

THE SECRETORY PATHWAYS OF RAT SERUM GLYCOPROTEINS AND ALBUMIN

Localization of Newly Formed Proteins within the Endoplasmic Reticulum

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ABSTRACT

These studies compare the secretory pathways of newly formed rat serum glycoproteins and albumin by studying their submicrosomal localization at early times after the beginning of their synthesis and also by determining the submicrosomal site of incorporation of *N*-acetylglucosamine, mannose, galactose, and leucine into protein. *N*-acetylglucosamine, mannose, and galactose were only incorporated *in vitro* into proteins from membrane-attached polysomes and not into proteins from free polysomes. Mannose incorporation occurred in the rough endoplasmic reticulum, was stimulated by puromycin but not by cycloheximide, and 90% of the mannose-labeled protein was bound to the membranes. Galactose incorporation, by contrast, occurred in the smooth microsome fraction and 89% of the radioactive protein was in the cisternae. Albumin was mostly recovered (98%) in the cisternae, with negligible amounts in the membranes. To determine whether the radioactive sugars were being incorporated into serum proteins or into membrane protein, the solubilized *in vivo*-labeled proteins were treated with specific antisera to rat serum proteins or to albumin. Immunoelectrophoresis of the ¹⁴C-labeled leucine membrane and cisternal proteins showed that the membranes contained radioactive serum glycoprotein but no albumin, while the cisternal fraction contained all of the radioactive albumin and some glycoproteins. The results indicate that newly formed serum glycoproteins remain attached to the membranes of the rough endoplasmic reticulum after they are released from the membrane-attached polysomes, while albumin passes directly into the cisternae.

INTRODUCTION

The liver synthesizes most of the serum proteins on membrane-attached polysomes, and these proteins pass into the endoplasmic reticulum (ER) (1-8). Serum proteins are to a large extent glycoproteins and they are thought to be synthesized by the sequential addition of carbo-

hydrate moieties to a protein core at different cellular locations. It has been suggested that *N*-acetylglucosamine is incorporated while the nascent protein is on the polysomes but that mannose and galactose are put on after the newly formed protein has left the polysomes and is on

the intracellular path which leads to secretion (9-16). Since *N*-acetylglucosamine and mannose are thought to be added to the glycoprotein at an early stage of glycoprotein biosynthesis, either at the polysomal or at the rough endoplasmic reticulum stage, we have studied the problem of whether these sugars which are closest to the protein core are added to the endogenous nascent protein during the time at which the protein is being vectorially passed to the cisternae of the ER, or whether the process occurs at a subsequent time and at a different location on the rough ER. These studies also compare the submicrosomal location of the newly formed glycoproteins with that of albumin which is not a glycoprotein, to determine whether the endoplasmic reticulum can differentiate between these two classes of serum protein.

The results indicate that both *N*-acetylglucosamine and mannose are incorporated into the rough ER and that mannose incorporation occurs immediately after the nascent protein is released from the polysomes, but that the released glycoprotein remains attached to the membrane of the ER. This is in contrast to albumin which passes directly into the lumen of the ER. When the newly formed glycoprotein passes to the smooth ER and the Golgi apparatus, other moieties of *N*-acetylglucosamine and galactose are incorporated into the glycoprotein and it leaves the membrane and is found, together with albumin, within the cisternae of the smooth microsomes.

EXPERIMENTAL PROCEDURES

Materials

The radioactive nucleotide sugars, uridine diphosphate (UDP)-*N*-acetylglucosamine-1-¹⁴C (40 mCi/mmole) GDP-mannose-¹⁴C (150 mCi/mmole) and UDP-galactose-¹⁴C (200 mCi/mmole) were obtained from New England Nuclear Corp., Boston, Mass. L-leucine-1-¹⁴C (25 mCi/mmole) or L-leucine-4-5-³H (36 Ci/mmole) were obtained from the same source. The GDP-mannose-¹⁴C was stored at -20°C in 70% ethanol to prevent degradation. Sodium deoxycholate (enzyme grade) was obtained from Mann Research Labs., Inc., New York and Soluene TM100 from Packard Instrument Co., Inc., Downers Grove, Ill. All other chemicals were analytical grade commercial products.

Methods

Microsomes and pH 5 enzyme were prepared as previously described (7). A high-speed super-

natant fraction was sometimes substituted for the pH 5 enzyme. This was made by centrifuging the microsomal supernatant at 105,000 *g* for 4 hr. Rough and smooth microsomes were obtained using the method of Dallner et al. (17). Microsomes were subfractionated into ribosomes, a membrane fraction, and a microsomal content fraction following the method of Ernster et al. (18) as previously modified (8). It is important in this procedure that the microsomes be washed thoroughly in 0.25 M sucrose after incubation and that microsomes from at least 5 g of liver be used for each 10 ml of suspension before the addition of the 0.26% sodium deoxycholate.

In Vivo Experiments

All injections were given under light Nembutal anesthesia, and the solutions were injected into the femoral vein.

In Vitro Incubation

For the *in vitro* incorporation of either leucine-¹⁴C or the ¹⁴C-sugars, the incubation mixture contained microsomes from 0.5 g liver and 0.1 ml of pH 5 enzyme or high-speed supernatant in 1 ml of incubation mixture. The mixture also contained 40 mM Tris pH 7.4, 1 mM adenosine triphosphate (ATP), 10 mM phosphoenolpyruvate, 100 μg/ml of pyruvate kinase, 0.25 mM guanosine triphosphate (GTP), 2.5 mM MgCl₂, 2.5 mM MnCl₂, 12.5 mM KCl, 1.7 mM cysteine, and 1 μCi/ml of the radioactive precursor. The amounts of radioactive precursor, MgCl₂, MnCl₂, and KCl were varied to determine the conditions which would optimally accommodate both protein synthesis and the incorporation of sugars into endogenous protein. The values given above were those found to give best results. The reactions were stopped by adding 5% trichloroacetic acid (TCA). The TCA-insoluble material was washed three times with cold 5% TCA, once with alcohol, twice with 2:1 (v/v) alcohol:ether, and once with ether. In the experiments with radioactive leucine the TCA precipitate was heated at 90°C for 20 min in 5% TCA before the alcohol extraction. The lipid-extracted material was dissolved in either 0.5 ml of formic acid, hydroxide of hyamine or soluene TM100, and the radioactivity was determined by adding 10 ml of Bray's Dioxane scintillation fluid and by counting in a Packard Tricarb liquid scintillation spectrometer (Packard Instrument Co., Inc.).

Chromatographic Analysis of

Radioactive Proteins

The TCA precipitates which had been washed with TCA and extracted with lipid solvents as

described above were suspended in 4 N HCl and hydrolyzed for 6 hr at 100°C. The radioactive sugars were recovered from the hydrolysate by paper chromatography after previous treatment with Dowex 50-H (19). The solvent systems used were butanol-pyridine-water (6:4:3 vol) and ethyl acetate-pyridine-*n*-butanol-butyric acid-water (10:10:5:1:5 vol). The sugars were detected by staining with AgNO₃ and the radioactive sugars by cutting the chromatographic paper into 1 cm strips and counting them in a Packard scintillation counter using Bray's Dioxane phosphor. In all cases the radioactivity recovered coincided with the locality on the chromatogram of the appropriate carrier sugar.

Immuno-electrophoresis and Radioautography

Electrophoresis was performed on cellulose acetate membranes using a barbital buffer, pH 8.6, of 0.075 ionic strength purchased from Beckman (Beckman Instruments Inc., Fullerton, Calif.). Rabbit antiserum to rat serum proteins was purchased from Kallestad and Hyland laboratories or was prepared in our laboratory. Rabbit antiserum to rat albumin was a gift from Dr. Edward Kaighn of the New York Blood Center.

Radioautography was done on Kodak X-ray no-screen film. Staining of proteins and precipitin bands was done with 0.2% Ponceau S dye in 5% sulfosalicylic acid and TCA. The stain was purchased from Beckman Instruments Inc.

Precipitation of Membrane and Microsomal Content Proteins with Specific Antisera

The submicrosomal membrane fractions were treated with 0.5% sodium deoxycholate to solubilize them and were then centrifuged at 105,000 *g* for 1 hr to remove any insoluble material. These solubilized membrane fractions and the soluble microsomal content fraction were then treated with rabbit antiserum to chicken serum and with chicken serum to clear the solution of nonspecific precipitations. The cleared material was then used to precipitate the serum protein using rabbit antiserum to rat serum protein or rabbit antiserum to rat albumin. A second precipitate with antiserum to chicken serum was used as a control. All of the immunological reactions were performed in 0.15 M NaCl buffered with 0.01 M Tris at pH 7.4. They contained sufficient rabbit antiserum to either rat serum proteins, chicken serum proteins, or to rat albumin to precipitate all of the antigen in 0.025 ml of ten times diluted chicken serum or 0.1 ml of 50 times diluted rat sera. The volume of antiserum used depended on the titer of the particular batch of antiserum. Incubations were carried out for 1 hr at 37°C and then overnight at 4°C as previously

described (3). The "control" values for the serum protein radioactivity in the membranes and cis-ternal fractions (Table VI) were 4% and 8% of the glucosamine radioactivity, 30% and 10% of the mannose radioactivity, 30% and 25% of the galactose radioactivity, and 7% and 2% of the leucine radioactivity.

Other Methods

Protein was determined by the method of Lowry, using bovine albumin as standard (20), and RNA by the orcinol method (21). Sephadex chromatography was done on G150 columns, 60 × 1 cm, using 0.05 M phosphate buffer, pH 7.4, with 0.1% Triton × 100.

RESULTS

In Vitro Incorporation of Sugars into Endogenous Microsomal Protein

To study at what submicrosomal location the sugars are incorporated into the newly formed glycoproteins and at what stage of protein synthesis and secretion this process occurs, an *in vitro* system which is capable of both protein synthesis and attachment of sugars to endogenous protein is needed. Protein synthesis was followed by measuring the incorporation of radioactive leucine into protein, and sugar incorporation was measured by incubating microsomes with the appropriate radioactive nucleotide-sugars. The effects of puromycin and cycloheximide were noted since the use of these substances allows the study of the incorporation of sugars in the absence of protein synthesis. These two inhibitors were chosen because both of them inhibit protein synthesis at the ribosomal level, but one of them, puromycin, causes a release of the resulting puromycyl peptide from the ribosome while cycloheximide allows the incompleting peptide chain to remain attached to the polysome.

The time course of incorporation of *N*-acetylglucosamine into microsomal protein is shown in Fig. 1. *N*-acetylglucosamine was incorporated into endogenous protein for only 5 min. Puromycin or cycloheximide added at either 0, 2, 5, or 10 min of incubation had little effect on the incorporation of *N*-acetylglucosamine when measured at 20 min of incubation.

Mannose was incorporated for 10 min into microsomal protein. Puromycin enhanced the incorporation of mannose into endogenous pro-

UDP-N-Ac-Glucosamine

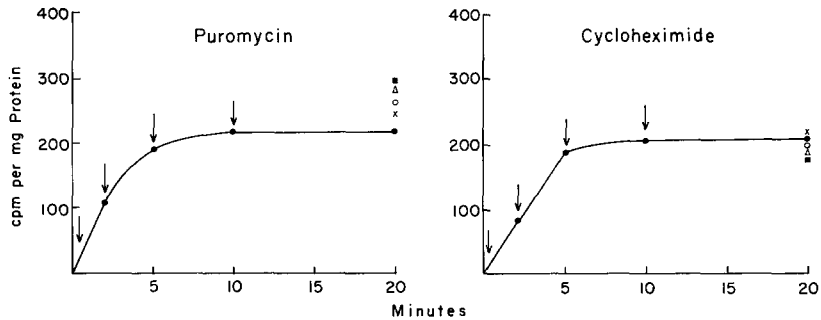


FIGURE 1 Time course of incorporation of *N*-acetylglucosamine into microsomal protein and effect of puromycin and cycloheximide. Microsomes were incubated with UDP-*N*-acetylglucosamine-¹⁴C, as detailed under Methods. Puromycin 5×10^{-4} M or cycloheximide (5×10^{-4} M) was added at various times of incubations as indicated in the above figures. All incubations were carried out at 37°C and were stopped at 20 min. The determination of incorporated radioactivity into protein is also described in Methods. Puromycin or cycloheximide was added at zero time (○), 2 min (■), 5 min (△), or 10 min (×) of incubation as indicated by the arrows.

GDP-Mannose

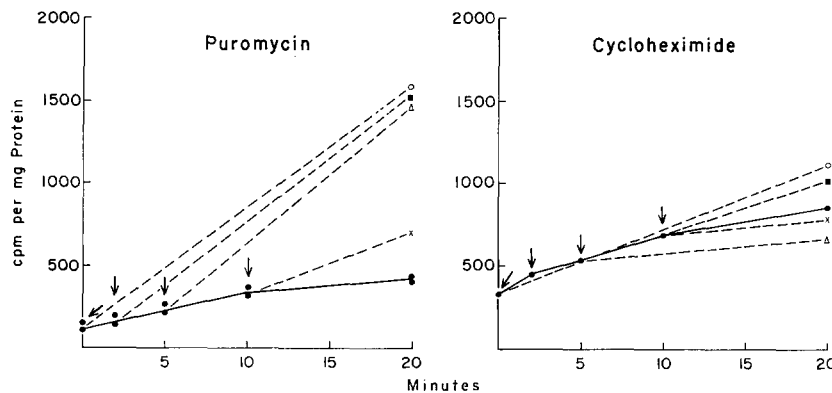


FIGURE 2 Effect of puromycin and cycloheximide on the in vitro incorporation of mannose from GDP-mannose into microsomal proteins. Microsomes were incubated as described in Fig. 1, except that GDP-mannose ¹⁴C was the radioactive precursor used and the incubation mixture also contained 0.15 μ moles per ml of D-glucosamine, D-galactose, *N*-acetylglucosamine, and D-galactosamine. Puromycin or cycloheximide was added at zero time (○), 2 min (■), 5 min (△), or 10 min (×) after incubation as indicated by the arrows.

tein. The earlier that puromycin was added to the incubation medium the greater was the enhancement as measured after 20 min of incubation. Cycloheximide had little or no effect on mannose incorporation (Fig. 2).

Galactose was also incorporated into microsomal protein in vitro for 10–15 min. Puromycin and cycloheximide had no effect on the attach-

ment of this sugar (Fig. 3). To show that these systems which incorporated sugars into protein were also capable of protein synthesis, the incorporation of leucine into protein was also followed. Under these conditions leucine incorporation occurred linearly for 12 min. Puromycin and cycloheximide when added at 9 min completely inhibited further incorporation.

UDP-Galactose

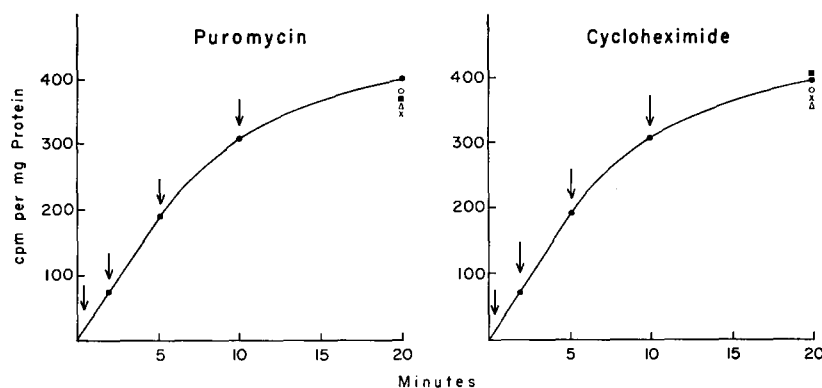


FIGURE 3 Effect of puromycin and cycloheximide on the in vitro incorporation of galactose into microsomal protein. The conditions of incubation have been given in Fig. 1 and under Methods. UDP-galactose- ^{14}C was the radioactive precursor used. Puromycin or cycloheximide was added at zero time (\circ), 2 min (\blacksquare), 5 min (\triangle), or 10 min (\times) after incubation as indicated by the arrows.

In Vitro Incorporation of Leucine and Sugars into Protein from Free and Membrane-Attached Polysomes and from Rough and Smooth Microsomes

To determine what microsomes are active in incorporating sugars into protein the liver microsomes were further fractionated into free and membrane-attached polysomes and into rough and smooth microsomes. These microsomal fractions were then incubated with either leucine or the appropriate nucleotide-sugars to determine their capability to incorporate these precursors into protein.

Free and membrane-attached polysomes were prepared as previously described (1), and each preparation was incubated with the in vitro system capable of supporting protein synthesis and the attachment of sugars to protein. Both free and membrane-attached polysomes were equally active in protein synthesis. The free polysomes were not capable, however, of incorporating *N*-acetylglucosamine, mannose, or galactose from the appropriate nucleotide-sugar into endogenous protein. The fraction which contains polysomes attached to the ER membranes incorporated these sugars into protein (Table I).

Rough and smooth microsomes were prepared as described by Dallner et al. (17). These microsomal fractions were also incubated with the

radioactive nucleotide-sugars. *N*-acetylglucosamine was incorporated into protein from both rough (59%) and smooth microsomes (41%). The majority (76%) of the mannose incorporation occurred in the microsomal fraction derived from the rough ER, and 82% of the galactose

TABLE I
The In Vitro Incorporation of Leucine, N-Acetylglucosamine, Mannose, and Galactose into Endogenous Protein of Free and Membrane-Attached Polysomes

Radioactive precursor	Incorporation into endogenous protein (cpm/mg RNA)		
	Free polysomes	Attached microsomes	Mixture
Leucine	659	747	581
UDP- <i>N</i> -acetylglucosamine	4	106	76
GDP-mannose	10	94	52
UDP-galactose	15	99	65

Free and attached polysomes (or an equal mixture as determined by RNA content) were incubated for 20 min at 37°C with the radioactive precursors indicated, as described in Methods. pH 5 enzyme instead of the 105,000 *g* supernatant was used in these experiments. The sample with GDP-mannose also contained 5×10^{-4} M puromycin.

TABLE II
*The In Vitro Incorporation of N-Acetylglucosamine,
 Mannose, and Galactose by Rough and Smooth
 Microsomes*

Nucleotide-sugar used as radioactive precursor	Radioactivity incorporated by microsomal fraction			
	Rough (cpm/mg protein)	Smooth (cpm/mg protein)		%
UDP- <i>N</i> -acetyl- glucosamine	120	59	83	41
GDP-mannose	285	76	88	24
UDP-galactose	35	18	152	82

Rough and smooth microsomes were prepared as described by Dallner et al. (17). The microsomal subfractions were separately incubated at 37°C for 20 min under optimal conditions for the incorporation of the radioactive sugar as described under Experimental Procedures. The sample incubated with GDP-mannose also contained 5×10^{-4} M puromycin. The values given under % were calculated from the sum of incorporation of both rough and smooth microsomes.

incorporation occurred in the smooth microsomes (Table II).

These experiments indicate that liver glycoproteins are synthesized exclusively on membrane-attached polysomes rather than on free polysomes. They also show that *N*-acetylglucosamine is incorporated into the protein in at least two sites on the ER: the first incorporation occurs in the region which contains polysomes (rough ER), and the second on the smooth ER. Mannose, on the other hand, is mostly inserted into protein in the rough ER while galactose is attached on protein in the smooth ER. Similar results have been obtained by Molnar et al. (15).

Localization of the Radioactive Proteins on Microsomal Subfractions

The microsomal fraction as isolated contains predominantly sealed vesicles derived from the ER, some with ribosomes attached to the outside of the membrane (22). Methods have been developed, using limiting amounts of deoxycholate, which can separate the microsomes into a ribosomal fraction, a membrane fraction, and the contents of the cisternae of the microsomal vesicle (18). In order to determine which of these microsomal subfractions contained the radio-

active proteins, microsomes were incubated with either leucine-¹⁴C or the appropriate radioactive nucleotide-sugars, and then the washed microsomes were subfractionated into the three microsomal subfractions.

When leucine-¹⁴C was the radioactive precursor, labeled proteins were found in the ribosomes, the membranes, and the deoxycholate (DOC) soluble or microsomal content fraction. The majority of the radioactivity was bound to the ribosomes, even after puromycin treatment. The ribosomal fraction obtained by this subfractionation method contains a substantial amount of membranes and, thus, this fraction is in reality a mixture of both ribosomes and membranes. The membranes and the soluble fraction contained a variable amount of labeled material from experiment to experiment. The range found in the membranes was from 8 to 30% of the radioactivity, with the same percentage variation in the cisternal fraction.

Less *N*-acetylglucosamine than leucine was found incorporated into the ribosomal fraction. The majority of the incorporation (42%) was found in endogenous protein which fractionated with the membrane fraction, and 23% of the radioactivity was found in the soluble fraction which represents the cisternal contents of the ER.

The ribosomal-membrane fraction contained 30% of the radioactive proteins labeled with mannose. The largest amount of the mannose protein radioactivity, 60%, was in the membrane fraction and only a small amount, 10%, was located in the soluble microsomal content.

The protein labeled with galactose had a very different microsomal distribution. No radioactivity was recovered in the ribosomal fraction, only 22% was found in the membrane, and the large majority (78%) was in the soluble microsomal content. The distribution of radioactive protein in the various microsomal subfractions presented in Table III is that from a representative experiment. Occasionally, however, as mentioned above, some variations from these figures were obtained, and for consistent results it is essential that the ratio of deoxycholate to amount of liver microsomes used be kept constant. Thus, not only is the final concentration of deoxycholate, 0.26%, important, but it is also important that liver microsomes from 5 g of liver be used per tube. If fewer microsomes were used, there was little or no recovery of a membrane fraction and, under these conditions, there was a

TABLE III
Submicrosomal Distribution of Newly Formed Protein Formed In Vitro

Radioactive precursor	% of microsomal radioactivity			% of radioactivity in diluted cisternal fraction	
	Ribosomes and membranes	Membranes	Cisternal fraction	Pellet	Soluble
	Leucine	65	17	18	55
UDP- <i>N</i> -acetylglucosamine	35	42	23	63	37
GDP-Mannose	30	60	10	65	35
UDP-Galactose	—	22	78	60	40

Microsomes from 6 g of liver were incubated with ¹⁴C-labeled precursor shown above for 20 min at 37°C. The tubes with GDP-mannose-¹⁴C also contained 5 × 10⁻⁴ M puromycin. The incubation conditions are listed under Experimental Procedures. The incubated microsomes were washed twice by resuspension and centrifugation in 0.25 M sucrose. The washed microsomes were then subfractionated, as described under Methods, into a ribosomal fraction, a membrane fraction, and the cisternal fraction. The cisternal fraction was diluted five times with water and centrifuged at 105,000 *g* for 15 hr to obtain a pellet and soluble fraction. The last two columns show the distribution of radioactivity in these fractions.

corresponding increase in the radioactivity found in the soluble cisternal fraction, and also the per cent of this deoxycholate-soluble fraction which could be pelleted, after a 15 hr 105,000 *g* spin, rose from 60% to nearly 90%.

The Effect of Sonication on the Release from Incubated Microsomes of Proteins Labeled with either Leucine, N-Acetylglucosamine, Mannose, or Galactose

Sonication of liver microsomes is thought to rupture the microsomal vesicle and to release in a soluble fraction the intravesicular contents (34). Thus, sonication of microsomes, which had been incubated in vitro with the various nucleotide-sugar precursors or with leucine, should release mostly that radioactive protein which is not bound to the microsomal membrane or to the ribosomes, but which is free within the microsomal cisternae. Taking into account the results of the previous experiments, which indicated that the protein labeled with galactose was mostly soluble in the cisternae of the microsomal vesicle, but that the proteins labeled with leucine, *N*-acetylglucosamine, or mannose were mostly bound to either ribosomes or microsomal membrane, we should expect that sonication will release galactose-labeled protein but will not release protein labeled with the other precursors.

The experiments described in Table IV show that sonication of microsomes caused the release of radioactive protein from microsomes labeled with leucine or with any of the sugars. However, those microsomes which are labeled with galactose-¹⁴C released the largest amount of radioactivity on sonication; they released 40% of the protein while those labeled with *N*-acetylglucosamine only released 18%, and those with mannose 21%. The microsomes labeled with leucine released the smallest amount, only 13%. This is probably because a large per cent of leucine-labeled material is not in the microsomal vesicle or on the membrane but is attached to the ribosomes as incomplete polypeptides.

Identification of the Proteins Located in the Membranes and in the Soluble Microsomal Content Fractions

To characterize the radioactive products formed when microsomes were incubated with leucine, UDP-*N*-acetylglucosamine, GDP mannose, or UDP galactose, the incubated microsomes were washed twice with 0.25 M sucrose and were then treated with 0.5% sodium deoxycholate to solubilize the nonribosomal material. The soluble deoxycholate fractions were chromatographed on Sephadex G150 columns. Microsomes labeled with either leucine, *N*-acetylglucosamine, man-

TABLE IV
Release of Radioactive Protein from Microsomes by Sonication

Radioactive precursor	% of radioactivity released from microsomes		
	Control, nonsonicated	Sonicated	Difference
Leucine- ³ H	1.9 ± 0.2	15.3 ± 0.5	13.4
¹⁴ C- <i>N</i> -acetylglucosamine	10.6 ± 12.5	28.5 ± 7.5	17.9
Mannose- ¹⁴ C	4.1 ± 3.8	25.3 ± 13.6	21.2
Galactose- ¹⁴ C	6.8 ± 5.6	47.1 ± 10.6	40.3

Microsomes were incubated *in vitro* with either UDP-*N*-acetylglucosamine-¹⁴C, GDP-mannose-¹⁴C, UDP-galactose-¹⁴C, or leucine-³H for 20 min at 37°C as described previously. The incubation mixture with GDP-mannose-¹⁴C contained 5×10^{-4} M puromycin added at zero time, and that with leucine-³H also contained puromycin but it was added after 5 min of incubation. The incubated microsomes were recovered by centrifugation at 105,000 *g* for 30 min, and then the microsomes were washed twice in 0.25 M sucrose and divided into two equal portions. One portion was sonicated with a Branson sonifier at setting number 5 for three 1-min pulses. The temperature was kept below 10°C by the use of an ice-salt bath. The control portion was not sonicated. The microsomes were then centrifuged at 105,000 *g* for 30 min, and the protein radioactivity was determined in both the pellet and the sonic extract. The values are the averages of four experiments ± SD.

nose, or galactose contained protein in the deoxycholate-soluble fraction that chromatographed close to the void volume of the G150 column. This could mean either that the labeled material is of very large molecular weight, perhaps in aggregated form, or that the labeled protein is attached to the membrane and thus appears as part of a larger complex. Another possibility is that some or all of the radioactive label is not part of a glycoprotein but is incorporated into some other membranous component such as glycolipid or lipoprotein. There is precedence for the latter argument as Tetas et al. have shown the presence of a lipid acceptor of sugars in liver (23). Tetas et al. also showed that hydrolysis of liver microsomal material at 80°C for 20 min in 0.1 N HCl would liberate the radioactive sugar from the lipid acceptor but would leave a residual radioactive protein. To test if the material which eluted at the void volume was affected by such acid hydrolysis, indicating that it may be a glycolipid, the deoxycholate-soluble material was hydrolyzed and chromatographed on Sephadex G150. The pattern obtained with the hydrolyzed material showed a marked change. The majority of the radioactivity was now eluted in a region which indicated a much smaller size, and there was only a small amount of radioactivity being eluted close to the void volume.

Hydrolysis in 0.1 M HCl for 20 min at 80°C may not only remove sugar from a lipid in the membrane but may also, however, remove a membrane-bound protein from the membrane. To test if under our incubation conditions lipid-sugars were being produced, we counted the radioactivity extracted by the alcohol, alcohol-ether, and ether extracts which remove lipids from the TCA precipitates. Alcohol in all cases removed the majority of the radioactivity. It removed 35% of the radioactivity from the TCA precipitates labeled with *N*-acetylglucosamine, 45% from those labeled with mannose, and 65% from those labeled with galactose. Smaller amounts were removed with alcohol-ether and ether. Chloroform:methanol (2:1, v/v) has been shown to remove the lipid-sugar from TCA precipitates after a 15-min treatment at room temperature (23). Repeated treatment with this solvent mixture removed 74% of the labeling with *N*-acetylglucosamine, 43% of the labeled mannose, 41% of the galactose- and only 15% of the leucine-labeled material (Table V). Thus, the incubated microsomes do incorporate the radioactive sugar into a compound which is extracted with lipid solvents, but substantial radioactivity remains insoluble to lipid solvents and is presumably incorporated into proteins. It should be noted that compounds other than lipids

TABLE V
Extraction of Microsomes Labeled with Radioactive Sugars or with Leucine with Chloroform: Methanol

Radioactive precursor	Total radioactivity	Radioactivity extracted with CHCl ₃ /Methanol(2:1)				Radioactivity remaining in extracted pellet
		1st	2nd	3rd	4th	
		%	%	%	%	
Leucine	34270	8	3	3	1	85
UDP- <i>N</i> -acetylglucosamine	3059	54	12	5	3	26
GDP-mannose	13160	38	8	6	5	57
UDP-galactose	2000	26	5	6	4	59

Microsomes were incubated for 20 min at 37°C with either leucine-¹⁴C, UDP-*N*-acetylglucosamine-¹⁴C, GDP-mannose-¹⁴C or UDP-galactose-¹⁴C. Those incubated with GDP-mannose-¹⁴C also had 5 × 10⁻⁴ M puromycin. The reaction was stopped by adding TCA to a final concentration of 5%. The pellet was washed three times with cold 5% TCA, and then the washed pellet was extracted with 2:1 CHCl₃/methanol for 15 min at room temperature each time.

TABLE VI
Per Cent of Total Protein Radioactivity in Microsomal Subfractions, Labeled *In Vivo*, which React with Antiserum to Rat Serum Proteins

Radioactive precursor	Protein radioactivity in microsomal subfractions (cpm/fraction)					
	Membranes			Cisternal fraction		
	TCA-ppt	Serum proteins		TCA-ppt	Serum proteins	
			%		%	
Leucine	218,000	16,324	7.5	262,500	159,000	61.0
Glucosamine	49,440	5420	11.0	91,300	27,200	30.0
Mannose	3815	634	16.6	6920	3020	44.0
Galactose	16,900	1610	10.5	25,500	7650	30.0

The following amounts of radioactive material were injected intravenously. D-glucosamine-6-³H, 10 μCi; D-galactose-³H, 10 μCi; D-mannose-1-¹⁴C, 10 μCi; and L-leucine-¹⁴C, 5 μCi. The livers from rats injected with the radioactive sugars were removed 60 min after injection, and those from rats injected with leucine were removed 20 min after intravenous injection. Membranes and microsomal cisternae were isolated as described in Experimental Procedures. The membranes were solubilized by treatment with 0.5% sodium deoxycholate, and the treated membranes were centrifuged at 105,000 g for 1 hr to obtain a clear supernatant which was then used for determining total protein radioactivity and the radioactivity in serum proteins by precipitation with specific antisera.

may be extracted by lipid solvents from a TCA precipitate. For instance, albumin and thyroglobulin, among other proteins, can be extracted by alcohol (13).

To determine whether this lipid-insoluble radioactivity was incorporated into serum proteins rather than some other microsomal protein, rats were injected with radioactive leucine, *N*-acetylglucosamine, mannose, or galactose and the isolated liver microsomes were subfractionated into a membrane and a microsomal cisternal fraction. The total TCA-precipitable protein radioactivity in these fractions was determined

and was compared to the amount of protein radioactivity which reacted with rabbit antiserum to rat serum proteins. In order to make this comparison, the membrane fractions had to be solubilized with deoxycholate before treatment with the specific antiserum. Samples from the solubilized membranes and the microsomal contents fraction were then taken for the determination of total protein radioactivity and for precipitation of serum proteins by specific antiserum. Of the total protein radioactivity in the membrane fraction, only 8% of the leucine-labeled material, 11% of the glucosamine-, 17%

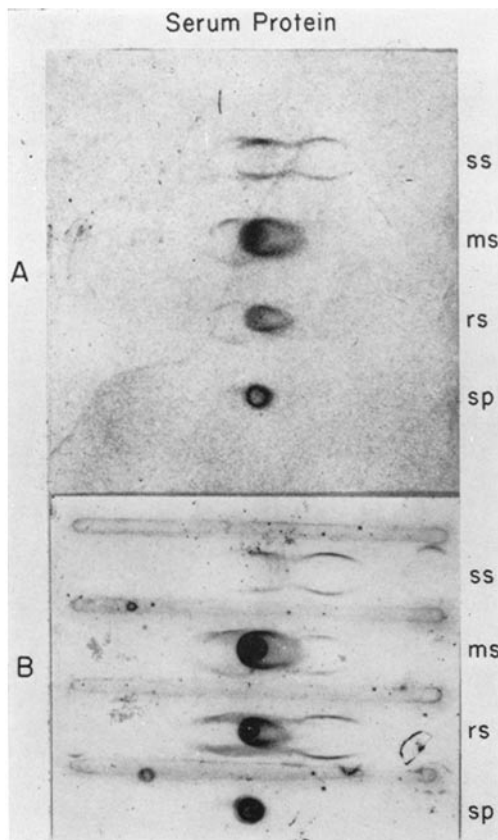


FIGURE 4 Immunoelectrophoresis and radioautography of leucine-¹⁴C-labeled serum proteins obtained from the microsomal membranes and cisternal fractions. Rats were injected intravenously with 10 μ Ci of leucine-¹⁴C, and the livers were removed after 20-20 min of injection. Microsomes for 5 g of liver were washed twice with 0.25 M sucrose and then treated with 0.26% sodium deoxycholate by the method of Ernster et al. (18) to separate the microsomes into ribosomal, membrane, and cisternal fractions.

The ribosomes and membrane fractions were further treated with 0.5% sodium deoxycholate to solubilize membranes. All of the deoxycholate-soluble fractions were then concentrated by dialysis in the cold against dry Sephadex G200. Immunoelectrophoresis was done on cellulose acetate membranes in 0.75 M barbital buffer, pH 8.6, at 150 v for 50 min. The antiserum was rabbit antiserum to rat serum protein. This is indicated in the figures. *A* is the radioautogram, and *B* is the Poinceau-stained precipitin pattern. *ss* is the cisternal material, *ms* and *rs* are deoxycholate-soluble fractions of the membrane and ribosomal fractions respectively, and *sp* is the pellet obtained from a 15-hr spin at 105,000 *g* of a five times diluted SS fraction.

of the mannose-, and 11% of the galactose-labeled proteins could be identified as serum proteins by immunological precipitation. A larger per cent of the total protein radioactivity in the cisternal fraction was precipitated as serum proteins. In the cisternae, 61% of the leucine-, 30% of the glucosamine-, 44% of the mannose-, and 30% of the galactose-labeled proteins were recovered as serum proteins (Table VI).

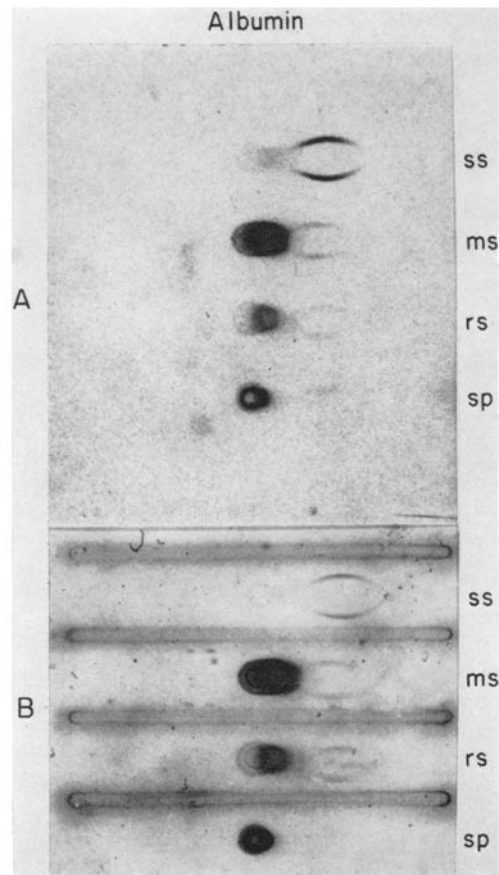


FIGURE 5 Immunoelectrophoresis and radioautography of ¹⁴C-labeled albumin from microsomal membranes and cisternae. Immunoelectrophoresis and radioautography were performed as in Fig. 4, except that antiserum to rat albumin was used. *A* is the radioautogram, and *B* is the Poinceau-stained immunoelectrophoresis pattern. *ss* is the cisternal proteins, *ms* and *rs* are the membrane and ribosomal deoxycholate-soluble material, and *sp* is the pellet obtained after a 15-hr 105,000 *g* spin of a five times diluted *ss* fraction.

TABLE VII
Intramicrosomal Location of In Vivo Labeled Albumin

Microsomal subfraction	Time after injection	Radioactivity (cpm/microsomal fraction)				Total protein radioactivity recovered as albumin
		Total protein		Albumin		
		<i>min</i>	%		%	
Ribosomal fraction	20	183,900	16.1	—	—	—
	60	135,140	17.1	—	—	—
Membranes	20	176,800	15.5	876	1.4	0.5
	60	193,940	24.6	—	—	—
Cisternal fractions	20	780,080	68.4	62,400	98.6	8.0
	60	459,540	58.3	7500	100.0	1.6

Rats were injected intravenously with 10 μ Ci of Leucine- 14 C, and the livers were removed at either 20 or 60 min after injection. The microsomes were subfractionated as described in the text. Total protein radioactivity represents the 5% TCA-insoluble proteins, and albumin was determined by immunological precipitations as described in Experimental Procedures. The per cent radioactivity represents the percentage of the microsomal radioactivity, either total protein or albumin, which was recovered in the submicrosomal fractions.

Further identification of the membrane-bound protein and the protein in the microsomal contents was done by immunoelectrophoresis and radioautography of these *in vivo* labeled proteins using antiserum to total rat serum proteins and antiserum to rat albumin. Insufficient incorporation into the microsomal protein by the various sugars could be obtained to perform radioautography. It was possible, however, using leucine- 14 C, to obtain radioautograms of the radioactive precipitin bands obtained from the microsomal membranes and cisternal proteins. These experiments showed that the membranes contained little or no albumin and that the only radioactive bands obtained were in the electrophoretic region usually occupied by glycoproteins. The cisternal proteins, however, showed several bands including albumin and glycoproteins (Fig. 4). Immunoelectrophoresis of the radioactive microsomal membrane and cisternal proteins with antiserum specific against albumin, rather than total serum protein, again showed that most of the albumin was in the cisternal space, with only a small amount in the membranes (Fig. 5). The amount of albumin located in the cisternal space, as opposed to the membrane, could be quantitated by measuring the protein radioactivity precipitated by antiserum specific to rat albumin. Rats were injected

intravenously with leucine- 14 C, the livers were removed 20 and 60 min after injection, and the radioactive proteins isolated from the solubilized microsomal membranes and cisternae fractions were precipitated with antiserum against rat albumin. Over 98% of the recovered radioactive albumin was in the microsomal cisternae after 20 and 60 min of injection. The amount of total radioactive microsomal protein which could be recovered as albumin in the microsomal cisternae was 8% after 20 min of injection and 1.6% after 60 min. A much smaller per cent of the membrane protein showed up as albumin with 0.5% at 20 min injection and it was not detectable after 60 min (Table VII).

DISCUSSION

Evidence has been presented by many laboratories which indicates that the sugars are added stepwise to the protein part of the molecule, beginning at the polysomal level and continuing while the protein passes through its intracellular route in the rough and smooth ER and the Golgi apparatus. Although there is some evidence to the contrary, the first sugar, *N*-acetylglucosamine, is thought to be added to the incompletely polypeptide chain while it is still attached to the polysomes. Mannose, the other sugar which is

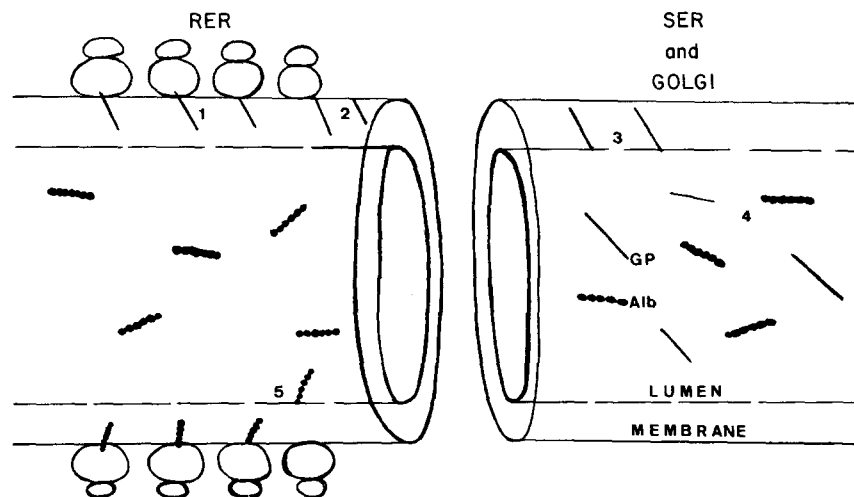


FIGURE 6 Scheme for the secretion of hepatic glycoprotein and albumin. (1) N-acetylglucosamine is attached while the protein moiety is still on the polysomes. (2) N-acetylglucosamine and mannose are attached while the protein is off the polysome but is still within the membranes of the RER. (3) More N-acetylglucosamine and some mannose are further attached on the smooth microsomes. (4) The protein leaves the membrane of the smooth microsomes and then enters the lumen. The terminal sugars are added when the glycoprotein reaches the Golgi apparatus. (5) Albumin (and other nonglycoproteins) are released directly from the polysomes through the ER membranes into the lumen. *RER*, rough endoplasmic reticulum; *SER*, smooth endoplasmic reticulum; *Alb*, albumin; *GP*, glycoprotein.

closest to the protein, has also been shown to be incorporated mostly in the rough ER; and the sugars situated at the terminal part of the chain are added in the smooth ER and the Golgi apparatus (9-16). Although albumin is not a glycoprotein, it still travels the same secretory route as glycoproteins in that it can be located in the rough and smooth ER and in the Golgi apparatus (29-33).

The experiments reported in this paper concentrate on the in vitro incorporation into endogenous protein of the sugars closest to the protein core, and these experiments attempt to distinguish between the immediate submicrosomal fate of newly formed hepatic glycoprotein and of albumin. The results suggest that albumin and glycoproteins may be segregated at an early stage of secretion, in that the nascent glycoproteins remain attached to the membranes of the rough ER, while mannose and other "core" sugars are being added, but that albumin passes directly from the attached polysomes to the contents of the endoplasmic reticulum.

All of the experiments were performed with endogenous protein acceptors, as one of the purposes of the study was to determine the localiza-

tion of the naturally occurring, newly formed serum proteins within the endoplasmic reticulum. Exogenous proteins can and have been added to in vitro systems as acceptors for sugars but this type of experiment, although it can localize the presence of the sugar transferases, cannot supply useful information on the location of the natural product within the cell organelle. If an exogenous substrate is added in soluble form, then the resulting product will probably also be recovered in soluble form. There are drawbacks, however, to studying the incorporation of labeled sugars into endogenous proteins. It is difficult to characterize the endogenous radioactive product, and thus in this study we have relied on the use of specific antisera to determine the presence of these proteins.

A scheme depicting the hypothetical intracellular pathways for albumin and the glycoproteins based on these and other studies is given in Fig. 6. The first steps in the scheme (steps 1 and 2, Fig. 6) either have been demonstrated previously or gather strong support from these experiments. Hepatic glycoproteins are made preferentially on membrane-attached polysomes (Table I). This agrees with the work done with rat liver,

mouse kidney, and myeloma cells which also indicates that glycoproteins are synthesized by membrane-attached polysomes (12, 25, 26). The majority of evidence now favors the view that the first sugar moiety, *N*-acetylglucosamine, of hepatic glycoproteins is incorporated into the incompleting polypeptide chain (9–16). The site of incorporation of mannose has not, however, been well elucidated. Molnar et al. show a preferential incorporation of mannose and *N*-acetylglucosamine into endogenous protein from rough microsomes while galactose is preferentially incorporated into the smooth microsomes (15). Wagner and Cynkin (27) and Caccam et al. (28) do not find incorporation into the rough ER. Our studies agree with those of Molnar et al. and also show that larger incorporation of mannose into endogenous protein can be obtained if puromycin peptides are caused to be released from the polysomes by treatment with puromycin. Presumably this makes available substrate for the enzyme which is housed in the membranes of the rough ER. Perhaps the reason for low enzymatic activity when exogenous substrates are used is that these substrates are not available to the enzyme which is within the membranes. Mannose incorporation is not affected by the inhibition of protein synthesis per se, since cycloheximide, which also inhibits protein synthesis but does not cause the release of incompleting peptides, has no effect on the incorporation of mannose into protein.

In the smooth microsomal fraction, other moieties of *N*-acetylglucosamine and galactose are added to the glycoprotein (steps 3 and 4 of Fig. 6 and Table III). This may occur in the smooth ER or the Golgi apparatus; our experiments do not differentiate between these cellular locations, but there is ample evidence that *N*-acetylglucosamine, galactose, and sialic acid are incorporated in the Golgi apparatus (35–38). The experiments given in Tables III and IV indicate that the glycoproteins, once labeled with galactose, are found within the cisternae of the smooth microsomes and are no longer bound to the membranes. Whether the glycoproteins are still bound to the membranes in the smooth microsomes before galactose is added (step 3, Fig. 6) is difficult to determine. Some protein labeled with *N*-acetylglucosamine is found in the cisternal space, but the per cent does not seem to be much larger than the amount of mannose-labeled proteins.

Therefore, for this reason, and since *N*-acetylglucosamine is added to proteins in both the rough and smooth microsomes, we have depicted step 3 in Fig. 6 as being a stage in which the glycoprotein is in the smooth microsomes but is still attached to the membranes. This stage is followed by the addition of galactose to the carbohydrate chain of the glycoprotein. At this stage the glycoprotein is found in the microsomal cisternae (step 4, Fig. 6, Tables III and IV), and is probably located in the Golgi apparatus since the terminal sugars, such as galactose and sialic acid, are known to be added to the glycoprotein in the Golgi apparatus (35–38). The secretory proteins are known to travel from the smooth ER to the Golgi apparatus, and thus the unfinished glycoproteins either leave the membranes of the smooth ER and travel to the Golgi apparatus while in the lumen of the ER, or they pass from the smooth ER to the Golgi apparatus while still attached to the membranes and then they are released into the lumen of the Golgi apparatus when the terminal sugars are added.

The localization of the various labeled proteins, either attached to the membranes or within the cisternae of the ER, was done by subfractionation of the microsomes by two methods: (a) use of low deoxycholate by the method of Ernster et al. (18) (Table III) or (b) sonication (Table IV). Although both of these methods fail to give clear-cut separations between microsomal membrane and cisternae, there being membranous material in both of these two cisternal microsomal subfractions, they do represent different approaches to subfractionation, one being disruption of the membrane with detergent and the other a physical disruption. Both of these methods give similar results (Tables III and IV), showing that the *N*-acetylglucosamine- and the mannose-labeled proteins are more tightly bound to the microsomal membrane than the galactose-labeled protein.

The steps described above are for serum glycoproteins. Albumin, which is not a glycoprotein, seems to have a different intracellular pathway in that the large majority of the newly formed albumin is always found in the cisternae of the microsomes, with little or none in the membranes. This evidence is gathered from several different experiments. Immunoelectrophoresis of the ¹⁴C-labeled leucine protein from the microsomal membrane and cisternal fractions shows that most of

the albumin-¹⁴C is located in the cisternae (Figs. 4 and 5). A quantitative determination of the amounts of radioactive albumin found in the microsomal membranes and cisternae after 20 and 60 min of intravenous injection of leucine-¹⁴C showed that 98.6% of the albumin was in the cisternal fraction (Table VII). There is much information from other laboratories which also shows that albumin is in the microsomal cisternae and is not attached to the membranes (33, 34).

The question arises whether the proteins labeled with these sugars, especially those found in the membranes, are secretory plasma proteins or are membrane constitutive proteins. The majority of the incorporation of sugars into the microsomal membrane goes into glycolipids (Table VI), part of which may be the lipid-sugar acceptor proposed by Tetas et al. since it can be hydrolyzed in 0.1 M HCl at 80°C for 20 min (23). There is, however, a nonlipid component in the membrane which is radioactive and which is probably protein (Table V). Further identity of the sugar-labeled proteins was established by immunological precipitation of these radioactive proteins with antiserum to rat serum proteins. In Table VI, it can be seen that about 30–60% of the proteins in the microsomal cisternae can be precipitated with antiserum to serum proteins, regardless of the radioactive tag used. Thus, these proteins are mainly serum proteins. The identity of the radioactive membrane proteins is more difficult to establish. Only 8–16% of these radioactive proteins react with antiserum to rat serum protein. There are several possible reasons or combination of reasons for this: (a) the radioactive proteins are not serum proteins, for instance they may only be partly serum proteins and partly some other membrane glycoprotein; (b) the radioactive proteins are serum proteins but are not precipitated by the antiserum either because they contain incompletely polysaccharide chains or because their binding to the membrane may hinder the immunologic reaction; (c) solubilization of the membrane with deoxycholate affects the immunological properties or reaction of the glycoproteins. Simkin et al. have shown that a guinea pig liver cell-free system incorporates labeled *N*-acetylglucosamine into protein found in sonic or Lubrol extracts from microsomes and that this labeled material reacts with antiserum to guinea pig serum glycoproteins (39).

Thus it is most probable that the incorporation into protein is mostly into incompletely serum glycoproteins.

A combination of radioautography and immunoelectrophoresis showed that ¹⁴C-labeled leucine proteins in the membrane specifically reacted with antiserum to rat serum proteins and that the immunoelectrophoretic pattern obtained by the membrane proteins differed from that obtained with the proteins from the cisternae (Fig. 4). These experiments show the following: (a) both the membrane and the cisternae of the ER contain serum proteins, but they contain different proteins. The membrane proteins have little or no albumin, and most of the albumin was found in the cisternal space (Figs. 4 and 5); (b) the serum proteins in the membrane cannot arise from contamination with cisternal proteins since if this were the case the radioactive protein profile of both fractions should be the same or at least the membranes should contain some of all of the cisternal proteins; (c) these experiments not only confirm the presence of serum proteins in the microsomal membranes and cisternae, but they also provide evidence for the hypothesis that albumin is in the cisternae of the ER, while the glycoproteins remain temporarily bound to the membranes.

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