The Synthesis of Acyl Phosphates in Aqueous Solution.

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Reaction of phosphate anions with carboxylic acid anhydrides in aqueous media leads to biologically interesting acyl phosphates. The mechanism of the reaction, which is strongly catalysed by pyridine, is believed to involve two-point attack on the two carbonyl-carbon atoms of the anhydride. Diacetylmethylamine, azlactones, and acyl dialkyl phosphates can also act as acyl donors in the reaction. Convenient preparations are given for acetyl phosphate and other acyl phosphates. An analogous reaction takes place between tetramethyl pyrophosphate and salts of dibasic phosphoric acids.

Interest in acyl phosphates increased when Negelein and Brömel isolated 3-phosphatoglyceroyl phosphate as one of the intermediates of glycolysis (*Biochem. Z.*, 1939, **303**, 132) and as a result of the work of Lipmann and his colleagues and others on acetyl phosphate (see, *e.g.*, *Adv. Enzymology*, 1946, **6**, 231). Acetyl phosphate itself was first prepared by Lynen (*Ber.*, 1940, **73**, 367) by condensing silver dibenzyl phosphate with acetyl chloride, followed by hydrogenolysis of the benzyl groups from the product. This and other, more direct methods (Lipmann and Tuttle, *J. Biol. Chem.*, 1944, **153**, 571; Stadtman and Lipmann, *ibid.*, 1950, **72**, 1312; Bentley, *J. Amer. Chem. Soc.*, 1948, **70**, 2183; Koshland, *ibid.*, 1951, **73**, 4103), straightforward though they seem, involve, at some stage, the very unstable free acetylphosphoric acid.

In attempts to synthesise dinucleotide coenzymes by exchange reactions with acyl phosphates, it was discovered that the latter were produced with remarkable facility by the action of carboxylic anhydrides on phosphates in aqueous solution (B.P. Appln. 20,335/1953). This was originally modelled on the well-known Chattaway technique of acylation of phenoxide ions (*J.*, 1931, 2495) since the pK_3' of orthophosphoric acid is that of a very weak acid (*ca.* 11 as compared with pK' *ca.* 10 for phenol), although, as it later turned out, this would appear not to be an appropriate analogy. Trisodium phosphate was found to be readily acetylated by an excess of acetic anhydride at 0°, but the reaction proceeded best in the presence of sufficient sodium hydrogen carbonate to keep the pH between 7 and 8 throughout. By employing concentrated solutions and using the more soluble potassium salts, a useful preparative method for acetyl phosphate resulted. The method did not appear to possess any scaling-up difficulties, a neutral solution being maintained throughout, and gave yields of 40% of purified product. An almost identical procedure has since been described by Kaufman (*Arch. Biochem. Biophys.*, 1954, **50**, 506) for the preparation of succinyl phosphate.

Under such conditions, it was hoped that alcoholic hydroxyl groups present in sugar phosphates and nucleotides would not be esterified; in fact, α-D-glucose 1-phosphate, D-galactose 6-phosphate, D-riboflavin-5′ phosphate, and adenosine-5′ phosphate were even more readily attacked than orthophosphate ions by acetic anhydride in aqueous solution at pH 7—8, to give the corresponding acetyl phosphates. The same was found for aneurin mono- and di-phosphate, as well as inorganic pyrophosphate. Anhydrides other than acetic anhydride also acylate orthophosphate ions similarly, although with varying degrees of facility. In these reactions the hydrolyses both of reagents (very rapid) and of products (slower, but quite appreciable) prevent reliable estimation of relative rates. However, Table 1 gives the approximate conversions of orthophosphate into acyl phosphate under standard conditions: there is no obvious correlation with the strength of the parent acid, which would be some measure of the electrophilic properties of the carbonyl-carbon atom, and the high conversions of the cyclic anhydrides are striking. However, the cyclic anhydrides (maleic and succinic) had no measurable action on D-riboflavin-5′ phosphate or inorganic pyrophosphate under the same conditions.

A study of the effect of pH on the action of acetic anhydride on orthophosphate ions (Table 2) was rendered difficult by the need to choose buffers which would not be expected

to participate in the reaction. Consequently slight change of pH during the run was sometimes unavoidable, and the final pH in these cases is given in parentheses. The approximate distributions of the three ionic species were calculated from the published values of the apparent pK's of orthophosphoric acid (Kumler and Eiler, *J. Amer. Chem. Soc.*, 1943, **65**, 2355, for pK_1 and pK_2; Morton, *Quart. J. Pharm. Pharmacol.*, 1930, **3**, 438, for pK_3) which were unfortunately measured at temperatures and ionic strengths different from those employed in the present work. However, the figures serve as a rough guide, and

TABLE 1. *Acyl phosphates formed from carboxylic anhydrides* (10 *mols.*) *under standard* (*not optimal*) *conditions* (M-$NaHCO_3$ *at* 0°).

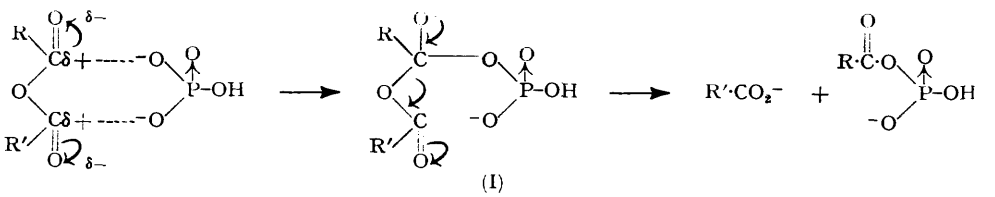
Anhydride	Conversion of HPO_4^{2-} (%)	10^3K_1 for parent acid	Anhydride	Conversion of HPO_4^{2-} (%)	10^3K_1 for parent acid
Benzoic	9	0·063	Phthalic	45	1·26
Chloroacetic	30	1·4	Succinic	60	0·066
Propionic	35	0·014	Maleic	85	15

TABLE 2. *Effect of* pH *on acetyl phosphate synthesis.*

Buffer	pH	Conversion (%) into acetyl phosphate in 30 mins.	Phosphate ions (%) initially present as: $H_2PO_4^-$	HPO_4^{2-}	PO_4^{3-}
M-Na_2CO_3	10·2 (9·7)	Nil	—	91	9
M-$NaHCO_3$ ..... + M-Na_2CO_3	9·15 (8·7)	Nil	—	99	1
M-$NaHCO_3$	7·95	22	7	93	—
M-NaOAc ..... + M-$NaHCO_3$ + AcOH ..... *	7·0 (6·85)	33	40	60	—
M-NaOAc ..... + M-$NaHCO_3$ + AcOH ..... *	6·1 (5·5)	20	83	17	—
M-NaOAc + AcOH	4·95 (4·75)	*ca.* 4	99	1	—
M-NaOAc + AcOH	4·05	Nil	100	—	—

* Prepared at 0° to minimise loss of CO_2.

show that the conversion falls drastically as the proportion of HPO_4^{2-} drops. The similar fall at higher pH's is due to the rapid increase in rate of hydrolysis of the acetic anhydride which, under these conditions, after 10 mins., was 95% complete at pH 8, 72% at pH 7, and only 60% at pH 6. Acetate ions had no measurable effect on the rate of orthophosphate liberation from lithium acetyl phosphate in M-sodium hydrogen carbonate, suggesting that the reverse change, $AcO^- + Ac{\cdot}PO_4^{2-} \longrightarrow Ac_2O + PO_4^{3-}$, does not occur to a significant extent. The observed conversions into acetyl phosphate therefore seem to be a function of the true rate of reaction at the starting concentration of acetic anhydride on which is superimposed the steadily changing effect of the hydrolysis of the latter. These facts suggest a mechanism for the reaction based on two-point attack of the doubly negatively charged phosphate anion on the two carbonyl-carbon atoms of the anhydride :

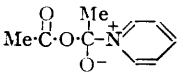
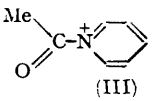


(I)

Such a mesomeric transition state would provide conditions for the necessary low activation energy of this ready reaction in a strongly hydrolytic medium.

When 2M-aqueous pyridine (pH 7·8) was used as buffering medium instead of sodium hydrogen carbonate, a remarkably rapid and almost quantitative conversion of orthophosphate into acetyl phosphate occurred in spite of the pronounced catalysis of the hydrolysis both of acetic anhydride (Bafna and Gold, *J.*, 1953, 1406) and of acetyl phosphate

(Koshland, *J. Amer. Chem. Soc.*, **1952**, **74**, 2286) by pyridine. The increased rate of synthesis is unlikely to involve the 1-acetylpyridinium ion (III) (cf. Koshland, *loc. cit.*; Gold and Jefferson, *J.*, **1953**, 1409) since in an overwhelmingly aqueous environment this highly reactive entity would almost certainly be attacked primarily by water. For the hydrolysis of acetic anhydride, Gold and Jefferson (*loc. cit.*) point out that the ion (II)

(II)   + AcO^-

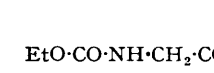
(III)

would approximate to the transition state in the rate-determining step of formation of (III) which then undergoes virtually instantaneous hydrolysis. Since the acetylation of phosphate ions has a reaction velocity at least comparable with that of the hydrolysis of acetic anhydride, and since the very large concentration of water will not be relevant for the catalysed hydrolysis until the ion (III) has been formed, the structure (II) might approximate to an activated acceptor of HPO_4^{2-} in the scheme outlined above. An alternative, but less likely, explanation would be that pyridine intervenes, not at the initial step, but in assisting the breakdown of (I) or some similar intermediate.

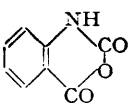
In further support of the proposed mechanism, acyl chlorides (*n*-butyryl and benzoyl) do not form measurable amounts of acyl phosphates in aqueous solution, and no evidence has been obtained of the reaction of acetic anhydride with dibenzyl phosphate, 2′ : 3′-*O*-*iso*propylidene adenosine-5′ benzyl phosphate, or betaine, which have only a single negatively charged oxygen atom.

In preparative procedures pyridine is superior to sodium or potassium hydrogen carbonate owing to the higher and more rapid conversions, and also to the greater ease of separation of the products. Dilithium acetyl phosphate and sodium D-riboflavin-5′ acetyl and monochloroacetyl phosphate have been prepared in this way. Benzoic anhydride, which reacted only sluggishly with orthophosphate in the presence of sodium hydrogen carbonate, reacted almost quantitatively in the presence of pyridine. On the other hand, succinic anhydride was without effect on D-riboflavin-5′ phosphate in aqueous pyridine or aqueous sodium hydrogen carbonate, conceivably owing to steric interference with the formation of the transition state.

(IV)

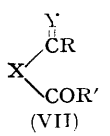


(V)

 (VI)

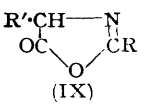
The mixed anhydride (IV) reacted readily with orthophosphate ions to give the corresponding glycyl phosphate derivative (V); but isatoic anhydride (VI) did not react. The more reactive *N*-carboxy-anhydrides of α-amino-acids might well be usable, however, as a source of free aminoacyl phosphates.

Some analogous compounds were also investigated. Diacetylmethylamine (VII; X = MeN, Y = O, R = R′ = Me) reacted sluggishly and incompletely with orthophosphate in aqueous pyridine, but not in aqueous sodium hydrogen carbonate.



(VII)

(VIII)



(IX)

Diacetylaniline (VII; X = PhN, Y = O, R = R′ = Me) on the other hand did not react to any measurable extent under either set of conditions. It is interesting that, since the completion of this work, Stadtman and White (*J. Amer. Chem. Soc.*, **1953**, **75**, 2022) have briefly reported that *N*-acetylglyoxaline (VIII; R′ = Me) acetylates orthophosphate ions in aqueous solution at pH 7·0 in 50% yield: glyoxaline has a relatively high acid strength (pK ~7·0) and its *N*-acyl derivatives (VIII) are known to be reactive anhydride analogues

(Wieland and Schneider, *Annalen*, 1953, **580**, 159), and probably acylate phosphate ions by a mechanism similar to that of the carboxylic anhydrides (VII; X = Y = O), all the structural features of the latter being present.

Azlactones (IX) are also related structurally, being cyclic iminoacyl analogues of (VII), and the compounds (IX; R = Ph, R′ = H; and R = Me, R′ = Ph·CH_2) reacted quantitatively with orthophosphate in aqueous pyridine to give benzoylglycyl phosphate (X) and *N*-acetylphenylalanyl phosphate (XI), respectively. In addition, the latter azlactone with adenosine-5′ phosphate gave 81% of the compound (XII).

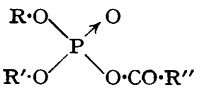
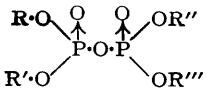
(X) Ph·CO·NH·CH_2·CO·O·PO_3^{2-} $\qquad$ CH_3·CO·NH·CH(·CH_2Ph)·CO·O·PO_3^{2-} (XI)

Adenosine-5′-O·PO(O^-)·O·CO·CH(CH_2Ph)·NHAc
(XII)

The phosphates (X) and (XI) reacted readily with aniline in aqueous solution, but not with glycine at pH 8—9. Under the same conditions, the adenosine compound (XII) gave *N*-acetylphenylalanylglycine, identified by paper chromatography, confirming Chantrenne's contention (*Compt. rend. Lab. Carlsberg, Sér. Chim.*, 1948, **26**, 297; *Nature*, 1949, **164**, 576) that only acyl phosphates with one ionisable acid group react with amino-acids in aqueous solution.

*iso*Propenyl acetate (VII; X = O, Y = CH_2, R = R′ = Me), although an active acetylating agent under anhydrous, acid-catalysed conditions, did not react with aqueous orthophosphate ions, or appreciably with aqueous hydroxylamine at pH 6, and therefore cannot be considered to be an anhydride analogue in the present sense.

So far only the reactions of wholly carboxylic anhydrides and their analogues have been discussed. There appears to be no marked reaction of salts of acyl phosphoric acids under the usual conditions, but this is not surprising since their negative charge would prevent approach of the attacking reagent. With neutral acyl phosphates such as (XIII; R = R′ = R″ = Me, or R = R′ = Ph·CH_2, R″ = Me) this objection does not arise and

(XIII)  $\qquad$  (XIV)

in both these cases acylation of orthophosphate ions has been observed rather than dialkylphosphorylation. This is explicable in terms of the inherently greater tendency in acetyl phosphates for the C–O rather than the O–P bond to be broken, just as acetic acid is much weaker than dimethyl or dibenzyl hydrogen phosphate. With tetramethyl pyrophosphate (XIV; R = R′ = R″ = R‴ = Me) in aqueous sodium hydrogen carbonate, reaction again occurred, as indicated by the disappearance of orthophosphate ions, to give, presumably, the salt of unsymmetrical dimethyl pyrophosphate (XIV; R = R′ = Me, R″ = R‴ = H). This reaction might conceivably be regarded as a model of phosphate transfer in biological systems where the negative charges of adenosine-5′ triphosphate, for example, should be capable of being screened by chelation or in some other way as a result of its enzymic environment.

Experimental

Analytical Methods.—Estimation of labile acyl groups. A procedure slightly modified from that of Lipmann and Tuttle (*J. Biol. Chem.*, 1944, **153**, 571), based on the formation of a purple-red complex from hydroxamic acids and Fe^{3+}, was employed: 3·5N-sodium hydroxide (0·4 ml.) was mixed with 4·0M-hydroxylamine hydrochloride (0·5 ml.), and the sample solution and additional water were added to a total vol. of 3·00 ml. After 10 min. at room temperature, a solution (3·0 ml.) of 0·063M-ferric chloride in N-hydrochloric acid was added. After a further 5 min. the colour was measured in a photo-electric absorptiometer with a green filter. A standard curve of optical density–concentration was prepared for each carboxylic anhydride. *iso*Propenyl acetate gave no colour under these conditions.

For estimation of the acyl phosphate content of a reaction solution, the unchanged carboxylic anhydride or anhydride analogue was first removed by ether. Sampling was carried out as rapidly as possible to avoid losses by hydrolysis.

Filtration of the precipitated solid was necessary in estimations of D-riboflavin-5′ acyl phosphates before measurement of the final colour. In this and all other cases, appropriate blanks were carried out.

Phosphorus estimation. The uptake of orthophosphate ions in reactions with various anhydrides was determined as the difference between the orthophosphate content by the Lowry and Lopez procedure (*J. Biol. Chem.*, **1946**, **162**, 421) and that originally present.

Total-phosphorus determinations were carried out by combusion of samples with perchloric–sulphuric acid, followed by estimation of the orthophosphate produced by Berenblum and Chain's method (*Biochem. J.*, **1938**, **32**, 295), except that, owing to their molybdate-sensitivity, combustion was not necessary in the case of acyl unsubstituted phosphates such as acetyl phosphate salts.

Riboflavin. The total riboflavin content was determined colorimetrically, with sodium D-riboflavin-5′ phosphate dihydrate as a standard.

Glycine estimation. The ninhydrin procedure of Moore and Stein (*J. Biol. Chem.*, **1948**, **176**, 367) was employed.

Acylations.—General procedure. The following conditions were used in most of the experiments which were carried out in order to screeen various anhydrides and anhydride-like substances for their action on orthophosphate ions in aqueous solution: 0·016M-Disodium hydrogen phosphate (1 mol.) in M-sodium hydrogen carbonate was cooled thoroughly in ice and, with vigorous mechanical stirring, the anhydride (10 mols.) was added, if necessary, in solution in dimethylformamide. Samples were withdrawn for analysis at appropriate intervals in order to follow the course of the acylation, which was usually optimal after 60 min.

When 2M-aqueous pyridine was used instead of bicarbonate, the production of acyl phosphate was faster and more nearly complete (~10 min.).

Solutions approximately five times as concentrated were usually employed with substituted phosphates, but an amount of solid sodium hydrogen carbonate corresponding to the excess of anhydride employed had then to be added. The aqueous pyridine procedure was generally preferred. The following examples illustrate the preparative usefulness of the reaction.

Dilithium and disilver acetyl phosphates. (*a*) A suspension of potassium hydrogen carbonate (50 g.) in water (100 ml.) and 2·5M-dipotassium hydrogen phosphate (20 ml.) were well stirred together and cooled in ice to 1°. Acetic anhydride (48 ml.) and more 2·5M-phosphate solution (80 ml.) were then added dropwise, separately but simultaneously, during 30 min. After an additional 1—2 hours' stirring, 31% aqueous lithium acetate dihydrate (100 ml.) was slowly added, followed carefully by 4M-lithium hydroxide (*ca.* 85 ml.) until a sample diluted 5—10-fold had pH 8·0—8·5. During all these procedures the mixture was kept below 5°. The resulting turbid liquid was then filtered through Hyflo Supercel, on an ice-cooled Buchner flask. The clear filtrate (*ca.* 420 ml.) was then treated, with good agitation, with cold absolute ethanol (4 l.). After settling for 2 hr. at 0°, the dilithium acetyl phosphate was filtered off and washed with alcohol and then with ether, and dried *in vacuo*. The white powder (31 g.) so obtained was about 63% pure. The crude dilithium salt (15 g.) was dissolved in ice-cold water (700 ml.) and treated with 25% aqueous silver nitrate (*ca.* 50 ml., equiv. to the inorganic phosphate content of crude lithium salt). After settling at 0—5° the dirty yellow precipitate of silver phosphate was filtered off (ice-cooled Buchner flask). The clear filtrate was treated with 25% aqueous silver nitrate (215 ml.), previously cooled to 0°, with agitation, the apparatus being protected from bright light. After 2 hours' settling at 0°, the crystalline salt was filtered off and washed with ice-cold water (3 × 25 ml.), cold absolute alcohol (3 × 150 ml.) and dry ether (2 × 150 ml.). The disilver acetyl phosphate (18·1 g., 40%) was obtained as light-sensitive, white, crystals of about 96% purity (Found: Ag, 60·7; P, 8·75; labile acetyl, 11·65. Calc. for $C_2H_3O_5PAg_2$: Ag, 61·0; P, 8·75; Ac, 12·1%).

(*b*) Pyridine (95 g. 1·2 moles) and 0·25M-dipotassium hydrogen phosphate (200 ml.) were stirred together at 0° to give a clear, single phase. Acetic anhydride (9·6 ml., 0·1 mole) was then added dropwise to the vigorously stirred solution during 5 min., causing the temperature to rise to 3·5°. After 35 min., 4·1N-lithium hydroxide (37 ml.) was added, giving (after 10-fold dilution with water) a practically clear solution of pH 7·6: 90% conversion was observed at this stage. Cold ethanol (2300 ml.) was then added slowly, and the precipitate allowed to settle for 2 hr. at 0° before being filtered off. Crude dilithium acetyl phosphate (6·85 g., containing 32·5 mmoles of labile acetyl) was obtained.

Sodium D-*riboflavin-5′ acetyl phosphate.* Monosodium D-riboflavin-5′ phosphate dihydrate (340 mg., 0·66 mmole) was dissolved in 40% aqueous pyridine (8 ml.), and the solution cooled to 0°. With vigorous stirring, acetic anhydride (0·33 ml., 3·4 mmoles) was added. After 5 min., ice-cold acetone (50 ml.) was added, and the precipitated product rapidly filtered off, washed with cold acetone and then dry ether, and dried rapidly *in vacuo*. The sodium D-riboflavin-5′ acetyl phosphate was obtained as an orange solid (330 mg.) of about 90% purity (Found: riboflavin, 63; labile acetyl, 6·45. Calc. for $C_{19}H_{22}O_{10}N_4PNa,4H_2O$: riboflavin, 63; Ac, 7·25%). It is stable for long periods at $<0°$ in the absence of light and moisture but decomposes slowly at room temperature.

Sodium D-*riboflavin-5′ chloroacetyl phosphate.* Monosodium D-riboflavin-5′ phosphate dihydrate (170 mg., 0·33 mmole) was dissolved in 40% aqueous pyridine (4 ml.), the solution cooled to 0°, and, with vigorous stirring, a solution of chloroacetic anhydride (560 mg. 3·3 mmoles) in dry acetonitrile (3 ml.) was added, half at once and the remainder dropwise during the next 5 min. Then cold acetone (25 ml.) was at once added, and the product isolated as for the corresponding acetyl compound. The orange solid (137 mg.) so obtained contained *ca.* 80% of sodium D-riboflavin-5′ chloroacetyl phosphate (Found: riboflavin, 65; labile $CH_2Cl{\cdot}CO$, 10·8. Calc. for $C_{19}H_{21}O_{10}N_4ClPNa,2H_2O$: riboflavin, 64; $CH_2Cl{\cdot}CO$, 13·1%). The impurity in both the above preparations was the unacylated compound. The chloroacetyl compound was even less stable than the acetyl compound, but it could be stored safely at $-10°$, at least for several days. The dry solid lost 20% of its content of the chloroacetyl compound in 5 hr. at 20°.

Reaction of Ethoxycarbonylaminoacetyl Ethyl Carbonate (IV) *with Orthophosphate Ions.*—A procedure based on those of Wieland *et al.* (*Annalen*, 1951, **573**, 99), Vaughan (*J. Amer. Chem. Soc.*, 1951, **73**, 3547), and Boissonas (*Helv. Chim. Acta*, 1951, **34**, 874) was used. *N*-Ethoxycarbonylglycine (3·7 g., 25 mmoles) was dissolved in dry acetonitrile (15 ml.), and triethylamine (2·5 g., 25 mmoles) was added. To this, cooled in ice–salt, with good stirring, ethyl chloroformate (2·7 g., 25 mmoles) in dry acetonitrile (5 ml.) was added dropwise. Triethylamine hydrochloride was soon precipitated and, after the halide addition was complete, the mixture was stored in ice–salt for 30 min. before use. This solution was used to acylate sodium phosphate (2·5 mmoles) in M-potassium hydrogen carbonate (40 ml.). Under these conditions the conversion was *ca.* 35% after 2 hr.

Reaction of 2-Phenyl-5-oxazolone (IX; R = Ph, R′ = H) *with Phosphate Ions.*—2-Phenyloxazolone (0·645 g., 4·0 mmoles) was added to disodium hydrogen phosphate (2·0 mmoles) in 16% aqueous pyridine (20 ml.) at 0°. Complete conversion of the orthophosphate into *N*-benzoylglycyl phosphate (X) occurred in 90 min.

Reaction of Benzoylglycyl Phosphate (X) *with Aniline.*—Aniline (10 mols.) was incubated with an ether-washed portion of the above solution of hippuryl phosphate (1 mol.) at 37° for 1 hr. The resulting white solid (56%) had m. p. 217—218°, undepressed on mixing with a pure, authentic specimen of benzoylglycine anilide (m. p. 217—218°).

Reaction of 4-Benzyl-2-methyl-5-oxazolone (IX; R = Me, R′ = CH_2Ph) *with Phosphate Ions.*—The azlactone (0·76 g., 4·0 mmoles) was added to sodium phosphate (2·0 mmoles) in 32% aqueous pyridine (20 ml.) at 0° with vigorous stirring, converting 93% of the phosphate ions into the product (XI) in 1 hr.

Reaction of α-Acetamido-β-phenylpropionyl Phosphate with Glycine.—The foregoing solution was washed with ether to remove any unchanged azlactone, and glycine (150 mg. 2·0 mmoles), dissolved in a little M-sodium hydrogen carbonate, was added. The solution was brought to pH 8·4 with N-sodium hydroxide, and M-sodium hydrogen carbonate (6 ml.) was then added to buffer the solution to pH 8·2. It was divided into two parts, one of which was stored at 1°, and the other at room temperature. After 72 hr., no change in the glycine content of either solution had occurred. All the original acyl phosphate (XI) had disappeared from the solution after 72 hr. at room temperature, while 50% of it remained in the refrigerated solution.

In a similar experiment, the phosphate also did not react with glycine.

Reaction of the Azlactone (IX; R = Me, R′ = CH_2Ph) *with Adenosine-5′ Phosphate.*—Synthetic adenosine-5′ phosphoric acid (245 mg., 0·7 mmole) was dissolved in 32% aqueous pyridine (7 ml.), and the azlactone (265 mg., 1·4 mmoles) added with vigorous stirring at 0°. After 1 hr. a portion (2 ml.) was analysed and 81% conversion of the adenylic acid into (XII) was found. This value was unchanged after a further 30 min.

Reaction of N-Acetylphenylalanyl Phosphate (XII) *with Glycine.*—The remaining solution of (XII) (containing *ca.* 0·4 mmole) was washed with ether and treated with glycine (37·5 mg., 0·5 mmole) and buffered as before to pH 8·2. The mixture was then allowed to remain at

room temperature for 1 hr.; by then all the reactant (XII) had disappeared and only 65% of the glycine (0·325 mmole) remained. The solution was acidified with 85% phosphoric acid (0·5 ml.), and the acylamino-acid and acylpeptide products were extracted with ethyl acetate (cf. Kenner and Stedman, *J.*, 1952, 2069). Concentration of the extract gave a residue (90 mg.) which was dissolved in water and subjected to paper chromatography in *n*-butanol saturated with 2N-ammonia (Kenner and Stedman, *loc. cit.*). This enabled the two components to be identified as *N*-acetylphenylalanine and *N*-acetylphenylalanylglycine.

Acetyl Dibenzyl Phosphate (XIII; R = R′ = CH_2Ph, R″ = Me).—A suspension of silver dibenzyl phosphate in ether was treated with acetyl chloride (cf. Lynen, *Ber.*, 1940, **73**, 367), and the resulting ethereal solution of acetyl dibenzyl phosphate (after filtration of silver halide) was concentrated and used directly.

Acetyl Dimethyl Phosphate (XIII; R = R′ = R″ = Me).—Trimethyl phosphate and an equivalent amount of 2N-sodium hydroxide in 60—90 min. at 60° gave sodium dimethyl phosphate (hydrolysis followed by titration of aliquot portions at intervals). The sodium salt was converted into the triethylammonium salt by passage of the hydrolysis solution, diluted with water to 0·067M, down a column of Amberlite IRC-50 in the triethylammonium form, concentration of the aqueous effluent *in vacuo*, and drying of the residue by evaporation with benzene. The dry, syrupy triethylamine salt (1 mol.) was suspended in dry ether and treated with acetyl chloride (1·5 mols.) at 0°. The resulting ethereal solution of acetyl dimethyl phosphate was filtered under anhydrous conditions from the base hydrochloride and concentrated *in vacuo*. The resulting viscous oil had a purity of 80—90% and was stored at 0° before use.

Reactions of Neutral Acyl Phosphates with Phosphate Ions.—The reactions of the phosphates (XIII; R = R′ = CH_2Ph, R″ = Me; and R = R′ = R″ = Me) with sodium phosphate under the usual conditions (aqueous bicarbonate or aqueous pyridine) were followed analytically by the methods of Lowry and Lopez and of Berenblum and Chain (*locc. cit.*), as well as by the labile acetyl determinations on the ether-washed solutions. These showed that orthophosphate ions are acylated, but not dialkylphosphorylated, by acetyl dialkyl phosphates.

Acetyl dimethyl phosphate was also found to acetylate the phosphate group of sodium D-riboflavin-5′ phosphate.

Reaction of Tetramethyl Pyrophosphate with Phosphate Ions.—Tetramethyl pyrophosphate (Toy, *J. Amer. Chem. Soc.*, 1949, **71**, 2268) (1·6 g., 6·8 mmoles) was added to sodium phosphate (0·67 mmoles) in M-sodium hydrogen carbonate (40 ml.) vigorously stirred at 0°. As indicated by estimations of phosphate, about 25% of the orthophosphate ions were converted into *PP*-dimethyl dihydrogen pyrophosphate (XIV; R = R′ = Me, R″ = R‴ = H) in 1 hr. However, hydrolysis supervened to a detectable extent after 2—3 hr., even at 0°. When aqueous pyridine was used as reaction medium, the maximum conversion was only *ca.* 10% and, on warming to room temperature, hydrolysis was complete in 30 min. It is, of course, to be expected that an unsymmetrical dialkyl pyrophosphate should have somewhat the same hydrolytic stability as an acyl phosphate, and it appears that, whereas the hydrolysis is pyridine-catalysed, the synthesis is not (cf. proposed reaction mechanism).

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