*MULTIPLE PROTEIN COMPONENTS OF MAMMALIAN CELL MEMBRANES**

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Because cell membranes are complex, relatively insoluble structures composed mainly of a mixture of proteins, carbohydrates, and phospholipids, little is known about their structure.[1] In studies utilizing procaryotic organisms it has been reported that detergent-treated *Mycoplasma* membranes[2] dissociated into "homogeneous" 3.3*S* "subunits" and that bacterial membranes dissociated into two major protein components.[3]

The more specialized membranes of eucaryotic cells have been widely studied. Green and his colleagues[4] isolated a "structural protein" of 22,500 molecular weight and have discussed the evidence for protein subunits as the basic structural components of membranes. A structural protein of similar size has been reported for *Neurospora* membranes[5] and for human red cell membranes.[6] Bakerman and Wasemiller[7] reported a structural unit of human erythrocyte membranes of 40,400 molecular weight consisting of a protein of 22,220 molecular weight with associated carbohydrate and lipid. The most recent evidence from Green's laboratory, however, indicates that "structural protein" preparations from mitochondria may consist of several proteins of molecular weight 50,000–65,000.[8] Maddy reported that butanol-solubilized erythrocyte membranes yielded an aggregate of about 300,000 molecular weight which dissociated into heterogeneous subunits.[9] Red cell membranes dissolved in 8 *M* urea plus nonionic detergent exhibited many protein bands upon electrophoresis.[10] Lenard and Singer[11] studied protein conformation in red cell and bacterial membranes by optical rotatory dispersion and circular dichroism. They found unusual properties with both preparations that suggested a common characteristic feature of membrane proteins.

Recently, several laboratories have presented evidence that the membrane envelope of arboviruses is composed of a single virus-coded protein associated with host cell phospholipids.[12] It is of great importance to know whether animal and human cell membranes are composed mainly of one or several "structural proteins." We have investigated this question extensively, employing gel electrophoresis under conditions in which essentially all membrane proteins can be dissolved and in which their molecular weights can be accurately estimated. It will be shown that the various cellular membranes of cultured human and animal cells and of mouse tissues are composed of a large number of proteins of differing molecular weights.

Materials and Methods.—Cells were grown in Eagle's minimum essential medium (MEM), containing 5% calf serum, and labeled in MEM containing H^3- or C^{14}-phenylalanine, valine, and tyrosine in place of the same unlabeled amino acids, plus 2% dialyzed calf serum. The H^3-labeled amino acids were l-phenylalanine 5,300 mc/mM; l-tyrosine 33,700 mc/mM; and l-valine 267 mc/mM. The C^{14}-labeled amino acids were l-phenylalanine 459 mc/mM; l-tyrosine 475 mc/mM; and l-valine 270 mc/mM. *In vivo* label-

ing of mouse tissues involved six intraperitoneal injections of a mixture of six C^{14} amino acids over a 3-day period.

Electrophoretic separations were carried out in 5% acrylamide gels containing 0.1% sodium dodecyl sulfate (SDS), by a modification of the techniques developed by Maizel and his colleagues.[13] The electrophoretic buffer consisted of 0.1 *M* tris(hydroxymethyl) aminomethane (Tris) acetate buffer (pH 9.0), with sodium acetate added to a concentration of 0.05 *M* in order to raise the anion concentration, 0.1% SDS, and 0.01% ethylenediaminetetraacetate (EDTA). Mercaptoethanol (0.1%) was added immediately before use. The polyacrylamide gel was polymerized in 0.1 *M* Tris acetate buffer (pH 9.0), containing 0.5 *M* urea and 0.1% SDS, 0.1% mercaptoethanol, and 0.001% EDTA. Gel polymerizing components in this buffer were 5% acrylamide, 0.167% bis acrylamide, 0.07% ammonium persulfate, and 0.035% N,N,N',N'-tetramethyethylenediamine. Samples were loaded onto 23 × 0.6-cm gel columns after being mixed with appropriate amounts of C^{14}-labeled and H^3-labeled proteins that were to be compared. Samples were applied under the electrophoresis buffer in sample buffer plus 20% glycerol in volumes of 0.3 ml or less. Sample buffer consisted of 0.01 *M* Tris acetate buffer pH 9.0, 0.1% SDS, 0.001% EDTA, 0.5 *M* urea, and 0.1% mercaptoethanol. Protein samples were exhaustively dialyzed against large volumes of this buffer and then were heated in this buffer for about 30 sec before application to the electrophoresis column. Electrophoretic separations were carried out at 3 v/cm constant voltage for about 16 hr. The gels were crushed sequentially on the linear fractionator designed by Maizel,[13] and the fractions were counted in a Beckman scintillation counter under conditions appropriate for discriminating H^3- and C^{14}-labeled proteins.

Microsomal membranes and mitochondria were isolated essentially as described by Mahler.[14] This is a composite technique derived from standard methods evolved in a number of laboratories. Our detailed procedures for isolating and washing membrane fractions will be described in entirety elsewhere.[15] The plasma membrane of cultured cells was stabilized and isolated by the Tris method of Warren *et al.*[16] Nuclear membranes were prepared as follows: Cells were allowed to swell in distilled water for 30 sec at 0°C. Sodium deoxycholate (DOC) was added to a final concentration of 0.1% and the cells were disrupted at 0°C in a tissue grinder with a loose-fitting Teflon pestle. Five to seven strokes were sufficient to disrupt all cells and provide clean, smooth nuclei free of all cytoplasmic tabs. The nuclei were quickly washed and centrifuged at 1500 × *g* and gently resuspended several times in distilled H_2O at 0°C. The nuclei were then resuspended in 0.15 *M* NaCl containing 0.5% DOC and homogenized again in the tissue grinder. This dissolved the nuclear membrane but left most of the nuclear contents as insoluble nucleoprotein which precipitated upon standing at 0°C for 10 min. This precipitate was removed by centrifugation for 5 min at 1600 × *g* and the dissolved membrane proteins in the supernatant were dialyzed against sample buffer. Red blood cell ghost plasma membranes were prepared by a modification of the hemolysis procedure of Kirk.[17]

For accurate molecular weight estimations, highly purified protein standards were labeled with C^{14} dimethyl sulfate. This is an adaptation of the method which Smith *et al.*[18] used to introduce radioisotopes into RNA by methylation *in vitro*. We have found that it can be used to label proteins *in vitro* and that it does not cause significant polypeptide degradation, nor does it markedly change the electrophoretic migration of the labeled protein in this SDS technique (as compared to unlabeled samples stained with amido schwarz). The protein to be labeled was suspended in 0.3 *M* sodium phosphate buffer pH 7.2 containing 0.2% SDS. Between 2 and 20 mg of protein was dissolved in 0.5 ml buffer and heated at 70°C for 1 min to disperse the SDS-denatured protein thoroughly. Dimethyl sulfate C^{14} (New England Nuclear, 2.15 mc/mM) was dissolved in benzene, and 70 μc in 0.05 ml benzene was added to the protein in a screw-cap test tube at 20°C and then shaken vigorously on a rotary mixer in a hood to emulsify the mixture. After 15 min at 20°C the solution was dialyzed for 24 hr against large volumes of electrophoretic sample buffer to remove benzene and C^{14} methanol.

Results.—(1) *Electrophoretic separation of proteins from membrane fractions*

labeled in cell cultures or in vivo with H^3 and C^{14} amino acids: In initial experiments employing gel electrophoresis in Maizel's phosphate buffer at pH 7.2,[13] we experienced difficulty in dissolving membranes completely and in obtaining reproducible electropherograms of labeled microsomal membrane proteins. We therefore tested a number of buffer systems at pH values from 3.0 to 11.2. We found that the pH 9.0 buffer system described above gave very satisfactory results. Apparently, all membranes were completely dissolved in it, electrophoretic results were reproducible, and more than 98 per cent of membrane protein applied to these gels can be recovered.

Figure 1*A* shows that thoroughly washed microsomes from L cells exhibited a large number of labeled proteins ranging in molecular weight from less than 15,000 to over 100,000. Molecular-weight reference points are shown. By using purified C^{14} methyl-labeled reference proteins, we have been able to confirm the report of Shapiro *et al.*[19] that, with these gel techniques that use SDS-containing buffers, the migration of many diverse proteins is inversely related to molecular weight, and that from molecular weight 15,000 to over 100,000 a straight line results when relative migration is plotted against the log of molecular weight.

Figure 1*B*, *C*, and *D* shows that microsomes, nuclear membranes, mitochondria, and plasma membranes are all nearly identical in the composition of their membrane proteins. No two electropherograms are exactly alike but the dual-label technique allows exact comparison on a single gel of two different membrane preparations when one is labeled with H^3 and the other with C^{14}. It can be seen that the ratio of the various proteins is relatively constant in the different membranes and that in all of them the most abundant proteins fall in the size range between 45,000 and 70,000 molecular weight. It is obvious that no single structural protein stands out as the dominant component of these membranes.

Figure 1*E* shows that soluble proteins of L cells are distinguishable from membrane proteins. Other experiments[15] demonstrate that insoluble nucleoproteins are readily distinguishable from nuclear membrane proteins shown in Figure 1*C*.

Figure 2*A* and *B* shows that mitochondrial proteins from HeLa cells, primary human amnion, and L cells are similar in protein composition, although a reproducible difference appears in one of the major peaks. This difference appears in all cellular membrane fractions.[15]

Figure 2*C* shows that microsomal membranes from the livers of mice labeled *in vivo* with C^{14} amino acids also exhibit multiple protein components, although some of these are in much different ratios than those seen in the membranes of mouse L cells in culture. Similar marked differences between the L cell and mouse kidney and liver have been repeatedly observed in mitochondria, microsomes, and other membranes.[15]

(2) *Aggregation controls:* In other studies[20] we have observed sharp separation of viral capsid proteins in this electrophoresis system despite the presence of a great preponderance of membrane proteins. Nor has evidence for aggregation ever been observed when purified enzyme proteins and virus proteins were coelectrophoresed with a great excess of membrane proteins.[15] While this tends to rule out nonspecific protein aggregation in SDS gels, it is necessary to show

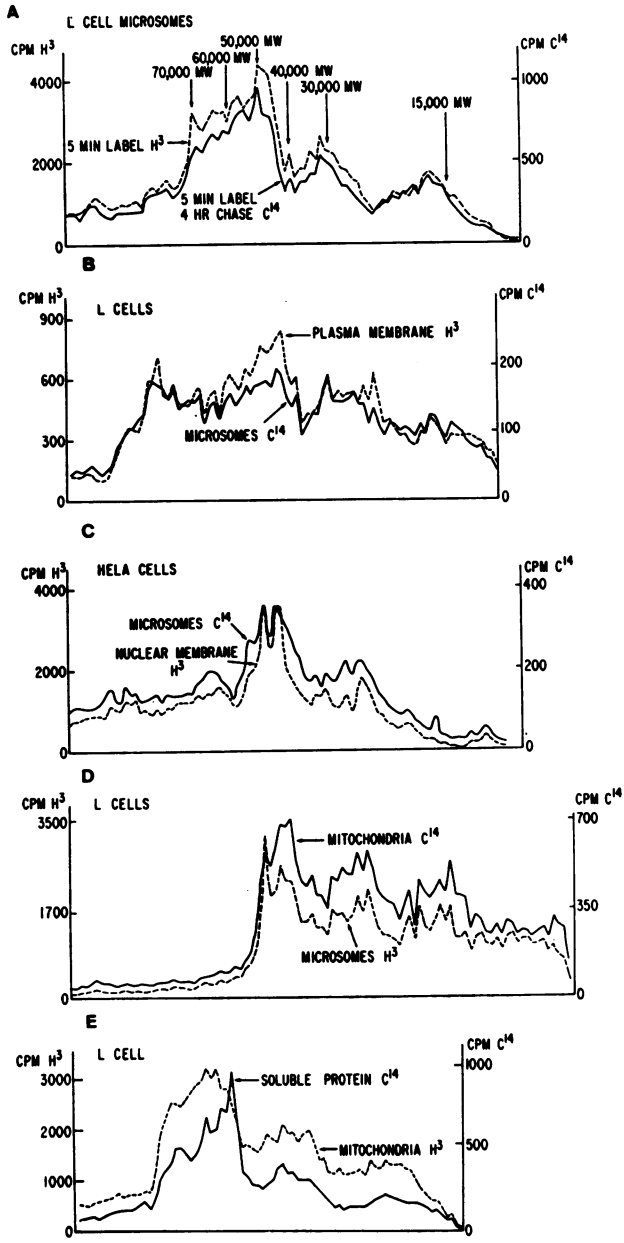

FIG. 1.—Polyacrylamide gel electropherograms of the proteins from various membrane fractions of cultured cells. The anode is on the right. Each graph shows data from about 90 fractions. The final one fourth of the gel at the origin end (to the left) is not shown because it contains negligible radioactivity. Except where otherwise indicated, cells were labeled with H^3 or C^{14} amino acids for 60 min prior to cell fractionation. L cell mitochondria and microsomes in (*D*) were labeled 20 hr with labeled amino acids added to complete Eagle's medium.

that these membrane proteins are really proteins of various sizes and not specific aggregates of membrane subunits. We have carried out the following controls to examine this possibility.

(a) *Different electrophoretic conditions:* We have failed to alter electrophoretic patterns of membrane proteins significantly by (*i*) using a pH 11.2 buffer system with SDS, (*ii*) using 8 *M* urea with 0.5 per cent SDS, 0.1 per cent DOC,

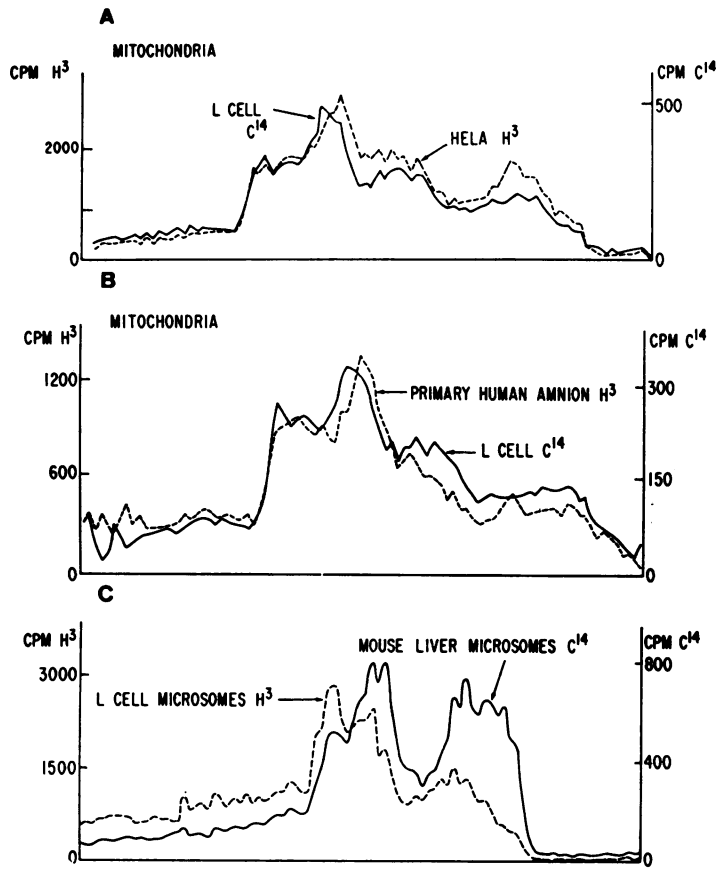

FIG. 2.—Polyacrylamide gel electropherograms of proteins of cellular membranes. Conditions as for Fig. 1. Mouse tissue membranes were labeled by repeated intraperitoneal injection of C^{14} amino acids.

and 0.1 per cent Triton X-100 nonionic detergent in both the gel and the buffer, (*iii*) carrying out electrophoresis at elevated temperature in a 50°C incubator, (*iv*) carboxymethylating reduced membrane proteins before electrophoresis to eliminate disulfide bridging, or (*v*) using prior lipid extraction of membrane protein preparations.

(b) *Re-electrophoresis of electrophoretically separated proteins:* Next we separated bovine cell (MBK) microsomal proteins by electrophoresis and collected 61 fractions. We thoroughly crushed selected fractions in a tissue homogenizer, eluted the proteins at 70°C for an hour in sample buffer, allowed additional time (days) for dissociation of any aggregates present, and re-electrophoresed the individual fractions to see if they ran true. It can be seen in Figure 3 that each fraction did actually contain from one to several discrete peaks at a molecular-weight position approximately corresponding to their position in the original gel. This is strong evidence that these really are individual polypeptide chains of differing molecular weights and that they are not equilibrium aggregates of membrane protein subunits.

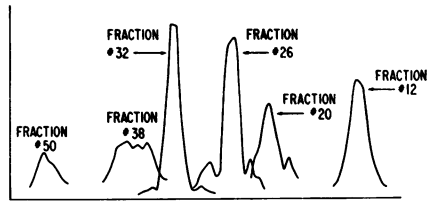

FIG. 3.—Re-electrophoresis of microsomal protein fractions eluted from acrylamide gels after electrophoretic separation. Conditions for recovery of proteins from the first gel are given in *Materials and Methods*.

(c) *DEAE column chromatography in 8 M urea and detergent:* We obtained chromatographic evidence for heterogeneity of membrane proteins by employing a nonionic solution that dissolved membranes completely: 8 *M* urea with 0.1 per cent of the nonionic detergent Triton X-100. This allowed ion-exchange chromatography of denatured membrane proteins on DEAE columns. Figure 4A shows an elution profile of microsomal membrane proteins from bovine kidney cells. A linear gradient to 0.5 *M* NaCl was employed. Figure 4*B*, *C*, and *D* shows electrophoretic reruns of DEAE-separated fractions. It can be seen that this chromatographic column effected a partial fractionation of the membrane proteins. These and other DEAE ion-exchange experiments[15] suggest that this system separates membrane proteins at least partially according to molecular weight.

It was essential to determine whether labeling time affected the above results, since uneven labeling rates and/or uneven rates of turnover could give a distorted protein pattern. However, we have found that membrane protein patterns are identical whether cells were labeled for several minutes, for several hours, or for four days.[15] Membrane protein turnover rates are unequal, but they are sufficiently slow and sufficiently similar that they have no significance for interpretation of the present results.[15] It should be noted in Figure 1*A* that membrane protein patterns are the same when a five-minute pulse is compared with a five-minute pulse followed by a four-hour chase. This precludes gross participation of mechanisms such as those seen with the small RNA viruses in which large precursor proteins are synthesized and are then cleaved into functional viral proteins.[20, 21]

Many of these membrane proteins carry covalently linked polysaccharides since they are readily labeled with H^3 mannose and galactose.[15] However, these sugars do not account for the molecular weight heterogeneity seen here (there is only a slight shift downward in molecular weights of membrane proteins after extensive periodate treatment[15]).

In this system human red blood cell plasma membrane protein behaves differently, and it is likely that RBC membranes are uniquely specialized.[15]

Discussion.—The evidence presented above for heterogeneity of membrane proteins might be viewed merely as evidence for contamination of all membranes with firmly bound nonmembrane proteins. However, since all the cellular membranes contained similar amounts of these proteins even after thorough washing, it does not seem reasonable to consider them as contaminants. Certainly the catalytic proteins of mitochondria[4] and of lysosomes are different from each other and from nuclear and plasma membrane enzymes; hence, the basic similarities seen here between the proteins of these membranes argues for the interpretation that all of these membranes possess a number of related or identi-

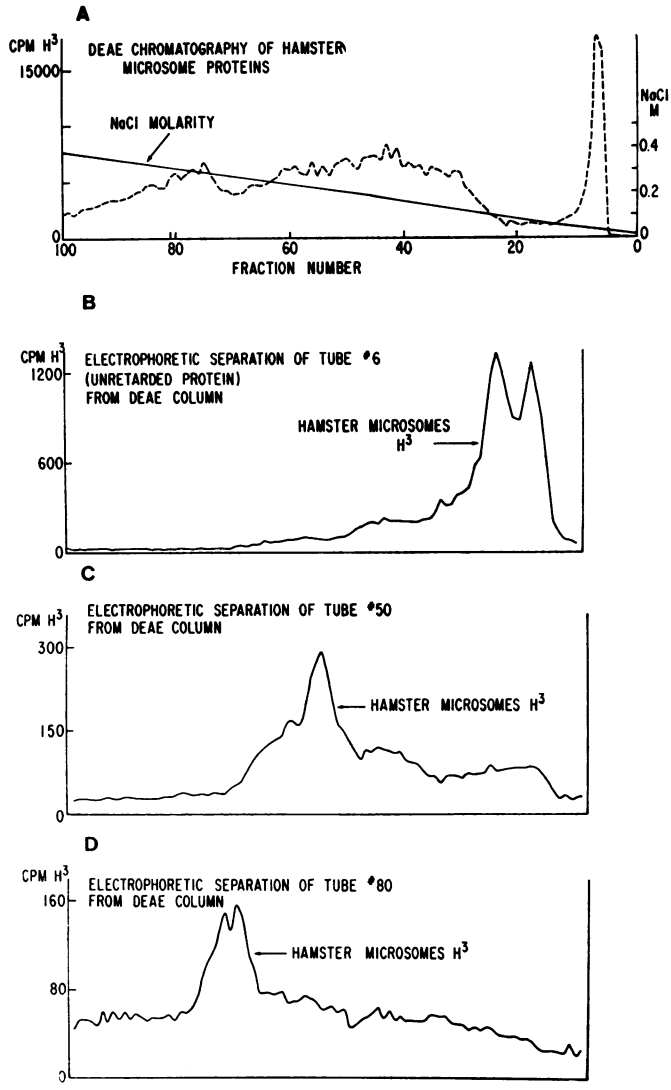

FIG. 4.—Column chromatography of membrane proteins on DEAE, followed by electrophoretic analysis of separated fractions. The membrane proteins were denatured and dissolved in 8 *M* urea plus 0.1% Triton. The elution profile in (*A*) is plotted from right to left to correlate with electropherograms. (*B–D*) Electrophoretic separations of the indicated tubes (fractions) from the DEAE column shown in (*A*).

cal proteins as part of their basic structure. This concept of multiple protein subunits is in harmony with the multiple functions of membranes, although it is at variance with current ideas of one or a few structural proteins.

The differences between L cell and mouse tissue membranes (Fig. 2*C*) and L cell and human cell membranes (Fig. 2*A* and *B*) are interesting, but further work will be needed to interpret their significance.

Summary.—We have solubilized and fractionated the membrane proteins of mouse tissues and of cultured animal and human cells under conditions in which over 98 per cent of the protein was recovered after electrophoresis.

Plasma membranes, microsomal membranes, mitochondria, and nuclear membranes are composed of a large array of proteins of widely differing molecular weights. The membrane proteins from different cell fractions of the same cell are remarkably similar in molecular weight and in their relative proportions. Differences were observed between the membrane proteins of L cells and HeLa cells, and those of L cells and mouse tissues.

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