ANALOGS OF PYRIMIDINE MONO- AND POLYNUCLEOTIDES.

II.* PHOSPHORYLATION OF N_1-(1,4)DIHYDRO-2-BUTYL)THYMINE WITH β-CYANOETHYL PHOSPHATE

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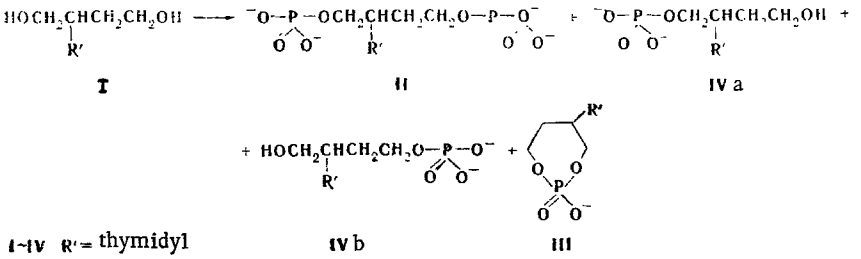
The phosphorylation of N_1-(1,4-dihydroxy-2-butyl)thymine with β-cyanoethyl phosphate in the presence of dicyclohexylcarbodiimide was investigated. The optimum conditions for the specific synthesis of the diphosphate, the isomeric monophosphates, and the cyclophosphate of N_1-(1,4-dihydroxy-2-butyl)thyimine were found. A number of side products were identified, and the fundamental ideas regarding the reaction mechanism are given. It is shown that the "pseudoglycoside" bond in the synthesized compounds is more resistant to acid hydrolysis than the analogous bond in the natural prototypes.

The chief reaction product in the phosphorylation of N_1-(1,4-dihydroxy-2-butyl)thymine (I) with β-cyanoethyl phosphate (CP) in the presence of dicyclohexylcarbodiimide (DCC) at a DCC:CP ratio of 4 is N_1-(1,4-dihydroxy-2-butyl)thymine diphosphate (II) [1].

The present paper is devoted to an investigation of the phosphorylation of I by means of β-cyanoethyl phosphate for the development of a method for the preparation of isomeric monophosphates (IV) and the cyclophosphate (III) of N_1-(1,4-dihydroxy-2-butyl)thymine (III).



I–IV R′ = thymidyl

Anion-exchange chromatography on QAE-Sephadex A-25 was used for the analytical determination and preparative isolation of these compounds. We found that a complex mixture of reaction products consisting of phosphate II-IV and a small amount of the analog of a dinucleotide (V) is formed in the phosphorylation of I. At CP:I and DCC:CP molar ratios of 6 and 1, respectively, the reaction does not proceed to completion, and 35% of unchanged I remains in the mixture. The chief reaction product is monophosphate IV (50%). An increase in the DCC:CP molar ratio to 1.5 leads to the complete disappearance of I and to the formation of diphosphate II (75%) as the chief reaction product. A further increase in the DCC:CP molar ratio to 5 lowers the yield of diphosphate II to 55% and increases the yield of cyclophosphate III extremely considerably.

The results of a study of the rate of formation of the products of phosphorylation of I with time at a DCC:CP molar ratio of 2 are presented in Fig. 1.

*See [1] for communication I.

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TABLE 1. Some Characteristics of the Products of Phosphorylation of N_1-(1,4-Dihydroxy-2-butyl)thymine

Compound	Empirical formula	Found, %			Calc., %		
		C	H	N	C	H	N
I							
II	$C_9H_{12}N_2Na_4O_{10}P_2 \cdot 2H_2O$	21,9	3.4	5,8	21,7	3.2	5,6
III	$C_9H_{16}N_3O_6P \cdot H_2O$	34,5	6,0	13,6	34,7	5,8	13,5
IV ‡	$C_9H_{21}N_4O_7P \cdot 3H_2O$	28,1	6,7	14,9	28,2	6,7	14,7
V	$C_{18}H_{34}N_6O_{13}P_2 \cdot 6H_2O$	30,5	6,5	11,5	30,4	6,5	11,8

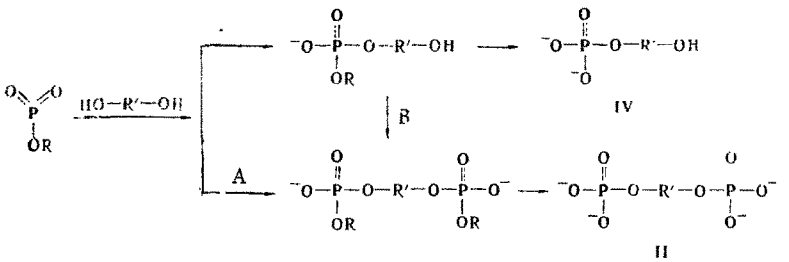
TABLE 1 (Continued)

Compound	UV spectra (pH 7)		K_{av}*	R_f*			Electrophoretic mobility†	
	λ_{max}, nm	ε		A	B	C	ph 7.5	pH 2,8
I	270	10600	0,46±0,02	0,72	0,78	0,66	0,57	—
II	271	10200	0,015±0,02	0,03	0,07	0,26	1,19	1,0
III	272	10800	0,16±0,02	0,48	0,6	0,48	0,9	1,36
IV ‡	272	9900	0,07±0,02	0,21	0,34	0,47	1,0	1,0
V	272	19500	0,05±0,02	0,16	0,3	0,38	1,10	1,36

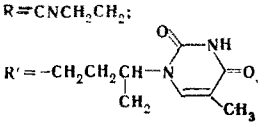
*See the experimental section.
†Relative to uridine 5'-monophosphate.
††PMR spectrum (water): δ 7.61 (6H, singlet), 1.78 (5-CH_3, singlet), 8.20 (OH, broad line), 3.9-4.7 (2'-H, multiplet), 3.1-3.9 (1'-H and 4'-H, multiplet), and 1.7-2.4 ppm (3'-H, multiplet).

A practically important result of these experiments is the exposure of the optimum conditions for the formation of phosphates II-IV. The preparation of monophosphate IV in 50% yield is possible for DCC:CP and CP : I molar ratios of 0.75-1 and 6, respectively, at 20-25°. Carrying out the reaction of DCC:CP:I molar ratios of 12:6:1 and at 30° makes it possible to reduce the reaction time from 24 h to 1 h and to retain the earlier yield of monophosphate IV (50%). The maximum yield of diphosphate II, which is 75%, is achieved under the following conditions: DCC:CP:I = 9:6:1, 20-25°, and a reaction time of 24 h.

Considering the role of the monomeric metaphosphate as the active phosphorylating particle [2], one should assume that the formation of diphosphate II occurs as a result of reaction of I with the monomeric metaphosphate via parallel (A) and consecutive (B) schemes:



Here and subsequently R = $CNCH_2CH_2$;



In fact, if one takes into account the fact that the contribution of each of the above-indicated paths for the formation of diphosphate II depends on the ratio of the rate constants of the parallel and consecutive reactions, its formation curve (Fig. 1) can correspond to both schemes. The experimental data provide evidence that in the initial

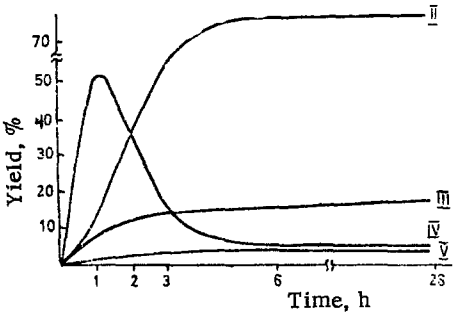


Fig. 1

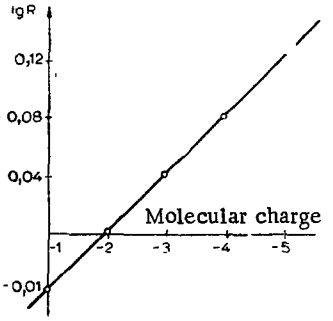


Fig. 2

Fig. 1. Dependence of the yields of the products of phosphorylation of N_1-(1,4-dihydroxy-2-butyl)thymine with β-cyanoethyl phosphate on the reaction time: II) N_1-(1,4-dihydroxy-2-butyl)thymine; III) N_1-(1,4-dihydroxy-2-butyl(thymine cyclophosphate; IV) N_1-(1,4-dihydroxy-2-butyl)thymine monophosphate; V) analog of a dinucleotide.

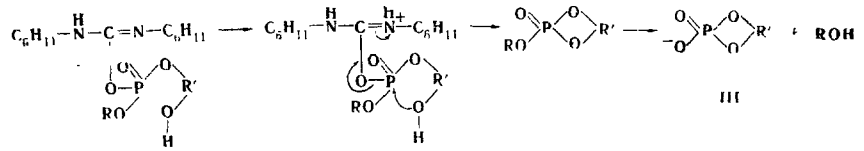
Fig. 2. Dependence of the logarithm of the relative mobility on the total negative charge.

step of the reaction the amount of diphosphate II formed is very small as compared with the amount of monophosphate IV. Thus, 15 min after the start of the reaction, IV:II = 16. An increase in the reaction time leads to an increase in the concentration of diphosphate II due to the decrease in the concentration of monophosphate IV. This makes it possible to assume that the formation of diphosphate II is realized primarily via a consecutive scheme.

In order to isolate III-V we attempted to use gel filtration on Sephadex G-10 in a 0.01 N ammonium carbonate buffer. It was found that these compounds cannot be isolated from one another by this method in view of the closeness of their elution parameters (Table 1). However, as seen from Table 1, this method can be used to establish the presence of the starting compound in the mixture.

We were able to accomplish complete separation of the products of the reaction under investigation by means of anion-exchange chromatography with columns filled with a DEAE-cellulose resin with the linear gradient of a triethylammonium bicarbonate buffer. It should be noted that the irritating action of the buffer on the mucous membranes of the eye, nose, and throat creates a certain amount of inconvenience in the preparative work. In the development of a preparative method for the separation of the products of phosphorylation we therefore turned to the use of anion-exchange chromatography with columns filled with QAE-Sephadex in the stepwise gradient of an ammonium carbonate buffer containing 10% ethanol at pH 8.5. The physicochemical constants of the isolated compounds are presented in Table 1.

The substance eluted with the 0.05 N ammonium carbonate buffer has an electrophoretic mobility in acidic media (pH 2.8) equal to the mobility of uridine 5'-monophosphate (5'-UMP) and somewhat lower than that of 5'-UMP in neutral media (pH 7.5). These results indicate the absence of dissociation of the secondary hydroxyl group of the investigated product. The PMR spectrum also constitutes evidence for the cyclic character of this phosphorylation product. The probable scheme of the formation of cyclophosphate III is presented below:



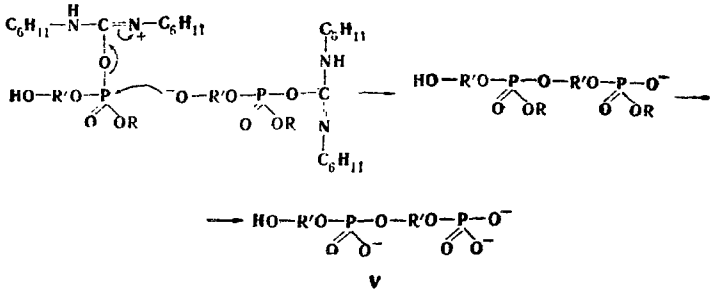
The product eluted with the 0.1 N ammonium carbonate buffer has an electrophoretic mobility in acidic and neutral media that is equal to the mobility of 5'-UMP. The results of elementary analysis for this compound are in good agreement with the calculated values

TABLE 2. Acid Hydrolysis of Products Eluted with the 0.25 N Buffer

Products obtained as a result of the hydrolysis	Normality of the buffer	Yield with respect to the results of ion-exchange chromatography, %	Electrophoretic mobility*
IV	0.1	25	1,0
V	0,25	45	1,1
II	0,3	30	1,19

*Relative to 5'-UMP.

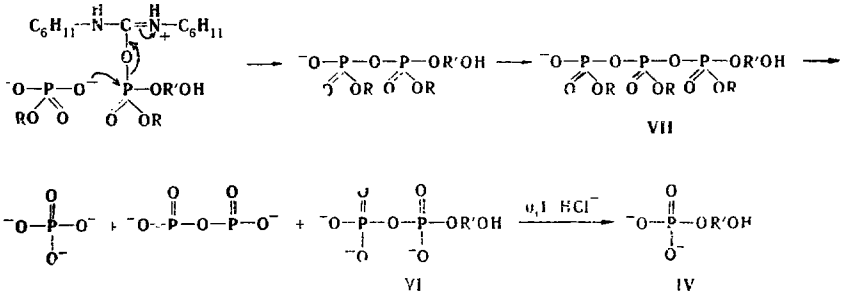
for IV. However, one cannot form a definite judgment regarding the structure of this substance from the presented data, inasmuch as it may exist in the form of two isomers. It is important to point out that by cyclization of it under the influence of dicyclohexylcarbodiimide we were able to obtain a substance that, with repeat to its physicochemical properties, is in complete agreement with cyclophosphate structure III; this is yet another proof in favor of structure IV. The product eluted with the 0.25 N buffer is electrophoretically homogeneous. Its electrophoretic mobility in neutral media is higher that in the case of IV but lower than that of II and is identical to the lability of II in acidic media but higher than in the case of IV. On this basis, it might be supposed that the product has two primary and one secondary dissociated hydroxyl groups attached to phosphate groups. The results of elementary analysis for this compound are in agreement with the calculated values for V. However, one cannot form a definitive judgment regarding the structure of this substance from the presented data, inasmuch as it may exist in the form of several isomers. Compound V is evidently formed as a result of intercondensation of two molecules of IV under the influence of dicyclohexylcarbodiimide.



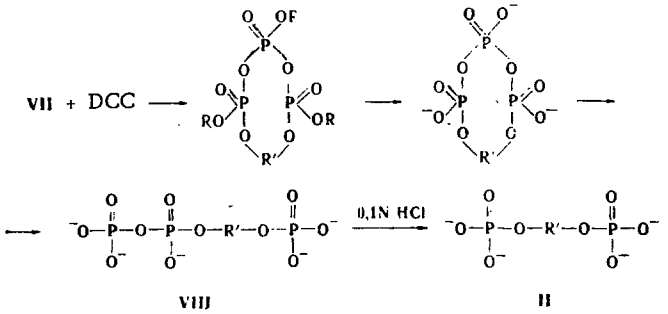
V

A characteristic feature of the investigated phosphorylation reaction is the presence of a small amount of monophosphate IV in the reaction products even under conditions of exhaustive phosphorylation, i.e., when excess β-cyanoethyl phosphate and dicyclohexylcarbodiimide are used and the reaction time is 24 h (Fig. 1). We suppose that this occurs as a result of the fact that a portion of phosphate IV is formed subsequently from pyrophosphate VI, which is a side product and is hydrolyzed under the conditions used to work up the reaction mixture. The formation of pyrophosphates was also previously observed by other investigators and is confirmed by persuasive literature data [3]. It is known that water is added to aqueous pyridine solutions to terminate the reation, during which the pyrophosphate bonds undergo considerable hydrolysis [3, 4]. However, if the pyridine is removed from the reaction mixture by evaporation and the residue is treated with aqueous alkali (during which the β-cyanoethyl protective group is removed), the pyrophosphates should be retained among the reaction products, inasmuch as they cannot undergo hydrolysis under these conditions. When the protective group is removed without preliminary treatment with water, the pyrophosphate particles are eluted along with V by means of the 0.25 N buffer. In order to ascertain the character of the compounds that were isolated along with V during the separation, the appropriate fraction was treated with acid with observance of the conditions under which hydrolysis of the pyrophosphate bonds occurs [5].

It is seen from the data in Table 2 that monophosphate IV is actually observed among the hydrolysis products. From this, it can be concluded with a high degree of probability that IV is formed in the acid hydrolysis of pyrophosphate VI.



It might be assumed that the appearance of diphosphate II is due to hydrolysis of a compound whose overall negative charge is three. However, electrophoretic analysis of the fraction under consideration prior to acid hydrolysis reveals, along with V, a substance with R = 1.30, in place of which diphosphate II appears after acid hydrolysis. The product with R = 1.30 is apparently five-charged anion VIII:



This assumption is confirmed by the observed linear dependence between the logarithm of the relative mobility and the total negative charges of the investigated substances (Fig. 2). The appearance of five-charged anions among the products eluted with the 0.25 N ammonium carbonate buffer, in which the presence of three-charged anions should be expected theoretically, constitutes evidence for the presence of labile components that are partially decomposed in neutral media under the electrophoresis conditions or during the treatment step preceding electrophoresis.

The results of a study of the rate of hydrolysis of III and IV in 0.1 N HCl at 100° are presented in Figs. 3 and 4. It is apparent from these data that the rate of hydrolysis of monophosphate IV is low under the indicated conditions. An analysis of the hydrolyzate provides evidence that dephosphorylation occurs in this case and starting I is isolated. As we have shown, as a result of the hydrolysis of cyclophosphate III, initially cyclic phosphates undergo ring opening to give intermediate IV, which then undergoes dephosphorylation, as in the preceding case. The fact that thymine is not detected in the hydrolysis products constitutes evidence for the relative low stability of the "pseudoglycoside" bond. A comparison of the relative resistance to acid hydrolysis of the thymidine 3',5'-cyclophosphate "analog" that we synthesized with its natural prototype provides evidence that the latter is hydrolyzed considerably more rapidly under the same conditions [5]. Compound V undergoes 21% hydrolysis at 100° in 1 N HCl, i.e., hydrolysis to approximately the same extent as thymidiyl oligonucleotides with natural 3',5'-phosphate bonds [5]; this indicates the great similarity between the reactivities of the analogs that we synthesized and those of the natural oligonucleotides.

EXPERIMENTAL

Column chromatography was monitored by means of a flow Uvicord-2 UV absorption meter at 254 nm. The fractions were selected with a collector electrically connected to a time relay and a recorder that registered the fractions. The eluent was fed into a column at a fixed rate by means of an LKB Varioperpex peristaltic pump.

Quantitative analysis was carried out with an SF-4A spectrophotometer with 1 cm thick quartz cuvettes.

Paper chromatography was carried out by the ascending method on Whatmann No. 1 paper in the following three systems: isopropyl alcohol—concentrated ammonium hydroxide—water

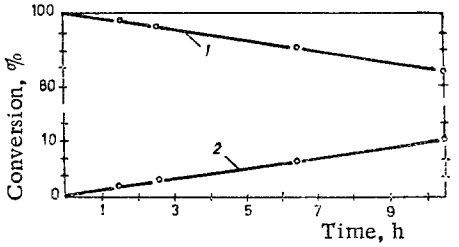


Fig. 3

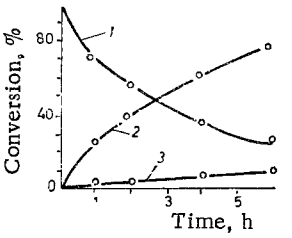


Fig. 4

Fig. 3. Acid hydrolysis of monophosphate IV in 0.1 N HCl at 100°C; 1) IV; 2) I.

Fig. 4. Acid hydrolysis of cyclophosphate III in 0.1 N HCl at 100°: 1) cyclophosphate III; 2) monophosphate IV; 3) I.

(7:1:2) (A), 1 M ammonium acetate–ethanol (3:7) (B), and isobutyric acid–concentrated ammonium hydroxide–water (66:15:33, pH 3.4) (C).

Electrophoresis was accomplished on FN 11 paper in a phosphate buffer at pH 7.5 and 700 V and in 0.1 M acetic acid at pH 2.83 and 900 V.

Sephadex G-10. Sephadex G-10 was swollen for 3 days in 0.01 N ammonium carbonate solution, after which it was freed of fine particles, suspended, and introduced into a glass column. A 68.7-cm long column with a diameter of 1 cm was used for the analytical experiments; a 180-cm long column with a diameter of 3.2 cm was used for the preparative experiments. The rates of elution from the columns were 76 and 25 ml/cm^2/h, respectively. The distribution coefficients (K_{av}) of the investigated products were calculated from the equation

$$K_{av}=\frac{V_e-V_o}{V_t-V_o},$$

where V_e is the elution volume of the substance, V_o is the outer volume of the column, which was assumed to be equal to the elution volume of polyuridylic acid, and V_t is the total volume of the column.

A-25 QAE-Sephadex Anion-Exchange Resin. This resin was swollen for 24 h in a tenfold volumn of 1 N ammonium carbonate, after which it was treated with the same volume of 0.01 N buffer containing 10% ethanol. The fine particles were then removed by decantation, and a suspension of the resin was introduced into a glass column.

Dowex 50 W × 4 Cation-Exchange Resin (200-400 Mesh). This resin was treated with 10 volumes of 10% NaOH, after which it was washed with distilled water and treated with 15% HCl (10 volumes). It was then washed successively with distilled water, 2 volumes of alcohol, and water. It was washed with 2 volumes of water prior to use.

β-Cyanoethyl Phosphate. This compound was synthesized by the method in [6] and was used in the form of a standard 1 M solution in absolute pyridine.

Monophosphate IV. A 9.65-g (0.045 mole) sample of I, 0.27 mole of β-cyanoethyl phosphate, and 56.1 g (0.27 mole) of dicyclohexylcarbodiimide were dissolved in 410 ml of absolute pyridine, and the mixture was shaken on a rocker for 3 h. Water (50 ml) was then added, and the precipitated dicyclohexylurea was removed by filtration after 1 h. The orange-yellow filtrate was vacuum evaporated with a rotary evaporater to 150 ml, an equal volume of 25% hydroxide was added, and the mixture was refluxed on a water bath for 3 h. The additional amount of precipitated dicyclohexylurea was removed by filtration, 30 ml of 25% ammonium hydroxide was added, and the mixture was again evaporated. The residue was dissolved in 100 ml of water. Chromatographic analysis of a sample of the reaction mixture with a column filled with 2 ml of QAE-Sephadex showed that the mixture consisted of 40% of starting I, 8.0% of diphosphate III, 50% of monophosphate IV, and 2% of cyclophosphate III. The inorganic phosphate and diphosphate II were precipitated from the basic solution by the addition of barium sulfate solution (100 g in 200 ml of water). The barium salts were removed by filtration and washed with water, and the combined filtrates were passed through a column containing a 550 ml of Dowex-50 H^+ resin. The eluate was evaporated several times in order to remove acetic acid. The residue was dissolved in 2.1 liter of

water, the solution pH was brought up to 7.5, and the solution was chromatographed with a column (80 cm long and 5 cm in diameter) filled with QAE-Sephadex. The components of the mixture were eluted with an ammonium carbonate buffer containing 10% ethanol with gradual raising of the ionic strength of the buffer. The rate of elution was 195 ml/h. Samples were collected every 5 min. The products eluted from the column were freed from ammonium carbonate by repeated vacuum evaporation. Starting I was eluted by means of a 0.01 N buffer, and monophosphate IV was eluted with a 0.1 N buffer. The solution containing IV was evaporated several times with absolute ethanol and absolute diethyl ether to give an oily residue, which was crystallized from absolute ethanol to give 5.96 g (41%) of IV. Cyclophosphate III and dimer V were similarly isolated from the appropriate fractions.

N_1-(1,4-Dihydroxy-2-butyl)thymine Cyclophosphate (III). A 0.058-mmole sample of IV in the H^+ form was dissolved in 10 ml of pyridine and dried by repeated evaporation with dry pyridine. The residue was dissolved in 4 ml of dry pyridine, and a solution of 72.4 mg (0.352 mmole) of dicyclohexylcarbodiimide in 2.3 ml of pyridine was added to it. After 87 h, 7 ml of water was added to the reaction mixture, and it was allowed to stand for 12 h. The precipitated dicyclohexylurea was removed by filtration, and the filtrate was evaporated twice with water and once with 1 N ammonium hydroxide. The residue was dissolved in 15 ml of water, the pH of the solution was brought up to 7.5, and the solution was applied to a column containing 4 ml of QAE-Sephadex. The solution was chromatographed as described above, and 82% of a substance that absorbs UV light at 254 nm was eluted with 0.05 N buffer. The eluate was evaporated with absolute ethanol and absolute diethyl ether to give an oily residue, which was crystallized from absolute ethanol to give a product in 79% yield.

Experiments were carried out at the following molar ratios in order to determine the optimum conditions for the synthesis of the principal products of phosphorylation (the mono- and diphosphate): DCC : CP —1, 1.5, 2.0, 3.0, and 5.0, and CP : I = 6 (for 24 h at 21-25°). Pyridine solutions of I (107 mg in 3.4 ml) and of dicyclohexylcarbodiimide (1.545 g in 4.38 ml) and a standard solution of β-cyanoethyl phosphate were prepared. A 0.34-ml sample of I (0.0312 mmole/ml), 0.3 ml of a solution of CP (1 mmole/ml), and calculated volumes of DCC (0.18 ml, 0.26 ml, 0.35 ml, 0.65 ml, and 0.69 ml) were introduced into volumetric test tubes, after which the volume in each test tube was brought up to 1.6 ml with absolute pyridine, and the mixtures were held at the indicated temperatures for 24 h with periodic shaking. The subsequent workup of the mixtures was similar to that presented above for the synthesis of the monophosphate.

In order to retain the pyrophosphate fractions, the phosphorylation reaction was stopped by evaporation of the pyridine, and the β-cyanoethyl protective group was removed with 25% ammonium hydroxide.

Acid Hydrolysis. The products to be subjected to hydrolysis (0.03 mmole) were refluxed in 5 ml of 0.1 N HCl. Samples for analysis were selected at equal time intervals. They were evaporated in order to remove the HCl, and the residue was dissolved in 15 ml of water. The pH of the solution was brought up to 7.5, and the solutions were chromatographed with a column filled with 4 ml of QAE-Sephadex, as described above.

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