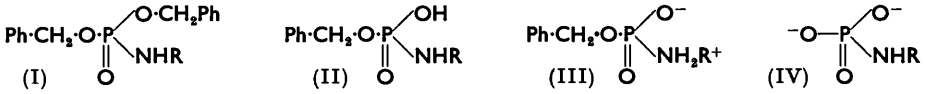
287. *Studies on Phosphorylation. Part XV.* The Use of Phosphoramidic Esters in Acylation. A New Preparation of Adenosine-5′ Pyrophosphate and Adenosine-5′ Triphosphate.*

By V. M. Clark, G. W. Kirby, and Sir Alexander Todd.

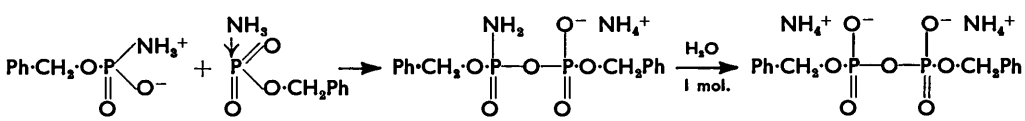
The monobenzyl esters of phosphoramidic and certain *N*-substituted phosphoramidic acids behave as selective acylating agents for phosphoric acid anions; alcoholic hydroxyl groups present are unaffected. By their use nucleoside polyphosphates can be readily synthesised from unprotected nucleotides. In particular adenosine-5′ phosphate (AMP) can be converted into adenosine-5′ pyrophosphate (ADP) and thence into adenosine-5′ triphosphate (ATP) in high yield.

While investigating the selective anionic debenzylation of the dibenzyl esters of phosphorus-containing acids it was shown [1] that the esters of certain phosphoramidic acids, *e.g.*, (I; R = H), gave rise to a series of mono-de-esterified products (II) which behaved, apparently, as zwitterions (III). It was suggested that such systems might behave as acylating agents, being closely related to the base–metaphosphate complexes investigated in preliminary fashion by Langheld,[2] who showed that ethyl metaphosphate (presumably in polymeric form) gave complexes with amines and amino-acids although



these complexes were not characterised. That the system (II/III) might have zwitterionic character is supported by the *X*-ray crystallographic work of Hobbs, Corbridge, and Raistrick [3] on the monosodium salt of phosphoramidic acid itself in which it was shown that the $^{+}NH_3 \cdot PO_3^{-}$ ion has a 3-fold symmetry, the arrangement of bonds around both the N and P atoms being tetrahedral.

Our initial experiments were directed towards the phosphorylation of alcohols by the monobenzyl ester of phosphoramidic acid (II/III; R = H); when its solution in benzyl alcohol was heated no detectable acylation of the solvent occurred but the crystalline diammonium salt of P^1P^2-dibenzyl pyrophosphate separated. In dioxan at 100°, separation of this salt began within a few minutes and in two hours it was formed in high yield. Re-examination of the original specimen of monobenzyl phosphoramidate, isolated as the hemihydrate some five years earlier [1] and stored in a stoppered tube without any special precautions, showed that this, too, with the passage of time had been largely converted into diammonium P^1P^2-dibenzyl pyrophosphate. The formation of this product is presumably attributable to interaction of the anionic oxygen atom of one molecule with the electrophilic phosphorus atom of the other, followed by hydrolysis of the remaining $>PO \cdot NH_2$ grouping and formation of the ammonium salt, as shown.



Detailed consideration of the mechanism of formation must await a kinetic analysis of this reaction but the occurrence of phosphorylation of an anionic species by the monoester of a phosphoramidic acid is not in doubt.

* Part XIV, *J.*, 1956, 3524.

[1] Clark and Todd, *J.*, 1950, 2031.
[2] Langheld, *Ber.*, 1910, **43**, 1857; 1911, **44**, 2076.
[3] Hobbs, Corbridge, and Raistrick, *Acta Cryst.*, 1953, **6**, 621.

These preliminary observations suggested that the monoesters of the phosphoramidic acids would react with phosphate anions to yield pyrophosphates and that alcoholic groups also present would be unaffected. This selectivity would make them particularly suited to the synthesis of nucleoside polyphosphates and unsymmetrical pyrophosphates of the nucleotide coenzyme type.

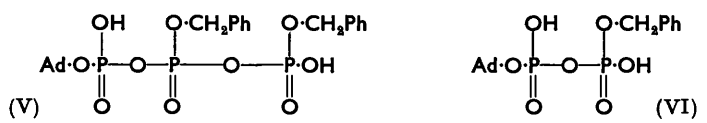
The early work of Stokes [4] indicated that preliminary addition of a proton is of importance in this acylation and our observations suggest that the site of the proton is also relevant to the course of reaction. Stokes showed that the dianion of phosphoramidic acid is extremely stable in aqueous solution, the acid yielding but 3% of its nitrogen as ammonia after treatment with hot concentrated potassium hydroxide for one hour; in contrast, the monoprotonated anion, $\{[NH_2 \cdot P(O)O_2^{--}][H^+]\}$ undergoes hydrolysis to orthophosphate merely on recrystallisation of its potassium salt. In ten minutes at 100°, 98% of the nitrogen is available as ammonia.

A proton, added to the anion of a phosphoramidic acid (IV), can become attached to an oxygen atom (cf. II) or to the nitrogen atom (cf. III), according to their relative basicities, but only in the latter case would the product be a phosphorylating agent. Substitution of the nitrogen atom by an aryl residue would be expected to decrease the possibility of adding a proton to nitrogen and in accordance with this we find that benzyl hydrogen *N*-phenylphosphoramidate gives very much lower yields of pyrophosphate in the presence of phosphate anions.

With the pyridinium salt of adenosine-5′ phosphate dissolved in dimethylformamide, a variety of *N*-substituted monobenzyl phosphoramidates gave the monobenzyl ester of adenosine-5′ pyrophosphate as the major product, although some di(nucleoside-5′) pyrophosphate was also formed, presumably through anhydride–anion exchange: [5] the amount of this by-product could be considerably diminished by adding small amounts of water. Uridine-5′ phosphate was phosphorylated in analogous fashion, yielding P^1-benzyl P^2-uridine-5′ pyrophosphate and di(uridine-5′) pyrophosphate. In none of these reactions could acylation of alcoholic groups be detected. The monoesters of *N*-benzyl-, *N-cyclo*hexyl-, and the unsubstituted phosphoramidic acid gave comparable results as phosphorylating agents, but the *N*-phenyl derivative was much less effective.

By using benzyl hydrogen phosphoramidate and the pyridinium salt of adenosine-5′ phosphate in dimethylformamide containing 5% of water it was possible, after removal of the benzyl group by catalytic hydrogenation, to isolate lithium adenosine-5′ pyrophosphate in 48% yield. Analogously, by using benzyl hydrogen *N-cyclo*hexylphosphoramidate and the tri-*n*-butylammonium salt of adenosine-5′ pyrophosphate in dry dimethylformamide, adenosine-5′ triphosphate has been obtained in 71% yield (isolated as its lithium salt). In the last reaction some higher adenosine polyphosphates are also produced.

From these observations it appears that monobenzyl phosphoramidates phosphorylate nucleotides in step-wise fashion: thus, adenosine-5′ phosphate (AMP) was converted into



Ad = adenosine-5′ residue

a monobenzyl ester of adenosine-5′ pyrophosphate (ADP) without appreciable formation of esters of adenosine-5′ triphosphate (ATP), and similarly, ADP yielded a benzyl ester of ATP as the major product although in both experiments an excess of phosphoramidate was present. The observations suggest that, under the particular experimental conditions, the dibenzyl ester (V) of ATP is unstable relative to the monoester (VI) of ADP.

[4] Stokes, *Amer. Chem. J.*, 1893, **15**, 198; cf. Muller, Rathly, and Rosenberg, *Biochim. Biophys. Acta*, 1956, **19**, 563.
[5] Corby, Kenner, and Todd, *J.*, 1952, 1234.

These investigations demonstrate the utility of monoesterified phosphoramidates in the phosphorylation of unprotected nucleoside phosphates and indicate that nucleoside phosphoramidates might be used in the synthesis of unsymmetrical pyrophosphates.

In a note published some time after completion of this work, Chambers and Khorana [6] recorded their preliminary observations that certain monosalts of phosphoramidic acid react with orthophosphoric acid to yield pyrophosphates and with AMP to yield a mixture of ADP, ATP, higher polyphosphates, and unchanged AMP in approximately equal amounts. They have also indicated the formation of ADP from the interaction of adenosine-5′ phosphoramidate and orthophosphoric acid. Baddiley, Buchanan, and Letters [7] have shown that *N*-phosphoryl derivatives of glyoxaline can be used in phosphorylation though, in this case, the systems are to be compared with mixed anhydrides, and, characteristically being less selective, acylate alcoholic groups as well as acid anions. The *di*esters of *N*-phosphorylglyoxaline are themselves phosphorylating agents whereas the *di*esters of phosphoramidic acid appear not to be.

Experimental

Benzyl Hydrogen Phosphoramidate.—Its hemihydrate [1] was obtained (Found: equiv., 196. Calc. for $C_7H_{10}O_3NP,\frac{1}{2}H_2O$: equiv., 196). After drying for 4 days (P_2O_5/0·1 mm.) the *anhydrous compound* was obtained (Found: C, 44·8; H, 5·4; N, 7·3%; equiv., 182. $C_7H_{10}O_3NP$ requires C, 44·9; H, 5·4; N, 7·5%; equiv., 187).

Benzyl Hydrogen N-*Benzylphosphoramidate.*—Dibenzyl *N*-benzylphosphoramidate [8] was monodebenzylated by lithium chloride according to Clark and Todd's method.[1] To the ice-cold solution of the crude lithium salt (2·5 g.) in water (40 c.c.) excess of hydrochloric acid (3N) was added whereupon *benzyl hydrogen* N-*benzylphosphoramidate* (1·7 g.) was precipitated; it was washed with water and dried *in vacuo* (P_2O_5). Recrystallisation from ethyl acetate gave prisms, m. p. 98—100° (varying with the rate of heating) (Found: C, 59·0; H, 5·7; N, 4·9. $C_{14}H_{16}O_3NP,\frac{1}{2}H_2O$ requires C, 58·8; H, 5·9; N, 4·9%).

Benzyl Hydrogen N-cyclo*Hexylphosphoramidate.*—Dibenzyl *N-cyclo*hexylphosphoramidate [8] was debenzylated by lithium chloride, and the free acid isolated (75% yield) as described above for the *N*-benzyl derivative. Recrystallised from ethyl acetate *benzyl hydrogen* N-cyclo*hexylphosphoramidate* was obtained as needles, m. p. 101—104° (Found: C, 58·0; H, 7·6; N, 5·5. $C_{13}H_{20}O_3NP$ requires C, 58·0; H, 7·4; N, 5·2%).

Benzyl Hydrogen N-*Phenylphosphoramidate.*—The lithium salt [1] of this acid was converted into the silver salt by treatment with aqueous silver nitrate, and a suspension of this salt in chloroform was then decomposed by passage of hydrogen sulphide. After 1 hr. the precipitate was filtered off and extracted with ethanol. Evaporation of the ethanol gave benzyl hydrogen *N*-phenylphosphoramidate as pale yellow crystals whose *cyclo*hexylamine salt, crystallised from ethanol–acetone, had m. p. 178—179°, undepressed on admixture with that obtained by Clark and Todd.[1]

Diammonium P^1P^2-*Dibenzyl Pyrophosphate.*—Tetrabenzyl pyrophosphate [9] (0·20 g.) was heated under reflux for 2 hr. in ethyl methyl ketone (2 c.c.) containing ammonium thiocyanate (57 mg.), and the *diammonium* P^1P^2-*dibenzyl pyrophosphate* which separated was filtered off, washed with ethyl methyl ketone until colourless, and dried *in vacuo* at room temperature (0·11 g., 78%). Recrystallised from aqueous acetone it formed plates, m. p. 220° (decomp.) (Found: C, 42·6; H, 6·0; N, 6·6. $C_{14}H_{22}O_7N_2P_2$ requires C, 42·9; H, 5·6; N, 7·1%).

Effect of Heat on Benzyl Hydrogen Phosphoramidate in Dioxan Solution.—Benzyl hydrogen phosphoramidate (0·10 g.) was heated at 100° in dioxan solution for 2 hr. The colourless crystals which separated were washed with dioxan, then with ether, and dried (0·07 g.). Recrystallised from aqueous acetone they formed plates, m. p. 216° (decomp.), whose aqueous solution was neutral, and whose infrared spectrum was identical with that of diammonium

[6] Chambers and Khorana, *Chem. and Ind.*, 1956, 1022.
[7] Baddiley, Buchanan, and Letters, *J.*, 1956, 2812.
[8] Atherton, Openshaw, and Todd, *J.*, 1945, 660.
[9] Kenner, Todd, and Weymouth, *J.*, 1952, 3675.

P^1P^2-dibenzyl pyrophosphate (Found: C, 42·6; H, 5·6; N, 7·0. Calc. for $C_{14}H_{22}O_7N_2P_2$: C, 42·9; H, 5·6; N, 7·1%).

Adenosine-5′ Pyrophosphate.—Adenosine-5′ phosphate (300 mg. of monohydrate) was converted into its dipyridinium salt by dissolution in water (2—3 c.c.), addition of excess (3 drops) of pyridine, evaporation at room temperature, and drying over phosphoric oxide. The salt, a brittle glass, was suspended in dimethylformamide (40 c.c.) containing water (2 c.c.), and benzyl hydrogen phosphoramidate (600 mg.; 3·5 mols.) was added. The suspension was slowly heated to 70° to effect solution and was then kept at 95—100° for 5 hr. Solvent was removed under reduced pressure at 40° and the residue dissolved in water, the pH being adjusted to 4—5 by initial addition of pyridine followed by acetic acid. Hydrogenation at room temperature and atmospheric pressure with 10% palladised charcoal (10 mg.) together with palladous oxide (60 mg.) brought about complete removal of benzyl groups; after 5 hr. the solution was filtered and adjusted to pH 5, and one-half of it subjected to ion-exchange chromatography on a Dowex-2 column (7·5 × 1·8 cm., Cl^- form). Elution with 0·003M-hydrochloric acid gave unchanged adenosine-5′ phosphate (42 mg.) and some orthophosphate: with 0·01M-hydrochloric acid containing 0·01M-lithium chloride a small quantity (5 mg.) of di(adenosine-5′) pyrophosphate was obtained followed by adenosine-5′ pyrophosphate (85 mg.). The elution was followed, and the yields were estimated, by measuring light absorption at 260 mμ. Each eluate was neutralised with lithium hydroxide and evaporated to small volume under reduced pressure at 50°, and the lithium salts were precipitated by addition of acetone–ethanol (3 : 1). Centrifugation of the adenosine-5′ pyrophosphate fraction followed by washing with acetone and drying (P_2O_5) gave the lithium salt of ADP in 48% yield (based on AMP). A portion of this lithium salt was converted into the free nucleotide by means of a Dowex-50 column in the acid form, the eluate being freeze-dried. Examination by paper electrophoresis at pH 4·8 and chromatography on Whatman No. 1 paper with butan-1-ol–acetic acid–water (5 : 2 : 3) showed the product to be indistinguishable from ADP and to contain no other ultraviolet-absorbing or phosphorus-containing component (Found: C, 26·6; H, 3·7; N, 15·2; P, 14·0. Calc. for $C_{10}H_{15}O_{10}N_5P_2,H_2O$: C, 27·0; H, 3·8; N, 15·7; P, 13·9%). Electrophoresis in 0·1M-potassium cyanide showed the synthetic material to travel appreciably slower than a mixture of adenosine-2′ : 5′ and adenosine-3′ : 5′ diphosphate.

Adenosine-5′ Triphosphate.—A solution of adenosine-5′ pyrophosphate (150 mg.) was obtained by decomposing an aqueous suspension of its barium salt with an equivalent amount of sulphuric acid (N/50); after removal of barium sulphate by centrifugation, the aqueous solution was treated with a slight excess of tri-*n*-butylamine in ether, and the resulting aqueous layer evaporated under reduced pressure at 40°, the residue being dried over phosphoric oxide. The glassy salt so obtained was dissolved in dry dimethylformamide (35 c.c.) containing benzyl hydrogen *N-cyclo*hexylphosphoramidate (490 mg., *ca.* 5 mols.), and the solution heated at 70° for 2 hr. After removal of solvent at 40°/1 mm. the resulting gum was suspended in water and brought into solution by dropwise addition of dilute aqueous ammonia; adjustment to pH 4 (by glacial acetic acid) was followed by hydrogenation at room temperature and atmospheric pressure with a palladous oxide catalyst (46 mg.). Hydrogen uptake was complete in 13 hr. The catalyst was filtered off, the pH adjusted to pH 9 by aqueous ammonia, and the solution subjected to ion-exchange chromatography on a column (7·5 × 1·8 cm.) of Dowex-2 resin (Cl^- form). Elution with 0·003M-hydrochloric acid gave adenosine-5′ phosphate (5 mg.) together with orthophosphate; with 0·01M-hydrochloric acid containing 0·01M-lithium chloride di(adenosine-5′) pyrophosphate (3 mg.) and unchanged adenosine-5′ pyrophosphate (13 mg.) were obtained; with 0·01M-hydrochloric acid containing 0·10M-lithium chloride adenosine-5′ triphosphate (127 mg.) was obtained. Elution with M-hydrochloric acid gave higher adenosine polyphosphates (20 mg.).

The ATP was isolated as its lithium salt (71%; cf. ADP), evaporation of the neutralised eluate being carried out at 40°. Adding barium acetate solution at pH 6 to an aqueous solution of the lithium salt precipitated the barium salt of ATP. After centrifugation it was washed first with 50% ethanol, then with 95% ethanol, and dried (over P_2O_5) (Found: N, 8·0; total P, 10·6; easily hydrolysed P,[10] 6·7%; *M*, 895, 878.[11] Calc. for $C_{10}H_{12}O_{13}N_5P_3Ba_2,6H_2O$: N, 7·9; total P, 10·5; easily hydrolysed P, 7·0%; *M*, 885). The lithium salt appeared chromatographically homogeneous in three different solvent systems (see below) and was

[10] Allen, *Biochem. J.*, 1940, **34**, 858.
[11] From absorbance at 260 mμ.

unresolved by electrophoresis at pH 4·8. After conversion of the synthetic material into its sodium salt by ion-exchange and treatment with 8-hydroxyquinoline to remove traces of heavy-metal impurities, inorganic phosphate was liberated from it by L-myosin [12] at a rate 82% of that observed for chromatographically pure ATP of natural origin.

Paper-chromatographic and Electrophoretic Data.—Electrophoretic buffer systems. A, acetate buffer at pH 4·8; B, 0·1M-potassium cyanide solution. Electrophoretic runs were carried out on Whatman No. 54 paper at 5 v/cm. for 7 hr.

Solvent systems for paper chromatography. C, butan-1-ol–acetic acid–water (5 : 2 : 3); D, propan-2-ol–1% aqueous ammonium sulphate (3 : 2) run on paper previously soaked in 1% ammonium sulphate solution and dried; E, *iso*butyric acid–N-ammonia–0·1M-ethylenediaminetetra-acetic acid [13] (100 : 60 : 1·6). Ascending chromatograms were run on Whatman No. 1 paper throughout, phosphorus being detected by the molybdate spray.[14]

Results are tabulated.

	Migration (cm.) in buffer			R_F in solvent	
	A	B	C	D	E
Adenosine-5′ phosphate	3·1	—	0·24	0·45	0·53
Adenosine-5′ pyrophosphate	6·8	8·8	0·12	0·33	0·39
Adenosine-5′ triphosphate	8·6	—	0·06	0·20	0·30
Di(adenosine-5′) pyrophosphate	4·1	—	—	—	—
Adenosine-(2′ or 3′)5′ diphosphate	—	11·8	—	—	—

Reaction of Benzyl Hydrogen Phosphoramidate with Adenosine-5′ Phosphate and Uridine-5′ Phosphate.—The nucleoside-5′ phosphate (10 mg.) as its pyridinium salt was heated in dimethylformamide solution (2—3 c.c.) with a variety of benzyl hydrogen phosphoramidates (3 mols.) for 3 hr. at 90—100°. The products were examined by paper chromatography in the butan-1-ol–acetic acid–water (5 : 2 : 3) system, the nucleotides being observed under filtered ultraviolet light (mercury lamp). Qualitative assessment gave the annexed results :

	Unchanged nucleoside-5′ phosphate	Monobenzyl ester of nucleoside-5′ pyrophosphate	Di-(nucleoside-5′) pyrophosphate
UMP in dry dimethylformamide	++	++++	+
AMP in dry dimethylformamide	++	+++	++
AMP in dimethylformamide containing 5% of water	+++	++++	Trace
AMP in dimethylformamide containing 15% of water	+++++	++	None detectable

Benzyl hydrogen phosphoramidate and the corresponding *N*-benzyl and *N-cyclo*hexyl derivatives gave comparable results, whereas the *N*-phenyl analogue gave much less pyrophosphate. Varying amounts of pyridine were added to a mixture of adenosine-5′ phosphate and benzyl hydrogen phosphoramidate (up to an excess of 7 mols.) without significantly affecting the result.

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[12] Perry, "Methods in Enzymology," Academic Press, New York, 1955, Vol. II, p. 582.
[13] Krebs and Hems, *Biochim. Biophys. Acta*, 1953, **12**, 172.
[14] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.