

Characterization of Ribonucleases and Ribonuclease Inhibitor in Subcellular Fractions from Rat Adrenals

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1. The presence of two RNA-degrading enzymes, one with optimum activity at pH 5.6 (acid ribonuclease) and the other with optimum activity at pH 7.8 (alkaline ribonuclease), in rat adrenals has been demonstrated. The acid ribonuclease was localized in the mitochondrial fraction whereas the alkaline ribonuclease was present in mitochondria as well as in the supernatant fraction. Freezing and thawing of mitochondria and treatment with Triton X-100 gave a three- to four-fold increase in acid-ribonuclease activity, whereas the mitochondrial alkaline-ribonuclease activity was practically unaffected. 2. The amount of free ribonuclease in the adrenal supernatant was small. Treatment of the supernatant fraction with *N*-ethylmaleimide resulted in release of large amounts of ribonuclease activity, indicating the presence of a ribonuclease inhibitor having reactive thiol groups. 3. Considerable amounts of free ribonuclease inhibitor in excess over the bound alkaline ribonuclease are present in the rat-adrenal supernatant fraction. The inhibitor is heat-labile and non-diffusible. A 400–500-fold purification of the ribonuclease inhibitor was achieved by ammonium sulphate fractionation, treatment with calcium phosphate gel and DEAE-cellulose chromatography. It is concluded that the adrenal inhibitor is protein in nature, similar to the inhibitor present in rat liver.

Roth (1956a, 1957) has reported the presence of an inhibitor for ribonuclease activity in rat-liver supernatant fraction. Shortman (1961) has demonstrated from purification studies of the rat-liver inhibitor that the inhibitor activity is associated with a fraction that is mostly protein. Eichel, Figueroa & Goldenberg (1961) have provided evidence for the presence of ribonuclease inhibitor in rat adipose tissue.

There have been a number of attempts to assess the possible role of the ribonuclease inhibitor in RNA metabolism and protein synthesis by determining the changes in the concentrations of ribonuclease and ribonuclease inhibitor after altered physiological states in the organism. Thus the changes in inhibitor activity have been studied in rat liver after whole-body X-irradiation (Roth, 1956b) or during regeneration after partial hepatectomy (Shortman, 1962b) and in different cancerous tissues (Brody, 1957; Roth, 1962). Giriya, Pradhan & Sreenivasan (1965) have reported on the correlation observed between altered protein-synthetic activity and the ribonuclease inhibitor concentrations in livers of rats maintained on protein-free diet for 3 weeks. Smeaton, Elliott & Coleman (1965) have found a powerful inhibitor of ribonuclease

activity in the soluble fraction of disrupted cell preparations of a strain of *Bacillus subtilis*. They have also provided preliminary evidence for the protein nature of this bacterial ribonuclease inhibitor.

Thus these observations call for a detailed understanding of the cellular inhibitors of ribonuclease from a wide variety of sources, with special reference to their intracellular location, specificity and mode of inhibition. The present work reports on the distribution of ribonuclease activity in the subcellular fractions of rat adrenals and the presence of an inhibitor of ribonuclease activity in the adrenal supernatant fraction. The inhibitor has been purified by chromatography on DEAE-cellulose columns.

EXPERIMENTAL

Animals. Livers and adrenals were obtained from male Wistar rats, maintained on the laboratory stock diet, and weighing 250–300g.

Preparation of subcellular fractions. The tissues were dissected and homogenized with 10 vol. of 0.25M-sucrose, previously chilled, in a Potter-Elvehjem glass homogenizer. The isolation of subcellular fractions was done according to the procedure of Schneider & Hogeboom (1945). The

homogenate was centrifuged at 500g in an International model PR-2 refrigerated centrifuge to remove nuclei and unbroken cells. Mitochondria were separated at 5000g for 15 min. These were washed twice with 10 vol. of 0.25M-sucrose and re-centrifuged under the same conditions. The resulting mitochondrial pellet was suspended in 10 vol. of 0.25M-sucrose for the assay of free ribonuclease activity. Total ribonuclease activity was determined after freezing and thawing a 1:10 suspension of mitochondria in water or after treatment with Triton X-100 (0.1%, v/v) at 4° for 20 min.

The supernatant obtained after sedimenting mitochondria was spun in a Spinco model L ultracentrifuge at 105000g for 1 hr. and the resulting supernatant fraction was used in the studies on the ribonuclease inhibitor.

Determination of ribonuclease activity. Ribonuclease activity was determined as described by Brody (1957), by following the release of acid-soluble nucleotides on incubation of 2.5 mg. of dialysed yeast RNA with a 1:10 homogenate or equivalent cell fraction in a final volume of 3.0 ml. Incubations at 37° were carried out in 20 mM-tris-HCl buffer, pH 7.8, for the determination of alkaline-ribonuclease activity and in 20 mM-acetate buffer, pH 5.6, for the assay of acid-ribonuclease activity. For the supernatant enzyme, preincubation of the supernatant fraction with *N*-ethylmaleimide (final concn. 3 mM) was carried out for 30 min. before incubation with the substrate. The reaction was stopped by the addition of 3.0 ml. of 10% (w/v) trichloroacetic acid containing uranyl acetate (0.5%), and the acid-soluble nucleotides were determined by measuring the extinction at 290 m μ in a Beckman model DU spectrophotometer, trichloroacetic acid giving negligible extinction at this wavelength. The amount of enzyme releasing acid-soluble nucleotides equivalent to 1 extinction unit under the above conditions is expressed as 1 ribonuclease unit.

Assay of ribonuclease inhibitor. The system for the assay of ribonuclease inhibitor (Roth & Wojner, 1961) consisted of 0.2 ml. of supernatant fraction, 0.15 ml. of 50 mM-EDTA, pH 7.8, 0.125 μ g. of crystalline pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), 1.0 ml. of veronal-acetate buffer (50 mM with respect to both sodium diethylbarbiturate and sodium acetate), pH 7.8, and 1.0 ml. of 1% RNA solution in a final volume of 3.0 ml. Incubation was carried out for 30 min. at 37°. The reaction was stopped by the addition of 3.0 ml. of precipitating reagent [*N*-HCl in 76% (v/v) ethanol containing LaCl₃·7H₂O (0.5%)]. Release of acid-soluble nucleotides was estimated by the change in extinction at 260 m μ . One unit of inhibitor is equivalent to the concentration causing 50% inhibition of 0.005 μ g. of crystalline pancreatic ribonuclease.

Purification of ribonuclease inhibitor. Sheep adrenals contained inhibitor activity comparable with that from rat adrenals. The inhibitor from sheep adrenals resembled the rat-liver inhibitor in all its characteristics, being heat-labile, non-diffusible and containing active thiol groups.

A 30g. portion of sheep adrenals was homogenized in 3 vol. of 0.25M-sucrose and the supernatant fraction obtained by spinning the homogenate at 105000g was treated with solid (NH₄)₂SO₄ to give 35% saturation. The precipitate was removed by centrifugation at 60000g in the Spinco model L ultracentrifuge for 20 min. Then (NH₄)₂SO₄ was added to the supernatant to give 55%

saturation and the mixture was centrifuged at 60000g for 20 min. The pellet was dissolved in 20 ml. of cold water and this was treated with calcium phosphate gel, prepared according to the method of Swingle & Tiselius (1951). The inhibitor was eluted from the gel with 20–25 ml. of 0.2M-phosphate buffer, pH 6.4. The eluate was diluted to give a phosphate concentration of 10 mM and NaCl and EDTA were added to give concentrations of 0.15M and 1 mM respectively. This solution was applied to DEAE-cellulose columns. The chromatography on DEAE-cellulose columns was essentially as described by Shortman (1961). The column was washed with 50 ml. of buffered saline [NaCl (0.15M), potassium phosphate buffer, pH 6.4 (10 mM), and EDTA (1 mM)]. The elution was carried out with a linear gradient of 0.15–1.5M-NaCl in 10 mM-phosphate buffer, pH 6.4, containing EDTA (1 mM). The eluate was collected in 5.0 ml. fractions.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS AND DISCUSSION

Two apparently distinct enzymes from various tissues that hydrolyse RNA have been described by Roth (1954), Stevens & Reid (1956), Maver & Greco (1956) and Zytko, De Lamirande, Cantero & Allard (1958). The activities of these have been differentiated on the basis of pH optima and stability to heat and acid, one enzyme being termed

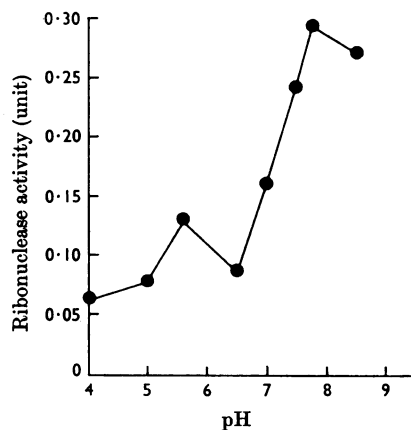


Fig. 1. Effect of pH on the ribonuclease activity of rat-adrenal homogenate. Samples of rat-adrenal homogenate equivalent to 50 mg. of tissue were incubated with 2.5 mg. of dialysed yeast RNA at 37° for 30 min. in veronal-acetate buffers in the pH range 4.0–8.5. The reaction was stopped by the addition of 3.0 ml. of 10% (w/v) trichloroacetic acid containing uranyl acetate (0.5%), and the acid-soluble nucleotides were determined by measuring the extinction at 290 m μ . One unit of ribonuclease activity is the amount of enzyme releasing acid-soluble nucleotides equivalent to 1 extinction unit under the above conditions. The plot represents the average of three different experiments.

alkaline ribonuclease (optimum pH 7.8–8.2, heat-stable and acid-stable) and the other acid ribonuclease (optimum pH 5.2–5.8, heat-labile and acid-labile). Fig. 1 illustrates the pH-activity curve of ribonuclease in adrenal homogenate. The curve reveals two peaks of activity, one at pH 5.6 and another at pH 7.8. These results indicate that rat adrenals contain both acid and alkaline ribonucleases with pH optima similar to those reported for the ribonucleases of rat liver (Roth, 1957).

The distribution of acid and alkaline ribonucleases in the subcellular fractions of rat liver and adrenals is shown in Table 1. The acid-ribonuclease activity is localized in the mitochondrial fraction, the activity in the supernatant fraction being negligible. Alkaline-ribonuclease activity is present in mitochondria as well as in the supernatant fraction. The ribonuclease activity in the supernatant fraction is low. But, on the addition of *N*-ethylmaleimide, a 3–4-fold increase in the activity is observed. The same extent of release of supernatant enzyme was obtained when *p*-chloromercuribenzoate was present in the incubation system at a final concentration of 0.4 mM. These results are similar to those reported for rat liver (Roth, 1958). Calculation of the distribution of alkaline-ribonuclease activity between mitochondria and supernatant shows that about 60% of the ribonuclease activity could be recovered in the supernatant fraction, after taking into account the ribonuclease activity released on treatment with *N*-ethylmaleimide, and about 20% in the mitochondria. Experiments showed that mitochondrial activity is not increased on treatment of mitochondria with *N*-ethylmaleimide. However, other treatments, such as freezing and thawing and

treatment with detergents like Triton X-100, increase the mitochondrial ribonuclease activity. The increase is mainly in the acid-ribonuclease activity, alkaline-ribonuclease activity being unaffected by these treatments (Table 1).

These results demonstrate that, as in rat liver, a major part of the ribonuclease activity in the adrenals is present in a latent form, the supernatant enzyme occurring in combination with an inhibitor, which could be inactivated by thiol-binding reagents, and the mitochondrial acid-ribonuclease activity present presumably as a membrane component.

The significance of the intracellular location of acid and alkaline ribonucleases of mitochondria is not known, though attempts have been made to study the characteristics of the two enzymes (Stevens & Reid, 1956; Zytka *et al.* 1958; Maver & Greco, 1962). Nodes, Reid & Whitcut (1962), working with purified enzymes, found minor differences in their specificities towards synthetic substrates. However, one difference between acid and alkaline ribonucleases is that, whereas acid ribonuclease is not inhibited by the rat-liver inhibitor, all the three mammalian alkaline ribonucleases tested, namely those of rat-liver mitochondria and supernatant as well as the pancreatic enzyme, are all inhibited (Shortman, 1962a).

Although mitochondrial alkaline-ribonuclease activity is present in a free form whereas the supernatant alkaline-ribonuclease activity is present along with the inhibitor presumably in combination, as inferable from observations with use of thiol-binding agents, it has been shown that purified alkaline ribonucleases of mitochondria and super-

Table 1. *Distribution of ribonuclease activity in rat-adrenal fractions and comparison with liver cell fractions*

Free ribonuclease was determined by following the release of acid-soluble nucleotides on incubation of a 1:10 homogenate or equivalent cell fraction with 2.5 mg. of dialysed yeast RNA. Total ribonuclease activity of mitochondria at pH 5.6 or 7.8 was determined in the same manner after freezing and thawing a 1:10 suspension in water (*) or after treatment with 0.1% (v/v) Triton X-100 (†). The supernatant activity was assayed with and without preincubation with *N*-ethylmaleimide (final concn. 3 mM). The activities are expressed as ribonuclease activity units/50 mg. of tissue or equivalent cell fraction. One unit of ribonuclease activity is the amount of enzyme releasing acid-soluble nucleotides equivalent to 1 extinction unit under the above conditions. The results are averages of four independent determinations.

Tissue	Ribonuclease activity (unit)							
	Homogenate	Mitochondria				Supernatant		
		Free activity	Free activity		Total activity		– <i>N</i> -Ethylmaleimide pH 7.8	+ <i>N</i> -Ethylmaleimide pH 7.8
			pH 5.6	pH 7.8	pH 5.6	pH 7.8		
Adrenals	0.305	0.100	0.108	0.398*	0.108*	0.095	0.280	
Liver	0.250	0.124	0.124	0.412†	0.112†	0.080	0.220	
				0.342*	0.118*			
				0.400†	0.121†			

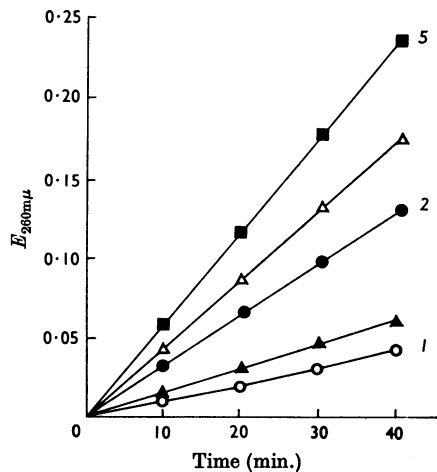


Fig. 2. Effect of *N*-ethylmaleimide on the inhibition of crystalline pancreatic ribonuclease by adrenal supernatant fraction. The assay system consisted of 10.0 ml. of 50 mM-veronal-acetate buffer, pH 7.8, 10 ml. of 1% dialysed yeast RNA, 1.0 μ g. of crystalline pancreatic ribonuclease, 4.0 ml. of 1:10 supernatant and, where indicated, *N*-ethylmaleimide (final concn. 3 mM). The total volume was 25 ml. Samples (4.0 ml.) were added after 0, 10, 20, 30 and 40 min. to 4.0 ml. of precipitating reagent [*N*-HCl in 76% (v/v) ethanol containing $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ (0.5%)]. The supernatant was diluted 1:50 and readings were taken at 260 $m\mu$. The plots represent the averages of three independent experiments. Curve 1, activity of adrenal supernatant alone; curve 2, activity of supernatant fraction preincubated with *N*-ethylmaleimide; curve 3, activity of crystalline pancreatic ribonuclease; curve 4, activity of crystalline pancreatic ribonuclease in the presence of supernatant; curve 5, activity of crystalline pancreatic ribonuclease in the presence of supernatant and *N*-ethylmaleimide.

nant are identical in their specificity towards substrates (Nodes *et al.* 1962; Beard & Razzell, 1964).

A time-course study of the action of *N*-ethylmaleimide on the adrenal supernatant fraction is shown in Fig. 2. The ribonuclease activity in the supernatant fraction is low (curve 1). On the addition of *N*-ethylmaleimide, a threefold increase in activity is observable (curve 2), indicating a bound form of ribonuclease in the supernatant fraction, and that this is released on inactivation of the inhibitor by thiol-binding reagents like *N*-ethylmaleimide. However, the supernatant fraction is not saturated with respect to ribonuclease, since considerable amounts of crystalline enzyme may be inactivated by the addition of supernatant fraction (curves 3 and 4). This inhibition is overcome in the presence of *N*-ethylmaleimide (curve 5).

The results of two typical experiments to test the heat-sensitivity of the inhibitor are given in

Table 2. Effect of heating the supernatant fraction on the activity of ribonuclease inhibitor

Samples (2 ml.) of adrenal supernatant fraction were placed in test tubes and heated in a water bath for 5 min. at the different temperatures. After heating, the samples were cooled and assayed for inhibitor activity. The system for the assay of ribonuclease inhibitor consisted of 0.2 ml. of supernatant fraction, 0.15 ml. of 50 mM-EDTA, pH 7.8, 0.125 μ g. of crystalline pancreatic ribonuclease, 1.0 ml. of 50 mM-veronal-acetate buffer, pH 7.8, and 1.0 ml. of 1% RNA solution in a final volume of 3.0 ml. The reaction was stopped after 30 min. incubation at 37° by adding 3.0 ml. of precipitating reagent [*N*-HCl in 76% (v/v) ethanol containing $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ (0.5%)]. Release of acid-soluble nucleotides was estimated by the change in extinction at 260 $m\mu$. The unit of inhibitor is equivalent to the inhibitor concentration causing 50% inhibition of 0.005 μ g. of crystalline pancreatic ribonuclease. The results are averages of two experiments.

Temperature	Inhibitor activity (units/mg. of protein)	Decrease of inhibitor activity (%)
37°	13.6	—
45	12.4	9
55	5.8	57
65	0	100

Table 3. Purification of sheep-adrenal ribonuclease inhibitor

The supernatant fraction was subjected to $(\text{NH}_4)_2\text{SO}_4$ precipitation and treatment with calcium phosphate gel according to the procedure described in the text. The eluate from calcium phosphate gel was adsorbed on DEAE-cellulose columns. The conditions of adsorption and elution are given in the text. One unit of inhibitor is equivalent to the inhibitor concentration causing 50% inhibition of 0.005 μ g. of crystalline pancreatic ribonuclease. The results are averages of two independent experiments carried out with separate batches of sheep adrenals.

Stage	Activity (units/ml.)	Specific activity (units/mg. of protein)	Recovery (%)
Supernatant	420	15	100
55% satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate	420	52	85
Eluate from calcium phosphate gel	630	174	58
Eluate from DEAE-cellulose column chromatography	5640	6300	80

Table 2. The inhibitor is relatively heat-sensitive, being completely destroyed after heating for 5 min. at 65°. Together with the fact that the inhibitor is

non-diffusible in crude as well as in purified preparations, the observation suggests that it may be protein in nature.

The inhibitor activity could be purified several hundred-fold by employing methods used for the purification of proteins. An analysis of the results of a standard purification procedure is presented in Table 3. Initial purification was carried out by combined ammonium sulphate precipitation and calcium phosphate gel treatment. The specific activity of the inhibitor preparation thereby increased 10–12-fold; the partially purified inhibitor was next passed through a column of DEAE-cellulose. All of the inhibitor activity was retained on the column. Elution was carried out with a linear gradient of 0.15–1.5M-sodium chloride buffered at pH 6.4 with tris. The highest inhibitor concentrations were obtained in fractions 13, 14, 15 and 16. These eluate fractions also contained most of the 280 μ -absorbing material. A 400–500-fold purification was thus attained.

Though an analysis of the purified inhibitor preparation has not been undertaken, previous studies also indicate that protein forms the major constituent in purified rat-liver inhibitor preparations. No other component was present to any considerable extent (Shortman, 1962a).

The protein nature of the inhibitor (Shortman, 1961), as well as its lability *in vivo* (Roth, 1956b; Shortman, 1962b; Giriya *et al.* 1965), lead one to speculate that the inhibitor may play an important role in the regulation of RNA metabolism, especially in view of recent reports implicating ribonuclease as the enzyme involved in the degradation of rapidly turned over RNA in bacterial (Artman & Engelberg, 1964) as well as in animal systems (Harris, 1963).

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