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Proteins Exposed on the Surface of Mammalian Membranes†

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ABSTRACT: The technique employing lactoperoxidase-catalyzed iodination for investigating protein distribution within erythrocyte stroma has been extended to examine the proteins of the surface membranes of mouse fibroblasts (L cells). The surface membranes were examined after four different conditions of iodination: (1) the intact cell, where only proteins exposed at the surface are susceptible to iodination by lactoperoxidase; (2) the intact cell in the absence of lactoperoxidase as a measure of nonspecific iodination; (3) the isolated surface membrane; and (4) the partially denatured surface membrane, where, presumably, most proteins are accessible. The surface membranes were solubilized in sodium dodecyl sulfate and the protein subunits were examined by disc gel electrophoresis. The results indicate that one group of polypeptides with a molecular weight of approximately 230,000 is in an exposed position on the exterior of the L cell surface membrane. Smaller amounts of radioactive iodine are associated with polypeptides of molecular weight 46,000–150,000 suggesting that a small portion of their chain is exposed. No radioactivity is associated with two polypeptides of large molecular weight (170,000 and 190,000), a polypeptide of molecular weight 39,500, and several smaller polypeptides of molecular weight range 15,000–32,000. All proteins are iodinated when surface membranes were partially denatured and subsequently iodinated. The fact that the degree of iodination increases manyfold in the isolated membranes supports the observation that only a few proteins are exposed on the surface of the intact L cell. This iodination technique was extended to the surface membranes of baby hamster kidney fibroblasts, BHK_{21}/C_{13}, before and after transformation by the Bryan strain of Rous sarcoma virus, C_{13}/B_4. Again the results indicate that in both cases a large molecular weight group of polypeptides is exposed on the outside of the surface membrane.

The paucity of information regarding the spatial arrangement of proteins in membranes can be attributed to inadequate techniques of protein vectorial analysis. The recent report of Phillips and Morrison (1971a) regarding the use of lactoperoxidase to iodinate membrane proteins by means of an enzyme–substrate complex which is impermeable to erythrocyte membranes (Phillips and Morrison, 1970) seemingly circumvents this problem. This technique has been extended

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to investigate the arrangement of the membrane proteins of the L cell, a mouse fibroblast. The L cell was selected for these studies because previous investigations have defined a number of chemical parameters of the isolated surface membranes (Weinstein *et al.*, 1969, 1970; Glick *et al.*, 1970). The proteins of these isolated membranes have been studied in detail by disc electrophoresis on sodium dodecyl sulfate–polyacrylamide gels (Greenberg and Glick,[1] 1972). In addition, L cells can be grown in suspension culture so the problems inherent in removing cells from a glass surface were not encountered (Codington *et al.*, 1970; Buck *et al.*, 1970).

This investigation reports on those proteins of the surface membrane which are in an exposed position on the exterior of L cells and on the exterior of BHK_{21}/C_{13} cells before and after virus transformation.

Materials and Methods

Cell Culture. Mouse fibroblasts (L cells) were grown and harvested as described previously (Glick *et al.*, 1970). Low passage baby hamster kidney cells (BHK_{21}/C_{13}) and BHK_{21}/C_{13} cells transformed by the Bryan strain of Rous sarcoma virus (C_{13}/B_4) were cultured as described (Buck *et al.*, 1970). All cultures were examined at routine intervals for *Mycoplasma* and were found negative.

Preparation of Cells. The washed L cells were suspended in 0.16 M NaCl (5×10^7 cells/ml) and divided into three aliquots. Two aliquots were iodinated before the surface membranes were prepared, and the remaining aliquot was used to prepare surface membranes before iodination. The washed BHK_{21}/C_{13} and C_{13}/B_4 cells were suspended in 0.16 M NaCl (5×10^7 cells/ml), and each was iodinated before the surface membranes were prepared.

Preparation of Surface Membranes. Surface membranes were prepared by the zinc ion procedure (Warren and Glick, 1969). The surface membranes were whole and were counted in a hemocytometer. The characteristics and purity of these preparations have been described (Warren *et al.*, 1966; Glick and Warren, 1969; and Glick *et al.*, 1970). Soluble material from the cytoplasm of the cell was prepared from the same cells from which the surface membranes were isolated (Glick and Warren, 1969).

Iodination Procedure. Washed cells or isolated surface membranes were suspended in 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.4). Lactoperoxidase was added to the reaction mixture to give a final concentration of 1×10^{-6} M. The lactoperoxidase activity was determined prior to each iodination by using the guaiacol assay method (Maehly and Chance, 1954). In each case 0.5 mCi of ^{125}I was added as NaI for the L cells. BHK_{21}/C_{13} and C_{13}/B_4 cells were iodinated with 0.25 mCi of ^{125}I. The reaction was initiated by the addition of 5 μl of 1.56 mM H_2O_2 to give a concentration of 7.8 μM in the reaction mixture. Twenty sequential additions of hydrogen peroxide were made after 15-sec intervals to reinitiate the reaction. This delay maintains negligible concentrations of free peroxide; consequently, membrane oxidation was minimized. The reaction was terminated by the addition of KI followed by washing the suspension with cold phosphate buffer six times and spinning at 60*g* for 5 min.

Iodination of Cells and Surface Membranes. Two aliquots of washed L cells (2.5×10^8 cells each) were centrifuged at 150*g* for 5 min at 5°. The pellets, resuspended in 0.1 M phosphate buffer (pH 7.4), were subjected to the iodination procedure as intact cells in the presence (1) and absence (2) of lactoperoxidase. Two preparations of surface membranes were then isolated and examined by disc gel electrophoresis. From the remaining aliquot of L cells, surface membranes (1.02×10^8 membranes) were prepared. One-half of the membrane preparation (3) was suspended in 0.1 M phosphate buffer (pH 7.4) and iodinated according to the procedure described. the other half (4) was extracted with lipid solvents, suspended in 0.1 M phosphate buffer (pH 7.4), and then iodinated.

BHK_{21}/C_{13} and C_{13}/B_4 cells (5.3×10^8 cells and 4.8×10^8 cells, respectively) were washed in 0.16 M NaCl and centrifuged at 150*g* for 5 min at 5°. The pellets were suspended in 0.1 M phosphate buffer (pH 7.4) and iodinated as described for intact L cells. Surface membranes were then prepared from the iodinated cells.

Extraction of Lipids. Lipids were extracted from the surface membranes by repeated addition (4 times) of 1.0 ml of chloroform–methanol (2:1, v/v) followed by evaporation under N_2. On the fifth addition, the organic solvent was removed, and the remaining material was then repeatedly extracted (4 times) with 1.0 ml of ether–ethanol (3:2, v/v). The resulting pellet was suspended in 0.1 M phosphate buffer (pH 7.4) and iodinated according to the above procedure. In addition, this partial denaturation–extraction process was performed on each of the other membrane preparations (1, 2, and 3) following iodination. The amount of radioactivity associated with the lipid extracts was determined in each case.

Disc Gel Electrophoresis. The lipid-extracted surface membranes were dissolved in 2% sodium dodecyl sulfate for electrophoresis on sodium dodecyl sulfate gels of 5% polyacrylamide. The method of solubilization and the conditions for electrophoresis on polyacrylamide gels have been described (Greenberg and Glick,[1] 1972). In each case 100 μg of membrane protein was applied to each gel. With the methods employed, the polypeptide patterns of the isolated membranes were reproducible. The gels were stained with Coomassie Blue as described by Fairbanks *et al.* (1971). Gel densitometry was performed using a Zeiss spectrophotometer equipped with Vicon linear transport accessory. The gels were scanned at 550 mμ. The radioactive iodide distribution within the gel was determined by slicing the gels laterally with a razor blade in a Canalco slicer into sections of approximately 1.5-mm thickness. The γ emissions from each slice were counted directly in a Model 300 Packard Auto-Gamma spectrometer.

Other Procedures and Materials. Proteins were determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. Hydrogen peroxide concentrations were determined using a molar extinction coefficient of 72.4 at 230 mμ optical density (Phillips and Morrison, 1971a). The molecular weight estimates for membrane polypeptides were calibrated from the mobilities of molecular weight markers relative to trypsin (Greenberg and Glick,[1] 1972). Lactoperoxidase was purchased from Calbiochem, and ^{125}I was obtained from NEN as NaI, carrier-free.

Results

Electrophoretic Pattern of Surface Membrane Polypeptides. Electrophoresis on sodium dodecyl sulfate gels of 5% polyacrylamide under the conditions described revealed that 22 or more polypeptides are present in the isolated surface membrane. The polypeptides were detected by staining with Coomassie Blue, followed by densitometric scanning of the gel. The pattern (Figure 1a) was reproducible with different mem-

[1] Submitted for publication.

TABLE I: Surface Membrane Polypeptides of the L Cell Grouped According to Molecular Weight as Determined by Electrophoresis on Sodium Dodecyl Sulfate–Polyacrylamide Gels.

Group	Relative Mobility	Approximate Molecular Weight[a]	No. of Polypeptides[b]
I	0.02	230,000	1–2
II	0.1	190,000	1
III	0.14	170,000	1
IV	0.18–0.7	46,000–150,000	10–14
V	0.75	39,500	1
VI	0.86–1.15	15,000–32,000	4–6

[a] Molecular weight estimates calibrated from the mobilities of molecular weight markers relative to trypsin. Details are described in the text. [b] Estimated from the optical density scan of the polypeptides on the gel stained with Coomassie Blue.

brane preparations of L cells. The protein subunits have been divided into six groups on the basis of the relative mobility of the polypeptides after electrophoresis on sodium dodecyl sulfate gels of 5% polyacrylamide. Under the conditions described, the relative mobilities are characteristic for proteins of known molecular weight (Greenberg and Glick,[1] 1972).

The six groups of polypeptides are further defined in Table I. The grouping was arbitrary but based on decreasing molecular weights.

Incorporation of ^{125}I. The proteins of the surface membranes of L cells were subjected to the iodination under four different conditions. Intact cells were treated (1) in the presence of lactoperoxidase and (2) in the absence of lactoperoxidase. Surface membranes were then isolated from these radioactive cells. Surface membranes were also isolated from cells which were not iodinated. These membrane preparations were subsequently iodinated either (3) directly or (4) after extraction with lipid solvents to partially denature the membrane.

Table II gives the counts per minute of radioactive iodine incorporated into the surface membranes in each of these four membrane preparations. The surface membranes isolated from intact cells after iodination in the presence of lactoperoxidase (1) contained 5.5×10^{-4} cpm per membrane. When the amount of radioactive iodine incorporated into these surface membranes was compared to the amount incorporated into surface membranes which were iodinated after isolation (3), the latter contained 73 times more radioactivity. Similarly, an increased amount was incorporated into the isolated membranes which were partially denatured (4) before iodination. The cells which were treated in the absence of lactoperoxidase (2) served as a control for nonspecific iodination.

Approximately 30% of the radioactivity was extracted from the surface membranes of the L cell by lipid solvents (Table II). After this lipid extraction, the surface membranes prepared from the whole cells treated in the presence (1) or absence (2) of lactoperoxidase showed a difference of only 2.1×10^{-4} cpm of ^{125}I incorporated per membrane. Thus only a small amount of membrane protein was iodinated in the whole cells.

TABLE II: Incorporation of ^{125}I.

Condition of Cell or Surface Membrane when Iodinated[a]	Cpm Per Cell $\times 10^{-2}$	Cpm Per Membrane[b] $\times 10^{-4}$	Cpm Per mg of Membrane Protein $\times 10^{3}$	% of Total Lipid Extract of Membrane[c]
L Cell				
(1) Intact cell	2.47	5.5	15	32
(2) Intact cell in the absence of lactoperoxidase	1.78	3.4	10	27
(3) Isolated surface membrane		401	1083	34
(4) Isolated surface membrane, lipid extracted		357	974	
BHK_{21}/C_{13}				
(1) Intact cell	1.33	ND[d]	10	16
C_{13}/B_4				
(1) Intact cell	1.71	4.0	11	12

[a] Intact cells were iodinated with ^{125}I in the presence (1) or absence (2) of lactoperoxidase. Surface membranes were isolated from these iodinated cells. Surface membranes were prepared from L cells and subsequently iodinated before (3) or after (4) extraction with lipid solvents. All of the membrane preparations were extracted with lipid solvents. Details are described in the text. [b] After extraction with lipid solvents. [c] Percentage of the total radioactivity found in the surface membranes. [d] ND: not determined.

The amount of radioactivity found in the whole cells indicated that there was considerable uptake of radioactive iodine by the cells. In contrast to the iodination of the proteins of surface membranes this amount was similar in the presence (2.47×10^{-2} cpm) or absence (1.78×10^{-2} cpm) of lactoperoxidase (Table II). Of this amount taken up by the whole cells, 36–39% was recovered in cytoplasmic fractions known to include soluble cell material.

The amount of ^{125}I taken up by the BHK cells before and after viral transformation (1.33×10^{-2} and 1.71×10^{-2}, respectively) was similar to that of the L cells (Table II). Again, only small amounts of radioactivity were found in the membranes isolated from the iodinated cells (Table II). These amounts, when expressed per milligram of membrane protein, are similar in the membrane preparations from the three cell lines.

Location of Surface Membrane Proteins. Proteins of the surface membranes were iodinated under the four different conditions described and examined by disc gel electrophoresis. The four preparations of surface membranes were extracted similarly with lipid solvents and dissolved in 2% sodium dodecyl sulfate prior to electrophoresis. The pattern obtained after visualizing the constituent polypeptides with Coomassie Blue (Figure 1a) was similar to the pattern obtained from the surface membranes without the iodination procedure. After iodination, the polypeptide pattern observed

by staining with Coomassie Blue was the same for all the surface membrane preparations.

In contrast, the pattern of radioactive iodine associated with each polypeptide band after electrophoresis was different depending upon the condition of iodination (Figure 1). When whole cells were iodinated (1) prior to the isolation of the surface membranes (Figure 1a) only group I polypeptides (Table I) were heavily iodinated. Group IV polypeptides had a small amount of radioactivity associated with them (Table III). However, when the isolated surface membranes, which appear as whole membranes, were iodinated (3) many additional proteins were radioactive (Figure 1b and Table III). Iodination of group I increased over 2-fold and significant radioactivity was associated with group III. Group V increased in radioactivity almost 20-fold, whereas groups IV and VI increased 4- to 10-fold.

The isolated surface membranes which were partially denatured before iodination (4) revealed the greatest number of proteins iodinated (Figure 1c and Table III). Group II was now heavily iodinated. It appears that all of the polypeptides were iodinated under this condition.

Treatment of intact L cells in the absence of lactoperoxidase (2) revealed no significant chemical iodination of the membrane proteins since the radioactivity of the polyacrylamide gel slices was just above background. The membrane proteins from the iodinated whole cells in the presence of the enzyme (1) were corrected for this amount.

The results, therefore, indicate that only group I polypeptides and, to a lesser extent, group IV are available for iodination at the surface of the L cell.

Proteins Exposed on the Surface of BHK Cells. BHK_{21}/C_{13} and C_{13}/B_4 cells were iodinated in the presence of lactoperoxidase. Surface membranes were isolated from the iodinated cells, dissolved in 2% sodium dodecyl sulfate, and examined by disc gel electrophoresis. Only group I polypeptides were iodinated under these conditions. Figure 2 shows this radioactivity superimposed on the densiometric scans of the gels after staining with Coomassie Blue. These results were similar to those obtained with the preparations of L cell membranes. Little or no radioactivity was detectable in the other polypeptides for either BHK_{21}/C_{13} (Figure 2a) or C_{13}/B_4, the virus-transformed cells (Figure 2b). This again indicates that the proteins exposed on the surface are of high molecular weight.

TABLE III: ^{125}I Associated with Membrane Polypeptides after Electrophoresis on Sodium Dodecyl Sulfate–Polyacrylamide Gels.[a]

Polypeptide Group[b]	Cpm[c]		
	Isolated Membranes from Iodinated Intact Cells	Isolated Membranes Subsequently Iodinated	Extracted Membranes Subsequently Iodinated
I	1330	3000	3220
II	0	0	1540
III	0	1850	1950
IV[d]	100–160	450–800	500
V	195	3300	2300
VI[d]	30–40	200–450	300–600

[a] Constant amount of protein (100 μg) was applied to each gel. [b] Polypeptide groups determined as in Table II. [c] Calculated from Figure 1. [d] Given as peak height range of radioactivity for number of polypeptides as indicated in Table II and does not represent the total amount of radioactivity in this area of the gel.

Discussion

The action of lactoperoxidase has been demonstrated by Morrison and Bayse (1970) to involve the iodination of exposed tyrosine and histidine residues on proteins. The human erythrocyte is impermeable to lactoperoxidase (Phillips and Morrison, 1970), consequently iodination catalyzed by lactoperoxidase will occur only on those membrane proteins which are exposed to the external environment (Phillips and Morrison, 1971a).

The distribution of radioactive iodine in surface membranes from iodinated mouse and hamster fibroblasts and in the isolated membranes which were subsequently iodinated indicates that this technique can establish the position of exposed proteins in the membranes of cells which have been grown in culture. The iodination of whole L cells and the baby hamster kidney cells, BHK_{21}/C_{13} and C_{13}/B_4, in the presence of lactoperoxidase reveals a significant amount of radioactive iodine associated with a membrane polypeptide of large molecular weight (230,000). Recently, Barber and Jamieson (1971), using the same technique, found a similar result with platelet membranes. Phillips and Morrison (1971a,b) have shown that two polypeptides of molecular weights of 90,000 and 62,000 are exposed on the erythrocyte surface.

A small amount of radioactivity is associated with polypeptides of molecular weight range 46,000–150,000 when the intact L cells are iodinated. This suggests that only a portion of their protein chain could be exposed (Figure 1a). In contrast, the incorporation of radioactive iodine into the isolated surface membranes gives a pattern suggesting the general iodination of all the polypeptides with the exception of a polypeptide of 190,000 molecular weight (Figure 1b). The restricted iodination of this protein subunit in the isolated surface membranes suggests a more internal position in the membrane. The iodination of the isolated membranes which have been partially denatured by lipid solvents reveals that all proteins are iodinated indicating that they are all accessible to lactoperoxidase. The fact that in the isolated membranes most of the proteins are iodinated while in the extracted membranes all are iodinated by a factor of at least five times compared to the intact cell, indicates that the iodination procedure reflects the degree of exposure of the membrane proteins.

A considerable amount of ^{125}I is taken up by the cells (Table II), and approximately 40% of this radioactivity is associated with the cytoplasmic contents of the cell. This high amount of radioactivity probably represents free ^{125}I, although no attempts were made to examine this question. Of the total radioactivity taken up per cell, only 2–3% is associated with the membranes. Approximately one-third of this radioactivity is extractable with lipid solvents suggesting that chemical iodination of membrane lipids does take place. However, Phillips and Morrison (1971a), found no radioactivity associated with the lipids of the erythrocyte stroma.

It has been shown that in the absence of hydrogen peroxide iodination of tyrosine does not occur; consequently, the enzyme does not have a specific iodination activity (Morrison and Bayse, 1970; Bayse and Morrison, 1971). In our experi-

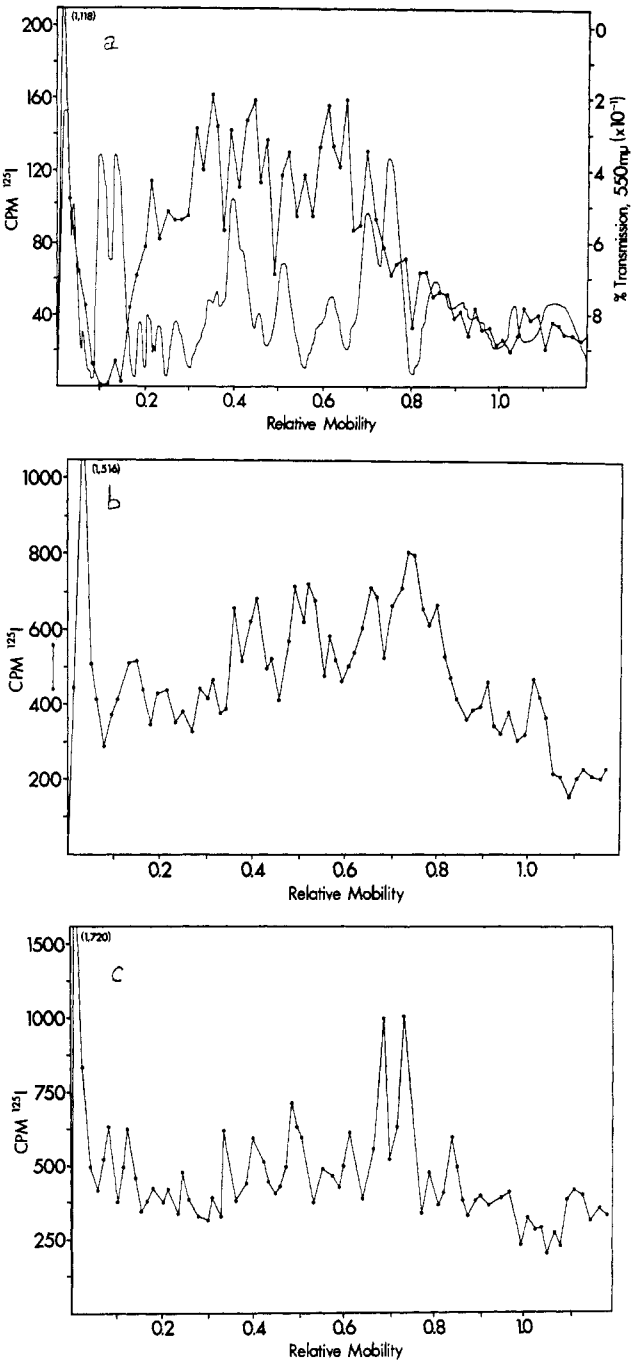

FIGURE 1: Distribution of ^{125}I in polypeptides of L cell membranes after electrophoresis on sodium dodecyl sulfate gels of 5% polyacrylamide. (a) Intact cells which were iodinated and the surface membranes subsequently isolated; (b) isolated surface membranes which were iodinated; (c) isolated surface membranes which were extracted by lipid solvents prior to iodination. In (a), the radioactivity (●-●-●) is compared to the densitometric scan (solid line) of the polypeptides after staining with Coomassie Blue.

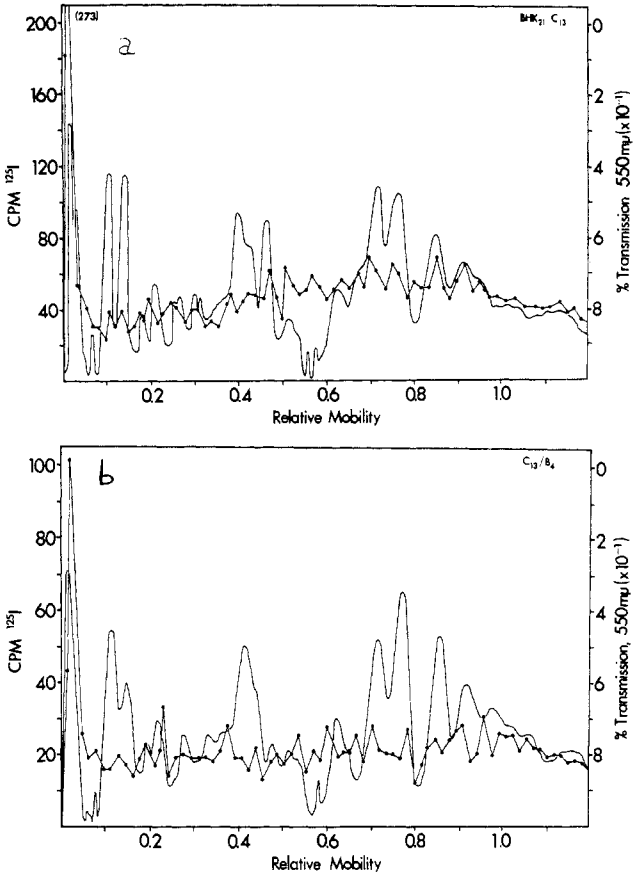

FIGURE 2: Distribution of ^{125}I in polypeptides of BHK cell membranes after electrophoresis on sodium dodecyl sulfate gels of 5% polyacrylamide. (a) BHK_{21}/C_{13}; (b) C_{13}/B_4. Intact cells were iodinated and surface membranes were subsequently isolated. The radioactivity (●-●-●) is compared to the densitometric scan (solid lines) of the polypeptides after staining with Coomassie Blue.

ments treatment of the cells in the absence of lactoperoxidase also clearly reveals no significant chemical iodination of the membrane proteins when examined by disc gel electrophoresis.

One must be cautious in interpreting the differences in the iodination patterns of the whole cells and isolated membranes. The fact that one protein appears to be exposed on the surface of the cells grown in culture does not necessarily indicate that this is the position of this protein(s) during the growth of the cell. The process of harvesting the cells from culture may cause removal or rearrangement of membrane components resulting in the exposure of proteins not present during growth. The BHK cells were harvested by trypsinization from a monolayer while the L cells were grown in suspension culture. Indeed, trypsinization has been shown to remove glycoproteins from the cell surface (Buck *et al.*, 1970) and to rearrange the glycoproteins on the erythrocyte stroma (Marchesi *et al.*, 1971). However, despite the different conditions of growth and harvesting which remove glycopeptides from the cell surface, a similar group of high molecular weight polypeptides appears to be exposed on the surface of the baby hamster kidney and the mouse fibroblasts.

One can suggest that the reason for the high degree of iodination of the isolated surface membranes which were not denatured (Table III) is a rearrangement of the proteins during the membrane-isolation techniques. The surface membranes were removed from the cells after treating with $ZnCl_2$ under hypotonic conditions (Warren and Glick, 1969). This process could cause a rearrangement or exposure of additional proteins on the cell surface. In view of recent observations suggesting the movement of proteins through fluid membranes (Blasie and Worthington, 1969; Glaser *et al.*, 1970; Marchesi *et al.*, 1971; Nicolson, 1971; and Taylor *et al.*, 1971), this possibility must be considered. On the other hand, the increased iodination may be the result of the exposed inner surface due to the lack of "resealing" of the membrane after the

contents of the cytoplasm have been expelled during preparation. Iodination of membranes prepared under milder conditions such as the Tris method (Warren and Glick, 1969) may answer this question.

Carraway *et al.* (1971) have found by diazotized sulfanilic acid labeling and trypsin digestion that the major membrane protein of erythrocytes which is readily accessible at the cell surface is a glycoprotein. Bretscher (1971) has extended these findings using the labeling reagent, *S*-formylmethionine sulfone methyl phosphate, to conclude that the polypeptide portion of this principle glycoprotein extends through the membrane barrier to the interior surface of the cell membrane. The high molecular weight protein that we find exposed on the surface of the L cell gives the staining characteristics of a glycoprotein with periodic acid–Schiff reagent (Greenberg and Glick,[1] 1972). The availability of such glycoproteins on outer cell surfaces suggests their increasing relevance in understanding membrane-associated phenomena.

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