ing the blank reading. The rates of glucose oxidation at room temperature and at 0° are shown graphically in Fig. 5. At room temperature the theoretical 5.0 moles of periodate was consumed after 23 hours and at 0° the theoretical amount of periodate consumed after 46 hours. Additional studies on other sugars and sugar alcohols revealed that the rates of reaction with periodate are dependent on the structure of the compound. The spectrophotometric method is a very simple one for following the periodate consumption and therefore for determining reaction velocity curves.

ROCHESTER, N. Y.

[CONTRIBUTION FROM THE BRITISH COLUMBIA RESEARCH COUNCIL, UNIVERSITY OF BRITISH COLUMBIA]

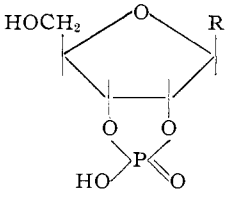
Cyclic Phosphates. II. Further Studies of Ribonucleoside 2′:3′-Cyclic Phosphates[1]

BY G. M. TENER AND H. G. KHORANA

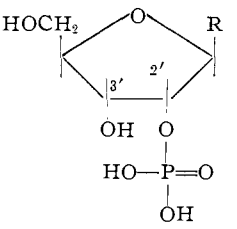
RECEIVED JUNE 2, 1955

A satisfactory method for the synthesis of monoalkyl esters of the ribonucleoside 2′- and 3′-phosphates is described which utilizes the facile, acid-catalyzed transesterification reaction between alcohols and ribonucleoside 2′:3′-cyclic phosphates. The latter compounds are prepared readily by the treatment of the yeast ribonucleotides with dicyclohexylcarbodiimide.

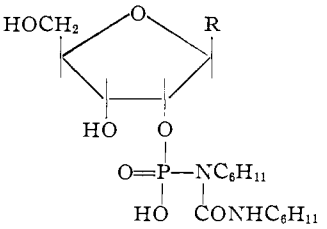
Pyrimidine ribonucleoside 2′:3′-cyclic phosphates (I, R = cytosine, uracil) were first encountered by Markham and Smith[2] as intermediates in the ribonuclease-catalyzed hydrolysis of ribonucleic acid (RNA) and their identification was accomplished through comparison with samples of these substances synthesized by Todd and collaborators.[3] Markham and Smith[4] were also able to isolate the cyclic phosphates corresponding to all the four ribonucleosides after mild alkaline hydrolysis of RNA. Recently, Lipkin and Talbert[5] have shown that the cyclic esters can be obtained in markedly improved yield by treatment of RNA with potassium *t*-butoxide in anhydrous *t*-butyl alcohol.



I



IIa



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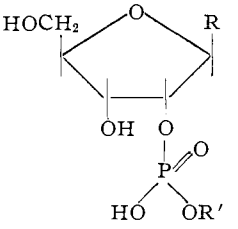

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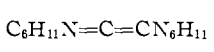

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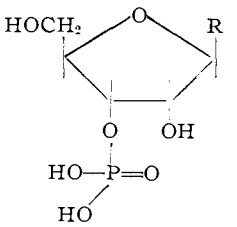
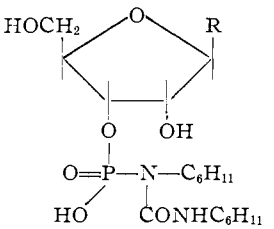
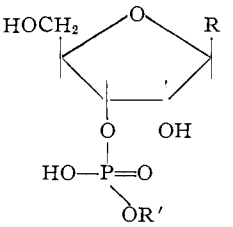


IIb



IIIb



IVb

The method employed by Brown, Magrath and Todd[3] for the chemical synthesis of the cyclic phosphates involved the reaction of the yeast ribonucleotides (II) (mixtures of ribonucleoside 2′- and 3′-phosphates) with trifluoroacetic anhydride and the yields reported were low. Recently a simple method for the purpose was reported[1] which consisted in treating the yeast ribonucleotides (II) with dicyclohexylcarbodiimide (DCC) (V) in aqueous pyridine at room temperature. The reaction converted the parent nucleotides quantitatively to mixtures of the cyclic phosphates (I) and phosphorylureas of the type III, the former predominating in the short period reactions. Compounds of the type III are reconverted to the cyclic phosphates under acidic or alkaline conditions and are, for all practical purposes, equivalent to the cyclic phosphates. Thus the method described[1] makes the cyclic phosphates readily available in quantity.

Thorough chemical study of the properties of the cyclic phosphates is desirable since the process of cyclization (II → I) represents a simple means for the activation of the phosphate group present in the yeast ribonucleotides (II). The energy stored in these labile esters of phosphoric acid (I), it is hoped, may be used to bring about, through transesterification reactions, the synthesis of 2′,5′- and 3′,5′-dinucleoside phosphates (IVa and IVb, respectively; R = purine or pyrimidine, R′ = purine or pyrimidine nucleoside) and polynucleotides. Ample enzymatic as well as chemical evidence has

(1) For the previous paper see C. A. Dekker and H. G. Khorana, THIS JOURNAL, **76**, 3522 (1954).

(2) R. Markham and J. D. Smith, *Nature*, **168**, 406 (1951).

(3) D. M. Brown, D. I. Magrath and A. R. Todd, *J. Chem. Soc.*, 2708 (1952).

(4) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(5) D. Lipkin and P. T. Talbert, *Chemistry and Industry*, 143 (1955).

recently accumulated to show the correctness of this view. Heppel and Whitfeld[6] have demonstrated the formation of the alkyl esters of ribonucleoside 3′-phosphates from the nucleoside cyclic phosphates and alkyl alcohols in the presence of ribonuclease. Heppel, Whitfeld and Markham[7] have in a similar fashion been able to synthesize dinucleoside monophosphates as well as di- and trinucleotides from the cyclic phosphates and appropriate ribonucleosides or nucleotides. It should be pointed out that these ribonuclease-catalyzed reactions are limited, in keeping with the well-established specificity of this enzyme, to the pyrimidine cyclic phosphates. The chemical synthesis of the monoalkyl esters of nucleoside 2′- and 3′-phosphates from the corresponding cyclic phosphates was recently demonstrated.[1] The base-catalyzed transesterification reaction used was however very sensitive to moisture and so gave only moderate yields of the alkyl esters. It has now been found that transesterification using acidic catalysis is much more efficient. Of a number of catalysts (Lewis and mineral acids) tried, hydrogen chloride has been found to be the most satisfactory. When the mixture of cytidine-2′:3′-cyclic phosphate (I, R = cytosine) and the cytidylylureas (III, R = cytosine) was dissolved in *n*-propyl alcohol containing 15% dioxane saturated with hydrogen chloride, all the starting material had disappeared within 15 minutes. Cytidine-2′(3′)-*n*-propyl phosphate (IV, R = cytosine; R′ = *n*-propyl) and a little cytidylic acid were shown to be the only products of reaction by paper chromatography. Transesterification reactions of the cyclic phosphates derived from uridine, adenosine and guanosine were equally rapid with propyl as well as benzyl alcohol. No cleavage of the N-glycosidic linkage in the purine nucleotides was detected in these experiments. The alkyl esters were separated readily from any unreacted cyclic phosphates and the small amounts of the free ribonucleotides both by paper and ion-exchange chromatography by established procedures. The yields of the alkyl esters were thus estimated to be 80–90%. Although only a few of the isomeric alkyl esters were separated, it appears certain that mixtures of 2′- and 3′-phosphate esters invariably are formed and that the 3′-isomer is always the predominating reaction product. These results are thus similar to those obtained in the base-catalyzed transesterification reaction.[1]

The R_f values, in three solvent systems, of the alkyl esters prepared, the parent ribonucleotides and the cyclic phosphates are compared in Table I. It will be seen that the solvent systems used separated the isomeric alkyl esters derived from adenosine only. The latter compounds were separated also on ion-exchange columns (*cf.* Brown and Todd, ref. 8). However, the ion-exchange procedures which readily separated cytidine-2′- and -3′-benzyl phosphates failed with the corresponding propyl esters and the analogous uridine compounds.

(6) L. A. Heppel and P. R. Whitfeld, *Biochem. J.*, **56**, ii (1954), and *ibid.*, **60**, 1 (1955).

(7) L. A. Heppel, P. R. Whitfeld and R. Markham, *ibid.*, **56**, iii (1954), and *ibid*, **60**, 8 (1955).

(8) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 2040 (1953).

The preparation of methyl esters of ribonucleotides by the base-catalyzed methanolysis of RNA has been recorded by Lipkin and Dixon.[9] Some alkyl esters of the yeast ribonucleotides have also been prepared by Brown and Todd[8] through the esterification of the nucleotides with diazoalkanes. The present transesterification method, which appears to be applicable to the primary as well as secondary alcohols, offers a simple and rapid method for the preparation of these substances. However, since mixtures of 2′- and 3′-esters are obtained, separation of the isomers will be necessary. Ion-exchange technique, which already has proven to be capable of separating some isomers, could very likely be improved to separate all the pairs.

Extension of this method to the synthesis of dinucleoside phosphates is in progress.

Experimental

The Reaction of Yeast Ribonucleotides with DCC: General Method.—To a solution of 1 g. of the yeast ribonucleotide in 10 ml. of 10% aqueous pyridine was added a pyridine solution (30 ml.) of DCC (4 g.). The mixture was left at room temperature for three hours and then diluted with 30 ml. of water. A precipitate of dicyclohexylurea was filtered off and the filtrate extracted with ether (4 × 50 ml.) and then lyophilized. In one typical experiment the yield from 1 g. of yeast cytidylic acid was 1.4 g. By paper chromatography the reaction products were shown to be mixtures of the cyclic phosphates and the phosphorylureas, containing traces of unreacted nucleotides. These mixtures of pyridine salts were used directly in the transesterification experiments.

Cytidine-2′- and -3′-Benzyl Phosphates.—To a solution of 194 mg. of the mixture of the cytidine cyclic phosphate and the cytidylylureas in 5 ml. of dry benzyl alcohol was added 1 ml. of dioxane saturated with hydrogen chloride. The

TABLE I

R_f VALUES OF RIBONUCLEOTIDES AND THEIR DERIVATIVES

Phosphate	Solvent systems A[10]	B[11]	C[12]
Uridine-2′(3′)-	0.06	0.83	0.18
Uridine-2′:3′-cyclic	.30	.75	.23
Uridine-2′(3′)-propyl	.45	.86	.39
Uridine-2′(3′)-benzyl	.55	.78	.47
Cytidine-2′(3′)	.07	.81	.10
Cytidine-2′:3′-cyclic	.31	.72	.27
Cytidine-2′(3′)-propyl	.54	.82	.32
Cytidine-2′-benzyl[13]	.54	.74	.39
Cytidine-3′-benzyl[13]	.54	.74	.39
Adenosine-2′(3′)	.08	.59 and 0.66	.10
Adenosine-2′:3′-cyclic	.42	.45	.56
Adenosine-2′(3′)-*n*-propyl	.56	.55 and 0.62	.31
Adenosine-2′(3′)-isopropyl	.50	.60 and 0.69	.25
Adenosine-2′(3′)-benzyl	.62	.46 and 0.60	.36
Guanosine-2′(3′)	.05	.83	.07
Guanosine-2′:3′-cyclic	.28	.64	..
Guanosine-2′(3′)-propyl	.47	.83	..
Guanosine-2′(3′)-benzyl	.72	.83	.68

(9) D. Lipkin and J. S. Dixon, *Science*, **116**, 525 (1952).

(10) Isopropyl alcohol:ammonia:water, 70:10:20 v./v., this is a modification of the system described by R. Markham and J. D. Smith (ref. 4).

(11) 5% disodium hydrogen phosphate–isoamyl alcohol, W. E. Cohn and C. E. Carter, THIS JOURNAL, **72**, 4273 (1950).

(12) *n*-Butyl alcohol:acetic acid:water, 4:1:5, v./v., S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(13) Separated on an ion-exchange column according to Brown and Todd (ref. 8).

solution was left at room temperature for 15 minutes and then evacuated for 10 minutes to remove as much of the hydrogen chloride as possible. Four ml. of dilute ammonium hydroxide was added to the solution and the benzyl alcohol extracted with ether (3 × 10 ml.). After removal of the excess of ammonia under vacuum the aqueous solution was applied to the top of a column (24 × 135 mm.) of Dowex 2[14] ion-exchange resin (200–325 mesh, formate form). The sample was washed in with 15 ml. of water and elution was carried out successively with 0.02 *N* formic acid (250 ml.), 0.1 *N* formic acid (750 ml.) and finally 250 ml. of 0.5 *N* formic acid. Fractions of twenty ml. each were collected, the elution being followed spectrophotometrically (optical density at 270 mμ). Cytidylic acid and cytidine-2′-benzyl phosphate emerged as separate peaks with 0.1 *N* formic acid and cytidine-3′-benzyl phosphate was eluted rapidly with the 0.5 *N* acid.[8] The proportions of the components eluted were found to be: cytidine-3′-benzyl phosphate, 65%; cytidine-2′-benzyl phosphate, 26%; unreacted cytidylic acid, 9%. The R_f values in three solvent systems are reported in Table I.

Adenosine-2′(3′)-propyl Phosphates.—Using a solution of the mixture of adenosine-2′:3′-cyclic phosphate and the adenylylureas in anhydrous *n*-propyl alcohol, the propyl esters were prepared as described above for cytidine benzy phosphates. The esters were isolated by paper chromatography on large sheets of Whatman paper No. 3MM in the solvent system A (Table I) and the eluted material further purified on an ion-exchange column as described above. The tubes containing the single broad peak eluted with 2.0 *N* formic acid were combined and lyophilized. On heating, the product decomposed above 164°, $\lambda_{max.}$ in 0.01 *N* hydrochloric acid, 257 mμ; ε 13,150.

Anal. Calcd. for $C_{13}H_{20}N_5O_7P \cdot 1H_2O$: C, 38.33; H, 5.44; P, 7.62. Found[15]: C, 38.33; H, 5.64; P, 7.63, 7.76.

By the procedure described above a number of ribonucleotide alkyl esters were prepared. The R_f values of the esters, the parent nucleotides and the cyclic phosphates are listed in Table I.

Acknowledgment.—This work was carried out under a consolidated grant from the National Research Council of Canada, Ottawa. We are grateful to Dr. G. M. Shrum for his generous encouragement of this work.

(14) The Dow Chemical Company, Midland, Michigan.

(15) Analyses by Dr. D. R. Idler, Fisheries Experimental Station, Vancouver, B. C.

VANCOUVER 8, B.C.

[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS]

N-Phosphoroamino Acids and Peptides

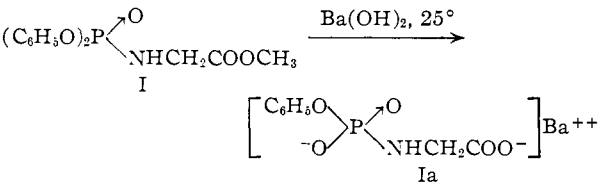
BY LEONIDAS ZERVAS AND PANAYOTIS G. KATSOYANNIS[1]

RECEIVED DECEMBER 2, 1954

A method for the synthesis of N-phosphoroamino acids and peptides is described. Di-*p*-nitrobenzylphosphorochloridate or di-*p*-iodobenzylphosphorochloridate was coupled with esters of amino acids or peptides; this was followed by removal of the *p*-nitrobenzyl and *p*-iodobenzyl groups through catalytic hydrogenation in alcoholic alkaline medium.

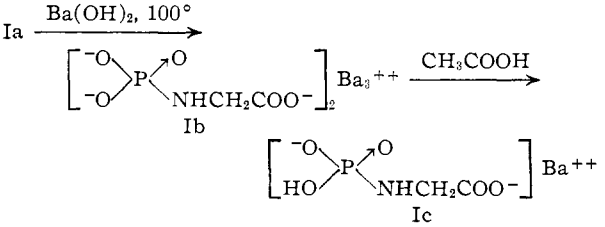
A number of phosphorylating agents have been used for the synthesis of N-phosphoroamino acids and peptides. With phosphorus oxychloride[2,3] the yields were low and the preparation of pure substances was difficult. The reaction of diphenylphosphorochloridate[4] (DPPCl) or diisopropylphosphorochloridate[5] with esters of amino acids yielded crystalline N-diphenylphosphoro or N-diisopropylphosphoro derivatives; however, it was not possible to split off the isopropyl groups without attacking the P–N bond[5] and saponification of the ester group in the case of the diphenyl compounds was not entirely successful.[4] In a few cases dibenzylphosphorochloridate[6] yielded crystalline dibenzylphosphoroamino acid esters; the benzyl groups can be removed easily by catalytic hydrogenation.[7] Non-esterified phosphoroamino acids have not been prepared by this method.

With DPPCl as the phosphorylating agent, we prepared N-phosphoroamino acids in some cases. The treatment of N-diphenylphosphoroglycine methyl ester (I), N-diphenylphosphoro-L-tyrosine ethyl ester (II) and N-diphenylphosphoroglycyl-L-tyrosine ethyl ester (III) with barium hydroxide resulted in saponification of the carboxyl ester group and splitting off of one phenyl group[8] as illustrated below



I Ia

Removal of the remaining phenyl group was accomplished without hydrolysis of the P–N bond by heating of the monophenyl derivative in a strongly alkaline solution (barium, sodium or potassium hydroxide)[9] as illustrated below for Ia



Ib Ic

In this case the insoluble barium salt (Ib) was con-

(1) This paper is based in part on the doctoral dissertation of P. G. Katsoyannis, Division of Natural Sciences (Chemistry Section), University of Athens, May, 1952. Present address: Department of Biochemistry, Cornell University Medical College, New York.

(2) C. Neuberg and W. Oertel, *Biochem. Z.*, **60**, 491 (1914).

(3) T. Winnick and E. M. Scott, *Arch. Biochem.*, **12**, 201 (1947).

(4) L. T. Sciarini and J. S. Fruton, THIS JOURNAL, **71**, 2940 (1949).

(5) T. Wagner-Jauregg, J. J. O'Neill and W. H. Summerson, *ibid.*, **73**, 5202 (1951).

(6) S. O. Li, *ibid.*, **74**, 5959 (1952).

(7) L. Zervas, *Naturwissenschaften*, **27**, 317 (1939); M. L. Wolfrom, C. S. Smith, D. E. Pletcher and A. E. Brown, THIS JOURNAL, **64**, 23 (1942); F. A. Atherton, H. T. Openshaw and A. R. Todd, *J. Chem. Soc.*, 382 (1945); S. O. Li, *Acta Chem. Scand.*, **4**, 610 (1950).

(8) This behavior toward alkali is similar to that of triphenyl phosphate (von Glutz, *Ann.*, **143**, 192 (1867); E. Baer, THIS JOURNAL, **69**, 1253 (1947), and of tribenzyl phosphate (W. Lossen and A. Koehler, *Ann.*, **262**, 196 (1891); our conditions are milder.

(9) Considerable hydrolysis of the P–N bond accompanied removal of the phenyl group by heating Ia in an aqueous solution; some hydrolysis occurred when a buffered solution (*p*H 7.5) was warmed.