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STEPS IN THE BIOSYNTHESIS OF MOSQUITO CELL MEMBRANE GLYCOPROTEINS AND THE EFFECTS OF TUNICAMYCIN

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Summary

A cultured cell line of the mosquito, *Aedes aegypti*, is sensitive to tunicamycin as expected from the ability of crude membrane preparations to catalyse the formation of *N*-acetylglucosamine-linked dolichyl pyrophosphate. Formation of dolichylphosphomannose was also detected and this reaction was totally insensitive to tunicamycin. Incorporation of radioactive mannose into total acid-precipitable glycoproteins was inhibited greater than 90% in whole cells by tunicamycin, while the incorporation of leucine and glucosamine was less affected. Separation of the radioactive hexosamines from acid hydrolysates of cells incubated with [^{14}C]glucosamine and tunicamycin showed predominant labelling of galactosamine, whereas in control cells not treated with the drug both glucosamine and galactosamine were labelled equally. Evidently, mosquito cells synthesise *N*-glycosidically linked carbohydrate chains assembled through tunicamycin-sensitive steps involving dolichyl pyrophospho-oligosaccharides, and *O*-glycosidically linked chains rich in *N*-acetylgalactosamine, the assembly of which is unaffected by tunicamycin. These results support structural evidence (Butters, T.D. and Hughes, R.C. (1981) Biochim. Biophys. Acta 640, 655—671) for the presence of high mannose *N*-glycans and *N*-acetylgalactosamine-rich *O*-glycans in mosquito cell glycoproteins. The absence of complex *N*-glycans was confirmed by the demonstration of negligible activities of *N*-acetylglucosaminyl-, galactosyl- and sialyltransferases responsible for assembly of the terminal sequences of *N*-glycans of mature mammalian glycoproteins.

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Abbreviations: SDS, sodium dodecyl sulphate; PPO, 2,5-diphenyloxazole.

Introduction

There is now extensive evidence implicating sugar-polyprenol phosphates [1—3] in the assembly of *N*-glycosidically linked glycan chains of glycoproteins [4,5]. Insect cells are interesting subjects for study of the regulation of glycoprotein synthesis because these cells do not synthesise sterols, including cholesterol [6], that in mammalian species may regulate glycosylation reactions by feedback inhibition of isoprenoid biosynthesis [7]. Insect cells do synthesise isoprenoid-derived compounds de novo from acetate or mevalonate [6], however, and presumably in amounts sufficient to support protein glycosylation. In our previous publications [8,9] we have presented evidence for the presence in mosquito cell glycoproteins of *O*-glycosidically linked carbohydrate chains and *N*-glycosidically linked 'high mannose' type chains. In the present paper we describe several aspects of the biosynthesis of these glycoproteins in the mosquito (*Aedes aegypti*) larval cell line, Mos 20A. We have used the drug, tunicamycin, as a probe [10—13] for the first step in *N*-glycan assembly, namely the formation of the *N*-acetylglucosaminyl pyrophosphate isoprenyl intermediate, and have analysed the activities of several specific glycosyl transferases utilizing lipids and modified mammalian glycoproteins as acceptors. Our results are in agreement with others [14,15] implicating isoprenoids as intermediates in protein glycosylation in insect cells and indicate the absence of glycosyl transferases responsible for the termination of 'complex' type glycan chains present in mammalian glycoproteins.

Materials and Methods

Cells. The established cell line Mos 20A of the mosquito *A. aegypti* was grown at 28°C in an insect culture medium supplemented with heat-inactivated fetal calf serum (15%, v/v) as described [9]. When required, the cells were displaced from the growth surface by vigorous shaking, washed in phosphate-buffered saline [9] by centrifugation and suspended in the same buffer for homogenization and analysis. In metabolic labelling experiments, cells were grown in Glasgow modified Eagle's medium (Flow Laboratories, Irvine, U.K.) supplemented with 10% fetal calf serum. BHK cells were grown in the same medium at 37°C.

Biochemical reagents. Tunicamycin was a generous gift from Dr. W.J. Cuthbertson and Glaxo Ltd., Greenford, Middlesex. It was kept as a 1 mg/ml solution in dimethyl sulphoxide at −20°C and diluted appropriately immediately before use. [^{14}C]Mevalonic acid lactone (22 mCi/mmol), [^{3}H]leucine, (53 Ci/mmol), [^{3}H]mannose (12 Ci/mmol), [^{14}C]mannose (3 mCi/mmol), [^{14}C]glucosamine (254 mCi/mmol), UDP-[^{14}C]galactose (347 mCi/mmol), GDP-[^{14}C]mannose (83 mCi/mmol), UDP-*N*-acetyl[^{14}C]glucosamine (323 mCi/mmol) and CMP-*N*-acetyl[^{14}C]neuraminic acid (9.1 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks, or New England Nuclear. Non-radioactive sugar nucleotides were from Sigma Chemical Co. Dolichol phosphate was obtained from Sigma (90% pure) and stored (2 mg/ml) in $CHCl_3/CH_3OH$ (2 : 1, v/v). Other rare plant polyprenoids were kindly provided by Dr. T. Chojnacki, Polish Academy of Sciences, Warsaw. Asialo-

fetuin and asialoagalactofetuin were obtained by treatment of fetuin (Sigma) with *Vibrio cholera* neuraminidase alone or followed by mild periodate oxidation to remove terminal galactose residues [16]. Ovalbumin was obtained from Sigma.

Glycosyltransferase assays. Cells were harvested and washed twice with cold saline and extracted with Triton X-100 (1%, w/v) by freeze-thawing until greater than 90% lysis had occurred. Nuclei and whole cells were removed by centrifugation at 8000 × *g* for 3 min and the clear supernatant was used in the transferase assays. The complete system for sialyltransferase activity contained in a final volume of 90 μl, 29 μM CMP-*N*-acetyl[^{14}C]neuraminic acid, 0.1% (w/v) Triton X-100, 1 mg of asialofetuin as exogenous acceptor, 0.2—0.3 mg of cell protein and 72 mM sodium cacodylate buffer, pH 6.6. UDP-galactose : glycoprotein galactosyltransferase was estimated in the presence of 1 mM UDP-[^{14}C]galactose, 12 mM $MnCl_2$, 2 mM $MgCl_2$, 5 mM dithiothreitol, 0.1% (w/v) Triton X-100, 0.20—0.25 mg of cell protein and 60 mM sodium cacodylate, pH 6.6. As exogenous acceptors, we used either 3 mg ovalbumin or 0.5 mg asialoagalactofetuin. The incubation mixture for UDP-*N*-acetylglucosamine : glycoprotein *N*-acetylglucosaminyltransferase contained 1 mM UDP-*N*-acetyl[^{14}C]glucosamine, 12.5 mM $MnCl_2$, 0.1% (w/v) Triton X-100, 0.2—0.3 mg of cell protein, 1 mg ovalbumin and 62 mM 2-(*N*-morpholino)-ethanesulphonate buffer, pH 6.3. The assay was terminated by the addition of 1 ml of 5% phosphotungstic acid in 2 M HCl, the mixture centrifuged and the pellet washed twice with 1 ml of 1% phosphotungstic acid in 10% (w/v) trichloroacetic acid, once with 1 ml ethanol/water (4 : 1, v/v) and once with 1 ml ethanol/ether (1 : 1). The resultant precipitate was dissolved in 0.5 ml 0.5 M NaOH overnight and together with 10 ml of gel scintillation mixture counted in an Intertechnique SL4000 scintillation counter.

Metabolic labelling. Nearly confluent monolayer cultures of Mos 20A cells in 35 mm diameter Falcon dishes were incubated at 28°C in medium (1 ml) containing 1 μCi/ml of [^{3}H]leucine, 2.5 μCi/ml of [^{14}C]glucosamine or 2.5 μCi/ml of [^{3}H]mannose. Leucine-free Eagle's medium was used in the first case. To some dishes, tunicamycin was added at various concentrations, usually to 0.5 μg/ml. At times, the cells were harvested by shaking, washed three times with phosphate-buffered saline followed by 2% phosphotungstic acid/10% perchloric acid at 2°C and the insoluble residues were dissolved in 0.5 M NaOH (1 ml). Portions were used to determine protein contents [17] and for radioactive counting.

Lipid intermediates. Mos 20A cells were grown to confluency, harvested by shaking and washed in phosphate-buffered saline and suspended in 10 mM Tris-HCl buffer, pH 7.4. The suspended cells were broken by Dounce homogenization and centrifuged at 3650 × *g* for 5 min at 2°C. The pellet obtained after further centrifugation at 180 000 × *g* for 1.5 h at 2°C was suspended in 0.05 M Tris-HCl (pH 7.4)/0.25 M sucrose and used for enzyme analysis. Standard incubations for the synthesis of lipid intermediate contained microsomes (780 μg of protein), Triton X-100 (0.089%), EDTA (2.2 mM), AMP (5 mM) and $MnCl_2$ (8.8 mM) in 22.2 mM Tris-HCl, pH 7.4 (total volume 0.220 ml). The reaction was started by addition of GDP-[^{14}C]mannose (5 μl, 25 nCi). Alternatively, the reaction mixture contained microsomes (50 μl, 780 μg of

protein), Triton X-100 (0.51%), $MnCl_2$ (8.8 mM), AMP (5 mM), ATP (1 mM), dithiothreitol (1 mM) in 22.2 mM Tris-HCl, pH 7.4. UDP-*N*-acetyl[^{14}C]glucosamine was added (10 μl, 50 nCi) to start the reaction (total volume 0.225 ml). When dolichol phosphate (usually 10 μg per 225 μl of reaction mixture) or different plant polyisoprenoids were added, the $CHCl_3/CH_3OH$ solvent was evaporated under a stream of N_2 and the lipids solubilised in 1% Triton X-100 before addition of other reactants. After incubation at 28°C for 15 min, the reaction was stopped by the addition (2 ml) of $CHCl_3/CH_3OH$ (2 : 1, v/v). Reaction products were extracted as described [12,25] with $CHCl_3/CH_3OH$ (2 : 1, v/v) followed by $CHCl_3/CH_3OH/H_2O$ (1 : 1 : 0.3, v/v). The protein residues were dissolved in 0.5 M NaOH and all fractions were counted for radioactivity.

Radioactive measurements were made using a Triton X-100/toluene-based scintillation fluid.

Thin-layer chromatography. Aliquots of lipid fractions were spotted onto plastic-backed silica gel plates (Eastman Kodak) and ascending chromatography performed in solvent A: $CHCl_3/CH_3OH/H_2O$ (60 : 25 : 4, v/v). The plate was sprayed with a $CHCl_3$ solution containing 10% PPO and set up for fluorography as described [9]. Free hexosamines were separated by chromatography in solvent B: *n*-propanol/ethyl acetate/H_2O/25% (v/v) NH_3 (60 : 10 : 30 : 10, v/v) for 5 h. Standards were detected with ninhydrin and radioactive hexosamines were located by scraping off 0.5 cm sections of the gel and eluting the sections with chromatography solvent. The eluates were dried and counted for radioactivity in Triton X-100/toluene-based scintillator.

Surface labelling. Lactoperoxidase-catalysed iodination of cell monolayers was as described [9]. Surface galactose or *N*-acetylgalactosamine groups were detected [18] by using galactose oxidase-NaB[^{3}H]H_4. The enzyme (Sigma) was added (50 units/ml) to washed monolayers and after incubation at room temperature for 30 min, the cells were suspended by shaking, washed and treated with NaB[^{3}H]H_4 (0.5 mCi/ml, The Radiochemical Centre) at 2°C for 10 min. The labelled cells were disrupted in 2% SDS/2% 2-mercaptoethanol and portions taken for electrophoresis.

SDS-polyacrylamide gel electrophoresis. Cells were disrupted by boiling for 5 min in 2% SDS/2% 2-mercaptoethanol and loaded (250 μg protein) onto slab polyacrylamide gels [9]. After electrophoresis the gels were stained with Coomassie blue and when indicated with ^{125}I-labelled lectins [9]. The gels were dried and developed using Fast tungstate film cassettes at −70°C to locate iodinated materials and metabolically labelled bands were detected over 2—7 days. Some gels were cut into 2-mm slices and dispersed for radioactive counting.

Alkaline hydrolysis and gel filtration. Glycopeptides were obtained from [^{14}C]glucosamine-labelled cells by digestion with pronase and isolated as described previously [9]. Lyophilized fractions of glycopeptide material were dissolved in 0.05 M NaOH/1 M $NaBH_4$ under N_2 at 45°C for 15 h [20]. Excess $NaBH_4$ was destroyed by addition of glacial acetic acid to pH 5 and the material was dried several times with CH_3OH to remove borate. The final residue was dissolved in Tris-HCl buffer (pH 7.8)/0.1% SDS for chromatography on Bio-Gel P6 as before [9].

Results

Assays using exogenous glycoprotein acceptors. The terminal sequence, NeuNAc-Gal-GlcNAc predominates in many mammalian glycoproteins containing *N*-glycans of the complex type [4]. Assembly of the sequence requires the sequential action of *N*-acetylglucosaminyl-, galactosyl- and sialyltransferases. Assays of these activities, using appropriate acceptors derived from fetuin and ovalbumin, with solubilized fractions of mosquito cells and BHK cells as positive control, are shown in Table I. No activity of galactosyl- or *N*-acetylglucosaminyltransferases was detected in the mosquito cells. A small but significant sialyltransferase activity was detected amounting to about 15% of the activity present in BHK cells. The low incorporation in incubation mixtures containing mosquito cell extracts was linear with time over 60 min. In order to assess the possible contribution of contaminating serum proteins to this low level of activity, an incubation mixture (90 μl) containing no cell homogenate, but supplemented with growth medium/10% serum (20 μl) was set up. A small but significant incorporation of radioactivity linear with time into asialofetuin was detected amounting to approx. 0.015 nmol/h. Therefore, although it cannot be ruled out that mosquito cells contain a weak CMP-*N*-acetylneuraminic acid : asialofetuin sialyltransferase activity, it seems likely that the low level of incorporation shown in Table I represents enzymes adsorbed by the cells from the serum used to grow the cells. Direct chemical analysis [9] of the mosquito cell glycoproteins showing the absence of any sialic acid is consistent with this interpretation.

Effects of tunicamycin on metabolism. Tunicamycin was added to monolayer cultures of mosquito cells together with radioactive precursors at 28°C. The drug had small but significant effects on the incorporation of [^{3}H]leucine (Fig. 1c) and at the highest concentration tested (5 μg/ml) incorporation was inhibited about 35% (Fig. 1d). After 24 h of incubation with tunicamycin, the cells remained intact and attached to the growth surface and showed no marked change in morphology, unlike BHK cells that become highly distended [21]. After prolonged incubation, however, mosquito cells became detached and non-viable.

Tunicamycin also has only a small inhibitory effect (20%) on the incorporation of [^{14}C]glucosamine into acid-precipitable glycoproteins of mosquito

TABLE I

GLYCOSYLTRANSFERASE ACTIVITIES OF MOSQUITO CELLS

Mosquito and BHK cells were harvested, extracted with Triton X-100 and the resultant supernatants obtained after centrifugation assayed for transferase activity using appropriate acceptors. Activities are given in nmol/mg protein per h. n.d., not determined.

Transferase:	Sialyltransferase	Galactosyltransferase		*N*-Acetylglucosaminyl-transferase
Acceptor:	Fetuin	Ovalbumin	Fetuin	Ovalbumin
Mosquito cells	0.057	< 0.1	<0.1	<0.1
BHK cells	0.415	10.9	n.d.	3.1

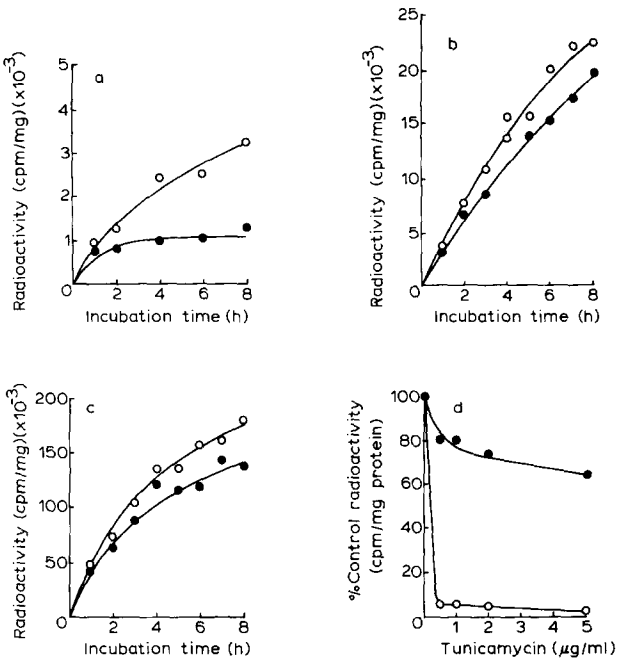

Fig. 1. Effects of tunicamycin on the uptake of precursors. Mosquito cells were labelled as described in Materials and Methods in the presence of tunicamycin (0.5 μg/ml) at 28°C for the times indicated and specific incorporations were determined after acid precipitation. (a) [^{3}H]Mannose, (b) [^{14}C]glucosamine, (c) [^{3}H]leucine, (d) cells were treated with the concentrations of tunicamycin shown at 28°C for 16 h before addition of radioactive mannose or leucine. Specific incorporation was measured after 2 h. a—c, (○) no tunicamycin; (●) plus tunicamycin. d, (○) [^{3}H]mannose; (●) [^{3}H]leucine.

cells (Fig. 1b). Glucosamine is rapidly converted metabolically to galactosamine in cells [4] and the mosquito cell glycoproteins contain both sugars [9]. Analysis of the patterns of incorporation of radioactivity from [^{3}H]-glucosamine into the hexosamines liberated from total glycoproteins by strong acid hydrolysis showed that about equal amounts of radioactivity were present in glucosamine and galactosamine residues of glycoproteins from untreated cells. In contrast, at least 80% of the radioactivity present in glycoproteins of cells pretreated with tunicamycin was recovered as galactosamine. We conclude that tunicamycin inhibits almost completely assembly of *N*-glycosidically linked oligosaccharides [9] as expected from its specificity, but has no inhibitory effect on the attachment [9] of *N*-acetylgalactosamine to polypeptides. The profound effect of tunicamycin on the incorporation of [^{3}H]-mannose (Fig. 1a, d) is consistent with this interpretation, since mannose is an important constituent of *N*-glycosidically linked carbohydrate chains of glycoproteins [4]. After an initial lag of about 1 h, tunicamycin prevented almost completely (greater than 90%) further incorporation of [^{3}H]mannose into the total glycoprotein fraction of mosquito cells (Fig. 1a).

Electrophoresis of glycoproteins from tunicamycin-treated cells. The effects of tunicamycin on incorporation of radioactive glucosamine or mannose into mosquito cell glycoproteins were confirmed by electrophoretic examination

of the glycosylated polypeptides synthesised in the presence or absence of the drug. A major glycoprotein fraction (band 4) of mosquito plasma membranes [9] with a molecular weight of 110 000 was labelled extensively with [^{3}H]-mannose (Fig. 2a) and the labelling was severely inhibited by tunicamycin (Fig. 2b). Residual labelling was detected mainly in polypeptides of lower molecular weights that were also present as minor labelled components in the untreated cells.

The results of [^{14}C]glucosamine labelling of mosquito cells are shown in Fig. 2c and d. In the absence of the drug, most of the radioactivity was present

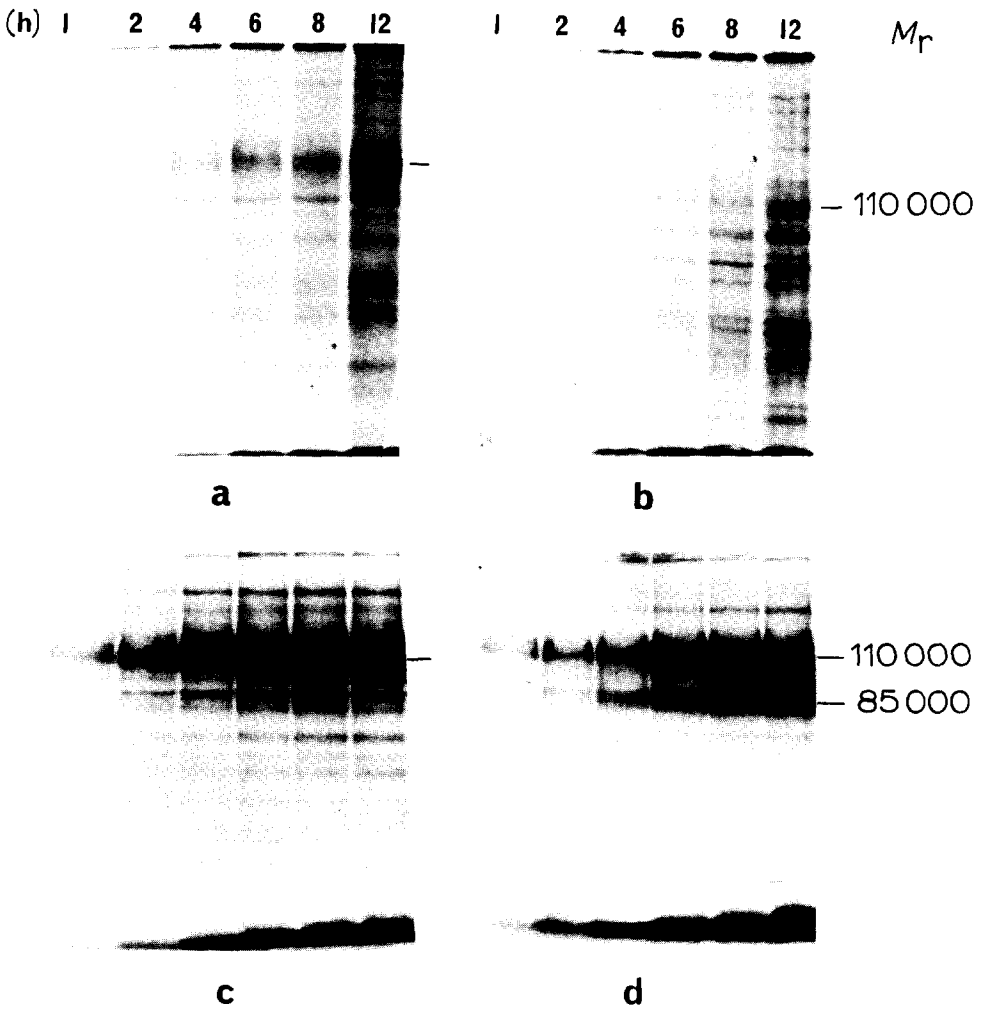

Fig. 2. SDS-polyacrylamide gel electrophoresis of metabolically labelled mosquito cells. Mosquito cells were pulsed (1—12 h) with [^{3}H]mannose or [^{14}C]glucosamine in the presence or absence of tunicamycin (0.5 μg/ml), and disrupted in SDS/2-mercaptoethanol before electrophoresis. The gels were dried and radioactive bands were located by fluorography. (a and b) [^{3}H]Mannose in control and treated cells, respectively; (c and d) [^{14}C]glucosamine in control and treated cells, respectively. The markers indicate the major M_r 110 000 glycoprotein species and a component of M_r 85 000 in drug-treated cells.

in the major plasma membrane glycoprotein fraction of molecular weight 110 000. This fraction contains [9] a mixture of glycoproteins with binding sites for concanavalin A (glycoprotein 4a) and for both concanavalin A and soybean agglutinin (glycoprotein 4b), respectively. Other minor labelled species were present in the regions expected [9] for plasma membrane glycoproteins (Fig. 2c). Tunicamycin clearly inhibited the labelling of [^{14}C]glucosamine of the major glycoprotein fraction as well as the minor bands (Fig. 2d). The overall labelling was hardly affected, however, and a new heavily labelled species appeared in the gel profiles migrating with an approximate molecular weight of 85 000 (Fig. 2d). The glycoprotein 4a contains [9] about 10% carbohydrate, containing both *N*-acetylglucosamine and *N*-acetylgalactosamine in a molar ratio of 1 : 1.6. If the former residues are linked to *N*-glycans, the synthesis of which is inhibited by tunicamycin, then the labelling with [^{14}C]-glucosamine presumably reflects an unperturbed synthesis of *O*-glycans containing *N*-acetylgalactosamine. The glycoprotein 4b contains [9] about 46% total carbohydrate, a major part of which is *N*-acetylgalactosamine. The new labelled species appearing in tunicamycin-treated cells incubated with [^{14}C]-glucosamine presumably represents a modified derivative of the major plasma membrane glycoprotein 4b lacking *N*-glycosidically linked *N*-acetylglucosamine and enriched in *N*-acetylgalactosamine present in glycans, the synthesis of which is unaffected by tunicamycin. In agreement with this interpretation, Fig. 3 shows that the majority of the *N*-acetylgalactosamine content of glycoproteins from tunicamycin-treated cells is present in base-labile [4,20] *O*-glycans.

Lectin binding properties of glycoproteins in tunicamycin-treated cells. The major glycoprotein species 4b of mosquito cell plasma membranes contains sugar sequences binding to concanavalin A and to soybean agglutinin [8,9]. The former lectin binds α-mannosyl residues of *N*-glycans in glycoproteins, while soybean agglutinin binding indicates terminal *N*-acetylgalactosamine in the mosquito glycoprotein [22]. When cell glycoproteins were separated by SDS-polyacrylamide gel electrophoresis and stained with ^{125}I-labelled concanavalin A, the major labelled species migrated with an apparent molecular weight of 110 000 (Fig. 4) as described earlier [9] for purified plasma membranes. Tunicamycin decreased the amount of reactive material migrating in this region of the gel as well as in most of the glycoprotein species detected in the untreated cell extracts (Fig. 4). Soybean agglutinin also labelled the glycoproteins of molecular weight 110 000 in the total cell extracts, confirming [9] that this glycoprotein fraction of plasma membranes is the major soybean agglutinin reactive species of mosquito cells. The extent of ^{125}I-labelled soybean labelling of the M_r 110 000 glycoprotein fraction decreased in parallel with ^{125}I-labelled concanavalin A labelling after treatment of the cells with tunicamycin and concurrently a new intensely labelled soybean agglutinin-binding species was detected migrating with a molecular weight of 85 000 (Fig. 4).

Turnover of lectin-binding glycoproteins. Since tunicamycin appears to prevent the synthesis of concanavalin A-binding sequences of mosquito cell glycoproteins, we could estimate the rates of turnover of individual glycoproteins of mosquito cells from experiments such as that shown in Fig. 4.

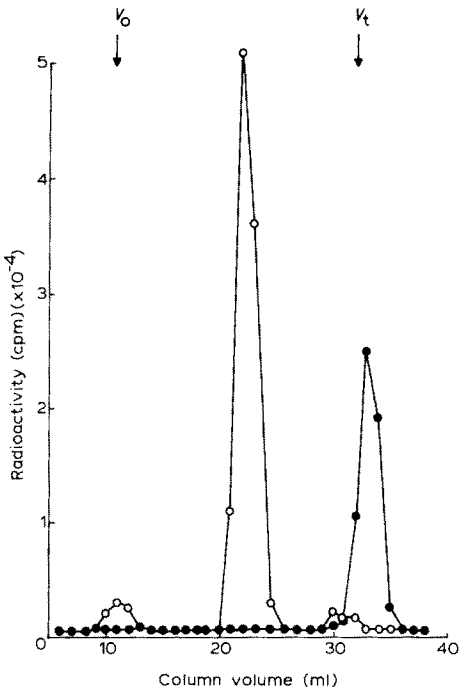

Fig. 3. Mosquito cells were treated with tunicamycin (0.5 μg/ml) at 28°C for 16 h then pulsed with [^{14}C]glucosamine for 2 h to label *N*-acetylgalactosamine residues. The glycoprotein fraction was treated with pronase to prepare a glycopeptide mixture that was separated by chromatography on Bio-Gel P6 (○), treated with alkaline $NaBH_4$ to release *O*-glycosidically linked sugars and re-chromatographed on Bio-Gel P6 (●). The arrows indicate the void volume (V_o) and total volume (V_t) of the column.

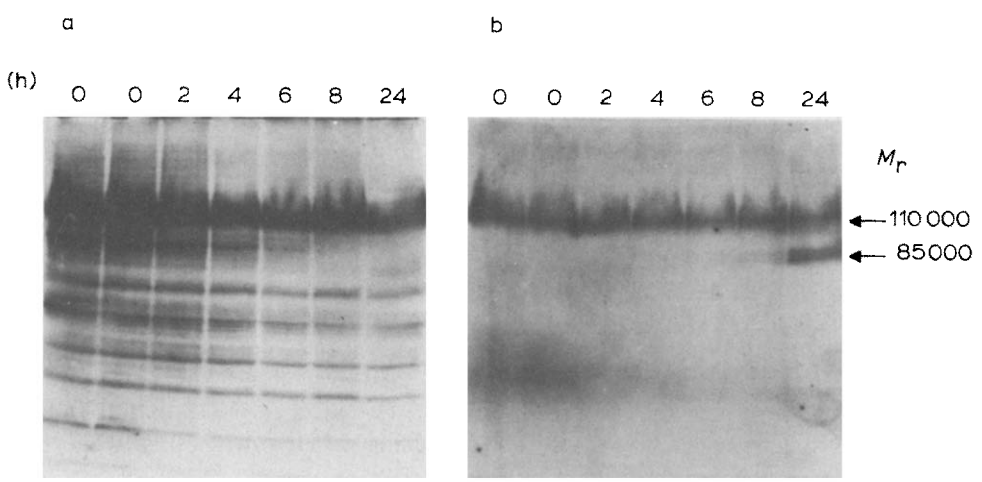

Fig. 4. SDS-polyacrylamide gel electrophoresis of tunicamycin-treated mosquito cells. Cells were grown at 28°C with or without tunicamycin (0.5 μg/ml) for the periods shown. Cells were dissolved in SDS and 2-mercaptoethanol and aliquots (250 μg protein) were used for electrophoresis. Gels were stained by ^{125}I-labelled concanavalin A (a) or ^{125}I-labelled soybean agglutinin (b) as described. The arrows indicate stained glycoproteins of M_r 110 000 and 85 000.

Selected bands were cut out from gels stained with ^{125}I-labelled concanavalin A and the amounts of glycoproteins carrying binding sites for the lectin were estimated as a function of time of treatment with tunicamycin (Fig. 5). The loss of concanavalin A binding activity in the various glycoprotein species proceeded at very different rates. Glycoprotein species 4 and 5 (see Ref. 9) exhibited the greatest turnover rates, while fractions 6, 8 and 10 of lower molecular weights showed slower rates of turnover.

The turnover of glycoproteins in tunicamycin-treated cells was also detected by surface labelling (Fig. 6). Several bands were labelled by lactoperoxidase-catalysed [^{125}I]iodination of intact cells as described previously [9] and some, but not all, of these were less labelled in cells treated for 16—24 h with tunicamycin (Fig. 6a). In particular, a polypeptide with a molecular weight of 94 000 was sensitive to tunicamycin and represents band 5 glycoprotein [9]. In contrast, band 4 glycoproteins of M_r 110 00, labelled with lactoperoxidase, showed little decrease, although the carbohydrate composition of these is affected, as shown by metabolic labelling (Fig. 2) and lectin binding studies (Fig. 4). The major soybean agglutinin-binding glycoprotein of band 4 is not labelled with lactoperoxidase [9] and changes caused by tunicamycin cannot be detected using this technique. However, Coomassie blue staining reveals a new band with a molecular weight of 85 000 in drug-treated cells (Fig. 6a) and labelling with galactose oxidase (Fig. 6b) shows a surface location for this component. Presumably, this component represents the soybean agglutinin-binding band appearing in tunicamycin-treated cells (Fig. 4), and probably (Fig. 5 inset) derived from the major soybean agglutinin-binding glycoprotein of molecular weight 110 000 in untreated cells.

Lipid intermediates. In mammalian cells sugars are transferred from GDP-mannose and UDP-*N*-acetylglucosamine to dolichol phosphate to form

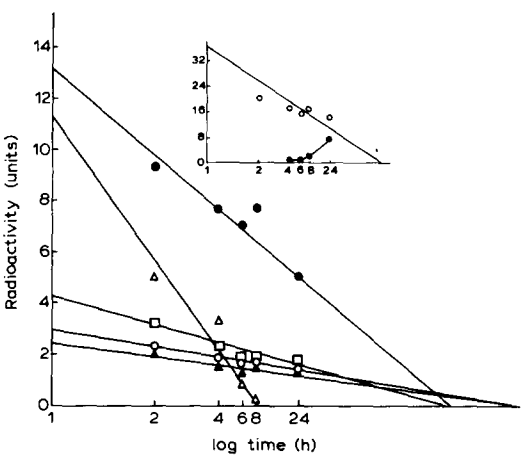

Fig. 5. Rates of turnover of glycoproteins in tunicamycin-treated mosquito cells. The ^{125}I-labelled lectin-stained gels shown in Fig. 4 were analysed for radioactivity associated with selected bands at the various times of incubation with tunicamycin. In the large-scale figure, the concanavalin A staining of bands corresponding to major plasma membraen glycoprotein species [9] is indicated: ●, 4; △, 5; □, 6; ○, 8; ▲, 10. Inset: the soybean agglutinin staining of band 4 of molecular weight 110 000 (○) and the new band of molecular weight 85 000 (●) are shown.

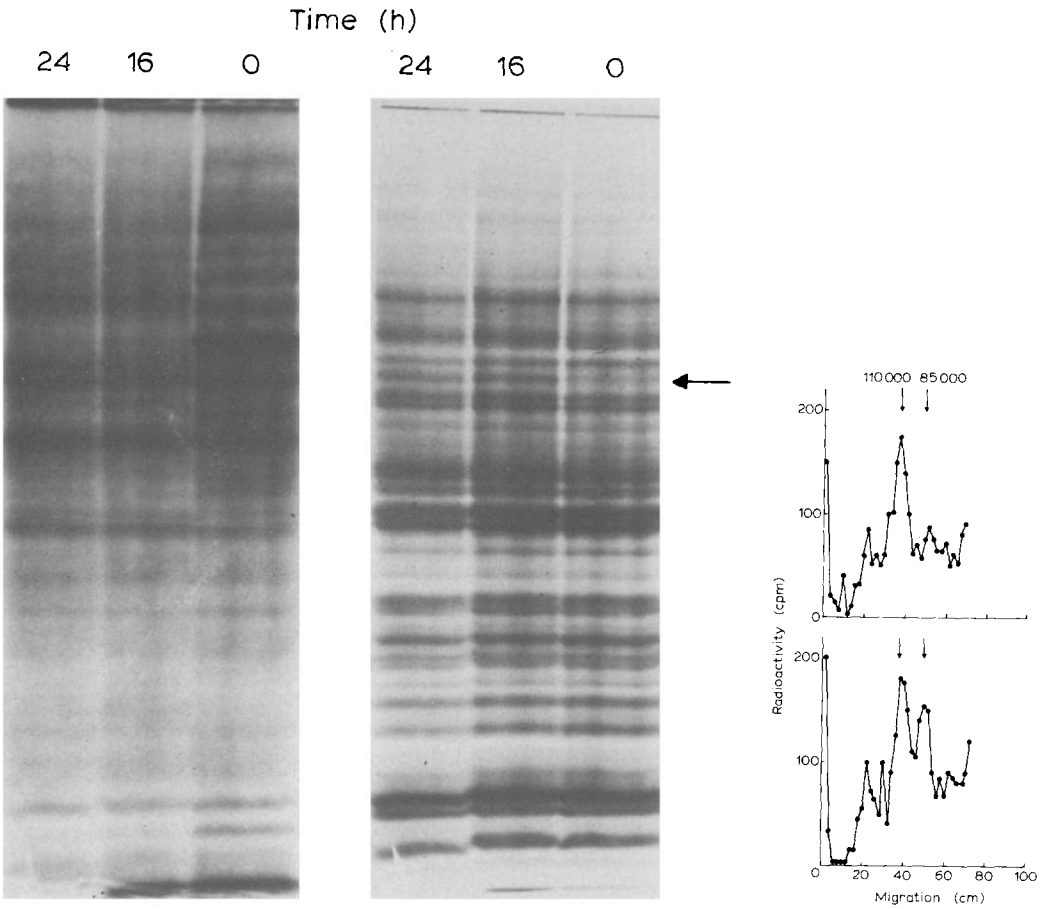

Fig. 6. Surface labelling of tunicamycin-treated mosquito cells. (a) Cell monolayers were [^{125}I]iodinated by lactoperoxidase after treatment at 28°C with tunicamycin (0.5 μg/ml) for 0, 16 and 24 h. SDS-polyacrylamide gel electrophoresis and radioautographic localization of surface-labelled polypeptides were performed (left panel) after staining with Coomassie blue (right panel). Note the new Coomassie blue-stained band in treated cells (arrowed). (b) Cell monolayers treated for 0 (top) or 24 h (bottom) with tunicamycin (0.5 μg/ml) were labelled with galactose oxidase-NaB[^{3}H]H$_4$ and examined by SDS-polyacrylamide gel electrophoresis. The arrows indicate components of molecular weights 110 000 and 85 000. Note the increase in the latter in tunicamycin-treated cells.

dolichylphosphomannose and dolichylpyrophospho-*N*-acetylglucosamine as first intermediates in synthesis of *N*-glycans of glycoproteins [1—3]. The presence of high mannose glycans in mosquito membrane glycoproteins [9] and the effects as described here of tunicamycin, a drug that specifically inhibits the latter reaction, suggested that similar intermediates were formed in Mos 20A cells. Direct evidence for this conclusion is shown in Fig. 7 and Table II. Incubation of microsomal preparations with GDP-[^{14}C]mannose at 28°C led to the rapid (Fig. 7a) formation of a compound soluble in $CHCl_3/CH_3OH$ (2 : 1, v/v) and in solvent A with the mobility (R_f = 0.61) of authentic dolichylphospho-β-mannose [23,24]. Furthermore, when cells were labelled with [^{14}C]mevalonate (10 μCi/ml) the putative dolichyl-phosphomannose was a

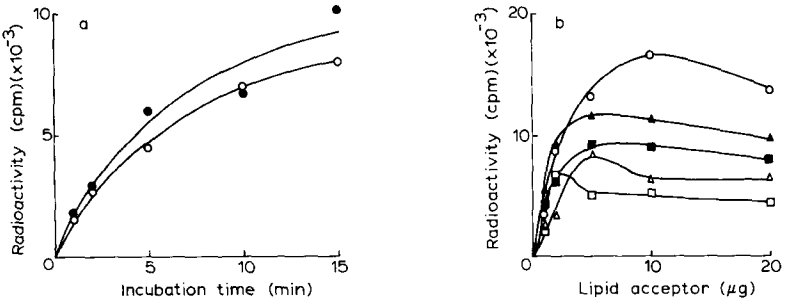

Fig. 7. (a) Transfer of [^{14}C]mannose from GDP-mannose into lipids catalysed by microsomal preparations of mosquito cells and the effect of tunicamycin. The complete mixture containing exogenous dolichol phosphate was incubated at 28°C for varying times. ○, no tunicamycin; ●, plus tunicamycin (10 μg/ml). (b) The assay mixtures contained various concentrations of dolichol phosphate (○), dihydrododecaprenyl phosphate (▲), dihydroheptaprenyl phosphate (■), solanesyl phosphate (△), or decaprenyl phosphate (□). No tunicamycin was present and incubation at 28°C was for 15 min.

major radioactive component of the $CHCl_3/CH_3OH$ cell extracts. The formation of dolichylphosphomannose required exogenous isoprenoid that at saturation produced 2000-fold enhancement of incorporation into lipids amounting to 282 pmol mannose/mg protein (Fig. 7b).

TABLE II

TRANSFER OF *N*-ACETYL[^{14}C]GLUCOSAMINE FROM UDP-*N*-ACETYLGLUCOSAMINE TO LIPID BY MICROSOMAL PREPARATIONS OF MOSQUITO CELLS

The complete mixture is comprised of microsomal protein (780 μg), 8.8 mM $MnCl_2$, 5 mM AMP, 1 mM ATP, UDP-*N*-acetyl[^{14}C]glucosamine (50 nCi), 0.089% Triton X-100 and 22.2 mM Tris-HCl buffer, pH 7.4, supplemented as indicated. Incubation of the total mixtures (225 μl) was at 28°C for 15 min and the incorporation of radioactivity into $CHCl_3/CH_3OH$ (2 : 1, v/v) extracts was estimated as described in Materials and Methods. Additions are given as final concentrations.

	Additions					
	Dithiothreitol (mM)	Triton X-100 (%)	Dolichol-phosphate (μg)	Deoxycholate (%)	Tunicamycin (μg/ml)	Radioactivity incorporated into lipids (pmol/mg protein)
Expt. A						
	—	—	—	—	—	—
	1	0.17	—	—	—	1.71
	1	0.17	10	—	—	1.67
	1	0.34	10	—	—	1.83
	1	0.51	10	—	—	2.86
	1	0.17	—	0.17	—	1.88
	1	0.17	—	0.34	—	0.37
Expt. B						
	1	0.51	—	—	—	3.25
	1	0.51	—	—	2	2.78
	1	0.51	—	—	5	2.25
	1	0.51	—	—	10	0.91
	1	0.51	10	—	—	2.96
	1	0.51	10	—	2	2.80
	1	0.51	10	—	5	2.22
	1	0.51	10	—	10	0.91

Various plant polyisoprenoids were examined for their ability to accept mannose from GDP-mannose. None of these acceptors were as active as dolichol phosphate in stimulating incorporation of radioactivity (Fig. 7b). It was clear that higher levels of incorporation were obtained by the presence of a saturated α-group and increased with increasing chain lengths from decaprenyl phosphate to dihydrododecaprenyl phosphate. Similar results to these have been demonstrated in the formation of lipid intermediates in yeast [26]. Tunicamycin at 10 μg/ml had no effect on the incorporation of mannose into polyisoprenoid lipids (Fig. 7a).

In contrast, the transfer of [^{14}C]glucosamine from UDP-*N*-acetylglucosamine into $CHCl_3/CH_3OH$ (2 : 1, v/v) extracts proceeded at the same low rates in the presence or absence of exogenous dolichol phosphate (Table II). The concentration of Triton X-100 in the reaction mixtures had a small effect on this incorporation with an optimal 2-fold enhancement at 0.51% detergent. Higher levels of Triton X-100 or the use of deoxycholate were no more or less effective. A reducing agent such as dithiothreitol was essential, since its omission from the reaction mixture completely abolished activity (Table II). Tunicamycin inhibited the reaction, although at much higher concentrations than those required to produce effects on glycosylation reactions in intact cells. Similar results were reported for membrane preparations of chick embryo fibroblasts [12].

Discussion

The results described in the present paper are entirely consistent with our earlier conclusions [9] concerning the structure of glycoproteins present in purified plasma membranes of mosquito cells. Thus, the negligible, or very low activities of *N*-acetylglucosaminyl-, galactosyl- and sialyl transferases active on the macromolecular acceptors, fetuin and ovalbumin, agree with the finding that the *N*-glycans of mosquito membrane glycoproteins are predominantly of the high mannose type and lack complex glycan chains terminated in the sequence, NeuNAc-Gal-GlcNAc.

The profound effect of tunicamycin on glycoprotein synthesis in mosquito cells confirms the presence of *N*-glycans containing the di-*N*-acetylchitoboise sequence, in agreement with the sensitivity of glycopeptides prepared from the major plasma membrane glycoproteins to *endo*-β-*N*-acetylglucosaminidase H of *Streptomyces griseus* [9]. The glycoprotein fraction of molecular weight 110 000 in whole cell homogenates or purified plasma membranes is a mixture of glycoproteins containing *N*-glycans with affinity for concanavalin A [9]. After tunicamycin treatment, the cells continue to produce the polypeptide moieties of these glycoproteins lacking *N*-glycans containing *N*-acetylglucosamine and mannose residues, but retaining *O*-glycosidically linked *N*-acetylgalactosamine. One glycoprotein (4a) [9] containing only 10% total carbohydrate, largely as *N*-glycans, is affected by tunicamycin, but its migration during SDS-polyacrylamide gel electrophoresis is little affected. The second major glycoprotein species (4b) containing 46% total carbohydrate and the majority of the *N*-acetylgalactosamine content of purified plasma membranes is also altered in cells treated with tunicamycin and its mobility changes to

indicate the loss of a substantial part of the total carbohydrate, but retaining glycans with affinity for soybean agglutinin. The difference in apparent molecular weight of 30 000 suggests that about 10—12 *N*-glycans are present, a value similar to that determined [9] earlier for the corresponding glycoprotein isolated from purified plasma membranes.

It is likely that the primary effect of tunicamycin on mosquito cell membrane glycoprotein biosynthesis is at the level of the first step in *N*-glycan assembly, namely transfer of *N*-acetylglucosamine 1-phosphate from UDP-*N*-acetylglucosamine to dolichol phosphate as in mammalian systems [13]. This reaction requires the presence in the incubation mixture of a reducing reagent, unlike mammalian systems [25], and is independent of the source of exogenous dolichol phosphate. The relative insensitivity of the reaction to the addition of exogenous dolichol phosphate has been reported previously [25] for rat liver membrane preparations and is ascribed to a close hydrophobic association of the transferase with endogenous lipids. Thus, significant stimulation of dolichylpyrophospho-*N*-acetylglucosamine formation by exogenous dolichol phosphate was observed only in the presence of high (1.125%) concentrations of Triton X-100 [25]. In contrast, the formation of dolichylphosphomannose by mosquito membrane preparations, as earlier shown in mammalian systems [24,25], was greatly stimulated by exogenous dolichol phosphate and several plant polyisoprenoid phosphates even at the lowest concentration (0.089%) of Triton X-100. If the reaction rates measured in cell-free systems represent the situation in vivo, then the formation of dolichylpyrophospho-*N*-acetylglucosamine is likely to be rate limiting, perhaps explaining the extreme sensitivity of the cells to relatively low concentrations of tunicamycin. Further work will be required to decide on this point and to show further the similarities in *N*-glycan structure [9] and biosynthesis in mosquito cells with well characterised mammalian and chick cells.

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