747. *Biosynthesis of Polynucleotides. Part I. The Mode of Action of Ribonuclease.*

By G. R. Barker, M. D. Montague, R. J. Moss, and Margaret A. Parsons.

The enzymic conversion of nucleoside cyclic phosphates into nucleoside alkyl phosphates has been studied and it has been found that only primary alcohols react. It is demonstrated that the above type of reaction takes place simultaneously with the enzymic degradation of ribonucleic acid.

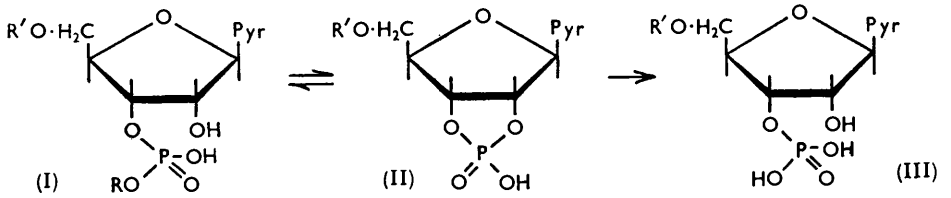
It is shown that the action of alkoxide ions on cyclic phosphates is analogous in certain respects to the action of ribonuclease and suggestions are made concerning the mechanism of the enzymic reaction. A nucleoside *iso*propyl phosphate differs in properties from the corresponding benzyl phosphate.

In this series of investigations it is proposed to study the processes whereby polynucleotides are formed and broken down *in vivo*. Chemical analogies are being sought for enzymic reactions involved, and syntheses of metabolic intermediates and their analogues are being undertaken. The present communication describes reactions catalysed by the enzyme ribonuclease; some of the results discussed have been briefly reported earlier.[1]

Ribonuclease is known to degrade ribopolynucleotides by converting pyrimidine nucleoside-3′ alkyl hydrogen phosphate residues into nucleoside-2′ : 3′ hydrogen phosphates which are subsequently hydrolysed to nucleoside-3′ dihydrogen phosphates.[2,3,4] These reactions, which may be represented by (I ⟶ II ⟶ III), take place when R and R′ are parts of a polynucleotide chain, or when R′ is hydrogen and R is a simple residue such as methyl, ethyl, or benzyl. In the simpler cases, moreover, the reverse reaction (II ⟶ I) has also been observed, and by this process both simple alkyl phosphates of nucleosides

[1] Barker and Parsons, *Chem. and Ind.*, 1955, 1009.
[2] Markham and Smith, *Biochem. J.*, 1952, **52**, 552.
[3] Brown and Todd, *J.*, 1953, 2040.
[4] Brown, Magrath, and Todd, *J.*, 1952, 2708.

and dinucleoside monophosphates have been obtained enzymically.[4] In order to provide a basis for the synthesis of potential inhibitors of ribonuclease we have studied the structural requirements for the formation of alkyl phosphates by this reverse reaction of ribonuclease. The isolation and characterisation of each compound formed would have been impracticable



in a general survey of this kind, but the chromatographic behaviour of various types of nucleotide derivative is sufficiently well known to enable us to identify the nature of the products by this means.

The cyclic phosphate of uridylic acid was incubated with the enzyme and various alcohols; and, in control experiments, the cyclic phosphate was incubated with the enzyme alone, and with the alcohol alone. Several nucleoside alkyl phosphates have been described previously, and shown to have higher R_F values than the parent nucleotide or cyclic phosphate in propan-2-ol–ammonia–water.[3, 6, 7] By using this solvent system nucleoside alkyl phosphates formed in the reaction were detected readily. No fast-moving spot was observed in control experiments, but alkyl phosphates were detected after incubation of the cyclic phosphate with the enzyme and methanol, ethanol, propan-1-ol, butan-1-ol, or benzyl alcohol. Absorbing material was eluted from appropriate areas of the chromatograms and the optical densities of the eluates were measured at 260 mμ. Table 1 shows the R_F values and the approximate yields of the products.

TABLE 1. *Conversion of cyclic phosphates into alkyl esters by ribonuclease and by alkoxide ions.*

	Nucleotide ester			
	From uridine-2′ : 3′ hydrogen phosphate by ribonuclease		From adenosine-2′ : 3′ hydrogen phosphate by base-catalysis	
Alcohol	Yield (%)	R_F in solvent A	Yield (%)	R_F in solvent A
Methanol	15	0·40	51	0·44
Ethanol	19	0·46	45	0·48
Propan-1-ol	8	0·48	21	0·59
Butan-1-ol	7	0·53	44	0·68
Benzyl alcohol	10	0·55	36	0·68

To confirm that the fast-moving spots were in fact alkyl phosphates, the product from the incubation with ethanol was examined further. Both in the above solvent system and also in *iso*pentyl alcohol–5% sodium phosphate, it had the same R_F values as the ethyl phosphate formed as a by-product in the preparation of the cyclic phosphate. The R_F value was unchanged after treatment of the material with 0·1N-hydrochloric acid at 18° for 2 hours, whereas uridine-2′ : 3′ hydrogen phosphate was completely destroyed under these conditions. The material was converted into uridylic acid by prolonged treatment with ribonuclease. The progress with time of the enzyme-catalysed reaction between uridine-2′ : 3′ hydrogen phosphate and ethanol is shown in Table 2.

In contrast to the above results, no alkyl phosphates were detected after incubation of uridine-2′ : 3′ hydrogen phosphate with the enzyme and propan-2-ol, butan-2-ol, *tert.*-butyl alcohol, or phenol. The possibility was considered that these results were due either

[5] Heppel and Whitfeld, *Biochem. J.*, 1955, **60**, 1.
[6] Heppel, Whitfeld, and Markham, *ibid.*, 1955, **60**, 8.
[7] Dekker and Khorana, *J. Amer. Chem. Soc.*, 1954, **76**, 3522.

to the inability of the chromatographic method to separate in these cases the alkyl (or aryl) phosphate from the cyclic phosphate, or to a rapid hydrolysis of the products to uridylic acid. Experiments described below rule out these possibilities for the reaction with propan-2-ol and it is therefore concluded that ribonuclease is able to catalyse the formation

TABLE 2. *Enzymic conversion of uridine-2′ : 3′ hydrogen phosphate into uridine ethyl phosphates.*

Time (hr.)	Uridylic acid (%)	Uridine-2′ : 3′ hydrogen phosphate (%)	Uridine ethyl phosphates (%)
0	20	80	0
2	40	57	3
3	46	47	7
3·75	48	39	13
4·5	52	32	16
5·5	57	24	19
20	100	0	0

of esters of nucleotides only with primary alcohols. The observation [6] that a 3′ : 5′-diester linkage is present in the enzymically produced dinucleoside monophosphate is in agreement with this conclusion.

The view had been expressed [7] that ribonuclease is concerned *in vivo*, not only with breakdown, but also with re-formation of ribopolynucleotides, and in agreement with this it has been recorded [8] that radioactive phosphorus is incorporated by certain tissues more rapidly into the dialysable fractions of ribonuclease digests of the tissue ribonucleic acids than into the non-dialysable " cores." The specificity we have observed would ensure the preservation of a 3′ : 5′-linked structure during re-synthesis of polynucleotides by by ribonuclease action. As far as we are aware, however, the reverse reaction (II ⟶ I) has only been observed with simple compounds and no direct evidence has been obtained that it does, in fact, take place concurrently with the degradation of polynucleotides by the enzyme. Important in this connection is the observation [9] that the degradation of ribonucleic acid by ribonuclease is inhibited by the nucleotides produced by alkaline degradation of the polynucleotide. It was considered that the enhanced reactivity of the enzyme towards freshly precipitated nucleic acid was the result of the elimination of nucleotides and small oligonucleotides during purification. Some doubt has been cast on the correct interpretation of these observations [10] and it was decided to investigate the problem further.

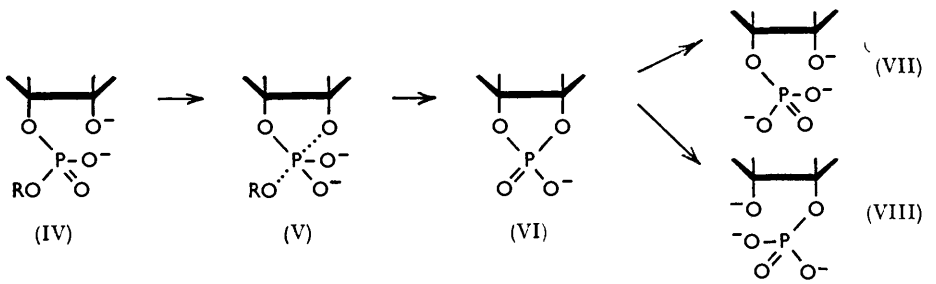
The activity of ribonuclease was measured by Kunitz's method [11] in which unchanged polynucleotide is precipitated by hydrochloric acid. Addition of mixtures of the 2′- and 3′-phosphate of adenosine, guanosine, cytidine, or uridine had no apparent effect on the activity of the enzyme measured by this method. This appears to be in contrast to Zittle's results,[9] but it is significant that he determined the activity of the enzyme by manometric measurement of carbon dioxide. This depends on the ability of the enzyme to liberate acidic dissociating groups, which takes place only in the second phase (II ⟶ III) of the degradation. On the other hand, Kunitz's method measures only the progress of the first phase of the reaction (I ⟶ II). We therefore examined the effect of nucleotides on the activity of ribonuclease as measured by continuous titration of acidic groups. The method used is essentially the same as that described by Edelhoch and Coleman.[12] They found that the activity of the enzyme, measured in this way, is reduced by the dialysable products of digestion. We examined the effects of various compounds of known structure and Edelhoch and Coleman's observations are consistent with our findings, outlined below. The enzyme was inhibited by the mixed 2′- and 3′-phosphates of adenosine, cytidine, and uridine, and by the nucleosides adenosine, cytidine, and uridine. No effect was observed

[8] Moldave and Heidelberger, *J. Amer. Cheœ. Soc.*, 1944, **76**, 679.
[9] Zittle, *J. Biol. Chem.*, 1946, **160**, 524.
[10] Cavalieri, *J. Amer. Chem. Soc.*, 1952, **74**, 1242.
[11] Kunitz, *J. Gen. Physiol.*, 1940, **24**, 15.
[12] Edelhoch and Coleman, *J. Biol. Chem.*, 1956, **219**, 351.

by adding the 5′-phosphate of adenosine, cytidine, or uridine. From these results we conclude that the inhibition observed by Zittle, by Edelhoch and Coleman, and by ourselves is due to suppression of the second phase (II ⟶ III) by reaction of the inhibitor with (II). Thus the nucleoside 5′-phosphates, being unable to react with (II) owing to the absence of a primary hydroxyl group, do not inhibit the enzyme. It thus appears that the reverse phase (II ⟶ I) can, in fact, take place during the action of ribonuclease on polynucleotides. It is interesting that Vandendriessche [13] has recorded that the two phases of the reactions catalysed by ribonuclease are affected to different degrees by certain inhibitors, but this effect cannot be related to our observations.

Apart from limitations imposed by the specificity of the enzyme, the reactions (I ⟶ II ⟶ III), catalysed by ribonuclease, are similar to those obtaining in the alkaline degradation of ribopolynucleotides.[3] We were interested to seek a chemical analogy of the reverse enzymic reaction (II ⟶ I), in order to throw light on the mechanism of the change. Two observations suggested that such an analogy might exist. First, that methyl phosphates of nucleosides are formed by interaction of the methoxide ion and ribopolynucleotides; [14] secondly, that potassium *tert.*-butoxide converts ribopolynucleotides largely into a mixture of cyclic phosphates.[15] In the first reaction, the alkoxide ion is believed initially to remove a proton from a sugar hydroxyl residue, thus promoting the formation of a cyclic phosphate, which is then attacked by the alkoxide ion, yielding an alkyl phosphate. The *tert.*-butoxide ion appears to be able to remove the proton from the sugar residue, but is unable to react with the cyclic phosphate formed. We therefore studied the action of various alkoxide ions on cyclic phosphates.

Adenosine-2′ : 3′ hydrogen phosphate, on treatment at room temperature with sodium methoxide (uridine-2′ : 3′ hydrogen phosphate behaved similarly with sodium methoxide), ethoxide, *n*-propoxide, *n*-butoxide, and benzyloxide, was largely converted into a mixture of the corresponding 2′- and 3′-alkyl phosphate (see Table 1). No alkyl phosphate was observed on chromatograms, however, with sodium *iso*propoxide or *tert.*-butoxide. In these instances the cyclic phosphate was recovered substantially unchanged apart from slight hydrolysis to adenylic acid. In order to test the sensitivity of the experimental procedure, the experiment with sodium *iso*propoxide was repeated on a larger scale. Elution of material from horizontal strips of the paper chromatogram indicated that not more than 2·5% of the cyclic phosphate had been converted into the *iso*propyl ester. It thus appeared possible that these reactions are analogous in mechanism to those catalysed by ribonuclease and it was decided to investigate this possibility further.



The suggestion has been made,[3] and supported by isotopic experiments,[16] that the alkaline hydrolysis of a compound such as (I) follows the course indicated by the partial structures (IV ⟶ V ⟶ VI ⟶ VII or VIII). A relatively stable intermediate was first postulated, but it is now thought that a transition-state complex such as (V) is concerned.[17]

[13] Vandendriessche, *Arch. Biochem. Biophys.*, 1956, **65**, 347.
[14] Lipkin and Dixon, *Science*, 1952, **116**, 525.
[15] Lipkin and Talbert, *Chem. and Ind.*, 1955, 143.
[16] Lipkin, Talbert, and Cohn, *J. Amer. Chem. Soc.*, 1954, **76**, 2871.
[17] Brown, Magrath, Neilson, and Todd, *Nature*, 1956, **177**, 1124.

It is immaterial to the present argument which view is correct, but it appears possible that a structure such as (V) may be involved, not only in the alkaline hydrolysis of (IV), but also in the reaction of an alkoxide ion with (VI). If so, the inability of (VI) to react with secondary or tertiary alkoxide ions may be due to steric resistance to the formation of (V). If this interpretation is correct, a compound (IV) in which R is secondary or tertiary should not show the lability to alkali which is characteristic of known compounds in which R is primary. Tener and Khorana [18] list the R_F value of the *iso*propyl esters of adenosine phosphates but give no further details of their properties. Using their method, we have prepared and characterised the mixed 2'- and 3'-*iso*propyl phosphate of uridine and have examined their behaviour towards alkali. As shown in Table 3, the material was hydrolysed by 0·5N-sodium hydroxide much more slowly than the corresponding benzyl esters. This supports the assumptions made above.

TABLE 3. *Hydrolysis of uridine-2' and -3' iso*propyl *and benzyl phosphates.*

	Uridylic acid present (%)			
	Hydrolysis by sodium hydroxide		Hydrolysis by ribonuclease	
Time (hr.)	Pr^i ester	CH_2Ph ester	Pr^i ester	CH_2Ph ester
0·5	—	65	—	52
1	4	85	32	72
2	—	98	43	80
5	4	100	53	80
9	6	—	68	—
15	—	—	67	—

Since the reactions of cyclic phosphates with alkoxide ions and with alcohols in the presence of ribonuclease show some similarities, it seemed possible that an intermediate such as (V) might be concerned in the enzyme-catalysed reactions. If this were the case, the above *iso*propyl ester would be expected to resist degradation also by the enzyme. However, this was found not to be the case. In Table 3 are compared the rates of production by the enzyme of uridylic acid from the mixed 2'- and 3'-*iso*propyl phosphate of uridine and from the corresponding benzyl esters. In each case, only the 3'-ester would be expected to react. Although decomposition of the *iso*propyl ester is slower than that of the benzyl ester, appreciable quantities of uridylic acid are formed.

From these results it appears that whereas ribonuclease is unable to convert (II) into (I) when R' is H and R is *iso*propyl, it is able to convert (I) into (II). This apparent anomaly may be due to the fact that the rate of conversion of (II) into (III) is not affected by the nature of R, and this reaction may predominate over the reaction (II ⟶ I) and prevent its detection experimentally.

From the above, it appears that the analogy between the enzyme and base-catalysed interconversions of cyclic phosphates and alkyl phosphates of nucleotides is not complete. However, the fact that the enzymic degradation of the above *iso*propyl ester takes place more slowly than that of the benzyl ester suggests that the mechanism (IV ⟶ V ⟶ VI) is operative at least in part. It may be significant in this connection that different fractions of ribonuclease show differences in their catalytic activities.[19] The possible existence of alternative pathways for the reaction will be considered in future work.

Long [20] has interpreted the reactions catalysed by ribonuclease in terms of a series of equations, according to which the enzyme combines with the phosphoryl residue of the substrate. Various groups in the enzyme molecule have been suggested as being concerned in the formation of such a complex, but no precise information is yet available. We believe that our results are in accord with the scheme envisaged by Long and we suggest tentatively a reason why ribonuclease catalyses the destruction or formation of only 3'-alkyl phosphates but not 2'-alkyl phosphates.

[18] Tener and Khorana, *J. Amer. Chem. Soc.*, 1955, **77**, 5349.
[19] Hakim, *Enzymologia*, 1956, **17**, 315.
[20] Long, *Science Progr.*, 1954, **42**, 266.

Heppel and Whitfeld [5] have shown that, provided an amino-group is present in the molecule, nucleoside-2′ phosphates and their esters have higher R_F values than the corresponding 3′-esters in the solvent system: saturated ammonium sulphate–propan-2-ol–M-sodium acetate (80 : 2 : 18 v/v/v). By this technique, and also using the *iso*pentyl alcohol–sodium phosphate system, we have separated the 2′- and 3′-alkyl phosphate

TABLE 4. *Proportions of isomeric alkyl phosphates formed by the action of alkoxide ions on adenosine-2′ : 3′ hydrogen phosphate.*

	2′-Alkyl phosphate			3′-Alkyl phosphate		
Catalytic base	R_F in B	R_F in C	%	R_F in B	R_F in C	%
Hydroxide	0·73	0·26	40	0·66	0·16	60
Methoxide	0·74	0·18	34	0·62	0·09	66
Ethoxide	0·77	0·25	32	0·68	0·11	68
n-Propoxide	0·76	0·19	25	0·62	0·06	75
n-Butoxide	0·75	0·13	25	0·63	0·05	75
Benzyloxide	0·66	0·11	20	0·56	0·02	80

produced by the action of alkoxide ions on adenosine-2′ : 3′ hydrogen phosphate. In Table 4 are given the relative proportions of each isomer formed. It is seen that the larger the attacking group, the higher is the proportion of 3′-ester. If it be assumed that an intermediate complex is formed in the enzymic reaction as suggested by Long [*e.g.*, (I; R′ = H, R = enzyme)], then by analogy with the above results it would be expected that such a complex would be formed only as in (I), and not by fission of the $C_{(3)}O$–P bond. Further reaction of this complex with an alcohol would not be governed by the nature of the incoming residue and a 3′-ester would be formed in all cases as is, in fact, found in the enzyme-catalysed reactions.

EXPERIMENTAL

Paper chromatography was carried out with the following solvent systems: A, propan-2-ol–ammonia–water; [2] B, *iso*pentyl alcohol–5% sodium phosphate; [21] C, saturated ammonium sulphate–propan-2-ol–0·5M-sodium acetate; [2] D, butan-1-ol–acetic acid–water.[22]

Enzyme-catalysed Reaction of Uridine-2′ : 3′ Hydrogen Phosphate with Alcohols.—In a typical experiment, uridine-2′ : 3′ hydrogen phosphate (2·3 mg.) and ribonuclease (crystalline, protease-free enzyme) (5 mg.) in 2M-aqueous ethanol (0·5 c.c.) were brought to pH 7·5 by adding dilute aqueous ammonia. One drop of toluene was added and the solution was incubated at 37°. Aliquot parts were removed periodically and chromatographed by ascending development, with solvent system A, authentic samples of uridylic acid and the cyclic phosphate being chromatographed at the same time. Control solutions, from which either ethanol or ribonuclease was omitted were treated in the same way. Ultraviolet absorbing spots were cut from the paper and eluted with water, and the optical densities of the eluates at 260 mμ were measured. Table 2 shows the progress of the reaction with time. No material having an R_F value higher than that of the cyclic phosphate was observed in control experiments. In the main experiments, material eluted from the fast-moving spots was chromatographed again on the same paper as the uridine ethyl phosphate obtained as by-product in the preparation of the cyclic phosphate and had identical R_F values with this material in solvent systems A (0·46) and B (0·87). The material was unaffected as judged chromatographically after being treated with 0·1N-hydrochloric acid at 18° for 2 hr.

Other uridine alkyl phosphates were obtained as fast-moving spots (for R_F values and yields see Table 1) under similar conditions to those described above, 2M-methanol, 3M-propan-1-ol, 3M-butanol-1-ol, and 2M-benzyl alcohol being used. No products were detected from 3M-propan-2-ol, 3M-butan-2-ol, 3M-*tert.*-butyl alcohol, or 3M-phenol. In experiments with the higher alcohols, the cyclic phosphate was brought into solution by gentle warming.

Reactions of Cyclic Phosphates with Sodium Alkoxides.—The following general method was used. To adenosine-2′ : 3′ hydrogen phosphate (5 mg.) in the appropriate alcohol (0·4 c.c.), an approximately molar solution of the sodium alkoxide in the alcohol (0·1 c.c.) was added. The

[21] Carter, *J. Amer. Chem. Soc.*, 1950, **72**, 1466.
[22] Partridge, *Biochem. J.*, 1948, **42**, 238.

solutions were kept in sealed tubes at room temperature for 20 hr. The solutions were then brought to pH 3·5 with aqueous acetic acid (2·5%, v/v) and chromatographed in solvent system A. The R_F values and the approximate yields, measured spectroscopically, of alkyl esters obtained from the cyclic phosphate of adenosine are shown in Table 1. The adenosine methyl phosphates are not as readily separated from the cyclic phosphate as are the other esters. In order to obtain the yield of the methyl phosphates, the material was eluted from the chromatogram and hydrolysed with 0·1N-hydrochloric acid at 18° for 2·5 hr. The solution was freeze-dried and the unchanged methyl phosphate was rechromatographed in solvent system A. Experiments showed that no measurable loss of the methyl phosphate was caused by the above treatment. No alkyl phosphates were observed under the above conditions when sodium *iso*propoxide or *tert.*-butoxide was used.

The mixed 2′- and 3′-alkyl phosphate obtained in the above experiments from papers developed with solvent system A were rechromatographed in solvent systems B and C. In each case two spots were observed and were separately eluted. The relative yields of isomers calculated from the optical densities of the eluates, measured at 260 mμ, are shown in Table 4.

Uridine-2′ : 3′ hydrogen phosphate (5 mg.) was treated with sodium methoxide, as described above except that the reaction time was 8 hr., and a spot was obtained on the chromatogram having the same R_F value (0·40) as the uridine methyl phosphate obtained in the enzymic reactions described above.

Uridine-2′ and -3′ iso*Propyl Hydrogen Phosphate.*—(*a*) To uridine-2′ : 3′ hydrogen phosphate (30 mg.) in propan-2-ol (1 c.c.) one drop of a saturated solution of hydrogen chloride in dioxan was added. After 10 min. the whole solution was applied to a sheet of Whatman filter paper (3MM) and irrigated with solvent system A. A component (R_F 0·48), travelling ahead of uridine and uridylic acid, was eluted with water and, after freeze-drying, yielded a material shown later to consist of a mixture of the ammonium salts of uridine-2′ and -3′ *iso*propyl hydrogen phosphate (Found: P, 8·1. Calc. for $C_{12}H_{22}O_9N_3P$: P, 8·1%).

(*b*) To uridine-2′ : 3′ hydrogen phosphate (120 mg.) in propan-2-ol (10 c.c.) was added a saturated solution of hydrogen chloride in dioxan (1 c.c.), and after 20 min. the solution was evaporated under reduced pressure to remove hydrogen chloride and brought to approx. pH 9 by adding dilute aqueous ammonia. Propan-2-ol was removed by extraction with ether. Excess of ether was removed under reduced pressure, the solution was diluted to approximately 2 l. and placed on a column (10 × 2·5 cm.) of Dowex-2 resin in the formate form. The column was eluted with a solution containing formic acid (0·01N) and ammonium formate (0·05N). A major component was eluted first and was shown by chromatography in solvent system A to contain the *iso*propyl ester. A small fraction containing the cyclic phosphate was eluted next, and uridylic acid was obtained by elution with ammonium formate (0·5N) and formic acid (0·5N). The solution containing the *iso*propyl ester was percolated through a column (10 × 1 cm.) of Amberlite IRC-50 resin in the hydrogen form, evaporated under reduced pressure, and finally freeze-dried, yielding a white hygroscopic powder (65 mg.). This was chromatographed in portions in solvent system A and the *ammonium salt* was eluted from appropriate areas of the paper and freeze-dried (Found: N, 11·02; P, 8·3. $C_{12}H_{22}O_9N_3P$ requires N, 10·9; P, 8·1%). Light absorption in H_2O: $\lambda_{max.}$ 261 mμ (ε9200), $\lambda_{min.}$ 231 mμ (ε 2700); in 0·01N-hydrochloric acid, $\lambda_{max.}$ 260 mμ (ε 9600), $\lambda_{min.}$ 230 mμ (ε 2300); in 0·01N-aqueous ammonia $\lambda_{max.}$ 260 mμ (ε 7100), $\lambda_{min.}$ 241 mμ (ε 5200). The material was not oxidised by sodium metaperiodate. In some experiments the ammonium salt was isolated by freeze-drying the eluate from the Dowex-2 resin, but in this case it was necessary to prepare the ammonium formate from ammonia and formic acid, since most samples of commercial ammonium formate contain traces of sodium formate. The *iso*propyl ester had R_F 0·6 in solvent system D; in solvent system B, complete separation from uridine-2′ and -3′ phosphates was not achieved.

Hydrolysis of Uridine-2′ and -3′ iso*Propyl Hydrogen Phosphate.*—(a) *By alkali.* The ester (3·1 mg.) was dissolved in 0·5N-sodium hydroxide (0·5 c.c.) and incubated at 37°. At intervals, aliquot parts were chromatographed in solvent system A, spots containing the *iso*propyl esters and uridylic acids were eluted with 0·1N-hydrochloric acid (5 c.c.) and the optical densities of the eluates at 260 mμ were measured. From these values, the percentage of the ester hydrolysed was calculated (see Table 3). The rate of hydrolysis of a mixture of uridine-2′ and -3′ benzyl hydrogen phosphate was measured in the same way.

(b) *By ribonuclease.* The ester (3·1 mg.) was dissolved in water (0·1 c.c.), and the solution was brought to pH 7·2 by adding dilute aqueous ammonia. Ribonuclease (10 μg.) in water

(0·1 c.c.) was added and the mixture incubated at 37°. Samples were chromatographed at intervals, and the degree of hydrolysis was determined as described above (see Table 3). The experiments were repeated with a mixture of uridine-2′ and -3′ benzyl hydrogen phosphate.

(c) *By Russell's viper's venom.* The ester (2 mg.) in water (0·1 c.c.) was incubated at 37° for 2 hr. with the venom (0·4 mg.). Chromatography of the resulting solution showed that the ester had been completely destroyed, and that uridylic acid was the main product. After incubation under the same conditions for 20 hr., uridine was the sole product of the hydrolysis.

TABLE 5. *Measurement of ribonuclease activity by precipitation.*

	Optical density of supernatant liquid at 260 mμ				
	Acid added				
Time (min.)	None	Adenylic	Guanylic	Cytidylic	Uridylic
0·25	0·30	0·29	0·30	0·30	0·30
0·75	0·32	0·34	0·35	0·37	0·36
2	0·39	0·42	0·41	0·42	0·42
5	0·46	0·47	0·47	0·50	0·50
10	0·50	0·51	0·52	0·54	0·55
15	0·53	0·52	0·54	0·57	0·58
20	0·55	0·53	0·56	0·59	0·60
25	0·57	0·54	0·58	0·62	0·63
30	0·59	0·56	0·59	0·64	0·64
40	0·61	0·59	0·59	0·65	0·64
50	0·61	0·61	0·61	0·67	0·65
60	0·62	0·60	0·61	0·67	0·66

(d) *By acetic acid.* The ester (2 mg.) was heated at 100° for 2 hr. with 80% acetic acid (1 c.c.). On chromatography of the resulting solution, no *iso*propyl ester was detected. Uridylic acid and uridine were both present in appreciable quantities.

Measurement of the Activity of Ribonuclease.—(a) *By precipitation.* Yeast ribonucleic acid (Pharmaco-Chemical Products Co., Ltd.) (purified by precipitation with acetic acid [11]) (160 mg.) was dissolved in 0·1N-sodium acetate (10 c.c.). The nucleotide under test (160 mg.) was added and the mixture was brought to pH 7·6 by adding dilute aqueous sodium hydroxide. In blank experiments, the same procedure was adopted but without the addition of nucleotide. The solutions were kept at 25° and ribonuclease (0·3 mg.) in water (2 c.c.) was added. The solutions were quickly mixed, and at intervals aliquot parts (1 c.c.) were withdrawn, immediately added to N-hydrochloric acid (2 c.c.), and set aside for 2 min. The suspension was centrifuged, an aliquot part (1 c.c.) of the supernatant was diluted to 100 c.c. with water, and the optical density at 260 mμ was measured. In experiments with nucleotides, the optical density at zero time in a blank experiment carried out simultaneously was subtracted from the observed optical density at each time. The values, corrected in this way for the absorption due to added nucleotide, are shown in Table 5.

(b) *By titration.* Yeast ribonucleic acid (0·8 g.) was suspended in water (20 c.c.) and the pH was brought to 7·8 (Cambridge pH meter) by adding 0·05N-sodium hydroxide. Various quantities of the nucleotide or nucleotide under test were added and the pH was again adjusted to 7·8. In blank experiments the same procedure was adopted but no addition was made. Ribonuclease (0·6 mg.) in water (2 c.c.) was added and the pH kept at 7·8 by adding 0·05N-sodium hydroxide. During the first approx. 30 min. the consumption of alkali is linear with respect to time, and the uptake of alkali after a fixed interval of time, chosen within this range, is proportional to the concentration of enzyme. The ratios of the slopes of the plots of consumption of alkali against time in experiments with added materials and in blank runs give a measure of the inhibition of the enzyme. The following ratios of slopes were obtained: adenylic acid (mixed 2′- and 3′-phosphate), 0·69; cytidylic acid (mixed 2′- and 3′-phosphate), 0·55; uridylic acid (mixed 2′- and 3′-phosphate), 0·64; adenosine, 0·81; cytidine, 0·40; uridine, 0·59; adenosine-5′ phosphate, 0·98; cytidine-5′ phosphate, 0·95; uridine-5′ phosphate, 0·98.

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