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An Arsenical Analogue of Adenosine Diphosphate

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An analogue of ADP was made in which the terminal phosphono-oxy group, $-O-PO(OH)_2$, has been replaced by the arsonomethyl group, $-CH_2-AsO(OH)_2$. This compound cannot form a stable analogue of ATP because anhydrides of arsonic acids are rapidly hydrolysed, so that any enzyme that phosphorylates ADP and accepts this analogue as a substrate should release orthophosphate in its presence. The analogue proves to be a poor substrate for 3-phosphoglycerate kinase (V/K_m is diminished by a factor of 10^2–10^3) and a very poor substrate for pyruvate kinase (V/K_m is diminished by a factor of 10^5–10^6). No substrate action was detected with adenylate kinase and creatine kinase.

Myers *et al.* (1963) made analogues of nucleoside diphosphates and triphosphates in which the terminal phosphono-oxy group, $-O-PO(OH)_2$, was replaced by the phosphonomethyl group, $-CH_2-PO(OH)_2$, and showed that extensive biochemical use could be made of them. Such work has been reviewed by Yount (1975) (see also Engel, 1977). Arsenate can often replace phosphate in enzyme reactions, for example in the enzymic oxidation of glyceral 3-phosphate (Needham & Pillai, 1937; Warburg & Christian, 1939), but is not built up to stable esters or anhydrides (see Braunstein, 1931), whereas organic arsenates, although unstable, may be good substrates for enzymes that act on the corresponding phosphates (Lagunas & Sols, 1968; Long & Ray, 1973). It therefore seemed likely that the analogue of ADP in which the terminal phosphate was replaced by the arsonomethyl group, $-CH_2-AsO(OH)_2$, could be made, and that any system that generates ATP from ADP and accepts this compound as ADP would be converted into a hydrolytic system ('uncoupled') by its presence. We have published a preliminary report of these ideas (Dixon *et al.*, 1977) and we now describe the synthesis and some properties of the ADP analogue.

Methods

Electrophoresis

This was performed on Whatman 3MM paper cooled by immersion in white spirit (Stoddard solvent) [containing 8% (v/v) pyridine for pH6.5] at 90–100V/cm for 15–20min in the following aqueous solutions: pH9.4, 1% (w/v) $(NH_4)_2CO_3$ adjusted with conc. NH_3 (sp.gr. 0.880); pH6.5, 10% (v/v) pyridine/0.3% (v/v) acetic acid; pH3.5, 0.5% (v/v) pyridine/5% (v/v) acetic acid; pH2.0, 2% (v/v) formic acid/8% (v/v) acetic acid.

Phosphates, phosphonates and arsonates were detected by their ability to bind Fe^{3+} (Wade & Morgan, 1953); the test is less sensitive for phosphonates and arsonates. Any spot detected by this method after electrophoresis is referred to below as 'phosphate-positive'.

Compounds containing adenine were detected by their ability to absorb u.v. light (Markham & Smith, 1949) with Kodagraph C13 contact paper (obtained from Kodak, London W.C.2, U.K.). Any spot detected by this method after electrophoresis is referred to below as 'u.v.-positive'.

Periodate treatments

Periodate uptake was measured by the method of Fields & Dixon (1968), i.e. residual periodate oxidized 1,2-di-(4-dimethylaminophenyl)ethane-1,2-diol (BDH, Poole, Dorset, U.K.) to 4-(dimethylamino)-benzaldehyde, which was determined spectrophotometrically. Phosphate elimination initiated by periodate oxidation was performed by the following modification of the method of Yu & Zamecnik (1960). To 0.25ml of a 40mM solution of the compound to be tested 0.25ml of 80mM-KIO_4 was added. After 5min, 115μl of cyclohexylamine (about 2M final concn.) was added, and incubation continued for 15min. A sample (10μl) was compared by paper electrophoresis at pH9.4 with samples withdrawn before cyclohexylamine addition and before periodate addition.

Enzyme incubations

(1) *Pyruvate kinase* (*EC* 2.7.1.40). Rabbit muscle enzyme (5μl of a 10mg/ml solution), obtained from Boehringer, London W5 2TZ, U.K., was added to 0.65ml of a solution containing 75mM-triethanolamine/HCl, pH7.6, 4mM-$MgSO_4$, 16mM-KCl, 8mM-tripotassium phosphoenolpyruvate (Clark & Kirby,

1966; see Webster *et al.*, 1976) and either 9mM-Na_2ADP (Boehringer) or 8mM-arsonomethyl analogue of ADP, at room temperature (about 18°C). Samples (50μl) were taken for immediate electrophoresis at pH3.5 before addition of the enzyme and at suitable times thereafter. The reaction with ADP was followed by the disappearance of phosphoenolpyruvate and the appearance of ATP; with the analogue the disappearance of phosphoenolpyruvate and the appearance of P_i were sought.

(2) *Creatine kinase* (*EC* 2.7.3.2). Rabbit muscle enzyme (0.2mg, Boehringer) was added to 0.9ml of a solution containing 55mM-glycylglycine buffer, pH7.0, 22mM-phosphocreatine (kindly given by Dr. A. A. Newton of this Department), 5.5mM-$MgSO_4$, 22mM-KCl and either 11mM-Na_2ADP or 11mM-arsonomethyl analogue of ADP, at room temperature (about 18°C). Samples (50μl) were taken for immediate electrophoresis at pH9.4 and at pH3.5 before addition of the enzyme and at suitable times thereafter. The reaction with ADP was followed by the disappearance of phosphocreatine and the appearance of ATP; with the analogue the disappearance of phosphocreatine and the appearance of P_i were sought.

(3) *Adenylate kinase* (*EC* 2.7.4.3). Rabbit muscle enzyme (25μl of a 2mg/ml solution; Boehringer) was added to 0.75ml of a solution containing 67mM-triethanolamine/HCl, pH7.6, 3.3mM-$MgSO_4$, 13.3mM-KCl and either 6.7mM-Na_2ADP alone or 6.7mM-Na_2ADP plus 6.7mM-arsonomethyl analogue of ADP. Samples (50μl) were taken for immediate electrophoresis at pH3.5 before addition of the enzyme and at suitable times thereafter. The reaction was followed by observing the formation of AMP and ATP, and with the analogue the formation of P_i was also sought.

Enzyme assays

(1) *Pyruvate kinase*. The reaction of ADP and its arsonomethyl analogue with rabbit muscle pyruvate kinase was investigated by following the decrease in A_{340} caused by oxidation of NADH in a coupled enzyme assay at 20°C, by assuming an absorption coefficient of 6220 litre·mol^{-1}·cm^{-1} (Horecker & Kornberg, 1948). Final concentrations in the cuvette were 90mM-triethanolamine/HCl, pH7.6, 10mM-KCl, 2.5mM-$MgCl_2$, 5mM-phosphoenolpyruvate (the salt with 1 molecule of cyclohexylamine; Clark & Kirby, 1966), 0.5mM-NADH (disodium salt, Boehringer), 40μg of pig heart lactate dehydrogenase (EC 1.1.1.27) (Boehringer) and 16μg of rabbit muscle pyruvate kinase/ml. This mixture was allowed to come to equilibrium in the cuvette for at least 15min, after which the solution was made 0.17mM or 0.33mM in ADP (disodium salt) or 0.17, 1.33, 5 or 10mM in its arsonomethyl analogue. Changes in A_{340} were followed in a Unicam SP.500 spectrophotometer with a Gilford 222-G attachment and with a potentiometric recorder for continuous monitoring.

(2) *Phosphoglycerate kinase* (*EC* 2.7.2.3). The reaction of ADP and its arsonomethyl analogue with yeast phosphoglycerate kinase (Boehringer) was investigated by following the increase in A_{340} due to the generation of NADH in a coupled enzyme assay at 20°C. Final concentrations in the cuvette were 98mM-triethanolamine/HCl, pH7.6, 0.9mM-EDTA, 3.3mM-Na_2HPO_4, 3.3mM-$MgSO_4$, 2.3mM-fructose 1,6-bisphosphate [tetrasodium salt obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K.], 33μg of rabbit muscle aldolase (EC 4.1.2.13) (Boehringer)/ml, 33μg of yeast glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (Boehringer)/ml, 33μg of rabbit muscle triose phosphate isomerase (EC 5.3.1.1) (Boehringer)/ml, 167μg of yeast 3-phosphoglycerate kinase/ml and 2.5mM-NAD^+ (disodium salt; Boehringer). This mixture was allowed to equilibrate in the cuvette for at least 20min to establish a baseline, and the reaction was started by making the solution either 0.33mM in Na_2ADP or 0.33mM in the arsonomethyl analogue (the salt with two molecules of cyclohexylamine). On occasion, this sequence of operations was modified as described in the Results section.

Syntheses

Arsonomethylphosphonic acid. Chloromethylphosphonic acid was made (Schwarzenbach *et al.*, 1949) by hydrolysis of $Cl–CH_2–POCl_2$, which was kindly given to us by Dr. S. G. Warren (Chemical Laboratory, University of Cambridge, U.K.) and had been made from formaldehyde and PCl_3. NaOH (21g, 0.525mol) was dissolved in 20ml of water, followed by As_2O_3 (11g, 0.055mol). While this solution was still hot from the heat of solution and neutralization, a solution of chloromethylphosphonic acid (13g, 0.1mol) in 15ml of water was added with stirring. During this addition the solution heated further, and after the addition it was heated to boiling, and placed in a stoppered flask which was incubated at 104°C for 4h. Electrophoresis showed almost complete conversion of the chloromethylphosphonic acid into arsonomethylphosphonic acid; preliminary experiments showed that the use of more dilute solutions led to lower yields and to the formation of hydroxymethylphosphonic acid. The solution was diluted to 500ml with water, passed through the acid form of a sulphonated polystyrene (Zerolit 225 SRC 13, 15–50 mesh), evaporated to dryness and placed in a desiccator overnight to remove HCl. It was dissolved in water, filtered, adjusted to pH10 with cyclohexylamine and evaporated to dryness. Residual water was removed by suspending in ethanol and re-evaporating to dryness. It dissolved on heating in

200ml of methanol, and crystallized on addition of 200ml of diethyl ether; yield 29.4g (57%) of $(HO)_2$-AsO-$CH_2PO(OH)_2$,$3C_6H_{11}NH_2$ (Found: C, 44.5; H, 8.6; N, 8.0; $C_{19}H_{45}AsN_3O_6P$ requires C, 44.1; H, 8.8; N, 8.1%).

The free acid was made by passing an aqueous solution of the salt through the acid form of a sulphonic resin (Zerolit 225 SRC 14). After concentration by rotary evaporation it crystallized from water on addition of acetone, and its elementary analysis gave C, 5.8; H, 2.8; CH_6AsO_6P requires C, 5.5, H, 2.7%. Titration of a sample of the free acid gave the following approximate pK values: 2.3, 4.4, 6.6 and 10.7. The compound gave a single spot positive in the phosphate test on electrophoresis at pH2.0, with a mobility 1.5 times that of orthophosphate (glucose was used as an immobile control to correct for electroosmosis).

2′:3′-Isopropylideneadenosine. This was made by treating adenosine with 2,2-dimethoxypropane in the presence of toluene-*p*-sulphonic acid by the method of Fromageot *et al.* (1967), but their method was modified by adding a volume of acetone equal to the volume of dimethoxypropane to prevent the formation of a sticky precipitate when the reactants were mixed.

Arsonomethylphosphonic acid, adenosine ester. Arsonomethylphosphonic acid (220mg, 1mmol) was dissolved in 10ml of freshly redistilled dimethylformamide (solution A). Isopropylideneadenosine (920mg, 3mmol) was dissolved in 10ml of the same solvent (solution B). Dicyclohexylcarbodi-imide (2.06g, 10mmol) was dissolved in a mixture of 10ml of dimethylformamide and 10ml of anhydrous pyridine (solution C). The three solutions were then mixed with stirring by adding A and C simultaneously to B. A white precipitate was immediately seen, as the arsenic compound is only sparingly soluble in the presence of pyridine. As coupling progressed, a more granular precipitate of the dicyclohexylurea was also seen. After 20h the precipitates were spun down in an MSE bench centrifuge (5min, 1000*g*) and the reaction was stopped by removing the solvents by rotary evaporation, followed by vacuum desiccation. The resulting solid was extracted with 5×10ml of water, the insoluble material being spun down each time as before. The extracts were pooled in a large round-bottomed flask and dried by rotary evaporation. A large flask is necessary because severe frothing occurs. The resulting glass was dissolved in 10ml of water and adjusted to pH6 with 1M-KOH. This precipitated most of the unchanged isopropylideneadenosine, which was spun down in a Sorvall Superspeed RC2B centrifuge (8×50 rotor, 20000*g*, 15min, 4°C). The precipitate was resuspended in 5ml of water and centrifuged as before. The supernatants were pooled and passed through a column (10cm×1cm) of the acid form of a sulphonic resin (Zerolit 225 SRC 15), the effluent being monitored at 260nm in a Unicam SP.500 spectrophotometer. The column was eluted with water until two peaks had appeared, and then with 0.5M-NH_3 until material giving another peak had been eluted. Fractions eluted with water were dried by rotary evaporation and redissolved in a small amount of water for characterization. Fractions eluted with NH_3 were stored at 4°C, except for samples for electrophoretic characterization, which were dried and redissolved in water.

As a result of electrophoretic analysis the material giving first peak (breakthrough) was discarded. The solution giving the second peak, which represented the desired product, was adjusted to pH7 with cyclohexylamine and dried by rotary evaporation. The resulting white solid, the salt with two molecules of cyclohexylamine, was precipitated from methanol with diethyl ether, washed with ether, and dried in a vacuum desiccator. The yield was 13%, calculated on the basis of a molar absorption coefficient of 15000 litre·mol^{-1}·cm^{-1} for ADP at 260nm at pH2 (Bock *et al.*, 1956).

The solution giving the final peak (NH_3 elution) was stored for 1 month and then rechromatographed on a similar column with water as eluent. The effluent was monitored at 260nm, and material eluted in the position of the desired product was collected and purified as described above.

Results

Characterization of product

Elementary analysis of the salt with two molecules of cyclohexylamine gave C 39.6, H 6.3, N 14.0%, whereas $C_{23}H_{43}AsN_7O_9P$ requires C 41.4, H 6.4, N 14.7%, but the values found expressed as atoms per 7N atoms give C 23.0, H 43.5. The u.v. spectrum of a 50μM solution (by weight) of this salt appeared very similar to that of a 50μM solution of commercial Na_2ADP. The observed absorption coefficient at 260nm for the analogue was 14900 litre·mol^{-1}·cm^{-1}, whereas that of ADP is 15400 litre·mol^{-1}·cm^{-1} (Bock *et al.*, 1956).

Passage of the product through the acid form of a sulphonic resin during the work-up proved sufficient to remove the protecting 2′,3′-isopropylidene group (cf. Tener, 1961), as shown in two ways. First, periodate oxidation as described above (40mM-KIO_4, 5min, room temperature) completely destroyed the original spot (u.v.- and phosphate-positive) seen on paper electrophoresis at pH9.4. This spot was replaced by one at the origin (only u.v.-positive) and by one with the mobility of arsonomethylphosphonic acid (only phosphate-positive), suggesting that periodate scission of the ribose ring had been followed by elimination of arsonomethylphosphonic acid. Addition of cyclohexylamine did not change this spot

Table 1. *Electrophoretic mobilities of the analogue and related compounds*
Mobilities are expressed relative to that of ATP (taken as unity) and measured from the position of glucose (taken as zero to correct for electro-osmosis).

Compound	Mobility (R_{ATP})			
pH ...	2.0	3.5	6.5	9.4
ADP analogue, i.e. Ado(5′)–O–PO(OH)–CH_2–$AsO(OH)_2$	0.08	0.33	0.92	0.88
ADP	0.76	0.83	0.98	0.95
AMP	−0.11	0.30	0.68	0.86
P_i	1.01	1.56	1.39	1.44
Arsonomethylphosphonic acid, i.e. $(HO)_2AsO$–CH_2–$PO(OH)_2$	1.51	1.4	1.85	1.6

pattern; evidently the NH_3 of the pH9.4 buffer was enough to cause the elimination. The second test of unmasking was the measurement of periodate uptake. On addition of 0.5ml of 1mM-ADP or analogue to 1.0ml of 1mM-$NaIO_4$ at room temperature, the time course of periodate uptake was similar for the two compounds. Uptake was complete in 10min, and was 1.1 molecules per molecule of analogue, and 1.0 molecule per molecule of ADP.

The electrophoretic mobility of the product at the four pH values described (Table 1) was consistent with that expected for the desired compound in view of the pK values of arsonic acids. Thus at pH2 there should be one full negative charge on the phosphonate group and one positive charge on the adenine; at pH3.5, a full negative charge on the phosphonate, nearly a full positive charge on the adenine and a partial negative charge on the arsonic acid; at the two higher pH values two negative charges would be expected. Thus at several pH values the analogue would be expected to show a mobility more like that of AMP than that of ADP.

Enzyme incubations and assays

On incubating the analogue with pyruvate kinase, adenylate kinase and creatine kinase in the systems described, no reaction could be detected even after several days, whereas in controls with ADP, reaction was detected within 15min.

In the coupled assay of pyruvate kinase, no reaction could be detected with the analogue at concentrations of 0.17mM and 1.33mM, but a very slow reaction (0.022μmol of NADH oxidized/h per mg of pyruvate kinase) was seen with 5mM analogue, and one approximately twice as fast (0.048μmol/h per mg of enzyme) with 10mM analogue. Addition of 0.33mM-ADP to the cuvette gave as rapid a reaction (220μmol of NADH oxidized/h per mg of pyruvate kinase) in the presence of 5mM analogue as in its absence.

In the coupled assay with phosphoglycerate kinase, an increase in A_{340} started on addition of the analogue to the cuvette. If the analogue was added to the cuvette before the phosphoglycerate kinase, no rise in A_{340} was seen until the enzyme was added; this indicated that the observed generation of NADH was not due to the action of glyceraldehyde 3-phosphate dehydrogenase with any contaminating arsenate. No NADH was produced if an equivalent amount of cyclohexylamine rather than the salt of the analogue with cyclohexylamine was added to the cuvette. Addition of 1μmol (by weight) of ADP gave an A_{340} in 3ml of 1.65. The expected value is 2.07 if the compound is pure, but the manufacturers' claim for enzymic purity is only 79%, which agrees with the result observed. Addition of 1μmol of the arsonomethyl analogue gave an absorbance above 3, whereas the expected value for a purely stoicheiometric reaction is 2.07. These values proved to be reproducible, so that the analogue must be operating catalytically rather than stoicheiometrically. The rate at which the reaction proceeded with the analogue was about one-hundredth of that with ADP.

Discussion

Synthetic route

The first problem in coupling 2′,3′-protected adenosine with arsonomethylphosphonic acid was that both ends of the latter would couple with the alcohol, and this would happen spontaneously even without coupling agent. We therefore decided to provide excess of the alcohol and allow this to happen. Esters of arsenic acid hydrolyse spontaneously (Braunstein, 1931) and glucose 6-arsenate has a half-life of about 30min in neutral solution (Long & Ray, 1973). The esters of the arsonic acid end of the molecule were therefore hydrolysed during the work-up.

Unfortunately in the method used the major product was material that was eluted from the sulphonic resin with 0.5M-NH_3. It was thus much more tightly bound to the resin than the desired product, which was eluted with water. On electrophoresis this compound proved to be positively charged at pH2, just positively charged at pH3.5 and negatively charged at pH6.5 and pH9.4. On staining, it proved to be both u.v.-

and phosphate-positive, and it was oxidized by periodate to arsonomethylphosphonic acid and adenine. We postulated that it might represent the compound containing two molecules of adenosine attached to one of arsonomethylphosphonic acid, and this theory was supported by the fact that on standing at 4°C for several weeks it decomposed to give the required product and an approximately equal amount (as judged by the A_{260} of the column effluent) of another u.v.-positive material that had to be eluted from the column with NH_3 (and was presumably adenosine). We have unsuccessfully attempted to accelerate the rate of decomposition of this material to the desired product by concentrating the solution and raising the pH. Because this compound is a major product, the immediate yield of the arsonomethyl analogue of ADP from arsonomethylphosphonic acid is only about 12–13%.

Attempts to improve the yield of the analogue by varying the durations of the couplings, the ratios and concentrations of the reagents and the solvents, and performing the reaction in a mixture of dimethylformamide and tributylamine, in which all reagents are completely soluble, were all unsuccessful, but did show that an excess of isopropylideneadenosine over arsonomethylphosphonic acid is necessary to prevent most of the latter from self-coupling.

In a further attempt to improve the yield we started with arsenosomethylphosphonic acid instead of arsonomethylphosphonic acid. The arsonic acid was reduced in methanolic HCl by passing SO_2 in the presence of a catalytic amount of I_2 for 20min, removing the HCl by rotary evaporation, the sulphate by precipitation with $Ba(OH)_2$, and the barium by passage down a sulphonic resin. Completeness of reduction was shown by electrophoresis at pH2; attempts at pH9.4 failed because the arsenoxide was spontaneously oxidized by air at this pH. The solution was concentrated to a viscous oil by rotary evaporation and vacuum desiccation, and the arsenoxide was used in a coupling as described above except that the incubation took 4h instead of 20h and the product was oxidized with H_2O_2 after extraction into water. With this procedure all of the product remained on the sulphonic resin during water elution and required 0.5M-NH_3 to elute it. Again, after several weeks at 4°C in this solution, a quantity of the desired product was produced, together with a second material (presumably adenosine).

Behaviour of analogue with enzymes

The analogue failed to be a good substrate for the four enzymes tested. It also failed to promote State-3 (Chance & Williams, 1955) respiration of succinate by rat liver mitochondria at concentrations up to 2mM, when addition of 0.25mM-ADP had a marked effect (C. J. R. Thorne, unpublished work). These results mean that it is not as useful as we had hoped as a tool for investigating phosphorylating systems. Since the analogue did not compete effectively with ADP in any of these systems, it was evidently poor at binding, and this was confirmed by its high K_m for the two enzymes that acted on it. This is in marked contrast with replacement of $-O-PO(OH)_2$ by $-CH_2-PO(OH)_2$ in glycolytic intermediates (Webster *et al.*, 1976, and references cited therein), or its replacement by $-O-AsO(OH)_2$ (Lagunas & Sols, 1968; Long & Ray, 1973), which leaves compounds as good enzyme substrates. Nevertheless it agrees with findings that the arsonomethylphosphonic acid prepared in the present work did not replace pyrophosphate as a substrate for isoleucyl- or tyrosyl-tRNA synthetase of *Escherichia coli* or valyl- or methionyl-tRNA synthetase of *Bacillus stearothermophilus* (A. R. Fersht & C. R. Dingwall, unpublished work) or for DNA polymerase I from *E. coli* (F. Sanger, unpublished work). Enzymes that bind pyrophosphate and its compounds may prove particularly sensitive to the spacing of the oxygen atoms that their substrates contain. The similarity of the two ends of arsonomethylphosphonic acid was shown by Falvello *et al.* (1977), whose X-ray analysis of crystals of its salt with two molecules of cyclohexylamine showed that the molecules were randomly orientated with respect to phosphorus–arsenic direction in crystals.

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