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Electrophoretic Study of the Polypeptides from Surface Membranes of Mammalian Cells†

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ABSTRACT: Surface membranes isolated from mouse fibroblasts (L cells) and baby hamster kidney cells before (BHK₂₁/C₁₃) and after (C₁₃/B₄) transformation by an RNA virus were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The optimal conditions for solubilization and electrophoresis of the isolated surface membranes were established. The molecular weight distribution of the polypeptides was determined by using proteins of known molecular weight comparably treated. All of the surface membranes examined show several polypeptides in the molecular weight regions of 230,000–200,000 and a spectrum from 190,000 to approximately 15,000. Polypeptides from the surface membranes of L cells in the molecular weight regions of 230,000, 210,000, 100,000, and 56,000–39,500 contain carbohydrate on the basis of staining with periodic acid–Schiff reagents. These polypeptides stained most intensely with Coomassie Blue.

Proteins and glycoproteins of the surface membrane represent enzymatic, structural, and receptor molecules responsible for many of the functional properties of animal cells. Receptors for viruses, hormones and drugs, cellular recognition sites, and histocompatibility antigens are among the specialized functional properties of animal cells which can be assigned to specific surface membrane components (see Kraemer, 1971, for review).

Some of these surface membrane molecules may be associated with the regulation of cell growth. Changes in the glycoprotein and glycolipid composition of the cell surface have been reported for cells after transformation by a number of oncogenic viruses (Wu *et al.*, 1969; Meezan *et al.*, 1969; Buck *et al.*, 1970, 1971; Hakomori and Murakami, 1968; Mora *et al.*, 1969).

Current concepts suggest that proteins and glycoproteins of the surface membrane may function in a mobile state in a fluid lipid bilayer (Singer and Nicolson, 1972). This dynamic nature of the surface membrane has been supported by recent findings demonstrating the mobility of the membrane components (Blasie and Worthington, 1969; Frye and Edidin, 1970; Marchesi *et al.*, 1971; Scott *et al.*, 1971; Taylor *et al.*, 1971).

The characterization of the polypeptides from surface

In contrast, the higher molecular weight polypeptides from the surface membranes of BHK₂₁/C₁₃ and C₁₃/B₄ fibroblasts, although staining intensely with Coomassie Blue, contain no detectable carbohydrate by the periodic acid–Schiff procedure. The polypeptides of 100,000 and 56,000–39,500 stain with these reagents in a manner similar to those of the surface membranes from the L cells. This lack of detectable carbohydrate in some of the polypeptide regions is discussed in relation to the trypsinization procedure which is used to remove the baby hamster kidney cells from the monolayer cultures. The polyacrylamide gel electrophorograms of the surface membranes of mouse fibroblasts and baby hamster kidney cells are similar. However, after virus transformation, the polypeptides of 210,000, 96,000, 82,000, and 56,000–46,000 are diminished when compared to those of the control membranes.

membranes has proceeded slowly, primarily due to the technical difficulties associated with surface membrane isolation with inherent problems of defining the final product (Warren and Glick, 1971), and the subsequent solubilization and fractionation of the membrane components (Fairbanks *et al.*, 1971; Neville and Glossmann, 1971; Trayer *et al.*, 1971). Methods have been developed permitting the isolation of whole surface membranes from animal cells grown in tissue culture (Warren and Glick, 1969). The isolated membranes have been characterized morphologically (Warren *et al.*, 1966) and chemically: lipids (Weinstein *et al.*, 1969), glycolipids (Weinstein *et al.*, 1970), and carbohydrates (Glick *et al.*, 1970). In this study the polypeptides from these mammalian cell membranes were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Materials and Methods

Cell Culture. Mouse fibroblasts (L cells), baby hamster kidney cells (BHK₂₁/C₁₃), and BHK₂₁/C₁₃ transformed by the Bryan strain of the Rous sarcoma virus (C₁₃/B₄) were grown and harvested as described previously (Warren and Glick, 1969; Buck *et al.*, 1970). The cultures were examined for the presence of *Mycoplasma* at routine intervals and were found to be negative.

Preparation of Surface Membranes. Surface membranes were prepared by the zinc ion procedure (Warren and Glick, 1969). The isolated whole membranes were counted in a hemocytometer and protein was determined by the method of Lowry *et al.* (1951).

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Solubilization of Surface Membranes. The surface membrane pellet in 0.1 ml of 35% sucrose was most readily solubilized in 0.01 M sodium phosphate buffer (pH 7.2), containing 2% sodium dodecyl sulfate, 5 M urea, 1% 2-mercaptoethanol, and 0.01% EDTA while maintaining 10 mg of the dodecyl sulfate/mg of membrane protein. This mixture is referred to as the solubilization buffer. Unless otherwise specified, membrane preparations contained 0.2 mg of protein in 0.1 ml of solubilizing buffer with a ratio of 10:1 of sodium dodecyl sulfate:membrane protein. Under these conditions the surface membranes were soluble within 15 min at 37° as evidenced by their disappearance in the phase-contrast microscope. The preparations were held for an additional 45 min at room temperature before applying to the gels. Dialysis against 1 l. of solubilizing buffer at room temperature for 17 hr did not change the solubility. The conditions for solubilization, time, temperature, ionic strength, and proportion of the dodecyl sulfate to membrane protein were rigorously maintained for all preparations. To follow the effect of simultaneously increasing the concentration of the dodecyl sulfate and other solubilizing agents, aliquots from the same membrane preparation were solubilized in various dilutions of the solubilizing buffer.

Lipid Extraction of the Surface Membranes. The lipids were extracted from the surface membranes as described (Weinstein *et al.*, 1969). The lipid-extracted material was evaporated to dryness under a stream of nitrogen. Solubilization of the lipid-extracted membranes with the solubilizing buffer required 30 min at 37° with vigorous shaking.

In one experiment, the membranes were extracted four times with 1-butanol. The organic phase from the butanol extraction was evaporated to dryness under vacuum in a Rotary Evapo-Mix (Buchler Instruments).

Electrophoresis Conditions. Electrophoresis was performed by a modification of a sodium dodecyl sulfate-gel electrophoresis system previously reported (Shapiro *et al.*, 1967). Polyacrylamide gels (0.5 × 11 cm) of either 5 or 10% acrylamide in 0.01 M sodium phosphate buffer (pH 7.2), containing 0.5 M urea, 0.001% EDTA, 0.2% sodium dodecyl sulfate, 0.7% ammonium persulfate, and 0.3% Temed¹ were utilized for all fractionations. A ratio of 25:1 (w/w) of acrylamide to bisacrylamide was maintained for all gels. The gels were layered with 2-butanol. An analytical disc gel electrophoresis apparatus (Canalco) with a constant current power supply (Buchler Instruments) was used for all fractionations. Reservoir buffers were 0.1 M sodium phosphate buffer (pH 7.2) with 0.5 M urea, 0.2% sodium dodecyl sulfate, 0.001% EDTA, and 0.01% 2-mercaptoethanol. Gels were prerun for 1 hr at 4 mA/gel before electrophoresis of the sample. Electrophoresis was for 15 hr at 4 mA/gel at room temperature.

Development of Gels. Following electrophoresis the membrane components in the gels were stained for protein or carbohydrate by the procedures described by Fairbanks *et al.* (1971). When a comparison of protein and carbohydrate components was made the gels were initially stained for carbohydrate with PAS reagents and scanned at 515 mμ on a Zeiss-PMQ II spectrophotometer fitted with a linear drive gel scanner (Vicon Industries) and stained subsequently with Coomassie Blue and scanned at 550 mμ.

Molecular Weight Estimation. Proteins of known molecular weights were treated with the same solubilizing buffer as the membrane preparations, while maintaining a 10:1 ratio of

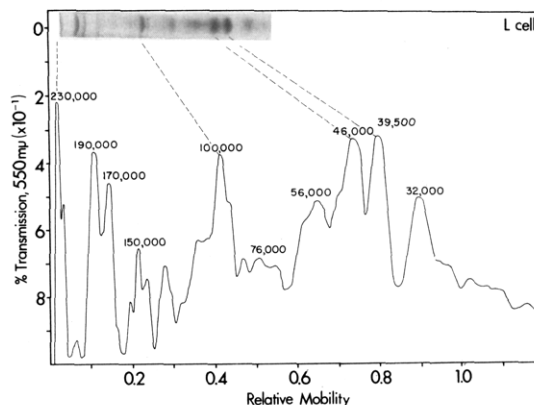


FIGURE 1: Polypeptides of the surface membranes of L cells. Isolated surface membranes were solubilized in buffer containing a 10:1 ratio of sodium dodecyl sulfate:protein. Membrane protein (50 μg) was fractionated on a sodium dodecyl sulfate gel of 5% polyacrylamide as described in Materials and Methods. After staining with Coomassie Blue, the gel was scanned at 550 mμ in an adapted Zeiss spectrophotometer. The mobility of each band was calculated relative to trypsin run in a parallel gel. The molecular weights, determined as described in Materials and Methods, are given for the major polypeptides and related to a photograph of the gel.

sodium dodecyl sulfate:protein. The relative migration of the components of the membrane preparations and protein standards were calculated relative to trypsin run in a parallel gel with the other protein standards. Similar values were obtained when solubilized trypsin was added to the same gel as the membrane components. A molecular weight calibration curve was constructed as described (Shapiro *et al.*, 1967) using the following protein standards: γ-globulin (mol wt 160,000), pepsin (mol wt 35,000), trypsin (mol wt 24,000), cytochrome *c* (mol wt 12,400), and insulin (mol wt 5,700), obtained from Mann Chem. Co., collagenase (mol wt 110,000) and ovalbumin (mol wt 46,000) purchased from Worthington Biochemicals, Inc., fetuin (mol wt 50,500) and bovine serum albumin (mol wt 66,000) obtained from Nutritional Biochemical Co. The molecular weights were described by Dunker and Rueckert (1969) or Sober (1968).

Results

Molecular Weights of Polypeptides. The calibration curves of proteins of known molecular weights in the sodium dodecyl sulfate-gels of 5% polyacrylamide showed that all of the proteins examined fell into a straight line when plotted as the log of molecular weight *vs.* relative mobility as described by Shapiro *et al.* (1967). In the 10% gels the proteins of lower molecular weight were slightly askew. Fetuin, a glycoprotein, fell slightly below the linear plot in gels of both concentrations of polyacrylamide.

Surface Membrane Polypeptides from L Cells. The separation of the polypeptides from the surface membranes of L cells by electrophoresis on sodium dodecyl sulfate-gels of 5% polyacrylamide is shown in Figure 1. The calibrated molecular weight values are included and related to the polypeptides of the gel.

Examination of the polypeptide distribution on gels of 5 and 10% polyacrylamide revealed molecular weights ranging from 230,000 to 15,000. The gels of 5% polyacrylamide fractionated all polypeptides from 250,000 to 20,000 molecular weights, however, the resolution of the polypeptides diminished for faster migrating components. The gels of 10% polyacrylamide permitted the migration of polypeptides with

¹ Abbreviations used are: Temed, *N,N,N',N'*-tetramethylethylenediamine; bis, *N,N'*-methylenebisacrylamide; PAS, periodic acid-Schiff.

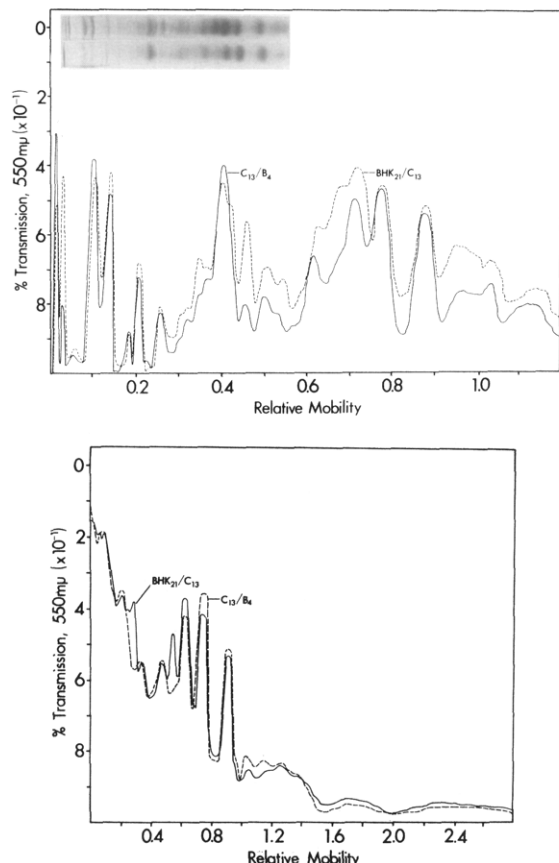


FIGURE 2: Comparison of the polypeptides from surface membranes of baby hamster kidney fibroblasts before (BHK₂₁/C₁₃) and after (C₁₃/B₄) transformation by the Bryan strain of Rous sarcoma virus. Membrane preparations were treated similarly by suspending in solubilizing buffer with a ratio of 10:1 of sodium dodecyl sulfate: protein. Protein (50 μ g) from each membrane preparation were fractionated on sodium dodecyl sulfate gels of 5% (a, top) and 10% (b, bottom) polyacrylamide as described in Materials and Methods. The gels were run in parallel and the absorbance scans of each were superimposed. For comparison, the heights of the last major absorbance peaks were adjusted to be approximately equal for both 5 and 10% gels. (a) Dashed line and upper photograph BHK₂₁/C₁₃; solid line and lower photograph, C₁₃/B₄; (b) solid line, BHK₂₁/C₁₃; dashed line, C₁₃/B₄.

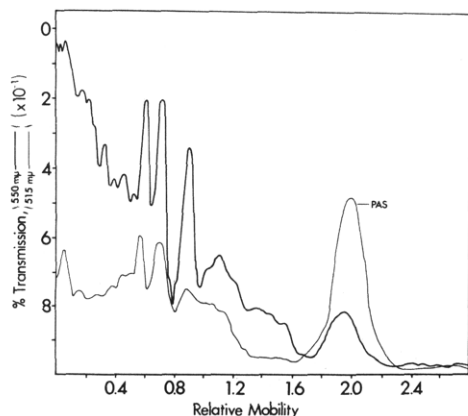


FIGURE 3: Carbohydrate and protein components of the surface membranes from L cells. Surface membranes were solubilized as described in Figure 1 and fractionated on a sodium dodecyl sulfate gel of 10% polyacrylamide. The gel was stained for carbohydrate using periodic acid-Schiff's reagents. The pattern (PAS) obtained was visualized by scanning on a Zeiss spectrophotometer at 515 $m\mu$. Subsequently, the gel was stained with Coomassie Blue and polypeptide pattern visualized by scanning at 550 $m\mu$.

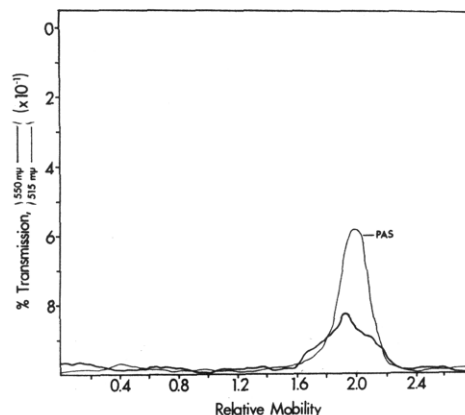


FIGURE 4: Sodium dodecyl sulfate-gel electrophoresis of material extracted with lipid solvents from the surface membranes. Surface membranes from L cells were extracted with lipid solvents and electrophoresis of the organic phase was carried out on sodium dodecyl sulfate gels of 10% polyacrylamide as described in Materials and Methods. The gels were stained for protein by Coomassie Blue, or for carbohydrate by the periodic acid-Schiff's (PAS) technique. The spectrophotometric scans of a Coomassie Blue stained gel and a PAS-stained gel are superimposed.

molecular weights from 130,000 to 5000 and after 15-hr electrophoresis, sharp resolution of the polypeptides was still retained for faster migrating components although the molecular weights could be slightly in error. The differences in molecular weight value for each of the individual absorption peaks of the polypeptides on gels of 10 and 5% polyacrylamide was not greater than $\pm 8\%$ for any of the polypeptides. The identity of the polypeptides separated on both gels was based upon the qualitative similarities in staining intensities as well as the location relative to standards.

Comparison of Surface Membrane Polypeptides before and after Virus Transformation. The separation of the polypeptides from surface membranes of BHK₂₁/C₁₃ and the virus-transformed C₁₃/B₄ fibroblasts after electrophoresis on the dodecyl sulfate gels of 5 and 10% polyacrylamide is shown in Figure 2a,b, respectively. The patterns represent individual gels from the same experiment and are superimposed. By the described procedures, the electrophoretic staining profiles obtained from similarly treated preparations are superimposable.

The majority of the surface membrane polypeptides from both cell lines have similar mobilities and absorption intensity maxima (Figure 2). The notable exceptions correspond to molecular weights of 56,000–46,000, 82,000, 96,000, and 210,000 on gels of 5% polyacrylamide (Figure 2a). In all of these molecular weight classes, the membrane fraction from the virus-transformed cells (C₁₃/B₄) showed reduced intensity of staining when compared to similar amounts of membrane from the nontransformed cells (BHK₂₁/C₁₃). The polypeptide pattern on 10% polyacrylamide gels (Figure 2b) further delineates this difference. The diminished absorbance again corresponds to polypeptides of 96,000–80,000 and 58,000–46,000.

These differences have been observed in all of the membrane preparations examined (four different preparations) and have remained the same after a number of electrophoretic runs. The differences remained constant for as long as the membrane fractions were stored (4 months) and upon reelectrophoresis for five times after storage at -20° . When the Coomassie Blue staining procedure was substituted by using Amido Black as described by Fairbanks *et al.* (1971) the same results were obtained. Lipid extraction did not alter the pattern.

Carbohydrate-Containing Components. Some of the poly-

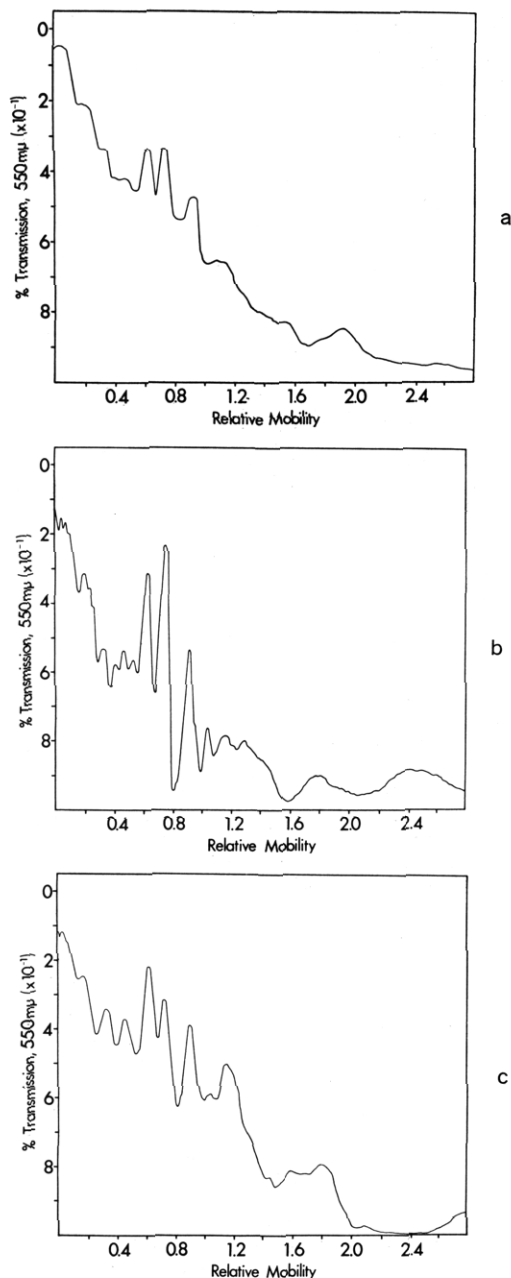


FIGURE 5: Spectrophotometric scans of electrophorograms showing the effects of varying the concentration of solubilizing buffer on the migration of membrane polypeptides. Surface membranes from L cells were solubilized in 0.01 M sodium phosphate buffer (pH 7.2,) containing: (a) 0.2% sodium dodecyl sulfate, 0.5 M urea, 0.1% 2-mercaptoethanol, and 0.001% EDTA; (b) 2% sodium dodecyl sulfate, 5 M urea, 1% 2-mercaptoethanol, and 0.01% EDTA; (c) 5% sodium dodecyl sulfate, 5 M urea, 10% 2-mercaptoethanol, and 0.01% EDTA. Ratios of 1:1, 10:1, and 25:1, respectively, were maintained for the amount of sodium dodecyl sulfate per mg of membrane protein. After electrophoresis of 50 μ g of protein, the sodium dodecyl sulfate gels of 10% polyacrylamide were stained with Coomassie Blue and scanned at 550 m μ on an adapted Zeiss spectrophotometer. Detailed procedures are described in Materials and Methods.

peptides from the surface membranes of L cells showed noticeable staining for carbohydrates by the PAS procedure. On gels of 10% polyacrylamide these polypeptides are 100,000, 56,000, 46,000, and 39,500 (Figure 3). On gels of 5% polyacrylamide the molecular weight regions of 230,000, 210,000, and 100,000 showed intensely staining, sharp bands. The lower molecular weight material was not stained with PAS reagents

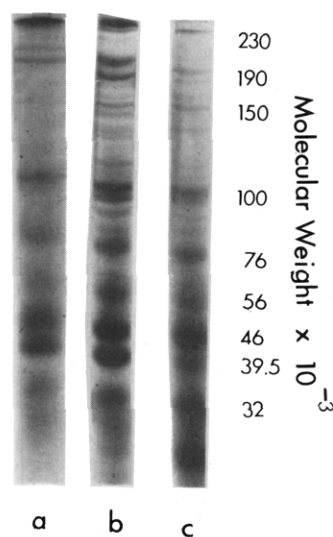


FIGURE 6: Electrophorograms showing the effects of varying the concentration of solubilizing buffer on the migration of membrane polypeptides. Surface membrane preparations from L cells were solubilized at ratios of (a) 1:1; (b) 10:1; (c) 25:1 of sodium dodecyl sulfate:protein as described in Figure 5, and were subjected to electrophoresis on sodium dodecyl sulfate gels of 5% polyacrylamide. The range of molecular weights is included.

probably due to the diffuseness of these polypeptides in gels of 5% polyacrylamide. The most rapidly migrating PAS-stainable region of the 10% gel with relative mobility, 1.8–2.2 (Figure 3) was extracted with lipid solvents (see Figure 4).

The carbohydrate-containing polypeptides from the surface membranes of the BHK₂₁/C₁₃ and the virus-transformed C₁₃/B₄ cells were similar, showing PAS staining in the 56,000–39,500 molecular weight regions. The polypeptide of 54,000 which was present only in the surface membranes of BHK₂₁/C₁₃ cells (Figure 2b) stained with PAS reagents. In contrast to the PAS-staining patterns of the L cell membranes, the membranes from both of these hamster fibroblasts showed none of this material in the 230,000 and 210,000 molecular weight regions. Even when 300 μ g of protein was placed on individual gels of 5% polyacrylamide no PAS-staining material was visualized in this region.

Effect of Lipid Extraction. One half of a preparation of surface membranes isolated from L cells was extracted with lipid solvents and the other half was not extracted. The polypeptides of the extracted and unextracted membranes, stained with Coomassie Blue, were identical after electrophoresis on gels of 10% polyacrylamide with the exception of a difference in the region with relative mobility of 1.8–2.2. The lipid extracted membranes showed no staining in this region. The polypeptides from similar amounts of protein from membrane preparations whether lipid extracted or not, were identical on gels of 5% polyacrylamide.

The lipid extract from the surface membranes migrated as low molecular weight material on gels of 10% polyacrylamide with a relative mobility of 1.8–2.2 and was stained with Coomassie Blue and PAS reagents (Figure 4). A comparable quantity of lecithin (50 μ g), similarly treated, had a similar relative mobility and stained with Coomassie Blue but was not stained by the PAS procedure.

In other experiments the membranes were extracted with 1-butanol and the organic phase extract and insoluble material were examined by electrophoresis on 10% polyacrylamide gels. The more rapidly migrating material (relative mobility of 1.8–2.2) which stained for carbohydrates was found mostly

in the butanol extract. The insoluble membrane pellet had a corresponding reduction in the intensity of staining with PAS reagents and Coomassie Blue.

Effect of Varying the Concentration of Solubilizing Buffer. Figures 5 and 6 show the electrophoretic profiles of the polypeptides from the surface membranes of L cells which were solubilized by varying the concentrations of the components of the solubilizing buffer. Ratios of 1:1, 10:1, and 25:1, respectively, were used for sodium dodecyl sulfate to membrane protein with 10- to 100-fold increases in 2-mercaptoethanol from a concentration of 0.1%. Urea was increased from 0.5 to 5 M and EDTA from 0.001 to 0.01%. The polypeptide distributions obtained under these conditions are shown in Figure 5a-c for gels of 10% polyacrylamide and Figure 6a-c for gels of 5% polyacrylamide. The polypeptides after electrophoresis on gels of both polyacrylamide concentrations showed enhanced resolution in the solubilizing buffer containing 2% sodium dodecyl sulfate, 5 M urea, 1% 2-mercaptoethanol, and 0.01% EDTA (Figures 5b and 6b). In higher concentrations of sodium dodecyl sulfate (5%) and 2-mercaptoethanol (10%), the polypeptide patterns were disrupted (Figures 5c and 6c). In lower concentrations of urea (0.5 M) and sodium dodecyl sulfate (0.2%) the migration of the higher molecular weight polypeptides was retarded (Figures 5a and 6a). When 5 M urea was omitted from the solubilizing buffer, the high molecular weight polypeptides did not enter the gel.

Discussion

The molecular weight distribution of the polypeptides from the surface membranes isolated from mouse and baby hamster kidney fibroblasts ranged from 230,000 to 15,000 (Figures 1 and 2). Polypeptides of comparable or slightly higher molecular weights (Trayer *et al.*, 1971; Fairbanks *et al.*, 1971) have been obtained for red blood cell stroma. With a discontinuous buffer system, Neville and Glossmann (1971) report a spectrum of molecular weights for the polypeptides from membranes of rat liver and kidney brush border as well as erythrocyte stroma, ranging from 310,000 to 10,000. In all cases the presence of high molecular weight polypeptides is characteristic of membrane proteins.

The polypeptide(s) of 230,000 appears to be more exposed to the external environment than the other membrane polypeptides (Poduslo *et al.*, 1972). It is possible that this class of polypeptides is exposed also to the internal environment of the membrane since Poduslo *et al.* (1972), utilizing the technique of lactoperoxidase-catalyzed iodination of proteins (Phillips and Morrison, 1971), find that additional radioactivity was incorporated into this polypeptide when the membranes were removed from the cells before iodination. Singer and Nicolson (1972) have proposed a fluid mosaic model for the structure of cell membranes. As a consequence of this model they suggest that certain proteins may span the thickness of the membrane and be exposed at both surfaces. It is possible that this class of high molecular weight polypeptides which we observe does span the membrane as well as being more exposed to the external surface.

That some of these high molecular weight polypeptides are exposed to the external environment through the carbohydrate moiety is suggested by the fact that after trypsinization of the cell membrane no carbohydrate is detectable in these polypeptides by the PAS-staining procedure. The polypeptides of lower molecular weights show the presence of carbohydrates. Trypsin has been shown to remove 20-30% of the carbohy-

drates from the cell surface (Buck *et al.*, 1970). The L cells, harvested from suspension culture without trypsinization, have carbohydrate-staining material in the high molecular weight polypeptides of the cell membranes (Figure 3), whereas BHK₂₁/C₁₃ and C₁₃/B₄ fibroblasts, grown as monolayer cultures are treated with trypsin in the process of harvesting and have no detectable carbohydrate in similar high molecular weight polypeptides.

The carbohydrate-containing portion of the polypeptides may influence the electrophoretic mobility since fetuin did not behave as predicted in the electrophoretic system. Studies of the dodecyl sulfate binding for glycoproteins show reduced sodium dodecyl sulfate-protein ratios (Pitt-Rivers and Ambesi Impiombato, 1968). Overestimates of the molecular weight for other glycoproteins have been described (Segrest *et al.*, 1971). However, no substantial change in relative molecular weights was observed in our system when molecular weight estimates from 5 and 10% gels were determined for a particular polypeptide although there was a slight shift to a higher molecular weight value in 5% gels for some of the polypeptides. Glossmann and Neville (1971) have demonstrated recently that the PAS-stained patterns do represent size separations of the glycoprotein subunits under their conditions of gel electrophoresis in a discontinuous buffer system.

The similarity of the polypeptide profile for the surface membranes of the mouse and baby hamster kidney fibroblasts may reflect common functional requirements of these membranes. However, even though the patterns were similar, it was possible always to visually associate a particular polypeptide profile with a specific surface membrane (Figures 1 and 2). The change in the polypeptide pattern of the surface membrane following virus transformation was particularly striking (Figure 2) since the amount of certain polypeptides was diminished, as shown by less intense staining with Coomassie Blue. Preliminary results indicate a similar finding for surface membranes from other cells transformed by oncogenic viruses (C. A. Greenberg and M. C. Glick, unpublished observations). These results show that even though there was some variation in the intensity of staining with Coomassie Blue after disc gel electrophoresis of surface membranes from different virus-transformed cells, the staining of these polypeptides was always less than those from the membranes of the normal counterparts. It should be noted that with the exception of the L cells these surface membranes have been prepared from cells which were treated with trypsin in the process of removing them from the monolayer cultures. Differences have been shown in the glycopeptides which are removed by trypsinization from the surface of the transformed cells when compared to their normal counterparts (Buck *et al.*, 1970, 1971). The exact relation that these differences in the glycopeptides and the polypeptides have to each other and to viral transformation is the subject of further investigations.

Acknowledgments

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Effects of 2,3-Diphosphoglycerate on the Oxygen Equilibria of the Half-Cyanmet Hybrid Hemoglobins[†]

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ABSTRACT: The effects of 2,3-diphosphoglycerate on the oxygen equilibria of the half-cyanmet hybrid hemoglobins, $\alpha_2^+(\text{CN})\beta_2(\text{O}_2)$ and $\alpha_2(\text{O}_2)\beta_2^+(\text{CN})$, have been studied. Although the phosphate decreases the oxygen affinity of both the derivatives, the effects are several times larger for $\alpha_2^+(\text{CN})\beta_2(\text{O}_2)$ than for $\alpha_2(\text{O}_2)\beta_2^+(\text{CN})$. The oxygen binding of the stripped hybrids are essentially noncooperative. On the addition of 2,3-diphosphoglycerate, the oxygen binding to $\alpha_2^+(\text{CN})\beta_2$ becomes cooperative, while the phosphate has no effect on the cooperativity in $\alpha_2(\text{O}_2)\beta_2^+(\text{CN})$. The oxygen

affinity of $\alpha_2^+(\text{CN})\beta_2(\text{O}_2)$ decreases more gradually with DPG concentration than that of normal hemoglobin. The response of $\alpha_2(\text{O}_2)\beta_2^+(\text{CN})$ to DPG suggests that the cyanmet β subunits change their conformation when the partner α subunits bind the oxygen molecules, *i.e.*, there exists a propagation of conformational changes from the α subunits to the β subunits. The present results do not follow both the original simple allosteric models of Monod, Wyman, and Changeux and of Koshland, Némethy, and Filmer and lead to some modifications of both the models.

Many studies with regard to the interaction of an unique allosteric effector, 2,3-diphosphoglycerate (DPG[†]) with hemoglobin have elucidated that it binds specifically to deoxy-

hemoglobin in a mole for mole ratio at physiological pH and salt concentration and profoundly lowers the oxygen affinity of hemoglobin (Benesch and Benesch, 1969). X-Ray studies (Perutz, 1970) and biochemical data (Benesch and Benesch,

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¹ Abbreviations used are: DPG, 2,3-diphosphoglycerate; bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane. The MWC and KNF models are those presented by Monod, Wyman, and Changeux

and by Koshland, Némethy, and Filmer, respectively. Molecular species of hemoglobin are represented by the formulae such as $\alpha_2^+(\text{CN})\beta_2(\text{O}_2)$, $\alpha_2\beta_2^+(\text{CN})$, etc., where $\alpha(\text{O}_2)$ and $\beta(\text{O}_2)$, α and β , and $\alpha^+(\text{CN})$ and $\beta^+(\text{CN})$ indicate the oxygenated, deoxygenated, and cyanmet subunits, respectively.