Nucleotide Exchange Reactions Catalysed by Ribonuclease and Spleen Phosphodiesterase

1. SYNTHESIS AND INTERCONVERSION OF SIMPLE ESTERS OF RIBOMONONUCLEOTIDES

BY L. A. HEPPEL* AND P. R. WHITFIELD

Agricultural Research Council Plant Virus Research Unit, Moltano Institute, University of Cambridge

(Received 16 September 1954)

Recent studies have considerably clarified the mechanism of hydrolysis of ribonucleic acids (RNA) by pancreatic ribonuclease and by spleen phosphodiesterase.

Digestion of RNA with ribonuclease results in the splitting of all pyrimidine-pyrimidine internucleotidic links and of those pyrimidine-purine internucleotidic links which involve C_{3'} of the pyrimidine nucleotide (Schmidt *et al.* 1951; Markham & Smith, 1952; Merrifield & Woolley, 1952; Volkin & Cohn, 1953). The first stage in the degradation involves the formation of pyrimidine nucleoside-2':3' phosphates (cyclic nucleotides) and the simultaneous splitting of the internucleotidic phosphoester bond distal to C_{3'} of the latter. Thus it is possible to isolate from early ribonuclease-digests of RNA pyrimidine cyclic nucleotides and small polynucleotides comprised of purine nucleotide(s) terminating in a pyrimidine cyclic nucleotide (Markham & Smith, 1952). Further treatment with ribonuclease hydrolyses these pyrimidine cyclic nucleotides giving pyrimidine nucleoside-3' phosphates only (Brown, Dekker & Todd, 1952; Markham & Smith, 1952).

Ribonuclease also splits simple esters of pyrimidine nucleoside-3' phosphates but not of pyrimidine nucleoside-2' phosphates to give the nucleoside-3' phosphate and an alcohol, the corresponding cyclic nucleotide appearing as an intermediate in the reaction (Brown & Todd, 1953). Purine nucleotide esters and purine cyclic nucleotides are not attacked by the enzyme (Markham & Smith, 1951*a*; Brown, Dekker & Todd, 1952; Brown & Todd, 1953).

Thus, in these reactions, ribonuclease can be regarded as a phosphodiesterase specific for esters of pyrimidine nucleoside-3' phosphates.

A phosphodiesterase fraction from spleen hydrolyses dinucleotides and simple esters of nucleoside-3' phosphates but not of nucleoside-2' phosphates to yield in each case a nucleoside-3' phosphate (Heppel, Markham & Hilmoe, 1953; Brown, Heppel & Hilmoe, 1954). This enzyme fraction, however,

* Present address: National Institutes of Health, Bethesda, Md., U.S.A.

differs from ribonuclease in two respects. In the first place, it attacks esters both of pyrimidine nucleoside-3' phosphates and purine nucleoside-3' phosphates. Secondly, evidence from kinetic studies and from the use of radioactive tracers indicates that the enzyme hydrolyses the nucleoside-3' phosphate esters to the corresponding nucleoside-3' phosphate without the intermediate formation of a cyclic nucleotide.

Hitherto, neither ribonuclease nor the spleen phosphodiesterase has been associated with any type of reaction other than the hydrolytic ones mentioned above. We now have evidence that both of these enzymes can participate in synthetic and exchange type reactions. It has been found that ribonuclease can catalyse the formation of certain alkyl esters of pyrimidine nucleotides either from the cyclic nucleotide and the appropriate alcohol or from the corresponding benzyl ester and the appropriate alcohol. Spleen phosphodiesterase has been shown to catalyse the formation of various alkyl esters of nucleoside-3' phosphates from the corresponding benzyl ester and the appropriate alcohol.

A preliminary report of this work has already been published (Heppel & Whitfield, 1954).

EXPERIMENTAL

Materials

Commercial yeast RNA was reprecipitated and deproteinized before use (Smith & Markham, 1950). For the preparation of 'core', the RNA was digested to completion (48 hr., 55°) with ribonuclease, *n*-NH₄OH being added at intervals to maintain the pH at neutrality. Chloroform was present to prevent bacterial growth. The digest was then dialysed for 48 hr. at 3° against running distilled water. Any insoluble material which formed was removed by centrifuging and the solution of non-dialysable polynucleotides was freeze-dried and stored at 3°. If the 'core' was to be used for the subsequent preparation of purine cyclic nucleotides (see below), the ribonuclease-digest of RNA was dialysed against running tap water and the polynucleotides precipitated by the addition of ethanol (final concentration of 75%, v/v).

Cyclic nucleotides. The 2':3' phosphates of cytidine and uridine were isolated from ribonuclease-digests of RNA as described by Markham & Smith (1952). The purine cyclic nucleotides were isolated by a slight modification of a previously published procedure (Markham & Smith, 1952). A solution of 'core' was mixed with excess solid BaCO_3 and enclosed in a cellophane dialysis bag. The bag was placed in a conical flask, covered with 10 vol. of distilled water and heated at 100° for 1 hr. The outer solution containing dialysable materials was concentrated by distillation *in vacuo* to a volume of about 5 ml. and chromatographed on sheets of Whatman no. 3MM filter paper (25×50 cm.) in the isopropanol-water- NH_3 system (solvent 1). The ultraviolet-absorbing band with the largest R_F value was cyclic adenylic acid and the band immediately behind it was cyclic guanylic acid. These bands were eluted and freeze-dried.

Cytidine-2':3' phosphate and adenosine-2':3' phosphate were also synthesized chemically by the method of Brown, Magrath & Todd (1952).

Simple esters of ribomononucleotides. Cytidine-2', cytidine-3', adenosine-2' and adenosine-3' benzyl phosphates were chemically synthesized (Brown & Todd, 1953; Brown *et al.* 1954). Cytidine methyl phosphate (isomeric mixture) was a gift from Dr D. M. Brown. Cytidine ethyl phosphate was obtained as a by-product in the chemical synthesis of cyclic cytidylic acid.

Adenosine methyl phosphate containing both the 2'- and 3'- isomers was synthesized as follows: a solution of diazomethane (4.2 g.) in ether (50 ml.) was added to a solution of 0.5 g. of yeast adenylic acid in 5 ml. of 50% (v/v) aqueous dimethylformamide. The mixture was shaken overnight at room temperature, after which the aqueous layer was chromatographed in solvent 1 and the adenosine methyl phosphate band eluted and concentrated.

Dinucleotides. Several dinucleotides were isolated from ribonuclease-digests of RNA by ion-exchange chromatography (Volkin & Cohn, 1953).

Reagent grade methanol and ethanol were commercial products.

Enzymes. Ribonuclease was obtained in crystalline form from pancreas (Kunitz, 1940).

The spleen phosphodiesterase fraction used in the present experiments was prepared as follows (all operations in a room maintained at 3°): calf spleen was dispersed in a Waring Blender with 3 vol. of cold 0.25M sucrose, and the mixture adjusted to pH 5.1 with acetic acid. A bulky precipitate formed which was collected by centrifuging, washed with cold (-10°) acetone and dried. The dried powder was extracted with 20 parts (v/w) of 0.2M acetate buffer (pH 6) and the solution fractionated with ammonium sulphate at pH 4.9 collecting a fraction which precipitated between 0.45 and 0.7 saturation. Refractionation was carried out with alkaline ammonium sulphate at pH 8.0, this time selecting the fraction precipitating between 0 and 0.62 saturation. The enzyme solution used in these experiments was dialysed against distilled water and contained 7 mg. of protein/ml.

As there is some evidence that the enzyme preparation obtained from spleen by the above method contains more than one phosphodiesterase, further purification of the fraction is being continued (by R. J. Hilme and L. A. Heppel) and a detailed description will be published later.

Methods

Chromatographic solvents. Solvent 1: isopropanol-water (70:30, v/v), with NH_3 in the vapour phase (Markham & Smith, 1952). This solvent was generally used for the separation of products after incubation with enzyme.

Solvent 2: saturated $(\text{NH}_4)_2\text{SO}_4$ -isopropanol-M sodium acetate (80:2:18, v/v/v) (Markham & Smith, 1951b). This solvent system differentiates between nucleoside-2' and nucleoside-3' phosphates, and between their secondary phosphate esters, provided that the base contains an $-\text{NH}_2$ group, compounds having a 2'-phosphate group then having larger R_F values than do the corresponding 3'-isomers.

Solvent 3: isopropanol, 170 ml.; conc. HCl, 44 ml.; water to 250 ml. (Wyatt, 1951).

Electrophoretic separation. The apparatus described by Markham & Smith (1952) was used and the separation was carried out on strips (56×9 cm.) of Whatman no. 3MM paper saturated with 0.05M ammonium formate buffer, pH 3.5.

Location of ultraviolet-absorbing materials. The purine- and pyrimidine-containing compounds were located on the chromatograms and electrophoresis strips by ultraviolet photography (Markham & Smith, 1949, 1951b).

Quantitative analysis. The relative proportions of the nucleotide esters, nucleotides and nucleosides were determined after chromatography by cutting out the spots, eluting them in 0.1N-HCl and reading the optical density at 260 m μ . against the appropriate blank. The molecular extinction coefficients ($\times 10^{-3}$) used were as follows (for pH 1.0 at 260 m μ): adenosine-3' phosphate, 13; adenosine-3' methyl phosphate, 13; adenosine-3' benzyl phosphate, 13.9; cytidine-3' phosphate, 6.8; cytidine-3' methyl phosphate, 6.8; cytidine-3' benzyl phosphate, 7.4.

General experimental procedure

Incubations were carried out at 38° in narrow, stoppered glass tubes (50×7 mm.), after which the mixture was chromatographed in solvent 1 on Whatman no. 3MM paper.

Table 1. R_F values of nucleotide derivatives in solvent 1

As the R_F values in solvent 1 of most nucleotides vary considerably according to the concentration of the NH_3 vapour in the tank, we have used the value R_0 , which is defined as the ratio of the distance moved by the substance to the distance moved by cytidylic acid. The latter has an R_F value in solvent 1 of approximately 0.25.

Compound	R_0 value
Cytidine	2.8
Cytidine-3' phosphate	1.0
Cytidine-3' methyl phosphate	2.8
Cytidine-3' ethyl phosphate	3.0
Cytidine-3' benzyl phosphate	3.7
Cytidine-2':3' phosphate	2.6
Adenosine-3' phosphate	1.0
Adenosine-3' methyl phosphate	2.8
Adenosine-3' ethyl phosphate	3.0
Adenosine-3' benzyl phosphate	3.7
Adenosine-2':3' phosphate	2.8
Guanosine-2':3' phosphate	2.1
Guanosine-3' methyl phosphate	2.1

When the period of incubation had been less than 1 hr. the mixture was placed on the paper and immediately rimmed with acetone which was allowed to diffuse into the applied spot. This procedure inhibited further reaction during the period of drying.

By comparing the chromatogram of the reaction products with that of the appropriate controls the presence of any new ultraviolet-absorbing component could generally be detected. The new compound was then eluted, concentrated and its chromatographic and electrophoretic behaviour compared with that of the chemically synthesized compound it was thought to be. The R_f values (distances moved by substances relative to cytidylic acid) in solvent 1 of the various substances encountered in this investigation are given in Table 1. Further identification was based upon an examination of certain other properties such as acid stability and the effect of enzymes.

RESULTS

Formation of alkyl esters from cyclic mononucleotides by ribonuclease

Digestion of 0.03M cytidine-2':3' phosphate with ribonuclease resulted in its complete hydrolysis to cytidine-3' phosphate and no other products were noted at any time. If, however, the incubation was carried out in the presence of methanol, part of the cyclic nucleotide was hydrolysed and part was converted into the methyl ester. Experimental conditions are given in Fig. 1, which illustrates the following reaction:

The identification of the product as cytidine-3' methyl phosphate was based on the following: (a) Its R_f value in solvent 1 was the same as that of chemically synthesized cytidine methyl phosphate. (b) It was stable to treatment for 4 hr. with 0.1N-HCl at room temperature, conditions under which cyclic mononucleotides, but not nucleoside alkyl phosphates, are hydrolysed. (c) Digestion with 150 μ g. of ribonuclease for 6 hr. at 37° converted it into cytidine-3' phosphate. (d) It was hydrolysed to cytidine-3' phosphate by spleen phosphodiesterase; cyclic nucleotides are hydrolysed to the nucleoside-2' phosphate by this enzyme (Whitfeld, Heppel & Markham, 1955). (e) It was unaffected by snake (*Crotalus adamanteus*) venom whereas cyclic cytidylic acid was converted into cytidine-3' phosphate under the same conditions.

Finally, the methyl ester was shown to be exclusively the 3'- isomer by chromatography in solvent 2. In this solvent, chemically synthesized cytidine methyl phosphate, which contains both the 2'- and 3'- isomers, moves as two substances, of which only the slower-moving is hydrolysed by ribonuclease. The latter, then, must be the 3'- isomer (Brown & Todd, 1953), and the enzymically

formed cytidine methyl phosphate has the same R_f value as this.

Optimum conditions for ester synthesis were not determined, but with a concentration of M methanol and 0.01M cyclic cytidylic acid, nearly 20% of the cyclic compound was converted into the methyl ester. With the same concentration of cyclic cytidylic acid but with only 0.25M methanol the synthesis of ester was still detectable. By increasing the methanol concentration to 8M the amount of cytidine-3' methyl phosphate synthesized from 0.03M cyclic cytidylic acid was found to be approximately equivalent to the amount of cytidine-3' phosphate resulting from the hydrolysis.

Fig. 1. Formation of cytidine-3' methyl phosphate from cyclic cytidylic acid and methanol by ribonuclease. Descending chromatogram run in solvent 1. The complete reaction mixture (col. 4) contained 2M methanol, 0.03M cyclic cytidylic acid, 0.015M phosphate buffer, pH 7.4, and 1 μ g. of ribonuclease in a total volume of 0.1 ml. Col. 3: no methanol. Col. 2: no enzyme. Col. 1: reference spots. Incubation was at 38°.

Cyclic cytidylic acid reacted also with ethanol in the presence of ribonuclease to form cytidine-3' ethyl phosphate.

Under experimental conditions comparable to those given in Fig. 1, cyclic uridylic acid could be converted into the methyl ester or into the ethyl ester by ribonuclease in the presence of the appropriate alcohol. The reaction products were identified

by procedures similar to those used in the identification of cytidine-3' methyl phosphate from reaction (1).

Formation of methyl esters of the pyrimidine nucleotides could also be demonstrated in an incubation mixture containing RNA, methanol and ribonuclease. A solution of RNA (1.0 g./7.0 ml.) in 14% (v/v) aqueous methanol, pH 7.0, was placed in a cellophan dialysis bag with 1.25 mg. of ribonuclease and dialysed for 1 hr. at room temperature against 120 ml. of 14% (v/v) aqueous methanol. The dialysate was concentrated *in vacuo* at 30° and chromatographed in solvent 1. When the chromatogram was compared with a chromatogram of the dialysate from a control incubation in which there was no methanol, the presence of an extra band of ultraviolet-absorbing material was detected. This extra band, which moved ahead of the pyrimidine cyclic nucleotides, was found to contain cytidine-3' and uridine-3' methyl phosphates.

Conversion of nucleoside benzyl phosphates into the corresponding alkyl phosphates by ribonuclease

When cytidine-3' benzyl phosphate was incubated with ribonuclease in the presence of methanol the formation of cytidine methyl phosphate could be demonstrated:

As in the case of reaction (1), the methyl ester formed was shown, by chromatography in solvent 2, to be the 3'- isomer exclusively.

Under favourable conditions, the amount of the benzyl ester which was converted into the methyl ester was almost equivalent to the amount which was hydrolysed to cytidylic acid. For instance, in one experiment, starting with 4.2 μ moles of cytidine benzyl phosphate, 5 M methanol, 13 μ moles of phosphate buffer, pH 7.4 and 1.2 μ g. of ribonuclease (total volume of 0.09 ml.), after 90 min. incubation at 38°, 67% of the benzyl ester had disappeared and there were formed 1.4 μ moles of methyl ester and 1.6 μ moles of cytidylic acid. Fig. 2 shows a chromatographic record of such an experiment run for 2 hr., at which time all the benzyl ester had disappeared.

At no stage in the reaction was any more than a trace of cytidine-2':3' phosphate detectable. It therefore seems unlikely that the methyl ester was formed by the production of free cyclic cytidylic acid followed by reaction (1). However, it is possible that on the enzyme surface cytidine-3' benzyl phosphate formed a bound cyclic nucleotide which in turn reacted with the methanol.

Synthesis of the ethyl ester could be shown in an

incubation mixture which contained cytidine-3' benzyl phosphate, ethanol and ribonuclease.

Cytidine-2', adenosine-2' and adenosine-3' benzyl phosphates could not be converted into the corresponding alkyl esters in the system, a result to be expected since none of them is hydrolysed by ribonuclease (Brown & Todd, 1953).

Fig. 2. Formation of cytidine-3' methyl phosphate from cytidine-3' benzyl phosphate and methanol by ribonuclease. Descending chromatogram run in solvent 1. Experimental conditions are given in the text; incubation was at 38° for 2 hr. Col. 1: reference spots. Col. 2: benzyl ester and methanol. Col. 3: benzyl ester and enzyme. Col. 4: complete reaction mixture (benzyl ester, methanol and enzyme).

Exchange reactions catalysed by spleen phosphodiesterase

The spleen phosphodiesterase preparation failed to catalyse reaction (1), and there was no synthesis of alkyl esters from the purine cyclic nucleotides and methanol. The enzyme, however, did catalyse reaction (2), forming cytidine-3' methyl phosphate from cytidine-3' benzyl phosphate and methanol. The reaction product was identified by the same procedures as those used for the identification of cytidine-3' methyl phosphate formed in reaction (1). As with ribonuclease, there was no synthesis of the alkyl ester starting with cytidine-2' benzyl phosphate.

Table 2. *Synthesis of adenosine-3' methyl phosphate from adenosine-3' benzyl phosphate and methanol by spleen phosphodiesterase*

Incubations were at 38° and the reaction mixture contained 0.012M Sørensen's phosphate buffer, pH 7.4.

Expt. no.	Concn. of enzyme (mg./ml.)	Concn. of methanol (M)	Time (min.)	Concn. (μ moles/ml.) of			
				Adenosine-3' benzyl phosphate		Adenosine-3' phosphate	Adenosine-3' methyl phosphate
				Initial	Final		
1*	0.7	0.5	90	11.6	7.7	3.5	0.4
	0.7	1.0	90	2.5	0.3	1.7	0.5
2	0.7	1.0	31	2.7	1.0	1.3	0.4
	2.8	1.0	31	2.0	0	1.5	0.5
3	2.8	1.0	31	2.1	0	1.7	0.4
	2.8	1.0	31	6.5	1.0	4.3	1.3
4	—	—	66	6.5	0.4	4.8	1.3
	2.8	4.7	31	2.2	0	0.8	1.4
5	2.8	5.5	240	10.7	0.4	3.6	6.7
6	2.8	6.0	360	7.6	0.4	2.4	4.8

* Temperature was 25° for this experiment.

In contrast to ribonuclease, spleen diesterase was active with purine nucleotide esters and it catalysed the formation of adenosine-3' methyl phosphate from the corresponding benzyl ester and methanol. Identification of the reaction product as adenosine-3' methyl phosphate was based on a comparison of its properties with those of chemically synthesized adenosine methyl phosphate. It was found that both compounds had (a) the same R_f value in solvent 1, (b) the same electrophoretic mobility at pH 3.5, namely 7.5 cm./2 hr. (20V/cm.), and (c) the same stability, which was much greater than that of cyclic adenylic acid, to 0.1N-HCl at room temperature. Finally, the enzymic product was shown to be the 3'- isomer exclusively, by chromatography in solvent 2. In this solvent, chemically synthesized adenosine methyl phosphate, which contains both the 2'- and 3'- isomers, moves as two substances of which only the slower-moving is hydrolysed by spleen phosphodiesterase. The latter, then, must be the 3'- isomer (Brown *et al.* 1954). The enzymically formed methyl ester was found to move in the same position as this isomer in solvent 2.

Experimental conditions and quantitative data for this reaction are presented in Table 2. It can be seen that, with 0.5M methanol and 0.01M adenosine-3' benzyl phosphate, of the benzyl ester which has disappeared, 90% has been hydrolysed and 10% converted into the methyl ester. However, on increasing the concentration of the methanol, the amount of the benzyl ester converted into the methyl ester exceeded the amount which was hydrolysed to adenylic acid.

Adenosine-2' benzyl phosphate was inactive in this system. Of several other alcohols which were tried as possible acceptors, ethanol was the only one found to be active (Table 3). *iso*Propanol

Table 3. *Synthesis of adenosine-3' ethyl phosphate from adenosine-3' benzyl phosphate and ethanol by spleen phosphodiesterase*

Incubation was at 38° and the reaction mixture contained ethanol (2.6M), phosphate buffer, pH 7.4 (0.012M), spleen enzyme (0.7 mg./ml.) and adenosine benzyl phosphate. Concentrations are expressed as μ moles/ml.

Time (min.)	Adenosine-3' benzyl phosphate	Adenosine-3' ethyl phosphate	Adenylic acid
0	9.4	0	0
30	5.1	2.3	2.0

proved to be ineffective and *n*-propanol was unsuitable because it completely inhibited the enzyme.

Formation of both adenosine methyl phosphate and guanosine methyl phosphate was demonstrated by incubating RNA 'core' (see 'Experimental') with spleen phosphodiesterase in the presence of methanol. Quantitative data, which are given in Table 4, indicate that ester formation exceeds hydrolysis, but that the presence of methanol inhibits the total reaction when compared with a control incubation containing no methanol. Rates of migration of the esters in solvent 1 are shown in Table 1.

A more specific example of the formation of nucleoside phosphate esters from polynucleotides by spleen diesterase was demonstrated by incubating the enzyme with the dinucleotide, adenylyl-uridylic acid, in the presence of methanol. Adenosine methyl phosphate, uridylic acid and a small amount of adenylic acid appeared as end-products of the reaction. If methanol was omitted from the system then uridylic and adenylic acids only were formed. In a typical experiment, 0.27 μ mole of

Table 4. *Formation of adenosine-3' and guanosine-3' methyl phosphates from RNA 'core' and methanol by spleen phosphodiesterase*

The experimental incubation mixture contained 0.05 ml. of 'core' ($\equiv$ 6 mg. of polynucleotide), 0.02 ml. of methanol, 0.015 ml. of 0.15M phosphate buffer, pH 7.4, and 0.015 ml. of spleen phosphodiesterase (2 mg./ml.). The control mixture contained water in place of methanol. Incubation was at 38°. Concentrations are expressed as μ moles/ml.

Time (hr.)	Experimental				Control	
	Adenosine methyl ester	Guanosine methyl ester	Adenylic acid	Guanylic acid	Adenylic acid	Guanylic acid
3	2.0	3.6	1.7	3.2	5.5	8.4
6	2.4	4.5	2.0	3.9	8.4	10.9
24	*	*	2.8	5.1	15.1	15.3

* Not analysed.

adenylyl-uridylic acid was digested with 30 μ g. of spleen diesterase in the presence of 500 μ moles of methanol (total volume of 0.1 ml.). After incubation at 38° for 135 min., 0.15 μ mole of methyl ester had been synthesized while only 0.02 μ mole of the dinucleotide had been hydrolysed to adenylic acid. Ethanol also acted as an acceptor in this reaction.

DISCUSSION

In recent years many different group transfer reactions have been discovered and for examples reference may be made to review articles on transpeptidation (Hanes, Connell & Dixon, 1952) and transglycosidation (Kalckar, 1954). Of the phosphatases, only the monoesterases have hitherto been known to participate in exchange reactions (Axelrod, 1948; Green & Meyerhof, 1952; Morton, 1953). Brawerman & Chargaff (1953*a, b*) effected the phosphorylation of nucleosides in a system containing a phosphate donor and either a malt diastase or a prostate phosphomonoesterase preparation.

The results described in this paper represent the first demonstration of transfer reactions which are catalysed by phosphodiesterases. Both ribonuclease and spleen phosphodiesterase participate in these reactions in which nucleotide residues act as donor molecules and certain simple alcohols, such as methanol and ethanol, act as acceptor groups. The mechanism of these transfer reactions can best be explained by postulating the intermediate formation of an activated nucleoside-3' phosphoryl group in which the energy of the phosphodiester is still maintained. This activated complex is formed by the enzyme from nucleoside-3' phosphate esters and, in the case of ribonuclease, also from pyrimidine nucleoside-2':3' phosphates. If a suitable acceptor molecule is present the activated complex then reacts with it to give the corresponding ester. In the absence of a suitable acceptor, and to a varying extent even in the presence of one, the complex reacts with water to yield the nucleotide, this constituting a simple hydrolysis reaction.

If the above interpretation is correct then the water present in the incubation mixture must compete against the alcohol as an acceptor for the activated complex. Furthermore, in transfer reactions the energy of the phosphodiester is largely maintained, whereas in the hydrolytic reactions the energy of one of the bonds is released. With this in mind, it is interesting to note that, in many of the experiments described, the amount of nucleotide ester which is involved in transfer reactions is equivalent to, or greater than, the amount of ester which is hydrolysed to the nucleotide, even though the concentration of the acceptor group is small if compared with that of the water present.

The present investigation has confirmed, by a somewhat different approach, previous reports on the specificity of ribonuclease and spleen phosphodiesterase. Thus, ribonuclease is known to hydrolyse the 3'- isomer, but not the 2'- isomer, of cytidine benzyl phosphate (Brown & Todd, 1953), and we have now shown that only the 3'- isomer of cytidine benzyl phosphate can participate in transfer reactions catalysed by this enzyme. Similarly, both hydrolytic (Brown *et al.* 1954) and exchange reactions catalysed by the spleen diesterase are limited to the nucleoside-3' phosphate esters only. With respect to pyrimidine cyclic nucleotides, Brown, Dekker & Todd (1952) found that hydrolysis by ribonuclease yielded the nucleoside-3' phosphate exclusively. We now observe that cytidine-2':3' phosphate reacts with methanol in the presence of ribonuclease to form only one of the possible isomers of cytidine methyl phosphate, namely the 3'- isomer.

It has been suggested (Heppel *et al.* 1953) that, in contrast to the action of ribonuclease, the hydrolysis of nucleotide esters to the nucleotide by the spleen phosphodiesterase does not involve the formation of a cyclic nucleotide as an intermediate. This view is supported by the results of the experiments reported in this paper. Whereas ribonuclease utilized both cyclic cytidylic acid and cytidine-3' benzyl phosph-

phate for the synthesis of cytidine-3' methyl phosphate, spleen diesterase catalysed the synthesis of the methyl ester from cytidine-3' benzyl phosphate only. It would appear that the activity of the latter enzyme does not involve the production or utilization of cyclic nucleotides. As will be shown later (Whitfield *et al.* 1955) cyclic nucleotides are hydrolysed by an enzyme present in the spleen preparation to give, quite unexpectedly, the nucleoside-2' phosphates.

Of the various alcohols which have been tried as possible acceptors in the transfer reactions, methanol and ethanol were found to be the only satisfactory ones. However, it will be shown in the following paper that compounds other than simple alcohols, namely ribonucleosides and cyclic nucleotides, can participate as acceptor molecules in these reactions.

It is unlikely that the ability to catalyse reactions of the type described in this paper is limited to two phosphodiesterases only. For instance the phosphodiesterase from intestinal mucosa which hydrolyses adenosine-5' benzyl phosphate (Brown *et al.* 1954) might well catalyse the formation of adenosine-5' methyl phosphate from the benzyl ester in the presence of methanol. A similar observation also applies to purified snake venom phosphodiesterase.

SUMMARY

1. A group of enzymic exchange reactions involving the transfer of ribomononucleotide residues has been described.

2. Ribonuclease catalyses the formation of cytidine-3' methyl phosphate from cytidine-2':3' phosphate and methanol. A similar reaction occurs with the uridine derivative and also with ethanol in place of methanol. Ribonuclease also catalyses the formation of cytidine-3' methyl phosphate from cytidine-3' benzyl phosphate and methanol, but is inactive towards cytidine-2' benzyl phosphate.

3. Spleen phosphodiesterase catalyses the formation of alkyl esters from both cytidine-3' benzyl phosphate and adenosine-3' benzyl phosphate in the presence of an alcohol, which may be either methanol or ethanol. With this enzyme, however, there is no synthesis of alkyl ester from either cytidine-2':3' phosphate and alcohol, or adenosine-2':3' phosphate and alcohol.

We are very grateful to Drs D. M. Brown and D. I. Magrath for advice and assistance in the chemical synthesis of cyclic mononucleotides and nucleoside benzyl phosphates. One of us (L. A. H.) participated in this work as a member of the National Institutes of Health, Bethesda, temporarily assigned to the Molteno Institute. Grateful acknowledgement is made to the John Simon Guggenheim Memorial Foundation for a Fellowship (L.A.H.), to the American Cancer Society for a Travel Aid Grant (to L.A.H.) and to the Australian Commonwealth Scientific and Industrial Research Organization for a Fellowship (P.R.W.).

REFERENCES

- Axelrod, B. (1948). *J. biol. Chem.* **172**, 1.
 Brawerman, G. & Chargaff, E. (1953*a*). *J. Amer. chem. Soc.* **75**, 2020.
 Brawerman, G. & Chargaff, E. (1953*b*). *J. Amer. chem. Soc.* **75**, 4113.
 Brown, D. M., Dekker, C. A. & Todd, A. R. (1952). *J. chem. Soc.* p. 2715.
 Brown, D. M., Heppel, L. A. & Hilmoe, R. J. (1954). *J. chem. Soc.* p. 40.
 Brown, D. M., Magrath, D. I. & Todd, A. R. (1952). *J. chem. Soc.* p. 2708.
 Brown, D. M. & Todd, A. R. (1953). *J. chem. Soc.* p. 2040.
 Green, H. & Meyerhof, O. (1952). *J. biol. Chem.* **197**, 347.
 Hanes, C. S., Connell, G. E. & Dixon, G. H. (1952). *Phosphorus Metabolism*, vol. 2, p. 95. Baltimore: The Johns Hopkins Press.
 Heppel, L. A., Markham, R. & Hilmoe, R. J. (1953). *Nature, Lond.*, **171**, 1151.
 Heppel, L. A. & Whitfield, P. R. (1954). *Biochem. J.* **56**, ii.
 Kalckar, H. M. (1954). *The Mechanism of Enzyme Action*, p. 675. Baltimore: The Johns Hopkins Press.
 Kunitz, M. (1940). *J. gen. Physiol.* **24**, 15.
 Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
 Markham, R. & Smith, J. D. (1951*a*). *Nature, Lond.*, **163**, 406.
 Markham, R. & Smith, J. D. (1951*b*). *Biochem. J.* **49**, 401.
 Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
 Merrifield, R. B. & Woolley, D. W. (1952). *J. biol. Chem.* **197**, 521.
 Morton, R. K. (1953). *Nature, Lond.*, **172**, 65.
 Schmidt, G., Cubiles, R., Zöllner, N., Hecht, L., Strickler, N., Seraidarian, K., Seraidarian, M. & Thannhauser, S. J. (1951). *J. biol. Chem.* **192**, 715.
 Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.
 Volkin, E. & Cohn, W. E. (1953). *J. biol. Chem.* **205**, 767.
 Whitfield, P. R., Heppel, L. A. & Markham, R. (1955). *Biochem. J.* **60**, 15.
 Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.