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THE SYNTHESIS AND DEGRADATION OF MEMBRANE PROTEINS

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Many studies in recent years have led to the concept that cellular membranes exist as a dynamic mosaic of proteins, lipids, and carbohydrates. Recent contributions from our laboratory have centered on the synthesis and degradation of membrane proteins, particularly those of the rat liver endoplasmic reticulum. This work has led to a general model of the membrane in which the protein constituents continually associate and dissociate from the membranes, and only in the dissociated state are they subject to degradation by normal intracellular degradative processes. In addition, the rates of synthesis and assembly of membrane proteins can be selectively modified by pharmacological agents.

Degradation of Membrane Proteins

Investigations of membrane protein degradation have provided several insights into their dynamic state as membrane components. Early studies by Omura (1), Schimke (2), and others show that membrane proteins are degraded in a random manner with time. There is also a marked heterogeneity in the degradation rates of these proteins (3) as illustrated in table 1. Furthermore, nearly all proteins examined, whether or not they are membrane-associated, show characteristic exponential decay curves from which half-lives can be determined (4).

In order to study the turnover of membrane proteins in a more general manner, a double-isotope labeling technique was developed (2) which permits a measure of the relative rate constants of degradation. This technique is illustrated schematically in fig. 1, which shows the theoretical labeling and decay patterns of two hypothetical proteins which are present in the animal tissue at the same steady-state concentrations, but one of which (*A*) is synthesized and degraded at twice the rate of the other (*B*). In this method, one isotopic form of an amino acid (^{14}C) is administered initially to the animal and allowed to decay a specified time, thereby defining a point on the exponential decay curve. Then, the second isotopic form (^{3}H) of the amino acid is administered to the same animal to establish an initial time point (which is at or near the maximum incorporation of the pulse label) on the decay curve, and the animal is sacrificed a short time thereafter. Thus, a protein which is turning over more rapidly will lose a greater relative amount of its incorporated ^{14}C-amino acid, and it will incorporate more of the ^{3}H-amino acid. Therefore, proteins which are turning over faster will have greater $^{3}H/^{14}C$ ratios, irrespective of what the absolute specific radioactivity of the protein is (see reference 2 for complete discussion, including assumptions and experimental verification).

Such an experimental approach has been used to determine the relative rates of degradation of proteins in the rat liver plasma membrane and endoplasmic reticulum (5) as examined by SDS[3] gel electrophoresis. In this method, proteins are broken into subunits by the detergent and separated according to the molecular size on gels, in such a way that small proteins and subunits migrate further into the gel than large ones (6). A study of the relative degradation rates of plasma membrane proteins is shown in fig. 2. As indicated by the variation in the $^{3}H/^{14}C$ ratios,

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[3] The abbreviations used are: SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

TABLE I

Half-lives of specific enzymes of rat liver endoplasmic reticulum

Data taken from reference 3.

Enzyme	Half-Life
Hydroxymethylglutaryl-coenzyme A reductase	2-3 hr
Cytochrome *c* reductase	60-80 hr
Cytochrome b_5	100-120 hr
NAD glucohydrolase	16 days

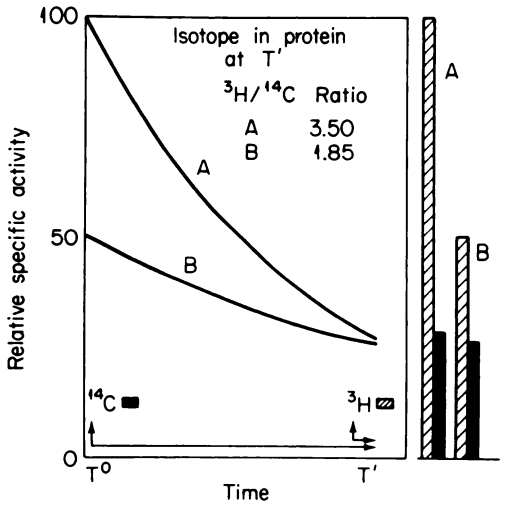

FIG. 1. *Theoretical labeling of two proteins with a 2-fold difference in degradation rates by a double-isotope technique.*

Proteins *A* and *B* are present at the same steady-state concentration, with *A* turning over at twice the rate of *B*. [Reprinted with permission of The American Society of Biological Chemists (2).]

there is a marked heterogeneity in the rates of degradation of the proteins. Furthermore, there is a correlation between the size of the protein or subunit and its relative rate of degradation. The larger proteins and subunits have higher $^3H/^{14}C$ ratios than the smaller ones, indicating a more rapid turnover rate for higher molecular weight proteins. In a control experiment, where both isotopic labels were administered at the same time, no systematic variation in $^3H/^{14}C$ ratios was observed.

A similar finding was also made for the endoplasmic reticulum. In these experiments, sufficient time was allowed for labeled plasma proteins to be secreted from the liver before membrane fractions were collected. Fig. 3 compares the $^3H/^{14}C$ ratios observed for those membrane proteins which are insoluble in 1% Triton X-100 (*top*) with a control in which both isotopic labels were administered simultaneously (*bottom*). An exception to the molecular size-turnover correlation is observed for the membrane proteins migrating in the 50,000 molecular weight range. These proteins, which appear to be major constituents of the endoplasmic reticulum, are turning over at a relatively rapid rate. Essentially, the same results are also observed for those membrane proteins which are soluble in 1% Triton X-100.

The correlation between the molecular size of a protein and its rate of degradation *in vivo* has also been found for the soluble cytoplasmic pro-

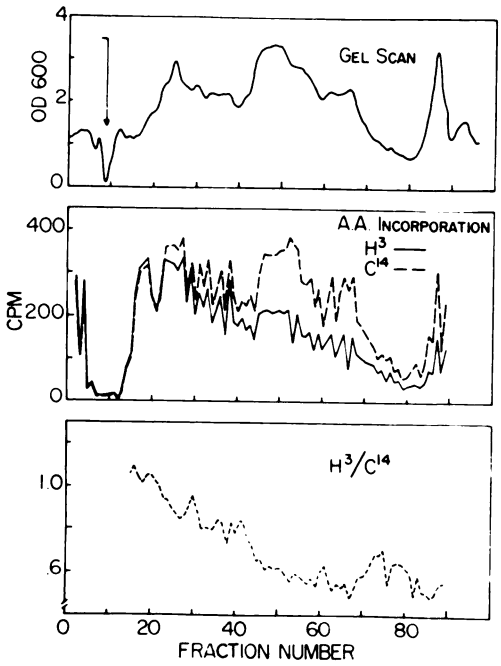

FIG. 2. *Electrophoretic patterns of double-labeled plasma membrane of rat liver.*

A male rat weighing 120 g was given 250 μCi of ^{3}H-L-leucine 4 days after administration of 100 μCi of ^{14}C-L-leucine. The rat was sacrificed 4 hr later and the plasma membrane fraction was isolated (5). The protein constituents were separated by SDS gel electrophoresis and the radioactivity of each gel fraction was measured (5). The *upper box* shows the optical scan of an analytical gel to which 225 μg of protein were applied, and subsequently stained with Acid-Fast Green. The *middle box* indicates the actual radioactivity measured when 5 mg of protein were applied to a 19 × 75 mm gel. The *lower box* indicates the calculated $^3H/^{14}C$ ratios obtained. [Reprinted with permission of The American Society of Biological Chemists (5).]

teins of the rat liver (7). Following a double-labeling procedure as described above, these soluble proteins were fractionated on a Sephadex G-200 column calibrated with molecular weight markers. Fig. 4 presents the $^{3}H/^{14}C$ ratios of the eluted fractions, clearly showing the molecular size-turnover correlation. When this experiment was repeated with soluble proteins that had been treated with SDS and fractionated in the presence of the detergent, the same correlation was again obtained. The range of $^{3}H/^{14}C$ ratios was virtually identical in both experiments, which indicates that multimeric proteins, although they tend to be larger, show the correlation expected for their subunits. These latter results would suggest that multimeric proteins first dissociate before undergoing degradation, and that it is the subunit size that influences this degradation phenomenon. In addition, Dice and Schimke have found the same correlation for proteins of rat liver ribosomes (8) as well as for cytoplasmic proteins from several other

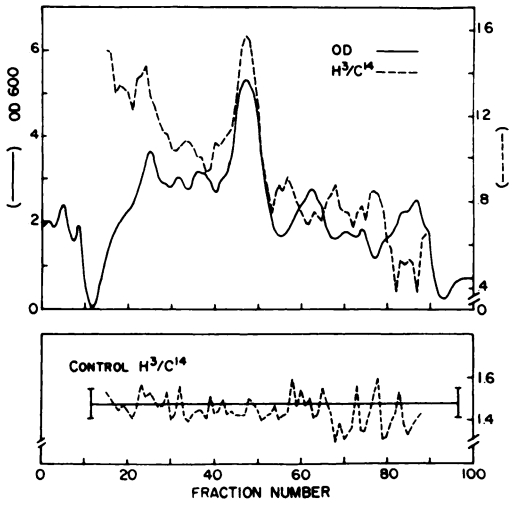

FIG. 3. *Relative turnover of Triton X-100-insoluble proteins of rat liver smooth endoplasmic reticulum.*

The labeling schedules were similar to those described in fig. 2. The smooth endoplasmic reticulum was isolated and the protein fraction insoluble in 1% Triton X-100 was examined (5), as described in fig. 2. The *upper box* shows the calculated $^{3}H/^{14}C$ ratios compared with the optical scan of an Acid-Fast Green-stained gel. The *lower box* shows the result of a control experiment in which a comparable animal was administered 50 μCi of ^{14}C-L-leucine and 250 μCi of ^{3}H-L-leucine simultaneously and sacrificed 4 hr later. [Reprinted with permission of The American Society of Biological Chemists (5).]

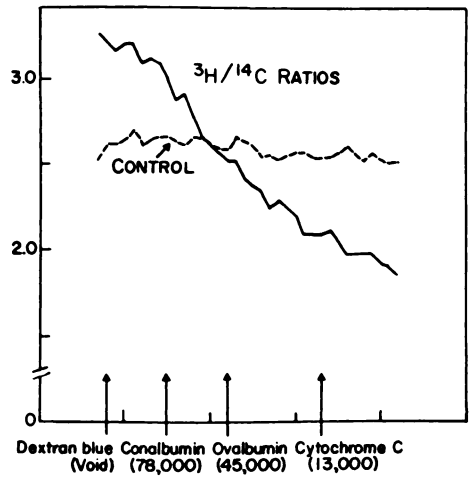

FIG. 4. *Fractionation of rat liver supernatant proteins on Sephadex G-200 in the presence of 0.1% sodium dodecyl sulfate.*

Male white Sprague-Dawley rats weighing 100–120 g each were given 100 μCi of ^{14}C-leucine in 2 ml of 0.85% NaCl ip. Four days later the animal was given 250 μCi of ^{3}H-leucine and sacrificed by decapitation 4 hr later. In a control experiment, an animal was given 50 μCi of ^{14}C-leucine and 250 μCi of ^{3}H-leucine simultaneously and sacrificed 4 hr later. The livers were perfused with ice-cold 0.25 M sucrose by the retrosplenic route and homogenized in 1 volume (w/v) in 0.05 M Tris-glycinate buffer, pH 8.9. The homogenate was centrifuged at 105,000g_{max} for 1 hr and the supernatant fraction (4 ml) was passed through a Sephadex G-25 column, 1.5 × 20 cm, equilibrated with the above buffer, to remove free amino acids. The column eluate to which SDS was added to a final concentration of 0.1% was sonicated for 30 sec and a sample containing about 30 mg of protein was then applied to a Sephadex G-200 column. The column was run at 4°C, with a flow rate of 10 ml/hr; 4–5 ml fractions were collected. Protein was precipitated from the eluate fractions by the addition of 1 ml of 50% TCA and trapped on glass filters. The filters were dissolved in 0.5 ml of NCS solubilizer (Amersham-Searle) and counted in a toluene-based scintillation fluid. The column was calibrated in a separate run with marker proteins and Blue Dextran (Pharmacia). ——, degradation experiment; ---, control experiment. [Reprinted with permission of Academic Press (7).]

tissues,[4] which suggests that this finding is a general phenomenon.

Recent experiments in our laboratory have also shown a relationship between a protein's rate of degradation *in vivo* and its susceptibility to proteolytic attack *in vitro*. This result is illu-

[4] J. F. Dice and R. T. Schimke, manuscript in preparation.

strated in fig. 5, in which cytoplasmic proteins were double-labeled with isotopes *in vivo*, as described above (so that rapidly-turning-over proteins have high $^3H/^{14}C$ ratios), and incubated with either pronase or trypsin. Such proteolytic digestion preferentially released trichloroacetic acid-soluble material having high $^3H/^{14}C$ ratios. As digestion proceeded toward greater solubilization, the ratios decreased progessively toward lower values. Thus, those proteins with high turnover rates *in vivo* are also degraded more rapidly *in vitro*. That the specificity for proteolysis is influenced greatly by the tertiary

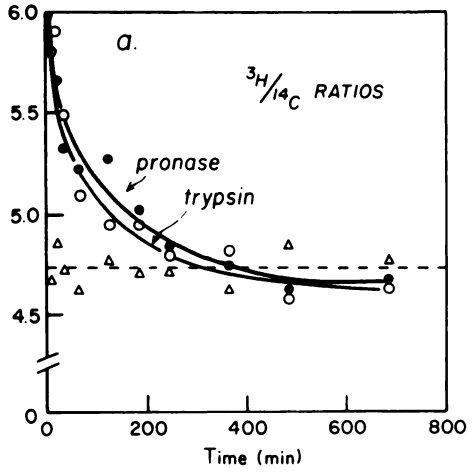

FIG. 5. *Degradation of rat liver soluble proteins in vitro.*

Double-labeled soluble proteins were prepared from rat liver as described above (fig. 4), except that the isotope doses were 100 μCi of ^{14}C-leucine followed by 500 μCi of ^{3}H-leucine. The soluble proteins were passed through a Sephadex G-25 column, 2 cm × 25 cm, and equilibrated with 0.15 M NaCl in 50 mM sodium phosphate buffer, pH 7.6, in order to remove free amino acids. The final soluble protein concentration was about 15 mg/ml, as determined by the method of Lowry *et al.* (9). Pronase or trypsin was added as a 1 mg/ml solution in distilled water to a final concentration of 100 μg/ml. The digestion was allowed to proceed at 20°C and 100-μl aliquots were removed at various times and mixed with 100 μl of 20% TCA in 400-μl plastic microfuge tubes (Beckman). The tubes were centrifuged for 2 min in a Beckman model 152 microfuge (15,000g_{max}). The upper 100 μl of each soluble fraction were dissolved in 0.5 ml of NCS solubilizer and counted in a toluene-based scintillation fluid. $^3H/^{14}C$ ratios of acid-soluble fractions were determined when double-labeled soluble proteins were incubated with either pronase (●——●) or trypsin (O——O). The *dotted line* indicates the results of a control experiment using soluble proteins which had received both ^{3}H- and ^{14}C-leucine 4 hr prior to sacrifice.

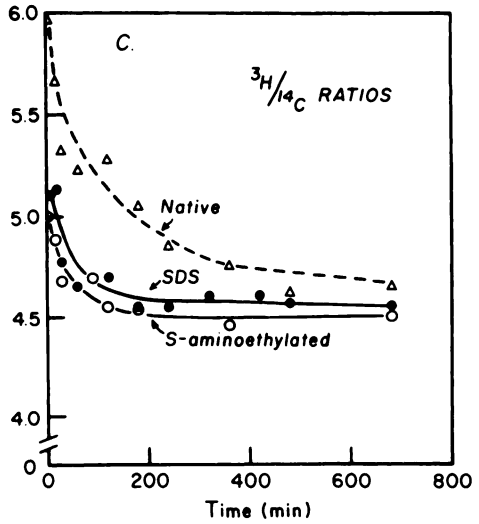

FIG. 6. *Comparison of in vitro degradation of native and of conformationally altered soluble proteins.*

The procedure is similar to that described above (fig. 5), except that the soluble proteins in some experiments were conformationally disrupted before the addition of protease. Protein conformation was altered by either the addition of SDS to a final concentration of 1% or by conversion of cysteine residues to S-aminoethylcysteine with the addition of ethyleneimine according to the procedure described by Cole (10). Pronase was added directly to soluble proteins in the presence of SDS to a final concentration of 100 μg/ml. Before pronase (100 μg/ml) was added to the S-aminoethylated proteins, small molecules were removed by dialysis against 2 M urea in 0.15 M NaCl and 50 mM sodium phosphate buffer, pH 7.6. The urea was necessary to prevent the soluble proteins from precipitating. The $^3H/^{14}C$ ratios of acid-soluble fractions were determined for double-labeled soluble proteins in their native conformations (Δ- - -Δ) or in altered conformations due to SDS (●——●) or S-aminoethylation (O——O).

structure of the protein is indicated in fig. 6. This experiment was carried out as above, except that the conformations of proteins were altered by SDS or S-aminoethylation before the addition of the proteolytic enzymes. Over most of the time course of the digestion, the radioactivity released with time showed no variation in $^3H/^{14}C$ ratios. Nevertheless, at short digestion times, soluble material of higher $^3H/^{14}C$ ratios was preferentially released, indicating that molecular size was still an important factor in proteolytic degradation reactions.

In contrast to soluble cytoplasmic proteins, however, the digestion of double-labeled intact endoplasmic reticulum by pronase did not show a correlation between turnover rates *in vivo* and

susceptibility to proteolytic degradation *in vitro*. Using experimental conditions similar to those employed for soluble proteins, a preliminary study indicates that membrane proteins appear to be nonspecifically degraded by proteases, as shown in fig. 7. Over most of the time course, the $^{3}H/^{14}C$ ratios of the trichloroacetic acid-soluble material released (*dotted line*) show no change. Furthermore, there is no significant alteration in the elctrophoretic pattern of the SDS-solubilized membrane proteins following the proteolytic digestion reaction. Thus, it would appear that membrane proteins may not be degraded as part of the membrane matrix.

This series of observations, then, leads to several general conclusions concerning the degradation of membrane proteins, which are shared in common with cytoplasmic proteins. Membrane proteins undergo random degradation, exhibit marked heterogeneity in their turnover rates, and show a molecular size correlation in their turnover rates. The lack of correlation between their rates of turnover *in vivo* and suceptibility to proteolytic attack *in vitro*, as contrasted with the existence of this correlation with cytoplasmic proteins, suggests that membrane proteins first dissociate from the membrane matrix before undergoing degradation by normal intracellular processes. Thus, membrane proteins, once dissociated from the membrane, appear to be subject to the same mechanism of degradation as are cytoplasmic proteins.

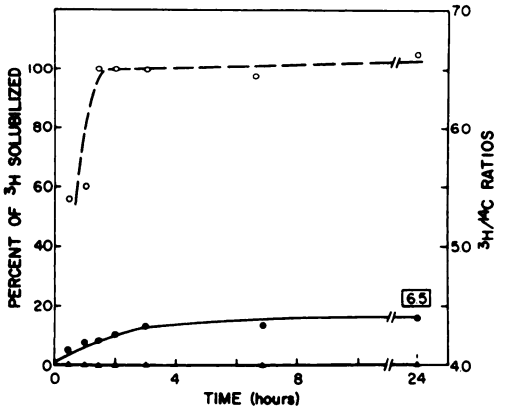

FIG. 7. *Susceptibility to pronase proteolysis of the endogenous proteins of the rat liver smooth endoplasmic reticulum.*

Experimental details of isotope administration, protease digestion, and radioactivity measurement are similar to those described for fig. 5. The smooth endoplasmic reticulum was isolated as described by Dehlinger and Schimke (5) and was used without further treatment in the proteolytic digestion reaction. The $^{3}H/^{14}C$ ratio of the membrane proteins was determined to be 6.5. O---O, $^{3}H/^{14}C$ ratios of released radioactivity; ●——●, ^{3}H (counts per minute) released; ▲——▲, no added pronase showing essentially no ^{3}H (counts per minute) released. [Results of Schimke and Dehlinger (3).]

Synthesis of Membrane Proteins

Much of our current understanding of the synthesis and assembly of membrane proteins comes from studies of drug-induced microsomal oxidase activities and associated proliferation of the rat liver endoplasmic reticulum (11). Following this approach, Dehlinger and Schimke (12) have recently analyzed the problem of membrane protein synthesis in a general way by employing some of the methodology utilized in studying degradation. In one series of experiments, different rats were treated for 16 hr with phenobarbital or 3-methylcholanthrene. Fours hours before sacrifice, the drug-administered animals received ^{3}H-leucine and control animals received ^{14}C-leucine. Liver endoplasmic reticulum was isolated from both animals and equal amounts were combined and examined by SDS gel electrophoresis. The $^{3}H/^{14}C$ ratios of the separated membrane protein fractions were compared with those of a saline-injected control animal as shown in fig. 8. If the drugs increased the rate of synthesis of all membrane proteins to the same extent, then the $^{3}H/^{14}C$ ratios should be the same in all proteins, similar to the appearance of the experimental control shown in fig. 8 (*top*). It is evident, however, that phenobarbital (*middle*) induces the preferential synthesis of certain membrane proteins, especially those in the 50,000 molecular weight region. Furthermore, 3-methylcholanthrene (*bottom*) induces a different pattern of membrane protein synthesis. Such experiments, then, indicate that the rates of synthesis of various membrane proteins are not constant, but can be selectively altered by the administration of pharmacological agents. These results confirm and extend the work of several other investigators who studied the induction of specific enzymes and changes in membrane morphology [reviewed by A. H. Conney (11) and R. T. Schimke (3)].

There is ample evidence that marked variations can occur normally in the rates of synthesis of various membrane proteins. For example, circadian rhythms can influence the

turnover rates of some membrane proteins (11), as in the case of hydroxymethylglutaryl-coenzyme A reductase, where the rate of synthesis varies markedly in a 24-hr period (14).

The cytoplasmic site of membrane protein synthesis has also been the focus of considerable interest. Early developmental studies indicated that the smooth endoplasmic reticulum arises from the rough endoplasmic reticulum (15, 16), possibly by ribosomal translocation, implying that membrane proteins are synthesized on membrane-bound polysomes. That this may be the case for NADPH-cytochrome *c* reductase and cytochrome b_5, but not all membrane proteins, is indicated by the work of Omura and Kuriyama (17).

Looking at this problem in a general manner, we have examined the incorporation of pulse-labeled membrane proteins into both the smooth and rough endoplasmic reticulum of the rat liver. Adult rats were sacrificed at short time periods following ^{3}H-leucine injection, and the membrane fractions isolated according to a double-gradient separation procedure (fig. 9). These fractions were extensively washed with buffered salt solutions to remove adsorbed soluble proteins, ribosomal proteins, as well as plasma proteins destined for secretion. The results of this experiment are shown in fig. 9. Isotopically labeled proteins are rapidly incorporated into the membrane fractions and reach a maximum within 20 min. There is a significant difference in the relative amount of label incorporated into each fraction, which persists for several hours even though the amount of label gradually decreases (possibly because of normal turnover). The rapid appearance of label in the different fractions suggests that membrane proteins have a relatively short processing time prior to insertion, and that both membrane-bound and free polysomes may be involved in membrane protein biogenesis, but not necessarily for the same protein. The fact that a difference in the specific radioactivity of the membrane fractions is maintained suggests that lateral diffusion of endoplasmic reticulum membrane proteins may not be as rapid as that observed for the plasma membrane (20). Furthermore, examination of the membrane proteins from the different fractions on SDS gels did not reveal any major differences in the label incorporation patterns between fractions; double-labeling studies (^{14}C-leucine for 3 hr with ^{3}H-leucine for 20 min) showed no major differences in label incorporation patterns with time.[5]

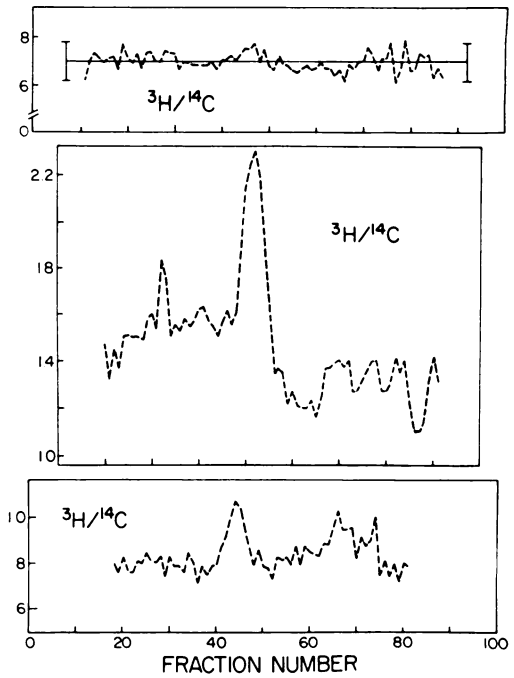

FIG. 8. *Relative rates of amino acid incorporation into smooth endoplasmic reticulum proteins of the rat liver in response to phenobarbital and 3-methylcholanthrene administration.*

The experimental animals were injected ip with either phenobarbital dissolved in water (100 mg/kg) or 3-methylcholanthrene dissolved in corn oil (25 mg/kg), with control animals receiving corresponding volumes of either water or corn oil. Twelve hours later, the experimental animals received 250 μCi of ^{3}H-leucine and the control animals received 75 μCi of ^{14}C-leucine. Four hours after radioisotope injection, the animals were sacrificed and the smooth endoplasmic reticulum fraction of the liver was isolated from each animal. The membrane fraction from a drug-injected animal was combined with the membrane fraction from the corresponding control animal and electrophoretically treated as described for fig. 2. The results for membrane proteins insoluble in 1% Triton X-100 are shown here. The *upper box* shows the control ^{3}H/^{14}C ratios for animals which received no drugs. The ^{3}H/^{14}C ratios in the *middle box* show the results of phenobarbital injection, and in the *lower box* shows the effects of 3-methylcholanthrene. [Reprinted with permission of The American Society of Biological Chemists (12).]

In conclusion, a general model for membrane turnover, which incorporates the findings of this laboratory, is presented in fig. 10. We propose

[5] J. M. Taylor and R. T. Schimke, unpublished observations.

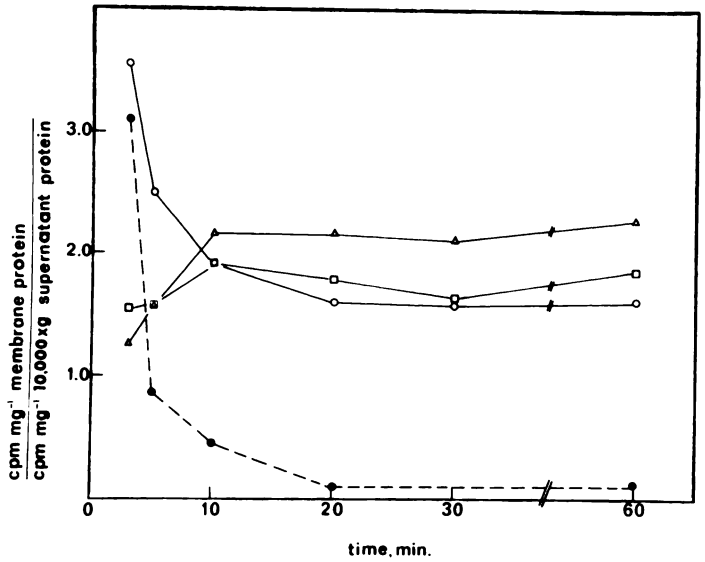

FIG. 9. *Relative rates of amino acid incorporation into smooth and rough endoplasmic reticulum proteins of the rat liver.*

Adult fasted (24 hr) rats, weighing 150 g, were injected ip with 1.5 mCi/kg of ^{3}H-L-leucine and then sacrificed at various time points indicated above. The SER and RER fractions were isolated from the 105,000g_{av} supernatant fraction according to Dallner (18), resuspended in 0.25 M sucrose, and further purified on two-step gradients in Beckman SW27 tubes by centrifuging for 18 hr at 96,000g_{av} and 4°C: the SER suspension was overlaid on 8 ml each of 1.11 M and 1.31 M sucrose in 10 mM HEPES, pH 7.5; the RER suspension was overlaid on 8 ml each of 1.31 M and 1.60 M sucrose in 10 mM HEPES, pH 7.5. Three membrane fractions were collected: SER I at the 1.11 M sucrose interface; SER II at the 1.31 M sucrose interface (below the 1.11 M sucrose only); and RER at the 1.60 M sucrose interface. They were characterized according to RNA/protein ratios and marker enzymes, with SER II appearing to be an intermediate fraction between SER I and RER. Typical RNA/protein ratios are 0.03, 0.07, and 0.35 for SER I, SER II, and RER, respectively. The membranes were then washed with 1.0 M NaCl and 10 mM EDTA, pH 7.5; 0.1 M Na_2CO_3, pH 9.3; and 0.1 M Na_2HPO_4-NaH_2PO_4, pH 7.5 (19). In the last wash solution, the membranes were sonicated for 45 sec at the low-power setting of a Branson sonifier. Between each wash, the membranes were collected by centrifugation at 105,000g_{av} for 1 hr at 4°C, and finally resuspended in 0.1 M sodium phosphate, pH 7.5. Membrane proteins and 10,000g_{max} supernatant proteins from the liver homogenate were then precipitated with 12% TCA, collected on glass fiber filters, washed with 5% TCA, and dried. The precipitated material was dissolved in 0.5 ml of NCS solubilizer and radioactivity was measured with a toluene-based scintillation fluid. Δ——Δ, SER I; □——□, SER II; ○——○, RER; ●- - -●, TCA-soluble material from the 10,000g_{av} supernatant fraction (containing free ^{3}H-L-leucine).

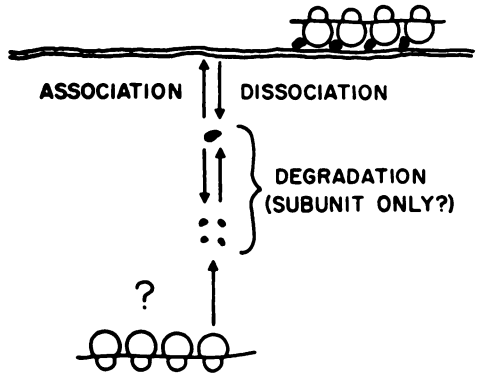

FIG. 10. *Proposed schematic model of membrane genesis and degradation.*

Some constituent membrane proteins are synthesized on membrane-bound polysomes and inserted directly into the membrane matrix, while others may be made on free polysomes and subsequently associate with the matrix by virtue of their physico-chemical properties. A finite free cytoplasmic pool exists for membrane proteins, with degradation occurring randomly in the dissociated state. The degradation rate of an individual membrane protein is determined by its properties as a substrate for the same protease system(s) acting on most or all intracellular proteins.

that a membrane is in a dynamic flux of association and dissociation, and that the protein components are degraded only in the dissociated state. This concept is in keeping with the known rapid exchange of membrane phospholipids (21), association-dissociation phenomenon of complex

organelles (ribosomes) (8), and the degradation data of multimeric protein complexes.[6] Such a model implies a finite cytoplasmic pool of membrane proteins. It also raises the question of whether membrane-associated proteins are synthesized on ribosomes intimately bound to the membrane or synthesized on so-called free polysomes, with subsequent insertion of protein into the membrane dependent on the properties of the protein following release from the ribosome, or perhaps as it is undergoing folding during peptide chain elongation.

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Discussion

Dr. Aust, *Michigan State U.:* We have studied the dissociation of the enzymes from the endoplasmic reticulum by using a simple model that you would apply to any monomer to an oligomer, and interestingly enough, by using the enzyme NADPH-cytochrome *c* reductase, because you can assay low levels, you do get a dissociation that is concentration-dependent. Our next experiment was to show that the dissociation was dependent upon molecular weight to fit your model. We did this by washing the same microsomes successively, taking the supernatant and concentrating it on a Diaflo membrane, subjecting it to SDS polyacrylamide gel, and we found, much to our surprise, that it looked just like microsomes. In other words, the low molecular weight species didn't come off after a high molecular weight species. Maybe we have to look for a different degradation phenomena or control of the degradation from some other place. While we are talking about SDS polyacrylamide gel experiments, I noticed two peaks in the center frame of one of your slides. We think we have identified the peak on the left side as NADPH-cytochrome *c* reductase. You will notice in your 3-MC microsomes that the peak did not show up since the enzyme is not induced. The center peak, the large one in the 40-50,000 molecular weight range, can be resolved into about five proteins, all of which contribute to about 10% or better of the total microsomal proteins. Only one of these is induced by 3-methylcholanthrene. At least three of them are induced by phenobarbital. I was wondering if you have resolved any of these, and if you have looked at incorporation ratios between tritium and ^{14}C in them?

Dr. Taylor: No, we haven't resolved that peak. You raise a good point in that the slides may have been a little misleading. What is being represented is the average degradation rates of the sum total of proteins in each fraction. In other words, each fraction might be composed of several different proteins. As you pointed out, there is more than one protein represented in the 40-50,000 molecular weight range; but we have not resolved them.