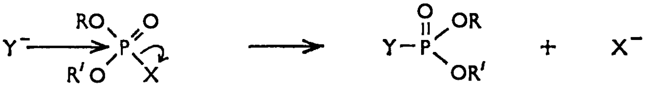
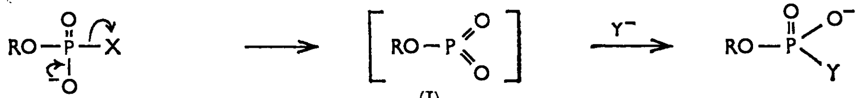
1026. *Studies in Phosphorylation. Part XXVIII.*[1] *The Formation of Pyrophosphates from Phosphoramidate Monoesters*[2]

By V. M. Clark and S. G. Warren

The initial stage of the conversion of benzyl hydrogen phosphoramidate (IV/V) hemihydrate into diammonium P^1P^2-dibenzyl pyrophosphate in dioxan solution follows second-order kinetics. The structure of the starting material and the mechanism of the reaction are discussed.

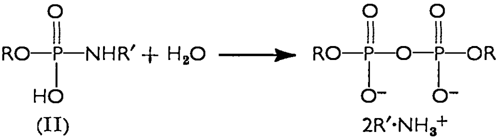
Biological phosphorylating agents, *e.g.*, ATP and phosphorocreatine, transfer a phosphoryl group [$(HO)_2PO^-$] to a variety of nucleophilic substrates with cleavage of a P–O or a P–N bond.[3] The route by which such a reaction can occur[4] involves either (i) a bimolecular displacement at phosphorus,[5] or (ii) the formation of a metaphosphate species (I) followed by attack of the substrate on this highly reactive intermediate.[6] Both routes are now well established.


(i)


(I)

(ii)

Phosphoramidate monoesters are of particular interest since they have been used in the synthesis of such compounds as ATP[7] and flavin adenine dinucleotide.[8] The formation (iii) of a pyrophosphate P^1P^2-diester when the hemihydrate of one of these compounds is heated in anhydrous dioxan can provide a kinetic distinction between the two pathways since the substrate and the phosphorylating agent are molecules of the same compound. Subsequent to our observations[2] on benzyl hydrogen phosphoramidate (II; R = $PhCH_2$, R′ = H), Hamer[1] showed that the pyrophosphoramidate (III; R = Me, R′ = cyclohexyl) was produced in a second-order reaction when *anhydrous* methyl hydrogen *N*-cyclohexylphosphoramidate (II; R = Me, R′ = cyclohexyl) was heated in



$$2\,RO{-}\underset{HO}{\underset{|}{\overset{O}{\overset{\|}{P}}}}{-}NHR' + H_2O \longrightarrow RO{-}\underset{O^-}{\underset{|}{\overset{O}{\overset{\|}{P}}}}{-}O{-}\underset{O^-}{\underset{|}{\overset{O}{\overset{\|}{P}}}}{-}OR \quad 2R'{\cdot}NH_3^+ \qquad \text{(iii)}$$

(II)

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[1] Part XXVII; N. K. Hamer, *J.*, 1965, 2731.

[2] Preliminary communication, V. M. Clark and S. G. Warren, *Proc. Chem. Soc.*, 1963, 178.

[3] R. Nordlie and H. A. Lardy in " The Enzymes," eds. P. D. Boyer, H. A. Lardy, and K. Myrbäck, Academic Press, 2nd edn., New York, 1962, vol. 6, p. 4. For recent reviews, see J. R. Cox and O. B. Ramsay, *Chem. Rev.*, 1964, **64**, 317; V. M. Clark, D. W. Hutchinson, A. J. Kirby, and S. G. Warren, *Angew. Chem.*, 1964, **76**, 704.

[4] V. M. Clark and A. R. Todd, *J.*, 1950, 2023; W. P. Jencks in Brookhaven Symposia in Biology, Brookhaven National Laboratory, 1962, vol. 15, p. 134.

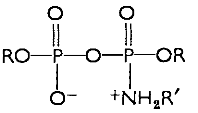
[5] I. Dostrovsky and M. Halmann, *J.*, 1953, 502, 508, 511, 516; G. O. Dudek and F. H. Westheimer, *J. Amer. Chem. Soc.*, 1959, **81**, 2641.

[6] W. W. Butcher and F. H. Westheimer, *J. Amer. Chem. Soc.*, 1955, **77**, 2420; G. di Sabato and W. P. Jencks, *ibid.*, 1961, **83**, 4400.

[7] V. M. Clark, G. W. Kirby, and Sir Alexander Todd, *J.*, 1957, 1497.

[8] J. G. Moffatt and H. G. Khorana, *J. Amer. Chem. Soc.*, 1958, **80**, 3756.

dry dioxan. Also, Moffatt and Khorana have reported the formation of a substituted pyrophosphoramidate in an analogous reaction sequence.[9]



$RO-P(=O)(O^-)-O-P(=O)(OR)(^+NH_2R')$ (III)

In our experiments, the *hemihydrate* of benzyl hydrogen phosphoramidate was heated in dry dioxan at 70°. After a short lag period, the disappearance of the benzyl hydrogen phosphoramidate followed second-order kinetics. This continued for one half-life of the starting material (Figures 1 and 2) with a rate constant of 26·8 l. mole^{-1} hr.$^{-1}$.

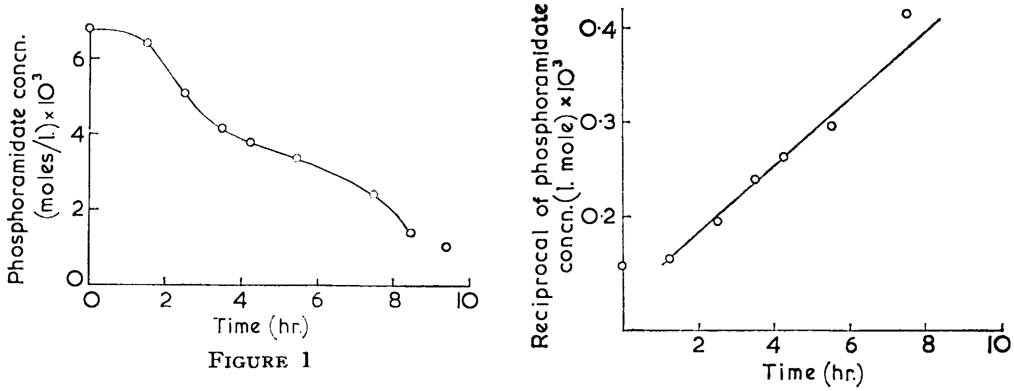


FIGURE 1

FIGURE 2

FIGURES 1 and 2. Typical run without added water

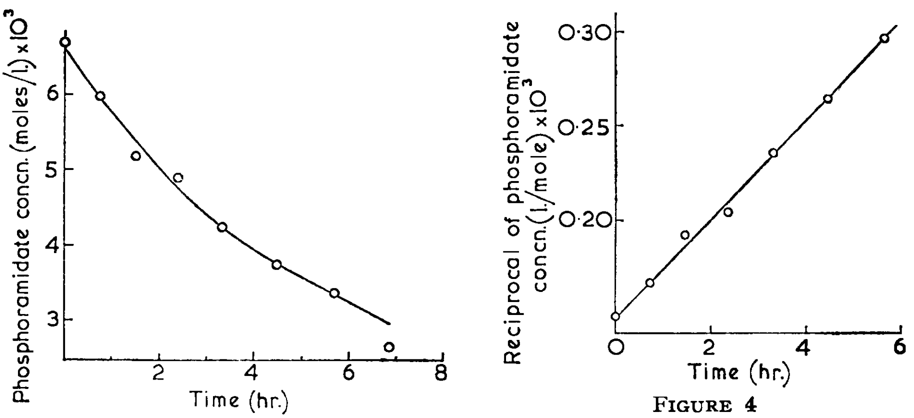


FIGURE 3

FIGURE 4

FIGURES 3 and 4. Typical run with added water (initial water concentration × 10^3 = 20 moles/l.)

When the reaction was carried out in the presence of *added* water, (20 × 10^{-3}M-water and 6·7 × 10^{-3}M-benzyl hydrogen phosphoramidate) the reaction was second-order from the start (Figures 3 and 4) with a rate constant of 26·0 l. mole^{-1} hr.$^{-1}$.

These kinetics are not compatible with metaphosphate formation but accord well with the selective behaviour of benzyl hydrogen phosphoramidate which can phosphorylate phosphates in the presence of alcohols.[7]

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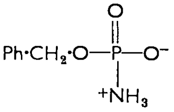
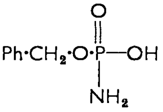
[9] J. G. Moffatt and H. G. Khorana, *J. Amer. Chem. Soc.*, 1961, **83**, 649.

The infrared spectrum of the solid monobenzyl ester hemihydrate shows characteristic NH_3^+ and PO_2^- vibrations best accounted for by the zwitterionic structure (IV). In solution in dimethylformamide or dimethyl sulphoxide, however, these vibrations are replaced by those of the NH_2 and P–O–H groupings, and the compound must have the uncharged structure (V) under these conditions.



$$Ph{\cdot}CH_2{\cdot}O{-}\overset{\displaystyle O}{\overset{\|}{\underset{\underset{\displaystyle ^+NH_3}{|}}{P}}}{-}O^-$$

(IV)

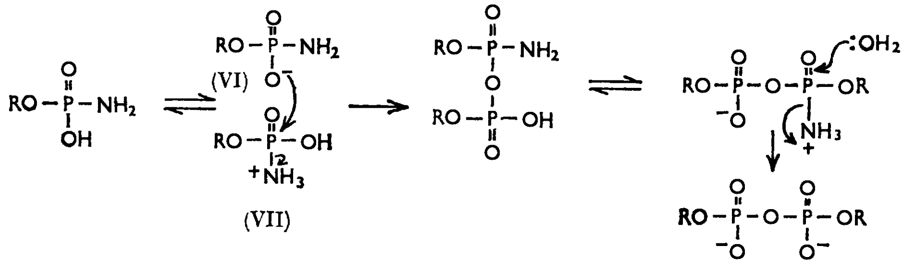


$$Ph{\cdot}CH_2{\cdot}O{\cdot}\overset{\displaystyle O}{\overset{\|}{\underset{\underset{\displaystyle NH_2}{|}}{P}}}{-}OH$$

(V)

As the formation of P^1P^2-dimethyl pyrophosphate from methyl hydrogen *N*-cyclohexylphosphoramidate is faster in ethanol than in dioxan,[1] the participation of some ionic form is indicated. The nucleophile is presumably the anion (VI) or the zwitterion (IV), whilst the electrophile must be the zwitterion or the cation (VII) since protonation of the nitrogen atom is required for cleavage of the P–N bond.[10]

The reaction is completed by hydrolysis (iv) of the pyrophosphoramidate. The adherence to bimolecular kinetics in the presence of added water indicates that this final hydrolysis is appreciably faster than the formation of the pyrophosphoramidate.* The more complex kinetics (see Figure 1) observed when the hemihydrate was heated in the absence of added water presumably result from the slower hydrolysis of the pyrophosphoramidate under these conditions.



(iv)

To sum up, cleavage of the P–N bond in phosphoramidates can follow first- or second-order kinetics. The former course, requiring a metaphosphate intermediate, may occur in certain solvolyses of phosphoramidic acids [11] and in the pyridine-catalysed reactions of phosphoramidic monoesters.[1] However, in the reactions of phosphoramidic diesters [10] and in the formation of pyrophosphates from phosphoramidic monoesters, the bimolecular pathway is followed.

EXPERIMENTAL

Paper chromatograms were run on Whatman No. 541 paper in solvent systems A: n-butanol : acetic acid : water (5 : 2 : 3), and B: propan-2-ol : ammonia : water (7 : 1 : 2); phosphorus-containing spots were detected by spraying with molybdate reagent.[12]

	Solvent system	
R_F values	A	B
Benzyl dihydrogen phosphate	0·72	0·30
P^1P^2-Dibenzyl pyrophosphate	0·86	0·66
Benzyl hydrogen phosphoramidate	—	0·44

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* Although benzyl dihydrogen phosphate was detected chromatographically in the reaction mixture, it was present in small and slowly increasing amounts, and, notwithstanding our previous suggestion,[2] does not appear to be an intermediate.

[10] (*a*) Z. Skrowarzewska and P. Mastalerz, *Roczniki Chem.*, 1955, **29**, 415; (*b*) J. F. P. Richter, Ph.D. Thesis, Cambridge, 1961.

[11] A. Lapidot and M. Halmann, *J.*, 1958, 1713; J. D. Chanley and E. Feageson, *J. Amer. Chem. Soc.*, 1958, **80**, 2686.

[12] C. S. Hanes and F. A. Isherwood, *Nature*, 1949, **164**, 1107.

Benzyl Hydrogen Phosphoramidate (*BHPA*).—This was prepared by the method of Clark, Kirby, and Todd,[13] and stored as its sodium salt.

Infrared Assay.—Spectra were taken in Nujol mulls on a Perkin-Elmer model 137 Infracord spectrophotometer (sodium chloride optics). A comparison of the spectra of BHPA and diammonium P^1P^2-dibenzyl pyrophosphate revealed a peak in each spectrum (at 1270 and 1200 cm.$^{-1}$, respectively) which corresponded to minimal absorption in the other. The ratios of the logarithms of the proportional absorption (I_0/I) at 1270 and 1200 cm.$^{-1}$ in spectra of 6 mg. of weighed mixtures of BHPA and compound (VII) were plotted against the molar ratio of the components of the mixture: a straight line was obtained (Figure 5).

Solvent.—Dioxan was dried by the method of Eigenberger [14] and passed down a column of molecular sieve (BDH type 4A; 7 ft. × ½ in.; 500 g.).

Kinetic Analyses.—Benzyl hydrogen phosphoramidate (80—130 mg.) was dissolved in dry dioxan (100 ml.) * at 70° ± 0·1° and kept at this temperature throughout the experiment. Aliquot portions (5 or 10 ml.) were withdrawn and the mixed solutes precipitated by vigorous shaking with excess of n-pentane (ten times the volume of the aliquot). The precipitate was collected by centrifugation and dried *in vacuo*. The infrared spectrum of a sample (about 5 mg.) was then recorded.

The concentration of phosphoramidate was calculated from the calibration graph, and this concentration and its reciprocal were plotted against time (Figures 1—4). For reactions with no added water (*e.g.*, Figure 2) the rate constant was calculated from the straight-line portion of the graph. The second-order rate constant was the same over a range of initial concentration of BHPA of 5·3—6·8 × 10^{-3} moles/l. The lower limit of this range was determined by the sensitivity of the method and the upper limit by precipitation of the product, which also prevented investigation of the reaction beyond about 85% completion. First-order plots were not linear.

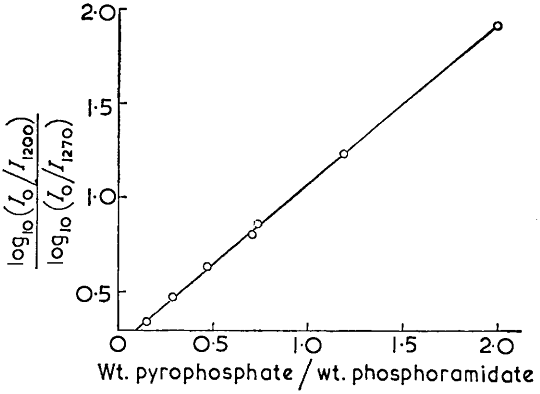

FIGURE 5. Calibration graph

The Structure of Solid BHPA.—Comparison of the N–H vibrational and deformation frequencies of BHPA (in Nujol mull) with those of sulphamic acid (Table 1), which has a zwitterionic structure,[15] shows that the phosphoramidate, too, possesses an NH_3^+ group.

The broad and shallow bands associated with the P–O–H group [16] were absent in the spectrum of BHPA although present in that of benzyl hydrogen *N*-cyclohexylphosphoramidate.[10b] The PO_2^- bands were present and confirmed the zwitterionic structure. The P–O–C (aliphatic) peak was single and sharp, as were those of the ionised phosphates, unlike the multiple bands of unionised phosphates in this region (Table 2).

The Structure of BHPA in Solution.—BHPA is insoluble in the usual spectroscopic solvents (and in cold dioxan): spectra were taken using dimethylformamide (DMF) and dimethyl

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* Although almost insoluble in dioxan at room temperature, it dissolved rapidly at 70° if finely ground.

[13] V. M. Clark, G. W. Kirby, and Sir Alexander Todd, *J.*, 1957, 1497.
[14] E. Eigenberger, *J. prakt. Chem.*, 1931, **130**, 75.
[15] A. M. Vuagnat and E. L. Wagner, *J. Chem. Phys.*, 1957, **26**, 77.
[16] J. T. Braunholtz, G. E. Hall, F. G. Mann, and N. Sheppard, *J.*, 1959, 868.

TABLE 1

NH_3^+ Bands (cm.$^{-1}$) in the infrared spectrum of BHPA

Vibration	Sulphamic acid [15]	BHPA
Degenerate stretch	3200s *	3200m *
Symmetrical stretch	3140s *	3100m *
Degenerate deformation	1570m	1603w
Symmetrical deformation	1446s	1472s
Degenerate " rock "	1000s	1003s
Overtones		
Twice degenerate deformation	3055s *	3050m *
Twice symmetrical deformation	2893m	2790s
Twice " rock "	2044w	2050w
Degenerate deformation + " rock "	2569w	2580m
Symmetrical deformation + " rock "	2465m	2445m

* Shoulder.

TABLE 2

The phosphoryl-region spectrum of BHPA

	Observed frequencies (cm.$^{-1}$)		
Compound	P=O	PO_2^- *	P–O–C
Dibenzyl hydrogen phosphate	1200	—	1000 †
Dibenzyl phosphoramidate	1236		1028 †
Lithium benzyl phosphoramidate	1199	1105	1040 ‡
Lithium benzyl phosphite	1205	1120	995 ‡
Benzyl hydrogen phosphoramidate	1268	1119	1000 ‡

* Imperfectly resolved doublets (cf. D. E. C. Corbridge and E. J. Lowe, *J.*, 1954, 493). † Single sharp peak. ‡ Multiplet.

sulphoxide (DMSO) as solvents. Comparison with spectra of dibenzyl phosphoramidate taken in the same two solvents showed the same NH_2 peaks, whilst the NH_3^+ and PO_2^- peaks were missing (Table 3). Fine structure was lacking. The possibility that a dimer might have been formed was eliminated by a cryoscopic molecular-weight determination † in dimethyl sulphoxide (Found: 183. Calc. for $C_7H_{10}NO_3P$: 187).

TABLE 3

The infrared spectrum of BHPA in solution

		Observed frequencies (cm.$^{-1}$)			
		DMF		DMSO	
Assignment	Model compound	A	Model	A	Model
(i) Antisymmetrical NH_2 stretch	B	3340	3300	3300	3250
(ii) Symmetrical NH_2 stretch	B				
(iii) H–N–H bend	B	1580	1560	1580	1580
(iv) Antisymmetrical NH_2 wag	B	1030	Obscured	Obscured	1060
(v) Symmetrical NH_2 rock	B	840	870	845	870
(vi) Overtone of (iii)	B	3150	3150	3150	3150
P = O stretch	B	1240	1235	1260	1230
P–O–C (aliphatic) *	B	960—1020	940—1040	910—990	930—1050
P–O–H stretch †	C			2800	2750

A = BHPA; B = dibenzyl phosphoramidate; C = phenyl dihydrogen phosphate.

* Multiplet or broad peak. † Broad and shallow.

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† Measured by Mr. D. Flory, University Chemical Laboratory, Cambridge.