56. *Studies in Phosphorylation. Part XXIV.*[1] *The Use of Phosphorohydrazidates as Phosphorylating Agents.*

By D. M. Brown, J. A. Flint, and N. K. Hamer.

When phenyl or benzyl hydrogen phosphorohydrazidate is oxidised by iodine or *N*-bromosuccinimide it gives an intermediate which phosphorylates alcohols and phosphate anions. In the latter case high yields of unsymmetrical pyrophosphates can be obtained. The mechanisms of these reactions are discussed.

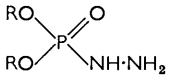
The phosphorohydrazidates, under non-oxidative conditions, show reactions comparable with those of phosphoroamidates.

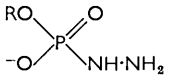
In recent years a number of phosphorylating systems have been devised which have, as their objective, the formation of phosphate and pyrophosphate diesters from monoalkyl phosphates under mild conditions. The problem of pyrophosphate synthesis has been largely solved but the phosphorylation of alcohols still presents difficulties in that the reactions involved are slow and often incomplete. It was hoped that a method might be evolved based on the oxidation of phosphorohydrazidates that would generate a powerful reagent under mild conditions. We have reported briefly that the oxidation of phosphorohydrazidates with iodine in pyridine showed promise as a phosphorylation method.[2] In the meantime other workers have found that oxidation of amino-acid hydrazides may be useful in the synthesis of polypeptides;[3] earlier papers, too, record conversion of carboxy- and sulphon-hydrazides into the corresponding chlorides by chlorine.[4,5]

It is our purpose here to give a general outline of the chemistry of the hydrazidates, particularly emphasising those reactions which are likely to be useful in phosphorylation procedures. As might be expected, the fully esterified phosphorohydrazidates (I) may be prepared in the same way as phosphoramidates and are thus relatively accessible. Treatment of a dialkyl phosphite with hydrazine and carbon tetrachloride,[6] or reaction of the appropriate phosphorochloridate with ethanolic or aqueous hydrazine,[7,8] generally give high yields. The latter method is easily adapted to the preparation of monoalkyl phosphorohydrazidates (II) since the necessary alkyl phenyl phosphorochloridates are readily prepared. The monobenzyl[6] and monophenyl[7] phosphorohydrazidates have already been described and other monoalkyl esters may be prepared by alkaline hydrolysis of the mixed alkyl phenyl esters, which proceeds rapidly. They may be simply isolated

[1] Part XXIII, Clark, Hutchinson, and Todd, *J.*, 1961, 722.
[2] Brown and Hamer, *Proc. Chem. Soc.*, 1960, 212.
[3] Wolman, Gallop, and Patchornik, *J. Amer. Chem. Soc.*, 1961, **83**, 1263.
[4] Carpino, *J. Amer. Chem. Soc.*, 1957, **79**, 96.
[5] Davies, Storrie, and Tucker, *J.*, 1931, 624.
[6] Klement and Knollmüller, *Chem. Ber.*, 1960, **93**, 834.
[7] Ephrain and Sackheim, *Ber.*, 1911, **44**, 3416.
[8] Audrieth, Gher, and Smith, *J. Org. Chem.*, 1955, **20**, 1288.

as their stable sparingly soluble zwitterions (III). Unlike the corresponding monoesters of phosphoramidic acid, these monoesters are not readily made by condensing a monoalkyl phosphate with hydrazine by use of dicyclohexylcarbodi-imide.[9] So far as the


(I)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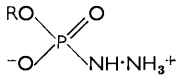
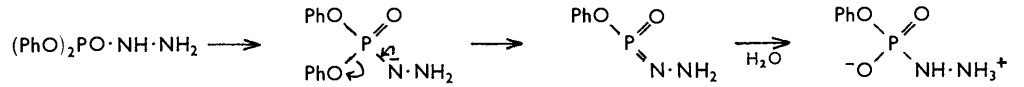

(III)

properties of the full esters are concerned, we notice that the diphenyl and dibenzyl phosphorohydrazidates are less stable than the corresponding phosphoramidates and decompose over a period of several months. The former gives phenol and the zwitterion



(III; R = Ph), while the latter gives a number of products which include dibenzylpyrophosphate, but not benzyl phosphorohydrazidate. The lability of the diphenyl ester is almost certainly due to the fact that the hydrazide group is basic and in the presence of moisture breakdown could occur by direct displacement of phenoxide by hydroxide ion or by the mechanism shown. The latter mechanism is analogous to the elimination of metaphosphate, and a closely related process has been observed in the hydrolysis of *N*-methylaminophosphorochloridates (IV).[10] Although it is not possible to estimate the importance of this pathway in the hydrolysis of diphenylphosphorohydrazidate itself, it is probable that it predominates in the hydrolysis of the esters (V) [8] and (VI) in which the other nitrogen atom is acylated. Both these compounds are hydrolysed extremely

(IV) $(Me\cdot NH)_2PO\cdot Cl$ $(PhO)_2PO\cdot NH\cdot NH\cdot PO(OPh)_2$ (V)

(VI) $Ph\cdot CH_2CO\cdot NH\cdot NH\cdot PO(OPh)_2$ $Ph\cdot CH_2CO\cdot NH\cdot NMe\cdot PO(OPh)_2$ (VII)

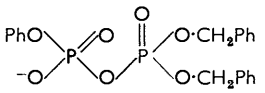
rapidly; one mol. of phenol is eliminated in less than five minutes in aqueous methanol, at pH 6, at room temperature. On the other hand, compound (VII) is stable under these conditions, providing strong support for the view that the elimination reaction is, in fact, occurring with compounds (V) and (VI).

As the hydrazide group is much more basic than the amide group it is not surprising that the zwitterionic forms of the monophenyl and monobenzyl esters are much more stable, and far less soluble in organic solvents, than the corresponding phosphoramidic acids. The apparent pK_a of the monophenyl compound in water is 5·6. Although these zwitterions dissolve in water and alcohol in the presence of organic bases the salts, with the exception of the cyclohexylamine salt, are unstable in the solid state and we have since found that the 1-ethylpiperidinium salt referred to in our previous Note was in fact the zwitterion. It is of interest that, while monophenyl hydrogen phosphorohydrazidate is hydrolysed to free phosphorohydrazidic acid by vigorous treatment with sodium hydroxide,[6,7] in dilute ammonia solution (during chromatography) hydrazine is liberated slowly and monophenyl dihydrogen phosphate is formed. Clearly, in the latter case, we are observing the hydrolysis of the small proportion of the zwitterion in equilibrium with the anion.

The reactions of phosphorohydrazidates with monoesters of phosphoric acid show, as might be expected, a close resemblance to those of the corresponding phosphoramidates.[11] Thus refluxing diphenyl or dibenzyl phosphorohydrazidate with a monoalkyl (or aryl) dihydrogen phosphate gave quite good yields (50—70%) of the symmetrical dialkyl

[9] Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," Wiley, New York, 1961.
[10] Grunden and Hudson, *Chem. and Ind.*, 1958, 1478; Samuel and Westheimer, *ibid.*, 1959, 51.
[11] Clark, Kirby, and Todd, *J.*, 1957, 1497.

(or diaryl) dihydrogen pyrophosphate (a similar result was observed by Richter [12] when phenyl dihydrogen phosphate was allowed to react with dibenzyl phosphoramidate under similar conditions). It seems probable that the intermediate is the mixed triester of pyrophosphoric acid, *e.g.*, (VIII) which then is attacked by a further molecule of the phosphate monoester.



(VIII)

The monoesters of phosphorohydrazidic acid in their zwitterionic form also react readily with monoalkyl dihydrogen phosphates, to give nearly quantitative yields of the mixed P^1P^2-diester of pyrophosphoric acid. In contrast with the corresponding phosphoramidic acids the zwitterionic forms do not rearrange smoothly to the symmetrical pyrophosphate ester in hot dioxan. In these conditions the phenyl compound gives no trace of hydrazinium diphenyl pyrophosphate but does undergo slight hydrolysis to monophenyl dihydrogen phosphate. The corresponding benzyl compound, on the other hand, does give dibenzyl dihydrogen pyrophosphate although considerable qualities of monobenzyl dihydrogen phosphate were also detected. These observations may be ascribed to a need for further protonation of the zwitterion before attack by a phosphate anion can occur; the difference noted between the phenyl and the benzyl ester may simply be a reflection of the lower solubility of the former.[6]

The oxidation of diphenyl and dibenzyl phosphorohydrazidates with iodine in aqueous pyridine to give quantitative yields of the corresponding phosphoric acids has already been reported.[2] It has since been found that *N*-chloro- and *N*-bromo-succinimide are equally effective and may be preferable for benzyl esters since they offer less chance of debenzylation. Even so, the oxidation of these diesters in pyridine in the presence of stoicheiometric amounts of an alcohol gave poor yields of the appropriate phosphate triester (possibly due to dealkylation). When the oxidation was carried out in the presence of phosphoric acid monoesters moderate yields of the symmetrical pyrophosphate diesters were obtained.

Oxidation of sodium phenyl phosphorohydrazidate hemihydrate or the corresponding benzyl compound in pyridine gave almost quantitative yields of the appropriate symmetrical pyrophosphate diester. However, when the zwitterionic form of benzyl hydrogen phosphorohydrazidate was oxidised in anhydrous pyridine only traces of dibenzyl dihydrogen pyrophosphate were produced, most of the phosphorus appearing (in the chromatographic solvent system used) as slow-running compounds suggestive of partially debenzylated polyphosphates. When the reaction solution was heated at 100° for 30 minutes before chromatography, to effect more complete debenzylation of the intermediate, the bulk of the phosphorus appeared as the cyclic trimetaphosphate ion (identified by comparison with an authentic sample). In the presence of monoalkyl or monoaryl dihydrogen phosphates oxidation of these phosphorohydrazidates gives almost quantitative yields of the mixed pyrophosphate diester in an extremely rapid reaction. Isolated yields of the various pyrophosphates are shown in Table 1. In the case of thymidine-5′

TABLE 1.

R in $RO{\cdot}PO(OH)_2$	Phosphorohydrazidate		Mixed pyrophosphate (%)
Ph	Ph	anhydrous	89
p-C_6H_4Cl	Ph	Na salt	82
Ph	$Ph{\cdot}CH_2$	zwitterion	75
Thymidine-5′	Ph	zwitterion	74

phosphate, two mol. of the phosphorohydrazidate were used and it was observed that 15% of dithymidine-5′ pyrophosphate was also produced.

[12] See Todd, *Proc. Chem. Soc.*, 1962, 199.

The phosphorylation of alcohols, however, is less successful unless the alcohol is present in large excess. Yields for stoicheiometric quantities are shown in Table 2. These yields

TABLE 2.

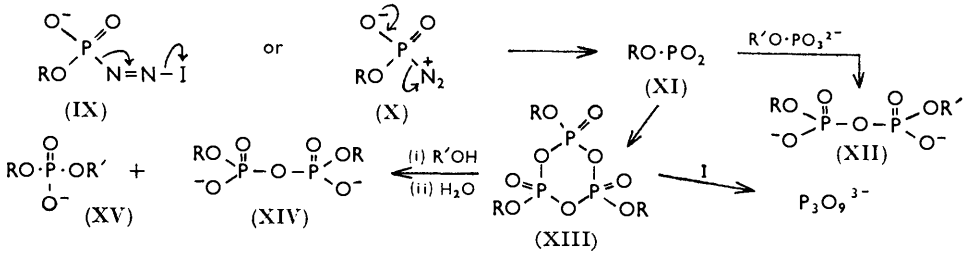
Alcohol	R in RO·(HO)PO·NH·NH_2	Oxidant	Yield (%) of diester (isolated)
Cyclohexanol	Ph (1 mol.)	Iodine	21
EtOH	Ph (1·5 mol.)	Iodine	50
Thymidine	Ph (2 mol.)	*N*-Bromosuccinimide	65
Ph·CH_2·OH	Ph (1 mol.)	Iodine	10
Ph·CH_2·OH	Ph·CH_2 (1 mol.)	Iodine	10
Ph·CH_2·OH	Ph·CH_2 1 mol.)	*N*-Bromosuccinimide	25

should be regarded as minimal since the methods of isolation (except in the case of thymidine) were far from quantitative. In the case of thymidine it was established that phosphorylation occurred at the 5′-position. The rather low yields together with the production of considerable amounts of the symmetrical dibenzyl and diphenyl dihydrogen pyrophosphates might, in part, be due to traces of water in the reaction mixture, but we shall show that an alternative explanation is more probable.

Although it is not yet possible to provide a complete description of the reaction mechanism for the diester phosphorohydrazidates the stoicheiometry and the nature of the products point to a phosphorohalidate as the intermediate phosphorylating species.

The reaction of the monoesters of phosphorohydrazidic acid requires rather more discussion. If we postulate as the initial product either (IX) or (X), these may be attacked by a nucleophile in the solution or may alternatively eliminate nitrogen in the manner indicated to give a metaphosphate ester (XI). We favour an explanation involving the initial formation of a metaphosphate ester,[12] the fate of the latter being dependent on the reactivity of other nucleophilic species present.

First, it accounts for the formation of trimeric metaphosphate anion when benzyl hydrogen phosphorohydrazidate is treated with iodine in pyridine and the solution subsequently heated. It seems probable that the trimetaphosphate is formed by debenzylation of the triester (XIII), although it is possible that debenzylation of the monomeric species followed by trimerisation occurs. In this connection we have argued that the



metaphosphate anion, PO_3^-, when formed in absence of hydroxylic compounds can trimerise.[13] Secondly, formation of a metaphosphate ester provides an explanation for the anomalously large quantities of diphenyl dihydrogen pyrophosphate formed when alcohols are phosphorylated with phenyl hydrogen phosphorohydrazidate and an oxidising agent. Thus the low reactivity of the alcohol would allow for essentially complete trimerisation of the metaphosphate to (XIII; R = Ph). Attack by alcohol with ring opening and subsequent hydrolysis during working up would then lead to the observed products (XIV) and (XV).

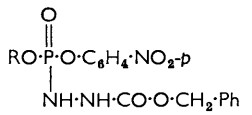
Finally the mechanism is consistent with those postulated for related reactions in which an alkyl dihydrogen phosphate is activated to phosphorylate alcohols and phosphate anions. In this connection we note that Weimann and Khorana [14] have observed a parallel

[13] Brown and Hamer, *J.*, 1960, 1155.
[14] Weimann and Khorana, *J. Amer. Chem. Soc.*, 1962, **84**, 4329.

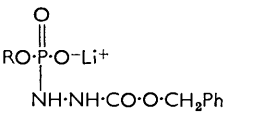
series of products in the reaction of dicyclohexylcarbodi-imide with alkyl phosphates and have, for example, isolated triethyl trimetaphosphate when compounds capable of phosphorylation were absent.

At present it is not possible to state unambiguously whether the monomeric or the trimeric alkyl metaphosphate is the important phosphorylating species. We tend to the view that the rapid and nearly quantitative reactions with strong nucleophiles such as alkyl phosphate anions involve the monomeric form, whereas with less reactive alcoholic hydroxyl groups it is probable (and reasonably consistent with our experimental findings) that the trimer is more important.

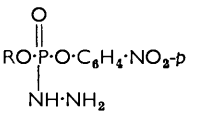
Since some degree of success had been achieved in phosphorylating hydroxyl groups of both simple alcohols and a nucleoside, it was considered that the oxidation of a nucleoside phosphorohydrazidate might yield oligonucleotides. This end has not been reached. When 3′-acetylthymidine was treated with *p*-nitrophenyl phosphorodichloridate followed by benzyl carbazate, the nucleoside derivative (XVI; R = 3′-acetylthymidine-5′) was



(XVI)



(XVII)



(XVIII)

obtained from which the nitrophenyl and acetyl groups could be removed by base, forming the salt (XVII; R = thymidine-5′). Unfortunately, although hydrogenolysis of this gave some of the desired thymidine-5′ phosphorohydrazidate detected on chromatograms, the major product was thymidine-5′ phosphate. As an alternative, 5′-tritylthymidine was treated with nitrophenyl phosphorochloridate and then with hydrazine. The product (XVIII; R = 5′-tritylthymidine-3′) was treated with aqueous acetic acid to remove the trityl group, but the mildest conditions used also led to loss of the hydrazine residue. Attempts were made to phosphorylate cyclohexanol by the ester (XVIII; R = 5′-trityl-thymidine-3′) and *N*-bromosuccinimide, but without success.

Experimental

Paper chromatograms and electrophoretograms were run on Whatman No. 1 paper. Solvent systems were: A, propan-2-ol–ammonia–water (7 : 2 : 1); and B, butan-1-ol–acetic acid–water (5 : 3 : 2). Phosphorohydrazidates were detected by spraying chromatograms with ammoniacal silver nitrate.

Diphenyl N^2-*Benzyloxycarbonylphosphorohydrazidate.*—To a solution of diphenyl phosphorochloridate (8·11 g.) and diethylaniline (5 ml.) in dry ether (50 ml.) was added a solution of benzyl carbazate (5·0 g., 1 mol.) in dry ether (100 ml.). When the mixture was set aside overnight a solid (3·4 g.) separated and it was collected. The mother-liquors were concentrated and a further quantity (10·85 g.) of the product was collected. Recrystallisation from ethanol gave the *product* as needles (7·5 g., 64%), m. p. 137—139° (Found: C, 60·3; H, 5·2; N, 7·4. $C_{20}H_{19}N_2O_5P$ requires C, 60·3; H, 4·8; N, 7·0%).

Diphenyl N^2-*Phenylacetylphosphorohydrazidate.*—Phenylacethydrazide (3·0 g.) was added to a solution of diphenyl phosphorochloridate (5·4 g.) in dry dioxan (30 ml.), and to this was added dry pyridine (4 ml.), whereupon the hydrazide dissolved and the solution became warm. After 4 hr. at room temperature the solution was poured into stirred 0·5N-hydrochloric acid (400 ml.) at 0°. The oil which was precipitated crystallised within 5 min. and was then collected, washed with cold water (25 ml.), and immediately dried *in vacuo*. The crude *product* (7·1 g.) had m. p. 106—110° and was recrystallised twice from methanol–water to which a few drops of concentrated hydrochloric acid had been added (this was necessary in order to prevent extensive hydrolysis with loss of phenol). It formed fine needles (3·4 g.), m. p. 116—117° (Found, in material dried at 0·1 mm. for 48 hr. at room temp.: C, 62·3; H, 5·1; N, 7·5; P, 7·9. $C_{20}H_{19}N_2O_4P$ requires C, 62·8; H, 5·0; N, 7·3; P, 8·1%).

Diphenyl N^1-*Methyl*-N^2-*phenylacetylphosphorohydrazidate.*—Phenylacethydrazide (1·0 g.) was heated at 50° for 1 hr. with dimethyl sulphate (0·85 g.) in dry dioxan (10 ml.). The resulting

solution was added to diphenyl phosphorochloridate (1·8 g.) in dry dioxan (10 ml.), followed by dry pyridine (3 ml.). The solution was worked up as above, to give the crude product as a slightly yellow solid (2·0 g.). This was placed in 2% sodium hydrogen carbonate solution (100 ml.) in order to hydrolyse unmethylated material, after which the *product* (0·8 g.) was filtered off. Recrystallisation from chloroform–light petroleum (b. p. 60—80°) gave colourless crystals (0·45 g.), m. p. 133—134° (Found: C, 63·6; H, 5·2; N, 7·2. $C_{21}H_{21}N_2O_4P$ requires C, 63·6; H, 5·3; N, 7·1%).

Sodium Phenyl Phosphorohydrazidate.—Diphenyl phosphorohydrazidate (5·4 g.), dissolved in propan-2-ol (10 ml.), was added to a hot solution of sodium hydroxide (1·6 g. in 10 ml.). The mixture was maintained at 100° for 15 min. in a stoppered flask and then a further 80 ml. of propanol were added. The sodium salt crystallised on cooling. It formed needles (4·0 g.) of the *hemihydrate* which were recrystallised from propan-2-ol–water (Found, in material dried at room temp. over P_2O_5 *in vacuo*, or in air: C, 33·0; H, 4·2. $C_6H_8N_2NaO_3P,0{\cdot}5H_2O$ requires C, 32·9; H, 4·1%). Drying at 95° yielded the anhydrous compound (Found: C, 34·3; H, 3·8. Calc. for $C_6H_8N_2NaO_3P$: C, 34·3; H, 3·8%).

Cyclohexylammonium Phenyl Phosphorohydrazidate.—The diphenyl ester (5·4 g.) was boiled under reflux with cyclohexylamine (5 ml.) and water (10 ml.), together with sufficient ethanol to ensure complete solution. After 1 hr. the solvent was completely removed under reduced pressure and the residual *salt* crystallised from ethanol–ether (Found: C, 50·0; H, 8·0; N, 15·2. $C_{12}H_{22}N_3O_3P$ requires C, 50·2; H, 7·7; N, 14·7%).

Phenyl Hydrogen Phosphorohydrazidate.—This was prepared according to the general method of Klement and Knollmüller [6] and had m. p. 194—195°. It was essentially stable when refluxed with dioxan, dry or containing water (1 mol.), for 5 hr. After 5 min. a sample was withdrawn and submitted to paper chromatography. A small amount of phenyl phosphate was present (R_F 0·26 in solvent A) in addition to the hydrazidate (R_F 0·50). The same substances were present after 30 min. and 5 hr. No diphenyl dihydrogen pyrophosphate was detected.

Dibenzyl Phosphorohydrazidate.—(*a*) Dibenzyl phosphite (5·25 g.) was dissolved in dry benzene (40 ml.), and *N*-chlorosuccinimide (2·8 g.) added. The mixture was kept for 1 hr. at room temperature, then the succinimide was filtered off and the bulk of the benzene removed *in vacuo*. The resulting phosphorochloridate was added with stirring to an ice-cold solution of hydrazine hydrate (10 ml.) in ethanol (50 ml.). After 10 min., water (100 ml.) was added and the product extracted in chloroform. The extract was dried, the chloroform removed, and the resulting oil crystallised from a small volume of ether. A further recrystallisation gave the product (3·5 g.) in leaflets, m. p. 50—51°. A subsequent preparation gave needles, m. p. 73—74°.

(*b*) When prepared by the method of Klement and Knollmüller,[6] the product had m. p. ranging from 69·5° to 75°. This is a case of dimorphism since the isopropylidene derivatives were identical and had m. p. and mixed m. p. 109°.

Benzyl Phenyl Phosphorohydrazidate.—Phenyl phosphorodichloridate (4·2 g.; freshly distilled) was dissolved in dry ether (20 ml.), and dry pyridine (1·65 ml.) added. After the solution had been cooled to 0°, benzyl alcohol (2·1 g.) in ether (10 ml.) was added dropwise with stirring. The mixture was maintained at 0° for 2 hr. and then transferred to a cold stirred solution of 64% hydrazine (6 ml.) in ethanol (50 ml.). After 3 min. chloroform (100 ml.) and 10% sodium chloride solution (50 ml.) were added. The chloroform layer was separated, washed with water (2 × 40 ml.), and dried ($MgSO_4$). Removal of solvent gave a viscous oil which crystallised on addition of ether to give the *product* (3·9 g.), m. p. 73—74°. Recrystallised from chloroform–ether it had m. p. 75—76° (Found, in material dried at 0·1 mm. for 48 hr.: C, 56·2; H, 5·5; N, 10·0; P, 11·3. $C_{13}H_{15}N_2O_3P$ requires C, 56·1; H, 5·4; N, 10·0; P, 11·2%).

Benzyl Hydrogen Phosphorohydrazidate.—To a hot solution of sodium hydroxide (0·8 g.) in water (7 ml.) was added a solution of the above phenyl ester (2·78 g.) in propan-2-ol (10 ml.), and the mixture was maintained at 100° for 5 min. (The sodium salt hemihydrate may be isolated as for the corresponding phenyl compound at this stage.) The propanol was removed under reduced pressure and the solution diluted to 10 ml. with water and then cooled to 0°. Acetic acid (2 ml.) was added and after 30 min. at 0° the colourless crystalline product was collected. It was washed with ice-cold water (2 × 2 ml.) and then ether. The benzyl hydrogen phosphorohydrazidate (1·45 g.) had m. p. 144—146°. It could not be recrystallised satisfactorily from any solvent without considerable decomposition and was very insoluble in most

organic solvents (Found, in material dried at 70°/0·1 mm. for 48 hr.: C, 41·7; H, 5·2; N, 13·9. Calc. for $C_7H_{11}O_3N_2P$: C, 41·6; H, 5·4; N, 13·8%).

Considerable difficulty was experienced in repeating the preparation of this substance by catalytic hydrogenolysis following Klement and Knollmüller's directions. A variety of paper chromatographically distinguishable products was formed, including the desired hydrazidate. Hydrogenolysis in presence of cyclohexylamine (2 mol.) over 10% palladium–charcoal at room temperature, however, gave moderate yields of the required compound, isolated most conveniently as the zwitterion.

Reaction of Benzyl Hydrogen Phosphorohydrazidate with Iodine.—The anhydrous phosphorohydrazidate (100 mg.) was added to a solution of iodine (250 mg.; dried over P_2O_5) in anhydrous pyridine (3 ml.). Nitrogen was evolved and the solution became warm, although if the mixture was kept at 0° nitrogen evolution was extremely slow. After 2 hr. at room temperature chromatography of the solution in solvent A showed that most of the phosphorus was present in slow-running compounds (R_F 0·0—0·2), together with small amounts of benzyl dihydrogen phosphate and dibenzyl dihydrogen pyrophosphate. If, however, the pyridine solution were heated at 100° for 30 min. to effect debenzylation of fully esterified substances it was found, by using Ebel's chromatographic system,[15] that ~80% of the phosphorus appeared as cyclic trimetaphosphate, identified by comparison with an authentic sample.

Reaction between Phenyl Dihydrogen Phosphate and Phenyl Hydrogen Phosphorohydrazidate.—Phenyl dihydrogen phosphate (174 mg.) and phenyl hydrogen phosphorohydrazidate (188 mg., 1 mol.) were refluxed together in dry dioxan (5 ml.) for 5 min. Paper chromatography showed that in less than this time the reaction had gone to completion. The dioxan was evaporated under reduced pressure, the product was taken up in warm water, and cyclohexylamine (0·5 ml.) was added. After 5 hr. at 0°, the fine needles of dicyclohexylammonium P^1P^2-diphenyl pyrophosphate (449 mg.; 85%) separated. It had m. p. 255—260° and an infrared spectrum identical with that of an authentic sample. Recrystallisation raised the m. p. to 257—261°.

In a similar reaction between *p*-chlorophenyl dihydrogen phosphate [16] (1 mmole) and phenyl hydrogen phosphorohydrazidate (1 mmole) during 15 min., paper chromatography showed quantitative conversion to P^1-*p*-chlorophenyl P^2-phenyl dihydrogen pyrophosphate (R_F in solvent A, 0·72). No diphenyl dihydrogen pyrophosphate (R_F 0·68) was present. The recrystallised dicyclohexylammonium salt formed platelets (202 mg., 36%), m. p. 265—269° (lit.,[17] 262°).

When benzyl dihydrogen phosphate (1 mmole) and phenyl hydrogen phosphorohydrazidate (1 mmole) were heated under reflux in pyridine (5 ml.) for 5 min. and the solution worked up as usual, dicyclohexylammonium P^1-benzyl P^2-phenyl dihydrogen pyrophosphate was obtained as platelets (309 mg.; 57%), m. p. 224—228°. It showed one spot on paper electrophoresis and on recrystallisation had m. p. 234—238°. The reaction proceeded satisfactorily, too, in dioxan to give a single product in high yield (Found: C, 54·9; H, 7·7; N, 5·0. $C_{25}H_{40}N_2O_7P_2$ requires C, 55·3; H, 7·4; N, 5·2%). This compound had been prepared earlier in this Laboratory by Dr. M. F. Stempien, by the phosphoramidate method and had m. p. 225°.

Reaction between Phenyl Dihydrogen Phosphate and Diphenyl Phosphorohydrazidate.—The diphenyl ester (264 mg., 1 mol.) and phenyl dihydrogen phosphate (348 mg., 2 mol.) were suspended in dry dioxan (5 ml.) and heated under reflux with exclusion of moisture. After 2 hr. solvent was removed under reduced pressure, the product dissolved in ethanol, and cyclohexylamine (2 ml.) added. The product separated and after 4 hr. at 0° it was collected (742 mg.). The solid was treated with a small amount of hot ethanol and filtered off. The insoluble material (352 mg., 67%) had an infrared spectrum identical with that of dicyclohexylammonium P^1P^2-diphenyl dihydrogen pyrophosphate. Recrystallised from water it had m. p. 259—261°.

From the filtrate there was obtained by fractional crystallisation cyclohexylammonium diphenyl phosphate (122 mg., 35%), m. p. 192—194°, and dicyclohexylammonium phenyl phosphate (74 mg.).

Likewise, benzyl dihydrogen phosphate (307 mg., 1·64 mmoles) and dibenzyl phosphorohydrazidate (244 mg., 0·83 mmole) were refluxed in dioxan (5 ml.) for 1 hr. Working up gave a cyclohexylammonium salt (361 mg.), m. p. 215—220°, whose infrared spectrum was practically

[15] Ebel, *Bull. Soc. chim. France*, 1953, 991.
[16] Maguire and Shaw, *J.*, 1953, 1479.
[17] Cramer and Wittmann, *Chem. Ber.*, 1961, **94**, 328.

identical with that of authentic dicyclohexylammonium P^1P^2-dibenzyl dihydrogen pyrophosphate. Chromatography showed that a trace of benzyl dihydrogen phosphate was also present. Recrystallisation from water gave the pure pyrophosphate, in needles (187 mg., 41%), m. p. 221—225°, which showed one spot (R_F 0·67 in A) on chromatography.

A similar experiment, in which *p*-chlorophenyl dihydrogen phosphate (2·0 mmoles) and dibenzyl phosphorohydrazidate (1·0 mmole) were heated together in dioxan (5 ml.) for 40 min. and worked up in the usual way, gave dicyclohexylammonium P^1P^2-di-*p*-chlorophenyl dihydrogen pyrophosphate (48%), m. p. 261—263°. It was identical with an authentic specimen in infrared spectrum and on chromatography.

Reaction of Diphenyl and Dibenzyl Phosphorohydrazidate with Iodine.—To a solution of iodine (508 mg., 2·0 mol.) in 50% aqueous pyridine (10 ml.) was added the appropriate hydrazidate, until the colour of the iodine was discharged. During the vigorous reaction, nitrogen was evolved and the mixture became warm. It was found that 136 mg. (1·03 mol.) of the diphenyl and 148 mg. (1·01 mol.) of the dibenzyl ester were required to discharge the colour. Chromatography showed complete conversion into diphenyl or dibenzyl phosphate as the sole product.

Oxidation of Sodium Phenyl Phosphorohydrazidate by Iodine.—Iodine (1·0 g.) was dissolved in dry pyridine (20 ml.), and sodium phenyl phosphorohydrazidate hemihydrate (0·4 g.) added. After 30 min. at room temperature a little aqueous hydrazine was added to remove the slight excess of iodine. Chromatography showed that P^1P^2-diphenyl dihydrogen pyrophosphate was the sole phosphorus-containing compound present. It was isolated as the dicyclohexylammonium salt which formed needles (400 mg.), m. p. 265° (decomp.).

Reaction of Cyclohexanol, Iodine, and Diphenyl Phosphorohydrazidate.—Freshly distilled cyclohexanol (158 mg., 1·58 mmoles) and iodine (760 mg., 3·0 mmoles) were dissolved in dry pyridine (5·0 ml.). Diphenyl phosphorohydrazidate (398 mg., 1·51 mmoles) in dry pyridine (5 ml.) and dry dioxan (2·0 ml.) were added to the stirred solution during 30 min. After a further 30 min. solvents were removed under reduced pressure and the resulting oil treated with dilute sodium hydroxide solution (5 ml.) at 90—100° for 15 min. The solution was acidified with hydrochloric acid and extracted with chloroform (3 × 20 ml.), the extracts were dried and evaporated to 10 ml., and cyclohexylamine was added. The crystalline product (148 mg.) yielded, on fractionation, the *cyclohexylammonium* salts of diphenyl phosphate (70 mg.) and of *cyclohexyl phenyl phosphate* (70 mg., 13%). The latter had m. p. 205—206° (Found, in material dried at 100°/0·5 mm. over P_2O_5: C, 60·6; H, 8·3. $C_{18}H_{30}NO_4P$ requires C, 60·9; H, 8·4%).

Reaction of Phenyl Dihydrogen Phosphate, Iodine, and Diphenyl Phosphorohydrazidate.—Phenyl dihydrogen phosphate (348 mg., 2·0 mol.) and iodine (508 mg., 2·0 mol.) were dissolved in dry pyridine (7 ml.) and to the gently swirled solution was added diphenyl phosphorohydrazidate (264 mg., 1·0 mol.) in pyridine (5 ml.) during 15 min. After 2 hr. the solution was worked up as usual, affording cyclohexylammonium salts (346 mg.). Chromatography showed that diphenyl phosphate and P^1P^2-diphenyl dihydrogen pyrophosphate were present. Trituration with ethanol left dicyclohexylammonium diphenyl pyrophosphate (182 mg., 35%), m. p. 252—255°, having an infrared spectrum identical with that of an authentic specimen.

Reaction of Phenyl Dihydrogen Phosphate, Sodium Phenyl Phosphorohydrazidate, and Iodine.—The phenyl phosphate (1·0 mmole), and iodine (2·0 mmole) were dissolved in dry pyridine (5 ml.), and the anhydrous sodium salt (1·0 mmole) was added. After nitrogen evolution had ceased the solution was heated at 100° for 15 min. Pyridine was removed *in vacuo* and the product, in water, was treated with cyclohexylamine. The cooled solution deposited dicyclohexylammonium P^1P^2-diphenyl pyrophosphate (467 mg.; 89%), m. p. 246—252°, raised to 258—261° by one recrystallisation.

Reaction of Benzyl Hydrogen Phosphorohydrazidate, Iodine, and Phenyl Phosphoric Acid.—A procedure similar to the above was followed with phenyl dihydrogen phosphate acid (260 mg.), benzyl hydrogen phosphorohydrazidate (300 mg.), and iodine (760 mg.) except that the solution was maintained at 20° during addition of the hydrazidate. After working up as before, there was obtained dicyclohexylammonium P^1-benzyl P^2-phenyl dihydrogen pyrophosphate (580 mg.), m. p. 232—235°, chromatographically identical with the authentic sample prepared as above.

Reaction of p-*Chlorophenyl Dihydrogen Phosphate, Sodium Phenyl Phosphorohydrazidate, and* N-*Bromosuccinimide.*—The *p*-chlorophenyl phosphate (1·0 mmole) and the sodium salt

(1·0 mmole) were suspended in dry pyridine (10 ml.), and *N*-bromosuccinimide (4 mmole) was added in small quantities, with shaking. Pyridine was removed and the product worked up in the usual way. Dicyclohexylammonium P^1-*p*-chlorophenyl P^2-phenyl pyrophosphate (460 mg., 82%) was obtained; it had m. p. 240°, raised to 265° by one recrystallisation. Before and after recrystallisation, the material showed only one phosphorus-containing spot on chromatography, with R_F 0·72 in system *A*, identical with that from an authentic specimen. A trace, only, of diphenyl dihydrogen pyrophosphate was present in the reaction mixture.

Reaction of Thymidine-5′ Phosphate, Phenyl Hydrogen Phosphorohydrazidate, and N-*Bromosuccinimide.*—Pyridinium thymidine-5′ phosphate (23·4 mg.) was dried by evaporation with pyridine and dissolved in the same solvent (2 ml.). The phosphorohydrazidate (17·5 mg., 2 mol.) and then *N*-bromosuccinimide (70 mg.) were added with swirling. The solution evolved nitrogen and became warm and yellow. After some time the solvent was removed and the product worked up by column chromatography on ECTEOLA-cellulose (chloride form) with gradient elution (0—0·2M-LiCl). The major peak contained material with $\lambda_{max.}$ 267 mμ, which constituted 88·5% of the applied optical density units. The lithium salt (21·2 mg.) was isolated in the usual manner and shown to contain the products by paper chromatography and electrophoresis. These were shown by hydrolysis experiments to be P^1P^2-dithymidine and P^1-thymidine-5′ P^2-phenyl dihydrogen pyrophosphate, present as 18% and 82%, respectively, of the isolated lithium salt.

Silver Ethyl Phenyl Phosphate.—To ethanol (0·3 ml., 1 mol.) in dry pyridine (20 ml.) was added iodine (3·1 g., 2 mol.; dried over P_2O_5 at 1 mm. for 48 hr.). When dissolution was complete phenyl hydrogen phosphorohydrazidate (1·9 g., 1·5 mol.) was added and the solution maintained at room temperature for 30 min. Paper chromatography showed that ethyl phenyl phosphate was the main product, contaminated by a smaller amount of diphenyl dihydrogen pyrophosphate and a little slow-running material. Pyridine was removed *in vacuo*, and the residue taken up in water (10 ml.), acidified with concentrated hydrochloric acid (2 ml.), and extracted with chloroform. The extract was neutralised with cyclohexylamine, the solvent removed, and the product, in water, percolated through a column of Dowex-50 (H^+) resin. The eluate was shaken with silver carbonate (3 g.). Filtration and evaporation of the filtrate gave *silver ethyl phenyl phosphate* (1·0 g.), needles, m. p. 115° (decomp.) (Found: C, 31·1; H, 3·4; Ag, 35·0. $C_8H_{10}AgO_4P$ requires C, 31·0; H, 3·2; Ag, 34·9%).

Benzyl Phenyl Hydrogen Phosphate.—Benzyl alcohol (1·54 mmoles) and iodine (3·7 mmoles) were dissolved in dry pyridine (10 ml.), then sodium phenyl phosphorohydrazidate (1·54 mmoles) was added in small quantities. The product was converted into a crude cyclohexylamine salt as above. Fractionation gave dicyclohexylammonium diphenyl pyrophosphate as plates (110 mg., 27%), m. p. 261—263°, and cyclohexylammonium benzyl phenyl phosphate (56 mg., 10%), m. p. 143—145° (lit.,[18] 145°) (Found: N, 3·8. Calc. for $C_{19}H_{26}NO_4P$: N, 3·9%).

Dibenzyl Hydrogen Phosphate.—Benzyl hydrogen phosphorohydrazidate (600 mg.) was added in 100-mg. portions to a cooled solution of benzyl alcohol (310 mg.) and iodine (1·52 g.) in dry pyridine (15 ml.) at 0°. Evolution of nitrogen was very sluggish and the mixture was left for 24 hr. at 0° before the excess of iodine was removed with a few drops of hydrazine. Pyridine was removed *in vacuo* and the residue taken up in dilute ammonia solution (15 ml.). Chromatography at this stage indicated that about 20% of the phosphorus was present as dibenzyl hydrogen phosphate. To this was added a solution of silver nitrate (2·5 g.) in distilled water to remove iodide ion. The precipitated silver iodide was filtered off, and the solution cooled to 0°, acidified with dilute sulphuric acid, and extracted with chloroform (4 × 30 ml.). The chloroform extracts were then shaken with 10% sodium carbonate solution (10 ml.) to extract the dibenzyl hydrogen phosphate; finally the aqueous layer was cooled and brought to pH 2 with dilute sulphuric acid. The oil which separated crystallised, to give needles (100 mg.) which after recrystallisation (82 mg.) had m. p. 77—79° (unchanged on admixture with an authentic sample).

When *N*-bromosuccinimide (1·05 g.) was substituted for the iodine the evolution of nitrogen was rapid. After 24 hr. at 0° the mixture was worked up as before, to give dibenzyl hydrogen phosphate (202 mg.).

Cyclohexyl Phenyl Hydrogen Phosphate.—Cyclohexanol (6·65 mmoles) was phosphorylated with iodine (13·3 mmoles) and phenyl hydrogen phosphorohydrazidate (6·65 mmoles) in pyridine, as in the previous preparation. Working up as usual gave dicyclohexylammonium

[18] Baddiley, Clark, Michalski, and Todd, *J.*, 1949, 815.

P^1P^2-diphenyl pyrophosphate (786 mg., 45%), m. p. 260—262°, and, after several recrystallisations, *cyclohexylammonium cyclohexyl phenyl phosphate* (502 mg., 21%), m. p. 209—212°. The latter had R_F 0·8 in solvent A (descending) (Found: C, 61·2; H, 8·0. $C_{18}H_{30}NO_4P$ requires C, 60·9; H, 8·4%).

Lithium Phenyl Thymidine-5′ Phosphate.—Thymidine (50 mg., 0·205 mmole) in dry pyridine (10 ml.) was treated with phenyl phosphorohydrazidate (77 mg., 0·409 mmole), followed by *N*-bromosuccinimide (282 mg., 1·64 mmoles). The mixture was shaken, and all the material dissolved to give an orange-coloured solution. Chromatography in system A and electrophoresis showed the presence of thymidine phenyl phosphate and of diphenyl dihydrogen pyrophosphate. Removal of pyridine and chromatography on ECTEOLA-cellulose with a lithium chloride gradient gave two peaks, containing 65% and 23%, respectively, of the added optical density units. Evaporation of solvent and precipitation from methanol solution with acetone gave the two lithium salts, 54·0 mg. and 35·7 mg., respectively. The latter product was shown to be dilithium diphenyl pyrophosphate by chromatography before and after acid hydrolysis.

The first product had $\lambda_{max.}$ 267 mμ, released thymidine quantitatively when treated with crude snake venom, and had R_F 0·66 in system A. The *p*-nitrophenyl ester of thymidine-3′ phosphate had R_F 0·68. Both migrated at closely similar rates on paper electrophoresis. Since the enzyme does not hydrolyse 3′-nucleotide esters the product must be lithium phenyl thymidine-5′ phosphate (Found: P, 6·5. Calc. for $C_{16}H_{18}LiN_2O_8P,5H_2O$: P, 6·4%).

Lithium Thymidine-5′ N^2-Benzyloxycarbonylphosphorohydrazidate.—To a stirred solution of *p*-nitrophenyl phosphorodichloridate [19] (1·1 mmoles) in dry dioxan (5 ml.) and dry pyridine (2·0 mmoles) was added a solution of 3′-acetylthymidine (1·06 mmoles) in dry dioxan (15 ml.) during 1 hr. After a further 1·5 hours' stirring, benzyl carbazate (1·07 mmoles) was added. Stirring was continued overnight, the mixture was then evaporated under reduced pressure, and chloroform (30 ml.) and water (30 ml.) were added. The organic layer was washed, dried, and evaporated, finally at an oil-pump, to give a hard glass of *3′-acetylthymidine-5′* p-*nitrophenyl* N^2-*benzyloxycarbonylphosphorohydrazidate* (Found: C, 50·5; H, 5·5; N, 10·8. $C_{26}H_{28}N_5O_{12}P$ requires C, 49·3; H, 4·4; N, 11·0%).

The glass was shaken with lithium hydroxide (185 mg.) in water (15 ml.) at room temperature for 2 hr. The pH was adjusted to 7 with dilute hydrochloric acid, and the solution evaporated *in vacuo*. The product was dissolved in the minimum amount of ethanol, precipitated with acetone, and centrifuged. This was repeated three times until the *product* (117 mg., 23%) was colourless (Found, in material dried over P_2O_5 at 0·1 mm.: C, 42·8; H, 5·2; N, 11·4. $C_{18}H_{22}LiN_4O_9P,1·5H_2O$ requires C, 42·8; H, 4·9; N, 11·1%). It was extremely hygroscopic and had R_F 0·58 in system A (descending). Attempts to remove the benzyloxycarbonyl group by hydrogenation with palladium black at room temperature led only to thymidine-5′ phosphate.

p-*Nitrophenyl 5′-Tritylthymidine-3′ Phosphorohydrazidate.*—To a stirred solution of *p*-nitrophenyl phosphorodichloridate (1·67 mmole) in dry dioxan (5 ml.) and dry pyridine (0·26 ml.) was added a solution of 5′-tritylthymidine (1·67 mmole) during 35 min. The mixture was left overnight with exclusion of moisture, and 95% hydrazine (0·6 ml.) was quickly added. The mixture was stirred for 75 min. and then evaporated under reduced pressure. The glassy product was dissolved in water and chloroform (125 ml. of each), and sodium chloride was added to break the emulsion. The organic layer was washed with water, dried ($MgSO_4$), and evaporated, to give the *product* as a pale yellow glass (959 mg., 82%) (Found, in material dried at 95°/0·7 mm. for 3 hr.: C, 60·3; H, 5·8; N, 9·9; P, 4·2. $C_{35}H_{34}N_5O_9P$ requires C, 60·1; H, 4·9; N, 10·0; P, 4·4%).

Dissolution in 80% acetic acid and boiling for 6 min. in an attempt to detritylate the compound gave thymidine-3′ phosphate as the sole product.

An attempt to phosphorylate cyclohexanol (excess) with the hydrazidate in pyridine, and *N*-bromosuccinimide, was unsuccessful. Thymidine-3′ phosphate and the corresponding *p*-nitrophenyl ester were the only products characterised by chromatography and electrophoresis.

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[19] Turner and Khorana, *J. Amer. Chem. Soc.*, 1959, **81**, 4651.