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Studies on Pyrophosphates II. A New Method for the Synthesis of Nucleotide Coenzymes and Their Analogs

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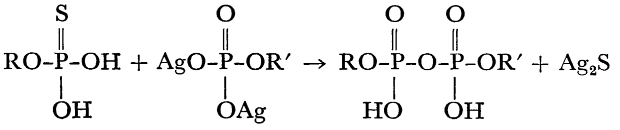
A new method for the synthesis of unsymmetrical pyrophosphates from the reaction of alkyl phosphorothioates and disilver salt of alkyl phosphates are described. Nucleotide coenzymes such as uridine diphosphate glucose, uridine diphosphate galactose, and flavin adenine dinucleotide was synthesized.

Information on the biological significance of a large number of nucleotide derivatives has increased rapidly and the domain of biochemistry of such compounds has been extended, one of them being nucleotide conenzymes which can be defined as unsymmetrical pyrophosphates having nucleoside residues. So far no biological function has been ascribed to symmetrical dinucleoside pyrophosphates, nor has any naturally occurring symmetrical pyrophosphate has been found. Consequently, development of the method for the selective synthesis of unsymmetrical pyrophosphates is of importance. The synthesis of nucleotide coenzymes were carried out by the use of reagents such as carbodiimide and related dehydrating reagents,[1] triesters of pyrophosphates,[2] α-pyridyl ester of phosphoric acid,[3] phosphoroamidates,[4] and *S*-ethyl phosphorothioates.[5] We have found a simple method for the selective synthesis of nucleotide coenzymes *via* nucleoside phosphorothioate intermediates[6] as shown in the following.

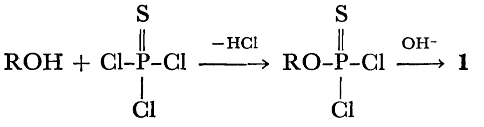


$$\mathrm{RO{-}\underset{OH}{\overset{S}{\overset{\|}{P}}}{-}OH + AgO{-}\underset{OAg}{\overset{O}{\overset{\|}{P}}}{-}OR' \rightarrow RO{-}\underset{HO}{\overset{O}{\overset{\|}{P}}}{-}O{-}\underset{OH}{\overset{O}{\overset{\|}{P}}}{-}OR' + Ag_2S}$$

(1) (2) (3)

In this paper results of the experiments for the synthesis of nucleotide coenzymes and their analogs are described in detail.

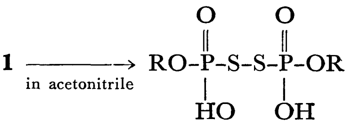
Preparation of Phosphorothioates. The starting material, phosphorothioate derivatives (**1**), was prepared by hydrolysis of the corresponding thiophosphorodichloridate (**4**) obtained from the reactions of alcohol or nucleoside with thiophosphoryl chloride.



$$\mathrm{ROH + Cl{-}\underset{Cl}{\overset{S}{\overset{\|}{P}}}{-}Cl \xrightarrow{-HCl} RO{-}\underset{Cl}{\overset{S}{\overset{\|}{P}}}{-}Cl \xrightarrow{OH^-} 1}$$

(4)

Alkyl thiophosphorodichloridates (**4**) are oily substances and purified by distillation (for their boiling points, see Experimental). Hydrolysis of **4** was performed in aqueous acetone at 0 °C in the presence of tertiary amines such as pyridine or triethylamine. Alkyl phosphorothioates (**1**) were extracted from the resulting mixture with ether and dried over sodium sulfate. After removal of ether at 5 °C, the phosphorothioates were dissolved in dry chloroform and stored in a refrigerator at 5 °C. They are stable under neutral and basic conditions at a temperature below 5 °C, but decompose in acidic medium even when kept at low temperature. Nucleoside phosphorothioates[7] were obtained by a similar procedure without isolating the intermediate **4**. Bis-triethylammonium salts of nucleoside phosphorothioates are stable solid substances. They are stable in dry pyridine at 5 °C, but unstable in acetonitrile or commercial pyridine, being converted into the corresponding P^1,P^2-bis-[nucleoside-5′-phosphoryl]disulfide (**5**).[8]



$$\mathrm{1 \xrightarrow[\text{in acetonitrile}]{} RO{-}\underset{HO}{\overset{O}{\overset{\|}{P}}}{-}S{-}S{-}\underset{OH}{\overset{O}{\overset{\|}{P}}}{-}OR}$$

(5)

R = nucleoside residue

Preparation of Disilver Salt of Phosphates. Disilver salts (**2**) were prepared from the corresponding disodium salts and silver nitrate. In order to avoid the formation of silver oxide, a solution of the sodium salts was added slowly to a solution of excess silver nitrate with vigorous stirring. Disilver salts of *p*-nitrophenyl phosphate and adenosine 5′-phosphate precipitated from the reaction mixtures. In the cases of glucose-1-phosphate, galactose-1-phosphate, and riboflavin 5′-phosphate, the silver salts precipitated by addition of methanol.[9]

Synthesis of Unsymmetrical Pyrophosphates. Several unsymmetrical pyrophosphates (**3**) were obtained when 1 equiv. of bis-triethylammonium salt of phosphorothioate (**1**) was treated with 1.2 equiv. of disilver salt of phosphate (**2**) in dry pyridine at room temperature for five hours. The results are

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[1] N. A. Hughes, G. W. Kenner, and A. R. Todd, *J. Chem. Soc.*, **1957**, 3733: E. P. Kennedy, *J. Biol. Chem.*, **222**, 185 (1956); R. J. Cremlyn, G. W. Kenner, and Sir A. Todd., *J. Chem. Soc.*, **1960**, 4511.

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[4] J. G. Moffatt and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 649 (1961); J. Josse and J. G. Moffartt, *Biochem.*, **4**, 2825 (1965).

[5] A. L. Nussbaum and R. Tiberi, *J. Amer. Chem. Soc.*, **87**, 2513 (1965); A. F. Cook, M. J. Holman, and A. L. Nussbaum, *ibid.*, **91**, 1522 (1969).

[6] T. Hata and I. Nakagawa, *ibid.*, **92**, 5516 (1970).

[7] T. Hata and I. Nakagawa, This Bulletin, **43**, 3619 (1970).

[8] The disulfides can be detected and distingushed from the corresponding pyrophosphates after the paper chlomatograms have been sprayed with the Grote reagent. J. G. Grote, *J. Biol. Chem.*, **93**, 25 (1931).

[9] Silver nitrate does not precipitate with the addition of methanol.

Table 1. Preparation of unsymmetrical pyrophosphates (**3**)

Phosphorothioate (1)		Disilver salt of phosphate (**2**)		Pyridine (ml)	Unsymmetrical pyrophosphate (**3**)			
R	mg (mmol)	R′	mg (mmol)		Yield (%)	R_f value (solv. A)	Spectral data $\lambda_{max}^{H_2O}$ ($\varepsilon \times 10^{-3}$)	(pH=7) $\lambda_{min}^{H_2O}$
C_2H_5	327 (1.0)	$(p)O_2N\text{-}C_6H_4$	508 (1.2)	20	74	0.68	290 (10.0)	242
n-C_3H_4	358 (1.0)	$(p)O_2N\text{-}C_6H_4$	508 (1.2)	20	85	0.70	290 (10.0)	242
n-C_4H_9	372 (1.0)	$(p)O_2N\text{-}C_6H_4$	508 (1.2)	20	84	0.72	290 (10·0)	242
iso-C_5H_{11}	386 (1.0)	$(p)O_2N\text{-}C_6H_4$	508 (1.2)	20	83	0.74	290 (10.0)	242
2′, 3′-*O*-Isopropylidene adenosine-(5′)	68 (0.1)	$(p)O_2N\text{-}C_6H_4$	51 (0.12)	2	54	0.65	263 (18.4)	235
2′, 3′-*O*-Isopropylidene uridine-(5′)	58 (0.1)	$(p)O_2N\text{-}C_6H_4$	64 (0.15)	1	86	0.56	267 (14.0)	237
n-C_4H_9	37 (0.1)	adenosine-(5′)	76 (0.12)	1	61	0.42	260 (15.4)	228

summarized in Table 1. The pyrophosphates were characterized by ratio of nucleoside base to phosphate and by ultraviolet absorption.

No symmetrical pyrophosphate was detected when only 1.2 equiv. of **2** was used. Satisfactory yields are maintained even when insoluble disilver salts containing crystal water were used.

Experimental

Methods. Paper chromatography was performed by the descending technique using Toyo Roshi papers No. 51 and No. 51A. The solvent system used was isopropyl alcohol-conc. aqueous ammonia–water (7 : 1 : 2 v/v) (Solvent A). The R_f values of different compounds are listed in Table 1. Paper electrophoresis was carried out with an apparatus similar to that described by Markham and Smith.[10] The buffer solution used was 0.05 M phosphate (pH=8.0). Estimation of the yields of unsymmetrical pyrophosphates was most frequently carried out spectrophotometrically after elution of the spot from paper chromatograms run on Solvent A.

Thiophosphoryl chloride was prepared by the procedure of Knotz.[11] It was distilled (bp 125 °C) prior to use. *p*-Nitrophenyl phosphate was prepared as described previously.[12] Commercial disodium salt of glucose-1-phosphate, galactose-1-phosphate, adenosine 5′-phosphate, and riboflavin 5′-phosphate were used.

*Alkyl Thiophosphorodichloridate (**4**).* These compounds were prepared by procedures given in literature. Boiling points of ethyl,[13] *n*-propyl,[14] and *n*-butyl[15] derivatives were 71 °C (23 mmHg), 82—82 °C (22 mmHg) and 91—93 °C (19 mmHg), respectively. Isoamyl derivative 115—116 °C (23 mmHg), was prepared by a modification of the procedure of Martin *et al.*[15].

*Alkyl Phosphorothioates (**1**). General procedure.* To 150 ml of aqueous acetone (1 : 2 v/v) was added alkyl thiophosphorodichloridate (0.1 mmol) under cooling at 0 °C in the presence of triethylamine (0.4 mmol). The mixture was concentrated to dryness under reduced pressure. Evaporation was repeated three times until triethylammonium chloride precipitated from the pyridine solution. The precipitate was then filtered off and the filtrate was concentrated. Chromatogram of the residue developed by Solvent A gave a single spot of compound **1**. The residue was dissolved in dry pyridine and a standard solution of **1** was prepared. The content of **1** was determined by phosphate measurement. The standard solution can be kept in a refrigerator for several weeks.

Disilver Salt of Adenosine 5′-Phosphate. To an aqueous solution of silver nitrate (0.2 mmol) was added disodium salt of adenosine 5′-phosphate (0.1 mmol) with vigorous stirring. The precipitate which soon appeared was collected by centrifuging. It was washed with water and then with methanol and finally with ether. The silver salt was kept in a desiccator under protection from light.

Disilver Salt of p-*Nitrophenyl Phosphate.* This was prepared in a similar way to that for adenosine 5′-phosphate.

Disilver Salts of Glucose-1-Phosphate and Galactose-1-phosphate. Experiments were carried out as for adenosine 5′-phosphate. The silver salts precipitated by addition of methanol.

Disilver Salt of Riboflavin 5′-Phosphate. The reaction was carried out under the same conditions as in the case of adenosine 5′-phosphate. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure until the precipitate appeared. After addition of methanol, red colored precipitate was collected and washed with methanol. The silver salt was dried over phosphorus pentoxide in a vacuum desiccator.

P^1-*Isoamyl* P^2-p-*Nitrophenyl Pyrophosphate.* To a suspension of disilver salt of *p*-nitrophenyl phosphate (508 mg, 1.2 mmol) in dry pyridine (20 ml) was added gradually a solution of bis-triethylammonium salt of isoamyl phosphorothioate (386 mg, 1.0 mmol) in dry pyridine (5 ml) with vigorous stirring. After 5 hr, the mixture was concentrated to dryness under reduced pressure at a temperature below 40 °C. The residue was shaken with four portions of a mixture of ethanol and concd. aqueous ammonia (2 : 1 v/v). The extracts were combined and concentrated. Water was then added and insoluble material was filtered off. The aqueous solution was neutralized by Dowex 50W-X8 (pyridinium form) and cyclohexylamine was added. The solution was concentrated to dryness. The residue was washed several times with chloroform and the extracts were combined and concentrated. Dicyclohexylammonium salt of P^1-isoamyl P^2-*p*-nitrophenyl pyrophosphate, separated by addition of dry ether and purified by reprecipitation, was obtained in an 83% yield based on isoamyl phosphorothioate. Mp 180—183 °C. Found: C, 48.06; H, 7.48; N, 7.95%. Calcd for $C_{23}H_{43}N_3P_2O_9$: C, 48.60; H, 7.57; N, 7.38%.

In a similar manner, P^1-ethyl P^2-*p*-nitrophenyl pyrophos-

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13) H. S. Booth, D. R. Martin, and F. E. Kendall. *J. Amer. Chem. Soc.*, **70**, 2523 (1948); "Inorganic Syntheses", Vol. IV, p. 75 (1953).

14) P. S. Pishchimuka, *Ber.*, **41**, 3854 (1908).

15) T. W. Martin, G. R. Norman, and E. A. Weilmuenster, *J. Amer. Chem. Soc.*, **67**, 1662 (1945).

phate, P^1-*n*-propyl P^2-*p*-nitrophenyl pyrophosphate and P^1-*n*-butyl P^2-*p*-nitrophenyl pyrophosphate were obtained. The data are given in Table 1.

Uridine Diphosphate Glucose. To a suspension of disilver salt of glucose-1-phosphate (monohydrate 59 mg, 0.12 mmol) in dry pyridine (0.5 ml) was added gradually a solution of bis-tri-*n*-butylammonium salt of 2′,3′-*O*-dibenzoyl uridine phosphorothioate (tetrahydrate 99 mg, 0.1 mmol) in dry pyridine (0.5 ml) with vigorous stirring. After 5 hr, the reaction mixture was filtered and the filtrate was passed through a column of Dowex 50W-X8 (pyridinium form). The eluate was treated with 0.2 M sodium hydroxide at room temperature for 30 min and then neutralized with Dowex 50W–X8 (pyridinium form). The solution was concentrated to a volume of 5 ml and applied to a column of Dowex 1–X8 (chloride form). Uridine 5′-phosphate was first eluted with 0.003 M hydrochloric acid in 0.01 M lithium chloride and then uridine diphosphate glucose with 0.003 M hydrochloric acid in 0.06 M lithium chloride. This was neutralized with lithium hydroxide and concentrated to a small volume. After addition of ethanol, dilithium salt of uridine diphosphate glucose was separated by addition of acetone. The precipitate was filtered and washed with acetone and then with dry ether. Uridine diphosphate glucose (dilithium salt) was obtained in 70% yield (43 mg). Found: C, 29.76; H, 4.93; N, 4.22%. Calcd for $C_{15}H_{22}N_2P_2Li_2O_{17}\cdot 2H_2O$: C, 29.31; H, 4.23; N, 4.57%. Determination of ratios of uridine, glucose, phosphorus gave 1.00 : 1.15 : 1.94; the calculated ratios 1 : 1 : 2.

Uridine Diphosphate Galactose. To a suspension of disilver salt of galactose-1-phosphate (monohydrate 59 mg, 0.12 mmol) in dry pyridine (0.5 ml) was added a solution of bis-triethylammonium salt of 2,′3,′-*O*-isopropylidene uridine 5′-phosphorothioate (48 mg, 0.10 mmol) in dry pyridine (0.5 ml) with vigorous stirring. After 5 hr, the reaction mixture was concentrated to dryness and the residue was treated with 10% sodium chloride (10 ml), hydrogen sulfide being passed into the solution. The precipitate was centrifuged and the supernatant liquid was concentrated to a small volume (*ca.* 5 ml). It was then treated with Dowex 50W-X8 (pyridinium form) and concentrated. The residue was dissolved in 80% acetic acid and heated at 100 °C for 10 min. After removal of acetic acid, the product showed the expected ultraviolet absorption characteristics ($\lambda_{max}^{H_2O}$ 262 mμ at pH=7), being homogeneous on paper electrophoresis (Mobility=0.78 relative to uridine 5′-phosphate). The yield of uridine diphosphate galactose was 63% (630 $O.D._{262}$) based on 2′,3′-*O*-isopropylidene uridine 5′-phosphorothioate.

Flavin Adenine Dinucleotide. To a suspension of disilver salt of riboflavin 5′-phosphate (40 mg, 0.06 mmol) in dry pyridine (0.25 ml) was added slowly a solution of bis-triethylammonium salt of 2′,3′-*O*-isopropylidene adenosine phosphorothioate (tetrahydrate 25.2 mg, 0.05 mmol) in dry pyridine (0.25 ml) with vigorous stirring. After 27 hr, the mixture was concentrated under reduced pressure. The residue was treated with 10% sodium chloride (10 ml), hydrogen sulfide being then passed into the solution. The precipitate was centrifuged and the supernatant liquid was concentrated to a small volume (*ca.* 5 ml). This was treated with Dowex 50W-X8 (pyridinium form) and concentrated to dryness. The residue was dissolved in 80% acetic acid and heated at 100 °C for 10 min. This was concentrated and flavin adenine dinucleotide was obtained by electrophoretic separation. The yield was 51% based on 2′,3′-*O*-isopropylidene adenosine 5′-phosphorothioate.

$\lambda_{max}^{H_2O}$ 264, 375, 450 mμ. $\lambda_{260}/\lambda_{375}=4.00$; $\lambda_{375}/\lambda_{450}=0.95$; $\lambda_{260}/\lambda_{450}=3.80$ (at pH=7).

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