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ISOLATION AND CHARACTERIZATION OF MOSQUITO CELL MEMBRANE GLYCOPROTEINS

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Summary

Plasma membranes have been purified from an established cell line, Mos 20A of *Aedes aegypti*, and analysed for glycoprotein and polypeptide constituents by isoelectric focusing and sodium dodecyl sulphate polyacrylamide gel electrophoresis. A major glycoprotein of molecular weight 110 000 carrying binding sites for concanavalin A and soybean agglutinin has been purified to homogeneity. Although located on the cell surface, the 110 kdalton glycoprotein is not labelled by lactoperoxidase-catalysed radioactive iodination of whole cells. Analysis indicates the presence of *N*-glycans, containing on average nine mannose residues, and the *N*-acetylglucosaminyl-β1,4-*N*-acetylglucosamine sequence. In addition, *O*-glycosidically linked *N*-acetylgalactosamine residues are present.

Introduction

Little is known of the structure and synthesis of insect cell glycoproteins, except that they lack sialic acid [1], a sugar occupying terminal positions in mammalian glycoproteins [2]. A deficiency in the activity of sialyltransferase(s) is responsible for this absence [3], analogous to certain mutant mammalian cell lines resistant to wheat germ agglutinin [4]. Mammalian cell mutants have been used to great effect to study glycoprotein biosynthesis and the biological roles of surface membrane carbohydrates [5] and it is of considerable interest to extend such studies to insect cells. Such knowledge may provide valuable insights into the biosynthetic control of surface carbo-

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hydrate structure and the roles of cell surface glycoproteins in cellular behaviour as well as contributing to the study of the fundamental common mechanisms involved in the replication of certain pathogens, e.g., arthropod-borne viruses that also infect mammalian cells including human cells [6] and rely on host cell glycosylation pathways for assembly of viral envelope glycoproteins [2,7,8].

Following our earlier report on the lectin binding properties of mosquito cells [9], we now describe a study of the structure and biosynthesis of the surface membrane glycoproteins of the mosquito (*Aedes aegypti*) larval cell line, Mos 20A, established in culture by Varma and Pudney [10]. The present paper describes the isolation of purified plasma membranes from these cells and the solubilization, isolation and characterization of the major lectin binding glycoprotein species of the cell surface. The following paper [11] describes steps involved in the assembly of the carbohydrate chains of the glycoprotein. A preliminary account of this work has appeared [12].

Materials and Methods

Solutions. Phosphate-buffered saline (pH 7.4) contains 10 g NaCl, 0.36 g KCl, 1.449 g Na_2HPO_4 and 0.36 g KH_2PO_4 dissolved in 1 l of water. Phosphate-buffered iodide contains KI in place of KCl. Tris-buffered saline contains 1.211 g Tris and 8.7 g NaCl, adjusted to pH 7.4 with HCl and made up to 1 l with water.

Cell culture. A cell line (Mos A) established [10] from larva of the mosquito, *A. aegypti*, was provided by Dr. M.R.G. Varma, London School of Hygiene and Tropical Medicine. The cells were grown at 28°C in monolayer culture in 25 cm^2 Falcon flasks or 1 ounce medical flats in the medium described by Varma and Pudney [10], supplemented with 15% heat-inactivated fetal calf serum (56°C for 30 min), penicillin (100 units/ml) and streptomycin (100 μg/ml). After 7—9 days at 28°C, approx. 10^7 cells per flask were obtained. The cells, which were loosely attached to the growth surface, were removed readily by shaking and re-inoculated at lower density (10^5 cells per 25 cm^2 flask) for sub-culturing.

Metabolic labelling. Flasks were inoculated with 10^6 cells and 24 h later one of the following isotopes was added: [6-^{3}H]mannose (2.5 μCi/ml, 3 mCi/mmol), [6-^{3}H]uridine (10 μCi/ml, 2.9 Ci/mmol), or [U-^{14}C]palmitic acid (5 μCi/ml, 501 mCi/mmol) prepared as described previously [13]. Cells were grown for a further 3—4 days before harvesting.

Surface labelling. Intact viable cells were labelled by lactoperoxidase-catalysed radioactive iodination [14]. Cell monolayers in a 60 mm diameter petri dish (approx. $5 \cdot 10^7$ cells) were washed with phosphate-buffered saline and labelled by incubation with phosphate-buffered saline (2 ml) containing 2.5 mM glucose, lactoperoxidase (40 μg, 42 U/mg), glucose oxidase (0.4 U, 1400 U/ml, Sigma) and 100 μCi of carrier-free $^{125}I^-$. After incubation for usually 10 min at room temperature, the cells were washed three times with phosphate-buffered iodide followed by phosphate-buffered saline, removed from the growth surface and pelleted by centrifugation at 1080 × *g* for 3 min at 3°C before storage at —20°C.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis. Disc gel electrophoresis in 10% polyacrylamide gels of samples (10—400 μg of protein) disaggregated by heating at 90°C for 5 min with 1% (w/v) sodium dodecyl sulphate (SDS), 1% (v/v) 2-mercaptoethanol in 10 mM sodium phosphate, pH 7.0, was carried out as previously described [13,14]. Other samples were suspended in 0.5 M Tris-HCl, pH 6.7, containing 1% (w/v) SDS and 1% 2-mercaptoethanol, and resolved by electrophoresis on 1 or 3 mm thick slab gels of 7.5% polyacrylamide. Two-dimensional gel electrophoresis was performed exactly as described by Pena et al. [15]. Samples (400 μg of protein) were lyophilized and resuspended in 10 mM cyclohexylaminopropanesulphonic acid, pH 11, containing 9.5 M urea, 2% (v/v) Nonidet P40 and 2 mM phenylmethylsulphonyl fluoride for isoelectric focusing. The second dimension utilized electrophoresis on 7.5% polyacrylamide of material exposed to SDS and 2-mercaptoethanol.

After electrophoresis, proteins were stained by 0.1% Coomassie blue in methanol/acetic acid/water (40 : 7 : 53, v/v) for 2 h at room temperature followed by destaining in methanol/acetic acid/water (5 : 7 : 88, v/v). Carbohydrate-containing material was stained by the periodate-Schiff's reagent after fixation of the gel for 4 h in methanol : acetic acid : water (40 : 7 : 53, v/v). The gel was incubated with 1% periodic acid in 7% acetic acid in the dark at room temperature for 2 h, washed overnight with 7% acetic acid and stained with the Schiff's reagent (R.A. Lamb, London) for 2 h at room temperature before thorough washing with 0.5% $Na_2S_2O_5$ in 0.1 M HCl. Stained gels were either photographed and scanned for light-absorbing material or dried prior to radioautography using Fast tungstate intensifying screens and Fuji RX film cassettes at −70°C. The calculation of molecular weights was made from a standard curve constructed from mixtures of commercially available proteins of known size and using reovirus structural polypeptides kindly given by Dr. Alan Hay.

Enzyme assays. 5′-Nucleotidase (EC 3.1.3.5), ouabain-sensitive ($Na^+ + K^+$)-ATPase (EC 3.6.1.3), succinate dehydrogenase (EC 1.3.99.1), NADPH-cytochrome *c* reductase (EC 1.6.99.1), acid phosphatase (EC 3.1.3.2), alkaline phosphatase (EC 3.1.4.1), β-glucuronidase (EC 3.2.1.31) and β-*N*-acetylhexosaminidase (EC 3.2.1.30) were assayed by using standard methods [13, 19]. In order to determine specific activities, membrane samples were solubilized in 0.5 M NaOH and suitable amounts were used for estimation of protein content [16] using bovine serum albumin as standard.

Chemical assays. Cholesterol was determined in samples saponified in methanolic HCl for 15 min at 60°C. Material extracted with hexane was mixed with a solution of $FeCl_3$ (420 mg/5 ml acetic acid) and concentrated H_2SO_4 for 45 min in the dark and the absorbance was subsequently measured at 500 nm or at 550 nm after the addition of *o*-phthalaldehyde (50 mg/100 ml acetic acid) and concentrated H_2SO_4. Cholesterol was used as a standard. The thiobarbituric acid method was used to estimate sialic acid in the presence of interfering chromophores. One set of samples was reduced with $NaBH_4$ to eliminate the absorption due to the sialic acid chromophore and the absorption at 549 nm due to interfering substances recorded. A duplicate set of samples remained unreduced and the sialic acid content was taken by difference. *N*-Acetylneuraminic acid was used as standard.

Gas-liquid chromatography. Monosaccharides were determined as methyl glycosides after methanolic HCL hydrolysis [17].

Binding of iodinated lectins to cell fractions. Lectins were labelled by the lactoperoxidase iodination method and purified by affinity chromatography as described earlier [9]. ^{125}I-labelled lectin was diluted with unlabelled lectin to a specific activity of $5 \cdot 10^6$ cpm/mg per ml and bovine serum albumin added (50 μg/ml). Lectin (0—80 μg) was added to 0.5 ml capacity cellulose nitrate centrifuge tubes containing cell fractions (50 μg). The reaction volume was made to 0.1 ml with phosphate-buffered saline. In some experiments, the appropriate sugar hapten was added to 20 mM final concentration. Tubes were incubated for usually 30 min at 2 or 38°C and then centrifuged in a Beckman angle 50 rotor operated at 100 000 × *g* for 45 min. The pellets were washed once with phosphate-buffered saline and then counted for radioactive iodine in a gamma-spectrometer to determine the amount of bound lectin.

Staining of gels with ^{125}I-labelled lectins. ^{125}I-labelled lectin was diluted with unlabelled lectin in 0.05 M sodium phosphate buffer, pH 6.5, containing 0.5 M NaCl to give a final concentration of $5—8 \cdot 10^7$ cpm/mg per 100 ml. Portions of this solution were incubated for 1—3 h at room temperature with gel slices or slabs previously washed with water followed by 0.5 M phosphate buffer, pH 6.5, and equilibrated in 0.05 M phosphate buffer (pH 6.5)/0.5 M NaCl. After incubation, the gel was washed thoroughly in phosphate/NaCl buffer and dried on a vacuum pump ready for radioautography.

Affinity chromatography. Concanavalin A or soybean agglutinin (each 5 mg) were coupled to CNBr-activated Sepharose 4B (1.8 g) by recommended procedures (Affinity Chromatography, Pharmacia, Sweden). The coupled gels were washed extensively with column buffer, 0.5% (v/v) sodium deoxycholate, 0.15 M NaCl, 10 mM Tris-HCl, pH 8.0, at 2°C and packed into small columns (capacity approx. 5 ml). Purified plasma membranes (2—8 mg of protein) were solubilized in 0.5% sodium deoxycholate in 10 mM Tris-HCl, pH 8, at 1 mg/ml for 30 min at 2°C [13]. After centrifugation at 100 000 × *g* for 45 min, the supernatants were adjusted to 0.15 M NaCl and applied to the lectin columns [13]. The column was eluted at 10—20 ml/h with column buffer and retarded material was desorbed with column buffer containing either 0.1 M α-methylmannoside for concanavalin A or 0.1 M galactose for soybean agglutinin. Fractions (1 ml) were collected.

Enzyme digests. Samples in 0.1 M Tris buffer, pH 8.0, containing 10 mM $CaCl_2$ and 0.01% (w/v) SDS, were treated with pronase (0.5 mg, Sigma) at 60°C for 24 h with addition of fresh pronase after 6 and 18 h. Incubation was terminated by boiling for 5 min and the soluble glycopeptide mixture was applied to a Bio-Gel P6 column (55 × 0.9 cm) using 0.15 M Tris buffer, pH 7.8, supplemented with 0.1% SDS as eluting buffer. Fractions (1 ml) were collected for analysis. Pooled fractions were desalted on a Bio-Gel P2 column (29 × 1.5 cm) using 0.02% NaN_3 as eluting buffer. Fractions (1 ml) were collected and estimated for radioactivity. Jack bean α-mannosidase was from Sigma (Type III, 20 units/mg) and human liver α-mannosidase [18] was kindly provided by Dr. B. Winchester, Queen Elizabeth College, London. Glycopeptide materials, desalted on Bio-Gel P2, were treated with α-mannosidase (0.0025—2 units) in citrate-phosphate buffer, pH 6.5, at 37°C for 2 days. The

reaction was terminated by boiling and Bio-Gel P6 chromatography was carried out as before. *Endo-β-N*-Acetylglucosamine H from *Streptomyces griseus* was obtained from Miles. Glycopeptide mixtures in citrate-phosphate buffer, pH 6.5, were treated with the enzyme (40 mU, 30 units/mg protein) at 37°C for 1 day followed by boiling and chromatography as before.

Results

Purification of plasma membranes. Mosquito cells growing in tightly packed arrays with epithelial morphology (Fig. 1a) were detached from the growth surface by vigorous shaking in Tris-buffered saline, washed three times with Tris-buffered saline by centrifugation at $1089 \times g$ for 5 min at 2°C and suspended in 10 mM Tris-HCl, pH 7.4, at $2 \cdot 10^8$ cells/ml. The cells were allowed to swell over 20 min at 2°C and were then broken in a tight-fitting Dounce homogenizer (12—15 strokes). This treatment was sufficient to break at least 95% of the cells. Each homogenate was centrifuged as above to remove unbroken cells and nuclei and the supernatants were adjusted to 1 mM EDTA and layered on top of sucrose gradients (20 ml) composed of 50, 40, 30 and 20% (w/w) sucrose in 10 mM Tris-HCl (pH 7.4)/1 mM EDTA. The addition of EDTA was necessary to prevent aggregation of cellular material. Gradients were centrifuged in a Beckman SW27 rotor for 16 h at 2°C and $80\,000 \times g$. A minor membrane band was present at the top of the 20% sucrose layer and major bands were present at the 20—30, 30—40 and 40—50% sucrose interfaces. Analysis of fractions from the gradient (Fig. 2a) showed 5'-nucleotidase, a presumptive marker enzyme for plasma membranes [19], to be concentrated in the first two membrane bands. In contrast, intracellular markers such as NADPH-cytochrome *c* reductase and succinate cytochrome *c* reductase, markers for endoplasmic reticulum and mitochondria, respectively, were concentrated at the 30—40% sucrose interface. Similarly, radioactivity associated with RNA by growth of cells with [^{3}H]uridine was also present predominantly at this interface (Fig. 2a). The presumptive lysosomal enzyme, acid phosphatase, was located about equally at the 40—50% interface and in the major plasma membrane-enriched fraction at the 20—30% interface (Fig. 2a). The plasma membrane-enriched bands between fractions [15—18] were purified further by re-centrifugation through discontinuous sucrose gradients (20 ml) formed by 40, 30 and 20% (w/w) sucrose solutions in 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and the two membrane fractions were centrifuged separately through narrow continuous sucrose gradients. Analysis (Fig. 3) showed a smooth profile in each case with mean densities of 1.08 and 1.12, respectively. These fractions were found to be enzymically and morphologically similar and were pooled. This combined plasma membrane fraction showed that the highest enrichment in cholesterol (4—5-fold) and in 5'-nucleotidase (3-fold) in 35 and 17% overall yields, respectively, compared with the homogenate (Table I). Electron microscopy (Fig. 1b) showed the presence of smooth membrane vesicles with no significant contamination by mitochondria, rough endoplasmic reticulum or lysosomal structures.

Fractionation of surface labelled cells. The activity of 5'-nucleotidase in mosquito cells is very low (Table I) compared to many mammalian cells [19]

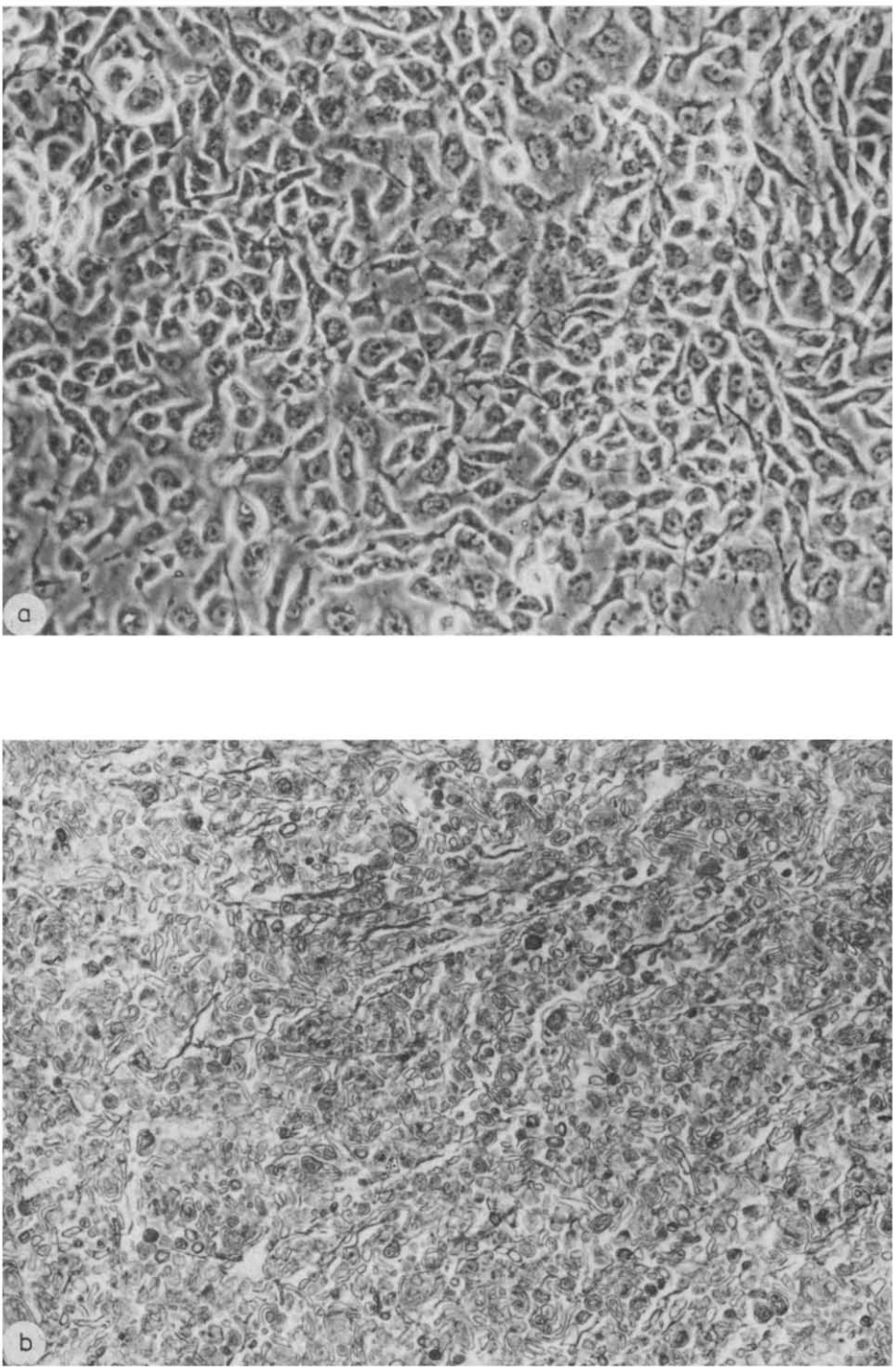


Fig. 1. (a) (top) Phase contrast photomicrograph of mosquito cells. Magnification, X265.(b) (bottom) Purified plasma membranes were fixed with glutaraldehyde at 0° C for 60 min, post-fixed in 0.25% uranyl acetate, pH 6.3, for 20 min, sectioned and embedded for electron microscopy. Magnification, X30 750.

and there is no direct evidence available for a strictly plasma membrane localization. Therefore, we monitored our fractionation scheme using cells labelled by the non-penetrating reagent, lactoperoxidase. High-density monolayer cultures were labelled with ^{125}I by lactoperoxidase-catalysed radioactive iodination and SDS-polyacrylamide gel electrophoresis showed a number of labelled polypeptide species (Fig. 2b). Trypsin treatment of intact, labelled cells removed all but one of these polypeptides, indicating a surface location (results not shown). A labelled band of molecular weight approx. 50 000 was not affected by trypsin.

Analysis of the fractions obtained from the discontinuous sucrose gradient centrifugation of a homogenate of labelled cells showed that the iodinated surface polypeptides were mainly present in the 20—30% sucrose compartments, supporting the previous conclusion that these fractions are derived from the cell surface membrane (Fig. 2b).

Polypeptides of purified plasma membrane. When mosquito cell plasma membranes were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions and the components analysed (Fig. 4), at least 11 bands were stained with Coomassie blue while at least 75 species were resolved by two-dimensional electrophoresis (Fig. 5). A polypeptide fraction of approximate molecular weight 110 000 (band 4) was the predominant Coomassie blue- and periodate Schiff-staining species and was also the band most prominently labelled by lactoperoxidase-catalysed radioactive iodination of the intact cells (Fig. 2b). This band accounted for 22, 64 and 25%, respectively, of the Coomassie blue- or periodate Schiff-stained material or labelled material recovered from the resolving gel after SDS-polyacrylamide gel electrophoresis. The M_r 110 000 glycoprotein fraction was clearly resolved by iso-electric focusing into at least six components (Fig. 5).

A low molecular weight component (less than 10 000) stained poorly with Coomassie blue but well with the periodate Schiff reagent and was labelled in membranes obtained from iodinated cells. When cells were first metabolically labelled with [^{14}C]palmitate and plasma membranes prepared, radioactivity was recovered exclusively migrating in this region of the gel. Further-

TABLE I

ANALYSIS OF TOTAL HOMOGENATES AND PLASMA MEMBRANES OF MOSQUITO CELLS

The cells were labelled by lactoperoxidase-catalysed radioactive iodination before fractionation. n.d., not determined.

	Total homogenates	Plasma membranes
5′-Nucleotidase (nmol/min per mg protein)	4.2	12.6
NADPH-cytochrome *c* reductase (μmol/min per mg protein)	1.5	0.5
Acid phosphatase (μmol/min per mg protein)	7.1	11.6
Succinate cytochrome *c* reductase (μmol/min per mg protein)	13.0	1.6
Cholesterol (μg/mg protein)	3.5	16.5
Ricin binding (μg/mg protein)	150	764
Lens culinaris lectin binding (μg/mg protein)	n.d.	356
^{125}I radioactivity (cpm/10^{-5} mg protein)	1.5	2.6

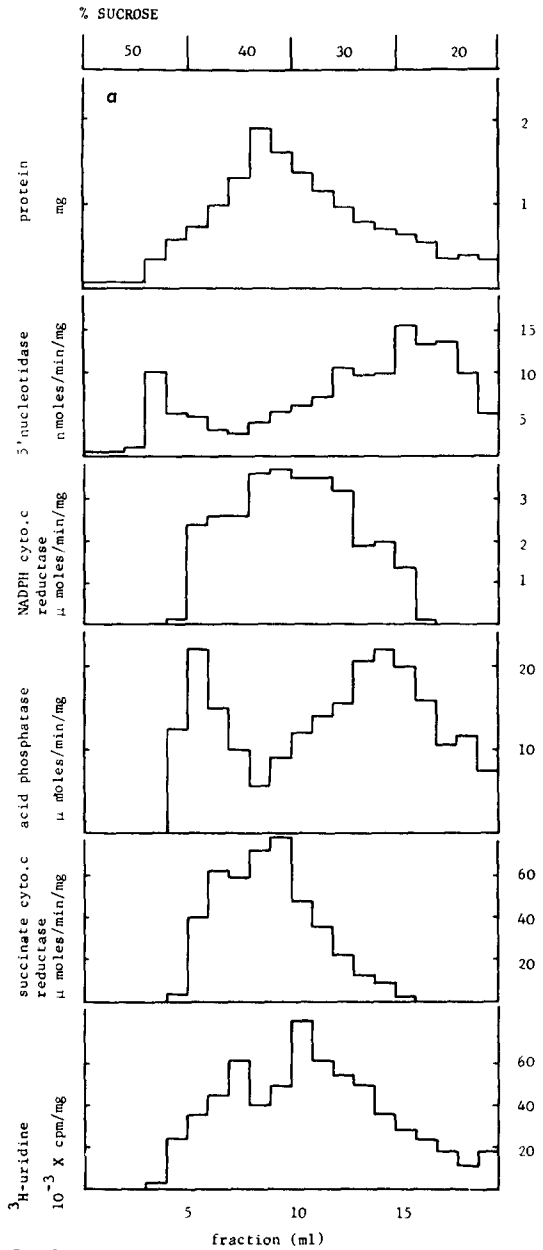


Fig. 2a.

more, when iodinated or [^{14}C]palmitate-labelled membranes were treated with $CHCl_3/CH_3OH$ (2 : 1, v/v) overnight at 2°C and the insoluble residues examined by SDS-polyacrylamide gel electrophoresis, radioactivity in the fast migrating material was completely absent. These results suggest that the low molecular material represents phospholipids and glycolipids of mosquito cell plasma membranes as reported for KB cells earlier [14].

Lectin binding components of the plasma membrane. We have shown [9] that intact mosquito cells bind several lectins to different extents and a pre-

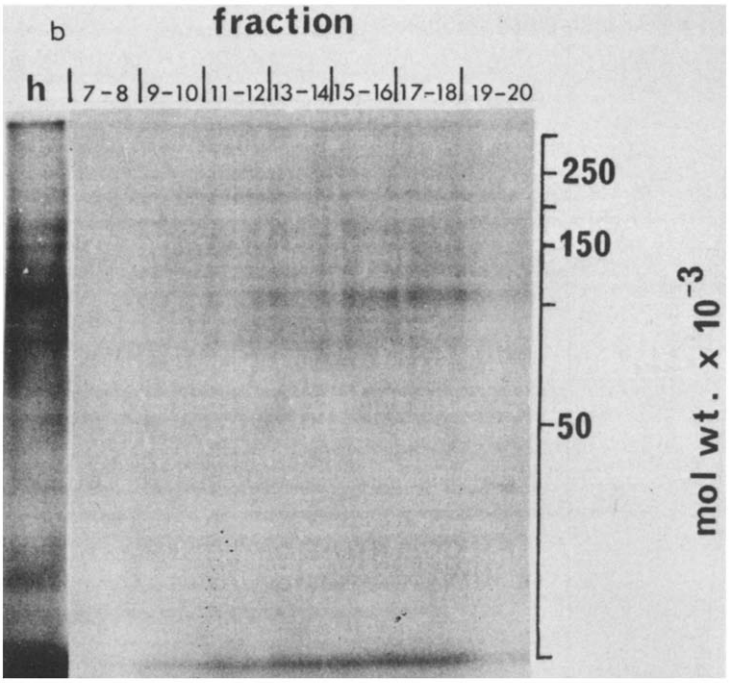


Fig. 2. (a) Analysis of mosquito cell homogenates on discontinuous sucrose gradients. The fractions were collected and assayed for activities as described in Materials and Methods. cyto., cytochrome. (b) SDS-polyacrylamide gel electrophoresis of ^{125}I-labelled whole cells and pooled fractions from the discontinuous sucrose gradient shown in a. Equal amounts of protein (250 μg) were applied and after electrophoresis the gel was dried and subjected to radioautoradiography. h, homogenate.

liminary survey of the lectin-binding properties of the isolated plasma membranes showed quantitatively similar results. Direct staining of plasma membrane components resolved by one- or two-dimensional techniques revealed many species binding concanavalin A (Fig. 5). The major glycoprotein fraction of molecular weight 110 000 was the predominant concanavalin A binding species. Interestingly, this fraction appeared to account also for most of the soybean binding capacity of the isolated membranes (Fig. 5).

Solubilization and affinity chromatography of membrane glycoproteins. Preliminary experiments showed that 0.5% sodium deoxycholate or Triton X-100 effectively solubilized all the polypeptide species of the isolated plasma membranes except for a non-glycosylated polypeptide of molecular weight 56 000. Material solubilized with 0.5% deoxycholate was fractionated on a concanavalin A-Sepharose 4B column. Two fractions were obtained (Fig. 6a), the first containing many polypeptides but not glycosylated or iodinated protein species (Fig. 6c). When the material eluted from concanavalin A-Sepharose 4B with α-methylmannoside was dialysed and fractionated on a soybean agglutinin-Sepharose 4B column, the majority of the glycosylated polypeptides and essentially all of the iodinated components were eluted unretarded (Fig. 6c). This fraction contained about 15—20% of the M_r 110 000 glycoprotein material (Fig. 6c). The material eluted with 0.1 M galactose (Fig.

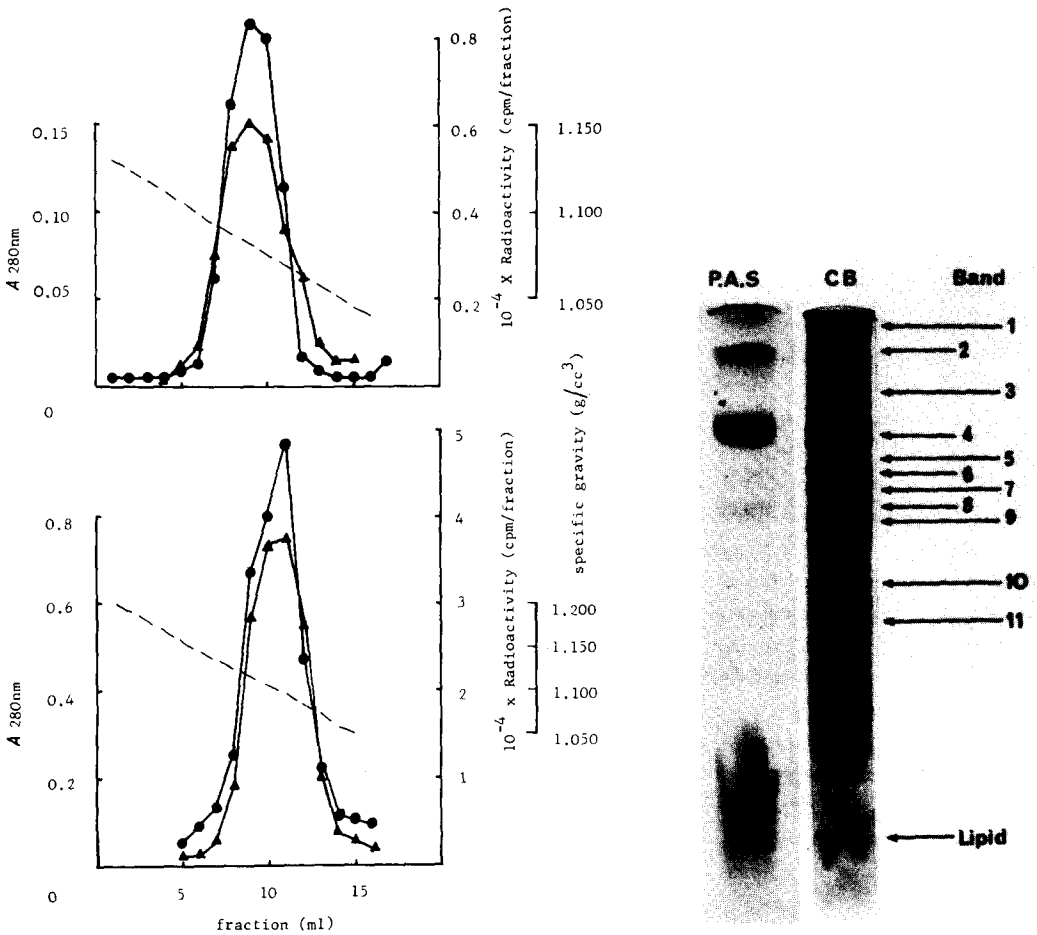


Fig. 3. Continuous sucrose density gradient ultracentrifugation of mosquito cell fractions. Membrane fractions at the 20—30% sucrose interface and in the 20% sucrose layer from Fig. 2a were applied separately to continuous sucrose gradients and analysed as described in Materials and Methods. ●, ^{125}I radioactivity; ▲, protein. The broken lines indicate sucrose concentrations.

Fig. 4. SDS-polyacrylamide gel electrophoresis of purified mosquito cell plasma membranes. PAS, periodate Schiff staining; CB, Coomassie blue staining. The band numbering is indicated.

6b) and gave a single band of molecular weight 110 000 staining with Coomassie blue or periodate Schiff reagent after SDS-polyacrylamide gel electrophoresis (Fig. 6c) but interestingly, this band was not labelled by lactoperoxidase-catalysed radioactive iodination of the whole cells (Fig. 6c). Therefore, we conclude that the M_r 110 000 fraction of plasma membranes is a mixture of glycoprotein species or subunits with different lectin affinities, some of which are labelled by lactoperoxidase iodination of whole cells.

Composition of the isolated membrane glycoprotein. The carbohydrate compositions of the purified glycoproteins obtained from a soybean column (Table II) are consistent with their behaviour during soybean agglutinin affinity chromatography. The glycoprotein(s) unretarded on this column contains

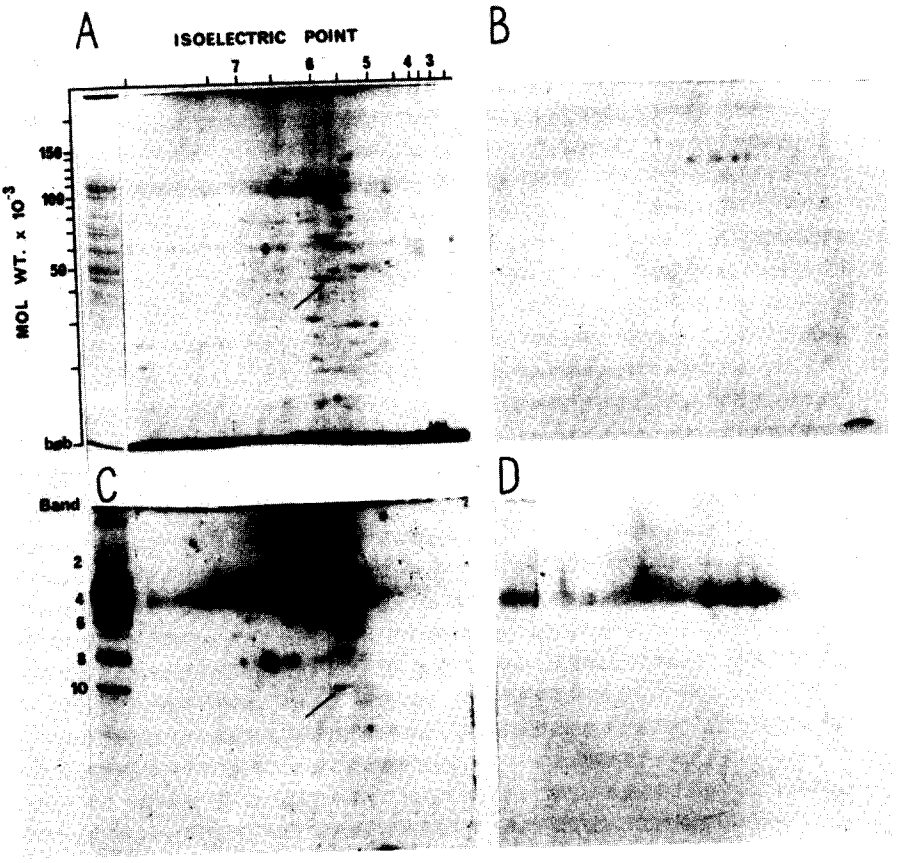


Fig. 5. Two-dimensional gel electrophoresis of purified mosquito cell plasma membranes. Membranes were analysed by iso-electric focusing followed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. A, Coomassie blue staining; B, periodate Schiff staining; C, ^{125}I-labelled concanavalin A staining; D, ^{125}I-labelled soybean agglutinin staining.

TABLE II

CARBOHYDRATE ANALYSIS OF MOSQUITO CELL PLASMA MEMBRANES AND GLYCOPROTEIN FRACTIONS ISOLATED BY LECTIN AFFINITY CHROMATOGRAPHY

	% total carbohydrate			Molar ratio, glycoproteins 4b **
	Plasma membranes	Glycoprotein		
		4a *	4b **	
Fucose	4.3	4.4	4.0	1.3
Mannose	34.7	40.5	29.4	9
Galactose	12.6	27.0	2.7	1
Glucose	13.6	17.2	11.1	3
Galactosamine	27.5	7.3	46.7	15
Glucosamine	7.3	4.5	6.2	2
Sialic acid	0.0	0.0	0.0	—
μg/mg protein	135.1	98.3	436.0	—

* Concanavalin A retarded and soybean agglutinin eluted fraction.
** Concanavalin A and soybean agglutinin retarded fraction.

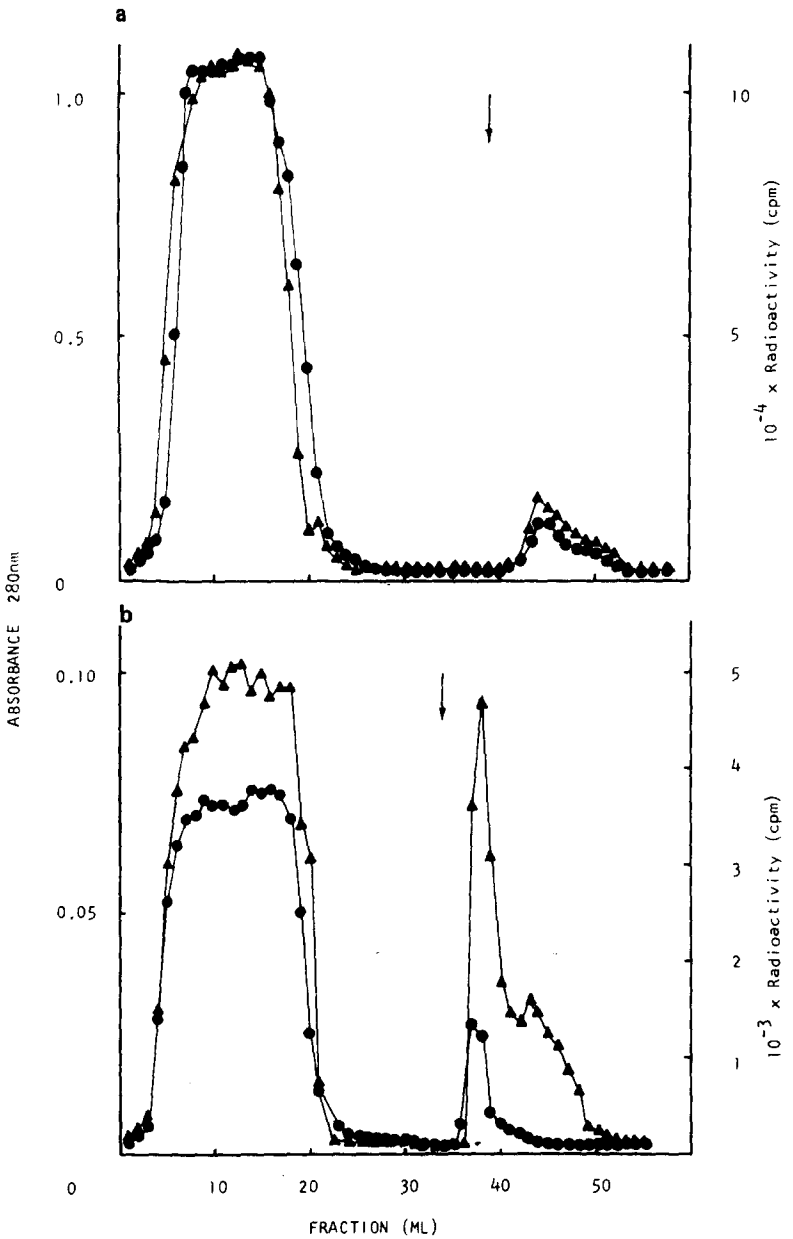


Fig. 6a and b.

little *N*-acetylgalactosamine compared with the starting plasma membranes, while the retarded glycoprotein is enriched, in agreement with the preferred affinity of soybean lectin for terminal *N*-acetylgalactosamine or galactose residues [20]. Although the unretarded glycoprotein 4a (Table II) contains a relatively large galactose content, presumably these residues are sub-terminal and not available for binding.

Inspection of the gross carbohydrate composition of glycoprotein 4b (Table II) showed the presence of mannose and *N*-acetylglucosamine in a molar

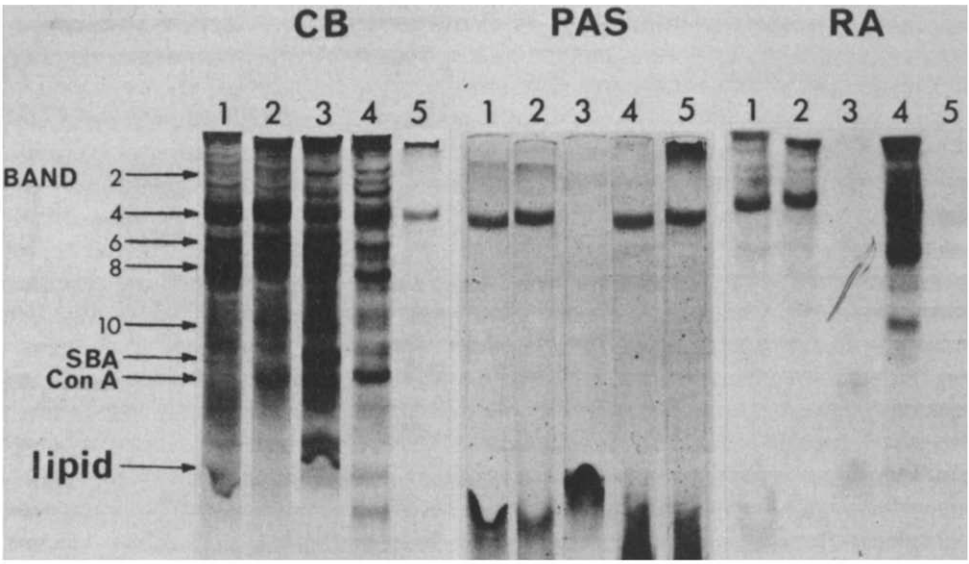


Fig. 6. (a) Purification of solubilized plasma membrane components using concanavalin A-Sepharose chromatography. Membranes from lactoperoxidase-labelled cells were used. The arrow shows the point at which elution with 0.1 M α-methylmannoside was started. ▲, protein; ●, ^{125}I radioactivity. (b) The retarded fraction from concanavalin A-Sepharose chromatography was fractionated on a soybean agglutinin-Sepharose affinity column. Elution with 0.1 M galactose was started as indicated (arrow). (c) SDS-polyacrylamide disc gel electrophoresis of fractions obtained from lectin affinity columns. 1, whole plasma membranes; 2, deoxycholate extracted supernatant; 3, concanavalin A eluted fraction; 4, concanavalin A retarded and soybean agglutinin eluted fraction; 5, concanavalin A and soybean agglutinin retarded fraction. In each case, 250 μg protein were used except track 5 (83 μg). CB, Coomassie blue staining; PAS, periodate Schiff staining; RA, radioautoradiography showing ^{125}I-labelled species; Con A, concanavalin A; SBA soybean agglutinin.

ratio of 9 : 2. In order to determine whether the mannose and glucosamine moieties of the mosquito glycoprotein 4b were present in *N*-glycans, we tested the effect of *S. griseus* β-*N*-acetylglucosaminidase H. This enzyme cleaves the di-*N*-acetylchitobiose inner unit of most mammalian *N*-glycans with the structure $(GlcNAc)_n(Man)_m$-GlcNAc-Asn, where m is greater than 3 [25]. The glycoprotein was prepared from cells labelled with [^{3}H]mannose and a glycopeptide fraction was obtained after treatment with pronase by chromatography on Bio-Gel P6 (Fig. 7a). As shown in Fig. 7c, enzyme treatment of this major mannose-containing glycopeptide fraction converted almost all of the radioactive material to a lower molecular size as indicated by its later elution from the Bio-Gel column. In order to detect terminal α-mannose residues in the major glycopeptide fraction, the [^{3}H]mannose-labelled material from Fig. 7a was treated with purified α-mannosidases from jack bean meal or human liver. Identical results were obtained with either enzyme preparation. Chromatography on Bio-Gel P6 of the reaction products showed that 56—58% of the total radioactivity was present in the peak eluting at the position expected for free mannose (Fig. 7b).

The glucose content of the major glycoprotein (Table II) was shown to be an integral constituent by metabolic labelling [29] with [^{3}H]galactose (Fig.

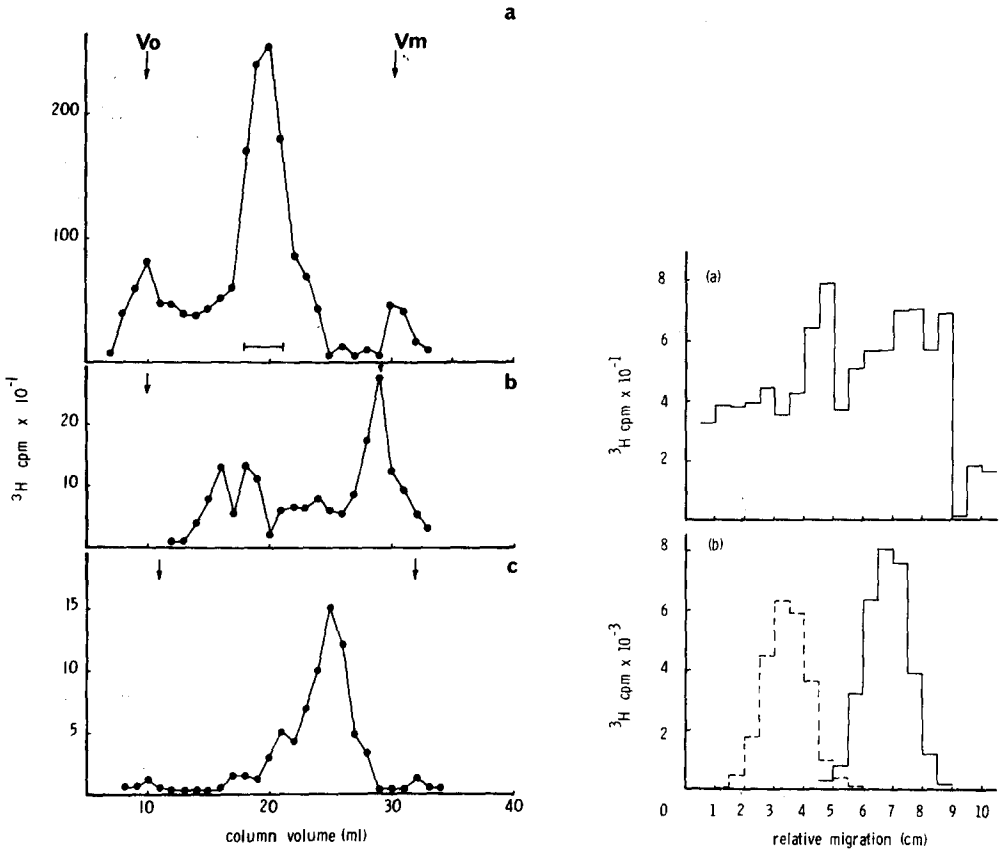


Fig. 7. Gel filtration of [^{3}H]mannose-labelled glycopeptides from mosquito cell plasma glycoprotein. The glycoprotein fraction was treated with pronase to prepare glycopeptides. The glycopeptides were then applied to a Bio-Gel P6 column (a). The fractions containing the majority of radioactivity (bar) were pooled, lyophilized, and a part was treated with α-mannosidase and rechromatographed on the Bio-Gel P6 column (b). Another sample was treated with *endo*-β-*N*-acetylhexosaminidase H and rechromatographed on the Bio-Gel P6 column (c). The void (V_o) volume of the columns is indicated. V_m is the point at which free mannose was eluted.

Fig. 8. Paper chromatography of acid hydrolysed glycopeptides. (a) A mosquito cell culture was labelled [29] with 250 μCi of [^{3}H]galactose (9.3 mCi/mmol) in glucose-free medium for 2 h at 28°C. The glycoprotein fraction was digested with pronase and glycopeptides were purified on a Bio-Gel P6 column and desalted on a P2 column as described in the text. Pooled fractions were hydrolysed in 2 M HCl for 2 h at 100°C and after drying under vacuum over P_2O_5 and KOH applied to Whatman 3MM paper and chromatographed in a solvent containing butanol/pyridine/water (6 : 4 : 3, v/v) for 20 h. The chromatogram was then cut into 0.5 cm strips and counted for radioactivity. (b) (— — —) [^{3}H]galactose standard, (———) [^{3}H]glucose standard.

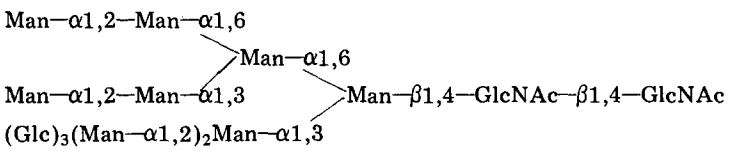
8). After acid hydrolysis, most of the radioactivity was present in glucose with a smaller amount in galactose.

Discussion

The complexity of protein and glycoprotein species in the mosquito cell plasma membrane is indicated by two-dimensional gel electrophoresis. The

heterogeneity of some glycoprotein species relates to net charge, resulting in several spots with identical mobility in SDS-polyacrylamide gel electrophoresis but different iso-electric points. Such heterogeneity is common in mammalian glycoproteins [2] containing variable sialic acid contents. Membrane glycoproteins of mosquito cells lacking sialic acid presumably differ in polypeptide net charge to produce the marked charge heterogeneity detected. A prominent polypeptide at $pI = 5.8$ and migrating with an apparent molecular weight of 45 000 corresponding to band 10 is resolved clearly by two-dimensional electrophoresis (Fig. 5, arrows). This component is stained well with concanavalin A and is also detected by lactoperoxidase labelling of whole cells. Properties similar to these are shown by glycoproteins carrying the major histocompatibility antigens of vertebrate cells. Recently, such antigents have been identified as cell surface receptors for Semliki Forest virus [40]. Semliki Forest virus is able to grow and produce infectious particles in a wide range of host cells, including mosquito cells. The possible relevance of the M_r 45 000 glycoprotein of mosquito membranes to Semliki Forest virus infection and its relation, if any, to vertebrate histocompatibility antigen remain to be determined.

In mammalian glycoproteins, carbohydrate is distributed between chains linked *O*-glycosidically to hydroxyamino acids and chains joined to asparagine through *N*-glycosidic linkages [2]. The latter chains are further divided into a 'complex' type having a variable amount of terminal galactose, *N*-acetylglucosamine, fucose and sialic acid and a 'high' mannose type [26,27]. Recently, a hybrid type containing structural features of complex and high mannose chains has been described [28] in avian cell glycoproteins. All chains have a common core region, namely Man-α1,3-(Man-α-1,6)Man-β1,4-GlNAc-β1,4-GlcNAc-Asn-(peptide), and appear to be assembled through a common intermediate [21—24,27] which is transferred as a whole to the polypeptide:



The sugars comprising the outer branches are removed to varying extents after attachment to the polypeptide and additional sugars may be transferred [29, 33]. To form complex glycans, all of the glucose and six mannose residues are removed before sequential addition of *N*-acetylglucosamine, galactose and sialic acid residues to each branch.

It is likely that the pure complex glycan type is confined to mammalian species, since the high mannose type predominates in avian cell glycoproteins [28] and in the products of eukaryotic micro-organisms such as yeasts [34]. The present data strongly suggest that the predominant *N*-glycans of the major glycoprotein of mosquito cell plasma membranes are of the high mannose type. The sensitivity to *endo*-β-*N*-acetylhexosaminidase of well defined specificity [25] confirms that it is the di-*N*-acetylchitobiose core region to which are attached at least three mannose residues in the sequence, Man-α1,3-Man-α1,6-Man-β1,4-GlcNAc [25]. The enzyme will not act on smaller

structures [25] and the rate of hydrolysis even of $(Man)_3(GlcNAc)_2$ is extremely slow. It seems probable, therefore, that all nine mannose residues are substituted onto the di-*N*-acetylchitobiose sequence in the manner demonstrated for the common intermediate of mammalian glycoproteins. Five of these mannose residues are released by α-mannosidase. It is interesting that four residues resist removal by this enzyme for which we can suggest two possible reasons. Either the α-mannosidases are specific for particular mannosidic linkages or some of the mannose residues are substituted by additional non-mannosidic residues. A rat Golgi α-mannosidase specific for α1,2-linked residues has recently been characterized [35]. The Jack bean enzyme releases the theoretical number of α-mannose residues including those attached to the Man-β1,4-GlcNAc-β1,4-GlcNAc sequence of oligosaccharides isolated from other glycoproteins, but the hydrolytic rate of α1,3 linkages is much less than that of α1,2 and α1,6 linkages [25]. It is interesting that three residues of glucose were detected in the purified major glycoprotein of mosquito cell plasma membranes. Glucose has not yet been unequivocally demonstrated as a component of *N*-glycans of membrane glycoproteins, although some soluble glycoproteins may contain glucose [36]. Usually, the glucose units of the common intermediate are very rapidly removed after formation of the linkage between oligosaccharide and polypeptide. Attempts to remove glucose from the mosquito glycoprotein by using αβ-glucosidases to expose additional mannose residues for release by α-mannosidase were unsuccessful. However, the glucose residues of the common intermediate of *N*-glycans of mammalian glycoproteins are also resistant and are susceptible only to highly specific glucosidases isolated from mammalian tissues [37—39].

The location and sequences of the chains containing the large number of *N*-acetylgalactosamine residues in the major mosquito glycoprotein are unknown. This residue usually [2] is linked directly to serine or threonine residues except in human blood group A substances in which α-*N*-acetylgalactosamine is also attached to the end of long oligosaccharide chains attached to serine or threonine residues of the polypeptide through additional *N*-acetylgalactosamine residues. The affinity of the mosquito glycoprotein for soybean agglutinin and the known specificity [20] of this lectin for terminal *N*-acetylgalactosamine suggests that these residues are exposed terminally, at least in part, in the glycoprotein and sharply distinguishes the *N*-acetylgalactosamine-containing chains of the mosquito glycoproteins from those of mammalian cells. The latter are heavily substituted with sialic acid residues and bind to soybean agglutinin only after treatment with neuraminidase [9]. As in mammalian glycoproteins, however, most of the *N*-acetylgalactosamine content of the major glycoprotein is base labile [11], in agreement with an *O*-glycosidic linkage to hydroxyamino acids and appears to be present in small oligosaccharides or as single *N*-acetylgalactosamine residues.

In summary, the major glycoprotein of mosquito cell plasma membranes contains high mannose type *N*-glycans similar to those identified in non-mammalian glycoproteins and as intermediate products in mammalian cells. The carbohydrate composition of the purified glycoprotein indicates the presence of about six to ten such glycans. In addition, approx. 100 *N*-acetylgalactosamine residues are present, probably in separate linkage to the polypeptide

chain. If all of these residues are linked directly to polypeptide, the composition of the latter must be unusual and very rich in hydroxyamino acids and, together with the high carbohydrate content, could explain the poor labelling of this surface glycoprotein by lactoperoxidase-catalysed iodination.

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