9. *Studies in Phosphorylation. Part XXVI.* The Mechanism of Phosphorylation by Monoesters of Phosphoramidic Acid.*

By N. K. Hamer.

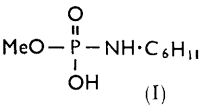
Methyl hydrogen *N*-cyclohexylphosphoramidate is transformed on heating into cyclohexylammonium P^1P^2-dimethyl P^1-*N*-cyclohexylpyrophosphoramidate. The course of the reaction in the presence of other nucleophilic species is investigated and provides evidence to suggest that, while in the absence of pyridine phosphorylation occurs by a direct displacement on phosphorus, in the presence of pyridine, amine exchange occurs and a metaphosphate ester intermediate may be the phosphorylating agent. The preparation of some salts of P^2-methyl dihydrogen P^1-*N*-cyclohexylpyrophosphoramidate is described.

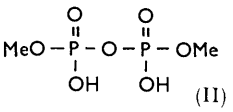
The phosphoramidic acids are well-established reagents for the preparation of both symmetrical and unsymmetrical esters of pyrophosphoric acid [1-3] and have also been used with success to prepare higher linear polyphosphate esters such as adenosine-5′-triphosphate.[1] However, except for the recent quantitative studies on the solvolysis of phosphoramidic acid itself and its *N*-aryl derivatives [4] there exists comparatively little evidence on the actual mechanism of phosphorylation. This is particularly so for the monoesterified phosphoramidic acids which, owing to their ability to phosphorylate phosphate anions preferentially in the presence of other nucleophiles, are the preferred reagents for the synthesis of the pyrophosphate coenzymes. In an attempt to obtain further information on the actual phosphorylation process the reactions of methyl hydrogen *N*-cyclohexylphosphoramidate [5] (I) were investigated under various conditions.

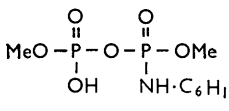
* Part XXV, Clark, Lord Todd, and Warren, *Biochem. Z.*, 1963, **338**, 591.

[1] Clark, Kirby, and Todd, *J.*, 1957, 1497.
[2] Chambers and Khorana, *J. Amer. Chem. Soc.*, 1958, **80**, 3749, 3756.
[3] Moffat and Khorana, *J. Amer. Chem. Soc.*, 1961, **83**, 649.
[4] Chanley and Feageson, *J. Amer. Chem. Soc.*, 1958, **80**, 2686; 1963, **85**, 1181; Halmann, Lapidot, and Samuel, *J.*, 1963, 1299.
[5] Brown and Hamer, *J.*, 1960, 1155.

Heating compound (I) alone in anhydrous dioxan or acetonitrile gave traces only (<5%) of the cyclohexylammonium salt of *sym*-dimethyl dihydrogen pyrophosphate (II), the remainder of the phosphorus appearing as a new compound which, with sodium hydroxide solution, gave methyl dihydrogen phosphate and compound (I) in equimolecular


(I)


(II)


(III)

amounts, and with dilute hydrochloric acid gave compound (II) together with some methyl dihydrogen phosphate. Moffat and Khorana [3] have made parallel observations with adenosine-5 hydrogen phosphoromorpholidate and it seems clear that this new compound should be assigned the pyrophosphoramidate structure (III). Even when 1—5 mol. of water were added to the dioxan solution substantial amounts of compound (III) were detectable at intermediate stages although they were, eventually, converted into the stable pyrophosphate ester (II). By following the rate of disappearance of free acid titrimetrically it was established that the rate of formation of compound (III) showed a second-order dependence * on the concentration of compound (I), the rate constants at 70° being 12·6 and 33·0 l. mole^{-1} hr.$^{-1}$ in dioxan and acetonitrile, respectively. (Benzyl hydrogen *N*-cyclohexylphosphoramidate, which exhibits similar behaviour in dioxan solution, had a rate constant of 14·6 l. mole^{-1} hr.$^{-1}$ at 70°.) When small amounts of pyridine were added to the dioxan solution and the reaction mixture analysed by paper chromatography at intervals it was found that the rate of formation of compound (III) was very greatly increased.

There exists considerable experimental evidence to suggest that compounds of type (I) do not phosphorylate alcoholic hydroxyl groups or carboxylic acids under conditions where monoalkyl phosphates are converted almost quantitatively into pyrophosphate esters, and it was therefore desirable to investigate also the reaction of these with compound (I). It was then found that, in dilute aqueous or very dilute ethanolic solution, compound (I) gave methyl dihydrogen phosphate and ethyl methyl hydrogen phosphate respectively as the sole products, the reaction being approximately of first order in substrate. In more concentrated ethanolic solution, however, considerable quantities of compound (III) were formed which broke down extremely slowly to give the phosphate diester mentioned above. Significantly, when the solvolysis of compound (I) was conducted in aqueous ethanol (50 mole %) rather more than 90% of the total phosphorus appeared as methyl dihydrogen phosphate (the rest as ethyl methyl hydrogen phosphate) a result that can be compared with the preferential phosphorylation of water observed for phosphoramidic acid itself under similar conditions. It seems clear therefore that while monoalkyl hydrogen phosphoramidates can phosphorylate alcohols this is unlikely, under normal experimental conditions, to compete effectively with their reaction with themselves (to give compounds of the type (III)) or water.

In the case of carboxylic acids, too, strong evidence has been obtained to show that they

	(I)	(II)	(III)	MeH_2PO_4	$C_6H_{11}\cdot NH\cdot COPh$
A	0·3	0·05	0·35	0·3	0·2
B	0·0	0·6	0·2	0·2	0·4

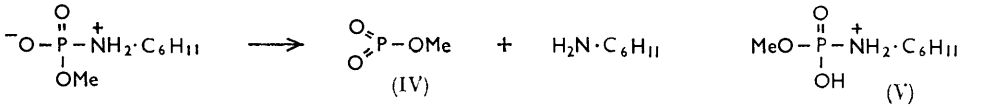
are phosphorylated on being heated with compound (I) in an inert solvent, both in the presence and absence of pyridine, but that in the latter case exchange reactions occur to give the carboxylic acid anhydride and compound (II). The Table shows the relative

* The authors of a recent Note [6] on the formation of diammonium dibenzyl pyrophosphate from benzyl hydrogen phosphoramidate hemihydrate have confirmed that the kinetic results reported there are undergoing re-interpretation.

[6] Clark and Warren, *Proc. Chem. Soc.*, 1963, 176.

amounts of phosphorus found as the various products and the molar fraction of *N*-cyclohexylbenzamide isolated when dioxan solutions of compound (I) (1 mol.), benzoic acid (4 mol.), and pyridine (10 mol. in B only) were heated at 70° for one hr. followed by treatment with excess of cyclohexylamine. If it be assumed that the methyl dihydrogen phosphate arises solely from the reaction of cyclohexylamine with benzoyl methyl hydrogen phosphate then the theoretical amounts of *N*-cyclohexylbenzamide formed should be 0·32 and 0·50, respectively, which are in fair agreement with those isolated.

The reactions recorded above substantiate the view that phosphorylation proceeds by way of a nucleophilic displacement on compound (I). In particular, if the reaction of compound (I) to give compound (III) is considered, it is difficult to see how the observed kinetic dependence, the increase in rate in acetonitrile solution compared with the less polar dioxan or the acceleration produced by pyridine, can be accommodated on the only reasonable alternative mechanism involving a metaphosphate ester (IV) as intermediate.[7–10] The preferential phosphorylation of water observed in the solvolysis of compound (I) in aqueous ethanol is also inconsistent with the intervention of a highly reactive (and there-



(IV) (V)

fore unspecific) intermediate such as compound (IV). On the basis of the second-order kinetics it seems probable that compound (III) arises from attack of one molecule of compound (I) on a second one (presumably in the zwitterionic form), or, possibly, by attack of the anion of compound (I) on the protonated species (V); attack of the anion on the zwitterion is, however, excluded. In the case of the former mechanism it is not yet possible to provide any detailed description of the actual nature of the transition state although several possibilities exist.[1,8] The latter mechanism is analogous to that postulated to account for the reactions of dialkyl phosphoramidates with hydrogen chloride [11] (giving dialkyl phosphorochloridates) or with phenyl dihydrogen phosphate [12] (giving, eventually, diphenyl dihydrogen pyrophosphate). The assumption throughout that the actual phosphorylating agent is protonated on nitrogen rests on the following evidence: (*a*) that in the case of adenosine-5′ hydrogen *N*-substituted phosphoramidates the reactivity increases with increasing basic strength of the parent amine; [3] (*b*) that diesters of phosphoramidic acid are relatively unreactive compared with the monoesters and phosphoramidic acid itself [this may be attributed to greater difficulty in protonation and, in addition, suggests that the first of the proposed mechanisms for the formation of compound (III) is to be preferred]; (*c*) that the observed solvent effect indicates that the transition state is more polar than that of compound (I) itself which exists in the non-zwitterion form both in the solid and in solution in organic solvents.

In view of the fact that syntheses with phosphoramidic acids are often conducted in the presence of pyridine and that this had been found to produce a definite acceleration in rate it was felt desirable to investigate further the nature of this catalysis. Accordingly the effects of pyridine, 2-picoline, 4-picoline and 2,6-lutidine (pK_a of conjugate acids in water 5·23, 5·97, 6·02,[13] and 6·9,[14] respectively) at the same concentration (0·5M) in dioxan solution were investigated. It was found that while pyridine and 4-picoline gave complete

[7] Todd, *Proc. Nat. Acad. Sci. U.S.A.*, 1959, **45**, 1389.
[8] Weimann and Khorana, *J. Amer. Chem. Soc.*, 1962, **84**, 4329.
[9] Clark, Hutchinson, Kirby, and Todd, *J.*, 1961, 715.
[10] Brown, Flint, and Hamer, *J.*, 1964, 326.
[11] Skrowaczewska and Mastalerz, *Roczniki Chem.*, 1955, **29**, 415.
[12] Richter, Ph.D. Thesis, Cambridge, 1961.
[13] Albert and Sergeant, "Ionisation Constants of Acids and Bases," Methuen, London, 1962, p. 145.
[14] Golumbic and Orchin, *J. Amer. Chem. Soc.*, 1950, **72**, 4145.

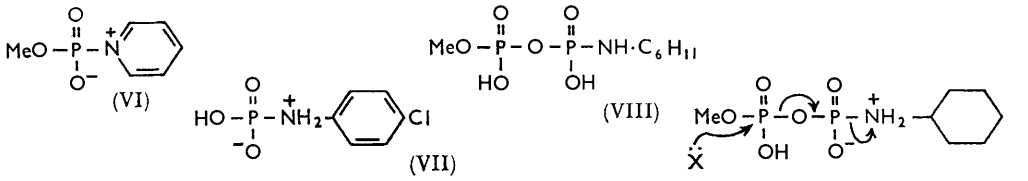
conversion into compound (III), with 2-picoline and 2,6-lutidine under the same conditions, the amounts of phosphorus present as compound (III) were 39% and 27%, respectively, compared with 47% in the absence of any base. Since the measured pK_a in water of compound (I) is 3·1 the suggested mechanism for the formation of compound (III) indicates that, in the absence of any direct participation by the base, the rate should decrease. The effect on the rate of the sterically hindered bases is therefore qualitatively explicable and the fact that it seems to be rather small is almost certainly a consequence of their decreased basic strength in dioxan. [This explanation is supported by the observation that compound (I) was stable in aqueous solution in the presence of a similar concentration of 2,6-lutidine.] The catalytic effect of pyridine and 4-picoline may be due to general base catalysis which is known, in certain cases, to be subject to steric hindrance, or it may involve the formation of an intermediate (VI). This latter explanation seems probable since it is well established that the monoanion of phosphoramidic acid phosphorylates amino-groups [15] and it has, in fact, been found that 1-naphthylamine displaces cyclohexylamine from compound (I). Further evidence to suggest that pyridine is involved in the phosphorylating agent is provided by the product distribution found when the solvolysis of compound (I) was conducted in aqueous ethanol (50 mole-%) in its presence. In this case 40% of the total phosphorus appeared as ethyl methyl hydrogen phosphate compared with 8% in the absence of pyridine.

It seems highly probable, therefore, that a compound of type (VI) is formed from compound (I) in the presence of pyridine but it cannot be assumed that the mechanism of phosphorylation by compound (VI) is necessarily similar to that of compound (I). Although no very positive evidence for the formation of methyl metaphosphate (IV) from compound (VI) has been obtained the intervention of such an intermediate certainly cannot be excluded. The product distribution formed from compound (I) in aqueous ethanol in the presence of pyridine indicates that the actual phosphorylating agent is relatively unspecific. Chanley and Feageson,[4] in their study of the product distribution formed in the solvolysis of *N*-*p*-chlorophenylphosphoramidic acid the zwitterion form (VII) of which might be expected to resemble compound (VI) in general reactivity, came to the conclusion that a metaphosphate ion intermediate was involved. Moreover, when compound (I) or the corresponding benzyl ester was heated at 100° in pyridine containing a little pyridine hydrochloride it was found that most of the phosphorus was converted into the cyclic trimetaphosphate ion. Since it is known with any certainty at which stage the dealkylation occurs this last observation does not provide any direct evidence of the formation of a metaphosphate ester (either monomer or trimer). What it does emphasise, however, is the close similarity between the reactions of compound (I) (and probably all compounds of this type) in pyridine solution and some other phosphorylation methods—notably the reaction of alkyl dihydrogen phosphates with dicyclohexyl carbodi-imide [8] and the oxidation of benzyl hydrogen phosphorohydrazidate,[10] both conducted in pyridine solution—which have been discussed in terms of metaphosphate ester intermediates. Both of these other phosphorylation methods also are characterised by the ability to phosphorylate monoesters of phosphoric acid almost quantitatively whereas the phosphorylation of alcohols is less satisfactory. It seems reasonable, therefore, to postulate that all these give rise to a common intermediate of the type (VI) which, in reaction with monoalkyl dihydrogen phosphates, react by a mechanism similar to that of compound (I) but with alcohols and other poor nucleophiles prefer to react by way of a metaphosphate ester intermediate.

Although none of the simple salts of compound (III) could be induced to crystallise the cyclohexylammonium salt [from compound (I)] was demethylated by treatment with sodium iodide in acetone to give the disodium salt of P^2-methyl dihydrogen P^1-*N*-cyclohexylpyrophosphoramidate (VIII). The reactions of this hitherto unreported class of

[15] Rathlev and Rosenberg, *Arch. Biochem. Biophys.*, 1956, **65**, 319.

compound are of some interest since although, by analogy with the monoesters of phosphoramidic acid, they should behave as pyrophosphorylating agents it is conceivable that,



under certain conditions, nucleophilic attack could occur in the manner shown (which would be of a general type recently discussed [16]) in which case they should act as phosphorylating agents. Preliminary experiments both here and elsewhere [17] indicate that such compounds normally behave as pyrophosphorylating agents. While the synthetic route to these compounds given here is not of general application it should provide a simple method for the preparation of the corresponding benzyl ester and hence to *N*-substituted pyrophosphoramidic acids.

Experimental

Paper chromatograms were run on Whatman No. 1 paper in propan-2-ol–ammonia–water (7 : 1 : 2). The amounts of phosphorus present in spots were estimated by using Usher's procedure.[18]

Methyl Hydrogen N-*Cyclohexylphosphoramidate.*—This was prepared by the method of Brown and Hamer [5] but for large quantities the following method is more convenient. Dimethyl *N*-cyclohexylphosphoramidate [19] (20·7 g.) was dissolved in warm methanol (60 ml.) and added to a hot solution of potassium hydroxide (11 g.) in water (30 ml.). The mixture was heated on a water-bath with vigorous stirring until a homogeneous solution was obtained and the heating continued for a further 2 hr. After being cooled the solution was brought to neutrality by the cautious addition of ethanolic hydrogen chloride (2N approx.) and the precipitation of potassium chloride completed by the addition of ethanol (200 ml.). The mixture was filtered, the filtrate evaporated to *ca.* 40 ml. and acetone (500 ml.) added. The potassium salt of the *product* crystallised out as plates (19·4 g.) (Found, in sample recrystallised from ethanol–acetone: C, 30·1; H, 7·6; N, 4·6. $C_7H_{15}KNO_3P,3H_2O$ requires C, 29·7; H, 7·4; N, 4·9%).

The free acid was obtained by treatment of this potassium salt (8·5 g.) in ethanol (30 ml.) at 0° with slightly less than the equivalent amount of ethanolic hydrogen chloride (freshly prepared and standardised) followed by the addition of dry ether (150 ml.) to precipitate all remaining potassium salts. The solution was dried ($MgSO_4$), evaporated at 0° *in vacuo* to a small volume and the viscous oil taken up in dry ether (40 ml.). Light petroleum (100 ml., b. p. 40—60) was added, and when the mixture was cooled the product separated as silky needles (4·2 g.) m. p. 91—92° which travelled as a single spot (R_F 0·70) on paper chromatograms (Found: Equiv. 194. Calc. 193).

Effect of Heat on Methyl Hydrogen N-*cyclohexylphosphoramidate.*—A solution of compound (I) (200—250 mg.) in dioxan or acetonitrile (10 ml.) was maintained at 70° in a thermostatted bath. At intervals 1 ml. portions were withdrawn, run into ice-cold distilled water (6 ml.), and titrated to pH 6·5 with 0·04N-sodium hydroxide solution. (It was first verified that, under these conditions, no further hydrolysis to methyl dihydrogen phosphate occurred.) Chromatography of the reaction mixture showed that, except for traces of dicyclohexylammonium dimethyl pyrophosphate (R_F 0·43), the product was a compound (R_F 0·75) which gave the characteristic pyrophosphate colour when the spot was developed. This compound was purified by graded elution with triethylammonium hydrogen carbonate solution from a Dowex 2 column but none of the salts obtained by replacing the cation could be obtained crystalline. On standing overnight in 10% sodium hydroxide solution it was degraded to methyl dihydrogen phosphate and compound (I) in equimolecular amounts.

[16] Clark, Hutchinson, Kirby, and Warren, *Angew. Chem.*, 1964, **76**, 704.
[17] Professor R. A. Smith, personal communication.
[18] Usher, *J. Chromatog.*, 1963, **12** (2), 262.
[19] Baumgarten and Setterquist, *J. Amer. Chem. Soc.*, 1959, **81**, 2132.

Disodium P^2-*Methyl* P^1-N-*Cyclohexylpyrophosphoramidate.*—A mixture of compound (I) (5·0 g.) and dioxan (3 ml.) was heated at 100° in a stoppered flask for 45 min. and then cooled, the gum taken up in dry acetone (15 ml.), and the solution made slightly alkaline by the addition of two drops of cyclohexylamine. After the mixture had stood overnight at 0° a small amount (200 mg.) of dicyclohexylammonium dimethyl pyrophosphate had separated and was filtered off. To the filtrate was then added a solution of sodium iodide (5·0 g.) dissolved in a minimal volume of dry acetone and the mixture refluxed for 8 hr. The solid which separated (3·2 g.) was washed well with acetone and recrystallised from ethanol–water to give the *product* (2·1 g.) as fine needles which gave a single spot (R_F, 0·55) on chromatograms (Found, in sample recrystallised twice: C, 24·4; H, 5·0; N, 4·0; P, 17·6. $C_7H_{15}NNa_2O_6P,2H_2O$ requires C, 23·9; H, 5·4; N, 4·0; P, 17·6%).

The corresponding barium salt crystallised when moderately concentrated solutions of the sodium salt and barium chloride were mixed.

Solvolysis of Methyl Hydrogen N-*Cyclohexylphosphoramidate.*—A solution of compound (I) (50 mg.) in ethanol–water (20 ml.; 76% ethanol v/v) was refluxed for 3 hr. then brought to pH 7 by the addition of cyclohexylamine. The solution was then concentrated to *ca.* 3 ml. and chromatographed, and the relative amounts of methyl dihydrogen phosphate and ethyl methyl hydrogen phosphate (the sole products) determined.

In another experiment pyridine (1 ml.) was added to the solution before the refluxing was carried out.

Reaction of Methyl Hydrogen N-*Cyclohexylphosphoramidate with Benzoic Acid.*—Benzoic acid (250 mg.) and compound (I) (98 mg.) in dry dioxan (3 ml.) was heated at 70° for 45 min. after which time the solution was cooled and cyclohexylamine (0·5 ml.) added. After 2 hr. at room temperature the mixture was analysed by paper chromatography and the relative amounts of phosphorus present in the various spots estimated. When the mixture was poured into water at 0° *N*-cyclohexylbenzamide crystallised, m. p. 144—146° (lit., 147°). In another experiment pyridine (0·4 ml.) was added and the mixture treated as before.

Effect of Added Bases on the Rearrangement of Methyl Hydrogen N-*Cyclohexylphosphoramidate.*—To 5 ml. portions of a solution of compound (I) (960 mg.) in dioxan (50 ml.) was added, from a burette, the appropriate base (2·5 mmole; dried and distilled) and the mixtures heated at 70° for 45 min. The relative amounts of phosphorus present as unchanged compounds (I) and (III) were then determined by paper chromatography (traces of dimethyl dihydrogen pyrophosphate were generally present but these were negligible).

Reaction of Methyl Hydrogen N-*Cyclohexylphosphoramidate with* 1-*Naphthylamine.*—To a solution of 1-naphthylamine (2·0 g.) in dioxan (10 ml.) was added compound (I) (400 mg.) and the mixture heated at 90° for 20 min. after which time water (10 ml.) and sodium hydroxide solution (10%; 1 ml.) were added. After extraction with ether (4 × 20 ml.) the aqueous extract showed, on paper chromatograms, the presence of a new phosphorus-containing spot (R_F, 0·85) which had a characteristic violet fluorescence in u.v. light. This compound was purified by graded elution from a column of diethylaminocellulose with ammonium hydrogen carbonate solution to give, after evaporation, the chromatographically pure ammonium salt (60 mg.). Treatment of this in water (1 ml.) with a few drops of 10% silver nitrate solution gave the crystalline silver salt which was identical with an authentic sample (prepared as described below).

Silver Methyl N-1-*Naphthylphosphoramidate.*—Dimethyl *N*-1-naphthylphosphoramidate was prepared by the general method of Atherton, Openshaw, and Todd [20] from dimethyl phosphite, 1-naphthylamine, and triethylamine in carbon tetrachloride. Recrystallised from chloroform–ether it formed prisms, m. p. 151—152° (Found: C, 57·7; H, 4·9; N, 5·7. $C_{12}H_{14}NO_3P$ requires C, 57·4; H, 5·6; N, 5·6%).

To a solution of this (1·25 g.) in methanol (8 ml.) was added a solution of potassium hydroxide (0·6 g.) in water (5 ml.) and the mixture was heated in a water-bath with vigorous stirring until a homogeneous solution was obtained. After being refluxed for a further hr. the mixture was cooled, brought to pH 6—7 with dilute sulphuric acid and extracted with ether (3 × 15 ml.). After evaporation of the aqueous extract the residue was taken up in warm ethanol, treated with a little animal charcoal, and filtered. After removal of the ethanol the sticky residue was taken up in water (8 ml.) and treated with a solution of silver nitrate (1·0 g.) in water (5 ml.). The amorphous precipitation which separated initially rapidly redissolved and was

[20] Atherton, Openshaw, and Todd, *J.*, 1945, 660.

followed, after 1 min. by the separation of the *product* as needles (0·5 g.) which were washed with water and dried *in vacuo* with the exclusion of light (Found: C, 36·1; H, 3·5; N, 4·1; Ag, 30·7. $C_{11}H_{11}AgNO_3P,H_2O$ requires C, 36·4; H, 3·5; N, 4·0; Ag, 31·4%).

Reaction of Methyl Hydrogen N-*Cylohexylphosphoramidate in Pyridine Solution.*—To an anhydrous solution of pyridine hydrochloride (0·5 g. approx.) in pyridine (10 ml.) was added compound (I) (200 mg.) and the mixture heated at 100° in a sealed flask for 3 hr. Analysis of the solution by paper chromatography both in the solvent system of Ebel [21] and one consisting of propan-2-ol–water (1 : 1) containing 2% of perchloric acid (60%) [22] showed that the major constituent was the cyclic trimetaphosphate anion identified by comparison with an authentic sample of the sodium salt.

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[21] Ebel, *Bull. Soc. chim. France*, 1953, 991.
[22] Dr. A. J. Kirby, personal communication.