Effect of Nucleoside 5'-Di- and 5'-Tri-phosphates on Pancreatic Ribonuclease Activity

By W. GAMBLE AND LEMUEL D. WRIGHT

*Graduate School of Nutrition, Cornell University, Ithaca, N.Y., and
Department of Chemistry and Science Research Institute, Oregon State University,
Corvallis, Oregon, U.S.A.*

(Received 4 January 1965)

1. ADP, ATP and GDP inhibited the phosphotransferase activity, the release of cyclic nucleotides from RNA, of ribonuclease. No significant inhibition was elicited by pyrimidine 5'-nucleoside diphosphates, CDP and UDP. 2. Inhibition by ADP, AMP, adenosine, adenine, NAD and NADP was insignificant at the concentrations tested. Small inhibition was observed with high concentrations of AMP and only when soluble RNA was the substrate. 3. Inhibition by ADP was found to be 'uncompetitive'. 4. Results seem to indicate that at least for optimum inhibition the polyphosphate of the purine nucleoside is essential. They further suggest that the inhibitor acts by combining with the enzyme only when the enzyme is bound to the substrate.

Experiments by several investigators have shown that adenosine monophosphates inhibit the cyclic phosphatase activity of ribonuclease (Davis & Allen, 1955; Dickman, Kropf & Aroskar, 1954; Ukita, Waku, Frie & Hoshino, 1961), but have little or no effect on the phosphotransferase activity (Dickman *et al.* 1954; Barker, Montague, Moss & Parsons, 1957). Ukita *et al.* (1961) reported the inhibition by ATP of the hydrolysis of cytidine 2',3'-(cyclic)-phosphate by ribonuclease. Preliminary experiments (Gamble & Wright, 1962) indicated an apparent inhibition of the phosphotransferase activity of ribonuclease by ADP. Therefore a study was undertaken to determine the exact nature of the inhibition. The present paper describes experiments which demonstrate the inhibition of the phosphotransferase reaction catalysed by ribonuclease by ADP and other nucleoside 5'-polyphosphates. Moreover, it is shown that the corresponding nucleoside monophosphates, nucleosides and free bases do not inhibit the phosphotransferase activity of ribonuclease. Under the conditions employed only purine nucleoside 5'-polyphosphates exhibit inhibition.

MATERIALS

Pancreatic ribonuclease preparations (5× crystallized or type III-A, 90-95% homogeneous) were from Sigma Chemical Co., St Louis, Mo., U.S.A. Similar results were obtained with both preparations. RNA (sodium salt; Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), yeast RNA (C grade, California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A., and Nutritional

Biochemicals Corp., 'purified' as described below) and soluble RNA (General Biochemicals Inc., Cleveland, Ohio, U.S.A.) were used as substrates. Trichloroacetic acid and KOH were reagent-grade chemicals from Mallinckrodt Chemical Works, New York, N.Y., U.S.A. ATP, NAD, CDP, IDP, UDP and GDP were purchased from Pabst Laboratories, Milwaukee, Wis., U.S.A. Adenine sulphate and adenosine were from Nutritional Biochemicals Corp. ADP was obtained from Nutritional Biochemicals Corp., Pabst Laboratories and General Biochemicals Inc. NADP was purchased from Sigma Chemical Co. GMP, 2'- and 3'-uridylic acid (mixed isomers), 2'- and 3'-cytidylic acid (mixed isomers) and 2'- and 3'-adenylic acid (mixed isomers) were products of Mann Research Laboratories Inc., New York, N.Y., U.S.A. All nucleotides were chromatographically pure and of reagent grade. Sodium pyrophosphate purchased from Fisher Scientific Co., New York, N.Y., U.S.A., was reagent grade. Buffers were 0.1 M-sodium acetate (pH 4.6-5.0), 0.1 M-potassium phosphate (pH 5.8-7.9), 0.05 M-sodium borate (pH 8.8-10.0) and 0.05 M-glycine (pH 10.6); the ionic strength of each was 0.15. Sodium acetate, boric acid and potassium phosphate were analytical reagent grade (Mallinckrodt Chemical Works). Glycine was purchased from Nutritional Biochemicals Corp.

METHODS

Purification of yeast RNA. A 2g. portion of crude yeast RNA was suspended in 100 ml. of 0.1 M-NaCl. The RNA was dissolved and the pH adjusted to 7.0 by adding dilute NaOH. To this solution was added 72 ml. of 95% ethanol. Then 70% ethanol was added until the solution became turbid. The mixture was allowed to stand overnight in the cold (0°). While cold, it was centrifuged at 2000 rev./min. until the supernatant was clear. After washing the precipitate once each with 70% and 95% ethanol it was allowed

to dry in air. This treatment rendered the RNA free of nucleotides; however, a small non-diffusible portion of the materials is not completely precipitated by 10% (w/v) trichloroacetic acid. The RNA so prepared was termed 'purified' RNA.

Nucleotide products. Two different solutions of nucleotides were used in the co-precipitation experiments. The first was a mixture of GMP, 2'- and 3'-uridylic acid, 2'- and 3'-cytidylic acid and AMP (substitution of 2'- and 3'-adenylic acid gave the same results), in equal amounts to give a 1% (w/v) solution. The pH was adjusted to 7.0 with NaOH. The second solution was prepared from nucleotides obtained from the hydrolysis of RNA by pancreatic ribonuclease: 1ml. of 2% (w/v) RNA, 2ml. of 0.1 M-phosphate buffer, pH 7.0, and 25 μ g. of ribonuclease were incubated at 30° for 18½ hr. The nucleotides were obtained by dialysis at 0° in water and subsequent freeze-drying of the diffusible material. A 1% (w/v) solution, adjusted to pH 7.0 with NaOH, was prepared from the resulting preparation.

Assay of ribonuclease. The procedure was a combination and modification of the methods of Schneider (1945), Schmidt & Thannhauser (1945), Scott, Fraccastoro & Taft (1956) and Kalnitsky *et al.* (1959). A portion (0.1ml.) of an aqueous solution of ribonuclease containing sufficient enzyme to give a final concentration of 10–13 or 25.8 μ g./ml. was added to a 15ml. plain centrifuge tube. Then 2ml. of appropriate buffer was added. RNA solution (1ml.) was added and the tube placed in a water bath at 30°. A blank was prepared simultaneously by replacing enzyme solution with 0.1ml. of water. Compounds to be tested for inhibition were dissolved in a portion of the buffer solution used. The reaction was stopped (usually after 4 min.) while the rate was linear by the addition of 2.5ml. of cold 10% (w/v) trichloroacetic acid to each test tube. The resulting mixtures were placed in an ice bath for 5 min. and then they were centrifuged at 0–4° until the supernatant was clear (approx. 30 min.). Precipitated RNA was washed, after decanting the supernatant, with 2.5ml. of cold trichloroacetic acid and again centrifuged in the cold. After removal of the trichloroacetic acid, the precipitates were washed with 5ml. of 95% (v/v) ethanol, centrifuged and the supernatant solution was decanted. Then to each tube 5ml. of 1N-NaOH was added, followed after 1 hr. by 1ml. of 6N-HCl. The tubes were centrifuged for 2–5 min. in the cold at about 2000 rev./min., and the solutions quantitatively transferred to 15ml. graduated centrifuge tubes; 2.5ml. of 1N-NaOH-6N-HCl (5:1, v/v) was added to each tube. After centrifuging for about 1 min. sufficient 1N-NaOH-6N-HCl (5:1, v/v) was added to bring the total volume to 10ml. A portion (0.1ml.) of each solution was mixed with 5ml. of water and the extinction was determined at 260 m μ by means of a Beckman model DU spectrophotometer. The observed extinction was inversely proportional to ribonuclease activity; that is, the greater the activity, the smaller the observed extinction value, since the extinction value represents the amount of substrate left, not the amount of nucleotides released. Similarly, the degree of inhibition was determined by comparing residual RNA values in the presence and absence of inhibitor. To determine the effect of products on the assay, solutions of nucleotides were added to the reaction mixture after the incubation period just before the addition of 10% (w/v) trichloroacetic acid (Table 1). The average error of the method was $\pm 7\%$.

For characterization of inhibition, maximum initial

velocities were determined by the method of Lineweaver & Burk (1934). The amounts of nucleotides released were plotted against time at 15 sec. intervals. An enzyme concentration of 10–13 μ g./ml. gave curves which were linear for about 5 min. and the activity then decreased. The maximum initial velocities were determined from these curves.

RESULTS

LePoutre, Stockx & Vandendriessche (1963) have shown that, in certain precipitation methods for the assay of ribonuclease activity, co-precipitation of nucleotides influenced the quantitative estimations. To investigate this point, experiments were performed in which various amounts of nucleotide products were added to the reaction mixture just before precipitation of the RNA (Table 1). There was no significant co-precipitation effect on the assay of ribonuclease activity. Fig. 1 shows the effect of ADP and ATP on ribonuclease activity over a period of 0 to 7 min. It is clear that the compounds decrease the activity of ribonuclease over the indicated time.

Results in Tables 2 and 3 and in Fig. 1 show that, under the same conditions and at similar concentrations of the compounds tested, ADP, GDP and ATP were the only compounds which elicited significant inhibition. At relatively high concentrations and only when soluble RNA was the substrate, AMP exhibited small but significant inhibition (Table 4). It should be added that, in other experiments with soluble RNA, inhibition by AMP was only 10%. A comparison of the inhibitions exhibited by various amounts of

Table 1. *Effect of nucleotide products on the assay of ribonuclease*

Nucleotide products used were a mixture (1) made from GMP, 2'- and 3'-uridylic acid, 2'- and 3'-cytidylic acid and AMP in equal amounts or (2) prepared from the hydrolysis of RNA by pancreatic ribonuclease. Concentrations of the products are those of the final solutions; concentrations of RNA are those of the employed solutions, not the final concentrations. Other conditions were as described in the Methods section.

	RNA (%, w/v)	Nucleotide products (%, w/v)	<i>E</i>	
			(1)	(2)
Blank	2	—	0.694	0.640
Ribonuclease	2	—	0.142	0.131
Blank	2	0.25	0.710	0.640
Ribonuclease	2	0.25	0.146	0.132
Blank	2	0.15	0.715	0.626
Ribonuclease	2	0.015	0.151	0.121
Blank	1	—	0.322	0.340
Ribonuclease	1	—	0.053	0.058
Blank	1	0.25	0.320	0.300
Ribonuclease	1	0.25	0.029	0.013

adenosine, AMP, ADP and ATP is shown in Table 3. When soluble RNA and 'purified' yeast RNA were the substrates, it was clearly shown that ADP was more inhibitory than ATP. However, with other RNA preparations (Tables 4 and 5) the

difference between inhibition by ADP and ATP was smaller or no longer apparent. The concentration of various RNA preparations were adjusted to give approximately the same extinction at 260m μ : similar results were obtained (Table 5). Therefore the difference in inhibition observed with ADP and ATP was not due to variation in absolute amount of material absorbing at 260m μ .

When pyrimidine nucleoside diphosphates (CDP, UDP; Table 2) were tested, no significant inhibition was observed compared with the inhibition obtained with similar amounts of ADP under the same conditions. Inhibition was small with purine nucleoside diphosphates having the second phos-

Table 2. *Influence of various compounds on the activity of ribonuclease*

Aqueous solutions (2%, w/v) of soluble RNA were used as substrate; ribonuclease, 25.8 μ g./ml.; 0.1 M-phosphate buffer, pH 7.0.

	Concn. (mm)	<i>E</i>	Inhibition (%)
Blank*	—	0.479	—
Ribonuclease	—	0.059	—
ADP	7	0.176	28
GDP	6	0.192	32
CDP	6	0.050	2
UDP	6	0.070	2
AMP	9	0.090	7
Blank	—	0.753	—
Ribonuclease	—	0.172	—
ADP	13	0.400	39
IDP	13	0.226	10
Blank	—	0.760	—
Ribonuclease	—	0.188	—
ADP	13	0.432	43
NAD	13	0.245	10
NADP	13	0.256	12
Pyrophosphate	28	0.115	-12
Blank	—	0.698	—
Ribonuclease	—	0.135	—
Adenine	29	0.162	5
Pyrophosphate	28	0.157	4

* In this experiment only, an aqueous 1.3% (w/v) solution of soluble RNA was used.

Fig. 1. Ribonuclease: ●, 10.3 μ g./ml.; △, 10.3 μ g./ml. (and ATP 5.26 mm); ○, 10.3 μ g./ml. (and ADP 6.8 mm). The substrate was soluble RNA (1.5%), which gave an average observed extinction of 0.550 in the absence of enzyme and inhibitors. The extinction values shown, which are corrected for zero-time controls, are relative values and do not indicate the absolute amount of RNA hydrolysed.

Table 3. *Comparison of the effects of adenosine, AMP, ADP and ATP on the activity of ribonuclease*

Aqueous solutions (2%, w/v) of 'purified' yeast RNA were used as substrates; ribonuclease, 25.8 μ g./ml.; 0.1 M-phosphate buffer, pH 7.19.

	Concn. of inhibitor (mm)	Adenosine		AMP		ADP		ADP*		ATP	
		<i>E</i>	Inhi- bition (%)	<i>E</i>	Inhi- bition (%)	<i>E</i>	Inhi- bition (%)	<i>E</i>	Inhi- bition (%)	<i>E</i>	Inhi- bition (%)
Blank	—	0.762	—	0.838	—	0.765	—	0.760	—	0.806	—
Ribo- nuclease	—	0.138	—	0.162	—	0.134	—	0.147	—	0.168	—
	26	0.139	0	0.213	8	0.416	45	0.537	64	0.362	30
	13	0.132	-1	0.182	3	0.305	27	0.383	38	0.303	21
	7	0.116	-4	0.174	2	0.231	15	0.283	22	0.216	8
	13	0.134	-1	0.150	3	0.140	1	0.163	3	0.146	2

* In this experiment only, an aqueous 2% (w/v) solution of soluble RNA was used.

Table 4. *Effects of AMP, ADP and ATP on ribonuclease activity as a function of substrate employed*

Substrates, as aqueous 2% (w/v) solutions: 1, soluble RNA; 2, RNA (sodium salt); 3, 'purified' yeast RNA (California Foundation for Biochemical Research); 4, 'purified' yeast RNA (Nutritional Biochemicals Corp.). Other conditions were as given for Table 3.

	Concn. (mm)	Substrate.....1		2		3		4	
		<i>E</i>	Inhibition (%)	<i>E</i>	Inhibition (%)	<i>E</i>	Inhibition (%)	<i>E</i>	Inhibition (%)
Blank	—	0.798	—	0.761	—	0.612	—	0.810	—
Ribonuclease	—	0.167	—	0.140	—	0.136	—	0.198	—
ATP	28	0.484	49	0.366	36	0.139	39	0.458	42
ADP	28	0.612	70	0.463	52	0.358	46	0.465	44
AMP	28	0.295	19	0.186	7	0.148	2	0.240	7

Fig. 2. Uncompetitive inhibition of ribonuclease (12.9 µg./ml.) by ADP. pH 7.0, 0.1M-phosphate buffer, *I* 0.15. ●, No ADP; ▲, 13 mM-ADP; ■, 20 mM-ADP. Soluble RNA was the substrate. Other conditions were as described in the Methods section. K_i 7 mM. K_m 1.32 mg./ml.

Fig. 3. Effect of pH on inhibition of ribonuclease (25.8 µg./ml.) by ADP: △, 1.3 mM; ●, 7 mM; □, 13 mM; ○, 26 mM. Substrate was 2% (w/v) soluble RNA. Incubations were at 30° for 4 min.

phate esterified with groups other than phosphate (Table 2). It can be seen in Tables 2 and 3 that adenosine, adenine, sodium pyrophosphate and IDP exhibited no significant inhibition.

By the Lineweaver-Burk reciprocal-plot method, the inhibition by ADP was shown to be 'uncompetitive' (Fig. 2). K_m at pH 7.0 was 1.32 mg./ml. This value is in agreement with the value 1.25 mg./ml. reported by Edelhoch & Coleman (1956). A plot of percentage inhibition versus pH revealed an apparent optimum pH for inhibition by ADP of about 5.7 (Fig. 3).

DISCUSSION

The phosphotransferase activity of ribonuclease was significantly inhibited by the purine nucleoside di- and tri-phosphates, ADP, GDP and ATP, but not by the pyrimidine nucleoside diphosphates, UDP and CDP. Adenine, adenosine and sodium

pyrophosphate did not significantly inhibit ribonuclease activity. Moreover, inhibition by AMP was absent, except in some experiments with large concentrations of AMP, and when soluble RNA was the substrate small inhibition was observed (Table 4). This would indicate that at least for optimum inhibition the polyphosphate of the purine nucleoside is essential. It appears that the presence of an amino group on the purine ring enhances inhibition, since IDP elicits less inhibition than ADP and GDP at similar concentrations. The lower magnitude of inhibition observed with NAD and NADP may indicate that esterification of the second phosphate of the nucleotide with groups other than phosphate diminishes the inhibitory

Table 5. *Effects of ATP, ADP and AMP on ribonuclease activity as a function of type and concentration of substrate*

In experiments with 1 and 2, ATP, ADP and AMP were 12 mM, 13 mM and 17 mM respectively. In experiments with 3 and 4, ATP, ADP and AMP were each 28 mM. In each experiment 0.1M-phosphate buffer, pH 7.0, was used. Substrates were: 1, aq. 2% (w/v) 'purified' yeast RNA (California Foundation for Biochemical Research); 2, aq. 0.67% RNA (sodium salt; Nutritional Biochemicals Corp.); 3, aq. 0.67% 'purified' yeast RNA (Nutritional Biochemicals Corp.); 4, aq. 0.7% soluble RNA (General Biochemicals Inc.).

	Substrate.....1		2		3		4	
	<i>E</i>	Inhibition (%)	<i>E</i>	Inhibition (%)	<i>E</i>	Inhibition (%)	<i>E</i>	Inhibition (%)
Blank	0.302	—	0.220	—	0.224	—	0.298	—
Ribonuclease	0.110	—	0.014	—	0.028	—	0.030	—
ATP	0.126	8	0.036	11	0.082	27	0.101	26
ADP	0.195	44	0.034	10	0.080	27	0.111	30
AMP	0.076	—17	0.017	1	0.026	—1	0.030	0

effect. Inhibition by ADP was shown to be 'un-competitive' [a type of inhibition described by Dodgson, Spencer & Williams (1956) and Ebersole, Gottentag & Wilson (1943)], which suggests the inhibition is due to a block of the reaction of the enzyme-substrate complex to yield products. That is, the data indicate that for inhibition to take place the ADP apparently must combine with the enzyme bound to the substrate. Thus, binding of the inhibitor to the free enzyme or the substrate separately would not bring about the observed effect. There is an apparent decrease in inhibition by ADP when the substrate concentration is decreased (Table 5). However, this decrease is not observed with ATP. Since the extent of inhibition varies when different RNA substrates are used (Table 5), the significance of this observation is not clear.

Ukita *et al.* (1961) reported that ATP competitively inhibits the cyclic-phosphatase activity of ribonuclease. Similarly, in unpublished experiments we have observed a competitive inhibition of the cyclic-phosphatase activity of ribonuclease by ADP. The experiments of this paper do not permit an explanation of the apparent difference in type of inhibition by ADP and ATP of the two reactions catalysed by ribonuclease.

This investigation was supported in part by Research Grant H-4138 (3) from the National Heart Institute, U.S. Public Health Service, Research Grant 3327 from the National Science Foundation and by funds made available

through the State University of New York. W. G. was a Postdoctoral Fellow of the National Heart Institute of the U.S. Public Health Service during this research. We are grateful for the technical assistance of Miss Elizabeth S. Kiersky.

REFERENCES

- Barker, G. R., Montague, M. D., Moss, R. J. & Parsons, M. A. (1957). *J. chem. Soc.* p. 3786.
 Davis, F. F. & Allen, F. W. (1955). *J. biol. Chem.* **217**, 13.
 Dickman, S. R., Kropf, R. B. & Aroskar, J. P. (1954). *Abstr. Pap. Amer. chem. Soc., 126th Meet., New York*, p. 71c.
 Dodgson, K. S., Spencer, B. & Williams, K. (1956). *Nature, Lond.*, **177**, 432.
 Ebersole, E. R., Gottentag, C. & Wilson, P. W. (1943). *Arch. Biochem.* **3**, 399.
 Edelhoch, H. & Coleman, J. (1956). *J. biol. Chem.* **209**, 351.
 Gamble, W. & Wright, L. D. (1962). *Fed. Proc.* **21**, 378.
 Kalnitsky, G., Hummel, J. P., Resnick, H., Carter, J. R., Barnett, L. B. & Dierks, C. (1959). *Ann. N.Y. Acad. Sci.* **81**, 542.
 LePoutre, L., Stockx, J. & Vandendriessche, L. (1963). *Analyt. Biochem.* **5**, 149.
 Lineweaver, H. & Burk, D. F. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
 Schneider, W. C. (1965). *J. biol. Chem.* **161**, 293.
 Scott, J. F., Fraccastoro, A. P. & Taft, E. B. (1956). *J. Histochem. Cytochem.* **4**, 1.
 Ukita, T., Waku, K., Frie, M. & Hoshino, O. (1961). *J. Biochem., Tokyo*, **50**, 405.