Membrane and Nonmembrane Proteins of Mammalian Cells. Organ, Species, and Tumor Specificities*

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ABSTRACT: We have used polyacrylamide gel electrophoresis to compare the protein components of cellular membranes from mouse tissues, cultured human and animal cells, and human erythrocytes.

The high-resolving power of this technique is based on protein separation by molecular weight and on the internal control afforded by utilizing protein mixtures labeled with different isotopes. The principal findings follow: (1) Membrane proteins from human and animal cells, with the sole exception of erythrocytes, have a very similar size distribution and most of the protein falls in a range of relatively high molecular weight. (2) Human erythrocyte membranes showed unique behavior. Their proteins were difficult to solubilize and to study by electrophoresis, but they do appear to differ from the other membrane proteins examined here. (3) The various membrane fractions from cultured cells are essentially indistinguishable from one another in their protein patterns, but the converse is true of the membrane systems of cells from mouse organs. The proteins of cell fractions represented by the soluble cytoplasmic supernatant and the nucleoprotein complex are in all cell types easily distinguishable from membrane proteins. (4) Membrane proteins are essentially indistinguishable between cultured cells of different species, while organ-specific differences were consistently found in the proteins of the mouse membranes we have studied. (5) No tumor-specific differences were found in the protein patterns of membranes. (6) A pattern of synthesis similar to that demonstrated *in vivo* was observed in finely diced organ pieces labeled in tissue culture medium.

Most biological membranes from diverse sources show general physical and chemical similarity. However, there is also considerable evidence that the various cellular membranes each contain a specific set of enzymes, consistent with their different roles in the cell. In addition, analogous membranes isolated from diverse cell types tend to have many similar enzymatic activities. This is in accord with parallel functional requirements relatively independent of cell type. The general subject of membrane specificity and diversity has been recently reviewed (Korn, 1969).

In addition to analysis of enzyme content, comparative membrane studies have also been carried out by physical and chemical analysis of major protein constituents. The strong similarity of "structural protein" preparations from a wide variety of membranes has been emphasized (Richardson *et al.*, 1963; Woodward and Munkres, 1966; Schneiderman and Junga, 1968; Green *et al.*, 1968). In addition, structural proteins with identical properties were found to be abundant in all the cellular membranes of *Neurospora*, and a mutation resulting in a single amino acid change in mitochondrial structural protein was likewise found to occur in the structural protein of every cell fraction (Woodward and Munkres, 1967; Woodward, 1968). However, observations on the nature of the structural protein fraction (Green *et al.*, 1968; Schnaitman, 1969) make it presently unclear what this fraction represents, either by itself or in relation to the total membrane protein.

An organ-specific protein antigen has been isolated from rat liver plasma membrane (Neville, 1968). It constitutes 10% of the total membrane protein, has a molecular weight of 70,000 and is specific for the plasma membrane. It is also found in mouse and guinea pig liver membranes, but is not found in chicken or in early embryonic rat liver membranes.

A homogeneous protein has been reported to constitute 25% of the total membrane protein isolated from the red blood cell ghosts of six different species (Marchesi *et al.*, 1969). This protein has a monomeric size of about 150,000 daltons. When preparations of this protein isolated from the six species are compared, they are found to have similar electrophoretic properties, identical amino acid composition, and show immunological cross-reactivity.

Membrane comparisons based on the entire spectrum of proteins give clearer indications of overall similarities and differences. The plasma membrane proteins of *Mycoplasma* were found to be highly strain specific (Rottem and Razin, 1967). In a comparative study of red cell membranes, it was found that the membrane proteins of pigs and sheep were quite similar, ox membranes showed less similarity, and rat and human membranes were both very different from all other species (Zwaal and Van Deenen, 1968b). No differences were found between the proteins of the smooth and rough membranes of rat liver (Manganiello and Phillips, 1965), but more recently (Schnaitman, 1969) it was found that there were eight proteins in common of the 15 total proteins in each membrane. In the latter study it was also shown that of the 12 proteins of the outer mitochondrial membrane only one was also found in the protein of the inner mitochondrial

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membrane, and four were identical with proteins of the smooth endoplasmic reticulum. We have shown that only minor protein differences are demonstrable between the various membranes of different cultured animal and human cells, that all are composed of a very complex array of proteins, and that liver microsomal proteins are considerably different from membranes of cultured cells (Kiehn and Holland, 1968).

In this paper we will show that in contrast to the membranes of cultured cells, differentiated mouse tissues usually show distinct protein differences between the various membranes of the cell and between analogous membranes derived from different organs. We will also describe experiments which suggest that the human erythrocyte ghost may be a uniquely different type of membrane, compared with all of the other membranes we have studied. Finally, because of the known membrane alterations associated with carcinogenesis (for references, see Wallach, 1968) we have searched for tumor-specific differences in the overall pattern of membrane proteins, but have failed to observe such possible differences.

Experimental Procedures

Most of the experimental procedures were described in the preceding paper (Kiehn and Holland, 1970).

The nuclear proteins of cultured cells were fractionated in the following ways. Cells were allowed to swell in distilled water for 30 sec at 0°. Sodium deoxycholate was added to a final concentration of 0.1% and the cells were disrupted at 0° 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°. The nuclei were then resuspended in 0.15 M NaCl containing 0.5% sodium deoxycholate 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° 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.

An alternative procedure which selectively removed all but the most tightly bound proteins from the nucleoprotein complex utilized nuclei purified as described in the preceding paper (Kiehn and Holland, 1970). The purified nuclei were suspended at pH 7.8 in 0.04 M Tris containing 0.5% sodium deoxycholate for 5 min at room temperature, cooled to 0–4° for 20 min, and the nucleoprotein precipitate was removed by centrifugation at 105,000*g* for 30 min.

Red blood cell ghosts were prepared by a modification of a method (Kirk, 1968) based on the standard procedure of Dodge and coworkers (1963). Red blood cells were suspended in a 1:10 dilution of 0.15 M NaCl–0.01 M Tris (pH 7.8)–0.001 M EDTA at 0°, pelleted at 2000*g* for 5 min, and the plasma and buffy coat were carefully aspirated. This washing treatment was repeated three times. The washed cells were then hemolyzed in a 1:20 dilution of 0.007 M NaCl–0.005 M Tris (pH 7.8)–0.001 M EDTA at 0°, pelleted at 2000*g* for 5 min, and the released hemoglobin and other intracellular constituents were aspirated. The stroma were then washed an additional five times by repetition of this treatment. The final wash solutions had no traces of hemoglobin and the final preparation of membranes was white and completely devoid of red pigment. The red blood cell stroma were labeled with radioactive dimethyl sulfate as described in the preceding paper (Kiehn and Holland, 1970).

Primary cultures of human and mouse tissues were established by washing excised tissues repeatedly in Hanks balanced salts solution (Hanks and Wallace, 1949), mincing the tissue thoroughly, dispersing the cells at room temperature with 0.25% trypsin in BSS,[1] washing the trypsinized cells repeatedly in BSS, and then resuspending them in minimal essential medium (MEM) for growth in culture bottles.

Permanent cell lines were purchased from Flow Laboratories and from Microbiological Associates.

Diced organ pieces were labeled *in vitro* in the following way: excised mouse organs were washed in BSS at 0°, diced into pieces 1–2 mm^2, washed again in BSS, and put up in MEM for labeling with radioactive amino acids according to our usual procedure (Kiehn and Holland, 1970).

Results

Comparative Studies of Proteins from Subcellular Fractions. Figure 1A compares the protein patterns of L-cell mitochondria and soluble cytoplasm. The soluble proteins of the cell are distinctly different than those associated with mitochondria and as will be further demonstrated, they are easily distinguishable from the proteins of any other cell fraction. The molecular weight markers included in Figure 1A show the size distribution of proteins in these cell fractions. This distribution is shifted to molecular weights slightly higher than we previously estimated (Kiehn and Holland, 1968). Our standard curve of protein molecular weight versus gel migration shown in the accompanying paper (Kiehn and Holland, 1970) is based on many more purified reference proteins than we had available for the initial study. The values quoted in that study which are now significantly different are ($\times 10^3$ daltons) 50, 60, and 70, which have been found to be, respectively, about 55, 67, and 82.

Figure 1B compares microsomal proteins with mitochondrial proteins of L cells and only minor differences are observed. Figure 1C shows the proteins of L-cell plasma membranes and the proteins of the microsomes left after separation of plasma membranes. Again, only small differences are noted.

Figure 2A compares the proteins of HeLa microsomes with HeLa nuclear membrane proteins obtained after precipitation of nucleoproteins in 0.15 M NaCl and 0.5% sodium deoxycholate as described in experimental procedures. The pattern of nucleoproteins obtained by this method is similar to that found for histones extracted from HeLa nuclei with 0.25 M HCl (Robbins and Borun, 1968). Very little, if any, histones contaminate the supernatant membrane proteins. This is in contrast to the considerable contamination of supernatant proteins shown in Figure 2B when nucleoprotein is precipitated in sodium deoxycholate in the absence of salt. Figure 2B shows the result of the fractionation of BHK-21 (baby hamster kidney line) nuclei by this procedure. Not only is the supernatant contaminated with histones but the remaining

[1] Abbreviations used are: BSS, balanced salts solution; MEM, minimal essential medium.

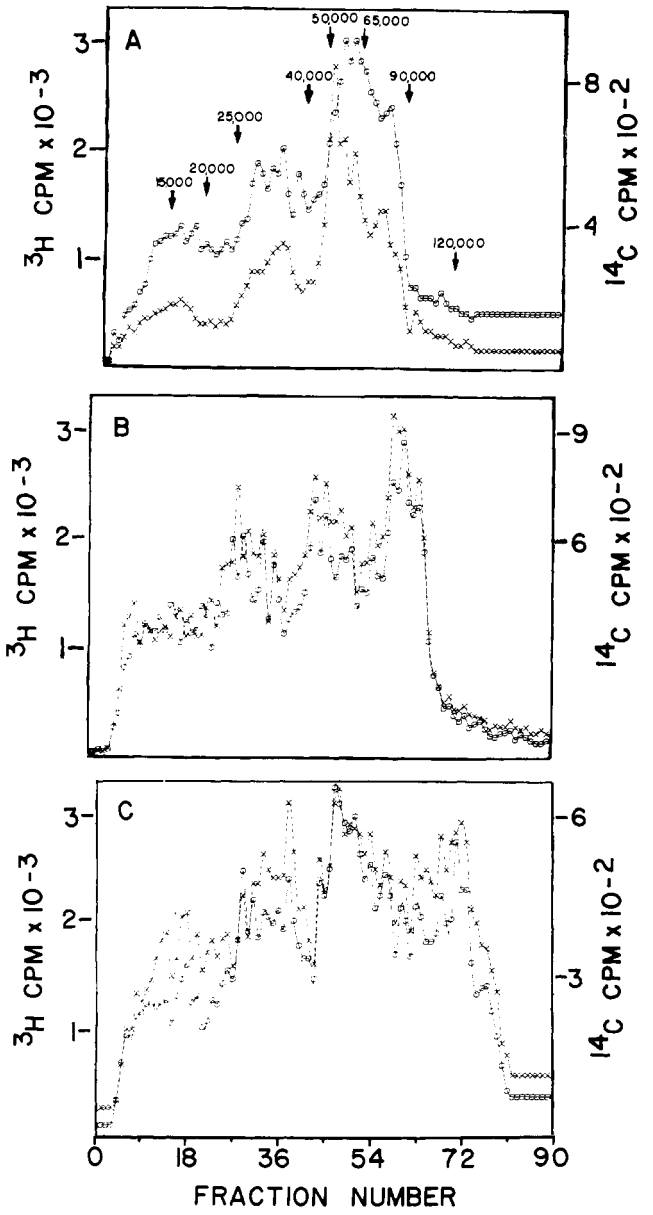

FIGURE 1: Proteins of L-cell subcellular fractions. (A) Proteins of mitochondria (—⊖—) and of soluble cytoplasm (--X--). Molecular weight markers are included. In this, and in all subsequent electropherograms, the anode is on the left. (B) Proteins of microsomes (—⊖—) and of mitochondria (--X--). (C) Proteins of plasma membrane (—⊖—) and of microsomes remaining after isolation of the plasma membrane (--X--).

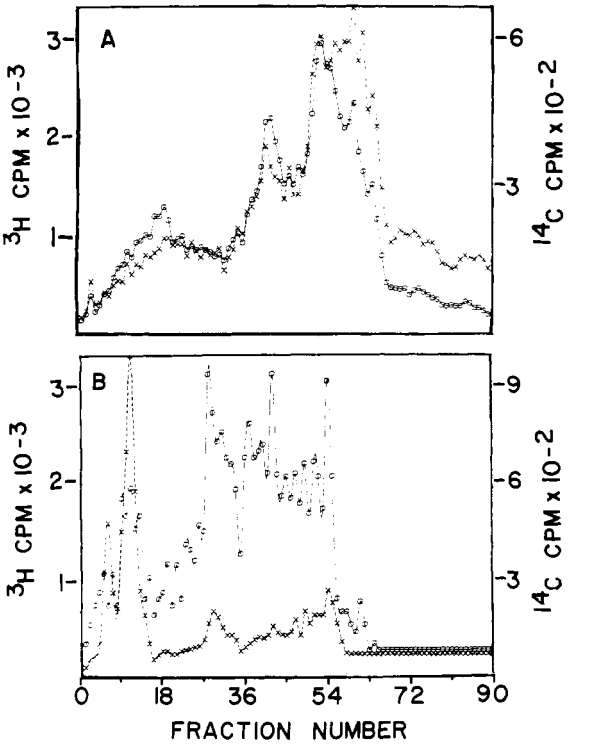

FIGURE 2: Nuclear proteins. (A) Proteins of HeLa nuclear membrane (—⊖—) and of HeLa microsomes (--X--). The nuclear membrane components were isolated by salt precipitation of nucleoprotein in sodium deoxycholate. (B) Result of fractionating hamster nuclei in 0.5% sodium deoxycholate in 0.04 M Tris (pH 7.8) without salt. Nucleoprotein precipitate (--X--) and supernatant proteins (—⊖—).

nucleoprotein precipitate is greatly enriched in arginine-rich histones, compared with salt-precipitated nucleoprotein. Robbins and Borun (1968) have identified the fastest moving peaks as the arginine-rich histones and the next more slowly moving major peak as lysine-rich histone.

Figure 3A shows a comparison of the proteins of mouse liver soluble cytoplasm and microsomes. As was the case with L cells, soluble proteins are clearly distinguished from membrane proteins. Molecular weight markers are included.

Figure 3B compares the microsomal and mitochondrial proteins of mouse liver. The differences observed in the three groups of highest molecular weight are very reproducible. It is also possible to distinguish microsomal proteins from mitochondrial proteins in kidney, heart, and lung tissue isolates. This is in contrast to the situation with cultured cell lines, where clear differences are not seen.

In Figure 3C,D liver nuclear proteins are compared with the proteins of microsomes and mitochondria, respectively, and are observed to be distinctly different.

Species Comparisons. We have examined the overall pattern of proteins in a variety of cultured cells and have found that several human cell types, including HeLa and primary amnion, L cells (mouse), BHK-21 (hamster), and chick embryo fibroblasts were indistinguishable. MBK (bovine) cells showed some very small but repeatable differences from the cell types mentioned above, but *E. coli* was very dissimilar. While differentiated mouse tissues will be shown to exhibit distinct individuality in protein patterns, taken together they would appear to conform much more closely to the other vertebrate cell types than to *E. coli*. Thus, the size distribution of proteins appears to be nearly identical for a variety of vertebrate species and conforms closely to the curve for HeLa proteins shown in the preceding paper (Kiehn and Holland, 1970; Figure 3).

Comparisons of organelle proteins between various species of cultured cells likewise results in the finding of only very small differences. For example, no differences were found in the nuclear proteins of human, rat, and rabbit cell lines.

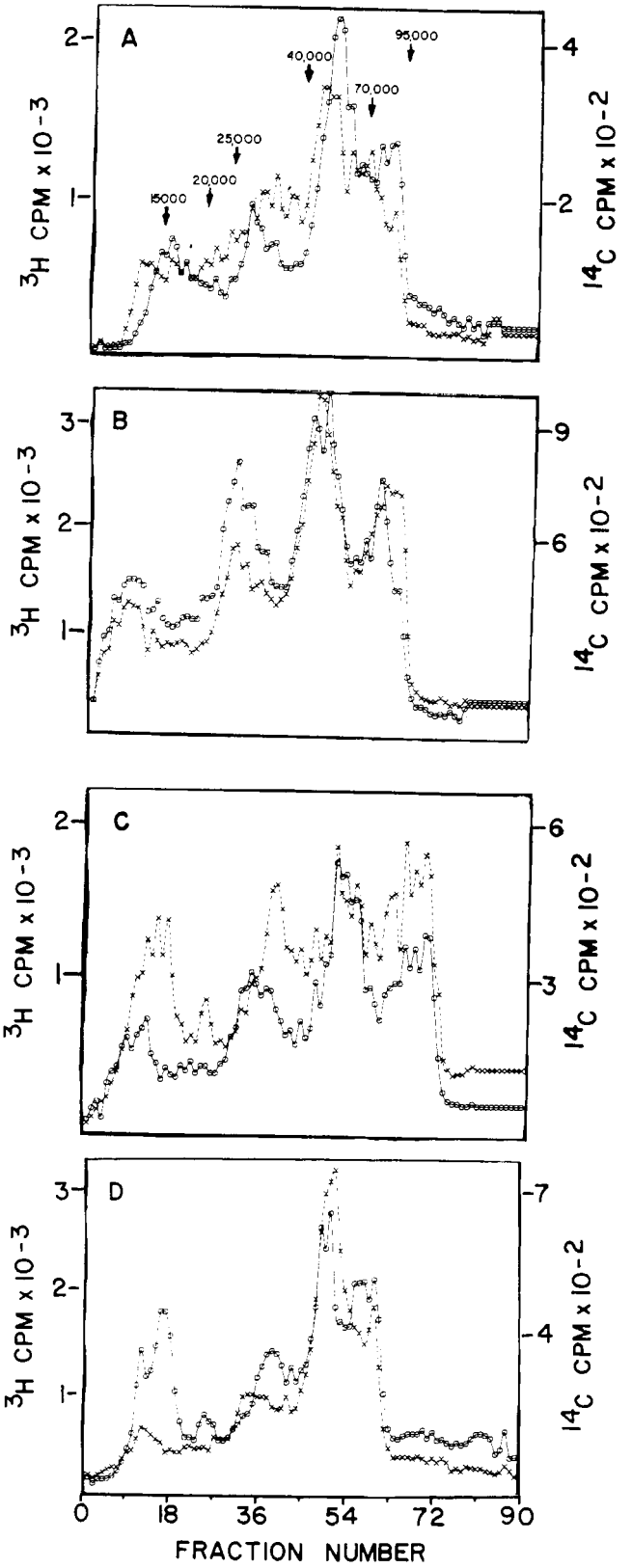

FIGURE 3: Proteins of mouse liver subcellular fractions. Proteins of mitochondria (—⊖—) and of soluble cytoplasm (--X--). Molecular weight markers are included. (B) Proteins of mitochondria (—⊖—) and of microsomes (--X--). (C) Proteins of microsomes (—⊖—) and of nuclei (--X--). (D) Proteins of nuclei (—⊖—) and of mitochondria (--X--).

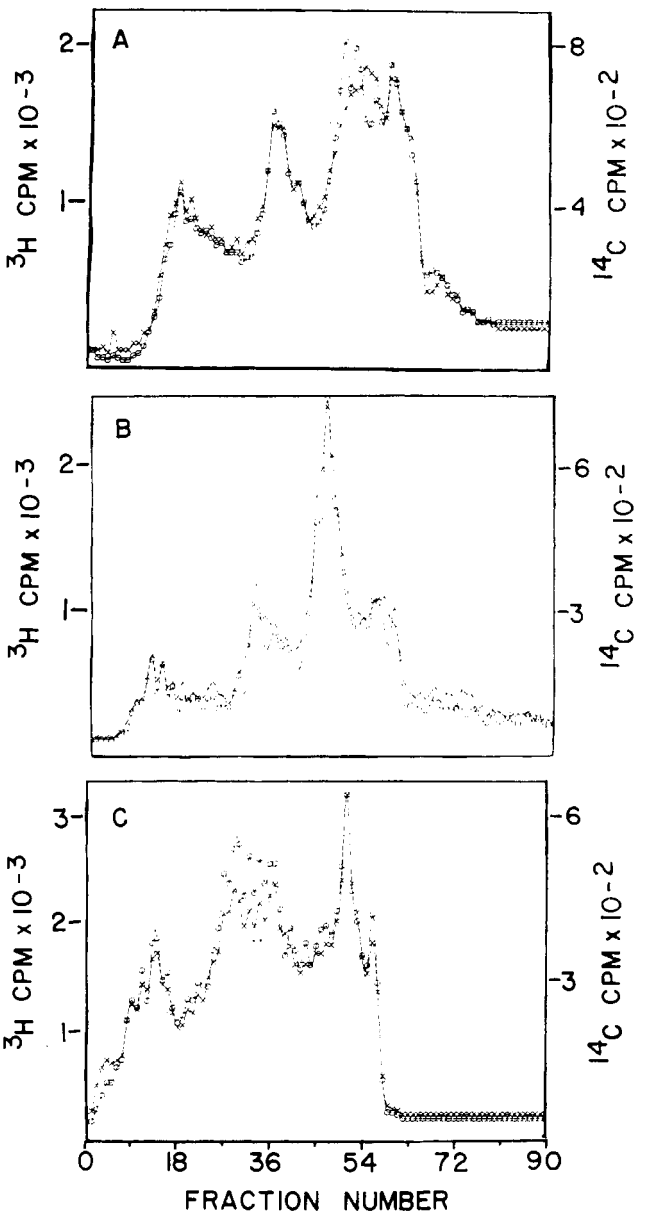

FIGURE 4: Control experiments on mouse organ subcellular fractions. (A) Proteins of kidney mitochondria isolated 4 hr after labeling with [^{14}C]amino acids (--X--) and isolated in a high concentration of unlabeled kidney and liver tissue 24 hr after labeling with [^{3}H]amino acids (—⊖—). (B) Proteins of liver microsomes isolated 4 hr (—⊖—) and 24 hr (--X--) after labeling. (C) Proteins of kidney soluble cytoplasm isolated 4 hr (—⊖—) and 24 hr (--X--) after labeling.

We reported (Kiehn and Holland, 1968) a significant difference between human and mouse L cells in mitochondrial proteins, and this was also displayed to a lesser degree in other cell fractions. Apparently this result was due a variant L-cell line, for after we lost this particular line by contamination, we were unable to again find this difference in experiments conducted with several new cultures of L cells.

Mouse Organ Differences. In mouse tissues, we have found that protein patterns are specific for the subcellular fraction and for the organ of derivation. No differences were found

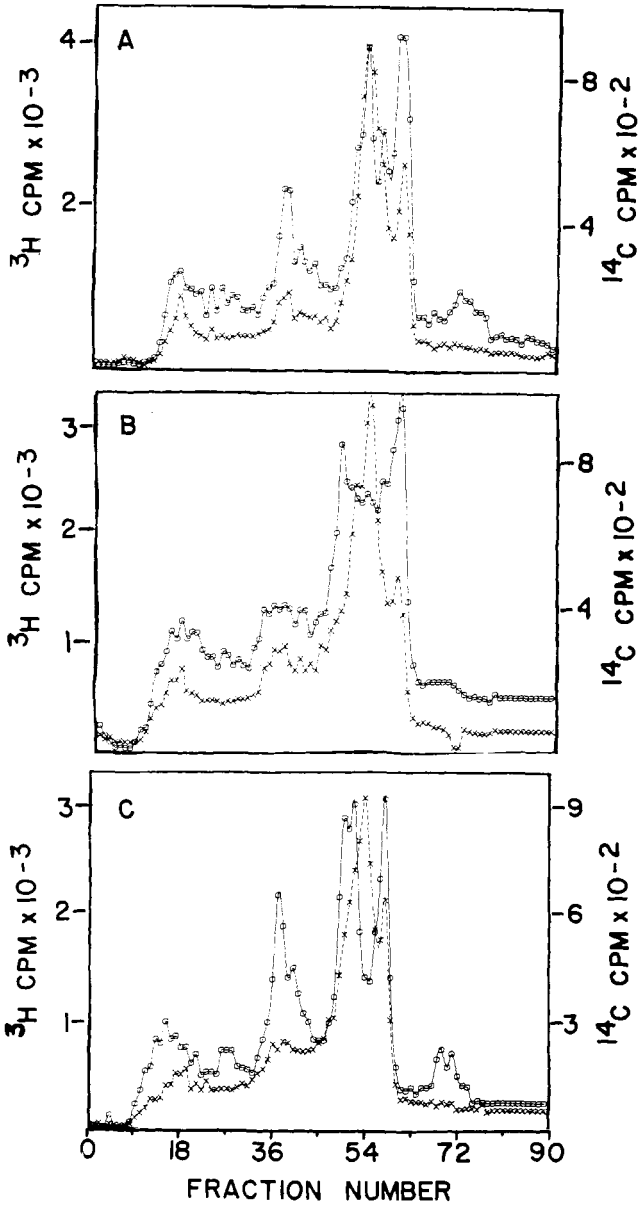

FIGURE 5: Organ specificity of mitochondrial proteins. Mouse liver mitochondrial proteins (--X--) are compared with those isolated from other organs (—⊖—): (A) kidney, (B) lung, and (C) heart.

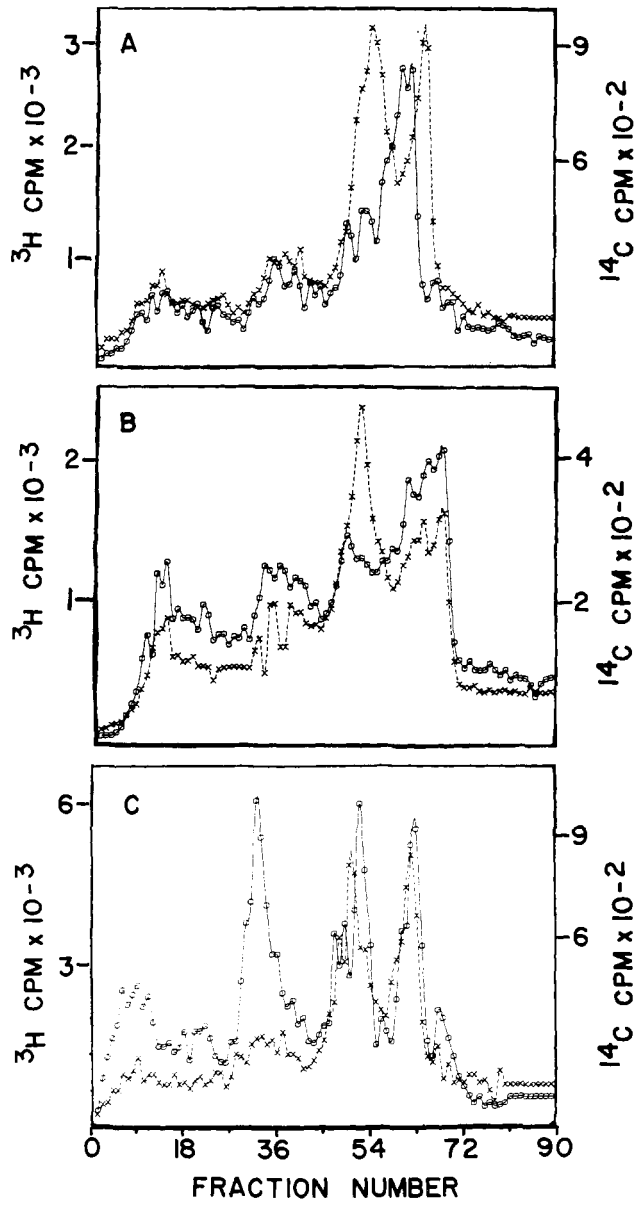

FIGURE 6: Organ specificity of microsomal proteins. Mouse liver microsomal proteins (--X--) are compared with those isolated from other organs (—⊖—): (A) kidney, (B) lung, and (C) heart.

between 2-week-old mice and 3–5-month-old mice, or whether sacrifice was 4 or 24 hr after intraperitoneal injection of radioactive amino acids. This was true when cell fractionation of labeled tissue was carried out in a high concentration of unlabeled kidney and liver homogenate.

Figure 4 shows some typical experiments in which such factors were ruled out as a cause of major variability.

Representative experiments are shown in Figures 5–7 using liver fractions for comparison and internal standardization. Figure 5A–C compares the mitochondrial proteins of liver with those of kidney, lung, and heart, respectively. Figure 6A–C, compares the microsomal proteins of liver with those of kidney, lung, and heart, respectively. Figure 7A compares the soluble proteins of liver with heart-soluble proteins. Figure 7B compares liver-soluble proteins with the soluble proteins of leg muscle which had been extracted at low ionic strength with 0.01 M Tris buffer (pH 7.0). This extraction procedure leaves muscle contractile proteins and other proteins as an insoluble precipitate, and yields a supernatant consisting largely of glycolytic enzymes (see White *et al.*, 1964). The observed number of protein species is roughly consistent with the limited array of enzymes in the glycolytic pathway. Finally, Figure 7C compares the nuclear proteins of liver and kidney.

All of the membranes and cell fractions of mouse tissues that we have compared have distinctly different protein patterns except that there is similarity between liver and kidney nuclei as is shown in Figure 7C. These were the only nuclei examined for organ differences. Due to the difficulty in obtaining brain proteins of high specific activity, only a few

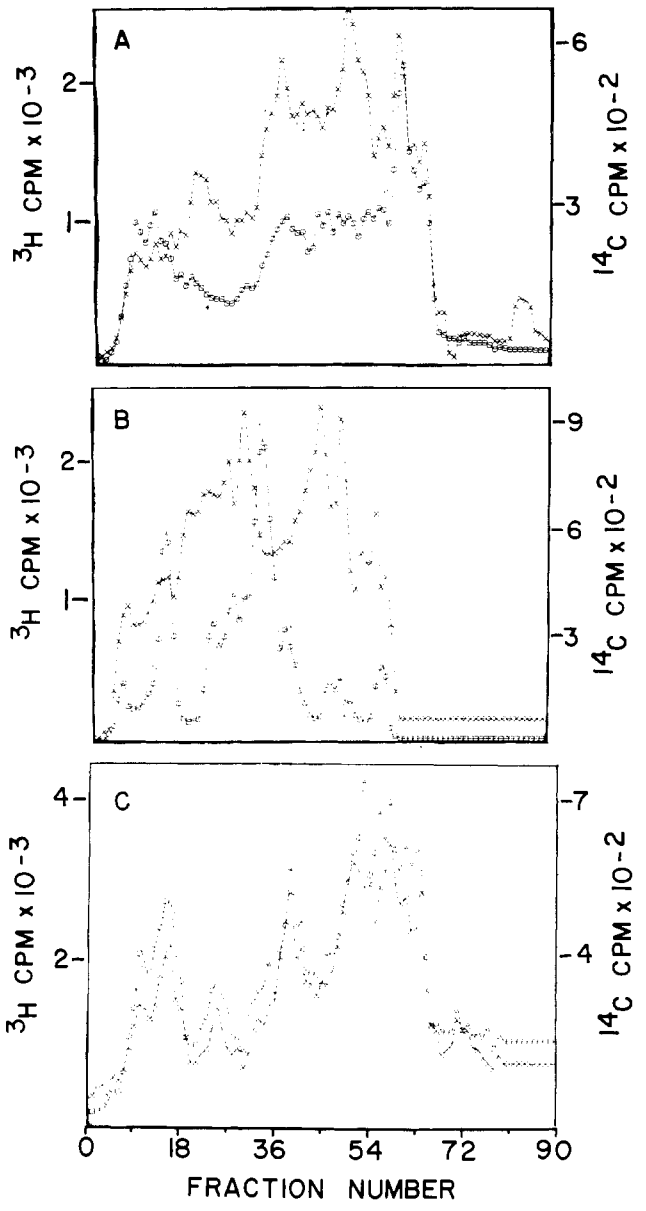

FIGURE 7: Organ specificity of soluble and nuclear proteins. Mouse liver proteins (--X--) are compared with those from other organs (—⊖—). (A) Soluble cytoplasmic proteins of liver and heart. (B) Liver soluble proteins and the soluble proteins of leg muscle extracted at low ionic strength as described in the text. (C) Nuclear proteins of liver and kidney.

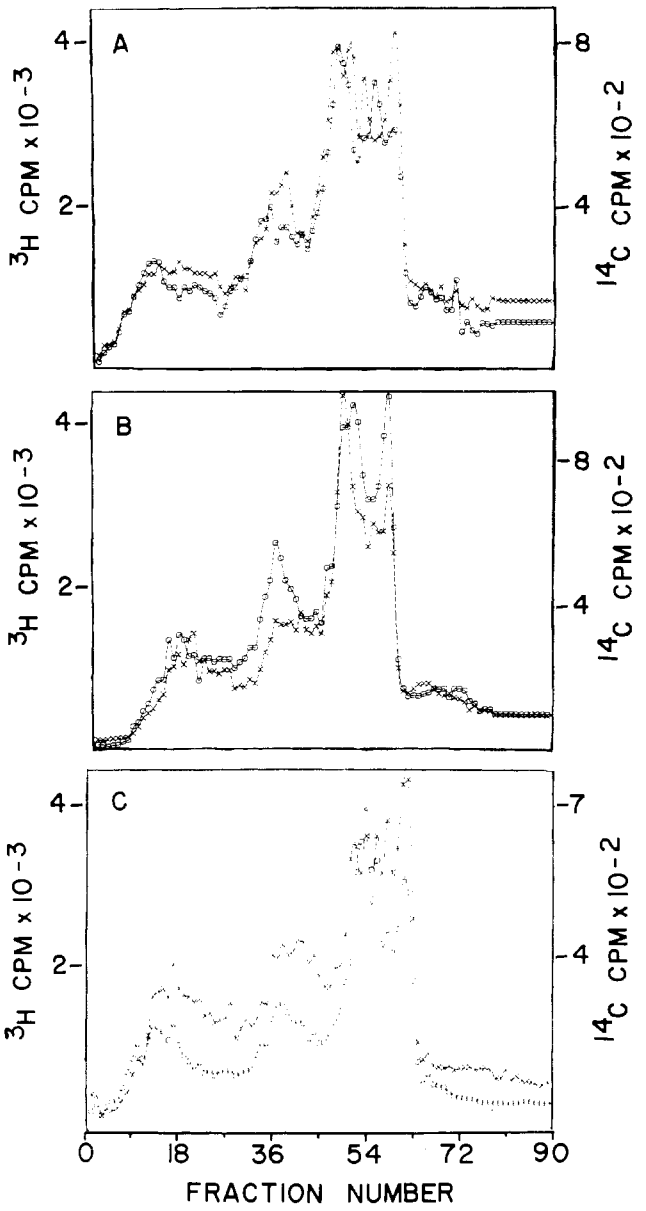

FIGURE 8: Effects of growth conditions on proteins of mouse subcellular fractions. Organ proteins (—⊖—) and proteins of undifferentiated cells (--X--). (A) Microsomal proteins of kidney and L cells. (B) Mitochondrial proteins of kidney and primary embryonic fibroblasts. (C) Microsomal proteins of liver and primary embryonic fibroblasts.

experiments were performed, but these suggested that brain fractions are also clearly distinct from liver fractions.

Effects of Growth Conditions on Protein Patterns. Figure 8A compares microsomal proteins of normal mouse kidney with those of the permanent L-cell line, which was originally capable of producing sarcomas in mice. The overall similarity in protein patterns is striking, especially in light of the long history of the L-cell line in culture. Figure 8B compares the mitochondrial proteins of normal kidney with those of cultured embryonic mouse fibroblasts. Again while there are specific differences, there is a notable overall similarity of proteins from differentiated tissues with the proteins of undifferentiated, actively growing cells. Figure 8C compares the microsomal proteins of liver with those of primary mouse embryo fibroblasts, and greater differences are observed than in Figure 8A,B.

Figure 9A compares the microsomal proteins of normal kidney with microsomal proteins isolated from primary mouse kidney cultures after 4 days *in vitro*. Figures 9B compares the mitochondrial proteins of normal and cultured kidney cells. Both exhibit distinct differences and there are also differences in the soluble cytoplasmic proteins, but nuclear proteins are quite similar to those of normal tissue. The altered protein patterns shown in Figure 9A,B which

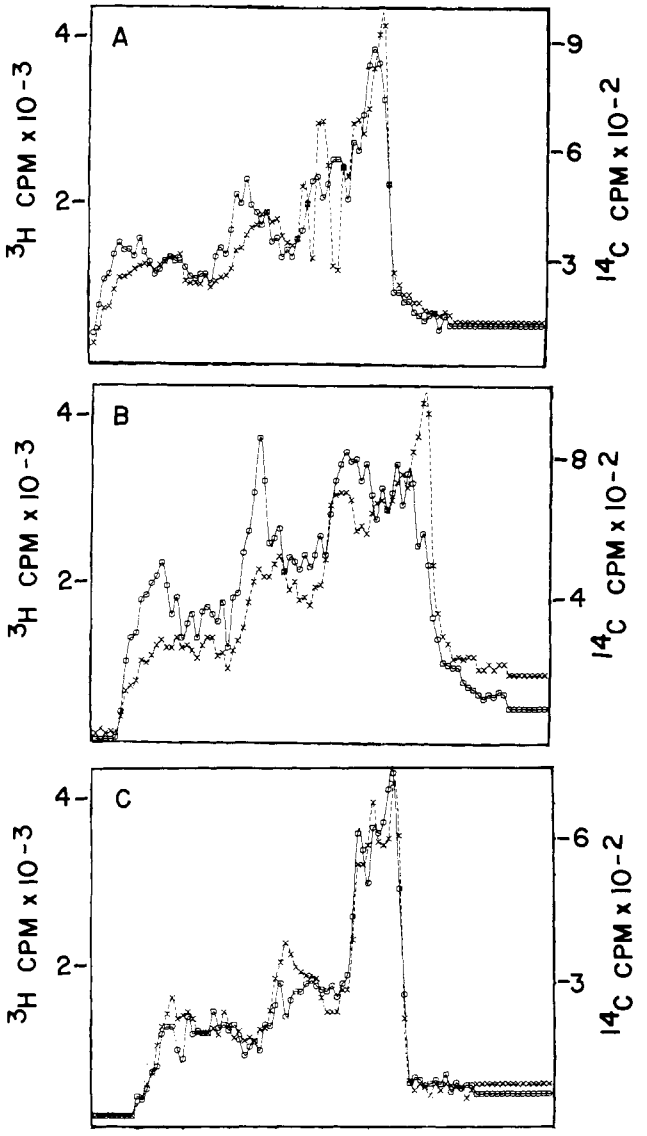

FIGURE 9: Effects of growth conditions on proteins of mouse subcellular fractions. (A) Microsomal proteins of kidney (—⊖—) and of primary kidney cultures after 4 days *in vitro* (--X--). (B) Same conditions as in part A except that mitochondrial proteins are compared. (C) Mitochondrial proteins of kidney labeled *in vivo* (--X--) and labeled *in vitro* in diced organ pieces (—⊖—).

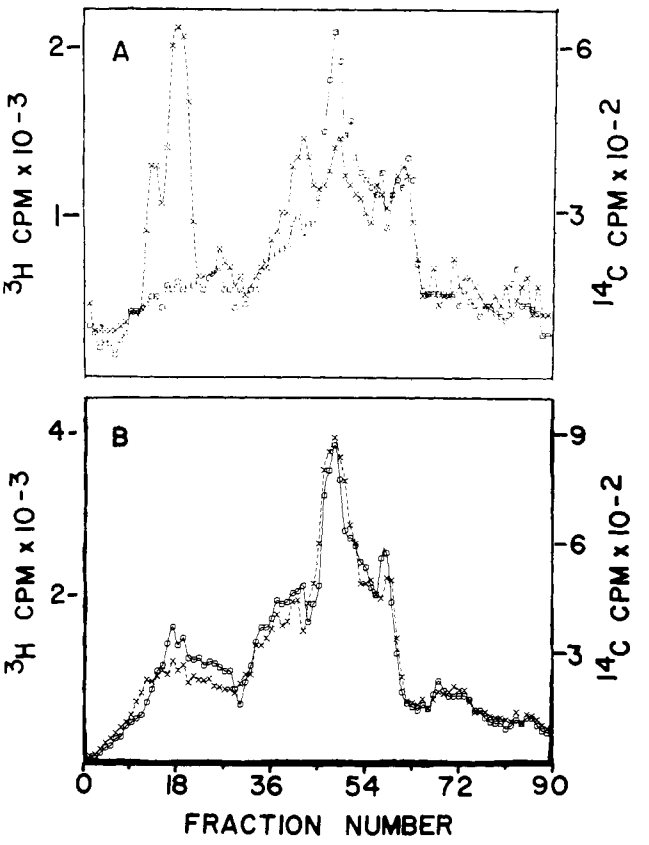

FIGURE 10: Effects of growth conditions on proteins of subcellular fractions. (A) Nuclear proteins of primary human amnion cultures which were actively growing (--X--) and which were older, confluent and not dividing (—⊖—). (B) Microsomal proteins of BHK-21 hamster cells (--X--) and of cultured cells derived from a polyoma virus induced hamster tumor (—⊖—).

accompany dedifferentiation in culture of mouse kidney cells are somewhat similar to the protein patterns of the mouse L cells and cultured embryonic mouse fibroblasts shown in Figure 8A,B.

Figure 9C compares the mitochondrial proteins of kidney labeled *in vivo*, with mitochondrial proteins isolated from diced kidney pieces labeled *in vitro*. Similar results were obtained with other organs, and in these cases the proteins labeled *in vitro* appear to be quite similar to those labeled *in vivo*.

Figure 10A compares human amnion nuclear proteins synthesized in growing primary cultures with those made in older, confluent cultures which were not actively dividing. It is obvious that histone synthesis is repressed in the cultures which are not multiplying but that high molecular weight proteins, similar to those of the nuclear membrane shown in Figure 2A, continue to be synthesized. It has been shown (Warren and Glick, 1968) that synthesis of the plasma membrane and of residual cell particulates continue at the same rates, whether or not cells are multiplying. It is well established that histone synthesis is coupled to DNA synthesis (for references, see Robbins and Borun, 1968).

We used BHK-21 (hamster) microsomes as a control to study the microsomal proteins of several cell lines which had been derived from hamster tumors. Figure 10B shows that only small differences exist between the microsomal proteins of BHK-21 and a polyoma-induced tumor line. The same result was found for Adenovirus 3, Rous sarcoma virus, and spontaneously induced hamster tumors.

Human Erythrocyte Membrane. The human red blood cell ghost was found to be fundamentally different from all of the other membranes we have studied. Though easily dissolved in our pH 9.0 sodium dodecyl sulfate buffer, the majority of the ghost protein usually did not enter the gel or entered it only marginally. This was verified by radioactivity measurements or by staining dissolved stroma that had not been labeled in dimethyl sulfate, which resulted in a single staining band at the origin.

With numerous preparations of blood from five different individuals, we generally found only 10–20% of the radio-

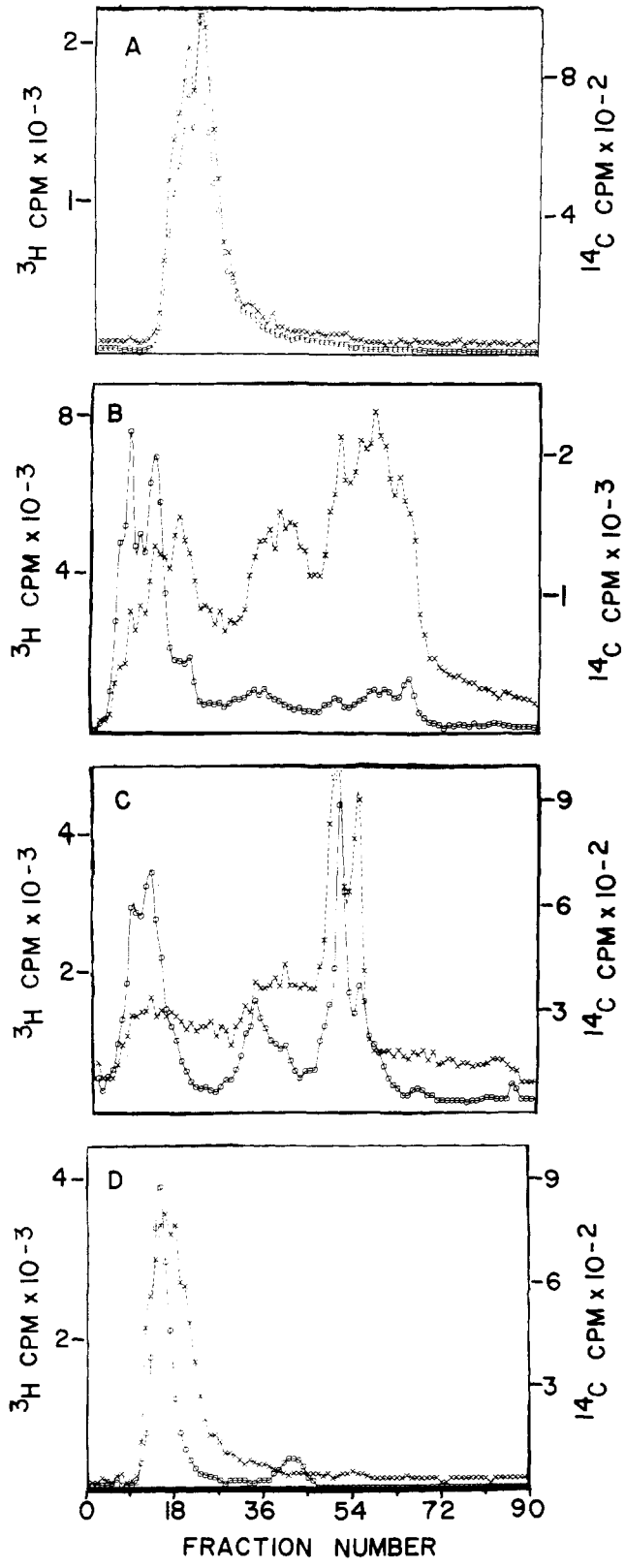

FIGURE 11: Human erythrocyte membrane proteins. (A) Blood type A (--X--) and type O (—⊖—). (B) Erythrocyte membrane proteins (—⊖—) and HeLa microsomal proteins (--X--). (C) The only preparation of erythrocyte membrane proteins from one individual (—⊖—) and microsomal proteins of primary mouse embryo fibroblasts (--X--). (D) Erythrocyte membrane proteins (--X--) and purified hemoglobin (—⊖—).

activity in the three-fourths of the gel (from the anode end) that we routinely analyze (see preceding paper, Kiehn and Holland, 1970). However in two preparations, red cell stroma proteins migrated to the extent of 32 and 41% of the applied radioactivity. A method (Mazia and Ruby, 1968) which eliminates ions in the preparation of stroma, and which results in membranes which are water soluble, did not appear to increase the proportion of migrating protein. However, when a sample of dissolved stroma which originally did not enter the gel was dissolved in 1:1:1 (v/v) phenol–glacial acetic acid–water solution, and then dialyzed back against large volumes of sample buffer, 77% of the protein migrated. In two paired experiments, with and without the strong denaturation step just described, a 41% migration was improved to 75%, and a 32% migration was improved to over 95%. The pattern of proteins which did migrate into the gel was not altered by the two- to threefold increase in migrating protein.

Figure 11A shows the membrane proteins of type A and type O red blood cells. The gel pattern, and trichloroacetic acid precipitable radioactivity, were resistant to periodate oxidation, but were completely sensitive to pronase digestion.

Figure 11B compares the proteins of erythrocyte membranes with the proteins of HeLa microsomes. It is observed that ghost protein is mainly represented by a heterogeneous collection of proteins of 10,000–20,000 daltons in molecular weight. Some high molecular weight protein is seen and this is variable; for example, in Figure 11A there is a complete absence of high molecular weight proteins in the two preparations.

Figure 11C shows a preparation of red blood cell stroma we obtained from one individual. It is included here because it is unique among all of the stromal preparations in having several distinct high molecular weight species of protein in addition to the usual small polypeptides. In all other properties it is similar to the other preparations of red cell ghosts. It is compared with the microsomal proteins of mouse embryo fibroblasts. This was the only sample we obtained from that individual.

Figure 11D compares stromal proteins with purified human hemoglobin. While the membrane proteins are in the same size range as hemoglobin, it is obvious that the pattern of stromal proteins cannot be due to hemoglobin contamination. Other experiments have shown that highly purified human hemoglobin labeled with [^{14}C]dimethyl sulfate coelectrophoresed with mouse red blood cell hemolysate labeled with [^{3}H]amino acids *in vivo*, and with human red blood cell hemolysate labeled with [^{3}H]dimethyl sulfate. This indicates that not only does hemoglobin represent almost the total intracellular protein of the erythrocyte, but that the dimethyl sulfate labeling procedure does not alter the migration of proteins. The latter finding has also been verified for other highly purified proteins by comparing the migration of methylated proteins with untreated proteins by staining with Amido Schwarz. In addition, our method of dimethyl sulfate labeling of proteins has never been observed to result in degradation. Finally, mitochondrial proteins from tissue culture cells were labeled with [^{3}H]dimethyl sulfate just as were the proteins of erythrocyte membranes. When coelectrophoresed with mitochondrial proteins labeled *in vivo* with [^{14}C]amino acids, they were found to display an almost identical size distribution of labeled material and almost all

of the applied radioactivity entered the gel, although there was not exact coincidence in certain peaks.

It is concluded that the pattern of stromal proteins is not due to hemoglobin contamination or to an artifact generated by the dimethyl sulfate labeling procedure.

Figure 12 shows gel filtration analysis on a P-200 column of HeLa microsomal proteins and phenol–acetic acid treated erythrocyte ghost proteins. The fraction at which the peak of deoxyribonuclease (31,000 daltons) eluted is shown by a marker. In agreement with the electrophoretic analysis of the same material, 80% of the ghost protein is of relatively low molecular weight and is therefore retarded, the ghost protein is mostly smaller than 31,000 daltons, and it is heterogeneous in protein components.

We have used Bio-Gel and Sephadex columns under a variety of conditions described in the preceding paper (Kiehn and Holland, 1970), in attempts to repeat the observation of Bakerman and Wasemiller (1967) that voided and retarded column fractions were in equilibrium with each other. Using dimethyl sulfate labeled stroma and *in vivo* labeled HeLa microsomes, we isolated the initial voided and retarded fractions, exhaustively dialyzed them against distilled water, redissolved them in sample buffer containing 1% sodium dodecyl sulfate, and allowed days for complete equilibration at room temperature. Analysis of each of the fractions on gel filtration columns resulted in the observation that there had been no equilibration of either of the fractions into the other fraction. This was true of both erythrocyte membrane proteins and HeLa microsomal proteins. Dr. Bakerman has informed us that his laboratory is now obtaining results similar to those reported here; that is, multiple protein components in mammalian cell membranes, and unusual characteristics of red blood cell stromal membrane proteins as compared with those of more typical types of cells (S. Bakerman, 1969, personal communication).

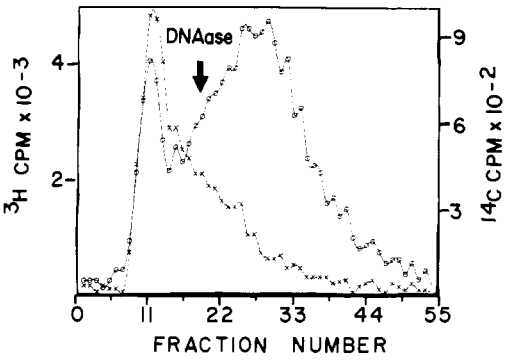

FIGURE 12: Gel filtration of membrane proteins. A 1.4 × 35.5 cm Bio-Gel P-200 column was equilibrated with pH 9.0 sodium dodecyl sulfate sample buffer, a mixture of phenol–acetic acid treated erythrocyte membrane proteins (—⊖—) and HeLa microsomal proteins (--×--) were eluted with sample buffer at 1.0 ml/hr and 1-ml fractions were collected. The fraction where deoxyribonuclease (31,000 daltons) elutes on this column is shown by a marker.

Discussion

We have found a general difference between the membranes of cultured cells labeled *in vitro* and the membranes of differentiated mouse cells labeled *in vivo*. It was usually not possible in cultured cells to discriminate between the protein patterns of the different subcellular membranes or even of membranes between diverse vertebrate species. In contrast, with mouse tissues the protein patterns of various types of cellular membranes were easily distinguishable and showed considerable organ-specific variation.

The membranes of cultured cells exhibit patterns of proteins which are more heterogeneous than membranes of differentiated cell types. This greater complexity could mask the specific differences between these membranes. It is a well-known phenomenon that tumors and cells grown *in vitro* show general tendencies to dedifferentiate and to exhibit metabolic convergence. This proclivity is probably responsible for the lack of specificity in the overall patterns of membrane proteins in cultured cells, not only because of the reflection in specific membranes of greater overall metabolic similarity, but possibly also because increased protein heterogeneity arising from general derepression masks specific differences.

The inability to discern tumor-specific differences in membrane proteins may also be explained by the phenomena described above. On the other hand, the unique properties of tumor cell membranes may not arise from major differences in protein components, as there is recent evidence for dramatic alterations in the carbohydrate constituents of tumor membranes (Hakomori *et al.*, 1968; Hakomori and Murakami, 1969; Wu *et al.*, 1969; Meezan *et al.*, 1969).

The erythrocyte ghost has been found to be different from every other membrane we have studied in that the membrane proteins only partially enter the polyacrylamide gel in pH 9.0 sample buffer containing sodium dodecyl sulfate. In our hands, in agreement with other workers (Bakerman and Wasemiller, 1967; Rosenberg and Guidotti, 1968). Sodium dodecyl sulfate is an efficient solvent for erythrocyte membranes, which suggests that the components in solution must be very large not to enter the gel (over 400,000 daltons). There is, in fact, considerable evidence of multiple subunits in red cell membranes, usually displaying extremely high molecular weights and having unique protein compositions (Maddy, 1964, 1966; Bakerman and Wasemiller, 1967; Blumenfeld *et al.*, 1967; Blumenfeld, 1968; Rosenberg and Guidotti, 1968; Zwaal and Van Deenen, 1968a; Copeland and Blumenfeld, 1969). In addition, there is evidence that some of the high molecular weight complexes are in equilibrium with lower molecular weight subunits (Morgan and Hanahan, 1966; Bakerman and Wasemiller, 1967; Blumenfeld *et al.*, 1967; Blumenfeld, 1968). The addition of a phenol–acetic acid solubilization step facilitated the migration of most of the membrane protein into the polyacrylamide gels, and this suggests that there may be high molecular weight aggregates of smaller subunits, though we could not find subunit equilibrium in sodium dodecyl sulfate solutions (Bakerman and Wasemiller, 1967; Blumenfeld *et al.*, 1967). Results similar to our findings have been recently reported (Berg, 1969). Berg found that most of the proteins of red blood cell stroma behaved in sodium dodecyl sulfate polyacrylamide gels as though they were of very high molecular weight. However, after prolonged extraction with salt solution most of these proteins migrated as low molecular weight polypeptides with a broad peak at about 20,000 daltons.

The heterogeneity of erythrocyte membrane proteins is well documented (Schneiderman, 1965; Azen *et al.*, 1965; Rosenberg and Guidotti, 1968; Zwaal and Van Deenen,

1968a,b; Cotman *et al.*, 1968; Copeland and Blumenfeld, 1969) but our findings are not in accord with many of the reported molecular weights for the constituent polypeptide chains. We have not observed in any preparation of stroma much protein of 22,000 daltons, reported to account for a considerable proportion of the total protein (Richardson *et al.*, 1963; Bakerman and Wasemiller, 1967; Schneiderman and Junga, 1968), or of 130,000–150,000 daltons, reported by other workers to account for 25% of the total protein (Marchesi *et al.*, 1969). Only a single preparation of stroma from one individual showed much protein around 50,000 daltons of molecular weight (Rosenberg and Guidotti, 1968), and the numerous preparations of four different individuals were consistent in that most of the protein that entered the gel at all was of low molecular weight (10,000–20,000 daltons). It is not possible at this time to reconcile these differences, but in view of the apparent extreme propensity of erythrocyte ghost proteins to aggregate they are not surprising. Caution is advisable in interpreting the results of any experiments on the proteins of this particular membrane. It seems clear, however, that the membrane proteins of the erthrocyte are different from the proteins of a variety of different cultured animal and human cells, and of mouse tissues.

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References

Azen, E. A., Orr, S., and Smithies, O. (1965), *J. Lab. Clin. Med. 65*, 440.

Bakerman, S., and Wasemiller, G. (1967), *Biochemistry 6*, 1100.

Berg, H. C. (1969), *Biochim. Biophys. Acta 183*, 65.

Blumenfeld, O. O. (1968), *Biochem. Biophys. Res. Commun. 30*, 200.

Blumenfeld, O. O., Goldstein, M., and Seifter, S. (1967), *Fed. Proc. 26*, 831.

Copeland, P. L., and Blumenfeld, O. O. (1969), *Fed. Proc. 28*, 404.

Cotman, C. W., Mahler, H. R., and Hugli, T. E. (1968), *Arch. Biochem. Biophys. 126*, 821.

Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963), *Arch. Biochem. Biophys. 100*, 119.

Green, D. E., Haard, N. F., Lenaz, G., and Silman, H. I. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 277.

Hakomori, S., Murakami, W. T. (1969), *Proc. Natl. Acad. Sci. U. S. 59*, 254.

Hakomori, S., Teather, C., and Andrews, H. (1968), *Biochim. Biophys. Res. Commun. 33*, 653.

Hanks, J. H., and Wallace, R. E. (1959), *Proc. Soc. Exptl. Biol. Med. 71*, 196.

Kiehn, E. D., and Holland, J. J. (1968), *Proc. Natl. Acad. Sci. U. S. 61*, 1370.

Kiehn, E. D., and Holland, J. J. (1970), *Biochemistry 9*, 1716.

Kirk, R. G. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 614.

Korn, E. D. (1969), *Ann. Rev. Biochem. 38*, 263.

Maddy, A. H. (1964), *Biochim. Biophys. Acta 88*, 448.

Maddy, A. H. (1966), *Biochim. Biophys. Acta 117*, 193.

Manganiello, V. C., and Phillips, A. H. (1965), *J. Biol. Chem. 240*, 3951.

Marchesi, V. T., Steers, E., Tillack, T. W., and Marchesi, S. L. (1969), *Fed. Proc. 28*, 567.

Mazia, D., and Ruby, A. (1968), *Proc. Natl. Acad. Sci. U. S. 61*, 1005.

Meezan, E., Wu, H. C., Black, P. H., and Robbins, P. W. (1969), *Biochemistry 8*, 2518.

Morgan, T. E., and Hanahan, D. J. (1966), *Biochemistry 5*, 1050.

Neville, D. M. (1968), *Biochim. Biophys. Acta 154*, 540.

Richardson, S. H., Hultin, H. O., and Green, D. E. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 821.

Robbins, E., and Borun, T. W. (1968), *Proc. Natl. Acad. Sci. U. S. 50*, 821.

Rosenberg, S. A., and Guidotti, G. (1968), *J. Biol. Chem. 243*, 1985.

Rotten, S., and Razin, S. (1967), *J. Bacteriol. 94*, 359.

Schnaitman, C. A. (1969), *Proc. Natl. Acad. Sci. U. S. 63*, 412.

Schneiderman, L. J. (1965), *Biochem. Biophys. Res. Commun. 20*, 763.

Schneiderman, L. J., and Junga, I. G. (1968), *Biochemistry 7*, 2281.

Wallach, D. F. H. (1968), *Proc. Natl. Acad. Sci. U. S. 61*, 868.

Warren, L., and Glick, M. C. (1968), *J. Cell. Biol. 37*, 729.

White, A., Handler, P., and Smith, E. L. (1964), *in* Principles of Biochemistry, New York, N. Y., McGraw-Hill, p 745.

Woodward, D. O. (1968), *Fed. Proc. 27*, 1167.

Woodward, D. O., and Munkres, K. D. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 872.

Woodward, D. O., and Munkres, K. D. (1967), *in* Organizational Biosynthesis, Vogel, H. J., Lampen, J. O., and Bryson, V., Ed., New York, N. Y., Academic, p 489.

Wu, H. C., Meezan, E., Black, P. H., and Robbins, P. W. (1969), *Biochemistry 8*, 2509.

Zwaal, R. F. A., and Van Deenen, L. L. M. (1968a), *Biochim. Biophys. Acta 150*, 323.

Zwaal, R. F. A., and Van Deenen, L. L. M. (1968b), *Biochim. Biophys. Acta 163*, 44.