Studies of the Effect of Proteolytic Enzymes on Ribosomes and Polysomes from Reticulocytes and Rat Liver*

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ABSTRACT: Mild treatment of polysomes from rabbit reticulocytes and rat liver with proteolytic enzymes leads to their dissociation into smaller units and into monomers. Liver ribosomal dimers which are insensitive to ribonuclease are split into monomers and subunits by trypsin and chymotrypsin. The polyuridylic acid induced stimulation of polyphenylalanine synthesis is markedly reduced by pretreatment of polysomes with small amounts of trypsin.

The inhibition so obtained is more marked than the effect produced on the incorporation of L-[^{14}C]-phenylalanine into proteins by polysomes in the absence of polyuridylic acid. These effects may be secondary to an alteration of the ribosomal binding sites for polyuridylic acid and messenger ribonucleic acid.

It is generally considered that the fundamental unit of protein synthesis is an aggregate of ribonucleoprotein particles held in position by messenger ribonucleic acid (m-RNA). It has been shown, for instance, that mild treatment with ribonuclease results in dissociation of reticulocyte polysomes; that the strand linking individual ribosomes to one another stains positively with uranyl acetate in electron micrographs; and that RNA with messenger activity can be isolated from polysomes upon their dissociation (Gierer, 1963; Risebrough *et al.*, 1962; Warner *et al.*, 1963; Wettstein *et al.*, 1963; Arnstein *et al.*, 1964). However, in a study of ribosomal particles from rat heart muscle it was found by Rabinowitz *et al.* (1964) that these aggregates are only partially broken down by ribonuclease treatment and are also partially broken down by incubation with chymotrypsin or trypsin. When ribonuclease and proteolytic enzyme are used simultaneously nearly all of the "heavy" material is converted to 70–80S particles. Furthermore, ribosomal clusters involved in the synthesis of collagen and γ-globulin are not totally broken down to monomers by RNAase alone but require proteolytic digestion in addition (Kretsinger *et al.*, 1964; Manner and Gould, 1965). It was therefore suggested that in some instances ribosomal particles may be held together by more than messenger RNA alone. In consequence, therefore, a detailed study of the behavior of well-characterized polysomes from reticulocytes and rat liver under conditions of mild proteolysis was undertaken.

Materials and Methods

Escherichia coli strain B was grown in a medium supplemented with 0.2% dextrose and 0.2% casamino acids as described by Gilbert (1963). The cells were harvested in the exponential phase (OD 550 mμ approximately 0.4) and ribosomes were isolated and prepared according to the method of Nirenberg and Matthaei (1961). Reticulocyte polyribosomes were obtained from rabbits made anemic by intraperitoneal injections of a neutral 2.5% solution of phenylhydrazine for 5 days. The blood was obtained by cardiac puncture, lysed in a hypotonic medium, and subjected to differential centrifugation for the isolation of polysomes according to the method of Arnstein *et al.* (1964). Reticulocyte polysomes were suspended in a medium which contained 0.25 M sucrose, 0.025 M KCl, 0.001 M $MgCl_2$, 0.05 M Tris–HCl, pH 7.6 (medium A_4). In general such preparations contained about two-thirds of the ribosomes in the form of aggregates. These ribosomes were similar in amino acid incorporating ability and sedimentation characteristics to those described by Arnstein *et al.* (1964). Polysomes from rat liver were prepared from deoxycholate-treated post-mitochondrial supernatant as described by Wettstein *et al.* (1963). In addition, liver ribosomal dimers and monomers were isolated by using a sucrose density gradient with a 2 M sucrose "step" at the bottom of the tube (Wettstein *et al.*, 1963) or by preincubation of ribosomes obtained by the method of Tashiro and Siekevitz (1965) in the amino acid incorporating system described by Nirenberg and Matthaei (1961).

Ribosomes were analyzed on linear sucrose density gradients. For *E. coli* and liver ribosomes the gradients were prepared with 15 and 30% sucrose solutions containing 0.05 M $MgCl_2$, 0.005 M Tris–HCl, pH 7.6, and 0.05 M KCl. For reticulocyte ribosomes the gradients were made with 15% sucrose and 30% sucrose but the solutions contained 0.0015 M $MgCl_2$, 0.01 M Tris–HCl,

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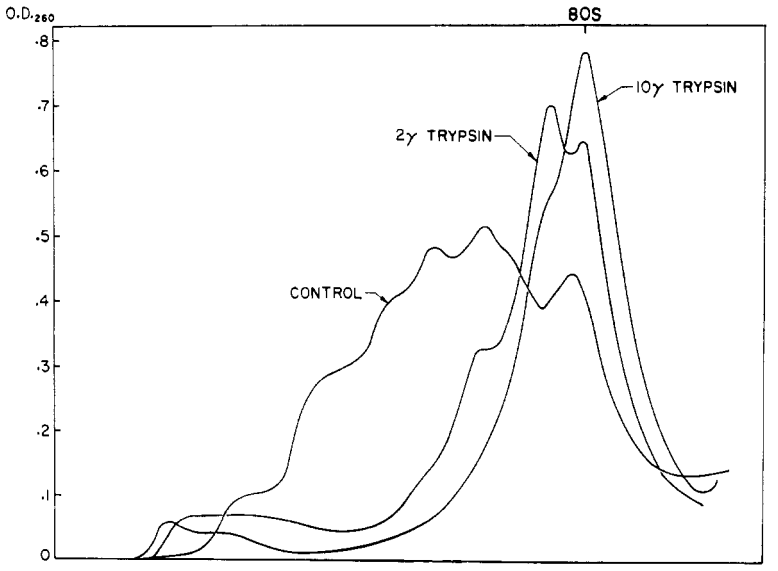

FIGURE 1: Effect of trypsin on the sedimentation profile of rat liver polysomes. The polysomes were prepared by the method of Wettstein *et al.* (1963). Samples were incubated with 2 and 10 μg of trypsin/A_{260} of RNP, respectively, in a medium containing 1 × 10^{-3} M $MgCl_2$, 5 × 10^{-2} M KCl, and 5 × 10^{-2} M Tris-HCl, pH 7.5, for 30 min at 0°. The reaction was terminated by the addition of a fivefold excess (w/w) of soybean trypsin inhibitor. A 0.2-ml aliquot containing 4 A_{260} of RNP, preincubated as described above, was layered on the top of a linear 5–20% sucrose density gradient as described in the Methods. The centrifugal run was for 1 hr at 39,000 rpm in the SW-39 rotor of the Model L-2 Spinco ultracentrifuge.

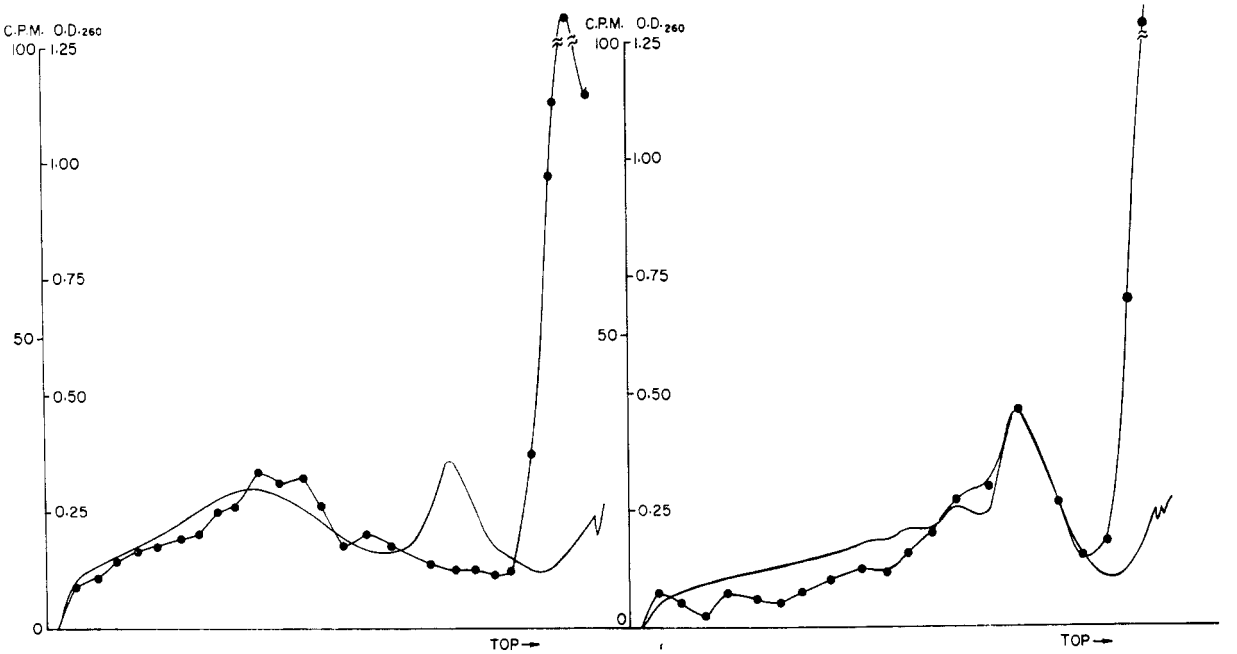

FIGURE 2: Effect of α-chymotrypsin on the sedimentation profile of reticulocyte polysomes. The ribosomes were labeled in intact reticulocytes by incubating 5 ml of packed reticulocytes in a medium containing: 10 μc of L-[^{14}C]-leucine (sp act. 41.8 μc/ml), 2 × 10^{-4} mole of each of the remaining 19 L-[^{12}C]amino acids, 5 × 10^{-2} M Tris-HCl, pH 7.6, 2.5 × 10^{-3} M KCl, 5 × 10^{-3} M $MgCl_2$, and 20 mg of glucose in a final volume of 7.5 ml for 15 min at 37° (Borsook *et al.*, 1957). The incubation was terminated by chilling the flask in ice and by the addition of 4 × 10^{-2} mole of L-[^{12}C]leucine. Polysomes were then isolated by the method of Arnstein *et al.* (1964) and an aliquot was incubated with 20 μg of α-chymotrypsin/A_{260} of RNP for 30 min at 0°; 4.6 A_{260} units of the treated ribosomes and untreated controls were layered on 5–20% sucrose density gradients, and spun in the SW-39 rotor of the Spinco L-2 ultracentrifuge at 30,000 rpm for 130 min at 0°. The left-hand panel shows the profile of the control sample; on the right is shown the profile after treatment with the proteolytic enzyme. Ten-drop fractions were collected on rectangular strips (3.25 × 1 in.) of Whatman 3MM paper and the radioactivity assayed by the method of Mans and Novelli (1960).

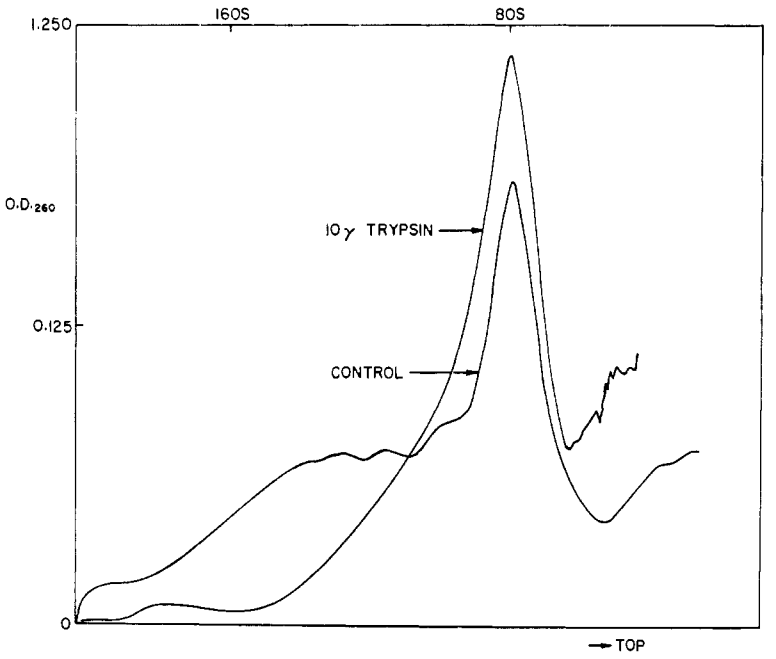

FIGURE 3: Dissociation of reticulocyte polysomes by trypsin; 4 A_{260} units of polysomes were incubated for 30 min at 0° in the presence of trypsin (10 μg of trypsin/A_{260} of RNP). The reaction was carried out in a medium containing 1×10^{-3} M $MgCl_2$, 2.5×10^{-2} M KCl, 5×10^{-2} M Tris–HCl, pH 7.6, and 0.25 M sucrose (medium A_4). The incubation was terminated by the addition of a fivefold excess of soybean trypsin inhibitor. An aliquot (3 A_{260} units) was analyzed by the sucrose density gradient method as described in the Methods. The centrifugal run was for 130 min at 30,000 rpm and 0° in the Spinco SW-39 rotor.

pH 7.6, and 0.01 M KCl. On the top of each gradient 0.2 ml of ribosomal suspension containing 2–5 A_{260} units was carefully layered and the gradients were spun at 30,000 rpm for 2 hr at 4° in the SW-39 rotor of the Model L 2 Spinco ultracentrifuge. In some instances the gradients were run at 39,000 rpm for 1 hr. The gradients were analyzed by running the contents through a Gilford multiple absorbance recorder (Model 2000).

The cell-free system for L-[^{14}C]phenylalanine incorporation into *E. coli* ribosomes was according to the method of Nirenberg and Matthaei (1961). Each tube contained in a final volume of 0.1 ml the following: 2 $A_{260m\mu}$ of ribonucleoprotein; 5×10^4 cpm L-[^{14}C]-phenylalanine; 5 μmoles of Tris–HCl buffer, pH 7.6; 1.5 μmoles of $MgCl_2$; 5 μmoles of KCl; 0.75 μmole of mercaptoethanol; 0.1 μmole of ATP;[1] 0.2 μmole of phosphoenol pyruvate; 0.025 μmole of GTP; 2.5 μg of pyruvate kinase (Boehringer); 0.1 mg of *E. coli* 105,000*g* supernatant protein; and 20 μg of polyuridylic acid (poly-U) when indicated. For reticulocyte ribosomes the cell-free system of Arnstein *et al.* (1964) was used. The system is similar to the one described above except that the $MgCl_2$ concentration used was 8 mM; 10 mM glutathione was used instead of mercaptoethanol and 1 mg of "pH 5" enzymes replaced the 105,000*g* supernatant. Each tube was assayed in duplicate; the incubations were carried out for 5–15 min at 35°. Ribosomes were also labeled with [^{14}C]leucine (specific radioactivity 41.8 μc/μmole) in intact reticulocytes by the method of Borsook *et al.* (1957). In experiments involving cell-free systems the amount of radioactivity incorporated into the ribosomes was assayed as follows. The reaction mixture was treated with 10% TCA containing 0.1% L-[^{12}C]phenylalanine at the end of the incubation, and 1 mg of bovine serum albumin was added. The precipitate was washed on Millipore membranes with cold and hot (90°) TCA. The filter and the protein were then dissolved in Bray's (1960) solution and counted in a Tri-Carb liquid scintillation counter with approximately 49% efficiency. Radioactivity in aliquots from sucrose density gradients was determined by the filter paper method of Mans and Novelli (1960).

Trypsin, α-chymotrypsin, and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. Estimations of peptide release following mild proteolysis of ribosomes were made by the quantitative ninhydrin method of Spies (1957). Polyuridylic acid was purchased from Miles Chemical Co. and L-[^{14}C]-phenylalanine (sp act. 333 mc/mM) from New England Nuclear Corp.

[1] Abbreviations used: ATP, adenosine triphosphate; GTP, guanosine triphosphate; TCA, trichloroacetic acid, SBTI, soy bean trypsin inhibitor; RNP, ribonucleoprotein.

TABLE I: Assay for Latent Ribonuclease Activity.[a]

Addn	Cpm/0.2-ml Aliquot
[^{32}P]RNA, medium A_4	298
[^{32}P]RNA, trypsin	297
[^{32}P]RNA, SBTI	288
[^{32}P]RNA, reticulocyte polysomes	250
[^{32}P]RNA, reticulocyte polysomes, trypsin; SBTI at end of incubation	233
[^{32}P]RNA, reticulocyte polysomes, SBTI, and trypsin	235
[^{32}P]RNA, *E. coli* ribosomes	337
[^{32}P]RNA, *E. coli* ribosomes, trypsin; SBTI at end of incubation	387
[^{32}P]RNA, *E. coli* ribosomes, SBTI, and trypsin	372

[a] The incubation mixture contained in a final volume of 0.2 ml: 2.5 A_{260} units of reticulocyte polysomes or *E. coli* ribosomes (where indicated), trypsin 5 μg, 1×10^{-3} M $MgCl_2$, 5×10^{-2} M Tris–HCl, pH 7.6, 5×10^{-2} M KCl, and 0.25 M sucrose. Each tube contained 1.5 μg of RNA from rat liver (sp act. 4397 cpm/μg) and the incubation was carried out for 30 min at 0°. SBTI (10 μg) was added either at the beginning or the end of the incubation as shown above. The reaction was terminated by the addition of 0.2 N $HClO_4$. Yeast RNA (1 mg) was added to the mixture which was then spun at 2500 rpm $\times$ 10 min and 0.2-ml aliquots of the supernatant solution were counted in a dioxane-based scintillation mixture.

TABLE II: Effect of Trypsin and SBTI on RNAase Activity.[a]

Addn	Cpm/0.2-ml Aliquot
[^{32}P]RNA, RNAase (1 μg)	1519
[^{32}P]RNA, reticulocyte polysomes, trypsin, RNAase; SBTI at end of incubation	1616
[^{32}P]RNA, reticulocyte polysomes, SBTI, trypsin, RNAase	1558
[^{32}P]RNA, trypsin, RNAase (1 μg)	1445
[^{32}P]RNA, SBTI, RNAase (1 μg)	1550

[a] Conditions for incubation and counting of radioactivity as in Table I.

Results

Proteolytic digestion of ribosomes was carried out at 0° for 30 min with trypsin or chymotrypsin; in the case of trypsin the reaction was terminated by adding a fivefold excess by weight of soybean inhibitor. The details of the procedure are given in the legends to Figures 1 and 3. Suitable controls were run to show that soybean trypsin inhibitor had no dispersive effect on the ribosomal aggregates, or in any way impaired their ability to synthesize protein. We have detected no activation of latent ribonuclease upon the proteolytic breakdown of ribosomes (Table I; Zak *et al.*, 1966). Under the conditions of our incubation (30 min at 0°) neither trypsin nor soybean trypsin inhibitor had any ribonuclease activity, and both failed to inhibit the breakdown of RNA by ribonuclease (Table II). Furthermore, when the ribosomes were centrifuged for 2 hr at 151,000*g* after mild tryptic digestion at 0° the supernatant solution showed no significant release of material reacting with ninhydrin (Table III). It is estimated from four such experiments that the amount of amino acid released does not exceed 1% of the amino acids present in the ribosomal protein.

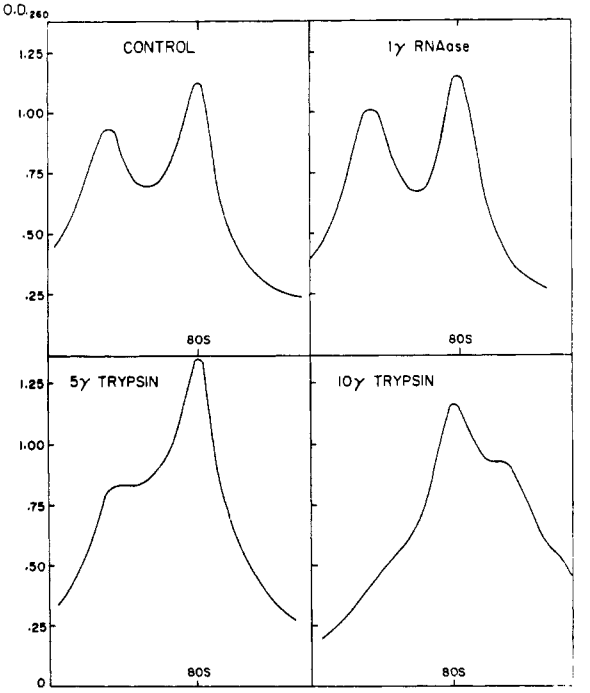

FIGURE 4: Dissociation of liver ribosomal dimers by trypsin. The dimers were obtained by sucrose density-gradient fractionation (Wettstein *et al.*, 1963) of liver ribosomes prepared by the method of Tashiro and Siekevitz (1965). Samples were incubated with 1 μg of RNAase/A_{260}, 5 μg of trypsin/A_{260}, and 10 μg/A_{260}, respectively, for 30 min at 0° in a medium containing 1×10^{-3} M $MgCl_2$, 5×10^{-2} M Tris–HCl, pH 7.6, and 5×10^{-2} M KCl. The tryptic digestion was stopped as usual and $4A_{260}$ units were analyzed on linear sucrose-density gradients as mentioned in the text. The gradients were run at 0° for 1 hr at 39,000 rpm in the SW-39 rotor of the Spinco L-2 ultracentrifuge. The sedimentation values are relative to that of *E. coli* monomers run under similar conditions.

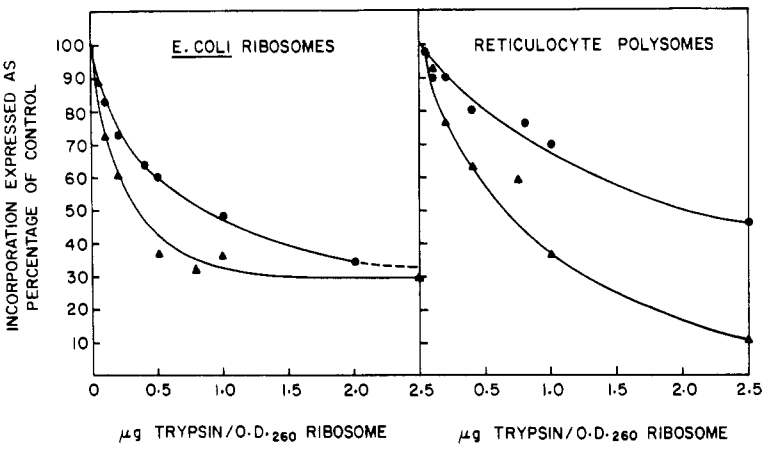

FIGURE 5: Effect of trypsin on the incorporation of L-[^{14}C]phenylalanine in *E. coli* monomers and reticulocyte polysomes in the presence and absence of poly-U. Both *E. coli* monomers and reticulocyte polysomes were preincubated in tubes containing 2 A_{260} units of ribosomes and trypsin at varying concentrations ranging from 0.1 μg of trypsin to 2.5 μg of trypsin/A_{260} of ribosome for 30 min at 0°. The final volume of the incubation mixture was 0.2 ml and was identical in composition with that mentioned in Figure 1. The reaction was stopped with soybean trypsin inhibitor. Control samples were incubated in the presence of trypsin inhibitor alone. The ribosomes, thus pretreated, were incubated for a second time in a complete amino acid incorporating system as mentioned in the text. The incubations were carried out for 5 min at 37°. Poly-U was added at a concentration of 10 μg/A_{260} of ribosome. ▲——▲, trypsin-treated ribosome plus poly-U. O——O, trypsin-treated ribosomes. [Control values (100%) are as follows: 2165 cpm/A_{260} of reticulocyte ribosomes; 12,219 cpm/A_{260} of reticulocyte ribosomes plus poly-U; 187 cpm/A_{260} of *E. coli* ribosomes; 2397 cpm/A_{260} of *E. coli* ribosomes plus poly-U].

TABLE III: Assay for Release of Material Reacting with Ninhydrin.[a]

Addn	A_{570} unit Ninhydrin Reaction
Reticulocyte polysomes	0.031
Reticulocyte polysomes, SBTI, trypsin (2 μg/A_{260} (8 μg/ml))	0.109
Reticulocyte polysomes, trypsin, incubate 30 min, then SBTI	0.112
E. coli ribosomes	0.100
E. coli ribosomes, SBTI, trypsin (2) μg/A_{260} (8 μg/ml))	0.159
E. coli ribosomes, trypsin, incubate 30 min, then SBTI	0.169

[a] The incubation mixture contained in a final volume of 0.25 ml the following. Reticulocyte polysomes or *E. coli* ribosomes 2.5 A_{260} units, 1×10^{-3} M $MgCl_2$, 5×10^{-2} M Tris–HCl, pH 7.6, 5×10^{-2} M KCl, and 0.25 M sucrose. Trypsin was added at a concentration of 2 μg/A_{260} of ribosome (8 μg/ml) and 10 μg of SBTI where indicated. After incubation for 30 min at 0° the volume of the mixture was increased to 1.6 ml with cold medium A_4 and the contents centrifuged at 50,000 rpm in the Ti-50 head of the Spinco L-2 ultracentrifuge for 2 hr at 0°. Aliquots (0.1 ml) of the supernatant solution were treated with ninhydrin reagent (Spies, 1957).

Figures 1–3 show that in general the effect of both trypsin and chymotrypsin is to cause dissociation of the ribosomal aggregates into monomers. Liver ribosomal dimers are not dissociated on incubation with 1 μg of RNAase/ml at 0° for 30 min, but treatment with trypsin at a concentration of 5 μg/A_{260} of ribosome at 0° for 30 min leads to breakdown of the dimer (Figure 4). At double this concentration of trypsin the dimeric structure is almost completely broken down and some subunits are also seen (Figure 4).

Figure 1 shows the behavior of rat liver ribosomal aggregates when submitted to mild proteolysis. At a concentration of 2 μg of trypsin/A_{260} of ribosome a considerable dissociation of the heavier particles has already occurred, and with 10 μg of trypsin/A_{260} of ribosome the ribosomes are almost completely in the monomer form although a small shoulder representing dimers remains.

Reticulocyte ribosomal aggregates also break down chiefly into monomers under conditions of mild proteolytic attack. Figure 2 shows the effect of 10 μg of α-chymotrypsin/A_{260} of ribosome at 0° for 30 min. The ribosomes were labeled first in intact reticulocytes with L-[^{14}C]phenylalanine. Whereas the label is chiefly associated with the polysome peak in the control preparation, the effect of chymotrypsin is to cause the label to be present mainly in the monomer peak.

Similarly, trypsin causes a considerable breakdown of reticulocyte polysomes (Figure 3). A marked flattening of the polysomes peak is observed with commen-

TABLE IV: Effect of Pretreatment with Trypsin on the Response of Reticulocyte Polysomes to Polyuridylic Acid.[a]

Duration of Pretreatment with Trypsin (5 $\mu g/A_{260}$), min	Complete System − Poly-U (cpm/A_{260})	Control %	Complete System + poly-U (cpm/A_{260})	Control %
0 (control)	2514.0		6321.4	
2	1332.0	52.9	1449.0	22.9
5	980.7	38.9	1005.4	15.9
10	504.0	20.0	615.5	9.7
15	388.7	15.4	569.4	9.0

[a] Tubes containing 2 A_{260} units of reticulocyte polysomes were preincubated in the presence of trypsin at a concentration of 5 $\mu g/A_{260}$ for 0–15 min at 0°. The reaction was run in duplicate and at the end of the pretreatment, a fivefold excess of soybean trypsin inhibitor was added. A second incubation was then carried out in a complete amino acid incorporating system containing 1 μc of L-[^{14}C]phenylalanine, 19 remaining unlabeled amino acids, and other factors as described in the text.

surate increase in the height of the monomer peak. We have also observed some degree of subunit formation at higher concentrations of trypsin.

Figure 5 compares the effect of varying amounts of trypsin on the response of *E. coli* monomers and reticulocyte polysomes to poly-U directed polyphenylalanine synthesis. It can be readily seen that in both cases the response to poly-U is more sensitive than the incorporation of L-[^{14}C]phenylalanine in its absence. It is also notable that this effect is manifest at as low a dose as 0.1 μg of trypsin/A_{260} of ribosome, a level at which we have previously shown that subunit formation is not present. A similar observation has been made by Kaji and Kaji (1965) with respect to *E. coli* ribosomes.

Furthermore, pretreatment of reticulocyte polyribosomes with small amounts of trypsin for varying intervals shows that the poly-U directed stimulation of polyphenylalanine synthesis is reduced within a few minutes of exposure to the proteolytic enzyme (Table IV).

Discussion

Although there is evidence to suggest that in some instances ribosomal aggregates are held together not only by a m-RNA strand but also by nascent peptide chains, it is clear that the complete dissociation of reticulocyte polysomes and liver ribosomal dimers by trypsin is due to structural alterations in ribosomal protein itself. The further breakdown of monomers to subunits under relatively high concentrations of proteolytic enzymes additionally supports the conclusion that the primary effect is on the protein moiety of the ribosome. Under the conditions of our assay system no evidence for activation of latent ribonuclease was found. Hence it is safe to conclude that the effects observed were due to mild proteolytic damage of the polysomes leading to their disaggregation.

In an earlier communication (Zak *et al.*, 1966) we showed that trypsin treatment of ribosomes from *E. coli* and reticulocytes causes marked reduction in their ability to bind poly-U, and to a lesser extent, an impairment in the binding of charged soluble RNA (s-RNA). Pretreatment of ribosomes with proteolytic enzymes affects their capacity to synthesize protein; synthesis of new peptide chains under the direction of natural m-RNA is less affected than that involving an artificial messenger such as poly-U (Figure 5). These findings suggest that tryptic digestion affects the binding of poly-U to ribosomes, presumably by damaging ribosomal protein, and that release of natural m-RNA from polysomes is also likely. It is also possible that aggregation of ribosomes into a polysomal structure renders them less susceptible to the effects of tryptic digestion.

Aepinus (1965) observed that liver polysomes are broken down with trypsin but not with chymotrypsin. He therefore concluded that the proteins involved in the stability of polysomes must be basic in nature. We have noted no such difference between the effects of trypsin and chymotrypsin (see Figures 1 and 2) and cannot substantiate this claim. Our observations on the effects of proteolytic enzymes on ribosomal aggregates from heart muscle (Rabinowitz *et al.*, 1965) are also different from those of Breuer *et al.* (1964) who noted no breakdown of heart muscle polysomes with chymotrypsin as judged by electron microscopy. Furthermore, unlike Wilson and Hoagland (1965), we have been able to dissociate rat liver ribosomal aggregates with proteolytic enzymes. These effects are obtained with proteolytic enzymes of very high purity (Worthington two times crystallized) with no detectable ribonuclease activity. Soybean trypsin inhibitor, added in excess, completely abolishes the effects of trypsin on ribosomes, and DFP–chymotrypsin when substituted for chymotrypsin produces no effect on ribosomal integrity. It is of interest that although liver ribosomal dimers are insensitive to ribonuclease, chymotrypsin or trypsin produces dissociation of dimers into monomers (Figure 4). Here again, a relatively mild disturbance in the

structure of ribosomal protein results in a dislocation of the bonds holding the monomers together.

The authors conclude that mild proteolysis of ribosomal aggregates from different tissues leads to damage of ribosomal protein with consequent disaggregation of ribosomes and their breakdown into monomers and subunits. The binding of m-RNA and s-RNA is affected and hence an adverse effect on the capacity of the ribosome to synthesize protein obtains.

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