Membrane and Nonmembrane Proteins of Mammalian Cells. Synthesis, Turnover, and Size Distribution*

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ABSTRACT: We have utilized polyacrylamide gel electrophoresis at pH 9.0 in sodium dodecyl sulfate to study the protein components of cellular membranes isolated from cultured human and animal cells, and from mouse tissues. With this technique, all cellular membrane proteins are readily dissolved and migrate electrophoretically as individual polypeptide chains at velocities which are a function of their molecular weight. We conclude that animal cell membranes consist of a complex array of proteins of widely varying molecular weights, with most of the membrane protein in polypeptides of 45,000–85,000 daltons. However, when the molecular weight distribution of proteins in whole cells and in membranes is expressed in terms of actual numbers of molecules, there is found to be a nearly inverse logarithmic relationship of polypeptide frequency with increasing molecular weight. We have studied kinetic aspects of the synthesis of proteins in whole cells and in subcellular fractions including the membrane systems. In established cell culture lines, the proteins in all subcellular fractions were found to be uniformly labeled within minutes by radioactive amino acids from the medium. In contrast, in all cell fractions the rate of turnover of individual proteins varies considerably, lending additional support to the conclusion that membranes are heterogeneous in protein composition. Finally, we have examined the formation at the host cell plasma membrane of the lipoprotein membranous envelope resulting upon infection by influenza virus. This structure exhibited only a few sharply resolved proteins, which demonstrates that the techniques employed are capable of resolving membrane "structural proteins" where such exist.

Information on the variety and sizes of membrane proteins is of obvious value in formulating meaningful structural models. It is generally held that biological membranes, rather than existing as mere structural permeability barriers, have a multitude of functional roles, such as providing catalytic surfaces for a variety of metabolic pathways, transporting and regulating the supply of nutrients and ions, and in cell recognition and communication. In accordance with these multiple functions, it is now well established that membranes contain a considerable array of enzymes. What is much less clear, however, is the degree to which enzymatic proteins contribute to the total content of membrane protein, and what roles these enzymes may have in maintaining the structural integrity of membranes.

Green and his coworkers (Green *et al.*, 1961) reported the isolation of an enzymatically inactive, homogeneous protein of apparent 25,000 mol wt from beef heart mitochondria which appeared to constitute 60% of the total protein of the mitochondrion. "Structural proteins" with almost identical properties were then isolated from beef liver mitochondria, beef liver microsomes, bovine erythrocyte membranes (Richardson *et al.*, 1963), chloroplasts (Richardson *et al.*, 1963; Criddle and Park, 1964), *Neurospora* mitochondria (Woodward and Munkres, 1966), *Neurospora* nuclei, microsomes and soluble cytoplasm (Woodward and Munkres, 1967), *Neurospora* plasma membrane (Woodward, 1968), and human red cell membranes (Schneiderman and Junga, 1968). Similarly, a protein component of a size identical with "structural protein" was reported to comprise a large fraction of the human red blood cell membrane (Bakerman and Wasemiller, 1967).

There is also much evidence from other studies that membranes are comprised of numerous proteins, with no single species predominating. It was shown that erythrocyte membranes contain up to 15 different protein species (Azen *et al.*, 1965; Schneiderman, 1965) and this has been confirmed in a number of laboratories. Plasma membranes isolated from several strains of *Mycoplasma*, the smallest cells known, displayed over 20 proteins (Rottem and Razin, 1967). The plasma membrane of rat liver cells was convincingly shown to have 15 major protein species and at least 10 minor species (Neville, 1967). We have reported the existence of a very large number of individual proteins in mouse microsomal membranes and in the nuclear membrane, plasma membrane, microsomes, and mitochondria of a variety of cultured human and animal cells (Kiehn and Holland, 1968). We found that most of the membrane protein was in the molecular weight range of 45,000–70,000 daltons. Schnaitman (1969), using techniques similar to ours with rat liver cells, found 23 different proteins comprising the inner mitochondrial membrane, 12 species in the outer mitochondrial membrane, and 15 proteins each in the smooth and rough microsomal membranes. Finally, the plasma membrane of *B. megaterium* was shown to contain at least 12 different proteins (Mirsky, 1969).

In conformity with evidence of considerable heterogeneity in whole membranes, subfractions of membranes also showed great heterogeneity and provided no evidence for a pre-

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dominant protein species (Takayama *et al.*, 1964; Haldar *et al.*, 1966). Later, Green and his coworkers (Green *et al.*, 1968; Lenaz *et al.*, 1968a) demonstrated that structural protein prepared by the usual methods was grossly heterogeneous, containing at least 12 separate species of protein. After additional steps of purification these workers concluded that there were six different noncatalytic proteins of highly similar molecular weight (50,000–65,000 daltons). Structural protein preparations from synaptic plasma membrane, erythrocyte membrane, brain mitochondria (Cotman *et al.*, 1968), *Bacillus* plasma membrane (Mirsky, 1969), rat liver mitochondria, and yeast mitochondria (Lejsek and Lusena, 1969) have also been shown to be heterogeneous. In the latter study, 15 different proteins were found in structural protein prepared by one of the standard methods (Richardson *et al.*, 1964) and 10 different species were found using the most recent modification (Lenaz *et al.*, 1968a) of these methods. In addition, it has been shown (Schnaitman, 1969) that no individual protein species similar in molecular weight to mitochondrial structural proteins (Green *et al.*, 1968) comprises more than 10–15% of the total protein of the mitochondrial membrane from which it was isolated.

In this paper and in the following paper (Kiehn and Holland, 1970), further evidence will be presented supporting the thesis that membranes contain a complex array of proteins in which no single species predominates. Further, in this paper experiments will be described which bear on the synthesis and turnover of membrane proteins.

Experimental Procedures

Cell Culture and Radioisotope Labeling. All cells were grown as monolayers in MEM[1] containing 5% calf serum and except where noted, were labeled in MEM containing [^{3}H]- or [^{14}C]phenylalanine, valine, and tyrosine in place of the same unlabeled amino acids, plus 2% dialyzed calf serum which had been frozen at −70° until just before use. There were no free amino acids in calf serum treated in this way as judged from the absence of incorporation competition of labeled amino acids in the presence of calf serum, relative to that without it. Except where noted, [^{3}H]amino acids were each used at a concentration of 100 μCi/ml and [^{14}C]amino acids were used at 5 μCi/ml. The ^{3}H-labeled amino acids were L-phenylalanine (5300 mCi/mmole), L-tyrosine (33,700 mCi/mmole), and L-valine (267 mCi/mmole). The ^{14}C-labeled amino acids were L-phenylalanine (459 mCi/mmole), L-tyrosine (475 mCi/mmole), and L-valine (270 mCi/mmole). *In vivo* labeling of mouse tissues involved an intraperitoneal injection of a mixture of six [^{3}H]- or [^{14}C]amino acids, and for turnover studies involved daily injections for 2 weeks followed by sacrifice after an additional 18 days.

Incorporation Kinetics. Incorporation of radiolabeled amino acids into free amino acid pools and proteins was determined by a modification of the procedure of Piez and Eagle (1958). Monolayers were instantly chilled by flooding with a large volume of saline at 0–4°, washed rapidly twice with the same solution, and drained, and the free amino acids were extracted with 10% trichloroacetic acid. The precipitated protein was dissolved in 0.1 M NaOH and was three-times precipitated in 5% trichloroacetic acid at 90°. Aliquots of the trichloroacetic acid extracted amino acid pool were plated in stainless steel planchets, dried, counted in a Nuclear-Chicago gas-flow spectrometer, and corrected for self-quenching. Trichloroacetic acid precipitated protein was collected on membrane filters and counted in the same fashion.

Protein concentrations were determined either by the Lowry method (Lowry *et al.*, 1961) or by the Biuret (Green and Cori, 1943) reaction. Cell counts were determined by a hemocytometer.

Polyacrylamide Gel Electrophoresis. Electrophoretic separations were carried out in 5% acrylamide gels containing 0.1% sodium dodecyl sulfate, by a modification of the techniques developed by Maizel and his colleagues (Maizel, 1966; Summers *et al.*, 1965). The electrophoretic buffer consisted of 0.1 M 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% sodium dodecyl sulfate, and 0.01% 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, 0.1% sodium dodecyl sulfate, and 0.001% EDTA. Gel polymerizing components in this buffer were 5% acrylamide, 0.16% bisacrylamide, 0.07% ammonium persulfate, and 0.035% *N,N,N',N'*-tetramethylethylenediamine. Samples were loaded onto 23 × 0.6 cm gel columns after being mixed with appropriate amounts of ^{14}C- and ^{3}H-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% sodium dodecyl sulfate, 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 at 70° 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 at room temperature. The gels were crushed sequentially on the linear fractionator designed by Maizel (1966) and the fractions were counted in a Beckman scintillation counter under conditions appropriate for discriminating ^{3}H- and ^{14}C-labeled proteins.

Estimation of Molecular Weights. For accurate molecular weight estimations, highly purified protein standards were radiolabeled with dimethyl sulfate. This is an adaptation of a method (Smith *et al.*, 1967) 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 dectectable polypeptide degradation, nor does it change the electrophoretic migration of the labeled protein in this sodium dodecyl sulfate technique (as compared with 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% sodium dodecyl sulfate. Between 2 and 20 mg of protein was dissolved in 0.5 ml of buffer and heated at 70° for 1 min to disperse the sodium dodecyl sulfate denatured protein thoroughly. [^{3}H]- or [^{14}C]dimethyl sulfate (New England Nuclear) was dissolved in benzene, and 70 μCi in 0.05 ml of benzene was added to the protein in a screw-cap test tube at 20° and then shaken vigorously on a rotary mixer in a hood to emulsify the mixture. After 15 min at 20° the solution was dialyzed for 24 hr against large volumes of electrophoretic sample buffer to remove benzene and [^{14}C]-methanol.

[1] Abbreviations used is: MEM, Eagles minimum essential medium.

A standard curve relating protein molecular weight to electrophoretic migration was prepared by plotting relative migration of standard proteins in the same gel. Bromophenol blue was used in all electrophoretic runs as a marker.

A curve relating polypeptide frequency to molecular weight in *E. coli* and HeLa cells was made in the following manner: uniformly labeled [^{3}H]proteins from HeLa cells were co-electrophoresed with uniformly labeled [^{14}C]proteins from *E. coli;* the resulting protein patterns were calibrated for molecular weight, divided into segments of 20,000 daltons, and for every segment the area under each curve was summed and divided by the segment-average molecular weight, giving the relative number of molecules per segment. This process was carried out over a molecular weight range of 0–240,000 daltons, which we defined as including 100% of the total cell protein (actually 97% of HeLa polypeptides and 99% of *E. coli* polypeptides). This analysis is based on the assumption, to be documented further in this paper, that the electrophoretic procedure always, or nearly always separates individual polypeptide chains.

Cell Fractionation. The basic cell fractionation procedure we have used follows Mahler's general scheme (Mahler and Cordes, 1966) with slight modifications. This scheme is a composite of a variety of standard techniques (for references, see Mahler and Cordes, 1966) and includes the procedure of Widnell and Tata (1964) for the separation and purification of nuclei, and Dallner's (1963) technique for the separation of smooth and rough membranes. Monolayer cells were chilled in 0–4° saline, scraped from the glass with a rubber policeman, and washed three times in saline. Mouse organs were chilled, finely diced, and washed in the same way. Throughout the fractionation procedures, the temperature is held at 0–4°.

Washed cells are swollen for 5–12 min in two to four volumes of distilled water containing 0.003 M $MgCl_2$ and 0.02 M Tris (pH 7.5). The cell suspension is then homogenized in a Dounce homogenizer with a tight-fitting pestle until over 95% of the cells are broken (10–25 strokes), rapidly made 0.25 M in sucrose, layered over an equal volume of 0.32 M sucrose also containing Tris and $MgCl_2$, and centrifuged at 700*g* for 10 min. The resulting pellet of crude nuclei is 20% contaminated on a protein basis by a few residual whole cells and about one-third of the total microsomes of the cell. The upper sucrose layer and the turbid interface between the layers are collected and centrifuged at 7000*g* for 10 min. The resulting pellet of mitochondria (and other small particles) is washed and centrifuged at 7000*g* for 10 min, first with 0.25 M sucrose and then three times with 0.05 M Tris at pH 7.8. The original postmitochondrial supernatant is respun at low speed to sediment residual small mitochondria (this appears to be necessary only with organ homogenates) and then the microsomes are pelleted by centrifugation at 105,000*g* for 100 min. The resulting supernatant is the soluble protein fraction. The microsomes are resuspended in 0.25 M sucrose containing 0.00015 M $MgCl_2$ (minimum concentration for polysome stability), layered over an equal volume of 1.5 M sucrose containing 0.00015 M $MgCl_2$, and centrifuged at 105,000*g* for 75 min, which pellets the rough vesicles. The supernatant is diluted to 0.3 M in sucrose and centrifuged at 105,000*g* for 100 min to pellet the smooth vesicles. The rough vesicles are resuspended in 0.25 M sucrose which is 0.00015 M in $MgCl_2$ and 0.5% in sodium deoxycholate. Ribosomes are pelleted by centrifugation at 105,000*g* for 60 min yielding a supernatant containing the components of the rough membranes.

Nuclei were purified by resuspension in 2.0 M sucrose and pelleting by centrifugation at 118,000*g* for 30 min. Whole cells and membranes float. In our hands, the omission of $MgCl_2$ in all solutions used for cell fractionation leads to higher recoveries of nuclei and these are free of cytoplasmic tabs as judged by phase-contrast microscopy. In fact, the initial pellet of "crude nuclei" is itself nearly free of microsomal material, compared to the large amounts of contaminating microsomes in the presence of $MgCl_2$. The absence of $MgCl_2$ in the fractionation media did not alter the pattern of proteins in any of the major subcellular fractions of cultured cells.

Microsomes not destined to be subfractionated were washed three times in 0.05 M Tris at pH 7.8 with intermediate high-speed centrifugation steps.

Plasma membranes were isolated by a small-scale adaptation of the Tris method of Warren and his coworkers (Warren *et al.*, 1966). Cells are treated in the same way as in our basic fractionation procedure except where noted. Cells are swollen at a concentration of 2.5×10^7/ml (we utilized 10^7 to 4×10^8 per ml without significant differences) in 0.05 M Tris at pH 7.4 containing 0.0025 M $MgCl_2$. After homogenization, the suspension is made 10% with respect to sucrose, and layered on top of a discontinuous sucrose gradient containing an equal volume of 50% sucrose as the bottom layer under two volumes of 30% sucrose. All sucrose solutions in the Warren procedure contain 0.005 M $MgCl_2$. This gradient is centrifuged at 1400*g* for 15 min. Plasma membrane ghosts are found in the 30% layer and in the fluffy material at the interface of the 30% and 10% layers. This material is pelleted by centrifugation at 9000*g* for 10 min. The resulting supernatant can be combined with the 10% layer of the original sucrose gradient and fractionated into mitochondria, microsomes, and soluble protein according to the above basic procedure; the cell nuclei are found in the pellet below the 50% sucrose layer of the original sucrose gradient. The pellet of plasma membranes is gently resuspended in a small volume of 30% sucrose, layered over a discontinuous sucrose gradient of equal volumes of 40, 45, 55, and 65% sucrose, and centrifuged at 200,000*g* for 100 min. The plasma membranes are found in a band within the 55% sucrose layer or at the interface of the 55 and 45% sucrose layers. The band of plasma membranes contains no whole cells and bands in the same place upon recentrifugation. The membranes are collected and washed in distilled water with high-speed centrifugation steps.

In the initial cell fractionation experiments mouse L cells were utilized and the procedures and recoveries were monitored by phase contrast microscopy and by the quantitation of protein in the various subcellular fractions and washes.

Results

Validity of Sodium Dodecyl Sulfate Gel Electrophoresis. In view of evidence that membrane proteins are highly insoluble and have marked tendencies to aggregate, we have intensively studied the possibility that a single species of structural protein might comprise a large proportion of the membrane protein and was not being resolved by our techniques. We therefore tried numerous variations (both individually and in combinations) in treatment of membrane proteins and in electrophoretic conditions. Some of these experimental

variations were already described in a previous paper (Kiehn and Holland, 1968).

None of the following treatments significantly altered the gel pattern of proteins: prior extraction of lipids by ether or by hot chloroform–methanol, a solubilization step in a 1:1:1 (v/v) mixture of phenol–glacial acetic acid–water, periodate oxidation of carbohydrates, carboxymethylation of protein thiol groups in 8 M urea, and reduction of thiols by dithiothreitol.

The following changes in our electrophoretic procedure did not result in a less complex pattern of membrane proteins: electrophoresis was carried out in Tris-acetate buffers from pH 5 to 9, and in sodium phosphate buffers from pH 7.2 to 11.2; electrophoresis was conducted at 50°; the urea concentration was raised to 8 M and sodium dodecyl sulfate was raised to 1.1% in gels and in samples; 0.5% sodium deoxycholate and 0.5 M dithiothreitol were included in samples and were diffused into preformed gels; and gels were continuously reduced during electrophoresis by a flow of mercaptoethylamine ions at a concentration of up to 0.2%.

Protein heterogeneity was also confirmed utilizing Sephadex and Bio-Gel columns over a range of pH from 9 to 13 in 8 M urea, including sodium dodecyl sulfate to 2% and sodium deoxycholate to 1%. An example is shown in Figure 12 of the following paper (Kiehn and Holland, 1970). The resolution of these columns is not nearly so high as with gel electrophoresis, but column fractions analyzed by gel electrophoresis do show that separation is by molecular weight. There is an exact correspondence of A_{280} and labeled protein in column patterns.

Considerable protein heterogeneity in membranes was also demonstrated employing a NaCl gradient to 0.5 M on DEAE-cellulose columns in 8 M urea plus 0.1% Triton X-100 at pH 8.5 (Tris). This column appeared to fractionate proteins by a combination of size and charge characteristics (see Figure 4, Kiehn and Holland, 1968). A major class of MBK (Maden bovine kidney line) microsome proteins electrophoresing as a peak between 55,000 and 70,000 daltons which was homogeneous upon electrophoresis, was split into at least five peaks with DEAE column chromatography.

Weber and Osborn (1969) have convincingly verified the reliability of sodium dodecyl sulfate gel electrophoresis for determination of molecular weight. Using 40 known proteins of high purity they found no evidence of aggregation. Other evidence that the heterogeneous gel patterns are composed of many different polypeptide chains, and not aggregates, was described earlier (Kiehn and Holland, 1968). Microsomal proteins were electrophoresed and separated into 61 fractions. The proteins were eluted from the gel fractions by homogenization followed by incubation for 1 hr at 70° in sample buffer, and allowing several days at room temperature for complete dissociation of any aggregates present. We have now reelectrophoresed 25 of the original 61 fractions, over the whole range of molecular weights, and have found that the eluted fractions run true to their original gel positions and are not in equilibrium with other species. One fraction of a very high molecular weight (about 300,000 daltons) was rather broad upon reelectrophoresis and therefore could have been in equilibrium with other high molecular weight species (monomers larger than 150,000 daltons), but proteins this large comprise less than 5% of the protein of membranes. Two fractions less than 15,000 daltons in molecular weight

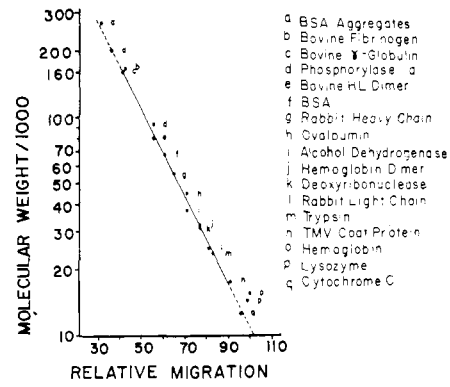

FIGURE 1: Relationship of molecular weight to migration distance in polyacrylamide gels for purified proteins. Abbreviations used are: BSA, bovine serum albumin; HL dimer, dimer of heavy and light chains of γ-globulin; TMV, tobacco mosaic virus. The gel origin is on the left and the anode is on the right.

also reran as broad peaks, but diffusion is highest with these smallest proteins and the gels do not separate them well according to molecular weight. In any case, these two fractions were not in equilibrium with dimers (or higher) of their original molecular weight.

Finally, over 95% of the protein from the membranes we have studied enters our sodium dodecyl sulfate polyacrylamide gels, a sole possible exception being the proteins of the human erythrocyte ghost, which will be discussed in the following paper (Kiehn and Holland, 1970).

Estimation of Molecular Weight. Figure 1 shows that when relative migration is plotted against the log molecular weight for a number of highly purified proteins, there is found a linear relationship, similar to that shown by Shapiro and coworkers (1967). The migration of proteins was standardized with the migration of bromophenol blue (relative migration = 100) in each gel, and then the relative migration of different proteins was determined by making comparisons within the same gel (made possible by differential isotope labels). The multichain molecules of γ-globulin and fibrinogen were employed as high molecular weight standards by avoiding exposure to mercaptoethanol and by leaving this and other reducing agents out of the electrophoretic system entirely. Fibrinogen was only partially present in its high molecular weight form under these conditions. A small amount of hemoglobin dimer was obtained when charged mercaptans were omitted from the electrophoresis buffer.

A lack of good correlation below 20,000 daltons was noted earlier (Shapiro *et al.*, 1967). Insulin, with polypeptide chains of 2300 and 3400 daltons, was not included in Figure 1, but it also deviates from the straight-line plot with a relative migration of 104.

The bovine serum albumin "dimer" was also found to deviate from out standard plot, unlike the bovine serum albumin monomer. We studied several electropherograms and observed repeatedly that the bovine serum albumin "dimer" was a small composite peak which electrophoresed as would be expected for bovine serum albumin "trimers" and "quatramers," and never as dimers or pentamers. That these are higher aggregates of the bovine serum albumin monomer was demonstrated by their disappearance when sulfhydryl

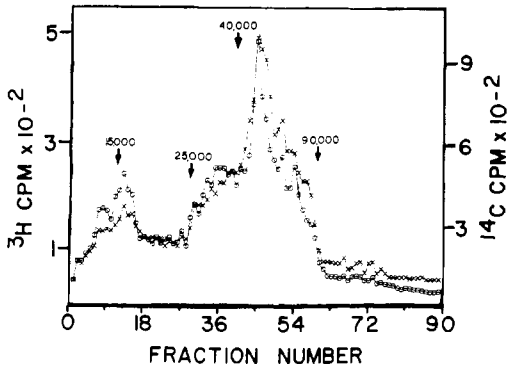

FIGURE 2: The distribution of proteins in whole L cells. In this, and in all subsequent electropherograms, the anode is on the left. Markers showing molecular weights are included. Cells labeled 90 min in [^{14}C]amino acids were directly dissolved in sample buffer (--X--). Cells labeled 90 min in [^{3}H]amino acids were fractionated as described in the text, and after dissolution in sample buffer the fractions were mixed back in the following proportions: nuclei 27%, mitochondria 22%, microsomes 16%, and soluble 35% (—○—).

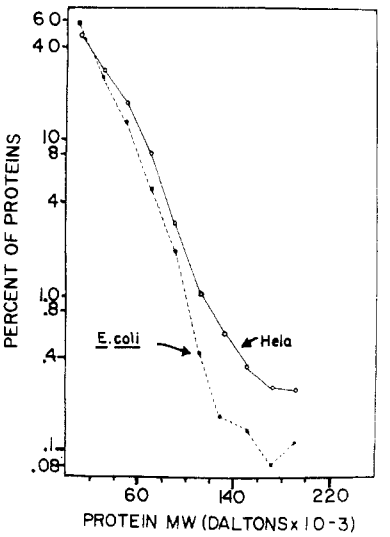

FIGURE 3: Relationship of polypeptide frequency to molecular weight. Frequency is expressed as per cent of total cell polypeptides occurring in molecular weight segments of 20,000 daltons, and is plotted at the average molecular weight for each segment.

reducing agents were used. In addition, there appeared to be no contaminating material in preparations of bovine serum albumin as judged by the electropherograms. This phenomenon is not likely to be an artifact of our pH 9.0 Tris buffer system or our dimethyl sulfate labeling procedure, because the same thing was independently observed using protein staining procedures with the original Maizel pH 7.2 phosphate buffer system (F. H. Gaertner, 1969, personal communication).

We also observed that while the physical spread of proteins can vary from gel to gel, within each individual gel the log relationship of molecular weight to migration holds true.

Fractionation Procedures. Using the described methods for the preparation of subcellular fractions, we found that 35% of the total protein of L cells was in the soluble cytoplasm, 27% in the nucleus, 22% in mitochondria, and 16% in the microsomes, the latter from which 1–5% of the total cell protein could be isolated as the plasma membrane.

Figure 2 shows a comparison between labeled whole cells which were directly dissolved in sample buffer, and "reconstructed" L cells which had been labeled, fractionated, and the resulting individual fractions mixed back in the proportions described in the preceding paragraph. It can be seen there has been no significant degradation or differential losses of proteins resulting from the fractionation procedure. Figure 2 also shows the general distribution of proteins by molecular weight. The plot of proteins in gels is always shown here with the anode (low molecular weight) on the left and the origin (high molecular weight) on the right. We routinely cut off the last one-fifth of gels (containing only proteins over 400,000 daltons in size) for convenience, but when whole gels were analyzed it was found that the declining slope of proteins seen in fractions above fraction 60 in Figure 2 continues to decline to the gel origin, at which there is practically no protein at all.

We carried out another type of experiment to determine whether degradation occurred during isolation. This involved the rapid homogenization in sodium dodecyl sulfate of whole mouse tissues, and we did not find evidence for protein degradation during fractionation. In addition, labeled mouse tissues and cultured cells were fractionated in the presence of high relative concentrations of unlabeled kidney and liver tissue. No differences were found in the protein patterns of cell fractions isolated in this way when compared with homologously labeled material fractionated without carrier and at relatively low cell concentrations.

The contribution of soluble proteins to the pattern of membrane proteins was considered to be low by a variety of observations. Firstly, the protein pattern of soluble proteins is distinctly different from that of membranes, as will be shown in the following paper (Kiehn and Holland, 1970). Secondly, sonication of membrane vesicles to the extent that their average size was notably reduced and 30% of the protein was rendered soluble (centrifugation at 165,000*g* for 2 hr) did not result in significant changes in the protein pattern of either the resulting soluble or insoluble fractions. Thirdly, the stated 35% of total cell proteins existing as soluble protein appears to represent a general maximum proportion. This was suggested by the following experiments. A suspension of L cells was swollen for only 2 min and homogenized, and the total cell particulates were rapidly pelleted, leaving 25% of the total protein in the supernatant. This figure could be raised to a maximum of 35% either by prolonged swelling, or by prolonged incubation of the homogenate at 4° prior to centrifugation. This suggests a general maximum dissociation level of protein from particulates in hypotonic buffers, and is not notably improved upon by extensive washing of the membranes. In addition, the protein pattern of the rapidly prepared soluble protein is identical with that obtained under conditions maximizing its proportion of the total protein. Therefore, we believe conditions are met which provide a definition of soluble and insoluble (particulate) proteins based not just on sedimentation criteria, but on their unique protein patterns.

Size Distribution of Proteins. Figure 3 shows that the frequency of polypeptide chains in HeLa and *E. coli* cells declines with increasing molecular weight in essentially a

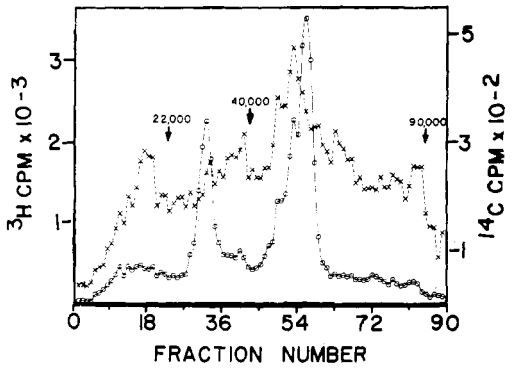

FIGURE 4: Comparison of the microsomes of normal cells with the microsomes of influenza-infected cells. Molecular weight markers are shown. Microsomes were isolated from BHK cells after labeling for 60 min with [^{14}C]amino acids (--X--), and from strain NWS, influenza-infected BHK cells labeled for 60 min with [^{3}H]amino acids (-O-).

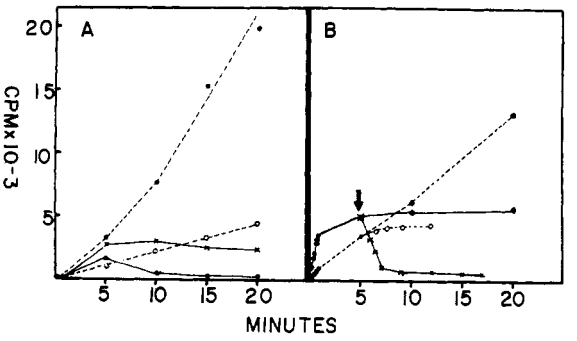

FIGURE 5: Incorporation of amino acids into the pools and proteins of L cells. Identical monolayers of 2 × 10^6 cells were incubated with radioactive amino acids and at each indicated time point, a culture was chilled and the incorporation into the free pool and into protein was measured at described in the text. (A) Uptake of [^{14}C]valine in the presence and absence of unlabeled valine in the medium. [^{14}C]Valine (209 mCi/mmole) was used at 1 μCi/ml (0.0048 mmole/l.) with or without the usual MEM concentration of unlabeled valine (0.4 mmole/l.). Incorporation in the absence of unlabeled valine into protein (--●--) and into the pool (—●—). Incorporation in the presence of unlabeled valine into protein (--O--) and into the pool (—X—). (B) Uptake and chase of [^{14}C]leucine in the presence of the normal MEM concentration of unlabeled leucine. [^{14}C]leucine (251 mCi/mmole) was used at 0.5 μCi/ml. The marker at 5 min shows when one set of cultures was drained of labeling medium and a large volume of unlabeled MEM added (200-fold excess of cold leucine) in order to chase [^{14}C]leucine. The incorporation of [^{14}C]leucine into the free pool (—●—) and into protein (--●--). The chased [^{14}C]-leucine remaining in the pool (—X—) and in protein (--O--).

logarithmic fashion. Only 10% or less of the protein chains in cells are larger than 60,000 daltons. Stable poliovirus-directed proteins fall on a similar curve (E. D. Kiehn, 1969, unpublished data). All cultured animal cells, including chick fibroblasts, and probably mouse tissues generally (Kiehn and Holland, 1970), follow the curve shown for HeLa cells, suggesting an evolutionary basis for the difference in protein size ranges between HeLa and *E. coli* cells shown in Figure 3.

The isolated membranes of cultured cells show patterns of proteins almost identical with that of whole cells, and therefore follow the frequency distribution curve shown in Figure 3 for HeLa cells. The identity of protein patterns between membranes and whole cells probably arises because general complexity masks individual proteins, and because in whole cells, the higher frequency of large molecular weight proteins in the soluble fraction (relative to that in membranes) is balanced by the relatively high frequency of low molecular weight proteins in the nucleus (Kiehn and Holland, 1970).

Figure 4 compares the proteins of normal BHK-21 (baby hamster kidney line) microsomes and microsomes from strain NWS influenza-infected BHK-21 cells. The normal membrane has an extremely complex array of proteins, but in the infected cell the synthesis of membrane proteins is directed by viral genes and the result is only three major protein components. This simplicity is typical of related membrane viruses and is being studied in detail (Holland and Kiehn, 1970). Figure 4 also illustrates that a membrane with only a few protein subunits is sharply resolved by our techniques, and that aggregation of viral membrane subunits does not occur in the presence of normal membrane proteins. The normal membrane in Figure 4 shows an especially widely spread pattern of proteins, which is a gel variation such as was described earlier, but the pattern is very typical of most of the membranes we have studied in complexity and in the size distribution of proteins.

Kinetics of Amino Acid Incorporation. Figure 5A illustrates the kinetics of exogenous [^{14}C]valine incorporation into the intracellular amino acid pool and into the proteins of L cells. Experiments are shown with and without unlabeled valine in the medium. The curves obtained with valine in the medium are the normal growth condition and show that the free valine pool equilibrates within 5 min and that incorporation into protein is linear. The absence of a significant concentration of valine in the medium exhausts the free valine pool in about 10 min, as seen by the slight 10-min lag in the maximal rate of incorporation into protein (due to competition from unlabeled valine in the pool) and by the rapid decline of [^{14}C]valine in the pool after 5 min. At 15 min there is hardly a measurable pool and the rate of valine incorporation into protein must be limited by the rate at which it can be transported into the cell. That the normal valine pool represents about 10-min protein synthesis is also inferred by the equilibrated pool size in Figure 5A. Figure 5B and other experiments (E. D. Kiehn, 1968, unpublished data) show that these results for valine are typical of aliphatic amino acids, that depletion of a required amino acid does not cause a change in the overall rate of protein synthesis for at least 30 min, and that thereafter the rate declines gradually. Our usual procedure for labeling cell proteins with amino acids involves deleting the cold homologous amino acids from the medium to achieve a very high specific activity of incorporation as exemplified in Figure 5A.

Figure 5B shows the details of [^{14}C]leucine incorporation from complete medium into the pool and into the proteins of L cells. The result of removing the [^{14}C]leucine medium and "chasing" with a large excess of cold leucine is also shown. Early time points show the very rapid uptake into the free pool, and the general results are about the same as seen for valine in Figure 5A. [^{14}C]Leucine in the free pool is chased in about 2 min and incorporation of [^{14}C]leucine into protein rapidly comes to a halt. After labeling under our usual conditions, where high specific activity labeled amino acids

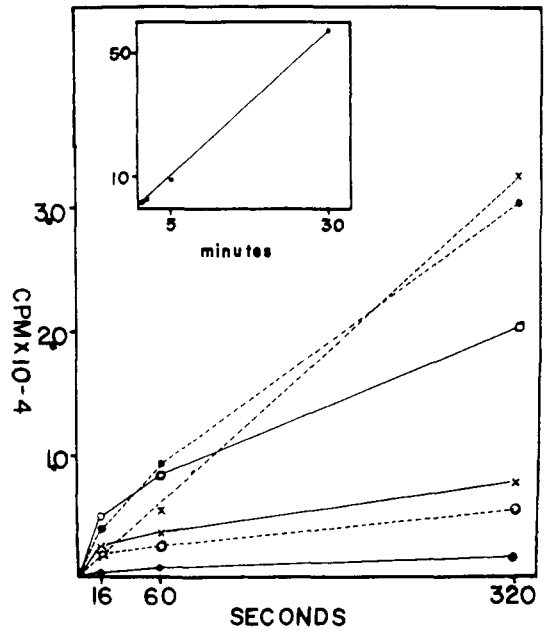

FIGURE 6: Incorporation of [^{14}C]leucine into subcellular fractions of L cells. Identical cultures of 20 × 10^6 cells were incubated in medium deleted of leucine for 6 min, labeled with [^{14}C]leucine at 1 μCi/ml in medium deleted for leucine, and then fractionated at the indicated times as described in the text. The inset figure shows the total incorporation at the various time points. The subcellular fractions are soluble cytoplasm (--×--), crude nuclei (--●--), mitochondria (—○—), rough vesicles and polysomes (—×—), polysomes removed from rough membranes (--○--), and smooth membranes (—●—); the membranes of the rough vesicles show the same kinetics of incorporation as the smooth membranes.

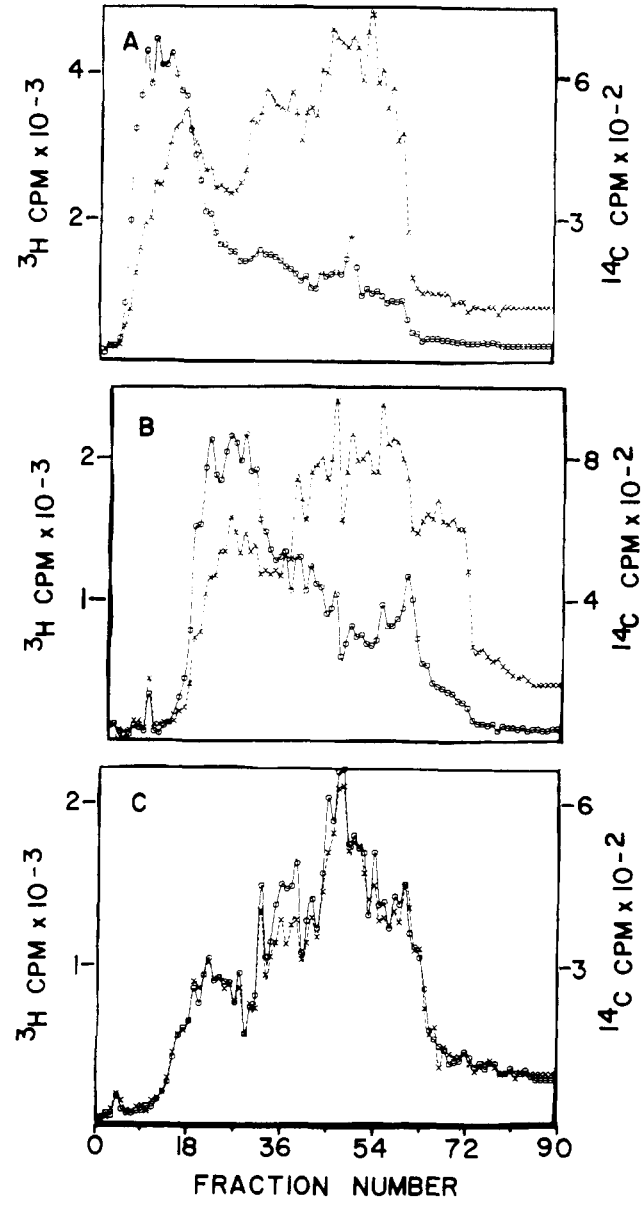

FIGURE 7: Incomplete polypeptide chains and their completion. (A) The protein patterns are compared between L cells labeled for 90 min with [^{14}C]amino acids (--×--) and cells labeled for 90 min with [^{3}H]amino acids in the presence of 200 μg/ml of puromycin (—○—). (B) The protein patterns of HeLa cells are compared between cells labeled 60 min with [^{14}C]amino acids (--×--) and for cells labeled for 10 sec with [^{3}H]amino acids (—○—). (C) The protein patterns of HeLa cells are compared between cells labeled 60 min in [^{14}C]-amino acids (--×--) and for cells labeled for 8 sec in [^{3}H]amino acids, immediately brought to 4° with a large volume of MEM containing a fivefold MEM concentration of amino acids, then warmed back to 37° for 20 min (—○—).

completely replace the usual cold amino acids in the medium, a chase is even more effective because only small quantities of the labeled amino acids are present and the free pools are very small as seen in Figure 5A.

Figure 6 shows the kinetics of [^{14}C]leucine incorporation into the subcellular fractions of L cells. The figure shows only the first 5 min in order to emphasize the initial labeling patterns but the slopes of incorporation established by the 5-min point are linear to the final 30-min point. The overall kinetics of incorporation shown in the inset represent the summed incorporations of the subcellular fractions. We have repeatedly found that the total rough vesicles, ribosomes, and mitochondria show labeling rates which are initially very high, in contrast to the continuous linearity seen with the isolated membranes of the rough vesicles, smooth membranes, and soluble cytoplasm. The high initial labeling rates of mitochondria may be due to contamination by rough vesicles, for, as a recent study shows, it is extremely difficult to remove contaminating rough vesicles from the mitochondria of cultured cells (Attardi *et al.*, 1969). Other experiments demonstrate that purified nuclei also show linearity of incorporation; the initial high rate of incorporation found in the crude nuclei shown in Figure 6 is probably due to contamination by rough vesicles. From this, and from several similar experiments, we have concluded that the various cell fractions attain the same specific activity of protein after about 30-min labeling.

Figure 7A shows the pattern of L-cell polypeptides labeled in a concentration of puromycin which caused a 98% inhibition in the rate of cellular protein synthesis. Polypeptides made during puromycin treatment are compared with the normal pattern of proteins and it can be seen that (consistent with the known action of puromycin) incomplete polypeptides accumulate in the cell. This puromycin pattern is very

similar to that shown in Figure 7B, where a 10-sec pulse of HeLa cells is compared with cells labeled 60 min. As expected, in the 10-sec pulse the label is found mostly in incomplete nascent polypeptides. The completion of labeled nascent chains is demonstrated in Figure 7C. HeLa cells were labeled for 8 sec, immediately chilled in excess unlabeled amino acids at 4° for 10 min, then warmed to 37° for 20 additional min. Incubation at 4° almost totally inhibits protein synthesis but allows the turnover of the free amino acid pool (E. D. Kiehn, 1969, unpublished data) and the success of this "chase" was verified by measuring the total label in protein at the end of the experiment, which agreed well with the expected incorporation for a pulse of 8 sec. The completed polypeptides labeled with the 8-sec pulse are compared with HeLa proteins labeled for 60 min.

Pulse-Chase and Steady-State Labeling. Figure 8A–D compares the proteins labeled in a 5-min pulse with those labeled by an identical pulse followed by a 4-hr chase, in the nucleus, mitochondria, microsomes, and soluble cytoplasm of L cells. Figure 9A shows a comparison of the proteins of whole HeLa cells labeled in a 2-min pulse with those labeled over a 4-day period under steady-state conditions. Figure 9B–D compares the patterns of proteins labeled in 5-min pulses with proteins labeled for 3 days under steady-state conditions in the nucleus, mitochondria, and microsomes of L cells. The experiments in Figures 8 and 9 demonstrate that the protein patterns of whole cells and of subcellular fractions are very similar whether pulsed for a very short time, pulsed, and then chased for a long period, or labeled under steady-state conditions. It is concluded that the great majority of proteins in the various subcellular fractions of cultured cells are uniformly labeled within a few minutes.

Nature of Newly Synthesized Proteins. It was of great interest to discern whether there exist normal cellular mechanisms involving the synthesis of giant proteins which are subsequently cleaved into smaller functional proteins. Such mechanisms have been demonstrated in the biosynthesis of insulin (Steiner and Oyer, 1967), and in enterovirus proteins (Maizel and Summers, 1968; Jacobson and Baltimore, 1968a,b; Holland and Kiehn, 1968). It has been found that the entire poliovirus genome may be translated into a huge polypeptide (>200,000 daltons) which is subsequently cleaved into the 12–14 stable proteins found in infected cells (Kiehn and Holland, 1970; Jacobson and Baltimore, 1968). During enterovirus growth, short pulses of radiolabeled amino acids, such as we have described in this paper, are found to label mostly very large proteins (the largest are over 125,000 daltons) which can then be "chased" into the usual gel pattern of stable viral proteins. Figures 8 and 9 provide no evidence that similar mechanisms exist in the general synthesis of cellular proteins. We have conducted numerous other experiments along these lines, utilizing pulses as short as 10 sec coupled with chases of 30–60 sec (in order to complete some of the labeled nascent chains), and these were totally without success in demonstrating the transient existence of giant proteins. However, Jacobson and Baltimore (1968b) have used high concentrations of amino acid analogs to inhibit the cleavage of pulse-labeled polio proteins in order to demonstrate even larger precursor proteins than was possible with usual pulse-labeling techniques. Using analogs during labeling, these workers also reported a slight skew to higher molecular weight in the proteins synthesized by uninfected HeLa cells.

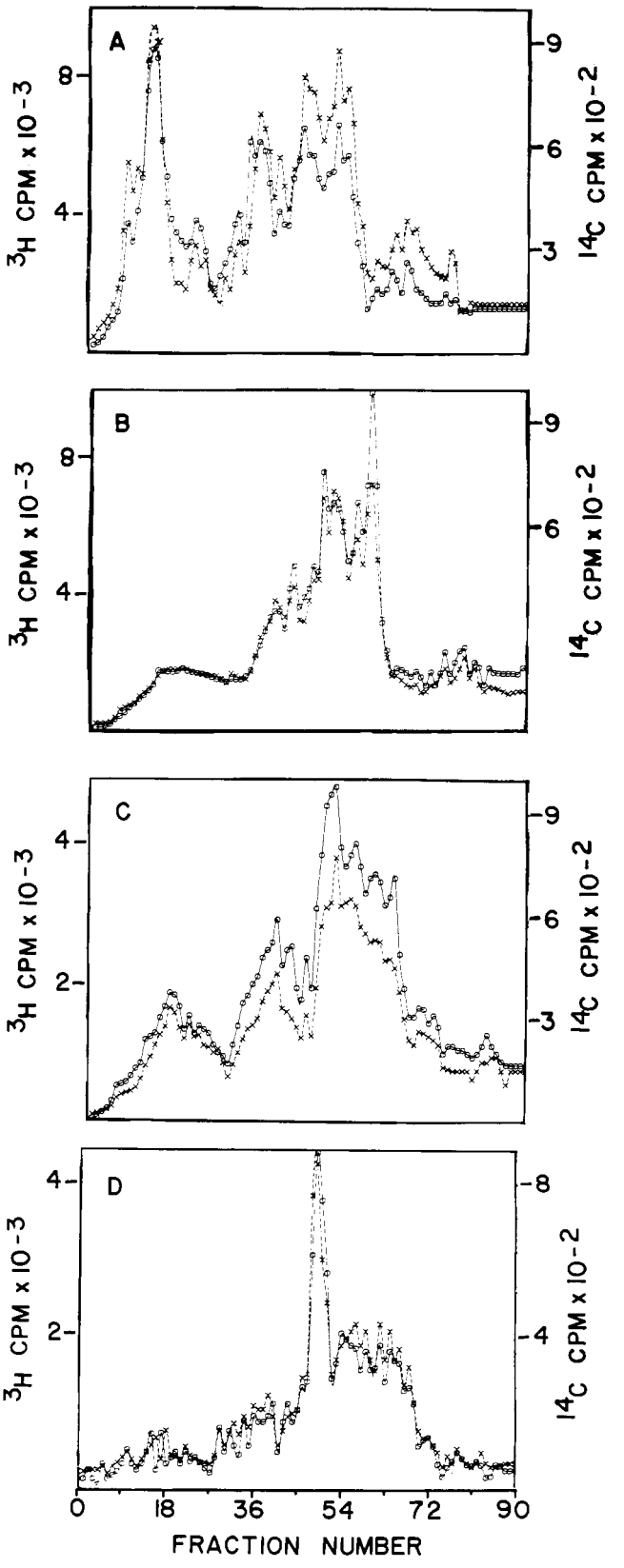

FIGURE 8: Pulse-chase comparisons of subcellular fractions of L cells. Comparison in protein patterns are made between a 5-min pulse of [^{3}H]amino acids (—O—) and a 5-min pulse of [^{14}C]amino acids followed by a 4-hr chase in MEM (--×--). (A) Nuclear proteins, (B) mitochondrial proteins, (C) microsomal proteins, and (D) soluble cytoplasmic proteins.

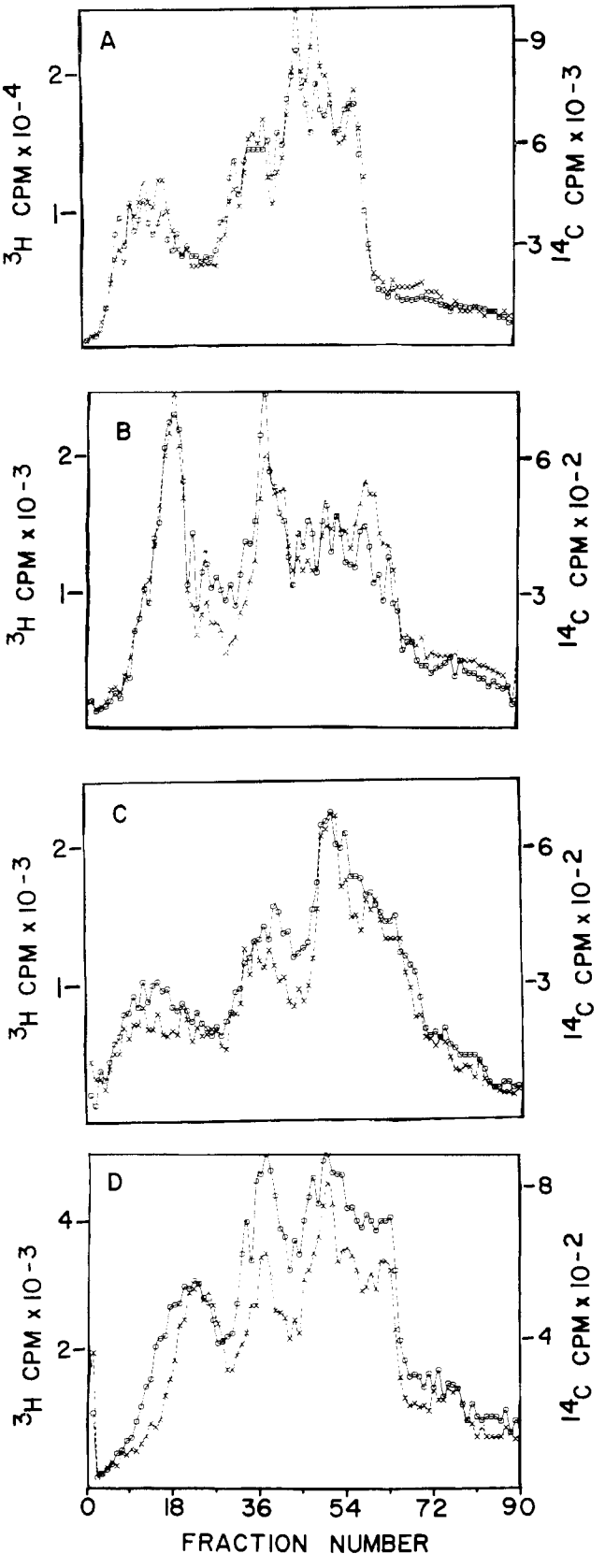

FIGURE 9: Comparisons of pulse-labeled proteins with steady-state labeled proteins. (A) Proteins of HeLa cells labeled for 2 min in [^{3}H]amino acids (—○—) are compared with proteins labeled with [^{14}C]amino acids under steady-state conditions for 4 days (--×--). For steady-state labeling, the usual concentration of labeled amino acids is dilated 50-fold into a large volume of MEM containing 10% calf serum, and daily additions are made of MEM vitamins and calf serum to 10%, with daily adjustment of pH to 7.2 with bicarbonate. (B–D) Comparisons of proteins from subcellular fractions of L cells labeled in a 5-min pulse of [^{3}H]amino acids (—○—), and a 3-day [^{14}C]amino acid steady-state label (--×--). (B) Nucleus, (C) mitochondria, and (D) microsomes.

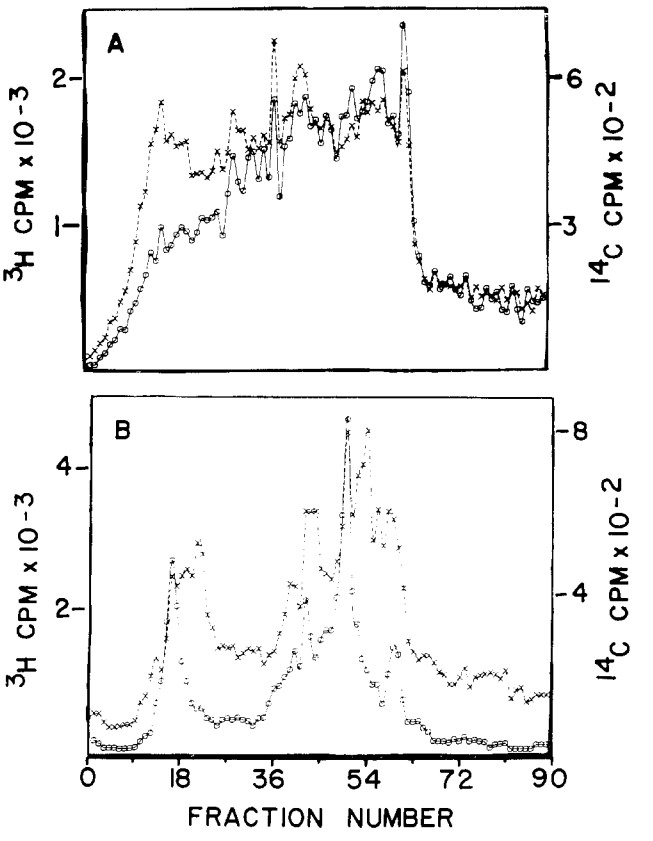

FIGURE 10: The pattern of proteins made in the presence of metabolic inhibitors. (A) The pattern of HeLa cell proteins labeled in 15 min with [^{14}C]amino acids (—×—) is compared with proteins labeled in 15 min with [^{3}H]amino acids (—○—) in the presence of 2.5 mM of DL-*p*-fluorophenylalanine, 3.3 mM of L-canavanine sulfate, 1.8 mM of DL-ethionine, and 5.4 mM of L-azetidine-2-carboxylic acid. (B) The patterns of L-cell mitochondrial proteins are compared between a 60-min label of [^{14}C]amino acids (--×--), and a 120-min label of [^{3}H]amino acids in the presence of 200 μg/ml of cycloheximide (—○—).

Figure 10A confirms this finding. This slight but reproducible skew is unlikely to be due to an analog effect directly on the protein synthesis apparatus itself, because analogs do not cause any shift to higher molecular weight of proteins synthesized under the direction of membrane viruses (J. J. Holland, 1969, unpublished data). However, it could be due to selective inhibition of synthesis of lower molecular weight proteins.

Mitochondrial Proteins. Figure 10B compares the pattern of L-cell mitochondrial proteins synthesized in the presence of a

high concentration of cycloheximide with the normal pattern of proteins in the mitochondrion. Cycloheximide is known to inhibit cytoplasmic ribosomes but not mitochondrial ribosomes (Clark-Walker and Linnane, 1966). The pattern of cycloheximide-resistant mitochondrial proteins may represent those proteins made on the mitochondrial ribosomes because under these conditions, relative to untreated controls, mitochondria have a specific activity three to five times higher than the other cell fractions. In addition, the pattern of cycloheximide-resistant soluble cytoplasmic proteins appears to be identical with untreated control cytoplasm, suggesting it merely represents residual synthesis on cytoplasmic ribosomes (E. D. Kiehn and J. J. Holland, 1969, manuscript in preparation).

Protein Turnover. The depletion of essential amino acids from the medium of cultured cells stops net protein synthesis and results in an extended "resting" period when the rates of synthesis and degradation of protein balance each other (Eagle *et al.*, 1958). Figure 11A compares the normal pattern of HeLa proteins with the proteins synthesized during amino acid depletion. In this experiment cells had been deprived of isoleucine for 70 hr and were morphologically similar to virus-infected cells (rounded up), which has been observed to be characteristic of amino acid deprivation (Eagle, 1955). Other experiments with both HeLa and L cells have involved depletion of tyrosine, phenylalanine, and valine for 24 hr. In all cases, the overall pattern of protein synthesis is the same in "resting" cells as it is in growing cells, although our experiments have not ruled out small or transient differences.

Figure 11B compares uniformly labeled HeLa proteins with proteins from cells which after labeling had been allowed to grow exponentially for an additional 6 days in unlabeled medium. There is observed a set of proteins having slower rates of degradation than most of the cellular proteins. Figure 11C shows the same phenomenon, but compares labeled cells which were incubated for 6 days in either normal growth medium or in starvation medium lacking five amino acids. An almost identical set of relatively stable proteins is observed, although differences exist in relative amounts of certain proteins. This may reflect a difference between resting and growing cells in the degradation rates of specific proteins, although the overall rate of protein degradation is the same for both conditions (Eagle *et al.*, 1958). Figure 11D compares the normal pattern of HeLa proteins with the proteins of labeled cells which were incubated for an additional 21 days in unlabeled starvation medium deleted of isoleucine. The results

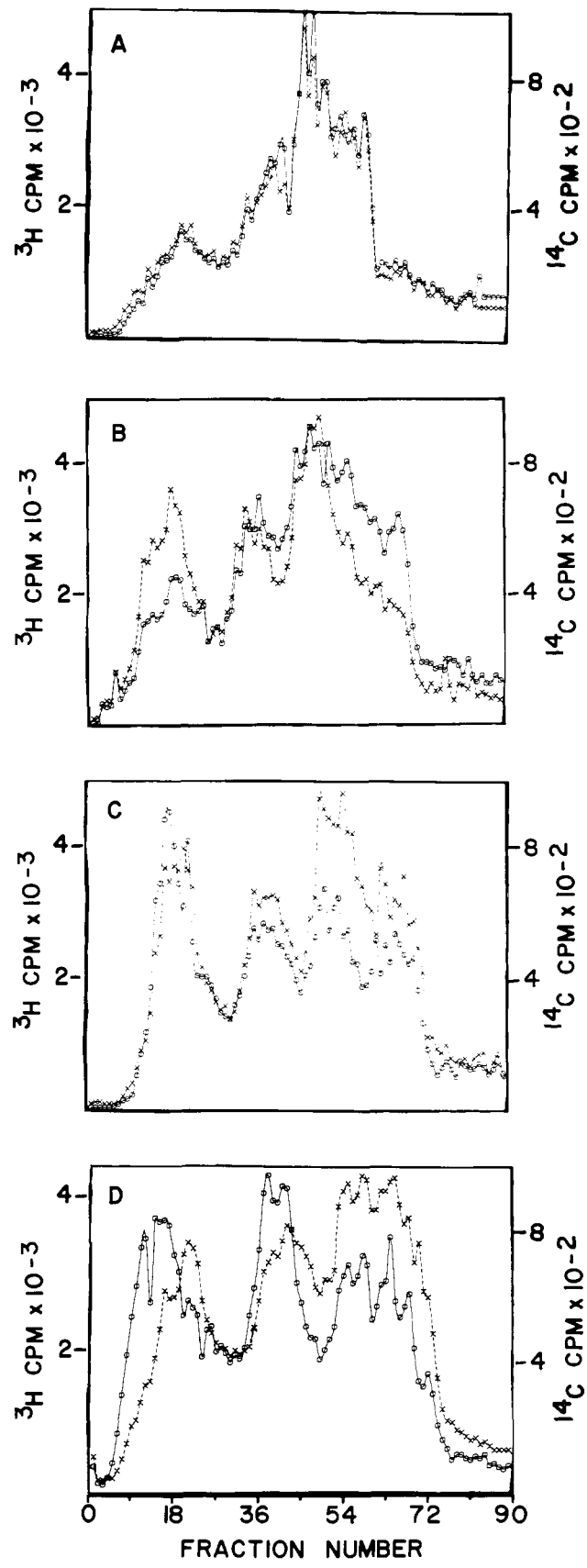

FIGURE 11: Protein turnover in HeLa cells. (A) The pattern of proteins labeled for 60 min with [^{3}H]amino acids (—O—) is compared with the proteins labeled in 60 min of [^{14}C]amino acids after the cells had been incubated for 70 hr in MEM lacking essential isoleucine (--X--). (B) The pattern of proteins labeled in 60 min with [^{3}H]-amino acids (—O—) is compared with proteins labeled for 60 min in [^{14}C]amino acids and then grown for an additional 6 days in unlabeled MEM (--X--). (C) The protein patterns are compared between cells labeled for 60 min with [^{14}C]amino acids and grown for 6 additional days in MEM (--X--), and cells were labeled for 60 min in [^{3}H]amino acids and incubated 6 days in MEM lacking essential leucine, isoleucine, tryptophan, arginine, and cystine (—O—). (D) The protein patterns are compared between cells labeled for 60 min in [^{14}C]amino acids (--X--) and cells labeled 9 hr in [^{3}H]amino acids and then incubated for 21 days in MEM lacking essential isoleucine (—O—).

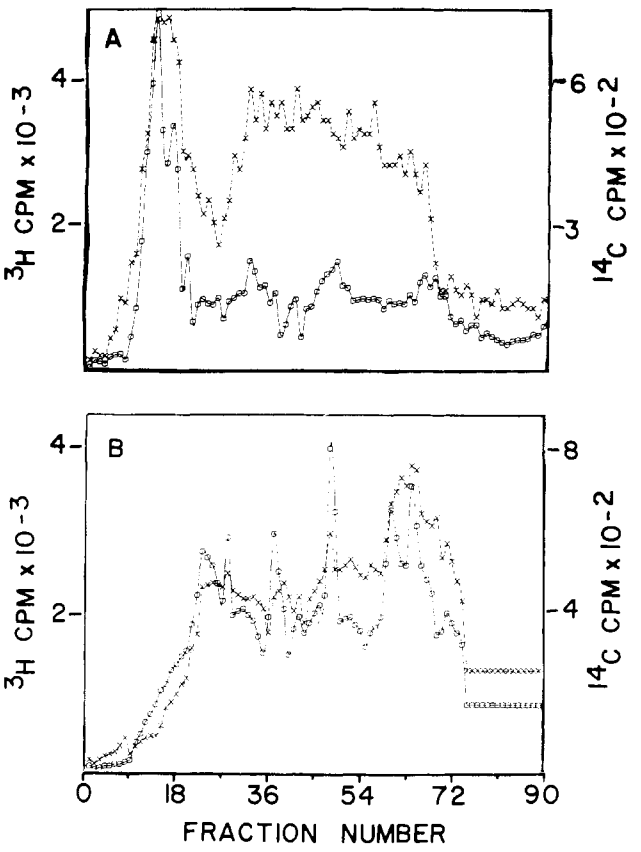

FIGURE 12: Protein turnover in subcellular fractions of mouse tissues. Mice were injected intraperitoneally with a mixture of six [^{14}C]amino acids, sacrificed 4 hr later, and subcellular fractions were isolated (--X--). Mice were injected daily with a mixture of six [^{3}H]amino acids for 2 weeks, sacrificed after an additional 18 days, and their tissues fractionated (---O---). (A) Liver nucleus and (B) kidney mitochondria.

are similar to Figure 11B,C but there is a relatively higher amount of medium-sized proteins and a unique peak of extremely small polypeptides.

Protein turnover was studied in mouse organs after daily injections of radiolabeled amino acids for 2 weeks, followed by an additional 18 days before sacrifice and cell fractionation. The specific activity of organ proteins after this 18-day turnover period was compared with the specific activity of the same organs measured soon after labeling. The general stability of organ protein was found to decrease in the following order: brain > heart > lung > kidney and liver.

Figure 12A compares uniformly labeled proteins of liver nuclei with those nuclear proteins remaining after the 18-day turnover period. The resulting pattern of relatively stable nuclear proteins appear to be the histone fraction. For examples of histone patterns, see the report of Robbins and Borun (1966) and the accompanying paper (Kiehn and Holland, 1970). We also examined the stable proteins of kidney nuclei and found an almost identical pattern. The metabolic stability of histones has been noted before (Gurley and Hardin, 1969), and these workers cited pertinent literature on this topic.

Figure 12B compares uniformly labeled proteins of kidney mitochondria with relatively stable proteins of this organelle remaining after the 18-day turnover period. The differences in the protein patterns shows that there are considerable variations in turnover rates between individual proteins in the kidney mitochondrion. For example, while we have not actually measured rates of turnover, it can be reasonably estimated from the gel profile of Figure 12B that the sharp protein peak of fraction 49 has only degraded at about one-half the rate of the adjacent peak at fraction 53.

The sort of heterogeneity of protein turnover shown in Figure 12B for kidney mitochondria is very typical of what we found in the mitochondria, microsomes, and soluble cytoplasm of the various mouse organs and of tissue culture cells. The only notable exceptions to this generalization were in the lung and kidney soluble cytoplasm. After 18-days turnover, these fractions both showed a lack of high molecular weight material but contained a huge accumulation of extremely low molecular weight polypeptides that were still trichloroacetic acid precipitable, and nondialyzable.

The above data show that membrane proteins are extremely heterogeneous and exhibit a wide range of molecular weights. We have studied the synthesis and turnover of these proteins under carefully controlled conditions and the results are in accord with membrane models which stress the dynamic complexity of these cellular structures.

Discussion

The complex heterogeneity of membrane proteins described in these two papers (Kiehn and Holland, 1970) is in disagreement with many earlier studies, but is similar to findings of several other workers. The size range we have reported for the proteins of various membranes of a number of human and animal cell types is close to that described for rat liver plasma membrane (Neville, 1967), human erythrocyte membrane (Rosenberg and Guidotti, 1968), and in the mitochondrial and microsomal membranes of rat liver (Schnaitman, 1969).

In addition, we have shown that this apparently common size distribution of proteins found in a variety of membranes is not a peculiar property of membranes but rather appears to reflect the general size range of protein in animal cells. The powerful sodium dodecyl sulfate electrophoresis procedure (Maizel, 1966; Summers *et al.*, 1965) allowed us to determine directly the distribution by size of polypeptide chains from a variety of sources. We found an inverse logarithmic relationship of polypeptide frequency to increasing molecular weight.

The relationship of the so-called structural protein fraction of other laboratories to the entire spectrum of membrane proteins seen here is unclear. In all of the membranes we have studied there is only a very small amount of protein of 22,000 daltons, the size originally reported by numerous workers for the structural protein of a variety of membranes. In our studies, proteins of this size are located in a "trough" between more abundant proteins of higher and of lower molecular weights. This can also be discerned in the experiments of Schnaitman (1969). The more recent studies of Green (Green *et al.*, 1968) give molecular weights of structural proteins (50,000–65,000 daltons) which are within our estimates for some of the major membrane proteins. Green and his colleagues suggest that several technical problems led his laboratory and numerous others erroneously to infer the existence of a homogeneous protein of low molecular weight underlying the structure of a variety of membranes (Green

et al., 1968; Lenaz *et al.*, 1968b). There are now many reports that structural protein preparations are quite heterogeneous in composition. Furthermore, it appears (Schnaitman, 1969) that the relative contribution of these proteins to the total membrane is much lower than previously believed, casting further doubt on the role of these proteins as the major structural determinants of membranes.

We have found that even in very short pulses of radiolabeled amino acids it was never possible to discern a nonuniform labeling of membrane proteins. This is probably because synthesis and assembly of membrane components is very rapid in cultured cells and the overall membrane complexity masks whatever differences might exist. This is perhaps not surprising since cultured cells have been shown to be continuously derepressed for membrane synthesis, in that the synthesis of membranes continues at a constant rate, whether or not the cells are growing and multiplying (Warren and Glick, 1968).

In contrast, it is possible to derepress the synthesis of rat liver smooth endoplasmic reticulum, and under these conditions specific enzymes are incorporated into the membrane at differing rates (Siekevitz *et al.*, 1967) and the overall protein patterns also show heterogeneity of synthesis (Arias *et al.*, 1969).

We have found no pulse-labeling evidence that the proteins in any cell fraction are synthesized as giant polypeptides which are subsequently cleaved into the stable spectrum of proteins. However, using amino acid analogs at concentrations which slow the rate of cleavage of poliovirus precursor proteins (Jacobson and Baltimore, 1968b), we were able to confirm the finding of these workers that analogs produce a slight shift to higher molecular weight in the normal pattern of cellular protein synthesis. Possibly, minor protein-cleavage mechanisms may exist in the maturation of at least some cellular proteins. It is conceivable that the normal cleavage mechanisms are much more rapid and less drastic than those involved in the synthesis of enterovirus proteins and therefore precluded detection by the usual pulse-labeling procedures.

It was observed in our studies that heterogeneity of protein turnover is a general phenomenon in specific membranes from a variety of sources. This has been previously observed in the smooth membranes of rat liver (Siekevitz *et al.*, 1967; Arias *et al.*, 1969). Taken together with evidence from the same workers of differing rates of incorporation into membranes for individual proteins, a complex multistep model of membrane synthesis and turnover is favored over more simple, single-step models which imply coordinated behavior for membrane components. However, the possibility is not ruled out that membranes are divided into specialized areas which are in fact synthesized and broken down as units. Evidence for such specialized areas of membranes are the unique chemical and physical properties of the tight junctions of plasma membranes (Benedetti and Emmelot, 1968), the nonrandom array of cell surface antigens (Boyse *et al.*, 1968), the marked heterogeneity of enzyme content and isotope incorporation between subfractions of rough-surfaced liver microsomal membranes separated on sucrose gradients (Dallman *et al.*, 1968), the latter membranes showing nonrandom enzyme distribution even after extensive sonication (Dallman *et al.*, 1969), and the discrete patches that exist on the plasma membranes of influenza-infected cells which represent the budding sites for progeny virus (Holland and Kiehn, 1970). The later-described membrane patches appear to consist entirely of virus-directed proteins, yet coexist in a continuous membrane structure with considerable areas containing normal membrane proteins. The simplicity of viral membrane systems, as compared with the enormous complexity of normal cellular membranes, may provide the most direct means of testing theories of membrane architecture and synthesis.

Finally it should be emphasized that studies such as these do not rule out the possibility that noncatalytic proteins might play a significant role in membrane structure. Our findings do show that no single protein or small group of proteins make up the bulk of membrane structure in cells (as they clearly do in influenza virus). If noncatalytic proteins do make up a considerable proportion of cellular membranes there must be many different species which interact to build membranes, or else there must be different noncatalytic proteins forming the basic structure of the various specialized areas of membranes (such as the inner and outer mitochondrial membranes).

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