Size Distribution of Membrane Proteins of Rat Liver and Their Relative Rates of Degradation*

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SUMMARY

The proteins associated with various membrane fractions, including smooth and rough endoplasmic reticulum, plasma membrane, and mitochondria of rat liver, were solubilized with sodium lauryl sulfate and separated by electrophoresis on sodium lauryl sulfate acrylamide gels. Smooth and rough endoplasmic reticulum have similar electrophoretic patterns. All other fractions have characteristic patterns in which no single or predominant protein occurs. The smooth endoplasmic reticulum fraction was fractionated by use of Triton X-100, which solubilized approximately 50% of the membrane proteins. The patterns of Triton X-100-soluble and -insoluble proteins are distinctive.

The relative rates of degradation of the proteins were studied by use of a double isotope technique in which 14C-leucine was administered to intact rats 4 days prior to administration of 3H-leucine. As indicated by the differences in 3H:14C ratios, there is a marked heterogeneity of turnover of proteins of both the endoplasmic reticulum and plasma membrane.

There is a general correlation between the relative degradation rates of membrane proteins and the molecular size of the protein (subunit) as separated by electrophoresis in sodium lauryl sulfate acrylamide gels. This general relationship was found also for the soluble protein fraction of rat liver, whether fractionated by sodium lauryl sulfate acrylamide gels, or by Sephadex G-200 columns in the presence of sodium lauryl sulfate.

A model in which membrane proteins associate and dissociate from the protein-lipid complex is favored to explain the heterogeneity of degradation, and the general correlation of size with degradation rate.

Several studies have shown that the protein components of liver microsomes and plasma membrane are turning over relatively rapidly (1-3). This finding has raised the question of whether the membrane is degraded and synthesized as a unit, or whether individual protein components can be introduced and removed from the membrane at different rates (asynchronously). Studies on the degradation rate constants of individual membrane proteins, cytochrome b5 (1, 2), cytochrome c reductase (1, 2), and NAD-glycohydrolase (4) indicate a heterogeneity of half-lives of these purified proteins ranging from 60 to 100 hours (cytochrome c reductase (2)) to 17 days (NAD-glycohydrolase (4)). The problems of isolation and identification of only a few protein components of membranes led Arias, Doyle, and Schimke (1) to determine relative rate constants of degradation of proteins solubilized from endoplasmic reticulum and separated by chromatography on DEAE-cellulose in the presence of Triton X-100. These studies likewise indicated marked heterogeneity of turnover of such solubilized proteins.

In the present study we have examined the relative degradation rates of protein constituents of rat liver microsomal and plasma membrane fractions as they are separated by electrophoresis on acrylamide gels in the presence of sodium lauryl sulfate. This method offers several advantages in the study of membrane turnover: (a) essentially all of the membrane proteins are solubilized by the detergent and can be resolved well by gels; (b) major protein bands can be identified and studied for unusual turnover characteristics; (c) since migration in SLS acrylamide gels is dependent on protein size (5), correlations between molecular size and degradation rate constants can be made; (d) differences in the protein components of various membrane types can be readily assessed.

METHODS

Cell Fractionation—All fractionation procedures were performed on livers from male, white Sprague-Dawley rats weighing 120 to 150 g each. The animals were fasted 18 hours before killing. Livers were perfused through the spleen with 10 ml of cold 0.25 M sucrose before excision, and homogenized with a glass homogenizer and Teflon pestle, three strokes at 700 rpm. For the isolation of plasma membrane, a Dounce homogenizer was used. The method of Leighton et al. (6) was used for isolating mitochondria and peroxisomes, except that the isopycnic centrifugation was done in 13-ml swinging bucket tubes (SB-283 rotor) in an International centrifuge, model B-60, rather than a zonal rotor. The method of Neville (7) as modified by Emmelot et al. (8) was used for preparing the plasma membrane fraction. Three microsomal fractions were prepared as

1 The abbreviations used are: SLS, sodium lauryl sulfate; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; LEH, light endoplasmic reticulum; TX, Triton X-100; ER, endoplasmic reticulum.
were required for gel electrophoresis experiments in order to loss of membrane proteins.

For preparing the soluble liver fraction, a perfused liver was homogenized with 1 volume (v/w) of 0.05 M Tris-glycine buffer, pH 8.9. The homogenate was centrifuged at 10,000 \( \times g \) for 20 min and the resulting supernatant spun at 105,000 \( \times g \) for 1 hour. The 105,000 \( \times g \) supernatant was drawn off and passed through a Sephadex G-25 column, 1.5 \( \times 20 \) cm, which had been equilibrated with the homogenization buffer. This procedure effectively removed free amino acid from the supernatant fraction. The resulting material was frozen until used.

**Enzyme Assays**—The following enzyme markers were used to monitor purity of membrane fractions (6). Catalase, a peroxisomal enzyme, was assayed by following the disappearance of \( \text{H}_2\text{O}_2 \) at 240 nm (10); cytochrome oxidase was assayed by the oxidation of reduced cytochrome c (11). Both assays were recorded with a Gilford model 2000 spectrophotometer. Alkaline nitrophenylphosphatase activity, characteristic of plasma membrane (8), was measured by appearance of p-nitrophenol at pH 10.8. In this assay, the sample was previously incubated at 37\(^\circ\)C in 0.05 M Tris-HCl, pH 8.9, for 30 min to inactivate glucose 6-phosphatase (12) prior to the addition of the substrate, p-nitrophenylphosphate. Glucose 6-phosphatase was assayed by the appearance of inorganic phosphate from glucose 6-phosphate at pH 6.5 (13).

**Isotope Administration**—Uniformly labeled \( ^{14} \text{C}-\text{leucine} \) (300 mCi per mm) and 4, 5, 6-\( ^{14} \text{C}-\text{leucine} \) (2000 mCi per mm) were obtained from Schwarz BioResearch. The isotope was diluted to 2 ml in 0.15 M NaCl and injected intraperitoneally in two 1-ml doses. In the double label experiments animals were fasted 18 hours, then given 100 \( \mu \text{Ci} \) of \( ^{14} \text{C}-\text{leucine} \). Food was restored 4 hours after isotope administration. Four days later this schedule was repeated; this time the animal was given 250 \( \mu \text{Ci} \) of \( ^{14} \text{C}-\text{leucine} \) and killed 4 hours later.

**Protein separation by acrylamide gel electrophoresis was carried out on discontinuous gels according to the procedure described by GAusfeld (16). Discontinuous gradient gels consist of regions of varying concentration of acrylamide, thus allowing for greater resolution of proteins of heterogeneous size. The gel stock solutions were similar to those used by Davis (17) for electrophoresis in Tris-glycine buffer, except that stocks for 12, 10, 7.5, and 4% acrylamide were prepared. The upper reservoir buffer (pH 8.9) consisted of 6 g of Tris and 4 g of glycine per liter; the lower reservoir buffer (pH 8.1) consisted of 12 g of Tris and 50 ml of 1 \% HCl per liter. The gels consisted of one part of acrylamide stock to three parts of 0.5 M Tris-\( \text{HCl} \) buffer, pH 8.9, containing 0.1% ammonium persulfate (w/v) and 0.08% \( N,N',N''\text{-}\text{tetramethylmethylenediamine} \) (v/v). Detergent was not incorporated into the gel itself, since SLS in the sample and upper buffer migrated into the gel ahead of the sample proteins. The gel solutions were carefully layered in the glass tubes, one over the other, before polymerization occurred. This resulted in a more gradual transition at the gel interfaces. Polymerization was retarded by addition of 0.15% (w/v) potassium ferricyanide to the acrylamide stocks.

We employed gel gradients of three types, the compositions of which are given in Table 1. The polyacrylamide resolving gel was overlayered with stacking gel (16) which was polymerized by exposure to light. Gels of two sizes were employed. Gels, \( 5 \times 75 \) mm, were used for display of protein patterns (analytical gels). Large (preparative) gels, \( 19 \times 75 \) mm, were employed for radioative samples, since such gels were capable of resolving an amount of protein (about 5 mg) required to obtain sufficient radioactivity for accurate counting. After washing the stacking gel surface with the upper reservoir buffer, samples of 200 to 300 \( \mu \text{g} \) of protein in 50 to 100 \( \mu \text{l} \) were applied to the small gels, and 5 \( \mu \text{g} \) in 1 to 2 ml, to the large gels.

The samples were mixed with a small amount of sucrose and tracking dye (bromphenol blue) and overlaid with upper buffer. Electrophoresis was performed in a Hoefer electrophoresis chamber. A current of 0.1 mA per small gel (1.5 mA per large gel) was applied until the tracking dye entered the stacking gel, at which time the current was raised to 0.2 mA per small gel (3 mA per large gel). When the dye had entered the re-

\[ ^3 \text{P. J. Dehlinger and R. T. Schimke, unpublished observations.} \]
Volume of acrylamide solutions used in preparing gradient gels

Lower case letters indicate 5 X 75 mm gels (analytical gels); upper case letters indicate the corresponding gradients of 19 X 75 mm gels (preparative gels). Gel a was used in comparing the electrophoretic patterns of six subcellular fractions. Gels b and C were employed in the double label studies on protein constituents.

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<tr>
<th>Gels</th>
<th>Volume of acrylamide solutions</th>
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<tbody>
<tr>
<td></td>
<td>4%</td>
</tr>
<tr>
<td>5 X 75 mm</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>0.1</td>
</tr>
<tr>
<td>b</td>
<td>0.1</td>
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<tr>
<td>c</td>
<td>0.1</td>
</tr>
<tr>
<td>19 X 75 mm</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.4</td>
</tr>
<tr>
<td>B</td>
<td>1.4</td>
</tr>
<tr>
<td>C</td>
<td>1.4</td>
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</table>

For sectioning, a large gel was placed in the shallow trough of a plexiglas mount and a slab of Dry Ice was placed over the mount to keep the gel frozen during sectioning. The gel was attached to the end of a 1-mm thread screw which was used to advance the gel along its length. Gel slices 1 mm thick were cut as the gel advanced through the cutting plane of a razor blade held perpendicularly to the long axis of the gel. Each slice was placed in a scintillation vial containing 0.5 ml of 30% H2O2, capped tightly, and incubated at 37°C for 48 hours. Following this means of dissolving the gel, 0.5 ml of "Tritiumine" (New England Nuclear) and 10 ml of modified Bray's solution (18) were added. The scintillation mixture cleared after being shaken at 37°C overnight. The samples were counted in a Packard Tri-Carb model 332C. A counting efficiency of approximately 15% for tritium and 55% for 14C was obtained for all concentrations of acrylamide present in the sample slices (i.e. from 4 to 12% acrylamide). Quenching was monitored routinely by a channels ratio method. Differential quenching from vial to vial was not encountered. Spillover of 14C counts into the tritium channel was subtracted from total counts in the tritium channel to yield net tritium counts. This spillover was about 35% of the total counts present in the 14C channel. There was no spillover of tritium counts into the 14C channel.

The small stained gels were scanned in a Gilford model 2000 spectrophotometer equipped with a gel-scanning attachment at a wave length of 600 nm. The isotope-labeling pattern was superimposed on the optical density tracing by matching main features of the short term isotope incorporation pattern with corresponding features of the optical tracing. Once the correspondence between the two patterns was established, the 3H:14C ratios were plotted on the background of optical density profiles. Typical data are those of Figs. 4 to 9. Proteins used as molecular weight markers (see Figs. 1 and 2) were obtained from commercial sources. About 20 μg of marker protein were applied to analytical gels and electrophoresis was carried out as described for membrane proteins.

Column Chromatography—Proteins of liver supernatant were fractionated on Sephadex G-200 in the presence of SLS. Sephadex G-200 was equilibrated with 0.05 M Tris-glycine buffer, pH 8.9, containing 0.1% SLS, identical with the upper buffer used in gel electrophoresis. A sample of the supernatant fraction containing 30 mg of protein was heated for 5 min at 100°C in the presence of 0.1% SLS before application to the column. Dextran blue and bromphenol blue were added to the sample to serve as end point markers. The flow rate was adjusted to about 12 ml per hour. Fractions of 5 ml were collected, and 1 ml of 50% (w/v) of trichloracetic acid was added to each fraction. The resulting precipitates were collected on glass filters, and incubated in 0.5 ml of "NCS" (Amer sham-Searle) solubilizer for 3 hours at 37°C. Ten milliliters of scintillation fluid (6 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per liter of toluene) were added to the vials and counted as described above for gel slices. The counting efficiency in this method was approximately 35% for tritium and 63% for 14C. Spillover of 14C into the tritium channel, above 13% of total 14C counts, was subtracted from total tritium counts to yield net tritium counts.

The column was calibrated with dextran blue (void volume), conalbumin (78,000 daltons), ovalbumin (45,000 daltons), and cytochrome c (13,000 daltons). The marker proteins were obtained from commercial sources.

Immunologic Techniques—Rabbit antiserum against whole rat serum and control rabbit serum were obtained from GIBCO, Grand Island, New York. The lyophilized antiserum was reconstituted with 1% Triton X-100 in water. The antiserum was as effective in precipitating rat serum in the presence of 1% Triton X-100 as in its absence. In an experiment designed to determine the equivalence point of precipitation of microsomal proteins solubilized by Triton X-100, 50-μl aliquots of TX-soluble SER, containing 0.26 mg of protein per aliquot, were added to increasing volumes of antiserum in 0.5 ml (total volume) plastic "microfuge" tubes (Beckman Instruments). The volume of antiserum ranged from 0 to 250 μl. In each tube the total serum volume was adjusted to 250 μl by the addition of an appropriate volume of control rabbit serum in 1% Triton X-100. The mixture was incubated for 2 hours at 37°C and the resulting precipitate pelletled by centrifugation in a Beckman model 152 microfuge. Incubation longer than 2 hours was avoided, since upon longer incubation nonspecific precipitation of microsomal protein occurred. The precipitate was washed two times with 300 μl of 1% Triton X-100. The tips of the tubes (containing the precipitates) were cut off, placed in glass scintillation vials, and incubated with 0.5 ml of NCS solubilizer overnight. Scintillation counting was performed as for the Sephadex G-200 column fractions. The microsomal material used in this experiment was obtained from a rat which had received 250 μCi of 3H-leucine intraperitoneally 4 hours before killing.

The TX-soluble SER proteins and the immunoprecipitate from this fraction were prepared for electrophoresis by heating the samples at 100°C for 5 min in the presence of 0.1% SLS and 50
TABLE II

Enzyme distributions in six subcellular fractions of rat liver

Preparation of fractions and assays is described under "Methods." That fraction containing the highest specific activity (units per mg of protein) was set at 100% and specific activity in other fractions was expressed as a percentage of that value. Undetectable activity is denoted by dashes. These values are typical of four-cell fractionation experiments.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Percentage of maximal specific activity of enzymes</th>
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<tbody>
<tr>
<td></td>
<td>Catalase</td>
</tr>
<tr>
<td>Peroxisomes</td>
<td>100</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>11.0</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>1.9</td>
</tr>
<tr>
<td>Rough ER</td>
<td>1.9</td>
</tr>
<tr>
<td>Smooth ER</td>
<td>1.0</td>
</tr>
<tr>
<td>Light ER</td>
<td>9.8</td>
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</table>

units, and to what extent they are undisassociated protein complexes which exist in the native membrane, or protein aggregates which form when membranes are treated with SLS. There is ample documentation that SLS, in the presence of a reducing agent such as mercaptoethanol, is effective in dissociating soluble oligomeric proteins, and that the electrophoretic mobility of the dissociated subunits in the presence of SLS is dependent on subunit molecular weight (5, 20). With the gel electrophoresis system described under "Methods," we have verified these findings on several known soluble oligomeric proteins, including hemoglobin, catalase, and lactate dehydrogenase. However, it cannot be assumed from this that the relatively insoluble membrane fractions will be completely dissociated under these conditions. Several factors which might prevent complete dissociation of the membrane proteins are considered below.

We have ruled out extensive protein aggregation on the basis of the observation that essentially all of the applied membrane sample enters the resolving gel, as judged visually on the stained analytical gels and by the distribution of radioactive counts on the large gels (see Fig. 5). Incomplete dissociation caused by disulfide linkages between protein subunits was examined by two methods: the SER was prepared for electrophoresis by heating the membrane sample at 100° for 5 min in the presence of 0.1% SLS and 50 mM dithiothreitol. In a second experiment, the SER fraction was reduced with mercaptoethanol in the presence of 8 M urea, pH 8.5, and carboxymethylated with excess iodoacetate acid (21), prior to solubilization in 0.1% SLS. In both cases, the electrophoretic patterns were similar to that of the SER fraction seen in Fig. 1. In addition, the same electrophoretic patterns were obtained when SER had been first washed with chloroform-methanol (2:1) or ethanol-ether (3:1) to remove lipid. Nor was the electrophoretic pattern altered when the SER fraction was precipitated with trichloroacetic acid prior to solubilization in SLS. Thus it appears that factors which might be expected to prevent complete dissociation of the membrane proteins, such as disulfide linkages or protein-lipid interactions, had no effect on the electrophoretic distribution of SER proteins. These considerations suggest that membrane proteins...
are indeed resolved on the basis of monomeric units by the electrophoresis procedure employed in these studies. The possibility that some of the protein bands result from stacking at the gradient discontinuities in the gel is unlikely, since only at the 4 to 7% acrylamide interface does a protein band exist (Fig. 1).

The general features seen in Fig. 1, including the distinctive patterns and the lack of common, major protein species, agree with previous electrophoretic studies comparing mitochondria and microsomes (22, 23) and various membranes (plasma membrane, smooth and rough ER, mitochondria, and Golgi membrane) of bovine liver (24). Another consistent finding has been the presence of major protein components in the molecular weight range of 45,000 to 70,000 in all subcellular membrane fractions (22, 23). In agreement with several studies which indicate similarities between rough and smooth microsomal fractions as based on enzyme activities (9, 25, 26) we find no difference between the electrophoretic patterns of these two fractions. Also noteworthy is the correspondence between the marker protein catalase and a major band of the peroxisomal fraction, of which catalase may represent up to 15% of the total protein (27).

The smooth endoplasmic reticulum fraction was further fractionated on the basis of solubility of proteins in 1% Triton X-100 as outlined under "Methods." This fractionation was undertaken to increase the resolution of proteins and to determine the extent to which the membrane components as displayed on acrylamide gels represent plasma proteins in transit through endoplasmic reticulum. This latter point becomes of particular importance in the studies on turnover of the membrane components. The electrophoretic patterns of TX-soluble and -insoluble fractions, each comprising approximately 50% of the total microsomal protein, are shown in Fig. 2. The two fractions have distinctive patterns; most of the proteins in the TX-soluble fraction have molecular weights greater than 50,000,

![Acrylamide gel electrophoretic patterns of total SER and TX-soluble and TX-insoluble subfractions. Fractionation of SER on the basis of solubility in Triton X-100 is described under "Methods." The three gels at the left were obtained with gels of Composition a (Table I). The two gels at the right show the same material on gels of Composition c (TX-soluble SER) and b (TX-insoluble SER). Approximately 225 μg of protein were applied to each gel.](http://www.jbc.org/)

![Precipitation of rat serum proteins by rabbit antirat serum. TX-soluble SER was prepared as described under "Methods." To 50 μl of TX-soluble SER (285 μg of protein) increasing volumes of rabbit antirat serum in 1% Triton X-100 were added. The total number of counts contained in the fractions (3250 cpm) was determined by precipitation of 50 μl of TX-soluble SER with 10% trichloroacetic acid. Washing and counting procedures are described under "Methods."](http://www.jbc.org/)

![Electrophoretic patterns of immunoprecipitate from SER. Serum proteins present in the TX-soluble SER subfraction were precipitated by a rabbit anti-rat serum antibody preparation. The immunoprecipitate obtained from 5 mg of TX-soluble SER treated with an excess of antibody (see Fig. 3) was washed, solubilized, and electrophoresed on a large gel, Composition C (Table I), and subsequently fractionated for radioactive counting as described under "Methods." Identical electrophoresis was performed with 5 mg of TX-soluble SER protein. The animal from which the SER was prepared had been injected with 250 μCi of [3H]-leucine 4 hours prior to death. The upper box shows the optical density scan of an analytical gel to which 225 μg of TX-soluble SER were added. The lower box shows the radioactivity patterns of the TX-soluble SER (——) and the immunoprecipitate from this fraction (--.--).](http://www.jbc.org/)
whereas the TX-insoluble fraction contains proteins of a more heterogeneous molecular weight distribution. The presence of 1% Triton X-100 in the SER fraction did not alter the electrophoretic pattern of the sample on SLS gels. A gel of predominantly 7% acrylamide was effective in fractionating the TX-soluble proteins. A gel containing predominantly 10% acrylamide was used to display more effectively the proteins of the TX-insoluble fraction.

As can be seen in Fig. 2, a major protein band of the TX-soluble fraction has migratory properties similar to serum albumin. This raises the question of the extent to which measurements of protein turnover in endoplasmic reticulum reflect the passage of serum proteins through the ER. Several techniques were used to identify secretory protein released from SER by treatment with Triton X-100. The studies were performed with microsomes from animals that had been injected with 3H-leucine 4 hours before killing. The results indicate that at most 12% of the radioactivity solubilized by 1% TX-100 represents serum proteins. One such technique was the use of an antiserum against rat serum. Preliminary results with Ouchterlony double diffusion and quantitative precipitation techniques indicated that 1% Triton X-100 did not alter the ability of this antiserum to precipitate serum components. As shown in Fig. 3, this antiserum was capable of precipitating labeled SER proteins to a plateau of 12% of the radioactivity solubilized by 1% Triton X-100. Addition of antiserum beyond 0.4 ml antiserum per mg of SER TX-soluble protein did not further increase the amount of labeled protein precipitated. That such precipitated protein is comparable to serum proteins was indicated by the existence of common precipitation bands between serum and solubilized membrane proteins as evidenced by Ouchterlony double diffusion (not shown). Fig. 4 shows the SLS acrylamide gel-labeling pattern of the immunoprecipitates compared with that of the total TX-soluble fraction from which the immunoprecipitate was derived. Four radioactive protein peaks are observed in the solubilized precipitate, one of which is a protein (or group of proteins) of molecular weight 60,000 to 70,000, and presumed to represent serum albumin. The identity of the other proteins is not known. We have used the method of Campbell, Gergur, and Kernot (28) for isolating serum albumin from liver microsomes and have found that the serum albumin isolated constitutes at most 2.5% of the TX-soluble microsomal radioactivity and 0.4% of the TX-insoluble radioactivity. These experiments, then, indicate that secretory proteins, which are presumed to be trapped within vesicles during homogenization, and not released by the washing procedure employed, constitute only a small portion of the proteins being studied by isotope labeling.

**Turnover of Membrane Subfractions**—We have employed a double isotope technique first described by Arias et al. (1) for measuring the relative turnover rates of the protein constituents of the Triton X-100 subfractions of SER and plasma membrane. In this method two isotopic forms of the same amino acid, 3H- and 14C-leucine, are used to establish two time points on the decay curve resulting from degradation of the protein. In the experiments reported here, an animal was injected initially...
Fig. 7. Relative degradation of TX-insoluble SER proteins. The proteins used from this experiment are labeled as described in Fig. 6. The control \( { }^{3} \text{H}: { }^{14} \text{C} \) ratios are those from the animal of Fig. 5. The protein peak is in the molecular weight range of about 50,000.

with \( { }^{14} \text{C} \)-leucine, followed 4 days later by administration of \( { }^{3} \text{H} \)-leucine, with killing after 4 hours. For any protein species the level of \( { }^{3} \text{H} \) radioactivity indicates short term incorporation, whereas the \( { }^{14} \text{C} \) radioactivity indicates the amount of decay after 4 days. Proteins that are turning over rapidly, i.e. synthesized rapidly (high \( { }^{3} \text{H} \) counts) and degraded rapidly (low \( { }^{14} \text{C} \) counts) will have high \( { }^{3} \text{H}: { }^{14} \text{C} \) ratios. If all proteins are turning over at the same rate the ratios will be constant. The assumptions and discussion of the validity of the method are discussed by Arias et al. (1).

To determine the limit of statistical error inherent in the double label method as applied to gel electrophoresis, a standard control experiment was performed in which an animal was given both \( { }^{3} \text{H} \)- and \( { }^{14} \text{C} \)-leucine simultaneously. The results are seen in Fig. 5, which shows the optical density tracing, the pattern of radioactivity, the typical counts obtained, and the standard error of ratios obtained in such a control experiment. The results of the experiments to determine the relative turnover rates among the proteins of the two Triton X-100 subfractions of SER are given in Figs. 6 and 7. The upper frames show the optical gel scan of the stained analytical gels and the \( { }^{3} \text{H}: { }^{14} \text{C} \) ratios determined from the preparative gels. The control \( { }^{3} \text{H}: { }^{14} \text{C} \) ratios are those from the control experiment just described. Several features of the \( { }^{3} \text{H}: { }^{14} \text{C} \) ratio patterns are common to both of the SER subfractions.

1. There is a marked heterogeneity in the ratios among the protein constituents, again supporting the concept of heterogeneity of degradation rate constants. There is a general correlation between the \( { }^{3} \text{H}: { }^{14} \text{C} \) ratios and the migration distance in the gel. Because migration in SLS acrylamide gel reflects subunit molecular weight, we infer that turnover rates, in general, are related to subunit molecular size.

2. Not all proteins conform to this size degradation rate correlation. In Fig. 6 two definite \( { }^{3} \text{H}: { }^{14} \text{C} \) peaks are seen. The nature of these proteins is not known, but on the basis of the exclusion of serum proteins as significant labels in these molecular weight ranges, it appears unlikely that they represent serum protein in transit. The major protein band in the TX-insoluble fraction is particularly interesting, since it is a protein (or group of proteins) with an unusually high turnover rate. Because of the studies of Omura and Sato (30) indicating that the carbon monoxide-binding protein, P-456, is a major protein constituent of the endoplasmic reticulum, amounting to approximately 16% of total microsomal protein of rabbit liver, it seems possible that this major protein species (or a component of it) represents the hemoprotein P-450. In support of this, we have purified partially the CO-binding pigment (30) from rat liver microsomes and found that the fraction contains a protein whose migration rate on SLS gels is identical with the major protein band of the TX-insoluble fraction.

The relative turnover rates of protein constituents of the plasma membrane are shown in Fig. 8. The results show several similarities with the SER subfractions. First, heterogeneity...
is indicated by the variation in $^3$H:$^1$C ratios. Second, the general correlation between $^3$H:$^1$C ratios and protein size is evident.

**Turnover of Supernatant Proteins**—The finding of a general correlation between subunit size and rate of degradation in the membrane fractions prompted an investigation of the characteristics of turnover of proteins in the supernatant fraction from rat liver. Experiments similar to those employed with the membrane fractions, but using supernatant fractions as the source of labeled protein, are shown in Fig. 9. The same general correlation between size and degradation rates exists with these proteins. Since in control experiments this systematic variation does not occur, it cannot be ascribed to a systemic error in counting. Furthermore the same results are obtained when the isotopes are reversed (i.e. when the $^3$H-leucine is administered before the $^1$C-leucine), and when the animals are fed throughout the experiment, as opposed to the 4-hour fast prior to isotope injection used in the experiments reported. The same results were obtained when the supernatant fraction was treated with 0.1% sodium lauryl sulfate and 50 mM dithiothreitol, indicating that disruption of the disulfide bonds has no effect on the size distribution of subunits.

We have also subjected the supernatant proteins to chromatography on Sephadex G-200 in the presence of 0.1% SLS, as seen in Fig. 10. The difference in actual $^3$H:$^1$C ratios compared with the corresponding gel electrophoresis experiment (Fig. 9) is caused by the difference in counting efficiencies between the two counting procedures employed (see "Methods"). The correlation between turnover rates and molecular weight is again evident, with an independent method for separating proteins on the basis of size.

**DISCUSSION**

The experiments reported in this paper were addressed to the question of how biologic membranes are degraded, and indirectly, how they are assembled. Studies of Arias et al. (1), Omura, Siekevitz, and Palade (2), and Kuriyama et al. (31) have indicated that the endoplasmic reticulum fractions of rat liver are turning over rapidly, with a mean half-life of approximately 2 to 3 days. In addition Arias et al. (1) and Widnell and Siekevitz (3) have found that plasma membrane turns over at the same rate as the endoplasmic reticulum. The lipid moieties of the endoplasmic reticulum likewise display rapid turnover (2, 32). The turnover characteristics of membrane components can suggest one of two general models for the assembly and breakdown of cellular membranes.

1. All of the major components of the membrane are synthesized and degraded at the same rate, indicating that the membrane is synthesized and degraded as a multiprotein unit; or
2. Turnover rates of membrane components are heterogeneous, suggesting that the membrane is in a continual state of flux, in which individual component proteins are assembled at different...
rates, and subsequently dissociate, or are degraded while remaining attached to the membrane, at rates characteristic for the individual proteins.

Fundamental to this problem is the question of the protein composition of cellular membranes. Are cellular membranes composed of a major structural protein to which lipid and enzymatic components are attached (33, 34), or are they a composite of enzymatic proteins in association with lipid elements in the membrane? In the former case the structural protein by itself might control the turnover rate of the membrane. The question of membrane composition has been investigated in several recent studies utilizing SLS acrylamide gel electrophoretic techniques similar to those employed in this study (22, 23). The findings of Fig. 1 are in agreement with these other investigations in suggesting that there is no protein which comprises a majority of the membrane protein, and no single major protein that is common to all membranes.

Our results show that membrane proteins as separated by SLS acrylamide gel electrophoresis are heterogeneous with respect to degradation rate constants. This finding is in agreement with our own earlier studies involving the isolated membrane proteins, cytochrome bs and cytochrome c reductase, and unidentified proteins solubilized by Triton X-100 and separated by chromatography on DEAE-cellulose (1), as well as the findings of Omura et al. (2) and Kuriyama et al. (31) on cytochrome bs and cytochrome c reductase turnover. In these studies the half-lives of cytochrome bs and cytochrome c reductase were of the order of 120 and 75 hours, respectively. More recently Bock, Siekevitz, and Palade (4) have studied a NAD-glycohydrolase solubilized from both endoplasmic reticulum and plasma membrane of rat liver, and have found its half-life to be approximately 17 days as isolated from either membrane fraction. All these studies support the concept that membranes are in a state of flux, in which the membrane constituents are degraded asynchronously. Furthermore studies by Arias et al. (1) and Kuriyama et al. (31) have shown that the rates of synthesis of the two identifiable proteins of membranes, cytochrome c reductase and cytochrome bs, can be affected differentially by administration of phenobarbital.

It should be noted that heterogeneity of turnover, by itself, does not preclude the possibility that the membrane fraction studied consists of several membrane types, each with a characteristic turnover rate. If this were the case an appropriate means of subfractionating the membranes into homogeneous types would eliminate the observed heterogeneity of turnover. We have attempted such a fractionation of the smooth endoplasmic reticulum by isopycnic centrifugation in 20 to 45% sucrose gradients. Four discernible fractions can be separated. These all had similar electrophoretic patterns on SLS acrylamide gels and similar mean rates of turnover as measured by the double isotope ratios of total membrane proteins. By the criterion of density, then, we did not find heterogeneity of membrane types within the SR fraction.

The identity of the proteins displaying a heterogeneity of turnover in plasma membrane and endoplasmic reticulum as separated by sodium lauryl sulfate-acrylamide gels is, of course, essentially unknown. The experiments described in Figs. 3 and 4 suggest that these do not represent simply those proteins in transport to the serum. Since these results are based in large part on immunologic reactivity of the solubilized endoplasmic reticulum protein, we cannot discount at present the possibility that protein destined for transport that is present in endoplasmic reticulum vesicles may not react with an antibody to the serum proteins. One major peak in the Triton X-insoluble fraction is of particular interest, since it constitutes such a large portion of the membrane protein, and has an "anomalously" high turnover rate (see Fig. 7). That this constitutes a major portion of the protein of this fraction, and has migratory properties similar to the solubilized form of the hemoprotein, cytochrome P-420, suggests that this band is, indeed, cytochrome P-450. This is supported further by our finding that isotopic amino acid incorporation is preferentially stimulated in this protein band in response to phenobarbital. It is well established that the level of P-450 is increased in response to phenobarbital (35). The role which this protein may have in the regulation of turnover in ER is a question we are now investigating.

Heterogeneity of turnover and, in particular, the correlation between degradation rate and molecular size of the protein components of membrane raise a number of questions concerning the mechanism of membrane degradation. Are the membrane proteins degraded in situ while associated with the membrane? Morrison and Neurath (36) have reported that erythrocyte membranes contain potent proteases, suggesting the presence of degradative enzymes within the membranes. Given the correlation between turnover rates and molecular size, this would require that the larger proteins (or subunits) are more accessible to the degradative enzymes present in the membrane. Such a membrane might be pictured as a core of smaller proteins encluttered by larger proteins. On the other hand, the degradative enzymes may be in the so-called soluble fraction of the cell, such that those proteins more peripheral on the membrane might be degraded more readily. Trypsin treatment of the endoplasmic reticulum preferentially releases the cytochrome bs, presumably indicating that cytochrome bs is more accessible. However cytochrome bs is actually turning over less rapidly (2).

Perhaps a more attractive explanation of the heterogeneity of membrane degradation involves the concept that the degradation proceeds through an initial dissociation of the protein from the membrane. Hence degradation of membrane proteins would proceed by the same protein degradative mechanism or mechanisms as the so-called soluble proteins. As a corollary of this proposal one might suggest that membrane proteins are actually in equilibrium between associated and dissociated forms. The heterogeneity of degradation, then, would be determined both by the equilibrium between associated and dissociated forms of the membrane protein, and by the normal controls of degradation of soluble proteins. It is of note that for both soluble and membrane proteins, degradation rates are related to molecular size. Further, we have found the correlation between size and degradation rates to occur with ribosomal proteins. In this connection, it does appear that ribosomal proteins have a cytoplasmic pool (37). The notion that membrane proteins can have independent existence in the cytoplasm is not new. Studies on the genesis of chloroplasts (38) and mitochondria (39, 40) indicate that at least some membrane proteins are synthesized at sites in the cytoplasm and are subsequently incorporated into the developing membrane. Whether dissociated proteins are in true equilibrium with those of the membrane is an intriguing problem, since
if so, it would suggest that self-assembly of membranes could occur without specific enzymatic processes or the juxtaposition of specific ribosomes with a growing membrane.

The correlation between molecular weight and general tendency for more rapid degradation of proteins of membrane fractions and soluble proteins is a new finding concerning the properties of protein degradation in rat liver. A number of studies have shown exponential decay or uptake of isotope into specific proteins (41–45), including the membrane-associated cytochrome $b_6$ and cytochrome $c$ reductase (2, 28), thereby implying that the degradation of any given molecule from among like molecules is a random event. This finding, plus the fact that specific proteins have characteristic half-lives, has led to the concept that it is the protein as a substrate which determines its rate of degradation (30). One simple interpretation of the correlation between size and degradation rate is that the larger molecule has a greater chance of a protease making an initial (rate-limiting) "hit," i.e. peptide bond cleavage.

An alternative possibility comes from the proposal of Segal et al. (46) that lysosomes act essentially as sieves, in which proteins may exit from lysosomes as well as enter and be degraded. If small molecules were to exit more readily from lysosomes, the general correlation between size and degradation rate could also be explained. It should be emphasized, however, that molecular size is by no means the sole determinant of degradation rate, since three soluble enzymes of rat liver, arginase (molecular weight 120,000, three subunits) (48), and lactate dehydrogenase-isozyme 5 (molecular weight 136,000, four subunits) (47), tyrosine aminotransferase (molecular weight 120,000, three subunits) (48), and lactate dehydrogenase-isozyme 5 (molecular weight 136,000, four subunits) (49) have half-lives of 4 days (40), 14 hours (41), and 16 days (50), respectively. Perhaps these differences in rates of turnover relate to the rate of dissociation of these different proteins into subunits. Thus molecular size is only one of several parameters which determine the susceptibility of a protein to degradation, in addition to the nature of peptide bonds exposed, the conformation of the protein as affected by interactions with small molecules and macromolecular ligands, etc. Knowledge of the role each of these parameters plays, and of the actual mechanism of protein degradation, awaits localization and isolation of intracellular degradative enzymes.

REFERENCES

42. KENNY, F. T., Science, 166, 523 (1967).
Size Distribution of Membrane Proteins of Rat Liver and Their Relative Rates of Degradation
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