550. *Phosphorylation through Glyoxalines [Iminazoles] and its Significance in Enzymic Transphosphorylation.*

By J. Baddiley, J. G. Buchanan, and R. Letters.

1-Phosphoglyoxaline (I; R = H) and its esters have been prepared. The esters phosphorylate alcohols and amines readily, and with acetic acid give esters of acetyl phosphate. They also react, often in aqueous solution, with phosphate ions or phosphoric esters yielding pyrophosphates. The possible significance of these observations in connection with certain enzymic reactions involving adenosine triphosphate is discussed. It is suggested that a glyoxaline residue in an enzyme might be the site of activity for transfer of nucleotide or phosphate groups.

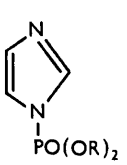
In certain enzymic reactions, particularly those involving cholinesterase [1] and chymotrypsin,[2] it has been suggested that acyl-enzyme intermediates occur. The glyoxaline ring of a histidine residue in the enzyme protein may be the site of acylation. In support of this view is the known reactivity of *N*-acylglyoxalines, *e.g.*, with amines,[3] thiols, alcohols, and inorganic phosphate ions [4] to form the respective acyl derivatives. Further, the effect of pH on the activity of chymotrypsin is consistent with the ionisation of glyoxaline residues and supports the suggested participation of these residues in the enzyme reaction.[5] Glyoxaline residues are also thought to be involved in the action of certain glycosidases.[6]

We have considered the possibility that a similar sequence involving the substitution of glyoxaline residues in the protein may take place during the very many enzymic reactions in which pyro- and tri-phosphates are required. The details of such a scheme are discussed more fully below. Briefly, our problem included the preparation of phosphorylated derivatives of glyoxaline and a study of their reactions. It was thought that some of these derivatives might show certain reactions associated with such compounds as adenosine triphosphate (ATP), *e.g.*, they might be phosphorylating agents or form pyrophosphates.

[1] Wilson, *Biochim. Biophys. Acta*, 1951, **7**, 466, 520.
[2] Hartley and Kilby, *Biochem. J.*, 1954, **56**, 288.
[3] Bergmann and Zervas, *Z. physiol. Chem.*, 1919, **50**, 108.
[4] Stadtman and White, *J. Amer. Chem. Soc.*, 1953, **75**, 2022.
[5] Hammond and Gutfreund, *Biochem. J.*, 1955, **61**, 187.
[6] Larner and Gillespie, *Arch. Biochem. Biophys.*, 1955, **58**, 252.

Related to these suggestions is the observation that glyoxaline and its derivatives catalyse the hydrolysis of di*iso*propyl phosphorofluoridate, a powerful cholinesterase inhibitor. It has been claimed [7] that this inhibitor acts through the intermediate formation of a di*iso*propyl phosphoryl derivative of a glyoxaline residue in the enzyme. The reactivity of such an intermediate is probable, since the di*iso*propyl phosphoryl group finally appears attached to the hydroxyl group in a serine residue on the enzyme. A study of the reactions of phosphorylated glyoxalines should clarify this theory.

When two mols. of glyoxaline in toluene or dioxan are mixed with one of diphenyl phosphorochloridate, the theoretical amount of glyoxaline hydrochloride was rapidly precipitated: the product, the diphenyl ester of 1-phosphoglyoxaline (I; R = Ph), was obtained as an oil on evaporation of the solvent but was used without isolation in the experiments described here. It reacted readily at room temperature with benzylamine, or when heated



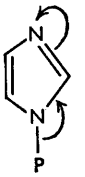
(I)

$(RO)_2PO{\cdot}NHR'$

(II)

$RO{\cdot}PO(NHR')_2$

(III)



(IV)

with aniline, to give the respective phosphoramidates (II) in high yield. With *cyclo*hexylamine a lower yield (44%) of the substituted phosphoramidate was obtained, and even with freshly distilled reagents and dry apparatus this reaction was always accompanied by the formation of varying amounts of *cyclo*hexylammonium diphenyl phosphate. When an excess of aniline was used the substituted phenyl phosphorodiamidate (III; R = R′ = Ph) was obtained.

Alcohols also reacted readily with the phosphorylating agent (I; R = Ph). *cyclo*Hexanol gave *cyclo*hexyl diphenyl phosphate in 85% yield at room temperature, and even the rather unreactive secondary hydroxyl group in pantolactone (D-ββ-dimethyl-γ-butyrolactone) was phosphorylated under these conditions.

Benziminazole reacted with diphenyl phosphorochloridate to give the diphenyl ester of 1-phosphobenziminazole. This reagent was slower than the glyoxaline compound in its action on amines. Although phosphoramidates were obtained in good yield when it reacted with benzylamine or aniline prolonged heating was necessary. Both reagents were readily hydrolysed by water.

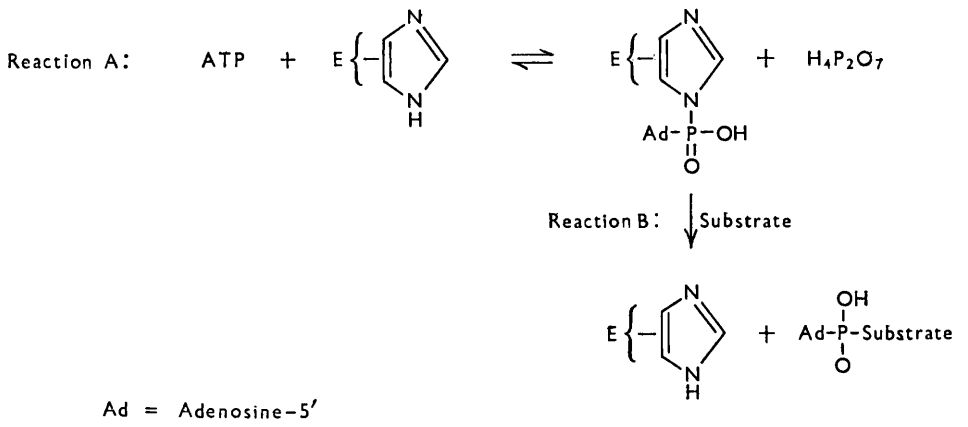
The reactivity of phosphorylated glyoxalines is much greater than that of the ordinary phosphoramidates which also contain an N–P linkage. We consider that this is a result of electronic displacements of the type shown in (IV) and associated in particular with the electron attraction of the unsubstituted nitrogen atom. It is interesting that creatine phosphate, a well-known enzymic phosphorylating agent, also has the system –P–N–C=N– in its structure.

The experiments described so far support the view that di*iso*propyl phosphorofluoridate reacts with glyoxaline residues in cholinesterase. Not only are such glyoxaline phosphoric amides readily formed, but they could phosphorylate hydroxyl groups (*e.g.*, serine residues) in other parts of the enzyme protein.

The chemical reactivity of phosphorylated glyoxalines may be related to certain enzymic reactions involving pyrophosphates. It is possible that the glyoxaline ring in a histidine residue in the enzyme might react with, say, adenosine triphosphate (ATP) to give an adenosine-5′ phosphate–(AMP) enzyme compound (reaction A). This would then react with one of a variety of substrates to give an AMP-substrate compound (reaction B). Such a scheme could apply to the enzymic activation of acetate to give acetyl coenzyme A,[8] in which acetyl-AMP is believed to be an intermediate.[9] In this connection it was found

[7] Wagner-Jauregg and Hackley, *J. Amer. Chem. Soc.*, 1953, **75**, 2125.
[8] Cf. Jones, Lipmann, Hilz, and Lynen, *ibid.*, p. 3285.
[9] Berg, *ibid.*, 1955, **77**, 3163.

that addition of acetic acid to a solution of the phosphate (I; R = Ph) gave acetyl diphenyl phosphate. This was not isolated but its presence was demonstrated by adding hydroxylamine to the mixture. Acethydroxamic acid was formed, and was identified



by paper chromatography and a ferric spray.[10] The formation of an acyl phosphate in this way is somewhat analogous to that of acetyl phosphate from 1-acetylglyoxaline and inorganic phosphate,[4] but in our case the reaction is one of phosphorylation of acetate rather than acetylation of phosphate and may be more analogous to the enzymic " activation " of acetate.

The reversibility of reaction A implies that phosphorylated glyoxalines should react with phosphoric esters to give pyrophosphates. If this is possible, then a phosphorylated substrate in reaction B should also yield pyrophosphates. One might imagine riboflavin-5′ phosphate or nicotinamide nucleotide, etc., as substrates in reaction B in the synthesis of flavin-adenine dinucleotide, diphosphopyridine nucleotide, and other nucleoside pyrophosphates. Strong evidence for the possibility of such reactions has now been obtained.

The diphenyl ester of 1-phosphoglyoxaline reacted readily with dibenzyl hydrogen phosphate at room temperature. The product, presumably *OO*-dibenzyl *O′O′*-diphenyl pyrophosphate, was not isolated but its presence was demonstrated by the addition of benzylamine whereupon dibenzyl *N*-benzylphosphoramidate (II; R = R′ = CH_2Ph) was produced in 61% yield.

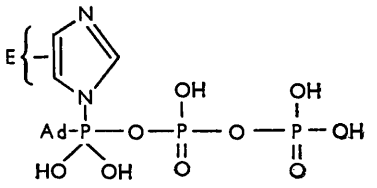
The dibenzyl ester (I; R = CH_2Ph) was prepared from glyoxaline and dibenzyl phosphorochloridate. This compound was less stable than the corresponding diphenyl ester since, although a 37% yield of the *N*-benzylphosphoramidate was obtained when treated with benzylamine, no crystalline esters were isolated after reaction with *cyclo*hexanol. When the ester was heated in water for a few minutes a complex mixture was obtained: paper chromatography showed the presence of mono- and di-benzyl phosphate, a trace of orthophosphate, and appreciable amounts of tetrabenzyl pyrophosphate. The presence of the last substance was confirmed by reduction of the reaction mixture with sodium in liquid ammonia:[11] in addition to orthophosphate, the reduced solution contained inorganic pyrophosphate.

Attempts to remove benzyl groups from the dibenzyl ester (I; R = CH_2Ph) by catalytic hydrogenation were unsuccessful. The substance is probably a catalyst inhibitor, since no hydrogen uptake was observed with several preparations. Partial debenzylation was effected by the action of sodium iodide in acetone.[12] The product was mainly the sodium benzyl derivative of 1-phosphoglyoxaline. Like the dibenzyl ester, this compound was unstable to water, giving a mixture of *OO′*-dibenzyl pyrophosphate and benzyl dihydrogen phosphate. The formation of these substances, and those from the hydrolysis of the dibenzyl ester of 1-phosphoglyoxaline are best explained by a partial hydrolysis of the

[10] Stadtman and Barker, *J. Biol. Chem.*, 1950, **184**, 769.
[11] Arris, Baddiley, Buchanan, and Thain, unpublished work.
[12] Zervas and Dilaus, *J. Amer. Chem. Soc.*, 1955, **77**, 5354.

N–P linkage to yield mono- or di-benzyl phosphate followed by reaction between these esters and unchanged reagent to give pyrophosphates.

The benzyl group from the sodium benzyl derivative was removed by sodium in liquid ammonia. The product, 1-phosphoglyoxaline (I; R = H), was isolated as its barium salt. When examined by paper chromatography in propan-1-ol–ammonia this product was found to contain a small quantity of the barium salt of phosphoramidic acid. This impurity was identified by comparison with a sample of ammonium phosphoramidate prepared by reduction of dibenzyl phosphoramidate with sodium in liquid ammonia. Both phosphoramidate and 1-phosphoglyoxaline give a yellow colour on paper after spraying with perchloric acid–molybdate. This colour is also given by inorganic orthophosphate [13] and indicates very rapid hydrolysis of phosphoramidic acid and the glyoxaline compound to orthophosphate in the presence of perchloric acid.



(V)

The barium salt of 1-phosphoglyoxaline was unstable in aqueous solution. In 5 minutes at 100° it was completely destroyed, the products being glyoxaline, orthophosphate, and appreciable amounts of inorganic pyrophosphate. The ready formation of pyrophosphates from this compound and from its benzyl esters supports the feasibility of reactions A and B in the general scheme for enzymic reactions involving ATP.

The main difficulty in the above, or any other sequence of events intended to explain the reactions of ATP, lies in the observation from several different cases [9, 14] that inorganic pyrophosphate is not in equilibrium with ATP in the presence of the enzyme but in the absence of substrate. However, it is possible that the inorganic pyrophosphate formed in the first stage of the above scheme is bound to the enzyme protein and is only released when the AMP-enzyme compound has been destroyed by the action of the substrate. The known requirements of these enzymes for magnesium ions may be related to this point. The inorganic pyrophosphate may be in semicovalent linkage with magnesium at certain stages in the above reactions. An alternative possibility is that an enzyme–ATP compound (also involving a glyoxaline residue) may be formed and the substrate would displace pyrophosphate from this directly. Enzyme–ATP complexes have been suggested as intermediates in transphosphorylation.[15, 16] It is difficult to formulate such complexes but we suggest (V) as one possible form.

Experimental

Diphenyl Ester of 1-Phosphoglyoxaline.—Diphenyl phosphorochloridate (2·68 g.) was added with shaking to a solution of glyoxaline (1·36 g.) in dry dioxan (40 c.c.). The hydrochloride (1·0 g.) was filtered off rapidly and the resulting ester solution was used directly in the following experiments.

(a) *Reaction with benzylamine.* Benzylamine (1·5 mols.) was added to a dioxan solution of the reagent (from 1 mol. of phosphorochloridate), and the solution was set aside at room temperature for 16 hr. with the exclusion of moisture. Solvent was removed under reduced pressure and the remaining oil was dissolved in chloroform. After being washed with N-sulphuric acid, then sodium hydrogen carbonate solution, the chloroform solution was dried (Na_2SO_4) and evaporated. The residue of diphenyl *N*-benzylphosphoramidate (65%) was recrystallised from aqueous methanol. It had m. p. and mixed m. p. 104°.

(b) *Reaction with aniline.* Diphenyl *N*-phenylphosphoramidate (61%) was obtained in a similar reaction with aniline. It had m. p. 124°. When the reaction was carried out by 4 hours' refluxing with an excess of aniline the product, which crystallised directly from the chloroform solution, was phenyl *NN'*-diphenylphosphorodiamidate (78%), m. p. and mixed m. p. 169°.

(c) *Reaction with* cyclo*hexylamine.* A 44% yield of diphenyl *N-cyclo*hexylphosphoramidate, m. p. 101°, was obtained under conditions similar to those described for benzylamine. Variable

[13] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.
[14] Kornberg and Pricer, *J. Biol. Chem.*, 1951, **191**, 535.
[15] Hoagland, *Biochim. Biophys. Acta*, 1955, **16**, 288.
[16] Hoagland and Keller, *Fed. Proc.*, 1955, **14**, 73.

amounts of *cyclo*hexylammonium diphenyl phosphate, m. p. 197°, were produced in this reaction. This salt crystallised from the dioxan solution of reactants within a few minutes of mixing.

(d) *Reaction with* cyclo*hexanol.* Diphenyl *cyclo*hexyl phosphate was obtained in 85% yield from *cyclo*hexanol (1·4 g.) and the reagent in dioxan at room temperature (40 hr.). The product was isolated in a manner similar to that described for benzylamine. It had m. p. 38° after crystallisation in the presence of light petroleum.

(e) *Reaction with pantolactone.* From D(−)-pantolactone (1·3 g.) a 27% yield of pantolactone 2-(diphenyl phosphate), m. p. 81°, was obtained when the reaction was carried out for 12 hr. at room temperature. The product was recrystallised from aqueous methanol and its m. p. was undepressed in admixture with authentic material.

Diphenyl Ester of 1-*Phosphobenziminazole.*—A dioxan solution of this substance was prepared from benziminazole (2·36 g.; resublimed at 100°/15 mm.) and diphenyl phosphorochloridate (2·68 g.) as described for the glyoxaline analogue. It reacted with aniline or benzylamine when its dioxan solution was refluxed for about 9 hr., yielding the substituted phosphoramidates (74% from benzylamine, 50% from aniline). With *cyclo*hexylamine only the *cyclo*hexylammonium salt (84%) was obtained.

Hydrolysis of the Diphenyl Ester of 1-*Phosphobenziminazole.*—The above reagent was prepared in acetonitrile and a little water was added. Within a few minutes crystals of *benziminazolium diphenyl phosphate* had separated. It had m. p. 158° (from alcohol) (Found: C, 61·8; H, 4·9; N, 8·3. $C_{19}H_{17}O_4N_2P$ requires C, 62·0; H, 4·6; N, 7·6%).

Formation of Neutral Pyrophosphoric Esters.—A solution of the diphenyl ester of 1-phosphoglyoxaline (from 2·68 g. of diphenyl phosphorochloridate) in toluene was added to one of dibenzyl phosphate (5·54 g.) in the same solvent, and the mixture was kept at room temperature for 1½ hr. The amount of *OO*-dibenzyl *O'O'*-diphenyl pyrophosphate formed was determined in a manner similar to that used by Corby, Kenner and Todd,[17] with substitution of benzylamine for *cyclo*hexylamine.

Benzylamine (3·2 g.) was added to the mixture and after 12 hr. the benzylamine salt was filtered off. The toluene solution was washed with dilute sulphuric acid, then sodium hydrogen carbonate solution, dried (Na_2SO_4), and evaporated *in vacuo*. The residue of dibenzyl *N*-benzylphosphoramidate (2·1 g., 61%) after recrystallisation from *cyclo*hexane had m. p. and mixed m. p. 84°.

Dibenzyl Ester of 1-*Phosphoglyoxaline.*—A solution of glyoxaline (1·36 g.) in dioxan (25 c.c.) was added at 0° to dibenzyl phosphorochloridate (from 2·62 g. of dibenzyl phosphite) in dioxan (20 c.c.). The mixture was shaken for a few minutes at 0° then allowed to reach room temperature. The hydrochloride (1 g. Theor., 1·05 g.) was filtered off as rapidly as possible and the solution was used without delay in the subsequent experiments.

Reaction between the Glyoxaline Diphenyl Phosphate and Acetic Acid.—A solution of the ester (from 2·68 g. of diphenyl phosphorochloridate) was mixed with acetic acid (5 c.c.). After 2 hr. at room temperature a sample was treated with hydroxylamine under standard conditions,[10] and the formation of acethydroxamic acid was demonstrated by a ferric reaction. Both the phosphate ester and acetic acid failed to react in this test. The solution of acethydroxamic acid was compared by paper chromatography in butanol–acetic acid–water (4 : 1 : 5) with an authentic sample. The preparations were indistinguishable, with R_F 0·64. The hydroxamic acid was detected by spraying the paper with ferric chloride in hydrochloric acid.

Hydrolysis of the Dibenzyl Ester of 1-*Phosphoglyoxaline.*—The ester (from 0·25 g. of dibenzyl phosphite) was heated with water (10 c.c.) for 5 min. at 100°, then examined by paper chromatography in propan-1-ol–ammonia–water (6 : 3 : 1). The following substances were detected: tetrabenzyl pyrophosphate, dibenzyl phosphate, benzyl hydrogen phosphate, and traces of orthophosphate (for R_F values, see Table).

	R_F (A)	R_F (B)
Tetrabenzyl pyrophosphate	0·98	—
Tribenzyl pyrophosphate	—	0·96
Dibenzyl hydrogen phosphate	0·90	0·89
OO'-Dibenzyl pyrophosphate	0·83	0·74
Benzyl dihydrogen phosphate	0·54	0·37
1-Phosphoglyoxaline	0·42	—
Phosphoramidate	0·19	—
Orthophosphate	0·18	—
Pyrophosphate	0·10	—

The formation of pyrophosphates in this reaction was confirmed by hydrogenolysis of the products with sodium in liquid ammonia. Water was completely removed from the mixture

[17] Corby, Kenner, and Todd, *J.*, 1952, 1234.

by evaporation *in vacuo* and the residue was dissolved in liquid ammonia (30—40 c.c.). Sodium was added without delay in small pieces until the blue colour persisted. Solvent was allowed to evaporate with occasional rocking, last traces being removed at the pump. Alcohol (5 c.c.), then water, were added to the residue, and the solution was passed through a column of Amberlite IR-120 resin (H^+ form). The eluate was evaporated and shown by paper chromatography to contain inorganic pyrophosphate and orthophosphate only.

Sodium Salt of 1-*Phosphoglyoxaline Benzyl Ester*.—The dibenzyl ester of 1-phosphoglyoxaline (0·01 mol.) was prepared in the usual manner but in dry acetone. Sodium iodide (1·5 g.; previously heated at 110° for 30 min.) was added and the solution was kept at 50—60° for 15 min., during which solid was deposited, then at room temperature for 2 hr. The solid (2·2 g., 85%) was filtered off and dried (P_2O_5). Benzyl iodide was detected in the filtrate. A solution of the sodium salt in water and a similar solution which had been heated for 5 min. at 100° were examined by paper chromatography in propan-1-ol–ammonia (*d* 0·880)–water (7 : 2 : 1). Both solutions were found to contain dibenzyl hydrogen phosphate, *OO'*-dibenzyl pyrophosphate, and benzyl dihydrogen phosphate (for R_F values, see Table). In addition a spot with R_F 0·58 was present in large amount in the unheated sample and in much smaller amount in the heated sample. This is probably the monobenzyl ester of 1-phosphoglyoxaline. It appears that considerable decomposition of this substance must have occurred during the debenzylation or subsequent operations.

1-*Phosphoglyoxaline*.—The above sodium salt (0·2 g.) was suspended in liquid ammonia, and pieces of sodium were added until the solution remained blue. Ammonia was allowed to evaporate, alcohol and water were added, and the solution was passed through a column of Amberlite IR-120 (H^+ form). The eluate was neutralised immediately with barium hydroxide solution. The mixture was centrifuged, and the clear solution was concentrated *in vacuo* below 40° and examined by paper chromatography. It contained the barium salt of 1-phosphoglyoxaline, together with a small proportion of barium phosphoramidate. The barium salt was isolated by precipitation with acetone (Found: C, 10·2; H, 2·0; Residue, 67·3. Calc. for $C_3H_3O_3N_2PBa,2H_2O$ + 10% of $BaPO_3NH_2$: C, 10·2; H, 2·0; Residue 68·7% calc. as barium pyrophosphate). The identity of the phosphoramidate was established by comparison on paper with a sample prepared from dibenzyl phosphoramidate by reduction with sodium in liquid ammonia as described above for the glyoxaline compound.

1-Phosphoglyoxaline was separated from phosphoramidate on a small-scale by paper chromatography in the form of a series of spots or a band on the paper. When this band was eluted the pure compound was obtained as its ammonium salt in solution. A drop of solution obtained in this way was hydrolysed with 0·1N-hydrochloric acid at 100° for 15 min. The presence of free glyoxaline was then shown by paper chromatography in ethyl acetate–pyridine–water (2 : 1 : 2), followed by spraying with diazotised sulphanilic acid. It had R_F 0·76, identical with authentic glyoxaline.

Neutral Hydrolysis of 1-*Phosphoglyoxaline*.—The pure ammonium salt eluted from a paper chromatogram was heated in water for a few minutes at 100°. The resulting solution was run on a paper chromatogram in propan-1-ol–ammonia–water and was found to have been completely converted into a mixture of inorganic ortho- and pyro-phosphate, the former in greater amount. It was shown that ammonium phosphoramidate was largely unchanged under similar conditions.

Paper Chromatography of Glyoxaline Derivatives and Pyrophosphates.—Chromatography was carried out by the ascending front method on Whatman No. 4 paper previously washed with dilute acetic acid and water. The solvent was propan-1-ol–ammonia (*d* 0·880)–water [(A) 6 : 3 : 1; (B) 7 : 2 : 1], and the molybdate spray [13] was used for detection of phosphoric esters. The R_F values in the Table were observed.

Paper Electrophoresis of 1-*Phosphoglyoxaline*.—Electrophoresis was carried out on Whatman No. 4 paper soaked in 0·1M-acetate buffer at pH 7 with a current of 10 mA and a gradient of 5·4 v per cm. The molybdate spray was used for detecting phosphates after the paper had been dried at 80° for 30 min. The following migrations were observed:

	Distance moved towards cathode (cm.)
Pyrophosphate	7·3
Orthophosphate	6·5
1-Phosphoglyoxaline	5·3

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