104. *Nucleotides. Part XLIII.* The Use of Cyanamide Derivatives in Pyrophosphate Synthesis. Syntheses of* P^1-*Adenosine-5′* P^2-*Uridine-5′ Pyrophosphate and of Cytidine-5′ Pyrophosphate.*

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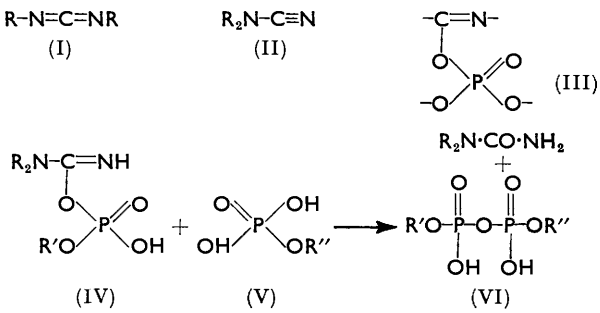
Condensations of phosphates to pyrophosphates can be effected in aqueous solutions by means of dimethylcyanamide or cyanamide. P^1-Adenosine-5′ P^2-uridine-5′ pyrophosphate has been synthesised by these methods and by the established phosphorochloridate and carbodi-imide procedures; the symmetrical P^1P^2-dialkyl pyrophosphates were invariably obtained as by-products. Cytidine-5′ pyrophosphate is obtained in satisfactory yield from cytidine-5′ phosphate by using dimethylcyanamide.

The synthesis of nucleotide coenzymes has been the object of much research in these laboratories during the past decade. Since all coenzymes of this group so far identified are either monoesters of pyro- or poly-phosphoric acids or unsymmetrical diesters of pyrophosphoric acid in which at least one esterifying group is a nucleoside derivative, the discovery and evaluation of new methods for synthesising unsymmetrical pyrophosphates has been a continuing feature of our researches. The preparation of pyrophosphates from phosphates by means of carbodi-imides [1] (I) is now a well-established procedure in the nucleotide field.[2,3] Further, several schemes of synthesis involving imidoyl phosphates

* Part XLII, *J.*, 1957, 3297.

[1] Khorana and Todd, *J.*, 1953, 2257.
[2] *E.g.*, Khorana, *J. Amer. Chem. Soc.*, 1954, **76**, 3517; Kenner, Todd, and Webb, *J.*, 1954, 2843.
[3] Kennedy, *J. Biol. Chem.*, 1956, **222**, 185; Hughes, Kenner, and Todd, *J.*, 1957, 3733.

(III) have been devised,[4] which bear some resemblance to the carbodi-imide method since the latter probably proceeds by way of an intermediate adduct of a phosphoric acid and the carbodi-imide. It seemed likely that dialkylcyanamides (II), which are isomeric with the corresponding dialkylcarbodi-imides (I) might generate with mono- or di-esters of phosphoric acids highly reactive adducts, such as (IV), of a type similar to that postulated for the



carbodi-imide reaction.[1] If so, dialkylcyanamides should be capable of forming pyrophosphates from mono- and di-esters of phosphoric acid. That this is the case was confirmed by the experiments described below.

In preliminary experiments it was shown that P^1P^2-dibenzyl pyrophosphate (VI; R′ = R″ = Ph·CH_2) is formed in *ca.* 50% yield when benzyl dihydrogen phosphate (2 mols.) is refluxed with dimethylcyanamide (1 mol.) in methyl cyanide. Dimethylcyanamide is readily available and has good solvent properties; when diluted with about 10% of water it gives a mixture which dissolves a number of nucleotides. In extending our experiments to the nucleotide field we therefore dispensed with added solvent and simply condensed adenosine-5′ phosphate with uridine-5′ phosphate by heating them in aqueous dimethylcyanamide. This condensation was selected as being analogous to coenzyme synthesis although the product (VI; R′ = adenosine-5′; R″ = uridine-5′), which had not previously been prepared, is not known to occur naturally or expected to have any unusual biological action. In a preparative experiment the reaction mixture contained 30% of water and was buffered with a little pyridine. The solution was heated for 13 hours at 92° and the products (see Table) were isolated by anion-exchange chromatography. The yield of each product was reckoned from the weights of lithium salt actually isolated by rather wasteful precipitations and therefore do not give a true estimate of the total amount of pyrophosphates formed. Paper chromatography showed that the total yield of pyrophosphates was *ca.* 50% under these conditions and that higher yields could be obtained by raising the temperature or reducing the amount of water in the medium. At temperatures below 80° reaction was too slow for preparative purposes. Condensation still occurred when 50% of water was present and even then hydrolysis of the dimethylcyanamide to *NN′*-dimethylurea was negligible when the reaction was carried out at 100°; in marked contrast massive quantities of *NN′*-di*cyclo*hexylurea are produced from di*cyclo*hexylcarbodi-imide when it is used in a similar fashion in large excess in presence of water.[2] Thus the dimethylcyanamide method, which has the merit of being easy to handle, may well be of considerable value for the preparation of fairly stable pyrophosphates.

Our success with dimethylcyanamide encouraged us to examine cyanamide itself as a reagent for pyrophosphate synthesis. Although commonly believed to have structure (II; R = H) it was not excluded that it might react in the carbodi-imide form (I; R = H).[5] The results obtained were, in fact, little different from those obtained with dimethylcyanamide, suggesting that it reacted as (II; R = H). Dimerisation of the cyanamide

[4] Atherton, Morrison, Cremlyn, Kenner, Todd, and Webb, *Chem. and Ind.*, 1955, 1183; Kenner, Todd, and Webb, *J.*, 1956, 1231; Chase, Kenner, Todd, and Webb, *J.*, 1956, 1371.

[5] Cf. Kahovee and Kohlrausch, *Z. phys. Chem.*, 1937, **37**, *B*, 421.

was a serious side reaction, however, and even when the reaction temperature was not allowed to exceed 75° isolation of the products was hampered by the presence of substantial amounts of dimer. For preparative purposes, therefore, dimethylcyanamide is superior.

Condensations of adenosine-5′ phosphate and uridine-5′ phosphate.

	Yield (%) * from experiment					
Product	a	b	c	d	e	f
P^1-Adenosine-5′ P^2-uridine-5′ pyrophosphate ...	17·1	13·8	25·6	14·6	10·6	17·0
P^1P^2-Diadenosine-5′ pyrophosphate	4·4	6·8	11·1	10·1	3·6	9·1
P^1P^2-Diuridine-5′ pyrophosphate	4·9	6·8	13·0	†	4·4	9·8

* Moles of dilithium salt, after anion-exchange chromatography, from 100 moles of each starting material. † None was detected.

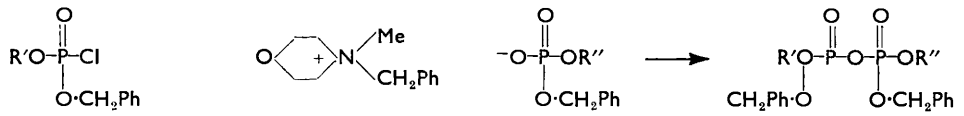
a Dimethylcyanamide method (at 92° with 30% of water). b Cyanamide method (at 75° with 30% of water). c Carbodi-imide method (at 20° with 10% of water). d From 2′ : 3′-*O*-*iso*propylideneuridine-5′ benzyl phosphorochloridate. e From 2′ : 3′-*O*-*iso*propylideneadenosine-5′ benzyl phosphorochloridate. f Like e, but debenzylation in methyl cyanide.

A comparative experiment was also made using di*cyclo*hexylcarbodi-imide with the same nucleotides. The variation in total yield between the three experiments (a, b, c in Table) is not very significant since optimum conditions were not observed, but the proportions of the three products deserve comment. It can readily be deduced that, in these reactions, if intermediates such as (IV) are produced equally readily from both the phosphates used as starting materials and if they react further equally rapidly with either starting material, then the yield of unsymmetrical pyrophosphate should be twice as great as that of each of the symmetrical esters. The experiments with cyanamide (b) and di*cyclo*hexylcarbodi-imide (c) conform to this pattern and too much weight should not be given to the slightly increased proportion of unsymmetrical product obtained by using dimethylcyanamide (a). Apparently the structures of adenosine-5′ phosphate and uridine-5′ phosphate are so similar that the above conditions are effectively fulfilled, and indeed this was not unexpected. On the other hand certain carbodi-imide condensations in which one of the reactants was a dipolar ion and the other a simple nucleotide have given results which deviate greatly from this pattern; in such cases one of the symmetrical pyrophosphates was formed in only negligible amount.[3]

Although the elementary analysis of the P^1-adenosine-5′ P^2-uridine-5′ pyrophosphate obtained from its dilithium salt, and its acidic hydrolysis to uridine-5′ phosphate and adenine, left little room for doubt as to its structure, an alternative synthesis by the phosphorochloridate route was undertaken. The 4-methyl-4-benzylmorpholinium salt (VIII; R″ = 2′ : 3′-*O*-*iso*propylideneadenosine-5′) of 2′ : 3′-*O*-*iso*propylideneadenosine-5′ benzyl phosphate was prepared from the dibenzyl ester and 4-methylmorpholine [6] and then brought into reaction with 2′ : 3′-*O*-*iso*propylideneuridine-5′ benzyl phosphorochloridate [7] (VII; R′ = 2′ : 3′-*O*-*iso*propylideneuridine-5′). The resulting gum, which was presumed to be mainly the fully esterified pyrophosphate (IX; R′ = 2′ : 3′-*O*-*iso*propylideneuridine-5′, R″ = 2′ : 3′-*O*-*iso*propylideneadenosine-5′), was treated with ammonium thiocyanate in ethyl methyl ketone solution and then with dilute acid in order to remove the benzyl [8] and *iso*propylidene residues. Anion-exchange chromatography of the product showed it to be a mixture of the desired material and P^1P^2-diadenosine-5′ pyrophosphate (see Table, expt. d). After this disconcerting outcome, the synthesis was carried out with the reverse orientation of groups (*viz.*, R′ = 2′ : 3′-*O*-*iso*propylideneadenosine-5′, R″ = 2′ : 3′-*O*-*iso*propylideneuridine-5′). In this case the results (Table, expt. e) were similar to those of " random " condensations with cyanamides or di*cyclo*hexylcarbodi-imide. The yield was improved, without altering greatly the proportions

[6] Baddiley, Clark, Michalski, and Todd, *J.*, 1949, 875.
[7] Kenner, Todd, and Weymouth, *J.*, 1952, 3675.
[8] Atherton and Morrison, B.P. 675,779.

of the products, by substituting methyl cyanide for ethyl methyl ketone as the solvent for debenzylation (Table, expt. f). The origin of these mixtures is not far to seek, and we have suspected for some time that syntheses may take this course.[9] Fully esterified pyrophosphates very easily undergo exchange reactions with anions of phosphates,[10] and



(VII) (VIII) (IX)

consequently the product (IX) as it is formed will compete with the phosphorochloridate (VII) for the salt (VIII). This exchange reaction either regenerates the reagents or leads to one symmetrical pyrophosphate and sets free the anion which may eventually be incorporated in the other symmetrical pyrophosphate. The assumption, for which there is at present no supporting evidence, that the anion of 2′ : 3′-*O*-*iso*propylideneadenosine-5′ benzyl phosphate is considerably more nucleophilic than the corresponding anion in the uridine series would account for the difference between the results of the two experiments (d and e). A pyrophosphate is of course less susceptible to exchange reactions when it is not fully esterified and bears a negative charge. Hence syntheses of the phosphorochloridate type should be more efficient when they avoid the fully esterified pyrophosphate (*e.g.*, IX) by starting from the salt of a monoalkyl instead of a dialkyl phosphate. In line with this, thymidine-5′ pyrophosphate and triphosphate have been obtained in high yields [11] whereas the older type of synthesis led to many by-products. It is of interest in this connection that coenzyme syntheses in which phosphorochloridates [12] were used also involved coupling with salts of phosphoric monoesters.

The utility of dimethylcyanamide as a reagent for preparing nucleoside pyrophosphates has also been tested in the synthesis of cytidine-5′ pyrophosphate. This seemed a particularly suitable case since earlier experiments had shown that the phosphorochloridate route gave very low yields of a very impure product. When cytidine-5′ phosphate was heated in aqueous dimethylcyanamide with 2 mols. of benzyl dihydrogen phosphate and the product debenzylated, cytidine-5′ pyrophosphate was obtained in *ca.* 40% yield.

Experimental

Dimethylcyanamide Method (*Experiment a*).—A solution of adenosine-5′ phosphate (69·4 mg.) and uridine-5′ phosphate (64·8 mg.) in water (2 c.c.), containing 5% of pyridine, and dimethylcyanamide (4 c.c.) was heated at 92° during 13 hr. No precipitate appeared. The residue from evaporation under reduced pressure was dissolved in water (30 c.c.), and passed through a column (3 cm. × 1 cm.²) of Dowex-2 resin (chloride form). The column was thoroughly washed with water, in order to remove any pyridine, and then developed with hydrochloric acid as follows: 0·003N-acid (1 l.) removed all the starting materials; 0·005N-acid (0·7 l.) removed P^1P^2-diadenosine-5′ pyrophosphate; 0·01N-acid (0·9 l.) removed P^1-adenosine-5′ P^2-uridine-5′ pyrophosphate; 0·035N-acid (1·5 l.) removed P^1P^2-diuridine-5′ pyrophosphate. The pyrophosphate solutions were separately neutralised with lithium hydroxide, concentrated under reduced pressure to less than 1 c.c., and diluted with acetone–ethanol (85 : 15). The precipitated lithium salts were centrifuged, washed with acetone, and dried; 6·0, 22·7, and 6·3 mg. respectively were obtained.

Cyanamide Method (*Experiment b*).—The same quantities of nucleotides as in experiment a were dissolved in water (1·5 c.c.) containing 5% of pyridine. Cyanamide (3 g.) was added and

[9] Curry, Ph.D. Thesis, Cambridge, 1952, p. 86.
[10] Mason and Todd, *J.*, 1951, 2267; Corby, Kenner, and Todd, *J.*, 1952, 1234.
[11] Mrs. B. Griffin, unpublished work.
[12] Christie, Kenner, and Todd, *J.*, 1954, 46; Michelson and Todd, *J.*, 1956, 3459.

the mixture was kept at 75° during 12 hr. before being worked up in the same way as before. The yields of lithium salts were 9·4, 18·4, and 9·3 mg. respectively.

Carbodi-imide Method (*Experiment c*).—The same quantities of nucleotides as in experiments a and b were dissolved in water (1 c.c.) and pyridine (10 c.c.). Di*cyclo*hexylcarbodi-imide (2 c.c.) was added and the mixture was shaken at room temperature during 40 hr. It was then poured into cold water (200 c.c.). After 2 hr. the di*cyclo*hexylurea was filtered off and the filtrate was concentrated to 20 c.c. Chromatography as before yielded 16·2, 34·1, and 16·7 mg. respectively of the pyrophosphates as their lithium salts.

Phosphorochloridate Method (*Experiment d*).—A mixture of 2′ : 3′-*O-iso*propylideneadenosine-5′ dibenzyl phosphate (1·02 g.; dried over phosphoric oxide) and 4-methylmorpholine (8·5 c.c.) was kept at 100° during 1 hr. before being evaporated under reduced pressure. The morpholinium salt was obtained as a resin by repeated dissolution in dry benzene and evaporation. A phosphorochloridate solution was prepared by adding *N*-chlorosuccinimide (0·24 g.) to a solution of 2′ : 3′-*O-iso*propylideneuridine-5′ benzyl phosphite (0·789 g.) in benzene (6 c.c.) and methyl cyanide (0·5 c.c.) and keeping the mixture for 2 hr. at room temperature. This phosphorochloridate solution was added rapidly to a stirred solution of the morpholinium salt in dry benzene (20 c.c.) and methyl cyanide (6 c.c.). Stirring was continued for 2 hr., during which a precipitate separated. After its removal the filtrate was evaporated at room temperature to a gum which was mixed with ethyl methyl ketone (30 c.c.) and dry potassium thiocyanate (0·8 g.). The mixture was boiled under reflux on a water-bath during 3 hr. The solid products were collected at a centrifuge, washed with acetone, and dissolved in $\frac{1}{7}$N-hydrochloric acid (21 c.c.). The solution was kept at room temperature for 24 hr. and aqueous barium hydroxide was added until the pH was 5. Barium bromide (0·6 g.) and then ethanol (180 c.c.) were added. The precipitate was collected and dried (0·57 g.); on paper chromatography (see below) it appeared to be almost free from mononucleotides. An aqueous solution of this barium salt was put on to a column (3 cm. × 8 cm.^{2}) of Dowex-2 resin (chloride form), which was washed with 0·01N-hydrochloric acid. The first 200 c.c. removed some adenosine-5′ phosphate, and this was followed in the next 1·8 l. by P^1P^2-diadenosine-5′ pyrophosphate, which was isolated as its lithium salt (0·125 g.) as in experiment a. Washing with 0·032N-hydrochloric acid (0·80 l.) removed P^1-adenosine-5′ P^2-uridine-5′ pyrophosphate (0·175 g. of lithium salt).

Phosphorochloridate Method (*Experiment e*).—A solution of 5′-deoxy-5′-iodo-2′ : 3′-*O-iso*propylideneuridine (0·724 g.) in dry benzene (16 c.c.) was boiled under reflux with silver dibenzyl phosphate (0·686 g.) during 30 min. The solution was filtered and evaporated to a yellow resin, which was heated with 4-methylmorpholine (8 c.c.) at 100° during 1 hr. The morpholinium salt was obtained as in experiment d and then dissolved in benzene (20 c.c.) and methyl cyanide (14 c.c.). It was then brought into reaction with a phosphorochloridate solution prepared from 2′ : 3′-*O-iso*propylideneadenosine-5′ benzyl phosphite (0·847 g.) and *N*-chlorosuccinimide (0·245 g.) in benzene (10 c.c.) and methyl cyanide (1 c.c.). Debenzylation was carried on for 2 hr. in boiling ethyl methyl ketone (30 c.c.) with ammonium thiocyanate (0·8 g.), and the subsequent operations followed the plan of experiment d. When the column was washed with 0·008N-hydrochloric acid, uridine-5′ phosphate appeared in the first 300 c.c. and P^1P^2-diadenosine-5′ pyrophosphate in the next 200 c.c., which were added to the 0·01N-acidic washings (1·5 l.); the combined solutions yielded 0·045 g. of lithium salt. P^1-Adenosine-5′ P^2-uridine-5′ pyrophosphate (0·129 g. of lithium salt) and P^1P^2-diuridine-5′ pyrophosphate (0·052 g. of lithium salt) were eluted by 0·03N-acid (1·4 l.) and 0·04N-hydrochloric acid (0·05M in lithium chloride) (1·7 l.) respectively.

Phosphorochloridate Method (*Experiment f*).—This experiment was like the foregoing, but with the following modifications. More silver dibenzyl phosphate (0·724 g.) was used and boiling was for 90 min.; the morpholinium salt was dissolved in benzene (10 c.c.) and methyl cyanide (10 c.c.); methyl cyanide (20 c.c.) was substituted for ethyl methyl ketone for the debenzylation. The weight of barium salt was 0·75 g. The P^1P^2-diadenosine-5′ pyrophosphate (0·115 g. of lithium salt) was eluted by 0·008N-acid (400 c.c.) and 0·01N-acid (800 c.c.), the P^1-adenosine-5′ P^2-uridine-5′ pyrophosphate (0·208 g. of lithium salt) by 0·02N-acid (1 l.), and the P^1P^2-diuridine-5′ pyrophosphate (0·115 g. of lithium salt) by 0·02N-acid (1·5 l.), 0·05M in lithium chloride.

Isolation and Identification of P^1-*Adenosine*-5′ P^2-*Uridine*-5′ *Pyrophosphate*.—The lithium salt (0·035 g.), obtained from a Dowex-2 column, was dissolved in water (5 c.c.) and added to a column (3 cm. × 0·3 cm.^{2}) of Dowex-50 resin (hydroxonium form), which was washed with

water (25 c.c.). Lyophilisation of the filtrate furnished the *pyrophosphate* as a white powder (Found, in material dried over P_2O_5 at room temperature: C, 32·6; H, 4·1; N, 13·8; P, 8·8. $C_{19}H_{25}O_{15}N_7P_2,2{\cdot}5H_2O$ requires C, 32·7; H, 4·3; N, 14·0; P, 8·9%).

The most convenient identification of this pyrophosphate (and its salts) was by paper electrophoresis at pH 4·5 (in a buffer containing 6 g. of sodium acetate and 4·5 c.c. of acetic acid per l.) at 220 v (4·7 v/cm.) for 16 hr. The distance of migration towards the anode was 8·5 cm., while the distances for P^1P^2-diadenosine-5′ and P^1P^2-diuridine-5′ pyrophosphates were 6·7 cm. and 10·9 cm. respectively.

A sample (*ca.* 1 mg.) of the lithium salt was heated with 0·1N-hydrochloric acid at 100° for 1 hr. Ascending paper chromatography in butan-1-ol–acetic acid–water (5 : 2 : 3 v/v) then separated three components, which were identified by comparison with authentic samples as unchanged pyrophosphate (R_F 0·06), uridine-5′ phosphate (R_F 0·18), and adenine (R_F 0·61). (This system of chromatography was also useful for following the progress of syntheses although it did not separate the unsymmetrical pyrophosphate from the two symmetrical ones.) These identifications were confirmed by paper electrophoresis at pH 4·5.

Cytidine-5′ Pyrophosphate.—A solution of cytidine-5′ phosphate (65 mg., 1 mol.) and benzyl dihydrogen phosphate (75 mg., 2 mols.) in aqueous pyridine (1·5 c.c. of a solution containing 5 parts of pyridine and 95 parts of water by volume) and dimethylcyanamide (4 c.c.) was heated at 110—115° for 16 hr. Solvents were removed under reduced pressure, the residue dissolved in 50% aqueous ethanol (10 c.c.), glacial acetic (0·1 c.c.) added, and the solution hydrogenated at atmospheric pressure and room temperature for 3 hr. in presence of palladium oxide (25 mg.) and 10% palladised charcoal (25 mg.). The filtered solution examined by paper chromatography and paper electrophoresis (acetate buffer, pH 4·8) indicated the presence of 3 nucleotide derivatives—cytidine-5′ phosphate, cytidine-5′ pyrophosphate, and another pyrophosphate, presumably P^1P^2-dicytidine-5′ pyrophosphate—as well as inorganic phosphate and pyrophosphate. The solution was then put on a Dowex·2 column (chloride form, 3 cm. × 1 cm.^{2}) which was well washed with water and eluted with increasing concentrations of hydrochloric acid. 0·0015N-Hydrochloric acid (1·75 l.) eluted cytidine-5′ phosphate and the presumed P^1P^2-dicytidine-5′ pyrophosphate together, with a peak after 900 c.c. Cytidine-5′ pyrophosphate was eluted with 0·003N-hydrochloric acid (1·8 l.) with the peak after 1 l.

The latter eluate was brought to pH 7 with lithium hydroxide and concentrated to *ca.* 3 c.c. A mixture of acetone (85 c.c.) and ethanol (15 c.c.) was added, and the white precipitate of lithium salt centrifuged off, washed with acetone (30 c.c.), and dried. The product was free from inorganic phosphate and ran as a single phosphorus-containing ultraviolet-absorbing spot on paper chromatography and on paper electrophoresis (Whatman No. 54 paper, acetate buffer pH 4·8). The yield was 31·2 mg. (37%). A solution of the lithium salt was absorbed on a small Dowex-50 column (hydrogen form; 1 cm. × 0·5 cm.^{2}). The aqueous eluate from the column was freeze-dried, to give *cytidine-5′ pyrophosphate* as a colourless glass (Found: C, 24·9; H, 4·2; N, 9·2. $C_9H_{15}O_{11}N_3P_2,2H_2O$ requires C, 24·6; H, 4·3; N, 9·6%).

An experiment on a somewhat larger scale gave a 42% yield of lithium salt.

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