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Phosphonomethyl Analogues of Hexose Phosphates

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The analogue of fructose 1,6-bisphosphate in which the phosphate group, $-O-PO_3H_2$, on C-6 is replaced by the phosphonomethyl group, $-CH_2-PO_3H_2$, was made enzymically from the corresponding analogue of 3-phosphoglycerate. It was a substrate for aldolase, which was used to form it, but not for fructose 1,6-bisphosphatase. It was hydrolysed chemically to yield the corresponding analogue of fructose 6-phosphate [i.e. 6-deoxy-6-(phosphonomethyl)-D-fructose, or, more strictly, 6,7-dideoxy-7-phosphono-D-arabino-2-heptulose]. This proved to be a substrate for the sequential actions of glucose 6-phosphate isomerase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Thus seven out of the nine enzymes of the glycolytic and pentose phosphate pathways so far tested catalyse the reactions of the phosphonomethyl isosteres of their substrates.

Dixon & Sparkes (1974) synthesized the analogues of dihydroxyacetone phosphate and of 3-phosphoglycerate in which the phosphate group $-O-PO_3H_2$ is replaced by the phosphonomethyl group $-CH_2-PO_3H_2$, as did also Goldstein *et al.* (1974) and Pfeiffer *et al.* (1974). These isosteres acted as substrates for four out of the five glycolytic enzymes tested; thus the analogue of dihydroxyacetone phosphate was a substrate for aldolase (EC 4.1.2.13) (Stribling, 1974) and for glycerol phosphate dehydrogenase (EC 1.1.1.8) (Stribling, 1974; see also Adams *et al.*, 1974; Cheng *et al.*, 1974; Goldstein *et al.*, 1974), and the analogue of 3-phosphoglycerate was a substrate for the combined action of 3-phosphoglycerate kinase (EC 2.7.2.3) and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (Dixon & Sparkes, 1974; Orr & Knowles, 1974). The phosphonomethyl analogue of dihydroxyacetone phosphate was not, however, a substrate or inhibitor of triose phosphate isomerase (EC 5.3.1.1) (Dixon & Sparkes, 1974).

Dixon & Sparkes (1974) mentioned the possibility that such analogues might be enzymically convertible into nucleotide and even nucleic acid analogues. We have therefore explored this possibility by using glycolytic enzymes to convert the analogue of 3-phosphoglycerate into an analogue of fructose 1,6-bisphosphate (Scheme 1). When this proved not to be a substrate for fructose bisphosphatase (EC 3.1.3.11) we hydrolysed it chemically to obtain the analogue of fructose 6-phosphate. With it we have shown that three further enzymes, glucose 6-phosphate isomerase (EC 5.3.1.9), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44), act on the phosphonomethyl analogues of their natural substrates. Thus two enzymic steps, a chemical hydrolysis and three further enzymic steps have been used to convert the phosphonomethyl analogue of 3-phosphoglycerate into that of ribulose 5-phosphate, although so far only the steps as far as the analogue of fructose 6-phosphate have been achieved on a preparative scale.

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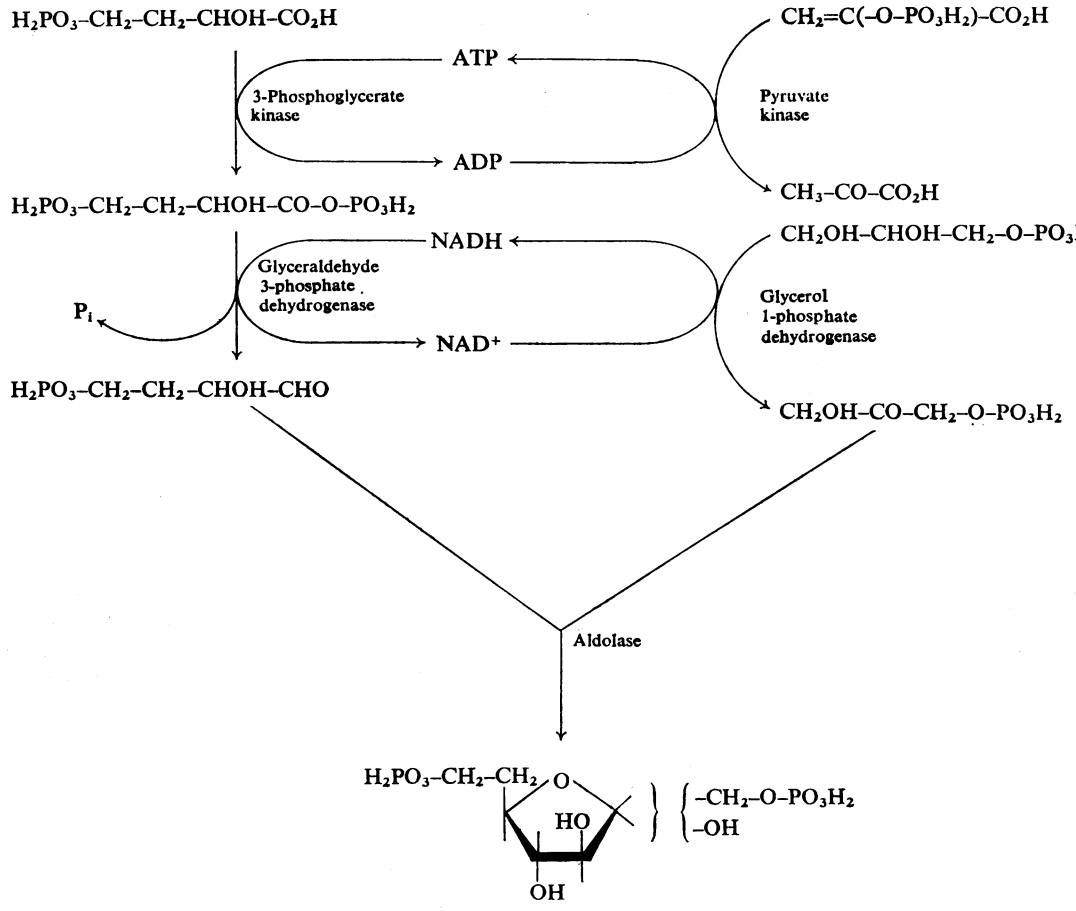
Experimental

Materials

Trisodium 2-hydroxy-4-phosphonobutyrate. The salt of the acid with two molecules of cyclohexylamine, prepared as described in the Appendix to this paper (Dixon & Sparkes, 1976), was converted into the free acid by passing an aqueous solution through a column of the acid form of a sulphonic resin (Zerolit 225 SRC 14). The pH of the effluent was adjusted to 9.0 with NaOH. On rotary evaporation an oil was obtained. This dissolved on repeated extraction with boiling methanol, and rotary evaporation gave the material as a white powder.

Paper electrophoresis showed no phosphonate other than the desired compound. Enzymic assay (see below) showed that 66–69% of the weight was $Na_2O_3P-CH_2-CH_2-CHOH-CO_2Na$; 67% was taken for calculating the number of moles added and hence yields were based on this. The material is hygroscopic.

Tripotassium phosphoenolpyruvate. The cyclohexylamine salt of phosphoenolpyruvic acid was made and converted into a solution of the tripotassium salt by the method of Clark & Kirby (1966), except that Zerolit 225 SRC 14 was used as the sulphonic resin. The solution was evaporated



Scheme 1. *Method of preparation of the 6-phosphonomethyl analogue of fructose 1,6-bisphosphate*

to dryness on a rotary evaporator, and the last traces of water were removed by repeated addition of ethanol and re-evaporation. The resulting white solid was crystallized from methanol on addition of ethanol. (Found: C, 12.3; H, 2.1; P, 10.4. Calc. for $C_3H_2K_3O_6P$: C, 12.8; H, 0.7; P, 11.0%.) The material is very hygroscopic, which probably accounts for the high H and slightly low C and P analyses.

Methods

Enzymic assay of 2-hydroxy-4-phosphonobutyrate. The extent of NADH oxidation was determined from the fall in E_{340}, assuming an extinction coefficient of $6.22 \times 10^3 M^{-1} \cdot cm^{-1}$ (Horecker & Kornberg, 1948) in a solution of 0.2M-triethanolamine/HCl (pH7.8) 32mM-EDTA (disodium salt), 40mM-$MgCl_2$, 2mM-hydrazine, 6mM-ATP, 0.47mM-NADH, 1.7mg of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase/ml and 83μg of yeast 3-phosphoglycerate kinase/ml (an assay modified from an unpublished method of P. F. Leadlay, W. J. Albery & J. R. Knowles). Some hours were required for the reaction to finish; during this time some oxidation of NADH occurred in a blank incubation without hydroxyphosphonobutyrate, and the change was corrected for this blank.

Enzymic oxidation of fructose 6-phosphate and its analogue. This was followed by observing the rise in E_{340} due to $NADP^+$ reduction in a solution containing 250mM-glycylglycine (adjusted with NaOH to pH7.4), 2.5mM-$MgCl_2$, 0.62mM-$NADP^+$, 20μg of yeast glucose phosphate isomerase (grade III; Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K.)/ml, 10μg of yeast glucose 6-phosphate dehydrogenase (type XII, Sigma)/ml and 46μg of yeast phosphogluconate dehydrogenase (type VI, Sigma)/ml (this assay is modified from that described by Cooper *et al.*, 1958).

The first and last of these enzymes were dialysed against three 50ml portions of a solution of 250mM-glycylglycine, adjusted to pH7.4 with NaOH, and 2.5mM-$MgCl_2$ at 4°C before use.

Assay for the investigation into the effect of the 6-phosphonomethyl analogue of fructose 1,6-bisphosphate on the action of fructose 1,6-bisphosphatase. The reaction of ox liver fructose 1,6-bisphosphatase (Nimmo & Tipton, 1975; the kind gift of J. S. Shindler & K. F. Tipton) with fructose 1,6-bisphosphate (tetrasodium salt; Sigma) was investigated in the presence of various concentrations of its 6-phosphonomethyl analogue by following the rise in E_{340} due to the generation of NADPH in a coupled enzyme assay at 30°C (a modification of the procedure described by Nimmo & Tipton, 1975). Final concentrations in the cuvette were 50mM-triethanolamine/HCl (pH7.4), 100mM-KCl, 2.1mM-$MgCl_2$, 0.1mM-EDTA (disodium salt), 0.15mM-$NADP^+$, 9μg of yeast glucose 6-phosphate dehydrogenase/ml, 16μg of yeast phosphoglucose isomerase/ml, and approx. 5μg of fructose 1,6-bisphosphatase/ml. The last two of these enzymes were dialysed against three 100ml portions of a solution containing 50mM-triethanolamine/HCl (pH7.4), 100mM-KCl, 2.1mM-$MgCl_2$ and 0.1mM-EDTA (disodium salt) at 4°C before use. The concentration of fructose 1,6-bisphosphate was varied between 5 and 50μM and that of the analogue between 0 and 500μM, and the reaction was started by addition of the former. The rate was measured several times at each combination of substrate and inhibitor concentrations.

Electrophoresis. This was performed on Whatman no. 3 paper, cooled by immersion in white spirit, in a solution of acetic acid (8%, v/v) and pyridine (2%, v/v) at pH3.5. Mobilities were determined with glucose as a marker for electro-osmosis. Phosphonates and phosphates were detected by their ability to bind Fe^{3+} (Wade & Morgan, 1953); the test is slightly less sensitive for phosphonates. Carbohydrates were detected on paper with alkaline $AgNO_3$ solution (Trevelyan *et al.*, 1950). Phosphonate analogues took longer to stain than the corresponding phosphate, but gave a similar colour. 2-Hydroxy-4-phosphonobutyrate stained very slowly to give a deeper brown colour than the other compounds tested. P_i gave a grey spot.

Determination of hexose derivatives. The anthrone reaction (Mokrasch, 1954) was used with incubation at 80°C for 4min instead of 3, because the 6-phosphonomethyl analogue of fructose 1,6-bisphosphate proved to give maximum colour with the longer treatment. The assay was standardized against fructose 6-phosphate (barium salt), itself standardized enzymically (see above) by the amount of $NADP^+$ that it would reduce in the presence of glucose 6-phosphate dehydrogenase (by this test it was 72% pure). The percentage yields stated are therefore based on the arbitrary assumption that the colour yields of the 6-phosphonomethyl analogues of fructose 1,6-bisphosphate and of fructose 6-phosphate are the same as for the phosphates. Mokrasch (1954) had found identical colour yields for these two phosphates, about 70% of the yield that we find (i.e. we get the same colour yield as his if we do not correct the weighed amount of fructose 6-phosphate by the results of the enzymic assay).

The arbitrary assumption is finally checked by the amount of $NADP^+$ that a given amount of the analogue of fructose 6-phosphate will reduce. This proves to be 70% of that expected from its anthrone colour; all yields stated should therefore probably be corrected by a factor of 0.7 unless it is stated that they are calculated on the basis of the enzymic assay.

Syntheses

(1) *6-Phosphonomethyl analogue of fructose 1,6-bisphosphate* (*Scheme* 1). For 0.5mmol of DL-2-hydroxy-4-phosphonobutyrate it was convenient to use an incubation volume of 20ml (and 1ml for control) and the following procedure refers to this scale. Samples of 1.0ml of yeast glyceraldehyde 3-phosphate dehydrogenase, 0.2ml of rabbit muscle aldolase, 0.2ml of yeast 3-phosphoglycerate kinase, 0.2ml of rabbit muscle pyruvate kinase (all 10mg/ml and from Boehringer, Uxbridge Road, London W5 2TZ, U.K.), and 25μl of rabbit muscle glycerol 1-phosphate dehydrogenase (14mg/ml; Sigma) were dissolved in 13ml of the incubation medium, which consisted of 10mM-$MgCl_2$, 10mM-2-mercaptoethanol, 10mM-KCl, 2mM-tren,3HCl [$(NH_2CH_2CH_2)_3N$,3HCl; Dixon *et al.*, 1972], adjusted to pH8.0 with KOH (since the substrates to be added buffer strongly, other buffering is not required). The resulting solution was dialysed at 4°C for 6h against 3×250ml of the incubation medium, in dialysis tubing (Visking size 2; from Medicell International, 49 Queen Victoria Street, London EC4N 4SA, U.K.) that had been boiled for 10min in 0.5% EDTA (disodium salt) solution.

Substrates and cofactors were then dissolved in the enzyme solution as follows (the concentrations are the final ones); 0.735g of DL-glycerol 1-phosphate (0.12M) (disodium salt, Sigma); 0.6g of tripotassium phosphoenolpyruvate (0.1M); 14.7mg of Na_2NAD^+ (1mM) (Boehringer); 25.2mg of Na_2ATP (2mM) (Boehringer). The volume of the solution was made up to 21ml with incubation medium and 1ml was removed for use as a control. The remaining 20ml was added to 188mg of trisodium DL-2-hydroxy-4-phosphonobutyrate (25mM) and the pH of both this solution and the control adjusted to 8.0 with KOH.

The reaction was allowed to proceed at room temperature (approx. 20°C) and its progress was followed by the anthrone reaction on 10μl samples from the experimental and control solutions.

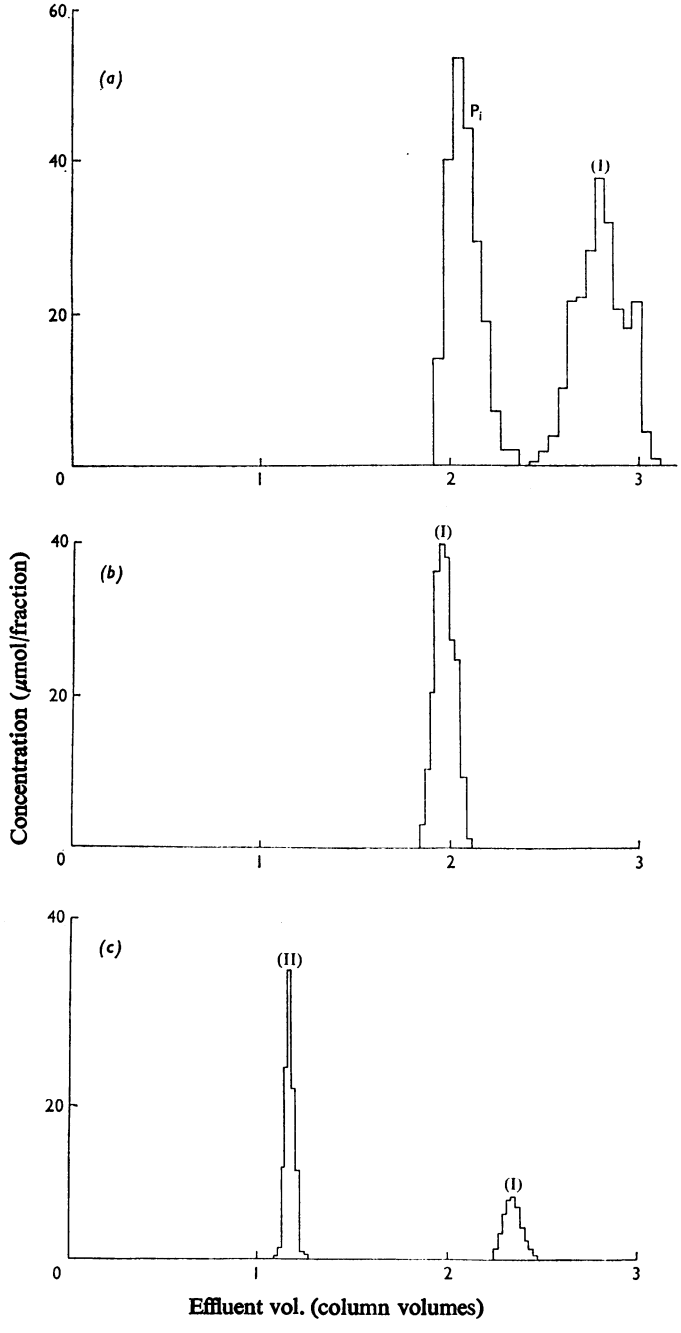


Fig. 1. *Analogue purification by chromatography on DEAE-cellulose*

(*a*) Preparative chromatography of the 6-phosphonomethyl analogue of fructose 1,6-bisphosphate (I), i.e. 6-deoxy-6-phosphonomethyl-fructose 1-phosphate; (*b*) re-run of the analogue peak from column (*a*) in a less acidic medium; (*c*) chromatography of a hydrolysate of the analogue peak from column (*a*) to obtain the analogue of fructose 6-phosphate (II), i.e. 6-deoxy-6-phosphonomethyl-fructose. The columns (53–98 cm × 3.0 cm, see the text) were eluted with (*a*) 0.1 M-pyridine/1 M-formic acid or with (*b* and *c*) 0.15 M-pyridine/0.4 M-formic acid. Phosphate was determined by the method of Fiske & SubbaRow (1925) and fructose phosphate analogues by the anthrone reaction. The analogue of fructose 1,6-bisphosphate (I) emerged in slightly different positions in columns (*b*) and (*c*), probably because of packing differences.

When the reaction had ceased (about 18h) the pH was adjusted to 2.65 with 50% (w/v) trichloroacetic acid. The solution was cooled in ice for 15min and the precipitated material was removed by centrifugation (5min, bench centrifuge, approx. 500*g*). Water was added to the solution to adjust its conductivity to that of the chromatographic buffer, which consisted of 0.1M-pyridine, 1M-formic acid and 0.03% toluene (pH2.65). The mixture was then applied to a column (53.5cm×3cm) of DEAE-cellulose (Whatman DE52) that had previously been equilibrated with this buffer. The column was eluted with the same solution at about 300ml/h and fractions (about 20ml) were collected. The fractions containing the analogue of fructose 1,6-bisphosphate (2.5–3.1 column volumes, see Fig. 1*a*; cf. Stribling, 1974) were detected by the anthrone reaction, which indicated a yield of 80–90% from the starting hydroxyphosphonobutyrate. They were pooled and evaporated on a rotary evaporator (water bath at 40–50°C). Water was added and the solution re-evaporated several times until all smell of pyridine had gone and a white solid remained. Paper electrophoresis indicated that P_i, glycerol 1-phosphate and hydroxyphosphonobutyrate were absent from this product. Nevertheless it still contained phosphoenolpyruvate. Attempts to resolve both this and the hydroxyphosphonobutyrate from the desired material on a single passage were made by varying the conditions, but were unsuccessful. In the preparation of the analogue of fructose 6-phosphate no further purification was necessary at this stage and the mixture was subjected directly to the hydrolysis procedure described below.

To obtain a pure sample of the analogue of fructose 1,6-bisphosphate this white solid was dissolved in water and the pH adjusted to 3.3. The solution was then diluted with water to adjust its conductivity to that of a second chromatographic buffer, which consisted of 0.15M-pyridine, 0.4M-formic acid and 0.03% toluene (pH3.3). This adjusted solution was applied to a column (98cm×3cm) of DEAE-cellulose (Whatman DE 52) that had previously been equilibrated with this buffer. It was eluted with the same buffer at about 250ml/h and fractions (approx. 18ml) were collected. The fractions containing the analogue of fructose 1,6-bisphosphate, detected as before, were at 1.8–2.1 column volumes (Fig. 1*b*). Recovery was quantitative, and these fractions were pooled and evaporated on a rotary evaporator (water bath at 40–50°C). Water was added and the solution re-evaporated several times until all smell of pyridine had gone and a white solid was obtained.

(2) *Phosphonomethyl analogue of fructose 6-phosphate.* A sample of about 150μmol of the 6-phosphonomethyl analogue of fructose 1,6-bisphosphate in 3ml of aq. 0.1M-pyridine/1.0M-formic acid (pH2.45) was heated in a sealed tube for 16h at 105°C. The pH of the solution, which had risen to about 2.7 during hydrolysis, was adjusted to 3.3 with aq. KOH and the conductivity was adjusted with water to that of the second chromatographic buffer (pH3.3) described above. The sample was applied to the repacked column (98cm×3cm) and chromatographed as before (Fig. 1*c*). The yield of material in the shifted position, 1.1–1.25 column volumes, judged by anthrone colour, was 50–60% of that submitted to hydrolysis. Buffer was removed by evaporation as before. Since no solid could be obtained, the product was kept as an aqueous solution at 0°C. Electrophoresis of samples of this solution and subsequent staining failed to indicate the presence of any phosphate-positive or sugar-positive material other than the fructose 6-phosphonate.

Also present in the eluate was some unhydrolysed fructose 1,6-bisphosphate analogue. It was found that, although the amount of this analogue could be diminished by lengthening the duration of the incubation at 105°C, the amount of fructose 6-phosphate analogue also diminished. This compound thus seems not to be completely stable to the conditions of hydrolysis; it was therefore more economical to hydrolyse the fructose 1,6-bisphosphate analogue for a shorter time, and to recover the unhydrolysed fraction for further hydrolysis.

Results

Development of the synthesis of a hexose phosphate analogue (*Scheme* 1)

Dixon & Sparkes (1974) had shown that 2-hydroxy-4-phosphonobutyrate could be converted into the phosphonomethyl analogue of glyceraldehyde 3-phosphate by the combined action of 3-phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase, as judged by NADH oxidation. Initial attempts to make this sequence preparative by trapping the aldehyde with hydrazine (with creatine phosphate and creatine kinase to regenerate ATP, and lactate and its dehydrogenase to regenerate NADH) were unsuccessful, so we decided to trap the aldehyde formed by the aldolase reaction, and to use glycerol phosphate and its dehydrogenase to regenerate NADH, since this would concurrently produce dihydroxyacetone phosphate to react with the aldehyde produced (dihydroxyacetone phosphate is unstable as well as expensive).

Preliminary experiments with a solution containing 100mM-DL-2-hydroxy-4-phosphonobutyrate (salt with two molecules of cyclohexylamine), 2mM-ATP, 2mM-NAD^+, 60mM-creatine phosphate, 120mM-DL-glycerol 1-phosphate, 10mM-$MgCl_2$, 160mM-2-mercaptoethanol and the enzymes mentioned under 'Syntheses' (with creatine kinase instead of pyruvate kinase) gave a yield of only 1.5%, as judged by the anthrone reaction (its standardization is described

under 'Methods'). This yield was increased to 6% on changing the pH to 8.0, introducing 2mM-tren,3HCl and lowering the concentration of 2-mercaptoethanol to 10mM, and then to 19% when the 2-hydroxy-4-phosphonobutyrate was added as the sodium salt and not as the cyclohexylammonium salt. We do not know why cyclohexylamine interfered with the reaction, but it is possibly relevant that under incubation conditions in which the cyclohexylamine salt gave 8.5% yield, 56% of the creatine phosphate had been hydrolysed, whereas under the same conditions the sodium salt gave a 19% yield for the hydrolysis of only 28% of the available creatine phosphate. Replacement of the creatine phosphate system with phosphoenolpyruvate and pyruvate kinase raised the yield to 29%. The above yields were with the addition of molar proportions of 2:2.4:1 (100mM, 120mM, 50mM) for DL-2-hydroxy-4-phosphonobutyrate, DL-glycerol 1-phosphate and phosphoenolpyruvate; raising the relative amounts of the last two to give molar proportions of 1:4.8:4 (25mM, 120mM, 100mM) increased the yield to 70% (as judged by the anthrone reaction), and for this it was necessary to increase both of them. In preparative runs yields of 50–60% have been obtained (based on the enzymic assay of the fructose 6-phosphate analogue).

Development of the synthesis of the fructose 6-phosphate analogue

At first we added fructose bisphosphatase to the incubation medium described above, in the hope that the product of Scheme 1 would be the analogue of fructose 6-phosphate, and that the overall reaction would be energetically more favourable. Electrophoresis showed, however, that the anthrone-positive product had a mobility close to that of fructose bisphosphate, i.e. fructose bisphosphatase failed to hydrolyse the product. On incubation of the isolated analogue with fructose bisphosphatase no production of electrophoretically different material was seen, even though a similar incubation gave complete conversion of fructose bisphosphate into fructose 6-phosphate. We therefore used chemical hydrolysis, which is fastest for monoesters of phosphoric acid in the pH range 2–7 (Bailly, 1942). We used the acid side of this range to minimize degradations of the carbohydrate moiety.

Preliminary investigation into the effect of the analogue of fructose 1,6-bisphosphate on the action of fructose 1,6-bisphosphatase

Plots of initial velocity against substrate concentration indicated that high concentrations of substrate inhibited the hydrolysis, but at lower substrate concentrations the velocity dependence was close to a hyperbola of K_m 2μM. This phenomenon made less certain the interpretation of the kinetic data obtained when various concentrations of analogue were added to the medium. Nevertheless the relevant double-reciprocal plots (Fig. 2) indicated that the analogue inhibited the hydrolysis of the normal substrate with an apparent K_I of 150μM, nearly two orders of magnitude greater than the K_m for substrate. The inhibition observed appeared to be largely competitive, as extrapolations of the linear portions of the reciprocal plots intersected on or near the vertical axis.

Enzymic oxidation of fructose 6-phosphate and its analogue

In the enzymic assay described for either fructose 6-phosphate or its analogue (about 0.2μmol in 1ml), two 'bursts' of NADPH production were seen if the glucose 6-phosphate dehydrogenase and the 6-phosphogluconate dehydrogenase were added consecutively. The first step was rapid with the normal substrate (essentially complete in 10min) and quite rapid with the analogue (complete in 1h). The burst on adding the 6-phosphogluconate dehydrogenase was slower; it was complete in about 2h with normal substrate, but required much longer with the analogue. Thus the phosphonomethyl analogue of 6-phosphogluconic acid appears to be a poor substrate for the dehydrogenase. Nevertheless the reaction did occur

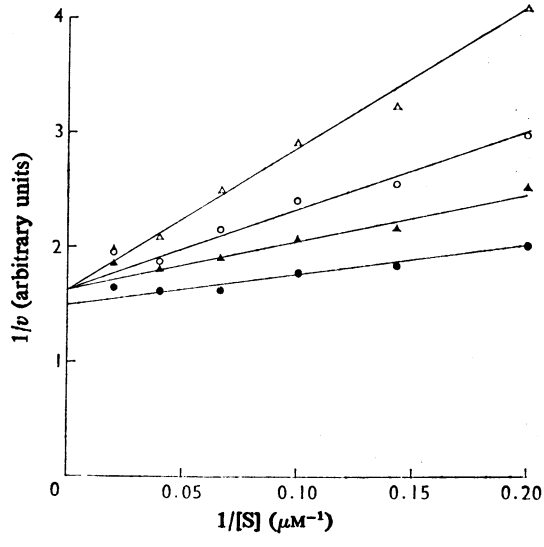


Fig. 2. *Double-reciprocal plots for fructose bisphosphatase in the presence of various concentrations of the 6-phosphonomethyl analogue of its substrate*

Substrate inhibition is apparent when 1/[S] is 0.02μM^{-1}. Lines for 50 and 150μM inhibitor also intersected on or close to the vertical axis. ●, control; ▲, 100μM; ○, 250μM; △, 500μM.

at a rate sufficient to suggest that the method could be adapted to a preparative scale in order to obtain the analogue of ribulose 5-phosphate.

Discussion

There are three main results in this paper. One is that the 6-phosphonomethyl analogue of fructose 1,6-bisphosphate can be enzymically prepared by the route shown in Scheme 1 and can be chemically hydrolysed to form the analogue of fructose 6-phosphate. The second is that fructose bisphosphatase fails to hydrolyse this analogue of fructose 1,6-bisphosphate and is competitively inhibited by it. The third is that the analogue of fructose 6-phosphate is a substrate for the sequential action of glucose 6-phosphate isomerase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. This last finding largely overlaps that of Adams *et al.* (1976) who synthesized the phosphonomethyl analogue of glucose 6-phosphate by a chemical method, found its kinetic constants as a substrate for glucose 6-phosphate dehydrogenase, and showed that the product was a substrate for 6-phosphogluconate dehydrogenase.

These results, together with the previous findings of Dixon & Sparkes (1974), Cheng *et al.* (1974) and Goldstein *et al.* (1974), show that out of nine enzymes of glycolysis and the pentose phosphate pathway tested, seven catalyse transformations of the phosphonomethyl analogues of their natural substrates. Only enzymes that do not move the phosphate group are counted; Stribling (1974) showed that the 1-analogue of fructose 1,6-bisphosphate inhibited fructose bisphosphatase. However, both remaining enzymes of the nine tested, namely triose phosphate isomerase (Dixon & Sparkes, 1974) and fructose bisphosphatase (tested in the present work with the 6-phosphonomethyl analogue of its substrate), appear strictly to discriminate between the $-CH_2-PO_3H_2$ and $-O-PO_3H_2$ groups.

For triose phosphate isomerase it appears that the analogue is not even an inhibitor, hence it is likely that this isomerase interacts closely with the oxygen atom replaced so that even the small hydrogen atoms of the methylene group inhibit binding. Changes in positions of other atoms, the change in pK of the group (Orr & Knowles, 1974; Dixon & Sparkes, 1974) and the possible absence of a favourable electronic interaction with the oxygen atom seem, even collectively, too small to account for the failure to bind.

With fructose bisphosphatase, however, binding does appear to occur, since inhibition is seen in kinetic studies. The reason for the total lack of hydrolysis of the analogue is not immediately apparent. It may be that on binding there needs to be an interaction between the oxygen normally present on C-6 and some part of the enzyme before catalysis can occur. Alternatively, the analogue may bind the 'wrong way round', again precluding catalysis.

The phosphonomethyl analogue of the ribose 5-phosphate moiety of nucleotides has been synthesized (Jones & Moffatt, 1968) and the derived dinucleotide analogue has been shown to bind to ribonuclease, but not to be hydrolysed by it (Richards *et al.*, 1971). The enzymic conversion of phosphonomethyl analogues of triose phosphates into analogues of hexose and pentose phosphates (with chemical hydrolysis of the 6-analogue of fructose 1,6-bisphosphate) may be helpful in avoiding the more difficult chemical synthesis. If derivatives can be made that can enter cells and release the phosphonate analogues inside, these might be converted there into nucleotide analogues that could preferentially inhibit nucleic acid metabolism (e.g. for therapeutic purposes). Of course it is possible that the failure of fructose bisphosphatase to work may prevent such a pathway from starting from triose phosphate analogues, although conceivably some other phosphatase could catalyse the necessary hydrolysis.

The greatly improved yield found when phosphoenolpyruvate replaced creatine phosphate as energy source may correlate with the greater Gibbs energy of hydrolysis of the former. Nevertheless the reactions of Scheme 1 appear to be exergonic even with creatine phosphate. Possibly the more favourable equilibrium of the first step allowed a faster rate of reaction of the second step. In view of the need for excess of glycerol phosphate and phosphoenolpyruvate over hydroxyphosphonobutyrate, some unwanted side reactions may use up NADH and ATP; hence the kinetics of the wanted reaction may be as important as the equilibrium position. The cheap and simple synthesis of phosphoenolpyruvate by Clark & Kirby (1966) makes it in any case the preferable energy source.

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APPENDIX

A Simplified Preparation of 2-Hydroxy-4-phosphonobutyric Acid

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2-Hydroxy-4-phosphonobutyric acid is the analogue of 3-phosphoglyceric acid in which the $-O-PO_3H_2$ group is replaced by $-CH_2-PO_3H_2$. Our previous method of making it (Dixon & Sparkes, 1974) by treatment of 2-amino-4-phosphonobutyric acid with HNO_2 had four disadvantages. These were associated with the fact that the HNO_2 was generated by addition of $NaNO_2$ to excess of HCl. Firstly, the excess of HCl slowed the reaction by overwhelming protonation of the amino groups (Taylor, 1928; Hughes *et al.*, 1958); paper electrophoresis showed that the reaction did not go to completion. Secondly, two products were formed; the unwanted one, possibly the chloro acid, had to be converted into the wanted one by boiling with alkali and the excess of alkali had then to be removed. Thirdly, evaporation of a solution of the product as the free acid in the presence of HCl gave an intractable glass, which only partly redissolved when neutralized with cyclohexylamine. Finally, the yield proved to be variable. All these difficulties are avoided in the following procedure, in which the substrate itself provides the acid necessary to convert $NaNO_2$ into HNO_2:

$$HPO_3^{-}-CH_2-CH_2-CH(-NH_3^{+})-CO_2H + NO_2^{-} \rightarrow HPO_3^{-}-CH_2-CH_2-CHOH-CO_2H + N_2$$

Method

DL-2-Amino-4-phosphonobutyric acid (Dixon & Sparkes, 1974) (4.6g) was suspended in water (300ml) and cooled to 10°C. A solution of $NaNO_2$ (7g in 20ml of water, about 4mol/mol of substrate) was added slowly with stirring. The substrate dissolved in about 10min and the solution was stirred at 20°C for 2h. Paper electrophoresis showed that the reaction was almost complete. Excess of the acid form of a sulphonic resin (Zerolit 225 SRC 14) was added and stirred with warming to 50°C until effervescence ceased (about 2h). The suspension was submitted to reduced pressure to remove dissolved N_2, and filtered through a bed (10cm × 3cm) of the same resin. The bed was washed with water. The effluent was evaporated to dryness to remove residual oxides of nitrogen, was redissolved in water, and was adjusted to pH6.5 with cyclohexylamine. On evaporation to dryness, addition of ethanol and re-evaporation, the product solidified. It was crystallized as described previously, i.e. by dissolving in methanol (150ml) and adding diethyl ether (200ml); yield 7.2g (70%).

Characterization

Elementary analysis gave: C, 49.3; H, 9.0; N, 7.3; P, 8.3% (Calc. for $C_4H_9O_6P,2C_6H_{13}N$: C, 50.3; H, 9.2; N, 7.3; P, 8.1%). The product possessed the same electrophoretic properties as the material prepared by the previous method, and acted as a substrate for 3-phosphoglycerate kinase, as described in the main paper (Webster *et al.*, 1976).