Studies on the Function of Intracellular Ribonucleases*

II. The Interaction of Ribonucleoprotein and Enzymes

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ABSTRACT

To determine the possible significance of *in vivo* or *in vitro* enzyme action in ribonucleoprotein systems, rat liver microsomes and ribonucleoprotein particles (RNP) prepared from them by deoxycholate treatment were incubated for 1 hour at 37° C. with crystalline pancreatic ribonuclease (RNase) or various RNase-free crystalline proteolytic enzymes. The extent of the degradation of the RNA of the microsomes and RNP was determined and the protein degradation estimated in both cases. With either microsomes or RNP, RNase (0.5 to 1.0 mg. per ml.) degraded from 75 to 95 per cent of the RNA, with little protein breakdown being apparent when microsomes were used but with significant protein degradation in the RNP. When microsomes were treated with proteolytic enzymes approximately 40 to 50 per cent of the original microsomal protein became non-sedimentable while at the same time 60 to 80 per cent of the RNA was also found to be non-sedimentable. Of the non-sedimentable RNA, approximately one-third was in the form of acid-precipitable RNA while the remainder was in the form of acid-soluble nucleotides. When RNP was treated with proteolytic enzymes, about 95 per cent of the RNA could no longer be sedimented. About half of this appeared as acid-precipitable RNA and half as acid-soluble nucleotides. Both microsomes and RNP contained significant RNase activity with RNP exhibiting about 10 times the specific activity of microsomes. Some of the characteristics of this RNase activity were determined and the results with proteolytic enzymes interpreted in light of this activity.

INTRODUCTION

It is fairly well established, by a large body of direct and indirect evidence, that microsomal ribonucleoprotein (RNP)[1] plays an important role in the synthesis of protein (1–5). As yet, the respective functions of the protein and ribonucleic acid portions of the molecule are not clear, although it has been suggested by many investigators that the RNA in some way supplies the coding information required for the manufacture of the extremely specific protein structure. The importance of the protein portion of the RNP has not been delineated, but one possibility is that it has a protective or stabilizing function, for example, suppressing the degradative action of ribonucleases (RNases) which are present in various portions of the cell. With respect to RNases, most studies of these enzymes have been concerned with their action on purified RNA or simple nucleotide substrates and little is known of their interaction with different types of ribonucleoprotein under various conditions. It is well to keep in mind that in the cell, RNA probably does not exist free but rather as ribonucleoprotein. Thus it would seem of importance to investigate the interactions of ribonucleo-

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[1] The abbreviation RNP will refer specifically to the small ribonucleoprotein particles obtained by treatment of microsomes with deoxycholate.

protein and RNases, as well as the action of other enzymes capable of degrading ribonucleoprotein.

Increasing interest in the function of RNases has been apparent in view of the report by Elson (6) that an inactive RNase is found associated with the ribonucleoprotein isolated from *Escherichia coli*. In addition, Siekevitz and Palade (7) and Dickman and Morrill (8) have reported considerable RNase activity associated with the RNP isolated from pancreas tissue. At the same time, Roth (9) presented data to indicate that RNP isolated from rat liver microsomes also has considerable amounts of RNase activity associated with it.

Palade and Siekevitz (10) have investigated the action of crystalline pancreatic RNase on isolated rat liver microsomes. They found that up to 85 per cent of the RNA present could be degraded. The use of RNase to study and remove basophilia of the cell in histological examinations (11, 12) is commonplace. While the work described in this paper was in progress, several papers by Tashiro and coworkers on the effects of RNase and trypsin on microsomes and RNP appeared (30–32).

Some aspects of Tashiro's work have been confirmed and extended in this paper. In addition the effects of various proteolytic agents on microsomes and RNP have been investigated in detail.

Materials and Methods

The rats used in these studies were males of the Wistar strain and ranged from 250 to 500 gm. in weight. No biochemical differences attributable to the variations in weight of the animals have been observed. All operations were conducted between 0 and 4°C. Crystalline pancreatic RNase, papain, and ficin were supplied by Worthington Biochemical Co., Freehold, New Jersey, cathepsin C, chymotrypsin, rennin, and pepsin were obtained from Nutritional Biochemicals Co., Cleveland, Ohio. A sample of highly purified bromelin was obtained from the Hawaiian Pineapple Co., Honolulu, through the courtesy of Dr. Ralph M. Heinicke. All of the above enzymes showed no detectable RNase activity when assayed in the test system described below. In more absolute terms they contained less activity per mg. of enzyme than 0.001 γ of crystalline pancreatic RNase, the lower limit of sensitivity of the RNase assay system utilized. Crystalline trypsin, from several sources, even trichloroacetic acid (TCA) purified, was found to be contaminated with RNase activity in varying amounts ranging from moderate to heavy and, therefore, could not be used in these studies.

Preparation and Properties of Microsomes.–The rats were etherized, exsanguinated, and the livers rapidly removed and rinsed with ice-cold 0.25 M sucrose solution. They were then homogenized with 9 parts by weight of 0.25 M sucrose solution in a Ten Broeck homogenizer, after which the homogenate was centrifuged in a Spinco model L ultracentrifuge at 5,000 *g* for 15 minutes. The supernatant fluid was centrifuged again under the same conditions and the mitochondrial fractions discarded. The pink fluffy layer was retained with the supernatant fluid. This was then centrifuged at 60,000 *g* for 60 minutes and the resulting reddish, translucent microsomal pellet was washed with 0.25 M sucrose by homogenization in a small Ten Broeck homogenizer. The washed microsomes were recentrifuged at 60,000 *g* for 30 minutes and the microsomal pellet generally made up in water to a specific volume by homogenization. No attempt was made in the preparative process to obtain a quantitative recovery of microsomes. Table I lists some of the biochemical properties of microsomes prepared by the above method. The values for RNA are about 25 per cent lower than those reported by Palade and Siekevitz (10). The higher values reported by them may possibly be accounted for by the fact that they used a centrifugal force of 105,000 *g* and would, therefore, collect more and smaller particles than in the present study in which

TABLE I

Chemical Properties of Microsomes and Ribonucleoprotein Prepared from Rat Liver

Sample	Average and range from 1 gm. wet weight of liver					
		RNA	Total N	Protein N	Total P	Total lipid
		mg.	*mg.*	*mg.*	*mg.*	*mg.*
Microsomes	Av.	2.55 ± 0.24* (10)‡	3.84 ± 0.49 (10)	2.85 ± 0.41 (10)	0.72 ± 0.09 (10)	15.8 ± 2.5 (10)
	Range	2.03 – 2.94	3.08 – 4.73	2.08 – 3.57	0.52 – 0.97	11.3 – 20.6
RNP§	Av.	1.16 ± 0.12 (12)	0.49 ± 0.08 (10)	0.21 ± 0.02 (8)	0.124 ± 0.01 (4)	
	Range	0.82 – 1.42	0.36 – 0.72	0.17 – 0.24	0.108 – 0.131	

* Average deviation.

‡ Number in parenthesis is the number of separate determinations.

§ Experiments on microsomes and RNP were separately performed.

60,000 *g* was used. However, the greater centrifugal force used by Palade and Siekevitz would be counteracted somewhat by the higher density of their sucrose solution which was 0.88 M compared to 0.25 M in this report. Another factor that might tend to lower the RNA values reported here is a possible difference in the extinction coefficient of the RNA used as a standard (see assay methods below and refer to Table II). Use of a Crestfield, Smith, and Allen preparation of RNA, (13) which is presumably purer than the commercial RNA used as a standard here, would increase the RNA values reported in this paper approximately 13.5 per cent. On the other hand, agreement on nitrogen (N) content of the microsomes with the results of Palade and Siekevitz was obtained, for example, in Table I, the value of 2.85 mg. of protein N per gm. of wet weight of liver agrees fairly well with the 3.09 mg. reported by these authors (10). These investigators did not include the pink fluffy layer in their microsome preparations.

Assay Methods.–RNA was determined by the method of Schneider (14) using Mejbaum's (15) modification of the orcinol color test. In most experiments spectrophotometric determination of the absorption at 260 mμ of the hot TCA extracts, alone, was used for the assay of RNA. Many experiments, in which both methods were used, showed that there was no significant difference in the values determined by either method. The standard used for RNA determination, by either method, was a sample of Nutritional Biochemicals sodium ribonucleate (Table II). When RNA samples prepared by different means, or obtained from different sources, are used, significant differences in the extinction of the dissolved or hydrolyzed material may be obtained. This is a factor that is not often mentioned when RNA assays are reported. It would be helpful in comparing RNA values among different investigators if the extinction coefficients at 260 mμ of the RNA samples used as standards were given. In Table II analytical data for various RNA samples are presented. It is obvious from the data in the table that considerable differences in the RNA content of a microsomal preparation might be obtained depending on the sample of RNA chosen as standard.

Phosphorus determinations were performed by the method of Dryer, Tammes, and Routh (16). Nitrogen was assayed by a micro Kjeldahl procedure.

RNase was assayed by use of the following technique. Duplicate tubes containing 1.0 ml. of a 1 per cent solution of Schwarz RNA, 1.0 ml. of buffer of the appropriate pH, and 1.0 ml. of enzyme plus other components were set up for each determination. The RNA was added last, at zero time, and the mixture incubated in a water bath at 37°C. for 30 to 60 minutes. Three ml. of N HCl in 76 per cent ethanol were then added with shaking and the precipitate of unhydrolyzed RNA allowed to stand for 8 to 10 minutes in order to flocculate. The mixture was then filtered through 9 cm. circles of Whatman No. 42 filter paper and the funnels were covered with watch-glasses. One ml. of the clear filtrate was diluted to 50 ml. with water and the absorption at 260 mμ determined in the Beckman spectrophotometer. A standard consisting of 0.015 γ of crystalline pancreatic RNase was run each time an assay was

TABLE II

Analytical Data on Various RNA Samples

The values represent the average of at least two separate determinations

Sample	Unhydrolyzed $E^{1\%}_{1cm.}$ 260 mμ in 0.01 N HCl	Hydrolyzed $E^{1\%}_{1cm.}$ 260 mμ in 0.01 N HCl	Orcinol $E^{1\%}_{1cm.}$ 570 mμ	Phosphorus	Nitrogen	N/P
				per cent	*per cent*	
Schwarz RNA	250.0	301.5	228.0	7.9	14.4	1.82
Nutritional Biochemicals sodium ribonucleate*	251.5	289.0	224.5	7.4	13.1	1.77
Purified Schwarz RNA‡	238.5	283.5	214.5	7.3	12.5	1.71
RNA prepared by NaOH extraction§	190.0	220.5	159.0	5.7	10.5	1.84
RNA prepared by NaOH extraction further purified‖	243.0	256.0	196.5	6.4	11.2	1.75
Crestfield, Smith, and Allen RNA¶	213.0	267.5	210.5	6.9	11.7	1.72

* Used as standard in this report.

‡ Exhaustively dialyzed in the cold against distilled water and then precipitated with ethanol, and washed with 95 per cent ethanol and ether.

§ Method of Di Carlo *et al.* (28).

‖ Purified as described under footnote ‡ above.

¶ Prepared as described in reference (13) but also washed with ether, exhaustively dialyzed, and lyophilized.

performed and under the test conditions gave an optical density increment of 0.100 to 0.110 in 30 minutes when 0.1 M acetate, borate, cacodylate (ABC) buffer (17) was employed. When necessary, tissue blanks were run by omitting RNA. In general, the contributions of the tissue to the optical density reading were negligible. One unit of RNase activity is defined as an optical density increment of 0.001 under the described conditions (1 hour incubation). A stock solution of crystalline pancreatic RNase containing 50 γ of enzyme and 1 mg. gelatin per ml. was prepared every 2 weeks. To obtain the enzyme standards this stock solution was diluted with 0.1 per cent gelatin solution just before use.

Preparation of Ribonucleoprotein.—The microsome pellets obtained after washing microsomes once with 0.25 M sucrose solution were homogenized in a freshly prepared 1 per cent water solution of sodium deoxycholate; 20 to 25 ml. of deoxycholate were used for microsomes obtained from 15 to 30 gm. of liver. The resulting translucent solutions were centrifuged at 105,000 *g* for 90 minutes. The RNP pellet was then homogenized in glass-distilled water and made up to the required volume. The properties of some typical RNP preparations are shown in the lower half of Table I. Consideration of the data indicates, that after treatment of microsomes with 1 per cent deoxycholate, over 50 per cent of the RNA is no longer sedimented. Palade and Siekevitz (10) studied the effect on microsomes of deoxycholate concentrations from 0.1 to 0.5 per cent at pH 7.5. At the highest concentration, in 3 experiments, they found approximately 15, 22, and 49 per cent of the RNA was non-sedimentable after deoxycholate treatment. The value from Table I of over 50 per cent RNA remaining in the deoxycholate extract was obtained by difference in different experiments on microsomes or RNP and is, therefore, probably not as valid as actual assays performed in any one experiment, particularly since there may be losses of RNA due to degradation by deoxycholate, or for other reasons. However, in a series of 5 experiments (not included in Table I) in which assays were performed on the microsomes, as well as the RNP residue and deoxycholate extract obtained from them, the following values were found for the amount of RNA not sedimenting; 43.6, 67.3, 40.3, 43.0, and 43.8 per cent of the original microsomal RNA (average 47.6). The average is in fairly close agreement with the one obtained by difference from Table I, since the recovery of total RNA was between 90 and 100 per cent. Judging by this recovery, it appears likely that deoxycholate treatment did not cause any significant breakdown of RNA. Whether the RNA in the deoxycholate supernatant solution was disassociated from, or associated with, protein is not known. In all experiments with deoxycholate the microsome pellet was homogenized with 1 per cent deoxycholate. It was noted, however, that relatively minor changes in the experimental prôcedure, for example, if instead of homogenization, equal volumes of microsomes suspended in water and 2 per cent deoxycholate are mixed, or if homogenizers with smaller or larger clearances are used, considerable variations in the composition of the RNP pellet and deoxycholate supernatant fluid might result.

From Table I it may be calculated that approximately 92 per cent of the protein N is removed from the microsomes by treatment with 1 per cent deoxycholate solution. Actual measurement in the 5 experiments previously mentioned gave the following values; 85.7, 84.0, 85.5, 80.4, and 90.5 (average 85.3) somewhat less than the calculated value. It is possible that 1 per cent deoxycholate causes some protein degradation. Another possible source of protein loss would be during the Schneider procedure for assay of RNA.

If the average protein N in the RNP is multiplied by 6.25 to convert to mg. of protein, the resulting RNA to protein ratio is 1.16:1.31 and the per cent of RNA in the RNP preparation is therefore 47 per cent. This is slightly higher, but in essential agreement with the value of approximately 40 per cent usually given for rat liver RNP (10, 18). The higher values reported here could be accounted for by more complete removal of the non-functional protein by the higher concentration of sodium deoxycholate used. It is possible that the value of 0.21 for protein N in RNP (Table I) is somewhat low. The value of 85 per cent loss of protein from microsomes should leave a protein N in RNP of 0.24. This, when added to the calculated N in 1.16 mg. of RNA, would not completely account for the total N content of the RNP. Also, based on 1.16 mg. of RNA, the total phosphorus content is somewhat high; the reasons for these discrepancies are not clear. One possibility is that the protein contains a high percentage of basic amino acids which would considerably increase its N content and also some phosphate groups which would increase the phosphorus content of RNP. Amino acid assays on microsomal protein have, indeed, indicated a rather high content of basic amino acids (19, 29).

RESULTS

The Action of Crystalline Pancreatic RNase on Microsomes and RNP:

Since the results of these experiments, in general, were similar to those described by Palade and Siekevitz (10) and Tashiro (30, 31) they will not be described in detail. When microsomes were treated with crystalline pancreatic RNase (0.5 mg. per ml.) either by direct incubation at 37°C. for 1 hour, or upon treatment at 4°C. in a dialysis sac for up to 72 hours, the degradation of the RNA ranged from 75 to 90 per cent. Very little degradation of microsomal protein was observed under these conditions.

The effect of crystalline RNase on RNP is presented in Table IV, line 8. RNA breakdown in these experiments was greater, on the average, than that observed with microsomes and approached completion in some cases contrary to the results of Tashiro (30) and Novikoff (33) who found that 15 to 20 per cent of the RNA in the RNP remained intact. At the same time, a significant reduction in the protein content (44.8 per cent) of the RNP was observed.

It is important to point out that the concentrations of RNase used in the above experiments, as well as those of Tashiro, are thousands of times greater than those normally found in liver homogenates or fractions. Under physiological conditions the RNP of cells may be protected, for the most part, from the action of intracellular RNases. Even in liver homogenates, in which there is considerable disruption of organized structures, the RNA *appears* to be little affected by the RNases normally present. Thus, assays for RNA content on liver homogenate incubated at 37°C. showed little change even during considerable periods of time. With microsomes stored at 4°C. for 24 hours, a 5 per cent loss in RNA has been noted in this laboratory. This loss may not be significant. The stability of RNA in liver homogenates is due, possibly, to the high content of RNase inhibitor in this tissue (20) and this stability may not be evident in other tissues. Tabachnik (21) has reported a rapid fall in the RNA content of epidermal tissue on standing.

The Action of Proteolytic Enzymes on Isolated Rat Liver Microsomes:

The occurrence of a wide variety of intracellular proteases, cathepsins, and peptidases suggests that cellular RNP may be subjected to the action of these enzymes, either under normal conditions, or possibly only during abnormal physiological states. For this reason, it was of interest to examine the effect of various proteolytic enzymes on microsomes and RNP. In addition, degradation of the protein of RNP might be an effective way to prepare RNA in the native state. Tashiro (30) has examined the effect of trypsin on RNP but since his sample of trypsin was admittedly contaminated with RNase (as were all samples tested in our laboratory) it is impossible to ascertain what portion of the effects may be due to the proteolytic enzyme and what portions due to the RNase content of RNP, and the contaminating RNase of the added trypsin.

In Table III the effects of several RNase-free proteolytic enzymes on microsomes are presented. The experimental details are given in connection with the table. Three crystalline and one highly purified proteolytic enzymes were used and, in general, the results were qualitatively the same with all of them.

The RNase activities of the microsome-enzyme mixtures are also reported in Table III, as well as the amount of protein not sedimenting after treatment with the various enzymes. This non-sedimentable protein is assumed to be partially degraded but this is not necessarily the case. In all the preparations of unheated microsomes treated with proteolytic enzymes, the RNase activity did not differ markedly from that of the controls, although papain-treated samples exhibited slightly lower activity. It may be assumed, therefore, that the proteolytic agents had little effect on the RNase activity associated with microsomes under the conditions of these experiments. Heating the microsomes or RNP at 80°C. for 5 minutes completely destroyed the RNase activity (in confirmation of the results of Tashiro (30, 31). In view of the usual stability of alkaline RNases to heat treatment, complete destruction by this relatively mild procedure is somewhat unusual and suggests that the RNase associated with microsomes or RNP is more sensitive to heat than is generally the case or, alternatively, differs from other liver alkaline RNases.

In the control samples of microsomes (Table III, line 1), 86 per cent of the RNA was recovered in the residue obtained by recentrifugation of the material incubated at 37°C. with buffer for a period of 1 hour. Most of the remainder of the RNA was found in the acid-soluble fraction and represented, therefore, degradation products of the RNA portion of the ribonucleoprotein or, possibly, acid-soluble combinations of nucleotides with peptides. In those experiments in which RNP was treated with proteolytic enzymes this fraction might be a rich source of nucleopeptides which could be formed by breakage of protein and RNA chains in the same localities. The acid-soluble material obtained with the control microsomes may also be produced from unstable portions of the RNA macromolecule by action of the TCA utilized in the Schneider assay procedure for RNA, portions possibly rendered unstable by the prior action of RNase associated with the microsomes.

Slightly less RNA was recovered in the residue

TABLE III

The Effect of Crystalline Proteolytic Enzymes on Rat Liver Microsomes

Enzyme added*	Other treatment	No. of experiments	RNase specific activity‡	Per cent of total RNA in			Protein non-sedimented‖
				Residue	Supernatant fraction	Acid-soluble fraction§	
			units/mg. N				*per cent*
None (control)		12	178	86.1 (77.5–92.3)	1.6 (0–3.7)	12.3 (4.6–22.3)	8.7 (1.0–13.8)
None (control)	Microsomes heated¶	3	0	78.4 (76.2–80.4)	8.1 (1.5–12.8)	13.2 (9.5–20.1)	6.9 (4.0–10.5)
Chymotrypsin		15	166	25.7 (13.0–43.8)	31.4 (11.3–44.7)	42.8 (29.8–56.8)	43.5 (36.7–51.8)
Ficin		9	158	26.8 (8.5–35.2)	27.2 (19.2–34.6)	45.2 (35.2–60.4)	47.2 (39.5–53.8)
Papain		4	140	38.5 (18.7–45.8)	21.6 (13.5–26.8)	41.9 (34.6–54.5)	41.4 (35.5–51.5)
Bromelin		4	167	18.7 (15.2–20.3)	29.8 (27.4–35.8)	51.7 (43.6–57.1)	48.7 (44.0–60.0)
Chymotrypsin	Microsomes heated¶	3	0	59.2 (56.2–63.7)	19.9 (6.7–28.2)	17.6 (15.6–29.7)	28.5 (23.9–31.6)

* Livers of two rats were pooled; total liver weight varied from 15 to 30 gm. Microsomes were made up to a volume of 20 to 25 ml. in distilled water. Usually, 5 ml. of microsomal suspension plus 5 ml. of 0.1 M acetate buffer pH 7.4, were incubated with enzyme (0.5 mg. per ml.) for 1 hour at 37°C. After cooling and centrifuging at 60,000 *g* for 1 hour, the supernatant fraction was decanted and made up to 10 ml. with distilled water. The residue was homogenized in water and made up to 10 ml. also. Controls were similar solutions without enzyme. Microsomes, kept at 0°C., throughout, were assayed to determine the degradation in the control samples.

‡ The definition of RNase units is given under Materials and Methods.

§ The RNA of the original microsome preparation was assayed. RNA assays were also performed on the residue and supernatant fraction obtained after incubation with enzymes or buffer alone. The RNA in the residue is expressed as a per cent of that found in the original microsomes. The RNA in the supernatant fraction is expressed similarly. The acid-soluble fraction is the difference between the sum of the RNA in the residue and supernatant fraction and the RNA in the original microsomes. That this actually is a valid representation of acid-soluble nucleotides was determined by measuring the extinction coefficient at 260 mμ of this fraction, in several experiments, and calculating the mg. of RNA corresponding to this extinction coefficient. When this was done, the sum of residue plus supernatant RNA, plus equivalent RNA of the acid-soluble material closely approximated the total RNA of the original microsomes.

‖ The state of the non-sedimented protein was not determined. It is presumed to be at least partially degraded to polypeptides or amino acids. All the N lost from the residue was considered to be protein N.

¶ The microsome preparation was heated to 80°C. for 5 minutes.

after incubation of heated control microsomes, the difference from unheated controls, approximately 8 per cent, appeared, however, as RNA in the supernatant fractions; the heat treatment apparently caused the separation of a small amount of the RNA from the microsomes.

Nearly all the protein originally found in the control microsomes was recovered from the pellets obtained by recentrifugation of the incubated preparations.

When microsomes were incubated with proteolytic enzymes for 1 hour at 37°C., the recovery of RNA in the microsome pellet subsequently obtained by recentrifugation, ranged from 18.7 per cent in the case of bromelin to 38.5 per cent for papain. This greatly reduced recovery of RNA in

the pellet, compared to controls, was not anticipated, and it suggested, in early experiments in which the RNA distribution was not determined, that breaking the protein chain caused considerable degradation of the RNA. Further experiments showed that somewhat less than one-third of the RNA that was no longer sedimentable appeared in the supernatant fraction, presumably as polynucleotide RNA, while the remainder of the non-sedimentable RNA was in the form of acid-soluble products (Table III, columns 5 and 6).

One of two possibilities would seem to provide the best explanation of these findings. First; the proteolytic enzymes, in partially degrading the polypeptide chain, facilitate separation of RNA from the protein and the RNA is subsequently degraded by RNase present in the preparations. If this is true, then reduced degradation of the protein should result in reduced separation of RNA and also reduced formation of acid-soluble products. In many experiments (not reported in Table III), this has been the general finding. For example, in several experiments in which a fairly inactive sample of papain was utilized, the amount of non-sedimented protein was considerably reduced and the per cent of the original RNA obtained in the supernatant and acid-soluble fractions was also less than that reported in Table III. Similar results were obtained with use of pepsin, rennin, and acetyl chymotrypsin, which caused little protein degradation under the conditions utilized.

The second possibility, which has been proposed earlier (22), is that the structure of RNA in microsomal RNA is discontinuous so that partial breaking of the protein chain effects rapid dissolution of the RNA. Actually, the two possibilities are not exclusive.

Pertinent to determination of which of these possibilities is the correct one, is the RNase activity of the microsome preparations. A specific RNase activity averaging 178 units per mg. N was found. This activity is approximately equivalent to that of 0.015 γ of crystalline pancreatic enzyme assayed under the same conditions, and is capable of degrading 10 mg. of yeast RNA to the extent of about 40 to 50 per cent in 1 hour's time. Each microsome preparation contained from 8 to 12 mg. of RNA and the RNase activity present in 4 ml. of microsomes was, therefore, more than sufficient to degrade the RNA to the extent observed, provided, of course, there is no protecting action of protein and other substances.

However, consideration of the data for the controls, which have the same amount of RNase present, shows that this quantity of RNase produces only minimal degradation of the RNA in the absence of added proteolytic enzyme. Since heating microsomes at 80°C. for 5 minutes completely destroys their associated RNase activity, it is possible to test the action of the proteolytic enzyme in the absence of RNase activity. It should be kept in mind, however, that the heat treatment may alter considerably the macromolecular structure of the RNA and/or protein so that this is not the most effective control for determining the effect of the RNase activity associated with the microsomes.

When heated microsomes were treated with chymotrypsin much less RNA was found in a non-sedimentable form, the difference being in the amount of acid-soluble material formed. Fully as much RNA was found in the fraction which is non-sedimentable but precipitable by TCA as when unheated microsomes were used. The difference, therefore, could be ascribed to RNase activity. It should be noted, however, that the digestion of the protein of the heat-treated microsomes appeared to be less than that of the native microsomes and this may be a factor in producing the observed difference in effect between heated and unheated microsomes treated with chymotrypsin.

In the last column of Table III it may be noted that from 41.4 to 48.7 per cent of the protein originally present in the microsomes was not sedimented after treatment with proteolytic enzymes. The extent of the degradation of this material has proved somewhat difficult to determine since Lowry's assay procedure for protein (23) did not distinguish, in this case, between partially degraded and intact protein. When the supernatant fraction containing this partially degraded protein is treated with hot TCA and RNA is extracted, the residue obtained, when compared to a control sample, should give some indication of the extent of the protein breakdown. Unfortunately, the TCA-precipitated protein is extremely difficult to dissolve, requiring treatment with 1 N NaOH at 100°C. for 5 to 10 minutes, a procedure which in itself, causes approximately a 30 per cent breakdown of protein. Thus the degradation of protein measured by this method is generally higher than that given in Table III, the data in Table III having been computed as indicated in the explana-

tion below the table, from the N determinations. In a few instances, however, the hydrolysis of protein calculated by one method checked fairly well with that determined by the second method and the non-sedimented protein probably represents at least partially degraded protein which is no longer susceptible to precipitation by TCA.

Several other experiments related to those described in Table III were carried out but are not illustrated. A mixture of two proteolytic enzymes, chymotrypsin and ficin gave results not significantly different from experiments employing either enzyme singly. As previously mentioned, crystalline pepsin, rennin, and acetyl chymotrypsin were also tried, but they had little action on the microsomes under the conditions used. A preparation of cathepsin C was only slightly active as a proteolytic agent and was heavily contaminated with RNase activity. Several experiments were carried out utilizing ribonucleoprotein contained in the supernatant fraction of the liver cell. The amount of RNA recovered before and after incubation with chymotrypsin was the same. Several factors may contribute to this lack of action on soluble RNA.

TABLE IV

The Effect of Various Enzymes on Rat Liver Ribonucleoprotein Particles

Enzyme added*	Other treatment	No. of experiments	RNase specific activity	Per cent of total RNA in			Protein non-sedimented
				Residue	Supernatant fraction	Acid-soluble fraction‖	
			units per mg. N				*per cent*
None (control)		10	1780 (1085–2250)	69.3 (61.1–76.8)	12.2 (10.9–13.9)	18.3 (4.3–26.4)	21.2 (12.8–27.5)
None (control)	RNP heated‡	2	0	49.8 (49.0, 50.5)	40.7 (38.6, 42.7)	9.6 (6.8, 12.4)	29.0
Chymotrypsin		10	1550 (855–2880)	6.6 (3.0–12.3)	46.6 (39.5–52.8)	46.9 (39.0–53.0)	86.2 (73.2–99.0)
Chymotrypsin	RNP heated‡	2	0	19.1 (16.8, 21.4)	60.2 (59.0, 61.3)	20.8 (17.3, 24.2)	68.5 (63.9, 73.2)
Papain		5	1510 (1145–1940)	8.5 (0.4–13.6)	47.3 (38.5–55.5)	45.3 (43.2–47.5)	68.4 (48.0–89.3)
Bromelin		5	1320 (700–2030)	3.9 (0.4–5.6)	41.5 (33.9–49.3)	54.6 (50.3–61.2)	84.5 (81.7–86.3)
Ficin		3	1090 (900–1190)	4.3 (2.8–5.7)	49.2 (46.1–51.3)	46.4 (43.0–49.4)	81.3 (80.2–83.1)
RNase		4	—	6.8 (5.4–8.7)	1.0 (0–2.0)	92.7 (89.3–94.6)	44.8 (38.2–48.2)
None	+ RNase inhibitor§	2	0	57.9 (57.4, 58.5)	42.0 (41.5, 42.5)	0	—
Chymotrypsin	+ RNase inhibitor§	2	560 (360, 760)	8.9 (8.4, 9.3)	66.0 (61.3, 70.7)	25.2 (20.9, 29.4)	—

* Livers of two rats were pooled; total liver weight varied from 15 to 30 gm. RNP was made up to a volume of 20 ml. Usually, 5 ml. of RNP suspension plus 5 ml. of 0.1 M acetate buffer, pH 7.4 were incubated with enzyme (0.5 mg. per ml.) for 1 hour at 37°C. After cooling and centrifuging at 105,000 *g* for 90 minutes, the supernatant fraction was decanted and made up to 10 ml. with distilled water. The residue was homogenized in water and made up to 10 ml. also. Controls were similar solutions without enzymes. RNP kept at 0°C., throughout, was assayed to determine the degradation in the control samples.

‡ RNP was heated at 80°C. for 5 minutes before being used.

§ RNase inhibitor was prepared as described in reference 34. Two ml. of purified inhibitor preparation was used per 5.0 ml. of RNP suspension.

‖ The procedure for determining these fractions was the same as described under note §, Table III.

First, the presence of considerable quantities of RNase inhibitor in the supernatant fraction keeps the activity of RNase at a low level. Second, the soluble RNA is of relatively low molecular weight and contains a considerable percentage of unusual bases and therefore may not be as susceptible to RNase action as microsomal RNA.

The Action of Proteolytic Enzymes on Isolated RNP:

In Table IV are given the results obtained upon treatment of RNP with various proteolytic enzymes; also included are several experiments utilizing RNase. The experimental details are given in connection with the table. It may be noted that the specific activity of RNase in the RNP control samples is exactly 10 times that found in microsomes. This increase in RNase activity in separated RNP was also observed by Tashiro (30). It is somewhat lower in the RNP treated with proteolytic enzymes, possibly due to partial degradation of the RNase by proteolysis, but it is still many-fold greater than the RNase activity of microsomes. Addition of partially purified RNase inhibitor (34) to RNP reduced the RNase activity to zero, but in the presence of chymotrypsin considerable RNase activity remained due, perhaps, to partial destruction of the inhibitor by the proteolytic enzyme.

The effects obtained by the action of different proteolytic enzymes acting on RNP were qualitatively the same and are listed in Table IV. Incubation with chymotrypsin, bromelin, papain, or ficin resulted in the loss of nearly all the RNA from the RNP pellet, with about half of the RNA appearing as such in the supernatant fraction and half as acid-soluble nucleotides. Considerably more protein was lost from the incubated RNP than was the case when microsomes were treated similarly.

Heating the RNP at 80°C. for 5 minutes led to separation of about 41 per cent of the RNA, which then appeared in the supernatant fraction. Very little acid-soluble nucleotides were formed from the heated RNP probably due to the complete destruction of RNase activity. When the heated RNP was treated with chymotrypsin an even larger fraction of RNA appeared in the supernatant fraction while the production of acid-soluble nucleotides was twice that of the heated control RNP (20.8 per cent compared to 9.6 per cent). This observation could be taken as evidence for a greater instability of the RNA when separated or partially separated from protein.

TABLE V

Ribonuclease Activity of Microsomes and Ribonucleoprotein Preparations

Each value is the average of at least two experiments.

Conditions	RNase specific activity		
	Microsomes	RNP	Deoxycholate supernate
	units per mg. N	*units per mg. N*	*units per mg. N*
Control	166	1399	239
Heated at 80°C. for 5 minutes	0	0	0
+ 3.3×10^{-4} M Cu^{++}*	0	405	215
+ 8 M urea*	—	1200	—

* Final concentration.

It is interesting to note that when RNP is treated with RNase inhibitor alone, no acid-soluble nucleotides were formed. Tashiro has noted a decreased spontaneous degradation of RNP when liver supernatant fraction containing RNase inhibitor was present (31).

The RNase activity associated with RNP is more than sufficient to account for the degradation of RNA observed in Table IV. In fact, it may be surmised that the protein portion of the RNP exerts a strong protective effect since after incubation of control samples for 1 hour at 37°C. only 18.3 per cent of the RNA was hydrolyzed to acid-soluble nucleotides.

Some studies of the characteristics of this RNase activity associated with microsomes and RNP were carried out and the results agree quite well, in general, with those of Tashiro (31). Only an alkaline enzyme appeared to be present. When RNP was used as an enzyme source, a single sharp peak of optimum activity was obtained at pH 6.75 in the presence of 0.1 M ABC buffers, while with 0.1 M sodium methyl arsenate buffers a single peak was obtained at pH 7.43. Similar results were noted with separated microsomes. In addition, in a series of 3 experiments in which RNP was prepared with use of 1 per cent deoxycholate and RNase assays were performed on the RNP as well as the deoxycholate supernatant, it was found that approximately 44 per cent of the total RNase activity and 57.7 per cent of the RNA of the original microsomal preparation was retained in the RNP. Since about 90 per cent of the protein of the microsomes is removed by deoxycholate, but only 56 per cent of the RNase activity, this accounts for

the sharp rise in specific activity of separated RNP compared to microsomes.

Some properties of the RNase associated with liver microsomes and RNP are shown in the data given in Table V. Contrary to the results obtained by Elson (6) with ribonucleoprotein isolated from *E. coli*, concentrated urea solutions had no significant effect on the RNase activity of any of the preparations. However, a considerable solubilizing effect of 8 M urea on the RNA used for assay was noted (See also Kalnitsky *et al.* (24)). The considerably higher specific RNase activity of RNP, compared to microsomes, is noteworthy and it is obvious that the 1 per cent deoxycholate used in these experiments preferentially removes protein other than RNase from the microsomes. Table V also lists the effects of cupric ion on the various preparations.

DISCUSSION

The effects of proteolytic enzymes on liver microsomes and RNP appear to be consistent with the interpretation that degradation of the protein of RNP causes a separation of the RNA, which is then attacked by the RNase present in the preparation. Alternatively, the RNA may, when the cell is broken up, absorb RNase which ruptures nucleotide linkages. Subsequent breakage of peptide bonds would facilitate dissolution of the RNA into acid-soluble products. The occurrence of discontinuities or breaks in the native RNA cannot be completely excluded at present.

The presence of considerable RNase activity in preparations of rat liver microsomes and especially RNP raises some interesting questions. First, is this RNase simply a contaminant, or is it a normal constitutent of the RNP as has been suggested by Elson (6)? Second, if a normal constituent, what are its properties and functions, and under what conditions does it operate: also how is it related to the other RNases present in the liver cell? Third, if a contaminant, what is its effect on the incorporation of amino acids into protein by microsomes or RNP? In this latter respect, the RNase might be predicted to have a profound effect on amino acid incorporation or protein synthesis since it can be expected to exert a deleterious effect on the integrity of high molecular weight RNA.

Some answers to the first two questions above may be deduced from Table V which lists some of the properties of the RNase associated with liver microsomes and RNP. Some indications may be obtained, also, from previous work; for example Schneider and Hogeboom (25) presented evidence which indicated that microsomes were capable of absorbing RNase. There is much evidence that protein synthesis and the incorporation of amino acids into protein can be inhibited or abolished by crystalline pancreatic RNase in relatively high concentrations (26, 27).

It would appear to be important, therefore, to eliminate all possible contamination with this enzyme from systems in which protein synthesis or amino acid incorporation is being studied. This is, of course, not a simple task since as soon as a cell is broken by homogenization its component parts become subject to the action of RNase present principally in the mitochondrial fraction.

In view of the deleterious effect of RNase on amino acid incorporation and protein synthesis, a possible role for RNase inhibitor is suggested, namely, to combine with any RNase that may leak from mitochondria or lysosomes and thus help maintain the complex structure of both the soluble RNA and the RNA associated with microsomes.

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