Solubilization and Fractionation of Glycoproteins and Glycolipids of KB Cell Membranes

By TERRY D. BUTTERS and R. COLIN HUGHES

National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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1. A fraction enriched in plasma membranes of human tumour KB cell line, a permissive cell for adenovirus type 5, was obtained. 2. Electrophoresis of the membranes in polyacrylamide gels with buffers containing sodium dodecyl sulphate showed that the membranes after reduction with 2-mercaptoethanol contained over 20 polypeptide species. Three polypeptides were glycosylated and had apparent mol.wts. of 92000, 72000 and 62000. 3. The glycoproteins and the specific receptors responsible for adenovirus adsorption to the membranes were readily extracted into solutions containing low concentrations of Triton X-100. Glycolipids and proteins were also made soluble. A membranous residue obtained after Triton X-100 extraction was enriched in several proteins that appeared to consist of polypeptides of lower molecular weight than the average of KB membrane polypeptides. 4. Sphingomyelin, cholesterol and triglycerides were similarly concentrated in the insoluble residue remaining after successive extractions of KB membranes with Triton X-100. Further, ceramide trihexoside was significantly less easily extracted from KB membranes than lactosyl ceramide. 5. The differences noted in the ease of extraction of membrane components are discussed. 6. The components of membranes made soluble by detergent extraction and containing the large part of the KB membrane glycoproteins were subjected to chromatography on Sepharose 6B and DEAE-cellulose and to isoelectric focusing in the presence of buffers containing Triton X-100. In general, the degree of separation into fractions enriched in individual glycoproteins was disappointing. Possible reasons for the poor fractionation of membrane components by chromatographic systems conveniently used for purification of proteins and glycoproteins of non-membranous origin are briefly discussed.

Glycoproteins and glycolipids are ubiquitous components of cellular membranes and appear to be involved in a number of important membrane-mediated processes (Winzler, 1970; Kraemer, 1971; Winzler, 1972; Hughes, 1973). They occur in many cells at the cell surface and interact with extracellular substances such as insulin (Cuatrecasas, 1971; Cuatrecasas & Illiano, 1971; Cuatrecasas, 1972) and 5-hydroxytryptamine (serotonin) (Michal *et al.*, 1972; Mester *et al.*, 1972; Bosmann, 1972) in the initial steps leading to regulation of intracellular events. Further evidence for the surface location of complex carbohydrates in intact cells is provided by the accessibility of these substances to lectins (Sharon & Lis, 1972) and to degradation by proteases (Steck *et al.*, 1971; Winzler, 1972). The first event in viral replication involves interaction of the virus with a surface-membrane receptor that in several cases appears to be a membrane glycoprotein (Winzler, 1970; Hughes, 1973) or glycolipid (Shortridge *et al.*, 1972). Cells containing such receptors are able to support a lytic viral infection (Philipson *et al.*, 1968; Lonberg-Holm & Philipson, 1969; Hughes & Mautner, 1973). Some progress has been made in characterization of the specific receptors for adenoviruses in

human tumour KB cells (Hughes & Mautner, 1973; A. Meager, T. D. Butters, V. Mautner & R. C. Hughes, unpublished work). However, a more detailed study required methods for the solubilization and fractionation of membrane components, particularly the glycoproteins, of KB cells.

Neutral detergents are often used to solubilize receptors from membranes and to isolate membrane components. However, few detailed data are available concerning the optimal conditions for the extraction of proteins, glycoproteins and glycolipids from membranes. Of the neutral detergents Triton X-100 and sodium deoxycholate are favourable extractants in view of their efficient solubilizing effects on proteins while maintaining intact many biological activities (Stoeckenius & Engelman, 1969; Tzagoloff & Penelsky, 1971; Razin, 1972; Meunier *et al.*, 1972). Neutral detergents offer the further advantage that proteins dissolved in them can be fractionated by conventional techniques including ion-exchange chromatography and isoelectric focusing (Sandermann & Strominger, 1972; Gregoriadis, 1972).

In the present paper we describe the effect of Triton X-100 and sodium deoxycholate solutions on

purified membranes of the human KB tumour-cell line. Although glycoproteins and glycolipids are extracted into solution under mild conditions by low concentrations of detergents, some differences are noted in the ease of extraction of these components. The specific receptors responsible for binding of adenovirus to KB cells (Hughes & Mautner, 1973) are readily solubilized. In contrast with the relative ease of extraction of membrane components containing carbohydrate, several proteins are very resistant to extraction. The components made soluble have been fractionated by several techniques and in certain cases relatively pure fractions enriched in particular glycoproteins of the membrane have been obtained.

Materials and Methods

Growth of cells

KB cells, derived from a human carcinoma of nasopharynx, were obtained from Flow Laboratories, Irvine, Ayrshire, U.K. and grown in monolayer cultures in Leibowitz L-15, Eagles BHK or Eagles BHK/Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] medium, supplemented with 10% (v/v) foetal bovine serum and 10% (v/v) tryptose phosphate broth which were also from Flow Laboratories. Penicillin and streptomycin were added to give final concentrations of 100 units/ml and 100 µg/ml respectively.

Labelling procedures

Where radioactively labelled membranes were required a proportionate number of the cells were grown to confluency from 24h cultures in medium containing one or a mixture of the following: D-[6-³H]glucosamine (3.3 µCi/ml; sp. radioactivity 5 Ci/mmol), L-[³⁵S]methionine (3.3 µCi/ml; sp. radioactivity 125 Ci/mmol), D-[¹⁴C]glucosamine (1 µCi/ml; sp. radioactivity 55 mCi/mmol), L-[³H]methionine (20 µCi/ml; sp. radioactivity 320 mCi/mmol). To determine the identity of the labelled sugar after incorporation, samples were hydrolysed in 4M-HCl at 100°C for 4h. The hydrolysates were dried, dissolved in water and chromatographed on Whatman 3MM paper in butan-1-ol-pyridine-water (6:4:3, by vol.) or in a solvent system described by Fischer & Nebel (1955). Standards of glucosamine, galactosamine, galactose, mannose, glucose and fucose were located by alkaline AgNO₃ (Smith, 1958) and corresponding unstained sections were cut out, eluted with water and the eluents counted for radioactivity.

When radioactive palmitic acid was used, it was first prepared as described by Robbins & MacPherson (1971). [U-¹⁴C]Palmitic acid of specific radioactivity 501 mCi/mmol (50 µCi) as a solution in benzene was

evaporated to dryness on a rotary evaporator, resuspended in 2ml of foetal calf serum and sonicated. Then 18ml of Eagles BHK medium containing 10% (v/v) tryptose phosphate broth was added. The mixture was passed through a 0.45 µm Millipore filter. About one-half of the total radioactivity was recovered in the filtrate, as described by Robbins & MacPherson (1971). The filtrate was used for labelling experiments. Cultures of KB cells (approx. 2 × 10⁵ cells/ml) were exposed to radioactive palmitate (0.133 µCi/ml) for 2 days before harvesting.

Preparation of plasma membranes

A method similar to procedures described by Warren *et al.* (1966) and Atkinson & Summers (1971) was developed. Monolayer cultures of KB cells (approx. 2 × 10⁸ cells) labelled appropriately, were rinsed with phosphate-buffered saline (Evans & Bruning, 1970) and scraped from the glass surface with a rubber 'policeman' into phosphate-buffered saline at 2°C. The cells were sedimented at 1086g at 2°C for 3min. The cell pellet was washed once with phosphate-buffered saline and finally taken up in 20ml of 10mM-Tris-HCl buffer, pH8, and left on ice to swell for 30min. The cells were then broken in a Dounce glass homogenizer [0.076mm (0.003in) clearance]. Homogenization of the cell pellet was monitored by phase-contrast light-microscopy until approx. 90% of the cells were broken. This usually required about 10–15 strokes. The homogenate was centrifuged at 1086g for 3min and the supernatant (approx. 20ml) was layered on top of a gradient containing 5ml each of 50, 30 and 20% (w/w) sucrose in 10mM-Tris-HCl buffer, pH8. The gradients were centrifuged in the Beckman L2 65B centrifuge with a SW 27 rotor for 16h at 2°C and approx. 80000g. A large membrane band at the 30–50% interface was harvested with a glass Pasteur pipette, diluted with cold 10mM-Tris-HCl buffer, pH8, and centrifuged at 12000g for 1h. The pellet was suspended in cold buffer (5ml) and layered on top of a similar discontinuous sucrose gradient (9ml) containing 3ml of each concentration of sucrose solution. The gradients were centrifuged as above in the smaller capacity buckets of the SW 27 rotor. The membrane band harvested from the 30–50% interface was purified further by ultracentrifugation through a continuous sucrose gradient (16ml) formed by running 20% (w/w) sucrose (8ml) into 40% (w/w) sucrose (8ml). The sample suspended in 10mM-Tris-HCl buffer, pH8 (2ml), was placed on top of the gradient and centrifuged in the SW 27 rotor as above. A single visible membrane band was formed. Fractions (0.9ml) were collected from the bottom of the gradient tube with a syringe fitted with a side arm. Each fraction was assayed for membrane-specific markers as described in the Results section.

The fractions containing membrane material were pooled, diluted in cold 10mM-Tris-HCl buffer, pH8, and sedimented at 12000g at 2°C for 1h. The membrane preparation was resuspended in 5ml of the same buffer and stored at -20°C.

Assays

Plasma-membrane enzyme markers were used to establish the purity of the preparation. 5'-Nucleotidase was assayed by the method of Michell & Hawthorne (1965). Na⁺-K⁺-activated ATPase (adenosine triphosphatase) activity was determined as described by Wallach & Kamat (1966). The amount of subcellular contamination by mitochondria and microsomal fractions was characterized by assaying for succinate dehydrogenase activity by the method of Arigoni & Singer (1962), as modified by Forsberg & Ward (1972), and glucose 6-phosphatase (Swanson, 1955) respectively.

Protein was determined (Lowry *et al.*, 1951) after first solubilizing membrane samples in 0.5M-NaOH. Bovine serum albumin was used as standard.

Radioactive counting of aqueous samples (0.5ml) was carried out in 5ml of Bray's (Bray, 1960) scintillation fluid or a toluene-Triton X-100-based scintillation fluid (Patterson & Green, 1964) with a Packard scintillation spectrometer. The efficiency of counting for the isotopes used were as follows: ³H, 21-30%; ¹⁴C, 76-85%; ³⁵S, 80-85%. In dual-labelling experiments the amount of spill-over of ¹⁴C into the ³H channel and ³⁵S into the ³H channel was found to be 16% and 19% respectively. In these cases the ³H values were adjusted accordingly. Paper strips were counted for radioactivity in 2ml of toluene containing 2,5-diphenyloxazole (0.4%) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%).

Adenovirus fibre binding

The details of the binding test with crystalline adenovirus type 5 fibre (Mautner & Pereira, 1971) labelled with ¹²⁵I have been described elsewhere (Hughes & Mautner, 1973). Briefly, membrane samples equivalent to 50-100µg of protein were suspended in Leibowitz L15 medium (0.02ml) with various amounts (up to 0.05µg) of ¹²⁵I-labelled fibre (sp. radioactivity 10⁶c.p.m./µg of protein). The incubation mixtures (0.05ml) were kept at 37°C for 1h. Ice-cold 10mM-Tris-HCl buffer, pH7 (0.5ml), was then added and each assay mixture was centrifuged at 2°C and 90000g for 1h. The pellets were washed by centrifugation, twice with cold buffer and finally the amount of bound ¹²⁵I-labelled fibre was determined in a Nuclear-Chicago gamma spectrometer.

Polyacrylamide-gel electrophoresis

Membrane samples (60-200µg of protein) were prepared for electrophoresis by heating at 90°C for 5min in 10mM-sodium phosphate buffer, pH7, containing 1% (v/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol. After cooling, one drop of tracking dye, 0.05% Bromophenol Blue, and one drop of glycerol were added and mixed. The samples were layered directly on to tubes (10cm×0.5cm) containing 10% (w/v) polyacrylamide and three-quarters the normal amount of cross-linker, prepared by the method of Weber & Osborn (1969). After an initial current of 1mA/gel for 1h, the current was set at 5mA/gel for 20h at room temperature by which time the tracking dye had moved three-quarters of the way through the gel.

Proteins were made visible by staining overnight in a 0.25% solution of Coomassie Brilliant Blue dissolved in methanol-acetic acid-water (40:7:53, by vol.), or in a 0.1% solution of Amido Black in the same solvent. Destaining was done in a 7.5% solution of acetic acid containing 5.0% methanol. Carbohydrate components were localized by the periodic acid-Schiff methods described by Zacharius *et al.* (1969) and Kobylka *et al.* (1972). Radioactivity was determined by solubilizing 2mm-thick gel slices in 1ml of 20% (v/v) H₂O₂ at 60°C overnight and counting the aqueous sample for radioactivity as described above.

Apparent molecular weights were determined by comparing the electrophoretic mobility with a standard calibration curve constructed by running in parallel the polypeptides of purified adenovirus (Russell & Skehel, 1972).

Extraction of membranes with detergents

In a typical experiment samples (0.05ml, containing 100µg of protein) of a radioactively labelled membrane preparation were sedimented at 33000 rev./min (100000g) for 30min at 2°C. The pellets were resuspended at 1mg/ml in 10mM-Tris-HCl buffer, pH8, containing 0.5% (v/v) Triton X-100, a polyoxyethylene *p*-t-octylphenol with an average length of 9-10 ethylene oxide units (mean mol.wt. 638) purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. The tubes were incubated at 37°C or 2°C for different times up to 24h, and then the samples were diluted to 0.5ml with Tris-Triton X-100 solution and centrifuged as above. The supernatants were withdrawn with a syringe. Portions (0.05ml) were assayed for radioactivity.

In other experiments, membrane samples containing 100µg of protein were treated for 30min at 37°C or 2°C in 10mM-Tris-HCl buffer, pH8, containing different concentrations (0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 5%) of Triton X-100. The supernatants

obtained after high-speed centrifugation were assayed as described above.

In large-scale experiments, plasma membranes (10–20mg of protein) were pelleted at 27000g for 1h at 2°C and resuspended (2–2.8mg/ml) in a solution of 0.5% Triton X-100 in 10mM-Tris-HCl buffer, pH8. After incubation for 30min at 2°C the soluble fraction (S1) was obtained by centrifugation. The pellets were re-extracted under the same conditions to obtain a second supernatant fraction (S2). The twice-extracted pellet was washed once with 10mM-Tris-HCl buffer, pH8, and finally suspended in the original volume of this same buffer.

Thin-layer chromatography of membrane lipids

A total lipid extract was obtained by two separate treatments of plasma membranes, labelled radioactively with [^{14}C]palmitate, with chloroform-methanol (2:1, v/v). The combined extracts were evaporated to dryness under N_2 , reconstituted with chloroform-methanol (2:1, v/v) and spotted on to fast-running pre-coated silica-gel plates (20cm $\times$ 20cm)

(E. Merck, Darmstadt, Germany). The plates were run in the two-dimensional system of Gray (1967). The radioactive lipids were located by radioautography with Kodak No-screen X-ray film. Radioactive lipids were scraped from the plates with a spatula into scintillation bottles. A toluene-Triton X-100-based scintillation fluid (5ml) was added and the radioactivity in each separated lipid component determined. Similar procedures were used for other extracts of KB cell membranes. The identity of each spot was tentatively made by comparison with published patterns (Gray, 1967) and by running authentic samples of several compounds, including glucosylceramide, lactosylceramide and globoside.

Results

The final step in purification of the KB cell membrane fraction is shown in Fig. 1. After centrifugation through a continuous sucrose gradient a single membrane band was visible. The band was radioactively labelled with both [^{35}S]methionine and [^3H]glucosamine and contained 5'-nucleotidase

Fig. 1. *Isopycnic banding of KB cell membranes on a continuous 20–40% (w/w) linear sucrose gradient*

Membranes labelled with [^3H]glucosamine and [^{35}S]methionine were placed on the gradient and centrifuged at 80000g overnight at 2°C. Fractions (0.9ml) were taken from the bottom of the gradient and analysed for 5'-nucleotidase activity ($\circ$), ^3H (not shown) and ^{35}S radioactivity (Δ). The peak tubes coincide with the visible membrane band sedimenting at a density of 1.15. Densities (---) were determined with a refractometer. The direction of sedimentation is to the left.

Table 1. *Purity of KB cell membrane fractions*

Membranes obtained as described in Fig. 1 were assayed. Each activity is expressed as specific activity of the final preparation and as the ratio of the same activity to that of the starting homogenate.

Enzyme	Specific activity	Activity ratio
5'-Nucleotidase	59.6*	4.8
Na ⁺ -K ⁺ -activated ATPase	24.4*	10
Succinate dehydrogenase	$1.43 \times 10^{-3}\dagger$	0.15
Glucose 6-phosphatase	0*	—
¹²⁵ I-labelled adenovirus fibre binding	0.15‡	33

* μmol of P_i liberated/h per mg of protein at 37°C.

† μmol of substrate utilized/min per mg of protein at 25°C.

‡ μg of fibre bound/mg of protein at 37°C at saturation.

Table 2. *Apparent molecular weights of KB plasma-membrane proteins and glycoproteins*

Membranes were solubilized in sodium dodecyl sulphate-2-mercaptoethanol and fractionated by electrophoresis in polyacrylamide gel as described in Plate 2. Proteins were located with Amido Black or Coomassie Blue and glycoproteins with a periodate-Schiff reagent. Molecular weights are determined from the mobility of each band compared with proteins of known molecular weight. The three major glycoproteins are termed PAS I, PAS II and PAS III respectively and are periodate-Schiff-positive bands.

Band no.	Protein (intensity of stain)	Glycoprotein (intensity of stain)	$10^{-3} \times$ Average apparent mol. wt.
1	++		135
2	++		120
3	++		100
4 (PAS I)	++	+++	92
5	++		86
6	++		82
7	++		78
8 (PAS II)	++	+	72
9	++		68
10 (PAS III)	+++	++	62
11	+		56
12	+		54
13	+		52
14	+		50
15	++		45
16	++		40
17	++		36.5
18	++		34
19	+++		28
20	++++		24
21	++		19.5
22	+++		17.5
23	+++		16
24	++		13

activity. Electron micrographs of thin sections of the purified membranes showed a population of predominantly smooth-surfaced membrane vesicles. Few ribosomes were present and nuclei, mitochondria and rough-surface endoplasmic reticulum were not detected (Plate 1). In agreement with the latter findings the purified membranes contained no detectable activities of marker enzymes for mitochondria or endoplasmic reticulum. The specific activities of 5'-nucleotidase and Na⁺-K⁺-activated ATPase, which are recognized markers of plasma membranes in many cells, were enriched approximately five- to eight-fold and 10–15-fold respectively in the purified fraction relative to the original cell homogenate (Table 1). Another marker of the surface membrane of KB cells, the specific binding of adenovirus type 5 fibre protein (Hughes & Mautner, 1973), was increased about 20–30-fold. The membrane preparation contained no detectable levels of glucose 6-phosphatase, indicating negligible contamination by intracellular microsomal membranes. However, contamination by other intracellular smooth membranes such as lysosomal membranes or Golgi elements is not ruled out.

The yield of purified plasma membrane accounted for 2–4% of the total protein of starting homogenates and approx. 10–15% of the total cellular 5'nucleotidase.

Polyacrylamide-gel electrophoresis

The components of purified KB cell membranes were separated by electrophoresis in polyacrylamide gel in 0.1% sodium dodecyl sulphate and stained for protein or carbohydrate. Dissociation of the membranes before electrophoresis was obtained by heating in 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol and typical profiles are shown in Plate 2. Table 2 shows the numbering of the various protein bands and their staining properties with Coomassie Blue and Amido Black. The number of bands obtained was very reproducible in ten different preparations of membranes. The KB cell membrane contains three major glycosylated polypeptides as shown by electrophoresis in sodium dodecyl sulphate-polyacrylamide gel under reducing conditions. The apparent molecular weights of these components were estimated as respectively 92000, 72000 and 62000 with reference to the non-glycosylated structural proteins of adenovirus type 5 (Russell & Skehel, 1972). [³H]Glucosamine-labelled membranes were also separated by polyacrylamide-gel electrophoresis and the distribution of radioactivity in these gels is shown in Fig. 2. The relative degrees of incorporation of radioactivity from [³H]glucosamine into the three major glycoproteins were dissimilar to their proportions as estimated by glycoprotein staining with a periodate-Schiff reagent (Table 2).

Fig. 2. Radioactivity profiles of ^3H -labelled glycoproteins after polyacrylamide-gel electrophoresis of (a) intact KB cell membranes and (b) delipidated membranes

Membranes prepared from KB cells grown in the presence of radioactive glucosamine were subjected to sodium dodecylsulphate-polyacrylamide-gel electrophoresis either before or after extraction with chloroform-methanol (2:1, v/v). The gels were sliced and each slice counted for radioactivity. The arrows 1 and 2 indicate the positions at which PAS I and PAS II (Table 2) are detected and arrow 3 indicates the position of the dye marker.

At least 80% of the radioactivity incorporated from [^3H]glucosamine into the purified KB membrane fraction was recovered after acid hydrolysis as glucosamine and a small proportion of galactosamine in the relative proportions 3.5:1. The proportion of radioactivity converted into sialic acids was not determined. The excellent recovery of radioactivity suggests that very little conversion of the radioactive precursor into neutral sugars had occurred. It is noteworthy that a fast-moving band located with the periodate-Schiff reagent (Plate 2) and assumed to be glycolipid (Lenard, 1970) was very poorly labelled with [^3H]glucosamine. This suggests that the glycolipids contain predominantly neutral hexoses and that glycolipids with larger carbohydrate chains containing hexosamines are not present in large amounts.

Solubilization of KB cell membranes with Triton X-100

A series of experiments was carried out to determine the optimal conditions for extraction of glycoproteins from KB cell membranes. During incubation of membranes labelled with [^{14}C]glucosamine and [^3H]methionine with various concentrations of Triton

Fig. 3. Solubilization of proteins and glycoproteins from KB cell membranes with Triton X-100

(a) Membranes labelled with [^{14}C]glucosamine (Δ , $\blacktriangle$) and [^3H]methionine ($\circ$, $\bullet$) were incubated at 37° ($\blacktriangle$, $\bullet$) or 2°C (Δ , $\circ$) for 30 min in the presence of the stated concentrations of detergent. The mixtures were centrifuged at $100000g$ for 1 h at 2°C and the proportions of each radioisotope present in the supernatant fractions were determined. (b) Binding of [^{125}I] fibre bound to the membrane residues obtained after Triton X-100 extraction as described above. $\bullet$, Binding at 37°C ; $\circ$, binding at 2°C .

X-100, radioactive proteins and glycoproteins were made soluble (Fig. 3). Solubility is defined by the presence of these components in supernatants obtained by centrifugation at $100000g$ for 1 h. We have consistently found that the proteins of KB cell membranes are solubilized less easily by Triton X-100 and by sodium deoxycholate (T. D. Butters & R. C. Hughes, unpublished work) than the glycoproteins. In a number of independent experiments, extraction at 37°C with 0.5% Triton X-100 removed 50–70% of the total [^{14}C]glucosamine radioactivity associated with glycoproteins and only 30–50% of the total [^3H]methionine radioactivity. Since a proportion of the latter amino acid is almost certainly present in glycoproteins, the differential rates of solubilization of glycosylated and non-glycosylated proteins are more divergent than would be immediately apparent from the results as expressed in Fig. 3. The optimal concentration of detergent for efficient solubilization is 0.5%. At higher concentrations no more radioactivity is extracted. Solubilization in 0.5% Triton X-100 proceeds rapidly at 2° and 37°C and is virtually

EXPLANATION OF PLATE I

Electron micrograph of purified KB plasma membranes

Plasma-membrane samples were prefixed with 5% glutaraldehyde in 0.2M-cacodylate buffer (pH 7.2) for 1 h at room temperature (22°C), and then washed for 8 h with frequent changes of fresh buffer. Thin sections were prepared by enrobing the pellets in 2% agar, small blocks were cut and processed as described by Ryter & Kellenberger (1958). The specimen blocks were dehydrated in ethanol and embedded in Epikote 812 resin. The silver sections, 0.07 μ m thick, were poststained with uranyl acetate and lead citrate. Magnification $\times 63280$.

EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of KB cell membranes

Details of sample preparation and staining are given in the Materials and Methods section. (a) Membranes stained with Coomassie Blue; a complete numbering of the stained bands is given in Table 2; (b) membranes stained with periodate-Schiff reagent; (c) structural proteins of adenovirus type 5. The viral polypeptides in (c) are from top to bottom a heavily stained band of hexon (mol.wt. 115000), penton (mol.wt. 70000), fibre (mol.wt. 62000), core protein (mol.wt. 46000), core protein 2 (mol.wt. 22000) and an unidentified polypeptide moving more quickly. The origins are at the top.

EXPLANATION OF PLATE 3

Polyacrylamide-gel electrophoresis of fractions obtained from KB cell membranes by extraction with Triton X-100

(a) and (d), First Triton X-100 extract; (b) and (e), second Triton X-100 extract; (c) and (f), twice-extracted membrane pellet. (a)–(c), Gels stained with periodate–Schiff; (d)–(f), gels stained with Amido Black; (g) is a similar preparation to that shown in gels (c) and (f) stained with Coomassie Blue. The origins are at the top.

EXPLANATION OF PLATE 4

Polyacrylamide-gel electrophoresis of fractions obtained from chromatography of Triton X-100 extracts on Sepharose 6B
(a) and (d), First eluate GP I; (b) and (e), second eluate GP II; (c) and (f), third eluate GPIII. (a)–(c), Gels stained with periodate–Schiff reagent; (d)–(f), gels stained with Coomassie Blue. The origins are at the top.

Fig. 4. Chromatography on Sepharose 6B of Triton X-100 extracts of purified KB cell membranes labelled with [^3H]glucosamine ($\circ$) and [^{35}S]methionine (Δ)

Chromatography and analysis of the fractions were carried out as described in the text. The fractions that were pooled are indicated by the cross-hatched boxes and the arrow indicates the total volume of the column.

completed within 1 h. A time of 30 min was used subsequently for large-scale extraction at 2°C of KB cell membranes with 0.5% Triton X-100. Fig. 3 also shows the capacity of KB cell membranes extracted with various concentrations of Triton X-100 to bind ^{125}I -labelled adenovirus fibre. The amounts of fibre bound to membranes extracted at either 37° or 2°C with detergent solutions of concentration greater than 0.25% were 15 and 30% respectively, compared with the binding capacity of unextracted membranes. The specific adenovirus receptors of KB cell membranes are therefore readily solubilized under the conditions used to remove a major part of the membrane glycoproteins and a significantly smaller proportion of the non-glycosylated proteins.

Fractionation of KB cell membrane extracts

KB cell membranes (10–20 mg of protein) were extracted twice with 0.5% Triton X-100 at 2°C for 30 min. Each supernatant obtained after centrifugation of the extraction mixtures and the final twice-extracted membrane residue were examined separately. Samples were solubilized in sodium dodecyl sulphate and 2-mercaptoethanol and subjected to electrophoresis in polyacrylamide gels (Plate 3). Replicate gels were stained with Amido Black and periodate-Schiff reagents. Clearly the bulk of all three of the major membrane glycoproteins of KB cell membranes were present in the first Triton X-100 extract (supernatant S1). The second Triton X-100 extract contains relatively little glycoprotein material, but some glycolipid is present. The twice-extracted membrane residue contains traces of glycolipid constituents but no detectable

glycoprotein. Consistently the proteins present in the residue obtained after two extractions with Triton X-100 contain polypeptide subunits of lower than average molecular size compared with the polypeptides of the original membrane preparation.

The material extracted into solution in the first supernatant (S1) was fractionated further by chromatography on Sepharose 6B in eluting buffers containing 0.5% Triton X-100. A typical elution profile is shown in Fig. 4. An excluded peak appeared to be considerably enriched in non-glycosylated proteins. The relative amounts of radioactivity derived from [^3H]glucosamine and [^{35}S]methionine in the total Triton X-100 extract and in the excluded peak E were 1.13 and 1.75 respectively. A broad included peak was eluted later from the column. Fractions across this peak contained the two radioisotopes in approximately the same ratio as in the starting material taken for chromatography. The peak was divided arbitrarily into three fractions GP I, GP II and GP III in order of elution from the column. These were concentrated by vacuum dialysis against 10 mM-Tris-HCl buffer, pH 7.2, at 2°C . The pooled fractions from Sepharose 6B chromatography were subjected to polyacrylamide-gel electrophoresis in sodium dodecyl sulphate after reduction with 2-mercaptoethanol (Plate 4). The basis of the separation of protein components of the first Triton X-100 extract (S1) on Sepharose 6B presumably is according to molecular size. Nevertheless, the three fractions contained polypeptide subunits of rather similar molecular weights as detected by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate after reduction (Plate 4). In contrast, the carbohydrate staining pattern shows that the major glycoprotein subunit PAS I (Table 2) of KB cell membranes occurs predominantly in Sepharose 6B pooled fraction GP I (Plate 4a). This fraction is very little contaminated with the other glycoprotein subunits PAS II and PAS III of lower molecular subunit size. These were found in Sepharose 6B pooled fractions GP II (Plate 4b) and to a small extent in GP III (Plate 4c). Subunit PAS I appeared to be present also in these last two fractions.

The glycolipids were concentrated in fractions GP II and GP III and were absent in fraction GP I.

Glycolipids in KB cell membrane extracts

When membranes labelled with [^{14}C]palmitate as described in the Materials and Methods section were extracted twice with 0.5% Triton X-100 in 10 mM-Tris-HCl buffer, pH 8, for 30 min at 2°C , 40–65 and 25–38% of the label was solubilized in the successive treatments. The membrane residue remaining after two extractions contained about 7–18% of the total label. During chromatography of the first Triton X-100 extract on Sepharose 6B under the conditions described in Fig. 4, the labelled lipids moved

Table 3. *Extraction of lipids from KB cell membranes*

Cells were labelled with [^{14}C]palmitate as described in the text. The radioactive lipids were extracted from whole membranes either with chloroform-methanol (2:1, v/v) (column a) or with Triton X-100 (columns b and c). The insoluble residue remaining after two separate Triton X-100 extractions was further extracted with chloroform-methanol. Each lipid extract was fractionated by the method of Gray (1967) as described in the text and the lipids were located by radioautography. Each spot was eluted and counted for radioactivity.

Spot	Probable identity	Radioactivity (% of total)			
		(a) Whole membrane	(b) First Triton X-100 extract (S1)	(c) Second Triton X-100 extract (S2)	(d) Residue
0	Origin	0.57	1.67	1.74	5.32
1		3.44	3.32	4.40	3.49
2	Sphingomyelin	3.40	2.33	4.05	3.52
3		4.18	1.38	1.66	3.19
4	Phosphatidylcholine	26.58	46.25	41.40	12.28
5	Phosphatidylinositol	4.12	2.51	1.71	0.00
6	Phosphatidylethanolamine	23.03	15.28	14.69	3.29
7	Ceramide trihexoside	8.65	1.76	1.36	7.06
8	Unknown	0.87	1.76	1.52	0.00
9	Ceramide dihexoside	0.78	0.31	0.18	0.00
10	Unknown	0.22	0.09	0.30	0.00
11	Ceramide monohexoside	1.85	13.59	17.59	1.40
12	Cholesterol triglycerides (free fatty acids)	22.31	9.75	9.40	60.46

with a micellar molecular size equivalent to about 100000, that is between tube numbers 86 and 102 (Fig. 4).

Separation of the ^{14}C -labelled lipids extracted by two successive treatments with Triton X-100 was done by two-dimensional t.l.c. For comparison, the lipid patterns obtained by similar two-dimensional t.l.c. of the twice-extracted membrane residue extracted into chloroform-methanol (2:1, v/v), and the total lipids extracted from KB cell membranes with chloroform-methanol (2:1, v/v) were also obtained. The radioactivity in each spot, located by radioautography, was determined and is expressed in Table 3 as a percentage of the total radioactivity recovered from each t.l.c. plate.

Discussion

It is now recognized that the apparent molecular weights of glycoproteins derived by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate are often higher than the true values, since glycoproteins migrate at rates that are not proportional to their molecular mass (Segrest *et al.*, 1971). Under the conditions used in the present paper a well-defined glycoprotein, orosmuroid, gave an apparent mol.wt. of 55000. The true value is 44100 (Jeanloz, 1972). This result suggests therefore that the estimated molecular weights of the KB membrane glycoproteins may be high by about 25%. This estimate is similar to that (18%) obtained for the erythrocyte membrane

glycoprotein and for porcine ribonuclease by Segrest *et al.* (1971), who used similar electrophoresis conditions to those we have used. It should be noted in this connexion that the molecular weights of very few membrane proteins have been estimated by hydrodynamic or some other independent method, for comparison with results obtained by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. There is therefore no way of knowing at present if the anomalous behaviour described (Segrest *et al.*, 1971) for membrane glycoproteins by using the latter method may not also apply to non-glycosylated membrane proteins.

By the criteria described in the present paper KB cell membranes contain three glycoprotein species with apparent mol.wts. of 92000, 72000 and 62000 respectively. It remains to be determined if these values represent the sizes of the native glycoprotein components of the unreduced membrane. Alternatively, the glycosylated polypeptides, detected in reduced membranes by polyacrylamide-gel electrophoresis in buffers containing sodium dodecyl sulphate, could form part of multi-subunit membrane components consisting either entirely of glycosylated polypeptides or of mixtures with non-glycosylated polypeptides. Certainly it has proved very difficult to purify KB-cell-membrane glycoproteins free either of each other or of non-glycosylated proteins. Thus although a partial separation of the largest glycoprotein subunit, PAS I, was obtained by chromatography on Sepharose 6B in buffers containing 0.5% Triton X-100 (Fig. 4), the fraction contained smaller

amounts of other glycoprotein subunits and was heavily contaminated with one or more non-glycosylated polypeptide species (Plate 4). It is possible of course that the difficulty lies in the means of solubilization of these membrane components for fractionation. Previous studies (Bonsall & Hunt, 1971; Shibuya *et al.*, 1968; Dunnick *et al.*, 1972) have shown that Triton X-100 acts to disrupt membranes at very low concentrations (0.15%). At higher concentrations of detergent, similar to those used in the present study, most of the membrane material was found as micellar complexes containing protein, lipid and detergent (Helenius & Söderlund, 1973; Simons *et al.*, 1973). At yet higher concentrations of detergent Engelman *et al.* (1967), Salton & Schmitt (1967) and Helenius & Simons (1971) have shown that dissociation of membrane proteins from lipids takes place. Before this occurs, however, at intermediate concentrations of detergents, discrete complexes of proteins and lipids together with detergent molecules exist in solution. These complexes could include mixed micelles containing lipid and detergent molecules or micellar aggregates of lipid, protein and detergent. The present results show that the glycolipids extracted from KB cell membrane with 0.5% Triton X-100 are eluted from Sepharose 6B (Fig. 4) as complexes of high molecular size, approx. 100000.

Since the micellar complexes formed probably contain several membrane components in mixture, the probability is rather remote of subsequently obtaining homogeneous fractions containing any particular membrane component by relatively mild methods that do not perturb the micellar structure.

Nevertheless, the simple fractionation scheme described in the present paper shows that some separation of membrane components is achieved. Thus the material eluted in fraction GP I from Sepharose 6B (Fig. 4) is clearly enriched in the glycoprotein species of molecular weight 92000 (Plate 4). However, attempts to separate the components present in fraction GPII obtained from Sepharose 6B chromatography have been totally unsuccessful, probably for the reasons previously stated. Thus no purification of any component was obtained by chromatography on DEAE-cellulose in buffers containing 0.5% Triton X-100 or by isoelectric focusing in a 5–50% (w/v) sucrose gradient made up with 0.5% Triton X-100 and an ampholine pH gradient of 3–6. Radioactive glycoprotein fractions were obtained that gave unchanged gel patterns similar to those in Plate 4.

It is noteworthy that the major glycoproteins of KB cell membranes appear to be relatively easy to extract with dilute solutions of non-ionic detergents. It seems likely that these glycoproteins like several other membrane glycoproteins (Hughes, 1973) are located in exposed positions on the outside of the surface membrane of KB cells. Certainly this is implied for the KB cell membrane glycoprotein that

carried the specific receptors for adenovirus (Hughes & Mautner, 1973). This is not to say, however, that the KB cell membrane glycoproteins are peripheral proteins in the sense used by Singer & Nicholson (1972). In their classification of membrane components, peripheral proteins are stated to interact with the membrane largely by electrostatic forces and are removed from the membrane structure relatively easily by manipulation of ionic or pH conditions. Glycoprotein constituents of cellular membranes are in general rather firmly embedded in the hydrocarbon interior of the membrane (Hughes, 1973), and presumably are made soluble through disruption of hydrophobic interactions with the membrane structure and a transfer of these interactions to detergent molecules, forming mixed micelles.

Similarly, there is considerable evidence to suggest that although the glycolipids of cellular membranes are readily available for reaction with surface-labelling reagents such as specific antibodies or galactose oxidase (Gahmberg & Hakomori, 1973) they nevertheless are intrinsic or integral membrane components. It is surprising to find (Table 3) that ceramide trihexoside is very poorly extracted from KB cell membranes by non-ionic detergents whereas lactosyl ceramide is totally extracted. Sphingomyelin, cholesterol and triglycerides are similarly concentrated in the insoluble residue remaining after successive extractions of membranes with detergent although the method of separation does not enable us to differentiate the latter two lipid types.

It is intriguing that several non-glycosylated polypeptides of relatively low molecular size are extremely resistant to extraction with non-ionic detergents. The significance of this finding to the structural integrity of the KB cell membrane remains to be determined. It may be mentioned that similar protein components that are relatively resistant to solubilization have been detected in other membrane systems (Cotman *et al.*, 1971; Evans & Gurd, 1972). These proteins appear to be present in specialized regions of the cell surface, in particular at points of intercellular contact. The junctional complexes of liver membranes also appear, like the residue of KB membranes (Table 3), to be enriched in triglycerides and cholesterol (Evans & Gurd, 1972). Poorly extractable protein fractions have not been reported previously for cultured cells, confluent or non-confluent, growing in monolayers. However, cellular junctions are known to form in cells in culture (Harris, 1970), and this fact possibly points to the origin of the poorly extractable KB-cell-membrane proteins.

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