CONTROLLED PROTEOLYSIS OF NASCENT POLYPEPTIDES IN RAT LIVER CELL FRACTIONS

II. Location of the Polypeptides in Rough Microsomes

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ABSTRACT

Rough microsomes were incubated in an in vitro amino acid–incorporating system for labeling the nascent polypeptide chains on the membrane-bound ribosomes. Sucrose density gradient analysis showed that ribosomes did not detach from the membranes during incorporation in vitro. Trypsin and chymotrypsin treatment of microsomes at 0° led to the detachment of ribosomes from the membranes; furthermore, trypsin produced the dissociation of released, messenger RNA-free ribosomes into subunits. Electron microscopic observations indicated that the membranes remained as closed vesicles. In contrast to the situation with free polysomes, nascent chains contained in rough microsomes were extensively protected from proteolytic attach. By separating the microsomal membranes from the released subunits after proteolysis, it was found that nascent chains are split into two size classes of fragments when the ribosomes are detached. These were shown by column chromatography on Sephadex G-50 to be: (*a*) small (39 amino acid residues) ribosome-associated fragments and (*b*) a mixture of larger membrane-associated fragments excluded from the column. The small fragments correspond to the carboxy-terminal segments which are protected by the large subunits of free polysomes. The larger fragments associated with the microsomal membranes depend for their protection on membrane integrity. These fragments are completely digested if the microsomes are subjected to proteolysis in the presence of detergents. These results indicate that when the nascent polypeptides growing in the large subunits of membrane-bound ribosomes emerge from the ribosomes they enter directly into a close association with the microsomal membrane.

INTRODUCTION

In the preceding article (1), we showed that the carboxy-terminal segment of nascent polypeptide chains in free hepatic ribosomes is protected from proteolytic attack by the large ribosomal subunits. The remaining amino-terminal portion of the chains is unprotected, presumably because it protrudes from, or is otherwise exposed at the surface of the large subunit. Since in liver rough microsomes the large ribosomal subunit is attached to the membrane (2) and the nascent chains are discharged directly into the microsomal lumen (3, 4), we investigated the accessibility of the amino-terminal portion of nascent chains of membrane-bound ribosomes to the attack of proteases, which were added to intact or detergent-solubilized rough microsomes. The results indicate

that in rough microsomes virtually the entire polypeptide chain is resistant to proteolysis; the carboxy-terminal segment due to shielding by the large ribosomal subunit, and the amino-terminal remainder due to protection by the microsomal membrane.

MATERIALS AND METHODS

A. *Preparation and Purification of Rough Microsomes*

Preparation of the rat liver postmitochondrial supernatant (S-17) and fractionation in a discontinuous sucrose density gradient was carried out as described in the preceding paper (1). The 1.6 M sucrose-TKM (TKM is 0.05 M Tris-HCl, pH 7.5; at 20°C, 0.025 M KCl; 0.005 M $MgCl_5$) layer containing crude rough microsomes was diluted with one volume of TKM and layered (8 ml) over 3 ml of 1.35 M sucrose-TKM. Purified rough microsomes were obtained as a pellet by centrifugation for 2 hr in a Spinco No. 40 rotor at 40,000 rpm.

B. *Preparation of Bound Ribosomes*

Bound polysomes were prepared from crude microsomes by treating the diluted 1.6 M sucrose-TKM layer with a detergent mixture (1% DOC and 4% Triton X-100) in the presence of RNase inhibitor as described previously (5). In this paper, the term *bound* polysomes (or ribosomes) will be used in reference to ribosomes prepared from rough microsomes by the use of detergents and, therefore, freed of membranes.

C. *Amino Acid Incorporation*

In vivo labeling of polypeptides was carried out as previously described (1). The in vitro incorporation system was similar to that used for free polysomes (1), except that the 1-ml incubation mixture received 0.4 ml of microsomes ($\backsim$5 mg of ribosomes) instead of polysomes.

D. *Analytical Procedures*

Measurements of radioactivity, sucrose density gradient analysis, controlled proteolysis, and column chromatographic analysis were essentially as described (1), except that when microsomes were exposed to proteolysis prior to column chromatography they were resuspended in 0.25 M sucrose-TKM.

E. *Electron Microscopy*

Pellets were fixed for 2 hr in 1% OsO_4 in 30% sucrose, stained in block with uranyl acetate, and embedded in Epon. Sections doubly stained with uranyl acetate and lead citrate were observed and photographed in a Philips EM 300 electron microscope.

F. *Source of Materials*

In addition to those given in the previous article (1), chemicals were obtained from the following sources: Triton X-100 was a gift from Rohm and Haas Co., Philadelphia, and sodium deoxycholate (DOC) was from Matheson, Coleman and Bell, Cincinnati, Ohio.

RESULTS

Endogenous Incorporation Activity of Rough Microsomes and Bound Ribosomes

For studying protease sensitivity of nascent polypeptide chains in rough microsomes and bound polysomes, it was necessary to investigate in more detail the endogenous in vitro amino acid–incorporating capacity of these preparations. We observed that purified rough microsomes have considerably higher activity per mg of RNA than total microsomes prepared from the mitochondrial supernatant (S-17) by a previously described procedure (5). During purification, the rough microsomes were centrifuged through a 1.35 M sucrose-TKM layer; this presumably removed contaminating smooth microsomes and lysosomes. The latter are known (6) to inhibit amino acid incorporation in vitro.

Experiments carried out in parallel with (*a*) free polysomes, (*b*) purified rough microsomes, and (*c*) bound polysomes prepared from the latter by detergent treatment showed that (Fig. 1): (*a*) the kinetics of incorporation were roughly similar for the three preparations; (*b*) the total amount of radioactive leucine incorporated was higher in free polysomes; (*c*) the activity of rough microsomes was nearly equal to that of bound polysomes, which indicates that neither the presence of microsomal membranes nor the detergent treatment affects the activity of endogenous templates.

Effect of In Vitro Incorporation of Amino Acids on Rough Microsomes

Samples of rough microsomes incubated for 20 min for amino acid incorporation in vitro were analyzed by zone sedimentation in sucrose density gradients, the conditions of centrifugation being so chosen as to sediment as a pellet all microsomal membranes and any ribosomes bound to them and

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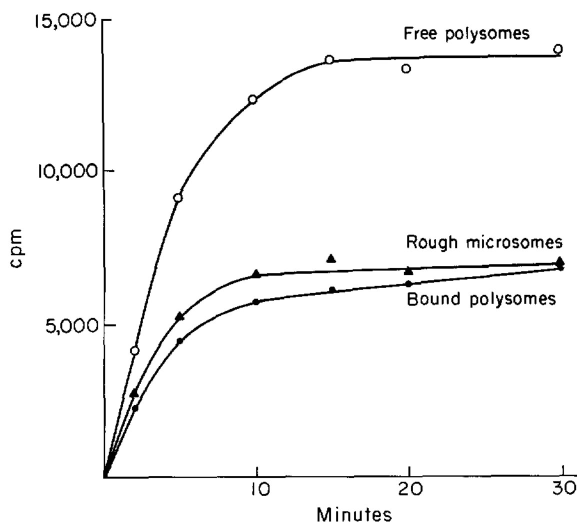

FIGURE 1 Kinetics of in vitro incorporation of leucine-^{14}C by free polysomes (—○—○—○—), rough microsomes (—▲—▲—▲—), and bound polysomes (—●—●—●—). The latter were prepared from purified rough microsomes by detergent treatment (see Materials and Methods, section B). Each experimental point represents an aliquot containing ~100 μg RNA.

to leave within the gradients unbound ribosomes and polysomes. In general, a small amount of free polysomes, accounting for less than 10% of the RNA of the original fraction, was found to contaminate samples of control (unincubated) rough microsomes. This amount did not increase after amino acid incorporation in vitro. Hence, we concluded that the number of ribosomes bound to the membranes was not affected by in vitro amino acid incorporation. In agreement with observations made on pure samples of free polysomes, the free polysomes contaminating the rough microsome fraction were converted during incorporation into monomers and dimers represented by two small peaks in the sedimentation profile of Fig. 4 *A*.

We then determined what proportion of nascent polypeptide radioactivity was released into the incubation medium when bound ribosomes or purified rough microsomes were incubated for 30 min in an in vitro incorporation system. To this intent, the incubation mixture was centrifuged for 2 hr at 40,000 rpm for sedimenting the ribosomes or microsomes and the radioactivity remaining in the supernatant was determined and taken to represent released polypeptides. The results showed that purified bound ribosomes released 15–20% of their radioactivity, while rough microsomes retained ~95–98% of their incorporated label. The distribution of radioactivity within the microsomes was also determined. The recovered microsomes were subfractionated by detergent treatment followed by sedimentation of detached ribosomes under the same conditions of centrifugation as above. The detergent-soluble supernatant—which represented the dissolved microsomal membranes and the content of the microsomal vesicles—was found to contain ~15–20% of the original microsomal radioactivity. This result is in agreement with previous reports (3, 7) which indicate that polypeptides released from membrane-bound ribosomes are retained within microsomal vesicles and are not discharged into the surrounding incubation medium.

Effect of Low Temperature Proteolysis on Nascent Polypeptides of Rough Microsomes and Bound Ribosomes

Preliminary control experiments indicated that microsomal membranes per se did not inhibit protease activity: a mixture of trypsin and chymotrypsin was equally active on labeled free polysomes, in the presence or absence of added microsomes.

Nascent polypeptides were labeled in vitro with leucine-^{14}C on bound ribosomes or on purified rough microsomes. The preparations were then treated with trypsin and chymotrypsin at 0°, and the kinetics of proteolysis of the labeled polypeptide chains were followed.

The results are given in Fig. 2 as percentages of initial radioactivity resistant to proteolysis, and in Fig. 3 as amounts of acid-insoluble radioactivity remaining in the subcellular components under investigation. As can be seen from Fig. 2, after

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5-hr proteolysis at 0° the protection of labeled chains in rough microsomes was extensive and amounted to ~80% of the original radioactivity. By contrast, in similarly treated bound polysomes

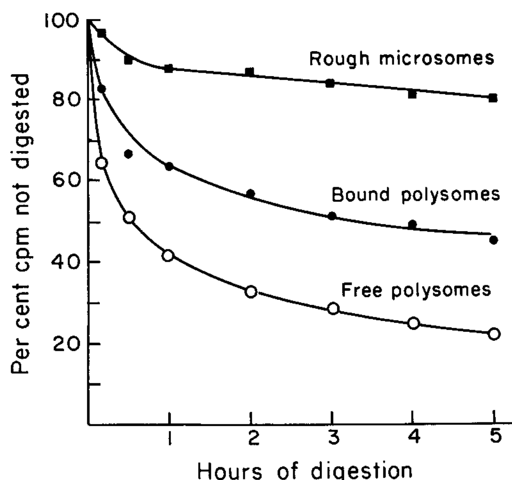

FIGURE 2 Kinetics of proteolysis of nascent polypeptides from rough microsomes (—■—■—■—), bound polysomes (—●—●—●—), and free polysomes (—○—○—○—). For all three preparations, incorporation of leucine-^{14}C in vitro was allowed to proceed for 20 min, at which time each sample was quickly frozen. After thawing, trypsin and chymotrypsin were added and the course of proteolysis at 0° was followed.

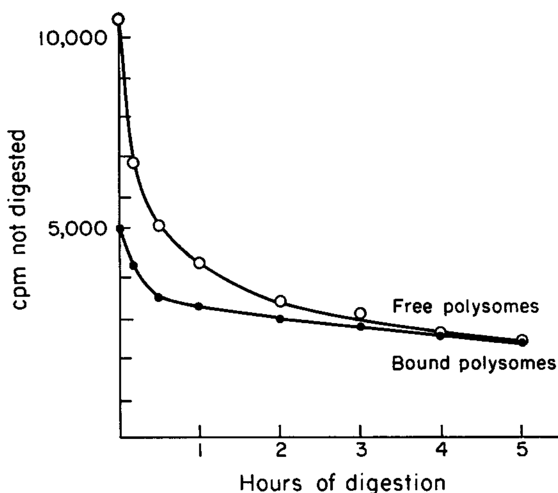

FIGURE 3 Kinetics of degradation of labeled nascent polypeptides from free and bound polysomes. Same experiment as in Fig. 2, except that amounts of residual, acid-insoluble radioactivity are plotted for each time point.

the protected fraction accounted for only ~40% of the initial value. The difference between the two preparations disappeared when the rough microsomes were exposed to the proteases at 0° in the presence of a mixture of Triton X-100 and DOC (Materials and Methods, section B). Not only did the level of protection by rough microsomes digested in the presence of detergent equal that provided by bound ribosomes (~40% of the initial radioactivity), but, in addition, the digestion kinetics became alike for both preparations and followed a curve similar to that given for bound polysomes in Figs. 2 and 3. On the basis of these results, we concluded that in rough microsomes most of the protection of nascent polypeptides against exogenous proteolytic attack was dependent on the integrity of the microsomal membranes. Fig. 2 shows, however, that the protection by microsomal membranes was not complete. The small amount of digestion detected could be accounted for by the presence (confirmed by electron microscopy) of ruptured microsomal vesicles in these preparations. Although the percentage of radioactivity protected in bound polysomes—and in rough microsomes digested in the presence of detergents—was nearly double that of the corresponding value for free polysomes (Fig. 2), the final amount of acid-insoluble, protease-resistant radioactivity (Fig. 3) preserved in each of these preparations was the same, irrespective of the initial value.

Viewed in the light of our previous results with free polysomes (1), this observation suggests that free and bound polysomes protect a segment of equal length in their nascent polypeptides.

The average size of free polysomes is significantly larger than the average size of bound polysomes (8). Hence, polypeptide chains made in free polysomes are expected to be longer than chains made in bound polysomes or in rough microsomes. Such a difference would account for the higher incorporation level observed in free polysomes (Fig. 1) and could explain the difference in the percentage of protection recorded between the two classes of ribosomes. This result does not represent an artifact arising from the conditions of in vitro incorporation, since we observed identical differences in levels of incorporation and protection between free and bound polysomes when the labeling of nascent polypeptide chains was carried out in vivo.

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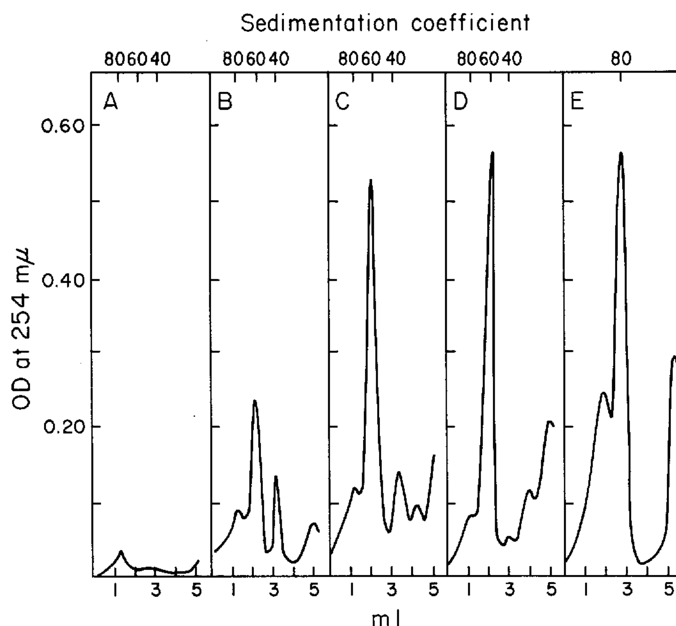

FIGURE 4 Sucrose gradient analysis of rough microsomes after proteolysis at 0°. All sedimentation profiles were obtained from rough microsomes previously incubated for 20 min for amino acid incorporation in vitro, then recovered by sedimentation, and resuspended in TKM. *A*, control not submitted to proteolysis. *B–D*, aliquots after incubation with trypsin (100 μg/ml) at 0° for 10 min (*B*), 2 hr (*C*), and 5 hr (*D*). *E*, ribosomal material released from an aliquot of rough microsomes treated with 0.5% DOC. Microsomal membranes have sedimented to the bottom of the tube. All gradients were from 5 to 20% sucrose in TKM and were centrifuged for 10 min (*A–D*) or 80 min (*E*) at 39,000 rpm in the Spinco SW 39 rotor.

Effect of Low Temperature Proteolysis on Rough Microsomes

Having assessed the relative purity of the rough microsomes and established that, under our conditions of incubation, completion of protein synthesis in vitro does not lead to ribosome detachment, we studied by density gradient centrifugation the effect of proteolysis at 0° on microsomes previously incubated for 20 min for amino acid incorporation in vitro. The effects of trypsin and chymotrypsin were studied separately and in combination. Fig. 4 *B* shows the sedimentation analysis of a sample incubated with trypsin at 0° for 10 min. A significant proportion of the bound ribosomes detached from the membranes and dissociated into subunits, as indicated by the appearance of prominent peaks in the subunit regions of the sedimentation profile. The detachment of ribosomes from membranes reached a maximum after 2 hr of incubation with trypsin at 0° (Fig. 4 *C*). The area under the subunit region of the sedimentation profile showed a very small increase when the incubation was prolonged to 5 hr (Fig. 4 *D*). As judged from their sedimentation coefficient, the detached ribosomal subunits were still hydrodynamically compact after 2 hr of proteolysis at 0°. As was the case with free polysomes, small subunits were more susceptible to degradation than large subunits and sedimented more slowly, but still as discrete particles after 2 hr of proteolysis.

After 5 hr of trypsin treatment, all bound ribosomes were detached from the membranes and dissociated into subunits. This conclusion was supported by the following findings: (*a*) the total area representing ribosomal material under the sedimentation profile did not increase when 0.5% DOC was added to the 5-hr microsomal digest to release any remaining attached ribosomes; (*b*) the amount of subunit material released by trypsin at 0° was equivalent to that released from a similar aliquot of rough microsomes treated with 0.5% DOC (Fig. 4 *E*) instead of

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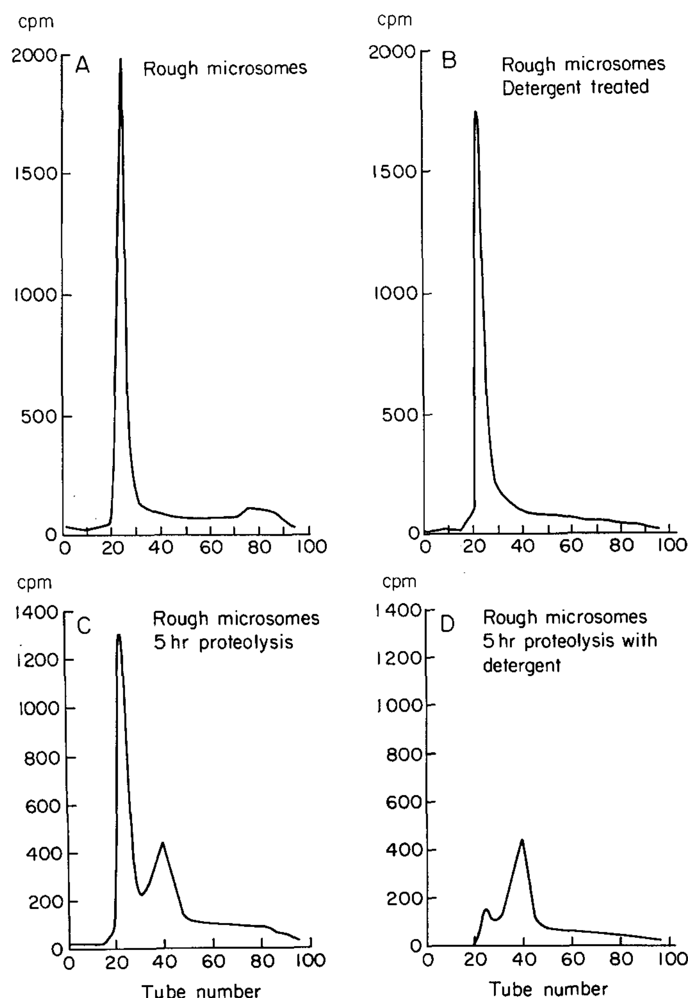

FIGURE 5 Gel filtration analysis of nascent polypeptides from purified rough microsomes labeled in vitro for 30 min with leucine-^{14}C. After incorporation, microsomes were recovered by sedimentation (4 hr at 40,000 rpm) and resuspended in 0.25 M sucrose-TKM. *A* and *B* received no proteases; *C* and *D* received trypsin and chymotrypsin (50 μg/ml of each). In addition, *B* and *D* received 1% DOC and 2% Triton X-100. After incubation for 5 hr at 0°, the samples were centrifuged and the ensuing pellets were prepared for gel filtration analysis on a Sephadex G-50 column by the urea-RNase method (1).

trypsin. Trypsin was equally effective in detaching ribosomes from unincubated microsomes; in this case, however, we observed no dissociation of the ribosomes into subunits.

Treatment of incubated rough microsomes with chymotrypsin alone at 0° also resulted in the detachment of ribosomes from the membranes, but, in this case, there was no further dissociation into subunits: the ribosomes remained as monomers.

Gel Filtration Analysis of Microsomal Nascent Polypeptides

Fig. 5 (*A–D*) presents the results of an analysis by G-50 Sephadex chromatography of leucine-^{14}C–labeled polypeptides contained in control and variously treated rough microsomes. Fig. 5 *A* shows the filtration pattern given by a control sample recovered from the incubation mixture and treated with 8 M urea and RNase (1). As in the case of free polysomes (1), nascent chains found

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in control rough microsomes had a minimum molecular weight of 10,000 daltons and were eluted from the column with the void volume at tube No. 25. The elution profile of a similar sample which, before centrifugation, was treated with a mixture of DOC and Triton X-100 (see Materials and Methods, section B) is shown in Fig 5 *B*. The bound ribosomes, recovered by centrifugation from the detergent-treated microsomes, contained ~80% of the total radioactivity in the nascent microsomal polypeptides, as estimated by comparing the areas under the peaks in patterns *A* and *B*. It is also apparent that the elution profile of the nascent polypeptides was not affected by detergent treatment.

As was mentioned before, 20% of the initial radioactivity remained soluble after detergent treatment of microsomes. This fraction probably represented secretion products which, upon their completion in vitro, were released from the ribosomes into the microsomal cavities.

The pattern obtained from labeled rough microsomes incubated with proteases at 0° for 5 hr is shown in Fig. 5 *C*. Two distinct peaks representing two different size classes of polypeptides are recognized. The major peak centered around tube No. 25 consists of relatively large polypeptides (larger than 10,000 mol wt) which were excluded from the column. The smaller peak at tube No. 40 represents radioactive fragments of smaller size which had been generated during proteolysis. From the elution position of the latter fragments, it is clear that they are of the same size as the fragments protected by the large subunits of free polysomes (1).

It should be noted that after proteolysis all microsomal membranes, as well as all ribosomal subunits released from these membranes, were recovered in a common pellet obtained from the incubation mixture by high-speed centrifugation. Therefore, the localization of each size class of polypeptides within the dissociated microsomal components remained to be established and was attempted in the following experiments.

Fig. 5 *D* shows the chromatogram of labeled peptides in bound ribosomes recovered from a sample of rough microsomes treated with detergents (DOC and Triton X-100) in addition to proteolytic enzymes. In this case, proteolysis was carried out in the presence of detergents which solubilized the microsomal membranes and rendered accessible the microsomal contents to the proteases. After proteolysis, the ribosomal material recovered by centrifugation for 4 hr at 105,000 *g* contained mainly labeled polypeptide fragments which eluted within the included volume of the column at tube No. 40, and only a small amount of larger excluded polypeptides which eluted at tube No. 25. No acid-insoluble radioactivity remained in the supernatant, which represented the dissolved membranes and the dispersed content of the microsomes. This result suggested that the protection of the large fragments observed in Fig. 5 *C* was due to the integrity of the microsomal membranes. When these membranes were dissolved by the addition of detergent, the polypeptide fragments in the larger size class became accessible to the enzymes and were digested. It should be observed that the peaks of radioactive fragments eluting at tube No. 40 covered equivalent areas in Fig. 5 *C* and Fig. 5 *D*. The finding suggests that the degradation of the excluded peptides proceeded to completion in the presence of detergents, without generating smaller fragments comparable in size to those eluting at peak position in tube No. 40. It follows that the included peak in Fig 5 *C* most probably contained nascent chain fragments protected by the large subunits of all the active attached ribosomes present in the original rough microsome fraction. This interpretation implies that proteolysis at 0° splits the nascent polypeptide chain in between the large subunit and the microsomal membrane as part of the detachment process.

To establish the localization of each size class of polypeptide fragments within the structural components of rough microsomes, we separated by several means the microsomal membranes from the detached ribosomal subunits at the end of proteolysis and investigated the distribution of nascent polypeptide fragments in these ensuing subfractions. Fig. 6 shows the results obtained by differential centrifugation.

Aliquots (2 ml) of protease-treated microsomes were diluted to 10 ml and centrifuged either for 30 min at 40,000 rpm in a Spinco No. 40 rotor (Fig. 6 *A*) or for 4 hr at 60,000 rpm in the A 321 rotor of the IEC centrifuge (Fig. 6 *B*). The first centrifugation was calculated to sediment most microsomal membranes and few ribosomal subunits, and the electron microscopy of the pellet (Fig. 7) confirmed that it consisted primarily of membranes still organized in closed vesicles. The

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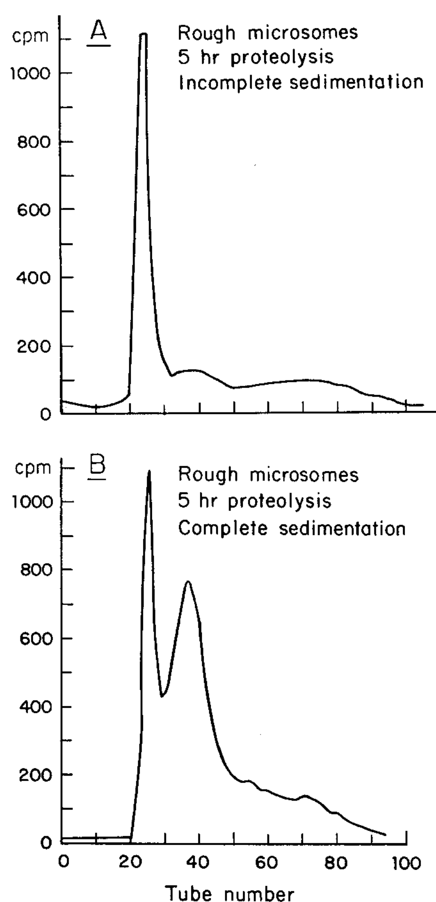

FIGURE 6 Gel filtration analysis of labeled peptides from subfractions of proteolyzed microsomes. After 5 hr of proteolysis at 0°, aliquots were centrifuged either for 30 min at 40,000 rpm (*A*) or for 4 hr at 60,000 rpm (*B*), and the pellets were processed for gel filtration analysis (1).

second centrifugation was sufficient to sediment both membranes and detached subunits. Gel filtration analysis of these pellets (after urea-RNase treatment [1]) showed that the first pellet (membrane-bound vesicles) contained mainly large excluded fragments (Fig. 6 *A*), while the second pellet (membranes and ribosomal subunits) contained both size classes of polypeptide fragments (Fig. 6 *B*).

After proteolysis, microsomal membranes were also separated from ribosomal subunits by flotation. Enough 2.3 M sucrose-TKM was added to proteolyzed microsomes for bringing the sucrose concentration to 1.75 M. The sample was transferred to a tube of the A 321 rotor of the IEC centrifuge, overlayed with 1 ml of 1.35 M sucrose-TKM and centrifuged for 2 hr at 60,000 rpm. Under these conditions, ribosome-free membranes ($\rho \simeq 1.2$ [9]) floated into the 1.35 M sucrose layer. After centrifugation, this layer was removed and the ribosomal material in the 1.75 M layer was sedimented (4 hr of centrifugation at 60,000 rpm) after dilution in TKM. These ribosomal particles contained mainly polypeptide fragments of the size shown (1) to correspond to ~39 amino acid residues (Fig. 8).

DISCUSSION

In discussing our results, we should consider the effect of proteolysis on bound ribosomes and on the membranes themselves, as well as on the association between these two microsomal components.

It was first shown by Tashiro (10) that rat liver ribosomes, prepared from microsomes by DOC treatment, are rapidly and extensively degraded during incubation with trypsin, 100 μg/ml at 37°, and it was subsequently reported that trypsin and other proteases have a similar effect at 37° on the attached ribosomes (11) of intact liver microsomes. Our results indicate that trypsin and/or chymotrypsin (50–100 μg/ml) at low temperature (0°) cause the complete detachment of ribosomes from microsomal membranes after 2- to 5-hr digestion. Under these conditions of proteolysis, ribosomes and membranes retain a considerable degree of structural integrity. Paralleling our observations with free ribosomes (1), large ribosomal subunits detached from microsomal membranes by proteolysis at 0° behaved hydrodynamically as discrete, compact particles which, even after several hours of incubation, retained digestion-resistant segments of nascent polypeptides. Another fraction of the same polypeptides, also resistant to proteolysis, remained associated with microsomal membranes.

We have studied these membranes with the electron microscope and have found that even after several hours of proteolysis at 0° most of them appear as seemingly intact, closed vesicles, freed of attached ribosomes. This observation is in agreement with previous reports in the literature which have shown that after proteolysis at 37° (12), or after overnight digestion at 0° (13), liver microsomes appear as closed vesicles bound by membranes devoid of ribosomes. Omura et al. (13) have indicated, however, that in spite of

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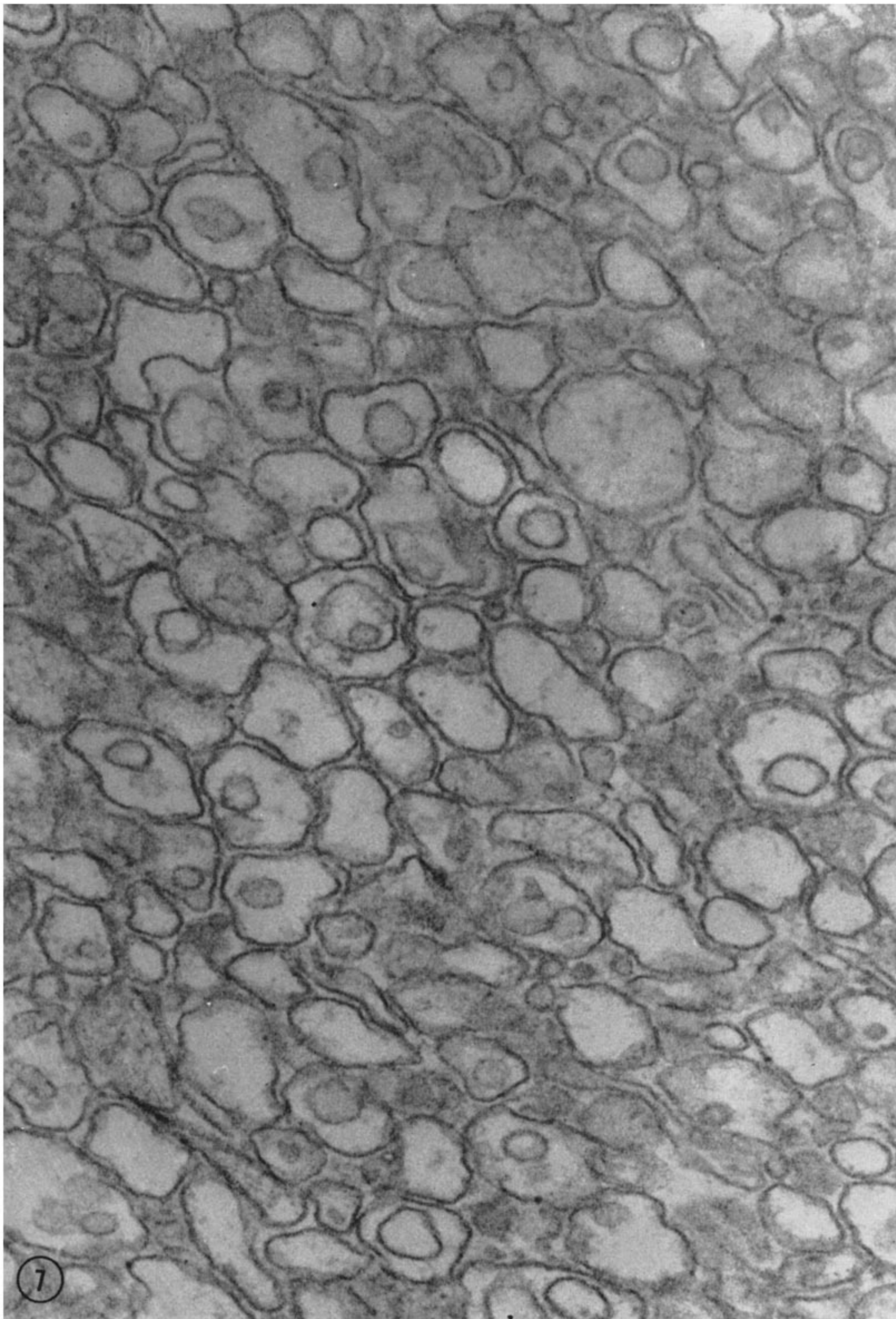

FIGURE 7 Microsomal vesicles recovered from a sample of rough microsomes subjected to 5 hr of proteolysis at 0°. Electron micrograph of a representative area of the pellet obtained after centrifugation for 30 min at 40,000 rpm. Fixation by OsO_4. × 100,000.

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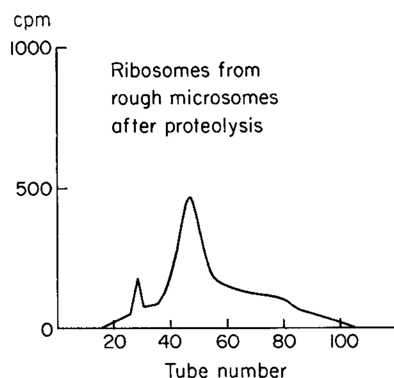

Figure 8 Chromatogram of labeled peptides in the ribosomal material obtained from rough microsomes after 5 hr of proteolysis at 0°. Microsomal membranes were separated from detached ribosomal subunits by flotation in heavy sucrose. Ribosomal particles were sedimented from the heavy sucrose layer after dilution with TKM (see text) and processed for gel filtration analysis (1).

their "intact" appearance a large protein fraction (30–40%) is lost from the microsomal vesicles during proteolysis at 0° concomitant with a loss of only 10–15% in phospholipids. Omura et al. (13) have also shown that some proteins, such as cytochrome b_5 and NADPH-cytochrome c reductase, are selectively "solubilized" by trypsinization while other enzymatic activities of the microsomal vesicles are not affected. Similar results have been obtained by Ito and Sato (14), who studied the effect of several proteases at 30° on liver smooth microsomes. Their results suggest that exogenous proteases have access to the outer aspect of the microsomal membranes only, since after proteolysis most of the microsomal vesicles remain impermeable to added macromolecules (mol wt $\simeq$ 40,000).

Turning to the question of how nascent microsomal polypeptides are affected by proteolysis which concomitantly dissects the large ribosomal subunits from their association with microsomal membranes, we should recall two features of these nascent polypeptides. The first is that—irrespective of in vivo or in vitro labeling—these polypeptides are long chains, equivalent in length to at least 10,000 daltons; therefore, they should have sizeable exposed or extraribosomal segments (1). The second feature is that even after 30 min of in vitro incorporation most of the nascent microsomal polypeptides remain bound to attached ribosomes. Only ~20% of the radioactivity incorporated in vitro was found to be solubilized by detergents and can be assumed to represent polypeptides released from attached ribosomes and segregated within microsomal vesicles. These segregated chains should be protected from digestion if microsomal membranes remain impermeable to the proteases.

Finally, a comparison of the results published in this paper and in its companion (1) shows that (in liver) bound ribosomes (prepared by detergents from rough microsomes) are similar to free ribosomes in the extent of protection they afford to nascent polypeptides. Approximately 60% of the radioactivity incorporated in vitro by bound ribosomes was digested during proteolysis at 0°. By analogy with respect to the results reported on free ribosomes, this fraction represents the exposed, extraribosomal segments of the ribosome-bound nascent chains, as well as the completed chains released in the incubation medium. After proteolysis, only a carboxy-terminal segment, equivalent in length to ~39 amino acid residues, remained undigested. Considering that bound and free liver polysomes are engaged in the synthesis of different proteins (15–18), the identical length of their protected segments should be taken as an expression of the structural similarity of these two types of ribosomes.

The results recorded with bound polysomes should be compared with those obtained with intact rough microsomes. In the latter, ~80% of the total radioactivity incorporated in vitro was protected from digestion. Trimming or degradation of labeled chains in microsomes was, therefore, much less extensive than in bound ribosomes. This higher level of protection cannot be accounted for entirely by polypeptides segregated in vitro in the microsomal cavity since these peptides contain only 20% of the initial radioactivity. Hence, we must conclude that: (*a*) in rough microsomes, protection from proteolysis was extended to the extraribosomal, amino-terminal segments of nascent polypeptides, and (*b*) the nascent polypeptides were clipped by proteases into two main fragments as part of the process of ribosome detachment. One set of the ensuing fragments was contained in the large ribosomal subunits detached from the membranes. These fragments were identical in length and amount to the nascent polypeptide segments protected by an equivalent sample of bound ribosomes. The other set of fragments was associated with residual microsomal

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membranes and was composed of polypeptides larger than the inclusion limit of G-50 Sephadex. This fraction should comprise completed segregated polypeptides, as well as amino-terminal segments of split nascent chains. The resistance of this set of fragments to proteolysis depended on the integrity of the microsomal membranes: when microsomes were digested in the presence of detergents, they were totally degraded, while the ribosome-protected fragments present in the mixture remained unaffected.

As already noted, protection of nascent polypeptide chains by rough microsomes was not complete: ~20% of the initial radioactivity was degraded during proteolysis of intact rough microsomes. This loss could be explained by partial degradation or trimming of the fragments of nascent chains exposed upon ribosome detachment, or by leakage from or into ruptured or otherwise damaged microsomal vesicles. Extensive trimming or degradation of membrane-associated polypeptide fragments apparently did not occur since their size was above the exclusion limit of the G-50 Sephadex column. Only one type of included fragment, corresponding to the length of 39 amino acid residues of the ribosome-protected segment, was observed.

Because of the extensive protection of the ribosome-bound nascent chains in rough microsomes, our results suggest that the amino-terminal end of the nascent polypeptide emerges from the large ribosomal subunit within or near the area through which this subunit binds to the microsomal membrane. Thus, no significant length of the growing chain becomes exposed to the enzymes, and it is probably only after degradation of proteins in the surface of the ribosome and/or membrane that the chain is reached and cut by the proteases, leading to release of the ribosome. If one assumes that the nascent polypeptide grows in an interior space of the large ribosomal subunit (see reference 1), an effective protection covering the entire nascent chain would be provided by structural arrangements of the type schematically depicted in Fig. 9. In this tentative model, the space within the large ribosomal subunit (through which the attachment to the membrane occurs) communicates—through a permanent or intermittent discontinuity of the membrane—with the cisternal space or the microsomal cavity. The transfer of peptides to the cisternal cavity, which follows natural (7) or puromycin-induced (3, 4) release, is explained by the structural restrictions which are imposed on the movement of the secretory product at the ribosome-membrane junction. In an alternative model, both segments of the growing polypeptide would be complexed to the ribosome surface and/or the surface of the microsomal membrane. Yet it seems unlikely that such a complexing could provide for the observed protection of the nascent chains since, as we and others have shown, proteases cause extensive degradation of the ribosomal and membrane proteins located at the surface of these structures. This degradation accounts for the change in electrophoretic pattern of ribosomal proteins (1) and for the loss of proteins from microsomal membranes (13).

A model like the one proposed in Fig. 9 is compatible with the data presented in this paper and with the known features of the process of in vitro transfer of secretory products into the cisternal cavity. These features—embodied in the concept of vectorial discharge—define a non-

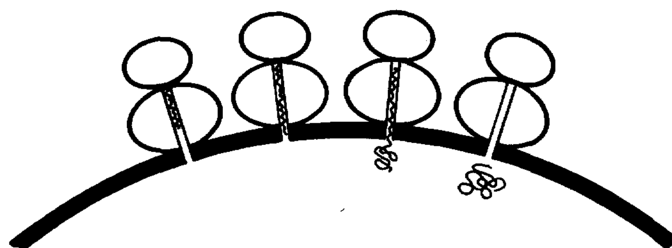

FIGURE 9 A model of the relationship of ribosomes, nascent polypeptides, and membranes of the endoplasmic reticulum, which accounts for the protection of nascent polypeptides from the attack of added proteases. A structural arrangement is proposed which is also compatible with the known features of the process of transfer of secretory polypeptides into the cisternal cavity.

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energy-requiring mechanism of transfer across the membrane, which is passive and undiscriminating and which can operate on completed polypeptides or on incomplete peptidyl-puromycin molecules. Transfer of this type in a model like the one here proposed should be independent of the length and possibly of the nature of the product synthesized in attached ribosomes. These predictions are now being tested in our laboratory.

The authors are indebted to Dr. George E. Palade for his generous support, advice, and criticism and to Dr. M. Adelman for his helpful comments on this manuscript. The expert technical assistance of Miss Belinda Ulrich is gratefully acknowledged.

This work was supported by grant 1R01 GM 16588-01 from the United States Public Health Service.

Received for publication 21 September 1969, and in revised form 3 November 1969.

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