Phosphorylated Sugars. Part 23.[1] Synthesis and Reactions of Phosphodiesters containing 2-Aminoethanol and a Polyfunctional Alcohol and their Behaviour during Acid- and Base-catalysed Hydrolysis

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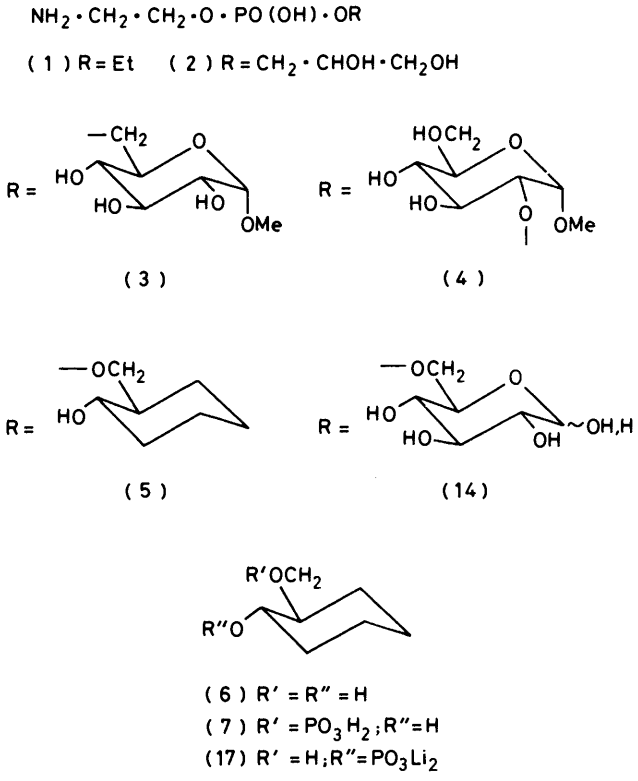
Phosphodiesters of 2-aminoethanol and of polyhydroxylated alcohols and sugars have been synthesised; their behaviour in M-HCl and in saturated (*ca.* 0.2M) $Ba(OH)_2$ solutions at 100 °C have been examined. With the exception of 2-aminoethyl ethyl phosphate which, in acidic medium, yielded 2-aminoethylphosphate, free 2-aminoethanol and phosphorylated polyols or sugars were the main products formed. Because of simultaneously and/or sequentially occurring reactions the structures of the phosphorylated derivatives formed gave no unambiguous information regarding the structure of the initial phosphodiester.

2-AMINOETHYL PHOSPHATE bound to glycerol is an ubiquitous component of phospholipids; it has also been identified as a component of the endotoxin of gram negative bacteria.[2] In the endotoxins of *Salmonellae* it appears to esterify position 7 of 3-deoxy-D-*manno*-oct-2-ulosonic acid, and also position 4 of L-*glycero*-D-*manno*-heptose, in the latter case as a pyrophosphate.[3,4] It has been observed that 2-aminoethyl phosphate and small amounts of free 2-aminoethanol were released upon mild acidic treatment of endotoxine.[2] The simultaneous release of 2-aminoethyl pyrophosphate and 2-aminoethyl phosphate, in mild acidic conditions, from the endotoxin of *Pseudomonas aeruginosa* was observed by Wilkinson,[5] and from that of *Bordetella pertussis* by R. Chaby (unpublished observation). Upon similar treatment free 2-aminoethanol (not 2-aminoethyl phosphate) and glycerol phosphate(s) were released from 2-aminoethyl hydrogen 2,3-dihydroxypropyl phosphate.[6,7] Simple phosphodiesters not carrying functional groups, *e.g.* dimethyl hydrogen phosphate, are not appreciably hydrolysed under such conditions.[8] It is known that phosphodiesters, such as ribonucleic[9] or teichoic acids[10] in which the esterifying groups bear free hydroxy-functions, are quite labile under both acidic and alkaline conditions, but the behaviour of analogous structures containing 2-aminoethanol [except for 2-aminoethyl hydrogen 2,3-dihydroxypropyl phosphate[6] and glycero(3)phosphocholine[11]] has not been investigated.

In view of the presence of 2-aminoethanol containing phosphodiesters and pyrophosphates in endotoxins, it appeared of interest to obtain information permitting predictions to be made of the behaviour of these molecular structures during acidic or basic hydrolyses used for structural studies. Accordingly, syntheses of phosphodiesters containing 2-aminoethanol as one of the esterifying groups were undertaken. The other esterifying group was chosen so as to give final products in which the steric position of the phosphate group and that of free hydroxy-groups were similar to those expected to be encountered in natural macromolecules. Thus, 2-aminoethyl ethyl hydrogen phosphate (1), 2-aminoethyl hydrogen 2,3-dihydroxypropyl phosphate (2), methyl 6-(2-aminoethylphosphoryl)-α-D-glucopyranoside (3), methyl 2-(2-aminoethylphosphoryl)-α-D-glucopyranoside (4), and 2-aminoethyl hydrogen *trans*-2-hydroxycyclohexyl phosphate (5) have been synthesised and their behaviour in acidic and alkaline media examined.

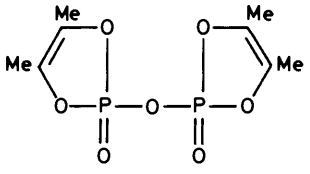
Phosphodiesters containing 2-aminoethanol as one of the ester groups have been obtained by the reaction of a



diesterified phosphoryl chloride with 2-(*N*-benzyloxycarbonylamino)ethanol followed by the removal of one of the esterifying groups,[12] by condensation of the silver salts of phosphono-2-phthaloylamino-,[13] t-butyloxycarbonylamino-,[14] and *N*-triphenylamino-[15] ethanol with iodo-derivatives, and from aziridine and monophenyl phosphoric acid[16] or phosphatidic acids,[17] in the latter case in chloroform solution. The low yield of the last

mentioned reaction was considerably improved when *N*-trityl aziridine was used instead of aziridine, the *N*-trityl group of the 2-aminoethanol derivative initially formed being removed by hydrogenolysis in acetic acid solution.[17] Attempted condensation of phosphodiesters with aziridine failed.[18]

The phosphodiesters (1)—(4) required for the present study were prepared as follows. Aqueous solutions or suspensions of salts of the appropriate phosphomonoesters were decationised with ion-exchange resins and



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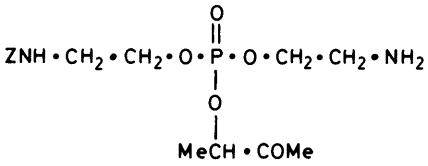

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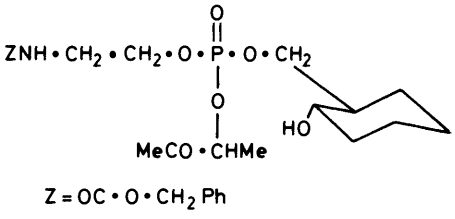

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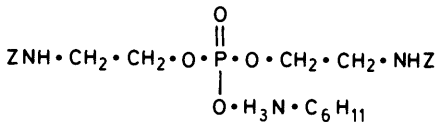
the free acid was neutralised with aziridine: upon concentration of the solution phosphodiesters containing 2-aminoethanol were readily formed albeit in very low (5—10%) yield. However, since the by-products are basic (polymers of aziridine) or acidic (unchanged phosphomonoester) the neutral phosphodiester was easily isolated by passage of the solution through appropriate ion-exchange columns. The unchanged phosphomonester was recovered and re-used.

trans-2-Hydroxymethylcyclohexanol (6), required for the synthesis of the phosphodiester (5), was easily obtained from the known[19] *trans*-2-hydroxycyclohexanecarboxylic acid: this, upon treatment with methanol and IR 120(H^+) resin gave the methyl ester which was reduced with borohydride to the corresponding diol (6). The synthesis of the phosphodiester (5) from dihydrogen *trans*-2-hydroxycyclohexylmethyl phosphate (7) (see below) and aziridine failed, none of the required phosphodiester being formed. The reason for this failure could not be established.

It has been shown by Ramirez and his colleagues[20] that unsymmetrical phosphodiesters can be obtained in a ' one-flask reaction ' by use of the pyrophosphate (8), the reagent being highly selective for the phosphorylation of primary *vs.* secondary alcohols. When this pyrophosphate was successively treated with *trans*-2-hydroxymethylcyclohexanol (6) and 2-(*N*-benzyloxy-



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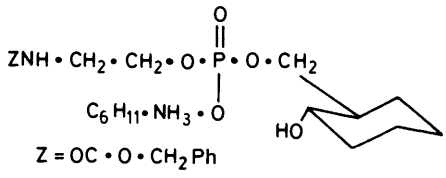

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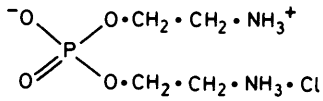
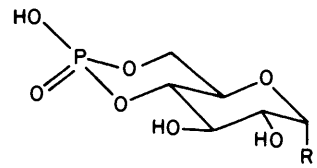


(13) • HCl



(15) R = H, OH in anomeric mixture
(16) R = OMe

carbonylamino)ethanol[21] in the presence of triethylamine, two phosphotriesters, (9) and (10), were formed in the ratio of 1 : 5; they were separated by column chromatography on silica gel. Structural assignments were based on their ^1H n.m.r. spectra and confirmed by transformation of the compounds into the phosphodiesters (11) and (12), isolated as the crystalline cyclohexylammonium salts. Following hydrogenolytic removal of the benzyloxycarbonyl groups, bis(2-aminoethyl) hydrogen phosphate (13) (*cf.* ref. 22) was crystallised as its hydrochloride while 2-aminoethyl *trans*-2-hydroxycyclohexyl hydrogen phosphate crystallised as the internal salt (5).

The isomeric phosphate esters (7) and (17) of *trans*-2-hydroxymethylcyclohexanol—being the expected breakdown products formed from the phosphodiester (5) upon alkaline treatment—were also required and synthesised

as follows. The primary hydroxy-group of the diol (6) was first selectively phosphorylated at 0 °C with 1.1 molar equivalents of diphenyl phosphoryl chloride in pyridine–dioxan solution. The phenyl groups were then removed by platinum-catalysed hydrogenolysis under pressure and the ester (9) was isolated as the Ba and Li salts. Only negligible phosphorylation (estimated to be *ca.* 5% by g.l.c.) of the secondary hydroxy-group occurred. To phosphorylate the secondary alcohol of the diol (6) the primary alcohol was first tritylated and the esterification was then carried out with phosphoryl chloride. After removal of the trityl group, the phosphate ester (17) was isolated by ion-exchange chromatography and recovered as the lithium-salt. The purity of both esters was controlled by g.l.c. after trimethylsilylation with bistrimethylsilyltrifluoroacetamide (BSTFA),[23] the isomers being cleanly separated in the conditions given in the Experimental section.

Alkaline Hydrolyses.—Hydrolyses were done at 100 °C with saturated aqueous $Ba(OH)_2$ solution (*ca.* 0.2M). This base was chosen since it is known [24,25] that hydroxides of di- and tri-valent cations are particularly efficient in promoting hydrolysis of phosphodiesters, and also because barium ions are relatively easy to remove when, as is often the case during structural analysis, the products of the hydrolysis are to be isolated.

For practical purposes 2-amino ethyl ethylphosphate (1) appeared to be completely stable to treatment with baryta (Table): at 100 °C after 72 h only 5% of the

TABLE

Approximate half-lives of 2-aminoethanol-containing phosphodiesters in acidic and basic media at 100 °C expressed in hours

Compounds	(1)	(2)	(3)	(4)	(5)	(14)
0.2M-$Ba(OH)_2$	∞	1/60	48	45/60	∞	*a*
M-HCl	38	2/60	3.25	3.50	20% in 80 h	1.45

[a] Not determined.

total phosphate appeared as inorganic phosphate; if, after a suitable work-up procedure, the hydrolysate was treated with a phosphomonoesterase, 7% of the total phosphate content was estimated as inorganic phosphate. Only traces of ethanol, as measured enzymatically, were present, and no 2-aminoethanol nor any 2-aminoethyl phosphate were detected upon paper chromatography or paper electrophoresis. It has been observed previously[26] that 2-aminoethyl phenyl phosphate was also stable in alkaline medium. It follows from these experiments that in 2-aminoethyl alkyl or aryl phosphates the NH_2 group does not attack the phosphorus atom to form a five-membered ring with simultaneous release of the esterifying group as is the case with analogous phosphotriesters.[27] It is noteworthy that 2-aminoethyl phosphoryl-L-serine has been reported to be completely destroyed when exposed to 0.5M-$Ba(OH)_2$ at 100 °C for 1 h.[28]

Because of the presence of a freely accessible, neighbouring hydroxy-group, 2-aminoethyl hydrogen 2,3-dihydroxypropyl phosphate (2) was found, as expected, to be very labile in baryta at 100 °C (Table): it was quantitatively destroyed within *ca.* 2 min to yield glycerol phosphate and free 2-aminoethanol. No 2-aminoethyl phosphate, nor inorganic phosphate, nor any cyclic phosphate esters could be detected in the hydrolysate. The glycerol phosphate mixture formed upon treatment of *sn*-glycero(3)phosphocholine with M-NaOH at 37 °C contained 44% of the α-isomer.[11] It has also been established previously [6] that upon treatment with 0.5M-NaOH at 100 °C, 2-aminoethanol was quantitatively released from the phosphate (2) within

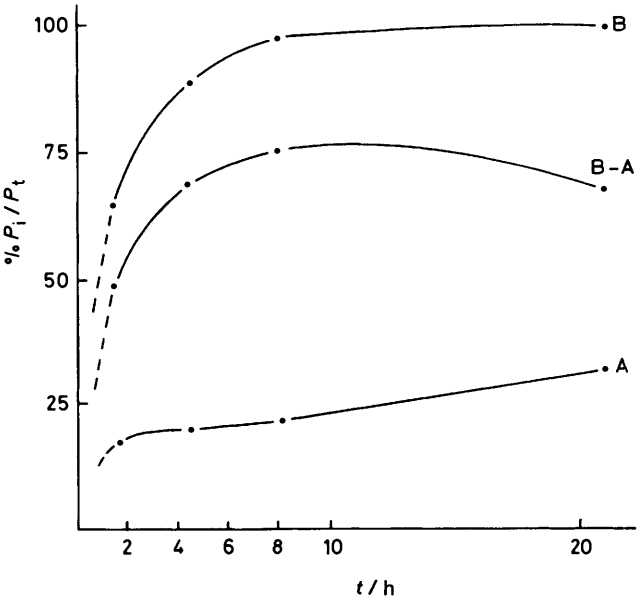


FIGURE 1 Kinetics of formation of inorganic phosphate (A), inorganic phosphate measured after treatment with phosphomonoesterase (B), and phosphomonoesters (B–A) during treatment of methyl 2-(2-aminoethylphosphoryl)-α-D-glucopyranoside (4) with 0.2M-barium hydroxide at 100 °C

3 h. In neither case has the formation of 2-aminoethyl phosphate been observed. The considerable enhancement of the rate at which 2-aminoethanol was released from the phosphate (2) when it was treated with baryta, rather than with 0.5M-NaOH, is similar to that reported [29] for the rate enhancement of the solvolysis of tetrabenzyl pyrophosphate and ethylene phosphate by calcium ions.

In methyl 2-(2-aminoethylphosphoryl)-α-D-glucopyranoside (4) the steric conditions leading to the base-catalysed transesterification reaction are less favourable, the vicinal free hydroxy-group on C-3 being in a position *trans* to that carrying the phosphate group: as a corollary, the rate of reaction was reduced compared to that of the phosphate (2); the half-life of the diester (4) in baryta at 100 °C was 40—50 min (Table). This was established by measuring on aliquots (i) the total phosphate content, (ii) the inorganic phosphate present, (iii) the inorganic phosphate released upon treatment with alkaline phosphomonoesterase (Figure). Analysis of the products formed revealed quantitative release of

2-aminoethanol: no 2-aminoethyl phosphate could be detected by paper chromatography or paper electrophoresis, the only phosphate containing products being (disregarding inorganic phosphate) derivatives of methyl α-D-glucopyranoside. Chromatographic analysis [30] revealed the presence of methyl glucoside-2, and 3-phosphates, these esters accounting for *ca.* 85% of the esterified phosphates. Unexpectedly, a phosphate ester eluting at the position of methyl α-D-glucopyranoside-4-phosphate, representing *ca.* 15% of the esterified phosphate, was also detected. The reaction sequence by which this compound was formed could not be established.

Not unexpectedly, the hydrolysis of the methyl glucoside derivative (3), which carries the 2-aminoethylphosphoryl-substituent in the position 6, and thus has no hydroxy-function adjacent to the phosphodiester grouping was very much slower than that of the diester (4): its half-life was *ca.* 48 h (Table) as measured by the inorganic phosphate released upon treatment with phosphomonoesterase. As in all previous cases only free 2-aminoethanol and no 2-aminoethyl phosphate was released as a result of the alkaline treatment. The only phosphorylated products formed were identified [30] as methyl α-D-glucopyranoside-6-, and -4-phosphates, formed in the ratio 1 : 5. The 6- and 4-phosphates were formed in the same ratio from the cyclic methyl α-D-glucopyranoside (16),[30] and phenyl β-D-glucopyranoside 4,6-(hydrogen phosphates) [31] upon hydrolysis with aqueous sodium hydroxide or barium hydroxide. This strongly suggests that the hydrolysis of the acyclic phosphodiester (3) proceeds *via* the cyclic 4,6-phosphodiester.

Finally, the phosphodiester (5), despite its formal resemblance to the methyl glucoside derivative (3), was found to be very resistant to alkaline hydrolysis: less than 10% of the phosphomonoesters were detected after 96 h (Table). Reasons for this unexpected behaviour are not known.

Acidic Hydrolyses.—These were mainly carried out in M-HCl at 100 °C, although in some instances 11 mM-acetic acid (pH 3.4) at 100 °C and trifluoroacetic acid of pH 3 at 50 °C were also employed, the latter conditions being not infrequently used in structural investigations on bacterial endotoxins.

In M-HCl 2-aminoethyl ethyl phosphate (1) was but slowly hydrolysed: its half-life was *ca.* 38 h (Table) as measured by the amount of inorganic phosphate formed in aliquots after treatment with alkaline phosphomonoesterase (Figure 2). It is reasonable to assume that the primary phosphorylated products should be ethyl phosphate and/or 2-aminoethyl phosphate: since, however, the enzymatically measured ethanol content of the hydrolysate was found to be the same before and after enzymic dephosphorylation, and since under the conditions used, the rates of hydrolysis of 2-aminoethyl phosphate and ethyl phosphate were very similar (Figure 3), it was concluded that the acid hydrolysis of the phosphodiester (1) M-HCl at 100 °C yielded mainly free ethanol and 2-aminoethyl phosphate. In fact, upon paper electrophoresis or paper chromatography 2-aminoethyl phosphate was identified but no ethyl phosphate was detected.

In contrast, and in agreement with previous work,[6] 2-aminoethyl hydrogen 2,3-dihydroxypropyl phosphate (2) was found to be rapidly hydrolysed; the reaction was complete within 15 min with an estimated half-life

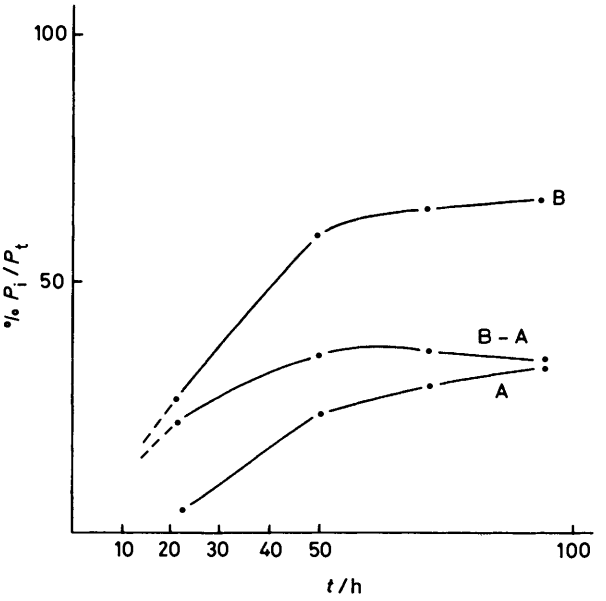


FIGURE 2 Kinetics of formation of inorganic phosphate (A), inorganic phosphate measured after treatment with phosphomonoesterase (B), and phosphomonoesters (B–A) during treatment of 2-aminoethyl ethyl phosphate (1) with M-HCl at 100 °C

of 2—3 min (Table); no inorganic phosphate was released. The products were glycerol 1- and 2-phosphates and free 2-aminoethanol: no 2-aminoethyl phosphate was found.

As regards the hydrolysis of the methyl glucoside derivatives (3) and (4), hydrolysis of the phosphodiester parallels the cleavage of the glycosidic bond. Consequently the results cannot be interpreted either mechanistically or kinetically, but since the phenomena to be described are those that can be expected to occur during hydrolytic fragmentation of macromolecules of natural origin, they are considered to be useful for structural studies. In fact, one of the major difficulties in quantitative biochemical analysis is the simultaneous occurrence of a number of reactions having similar, or at least comparable rates.

Upon treatment of methyl 2-(2-aminoethylphosphoryl)-α-D-glucopyranoside (4) with M-HCl, 2-aminoethanol was released but no 2-aminoethyl phosphate. From the amounts of inorganic phosphate estimated in aliquots before and after treatment with phosphomonoesterase, it can be calculated (Figure 4) that the amount of phosphomonoesters present in the reaction mixture never accounts for more than 25—30% of the total phosphate present in the sample, the highest

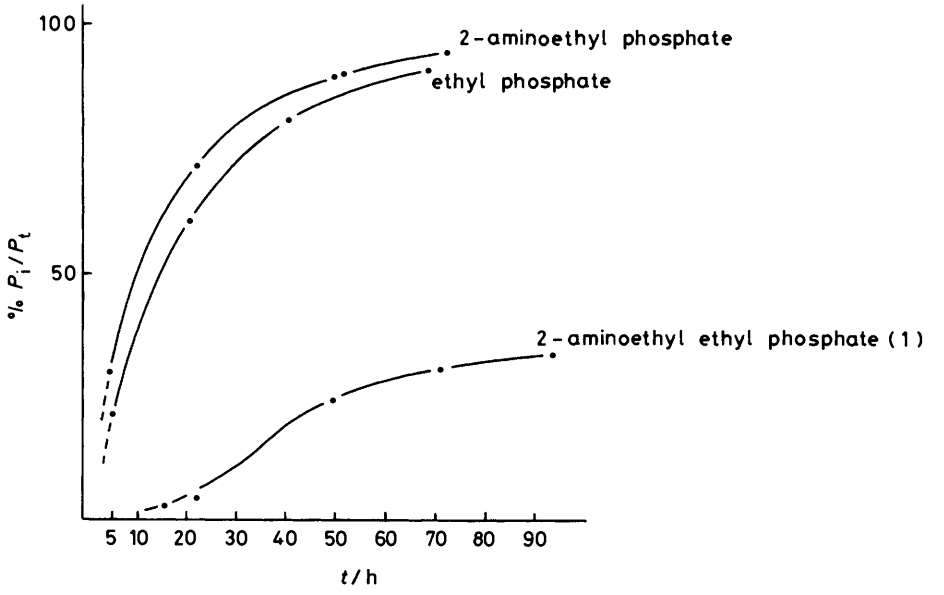


FIGURE 3 Kinetics of formation of inorganic phosphate from 2-aminoethylphosphate, ethyl phosphate, and 2-aminoethyl ethyl phosphate (1) during hydrolysis with M-HCl at 100 °C

value being reached between 4—8 h of hydrolysis. Although it is reasonable to assume that analogous phosphodiesters of the common aldohexoses will behave similarly, extrapolation of these data to other sugar derivatives should be made with caution, in view of the

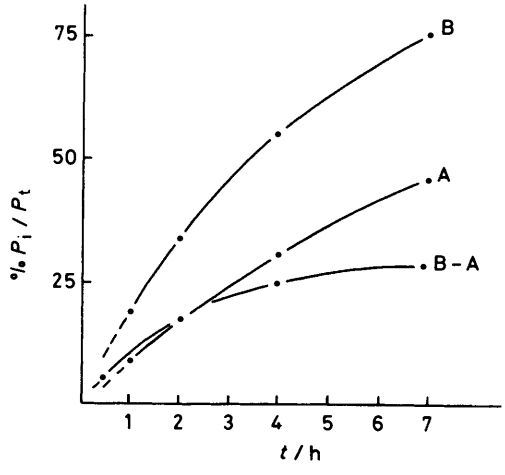


FIGURE 4 Kinetics of formation of inorganic phosphate (A), inorganic phosphate measured after treatment with phosphomonoesterase (B), and phosphomonoesters (B–A) during treatment of methyl 2-(2-aminoethylphosphoryl)-α-D-glucopyranoside (4) with M-HCl at 100 °C

considerable number of variables (position of the substituent on the sugar, steric effects, stability of the sugar to acid, *etc.*) involved. Because of the relatively small amounts of phosphodiesters present in the hydrolysate even during the peak period, no attempt was made to identify them individually.

Hydrolysis of the phosphate group in methyl 6-(2-aminoethylphosphoryl)-α-D-glucopyranoside (3) in M-HCl at 100 °C was rather slow: the half-life was just over 3 h (Table); traces of 2-aminoethanol containing phosphodiester(s), presumably 6-(2-aminoethylphosphoryl)-D-glucose (14), have been detected by chromatographic methods even after 7 h of hydrolysis. No 2-aminoethyl phosphate nor any cyclic phosphodiesters appeared on chromatograms. The main products were phosphomonoesters, only small amounts of inorganic phosphate being released (Figure 5). Although the influence of the 2-aminoethylphosphoryl group on the rate of hydrolysis of the glycosidic bond present in the glucoside (3) is not known, it can be assumed that at

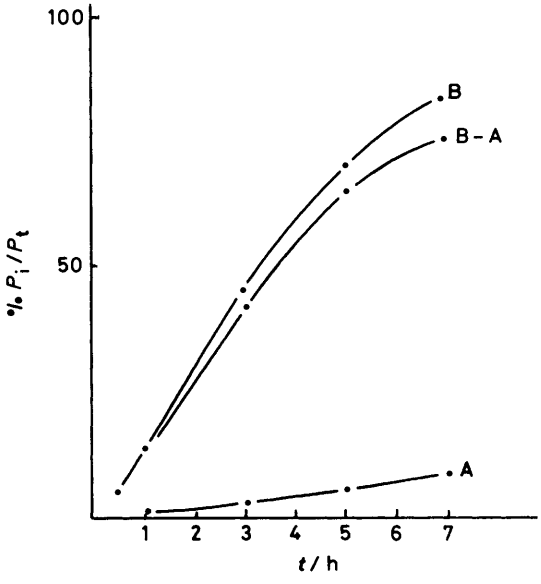


FIGURE 5 Kinetics of formation of inorganic phosphate (A), inorganic phosphate measured after treatment with phosphomonoesterase (B), and phosphomonoesters (B–A) during treatment of methyl 6-(2-aminoethylphosphoryl)-α-D-glucopyranoside (3) with M-HCl at 100 °C

least part of this glucoside will be transformed into 6-(2-aminoethylphosphoryl)-D-glucose (14) before the cleavage of the phosphodiester group. Indeed, in the phosphomonoester fraction, isolated after 4 h of hydrolysis, the total amount of esterified phosphate was equivalent to the sample's glucose content when this was estimated by methods that do not detect glucosides. The phosphomonoester fraction (contaminated with the sample's inorganic phosphate) required for this study was isolated by ion-exchange chromatography. The total glucose content was determined with the glucostat reagent after enzymic dephosphorylation, while glucose-6-phosphate was specifically estimated with glucose-6-phosphate dehydrogenase; the latter represents about 85% of the phosphomonoesters. The remaining 15% could be reasonably assumed to be either glucose-5-phosphate derived from the furanose or straight-chain forms of 6-(2-aminoethylphosphoryl)glucose *via* the 5-membered cyclic 5,6-phosphate, or glucose-4-phosphate produced *via* the 6-membered cyclic 4,6-phosphate and hydrolysis of the cyclic esters and the glucosidic bond. Since it was found that the amount of formaldehyde formed upon periodate treatment of the mixed phosphomonoesters corresponded to the amount of phosphomonoesters which were not glucose-6-phosphate, the presence of glucose-5-phosphate in the mixture could be excluded. To confirm the presence of glucose-4-phosphate an aliquot of the mixed phosphomonoesters was treated with baryta: it was expected that any glucose-4-phosphate would be transformed into ' gluco-isosaccharinic acid ' (a mixture of 2-*C*-hydroxymethyl-3-deoxypentonic acids) (*cf.* ref. 32). That this was indeed the case could be ascertained by treating the base-degraded material first with periodate and then with thiobarbituric acid:[33] a red dye[34] was formed which had an absorption maximum at 550 nm indicating that ' β-formylpyruvate ' (2,4-dioxobutanoic acid) had been produced as a result of the periodate oxidation. We have shown previously[35] that isosaccharinic acid, when treated with periodate and thiobarbiturate yielded ' β-formylpyruvate ' and the red dye, absorbing at 550 nm. Although the presence of glucose-2- and -3-phosphates in the mixed phosphomonoester fraction could not be rigorously excluded, the above mentioned experiments made their presence in large proportions unlikely and it can be concluded that glucose-6-phosphate and glucose-4-phosphate were the main products of the reaction. As with sugars, the rate of hydrolysis of phosphate esters of the secondary alcohols is faster than that of the primary alcohol, which leads, in this case to a diminished yield of glucose-4-phosphate; the ratio of glucose-6-phosphate to glucose-4-phosphate can be estimated to be 4—5 to 1.

It was conceivable that the glucose-4-phosphate present in hydrolysate was formed *via* the 6-membered, cyclic 4,6-phosphate of methyl glucoside or glucose, by a reaction analogous to the well known[36] intramolecular transesterification reaction of certain acyclic phosphodiesters which lead, *via* 5-membered cyclic phosphodiesters, followed by ring cleavage, to mixtures of phosphomonoesters. To test this hypothesis methyl α-D-glucopyranoside 4,6-(hydrogen phosphate) (16)[37,38] was treated with M-HCl at 100 °C and the products formed were estimated after 1, 2, 3, 4.5, and 6 h of hydrolysis as follows. Phosphomonoesters were determined as the difference in inorganic phosphate present in the sample after and before treatment with phosphomonoesterase (Figure 6a). Glucose 6-phosphate was estimated with glucose-6-phosphate dehydrogenase (glucose estimation kit of Boehringer, Mannheim but omitting ATP) (Figure 6b). Free glucose was determined with the same system containing ATP: the free glucose content was calculated from the glucose-6-phosphate content measured by the hexokinase/glucose 6-phosphate dehydrogenase reaction carried out in the presence and absence of ATP (Figure 6b). Finally, to ensure that most of the sample's glucose content had survived the acidic treatment and was present at the time when essentially no more phosphodiester was left (6 h), the glucose content was estimated with the glucostat reagent after the sample had been treated with phosphomonoesterase (Figure 6c). It can be seen from Figure 6a that after 6 h of hydrolysis the sample's phosphate content was present either as inorganic phosphate (32%) or as phosphomonoester (65%); 13% of the latter are undoubtedly glucose 6-phosphate (Figure 6b). It follows that, if it is assumed that no glucose 2-, or 3-phosphate, nor any phosphorylated breakdown product of glucose was present, 52% of the phosphomonoesters were glucose 4-phosphate. Because of the uncertainty of this approximation, the glucose 4-phosphate content was also calculated from the sample's glucose content in the following way. After 6 h of hydrolysis and treatment with phosphomonoesterase, 85% of the sample's glucose content still survived (Figure 6c): 41% of this was accounted for by the sample's glucose 6-phosphate (13%) plus free glucose (28%) contents (Figure 6b). The glucose 4-phosphate present in the sample appeared then to be 44%, *i.e.* of the same order of magnitude as estimated by the first method. It follows that, in the conditions of acidic hydrolysis employed methyl α-D-glucopyranoside 4,6-(hydrogen phosphate) forms, as principal products, glucose-4- and glucose-6-phosphate in the ratio of 4—5 to 1. As methyl 6-(2-aminoethylphosphoryl)-α-D-glucopyranoside (3) yielded the same products in an approximately reciprocal ratio, it can be concluded that the cyclic glucose and/or methyl-glucoside 4,6-(hydrogen phosphate)s (15) and (16) are not major intermediates during the hydrolytic breakdown of the acyclic 2-aminoethanol containing phosphodiester (3).

These conclusions were corroborated by the analysis of the phosphomonoesters formed upon hydrolysis of compound (5), which cannot assume a ' straight-chain ' form as is the case with 6-(2-aminoethylphosphoryl)-D-glucose (14), and should, therefore, exemplify the hydrolysis of an ' acid fast ' 6-(2-aminoethylphosphoryl)-glucopyranoside. Hydrolysis of the phosphodiester (5) was very slow (Table): after 80 h 20% of the total

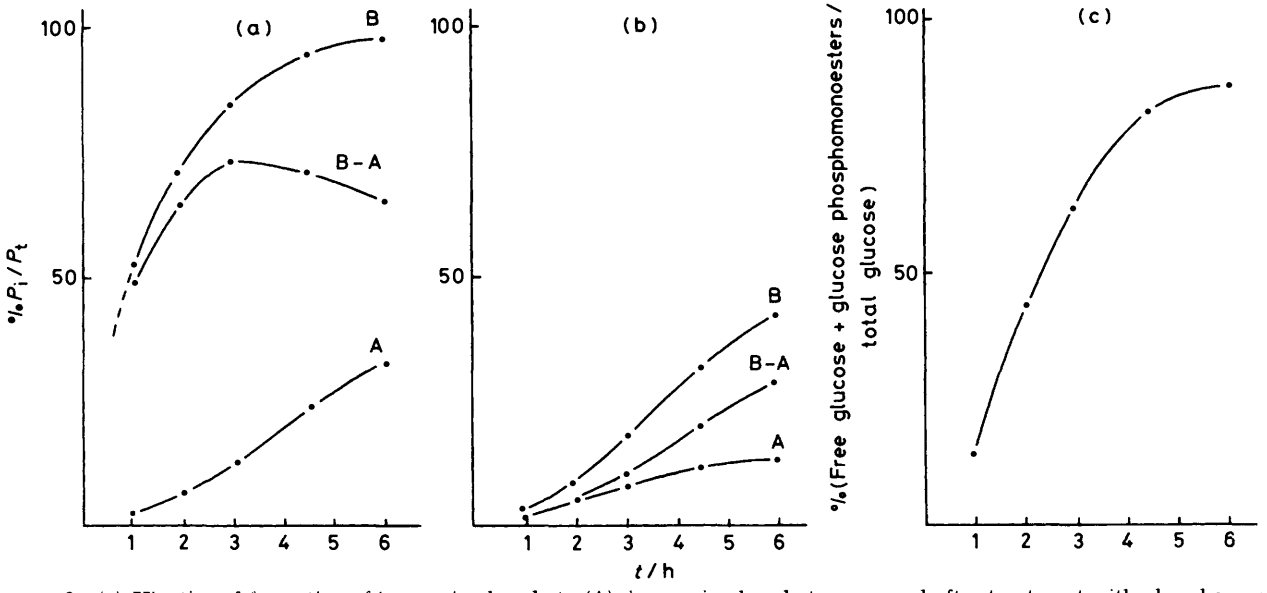


FIGURE 6 (a) Kinetics of formation of inorganic phosphate (A), inorganic phosphate measured after treatment with phosphomonoesterase (B), and phosphomonoesters (B–A) during treatment of methyl α-D-glucopyranoside 4,6-(hydrogen phosphate) (16) with M-HCl at 100 °C (b) Kinetics of formation of glucose 6-phosphate (A), glucose + glucose 6-phosphate (B), and free glucose (B–A) from compound (16) in the same conditions (c) Kinetics of formation of phosphomonoesters + free glucose from compound (16) under the same conditions

phosphate content was inorganic phosphate and 30% was present as phosphomonoester as estimated by the inorganic phosphate released by phosphomonoesterase. Product analysis (carried out after 24 h of hydrolysis) was done by g.l.c. of the pertrimethylsilylated [23] material, identification of the products being made by comparison of the retention times with those of synthetic products. The ratio of the phosphate of the secondary alcohol to that of the primary alcohol was found to be 3 to 1, *i.e.* similar to that found for methyl α-D-glucopyranoside 4,6-phosphate (16) and the reverse of that found for the acyclic phosphodiester (3).

The formation of a six-membered, cyclic 4,6-phosphate of glucose or methyl glucoside as the main intermediate in the acid-catalysed hydrolysis of the acyclic phosphodiesters (3) being thus ruled out, the possibility that the first step of the breakdown of compound (3) was hydrolysis of the glucosidic bond, and that the molecular species undergoing cleavage of the phosphodiester group was free 6-(2-aminoethylphosphoryl)-D-glucose (14) was investigated. Indeed, when this compound (synthesised from glucose 6-phosphate and aziridine) was treated with M-HCl at 100 °C, besides free 2-aminoethanol and a trace (6% after 4 h 15 min) of inorganic phosphate, the only phosphomonoester formed appeared to be glucose 6-phosphate: it accounted for 94% of the sample's phosphate and glucose content (Figure 7).

The half-life (Table) of 6-(2-aminoethylphosphoryl)-glucose (14) was *ca.* 90 min in M-HCl at 100 °C in sharp contrast with the 38 h measured for 2-aminoethyl ethyl phosphate (1); cleavage of the former was, therefore, very probably not a direct hydrolysis. The following reaction sequence (Scheme) could explain the experimental results. The phosphodiester (14) in the straight chain or the furanose form first undergoes intramolecular transesterification to yield the five-membered, cyclic 5,6-phosphate. Opening of the ring yields the 5- and 6-phosphates of glucose. The latter is stabilised by the

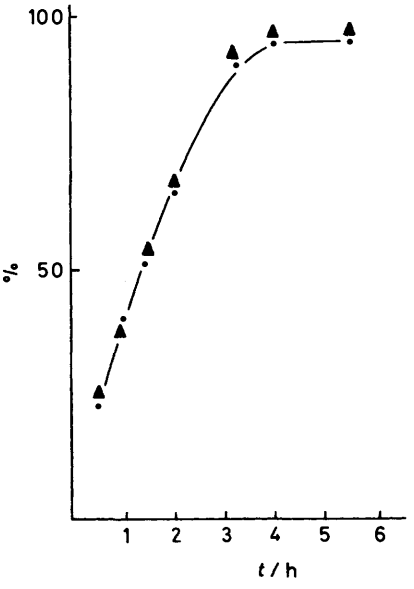
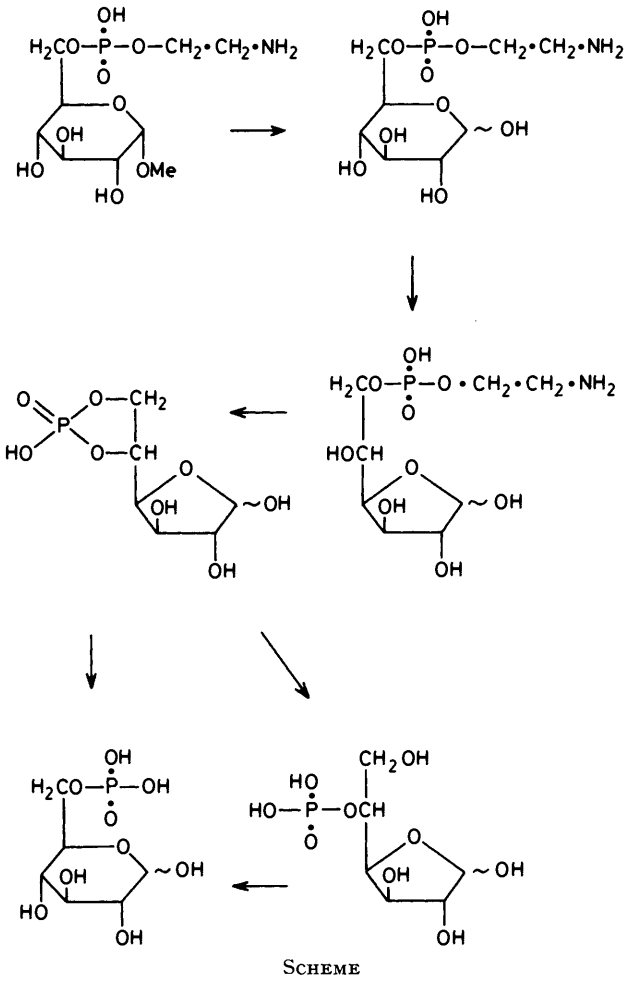


FIGURE 7 Kinetics of formation of glucose 6-phosphate (● — ●) and of phosphomonoesters (▲ — ▲) from 6-(2-aminoethylphosphoryl)-D-glucose (14) during hydrolysis with M-HCl at 100 °C

re-formed pyranose ring (glucose 6-phosphate is quite stable under the conditions used) while the 5-phosphate undergoes acid-catalysed phosphate migration to yield mainly, if not exclusively, the phosphate ester of the primary alcohol as is the case with glycerol-2-phosphate [39]

ultimately leading to the formation of glucose 6-phosphate as the sole detectable phosphomonoester among the final products. The glucose 4-phosphate found in the hydrolysate of compound (3) was thus probably derived from a small amount of methyl glucoside 4,6-phosphate (16), formed by intramolecular transesterification before hydrolysis of the glycosidic bond.



SCHEME

Finally, the stability of 2-aminoethyl ethyl phosphate (1) and of 2-aminoethyl hydrogen 2,3-dihydroxypropyl phosphate (2) was examined under conditions used for the selective cleavage of the glycosidic bond of the 3-deoxyoct-2-ulosonic acid present in the endotoxin of gram negative bacteria, *i.e.* treatment with 11mM-acetic acid of pH 3.4 at 100 °C for 1 h and treatment with aqueous trifluoroacetic acid of pH 3 at 50 °C for 144 h: neither compound was hydrolysed under these conditions.

As a result of these studies it would appear on the one hand that only phosphodiesters of the type represented by 2-aminoethyl ethyl phosphate (1), *i.e.* possessing no functional group other than the NH_2 group, are likely to release 2-aminoethylphosphate upon acidic hydrolysis, and, on the other hand, that the behaviour and the breakdown products of phosphodiesters exemplified by methyl 6-(2-aminoethylphosphoryl)-α-D-glucopyranoside (3) are not easily predicted on the sole basis of the steric arrangement of the phosphodiester grouping with respect to nearby functional groups.

EXPERIMENTAL

Evaporations were carried out under reduced pressure at 40 °C. Salts were dried *in vacuo* and then equilibrated with ambient humidity; other products were dried *in vacuo*; solutions in organic solvents were dried with Na_2SO_4. M.p.s were determined on a Kofler hot plate and are uncorrected; optical rotations were measured with a Perkin-Elmer model 141 polarimeter. G.l.c. was performed on a Varian Aerograph 1 800 instrument equipped with a flame ionisation detector and a stainless-steel column [3.2(O.D.) × 1 500 mm] packed with 3% SE 30 on Varaport 30 (100—120 mesh). Paper chromatography was performed on Whatman 3 MM paper with propan-2-ol–concentrated ammonium hydroxide–water (7 : 1 : 2, v/v, descending) for 17 h at room temperature; R_F values are given with respect to picric acid unless otherwise stated. T.l.c. was performed on silica gel (Merck 60 PF 254). ^1H N.m.r. spectra were taken with a Varian T 60 instrument with tetramethylsilane as internal standard.

2-Aminoethyl Ethyl Hydrogen Phosphate (1).—A suspension of ethyl (dilithium phosphate) [40] (4 g) in water (20 ml) was stirred with Amberlite IR 120 (H^+) resin (5 ml) until it had dissolved; the mixture was then poured into a column (12 × 3.5 cm) of the same resin and the column washed with water until the pH of the effluent reached 4—5. The pH of the effluent (*ca.* 300 ml) was brought to 7.4 with aziridine distilled immediately before use. The water was removed and the residue was taken up in water (50 ml); the pH of the solution was adjusted to 7.4 with dilute aqueous LiOH and the mixture was passed successively through columns of Dowex 1 × 8 (carbonate form, 3.5 × 12 cm) and Amberlite IR 120 (H^+) (3.3 × 12 cm) resins. Both columns were washed with water until free of phosphorus (11) and the neutral solution was brought to dryness. Ethanol was evaporated twice from the residue (*ca.* 500 mg) which was then dissolved in ethanol (5 ml). Acetone was added until incipient crystallisation. The *product* (400 mg) collected, washed with acetone, and dried had m.p. 238 °C, R_F 0.67 (Found: C, 28.6; H, 7.2; N, 8.3; P, 18.3. $C_4H_{12}NO_4P$ requires C, 28.4; H, 7.1; N, 8.3; P, 18.3%).

2-Aminoethyl 2,3-Dihydroxypropyl Hydrogen Phosphate (2).—An aqueous solution (20 ml) of disodium 2,3-dihydroxypropyl phosphate [39] (2 g) was allowed to percolate through a column (10 × 1.8 cm) of Amberlite IR 120 (H^+) resin; the resin was washed free of acid with water (*ca.* 200 ml) and the pH of the solution brought to 7.4 with aziridine. Solvents were removed and the residue was dissolved in water (20 ml), to give a solution the pH of which was brought to 7 with dilute LiOH. The solutions were passed through Dowex and Amberlite resins as described above and the columns were washed free of phosphorus with water (*ca.* 600 ml) (monitoring with Ninhydrin is too sensitive). The concentrated (to *ca.* 20 ml) effluent was treated with a mixture of Celite–charcoal, filtered, and brought to dryness. The *product* (150 mg), homogeneous by paper chromatography (R_F 0.53) could not be induced to crystallise. Upon treatment with periodate it gave 1 mol equiv. of formaldehyde per mole of phosphorus (absence of the 1,3-dihydroxypropyl isomer) (Found: C, 24.95; H, 6.7;

N, 5.8. $C_5H_{14}NO_6P\cdot1.5H_2O$ requires C, 25.0; H, 7.1; N, 5.8%). In some cases the effluent of the IR 120 (H^+) resin was weakly acidic (pH *ca.* 3): upon paper electrophoresis small amounts of glycerophosphate could be detected; it is likely that it was formed on the slightly alkaline Dowex column; it was eliminated by addition of small amounts (2—5 ml) of Amberlite 45 (OH^-) resin to the pooled final effluents.

Methyl 6-(2-*Aminoethylphosphoryl*)-α-D-*glucopyranoside* (3).—An aqueous (20 ml) suspension of methyl α-D-glucopyranoside 6-(biscyclohexylammonium phosphate) [38] (2 g) was stirred with Amberlite IR 120 (H^+) resin (20 ml) until the salt had dissolved; the resin was filtered off and washed with water. The pH of the pooled filtrate and washings (*ca.* 100 ml) was brought to 7.4 with aziridine and the mixture treated as described for compounds (1) and (2). The pooled effluents (*ca.* 500 ml) of the ion-exchange columns (monitoring was done with the phenol–sulphuric acid reagent [41] for neutral sugars) were brought to dryness, the residue was dissolved in methanol (10 ml), and the *product* (80 mg) precipitated by addition of diethyl ether (*ca.* 100 ml). When dried it had m.p. 142 °C, $[\alpha]_D^{22}$ +88° (*c*; 0.53, water) and R_F 0.48 (Found: C, 32.5; H, 6.6; N, 4.2. $C_9H_{20}NO_9P\cdot H_2O$ requires C, 32.2; H, 6.6; N, 4.2%).

Methyl 2-(2-*Aminoethylphosphoryl*)-α-D-*glucopyranoside* (4).—This compound was prepared in the same way as its isomer described above starting from methyl α-D-glucopyranoside 2-(biscyclohexylammonium phosphate) [38] (500 mg) and arizidine. The *product*, precipitated from methanol (2 ml) with ether (50 ml) had m.p. 185—190 °C (decomp.), $[\alpha]_D^{23}$ + 39.8° (*c*, 1.9, water) and R_F 0.54 (Found: C, 30.3; H, 6.65; N, 4.1. $C_9H_{20}NO_9P\cdot2H_2O$ requires C, 30.6; H, 6.8; N, 4.0%.

trans-2-*Hydroxymethylcyclohexanol* (6).—A mixture of *trans*-2-hydroxycyclohexanecarboxylic acid [19] (10 g), Amberlite IR 120(H^+) resin (washed free of water with methanol and dried), and anhydrous methanol were heated under reflux and stirring for 2 h, the progress of the esterification being followed by t.l.c. with ethyl acetate as solvent and the R_F of the ester being 1.36 with respect to the acid. The cooled mixture was filtered and the resin was washed with methanol. The syrup (10 g) remaining after removal of the solvent was dissolved in water (100 ml) and to the cooled (ice–water) solution was added, dropwise in *ca.* 30 min, $NaBH_4$ (5 g) in water (100 ml). Stirring was continued for a further hour before the solution was extracted with ethyl acetate (3 × 200 ml). The dried extract was freed from solvent and the residue (8.5 g) distilled under water-pump vacuum to yield the *diol*, b.p. 146 °C at 16 mmHg. The product for analysis was redistilled twice (Found: C, 64.4; H, 10.6. $C_7H_{14}O_2$ requires C, 64.6; H, 10.8%).

Dihydrogen trans-2-*Hydroxycyclohexylmethyl Phosphate* (7).—To a solution of the preceding diol (3.5 g) in a mixture of pyridine (9 ml) and dioxan (15 ml) kept in an ice–salt mixture was added, dropwise in *ca.* 1 h, diphenylphosphoryl chloride (8.1 ml) in dioxan (33 ml) with magnetic stirring. Stirring and cooling were continued for a further hour after which the mixture was allowed to reach room temperature and kept overnight. The syrup remaining after removal of the solvents was dissolved in chloroform (300 ml) and the solution was washed successively with aqueous sulphuric acid, 5% aqueous sodium sulphate (× 2), water, saturated aqueous sodium hydrogen carbonate and water. The organic layer was dried and the solvent was removed to leave a syrup (8—9 g). The phosphorylation can be followed visually by t.l.c. using benzene–ethyl acetate–methanol (70 : 30 : 5, v/v); the R_F of the diphenyl phosphate is 1.9 with respect to that of the diol. The above syrup was dissolved in methanol (100 ml) and hydrogenated overnight under pressure (30 bar) with Pt (500 mg of PtO_2) as catalyst. After removal of the catalyst an equal volume of water was added to the filtrate, and the pH of the acidic solution was brought to 7.5 with aqueous barium hydroxide; the volume was then reduced to *ca.* 50 ml. The *barium salt* of the title compound (6.2 g) was precipitated with ethanol (200 ml) and dried (Found: C, 36.2; H, 6.1; P, 13.2. $C_7H_{13}BaO_5P\cdot0.5H_2O$ requires C, 36.4; H, 6.1; P, 13.4%). To prepare the lithium salt the barium salt (1 g) was suspended in water (10 ml), Amberlite IR 120 (H^+) resin (10 ml) was added, and the mixture stirred until dissolution of the solids was complete. The resin was removed and washed, the pH of the filtrate and washings was brought to 7.1 with aqueous lithium hydroxide and the solution was concentrated to a syrup. The lithium salt (600 mg) was precipitated with ethanol and had R_F 0.52.

trans-2-*Hydroxymethylcyclohexyl Dilithium Phosphate* (17).—To the diol (6) (130 mg) was added a solution of triphenylmethyl chloride (300 mg) in pyridine (3 ml) and the mixture was left at room temperature overnight. Phosphoryl chloride (200 mg) in pyridine (2 ml) was added to the cooled (ice–salt) mixture which was then allowed to reach room temperature in *ca.* 2 h. The mixture was diluted with water (3 ml) and brought to dryness. The residue was dissolved in water (5 ml) and ethanol (5 ml) and Amberlite IR 120(H^+) resin (5 ml) was added. The mixture was kept on a boiling water-bath for 10 min and then cooled. The resin was filtered off and washed with water and the pH of the pooled filtrate and washings was brought to 7.1 with aqueous lithium hydroxide. Ethanol was distilled off and the remaining aqueous suspension was extracted with ether (2 × 25 ml) to eliminate triphenylmethanol. The volume of the aqueous phase was brought to 50 ml with water after which its pH was adjusted to 8 with dilute aqueous lithium hydroxide; it was then passed through a column (1.2 × 10 cm) of Dowex 1 × 8 (OH^-) resin. The resin was eluted with 10 mM-hydrochloric acid, inorganic phosphate being eluted first followed by the ester (17). Fractions (5 ml) containing the latter were pooled and their pH was brought to 7.2 with aqueous lithium hydroxide. The solution was concentrated to a syrup which was triturated with a mixture of ethanol and ether (1 : 1) and the precipitate (the product + LiCl) was recovered by centrifugation. It was then re-suspended in a mixture of ethanol–acetone (3 : 7, v/v) and centrifuged; this operation was repeated until the product (80 mg) was free of chloride ions. When dry it had R_F 0.48 (Found: C, 34.8; H, 6.0; P, 13.1. $C_7H_{13}Li_2O_5P\cdot H_2O$ requires C, 35.0; H, 6.25; P, 12.9%). In contrast to its isomer (7) the lithium salt of this phosphate was soluble in ethanol and insoluble in acetone and ether.

2-*Aminoethyl Hydrogen* trans-2-*Hydroxycyclohexyl Phosphate* (5) *and Bis*-(2-*aminoethyl*) *Hydrogen Phosphate* (13).—(a) *Condensation.* To a stirred solution of the diol (6) (1.3 g, 10 mmol) in dichloromethane (10 ml) containing triethylamine (1 g, 10 mmol) was added acetoin enediol cyclopyrophosphate (CEP-OCEP) [20] (2.82 g, 10 mmol) in dichloromethane (10 ml). The mixture was stirred for 1 h after which a solution of *N*-benzyloxycarbonylaminoethanol [21] (1.95 g, 10 mmol) and triethylamine (1 g) in dichloromethane (10 ml) was added and the reaction was allowed to proceed

overnight. The mixture was then diluted with dichloromethane (200 ml) and successively extracted with aqueous 5% sodium carbonate (3 × 50 ml), 5% hydrochloric acid (2 × 50 ml), and water (2 × 50 ml). Solvents were removed from the dried and filtered organic phase which left a yellow oil (3.2 g). T.l.c. with ethyl acetate as solvent gave two spots (detected with 10% sulphuric acid in ethanol and heating) having R_F 0.1 and 0.15.

(b) *Separation of the Esters* (9) *and* (10). The oil (1 g) dissolved in ethyl acetate (2.5 ml) was applied to a column (2.5 × 28 cm) of silica gel (Merck PF 254) prepared in ethyl acetate under pressure (80 bar). Elution with the same solvent under pressure (40 bar) gave as the first product the compound (200 mg) with R_F 0.15. Its n.m.r. spectrum [δ (60 MHz) ($CDCl_3$) 7.8—7.6 (10 H, m, ArH), 6.0 (2 H, br s, NH), 5.6 (4 H, s, CH_2Ph), 5.2 (1 H, m, PO–C*H*–CH_3), 4.8—4.4 (4 H, m, PO–CH_2), 4.1—3.8 (4 H, m, CH_2–N), 2.6 (3 H, s, CH_3CO), and 1.8 (3 H, d, *J*, 4 Hz, CH_3CH)] indicated that it was the symmetrical triester (9) containing two benzyloxycarbonamidoethyl residues. It was closely followed by the second compound (650 mg) having R_F 0.10 the n.m.r. spectrum of which [δ (60 MHz) ($CDCl_3$) 7.8—7.6 (5 H, m, ArH), 6.0 (1 H, br s, NH), 5.6 (2 H, s, CH_2Ph), 5.2 (1 H, m, POC*H*CH_3), 4.8—4.4 (4 H, m, POCH_2), 4.1—3.8 [3 H, m, CH_2–N and *H*C–(C,C,C)], 3.2 (1 H, s, OH), 2.6 (3 H, s, CH_3CO), 2.1—1.8 (3 H, d, CH_3CH) and (9 H, m, cyclohexane protons) indicated that it was the triester (10).

(c) *Hydrolysis of the triesters* (9) *and* (10) *to the diesters* (11) *and* (12). To a solution of the symmetrical triester (9) (200 mg) in acetonitrile (2 ml) were added water (4 ml) and triethylamine (200 mg). The mixture was kept at 70 °C overnight; solvents were removed, the residue was dissolved in water (10 ml), and the solution was extracted with dichloromethane (2 × 25 ml); the dried phase was acidified (5% hydrochloric acid) and again extracted with dichloromethane (2 × 25 ml); the dried organic phase was neutralised with cyclohexylamine in ethanol before being brought to dryness. The residue was dissolved in the minimum amount of ethanol: upon addition of ethyl acetate (10 ml) the symmetrical *diester* (11) (120 mg) crystallised, m.p. 193 °C (Found: C, 56.2; H, 7.3; N, 7.5. $C_{26}H_{38}N_3O_4P$ requires C, 56.6; H, 6.9; N, 7.6%), R_F 0.98. The diester (11) (100 mg) was dissolved in ethanol, treated with Amberlite IR 120(H^+) resin, and the filtered solution was hydrogenated over a 10% Pd-on-carbon catalyst (*ca.* 1 h). To the filtered and concentrated solution was added 1 mol equiv. of hydrogen chloride in ethanol and ethyl acetate (5 ml) to give the crystalline hydrochloride of the diester (13) (30 mg), m.p. 248 °C (Found: C, 21.6; H, 6.5; N, 12.6. $C_4H_{13}N_2O_4P$·HCl requires C, 21.7; 6.50; N, 12.6%), R_F 0.36.

The unsymmetrical triester (10) (650 mg) was treated with triethylamine as described for the symmetrical triester. The crystalline (ethanol–ethyl acetate) product (600 mg) had m.p. 118 °C and R_F 0.98 (Found: C, 56.6; H, 8.2; N, 5.9. $C_{23}H_{39}N_2O_7P$ requires C, 56.8; H, 8.0; N, 5.8%). Hydrogenation under the same conditions as given for the diester (11) followed by the same work-up procedure but without addition of hydrogen chloride, gave the crystalline asymmetrical diester (12) (270 mg), m.p. 234 °C (Found: C, 42.8; H, 8.0; N, 5.6. $C_9H_{20}NO_5P$ requires C, 42.7; H, 7.9; N, 5.5%), R_F 0.75.

D-*Glucose* 6-(2-*Aminoethyl phosphate*) (14).—An aqueous solution (10 ml) of D-glucose 6-(disodium phosphate) (900 mg) was stirred with Amberlite IR 120 (H^+) resin (20 ml) for a few minutes after which the resin was filtered off and washed with water. The pH of the acid solution was brought to 7.4 with freshly distilled aziridine and the solvent was removed. The dry residue was extracted with ethanol (2 × 50 ml) and the solvent was removed from the extract. The residue was dissolved in water (20 ml), and the pH of the solution was brought to 7.5 with aqueous lithium hydroxide; the solution was then passed successively through columns of Amberlite IR 400 (OH^-) (3 × 20 cm) and IR 120 (H^+) (2 × 25 cm) resins. The columns were washed until the effluent gave a negative test for neutral sugars.[41] Solvent was removed from the effluent and the residue was taken up in water (1 ml); methanol (5 ml) and acetone (30 ml) were then added. The precipitated *diester* (70 mg) was recovered by centrifugation and dried. It had m.p. 140 °C; $[\alpha]_D$ +18° (equil. after 4 h, *c* 1, water) (Found: C, 29.5; H, 5.95; P, 9.5. $C_8H_{18}NO_9P·H_2O$ requires C, 29.9; H, 6.2; P, 9.7), R_F 0.22.

Alkaline Degradation of the Glucose 4-*Phosphate formed during the Acidic Hydrolysis of Methyl* 6-(2-*Aminoethylphosphoryl*)-α-D-*glucopyranoside*.—A sample (10 ml) of the hydrolysate containing *ca.* 5 mg/ml of phosphate esters was brought to dryness. The residue was taken up in aqueous barium hydroxide (0.4 M; 3 ml) and nitrogen was bubbled through the mixture; the sealed flask was then kept for 48 h. IR 120 (H^+) resin was then added to remove the barium ions and the resin was filtered off. The pH of the acid solution was brought to 10 with concentrated ammonium hydroxide and the solvent was removed. The residue was dissolved in water (10 ml) and the treatment with ammonium hydroxide repeated to ring-open any lactone present. The residue was dissolved in water (1 ml); to an aliquot (0.1 ml) of it was added sodium periodate (0.025 M in 0.125 M-sulphuric acid; 0.1 ml) and 20 min later aqueous sodium arsenite (2% in 0.5 M-HCl; 0.15 ml) followed by the thiobarbiturate reagent.[42] The mixture was kept at 100 °C for 10 min, cooled, diluted with 0.3M–HCl (1 ml) and the absorption measured in the visible range; a band ($\lambda_{max.}$ 550 nm), indicating the presence of isosaccharinic acid in the base-treated hydrolysate, was observed.

Estimation of Phosphomonoesters.—(a) *In acidic hydrolysates*. To a sample (1 ml) of the hydrolysate (M with respect to HCl) and containing *ca.* 10 μmol of the substrate to be hydrolysed, were added trishydroxymethylaminomethane (Tris) (0.5 M; 1.1 ml) and water (0.9 ml) to give, after thorough mixing, a solution of pH 8. Alkaline phosphatase suspension (Boehringer, Marburg), from calf intestine (20 μl) was added and the mixture kept for 2 h at 37 °C [compounds (1) and (3)] or overnight at 20—22 °C [compounds (2) and (4)]. The inorganic phosphate formed was estimated either by the methods of Macheboeuf and Delsal [43] or Chen *et al.*[44]

(b) *In alkaline hydrolysates*. Except for those of 2-aminoethyl ethyl phosphate (1), the samples were decationised [IR 120 (H^+) resin], and the volume of the filtered solution was brought to 5 ml with water. Trishydroxymethylaminomethane (0.5 M; 0.5 ml) was added and the sample was treated with alkaline phosphate as above. Hydrolysates of 2-aminoethyl ethyl phosphate were acidified with a very slight excess of sulphuric acid, filtered, the volume was brought to 5 ml with water and then treated as described above with Tris and phosphatase. The total amount of phosphate present in each aliquot was estimated.

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