STUDIES ON THE FUNCTION OF INTRACELLULAR RIBONUCLEASES

V. Ribonuclease Activity in Ribosomes and Polysomes Prepared from Rat Liver and Hepatomas

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ABSTRACT

The RNase activity and properties of ribosome and polysome preparations from normal rat liver and some hepatomas have been examined. Polysome and ribosome preparations from the Novikoff, McCoy MDAB, and Dunning hepatomas had considerably higher specific RNase activity than corresponding preparations from normal rat liver, Novikoff ascites, or Morris 5123 hepatomas. The optimum pH of the RNase was approximately 8.5 for all samples tested, and the samples showed no evidence of latent RNase activity when treated with 3 M sodium chloride, EDTA, urea, or *p*-chloromercuribenzenesulfonic acid. The RNase activity appeared to be associated principally with breakdown products and/or subunits smaller than 80S. In the presence of Mg^{++} ions, subunits could reaggregate to form monomer ribosomes indistinguishable from the natural products, but some of the reassociated ribosomes could contain RNase activity which had been bound to the smaller particles. Similar results were obtained with spermine. In the hepatomas, evidence was obtained for the preexistence of considerable amounts of the smaller, RNase-containing subunits in the cell. When a small amount of crystalline bovine pancreatic RNase was added to partly dissociated ribosomes, the RNase was found only in association with the smaller subunits, and little or no enzyme was taken up by ribosomes or polysomes. The results have led to the conclusion that RNase is not a normal constituent of the ribosome or polysome, but that RNase may become associated with these particulates if dissociation and reassociation take place. Some implications of these findings for the stability of messenger RNA and for the mechanism of its breakdown are discussed.

INTRODUCTION

It seems clear that any appreciable intracellular hydrolysis of messenger RNA (mRNA), soluble RNA (sRNA), or even ribosomal RNA (rRNA) will interfere with the orderly and efficient synthesis of protein. It appears strange, therefore, that intracellular ribonucleases (RNases) and other RNA-degrading enzymes are so widespread and are even found in association with the ribosomal structures which are so intimately involved in protein synthesis. The probable answer to this dilemma is that, under some conditions, it is necessary to dispose of presently operating mRNA (and possibly also sRNA and rRNA) and replace it with new and different

messages or other RNA types. If this is true, a function of intracellular RNase becomes clear, i.e., to degrade the unwanted RNA. The question then becomes: Is there an orderly process by which mRNA can be destroyed at the requisite time and place, or is the process a random one depending upon the concentration and location of the RNase and possibly other factors as well?

In this paper, we have examined the RNase activity of ribosome and polysome preparations from normal rat liver and several transplantable rat hepatomas of different growth rates. We have also investigated various factors affecting the RNase activity of these preparations in an effort to answer the basic question posed above.

We have reached the tentative conclusions that RNase is not a normal constituent of the ribosome or polysome and that there is no orderly controlled turnover of mRNA or other RNA components of the cell. Intracellular RNase may, however, become associated in a number of ways with the ribosome, depending on the random dissociation and reassociation of this particulate, and to an extent conditioned by the quantities of RNase and RNase inhibitor in the cell, which in turn may be controlled by a host of factors. Ribosomes that have RNase associated with them are abnormal and may degrade mRNA with which they come in contact in a random manner; the amount of these ribosomes in the total ribosome population will therefore determine the rate of breakdown of messenger RNA and possibly its rate of synthesis.

MATERIALS AND METHODS

The methods for obtaining the ribosome and polysome preparations, the treatment of the tissues, and the buffers used have been described in the previous paper.

PREPARATION OF "METAL-FREE" RNA USED AS SUBSTRATE FOR RNASE ASSAY: Schwarz RNA (42 g) was dissolved in 700 ml of glass-distilled water, and the pH was adjusted to 7.0. The solution was dialyzed against 5 changes of 2.5 volumes of 0.025 M EDTA (pH 7.0) for 24 hr and then against 5 changes of 5.0 volumes of water for 72 hr. The concentration of RNA in the dialyzed solution was determined by absorbance measurements at 260 mμ before and after the dialysis, and a 1% solution was prepared and used for the assay below.

RNASE ASSAY: In a final volume of 1.5 ml, the incubation mixture contained 0.5 ml of enzyme sample, 0.5 ml of buffer (0.05 M Tris pH 7.6 containing 0.025 M KCl and 0.005 M $MgCl_2$, and 0.5 ml of 1% purified RNA. The assay was run in duplicate when possible. The RNA was added last, and the incubation was carried out at 37°C for 60 min. Then 1.5 ml of precipitating agent (1 M HCl in 76% ethanol + 0.5% $LaCl_3$) was added, the mixture shaken well, and allowed to stand for 10 to 15 min. It was then filtered through a 7 cm circle of Whatman No. 42 filter paper, and a watch glass was used to cover the funnel. One ml of the clear filtrate was added to 9.0 ml of water in a test tube. The diluted filtrate was mixed well, and the absorbancy at 260 mμ was determined. Appropriate enzyme and tissue blanks were run. A unit of RNase activity is defined as an absorbancy increment of 0.001, and the specific activity as units per milligram of protein. When desirable, a standard containing 0.0015 μg of crystalline pancreatic RNase (prepared in 0.1% gelatin solution) was run under the same conditions. Unless otherwise stated, the assay was carried out at pH 7.6.

TABLE I

Degradation of Ribosomal RNA of Rat Hepatomas During RNase Assay

	Absorbancy at 260 mμ	Radioactivity	
	Acid-soluble fraction	Acid-soluble fraction	Acid-insoluble fraction
		CPM	
McCoy MDAB ribosomes			
Before incubation	0.111	1770	186,000
After incubation	0.381	2010	188,000
Novikoff ribosomes			
Before incubation	0.109	2760	143,000
After incubation	0.296	3450	134,000

PREPARATION OF P^{32}-LABELED RIBOSOMES: One mc of carrier-free P^{32} was dissolved in 0.8 ml of 0.9% saline, and 0.2 ml of this solution was injected intraperitoneally into several rats on the second and third day after the transplantation of MDAB and Novikoff hepatomas. The animals were sacrificed on the fourth day, and a ribosomal preparation was obtained from the tumors. In the RNase assay, the acid-soluble fraction was separated from the precipitated materials by centrifugation at 500 *g* for 10 min. The precipitate was suspended in a known volume of water, and 0.2 ml of this suspension, and 0.2 ml of the acid-soluble fraction were plated on stainless steel planchets and dried. Counts were made in a windowless gas-flow counter. The results are shown in Table I.

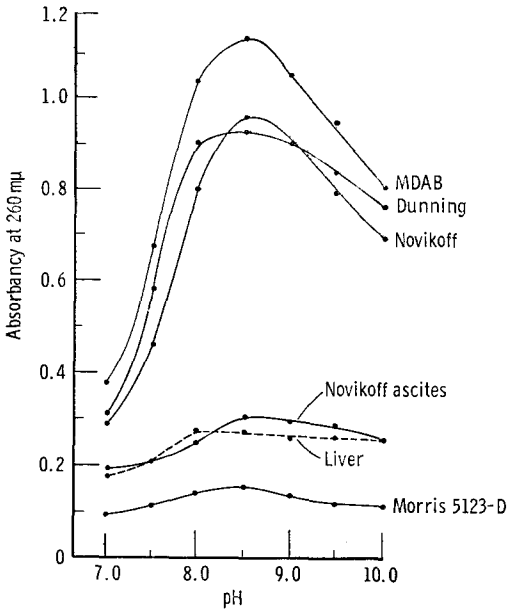

FIGURE 1 Effect of pH on RNase activity of various polysome preparations. Assay as described in the text, except that the buffers did not contain Mg^{++} in this experiment. Tris-HCl buffers, 0.05 M, were used.

RESULTS AND DISCUSSION

RNASE ASSAY AND ACTIVITY OF POLYSOME PREPARATIONS: In early experiments, some difficulty was encountered in achieving proportionality between RNase activity and the amount of ribosome or polysome preparation. Since the absorbancy of acid-soluble materials obtained on precipitation of the incubation mixture was lower in some cases than that of the blank, it appeared likely that the high ribonucleoprotein content of the samples was introducing coprecipitation or adsorption errors. (For a discussion of this problem, see references 1–3; for some of the experimental results with polysome and ribosome preparations, see reference 3.) We found that reducing the amount of protein of the polysome preparation to the following limits gave good proportionality: Novikoff hepatoma, 2 to 20 μg of protein per milliliter; Dunning hepatoma, 20 to 100 μg of protein per milliliter; McCoy MDAB hepatoma, 10 to 100 μg of protein per milliliter. Polysome preparations obtained from normal rat liver and Morris 5123-D hepatoma usually gave negligible RNase activity, although a small but significant activity was observed in some cases. Although considerable variability was encountered from one hepatoma polysome preparation to the next, normal liver and Morris 5123-D hepatomas generally had little or no activity, whereas the other hepatomas listed in Table I always had high activity. When tests were made for latent RNase activity (4) by addition of *p*-chloromercuribenzenesulfonic acid (0.6 mM) to the polysomes over a range of pH values from 7.0 to 10.0, little evidence was obtained for this type of activity.

OPTIMUM PH: The optimum pH for RNase activity associated with polysome preparations from normal rat liver and various hepatomas is illustrated in Fig. 1. Under the conditions used, the optimum pH was around 8.5, for all the samples. Fig. 1 also illustrates the relative RNase activity of the polysome preparations from liver and from different tumors, and it may be noted that the polysome preparation from the Novikoff ascites hepatoma has RNase activity in the same low range as the polysome preparations from liver and Morris 5123-D hepatoma.

SOURCE OF ACID-SOLUBLE PRODUCTS: One important question is the extent of the degradation of ribosomal RNA during incubation of a ribosomal preparation with yeast RNA in the RNase assay. This was tested by preparing ribosomes labeled with P^{32} (see Methods). After incubation for 1 hr as described in the section on RNase assay, approximately 99% of the radioactivity remained with the acid-insoluble fraction; this fact indicates immediately that the ribosomes were not significantly degraded to acid-soluble products by the RNase associated with them. Of course, it is possible that, in the absence of the yeast RNA, there would be a greater degradation of ribosomal RNA, but evidence from other experiments indicates that even the tumor ribosome preparations, which have relatively high RNase activity, are remarkably stable.

DISTRIBUTION IN A SUCROSE DENSITY GRADIENT AND RNASE ACTIVITY OF A POLYSOME PREPARATION OBTAINED FROM MCCOY MDAB HEPATOMA: Fig. 2 *A* shows the distribution of material in a polysome preparation from the McCoy MDAB hepatoma as well as the distribution of RNase associated with this preparation. RNase activity was detected only in fractions containing degradation products of ribosomes or particles having sedimentation rates less than that of monomer ribosomes. It is not clear whether

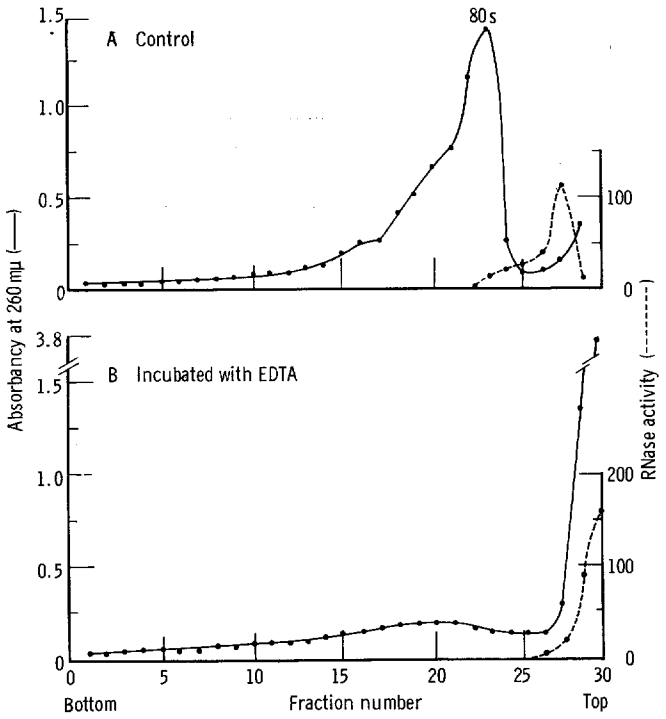

FIGURE 2 *A* Distribution in a linear sucrose density gradient (5 to 20% in TKM buffer) of RNase activity and particulate material in a polysome preparation obtained from McCoy MDAB hepatoma. Centrifuged for 2 hr at 25,000 RPM in a Spinco SW 25 rotor.

FIGURE 2 *B* A polysome preparation obtained from McCoy MDAB hepatoma first treated with 5 mM EDTA and incubated for 20 min at 37°C before centrifugation.

these small particles were derived from ribosomes or were components in the supernatant fraction which were brought into the polysome fraction as contaminants. It seems unlikely that they are contaminants, since they would not be expected to sediment through 0.5 and 2.0 M sucrose layers; and even if some did so, they would be removed by the second centrifugation that is employed. It appears reasonable to assume, therefore, that these smaller particles are degradation products of the ribosomes. If this is true, then a further breakdown of ribosomes might give increased RNase activity. This was tested by treating the polysome preparation with 5 mM EDTA for 20 min at 37°C and then by density gradient centrifugation through a gradient containing 1 mM EDTA. Fig. 2 *B* shows the distribution of particulate material and RNase activity under these conditions.

The treatment with EDTA resulted in complete degradation of the polysomes and ribosomes, but did not uncover any more RNase activity than was found in the control. This implies that there is no "latent" RNase in the ribosomes or polysomes of MDAB hepatoma, and that the RNase activity associated with the polysome preparation is not associated directly with the polysomes but rather with some subunit or degradation product of the ribosomes.

RNASE ACTIVITY ASSOCIATED WITH A RIBOSOME PREPARATION OBTAINED FROM MCCOY MDAB HEPATOMA: Since the RNase activity appeared to be associated principally with small particulates, a ribosome preparation from McCoy MDAB hepatoma was allowed to stand for 1 wk in the cold to favor breakdown. To obtain maximum possible separation in the density gradient, a thin layer of this preparation (0.25 ml containing 0.25 mg protein) was laid over the gradient. In this case, TKM buffer pH 8.5 was used, and the centrifugation was carried out for 8, instead of 4, hr. The results of this experiment are shown in Fig. 3.

A large peak of monomer ribosomes remained,

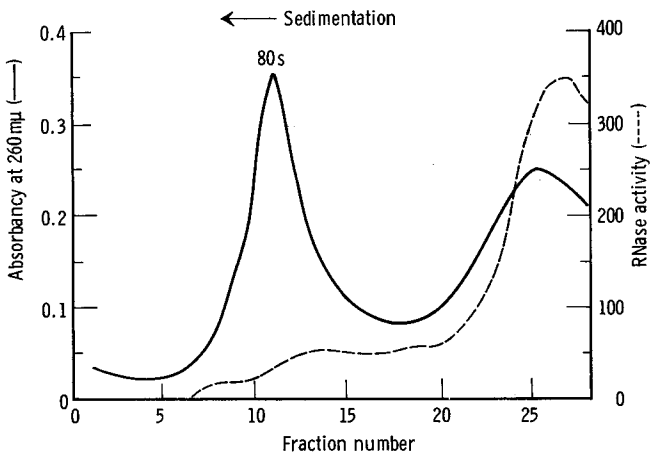

FIGURE 3 Distribution in a linear sucrose density gradient (5 to 20% in TKM buffer pH 8.5) of RNase activity and particulate material in a ribosome preparation obtained from McCoy MDAB hepatoma. Preparation had been allowed to stand in the cold for 1 wk before centrifugation.

and various degradation products, probably consisting of subunits and smaller pieces of ribonucleoprotein, were found in a second peak of low sedimentation rate. Almost all the RNase was found associated with this second peak.

In addition to storage in cold, Mg^{++}-free, distilled water as a method for breakdown of polysomes and ribosomes, EDTA may be used (see preceding paper for effects of EDTA on ribosomes and polysomes); similar results have been obtained using EDTA with both McCoy MDAB and Novikoff hepatoma ribosomes. In the same way, treatment of a ribosome preparation with 4 M urea or with 3 M NaCl (3) did not release any additional RNase activity in the case of McCoy MDAB or Novikoff hepatomas, and in every instance the enzyme activity was associated with the breakdown products and subunits.

THE EFFECT OF MG^{++} IONS ON RNASE ACTIVITY OF A RIBOSOMAL PREPARATION: A postmitochondrial fraction was prepared from Novokoff hepatoma by homogenizing the tissue with 2.5 volumes of 0.25 M sucrose solution in TK buffer pH 7.6 and by centrifuging the homogenate at 15,000 *g* for 15 min in a Spinco Model L preparative ultracentrifuge. The supernatant fraction was divided into 2 equal parts, and a solution of magnesium acetate was added to one to give a final concentration of 5 mM. The 2 samples were layered over a sucrose density gradient 0.7 to 2.0 M and centrifuged at 25,000 RPM for 20 hr. (The gradient for the Mg^{++}-containing sample also contained 5 mM Mg^{++}.) The results are shown in Fig. 4. In the absence of added Mg^{++} ions, the polysomes dissociated to form ribosomes and ribosomal subunits which was shown in earlier experiments. In the control (Fig. 4 *A*), therefore, no boundary for polysomes was observed. Region *a* contains soluble RNA plus smaller RNA fragments; region *b*, ribosomal subunits having sedimentation coefficients from 30 to 60S; region *c*, monomer ribosomes of 70 to 80S; and finally, region *d*, polysomes. Practically no RNase activity was associated with monomer ribosomes in region *c* in the control, and some significant activity was detected which was associated with the subunits of the ribosomes in region *b*. In the presence of Mg^{++} ions, the subunits were aggregated to form monomer ribosomes, and some of the ribosomes were clustered with, presumably, still existing messenger RNA to form polysomes in region *d*. This implies that all of the ribosomal preparations used in the preceding experiments contained not only "natural" monomer ribosomes but also unnatural or artifactual ribosomes which were aggregates of dissociated ribosomes, the aggregates being produced by the added Mg^{++} ions. When the ribosomal subunits were aggregated, the RNase activity which was associated with them was lost, presumably because it is now sterically hindered by the large bulk of the ribosome.

EFFECT OF SPERMINE ON RNASE ACTIVITY IN RIBOSOMES: When an experiment similar to the above was carried out with addition of 0.5 mM spermine, similar and even more dramatic results were obtained. Dissociated particles were aggregated to a considerable extent, and the RNase activity completely disappeared (3). At the concentration used, it was demonstrated that

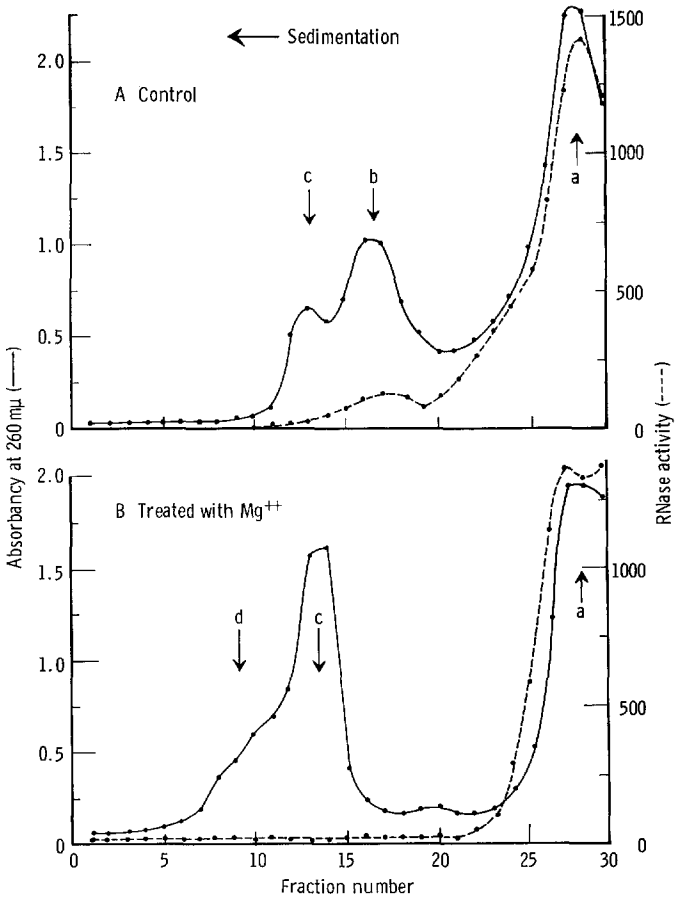

FIGURE 4 The effect of added Mg^{++} on the distribution, in a sucrose density gradient, of postmitochondrial materials and RNase activity prepared from Novikoff hepatoma. The conditions as well as the significance of the different marked peaks are described in the text.

spermine did not inhibit the activity of bovine or guinea pig pancreatic RNase (5), nor did it inhibit rat liver ribosomal RNase.

The action of spermine or added Mg^{++} to aggregate the particles suggests that if it is desired to preserve the integrity of polysomes by adding Mg^{++} ions to the homogenizing medium, some unnatural 80S particles (containing RNase) are obtained in the preparation. If, on the other hand, it is desired to avoid this aggregation by not adding Mg^{++} ions, then it is not possible to obtain a polysome preparation. In other words, under the usual conditions of isolation of these particulates, it is not possible to isolate polysomes without contaminants of artifactual aggregated ribosomal subunits. It appears, in addition, that there must be a considerable amount of ribosomal subunits in intact cells of Novikoff (and McCoy MDAB) hepatomas, since it has been shown that these 80S ribosomes maintain their integrity in the absence of added Mg^{++} ions for 1 wk (in the cold). The ribosomal subunits in peak *b* in Fig. 4 *A* must, therefore, have preexisted in the cell, since they would not be expected to be formed in 1 day.

ADSORPTION OF PANCREATIC RNASE BY RIBOSOMES: Fig. 4 shows that high RNase activities were detected in the *a* region. It is possible that the 80S ribosomes in intact cells of Novikoff hepatoma do not contain RNase, but that the RNase present in the soluble fraction is adsorbed by the 80S ribosomes when they dissociate owing to changes in ionic strength, Mg^{++}, or ATP concentration in the cytoplasm. To obtain a further understanding of this process, the interactions between isolated RNase and ribosomes were studied.

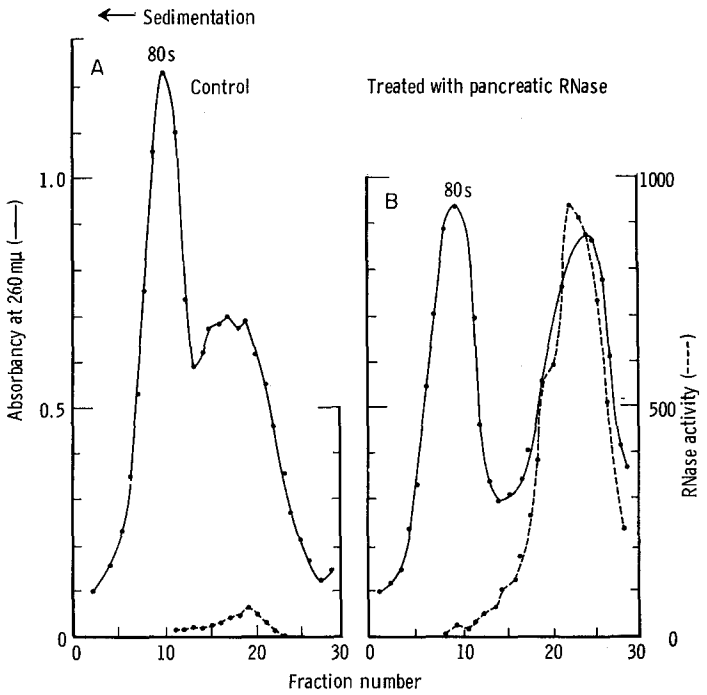

FIGURE 5 The effect of crystalline bovine pancreatic RNase on ribosomes prepared from Novikoff hepatoma. Ribosomes having approximately 0.9 mg protein in 0.45 ml of TK buffer pH 7.6 were treated with 0.45 ml of an aqueous solution of bovine pancreatic RNase containing 1 μg of enzyme per milliliter. The mixture was kept in the cold for 30 min before it was layered over a sucrose density gradient (5 to 20% made in the same TK buffer) and centrifuged at 25,000 RPM for 8 hr in the SW 25 rotor. The control sample contained 0.45 ml of water in place of the RNase solution.

The ribosomes used in the experiment were first isolated as a polysome preparation. The polysome preparation was then suspended in TK buffer which did not contain Mg^{++} ions, and therefore the polysomes dissociated to form monomers and subunits. This technique of preparation of ribosomes, instead of the conventional one described in the Methods section, was used, to minimize the amount of ribosomal RNase introduced into the ribosomal sample. Fig. 5 *A* shows that this was successful. The amount of RNase associated with the subunits is much less than the amount ordinarily found. As described below, incubation with pancreatic RNase degraded the subunits to form smaller particles; this fact is indicated by the shift of the absorbancy peak from fraction number 18 in Fig. 5 *A* to fraction number 23 in Fig. 5 *B*. The decrease in absorbancy of the 80S peak after the enzymic treatment is due to the movement of the dissociated subunits away from the 80S peak. All added pancreatic RNase appeared to be associated with the degraded ribosomal fragments, and no RNase activity was found in fractions containing 80S particles.

Recently, Hess and Horn (6) observed that bovine pancreatic RNase almost completely dissociated 50S subunits of *Streptococcus pyrogenes* and did not at all affect the 80S particles. The results reported in this paper and those of Hess and Horn indicate again that the dissociation of intact ribosomes is a prerequisite for significant enzymic attack on ribosomal RNA; this indication had been speculated earlier by Shigeura and Chargaff (7). Several other investigators have noted the insensitivity of bacterial, plant, or animal cell ribonucleoprotein to moderate amounts of RNase (8–10). Siekevitz (11) has demonstrated that calf liver ribosomes can absorb all crystalline RNase added to a suspension of these particles if the proper concentration of enzyme is used. In the presence of very large amounts of RNase, enzyme may be adsorbed on intact 70 or 80S ribosomes and may cause almost

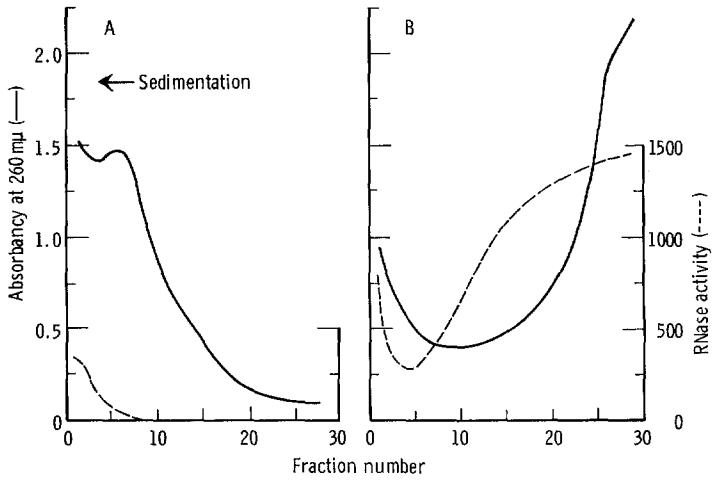

FIGURE 6 *A* Distribution of ribosomal subunits and RNase activity obtained from the Novikoff hepatoma in the absence of added Mg^{++} ions. Centrifugation at 25,000 RPM for 10 hr in a Spinco SW 25 rotor.
FIGURE 6 *B* Distribution of ribosomal subunits and RNase activity obtained from Novikoff hepatoma in the absence of added Mg^{++} ions after treatment with high-speed supernatant fraction from the same source. Conditions the same as described for Fig. 6 *A*.

complete breakdown of the particles (12). Neu and Heppel (13) also demonstrated that, under certain conditions, larger particles of *E. coli* (70S, 100S) could absorb RNase.

ABSORPTION OF SUPERNATANT FRACTION RNASE BY A PREPARATION OF RIBOSOMES OBTAINED FROM HEPATOMA: The absorption of bovine pancreatic RNase by ribosomal subunits suggests that RNase present in the supernatant fraction of Novikoff hepatoma might also be adsorbed by ribosomes or ribosomal subunits isolated from the same source. To test this possibility, a ribosomal preparation was obtained in TKM buffer, and the final pellet was suspended in TK buffer, pH 7.6, by homogenizing the particles in a Ten Broeck homogenizer. To insure some breakage of the 80S particles to smaller units, we applied about 15 strokes. This was favored also by lack of added Mg^{++}. This ribosome fraction and the supernatant fraction from Novikoff hepatoma were incubated together at 4°C for 10 min and then centrifuged for 10 hr at 25,000 RPM. The prolonged centrifugation time was employed to avoid overlap of peaks if possible.

In the control run, Fig. 6 *A*, all ribosomal particles were sedimented at the lower part of the centrifuge tube, and only a modest amount of RNase activity was associated with them. These particles were degraded rapidly during 10 min of incubation and during subsequent contact with the supernatant fraction as shown in Fig. 6 *B*. The amount of materials absorbing at 260 mμ at the lower part of the tube decreased remarkably, and RNase activity was detected throughout the tube. The enzyme appears to be bound to the dissociating ribosomes; if it were not bound and existed in free form, it could not sediment below fraction 18 to 20 under the conditions used in this centrifugation.

ADSORPTION OF SUPERNATANT FRACTION RNASE BY A POLYSOME PREPARATION: When similar experiments were carried out with a polysome preparation from Novikoff hepatoma, it was found that the polysomes and ribosomes were essentially unaffected by this treatment. Some of the RNase activity of the supernatant fraction did bind, however, to monomer and dimer units, but the largest portion was bound to subunits as observed with other systems.

GENERAL DISCUSSION AND CONCLUSIONS

Intracellular RNase in the liver cell probably does not exist free, but is, for the most part, bound by RNase inhibitor and, to a small extent, attached to ribosomal subunits. Under normal conditions, therefore, it would not be expected to attack mRNA, intact 80S ribosomes, or polysomes, and RNase does not appear to be a normal structural component of the latter two particulates.

Under some conditions, dissociation and reassociation of ribosomal particles and subunits may take place (14). Dissociation would be favored by absence of Mg salts and agents which, generally, complex Mg^{++} ion. Reassociation would be favored by the reverse of these conditions or by addition of spermine. Occasionally, a ribosome will be reformed from a subunit which contains bound RNase. The 80S particle so formed would be indistinguishable from the "native" 80S ribosome but would contain RNase. The RNase would be prevented, probably by stereochemical hindrance, from attacking the ribosome, although it may attack yeast RNA. The artifactual ribosome may become attached to an mRNA strand and, in this case, since the location of the RNase is probably close to that of the attachment of the mRNA strand, the strand may be cut by the enzyme.

Thus, the breakdown of mRNA in the liver cell is seen as a process occurring by chance; the extent and rapidity of the breakdown depends on the concentration of artifactual ribosomes. This, in turn, obviously depends upon the concentration of RNase as well as that of RNase inhibitor. In the liver, the RNase concentration is low and inhibitor concentration is high, and therefore it would be expected that the concentration of artifactual ribosomes would be low and the breakdown of mRNA a rare event. This is supported by some experimental observations which suggest that mRNA in liver is relatively stable (15), although there are a number of reports to the contrary.

Rapidly proliferating hepatomas such as Dunning, Novikoff, and McCoy MDAB have much higher RNase activity associated with their ribosomes and polysomes than normal rat liver or the slowly growing Morris 5123-D hepatoma. The rapidly proliferating hepatomas also contain amounts of RNase inhibitor much lower than normal (16). Thus, they undoubtedly contain a higher proportion of artifactual ribosomes with subsequently more rapid destruction and turnover of their mRNA, and this may account, in part, for the preponderance of monomer and dimer units observed in these tumors. (See previous paper and reference 17.)

Finally, the recent work of Neu and Heppel (13, 18, 19) on *E. coli* ribosomal RNase has led to somewhat similar conclusions with respect to this different system.

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