Biochem. J. (1983) **213**, 211–215
Printed in Great Britain

The arsonomethyl analogue of 3-phosphoglycerate*

Stephen R. ADAMS, Michael J. SPARKES and Henry B. F. DIXON
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

(*Received 29 November 1982/Accepted 28 March 1983*)

4-Arsono-2-hydroxybutanoic acid, the analogue of 3-phosphoglycerate in which $-CH_2-AsO_3H_2$ replaces $-O-PO_3H_2$, was synthesized. It proved to be a substrate for phosphoglycerate kinase. Its Michaelis constant was only slightly higher than that of the natural substrate, but its catalytic constant was about 1300 times smaller.

Many enzymes act on analogues of their natural substrates in which the phosphonomethyl group, $-CH_2-PO_3H_2$, replaces $-O-PO_3H_2$ (see Engel, 1977). Breaking of the C–P bond is difficult, so that these analogues will not undergo phosphotransferase or hydrolysis reactions, and much biochemical use can be made of this. Richards *et al.* (1971), for example, used a dinucleotide analogue in which $-CH_2-$ replaced $-O-$ to bind to ribonuclease, since the enzyme could not catalyse its hydrolysis.

A different analogue of the phospho group, the arsono group, $-AsO_3H_2$, has the property that its esters hydrolyse spontaneously. This has been applied in biochemistry (Braunstein, 1931) mainly to arsenate itself. Enzymes often transform analogues of their substrates in which $-O-AsO_3H_2$ replaces $-O-PO_3H_2$ (see, e.g., Lagunas, 1980; Lagunas & Sols, 1968; Long & Ray, 1973), but the usefulness of such analogues is limited by their rapid hydrolysis. The arsonomethyl ($-CH_2-AsO_3H_2$) analogues of substrates, however, might be transformed by enzymes, but could not be stably incorporated into analogues of phosphoric diesters, so would have different effects on metabolism. Such an analogue of ADP was made in this laboratory (Webster *et al.*, 1976) and proved to be a poor substrate for enzymes. Since the substitution of $-CH_2-$ for $-O-$ is poorly tolerated by enzymes that handle diphosphate or its residues, we decided to make 4-arsono-2-hydroxybutanoic acid, an isosteric analogue of 3-phosphoglycerate, and study its interaction with enzymes. The synthesis used was an adaptation of one used for the corresponding phosphonomethyl analogue (Dixon & Sparkes, 1974, 1976). The degree of similarity of $-O-PO_3H_2$, $-CH_2-PO_3H_2$ and $-CH_2-AsO_3H_2$ groups is discussed in the accompanying paper (Kamiya *et al.*, 1983).

* Dedicated to Academician A. E. Braunstein in honour of his 80th birthday on 26 May 1982, with respect and affection.

Methods

Paper electrophoresis was used for assessing the products of reactions. We used the systems specified by Webster *et al.* (1978) for electrophoresis; arsenates were detected on paper by their Fe^{3+}-chelating power (Wade & Morgan, 1953).

The rate of reaction catalysed by phosphoglycerate kinase (EC 2.7.2.3) was measured by following the fall of absorbance at 340nm due to the oxidation of NADH in a coupled assay with glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12) (Scheme 2). The assay mixture (total volume 2.5ml) contained 160mM-triethylenetetramine, 32mM-EDTA (disodium salt), 40mM-$MgCl_2$, 2mM-hydrazine, 6mM-ATP, 0.14mM-NADH, 200μg of rabbit glyceraldehyde-phosphate dehydrogenase (Boehringer)/ml and 0.16 or 20μg of yeast phosphoglycerate kinase (Boehringer)/ml, the two enzymes being added as suspensions in $(NH_4)_2SO_4$ solution. The pH of the solution was 8.5.

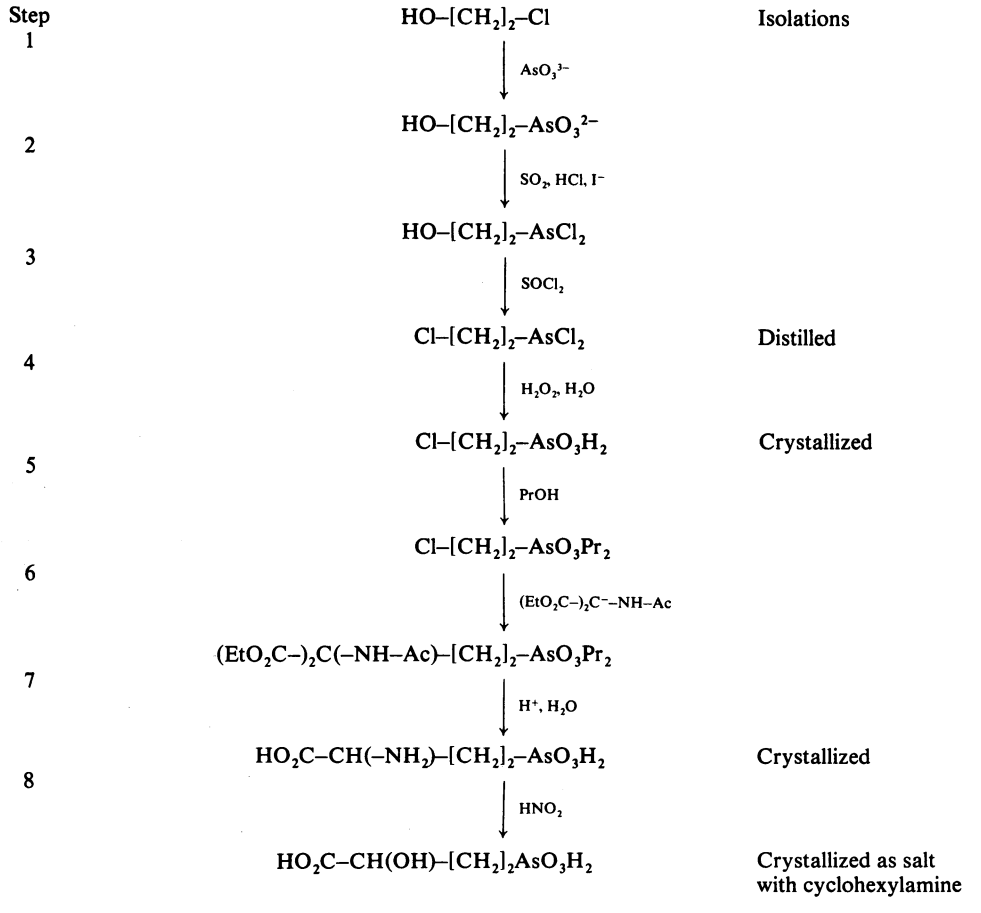
Rates were studied with (2*R*)-3-phosphoglycerate (0.08–2mM) and with (2*RS*)-4-arsono-2-hydroxybutanoate (2–30mM), both as their bis-(cyclohexylammonium) salts. This salt of 3-phosphoglycerate was prepared from the barium salt by passage of a solution through the sulphonic resin Zerolit 225 (H^+ form), adjustment to pH6 with cyclohexylamine, rotary evaporation, and crystallization from methanol on addition of diethyl ether.

Synthesis (*Scheme 1*)

2-Chloroethylarsonic acid, $Cl-CH_2-CH_2-AsO_3H_2$.

The method of Gough & King (1928), Nekrassow &

Nekrassow (1928) and Scherlin & Epstein (1928) was adapted as follows. NaOH (120g, 3mol) and As_2O_3 (100g, 0.5mol) were dissolved in water (300ml), and the solution was cooled to 20°C. 2-Chloroethanol (75ml, 90g, 1.1mol) was added slowly with stirring and cooling in a fume cupboard at such a rate that the temperature was maintained at 20°C. Cooling was required for a further 30min to prevent the temperature from rising above 20°C; the mixture was then left at this temperature overnight. Then 1.15 litres of 10M-HCl was added, and the precipitated NaCl was filtered off. KI (0.5g) was added to catalyse reduction and SO_2 (about 2mol) was bubbled into the solution, which was then extracted seven times with 250ml portions of chloroform. The extracts were combined, concentrated by rotary evaporation, dried over Na_2SO_4 and further evaporated to give a mixture of $AsCl_3$ (from the unchanged arsenite) and dichloro-(2-hydroxyethyl)arsine, $HO–CH_2–CH_2–AsCl_2$. This was dissolved in 300ml of chloroform, and the solution was cooled in ice to below 20°C while 120ml of thionyl chloride was added. This mixture was left to stand overnight while it gave off gaseous HCl and SO_2. The chloroform was then distilled off at atmospheric pressure. When nothing more distilled at 61°C, the mixture was subjected to distillation at water-pump vacuum (about 12mmHg, about 1.6kPa). A fraction distilling below 90°C (mainly near 25°C) and containing $AsCl_3$ and the excess of thionyl chloride was discarded, and the fraction distilling at 93–94°C, dichloro-(2-chloroethyl)arsine, $Cl–CH_2–CH_2–AsCl_2$, was collected. The yield was 64g (30%). After the distillation all residues and glassware were treated with alkaline H_2O_2 to render $AsCl_3$ and dichloro-(2-chloroethyl)arsine less toxic and less volatile before removal from the fume cupboard. The product was suspended in water, and



Scheme 1. *Synthesis of 4-arsono-2-hydroxybutanoic acid*

H_2O_2 was added until an excess was present; the solution was filtered and evaporated to dryness on a rotary evaporator with the bath at 50°C to minimize decomposition while the HCl was removed. The residue was dissolved in hot acetone and crystallized on cooling and addition of diethyl ether. The yield was 30g (16%). The residual liquid proved by paper electrophoresis to contain arsenic acid, H_3AsO_4, as well as chloroethylarsonic acid.

2-Amino-4-arsonobutanoic acid, $H_2O_3As–CH_2–CH_2–CH(NH_2)–CO_2H$.

2-Chloroethylarsonic acid (7.54g, 0.04mol) was esterified by boiling in 150ml of propan-1-ol under reflux, and slowly distilling off, up a fractionating column, the azeotrope of propanol and water until the boiling point reached that of propanol. This solution was concentrated to about 50ml and added to 40ml of 100% ethanol in which there had been dissolved 1g of sodium (0.04mol) and 8.68g (0.044mol) of diethyl acetamidomalonate. A precipitate, presumably NaCl, appeared at once, and the solution was left to stand for 2h. After filtration through glass-fibre paper the solvent was removed by rotary evaporation and the residue was boiled under reflux with 150ml of 6M-HCl for 1h. The solution was evaporated to dryness.

Amino acids were separated from other acids by adsorption on the sulphonic resin Zerolit 225 SRC 15 (H^+ form). The sample was dissolved in about 150ml of water, passed through a 20cm × 2.5cm column of the resin, and was washed through with about 120ml of water. The amino acids were displaced by washing with 0.5M-NH_3. The effluent was evaporated to dryness, dissolved in water and diluted until the conductivity was below that of a solution of 25mM-pyridine and 50mM-formic acid (volume about 750ml). Half this solution was worked up at a time, as follows. It was passed through a column (52cm × 3.6cm diam.) of the same resin equilibrated with a solution of 25mM-pyridine and 50mM-formic acid, and eluted with this solution. The fractions that contained the product, which emerged before glycine, were identified by paper electrophoresis at pH2.0 and staining with ninhydrin. They were combined and evaporated to dryness. These fractions represented about 0.6–1.3 column volumes from the start of application of the sample. The pooled material from two such columns crystallized and was repeatedly evaporated after addition of water until no smell of pyridine remained. It was stirred with acetone and filtered off. The yield was 2.8g (11.4mmol, 29% on 2-chloroethylarsonic acid).

Elemental analysis gave C, 20.37; H, 4.77; N, 6.05%. The calculated values for $C_4H_{10}AsNO_5$, H_2O are: C, 19.59; H, 4.94; N, 5.71%. The best characterization was the X-ray crystallography (Kamiya *et al.*, 1983) which showed that the compound was a hydrate with one molecule of water of crystallization.

4-Arsono-2-hydroxybutanoic acid, $H_2O_3As–CH_2–CH_2–CH(OH)–CO_2H$.

2-Amino-4-arsonobutanoic acid (1.15g, 5mmol) was suspended in 20ml of water and cooled to 5°C. While it was stirred, a solution of 1.75g (25mmol) of $NaNO_2$ in 20ml of water was run in. The amino acid gradually dissolved, and the solution was left to warm to room temperature (20°C) and was kept for 2h. Paper electrophoresis showed complete disappearance of ninhydrin-positive material and appearance of a new Fe^{3+}-chelating spot. The solution was stirred at 50°C with the sulphonated polystyrene resin Zerolit 225 SRC 14 (H^+ form) until evolution of N_2 ceased (about 20min), and the suspension was filtered through a bed of the same resin, and washed through with water. The solution was evaporated to dryness on a rotary evaporator, water was added and the solution was re-evaporated a few times, to remove oxides of nitrogen. The residue was diluted with water, adjusted to pH7 with cyclohexylamine, and re-evaporated. It crystallized from 96% ethanol on addition of diethyl ether. The yield was 1.15g (54%). Elemental analysis gave: C, 44.0; H, 8.0; N, 6.4%. The calculated values for $C_4H_9AsO_6$,$2C_6H_{13}N$ are: C, 45.0; H, 8.2; N, 6.6%. Paper electrophoresis showed only the one spot capable of binding Fe^{3+} when performed at pH2, at pH3.5 and at pH6.5.

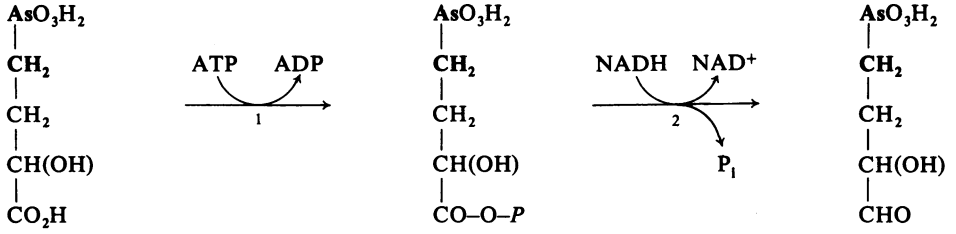
Results

The arsonomethyl analogue of 3-phosphoglycerate proved to oxidize NADH provided that both phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase were added. We infer from this that it interacts with these enzymes as shown in Scheme 2. With the enzyme concentrations given in the Methods section, the rate was found not to change when the concentration of the dehydrogenase was doubled, and controls gave undetectable rates in the absence of the kinase. The system was therefore suitable for measuring kinetic characteristics, and their values, derived from direct linear plots (Eisenthal & Cornish-Bowden, 1974), are given in Table 1. Michaelis kinetics were shown by the analogue, but not by 3-phosphoglycerate, as previously noted by Orr & Knowles (1974); the characteristics given for it are those of the Michaelis kinetics approached at low substrate concentrations.

Discussion

Synthetic route

Despite the many authors who have prepared 2-chloroethylarsonic acid by the route used (Scheme



Scheme 2. *Reactions in which 4-arsono-2-hydroxybutanoic acid replaces 3-phosphoglycerate*
1, Phosphoglycerate kinase; 2, glyceraldehyde-phosphate dehydrogenase. **Bold** type indicates the atoms that differ from those of the natural compounds.

Table 1. *Comparison of 3-phosphoglycerate and its arsonomethyl analogue, H_2O_3As–CH_2–CH_2–$CH(OH)$–CO_2H, as substrates for phosphoglycerate kinase*
The measurements were made at pH 8.5 in 6 mM-ATP. Reproducibility suggested that the values shown have probable errors of less than 10%. The catalytic constants are derived from specific limiting rates by using a value of 47 kg/mol for the molar mass of the enzyme (Krietsch & Bücher, 1970).

Substrate	Michaelis constant K_m (mM)	Catalytic constant k_0 (s^{-1})	Specificity constant k_0/K_m ($M^{-1} \cdot s^{-1}$)
(2*R*)-3-Phosphoglycerate	1.15	150	130000
(2*RS*)-4-Arsono-2-hydroxybutanoate	6.1	0.12	20

1, first four steps), and who found in step 2 that a phase of dichloro(hydroxyethyl)arsine separated when it was formed in aqueous solution, we have seldom found it to do so, even when made from the crystalline cyclohexylammonium salt of hydroxyethylarsonic acid. We have therefore modified the published method by adding a large excess of HCl so that we can extract the dichloro(hydroxyethyl)arsine from aqueous solution without its being hydrolysed to the arsenoxide.

Esterification of arsonic acids (Scheme 1, step 5) is best achieved with propanol, because its azeotrope with water boils at a much lower temperature than propanol itself. The route thereafter follows a pathway similar to the synthesis by two of us of the phosphonomethyl analogue (Dixon & Sparkes, 1974, 1976).

Substrate properties

The results in Table 1 show that replacement of $-O-PO_3H_2$ by $-CH_2-AsO_3H_2$ has little effect on the K_m of phosphoglycerate kinase (at a fixed ATP concentration). This is all the more remarkable as the analogue was used as the *RS*-compound, and as even at the high pH used (8.5) not all of it would exist as the trianion, shown by Orr & Knowles (1974) to be the reactive form of the substrate.

Despite the low K_m, however, the analogue is only a poor substrate with respect to its catalytic constant, which is 1000-fold lower than that for phosphoglycerate. We may speculate that the binding is tight, but that the carboxy group is slightly displaced so that its phosphorylation by ATP is slowed.

Although the arsonomethyl analogue of 3-phosphoglycerate proves to be only a poor substrate for phosphoglycerate kinase, the facts that it is one and that it binds well to the enzyme suggest that further investigation of such compounds as possible metabolic inhibitors is justified.

We thank many colleagues, especially Dr. D. M. Brown, Dr. Olga Kennard, Dr. A. J. Kirby and Dr. G. A. Smith, for advice and discussion, and the Science and Engineering Research Council for financial support.

References

Braunstein, A. E. (1931) *Biochem. Z.* **240**, 68–93
Dixon, H. B. F. & Sparkes, M. J. (1974) *Biochem. J.* **141**, 715–719
Dixon, H. B. F. & Sparkes, M. J. (1976) *Biochem. J.* **155**, 440–441
Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720
Engel, R. (1977) *Chem. Rev.* **77**, 349–367
Gough, G. A. C. & King, H. (1928) *J. Chem. Soc.* 2426–2447
Kamiya, K., Cruse, W. B. T. & Kennard, O. (1983) *Biochem. J.* **213**, 217–223
Krietsch, W. K. G. & Bücher, T. (1970) *Eur. J. Biochem.* **17**, 568–580
Lagunas, R. (1980) *Arch. Biochem. Biophys.* **205**, 67–75

Lagunas, R. & Sols, A. (1968) *FEBS Lett.* **1**, 32–34

Long, J. W. & Ray, W. J. (1973) *Biochemistry* **12**, 3932–3937

Nekrassow, W. W. & Nekrassow, A. S. (1928) *Ber. Dtsch. Chem. Ges.* **61**, 1816–1821

Orr, G. A. & Knowles, J. R. (1974) *Biochem. J.* **141**, 721–723

Richards, F. M., Wyckoff, H. W., Carlson, W. D., Allewell, N. M., Lee, B. & Mitsui, Y. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 35–43

Scherlin, S. M. & Epstein, G. (1928) *Ber. Dtsch. Chem. Ges.* **61**, 1821–1825

Wade, H. E. & Morgan, D. M. (1953) *Nature (London)* **171**, 529–530

Webster, D., Jondorf, W. R. & Dixon, H. B. F. (1976) *Biochem. J.* **155**, 433–441

Webster, D., Sparkes, M. J. & Dixon, H. B. F. (1978) *Biochem. J.* **169**, 239–244