RIBONUCLEASES OF RAT LIVER

I. PARTIAL PURIFICATION AND PROPERTIES*

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There is increasing evidence for the presence of different specific nucleases in mammalian tissues which are able to break the internucleotide linkages of ribonucleic acid. The pancreatic enzyme first crystallized by KUNITZ is the best known of the ribonucleases[1]. The pancreatic ribonuclease, despite its homogeneity as observed by sedimentation studies[2], has been shown by ion exchange chromatography to contain at least two protein components[3–6]. The presence in spleen of another specific polynuclease has been reported by MAVER AND GRECO[7] and by HILMOE AND HEPPEL[8]. The mode of action of this spleen polynuclease on RNA appears different from that of the pancreatic ribonuclease. The presence and intracellular distribution in rat liver of two ribonucleases differing in their properties has also been reported[9–13] as well as their presence in various tissues of mouse and rat[14]. Their intracellular distribution in liver cell particulates isolated by a more elaborate procedure of differential centrifugation has shown that acid ribonuclease is localized exclusively in the mitochondrial fractions whereas the alkaline enzyme was present in both the mitochondrial and microsomal fractions[15].

The behavior of the acid and alkaline ribonucleases under various physiological and pathological conditions has been studied in rat liver. The effect of fasting[16], cortisone administration[17], liver regeneration and azo dyes feeding[18] on the levels and intracellular distribution of these enzymes have been examined. Similar studies in primary liver tumors induced by azo dyes as well as in transplanted hepatoma have been carried out[17,19]. The variations in the levels and intracellular distribution of the liver ribonucleases under the conditions mentioned above have prompted us to investigate the mode of action of these enzymes on ribonucleic acid. This seemed of interest also in view of the different modes of action of spleen and pancreas nucleases on RNA. A better understanding of the physiological role of ribonucleases might be obtained by such a study.

It was necessary for this purpose to obtain preparations in which each liver

* This investigation was supported by a grant-in-aid from the National Cancer Institute of Canada.
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Abbreviation: Ribonuclease, RNAase; Ribonucleic acid, RNA; adenosine 2′-phosphate, A2p; adenosine 3′-phosphate, A3p; cyclic 2′–3′-adenosine monophosphate, Ap!; cyclic 2′–3′ uridine monophosphate, Up!

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ribonuclease would be freed from one another. Methods for preparing purified acid and alkaline ribonuclease are reported. Properties which differentiated these two liver ribonucleases are discussed.

METHODS

Substrate

The substrate was prepared from deproteinized commercial yeast RNA sodium salt by precipitation of a 20% solution with two volumes of glacial acetic acid. The precipitate was redissolved in water and dialyzed for 24 hours with multiple changes of distilled water. The dialyzed solution was treated in the cold with two volumes of absolute alcohol. The precipitate obtained was dried in vacuo and used as substrate.

Preparation of homogenate and mitochondrial fraction

White rats (Wistar) were stunned and decapitated. The liver was rapidly excised, placed on ice for two minutes and then blotted on filter paper. The liver was mashed in a plexiglass squeezer and an exact amount of the pulp obtained was weighed. The pulp was homogenized in 0.25 *M* sucrose in a glass homogenizer fitted with a Teflon pestle to give a final 10% homogenate. Mitochondrial fraction was obtained from this homogenate by differential centrifugation according to the method of HOGEBOOM AND SCHNEIDER[20].

Heating assays

Liver homogenates (10%) prepared in sucrose, isolated mitochondrial preparations or water extracts were used as crude sources of enzyme. Solutions of crystalline pancreatic ribonuclease (Worthington) containing 0.25 or 0.5 mg of enzyme per liter were also used for purpose of comparison. Two ml of the various sources of enzyme mentioned above were immersed for periods of 1 to 5 minutes in boiling water and then rapidly cooled. The mixture was then decanted or filtered and determinations of activity were carried out on the clear supernatant.

Determination of ribonuclease activity

The ribonuclease activity was determined spectrophotometrically as previously described and was expressed in arbitrary units[9].

Preparation of purified alkaline RNAase

The alkaline RNAase was prepared by a modification of the method of KUNITZ[1]. The preparation proceeds as follows: (1) 200–300 g of rat liver were homogenized, in the cold, with a glass homogenizer and Teflon pestle in an equal volume of distilled water. This homogenate was made 0.25 *N* with respect to sulfuric acid by addition of an equal volume of 0.5 *N* sulfuric acid solution. The homogenate was placed in the refrigerator for a period of 8 to 24 hours with intermittent stirring. When the extraction was carried out during a period of 24 hours, a colloidal suspension was formed which could not easily be clarified. Following extraction, the preparation was filtered through eight layers of cheese cloth. The supernatant was preserved and the remaining pulp was reextracted for 1 hour with an equal volume of 0.25 *N* sulfuric acid solution and then filtered through cheese cloth. The residue was discarded. The two supernatants were combined and centrifuged at 1000 *g* during 20 min in a Serval SS-1 centrifuge. The clear supernatant was used for the preparation of the enzyme concentrate. (2) The supernatant was brought to 0.6 saturation with solid ammonium sulfate and the precipitate formed was centrifuged and discarded. Ammonium sulfate was then added with stirring to the supernatant to obtain 0.8 saturation and left standing overnight in the refrigerator. The precipitate was collected by centrifugation and dissolved in 200–250 ml of distilled water. The solution was dialyzed during 24 hours against multiple changes of distilled water. (3) The dialyzed solution was again treated as in (2). The repetition of the ammonium sulfate treatment eliminated much protein without important loss of enzymic activity. (4) The final dialyzed solution was then heated for 5 minutes in boiling water and rapidly cooled. Heating insured an almost complete destruction of the acid ribonuclease as was indicated by preliminary assays on crude sources of enzyme (results Table I). After heating, the ammonium sulfate treatment as outlined in (2) was repeated again. However, once the precipitate was obtained with 0.8 saturation of ammonium sulfate, it was dissolved in water and this solution was treated in the cold with two volumes of absolute alcohol instead of being dialyzed. The precipitate was collected by centrifugation, dissolved with the smallest possible volume of distilled water, and subjected to lyophilization. The yield of such a preparation was 1.0 to 1.2 g of dry products.

Preparation of acid RNAase

An acetone powder was prepared from 250–300 g of rat liver, yielding 40 g of dry substance[21]. Acid

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ribonuclease was extracted from this powder by the following method based on the few indications given by HEPPEL *et al.*[22].

Forty g of acetone powder were extracted in the cold with 300–400 ml of 0.2 *N* acetate buffer pH 6.0 during four hours. All the following operations were performed in the cold. The suspension was centrifuged, the supernatant preserved and the precipitate reextracted during 30 minutes. The suspension was centrifuged, the precipitate discarded, and the supernatant combined with that of the first extraction.

The combined supernatants were adjusted at pH 5.0 by slow addition of 0.5 *N* sulfuric acid, and solid ammonium sulfate was added to give a solution of 0.6 saturation. The pH was continuously adjusted during addition of ammonium sulfate. The precipitate formed was centrifuged and discarded. The supernatant was then brought to 0.8 saturation with solid ammonium sulfate and the pH adjusted to 5.0. This was left overnight in the refrigerator. The precipitate formed was collected by centrifugation and dissolved in 200 ml of water. This solution was adjusted and maintained at pH 8.0 with ammonium hydroxide during addition of solid ammonium sulfate to 0.6 saturation. The precipitate was centrifuged and discarded. The supernatant was brought to 0.8 saturation of ammonium sulfate, the pH being continuously adjusted at 8.0. This was left overnight in the refrigerator. After this period, the precipitate was centrifuged and dissolved in water.

The precipitations at 0.6 and 0.8 saturation of ammonium sulfate at pH 8.0 were then repeated. This eliminated large amounts of alkaline RNAase. The final precipitate dissolved in distilled water was used as a source of purified acid ribonuclease.

RESULTS AND DISCUSSION

Effect of heating on liver ribonuclease activity of crude sources

Table I summarizes the results of the assays on the effect of heating on acid and alkaline ribonucleases. The results are the mean of four or five different assays and expressed as percentage of activity remaining after heating, the activity of the non-heated preparations being taken as 100 %. The results show that the behavior of acid and alkaline ribonucleases of rat liver was different upon heating. In whole liver homogenate, only 10% of acid ribonuclease activity remained following heating in boiling water, whereas the homogenate retained about 50 % of its original alkaline ribonuclease activity. Similar results were obtained when mitochondrial preparations were used as crude source of enzyme. Acid and alkaline ribonucleases retained 5 and 50 % respectively of their original activity. Assays carried out on extracts of homogenates and mitochondrial preparations showed essentially the same behavior. The

TABLE I

EFFECT OF HEATING ON RIBONUCLEASE ACTIVITY

*Period of heating**	*Liver homogenate*		*Liver mitochondria*		*Crystalline pancreatic enzyme*	
	Acid pH 5.5	*Alkaline pH 8.1*	*Acid pH 5.5*	*Alkaline pH 8.1*	*pH 5.5*	*pH 8.1*
min.	%**	%	%	%	%	%
0	100.0	100.0	100.0	100.0	100.0	100.0
1	13.5 ± 2.9***	39.6 ± 8.1	9.1 ± 2.6	48.8 ± 4.7	76.9	95.6
3	13.4 ± 3.6	31.2 ± 8.8	5.5 ± 0.2	46.5 ± 3.6	50.0	73.5
5	10.8 ± 6.6	43.8 ± 4.7	5.6 ± 2.7	46.0 ± 6.3	65.4	

* In boiling water.

** Percentage of residual activity calculated from values of the original unheated preparations taken as 100 %.

*** Standard deviation.

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crystalline pancreatic ribonuclease (Worthington) showed after heating a loss of 25% of its original activity in our conditions.

The loss of activity of the pancreatic enzyme is somewhat larger than that previously reported by McDONALD[23]. However, this can be easily explained by differences in the conditions of assays such as pH, salt concentration of the enzyme preparations, or the degree of purification of these preparations. These results showed essentially that the liver ribonucleases did not differ only by their optimum pH of activity[9,10] but also by their heat stability. The fact that acid ribonuclease is almost completely destroyed by heating proved of great help in separating this liver enzyme from the alkaline ribonuclease.

Purification of alkaline ribonuclease

Table II summarizes the progress of purification obtained at the various steps of the preparation procedure. One unit of activity is defined as the amount of enzyme which will cause an increase in the optical density of the incubation mixture equal to 2.0 during a 30 minute period of incubation. The activity was measured as previously described[9] except that the final concentration of substrate was 0.35% instead of 0.075%.

TABLE II

TOTAL AND SPECIFIC ACTIVITIES OBTAINED AT THE VARIOUS STEPS OF THE PURIFICATION PROCEDURE OF ALKALINE RIBONUCLEASE

	pH 5.7		*pH 7.8*		*Ratio*
	Total activity	*Spec. act.* act/mg N*	*Total activity*	*Spec. act. act/mg N*	*Sp. act. pH 7.8/ pH 5.7*
1) Original homogenate	170,750	29	341,500	59	2
2) First precipitate 0.8 saturation dissolved in water	9,384	109	37,930	318	2.9
3) 24 hours, dialysis	12,000	140	46,200	537	3.8
4) Second precipitation	7,728	563	30,139	2,216	4
5) Lyophilized	2,851	363	15,552	2,017	5.5

* Specific activity.

The total activity is the number of units present in the original suspension as a whole. The specific or activity per mg of nitrogen of the original suspension was calculated from four standard assays of ribonuclease activity with the amount of liver used in the original suspension. The activity of the alkaline ribonuclease preparation was assayed at both pH 5.7 and 7.8 at each step of the procedure. The results show that whereas the total activity of the alkaline ribonuclease decreased by about 22 times, the specific activity increased by 35 times. Comparatively the total activity at pH 5.7 decreased by 60 times and the specific activity increased only by 13 times. The ratio of the specific activity at pH 7.8 over pH 5.7 increased from 2 to 5.5 as indicated in the last column of Table II.

The specific activity of the lyophilized preparation at pH 5.7 was still 18% of the activity at pH 7.8. However, this activity does not seem to be due to contamination by acid ribonuclease since heating did not affect this activity. It would rather seem that alkaline ribonuclease is still able to destroy ribonucleic acid at pH 5.7. The purified preparation of alkaline RNAase was tested for the presence of various enzymes,

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i.e. unspecific acid and alkaline phosphatases[24], ATPase[16], and unspecific diesterase[25]. The lyophilized preparation was freed from these enzymes.

Purification of acid ribonuclease

Table III summarizes the progress of purification obtained at the various steps of the preparation procedure. The total final activity at pH 5.7 dropped 12 times whereas the specific activity increased by about 36 times. As this proceeded the total activity at pH 7.8 decreased by 55 times whereas the specific activity increased only by about 8 times. This purification of acid ribonuclease is illustrated by a drop in the ratio of specific activity at alkaline to acid pH from 2.0 to 0.43. The activity of this preparation was decreased by 70–75% upon heating at pH 5.7 but to a less extent at pH 7.8 indicating the presence of alkaline ribonuclease. Nevertheless, the great decrease in the ratio of alkaline over acid enzyme activity in the final preparation indicated that acid ribonuclease has been concentrated 5 times as compared to the original activity of alkaline ribonuclease. The purified preparations of acid RNAase were also assayed for the presence of the other enzymes mentioned above. The preparation was freed from alkaline phosphatase, ATPase and unspecific diesterase, but contained traces of acid phosphatase.

TABLE III

TOTAL AND SPECIFIC ACTIVITIES OBTAINED AT THE VARIOUS STEPS OF THE PURIFICATION PROCEDURE OF ACID RIBONUCLEASE

	pH 5.7		*pH 7.8*		*Ratio*
	Total activity	*Spec. act.* act/mg N*	*Total activity*	*Spec. act. act/mg N*	*Sp. act. pH 7.8/pH 5.7*
1) Original homogenate	170,750	29	341,500	59	2
2) After centrifugation from solid particles	85,027	169	82,700	164	1
3) 1st precip. 0.8 saturation pH 5 dissolved in water	39,936	577	35,942	519	0.9
4) 1st precipitate 0.8 saturation pH 8.0 dissolved in water	18,244	1,087	10,560	629	0.58
5) 2nd precipitate 0.8 saturation pH 8.0 dissolved in water	14,640	1,045	6,240	446	0.43

* Specific activity.

Properties of purified ribonuclease preparations from liver

The pH-activity curves of acid and alkaline ribonuclease preparations are given in Fig. 1. The curves indicate that separation of these enzymes was achieved. A sharp optimum is obtained for acid ribonuclease with low activities at alkaline pH values. On the other hand, the optimum pH of the alkaline RNAase is rather broad and low activity was observed at acid pH values. This, as stated above, is taken as indication that alkaline ribonuclease is still active at acid pH. The optimum pH of purified preparations, 5.7 and 7.5–8.0 corresponds to the pH already reported for homogenates[9, 10].

The effect of heat was studied on the purified preparations of enzymes (Table IV)

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and essentially the same results were obtained as with crude sources of enzymes (Table I). The purified preparation of acid ribonuclease retained only 29% of its original activity after heating whereas the alkaline one retained 80%. The heat lability

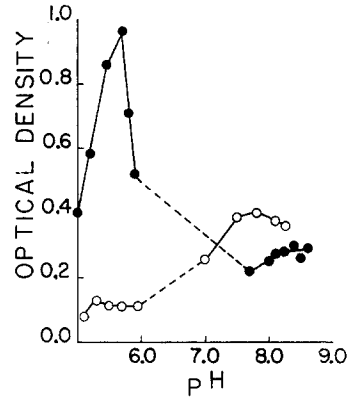

Fig. 1. pH-activity curve of purified preparations of acid and alkaline ribonucleases. Acid ●, alkaline ○.

TABLE IV

EFFECT OF HEATING* ON RNAASE ACTIVITIES OF PURIFIED PREPARATIONS

Purified preparations	*Percentage of original RNAase activity following heating at pH 7*	
	pH 5.7	pH 7.8
Acid ribonuclease	29 ± 3	90 ± 4
Alkaline ribonuclease**	80	92

* Period of 5 minutes in boiling water.
** Previously heated once during the purification procedure (see text).

of acid and alkaline RNAase seemed to differ according to the conditions of heating assays as mentioned above. The preparations of acid RNAase as reported here retained only 29% of the original activity after heating in our conditions. The presence of contaminating alkaline RNAase in our preparations is indicated by the high percentage of activity retained at pH 7.8. ROTH has reported that heating for 5 minutes at 70° C destroyed acid RNAase measured at pH 5.8 but had very little effect on alkaline RNAase measured at pH 7.8[26], whereas MAVER AND GRECO have observed only a slight loss of activity of both liver nucleases upon heating[19].

Another interesting difference between these enzymes was that alkaline ribonuclease retained most of its activity after lyophilization of its solution, whereas the acid enzyme activity was half destroyed under the same conditions. Furthermore, the acid ribonuclease was very labile and its activity was very rapidly lost in solutions even kept in the refrigerator.

The methods of preparation of purified liver ribonucleases differ from those already reported by MAVER AND GRECO[19] and by ROTH[26]. The methods presently reported permit the preparation of alkaline ribonuclease freed from the acid one with a specific activity 35 times greater than the original specific activity. This is less than the purification obtained by MAVER AND GRECO and by ROTH[19, 26]. However, preparation of acid ribonuclease, though slightly contaminated by alkaline ribonuclease, had a

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specific activity 36 times greater than that of the original homogenate. The methods outlined by these authors do not seem as effective as the present one for the preparation of acid RNAase, but superior for the preparation of alkaline ribonuclease.

Preliminary results on the specificity of acid and alkaline ribonuclease

The specificity of purified acid and alkaline ribonucleases are presently studied. Preliminary assays on the action of these enzymes on purine and pyrimidine cyclic mononucleotides have been carried out since it has been shown that pancreatic ribonuclease splits the cyclic pyrimidine derivatives without attacking the purine ones[27, 28]. It was thought of interest to establish whether either of the liver enzymes would possess this property. The assays were carried out as follows: purified acid and alkaline ribonuclease (2 to 3 units of activity) were incubated with 15 mg of Ap! or Up! at 37° C during periods of 17 and 40 hours. The pH was continuously adjusted at the optimum pH's 5.7 or 7.8 by careful addition of 0.01 *N* NaOH at short intervals throughout the incubation period. Paper chromatography (Whatman No. 4) was carried out on the reaction products using as identification guides the 2', 3' and the cyclic 2'-3' uridine or adenosine monophosphates. Controls were run in the same conditions of incubation but without the enzymes. The solvents used were the following: Saturated ammonium sulfate:isopropanol:water (79:2:19, v/v/v) or isopropanol: water (70:30) or 5% Na_2HPO_4: isoamyl alcohol.

TABLE V

PAPER CHROMATOGRAPHY OF CYCLIC MONONUCLEOTIDES INCUBATED WITH LIVER RIBONUCLEASES
R_F* VALUES OF THE VARIOUS SPOTS

Incubation time in hours	*0*	*17*	*40*
Adenylic acid			
Controls			
Ap!	0.11–0.12	0.11–0.12	0.11–0.12
A3p	0.18–0.19	0.18–0.19	0.18–0.19
A2p	0.28–0.31	0.28–0.31	0.28–0.31
Incubation mixtures			
Ap! and acid RNAase pH 5.7		0.11 0.28–0.31	0.28
Ap! and heated acid RNAase pH 5.7		0.11	0.11
Ap! and alkaline RNAase pH 7.8		0.12	0.11
Uridylic acid			
Controls			
Up!	0.52–0.55	0.55	0.55
Uridylic acid**	0.68–0.70	0.68	0.70
Incubation mixtures			
Up! and acid RNAase pH 5.7		0.55	0.55 0.70
Up! and heated acid RNAase pH 5.7		0.55	0.55
Up! and alkaline RNAase pH 7.8		0.50	0.51

* Solvent; saturated ammonium sulfate: isopropanol: water (79:2:19, v/v/v).

** Commercial uridylic acid containing both U2p and U3p. The solvent system used did not permit the separation of these isomers. Only one spot was then identified on the chromatogram.

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The results are summarized in Table V. R_F values of the various control mononucleotides and of the various spots obtained by paper chromatography of the incubation mixtures are given. The results show that acid RNAase splits Ap! to give A2p as indicated by the R_F values of the spots present in the chromatogram of the 17 hour incubation mixture. No A3p could be detected. The 40-hour incubation mixture showed only one spot with R_F value of 0.28 indicating a complete breakdown of Ap!. The alkaline ribonuclease had no effect on Ap! even after 40-hour incubation as indicated by the single spot with an R_F value of Ap!. Heated acid RNAase was also without effect on the Ap!, a fact which illustrates again that the activity of this enzyme was completely destroyed by heating.

Assays carried out with Up! gave similar results. Acid RNAase splits Up! to uridylic acid but the reaction seemed to be slower than with Ap! since traces of Up! could still be demonstrated after 40 hours of incubation. Alkaline RNAase and heated acid RNAase were without effect on Up!.

These results show an important difference between the acid and alkaline ribonuclease of liver. They also show that liver alkaline ribonuclease would be different from the pancreatic ribonuclease which split pyrimidine cyclic mononucleotides, whereas the liver enzyme did not.

The qualitative data presented here are presently studied from a quantitative point of view and these results will be the subject of a full report. The action of the purified liver enzymes on RNA and on the core left after exhaustive digestion of RNA by pancreatic ribonuclease is also investigated.

SUMMARY

Methods for the purification of both the acid and alkaline ribonucleases of rat liver are reported. It was possible to obtain a purified alkaline ribonuclease freed from acid ribonuclease activity. However, the preparation of acid ribonuclease was contaminated by the presence of alkaline ribonuclease activity.

Some characteristics of both enzymes are reported. Preliminary results on the specificity of these enzymes showed that acid RNAase splits cyclic mononucleotides (purine or pyrimidine) whereas alkaline RNAase does not.

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Received July 26th, 1957

ON THE THERMAL MELTING AND RECRYSTALLIZATION OF RIBONUCLEASE CRYSTALS AND THE HETEROGENEITY OF RIBONUCLEASE*

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Because of the instability of most proteins at elevated temperatures, studies of the crystallization of proteins have generally been conducted at room temperature or lower, while studies at elevated temperatures have been directed to the question of heat-denaturation. This limitation has generally precluded studies of thermal phase transformations of protein crystals; however, for the relatively few heat-stable proteins, such studies should be possible. We have observed melting and recrystallization for one such protein, bovine pancreatic ribonuclease, and found in the results evidence concerning the heterogeneity of ribonuclease.

Samples of ribonuclease crystals of several different modifications were grown in aqueous alcohol or glycol solutions, as described by KING, MAGDOFF, ADELMAN AND HARKER[1]. The crystals were heated gradually in their mother liquors, and were observed to melt to irregular masses of soft, transparent gel over narrow temperature ranges in the region 50–75° C. Generally, the melting was not instantaneous, but proceeded at a rate dependent on the temperature. The "melting points" determined in this work were generally the temperatures at which five to ten minutes was required for melting. These melting points were generally characteristic of the crystal modification used, and were higher for the densely-packed forms, such as form II, or those containing complexed metals, such as form I, than for other forms. No melting was observed if the mother liquor was replaced by a solution more concentrated in alcohol; however, the crystals lost their birefringence gradually on heating above 70° C.

After melting had been observed, the samples were incubated at 25° C, and in most cases, crystals were observed to grow in the mother liquor above the masses of gel. Such crystals were generally of the same modification as had been used for the melting experiments, with the exception that form VII usually gave form I.

* Contribution No. 11 from the Protein Structure Project.

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