

CONTROLLED PROTEOLYSIS OF NASCENT POLYPEPTIDES IN RAT LIVER CELL FRACTIONS

I. Location of the Polypeptides within Ribosomes

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ABSTRACT

Free ribosomes containing nascent polypeptide chains labeled *in vitro* were submitted to proteolysis at 0° by a mixture of trypsin and chymotrypsin. Sucrose gradient analysis showed that polysome patterns are retained even after 24 hr of proteolysis in the cold, while messenger RNA-free ribosomes (generated progressively during *in vitro* incorporation) are, within 2 hr, completely dissociated into subunits by trypsin. Although ribosomes and subunits are not extensively degraded into smaller fragments during low temperature proteolysis, changes in the acrylamide gel electrophoresis pattern showed that most ribosomal proteins are accessible to and are partially degraded by the proteases. Ribosome-bound nascent polypeptides are partially resistant to proteolysis at 0°, although they are totally digested at 37° or when the ribosomal subunit structure is disrupted by other means. Radioactivity incorporated into nascent chains during incubation times shorter than 3 min was mostly resistant to digestion at 0°. A larger fraction of the initial radioactivity became degraded in ribosomes which incorporated for longer times. In these ribosomes, the amount of radioactivity which was resistant to proteolysis was constant and independent of the initial value, which reflects the labeled length of the nascent chains. These results suggest that the growing end of the nascent polypeptide is resistant to digestion and is protected from proteolytic attack by the ribosomal structure. A pulse and chase experiment confirmed this suggestion, showing that the protected segment is located at the carboxy-terminal end of the nascent chain. The protected segment was contained in the large ribosomal subunit and had a length of ~39 amino acid residues, as estimated by chromatography on Sephadex G-50.

INTRODUCTION

Membrane-bound ribosomes exist in the liver cell in the form of polysomes (1, 2) which are attached to the outer surface of the membranes of the endoplasmic reticulum (ER) through their large ribosomal subunits (3). The first step in the process of protein secretion is believed to be the transfer of the secretory polypeptides from its site of synthesis

in an attached ribosome into the cavity of the ER (4, 5). An understanding of the mechanism which *in vivo* accounts for this unidirectional transfer can be gained from studying microsomal systems *in vitro* in which the transfer mechanism is still functional (6, 7). With these model systems, it has been learned that the transport site into the

cisternal cavity is close to, or is provided by, the ribosome-membrane junction. An analysis of the conditions under which the *in vitro* transfer is operative supports the idea that the vectorial release relies mainly on structural restrictions imposed by this junction on the direction of movement of the nascent polypeptide (7, 8).

So as to gain more information on the structural interrelationships of the nascent chains, ribosomes, and membranes of the endoplasmic reticulum, we have carried out experiments involving controlled proteolysis of the polypeptides growing in free ribosomes and in rough microsomes. The susceptibility of labeled nascent chains to digestion at 0° by added proteases was taken as an index of their accessibility to the enzymes. This approach is similar to that used by Malkin and Rich (9) in their study of the free polysomes of reticulocytes. In this paper, we present the results of experiments bearing on the location of nascent polypeptides within hepatic ribosomes. In the following paper (10), we examine the relationship of the nascent chains of bound ribosomes to the microsomal membrane.

MATERIALS AND METHODS

A. Fractionation of Liver Cells

Rats were decapitated without anesthesia by a guillotine (Harvard Apparatus Co., Dover, Mass.). The livers were quickly removed and chilled in several volumes of ice-cold 0.25 M sucrose TKM (TKM is 0.05 M Tris-HCl, pH 7.5, at 20°C; 0.025 M KCl; 0.005 M MgCl₂). All subsequent operations were performed in the cold (~4°C). The livers were weighed, minced with scissors in 2 volumes of ice-cold 0.25 M sucrose-TKM, and homogenized in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle. A postmitochondrial supernatant (S-17) was prepared by centrifuging the homogenate for 10 min at 17,000 *g*_{max} in the No. 40 rotor of the Spinco L centrifuge.

B. Preparation of Free Ribosomes

A previous procedure (2) to fractionate S-17 was modified as follows: 5 ml of S-17 were layered over a three-layer discontinuous sucrose gradient, containing 3 ml of 2.0 M sucrose, 2 ml of 1.60 M sucrose, and 2 ml of 1.35 M sucrose, all in TKM. After the gradients were centrifuged for 24 hr in a Spinco No. 40 rotor at 40,000 rpm (105,000 *g*_{av}), the top 7 ml of the gradients were removed with a syringe and discarded. The following 2 ml were collected for the preparation of rough microsomes and bound ribosomes (see following paper [10]). The pellet which contained the

free ribosomes present in the postmitochondrial supernatant (11) was rinsed with water and stored at -20°C.

C. Preparation of Supernatant Fractions Used for *In Vitro* Amino Acid Incorporation

10 ml of S-17 were centrifuged for 4 hr in a Spinco No. 40 rotor at 40,000 rpm to obtain a high-speed supernatant (S-105) which was kept frozen at -20°C and used as a source of RNase inhibitor (2). The pH 5 fraction was prepared from fresh S-105 (12) and dissolved in a 0.1 M Tris-HCl, pH 7.5, at 20°C. A G-100 Sephadex fraction (12) was prepared from fresh S-105 by gel filtration on a column equilibrated and eluted with 0.1 M Tris-HCl, pH 7.5, at 20°C.

D. Amino Acid Incorporation *in Vitro*

The incorporation mixture contained in 1 ml: 0.4 ml ribosomes (5 mg of ribosomes/ml) dissolved in a mixture (9:1) of water and G-100 Sephadex fraction, 0.2 ml of G-100 Sephadex fraction; 0.1 ml of pH 5 fraction, 5 µl of pyruvate kinase; 50 µl L-(³H) 4-5 leucine (50 µc/ml) or 50 µl L-(¹⁴C) leucine (5 µc/ml); 1.0 µmole ATP; 0.5 µmole GTP; 10 µmoles 2-phosphoenolpyruvic acid; 50 µmoles Tris-HCl, pH 7.5, at 20°C; 100 µmoles KCl; 5 µmoles MgCl₂. The components were mixed in the cold and the reaction was started by transferring them to a water bath at 37°. The time to warm the tubes to approximately 30° was observed to be 30 sec, and this was taken as zero time. Aliquots for the low-temperature proteolytic digestion were quickly cooled in ice or frozen in dry ice-acetone and thawed just before use. For measuring incorporation kinetics, samples of 100 µl were removed at the indicated time and pipetted onto Whatman 3 MM filter paper discs, which were transferred after 10 sec into a beaker containing ice-cold 10% TCA. After hot acid hydrolysis and phospholipid extraction (13), the residual radioactivity due to protein and polypeptides was determined by liquid-scintillation counting in 10 ml of toluene-Liquifluor (40 ml of Liquifluor and 960 ml of toluene).

For measuring the release of radioactive polypeptides into the incubation medium, aliquots of the incubation mixture were rapidly frozen (to stop incorporation), thawed, and then centrifuged for 1 hr at 60,000 rpm (A-321 rotor, IEC-centrifuge) for sedimenting the ribosomes. The radioactivity in the released polypeptides was measured in 100-µl aliquots of the supernatants by the filter disc procedure described above.

E. Amino Acid Incorporation *in Vivo*

Ether-anesthetized rats received by injection into the portal vein 0.5 ml of a neutral solution containing

200 μ C of L-(3 H) leucine. After 2 min, the liver was excised and homogenized in 0.25 M sucrose-TKM for cell fractionation.

F. Sucrose Density-Gradient Analysis

Linear sucrose density gradients (14) ranging from 10 to 40% w/v sucrose in TKM were prepared in the Spinco SW 25 tubes (30 ml) and centrifuged at 25,000 rpm (64,000 g_{av}). Gradients (5.1 ml) ranging from 5 to 20% w/v sucrose in TKM were prepared in the Spinco SW 39 tubes and centrifuged at 39,000 rpm (131,000 g_{av}). Samples were layered on top of the gradients, and centrifugation was carried out at approximately 2°C for the times indicated in the text. The centrifuges were stopped without braking, and optical density through the gradients was monitored at 254 $m\mu$ with an ISCO model D fractionator and UV analyzer (Instrument Specialties Co., Lincoln, Neb.). In all figures of gradient analyses, the direction of sedimentation is from right to left.

G. Course of Degradation of Labeled Polypeptides

A solution of trypsin and chymotrypsin (3 mg of each dissolved just before use in 10 ml of ice-cold TKM) was added, to a final concentration of 50 μ g/ml of each enzyme, to cooled or thawed incorporation mixtures (see section D) or to suspensions of ribosomes in TKM (section B) labeled in vivo (section E). The course of digestion at 0°C was followed by the measurement of radioactivity in 100- μ l aliquots by the filter paper disc procedure.

H. Preparation of Samples for Column Chromatography

Polysomes labeled in vitro for 20 min were recovered from the incorporation mixture by sedimentation and resuspended in an equivalent volume of TKM. In proteolysis experiments, aliquots (treated as in section G) and their controls (kept for equivalent times at 0°) were diluted to 10 ml with TKM and centrifuged either for 4 hr at 40,000 rpm (Spinco No. 40 rotor) or for 2 hr at 60,000 rpm (A-321 rotor, IEC centrifuge). The pellets were dissolved in 2 ml of 8.0 M urea, which stops proteolysis (15), but does not inhibit pancreatic RNase activity (16). To each dissolved pellet, 0.5 mg of pancreatic RNase was then added and the solution was incubated for 15 min at 37°. At the end of the incubation, 1 mg of DNP-lysine was added as a marker, and the mixture was centrifuged for 10 min at 1000 g . The supernatant was loaded onto the column described below.

I. Column Chromatography

Characterization of the polypeptide fragments by gel filtration was carried out, as described by Malkin

and Rich (9), in a G-50 Sephadex column, equilibrated with 8 M urea and 10 mM mercaptoethanol. The sample load was 2 ml. Elutions proceeded at a flow rate of about 20 ml/hr, and 2-ml fractions were collected. For each fraction, the radioactivity was measured in a 1-ml aliquot, counted with 10 ml of Bray's solution (17) in a liquid-scintillation spectrometer; the other 1 ml was used for optical density measurements. The column was calibrated with: blue dextran 2000 (2.0 mg in 2 ml) for determining the exclusion volume; bovine ACTH (adrenocorticotrophic hormone), 5.0 mg in 2 ml; oxidized insulin, chain B (20 mg in 2 ml), and DNP-lysine (1 mg in 2 ml) for determining positions in the included volume. All markers were dissolved in 8.0 M urea, 0.01 M mercaptoethanol.

J. Electrophoretic Analysis of Ribosomal Proteins

Ribosomal subunits dissociated by EDTA (5 μ moles/mg of ribosomes) from free polysomes or generated by proteolysis were separated in 5–20% sucrose gradients prepared in 0.050 M Tris-HCl pH 7.5 and 0.025 M KCl for controls and in TKM for digested specimens. The gradients were centrifuged in the SB 283 rotor (IEC centrifuge) for 150 min at 40,000 rpm, and the separated ribosomal subunits were collected and sedimented by centrifuging for 4 hr at 60,000 rpm in an A-321 rotor (IEC centrifuge). The sedimented subunits (1 mg of small subunits, or 3 mg of large subunits) were dissolved in 1 ml of water, and their proteins were extracted overnight at 0° by the addition of 1 ml of 8 M urea — 4 M LiCl — 0.1 M mercaptoethanol. The RNA was then separated by low-speed centrifugation, and the proteins in the supernatant were precipitated by 10% TCA. After two washes with ether, the proteins were dissolved in 0.5 ml of 8 M urea — 0.6 M sucrose containing 2 mg/ml of dithiothreitol, and 50- μ l samples were used for electrophoresis in polyacrylamide gels, which was carried out as recently described (18). Gels were stained with amido black and destained by washing with 7% acetic acid.

K. Source of Materials

Male albino rats of the Sprague-Dawley strain were used. The animals weighed between 150 and 200 g, were maintained on a Purina Chow diet, and were fasted for 18 hr. Chemicals were obtained from the following sources: uniformly labeled L-(14 C)-leucine (Sp. act: 274 mc/mmol) and Liquifluor, from New England Nuclear Corp., Boston, Mass.; L-leucine-(3 H) (Sp. act: 18 c/mmol) from Nuclear Chicago Corp., Chicago, Ill.; GTP and ATP and bovine pancreatic ribonuclease, from Sigma Chemical Co., St. Louis, Mo.; 2-phosphoenol-pyruvic acid and pyr-

uvate kinase (178 EU¹/mg at a concentration of 10 mg/ml), Calbiochem, Los Angeles, Calif. 3 X crystallized trypsin and α -chymotrypsin, from Worthington Biochemical Corp., Freehold, N.J.; Sephadex G-50 and Blue Dextran 2000, Pharmacia, Piscataway, N.J.; *N*-dinitrophenyl-L-lysine (DNP-lys), Nutritional Biochemicals Corp., Cleveland, Ohio. ACTH and the oxidized B chain of porcine insulin, Mann Research Laboratories, New York.

RESULTS

Amino Acid Incorporation In Vitro

As is shown in Fig. 1, in vitro incorporation by free polysomes proceeded rapidly during the first 10 min of incubation and then leveled off at ~20 min. Release of labeled polypeptides from the ribosomes began after a lag of ~1 min and reached a plateau at ~20 min when active incorporation ceased. At this time, ~20–25% of the total radioactivity was found in the supernatant after sedimenting the ribosomes.

A gradual breakdown of polysomes (Fig. 2) occurred during amino acid incorporation in vitro. After 4 min (Fig. 2, middle), a decrease of the number of heavy polysomes and a correlated increase in the number of monomers and dimers in the incubation mixture became apparent by comparison with the zero time control (Fig. 2, left).

After 20 min (Fig. 2, right), heavy polysomes had been almost completely converted into monomers and dimers. Since this conversion did not occur in the absence of an energy supply and clearly differed from the random fragmentation of polysomes caused by RNase action (19), we concluded that it depended on amino acid incorporation in vitro and was probably caused by the sequential release of monomers from the mRNA at the end of the readout process. It should be emphasized that at all times studied, no spontaneous dissociation of ribosomes into subunits was detected under our conditions, and that, as mentioned before, most of the polypeptides were not spontaneously released from ribosomes after the readout process.

Effect of Low Temperature Proteolysis on Free Ribosomes

It has been reported that reticulocyte polysomes are preserved after low-temperature proteolysis in the absence of nucleases, while under the same

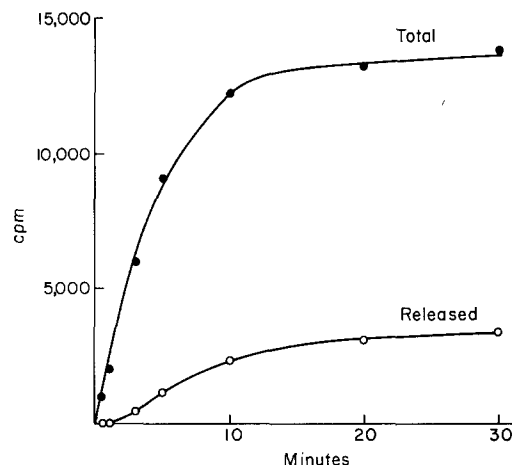


FIGURE 1 Incorporation of leucine-¹⁴C by free polysomes. —●—●—●—, total acid-insoluble radioactivity in 100- μ l aliquots of incubation mixture. —○—○—○—, "acid-insoluble" radioactivity measured in 100 μ l of supernatant after sedimenting the ribosomes (see Materials and Methods, section D). This radioactivity is ascribed to polypeptides released from ribosomes.

conditions monomers are dissociated into subunits (9). Fig. 3 shows that the incubation of free hepatic polysomes with a combination of trypsin-chymotrypsin for 5 hr (Fig. 3 B) and 20 hr (Fig. 3 C) at 0° results in the following changes: (a) A slow, progressive reduction in heavy polysomes by comparison with the control (Fig. 3 A). We assume that this change is due to the RNase known to contaminate commercial trypsin and chymotrypsin since polysomes are not degraded when incubated in pronase (9). (b) A slow production of subunits which after 20-hr digestion account for ~15% of the total ribosomes in the gradient. These subunits are probably derived from the ribosomes which in vivo are not involved in polysome formation. (c) An increase in the amount of monomers which, like polysomes, resist dissociation into subunits. We presume that they derive from polysomes by RNase action and that their resistance is due to their association with short fragments of mRNA.

This assumption is supported by the results of experiments (Fig. 4) in which messenger-free ribosomes (generated from polysomes after completion of amino acid incorporation in vitro [Fig. 4 A]) were digested at 0° with trypsin-chymotrypsin. In this case, 5-hr proteolytic treatment caused a complete dissociation of the ribosomes into subunits (Fig. 4 D). Fig. 4 also shows that this

¹ Enzyme Units.

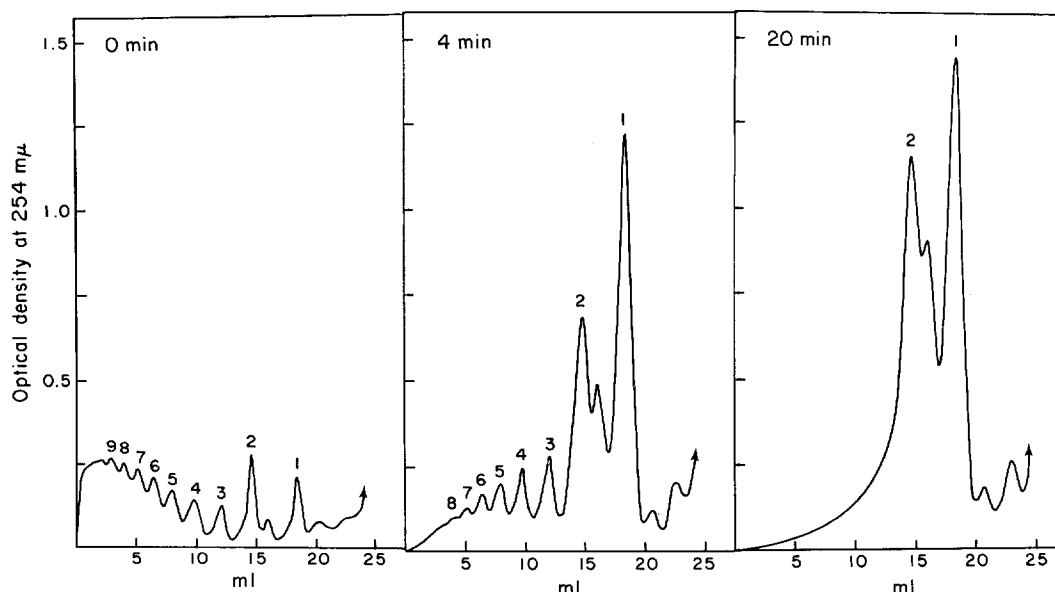


FIGURE 2 Change in the sedimentation profile of free polysomes during amino acid incorporation in vitro. At the times indicated, samples taken from the incubation mixture were rapidly cooled to stop incorporation. Aliquots containing ~ 1 mg of ribosomes were layered onto 10–40% sucrose gradients in TKM and centrifuged for 4 hr at 25,000 rpm in the SW 25 Spinco rotor. Numbers over the peaks correspond to the presumed number of ribosomes in each polysome class.

effect was due to trypsin since incubation in trypsin alone dissociated the ribosomes into subunits (Fig. 4 B), whereas incubation in chymotrypsin only did not (Fig. 4 C).

The kinetics of tryptic dissociation of mRNA-free ribosomes (Fig. 5) show that a considerable degree of dissociation (into subunits) occurred after 10 min of digestion (Fig. 5 B); that the process was nearly complete after 2 hr (Fig. 5 C); and that prolonged incubation (Fig. 5 C and D) resulted in progressive degradation of the small subunit.

Effect of Low-Temperature Proteolysis on Nascent Polypeptides

In their experiments on rabbit reticulocytes labeled in vitro as intact cells, Malkin and Rich (9) have demonstrated that nascent polypeptide chains comprise ribosome-bound segments which are resistant to low-temperature proteolysis and consist of 30–35 amino acid residues. Our experiments show that similar segments exist in the nascent polypeptide chains of rat liver ribosomes.

Initially, we subjected to digestion at 0°, by a mixture of trypsin and chymotrypsin, aliquots of amino acid incorporation mixtures containing rat

liver free ribosomes extensively labeled in vitro in their nascent polypeptide chains. The percentage of initial radioactivity of the sample which remained acid-insoluble with increasing digestion time was determined by filter paper assay. Fig. 6 gives the results of such an experiment: 40% of the radioactivity became acid-soluble during the first 5 min of incubation, then the rate of digestion rapidly slowed down so that $\sim 20\%$ of the initial radioactivity remained resistant to proteolytic digestion after 5 hr. This residual fraction decreased very slowly on more prolonged digestion: after 24 hr, it was reduced to only $\sim 15\%$ of the original value. Similar results were obtained whether the ribosomes had been frozen or not after the in vitro incorporation.

The addition of fresh solution of trypsin and chymotrypsin after 5-hr digestion did not affect the level of residual radioactivity; therefore, the results were not due to protease inactivation. Further incubation at 37° for 1 hr, however, reduced the acid-insoluble radioactivity to less than 5% of the initial value (Fig. 6). Since the residual radioactivity at 0° was ribosome-bound (see below), and since ribosomes are known to be destroyed by trypsin at 37° (20), the result suggested

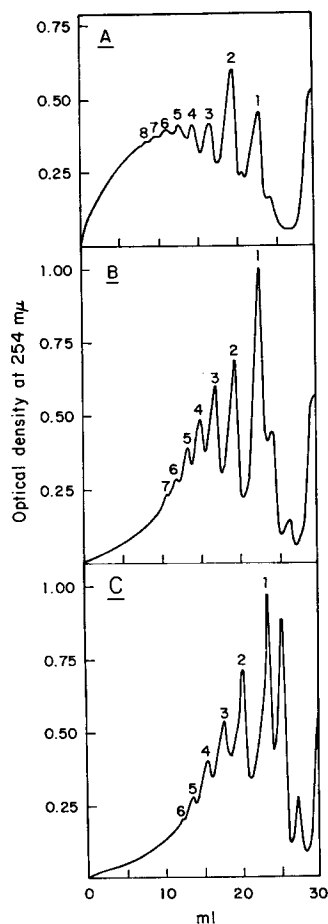


FIGURE 3 Effect of low-temperature proteolysis on the sedimentation pattern of free polysomes. *A*, control polysomes resuspended in TKM and kept at 0°C for 20 hr. *B* and *C*, similar polysomes incubated with trypsin and chymotrypsin at 0°C for 5 and 20 hr, respectively (see Materials and Methods, section G). Samples layered on gradients similar to those of Fig. 2 were centrifuged for 3 hr at 25,000 rpm.

that susceptible linkages in the residual polypeptide became accessible to the enzymes at 37° because of the loss of ribosomal protection. We also found that previous dissociation of ribosomes through chelation of Mg^{++} with EDTA (21) resulted in the extensive digestion of the labeled nascent polypeptides by the same proteases at 0°, presumably on account of a change in conformation undergone by the large ribosomal subunits upon Mg^{++} removal (22, 23).

Additional experiments were carried out to determine the length and the position of the protected

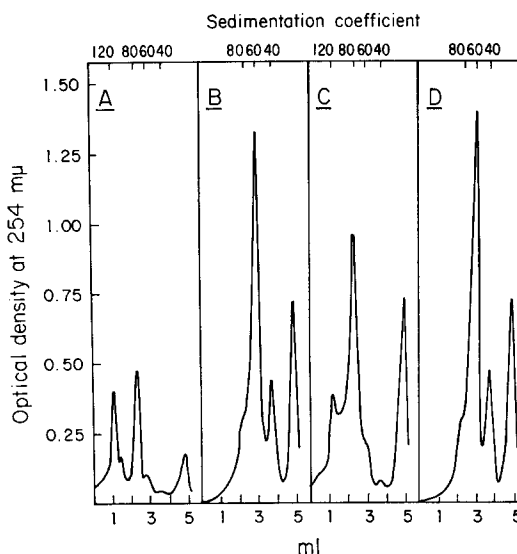


FIGURE 4 Effect of low-temperature proteolysis on ribosomes released from polysomes during amino acid incorporation. After 30 min of amino acid incorporation in vitro, ribosomes were recovered by sedimentation and resuspended in TKM. Sedimentation profiles from: ribosomes kept at 0°C for 5 hr with: *A*, no additions; *B*, trypsin (100 $\mu g/ml$); *C*, chymotrypsin (100 $\mu g/ml$); and *D*, a combination of trypsin and chymotrypsin (50 $\mu g/ml$ of each). Centrifugation in 5–20% sucrose-TKM gradients for 80 min at 39,000 rpm in the SW 39 Spinco rotor.

polypeptide fragment. These results are presented in two different ways. In Fig. 7, the percentage of initial radioactivity not degraded to acid-soluble products is given as a function of the time of proteolysis of samples labeled in vitro for different periods of time. All members in the resulting family of curves showed an initially fast rate of degradation which leveled off at different times at a level that depended on the extent to which amino acid incorporation had been allowed to proceed. Thus, after a very short incorporation (30 sec or 1 min), when a small number of labeled amino acid residues had been added to the carboxy terminal of the nascent chains, most of the label was resistant to digestion. When chains were allowed to grow for a longer time, a larger fraction of their initial radioactivity became digestible by the proteases. This fraction amounted to 80% (of the initial value) after 20 min (Fig. 6), i.e., at a time when incorporation had leveled off.

In Fig. 8, the actual amount of acid-insoluble radioactivity measured after each proteolysis in-

terval is given. This type of plotting brings forward a difference between samples which have incorporated amino acids for either more or less than 3 min.

After 3 min (or more) incorporation, the amount of *residual* radioactivity was independent of the amount of *initial* radioactivity and was practically equal, $\sim 3,000$ cpm, in all samples. After 30 sec or 1 min incubation, however, when

the amount of radioactivity was below 3,000 cpm, the proteolysis curves did not converge to a common value and lay entirely within the level of protection from digestion indicated by the first set of curves.

The results suggest that in a sufficiently long nascent polypeptide two segments can be defined, on the basis of their susceptibility to proteolysis. One segment is of variable length, is accessible to proteolytic enzymes, and is rapidly digested to acid-soluble peptides; the other is largely protected from digestion, accounts for the residual radioactivity, and its length appears to be independent of the extent to which the nascent chains have grown beyond the protection. In our *in vitro* conditions, it took an average time of ~ 3 min to finish this segment.

Figs. 7 and 8 show that the protection of this segment was extensive, but not complete. A small amount of radioactivity in samples incubated for less than 3 min was already susceptible to proteolysis. This lack of total protection after short periods of incubation cannot be explained by nascent peptide release from ribosomes, since release becomes significant only after the first minutes of incubation (Fig. 1).

Two possible explanations could be considered at present: (a) the existence of a small fraction of damaged ribosomes which remain active in amino acid incorporation but no longer confer protection; or (b) differential readout rates among polysomal ribosomes: some of these ribosomes, for instance those near the beginning of the mRNA, may have available longer stretches of unoccupied template and may synthesize at a faster rate than others.

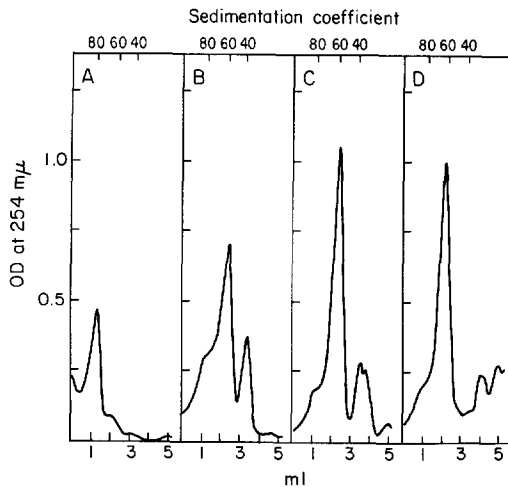


FIGURE 5 Time-course of dissociation by trypsin of messenger RNA-free ribosomes. Ribosomes incubated for 30 min for amino acid incorporation were recovered by sedimentation and resuspended in TKM. Sedimentation profiles from: A, control ribosomes kept for 5 hr at 0° ; B, C, and D, ribosomes incubated with $100 \mu\text{g/ml}$ of trypsin for 10 min (B), 2 hr (C), and 5 hr (D). Gradients similar to those described in Fig. 4 were centrifuged for 100 min at 39,000 rpm in the Spinco SW 39 rotor.

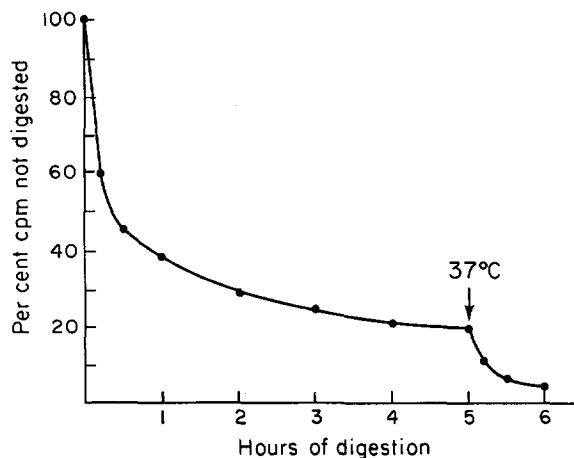


FIGURE 6 Course of proteolysis of *in vitro* labeled nascent polypeptides synthesized on free ribosomes. Incorporation of leucine- ^{14}C by free polysomes was allowed to proceed for 20 min, at which time the mixture was quickly frozen. The thawed sample received trypsin and chymotrypsin (see Materials and Methods, section G), and was incubated for 5 hr at 0°C and for an additional hr at 37° . The acid-insoluble radioactivity remaining at different times was measured as described in Materials and Methods, sections G and D.

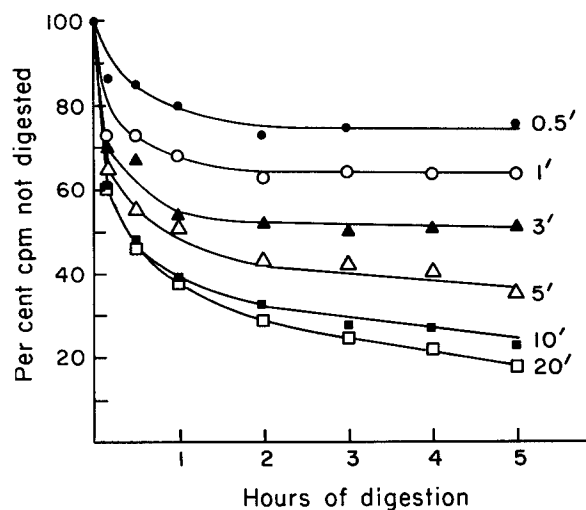


FIGURE 7 Course of proteolysis of nascent polypeptides labeled *in vitro* for different periods of time. At the times indicated, samples were removed from the incorporation mixture and quickly frozen. Digestion of labeled polypeptides was followed as described for Fig. 6. The results are given as percentage of initial radioactivity.

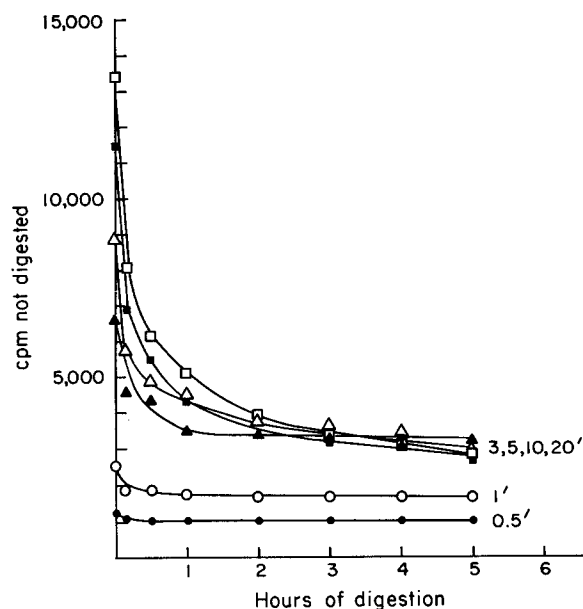


FIGURE 8 Same experiment as in Fig. 7, except that actual amounts of acid-insoluble radioactivity are plotted for each time-point.

Were this the case, a fraction of radioactive peptides—sufficiently long to possess exposed radioactive segments—may be produced even after short incubation times.

Location of the Proteolysis-Resistant Fragment on Nascent Polypeptides

The existence of the two segments defined in the previous section and their relative position on the nascent polypeptide were more firmly established by a pulse-chase experiment (Figs. 9 and 10).

We labeled small segments of nascent chains

(pulse) and studied the effect of proteolysis on these segments before and after growth in a nonradioactive medium (chase). Free polysomes were incubated for 30 sec in an amino acid incorporation system in the presence of ^{14}C -labeled leucine. The mixture was rapidly cooled to near 0°C and was divided into three samples. One of the samples was kept in ice, whereas the other two were reincubated at 37°C for another 20 min. Before reincubation, one of these samples received a 1000-fold excess of unlabeled leucine. At the end of the reincubation, the latter sample is expected to have polypeptide

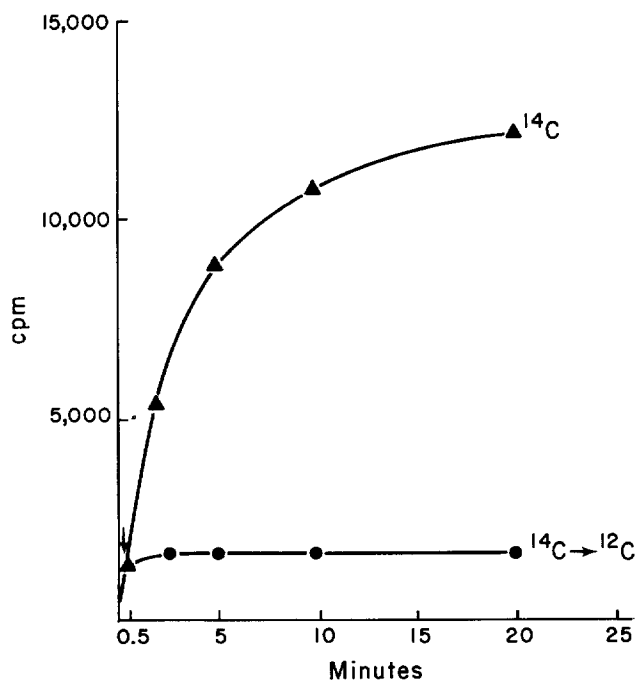


FIGURE 9 *Kinetics of incorporation of leucine- ^{14}C by free polysomes in a pulse-chase experiment.* Polysomes were incubated for 30 sec in an amino acid incorporation mixture containing leucine- ^{12}C . The mixture was rapidly cooled to 0° and divided into three samples. One sample was kept at 0° , and from this a 0.1-ml aliquot containing 100 μg of ribosomes was assayed for acid-insoluble radioactivity incorporated during the pulse (arrow). A second sample was rewarmed to 37° and the subsequent incorporation ($\text{---}\blacktriangle\text{---}\blacktriangle\text{---}\blacktriangle\text{---}$) was followed in similar aliquots. To the third sample, a 1,000-fold excess of leucine- ^{14}C was added, and incorporation at 37° was similarly followed ($\text{---}\bullet\text{---}\bullet\text{---}\bullet\text{---}$).

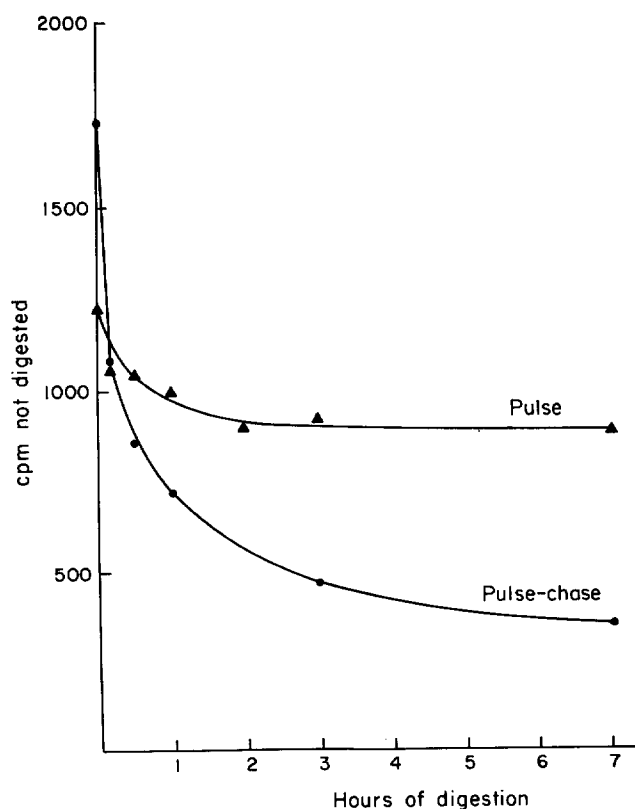


FIGURE 10 *Proteolysis of nascent polypeptides labeled in a pulse-chase experiment.* The samples, taken from the experiment in Fig. 9, contained nascent chains labeled during a 30-sec pulse with ($\text{---}\bullet\text{---}\bullet\text{---}\bullet\text{---}$) or without ($\text{---}\blacktriangle\text{---}\blacktriangle\text{---}\blacktriangle\text{---}$) a subsequent 20-min chase, and were subjected to proteolysis with trypsin and chymotrypsin at 0° .

chains in which the labeled segment is separated from the carboxy terminal end by an unlabeled portion synthesized during the chase period. The data on the kinetics of incorporation are shown in Fig. 9. When amino acid incorporation was stopped after 30 sec, there were 1200 cpm of acid-insoluble radioactivity in a 0.1-ml aliquot (Fig. 9, arrow). After 20 min of incubation with undiluted label, a similar aliquot contained 12,600 cpm. The corresponding value in the sample, in which the leucine- ^{14}C was diluted with an excess of unlabeled amino acid before reincubation, was 1,700 cpm. Thus, the chase was largely effective in reducing the incorporation of labeled leucine during the subsequent 20-min incubation.

Fig. 10 shows the time-course of proteolysis in aliquots of pulsed and pulse-chased samples. The considerable protection of radioactivity observed in the nonchased sample contrasts with the extensive degradation after the chase. This degradation must be attributed to the relocation of the labeled segments, during growth in the chase medium from a carboxy terminal position to a region distant from this terminus. Therefore, the results permit the unambiguous location of the resistant segment at the carboxy terminal end of the nascent polypeptide.

The small fraction of initial radioactivity which remained undigested in the chased sample was probably due to: (a) polypeptide chains which were completed during the 30-sec pulse and consequently could not be chased. In these completed but mostly unreleased chains, the labeled segment remains carboxy terminal and therefore protected; (b) the inactivation during the cooling and dilution step of some previously active (and therefore labeled) free polysomes, which would prevent them from being chased; (c) presence within the chains of some labeled leucine incorporated during the chase since, as was mentioned, the chase was not totally effective and resulted in a slightly higher level of radioactivity than in the pulse.

Determination of the Approximate Length of the Proteolysis-Resistant Segment in Nascent Polypeptides

Like Malkin and Rich (9), we used gel filtration in dissociating solvents to measure the length of the protected polypeptide segment.

Free hepatic polysomes were labeled *in vivo* for 2 min or *in vitro* for 20 min. Samples were cooled to $\sim 0^\circ$ and incubated at this temperature for 5

hr—with or without proteolytic enzymes. Ribosomes containing labeled polypeptides were recovered from this mixture by high-speed sedimentation, and were treated with 8 M urea and ribonuclease, as indicated under Methods. Aliquots of this digest were loaded on a G-50 Sephadex column which, according to the manufacturer, has an inclusion limit of $\sim 10,000$ mol wt for dextrans when eluted with buffers. Proteins with a molecular weight of 16,000 or larger are known to be excluded and to appear in the void volume when the columns are run in 8 M urea (9). This was the case with the labeled polypeptides present in ribosomes not exposed to proteolytic enzymes. It is interesting to note that even after 2 min of *in vivo* labeling all nascent chains still present in polysomes were of sufficient length to be excluded from the G-50 Sephadex column. A likely explanation of this observation is that, during the initial stages of cell fractionation, growth of nascent polypeptide chains continues to some extent, while initiation of new chains is no longer possible. Likewise, all nascent chains labeled *in vitro* eluted with the void volume from the G-50 Sephadex column (peak centered at tube No. 25 in Fig. 11 A), regardless of the time they were allowed to grow *in vitro* (from 30 sec to 20 min). Therefore, *in vitro* initiation of new chains did not occur in our incorporation system; we only observed elongation of nascent chains which had previously grown to a minimum length equivalent to at least 10,000 daltons. After 5 hr of proteolysis in the cold, however,—when according to previous experiments (Figs. 7 and 8) digestion had reached a plateau—the amount of radioactive peptides excluded from the column was greatly reduced (Fig. 11 B). Instead, labeled chains still sedimentable with ribosomal particles were eluted within the included volume of the column as a smaller peak centered on tube No. 40 (Fig. 11 B). Thus, proteolysis shortened the length of all nascent polypeptides to that of a fragment of discrete size considerably smaller than 16,000 daltons.

Further experiments showed that the resistance of this fragment to proteolysis was lost if digestion was carried out under conditions known to affect ribosomal structure, such as incubation with 2 M urea (Fig. 11 C), or at 37°C (Fig. 6). After 4 hr of proteolysis at 0° , the ribosomal material containing the resistant fragment was sedimented. The pellet was resuspended in 2 M urea in 0.1 M Tris-HCl, pH 7.5, and subjected to another hour of proteolysis at 0° . At this concentration of urea,

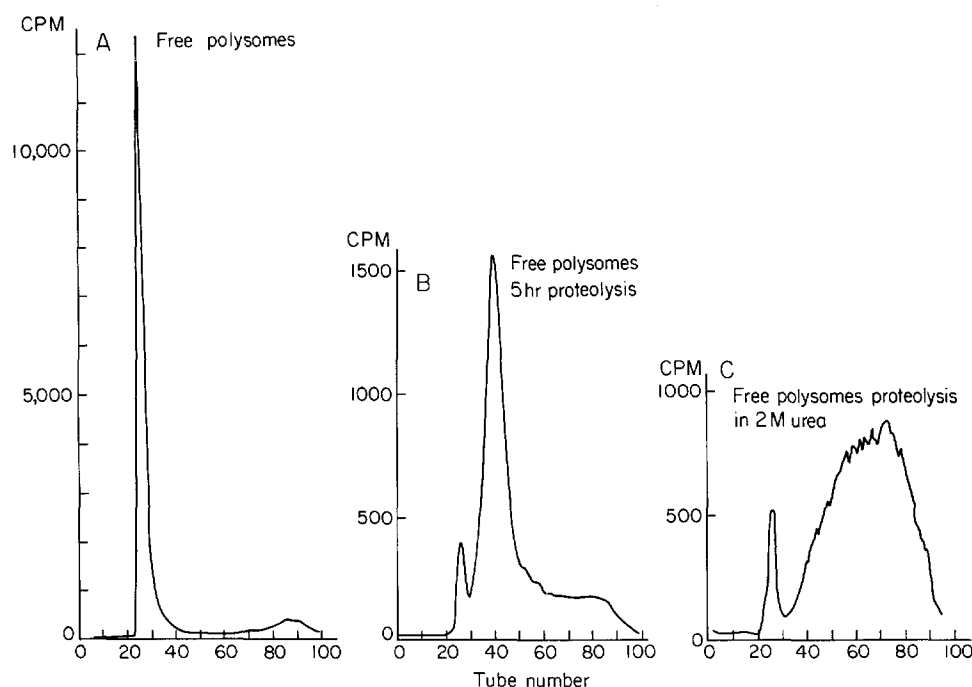


FIGURE 11 G-50 Sephadex column chromatograms of nascent polypeptides labeled *in vitro* in a free polysome system. After leucine- ^{14}C incorporation with or without subsequent proteolysis, all samples were processed by the urea-RNase method (see Materials and Methods, section H). *A*, nascent polypeptides from control free polysomes labeled *in vitro* for 20 min and kept in TKM for 5 hr at 0° . *B*, proteolysis-resistant fragment from similar polysomes incubated at 0° for 5 hr with the mixture of trypsin and chymotrypsin. *C*, degradation of the proteolysis-resistant fragment after 4 hr of incubation at 0° with trypsin and chymotrypsin, and 1 hr of digestion in 2 M urea.

trypsin and chymotrypsin are still fully active (15). Although ribosomal subunits remain as compact particles in 2 M urea (23), we found by sedimentation analysis that in this condition they are extensively degraded by proteolysis into soluble products and slow sedimenting fragments. The chromatographic analysis of the whole mixture—subsequently brought to 8 M urea and treated with ribonuclease—showed the rapid degradation of the resistant fragment to heterogeneously sized polypeptides, eluting as a broad band between tubes Nos. 50 and 80 (Fig. 11 *C*).

Finally, we considered the possibility that the resistant segment was digested in 2 M urea not because of the degradation of the ribosomal subunits, but because 2 M urea produced a change in the secondary structure of the polypeptide which, in itself, made it protease sensitive. To check this possibility, we prepared intact nascent polypeptides from labeled ribosomes by the standard procedure of 8 M urea-ribonuclease treatment. The

preparation was dialyzed against TKM solution to eliminate the urea, and centrifuged to eliminate aggregates. When the supernatant containing labeled nascent chains was treated with trypsin and chymotrypsin at 0° , a total and rapid loss of acid-insoluble radioactivity occurred, indicating that unprotected nascent polypeptides can be totally digested in TKM containing no urea. We concluded that the fragment was resistant to proteolysis because of its protection by ribosomal structure.

Localization of the Protected Fragment in the Large Ribosomal Subunit

It should be recalled (Figs. 4 and 5) that liver ribosomes preincubated for amino acid incorporation are almost completely dissociated into subunits by proteolysis at 0° . For determining the location of the protected segment, large and small subunits generated by a 2-hr proteolysis of ribosomes were

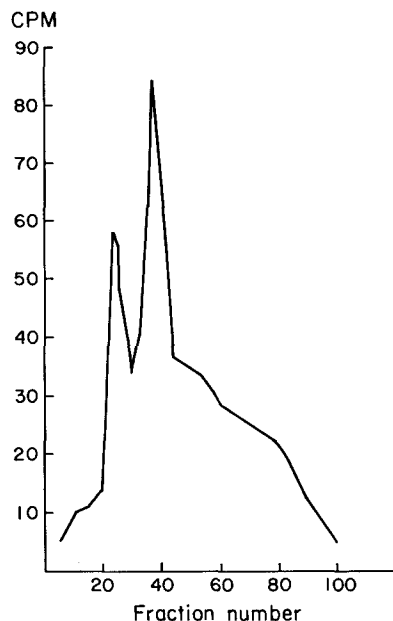


FIGURE 12 Gel filtration analysis of labeled peptides from a preparation of large ribosomal subunits obtained after proteolytic dissociation. After 2 hr of proteolysis, some polypeptides in the large subunits are still eluted with the void volume. The proteolysis-resistant fragments elute at tube No. 40. Small ribosomal subunits contained no radioactive peptides after proteolysis.

separated by zone centrifugation. An aliquot of the incubation mixture was loaded on a 5–20% sucrose-TKM density gradient in tubes of the SW 25 Spinco rotor. After 8-hr centrifugation at 25,000 rpm, the regions of the gradient containing the subunits were collected separately and sedimented (4 hr at 60,000 rpm) in the 321 rotor of the IEC centrifuge. The radioactivity was measured in an aliquot of each sample after the pellets were suspended in 8 M urea and treated with ribonuclease. Only the material from the large subunits was radioactive and was chromatographed in G-50 Sephadex. Fig. 12 shows that after 2 hr of proteolysis some excluded polypeptides are still found in the large subunits, which also contains the protected fragments generated from the digested chains.

Length of the Protected Segment

Fig. 13 gives the elution patterns of some of the probe molecules used to calibrate the G-50 Sephadex columns. Their elution positions were highly reproducible: the peaks were displaced by

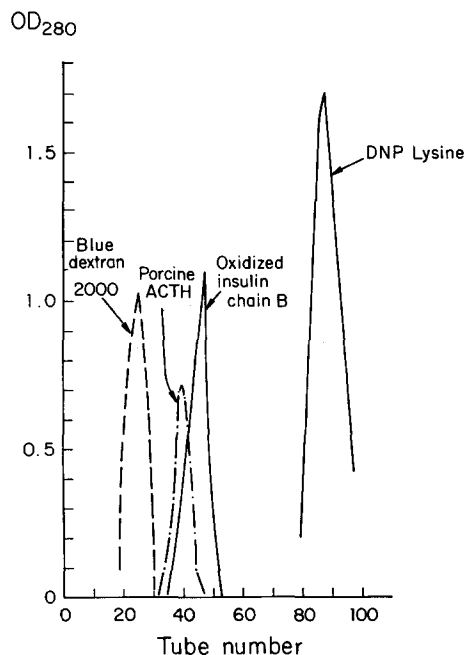


FIGURE 13 Chromatograms of probe molecules used to calibrate the G-50 Sephadex column. All samples were dissolved in 8 M urea, 0.01 M mercaptoethanol (see Materials and Methods, section I).

not more than one tube in different runs. Porcine ACTH, which consists of 39 amino acids, was eluted at tube No. 40, well resolved from the B chain of insulin which has 30 amino acids and emerged at tube No. 48. Since the protected segment of nascent polypeptides was eluted at tube No. 40, its approximate length is estimated at ~39 amino acid residues.

Effect of Proteolysis on the Ribosomal Proteins of the Large Subunits

Fig. 14 A shows the electrophoresis pattern on polyacrylamide gel of a protein fraction obtained from the large subunits separated by EDTA from rat liver free ribosomes. This control pattern which is characterized by sharp bands, and is similar to the patterns published by other authors (18, 24), should be compared with the pattern produced by the proteins of subunits generated from ribosomes which were proteolyzed after in vitro protein synthesis (Fig. 14 B). In the latter, some bands are absent at their normal positions (Fig. 14 B, top part); other bands are not displaced but appear broadened or blurred; finally, new

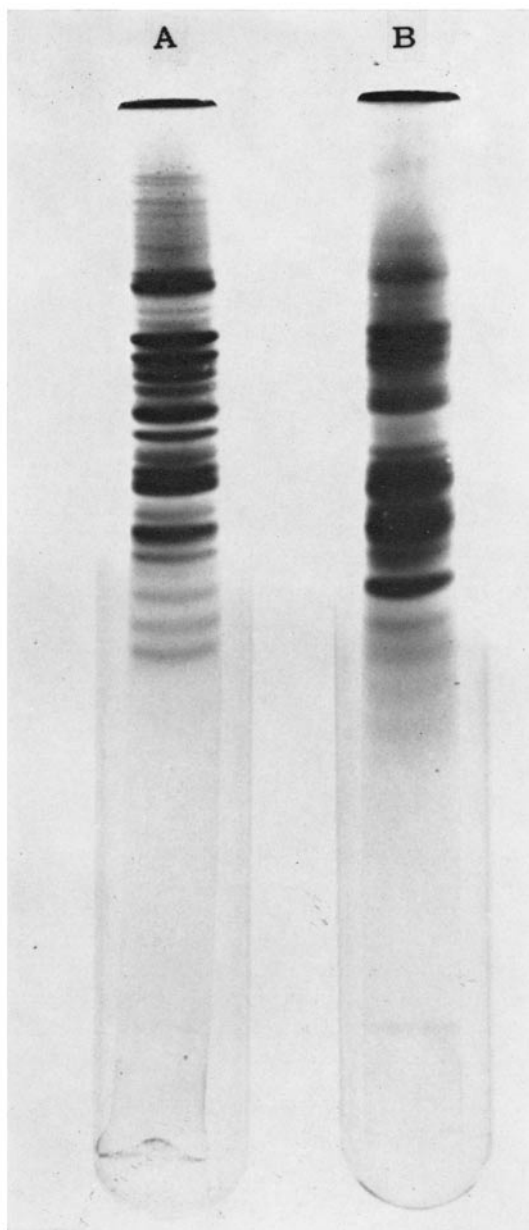


FIGURE 14 Effect of proteolysis on ribosomal proteins from large subunits. Polyacrylamide gel electrophoresis patterns from proteins of: *A*, control large subunits obtained by EDTA treatment of free polysomes; *B*, large subunits produced by proteolysis (5 hr at 0°C) of free polysomes. Migration is toward the bottom (cathode).

faster bands (Fig. 14 *B*, bottom part), which may correspond to degradation products, can be detected. When trypsin and chymotrypsin were

added to the subunits immediately after urea, a pattern identical to the control (Fig. 14 *A*) was obtained. This result demonstrates the effective inactivation of the proteases by 8 M urea, and indicates that the extensive degradation of ribosomal proteins shown in Fig. 14 *A* occurs before the disassembly of the subunits produced by urea. Thus, many ribosomal proteins are accessible to the proteases during the incubation of ribosomes. Under the same conditions, however, the carboxy terminal segment of the nascent polypeptide was inaccessible to the proteases and remained undigested (Fig. 11 *B*) over a length of ~39 amino acid residues.

DISCUSSION

The Protection of Nascent Polypeptides in Rat Liver Free Ribosomes

Our results on the partial resistance of nascent polypeptides to low-temperature proteolysis confirm the observations made by Malkin and Rich (9) on reticulocyte polysomes and extend them to rat liver free polysomes. These authors characterized a proteolysis-resistant fragment in nascent globin chains labeled by incubation of intact cells with radioactive amino acids. In our experiments, the nascent chains were labeled in isolated rat liver free polysomes in an in vitro amino acid-incorporating system. In this experimental set up, it was possible to label nascent polypeptides for times as short as 30 sec, and to study the effect of proteolysis on these short radioactive segments before and after they were relocated by further growth in non-radioactive medium. The results demonstrate that the segment resistant to proteolysis is located near the carboxy-terminal of the nascent chain. We estimate that in rat liver polysomes this segment contains approximately 39 amino acid residues, since by gel filtration analysis it elutes at the same position as porcine ACTH (Fig. 13). By the same method, the proteolysis-resistant segment in reticulocyte ribosomes has been estimated to contain 35 amino acids (9). In the extended configuration (3.6 Å per amino acid residue), a polypeptide of 39 amino acids has a length of ~140 Å, a value which should be compared with the diameter of the large ribosomal subunit with which the polypeptide is associated. In unfixed, negatively stained preparations of large subunits from rat liver ribosomes, the apparent diameter is ~175 – 200 Å (Y. Nonomura et al. Manuscript in preparation),

while in positively stained ribosomes examined in sections of material fixed in OsO_4 it varies between ~ 150 and 200 \AA (25).

Several observations indicate that the resistant segment is protected against protease attack at 0° as long as it maintains its intimate association with the large ribosomal subunit. When we disassembled the ribosomes by treatment with 8 M urea and thereby dissociated the labeled nascent polypeptides from the large subunits, we found that these polypeptides were completely digested by the proteases in TKM at 0° . Furthermore, when the ribosomes were incubated with the proteases at 37° , a condition which is known to produce extensive ribosome degradation (20), rapid digestion of the labeled nascent polypeptides ensued. As was the case with reticulocyte ribosomes, conditions which cause pronounced changes in the organization of liver ribosomal subunits, like treatment with EDTA, also permitted more extensive degradation of nascent chains by proteases at 0° . As recently shown (22, 23), complete Mg^{++} removal leads to a decrease in the sedimentation coefficient at the large subunits of liver ribosomes, presumably on account of profound conformational changes and of loss of the 5 sRNA molecule.

Taken together, our observations suggest that, during digestion in TKM at 0° , the carboxy-terminal segment of the nascent polypeptide is not accessible to the added proteases, owing to a relative preservation of ribosome structure, and that extensive ribosomal disorganization is necessary for complete degradation of nascent chains. As we showed by sedimentation analysis, the polysome pattern of rat liver free ribosomes was largely preserved after incubation for 20 hr with trypsin and chymotrypsin at 0° , whereas a similar treatment of mRNA-free ribosomes (monomers and dimers) caused their complete dissociation into subunits. However, the sedimentation coefficient of the ensuing large subunits was not greatly changed, a finding which suggests that they retained their compact conformation. By isolating the large subunits by zone sedimentation, we showed that they contained the proteolysis-resistant fragment of the nascent polypeptide. Our electrophoretic analysis showed, however, that the structural proteins of these subunits were degraded to a varied extent. Since they are accessible to proteases, it can be inferred that they reach, at least partially, the surface of the large subunits. A similar suggestion has been advanced for *E. coli*

ribosomes from spectroscopic studies (26). In contrast, the proteolysis-resistant carboxy-terminal segment of the nascent polypeptide chain must be buried beyond protease reach within the structure of the large ribosomal subunit. We, therefore, assume that this segment is protected from protease attack because it grows within a channel recessed in the surface of the ribosome or in a tunnel through the interior of the large subunit. In fact, a recent estimate of the degree of hydration of hepatic ribosomal subunits (23) obtained from their hydrodynamic properties indicates that large subunits are more hydrated than small subunits, which is an expected result if the former have an interior space accessible to water. Support for the existence of a space within the large ribosomal subunit can also be drawn from electron microscopic observations of liver ribosomes. In sectioned, OsO_4 -fixed hepatocytes large ribosomal subunits frequently appear as hollow structures with a light central region (25). Similarly, a central region accessible to phosphotungstate or uranyl salts can be observed in negatively stained, large ribosomal subunits isolated after EDTA treatment of ribosomes (Y. Nonomura et al. Manuscript in preparation).

It is interesting to note that other agents involved in peptide chain elongation are also thought to be located in the interior of the ribosome. Thus, it has been shown (27) that polynucleic acid bound to 30 S subunits of *E. coli* ribosomes can be released by Mg^{++} depletion, even after formaldehyde fixation; this does not apply to poly-U (polynucleic acid) bound to monomers subsequently fixed in formaldehyde, presumably because the templates are trapped in between the subunits cross-linked by the fixative. Similarly, it has been shown in *E. coli* ribosomes that aminoacyl-tRNA bound to 30 S subunits in response to a template can be subsequently deacylated with alkali (28) or degraded with RNase (29). But both effects are prevented if a 50 S subunit is subsequently bound to the 30 S template-aminoacyl-tRNA complex (28, 29). Finally, the peptidyl-synthetase (transferase) activity has been shown to be an integral part of the large ribosomal subunit (30).

Thus, a location of the growing end of the peptidyl-tRNA molecule in the interior of the large ribosomal subunit appears compatible with the topological requirements so far established for amino acid transfer.

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