triatoma infestans (Hemiptera, Reduviidae) incorporated radioactive sugars into cuticular chitin on both "in vivo" and "in vitro" experiments. The simultaneous formation of lipid-linked sugars behaving as N-Acetylglucosamine and NN'diacetylchitobiose-linked to dolichyl diphosphate has also been reported (23,25,26).

Our own as well as others' data (27,28) suggested a possible role for the lipid-linked sugars in the biosynthesis of nascent (insect) chitin. However, although "in vitro" glycosylation of insect proteins starting from dolichyl

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linked sugars has been obtained (25,29), direct assays to transfer a lipid-bound sugar moiety into insect chitin has so far been unsuccessful (25,30).

We now report the inhibition of insect chitin synthesis by tunicamycin, a nucleoside antibiotic which specifically blocks the biosynthesis of dolichyl diphosphate N-Acetylglucosamine (31).

MATERIALS AND METHODS. The materials and methods not explicitly mentioned here were exactly as previously described (23,26,29). All the chemicals were analytical grade as obtained from commercial sources. D-[U-^{14}C]Glucose (260 Ci/mol) and D-[U-^{14}C]glucosamine (254 Ci/mol) were from Amersham. Radioactive standards of liver and insect lipid-linked sugars were prepared as described (32,33). Tunicamycin and polyoxin D were generous gifts from Lilly Laboratories and Kaken Chemical Co. respectively. Streptomyces chitinase-chitobiase (10) was from Sigma.

Animals: Triatoma infestans larvae (V° stage) were kindly supplied by Dr.E.Segura from the M.Fatala Chaben Chagas Institute of Disease, Buenos Aires. Galleria mellonella larvae were from Carolina Biol.Co.

Tissue culture: After first anesthesizing the insect with cold it was pinned down in a paraffin dish and dissected at 0-4°C under binocular microscope. After cutting the abdominal edge with fine scissors, the abdomen was opened with forceps. After flooding the abdominal cavity with incubation medium (see below) the internal organs were removed. Fat bodies and the tergal (dorsal) part of the abdomen were then eliminated. The sternal (ventral) part was separated from the thorax, freed of the anal plate and cut into halves. A pool of 4 to 6 left moieties was incubated in a 35 x 10 mm tissue culture dish (Falcon) with medium. The right counterparts were incubated in the same manner for the antibiotics test.

The presence of epidermis tightly bound to cuticle and the viability of the cells (up to 8 hs) were tested by Trypan blue staining.

Incorporation of labelled sugars into glycoconjugates. Abdominal pieces were maintained in the tissue culture dishes containing 1 ml of chilled cold Grace's insect tissue culture medium (Grace Grand Island Biol.Co) supplemented with 8% locust haemolymph (pH 7.3). The radioactive sugar (3-5 µCi) was added and the dish incubated at 26°C. After incubation the dishes were quickly chilled and the culture medium was sucked off. The abdominal pieces were twice washed with cold Grace's medium after which 2 ml of methanol were added. Both cuticle pieces and the methanol extract were transferred into a large clearance glass-glass Kontes homogeneizer and chloroform was added up to a chloroform:methanol ratio of 3:2 (Solvent I). Homogeneization was carried out to obtain a complete cuticle disaggregation. After centrifugation, the insoluble material was extracted several times, first with the same Solvent I, then with chloroform:methanol:4 mM $mgCl_2$ 1:16:16 (Solvent III) and finally with methanol to eliminate water. The chloroform:methanol extracts were pooled, partitioned by Folch's method (34) and processed as previously described (29) to obtain washed lipophyllic (lower) phases. Aqueous (upper) phases were pooled together with Solvent III extracts. The partially delipidated material was twice extracted with Leloir's solvent (35) chloroform: methanol:water 1:1:0.3 (Solvent II) and then with methanol. The insoluble material was processed as described in legend to Figure 1.

RESULTS. When abdominal epidermis from just moulted Triatoma imagos were incubated with [^{14}C]glucosamine, radioactivity was incorporated into three fractions: the hydrophylic, the lipophylic and the insoluble (non-aqueous and aqueous solvents) (Figure 1). The bulk of the labeled hydrophylic material

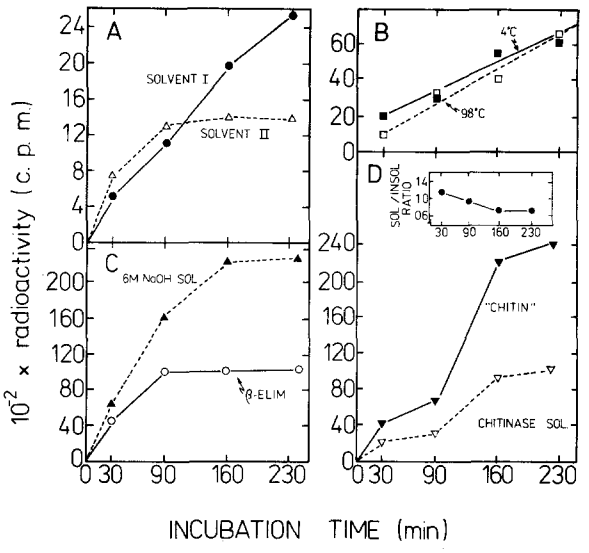

FIGURE 1.

Time-Dependent Incorporation of [^{14}C]-glucosamine into Glycoconjugates.
Triatoma epidermal cultures were incubated for the indicated times and processed as described in Methods.
Panel A: Incorporation into Solvent I- (●) Solvent II-soluble (△) materials (lipid-linked sugars) (see Methods). Panel B: Incorporation into TCA-soluble material. The delipidated material was suspended in 10% trichloroacetic acid and extracted for 7 min at 4°C (■) or at 98°C (□). Panel C: The TCA-insoluble pellet was washed several times (25,29) and then treated with 0.1 M NaOH for 24 hs at 37° C (β-elimination) (37). After centrifugation, the soluble material was counted ( ) and the pellet was treated with 6 M NaOH for 3 hs at 98°C to liberate sugar chains from glycoproteins (▲) (37). Panel D: The 6 M NaOH insoluble material (▼) was extensively water-washed, up to neutral pH and then incubated with chitinase from Streptomyces griseus (Sigma). The incubations were carried out for 48 hs at 21°C in a toluene atmosphere with: 0.2 M citric acid; 0.4 M Na_2HPO_4 buffer at pH 5.3; 0.8 mM EDTA. New enzyme was added every 12 hs. The bulk of digested material (▽) was identified as Acetylglucosamine. The inset shows the time course of ratio between the chitinase-soluble and -insoluble material.

was glucosamine, N-acetylglucosamine, UDP-N-acetylhexosamines and several non-identified oligosaccharide-like substances (not shown).

The lipophylic substances: Figure 1, panel A shows that radioactivity was incorporated into two lipophylic fractions. The amount of material soluble in the organic phase of Folch's partition (see Methods) was linear over 230 min. It has been resolved in neutral and anionic substances by DEAE-cellulose column chromatography (29,35) (not shown). The identity of the neutral substance was not investigated. The charged material behaved, according to several criteria (29) as the previously described insect lipid diphosphate N-acetylglucosamine (23,33) and insect lipid diphosphate NN'-diacetylchitobiose

(26). Figure 1, A also shows the kinetic of radioactivity incorporation into the material extracted with Solvent II (see Methods) that behaved (not shown) as a lipid-diphosphate oligosaccharide similar to the previously described insect dolichyl diphosphate derivatives (26,29).

The insoluble material: The particulate material insoluble in Solvents I, II and III and in Methanol was suspended in 10% trichloroacetic acid. The amount of TCA soluble (unidentified) labeled substances linearly increased with incubation time (Fig. 1, B). This and the fact that the same result was obtained at 4°C as well as at 98°C would indicate that soluble substances were not trapped by the partially denaturated proteins (36).

The TCA insoluble pellet was treated with 0.1 M NaOH in order to β-eliminate (37) sugar chains linked to SER and/or THR through O-glycosidic linkages. Figure 1 panel C shows that the amount of β-eliminated material increased over 90 min of incubation and then remained constant suggesting an equilibrium between synthesis and degradation and/or transformation. Experiments are in progress to identify this material. Finally, the mild alkali-resistant material was treated with 6 M NaOH to remove the bulk of proteins and labeled glycopeptides (Fig. 1, C). This treatment yielded an insoluble pellet enriched in partially de-acetylated chitin (11) (Fig. 1, D).

It was assumed that radioactivity in this material was representative of the total biosynthesized chitin since after acid treatment (23) 95% of the label was recovered as glucosamine.

The chitin-like material: The above described chitin-enriched fraction was washed, neutralized and subjected to a chitinase-chitobiase digestion. Depending on the experiments only 20-50% of the labeled material became soluble probably because of the above mentioned partial N-de-acetylation of the chitin chains (11).

Figure 1, panel D shows the time course of the radioactivity incorporation into chitinase-sensitive material. The proportion of the GlcNAc release, slowly decreased with time (Fig. 1, D, inset) suggesting slight differences between early and late chitinous material deposition.

Table 1: Tunicamycin effect on chitin synthesis.

EXPERIMENT A[a]	Radioactivity incorporated from 3 μCi [^{14}C]Glc (c.p.m.)		
	CONTROL	TUNICAMYCIN[b]	POLYOXIN[c]
UPTAKE	335,083 (100)	279,124 (100)	304,997 (100)
β-ELIMINATED	4,056 (1.2)	4,388 (1.5)	6,314 (2.2)
6N KOH-SOLUBLE	20,508 (6.1)	16,763 (6.0)	13,329 (4.7)
"CHITIN"	40,795 (12.1)	32,087 (11.4)	27,884 (9.1)
CHITINASE DIGESTED	8,974 (2.6) (21.9)d	4,863 (1,7) (15.1)d	4,182 (1.3) (14.9)d

EXPERIMENT B	Radioactivity incorporated from 5 μCi [^{14}C]GlcN (c.p.m.)			
	TRIATOMA, imagos[a]		GALLERIA, larvae[e]	
	CONTROL	TUNICAMYCIN[b]	CONTROL	TUNICAMYCIN[b]
UPTAKE	155,510(100)	177,702(100)	51,906(100)	35,662(100)
6N KOH-SOLUBLE	10,576(6.8)	7,881(4.4)	12,830(24.7)	7,684(21.5)
"CHITIN"	36,274(23.3)	25,827(14.5)	2,138(4.1)	1.060(2.9)
CHITINASE DIGESTED	6,913(4.4) (19.0)d	3,070(1.7) (11.8)d	-	-

Epidermal cultures were incubated for 3 hs and processed as indicated in legend to Fig.1. Data are the mean of triplicate (a) or duplicate (e) experiments. (b) One μg/ml (c) Two μg/ml (d) percent of "chitin".

The effect of antibiotics on sugars uptake: The uptake of [^{14}C]-labeled glucose and glucosamine did not substantially decrease when polyoxin D, a competitive inhibitor of fungal chitin synthase (38) was added to the incubation medium (Table 1, Exp. A). As also indicated in Table 1, the addition of tunicamycin slightly inhibited glucose (Exp. A) but not glucosamine (Exp. B) uptake.

The antibiotic effect on glycoconjugate biosynthesis: Polyoxin inhibited the incorporation of radioactivity into both strong alkali-sensitive and -resistant fractions (Table 1). The bulk of solubilized material represents the biosynthesized N-glycosylated proteins (39). Polyoxin D inhibition on the biosynthesis of alkali-resistant fraction seems to confirm that most of this labeled chitin-like material was, in fact, chitin. The competitive inhibition of fungal chitin biosynthesis by polyoxins is a well-known fact (7, 38) whereas the inhibition of insect chitin synthesis by polyoxin D has also been reported (11,16).

As expected, tunicamycin slightly inhibited the N-glycosylation of proteins (Table 1,B). This antibiotic specifically blocks the dolichyl diphosphate N-acetylglucosamine formation (31) thus inhibiting the glycoprotein biosynthesis.

Inhibition of Triatoma chitin biosynthesis by Tunicamycin: When [^{14}C]glucose was the precursor, little or no inhibition of the synthesis of chitin-like material could be detected in the presence of 1 μg/ml of tunicamycin (Table 1, A). However, the percentage of the radioactivity released by chitinase slightly decreased. This effect was also detected when Polyoxin D was used instead of tunicamycin (Table 1). When [^{14}C]glucosamine was utilized as chitin precursor the inhibitory effect of tunicamycin became more evident. Table 1, Exp. B shows that the synthesis of Triatoma cuticular chitinous material decreased by 37%. Galleria mellonella larval epidermal preparations were tested to confirm this inhibitory effect: Table 1, Exp. B shows that chitin synthesis was also 30% inhibited. As shown in Table 1, the percentage of radioactivity released by chitinase from the Triatoma "chitin" synthesized in the presence of tunicamycin decreased significantly.

DISCUSSION. A method of short-term culturing is described for the quantitative and qualitative analysis of chitin and glycoconjugates biosynthesis by epidermal tissue of Triatoma infestans. The results in this communication indicate that Triatoma chitin synthesis is inhibited by tunicamycin.

It has been reported that tunicamycin has no effect on the insect chitin synthase itself (12,24) whereas the Neurospora crassa enzyme was competitively inhibited by this antibiotic (40). In our system the latter possibility cannot be discarded but it is also likely that tunicamycin blocks the synthesis of a lipid-linked saccharide intermediate involved in the formation of a chitin-protein complexes (3,4,16,23).

It is interesting to point out that the "chitin" synthesized in the presence of tunicamycin appeared to be more resistant to chitinase treatment (Table 1). This result was obtained in a 3 hr-experiment, time in which digestion of normal "chitin" by chitinase has also diminished (Fig. 1, D, inset). Thus, both "tunicamycin" and "time" effects were additive. These

results suggest that radioactivity is incorporated into two different pools (or domains) of chitin.

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