BRIEF NOTES

FATE OF POLYPEPTIDES SYNTHESIZED ON ROUGH MICROSMAL VESICLES IN A MESSENGER-DEPENDENT RABBIT RETICULOCYTE SYSTEM

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In eucaryotic cells, the mechanism whereby proteins newly synthesized in the cytoplasm are transferred across cellular membranes is generally thought to involve synthesis of these proteins on ribosomes attached to membranes, primarily of the rough endoplasmic reticulum (RER). Proteins falling into this category, therefore, include those destined for secretion, for transport to inner compartments of other cellular organelles, e.g., lysosomes, Golgi complex, peroxisomes, and mitochondria, and those membrane proteins which form the tightly bound class of integral proteins or which are deposited asymmetrically toward the noncytoplasmic face of membranes. Such conclusions derive originally from studies (14, 17-19) which analyzed the directional discharge of polypeptides synthesized in vivo and in vitro on RER. This process has since become the subject of a large literature (recently reviewed, references 14 and 22), and now some of the details pertaining to the mechanism of this process have been elucidated, especially by experiments designed to test the “signal hypothesis” (2, 3, 5).

It still remains to be determined, however, whether transfer of nascent polypeptides into or across membranes is the underlying feature common to all proteins synthesized on bound ribosomes. It has been suggested, for example, that a large fraction of the proteins synthesized by RER in nonsecretory tissues is released from bound ribosomes directly to the cytosol (1). Serine dehydratase, an inducible enzyme located exclusively in the cytosol, is apparently synthesized on RER in rat liver (12). Rothman (20) has argued, on kinetic grounds, that secreted hydrolases in the pancreas may be released into both the ER and the cytosol after their synthesis on bound ribosomes. There has even been a recent proposal (9), based on cytochemical observations in rat liver, that newly formed albumin is released from bound ribosomes to the cytosol and cytoplasmic surface of RER where it is subsequently transported (but see references 4, 7, and 19). In all of these studies, however, the question is whether or not such polypeptides represent bona fide populations of proteins which are normally released from bound ribosomes directly to the cytosol or to the cytoplasmic side of RER in vivo.

The present study, therefore, considered this question for a tissue (rat liver) whose RER is well known to function in the synthesis of a heterogeneous population of both secreted and nonsecreted proteins. The fate of polypeptides synthesized in vitro on hepatic rough microsomes (RM) was followed under conditions which led to synthesis and release of primarily full-sized products. Such conditions were achieved by incubating RM in a potent messenger-dependent system derived from rabbit reticulocytes. The results described here clearly indicate that most, if not all, of these proteins are released either deep into or across this membrane.

MATERIALS AND METHODS

Male rats (110-130 g, Sprague-Dawley strain) which had been maintained on standard food pellets fed ad lib. were used. A detailed description of the methods used for purification of RM, isolation of messenger RNA, protein synthesis in vitro, product analysis, gel electrophoresis, and electron microscopy is provided in earlier publications (23, 24). Additional experimental details are described in the legends to figures and tables.
RESULTS AND DISCUSSION

RM were prepared by a procedure (24) which minimises contamination by free ribosomes (~10%) and retains the structural integrity of bound polyribosomes by maintaining a high pH and relatively high ionic strength during their isolation. Electron microscopy (Fig. 1) revealed that RM preparations consisted primarily of unbroken vesicles with ribosomes attached. RNA isolated from this fraction was very active in the rabbit reticulocyte cell-free system (Table I). Nuclease pretreatment of the latter resulted in a loss of ~98% of its endogenous messenger activity, but addition of RM RNA stimulated activity some 30-fold above background. Polypeptides reacting with antialbumin antiserum accounted for approx. 3% of the total translation products obtained in the presence of RM RNA; and, when adjustments are made to account for the low methionine content of albumin (six residues in 553 total, reference 16) relative to total protein, this value approaches 10–15%, i.e., similar to estimates made from in vivo labeling experiments for the percent of total protein synthesis which is albumin (see references 8 and 13).

When RM vesicles themselves were incubated in the messenger-dependent reticulocyte system (Fig. 2), active elongation of the polypeptide chain occurred. However, there was little or no initiation by reticulocyte ribosomes nor reinitiation by RM ribosomes since aurintricarboxylic acid, an inhibitor of peptide initiation (10), had little effect on the activity of RM (Fig. 2). Identical results were obtained with edeine, another inhibitor of initiation (6) (data not shown). Nevertheless, inclusion of reticulocyte lysate in the incubation medium was critical for active and complete elongation of polypeptides by RM ribosomes. Incorporation of [35S]methionine (15 μCi, 300 Ci/mmol) into polypeptides which were insoluble in hot TCA or which reacted with anti-albumin antiserum was measured (24). The results given are an average of three determinations. Incorporation of radioactive precursor into both total TCA-insoluble product and albumin proceeded linearly for more than 60 min.

RNA was isolated from RM by phenol extraction as described previously (24). Samples containing 50 μg of RNA were incubated with the rabbit reticulocyte lysate (+nuclease pretreatment) under conditions of protein synthesis (24). Nuclease pretreatment of the lysate was performed as follows (simplified from reference 15): aliquots (0.8 ml) of freshly thawed lysate were incubated at 20°C for 15 min with 8 μl of micrococcal nuclease (7,500 U/ml in H2O, 29,000 U/mg protein, obtained from P-L Biochemicals, Inc., Milwaukee, Wis.) and 8 μl of 0.1 M CaCl2. Enzyme activity was terminated upon addition of 16 μl of 0.1 M 1,2-Di(2-aminoethoxy)-N,N,N',N'-tetraacetic acid (EGTA), and the lysate (50 μl) was then used immediately for protein synthesis. Incorporation of [35S]methionine (15 μCi, 300 Ci/mmol) into polypeptides which were insoluble in hot TCA or which reacted with anti-albumin antiserum was measured (24). The results given are an average of three determinations. Incorporation of radioactive precursor into both total TCA-insoluble product and albumin proceeded linearly for more than 60 min.

![Figure 1](image1.png) **Figure 1** Electron micrograph of RM. RM were pelleted as described in Fig. 2, prepared for electron microscopy as described (23), and examined and photographed with a Philips EM model 201. × 33,500. Bar, 0.5 μm.

### Table I

| Addition          | TCA-insoluble polypeptides |
|-------------------|---------------------------|
|                   | Albumin                   |
| No nuclease pretreatment | [35S]Methionine incorporated/60 min/reaction tube |
| None              | 38,775 68                 |
| RNA from RM       | 67,305 653                |
| Nuclease pretreatment | 785 16               |
| RNA from RM       | 23,410 720                |

In order to investigate directional discharge of...
FIGURE 2 Effect of aurintricarboxylic acid (ATA) on the activity of polyribosomes attached to rough microsomal (RM) vesicles. RM were isolated (24) and sedimented at 2°C (130,000 gav for 20 min in the Beckman type 65 rotor, Beckman Instruments, Palo Alto, Calif.) through a 2-ml cushion containing 1.0 M sucrose, 200 mM Tris acetate, pH 8.5, 50 mM KCl, 10 mM Mg acetate, and suspended in 10 mM Tris acetate, pH 7.6, 20 mM KCl, 1 mM Mg acetate, and 20% glycerol (wt/vol). Aliquots (10 μl containing approx. 50 μg of RNA) were incubated in the nuclease-treated reticulocyte lysate mixture (100 μl final volume) in either the presence (●) or absence (○) of ATA (90 μM). Incorporation of [35S]methionine ([35S]met.) (15 μCi, 300 Ci/mmol) into total TCA-insoluble polypeptides was assayed at the times indicated. Values obtained in the absence of RM (minus mRNA controls) were subtracted from the values presented above (maximum of 0.9 × 10^6 cpm). In this experiment, ATA inhibited completely the activity of purified rat liver poly A^+ -RNA in the reticulocyte lysate.

The entire population of polyptides synthesized on rat liver RM, it was first necessary to establish the fidelity of the in vitro system. This was checked by following the fate of albumin since it is well known that this protein is transferred across the ER membrane to the intracisternal space during its synthesis on bound ribosomes in vivo (4, 7, 19). Figure 3 and Table II demonstrate that the majority (88%) of completed albumin chains synthesized by RM remained with the vesicles after their release from RM ribosomes. A small amount (12%) was released to the extravesicular, i.e., "cytoplasmic," compartment, and none was recovered in association with ribosomes. Moreover, results from two kinds of experiment (data not

FIGURE 3 Vectorial discharge of albumin synthesized in vitro on RM vesicles. RM vesicles (approx. 50 μg of RNA) were incubated in the messenger-dependent reticulocyte lysate (Fig. 2), and after 60 min detergent (500 μl of phosphate-buffered saline ±1% Triton X-100 and 0.1% deoxycholate) was either included (+D) or omitted (−D), and reaction mixtures were centrifuged for 30 min at 130,000 gav (2°C) to yield a supernate (S) and pellet (P). Labeled albumin was precipitated from the various fractions with antiserum, electrophoresed in a 10% polyacrylamide slab gel containing SDS, and autoradiographed (4 days) (23, 24). Rat serum albumin co-migrated in these gels to a position indicated by the arrows. Recovery of albumin from total (unfractionated) reaction products after incubation in the presence or absence of RM vesicles is shown above by T and C, respectively. The various subfractions represent different compartments to which RM ribosomes can release radioactive product; −D,S (extravesicular space), +D,P (retained on ribosomes), and the difference between −D,S (extravesicular space) and +D,S (extravesicular space + solubilized vesicles) represents product discharged either into the vesicle membrane itself or into the intravesicular space.
RM vesicles (containing approx. 50 µg of RNA) were incubated in the mRNA-dependent reticulocyte lysate for 60 min (see Fig. 2), at which time subfractionation was performed in the presence or absence of detergent (Fig. 3). Release of TCA-insoluble polypeptides (868,000 cpm recovered) and albumin (13,000 cpm recovered in immunoprecipitates) into the various compartments listed above was analyzed as described in Fig. 3. For quantitative comparisons of incorporation into albumin, densitometer tracings were performed on autoradiographs of the full-sized polypeptide in polyacrylamide (SDS) gels (see reference 24). Exposure times were such that band densities on the film were proportional to the amount of radioactivity in the gels.

shown) suggested that newly synthesized albumin was not merely binding nonspecifically to the outside of RM vesicles after polypeptide chain termination. First, when RM vesicles were added to a reticulocyte lysate containing radioactive albumin previously synthesized by the use of 1.0-µg membrane-free poly A⁺-RNA, less than 10% of the labeled albumin was recovered with the RM vesicles after subfractionation, i.e., it was recovered in the extravesicular compartment. Secondly, addition of excess, unlabeled serum albumin (250 µg) to reaction mixtures before subfractionation did not alter the distribution of radioactive albumin synthesized by RM, i.e., it was still recovered in the vesicular compartment. Albumin made by RM was released only under conditions which solubilized the membrane (detergent, Fig. 3).

Table II compares the distribution of total TCA-insoluble radioactive polypeptides with respect to their release to the vesicular vs. the extravesicular compartment. In contrast to the distribution recorded for albumin, a relatively large fraction (40%) of TCA-insoluble radioactivity was recovered in polypeptides released directly to the medium surrounding the vesicles. The experiment described in Fig. 4, however, provides strong evidence that this fraction probably arose spuriously and does not represent a population of polypeptides which is normally released directly to the cytosol in vivo. Radioactive polypeptides recovered in the extravesicular space were composed almost entirely of very low molecular weight material which migrated at the front in 13.5% polyacrylamide (SDS) gels. This is in marked contrast to the situation for products discharged into the vesicles themselves where >80% of the radioactivity was present in polypeptides covering a broad range of molecular weights typi-

| Compartment | TCA-insoluble polypeptides | Albumin |
|-------------|---------------------------|---------|
| Extravesicular | 40% Recovery | 12 |
| Vesicular | 37% | 88 |
| Ribosomal | 23% | <1 |
FIGURE 5 Sensitivity to proteolysis of polypeptides synthesized by RM or by membrane-free RNA. RM (approx. 50 μg of RNA) or phenol-extracted cytoplasmic RNA (40 μg) was incubated in the messenger-dependent reticulocyte lysate for 60 min (Table I, Fig. 2). Reactions were terminated by placing in ice water, and inactivated RM were added to those reaction tubes containing membrane-free RNA (to equalize protein content). Aliquots (10 μl) were removed and incubated (26°C, 30 min) with 5 μl of H2O ± 10 μg each per milliliter of trypsin and a-chymotrypsin (bovine pancreatic). An equal volume of double strength SDS sample buffer (Fig. 4) was then added. Samples were co-electrophoresed in 11.5% polyacrylamide (SDS) slab gels, and, after staining and destaining, slices (1 mm) were obtained, and their radioactivity was assayed in scintillation fluid (23). No protease (○—○); plus protease (●—●); protein synthesis in the absence of added liver RNA (▲—▲).

The most widely accepted criterion for establishing whether or not polypeptides synthesized on bound ribosomes have been discharged across the membrane is to subject RM vesicles to limited proteolysis (2, 3, 21). It has previously been demonstrated (3) for a number of secreted proteins that they are largely protected (40-70%) from the action of trypsin and chymotrypsin when their synthesis occurs on RM, but not on detached (membrane-free) polyribosomes. Protection from hydrolysis presumably arises because the polypeptides have been discharged into the confines of RM vesicles, i.e., beyond the side of the membrane peripheral to the cytoplasm, and thus remain inaccessible to the proteases. In the present study, similar protection was observed for most of the products synthesized on RM whereas products synthesized with the use of membrane-free messenger RNA were almost all degraded (Fig. 5). Certainly, there was a limited amount of degradation which occurred to the total product synthesized by RM, but the important observation (Fig. 5) is that the action of proteases in no way altered the pattern of these polypeptides as resolved on SDS-gels, i.e., all polypeptides were apparently equally resistant to the action of the enzymes.

It is concluded, therefore, that the majority of proteins normally synthesized on rat liver RER are not only discharged unidirectionally into the membrane, but are discharged at least to a depth such that on average they remain inaccessible for enzymic proteolysis. As a consequence of this finding, it might be expected that proteins which normally exist as peripheral components at the cytoplasmic surface of membranes, i.e., exposed and therefore available for hydrolysis, are inserted into the membranes after their synthesis on ribosomes free in the cytoplasm (11, 22).

SUMMARY
This paper describes a system for measuring protein synthesis in vitro on hepatic RM. RM were
incubated in a potent messenger-dependent rabbit reticulocyte lysate under conditions where polypeptide chain elongation on RM polyribosomes led to completion and release of full-sized products. When subfractionation of reaction mixtures was subsequently performed, virtually all the products, including albumin, were recovered in the vesicles, not in the extravesicular space (cytoplasmic compartment). Polypeptides synthesized by RM were relatively resistant to proteolysis when reaction mixtures were incubated with trypsin and chymotrypsin, whereas these enzymes degraded most of the product synthesized by the use of membrane-free RNA.

The results are interpreted as indicating that most, if not all, proteins normally synthesized on rat liver RER are passed either deep into or across this membrane after their release from bound ribosomes. Few, if any, proteins appear to be released directly to the cytosol.

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