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The Effect of Ribonuclease on Rat-Liver Ribosomes

By R. BRENTANI, M. BRENTANI and I. RAW
Department of Biochemistry, School of Medicine, University of São Paulo, Brazil
AND
J. L. M. CUNHA and N. WROTSCHINCKY
Laboratory of Biochemical Research, Institute Adolfo Lutz, São Paulo, Brazil

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1. Rat-liver ribosomes lose about 50% of their amino acid-incorporating activity when preincubated with ribonuclease. 2. This preincubation results also in loss of about 50% of the original protein content and 75% of the RNA. 3. Ribosomes sedimented by ultracentrifugation, after preincubation with ribonuclease, show negligible contamination by crystalline enzyme. 4. Washing of ribosomes treated with ribonuclease releases further protein, restoring the original RNA/protein ratio. 5. The washed particle is again capable of promoting amino acid incorporation. 6. Examination of ribosomes treated with ribonuclease in the analytical ultracentrifuge reveals destruction of ribosomes, disappearance of dimers and a decrease in the sedimentation coefficient of monomers. 7. Washed ribosomes consist of even smaller particles with a sedimentation coefficient 60S.

Since the recognition of a template RNA, which is needed for protein synthesis by ribosomes (Brenner, Jacob & Meselson, 1961; Gros *et al.* 1961), procedures have been devised to deplete these particles of any endogenous coding component, and thus prepare them to receive exogenous messenger. In this way *Escherichia coli* ribosomes, after preincubation, were able to synthesize tobacco-mosaic virus 'coat protein' (Tsugita, Fraenkel-Conrat, Nirenberg & Matthaei, 1962) and phage f2 coating protein (Nathans, Notami, Schwartz & Zinder, 1962). Sonic disruption has also been used (Ogata, Watanabe, Morita & Sugano, 1962; Ogata *et al.* 1963; Decken & Campbell, 1964). In a previous communication (Brentani, Brentani & Raw, 1964) we employed a different method, consisting in treating rat-liver ribosomes with crystalline ribonuclease (EC 2.7.7.16). The fact that some preparations did not lose their amino acid-incorporating activity, despite preincubation with high concentrations of enzyme, led us to a detailed study of the properties of the resulting particle.

MATERIALS AND METHODS

Chemicals. L-[U-^{14}C]Leucine, sp. activity 131 mc/m-mole, was obtained from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.

Phosphoenolpyruvate was synthesized as the cyclohexylammonium salt by the method of Clark & Kirby (1963) and converted into its potassium salt in the following way: 534mg. of the monocyclohexylammonium salt (mol.wt. 267, approx. 92% pure) was dissolved in 10ml. of twice-glass-distilled water. Then 0·8ml. (packed volume) of water-washed Dowex 50-H (X4; 200–400 mesh; analytical grade) was added and the mixture gently stirred. The suspension was poured into a small Buchner funnel and the filtrate collected. The resin was washed with 16ml. of water and the original filtrate was combined with this. The pH was adjusted to 7·4 with 3N-KOH and the volume made up to 18·4ml. with water (0·1N final concn.).

Leucine, ATP, GTP, pyruvate kinase (type II), ribonuclease type A (five-times crystallized) and soya-bean trypsin inhibitor were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Yeast RNA was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

All other components of incorporation media were analytical reagents and all solutions were prepared freshly from twice-glass-distilled water.

Animals. The rats used were Wistar-strain albinos from a closed colony bred at this Institute. Adult rats of both sexes, unstarved to avoid microsomal degradation (Petermann & Hamilton, 1958), were killed by a blow on the neck and their livers were rapidly removed and chilled before preparation of subcellular fractions.

Preparation of ribosomes. Ribosomes were prepared as described by Korner (1961) by treating the postmitochondrial supernatant with 5% sodium deoxycholate in 0·03M-tris–chloride buffer, pH 8·2, and collected by centrifugation at 105000$g_{av.}$ The ribosomal pellet was rinsed,

without detachment from the tube walls, first with 0·44 M-sucrose and then with 0·045 M-tris buffer, pH 7·5, containing $MgCl_2$ (7·5 mM), KCl (0·12 M) and NaCl (0·075 M) (medium B), and finally resuspended with manual homogenization in the same tris buffer solution.

Preparation of ribonuclease-treated ribosomes. Ribonuclease-treated ribosomes were prepared by addition of different enzyme concentrations to ribosomal suspensions in medium B, and the mixture was incubated at 37° for 20 min. After incubation the ribosomes were sedimented at $105000g_{av.}$ for 60 min., the supernatant was carefully decanted, the tube walls were wiped with tissue paper and the ribosomal pellet was rinsed (without detachment from the tube walls) with medium B and finally resuspended by gentle manual homogenization in the same medium.

Preparation of washed ribosomes. Ribonuclease-treated ribosomes, prepared as described above, were resuspended in medium B and centrifuged again at $105000g_{av.}$ for 60 min. The final pellet was taken up in medium B, the precautions already described being taken to avoid pellet contamination by supernatant constituents.

Preparation of 'pH 5 enzyme'. 'pH 5 enzyme' was prepared as described by Rendi & Campbell (1959), and suspended in 0·25 M-sucrose.

Analytical procedures. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine plasma albumin (Pentex Inc., Kankakee, Ill., U.S.A.) as standard. For the determination of RNA, particles were extracted as described by Korner (1959) and the RNA concentration was estimated by the orcinol reaction (Dische & Schwarz, 1937) with yeast RNA as standard.

Conditions of incubation for amino-acid incorporation. Ribosomes were incubated under the conditions stated in Table 2 for 120 min. at 37° in a Dubnoff shaking water bath.

Measurement of radioactivity in protein. Protein precipitated with trichloroacetic acid was washed twice with cold 5% (w/v) trichloroacetic acid, once with 5% (w/v) trichloroacetic acid at 70°, once with ethanol–ether (3:1) and once with ether. The protein precipitated was dried in a water bath at 40° and dissolved in conc. formic acid.

The extinction of diluted portions were read at 260 mμ and 280 mμ and protein was estimated by the method of Warburg & Christian (1941). Appropriate dilutions were made on the concentrated samples so that 1 ml. of the final solution contained 0·5 mg. of protein. Portions (1 ml.) were pipetted on to plastic planchets, dried in an oven at 150° and counted at infinite thinness in a thin-window low-background gas-flow counter (Nuclear–Chicago Corp.). A standard planchet containing 0·01 μc gave 2000 counts/min. The results were expressed in counts/min./mg. of protein (sp. activity) and the final results expressed as percentages of normal values.

Ultracentrifugal analysis. Ribosomes were suspended directly in 0·01 M-potassium phosphate buffer, pH 7·0, containing 1 mM-$MgCl_2$. Ultracentrifugal analyses were performed in a Spinco model E analytical ultracentrifuge equipped with schlieren optics and a R.T.I.C. temperature control.

Determination of ribonuclease activity. The determination was performed according to Rabinovitch & Dohi (1957) with yeast RNA and purified as described by the same authors, enzymic activity being expressed in Shortman (1961) units.

RESULTS

Usually, from 27 g. of liver, we obtained 75 mg. of ribosomal protein and 61·5 mg. of ribosomal RNA (RNA/protein ratio 0·82). These particles were able to incorporate 577 ± 109 counts/min./mg. of protein.

Effect of ribonuclease on ribosomes. In Table 1 the effects of enzymic digestion on the recovery of protein and RNA from treated ribosomes are shown. We can see that loss of RNA is greater with increasing concentrations of enzyme, as could be expected, but also that a great loss of protein occurs, independent of the enzyme concentration employed. That this loss cannot be attributed to trypsin or other proteolytic contaminants of commercial ribonuclease is demonstrated, in the same Table, by experiments in which trypsin inhibitor was present in the incubation mixture and boiled ribonuclease was utilized.

Table 2 shows the amino acid-incorporating activity and the RNA/protein ratio of the treated ribosomes. The loss of RNA results in a decrease in the ratio, and the incorporating activity is also decreased in relation to normal values.

The possibility that treated ribosomes incorporate less amino acid than normal particles because the former are still contaminated with traces of added crystalline enzyme was checked by an experiment where the ribonuclease activity of normal and ribonuclease-treated ribosomes was estimated (treated ribosomes had activity 1·6 Shortman units; normal ones had activity 0·4 unit)

Table 1. *Effect of different concentrations of ribonuclease on the recovery of protein and RNA*

Ribosomal suspension (1 ml.) in medium B, containing 5 mg. of protein, was treated with ribonuclease, in the concentrations specified, at 37° in a Dubnoff shaking water bath for 20 min., and treated ribosomes were prepared, as described in the Materials and Methods section, for RNA and protein estimations.

Concn. of enzyme (μg./ml.)	Protein not recovered in 105000*g* sediment (% of initial value)	RNA not recovered in 105000*g* sediment (% of initial value)
0	18	23
5×10^{-3}	40	23
5×10^{-2}	32	28
50	44	55
50*	43	59
50†	44	58

* In the presence of trypsin inhibitor, in the same concentration as ribonuclease.

† With boiled ribonuclease as enzyme. Ribonuclease, dissolved in 1 mN-HCl, was boiled in a water bath for 10 min. and used after cooling to room temperature.

Table 2. *Effect of different concentrations of ribonuclease on the recovery of protein and RNA*

Ribosomes (20mg./ml. in the first two experiments and 5mg./ml. in the third) were treated with ribonuclease and washed as described in the Materials and Methods section. Amino acid incorporation was tested in a medium containing: 1μmole of ATP, 20μmoles of phosphoenolpyruvate (potassium salt), 0·6μmole of GTP, 30μg. of pyruvate kinase (EC 2.7.1.40), 7·5μmoles of tris buffer, pH 7·5, 1·25μmoles of $MgCl_2$, 20μmoles of KCl, 12·5μmoles of NaCl, 110μmoles of sucrose, 0·5μc of [^{14}C]leucine, 4mg. of ribosomal protein and 1·5mg. of 'pH 5 enzyme', in a final volume of 1·5ml. After 120min. incubation at 37°, the reaction was stopped by the addition of 1·7ml. of 12% (w/v) ice-cold trichloroacetic acid containing unlabelled 1% leucine. After standing overnight, the protein was washed and the radioactivity determined as described in the Materials and Methods section. Results are the averages of duplicate determinations.

Expt.	Treatment	Protein not recovered in 105000g sediment (% of initial value)*	RNA not recovered in 105000g sediment (% of initial value)	Pellet RNA/protein ratio (% of initial value)	Sp. activity (counts/min./mg. of protein as % of initial value)
1	Ribonuclease (1:20)†	41	60	45	40
	Same experiment, washed	66	60	124	205
2	Ribonuclease (1:20)	47	55	74	47
	Same experiment, washed	65	55	180	167
3	Ribonuclease (1:20)	40	60	77	43
4	Ribonuclease (1:5)	43	59	55	—
	Same experiment, washed	62	56	117	—
5	Normal ribosomes + 1·6 Shortman units of ribonuclease (4mμg.)	—	—	—	96

* Values given are corrected for loss due to preincubation alone (see Table 1).
† 1mg. of enzyme: 20mg. of ribosomal protein.

and 1·6 units were added to normal ribosomes. We can see, in Table 2, that in the presence of such a concentration of enzyme ribosomes incorporate 96% of the normal amount of amino acid.

Fig. 1 shows the profile of normal ribonuclease-treated and washed ribosomes in the analytical ultracentrifuge. Normal ribosomes (Fig. 1*a*) appear as a mixed population of dimers and monomers with sedimentation coefficients 110s and 80s respectively.

Treated ribosomes (Fig. 1*b*) show only one 70s peak, migrating slightly more slowly than normal monomers. We can see that enzymic digestion resulted also in the destruction of many ribosomes (as judged by the different areas under the curves in Figs. 1*a* and 1*b*).

Effect of washing on treated ribosomes. In Table 2 are shown the results of washing treated ribosomes. Although no alteration was observed in RNA content, further loss of protein occurred, thus restoring the particle's original RNA/protein ratio. This was accompanied by a significant rise in amino acid-incorporating activity, which became equal to that of normal ribosomes. The analytical profile of washed ribosomes (Fig. 1*c*) consisted of a single 60s peak.

DISCUSSION

The smallest concentration of ribonuclease used in the present paper is sufficient to disintegrate rat-liver polysomes completely without affecting monomers (Noll, Staehelin & Wettstein, 1963). Ultracentrifugal analyses (Fig. 1) show that the decrease in RNA or protein content of the ribosomal fractions is not due to loss of monomers and higher aggregates, but to destruction of polysomes and decrease in size of monomers. Such a conclusion has already been put forward by Manner & Gould (1965) for lymph-node polysomes and by Aepinus (1965) for rat-liver polysomes, with a different approach.

Ultracentrifugal analyses (Fig. 1) show also that the decrease in RNA and protein contents is not related to selection of particles from a heterogeneous population but to destruction of ribosomes and decrease in size of the remaining particles, together with disappearance of dimers. Further, the washing of ribonuclease-treated ribosomes releases more protein with a decrease again in the size of monomers, yielding smaller particles that can, however, still conduct amino acid incorporation.

It can also be seen in Table 2 that the amino acid-incorporating activity of the ribosomal fraction is independent of its RNA content, as already pointed out for *E. coli* (Raacke & Fiala, 1965) and yeast (Dietz & Simpson, 1964). On the other hand, it is markedly dependent on protein content, as judged by the RNA/protein ratio. The possibility that the lost protein presented some kind of inhibitory activity cannot be excluded, despite the fact that normal ribosomes incorporate amino acid in the presence of this component. However, our

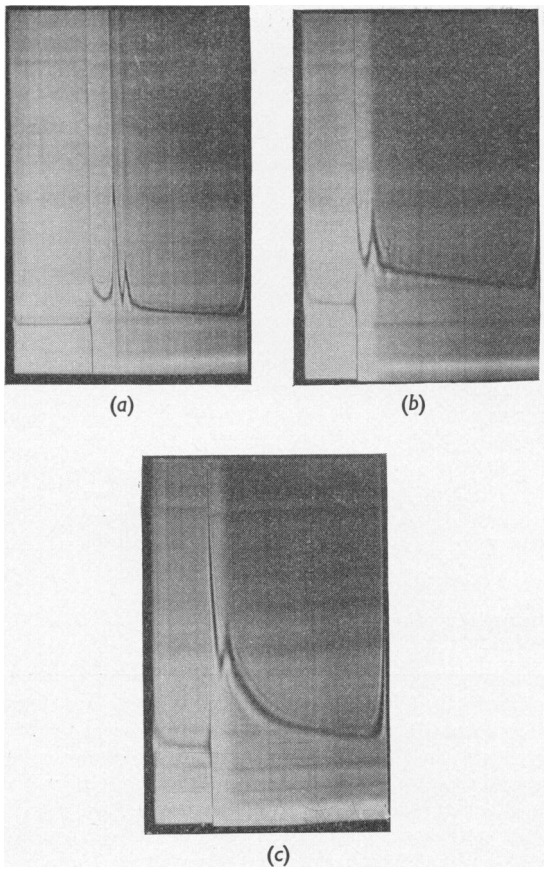

Fig. 1. Ultracentrifugal patterns of ribosomes. Ribosome concentrations: (*a*) normal, 32 mg./ml.; (*b*) ribonuclease-treated, 18 mg./ml.; (*c*) washed, 10 mg./ml. Ribosomes were suspended in 0·1 M-potassium phosphate buffer, pH 7·0, containing 1 mM-$MgCl_2$, and spun at 29 500 rev./min. in the An-E rotor at 4°. Pictures were taken 4 min. after full speed was reached.

results support the hypothesis of a distribution of RNA and protein in layers throughout the particle as suggested by Roth (1958).

A most likely interpretation is that all particles contain fragments of messenger RNA that can still code for amino acid incorporation (such a conclusion has been forwarded by Raacke & Fiala, 1965, for preincubated *E. coli* ribosomes), and that these fragments are distributed throughout the particle and can stimulate amino acid incorporation whenever exposed by protein removal.

The localization of messenger RNA inside ribosomes was suggested by Takanami & Zubay (1964), who demonstrated attachment of polyuridylic acid to *E. coli* ribosomes in such a way as to leave fragments, 27 nucleotides long, that were resistant to treatment with ribonuclease.

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