

# STUDIES ON THE FUNCTION OF INTRACELLULAR RIBONUCLEASES

## III. The Relationship of the Ribonuclease Activity of Rat Liver Microsomes to their Biological Activity

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### ABSTRACT

Attempts have been made to prepare rat liver microsomes and ribosomes free of RNase activity. Washing of microsomes with a large number of reagents, as well as preparation of microsomes by homogenizing the liver in the presence of a variety of reagents chosen to remove or inhibit RNase activity, failed to abolish completely the enzyme activity. However, when rat liver was homogenized in the presence of optimal concentrations of ATP the microsomes subsequently obtained showed no RNase activity. The composition of such microsomes was compared to controls prepared without the use of ATP. Preparation of microsomes with the use of ATP apparently repressed but did not remove the RNase activity for, when such microsomes were treated with 1 per cent deoxycholate to obtain ribosomes, the latter exhibited normal RNase activity. A possible explanation for these results based on several experiments is given. The incorporation of  $C^{14}$  of L-leucine- $C^{14}$  into control and ATP-treated microsomes was measured. Repression of RNase activity by use of ATP or with RNase inhibitor, significantly reduced the incorporation. As a result of these and other experiments it is tentatively concluded that an alkaline RNase is a normal constituent of rat liver ribosomes and plays a role in the biological activity of these particles.

### INTRODUCTION

A correspondence of intracellular ribonuclease (RNase) activity with proliferative growth has been noted by several investigators (1-3). The suggestion has been made by many workers that intracellular RNases may have a synthetic function (1, 4, 5) as well as the known degradative

one. It has also been suggested that RNase may function to break down the ribonucleoprotein complex, releasing biologically active protein from synthetic sites in the various cell particulates (6, 9).

Recently it has been demonstrated that micro-

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somes and ribonucleoprotein particles (ribosomes)<sup>1</sup> prepared from rat liver contain an alkaline RNase (7, 8). The ribosomes contain about 10 times the specific RNase activity of the microsomes from which they are prepared (8). The association of a latent RNase with ribosomes from *Escherichia coli* has also been reported (9), and some of the properties of this enzyme have been studied (10).

Since it is fairly well agreed that ribosomes play an important role in protein synthesis (11-13), these particles provide a useful system for studying the relationship between RNase activity and protein synthesis and possibly for determining the function of intracellular RNases.

The presence of this RNase activity in microsomes and ribosomes raises several important questions. First, is the enzyme a normal constituent of microsomes or ribosomes, or is it a contaminant adsorbed from other cell structures during homogenization? If a normal constituent, what is its function, and if a contaminant, would it interfere with the biological activity of microsomes or ribosomes? Efforts have been made to obtain answers to these questions by attempting to prepare rat liver microsomes and ribosomes free of RNase activity and by determining the biological activity of such RNase-free particles.

Dickman and Morrill have reported that RNase activity can be removed from mouse pancreas microsomes by washing them with a variety of reagents (14). These same reagents have been tested in this report and found to be ineffective with rat liver microsomes. It has been demonstrated recently that ATP will release enzymes including RNase from pancreas ribosomes (15).

In the experiments presented in this paper, ATP was found to reduce the RNase activity of rat liver microsomes to negligible values under certain conditions, but it appeared to do so by only repressing the activity, not removing the enzyme from the particle. A possible mechanism of this inhibition of activity is discussed below.

In searching for methods to reduce or remove the RNase activity from microsome preparations, two approaches may be used:

(a) The microsomes may be prepared by stand-

<sup>1</sup>In accordance with the suggestion in, *Microsomal Particles and Protein Synthesis*, (R. E. Roberts, editor), New York, Pergamon Press, 1958, viii, the term ribosome is used to indicate the ribonucleoprotein particles of 20 to 100 S associated with the endoplasmic reticulum of rat liver and is also used for similar particles from other sources.

ard techniques and then treated with various agents designed to either leach out or remove RNase activity, or suppress it by combining with, or otherwise inhibiting the enzyme. The disadvantage of this approach is that if contaminant RNase has combined with microsomal ribonucleoprotein it may have already exerted a deleterious action on RNA or ribonucleoprotein structure. Removal would only be expected to prevent further degradation during incubation or other treatment.

(b) The second approach, which is the preferable one, is to homogenize the tissue in the presence of reagents that will prevent the adsorption of cellular RNase by microsomes, or reagents that will preferentially combine with, adsorb, or inactivate free RNase activity. Microsomes or ribosomes prepared in this manner would be initially free of RNase activity and therefore not subjected to any degradative action during the preparative procedure or later.

The sections (a) and (b) of the Results correspond to the above two approaches, respectively.

It has been assumed thus far, that the RNase in microsomes is a contaminant, but the possibility that it is an integral part of ribonucleoprotein, necessary for its proper functioning, should not be overlooked. In fact this latter possibility seems more probable from the data in this report as well as others (10).

## MATERIALS AND METHODS

Microsomes and ribosomes were prepared essentially as previously described (8) with the following exceptions: Loose TenBroeck homogenizers were utilized to prepare the homogenates and, to remove mitochondria, a single centrifugation for 35 minutes at 6600 *g* was employed instead of two 17½ minute centrifugations at the same centrifugal force. These conditions reduced the yield of microsomes considerably over that previously described (8). However, all experiments were strictly comparative, so the absolute yield of microsomes was not a significant factor. In general the analytical values for the control samples seldom varied more than 10 to 15 per cent in all the experiments.

Varying quantities of liver from male Wistar rats of 200 to 400 gm. weight were used, except in the experiments dealing with the effect of ATP on the incorporation of labeled leucine into microsomes, in which the amount of liver was

always 10 gm. Each experiment was performed at least twice and in some cases 5 to 6 times, but usually with slight variations in the procedure or concentrations of reagents used. For this reason, in some cases, selected experiments are discussed, and significant variations in the procedure or results are commented upon. The methods used in the incorporation studies are described in connection with these experiments. The analytical methods for RNA, nitrogen (N), protein, and RNase activity were the same as those previously described (8) except that in the assay for RNase activity, carried out at pH 7.4, the precipitant consisted of 5 per cent trichloroacetic acid containing 0.5 per cent aluminum chloride. Crystalline pancreatic RNase was obtained from Worthington Biochemical Co., Freehold, New Jersey. Heparin and treburon were the same samples as described previously (18). Protamine sulfate was obtained from Nutritional Biochemicals Co., Cleveland, Ohio, and salmine from Delta Chemical Works, New York. Histidylhistidine was a product of Mann Research Laboratories, New York.

## RESULTS

### A. Attempts to Remove RNase Activity from Microsomes by Treatment with Various Reagents

1. Duponol (sodium dodecyl sulfate): Identical quantities of microsomes were homogenized in 0.1, 0.3, 0.5, and 1 per cent water solutions of duponol. The homogenates were centrifuged at 105,000 *g* for 90 minutes and the residues made up to a definite volume and analyzed. Duponol was quite effective in removing RNase activity, especially at the two higher concentrations. However, it also removed most of the RNA and N at these concentrations. An effective detergent would be one that would give a preparation of ribosomes containing 40 to 45 per cent RNA, with little RNase activity.

2. Deoxycholate plus heparin: Identical quantities of microsomes were treated as described under experiment 1 with different concentrations of water solutions of sodium deoxycholate, all containing 1 mg. of heparin per ml. of solution. The results of a typical experiment are illustrated in Table I. One per cent deoxycholate plus heparin proved quite effective in removing or suppressing the RNase activity of ribosomes. The yield of particles was considerably reduced, however.

Normally, when microsomes are treated with 1 per cent deoxycholate, 55 to 60 per cent of the microsomal RNA is recovered in the ribosomes (8). The presence of 1 mg. per ml. of heparin reduced this recovery to 25 to 30 per cent.

3. Deoxycholate plus treburon: When treburon, a synthetic sulfated polysaccharide and good RNase inhibitor (18), was substituted for heparin, the RNase activity of the resulting ribosomes was reduced to about one-third that of controls. The recovery of ribosomes was again considerably reduced as in the deoxycholate plus heparin treatment.

4. Amberlite XE 64 resin: This resin has been extensively used for the chromatographic separation of pancreatic RNase (19). Aliquots of microsomes were homogenized with 0.25 M sucrose and sucrose containing different quantities of suspended resin. The resin was removed by centrifugation at 5,000

TABLE I  
*The Effect of Treatment of Microsomes with Various Concentrations of Sodium Deoxycholate plus Heparin*

Sample and conditions*	RNase specific activity	RNA	Nitrogen
	<i>units per mg. N</i>	<i>mg. per ml.‡</i>	<i>mg. per ml.§</i>
Residue from microsomes treated with 0.1 per cent deoxycholate	267	1.07	1.42
Residue from microsomes treated with 0.3 per cent deoxycholate	351	0.71	0.50
Residue from microsomes treated with 0.5 per cent deoxycholate	470‡	0.55	0.23
Residue from microsomes treated with 1.0 per cent deoxycholate	0	0.25	0.095

\* The experimental procedure is described in the text. All sodium deoxycholate solutions contained 1.0 mg. of heparin per ml. of solution.

‡ The increase in specific RNase activity is due to removal of most of the protein. The values for RNase specific activity in this table are to be compared with an average value of 1780 for a series of 10 control ribosome samples (8).

§ The residues obtained after treatment of the microsomes with the above concentrations of deoxycholate were made up to a total volume of 10 ml. in distilled water and 1 ml. samples of this utilized for analysis of RNA and nitrogen.

*g* for 10 minutes and the microsomes then collected by centrifugation at 60,000 *g* for 35 minutes. In a series of experiments in which 3 different concentrations of resin were used, the adsorbent removed from 0 to 67 per cent of the RNase activity from the microsomes. There was no significant change in the RNA content of the preparations treated with resin.

5. The following reagents or treatments were ineffective in removing RNase activity from rat liver microsomes.

(a) Washing with sucrose, pH 9.0.

(b) Washing with sucrose, pH 9.0 plus 0.01 M ethylenediaminetetraacetic acid (EDTA).

(c) Washing with 0.1 M ammonium chloride, pH 9.0.

The difference in behavior of liver microsomes and pancreas microsomes treated with these reagents (14) is unexplained but it appears probable that pancreas microsomes have much larger quantities of RNase associated with them since the pancreas is concerned with the synthesis of this extracellular digestive enzyme. As an extracellular enzyme one would expect it to be easily removed from its site of synthesis.

(d) Homogenization of microsomes with tween 80, triton X-100, and triton X-305 in concentrations ranging from 0.1 to 1.0 per cent.

(e) Recently bentonite has been used with success to remove RNase from yeast and tobacco mosaic virus preparations (16, 17). However, treatment of sucrose suspensions of microsomes with bentonite caused aggregation of the microsomal particles which thereby sedimented at the low centrifugal forces used to separate the bentonite.

#### B. Attempts to Prepare RNase-Free Microsomes by Homogenization of Rat Liver in the Presence of Various Reagents

In the following experiments equal portions of liver were homogenized in 0.25 M sucrose containing various substances, and microsomes prepared and analyzed as before. The control was a similar portion of the same liver homogenized in 0.25 M sucrose solution.

6. Heparin: When rat liver was homogenized in 0.25 M sucrose containing 1 mg. of heparin per ml., the microsomal pellet was small and whitish-opalescent. It contained no RNase activity. However, no RNA or protein was present either, and

the pellet was, therefore, probably mostly lipid or polysaccharide. It was not investigated further. Use of 0.5 mg. of heparin per ml. of sucrose solution resulted in the retention of about one-third of the control amount of RNA in the microsomal pellet with a corresponding decrease in RNase activity.

7. Histidine or histidine plus EDTA: Homogenization of liver in 0.25 M sucrose containing the reagents listed in Table II gave results which were typical of many other experiments for which the analytical data are not given. In a large series of experiments there has been a good correlation of RNase activity with the RNA content of microsomal preparations. Removal of RNA, by any means, usually was accompanied by a corresponding reduction in RNase activity. This has been a fairly consistent finding with the exceptions noted in experiments 4, 8*h*, and 8*i*.

8. Homogenization of liver with the following solutions either failed to affect significantly the RNase activity of the microsome preparations or adversely affected them as described below.

(a) 0.25 M sucrose pH 9.0.

(b) 0.1 or 0.2 M phosphate buffer pH 7.0 (no sucrose).

(c) 0.25 M sucrose containing either 5.5 mM lysine or arginine neutralized to pH 7.3.

(d) 0.25 M sucrose containing 3.4 mM histidyl-histidine.

TABLE II

*The Effect of Histidine and Histidine plus EDTA on the RNase Activity and Composition of Rat Liver Microsomes*

Conditions*	RNase specific activity	RNA	Nitrogen	Protein
	units per mg. N	mg. per ml.	mg. per ml.	mg. per ml.
Control	160	0.80	0.94	6.87
6.5 mM histidine	102	0.68	0.88	6.01
6.5 mM histidine plus 3 mM EDTA	96	0.46	0.73	5.35

\* Microsomes were prepared from 7 gm. of liver in 0.25 M sucrose for the control or sucrose plus the final concentrations of histidine or histidine plus EDTA indicated in the table for the experimentals and the microsomal pellet in each case was made up to a final volume of 10 ml.

(e) 0.25 M sucrose containing 1 mg. per ml. of an insoluble complex of heparin-protamine.

(f) 0.25 M sucrose containing 1 mg. of protamine per ml. The results obtained with this solution were identical to those described in experiment 6. Smaller quantities of protamine gave increasing recoveries of RNase, RNA, and protein. Salmine substituted for protamine gave similar results.

(g) 0.25 M sucrose containing 0.018 M calcium chloride solution. The addition of this concentration of calcium chloride apparently caused aggregation of the microsomes because no RNA or RNase activity was recovered in the pellet. This effect has been studied by Gross and Pearl (25).

(h) 0.25 M sucrose, pH 9.0 plus 0.01 M EDTA. Homogenization of liver in this solution had no effect on RNase activity but reduced the RNA content of the microsomes to approximately one-quarter that of controls.

(i) 0.25 M sucrose containing increasing concentrations of XE 64 resin. No significant effect on the RNase activity of microsome preparations was noted, but with increasing concentrations of resin, increasing amounts of RNA, N, and protein were removed from the microsomal pellet.

(j) 0.25 M sucrose containing various quantities of bentonite. Aggregation of microsomes took place; no microsomal pellet was obtained even when small quantities of bentonite were used.

#### *The Effect of ATP on the RNase Activity of Rat Liver Microsomes*

In the following experiments with ATP, similar 10 gm. portions of pooled liver from 2 or 3 rats

were homogenized in either 0.25 M sucrose solution, or sucrose solution which was 1.8 or 3.6 mM with respect to ATP (1 mg. or 2 mg. ATP per ml.). Microsomes were prepared as before and washed once with 0.25 M sucrose solution. The results of a group of experiments are given in Table III. Consideration of the data in the table, shows that microsomes prepared in sucrose solution containing 1.8 mM ATP have negligible RNase activity compared to controls. In many experiments the RNase activity of this preparation was zero; in a few, slight activity of doubtful significance was observed, and in one or two experiments activity was significant but no more than 20 to 30 per cent of controls. The RNA content was slightly elevated and N and protein content significantly increased (35 to 45 per cent) compared to control samples. The increase in N is accounted for, within experimental error, by the N contained in the extra 3.9 mg. of protein and 0.24 mg. of RNA. The reason for this increase in protein and RNA is unknown. It appears possible that the solutions containing ATP in some way cause additional protein to aggregate with, and subsequently separate with the microsomes. Evidence for this possibility is presented below.

The microsomes prepared with the use of 3.6 mM ATP, almost without exception, contained no detectable RNase activity. The RNA content of these microsomes was significantly lower, but the N and protein contents were not significantly different from the controls. In the experiments summarized in Table III, the sucrose solutions containing the ATP were not adjusted to neutral

TABLE III  
*The Effect of ATP on the RNase Activity and Composition of Rat Liver Microsomes*

Average values and range for 8 experiments				
Sample*	RNase specific activity	RNA	Nitrogen	Protein
	<i>units per mg. N</i>	<i>mg. per ml.</i>	<i>mg. per ml.</i>	<i>mg. per ml.</i>
0.25 M sucrose (control)	208 (164-290)	1.50 (1.27-1.63)	2.00 (1.75-2.48)	11.4 (9.4-15.2)
0.25 M sucrose plus 1.8 mM ATP	28 (0-68)	1.74 (1.56-2.24)	2.87 (2.50-3.65)	15.3 (12.2-18.6)
0.25 M sucrose plus 3.6 mM ATP	0 (0)	1.10 (0.74-1.37)	2.18 (1.64-2.65)	12.0 (9.3-13.4)

\* For each sample 10 gm. of rat liver were homogenized with 9 volumes of the respective solutions in the cold. Microsomes were resuspended by homogenization in 35 ml. of ice cold 0.25 M sucrose solution. Of this 35 ml., 10 ml. were utilized for amino acid incorporation experiments and the remainder was centrifuged at 60,000 g for 35 minutes and the pellet made up to 5.0 ml. with glass-distilled water for the above assay.

pH before use, consequently the pH of the resulting homogenates was lowered from 6.5 for the controls to 5.9 or 5.6 with the lower and higher concentrations of ATP, respectively. This lowering of pH appears to be essential for the optimum reduction in RNase activity since the use of neutral ATP solutions, in several experiments, resulted in only a 50 per cent decline in RNase activity. Otherwise, the neutralized ATP solution gave the same results as the unneutralized solution.

It is known that ATP has the ability to chelate magnesium and other ions under certain conditions, and the results observed may be due in part to this effect. However, as previously mentioned, EDTA was not effective in removing RNase activity under the conditions described. Homogenization of liver in sucrose containing 1.8 mM guanosine triphosphate reduced the RNase activity of microsomes about 50 per cent without affecting the other measured components. 5'-Adenylic acid (6 mM) when unneutralized, caused most of the microsomal components to sediment with the mitochondria. This was thought to be due to lowering of the pH but when neutral 5'-adenylic acid was used, similar effects were noted.

#### *The RNase Activity of Ribosomes Prepared from ATP-Treated Microsomes*

It would appear from the data in Table III that homogenization of liver in the presence of

ATP was a successful method for removing RNase activity from microsomes. However, when microsomes prepared in this manner were treated with a 1 per cent water solution of sodium deoxycholate and the ribosomes subsequently isolated, they were found to contain approximately the same RNase activity as control samples not treated with ATP. The results of two typical experiments are depicted in Table IV which also gives the experimental details. Not enough sample was available to carry out a complete assay for RNA, N, and protein on microsomes, but the values in Table III will give a good approximation of the results missing in Table IV. Examination of the data in Table IV indicates a 7- to 9-fold increase in the specific RNase activity of ribosomes when compared to microsomes, in agreement with previous results (8). There is, however, no significant difference between the RNase activity of the controls and the samples prepared with use of ATP. This suggests that the RNase activity of microsomes had been suppressed by ATP but the enzyme not removed from the particles.

In view of the increased protein content of microsomes prepared in the presence of 1.8 and 3.6 mM ATP, it was considered possible that part of this extra protein might consist of RNase inhibitor (20), which in some way was caused by the ATP to adhere to the microsomes. On treatment with deoxycholate the RNase inhibitor pro-

TABLE IV  
*The RNase Activity and Composition of Ribosomes Obtained from Microsomes Prepared with Use of ATP*

Sample and conditions*	RNase specific activity		RNA		Nitrogen		Protein	
	units per mg. N		mg. per ml.		mg. per ml.		mg. per ml.	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Microsomes: 0.25 M sucrose (control)	250	220						
Microsomes: 0.25 M sucrose + 1.8 mM ATP	14	0						
Microsomes: 0.25 M sucrose + 3.6 mM ATP	0	0						
Ribosomes (control)	1800	1586	0.66	0.48	0.30	0.29	1.42	1.68
Ribosomes (1.8 mM ATP)	1520	1790	0.30 ‡	0.93	0.48	0.43	2.46	2.49
Ribosomes (3.6 mM ATP)	1544	1420	0.44	0.49	0.25	0.29	1.25	1.88

\* Liver from 2 to 3 rats was pooled and three 10 gm. samples homogenized with 9 volumes of the respective ice cold solutions above, using the same homogenizer. Microsomes were prepared and a portion reserved for RNase assay. The remaining microsomes were homogenized with 8 ml. of a cold 1 per cent water solution of sodium deoxycholate. These solutions were centrifuged at 105,000 g for 90 minutes and the resulting pellets made up to 5 ml. with glass-distilled water and analyzed.

‡ This value is probably in error.

tein may be removed, along with most of the microsomal protein.

The validity of this hypothesis may be tested in several ways. First, if RNase inhibitor is bound to the microsomes in the presence of ATP, addition of sulfhydryl reactants (21) may inactivate the inhibitor and release the suppressed RNase activity. To test this possibility, microsomes prepared in sucrose containing 1.8 or 3.6 mM ATP were treated, in separate experiments with 0.4 mM *p*-chloromercuribenzenesulfonic acid (CMS), *p*-chloromercuribenzoic acid (CMB), or Pb<sup>++</sup>, and compared to control microsomes treated similarly. In a series of five experiments, in no case, did any of the sulfhydryl reactants release appreciable RNase activity from the ATP-treated microsomes. It is, of course, possible that the release of RNase activity may not occur in the presence of ATP. It appeared that some activity was released from the control microsomes on treatment with the above sulfhydryl reactants but the nature of this release of RNase activity from control microsomes was not investigated further.

In a second type of experiment, a rat liver was divided into two similar portions. One portion (A) was homogenized in 0.25 M sucrose (control), and the second (B) in sucrose plus 2.7 mM ATP. Supernatant fraction was prepared from each and the supernate from A was divided into two portions. To one (A<sub>1</sub>) ATP to a final concentration of 2.7 mM was added. All three supernates were assayed for RNase inhibitor as previously described (21). The amount of inhibitor in A and A<sub>1</sub> was approximately the same, while in B the amount was considerably reduced. This experiment may be taken as evidence for the suggestion that

homogenization of rat liver in the presence of ATP causes at least partial adsorption of RNase inhibitor on the microsomes, along with, possibly, other proteins or nucleoproteins.

#### *The Incorporation of Amino Acids into Microsomes Prepared with Use of ATP*

The procedures described by Greengard and Campbell (22) were utilized with some modifications. A 10 ml. aliquot of washed microsomes was centrifuged at 60,000 *g* for 35 minutes and then made up to 2.0 ml. The incubation mixtures contained 0.7 ml. of microsome suspension in buffer (mixture X) (26), 0.3 ml. of rat liver supernatant fraction, 0.2 ml. of phosphoenolpyruvate solution containing 10 μM of phosphoenolpyruvate and 2 μM of ATP and 0.1 ml. of uniformly labeled L-leucine-C<sup>14</sup> (1 μc.). For the controls, the leucine was added immediately prior to precipitation of the mixture with 5 per cent perchloric acid. The supernates prepared in sucrose containing ATP contained, of course, a small additional quantity of ATP. The mixtures were incubated for 1 hour at 37° under a slow stream of 95 per cent N<sub>2</sub> plus 5 per cent CO<sub>2</sub> gas. After thorough washing and removal of nucleic acids, aliquots of the precipitates were placed in planchets and dried. An adsorption curve made with increasing amounts of microsomal protein was prepared for correcting the samples for self-absorption. With the amount of protein on the planchets (1 to 2 mg.) this correction proved to be negligible. The samples were counted in a gas-flow counter.

The results of several typical experiments are illustrated in Table V. Repression of RNase

TABLE V  
*The Incorporation of C<sup>14</sup> of L-Leucine-C<sup>14</sup> into Rat Liver Microsomes Prepared with Use of ATP*

Sample and method of preparation*	Counts per minute per mg of protein				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5†
Control (no ATP)	510	400	541	850	433
Control plus RNase inhibitor‡				289	
Control (1.8 mM ATP utilized in preparation of microsomes)	173	183	266		329
Control (3.6 mM ATP utilized in preparation of microsomes)	153	119	129		154

\* The experimental details are given in the text.

† Neutralized ATP was used. The RNase activity of the microsomes was approximately 50 per cent of the control activity.

‡ A partly purified preparation (0.3 ml.) (21) was utilized.

activity, whether by preparation of microsomes in the presence of ATP, or by addition of RNase inhibitor, resulted in a marked decrease in the incorporation of radioactivity of microsomes. The magnitude of the incorporation of radioactivity into the control microsomes varied over fairly wide limits with different preparations. Also, the magnitude of the decline in incorporation of the radioactive isotope into the microsomes prepared with use of ATP varied, and in some experiments it was less than in the ones illustrated. In spite of this, in a series of 14 experiments there were no important deviations from these results.

Addition of 1  $\mu$ g. of crystalline pancreatic RNase to the incubation mixture resulted in a small decline in activity, while with 5  $\mu$ g. the decline in incorporation was approximately 50 per cent. When small amounts of pancreatic RNase (0.03  $\mu$ g.) were added to the microsome samples prepared with the use of ATP, there was a small increase in the incorporation. However, this is not considered significant since it occurred with control samples also.

#### DISCUSSION

From the evidence presented in the foregoing sections the following tentative conclusions may be drawn.

1. An alkaline RNase is a normal constituent of rat liver ribosomal nucleoprotein and,
2. The activity of this RNase is essential to the optimal biological functioning of the ribosomes.

The two main lines of evidence for these conclusions are: an excellent correlation between the amount of RNA in microsomes and the RNase activity of these particles. Although not mentioned in the preceding results, this correlation also holds quite well for ribosomes. Thus, in several experiments in which ribosomes were treated in various ways resulting in a decrease in their RNA content, there was a corresponding decrease in their RNase activity. It should be also noted that Spahr and Hollingworth (10) recently investigated the properties of a RNase from *E. coli* ribosomes and concluded that the RNase is an integral part of the particles. With respect to rat liver ribosomes or microsomes, it is quite possible that part of the

RNase activity associated with these particles is adsorbed on homogenization of the cell, but a portion of the activity may be normally present. It is interesting to observe, however, that there appears to be no acid RNase present in these particles, although it would be expected that the processes used in preparation of the fractions would favor release of at least some of this lysosomal enzyme. The second line of evidence is: repression of the RNase activity of microsomes causes a decrease in the incorporation of the radioactivity of  $C^{14}$  L-leucine. This evidence would be stronger if it would be possible to restore this activity by addition of RNase, but it is possible that crystalline pancreatic RNase does not have the correct specificity or protein structure and that one must add back the ribosomal enzyme itself.

It is quite obvious that further evidence is needed to establish the above conclusions on firmer footing. Since the incorporation of amino acids into separated ribosomes may now be readily accomplished (23, 24), studies with this system might be particularly useful. By using ribosomes, most of the lipid components and a large part of the varied enzyme content of microsomes would be eliminated.

There is a need for further knowledge of the properties of the RNase associated with ribosomes, particularly its specificity. It would be important also to isolate and purify this RNase so that its relation to ribosome systems may be assessed. These problems are presently being studied in our laboratory.

It is of interest to speculate on a possible role of ATP in the final stages of the protein synthetic process. Although the details of the activation of amino acids and their transfer to soluble RNA are being rapidly elucidated (13), the manner in which the individual amino acid residues, when arranged on the matrix RNA, are joined together, and the method by which the final assembled protein is removed from association with the matrix RNA is still unknown, although many suggestions have been offered. It is quite possible that ATP plays an important role in these processes and that its apparent relation to RNase activity in microsomes is somehow involved.

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