587. *Nucleotides. Part XXII.* Syntheses of* P^1P^2*-Diadenosine-5′ and* P^1P^2*-Diuridine-5′ Pyrophosphates.*

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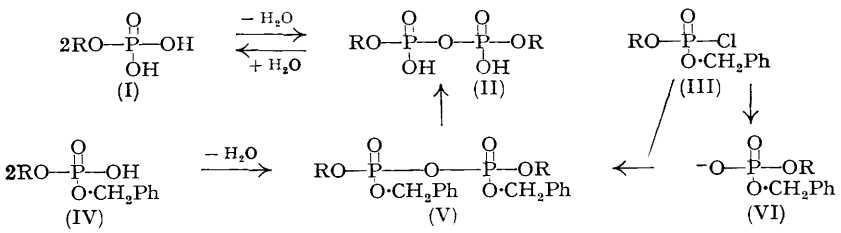
Methods suitable for the preparation of P^1P^2-diesters (II) of pyrophosphoric acid, developed in earlier model experiments, have been applied to the synthesis of P^1P^2-diadenosine-5′ pyrophosphate and P^1P^2-diuridine-5′ pyrophosphate. These syntheses demonstrate the applicability of the methods in the nucleotide field and indicate the feasibility of synthesis of dinucleotide coenzymes. Four different types of pyrophosphate synthesis have been used and their relative merits are discussed from the standpoint of ultimate application to the synthesis of such natural coenzymes as flavin–adenine dinucleotide and uridine–diphosphate–glucose. The synthetic pyrophosphates are hydrolysed to the parent nucleosides by the crude venoms of Russell's viper and diamond-back rattlesnake.

Naturally occurring nucleotide derivatives, which exercise coenzyme function, fall into two structural groups. The first group, which includes such substances as adenosine diphosphate and adenosine triphosphate, consists of monoesters of pyrophosphoric and triphosphoric acid. The synthesis of such nucleotides has been described in earlier papers of this series (Part I, *J.*, 1947, 648; Part II, *J.*, 1949, 582; Part IV, *J.*, 1949, 2487; Part XV, *J.*, 1952, 3665; Part XVII, *J.*, 1952, 3675). The second group, whose members are unsymmetrical P^1P^2-diesters of pyrophosphoric acid, is of considerable importance since it includes such coenzymes as flavin–adenine dinucleotide (FAD), uridine–diphosphate–glucose (UDPG), coenzymes I and II (DPN and TPN), and coenzyme A. The synthesis of such substances presents many difficulties, but during recent years we have developed, in model experiments, methods which should be applicable to this end, and which have been described partly in earlier papers of this series and partly in our cognate series entitled "Studies on Phosphorylation" (Parts I—XI, *J.*, 1945—1953). The present paper describes experiments in which we have tested their practical utility in the nucleotide field by the synthesis of P^1P^2-diadenosine-5′ pyrophosphate (II; R = adenosine-5′ residue) and P^1P^2-diuridine-5′ pyrophosphate (II; R = uridine-5′ residue).

* Part XXI, *J.*, 1953, 2040.

This task was relatively easy, owing to the favourable solubilities of the intermediates and the symmetry and stability of the products, but its successful completion demonstrates the feasibility of synthesising the natural dinucleotide coenzymes. In all, four distinct methods have been employed, the pyrophosphate being formed by the use of carbodiimides, by exchange reactions using trifluoroacetic anhydride or tetraphenyl pyrophosphate, and by employing nucleoside phosphorochloridates (chlorophosphonates). The use of each method will be described in turn before discussing their relative potentialities for coenzyme synthesis.



The most direct method of preparing diesters of pyrophosphoric acid is by condensation of two molecules of a monoester of phosphoric acid (I) by means of di*cyclo*hexyl carbodiimide (Khorana and Todd, *J.*, 1953, 2257), which has the advantage over other dehydrating agents of attacking acidic groups preferentially. It is not, however, necessary to use free phosphoric acids, for tetrabenzyl pyrophosphate is formed by reaction between carbodiimides and pyridinium, although not triethylammonium, dibenzyl phosphate. Some nucleotide coenzymes, *e.g.*, uridine–diphosphate–glucose, are very labile to acids, and therefore our model experiments were carried out with pyridinium uridine-5′ hydrogen phosphate. When a slight excess of di*cyclo*hexyl carbodi-imide was added to a solution of the pyridinium salt in dimethylformamide, di*cyclo*hexylurea rapidly crystallised; paper chromatography then showed that 60% of the uridine-5′ phosphate had been converted into a new substance with the same R_F as uridine-5′ pyrophosphate and as uridine–diphosphate–glucose. By using a large excess of the carbodi-imide, almost all the uridine-5′ phosphate could be brought into reaction, but some of it was converted into yet another material, probably of higher molecular weight. Formation of this by-product was avoided when pyridine was used as solvent, and the small amount of unchanged starting material was removed by repeated precipitation of the pyridinium salt from aqueous solution with acetone. The crystalline product was shown by analysis and electrometric titration to be monopyridinium P^1P^2-diuridine-5′ pyrophosphate.

The synthesis of diadenosine-5′ pyrophosphate was also achieved in a direct fashion, but as an unexpected result of another investigation. Brown, Magrath, and Todd (*J.*, 1952, 2708) showed that uridylic and cytidylic acids can be partially esterified with simple alcohols by means of trifluoroacetic anhydride. This result offered a possible route to dinucleoside esters of phosphoric acid, and this was tested in an attempt to synthesise the already known adenosine-5′ uridine-5′ phosphate (Elmore and Todd, *J.*, 1952, 3681). A solution of 2′ : 3′-*O*-*iso*propylideneuridine in dioxan was added to the reaction mixture of adenosine-5′ phosphate with trifluoroacetic anhydride. Paper chromatography of the product, after ethanolysis of trifluoroacetyl groups, revealed the presence of only one new substance and this did not contain a uracil residue. Moreover, repeated applications of trifluoroacetic anhydride to adenylic acid alone produced the same new substance. The possibility that it was adenosine-5′ ethyl phosphate, formed during the ethanolysis, was disproved by substitution of benzyl for ethyl alcohol; the R_F of the product was unchanged and quite distinct from that of adenosine-5′ benzyl phosphate (Baddiley and Todd, *J.*, 1947, 648). Anion-exchange chromatography with formic acid elution gave an efficient separation of unchanged adenylic acid from the new material. The analysis of the free acid and the results of electrometric and periodate titrations left no doubt that it was P^1P^2-diadenosine-5′ pyrophosphate (II; R = adenosine-5′ residue).

The above two direct syntheses represent a departure from our usual practice, which

has been to build up completely esterified nucleotides containing protective benzyl groups, which are removed in the final stages. The alternative synthesis of P^1P^2-diuridine-5′ pyrophosphate now to be described is derived from our earlier studies, in particular the preparation of benzyl 2′ : 3′-*O*-*iso*propylideneuridine-5′ phosphorochloridate (chlorophosphonate) (III; R = 2′ : 3′-*O*-*iso*propylideneuridine-5′ residue) (Corby, Kenner, and Todd, *J.*, 1952, 3669; Kenner, Todd, and Weymouth, *J.*, 1952, 3675). Toy (*J. Amer. Chem. Soc.*, 1948, **70**, 3882) has shown that phosphorochloridates can be converted into symmetrical tetraesters of pyrophosphoric acid in two ways, both of which have been used successfully in the present instance. The simpler method is to add water, in small excess, and then one equivalent of base to a solution of the phosphorochloridate in methyl cyanide. The pyrophosphate (V) is then formed by reaction between the anion (VI), derived from hydrolysis of the phosphorochloridate, and a further quantity of the chloride. The alternative and, in our experience, slightly superior procedure is to carry out the two steps separately. The further stage of removing the two benzyl groups could be accomplished by heating the pyrophosphate (V) with lithium chloride in ethoxyethanol (Anand, Clark, Hall, and Todd, *J.*, 1952, 3665), but the product contained uridine-5′ phosphate (I; R = uridine-5′ residue). This could only have arisen from the debenzylated pyrophosphate (II), since diesters (IV) of phosphoric acid are not attacked by lithium chloride (Clark and Todd, *J.*, 1950, 2031). In other words, hot ethoxyethanol causes appreciable cleavage of the pyrophosphate link in addition to partial removal of *iso*propylidene residues. We therefore turned to use of the thiocyanate anion for debenzylation in non-hydroxylic solvents (Morrison and Atherton, B.P. 675,779). Two hours' reaction with potassium thiocyanate in boiling methyl cyanide was sufficient to cause complete debenzylation without untoward side-reactions. The two *iso*propylidene groups were then removed with dilute hydrochloric acid. Final purification was achieved by anion-exchange chromatography with hydrochloric acid elution. The 0·1N-acid fractions contained the pyrophosphate (II; R = uridine-5′ residue), which was recovered as its dilithium salt by precipitation with acetone after neutralisation of the concentrated liquors with lithium hydroxide.

Fully esterified pyrophosphates (V) can also be prepared by exchange reactions between diesters of phosphoric acid (IV) and a suitable reactive pyrophosphate or other anhydride (Corby, Kenner, and Todd, *J.*, 1952, 1234). Tetraphenyl pyrophosphate was allowed to react with monobenzyl triethylammonium 2′ : 3′-*O*-*iso*propylideneuridine-5′ phosphate in methyl cyanide solution. Successive treatment of the resultant mixture with potassium thiocyanate and dilute hydrochloric acid afforded P^1P^2-diuridine-5′ pyrophosphate, but in only moderate yield. It was even lower in a similar experiment starting with the corresponding potassium salt and trifluoroacetic anhydride in dimethylformamide. These exchange reactions with nucleotides were thus much less successful than those with simple model compounds, possibly owing to the greater difficulty encountered in excluding moisture.

Syntheses of the natural pyrophosphate coenzymes present three major difficulties in addition to those encountered in the present work. Nevertheless it is possible to draw some conclusions about the relative utility of the four types of synthetic method described above. The coenzymes are not symmetrical esters of type (II), but have two different attached groups. Only the method using reaction between a phosphorochloridate (III) and an anion (VI) leads to such structures in other than a random way. It gave a very satisfactory yield (48%) of pure P^1P^2-diuridine-5′ pyrophosphate, but suffers from being limited to inert solvents such as benzene or methyl cyanide. Intermediates in the synthesis of coenzymes containing quaternary nicotinamide or riboflavin residues are not likely to be freely soluble even in methyl cyanide. To some extent this difficulty can be overcome by protection of polar groups with hydrophobic residues, but subsequent removal of these protecting groups is hindered by instability of the coenzymes, the third obstacle. For instance, flavin–adenine dinucleotide is very labile to alkali (Forrest and Todd, *J.*, 1950, 3295) and uridine–diphosphate–glucose to acid (Caputto, Leloir, Cardini, and Paladini, *J. Biol. Chem.*, 1950, **184**, 333). The carbodi-imide method is superior in these last two respects, since no protecting groups are necessary and the solvent may be pyridine

or dimethylformamide. The conversion of uridine-5′ phosphate into the pyrophosphate was high (60%), but the yield of an unsymmetrical ester would presumably be much lower. The other two types of synthesis described offer less satisfactory solutions of these problems and the yields obtained were much lower.

The following observations on the synthetic P^1P^2-diadenosine-5′ and P^1P^2-diuridine-5′ pyrophosphates are worthy of note. To acid and, particularly, alkali, both pyrophosphates were rather more stable than we had expected. For instance, the uridine compound suffered only 4% decomposition in 0·1N-sodium hydroxide during 80 min. at 100°. Both substances (II; R = adenosine-5′ or uridine-5′ residues) were hydrolysed to the nucleosides by the crude venoms of Russell's viper (*Vipera russelli*) and diamond-back rattlesnake (*Crotalus adamanteus*). This is explicable if the venoms contain a non-specific pyrophosphatase capable of degrading even these unnatural substances to the simple mononucleotides, which would then be attacked by the well-known 5′-nucleotidase present in the venoms. In addition to adenosine-5′ and uridine-5′ phosphates, the corresponding nucleoside-5′ monoesters of pyrophosphoric acid were also hydrolysed to the nucleosides. The cleavage of cozymase by snake venoms has, of course, already been reported (Chain, *Biochem. J.*, 1939, **33**, 407; Zeller and Epperson, cited in Sumner and Myrbäck, "The Enzymes," Academic Press, New York, 1951, Vol. I, Part 2, p. 1008).

Experimental

Barium Uridine-5′ Hydrogen Phosphate [with Dr. J. Davoll].—A solution of 5′-deoxy-5′-iodo-2′ : 3′-*O-iso*propylideneuridine (4·0 g.; Levene and Tipson, *J. Biol. Chem.*, 1934, **106**, 113) in dry benzene (50 c.c.) was refluxed for 90 min. with silver dibenzyl phosphate (4·0 g.). The solution was filtered and washed with water, sodium thiosulphate solution, sodium hydrogen carbonate solution, and again water. Evaporation of the dried (Na_2SO_4) benzene left a colourless resin (5·6 g.), which was dissolved in 50% aqueous ethanol (200 c.c.) and shaken for 15 hr. with hydrogen (1 atm.) and a mixture of palladous oxide (0·04 g.) and 10% palladised charcoal (0·04 g.). *Barium uridine-5′ hydrogen phosphate* (3·69 g., 81%) was precipitated by addition of ethanol (120 c.c.) to the filtered solution after it had been concentrated to 25 c.c. and neutralised (pH 5—6) with aqueous barium hydroxide [Found, in air-dried material: C, 24·2; H, 3·7; N, 6·2. $(C_9H_{12}O_9N_2P)_2Ba,6H_2O$ requires C, 24·1; H, 4·0; N, 6·2%].

Pyridinium P^1P^2-*Diuridine-5′ Hydrogen Pyrophosphate* (*Carbodi-imide Method*).—An aqueous M-solution of pyridinium sulphate was added drop by drop to a solution of barium uridine-5′ hydrogen phosphate (1·0 g.) in water (20 c.c.) until no further precipitate was formed. The filtered solution was evaporated, dissolved in dimethylformamide, and re-evaporated, giving the pyridinium salt as a stable glass. This was dissolved in dry pyridine (10 c.c.) and treated with a solution of di*cyclo*hexyl carbodi-imide (0·30 g.; Schmidt, Hitzler, and Lahde, *Ber.*, 1938, **71**, 1933) in pyridine (3 c.c.). After a few min. an oil began to separate and after 15 min. it contained crystals of di*cyclo*hexylurea. Water (5 c.c.) was then added and the mixture kept for 1 hr. The filtered solution was concentrated to 5 c.c. and poured into acetone (45 c.c.). The precipitated oil (0·60 g.) was collected by centrifugation and twice reprecipitated from aqueous acetone; it then started to crystallise. By slow crystallisation from aqueous acetone the *monopyridinium* salt (0·053 g.) was obtained as rosettes of flat needles, m. p. 216—218° (corr.; decomp.) (Found, in material dried at 50°: C, 38·3; H, 4·1; N, 9·9; P, 8·7. $C_{23}H_{29}O_{17}N_5P_2,\frac{1}{2}H_2O$ requires C, 38·4; H, 4·2; N, 9·75; P, 8·6%). Neither free nor labile orthophosphate was present and the substance consumed 2·07 mols. of sodium metaperiodate during 18 hr. at 20°. Ultra-violet absorption in 0·01N-HCl: max. 257—258 mμ (ε 23,700), min. 230—231 mμ (ε 7500). A solution of 4·35 mg. in 1 c.c. of water had pH 2·56 and, on back-titration from pH 10, showed a break in the curve at a mean pH of 5·6 requiring 0·84 equiv. of acid, corresponding to re-formation of the pyridinium ion (pK 5·3) (secondary phosphoryl dissociations have pK *ca.* 6·8).

A greater yield of this crystalline monopyridinium salt could probably have been obtained, for about 90% of the nucleotidic material in the original oil was the desired pyrophosphate, the remainder being uridine-5′ phosphate. A four-fold increase in the proportion of carbodi-imide (to 5 equivs.) caused conversion of 85% of the phosphate into pyrophosphate.

P^1P^2-*Diadenosine-5′ Pyrophosphate* (*Trifluoroacetic Anhydride Method*).—Adenosine-5′ phosphate (0·768 g.) was kept overnight with trifluoroacetic anhydride (5 c.c.). Evaporation under reduced pressure then left a frothy glass, which was treated in the same way with two

further portions of anhydride (4 and 3 c.c.). The final glass was kept for a short time with ethanol (5 c.c.) before evaporation and dissolution in dilute aqueous ammonia. The solution (pH 8) was put on a column (11 × 2·5 cm.) of Dowex-2 resin (250—500 mesh; formate cycle). Unchanged adenosine-5′ phosphate was eluted by 0·1N-formic acid, and the product by 0·5N-formic acid from which it was recovered by evaporation under reduced pressure and freeze-drying. When the residue (0·143 g., 17%) was dissolved in water (1 c.c.), P^1P^2-*diadenosine-5′ pyrophosphate* separated rapidly as a dense, amorphous, rather insoluble solid (Found: C, 32·2; H, 4·8; N, 18·7; P, 8·4. $C_{20}H_{26}O_{13}N_{10}P_2,4H_2O$ requires C, 32·1; H, 4·6; N, 18·7; P, 8·3%). It could not be crystallised but gave a single spot on paper chromatography in *n*-butanol–acetic acid–water (5 : 2 : 3; R_F = 0·09) and in *iso*propanol–1% aqueous ammonium sulphate (75 : 25; R_F = 0·06). Ultra-violet absorption: (1) in 0·01N-HCl: max. 257—259 mμ (ε 27,700), min. 230—231 mμ (ε 7300); (2) in 0·01N-NaOH: max. 259—260 mμ (ε 24,600), min. 229 mμ (ε 5100). In 16 hr. at 20° the substance consumed 2·25 mols. of 0·025M-sodium metaperiodate (calc. on mol. wt. 748). On potentiometric titration of a solution of 4·3 mg. in water (1 c.c.) with 0·05N-sulphuric acid from pH 8 no group was detected in the range to pH 5 characteristic of secondary phosphoryl. The pyrophosphate was stable to 0·5N-sodium hydroxide at room temperature overnight, but was slowly hydrolysed by the same reagent at 100°, adenine being the only product detected in ultra-violet light on a paper chromatogram. On the other hand hydrolysis to adenosine-5′ phosphate and adenine was practically complete after 1 hr. in 0·1N-hydrochloric acid at 100°.

Potassium Benzyl 2′ : 3′-O-isoPropylideneuridine-5′ Phosphate.—Benzene (20 c.c.) was refluxed for 2½ hr. together with 5′-deoxy-5′-iodo-2′ : 3′-*O-iso*propylideneuridine (1·17 g.), silver dibenzyl phosphate (1·17 g.), and anhydrous calcium sulphate (1 g.). The filtered solution was evaporated to a colourless resin, which was treated with potassium thiocyanate (0·43 g.) in boiling methyl cyanide (10 c.c.) for 2 hr. The colourless *potassium benzyl 2′ : 3′-O-iso-propylideneuridine-5′ phosphate* (0·96 g.), m. p. 288° (corr.; decomp.), which began to separate after 1 hr., was filtered from the cold solution (Found: in material dried at 80°: C, 46·2; H, 4·3; N, 5·9. $C_{19}H_{22}O_{19}N_2PK$ requires C, 46·4; H, 4·5; N, 5·7%). The triethylammonium salt was prepared from this potassium salt by acidifying its aqueous solution with dilute hydrochloric acid and evaporating the dried (Na_2SO_4) chloroform extract of the free acid with excess of triethylamine.

Ammonium Benzyl Phosphite.—This substance, used in the preparation of benzyl 2′ : 3′-*O-iso*propylideneuridine-5′ phosphite, is more conveniently prepared by the following method (personal communication from Dr. F. R. Atherton) than by that previously published. A solution of dibenzyl phosphite (25 g.) and dried ammonium thiocyanate (12·5 g.) in ethyl methyl ketone (85 c.c.) is refluxed for 2 hr. On addition of ethyl acetate (25 c.c.) and cooling, the ammonium monobenzyl phosphite (12 g.) crystallises in colourless needles, m. p. 153—155° (corr.), and is washed with ethyl acetate.

Dilithium P^1P^2-*Diuridine-5′ Pyrophosphate* (*Phosphorochloridate Method*).—(*a*) A solution of benzyl 2′ : 3′-*O-iso*propylideneuridine-5′ phosphite (0·869 g.) and *N*-chlorosuccinimide (0·270 g.) in benzene (6 c.c.) and methyl cyanide (0·5 c.c.) was kept for 1½ hr. at 20° before addition to a solution of triethylammonium benzyl 2′ : 3′-*O-iso*propylideneuridine-5′ phosphate (1·10 g.) in benzene (20 c.c.) and methyl cyanide (4 c.c.). Triethylammonium chloride began to separate after a few min. and was filtered off (0·110 g.) after 1 hr. The filtrate was evaporated to dryness and then heated for 2 hr. with potassium thiocyanate (0·80 g.) in boiling methyl cyanide (25 c.c.). The precipitated bright yellow solid was dissolved as completely as possible in N/7-hydrochloric acid (15 c.c.) and kept overnight at 20°. The solution was filtered and brought to pH 3·5 with barium hydroxide solution. The barium salt of the product (1·16 g.) was then precipitated as a white powder by the addition of barium bromide (0·60 g.) and ethanol (2 vols.). Paper chromatography in *iso*propanol–1% ammonium sulphate solution (67 : 33), followed by elution of the spots, showed that the ultra-violet absorption at 260 mμ was distributed amongst the components as follows: P^1P^2-diuridine-5′ pyrophosphate (R_F 0·19) 83%, uridine-5′ phosphate (R_F 0·35) 11%, and unidentified material (R_F 0·10) 6%. The solution of the crude barium salt (1·05 g.) in water (20 c.c.) was brought to pH 8 with aqueous ammonia and then passed slowly through a column (8 × 2·5 cm.) of Dowex-2 (previously cycled three times with 0·5N-sodium hydroxide and N-hydrochloric acid). After being washed with demineralised water (300 c.c.) the column was developed by successive elution with 0·005N-hydrochloric acid (240 c.c.), 0·01N-acid (400 c.c.), 0·025N-acid (400 c.c.), 0·05N-acid (400 c.c.), 0·1N-acid (600 c.c.), and 0·3N-acid (300 c.c.). In all 200 fractions (11·7 c.c. each) were collected, giving sharp separation of the uridine-5′ phosphate, the pyrophosphate, and the unidentified material, which were eluted by

0·025N-, 0·1N-, and 0·3N-hydrochloric acid respectively. Fractions 110—180, containing the pyrophosphate, were combined, neutralised (pH 7) with lithium hydroxide, and concentrated to 40 c.c. Addition of acetone (200 c.c.) then precipitated an oil, which solidified when stirred with ethanol. The solid was redissolved in water (8 c.c.), and the *dilithium* P^{1}P^2-*diuridine*-5′ *pyrophosphate* (0·57 g.) reprecipitated as an oil by acetone (200 c.c.) and solidified by stirring it with ethanol (Found, in material dried at 100°: C, 33·4; H, 3·8; N, 8·5; P, 9·7. $C_{18}H_{22}O_{17}N_4P_2Li_2,\frac{1}{2}H_2O$ requires C, 33·2; H, 3·5; N, 8·6; P, 9·5%). The hygroscopic solid gave no turbidity with silver nitrate solution, and contained neither free nor labile orthophosphate. It consumed 1·92 mols. of sodium metaperiodate during 18 hr. at 20°. Ultraviolet absorption in H_2O: max. 261 mμ (ε 17,300), min. 231 mμ (ε 4300) (calc. on mol. wt. 651). Electrometric titration with 0·05N-sulphuric acid of a solution of 3·3 mg. in water (1 c.c.), made alkaline with 0·1N-sodium hydroxide, showed no titratable group between pH 8·5 and pH 4. Lability of the pyrophosphate to alkaline and acid hydrolysis was studied by treating 5-mg. portions with 0·2 c.c. of reagent, and examining the solutions by paper chromatography in *iso*propanol–1% aqueous ammonium sulphate solution (67 : 33) after suitable reaction periods. The extent of decomposition was then determined by elution of the spots produced, and measurement of the relative optical densities at 260 mμ. In sodium hydroxide no decomposition was detected after 7 hr. at 100° in 0·001N-, 2% after 7 hr. at 100° in 0·01N-, 4·2% after 1⅓ hr. at 100° and 1·4% after 120 hr. at 23° in 0·1N-alkali. In hydrochloric acid decomposition occurred to the extent of 1% after 3½ hr. at 100° in 0·001N-, 2·8% after 2 hr. at 100° in 0·01N-, 47·4% after 1⅓ hr. at 100° and 4·7% after 180 hr. at 23° in 0·1N-acid.

(*b*) A mixture of benzyl 2′ : 3′-*O*-*iso*propylideneuridine-5′ phosphite (0·445 g.) and *N*-chlorosuccinimide (0·146 g.) in benzene (5 c.c.) was kept at room temperature for 2 hr. A solution of water (20 mg.) in methyl cyanide (0·5 c.c.) was then added to the mixture, which was stirred vigorously for 30 min. after slow addition of triethylamine (0·15 c.c.). The solvent was evaporated and replaced by methyl cyanide (10 c.c.). The solution was now refluxed for 2 hr. with potassium thiocyanate (0·165 g.); potassium chloride separated and caused severe bumping. The cooled mixture was diluted to 50 c.c. with acetone, and the mixed potassium salts (0·423 g.) were centrifuged off. The solution of these salts in N/7-hydrochloric acid (12 c.c.) was kept overnight at 20° and then neutralised (pH 8) with aqueous barium hydroxide. Addition of barium bromide (0·1 g.) and ethanol (4 vols.) then precipitated barium *P*^{1}*P*^{2}-diuridine-5′ pyrophosphate (0·273 g.), which was shown by paper chromatography to be about 85% pure; reprecipitation of the barium salt from water (2 c.c.) at pH 4 by addition of ethanol gave a 75% recovery of material substantially free from uridine-5′ phosphate, but still containing an unidentified impurity. Several similar runs were made under varied conditions, and the results showed that the proportion of water was not critical between the theoretical and a 200% excess (100% excess was used above), that the dibenzyl di-2′ : 3′-*O*-*iso*propylideneuridine-5′ pyrophosphate could be washed with sodium hydrogen carbonate solution and water with a loss of some 30%, and that debenzylation by lithium chloride in ethoxyethanol during 2 hr. at 100° was unsatisfactory owing to considerable cleavage of the pyrophosphate link.

Dilithium P^{1}P^2-*Diuridine*-5′ *Pyrophosphate* (*Trifluoroacetic Anhydride Method*).—Trifluoroacetic anhydride (0·105 c.c.) was added to a suspension of potassium monobenzyl 2′ : 3′-*O*-*iso*propylideneuridine-5′ phosphate in dimethylformamide (8 c.c.). After 15 min. any unchanged anhydride was removed under reduced pressure and potassium thiocyanate (0·39 g.) was added. The solution was kept at 70—80° for 2 hr. before being poured into dry ether (200 c.c.). The gelatinous precipitate was collected by centrifugation and dissolved in N/7-hydrochloric acid (7 c.c.). The next day the mixed barium salts of uridine-5′ phosphate and *P*^{1}*P*^{2}-diuridine-5′ pyrophosphate were precipitated by successive additions of barium hydroxide solution (to pH 3·5), barium bromide (0·30 g.), and ethanol (3 vols.). The solution of the salts in water (4 c.c.) was brought to pH 8 by aqueous ammonia before being put on a column (2 × 1·5 cm.) of Dowex-2 resin which was eluted successively with 200-c.c. portions of water, 0·025N-, 0·05N-, and 0·1N-hydrochloric acid. The second major component was in 160 c.c. of the last eluate, the optical density at 260 mμ (5·94) corresponding to the presence of 25 mg. of *P*^{1}*P*^{2}-diuridine-5′ pyrophosphate. The lithium salt (15 mg.), obtained by neutralisation to pH 7 with lithium hydroxide, evaporation to small bulk, and precipitation by ethanol, contained less than 1% of uridine-5′ phosphate, as detected by paper chromatography.

Barium P^{1}P^2-*Diuridine*-5′ *Pyrophosphate* (*Pyrophosphate Exchange Method*).—A solution of triethylammonium benzyl 2′ : 3′-*O*-*iso*propylideneuridine-5′ phosphate (1·03 g.) in dry benzene (5 c.c.) was evaporated to a gum, which was redissolved in dry methyl cyanide (10 c.c.). Tetraphenyl pyrophosphate (0·508 g.) was added and the solution was kept at 20° for 1 hr. It was

then boiled for 3 hr. with potassium thiocyanate (0·348 g.). The precipitated solid was collected and dissolved in N/7-hydrochloric acid (7 c.c.). The solution was kept at 20° for 15 hr. before addition of barium hydroxide solution (to pH 4) and barium bromide (0·6 g.). After filtration 2 vols. of ethanol were added to the filtrate, and the flocculent precipitate (0·444 g.) collected. It was redissolved in water (2 c.c.) and reprecipitated by an equal vol. of methanol in presence of barium bromide at pH 3·5. Two further repetitions of the process afforded the pure *barium* salt (0·120 g.) (Found, in material dried at 50°: C, 27·5; H, 3·6; N, 6·8; P, 7·8. $C_{18}H_{22}O_{17}N_4P_2Ba,H_2O$ requires C, 27·6; H, 3·1; N, 7·1; P, 7·9%). It ran as a single spot on a paper chromatogram in *n*-butanol : acetic acid : water (50 : 20 : 30) at the same speed as the authentic dilithium salt (R_F 0·13). Measurements of optical densities at 260 mμ of eluates of appropriate areas of the paper showed the amount of uridine-5′ phosphate contaminant to be less than 0·5%.

Action of Snake Venoms on Synthetic Phosphates and Pyrophosphates.—Aqueous solutions of the venom of Russell's viper (*Vipera russelli*) and diamond-back rattlesnake (*Crotalus adamanteus*) were incubated with the following substances in 0·25M-glycine–ammonia buffer (pH 9) at 37° overnight: adenosine-5′ phosphate, adenosine-5′ pyrophosphate, P^1P^2-di-adenosine-5′ pyrophosphate, uridine-5′ phosphate, uridine-5′ pyrophosphate, P^1P^2-diuridine-5′ pyrophosphate. In every case complete degradation to the parent nucleoside (detected by paper chromatography) occurred.

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