Surface Labelling of Human Tumour KB Cells

IODINATION AND FRACTIONATION OF MEMBRANE GLYCOPROTEINS

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1. Human tumour KB cells growing in suspension culture were labelled by lactoperoxidase-catalysed iodination. Several major radioactively labelled proteins were detected by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. 2. After reduction with 2-mercaptoethanol the major radioactive electrophoretic bands migrated as substances with apparent molecular weights of about 90 000, 70 000, 60 000, 50 000 and 34 000 and corresponded closely to the positions at which the major glycosylated polypeptide subunits of KB-cell homogenates migrated during electrophoresis under the same conditions. 3. All the iodinated protein bands except one were present in purified preparations of KB plasma membranes. 4. Most of the 50 000-molecular-weight species, supposedly a surface protein component labelled during iodination of intact and viable KB cells by a non-penetrating enzyme reagent, appeared in a crude nuclear pellet during fractionation. 5. The glycoprotein nature of the major external iodinated species of KB cells was confirmed by adsorption chromatography of these substances, dissolved in low concentrations of Triton X-100, on a lectin-Sephadex column. Two major enzyme markers of the KB plasma membrane, 5'-nucleotidase and alkaline phosphatase, were also found to be glycoproteins. 6. Enzyme-catalysed incorporation of radioactive iodine into a fraction of low molecular weight and soluble in chloroform-methanol mixtures also occurred during lactoperoxidase treatment of intact KB cells. The partial characterization of this fraction is briefly described.

Most accepted models of cellular plasma membranes incorporate the principle that the two faces of this structure are dissimilar (Robertson, 1969). In the best-studied example, the human erythrocyte membrane, certain polypeptide species exist exclusively at the cytoplasmic side, for example spectrin and glyceraldehyde 3-phosphate dehydrogenase, whereas others such as acetylcholinesterase occur only on the external face. All of these components are defined as extrinsic membrane proteins in the nomenclature of Singer & Nicolson (1972) and are attached to the lipid matrix of the membrane largely by electrostatic interactions. A third class of membrane polypeptides (integral membrane components) spans the total width of the membrane to interact within the hydrophobic interior of the lipid bilayer and emerge at both external and cytoplasmic faces. In these cases, however, the asymmetry of the membrane structure is preserved by substitution with carbohydrate units of the peptide segments emerging on the external side of the membrane. These studies have been reviewed (Wallach, 1972; Hughes, 1973; Steck, 1974; Juliano, 1973).

The disposition of protein and glycoprotein subunits within membrane structures as well as the

arrangements of various classes of lipids and glycolipids are important parameters that presumably are related directly to membrane function. For instance protein subunits that pass through the membrane are quite possibly involved in transport phenomena (Pinto da Silva & Nicolson, 1974). In addition the interesting findings (see Steck, 1974) that so far all of the proteins shown to be situated at the external face of the human erythrocyte membrane bear carbohydrate strongly suggests a causal relationship between glycosylation and the ultimate cellular relocation of nascent polypeptide species from intracellular biosynthetic sites to the outer cell surface. However, relatively little is known about the topography of the plasma membrane of cells other than the erythrocyte to test the generality of these conclusions.

In this paper we have used lactoperoxidase to substitute with radioactive iodine the tyrosine residues of protein species exposed at the external surface of intact and viable cells of the human tumour KB cell. The composition of the KB-cell plasma membrane has been characterized thoroughly (Butters & Hughes, 1974). We wished to examine the disposition of the various protein subunits within this membrane and in particular to determine which of these com-

ponents, proteins and glycoproteins, are exposed at the external surface of the intact cell. Since the enzyme lactoperoxidase is unable to penetrate the plasma membrane to react with intracellular proteins (Hubbard & Cohn, 1972; Kreibach *et al.*, 1974) it is possible to identify surface-located membrane components for comparison with the total composition of the plasma membrane. We have found first that only a relatively small number of the protein subunits of plasma membranes are accessible to enzymic iodination of intact KB cells. Secondly, most if not all of these externally located protein subunits appear to be glycosylated. The significance of our findings, which in general are similar to those obtained with the human erythrocyte, is discussed.

Materials and Methods

Culture of KB cells

KB cells, derived from a human carcinoma of nasopharynx, were obtained from Flow Laboratories, Irvine, Ayrshire, U.K., and grown as a routine in monolayer cultures in Eagle's Minimal Essential Medium (Glasgow modification, obtained from Flow Laboratories) containing 100 i.u. of penicillin/ml and 100 µg of streptomycin/ml. Foetal bovine serum (Flow Laboratories) and tryptose phosphate broth were added to 10% (v/v) as described by Butters & Hughes (1974).

Suspension cultures of KB cells were obtained by the addition of trypsin-treated monolayer cells to Eagle's medium containing ten times the normal amount of sodium phosphate (0.14 g/l) used for monolayer cultures, and no Ca^{2+} . This medium was supplemented with 10% (v/v) calf serum (Flow Laboratories) and contained penicillin (100 i.u./ml) and streptomycin (100 µg/ml).

Iodination experiments

Iodination of cells from suspension or monolayer culture was carried out essentially as described by Hubbard & Cohn (1972) by lactoperoxidase-catalysed iodination in which H_2O_2 was generated by glucose oxidase and glucose.

KB cells (1×10^6 – 2×10^8) from suspension cultures were washed by centrifugation at 1080g for 3 min at 2°C, in phosphate-buffered saline (8.09 g of NaCl, 0.29 g of KCl, 0.29 g of KH_2PO_4 , 1.159 g of K_2HPO_4 , 0.19 g of CaCl_2 and 0.19 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ dissolved in 1 litre of water, pH 7.4), and suspended in 1 ml of phosphate-buffered saline containing 100 µCi of carrier-free ^{125}I (The Radiochemical Centre, Amersham, Bucks., U.K.), 2.5 mM-glucose, 0.5 nmol of lactoperoxidase [Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.; 42 units/mg] and 0.4 unit of glucose oxidase (Sigma Chemical Co.; 1400 units/ml). After incubation for various times, usually 10 min at room temperature, the cells were

washed four times in phosphate-buffered iodide solution (a solution of similar composition to phosphate-buffered saline except that 8.09 g of NaI/litre replaced NaCl) and once in phosphate-buffered saline. The final cell pellet was then counted for ^{125}I in a Packard gamma counter, and the values obtained were used as a measure of apparent incorporation. As discussed below, however, a large proportion of radioactivity present in such cell pellets is present as non-covalently bound iodine. Cells in monolayer culture were washed directly with phosphate-buffered saline and labelled as above, keeping the cell sheet intact. After labelling, the cells were scraped from the culture vessel with a rubber 'policeman' and treated as described above.

Iodinated cells were then made 1% (v/v) in sodium dodecyl sulphate and 1% (v/v) in 2-mercaptoethanol, treated at 90°C for 5 min and then stored at –20°C before analysis by polyacrylamide-gel electrophoresis. In other experiments labelled cells (4×10^7) were mixed with unlabelled cells (2×10^8) ready for membrane fractionation.

In yet other experiments, iodinated cells were suspended (10^7 per ml) in phosphate-buffered saline, pH 7.4, and treated with trypsin (20 µg/ml; Sigma Chemical Co.) for 10 min at room temperature before addition of denaturing reagents.

Purification of plasma membranes

Plasma membranes from KB cells grown in either suspension or monolayer were prepared as described for monolayer cultures (Butters & Hughes, 1974). Briefly, cells were washed in phosphate-buffered saline and suspended in 10 mM-Tris-HCl buffer, pH 8, at 2°C. After 30 min the swollen cells were broken in an all-glass Dounce homogenizer fitted with a tight pestle. The homogenate was centrifuged at 1086g for 3 min and the supernatant decanted. The pellet containing nuclei and a small number of unbroken cells was resuspended in 10 mM-Tris-HCl buffer, pH 8, and further homogenized. The supernatant and the resuspended 'nuclear' fraction (approx. 20 ml of each) were centrifuged through a sucrose gradient containing 5 ml each of 50, 30 and 20% (w/w) sucrose in 10 mM-Tris-HCl buffer, pH 8, for 16 h at 2°C in the Beckman L2 65B centrifuge with a SW27 rotor at approx. 80000g. The prominent membrane band at the 30–50% sucrose interface was harvested by centrifugation at 12000g for 1 h, resuspended in 10 mM-Tris-HCl buffer, pH 8, and layered on top of a similar discontinuous sucrose gradient (9 ml) containing 3 ml of each concentration of sucrose solution. After centrifugation as above, the membrane fraction at the 30–50% sucrose interface was harvested and further centrifuged through a 20–40% continuous sucrose gradient (16 ml) as above (Fig. 2b). Fractions (0.9 ml) were collected from the bottom of the gradient and assayed for membrane

markers and ^{125}I . 5'-Nucleotidase activity was measured as described by Butters & Hughes (1974). Alkaline phosphatase was estimated by the method of Perkerthy *et al.* (1972). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Polyacrylamide-gel electrophoresis

The methods and the proteins used as molecular-weight markers have been fully described by Butters & Hughes (1974). Usually, samples (100–400 μg of protein) were made up to 1% (w/v) sodium dodecyl sulphate, 1% (v/v) 2-mercaptoethanol and heated for 5 min at 90°C. In some experiments reducing agent was omitted. Resolution of disaggregated samples was achieved in a 10% (w/v) polyacrylamide disc gel by the method of Weber & Osborn (1969). Protein was stained with Coomassie Blue, and carbohydrate material was detected by the periodate-Schiff reagent, slightly modified from a procedure described by Kobylka *et al.* (1972). Gels were fixed in a 40% (v/v) solution of methanol containing 7% (v/v) acetic acid for 6 h rather than overnight as recommended by the authors, since we have observed losses of certain glycoprotein materials during prolonged fixations. Radioactivity of ^{125}I was determined by directly counting 2 mm-thick gel slices. Slab-gel electrophoresis in a discontinuous-buffer system was performed by methods described by Maizels (1971). Dried gels were exposed to a Kodak no-screen X-ray film for 3–5 days and subsequently developed.

Lipid extraction

Purified membranes (1–2 mg) isolated from KB cells labelled with ^{125}I or with [^{14}C]palmitate as described by Butters & Hughes (1974) were extracted twice with chloroform-methanol (2:1, v/v). The combined extracts were analysed by two-dimensional thin-layer chromatography on pre-coated silica-gel plates as described by Butters & Hughes (1974). Radioautography was performed by using Kodak no-screen X-ray film.

Affinity chromatography

Iodinated plasma membranes (2.8 mg of protein/ml; 1.69×10^6 ^{125}I c.p.m. per ml) were solubilized in 0.5% Triton X-100 for 30 min at 2°C. Under these conditions approx. 70% of the label was extracted into a clear supernatant after centrifugation at 12000g for 1 h at 2°C. The extract (0.5 ml) was passed through a column (5 cm $\times$ 1 cm) of *Lens culinaris* lectin coupled to Sepharose 4B (Hayman & Crumpton, 1972) equilibrated with 0.5% Triton X-100 in 10 mM-Tris-HCl buffer, pH 8, containing 1 mM- CaCl_2 and 1 mM- MnCl_2 . Fractions (1 ml) were collected at 4 ml/h at 2°C. The retarded material was eluted with a solution of 1% α -methyl mannoside dissolved in the above equilibration buffer. Samples of each fraction were

assayed for ^{125}I , 5'-nucleotidase and alkaline phosphatase activity. Pooled fractions were dialysed under pressure to a small volume against 0.5% Triton X-100 in 10 mM-Tris-HCl buffer, pH 8, and subjected to sodium dodecyl sulphate-polyacrylamide-gel electrophoresis.

Results

Conditions of iodination

In preliminary experiments, washed KB cells from suspension cultures were resuspended in phosphate-buffered saline at cell densities ranging from 4×10^6 to 2×10^8 cells/ml and incubated for 10 min at room temperature with the complete standard iodinating mixture described in the Materials and Methods section. After incubation the cells were disrupted in 1% sodium dodecyl sulphate-1% 2-mercaptoethanol and samples containing equivalent amounts of radioactivity were examined by slab-gel electrophoresis (Plate 1). About twelve radioactive bands were consistently obtained in a variety of conditions. Only minor differences in the relative intensities of the various bands were apparent with a 50-fold range of cell densities (4×10^6 – 2×10^8 cells/incubation mixture) or a fourfold increase in the amount of ^{125}I in the standard incubation mixture. Maximum labelling appeared at a cell density of about 1×10^7 cells/ml and these conditions have been adhered to in subsequent experiments. The relatively poor labelling of very dense cell suspensions (greater than 1×10^8 cells/ml) was probably due to the fact that the final volume of these incubation mixtures was close to the packed volume of such large numbers of KB cells and consequently to the restricted diffusion of enzyme reagents.

Other preliminary experiments showed that maximum iodination of intact KB cells required the addition of all ingredients of the complete mixture. Thus, in the absence of lactoperoxidase, incorporation was less than 1% of the total incorporation in the complete system. Considerable iodination (about 10% of that obtained in the complete system) took place in incubation mixtures lacking either glucose oxidase or glucose, indicating the presence of an endogenous supply of an H_2O_2 -generating system in these cells.

Under standard conditions maximum incorporation of radioactive iodine into KB cells required about 10 min, with less than a 10% increase in ^{125}I incorporation occurring over the next 30 min. The patterns of components labelled for different times also were rather similar. Minor quantitative variations can be detected. Thus a component of apparent mol.wt. 60000 appeared to account for a far larger part of the radioactive iodine incorporation after 10 min (our standard incubation time; see Plate 1a, track 2) than during either shorter or longer labelling periods.

Fig. 1

Labelled cells were also subjected to mild trypsin treatment, by incubation at room temperature for 10 min in the presence of 20 μ g of trypsin/ml. Analysis of the cell pellet remaining after such treatment by polyacrylamide-gel electrophoresis revealed that the major iodinated band of 60000 mol.wt. had been removed completely. Proteolysis under these conditions had a lesser, although definite, effect on other iodinated bands. We have not studied the effects of more drastic conditions of proteinase treatment.

Labelled components

It could be shown by disc-gel electrophoresis (Fig. 1a) and very clearly when radioautographs of slab-gel electrophoresis runs were prepared (Plate 1) that several discrete bands migrating with apparent molecular weights of about 90000, 70000, 60000 and 34000 were consistently obtained after disaggregation of iodinated KB cells in 1% sodium dodecyl sulphate-1% 2-mercaptoethanol. Other minor iodinated bands were also noted, including a component of apparent mol.wt. 50000 and several faster-moving components. In all cases a large amount of radioactive iodine was found associated with a fraction migrating with or close to the dye marker. The molecular size of material in this region of the gel is difficult to estimate but cannot be greater than about 10000 if of peptide origin. Some properties of this material accounting for up to 20% of the total counts recovered after gel electrophoresis of cell homogenates or of purified membrane fractions will be described in a later section. It should be noted that most of the 125 I entered the gel during electrophoresis. However, very poor recoveries (20-40%) of radioactivity were obtained after both disc-gel and slab-gel electrophoresis of total cell homogenates. This low recovery cannot be due, however, to poor disaggregation of membrane samples and we conclude that much of the

Fig. 1. Polyacrylamide-gel electrophoresis of fractions obtained from lactoperoxidase-labelled KB cells

Cells were fractionated as described in Table 1 and samples (100-200 μ g of protein equivalents) were solubilized in 1% sodium dodecyl sulphate-1% 2-mercaptoethanol and subjected to electrophoresis in 0.1% sodium dodecyl sulphate containing buffer. The migration is left to right. Disc gels after electrophoresis were sliced into 2 mm widths and counted directly for 125 I in an automatic gamma spectrometer. (a) Cell homogenate; (b) 'nuclear' pellet from centrifugation at 1086g followed by sedimentation through a sucrose gradient; (c) crude membranes from first sucrose gradient; (d) major membrane band from second sucrose gradient; (e) major plasma-membrane fraction from the continuous sucrose gradient shown in Fig. 2 (b). 1-7, Subunits with apparent mol.wts. 135000; 90000-120000; 78000; 60000-70000; 50000; 40000; 34000 respectively. BD, Dye marker].

EXPLANATION OF PLATE I

Radioautography showing the separation by slab-gel electrophoresis of KB-cell surface components labelled by lactoperoxidase-catalysed iodination

(a) Intact and viable cells from suspension culture were washed extensively and incubated at the cell densities indicated, in a standard mixture containing lactoperoxidase, glucose oxidase, glucose and [125 I]iodide for 10 min at room temperature. After incubation the cells were dissolved in 1% sodium dodecyl sulphate–1% 2-mercaptoethanol and run in a slab of polyacrylamide gel in 0.1% sodium dodecyl sulphate. Radioautography of the developed slab gel was performed as described in the Materials and Methods section. Tracks 1, 4×10^6 cells/ml; 2, 1×10^7 cells/ml; 3, 5×10^7 cells/ml; 4, 1×10^8 cells/ml; 5, 2×10^8 cells/ml; 6, 1×10^7 cells/ml. In track 6, the incubation mixture contained $400 \mu\text{Ci}$ of ^{125}I , whereas the other incubation mixtures contained $100 \mu\text{Ci}$ of the radioisotope. (b) Intact cells (1×10^6) in monolayer culture were labelled with $250 \mu\text{Ci}$ of isotope. BD, Bromophenol Blue dye marker.

EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of plasma-membrane fractions obtained from KB cells growing (a) and (c) in suspension, or (b) and (d) in monolayer culture

Details of membrane preparations and conditions of electrophoresis are given in the text. Membrane material obtained from the 30–50% (w/w) sucrose interface (see Fig. 2*b*) was used in each case. Proteins were stained with Coomassie Blue (*a–b*) and glycoproteins with a modified periodate–Schiff reagent (*c–d*). The arrows indicate approximate molecular weights. The origins are at the top. BD, Bromophenol Blue dye marker.

EXPLANATION OF PLATE 3

Polyacrylamide-gel electrophoresis of purified KB-cell plasma membranes solubilized with 1% sodium dodecyl sulphate alone or with 1% sodium dodecyl sulphate-1% 2-mercaptoethanol

Electrophoresis was performed in 0.1% sodium dodecyl sulphate in the absence of reducing agent. (a) and (b), Profiles of glycoprotein bands detected by periodate-Schiff staining in the absence and the presence respectively of reducing agent. (c) Profiles of ^{125}I radioactivity obtained by slicing gels into 2mm widths and counting in a gamma spectrometer. ---- Sodium dodecyl sulphate alone; —, sodium dodecyl sulphate-2-mercaptoethanol. The positions of the glycoprotein bands referred to in the text with apparent mol.wts. 120 000-140 000 and 60 000-70 000 are indicated by arrows. BD, Dye marker.

Fig. 2. Centrifugation of membrane fractions obtained from iodinated KB cells through continuous sucrose gradients

The labelled cells were homogenized and subjected to preliminary centrifugation steps to eliminate unbroken cells, nuclei and other intracellular components. The partially purified plasma membranes were finally placed on continuous 20–40% sucrose gradients (16 ml) and centrifuged to equilibrium. Fractions (1 ml) were collected and assayed for ^{125}I radioactivity (—) and for 5'-nucleotidase activity (○). The direction of sedimentation is to the left. The origin of the light (a) and heavy (b) membrane bands is described in the text. ----, Sucrose densities.

^{125}I lost during electrophoresis is inorganic and not associated covalently with membrane components.

Membrane fractionation of iodinated cells

KB cells (5.2×10^8) from suspension culture were mixed with cells (4×10^7) labelled by lactoperoxidase-catalysed iodination in the standard incubation mixture, except that 500 μCi was used instead of 100 μCi of ^{125}I iodide. Cells were homogenized and fractionated as described in the Materials and Methods section which is essentially the method described by Butters & Hughes (1974).

It has been noticed in recent preparations that in

the initial centrifugation steps through discontinuous sucrose gradients, the prominent membrane band at the interface of 30 and 50% sucrose solutions often splits into two distinct bands. On re-centrifugation in the same discontinuous sucrose gradient (20, 30 and 50%) in the smaller-capacity buckets of the Beckman SW27 rotor the two bands are again evident. One band just penetrates the 30% sucrose layer after overnight centrifugation at 80000g and the major band deposits at the 30–50% sucrose interface as described above. Both membrane fractions contain typical plasma-membrane enzymes, for example 5'-nucleotidase (Table 1). The heavier membrane band is always more enriched in these markers, however, and accounts for at least 70–75% of the total homogenate activity of this enzyme. However, we show in Fig. 2 the separation of these two membrane fractions during the final step of membrane fractionation by centrifugation through continuous 20–40% sucrose gradients.

The extent of purification of a plasma-membrane enzyme marker obtained in the fractionation of KB cells from suspension culture is indicated in Table 1. Very similar results were obtained by Butters & Hughes (1974) for cells from monolayer cultures. Further, the polypeptide compositions of pure plasma membranes obtained from monolayer or suspension culture cells are very similar as shown by polyacrylamide-gel electrophoresis (Plate 2).

The heavy plasma-membrane band (Fig. 2b) containing most of the 5'-nucleotidase activity co-centrifuged with a peak of ^{125}I radioactivity. However, it is striking that the extent of purification of an enzyme marker 5'-nucleotidase, a sixfold increase over the unfractionated homogenate, as found earlier for monolayer cells (Butters & Hughes, 1974), was not paralleled by a similar purification of the radioactive iodine in this fraction relative to whole-cell homogenates. Indeed if total ^{125}I counts are considered, the specific radioactivity in the original KB-cell homogenate is considerably higher than in the plasma-membrane fraction. These calculations are somewhat misleading, however, since it can be shown by polyacrylamide-gel electrophoresis in dissociating conditions that only a small proportion of the radioactivity present in total cell homogenates is associated with identifiable KB-cell components. Thus the recovery of radioactive counts after electrophoretic separations of total KB-cell homogenates is only a few per cent of the total radioactivity applied to the gel. The proportion of radioactivity tightly associated, presumably covalently, with components of purified plasma membranes is higher. Thus up to 65% of the radioactivity present in solubilized membranes applied to gels is recovered after electrophoresis in dissociating conditions in identifiable membrane components. If these points are taken into consideration therefore the specific radioactivity of the purified plasma-membrane

Table 1. *Contents of 5'-nucleotidase and ^{125}I in fractions of KB-cell homogenates*

5'-Nucleotidase activity units are μmol of P_i liberated/h per mg of protein at 37°C . Total ^{125}I activities are given as c.p.m. per mg of protein in each fraction and membrane-bound ^{125}I as c.p.m. per mg of protein calculated from the % of radioactivity recovered in identifiable membrane components after polyacrylamide-gel electrophoresis. N.D., Not determined.

Fraction	Sp. activity of 5'-nucleotidase (units)	$10^{-6} \times \text{Specific radioactivity}$	
		Total ^{125}I	Membrane- bound ^{125}I
Homogenate	9.5	0.22	0.05–0.08
1086g pellet	5.8	0.26	N.D.
1086g supernatant	13.0	0.19	N.D.
Membranes from first sucrose gradient	28.0	0.087	0.055
Membranes from second sucrose gradient			
Minor band	9.1	0.011	N.D.
Major band	46.0	0.082	N.D.
Membranes from third sucrose gradient			
Minor band	8.7	0.010	N.D.
Major band	61.0	0.061	0.041

fraction is similar to that of unfractionated cell homogenates (Table 1). It is still the case, as shown in Table 1, that there is a considerably smaller degree of purification of radioactive iodine than of 5'-nucleotidase in the plasma-membrane fraction. This is presumably due in part to losses of iodinated material loosely bound to membranes during disruption and fractionation of the cells.

Many of the iodinated protein components identified in whole-cell homogenates (Fig. 1a) were also present (Fig. 1e) in the most highly purified plasma-membrane fraction obtained after centrifugation through a continuous sucrose gradient. The composition of these two fractions is, however, not identical. In particular an iodinated component with apparent mol.wt. 50000 present as a minor species of whole-cell homogenates (band 5, Fig. 1a) is enriched in the 'nuclear' fraction (Fig. 1b) and is almost absent from purified plasma membranes (Fig. 1c). The 'nuclear' fraction clearly contains other iodinated bands that are also detected in cell homogenates as well as in plasma-membrane fractions. It seems likely that these components represent contamination of the 'nuclear' fraction with plasma membranes, since about 25% of the total 5'-nucleotidase activity of the cell homogenate is also found in this fraction. Some contamination would be contributed by the small number of unbroken cells surviving homogenization that are removed together with nuclei during the initial centrifugation step of 1086g and are also pelleted through a discontinuous sucrose gradient. It seems likely, however, that the 50000-molecular-weight component is enriched in some subcellular fraction, presumably derived from the cell-surface membrane, that sediments independently of the bulk of the plasma membranes in association with heavy nuclear debris and broken cells. However, we have not characterized this material further.

Solubilization of iodinated plasma membranes

Plasma membranes prepared from iodinated KB cells were extracted with Triton X-100 as described by Butters & Hughes (1974). After incubation of membranes (2.8mg of protein/ml; 1.69×10^6 ^{125}I c.p.m. per ml) in 0.5% Triton X-100–10mM-Tris–HCl, pH8, for 30min at 2°C , 75–80% of the total radioactivity remained in the supernatant after centrifugation at 100000g for 1h. Under similar conditions of extraction we have found (Butters & Hughes, 1974) that 50–70% of the total [^{14}C]glucosamine radioactivity associated with membrane glycoproteins or glycolipids is made soluble and only 30–50% of the membrane non-glycosylated proteins.

Affinity chromatography

To examine the nature of the iodinated substances solubilized with Triton X-100 from KB-cell plasma membranes, we have used affinity chromatography on a column of *Lens culinaris* lectin coupled to Sepharose (lectin–Sepharose column). The lectin shows specificity for glycoproteins containing α -glycosidically linked mannosyl residues (Sharon & Lis, 1972). Several such separations with different batches of solubilized iodinated KB membranes gave consistent results, and a typical fractionation is shown in Fig. 3. About one-half to two-thirds of the total ^{125}I radioactivity of the fraction solubilized from plasma membranes with Triton X-100 passed through the column without retardation. A large part (greater than 90%) of the radioactivity recovered after gel electrophoresis of this fraction originated in a fast-moving peak close to but not coincident with the dye marker (Fig. 4a). Less than 10% of the radioactivity recovered from the gel was present in high-molecular-weight protein bands. The overall recovery of radioactivity was poor, indicating that a substantial amount of radio-

Fig. 3. Affinity chromatography on a lectin column of iodinated components solubilized from KB-cell plasma membranes with Triton X-100

Membranes containing about 1.5mg of protein and 0.9×10^6 c.p.m. of ^{125}I were extracted with detergent at 2°C as described in the text. The material made soluble, representing 80% of the total, was applied to a column of *Lens culinaris* lectin coupled to Sepharose 4B. The column was equilibrated with 0.5% Triton X-100–10mM-Tris-HCl (pH8)–1mM- CaCl_2 –1mM- MnCl_2 and initial elution at 2°C was with the same buffer. Fractions (1ml) were collected and assayed for ^{125}I radioactivity (-----), 5'-nucleotidase activity (○) and alkaline phosphatase activity (●). At fraction 12 elution with 1% α -methyl mannoside, dissolved in the equilibration buffer, was started.

Fig. 4. Polyacrylamide-gel electrophoresis of iodinated fractions obtained by affinity chromatography of Triton X-100-solubilized KB-cell plasma membranes

(a) Fraction obtained in the breakthrough volume from a lectin-Sepharose column. (b) Fraction adsorbed to the column and eluted with α -methyl mannoside. BD, Dye marker.

activity was lost during electrophoresis. Our interpretation is that this represents inorganic iodine that is removed from membrane fractions only under severe dissociating conditions such as treatment with

sodium dodecyl sulphate and subjection to electrophoresis.

A clear-cut difference was obtained in the profile of radioactivity recovered after gel electrophoresis of soluble KB membrane components retarded on the lectin column and eluted with α -methyl mannoside. Thus essentially all of the radioactivity migrated (Fig. 4b) as iodinated protein subunits of relatively high apparent molecular weights and almost no radioactive counts were found in material near the dye front.

The recovery of ^{125}I and enzyme activities from the lectin column was excellent and always greater than 80%. We therefore conclude that, except for the fast-migrating iodinated material present in the unretarded fraction, the origin of which is considered in a later section, most ^{125}I present in the plasma-membrane fraction solubilized by Triton X-100 is associated with glycoprotein subunits. In agreement with this view, all of the iodinated bands found in whole plasma membranes (Fig. 1e) are recognizable in the absorbed fraction from affinity chromatography (Fig. 4b).

Subunits of the major iodinated glycoprotein of KB-cell plasma membranes

All of the polyacrylamide-gel-electrophoretic separations considered up to now were obtained by using membrane samples solubilized in 1% sodium dodecyl sulphate–1% 2-mercaptoethanol. Although the electrophoresis itself was carried out in the absence of reducing agent, Fish *et al.* (1970) have shown that re-formation of disulphide bonds between

polypeptide subunits does not occur to any significant extent. The profiles obtained under these conditions of electrophoresis therefore relate to the fully disaggregated and reduced subunits. When electrophoresis of the iodinated KB plasma membranes was carried out on membranes not treated with 2-mercaptoethanol a different pattern of radioactivity was obtained during electrophoresis (Plate 3). Thus the major iodinated species now migrated with a mobility equivalent to a protein of mol.wt. 120 000–140 000. Direct staining of disc gels of plasma membranes disaggregated in sodium dodecyl sulphate in the absence of 2-mercaptoethanol (Plate 3a) revealed a major glycoprotein subunit in this region of the gel. In parallel gels run with plasma membranes treated with detergent plus reducing agent and stained with periodate–Schiff reagents a band of this mobility was clearly missing (Plate 3b) and the major glycoprotein subunit of mol.wt. about 60 000–70 000 corresponded closely to the major peak of radioactive iodine (Plate 3c).

Chloroform-methanol extraction of iodinated membranes

As mentioned above a substantial part of the ^{125}I radioactivity detected after gel electrophoresis of whole-cell homogenates migrated as a fraction of very low apparent size and almost coincident with the dye front. A similar iodinated material was present also in purified plasma membranes. Since this radioactive peak migrated to the region of the gel in which membrane lipids and glycolipids are found we studied the extractability of membrane-associated ^{125}I radioactivity with chloroform-methanol. As control, plasma membranes isolated from KB cells previously pulsed for prolonged periods with [^{14}C]palmitate to label membrane lipids (Butters & Hughes, 1974) were used. Extraction of isolated membranes with a chloroform-methanol (2:1, v/v) mixture at 2°C overnight removed respectively 12–13% and greater than 90% of ^{125}I and ^{14}C from appropriately labelled plasma-membrane fractions. Gel electrophoresis of the delipidated membrane residue (Fig. 5b) showed that a large part of the fast-migrating peak of ^{125}I radioactivity present in the original plasma membranes (Fig. 5a) and moving in the same region of the gel as ^{14}C -labelled material (Fig. 5c) had been extracted by a lipid solvent.

Further examination of ^{125}I -labelled substances extracted into chloroform-methanol was carried out by two-dimensional thin-layer chromatography on silica-coated plates as described by Butters & Hughes (1974). The radioautogram of the developed plate is shown in Fig. 6. Several mobile radioactive spots were detected that in the two solvents used suggests a lipophilic character for these substances. The ^{125}I radioactive spots were, however, not coincident with known [^{14}C]palmitate-labelled lipids and

Fig. 5. Profiles of ^{125}I and ^{14}C obtained on gel electrophoresis of iodinated KB-cell plasma membranes and [^{14}C]palmitate-labelled KB-cell plasma membranes

Plasma membranes purified from KB cells labelled previously by lactoperoxidase-catalysed iodination or by growth in ^{14}C -labelled palmitate were solubilized in 1% sodium dodecyl sulphate–1% 2-mercaptoethanol and subjected to polyacrylamide-gel electrophoresis. Gels were sliced into 2 mm widths and radioactivity present in each slice was determined as described in the Materials and Methods section. (a) ^{125}I -labelled plasma membranes; (b) delipidated ^{125}I -labelled plasma membranes after treatment with chloroform-methanol (2:1, v/v); (c) ^{14}C -labelled plasma membranes. BD, Dye marker.

the identity of the former cannot be assigned as present. Presumably iodination of unsaturated bonds in fatty acids, if this takes place, may occur by a non-enzymic free-radical mechanism analogous to peroxidation (Welton & Aust, 1972). If such reactions occur it must be assumed from the definite lack of

Fig. 6. Two-dimensional thin-layer chromatography of chloroform-methanol extracts of labelled KB plasma membranes

Membranes prepared from iodinated cells or cells grown in the presence of [^{14}C]palmitate to label cellular lipids were extracted with the solvent as described in the text. The extracts were applied to silica thin-layer plates and separated in the solvent system described in the Materials and Methods section. Radioautographs of the plates were prepared after chromatography and used to trace the position of ^{14}C -labelled spots (■) and ^{125}I -labelled spots (□) respectively. The direction of solvents 1 and 2 is arrowed. SM, Sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; CTH, ceramide trihexoside; CDH, ceramide dihexoside; CMH, ceramide monohexoside; FFA, free fatty acids, cholesterol and triacylglycerols.

reactivity of proteins at the cytoplasmic face of erythrocytes (Steck, 1974) that penetration of the iodinating species within the lipid bilayer is severely restricted (probably the large excess of lipid acts as a trap) and the lactoperoxidase iodination system can be considered as essentially a non-penetrating reagent.

Comparison of KB cells grown in suspension or monolayer culture

Butters & Hughes (1974) described the preparation of a plasma-membrane fraction from homogenates of KB cells grown in monolayer culture. Since identical methods have been applied directly in the present studies with suspension cultures of KB cells, we decided to compare the composition and iodination patterns of plasma membranes prepared from cells grown under these two different culture conditions. The pattern of lactoperoxidase-catalysed labelling of monolayer KB cells (Plate 1b) is shown for comparison with cells from suspension culture (Plate 1a) together with the polypeptide compositions of KB-cell plasma membranes prepared by using either of the two methods to cultivate the cells (Plate 2).

Discussion

The plasma-membrane fraction obtained in the present work from KB cells growing in suspension culture is similar in general composition to preparations described by Butters & Hughes (1974) for cells grown in monolayer. The KB plasma membrane contains three major classes of glycoprotein subunits with apparent molecular weights of about 90000, 70000 and 60000. A smaller glycoprotein subunit of apparent mol.wt. 34000 is not detected readily by staining of sodium dodecyl sulphate-polyacrylamide gels, probably owing to losses of material from the gel during periodate-Schiff staining by the methods of Kobylka *et al.* (1972), but was clearly labelled with a radioactive glucosamine precursor (Butters & Hughes, 1974).

In contrast with similar polypeptide compositions, patterns of labelling with lactoperoxidase of cells grown to high cell densities in suspension or monolayer cultures were found to be distinctly different. In particular several bands (see Plate 1b) present as major iodinated species in monolayer cells represent only a small part of the overall iodination of suspension-culture cells. Clearly proteins are exposed to different extents at the surface of KB cells grown under different conditions. Some specialization of the cell surface might be expected in monolayer culture, for example to provide anchorage of cells to the growth surface. Such differences of the cell surface due to growth requirements may be amenable to detection by a labelling procedure such as lactoperoxidase.

As appears to be the case for the human erythrocyte membrane, we conclude from our present results that most of the proteins containing iodlatable tyrosine residues that are exposed at the outer face of the plasma membrane of KB cells carry carbohydrate units. Thus the major iodinated polypeptide species are extracted with Triton X-100 from plasma membranes and in the soluble form show affinity for a column containing *Lens culinaris* lectin. This lectin binds to carbohydrate chains containing glucose and mannose residues, and the latter sugar particularly is a common constituent of many glycoproteins, including presumably those of the KB plasma membrane. It is not impossible but somewhat unlikely that the iodinated species detected may in fact include proteins, adsorbed to the lectin column by virtue of their aggregation with glycoproteins. It should be noted, however, that the large majority of the total protein material extracted from KB membranes with detergent passes unretarded through the affinity column, and these proteins contain little radioactivity.

Two enzyme markers of the KB plasma membrane, namely alkaline phosphatase and 5'-nucleotidase, appear also to be glycoproteins. Although these enzymes are co-purified with the iodinated glycoproteins during membrane fractionation of lactoperoxi-

dase-labelled cells, solubilization in detergents and affinity chromatography of the solubilized material on the lectin-Sepharose column, we do not know if either of these enzymes is present at the external surface of intact KB cells, thereby becoming iodinated with the lactoperoxidase system. It is noteworthy, however, that the 5'-nucleotidase activity of KB cells behaves as a component of about 130000 during gel filtration on Sepharose 6B in dilute solutions (0.5%) of Triton X-100 (T. D. Butters, unpublished work). The rat liver enzyme (Evans & Gurd, 1973) has a similar molecular weight but may consist of two glycoprotein subunits of mol.wt. about 70000-75000. These latter approximate values are similar to the major iodinated subunit of the KB plasma membrane. We suggest therefore that 5'-nucleotidase of KB cells, like that of liver (Farquhar *et al.*, 1974), may be located externally on the cells in a location accessible to lactoperoxidase labelling and that the native enzyme is a dimer of two glycoprotein subunits (not necessarily similar) of mol.wt. 65000. The evidence described above that the major iodinated species of KB plasma membranes may be a dimeric molecule containing two subunits of mol.wt. 60000-70000 certainly is consistent with this possible interpretation. Direct enzymic analysis of a highly purified dimeric molecule and proof of its glycoprotein structure would be required to decide satisfactorily on this point, however.

Evans (1974) has recently presented some evidence that another enzymically active glycoprotein, nucleotide pyrophosphatase, may be present at the exterior of hepatocytes and available to lactoperoxidase iodination. Although the monomeric molecular weight of this liver enzyme (130000) is not dissimilar to minor iodlatable species present on the KB cell surface direct analysis of cell homogenates shows that this activity is very low or undetectable in KB cells (T. D. Butters, unpublished work).

All of the major glycoprotein bands detected in the purified KB plasma membrane appear to react with lactoperoxidase, a non-penetrating reagent, when this enzyme is added to suspensions of intact viable cells. Thus the major iodinated bands obtained after gel electrophoresis in dissociating conditions of disaggregated and reduced plasma membranes prepared from lactoperoxidase-labelled intact KB cells migrate very similarly to the major glycoprotein subunits of these membranes. It is possible, but perhaps unlikely, that copies of these glycoprotein species are present at both faces of the KB-cell surface membrane. The experiments described here would not rule out this possibility. The limited experience gained by using the erythrocyte membrane strongly suggests, however, that components are distributed asymmetrically at either the external face or cytoplasmic face of the membrane and do not express equivalent structural features at both sides. This is probably true for both

extrinsic components such as acetylcholinesterase, spectrin and glyceraldehyde 3-phosphate as well as the integral protein components such as the major erythrocyte membrane glycoprotein. In the latter case the C-terminal peptide sequence of the polypeptide moiety emerges exclusively at the cytoplasmic face of the membrane.

With regard to minor glycoprotein components of the KB plasma membrane, our experiments are too imprecise to decide whether all of these are present, like the major glycoproteins, at the external face of the membrane and therefore become iodinated. Perhaps other labelling techniques specific for carbohydrate moieties, such as galactose oxidase plus $B^3H_4^-$ (Steck & Dawson, 1974), will help to assign these components to a particular face of the membrane.

The apparent unreactivity to the iodination system of non-glycosylated protein subunits at the external surface of the KB cells is a surprising feature of the present work, and extends the situation described for the human erythrocyte (Steck, 1974) to a nucleated human cell. It is not known at present whether such non-glycosylated proteins are indeed totally absent from this membrane face. Several explanations could be found for the apparent unreactivity of these components to lactoperoxidase. Thus putative non-glycosylated proteins from the external face of the KB cell may be present in a fraction that is not available to our present analysis, for example, the 'nuclear' fraction or the iodinated material (about 20% of total) not solubilized from plasma membranes by Triton X-100. We believe this explanation to be unlikely, however, since in no case was the absence of KB membrane glycoproteins including 5'-nucleotidase demonstrated in these fractions. Often the proportions of total incorporated ^{125}I in these fractions closely corresponded to the residuum of cellular glycoproteins contained in them. Similarly, the possibility that externally located peptide sequences of protein subunits may contain no iodlatable tyrosine residue seems *a priori* unlikely. It is of course possible that the relatively large number of exposed glycoproteins on the KB-cell surface containing unknown quantities of bulky carbohydrate groups may render certain iodlatable exposed peptide sequences unavailable to a large reagent such as lactoperoxidase. Conceivably proteins situated at the external face of the KB plasma membrane may be protected from iodination in this manner. Finally, it may be noted that iodinated peptides of relatively low molecular weight (approx. 10000) may have escaped detection by the methods described in this paper.

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