The Reaction of Ribonuclease Inhibitor with Modified Ribonucleases

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1. The effect of chemical modification of ribonuclease on its reaction with ribonuclease inhibitor has been studied. 2. Removal of free amino groups from the enzyme with nitrous acid or by acetylation did not affect the reaction. Some changes altered the stoichiometry of the reaction and ribonuclease S was found to be inhibited linearly by increasing amounts of ribonuclease inhibitor, in contrast with ribonuclease A, which is inhibited in a non-linear way. One derivative of ribonuclease containing dimethylaminonaphthalenesulphonyl groups actually reacted with ribonuclease inhibitor to a greater extent (and linearly) than did the unaltered enzyme. 3. The positively charged histidine at the active site and the active enzyme did not appear to be necessary for the reaction since 1-carboxymethylhistidine-119-ribonuclease reacted with ribonuclease inhibitor to almost the same extent as the native enzyme. In general, any significant change in the conformation of ribonuclease was accompanied by a loss in its ability to combine with inhibitor. The presence of 8M-urea also prevented reaction of ribonuclease with inhibitor. 4. Some characteristics of the reaction of ribonuclease inhibitor, ribonuclease and deaminated ribonuclease with RNA and deaminated RNA were investigated.

A protein ribonuclease inhibitor discovered by Pirotte & Desreux (1952) has been partly purified and many of its properties have been studied (Roth, 1956, 1958; Shortman, 1961, 1962). It has been shown to be widespread in mammalian species, but as yet there is little evidence that a similar inhibitor exists in lower forms (Roth, 1962).

At the present time, its function and relationship to RNA metabolism are unknown, although an obvious one is to prevent degradation of macromolecular RNA in certain specific cell structures or locations (Hymer & Kuff, 1964; Blobel & Potter, 1966).

Little is known also about the mechanism of action of ribonuclease inhibitor, but it seems clear that it combines primarily with ribonuclease rather than with the enzyme's substrate, RNA. In the present work the combination of ribonuclease inhibitor with various modified ribonucleases has been studied, as well as other factors concerning the reaction between enzyme and inhibitor, in the hope of learning something about the structural prerequisites for inhibitor action.

MATERIALS AND METHODS

Assay of ribonuclease inhibitor. Since the inhibitor assay is quite sensitive, unpurified inhibitor is adequate for studying the reactions. For this reason, high-speed supernatant fraction prepared from a 10% (w/v) homogenate of

rat liver in 0.25M-sucrose solution by centrifugation at 60000g for 1 hr. was utilized in most experiments. The supernatant fraction contained approx. 86 mg. of protein and 0.75 mg. of RNA/ml. Generally, Fischer strain rats of either sex weighing 150–200 g. were employed. The animals were anaesthetized and exsanguinated and the livers were rapidly removed and rinsed in ice-cold sucrose solution. A portion was homogenized in 9 vol. of ice-cold 0.25M-sucrose solution in a TenBroeck homogenizer and then centrifuged in a Spinco model L ultracentrifuge as indicated above. The supernatant fraction was always prepared immediately before use.

Duplicate assay tubes were set up at, usually, three amounts of ribonuclease inhibitor with the ribonuclease derivative as well as separate assays with untreated bovine crystalline pancreatic ribonuclease A as controls. The tubes contained 1 ml. of 29 mM-veronal-acetate buffer, pH 7.8, 0.05, 0.1 or 0.2 ml. of high-speed supernatant fraction, 0.15 ml. of 0.05M-EDTA, pH 7.8 (unless otherwise indicated), and usually 0.1 μ g. of ribonuclease or ribonuclease derivative plus water to make a total volume of 2.0 ml. To this was added serially 1.0 ml. of a 1% (w/v) solution of Schwarz RNA (or other RNA) that had been adjusted to pH 7.0. The tubes were incubated at 37° for 30 min. in a water bath and the reaction stopped by adding 3.0 ml. of N-HCl in 76% (v/v) ethanol containing $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ (0.5%) and mixing. The solutions were filtered through 9 cm. circles of Whatman no. 40 filter paper with watch glasses covering the funnels. Then 1 ml. of the clear filtrate was diluted to 50 ml. and the E_{260} value determined in the Beckman DU spectrophotometer. Deviations in the amounts of material or order of addition are noted in the Results and Discussion section.

A substrate blank, with enzyme omitted, and an enzyme standard (0.1 μ g.) were run with each experiment. The enzyme has no significant extinction under the conditions used (0.1 μ g.).

Ribonuclease derivatives that had less than 1% the activity of ribonuclease A were added (0.1 μ g.) to the mixture and incubated at room temperature for, generally, 15 min. After this, 0.1 μ g. of crystalline ribonuclease A was added and the assay carried out as described. It is assumed that, if the inactive ribonuclease derivative combines with ribonuclease inhibitor in the high-speed supernatant fraction, then the subsequent inhibition of ribonuclease A measured will be less than in controls. This assumption is, of course, unproved and it is possible that ribonuclease A would displace a ribonuclease derivative from a weak combination with ribonuclease inhibitor.

Enzyme preparations. The enzyme ribonuclease A [polyribonucleotide 2-oligonucleotidotransferase (cyclizing), EC 2.7.7.16] was a product of Worthington Biochemical Corp., Freehold, N. J., U.S.A. A stock solution was prepared by dissolving 5.0 mg. of crystalline enzyme in 100 ml. of 0.1% gelatin solution. This was frozen in small portions, one of which was thawed before use and diluted to the appropriate concentration with 0.1% gelatin solution.

Chemically and otherwise modified ribonucleases were prepared as described and diluted similarly with 0.1% gelatin solution to the required concentration.

Ribonuclease S was a gift from Dr Frederick Richards (Richards & Vithayathil, 1959). Under our conditions of assay this sample had only about 5% of the activity of ribonuclease A (see Table 1). Alanine-ribonuclease was obtained from Dr Michael Sela; this derivative (PAR4E) was prepared as described by Wellner, Silman & Sela (1963). The activity of this derivative is markedly influenced by the type of substrate and the conditions of assay (Wellner *et al.* 1963). The alanine content of PAR4E was approx. 142 extra residues/mol. compared with the original ribonuclease.

Oxidized ribonuclease was prepared according to the directions of Hirs (1956). DNS-ribonucleases* were prepared by Dr F. H. White, jun., and DNS-ribonucleases I, II and III correspond to peaks I, II and III from the CM-Sephadex column (White, 1964). Ribonuclease B was also obtained from Dr White and had been made by chromatography of a commercial (Sigma) preparation of ribonuclease on a CM-Sephadex column. The Sigma ribonuclease separated in one large peak (A) and one small peak (B) in a pattern that was nearly identical with that originally observed when ribonuclease was chromatographed on Amberlite XE-64 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) (Hirs, Moore & Stein, 1953). The activity of this sample of ribonuclease B was indistinguishable from that of the ribonuclease A sample used.

Thiolated (7.5 hr.) CM-ribonuclease has been described by White & Sandoval (1962).

Deaminated ribonuclease was prepared according to the directions of Vunakis, Leikhim, Delaney, Levine & Brown (1960), with a treatment time of 20 min. Acid- and base-denatured ribonucleases were prepared according to the methods of Bigelow (1961). To destroy all enzyme activity at pH 1, placing the enzyme in a boiling-water bath for 30 min. was required. Methylated ribonuclease was pre-

pared by the general method of Chibnall, Mangan & Rees (1958) by using dry HCl in methanol. Reduced ribonuclease was prepared according to the directions of White (1960). The reduced enzyme was not isolated, but a sample of the solution containing the reduced material was diluted directly with 0.1% gelatin solution to give the required concentration of enzyme. 1-CM-histidine-119-ribonuclease was a gift from Dr John P. Hummel (Yang & Hummel, 1964).

RNA samples. Schwarz RNA refers to a relatively crude preparation of total yeast RNA (lot 6080) obtained from Schwarz Bioresearch Inc. (Orangeburg, N.Y., U.S.A.) prepared by alkaline extraction of yeast. It was used unpurified unless otherwise stated.

CSA-RNA refers to a sample prepared by us from yeast grown in the laboratory and extracted with sodium dodecyl sulphate as described by Crestfield, Smith & Allen (1955). Some properties of the sample used have been reported (Roth, 1960).

Deaminated RNA was prepared by a modification of the method of Fletcher, Gulland & Jordan (1944). A 10 g. sample of Schwarz crude RNA (lot 6080) was dissolved in a minimum quantity of water by adjustment of the pH to 7 with dilute NaOH. Then 20 g. of KNO₂ dissolved in 10 ml. of water was added, plus an equivalent quantity of acetic acid (14.1 g.), slowly over a period of 2 hr. The reaction mixture was left for 48 hr. at room temperature and 24 hr. in the refrigerator. The solution was then dialysed in the cold for 48 hr. against several changes of distilled water, and precipitated with 2 vol. of ethanol containing potassium acetate (3%, w/v). The precipitate was dissolved in a small quantity of water and dialysed overnight and the resulting solution made up to 200 ml. The yield was 2.58 g. of material containing 10.5% of N, compared with 13.3% (micro-Kjeldahl) for the untreated RNA. A 1% solution of the deaminated RNA gave a negligible extinction (compared with E_{280} approx. 0.150 for untreated RNA; see Table 6) when precipitated with HCl-ethanol-LaCl₃ reagent, but use of 10% (w/v) trichloroacetic acid or 10% (v/v) HClO₄ as precipitants resulted in complete or nearly complete hydrolysis of the sample, as judged by the fact that such treatment gave an extinction nearly equivalent to that of the unhydrolysed sample.

Ribonuclease S protein and reduced CM-ribonuclease were obtained from Mann Laboratories, New York, N.Y., U.S.A. Acetylated ribonuclease was prepared by treating ribonuclease A with an excess of acetic anhydride for 24 hr. at 37°. The acetylated enzyme was precipitated with acetone, centrifuged, washed with acetone and dried in a vacuum desiccator. The acetylated product was much less soluble than the original enzyme and it retained considerable activity (Table 1), unlike the product described by Brown, Durieux, Delaney, Leikhim & Clark (1959).

Carbamoyl-ribonuclease was prepared essentially by the method of Stark, Stein & Moore (1960). Two preparations were made. For the first (carbamoyl-ribonuclease I), 6 mg. of ribonuclease A was incubated with 2 ml. of 1.0 M-KCNO, pH 8.0, at 50° for 1 hr. The enzyme was precipitated with 3 vol. of cold 95% (v/v) ethanol, centrifuged, washed with ethanol and dried in a vacuum desiccator. The second preparation (carbamoyl-ribonuclease II) was prepared similarly with 0.5 hr. heating.

Iodinated ribonuclease was prepared according to the directions of Cha & Scheraga (1963). The proportions were 6 m-moles of I₂/m-mole of ribonuclease.

* Abbreviations: DNS, 1-dimethylaminonaphthalene-5-sulphonyl; CM, carboxymethyl.

The enzyme activity of various derivatives is compared with that of ribonuclease A (100%) in Table 1. All experiments were performed several times and typical results are illustrated.

RESULTS AND DISCUSSION

Derivatives not combining with inhibitor. Many of the ribonuclease derivatives did not appear to combine with ribonuclease inhibitor. Among these were the following ribonucleases: oxidized, alanyl-,

base-denatured, acid-denatured, methylated, carbamoyl- (I and II), reduced and reduced carboxy-methylated. All of these derivatives, with the possible exception of alanyl-, carbamoyl- and methylated ribonucleases, involve rather extensive changes in the conformation of the ribonuclease molecule with considerable loss in the specific protein structure required for enzyme activity. With alanyl-ribonuclease, the basic enzyme structure may be relatively unaltered, but the presence of a number of relatively long alanyl side chains would certainly be expected to affect the molecular dimensions of the enzyme, and hence probably its ability to combine with another protein inhibitor. Completely methylated ribonuclease may be inactive, not so much from a change in configuration, although this apparently does occur (Vithayathil & Richards, 1961), but because carboxyl groups become unavailable for salt linkages. Carbamoylation of ribonuclease and subsequent production of a derivative that does not combine with ribonuclease inhibitor is less expected, since partial loss of the free amino groups by treatment with nitrous acid does not affect either the ribonuclease enzyme activity or its ability to combine with ribonuclease inhibitor.

Derivatives showing altered reactivity to ribonuclease inhibitor. Ribonuclease S reacted with ribonuclease inhibitor but in a somewhat different way from ribonuclease A. The results of a typical experiment are illustrated in Table 2. An attempt was made in this experiment to obtain ribonuclease activity from ribonuclease S equivalent to the control ribonuclease A activity. However, 0.24 μ g. of ribonuclease S gave only about one-half the activity of 0.04 μ g. of ribonuclease A in this instance. It is possible that equivalent weights of ribonuclease S and the unaltered enzyme would be inhibited to the same extent by a definite quantity of ribonuclease inhibitor. This was not tested since the activity of 0.04 μ g. of ribonuclease S is somewhat marginal for the inhibitor assay.

Table 1. *Enzyme activity of various ribonuclease derivatives*

The same quantities (0.015 μ g.) of ribonuclease A and the samples shown were assayed for ribonuclease activity as described in the Materials and Methods section. The increase in extinction of the acid-soluble products obtained with the ribonuclease sample was taken as 100% activity.

Sample	Activity (% of ribonuclease A activity)
Ribonuclease S	10-25
Alanyl-ribonuclease (PAR4E)	10
Oxidized ribonuclease	0
DNS-ribonuclease I	85-95
DNS-ribonuclease II	100
DNS-ribonuclease III	80-85
Ribonuclease B	100
Thiolated (7.5 hr.) CM-ribonuclease	80-85
Deaminated ribonuclease	100
Base-denatured ribonuclease	0
Acid-denatured ribonuclease	<1
Methylated ribonuclease	<1
Reduced ribonuclease	0
Iodinated ribonuclease	30-40
Carbamoyl-ribonuclease I	0
Carbamoyl-ribonuclease II	10
Reduced CM-ribonuclease	0
Ribonuclease S protein	0
Acetylated ribonuclease	60-80
1-CM-His-119-ribonuclease	0

Table 2. *Inhibition of ribonuclease S by ribonuclease inhibitor*

The indicated quantities of high-speed supernatant fraction (SF) were incubated with 0.04 μ g. of ribonuclease A or 0.24 μ g. of ribonuclease S and the inhibition of RNA hydrolysis was measured as described in the Materials and Methods section.

Sample	Increment in E_{260}	Inhibition (%)
Ribonuclease A (0.04 μ g.)	0.251	—
Ribonuclease A (0.04 μ g.) + SF (0.05 ml.)	0.056	77.5
Ribonuclease A (0.04 μ g.) + SF (0.10 ml.)	0.003	99.0
Ribonuclease S (0.24 μ g.)	0.137	—
Ribonuclease S (0.24 μ g.) + SF (0.05 ml.)	0.107	21.9
Ribonuclease S (0.24 μ g.) + SF (0.10 ml.)	0.084	39.7
Ribonuclease S (0.24 μ g.) + SF (0.20 ml.)	0.027	80.3

Table 3. *Inhibition of DNS-ribonuclease III by ribonuclease inhibitor*

The indicated quantities of high-speed supernatant fraction (SF) were incubated with ribonuclease A or DNS-ribonuclease III and the inhibition of RNA hydrolysis was measured as described in the Materials and Methods section.

Sample	Increment in E_{280}	Inhibition (%)
Ribonuclease A (0.1 μ g.)	0.462	—
Ribonuclease A (0.1 μ g.) + SF (0.05 ml.)	0.421	8.9
Ribonuclease A (0.1 μ g.) + SF (0.10 ml.)	0.326	27.3
Ribonuclease A (0.1 μ g.) + SF (0.20 ml.)	0.106	77.1
DNS-ribonuclease III (0.1 μ g.)	0.379	—
DNS-ribonuclease III (0.1 μ g.) + SF (0.05 ml.)	0.279	32.2
DNS-ribonuclease III (0.1 μ g.) + SF (0.10 ml.)	0.116	69.3
DNS-ribonuclease III (0.1 μ g.) + SF (0.20 ml.)	0.023	94.0

The inhibition of ribonuclease S by increasing amounts of ribonuclease inhibitor gave a linear relationship, in contrast with the results obtained with unaltered ribonuclease, in which case non-linearity is observed (Roth, 1956, 1958) with the assay conditions described herein.

DNS-ribonuclease III, which had about 85% of the activity of ribonuclease A, was inhibited to a greater extent by ribonuclease inhibitor than the normal enzyme. A typical experiment is shown in Table 3. The inhibition of DNS-ribonuclease by increasing amounts of inhibitor was linear, in contrast with the results obtained with ribonuclease A also illustrated in Table 3. Since DNS-ribonuclease III is more sensitive to ribonuclease inhibitor and gives a linear response, its use in assaying for the inhibitor would appear to be advantageous in comparison with the use of ribonuclease A.

Iodinated ribonuclease also appeared to be inhibited to a greater extent by ribonuclease inhibitor than ribonuclease A. Since the enzyme activity of the iodinated derivative is only one-third that of ribonuclease A, the iodinated product may have been a mixture of normal active and inactive iodinated molecules. The presence of smaller amounts of normal enzyme would be expected to lead to greater inhibition, and this would therefore not be any indication of the effect of iodination on the combination between enzyme and inhibitor. This is the only case described in which a homogeneous product for testing would appear to be essential. In all other cases either a relatively homogeneous product was used or the reactivity with ribonuclease inhibitor was either unchanged or completely lost.

It is difficult to visualize how the addition of DNS groups to ribonuclease facilitates the reaction between enzyme and inhibitor. One possibility is that the presence of additional charged groups on the DNS derivative may enable the inhibitor to

Fig. 1. Inhibition of 7.5-hr.-thiolated ribonuclease and ribonuclease A by increasing amounts of ribonuclease inhibitor (in supernatant fraction prepared from rat liver). ○, Ribonuclease A; ●, 7.5 hr.-thiolated ribonuclease. Assay conditions were as described in the Materials and Methods section.

bind more tightly to the ribonuclease. It is apparent that studies of conformational changes that occur during the reaction between ribonuclease and ribonuclease derivatives with ribonuclease inhibitor would be useful. In contrast with DNS-ribonuclease III, the inhibition of DNS-ribonuclease I and DNS-ribonuclease II by ribonuclease inhibitor was not significantly different from the results obtained with ribonuclease A.

The 7.5-hr.-thiolated ribonuclease presents an interesting variation, and the results of five experiments are averaged and plotted in Fig. 1. The curve for the inhibition of ribonuclease A by ribonuclease inhibitor exhibited the usual sigmoid shape (Wojnar & Roth, 1964) and rapid falling off with large amounts of supernatant fraction. In

common with the two ribonuclease derivatives just mentioned, the 7.5-hr.-thiolated ribonuclease showed a nearly linear response to increasing amounts of supernatant fraction up to about 0.15 ml. and then levelled off; but even with large amounts of supernatant fraction, the inhibition never rose above 80%. A blank with thiolated enzyme alone, or enzyme plus RNA with RNA added after the precipitating agent, showed no difference from ribonuclease A similarly treated.

Ribonuclease derivatives showing essentially the same reaction with ribonuclease inhibitor as ribonuclease A. Among these is included ribonuclease B, deaminated ribonuclease, DNS-ribonuclease II, DNS-ribonuclease I, acetylated ribonuclease and 1-CM-histidine-119-ribonuclease. Of these derivatives, deaminated ribonuclease, DNS-ribonuclease II, DNS-ribonuclease I and acetylated ribonuclease have principally modifications of the amino groups and probably do not have extensive conformational changes. It might be tentatively concluded from these results that the presence of some, or possibly all, free amino groups (either *N*-terminal or ω -lysine) is not essential for the reaction between enzyme and inhibitor.

The case of the 1-CM-histidine-119-ribonuclease is perhaps the most interesting. This derivative, which differs from ribonuclease A only in having a CM group on histidine-119, which has been located at the active site of the enzyme, is inactive enzymically and yet reacted with ribonuclease inhibitor to between 1.0 and 0.5 times the extent of native ribonuclease A. The results of a typical experiment are illustrated in Table 4, and indicate that somewhat more than 0.015 μ g. of 1-CM-histidine-119-

ribonuclease is required to combine with the ribonuclease inhibitor in 0.03 ml. of supernatant fraction. This amount of supernatant fraction by itself essentially completely inhibits the activity of 0.015 μ g. of ribonuclease A. The 1-CM-histidine-119-ribonuclease was the only derivative of ribonuclease tested that, although lacking in enzyme activity, was still capable of combining with ribonuclease inhibitor.

Effect of urea on the reaction between ribonuclease inhibitor and ribonuclease. 8M-Urea causes an unfolding of the ribonuclease molecule and presumably the enzyme is activated in this condition. On addition of either phosphate or RNA at least a partial refolding occurs and activity is restored (Nelson, Hummel, Swenson & Friedman, 1962). If the reaction between ribonuclease inhibitor and ribonuclease depends on the formation of hydrogen bonds between the two protein molecules, it would be expected that the reaction would be lessened or prevented by urea. This was tested by preparing solutions of all the reagents, RNA, EDTA and ribonuclease in 8M-urea. Under these conditions little or no inhibition of ribonuclease inhibitor could be demonstrated. The presence of 8M-urea decreased the activity of the ribonuclease approx. 70% compared with that obtained in water. A typical experiment is illustrated in Table 5. Addition of water or supernatant fraction increases enzyme activity somewhat, presumably by lowering the urea concentration. This accounts for the increased extinctions measured when supernatant fraction was added. When these increases are taken into account there is no significant inhibition.

Use of deaminated RNA in the ribonuclease-

Table 4. *Reaction of 1-CM-histidine-119-ribonuclease with ribonuclease inhibitor*

In part B, the indicated quantities of 1-CM-histidine-119-ribonuclease were incubated with the supernatant fraction (SF) for 30 min. at room temperature (23°) and then 0.015 μ g. of ribonuclease A was added and the ribonuclease activity determined as described in the Materials and Methods section. The amount of inhibition was obtained by comparison with the activity of 0.015 μ g. of ribonuclease A without inhibitor (first line).

	Sample	Increment in E_{260}	Inhibition (%)
A	Ribonuclease A (0.015 μ g.)	0.152	—
	Ribonuclease A (0.015 μ g.) + SF (0.03 ml.)	0.008	94.7
	Ribonuclease A (0.015 μ g.) + SF (0.05 ml.)	0.003	98.0
B	1-CM-His-119-ribonuclease (0.005 μ g.) + SF (0.03 ml.) + ribonuclease A (0.015 μ g.)	0.020	86.5
	1-CM-His-119-ribonuclease (0.010 μ g.) + SF (0.03 ml.) + ribonuclease A (0.015 μ g.)	0.054	64.4
	1-CM-His-119-ribonuclease (0.015 μ g.) + SF (0.03 ml.) + ribonuclease A (0.015 μ g.)	0.088	42.1
	1-CM-His-119-ribonuclease (0.030 μ g.) + SF (0.03 ml.) + ribonuclease A (0.015 μ g.)	0.158	0
	1-CM-His-119-ribonuclease (0.050 μ g.) + SF (0.03 ml.) + ribonuclease A (0.015 μ g.)	0.165	0
	1-CM-His-119-ribonuclease (0.005 μ g.) + SF (0.05 ml.) + ribonuclease A (0.015 μ g.)	0.003	98.0
	1-CM-His-119-ribonuclease (0.010 μ g.) + SF (0.05 ml.) + ribonuclease A (0.015 μ g.)	0.007	94.6
	1-CM-His-119-ribonuclease (0.015 μ g.) + SF (0.05 ml.) + ribonuclease A (0.015 μ g.)	0.007	94.6
	1-CM-His-119-ribonuclease (0.030 μ g.) + SF (0.05 ml.) + ribonuclease A (0.015 μ g.)	0.087	57.2
	1-CM-His-119-ribonuclease (0.050 μ g.) + SF (0.05 ml.) + ribonuclease A (0.015 μ g.)	0.154	0

Table 5. *Reaction between ribonuclease and ribonuclease inhibitor in 8M-urea*

All materials (RNA, buffer, ribonuclease, EDTA) were prepared in 8M-urea solution. The supernatant fraction (SF) was not, and, since when it (or water) alone was added to the 8M-urea solution an increase in extinction was obtained, a correction for this is made in the third column. The three corrected activities in the third column are not less than the activity of 0.10 μ g. of ribonuclease without addition of supernatant fraction. Similar results were obtained when 0.05, 0.10 and 0.20 ml. of supernatant fraction were added to 0.05 and 0.10 μ g. of ribonuclease. The high extinction of the RNA blank is typical of measurements made in 8M-urea.

Sample	<i>E</i> ₂₆₀			Inhibition (%)
	Uncorrected	Corrected for RNA blank	Corrected for SF	
RNA (blank)	0.370	—	—	—
RNA + ribonuclease A (0.05 μ g.)	0.480	0.110	—	—
RNA + ribonuclease A (0.10 μ g.)	0.602	0.232	—	—
RNA + ribonuclease A (0.15 μ g.)	0.705	0.335	—	—
RNA + ribonuclease A (0.10 μ g.) + SF (0.05 ml.)	0.640	0.270	0.245	0
RNA + ribonuclease A (0.10 μ g.) + SF (0.10 ml.)	0.690	0.320	0.251	0
RNA + ribonuclease A (0.10 μ g.) + SF (0.20 ml.)	0.750	0.380	0.262	0
RNA + ribonuclease A (0.10 μ g.) + water (0.20 ml.)	0.705	0.335	—	—

Table 6. *Some effects of deaminated RNA in the ribonuclease inhibitor system*

RNA and deaminated RNA were used as 1% solutions. The order of addition was always buffer, ribonuclease A (if used), supernatant fraction (SF) (if used) and finally the RNA sample.

	Sample	<i>E</i> ₂₆₀		Inhibition (%)
		Uncorrected	Corrected for blank	
Expt. 1	RNA (blank)	0.159	—	—
	RNA + ribonuclease A (0.1 μ g.)	0.505	0.346	—
	Deaminated RNA (blank)	0.015	—	—
	Deaminated RNA + ribonuclease A (0.1 μ g.)	0.026	0.011	—
	Deaminated RNA + ribonuclease A (1.0 μ g.)	0.183	0.168	—
Expt. 2	Ribonuclease A (0.1 μ g.) (RNA is substrate)	0.587	0.428	—
	Ribonuclease A (0.1 μ g.) + deaminated RNA (0.1 ml.)	0.417	0.258	39.6
	Ribonuclease A (0.1 μ g.) + deaminated RNA (0.2 ml.)	0.328	0.169	60.2
	Ribonuclease A (0.1 μ g.) + deaminated RNA (0.4 ml.)	0.232	0.073	83.0
Expt. 3	Deaminated RNA (blank)	0.013	—	—
	Deaminated RNA + ribonuclease A (0.1 μ g.)	0.039	0.026	—
	Deaminated RNA + ribonuclease A (0.1 μ g.) + SF (0.1 ml.)	0.027	0.014	44.5
	Deaminated RNA + ribonuclease A (0.1 μ g.) + SF (0.2 ml.)	0.016	0.003	88.5
Expt. 4	Deaminated RNA + ribonuclease A (0.1 μ g.)	—	0.023	—
	Deaminated RNA + ribonuclease A (0.5 μ g.)	—	0.139	—
	Deaminated RNA + ribonuclease A (1.0 μ g.)	—	0.234	—
	Deaminated RNA + deaminated ribonuclease (0.1 μ g.)	—	0.013	—
	Deaminated RNA + deaminated ribonuclease (0.5 μ g.)	—	0.066	—
	Deaminated RNA + deaminated ribonuclease (1.0 μ g.)	—	0.144	—

ribonuclease inhibitor system. Deaminated RNA is only slowly attacked by ribonuclease (Takemura, 1951; Schuster & Schramm, 1958), although relatively large quantities of enzyme will produce significant hydrolysis (Expt. 1 in Table 6). Because of this it is difficult to assess the action of ribonuclease inhibitor at equivalent enzyme concentrations. Deaminated RNA also inhibits the

hydrolysis of normal RNA by ribonuclease, and this introduces a further complication in assessing relationships among the reaction components (Expt. 2 in Table 6). If the small activity of 0.1 μ g. of ribonuclease on deaminated RNA is used as a basis for measuring inhibitor activity (Expt. 3 in Table 6) the inhibition is essentially the same as when normal RNA is used as substrate. Thus,

although the deaminated RNA competes very successfully with normal RNA for ribonuclease, if enzyme and ribonuclease inhibitor are first mixed the combination between them is strong enough to prevent subsequent reaction of ribonuclease with deaminated RNA. Finally, the reaction of deaminated ribonuclease with deaminated RNA was studied and, as illustrated in Expt. 4 in Table 6, deaminated ribonuclease hydrolyses deaminated RNA at about one-half the rate of the normal enzyme under similar conditions.

Reaction of ribonuclease inhibitor with RNA. It had previously been noted that when RNA is added to ribonuclease inhibitor before enzyme no inhibition of ribonuclease can subsequently be demonstrated (Roth, 1956, 1962). Since it has been shown that commercial samples of RNA contain heavy-metal impurities that inactivate ribonuclease inhibitor (Wojnar & Roth, 1964), it was at first believed that these impurities might be inactivating the inhibitor, particularly if the latter was not combined with enzyme. It was observed, however, that metal-free Schwarz RNA or a preparation of CSA-RNA that had little heavy-metal ions in it

(Wojnar & Roth, 1964), when added to inhibitor before enzyme, also prevented subsequent action of ribonuclease inhibitor on ribonuclease. In an attempt to understand the mechanism of the inactivation of ribonuclease inhibitor by prior addition of RNA preparations, some additional tests were carried out.

In one series of experiments (not illustrated) Schwarz RNA in amounts ranging from 0.025 mg. to 1 mg. was added to the test system before the other compounds. As little as 0.25 mg. of RNA significantly decreased the inhibition subsequently measured when small quantities (0.1 ml. of supernatant fraction) of ribonuclease inhibitor were utilized. With larger quantities of ribonuclease inhibitor, prior addition of increasing amounts of RNA was required to give an effect.

Several experiments were carried out with CSA-RNA and a Schwarz RNA sample that had been dialysed free of metal ions (Wojnar & Roth, 1964). The results of typical experiments are shown in Expts. 1 and 2 in Table 7. As has been previously noted (Wojnar & Roth, 1964), ribonuclease inhibitor activity is nearly the same with Schwarz

Table 7. *Effect of order of addition on ribonuclease inhibitor activity*

The order of addition of the components to buffer and EDTA (when present) is indicated in the Table. As is apparent from the Table, 0.1 μ g. of ribonuclease A gives about the same extinction increment with all RNA samples (0.461–0.494). SF, High-speed supernatant fraction.

	Substrate	Sample and order of addition	EDTA	Increment in E_{260} corrected for blank	Inhibition (%)
Expt. 1	Schwarz RNA	Ribonuclease A (0.1 μ g.) + RNA (control)	+	0.470	—
	Schwarz RNA	Ribonuclease A (0.1 μ g.) + SF (0.1 ml.) + RNA	+	0.245	47.8
	Schwarz RNA	Ribonuclease A (0.1 μ g.) + SF (0.1 ml.) + RNA	—	0.330	29.8
	CSA-RNA	Ribonuclease A (0.1 μ g.) + RNA (control)	+	0.483	—
	CSA-RNA	Ribonuclease A (0.1 μ g.) + SF (0.1 ml.) + RNA	+	0.190	60.5
	CSA-RNA	Ribonuclease A (0.1 μ g.) + SF (0.1 ml.) + RNA	—	0.188	60.6
	Schwarz RNA	SF (0.1 ml.) + RNA + ribonuclease A (0.1 μ g.)	—	0.492	0
	Schwarz RNA	SF (0.1 ml.) + RNA + ribonuclease A (0.1 μ g.)	+	0.197	58.0
	CSA-RNA	SF (0.1 ml.) + RNA + ribonuclease A (0.1 μ g.)	—	0.588	0
	CSA-RNA	SF (0.1 ml.) + RNA + ribonuclease A (0.1 μ g.)	+	0.320	33.8
Expt. 2	Schwarz RNA	Ribonuclease A (0.1 μ g.) + RNA (control)	+	0.461	—
	Schwarz RNA	Ribonuclease A (0.1 μ g.) + SF (0.1 ml.) + RNA	+	0.189	59.0
	Schwarz RNA	Ribonuclease A (0.1 μ g.) + SF (0.1 ml.) + RNA	—	0.314	31.9
	Schwarz metal-free RNA	Ribonuclease A (0.1 μ g.) (control)	+	0.712	—
	Schwarz metal-free RNA	Ribonuclease A (0.1 μ g.) + SF (0.1 ml.) + RNA	+	0.202	71.6
	Schwarz metal-free RNA	Ribonuclease A (0.1 μ g.) + SF (0.1 ml.) + RNA	—	0.137	80.5
	Schwarz RNA	SF (0.1 ml.) + RNA + ribonuclease A (0.1 μ g.)	—	0.494	0
	Schwarz RNA	SF (0.1 ml.) + RNA + ribonuclease A (0.1 μ g.)	+	0.329	28.6
	Schwarz metal-free RNA	SF (0.1 ml.) + RNA + ribonuclease A (0.1 μ g.)	—	0.325	54.4
	Schwarz metal-free RNA	SF (0.1 ml.) + RNA + ribonuclease A (0.1 μ g.)	+	0.417	41.4

RNA and CSA-RNA, provided that adequate EDTA is present. In the absence of EDTA, inhibition with Schwarz RNA is much less and with CSA-RNA is unchanged. Similar results were obtained when Schwarz RNA was compared with Schwarz metal-free RNA. If either Schwarz RNA or CSA-RNA was added to ribonuclease inhibitor before ribonuclease, and EDTA was absent, no subsequent inhibition was obtained (Expt. 1). However, prior addition of Schwarz metal-free RNA decreased only by a small amount, and occasionally not at all, the subsequent reaction between ribonuclease inhibitor and ribonuclease. If EDTA was present, on the other hand, some or nearly all ribonuclease inhibitor activity was retained regardless of the kind of RNA substrate added before the reaction between ribonuclease and ribonuclease inhibitor was initiated.

These experiments suggest that dialysis or EDTA have removed, in part at least, some component, the prior addition of which prevents reaction between ribonuclease and ribonuclease inhibitor. The possibility that this component may be bivalent metal ions such as Mg^{2+} and Ca^{2+} was tested and found to be not tenable, since addition of Ca^{2+} or Mg^{2+} to a mixture of either Schwarz RNA or Schwarz metal-free RNA and inhibitor and subsequent addition of ribonuclease led to normal inhibition.

Thus the nature of the interference of some RNA substrates with the reaction between ribonuclease and ribonuclease inhibitor remains unanswered, though it is possible that it may be caused by a small nucleotide.

Summing up the available information, it appears likely that ribonuclease inhibitor is an acidic protein. The combination of it with ribonuclease may depend on interaction of some of these acidic groups with, most likely, positively charged guanidino side chains of ribonuclease arginine residues. More important, however, appear to be specific interactions of the proteins through hydrogen-bonding and possibly Van der Waals attractions. The active site and its positive charge do not seem to be necessary for the formation of the inhibitor-enzyme complex, but the specific ribonuclease conformation appears to be essential since any considerable change abolishes the interaction. One or more free thiol groups on the inhibitor (Roth, 1956, 1958; Shortman, 1961) are also important to the combination in an, as yet, unknown way. Further studies with purified inhibitor and ribo-

nuclease and its derivatives may yield additional information.

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REFERENCES

- Bigelow, C. C. (1961). *J. biol. Chem.* **236**, 1706.
 Blobel, G. & Potter, V. R. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 1283.
 Brown, R. K., Durieux, J., Delaney, R., Leikhim, E. & Clark, B. J. (1959). *Ann. N.Y. Acad. Sci.* **81**, 524.
 Cha, C.-Y. & Scheraga, H. A. (1963). *J. biol. Chem.* **238**, 2958.
 Chibnall, A. C., Mangan, J. L. & Rees, M. W. (1958). *Biochem. J.* **68**, 114.
 Crestfield, A. M., Smith, K. C. & Allen, F. W. (1955). *J. biol. Chem.* **216**, 185.
 Fletcher, W. E., Gulland, J. M. & Jordan, D. O. (1944). *J. chem. Soc.* p. 33.
 Hirs, C. H. W. (1956). *J. biol. Chem.* **219**, 611.
 Hirs, C. H. W., Moore, S. & Stein, W. H. (1953). *J. biol. Chem.* **200**, 493.
 Hymer, W. C. & Kuff, E. L. (1964). *Biochem. biophys. Res. Commun.* **15**, 506.
 Nelson, C. A., Hummel, J. P., Swenson, C. A. & Friedman, L. (1962). *J. biol. Chem.* **237**, 1575.
 Pirotte, M. & Desreux, V. (1952). *Bull. Soc. chim. Belg.* **61**, 167.
 Richards, F. M. & Vithayathil, P. J. (1959). *J. biol. Chem.* **234**, 1459.
 Roth, J. S. (1956). *Biochim. biophys. Acta.* **21**, 34.
 Roth, J. S. (1958). *J. biol. Chem.* **231**, 1085.
 Roth, J. S. (1960). *J. biophys. biochem. Cytol.* **7**, 443.
 Roth, J. S. (1962). *Biochim. biophys. Acta*, **61**, 903.
 Schuster, H. & Schramm, G. (1958). *Z. Naturf.* **137**, 697.
 Shortman, K. (1961). *Biochim. biophys. Acta*, **51**, 37.
 Shortman, K. (1962). *Biochim. biophys. Acta*, **55**, 88.
 Stark, G. R., Stein, W. H. & Moore, S. (1960). *J. biol. Chem.* **235**, 3177.
 Takemura, S. (1951). *J. chem. Soc. Japan*, **72**, 674.
 Vithayathil, P. J. & Richards, F. M. (1961). *J. biol. Chem.* **236**, 1380.
 Vunakis, H. W., Leikhim, E., Delaney, R., Levine, L. & Brown, R. K. (1960). *J. biol. Chem.* **235**, 3430.
 Wellner, D., Silman, H. I. & Sela, M. (1963). *J. biol. Chem.* **238**, 1324.
 White, F. H., jun. (1960). *J. biol. Chem.* **235**, 383.
 White, F. H., jun. (1964). *J. biol. Chem.* **239**, 1032.
 White, F. H., jun. & Sandoval, A. (1962). *Biochemistry*, **1**, 938.
 Wojnar, R. J. & Roth, J. S. (1964). *Biochim. biophys. Acta*, **87**, 17.
 Yang, S. & Hummel, J. P. (1964). *J. biol. Chem.* **239**, 3775.