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Phosphonomethyl Analogues of Phosphate Ester Glycolytic Intermediates

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Analogues of dihydroxyacetone phosphate and of 3-phosphoglycerate were made in which the phosphate group, $-O-PO_3H_2$, is replaced by the phosphonomethyl group, $-CH_2-PO_3H_2$. The analogue of dihydroxyacetone phosphate is a substrate for aldolase and glycerol 1-phosphate dehydrogenase (Stribling, 1974), but not for triose phosphate isomerase. The analogue of 3-phosphoglycerate oxidizes NADH under the combined action of 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase if ATP is added. Thus four out of the five glycolytic enzymes tested handle the phosphonomethyl compounds like the natural phosphates.

Analogues of glycolytic intermediates in which the phosphate group, $-O-PO_3H_2$, is replaced by the phosphonomethyl group, $-CH_2-PO_3H_2$, have been little studied. Stubbe & Kenyon (1972) showed that such an analogue of phosphoenolpyruvate was a substrate for enolase (see also Nowak *et al.*, 1973). Shopsis *et al.* (1972) studied the effect of the analogue of glycerol 1-phosphate (Kabak *et al.*, 1972) on bacterial growth, but it is not known whether enzymes that metabolize glycerol 1-phosphate also handle the analogue.* If analogues of this kind were to be metabolized in all respects like the natural intermediates, except for reactions in which phosphate acts as a leaving group, as seemed possible in view of the similar sizes, shapes and acidities of the groups, a nucleic acid analogue should result in which the internucleotide link could not be hydrolysed by most known nucleases. A synthetic dinucleotide analogue of this kind has been shown to bind to pancreatic ribonuclease and not to be split by it (Richards *et al.*, 1971).

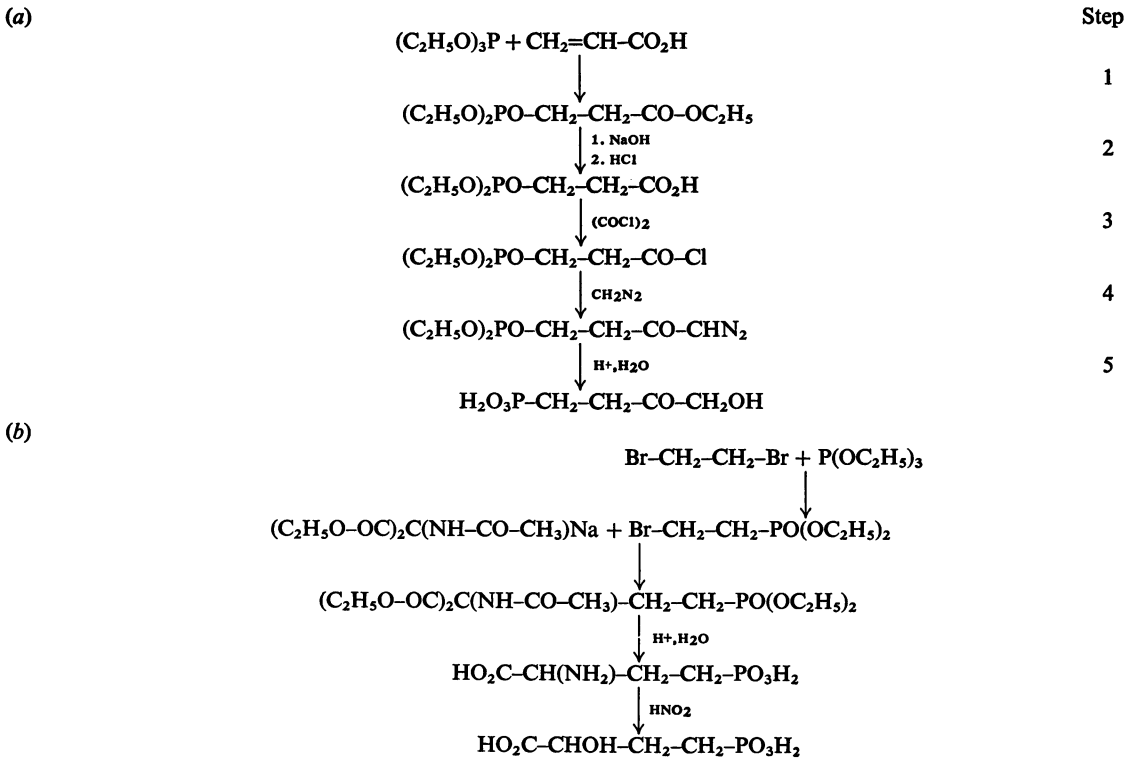
We therefore made 4-hydroxy-3-oxobutylphosphonic acid, the methylene analogue of dihydroxyacetone phosphate,* and studied some of its properties. When it proved not to be a substrate for triose phosphate isomerase (EC 5.3.1.1), and therefore not to be a potential precursor for hexose derivatives with a phosphonomethyl group on C-6, we prepared 2-hydroxy-4-phosphonobutyric acid, the methylene analogue of 3-phosphoglycerate, and have shown that it substitutes for 3-phosphoglycerate in undergoing enzymic phosphorylation and reduction. Another synthesis of this compound has been published (Pfeiffer *et al.*, 1974).

* See note added in proof.

Experimental

Synthesis of $CH_2OH-CO-CH_2-CH_2-PO_3H_2$ (Scheme 1a)

The first step was a modification of the method of Kamaĭ & Kukhtin (1956). Acrylic acid (13.7ml, 0.2mol) was added to triethyl phosphite (38ml, 0.22mol), the first 5ml rapidly and the rest with cooling so that the temperature did not exceed 70°C. The mixture was then heated to 100°C for 30min and cooled. The product was not isolated; part of it may have already been the carboxylic acid rather than its ester. The whole reaction mixture was dissolved in 200ml of water, and adjusted to pH12 with 4M-NaOH in a pH-stat until uptake was complete (about 30min). The solution was then extracted three times with 100ml of ethyl acetate, the aqueous phase was adjusted to pH2 with 12M-HCl, and this was extracted with 4×100ml of ethyl acetate. These four extracts were combined, dried with Na_2SO_4, and evaporated to dryness in a rotary evaporator. The yield of syrup was 34.6g [82% if it was entirely $(C_2H_5O)_2PO-CH_2-CH_2-CO_2H$]. Oxalyl chloride (7.62g, 0.06mol) was added to 5.35g (0.03mol) of the syrup over 2h in portions with cooling, and the mixture was left at room temperature (20°C) for a further 1h. The excess of oxalyl chloride was removed by rotary evaporation and the acid chloride formed was dissolved in 15ml of ether. For step 4 of Scheme 1(*a*) 10g of *N*-methyl-*N*-nitrosourea (caution, carcinogen), made from methylamine hydrochloride, urea and nitrous acid (Arndt, 1943*b*) was treated with KOH (Arndt, 1943*a*, Note 3) without distillation to yield an ethereal solution of diazomethane (another carcinogen). The solution of the acid chloride was added to the diazomethane solution slowly with stirring, and the excess of diazomethane was destroyed by addition of acetic acid



Scheme 1. *Synthetic routes to the phosphonomethyl analogues of (a) dihydroxyacetone phosphate and (b) 3-phosphoglycerate*

until effervescence stopped. The ether was removed by rotary evaporation, and the product was dissolved in 100ml of water. The diazoketone was decomposed by stirring the solution with a sulphonated polystyrene (Zerolit 225, SRC 14, 8% cross-linking, 100–200 mesh) (30ml of packed bed) and heating to 60°C until bubbling ceased. Resin rather than HCl was used to decompose the diazoketone to avoid the possibility that the final product would contain CH_2Cl–CO–CH_2–CH_2–PO_3H_2, which might be damaging to enzymes (cf. de la Mare *et al.*, 1972). The resin was filtered off, rinsed with water, and the solution was mixed with an equal volume of 12M-HCl, boiled under reflux for 4h and evaporated to dryness on a rotary evaporator. Residual HCl was removed in a vacuum desiccator.

The crude product was dissolved in water (50ml), divided into two portions, and each worked up as follows. It was adjusted to pH5 with 1M-NaOH and chromatographed on a column (44cm×2cm) of strongly basic resin (Zerolit FF-IP SRA 71, 8% cross-linking, 100–200 mesh; acetate form), as described by Dixon (1964) for keto acids, in a buffer of 0.6M-sodium acetate–0.2M-acetic acid. The product was found by spotting samples from the tubes of column effluent on paper and staining with 5mM-2,4-dinitrophenylhydrazine in 2M-HCl. Tubes containing the product, which emerged at about 1½–2 column volumes, were combined and passed through a column of the acid form of a sulphonated polystyrene resin (Zerolit 225, SRC 15) and evaporated to dryness. The product was dissolved in 20ml of water, adjusted to pH9 with cyclohexylamine and evaporated to dryness. Traces of water were removed by adding 10ml of ethanol and re-evaporating. The product was suspended in 50ml of ethanol, filtered and washed with ethanol (yield 2.1g; 23% from stage 2, 19% overall). The low yield and the need for chromatography to achieve an electrophoretically homogeneous product may reflect breakdown of the compound in the harsh conditions needed to hydrolyse the phosphonate ester groups.

Enzymic characterization of the compound is given by Stribling (1974). Elementary analysis gave C, 52.8; H, 9.85; N, 6.8; $C_4H_9O_5P$,$2C_6H_{13}N$ requires C, 52.4; H, 9.6; N, 7.6%. Titration of 200mg in 10ml of water with 1M-HCl from an initial pH of 8.7–8.9 showed a pK of 7.1 and an equivalent weight of 375; the formula weight is 366. On electrophoresis (at about 110V/cm, for 15min) in 10% (v/v) pyridine—

0.3% (v/v) acetic acid (pH6.5) on Whatman 3MM paper cooled in white spirit containing 8% (v/v) pyridine, the substance gave a single spot of mobility 0.9 times that of aspartic acid and 0.8 times that of P_i (neutral amino acids being used as indicators of electro-osmosis). This spot was revealed by staining for carbonyl group by spraying with 5mM-2,4-dinitrophenylhydrazine in 2M-HCl, and for the ability to bind Fe^{3+} ions by the test of Wade & Morgan (1953) for phosphates, here applied to phosphonates. When toluene was used instead of white spirit as coolant, extraction of pyridine from the buffer on the paper lowered the pH of this buffer as judged by the mobility of histidine, even though 8% pyridine was also added to the toluene (Dixon, 1972). A check by the same method with the present system showed that the pH was 6.5 during electrophoresis.

Synthesis of HO_2C–$CHOH$–CH_2–CH_2–PO_3H_2 (Scheme 1b)

2-Amino-4-phosphonobutyric acid was prepared by a modification of the method of Chambers & Isbell (1964). Sodium (0.1mol), diethyl acetamidomalonate (0.1mol) and diethyl 2-bromoethylphosphonate (0.1mol, made by the Arbuzov reaction described by Kosolapoff, 1948) were dissolved, in that order, in ethanol (100ml) and the mixture was left at 20°C overnight. The NaBr formed was filtered off and much of the ethanol was removed by rotary evaporation. The residue was boiled under reflux in 6M-HCl for 12h, evaporated to dryness, dissolved in water (200ml), passed through a column (25cm×3cm) of the acid form of a sulphonic resin (Zerolit 225, SRC 15) and washed through with water. The product was eluted from the resin with 0.5M-formic acid until the washings were only faintly ninhydrin-positive (10μl samples spotted on paper; about 5 litres required). The effluent was evaporated to dryness. The product started to crystallize when the volume reached about 200ml, and was washed with acetone (yield: 14.0g, 76%).

The 2-amino-4-phosphonobutyric acid (5.7g) was dissolved in 100ml of 2M-HCl, cooled to 5°C and a solution of 4.25g of $NaNO_2$ in 7ml of water was added. After 1h the solution was warmed to 50°C and maintained at this temperature until effervescence stopped, when it was evaporated to dryness. Because paper electrophoresis showed the presence at this stage of two compounds, one possibly the 2-chloro acid, the residue was dissolved in 100ml of water, adjusted to pH12.5 with 1M-NaOH and boiled under reflux for 1h. The solution was cooled, passed through a column (15cm×3cm) of the acid form of sulphonated polystyrene resin (Zerolit 225, SRC 14), washed through with water and evaporated to a thick syrup. This syrup was dissolved in 40ml of water and adjusted with cyclohexylamine to pH6.5 and evaporated to dryness. When dissolved in 150ml of methanol the salt with two molecules of cyclohexylamine crystallized on addition of 200ml of diethyl ether (this same salt also crystallized even when the composition of the solution was that of the salt with three molecules of base, obtained by adjusting the aqueous solution to pH9). The crystals were washed with methanol–ether (3:4, v/v) and then with ethanol until chloride-free, to remove traces of cyclohexylamine hydrochloride (yield: 8.75g, 73%).

Elementary analysis gave C, 50.6; H, 9.2; N, 7.2; $C_4H_9O_6P,2C_6H_{13}N$ requires C, 50.25; H, 9.2; N, 7.3%. Titration from an initial pH of 5.7–5.8 with 1M-NaOH showed a pK of 7.7 and an equivalent weight of 400; the formula weight is 382. The titration is not very accurate because the titrations of cyclohexylamine and the phosphonate group slightly overlap; Orr & Knowles (1974) report a pK of 7.45 in 0.1M-NaCl. On electrophoresis at pH6.5 (see above for details) a single spot capable of binding Fe^{3+} ions was found; its mobility was 1.33 times that of aspartic acid and 1.2 times that of P_i.

Enzyme assays

Chicken muscle triose phosphate isomerase (Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) was assayed at pH8.0 by the first method of Plaut & Knowles (1972), which involves measurement of NAD^+ reduction in the presence of glyceraldehyde 3-phosphate dehydrogenase.

The reduction of 3-phosphoglycerate to glyceraldehyde 3-phosphate was followed by observing the fall in extinction at 340nm due to NADH oxidation in a solution of triethylenetetramine dihydrochloride (160mM; Dixon *et al.*, 1972), EDTA (disodium salt) (32mM), $MgCl_2$ (40mM), hydrazine (2mM), ATP (6mM), NADH (0.14mM), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (200μg/ml, about 25nkat/ml) and yeast 3-phosphoglycerate kinase (40μg/ml, about 250nkat/ml) (both enzymes from Boehringer, London W5 2TZ, U.K.). The solution had pH 7.4–7.6, and the rate was measured at 20°C. This is the method of P. F. Leadlay, W. J. Albery & J. R. Knowles (unpublished work), and the hydrazine displaces the equilibrium in the desired direction.

Results and Discussion

The analogue of dihydroxyacetone phosphate, i.e. CH_2OH–CO–CH_2–CH_2–PO_3H_2, proved to be a substrate for both glycerol 1-phosphate dehydrogenase and aldolase (Stribling, 1974; see also Adams *et al.*, 1974). Aldolase has previously been found to be highly specific for dihydroxyacetone phosphate (Meyerhof *et al.*, 1936); although hydroxyacetone phosphate is a substrate, it is a poor one (Rose & O'Connell, 1969). Thus the prediction that the

phosphonomethyl group would closely resemble the phosphate group was confirmed for these two enzymes.

This analogue, however, failed to be a substrate for chicken muscle triose phosphate isomerase (EC 5.3.1.1), and at a concentration of 10mM it did not appreciably inhibit the action of this enzyme on 0.03mM-dihydroxyacetone phosphate. It was similarly inactive (S. Artavanis & J. I. Harris, personal communication) with the triose phosphate isomerase from *Bacillus stearothermophilus*. Inactivity could be explained either if the substance were not a substrate for the isomerase, or if the product formed by the isomerase were not a substrate for the glyceraldehyde 3-phosphate dehydrogenase used in the coupled assay. This second possibility is unlikely for two reasons. The first is that this dehydrogenase is not highly specific with respect to its aldehyde substrate (Harting & Velick, 1954); the second is that the dehydrogenase proved to work with the corresponding analogue in the reverse direction (see below). This conclusion is supported by the observation (S. G. Waley, unpublished work) that although phosphoglycollate, H_2PO_3–O–CH_2–CO_2H, is a powerful inhibitor of the isomerase (Wolfenden, 1970; Waley, 1973), its phosphonomethyl analogue, 3-phosphonopropionate, H_2PO_3–CH_2–CH_2–CO_2H, has an affinity about a thousand times lower.

We do not know the basis for this discrimination by triose phosphate isomerase. Since the pK of the analogue (7.1) differs by only 1.1 units from that of the natural substrate (6.0; Plaut & Knowles, 1972), no more than 13-fold discrimination could be achieved at any pH by preference for a particular ionic form. The enzyme may interact with the oxygen atom that is absent from the analogues. Alternatively the difference in binding may be due to the small differences in geometry, including the possibly greater preference of the methylene group for the staggered conformation (pointed out to us by W. N. Lipscomb; see Lehn, 1971). Other cases are known where substitution of methylene for oxygen prevents enzymic action even though the group is not directly involved in the reaction; thus such a substitution between the terminal and central phosphorus atoms of ATP prevents the analogue from activating some (but not most) of the amino acids (Papas & Case, 1970).

Since the analogue of glyceraldehyde 3-phosphate would be required for enzymic production of hexose 6-phosphate analogues, and since it could not be made enzymically by triose phosphate isomerase, we synthesized the analogue of 3-phosphoglycerate, i.e. H_2PO_3–CH_2–CH_2–CHOH–CO_2H. It proved able to replace 3-phosphoglycerate in oxidizing NADH when ATP, 3-phosphoglycerate kinase (EC 2.7.2.3) and glyceraldehyde 3-phosphate dehydrogenase were added. In the reaction mixture described with 40μg of kinase/ml and 200μg of dehydrogenase/ml, the rate of NADH oxidation by 1mM-D-3-phosphoglycerate was 2.7μM·s^{-1} and that with 1mM-DL-analogue was 0.2μM·s^{-1}. The dehydrogenase was largely limiting for the natural substrate and the kinase for the analogue; a detailed study of the action of the kinase on the analogue is reported by Orr & Knowles (1974). Evidently both enzymes can handle the phosphonomethyl analogues of their natural substrates.

Detailed kinetic comparisons of the analogues with natural substrates have been made for several glycolytic enzymes (Stribling, 1974; Orr & Knowles, 1974; Adams *et al.*, 1974). Although this comparison does not yet extend to glyceraldehyde 3-phosphate dehydrogenase, it is already clear that the isosteres work well enough with four out of the five glycolytic enzymes tested to permit these enzymes to be used for preparative conversions of the analogues. We plan to study what further conversions of the compounds prepared can be effected by glycolytic enzymes.

Note Added in Proof (Received 21 May 1974)

Cheng *et al.* (1974) have shown that the analogue of glycerol 1-phosphate is a substrate for glycerol phosphate dehydrogenase. Goldstein *et al.* (1974) have published another synthesis of the analogue of dihydroxyacetone phosphate and shown that it is also a substrate for this enzyme.

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