Stereochemical Course of Phosphokinases. The Use of Adenosine [γ-(*S*)-^{16}O,^{17}O,^{18}O]Triphosphate and the Mechanistic Consequences for the Reactions Catalyzed by Glycerol Kinase, Hexokinase, Pyruvate Kinase, and Acetate Kinase†

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ABSTRACT: We report the synthesis of adenosine [γ-(*S*)-^{16}O,^{17}O,^{18}O]triphosphate, an isotopically labeled species of ATP that is chiral at the γ-phosphoryl group, the configuration of which has been confirmed by independent stereochemical analysis. This molecule has been used as a substrate in the reactions catalyzed by glycerol kinase and by acetate kinase. The resulting samples of isotopically labeled *sn*-glycerol 3-phosphate and of acetyl phosphate have been used as substrates in the alkaline phosphatase mediated transfer of the chiral phosphoryl groups to (*S*)-propane-1,2-diol, whence the configuration at phosphorus has been determined [Abbott, S. J., Jones, S. R., Weinman, S. A., & Knowles, J. R. (1978) *J. Am. Chem. Soc. 100*, 2558]. It is shown that glycerol kinase and acetate kinase (and, by virtue of an earlier correlation, pyruvate kinase and hexokinase) proceed by pathways that result in *inversion* of the configuration at phosphorus. The stereochemical approach provides an access to the otherwise cryptic events that are involved in phosphoryl-group transfer within the ternary complexes of these kinases and their substrates.

Despite the large body of experimental information on the mechanistic pathways followed by the phosphokinases (Morrison & Heyde, 1972), controversy still rages over the existence, transitory or otherwise, of phosphoryl–enzyme intermediates (Spector, 1973). For some systems, such as hexokinase (Colowick, 1973) or pyruvate kinase (Kayne, 1973), the battery of kinetic methods (including steady-state kinetics, inhibition studies, flux kinetics, isotope exchange in partial reactions, and pulse–chase experiments) has combined to suggest that the phosphoryl group is transferred between substrates within a ternary complex, even if the details of this transfer reaction are unknown. Yet for other enzymes, such as acetate kinase, the isolation and characterization of a phosphoryl–enzyme (Anthony & Spector, 1972; Todhunter & Purich, 1974) and the demonstration of its chemical competence [i.e., its ability specifically to transfer the phosphoryl group to either of the two acceptors, even if not always at a rate that satisfies the criterion of kinetic competence (Anthony & Spector, 1971)] have led to mechanistic proposals that involve binary complexes and an obligatory phosphoryl–enzyme intermediate. Even for these apparently satisfactory cases, however, there are disturbing lacunas in our knowledge, and experimental findings exist that conflict with the simple view. For instance, hexokinase *can* form a phosphoryl–enzyme (Cheng et al., 1973) (even though it is believed to be the product of a dead-end reaction), and there was no way of knowing if a transient phosphoryl–enzyme was an intermediate in the transfer of the phosphoryl group within the ternary complex. Analogously, the various kinetic studies on acetate kinase are in some conflict (Purich & Fromm, 1972; Janson & Cleland, 1974b; Webb et al., 1976; Skarstedt & Silverstein, 1976), and the simple ping-pong mechanism has required considerable modification to accomodate most (never mind all) of the kinetic results.

In the face of such uncertainty, we have recently developed a stereochemical approach that allows us to define the stereochemical consequence at phosphorus of kinase-mediated reactions (Abbott et al., 1978, 1979; Blättler & Knowles, 1979). We expected that the results from such studies would remove some of the mechanistic ambiguities suffered by these enzymes and provide a focus for consideration of the catalysis of phosphoryl transfer. We report here the synthesis of [γ-^{16}O,^{17}O,^{18}O]ATP of one configuration at its γ-phosphorus and the use of this molecule to define the stereochemical course of glycerol kinase, hexokinase, pyruvate kinase, and acetate kinase.

Experimental Section

Methods. ^{1}H and ^{31}P NMR spectra were recorded on a Varian XL-100 instrument. The ^{31}P chemical shifts are relative to external 85% H_3PO_4 (downfield negative). Gas chromatography was performed on a Varian Aerograph 1400 instrument, using 6-ft glass columns and a standard flame ionization detector. Ion-exchange chromatography was done using DEAE-cellulose (DE-52 from Whatman), Dowex 1 (200–400 mesh; 8% cross-linked) or Dowex 50 (100–200 mesh; 8% cross-linked). High-pressure liquid chromatography, and mass spectrometry in both normal and linked scan modes, was done as described earlier (Abbott et al., 1978, 1979). Assays of ATP and of *sn*-glycerol 3-phosphate were done as described by Bergmeyer (1974); acetyl phosphate was assayed according to Whitesides et al. (1975). The absolute configuration at phosphorus in samples of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol was determined as described earlier (Abbott et al., 1978, 1979).

Materials. Enzymes and cofactors were obtained from Sigma: glycerol kinase (from *Escherichia coli*; lyophilized powder; 500 units/mg); alkaline phosphatase (from *E. coli*; ammonium sulfate suspension; 20–30 units/mg); acetate kinase (from *E. coli*; ammonium sulfate suspension; 170 units/mg; dialyzed against 50 mM *N*-ethylmorpholinium acetate, pH 7, overnight); 3-phosphoglycerate kinase (from yeast; lyophilized powder; 1000 units/mg); glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle; crystalline suspension in aqueous ammonium sulfate; 49 units/mg); α-glycerophosphate dehydrogenase (from rabbit muscle; crystalline suspension in aqueous ammonium sulfate; 1650 units/mg); hexokinase (from

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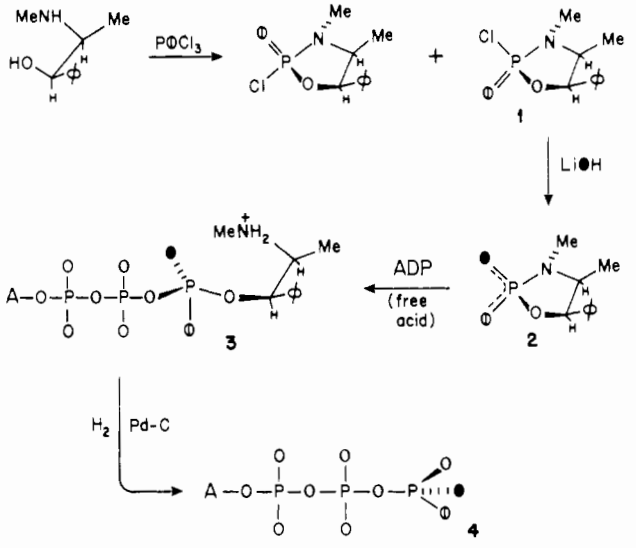
Scheme I: Synthetic Route to Adenosine [γ-(*S*)-^{16}O,^{17}O,^{18}O] Triphosphate[a]



[a] ^{16}O is O; ^{17}O is ⊕; ^{18}O is ●.

yeast; crystalline suspension in aqueous ammonium sulfate; 500 units/mg); glucose-6-phosphate dehydrogenase (from yeast; crystalline suspension in aqueous ammonium sulfate; 300 units/mg); NAD^+; NADH (disodium salt); $NADP^+$ (sodium salt); ADP (sodium salt).

Adenosine [γ-(*S*)-^{16}*O*,^{17}*O*,^{18}*O*]*Triphosphate* (**4**). This material was synthesized by a modification of our published route (Abbott et al., 1979) (see Scheme I). The two chloro adducts deriving from the reaction of $P^{17}OCl_3$ (prepared from PCl_5 and $H_2{}^{17}O$: 41.5% atom excess) and (−)-ephedrine were prepared as described earlier (Abbott et al., 1979), and the major isomer **1** was isolated by preparative thin-layer chromatography on silica using ethyl acetate as the solvent. The purified chloro adduct **1** (725 mg, 2.96 mmol) was dissolved in dry dioxane (2 mL), and this solution was added to aqueous Li^{18}OH [2 mL of a 3 M solution, prepared from $H_2{}^{18}O$ (2 mL of 97.3% atom excess) and lithium metal (42 mg, 6 mmol)]. After 10 min at room temperature, the proton-decoupled ^{31}P NMR spectrum of the reaction mixture showed a signal at −21.38 ppm (due to compound **2**) and a signal at +0.82 ppm (due to the acyclic monoester deriving from P–N bond cleavage of **2**) in a ratio of 97:3. The reaction mixture was freeze-dried, and the resulting white solid was then redissolved in dry dioxane and freeze-dried, twice. The cyclohexylammonium salt of **2** [prepared by passing the above product through a column of Dowex 50 (cyclohexylammonium form)] has a melting point of 174–177 °C after three recrystallizations from water–acetone. This material shows a singlet at −25.83 ppm in the proton-decoupled ^{31}P NMR (in water–acetone, 1:1 v/v).

To the lithium salt of **2** (2.87 mmol), prepared as above, was added a solution of ADP (free acid; 2.56 g, 6 mmol) in dimethyl sulfoxide (35 mL; freshly distilled from CaH_2) which had been stored for 24 h over 4-Å molecular sieves. After 24 h at room temperature, the reaction mixture was diluted with water to 500 mL. This solution, after neutralization with solid NH_4HCO_3, was applied to a column (1800 mL) of DEAE-cellulose equilibrated with 50 mM triethylammonium bicarbonate, pH 7. The column was eluted first with 50 mM triethylammonium bicarbonate (2 L) and then with a linear gradient of 50–400 mM triethylammonium bicarbonate, pH 7 (4.5 L plus 4.5 L). The product **3** was eluted at a buffer concentration of 110–150 mM. Unreacted ADP (2.77 mmol; 46%) was also recovered. The fractions containing **3** were evaporated to dryness at room temperature under reduced pressure, and the triethylammonium bicarbonate was removed by two evaporations of added 2-propanol. The product **3** shows three signals in the proton-decoupled ^{31}P NMR (in ethanol–water, 1:1 v/v, pH 7) at 11.83 (d, J = 21.5 Hz, P_α), 12.92 (d, J = 17.6 Hz, P_γ), and 22.28 (dd, J = 21.5 and 17.6 Hz, P_β) ppm.

The derivative **3** was converted into the labeled ATP (**4**) by hydrogenolysis over 10% Pd/C in ethanol–water (1:1 v/v; 50 mL) for 5 h at 3.4 atm. The reaction mixture was filtered through Celite, and the catalyst was washed thoroughly with ethanol–water–concentrated ammonia (50:48:2 v/v). The combined filtrate and washings were neutralized with CO_2 and then evaporated to dryness. The product **4** showed one spot on thin-layer chromatography [polyethylenimine plates (Polygram Cel-300), eluted with 0.75 M KH_2PO_4 adjusted to pH 3.5 with concentrated HCl], and its proton-decoupled ^{31}P NMR (in ethanol–water, 1:1 v/v, pH 7) showed signals at 6.00 (d, J = 19.2 Hz, P_γ), 11.18 (d, J = 19.9 Hz, P_α), and 21.43 (t, J = 19.7 Hz, P_β) ppm. The yield (determined by enzymic assay) was 1.18 mmol (40% from **1**), and this product was used without further purification.

Proof of the S Configuration of **4** *at the γ-Phosphorus.* The γ-phosphoryl group of **4** was transferred with retention of configuration to (*S*)-propane-1,2-diol by using alkaline phosphatase (Jones et al., 1978). The following were contained in the reaction mixture (2.68 mL): the labeled ATP, **4** (as the sodium salt; 255 μmol); (*S*)-propane-1,2-diol (1.27 mL); $Mg(OAc)_2$ (8 μmol); $Zn(OAc)_2$ (80 nmol); alkaline phosphatase (46 units); 0.3 M $KHCO_3$–K_2CO_3 (1:1; 1.27 mL). After 24 h at room temperature (at which time only a trace of ATP was detectable by TLC), the mixture was diluted to 20 mL with water, and the nucleotides were removed by adsorption onto charcoal. The filtrate was passed through a column (10 mL) of Dowex 50 (H^+ form), neutralized with triethylamine, and the mixture of 1- and 2-phospho-(*S*)-propane-1,2-diol (45 μmol; in 4.0:1 ratio as estimated from the ^{1}H NMR spectrum) was isolated by gradient elution (50–300 mM triethylammonium bicarbonate, pH 7) from a column (50 mL) of Dowex 1 (HCO_3^- form) preequilibrated with 50 mM triethylammonium bicarbonate, pH 7. (A control incubation and workup demonstrated that, under the conditions used, less than 3% of the recovered phosphopropanediol derived from *non*-enzyme-catalyzed alcoholysis of ATP.) The mixture of phosphopropanediols was then subjected to stereochemical analysis (see Table I) according to the method reported earlier (Abbott et al., 1978, 1979).

Glycerol Kinase Reaction. [γ-(*S*)-^{16}O,^{17}O,^{18}O]ATP (**4**) was used to phosphorylate glycerol in the reaction catalyzed by glycerol kinase. The following were contained in the reaction mixture (63 mL): labeled ATP (**4**) (615 μmol); glycerol (26 mmol); glycerol kinase (346 units); 2-mercaptoethanol (1.7 mmol); $MgCl_2$ (360 μmol); in 50 mM triethanolamine hydrochloride buffer, pH 8.32. The reaction was stopped after 1 h at room temperature by lowering the pH to 2 with Dowex 50 (H^+ form). After 1 min, the solution was neutralized with solid NH_4HCO_3, the resin was removed by filtration, and the nucleotides were removed by adsorption onto charcoal. The product *sn*-glycerol 3-phosphate (586 μmol; 95% yield based on **4**) was isolated by chromatography on a column (50 mL) of Dowex 1 (HCO_3^- form) as described above. It was then converted to the disodium salt by passage through a column of Dowex 50 (Na^+ form).

The transfer of the phosphoryl group from *sn*-glycerol 3-phosphate to (*S*)-propane-1,2-diol was effected with retention

Table 1: Results from Linked-Scan Metastable Ion Mass Spectrometry of the Isotopically Labeled Trimethyl Phosphate Ions Derived from [^{16}O,^{17}O,^{18}O]Phospho-(*S*)-propane-1,2-diol[a]

	ratio of granddaughter ion intensities at *m/z* 111 and 113[b]		percentage of (*S*)-phospho compound derived from the ratio of granddaughter ion intensities		
	anti[c]	syn[c]	anti[c]	syn[c]	av[d]
ideal case for					
(*S*)-phosphopropanediol	0.5	0	100	100	100
racemic mixture	0.25	0.25	50	50	50
(*R*)-phosphopropanediol	0	0.5	0	0	0
observed for phosphopropanediol from synthetic labeled ATP (**4**)	0.384	0.123	76.8	75.4	76.1[e]
predicted[f] for (*S*)-propanediol from synthetic labeled ATP (**4**)	0.386	0.123	77.2	75.5	76.4

[a] Samples of [^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol were analyzed as described by Abbott et al. (1978, 1979). After cyclization and methylation, the resulting syn and anti cyclic triesters were separated by high-pressure liquid chromatography and subjected to methanolysis. Linked-scan metastable ion mass spectrometry of the resulting acyclic triesters allowed the fragmentations from the labeled trimethyl phosphate ion of *m/z* 143 (which contains *both* ^{17}O and ^{18}O) to be investigated (Abbott et al., 1978, 1979). [b] The metastable fragmentation of the labeled trimethyl phosphate ion at *m/z* 143, which can lose [^{16}O]formaldehyde (to *m/z* 113), [^{17}O]formaldehyde (to *m/z* 112), or [^{18}O]-formaldehyde (to *m/z* 111), was measured. [c] That is, results deriving from investigation of the anti or the syn cyclic triesters after their separation by high-pressure liquid chromatography and subsequent methanolysis. [d] Average of the results from the separate measurements on the syn and the anti cyclic triesters. [e] This value should be compared with the predicted value of 76.4% and shows that the original labeled ATP, **4**, is indeed 99 ± 10% *S* at phosphorus. [f] Predicted results on the basis of the known isotopic composition of the labeled samples used in the synthesis of [γ-(*S*)-^{16}O,^{17}O,^{18}O]ATP, the measured enantiomeric purity of the (*S*)-propane-1,2-diol used, the measured cross-contamination of the syn and anti cyclic triesters after separation, and the measured ratio of 1- to 2-phosphopropanediol. The use in the analysis only of the ratio of granddaughter ion intensities at *m/z* 111 and 113 obviates any need for corrections for "downward cross-talk" in the mass spectrometer or for interference from natural-abundance ^{13}C and ^{2}H. For a full explanation of these effects, see Abbott et al. (1979).

of configuration by alkaline phosphatase (Jones et al., 1978). The following were contained in the reaction mixture (4.8 mL): the sample of isotopically labeled *sn*-glycerol 3-phosphate (disodium salt) from the glycerol kinase reaction (586 μmol); (*S*)-propane-1,2-diol (2.4 mL); $Mg(OAc)_2$ (1.8 μmol); $Zn(OAc)_2$ (18 nmol); alkaline phosphatase (9.1 units); 0.3 M $KHCO_3$–K_2CO_3 (1:1; 2.4 mL). After 50 h at room temperature, the reaction was stopped as described for the glycerol kinase reaction. The products 1- and 2-phospho-(*S*)-propane-1,2-diol along with the remaining *sn*-glycerol 3-phosphate were isolated together from a column (50 mL) of Dowex 1 (HCO_3^- form) as described above. The mixture was analyzed by GLC (on 3% OV-1; 150 °C) of a silylated sample [using *N,O*-bis(trimethylsilyl)trifluoroacetamide in pyridine] and found to contain approximately equal amounts of glycerol phosphate and the phosphopropanediols. The glycerol phosphate was oxidized by treating the mixture with sodium periodate (500 μmol) in water (2 mL). After 15 min at 0 °C, excess periodate was destroyed by the addition of ethylene glycol (100 μL), and after 5 min at 0 °C, the mixture was applied to a column (10 mL) of Dowex 1 (HCO_3^- form). Gradient elution (as above) gave the phosphopropanediols in 12% yield (on the basis of the amount of ATP originally used). Conversion of this material to the bis(cyclohexylammonium) salt and crystallization from water–acetone (1:10 v/v) gave 1- and 2-phospho-(*S*)-propane-1,2-diol (in 4.0:1 ratio) for stereochemical analysis.

Acetate Kinase Reaction. Because acetyl phosphate is rather unstable in aqueous solution, the acetate kinase catalyzed phosphorylation was done 3 times on a small scale, to minimize loss of product due to hydrolysis. The following were contained in the reaction mixture (55 mL): labeled ATP (**4**) (220 μmol); KOAc (220 mmol); $MgCl_2$ (550 μmol); acetate kinase (550 units); in 100 mM *N*-ethylmorpholinium acetate buffer, pH 7.0. After 1 h at room temperature, the mixture was cooled to 0 °C, and all subsequent operations were conducted at 4 °C. The cooled mixture was acidified with Dowex 50 (H^+ form) to pH 3.5, the resin was then removed by filtration, and the solution was washed with ether to remove acetic acid. This procedure (acidification, filtration, and ether washing) was repeated 5 times. The pH of the solution was then raised to 6.5 with triethylamine. This solution was freed from nucleotides by filtration through charcoal, and the filtrate was applied to a column (50 mL) of DEAE-cellulose equilibrated with 50 mM triethylammonium bicarbonate, pH 6.5, which was eluted with a linear gradient (50–250 mM) of triethylammonium bicarbonate, pH 6.5. Fractions containing acetyl phosphate were concentrated under reduced pressure, and the buffer was removed by two evaporations of added 2-propanol. The acetyl phosphate was thus isolated as its crystalline bis(triethylammonium) salt in 65–75% yield (estimated by enzymic assay) on the basis of the labeled ATP used.

For the transfer of the phosphoryl group from acetyl phosphate to propanediol, the following were contained in the reaction mixture (1 mL): a sample of labeled acetyl phosphate from *one* of the three acetate kinase incubations (130–150 μmol of the bis(triethylammonium) salt); (*S*)-propane-1,2-diol (0.5 mL); $Mg(OAc)_2$ (9 μmol); $Zn(OAc)_2$ (90 nmol); alkaline phosphatase (50 units); 0.3 M $KHCO_3$–K_2CO_3 (1:1; 0.5 mL). After 45 min at room temperature, the reaction was stopped and the product isolated as described above. The combined yield of 1- and 2-phospho-(*S*)-propane-1,2-diols from the three transfer reactions was 93 μmol (14% on the basis of the amount of labeled ATP originally used). Conversion of this material to the bis(cyclohexylammonium) salt and crystallization from water–acetone (1:10 v/v) gave 1- and 2-phospho-(*S*)-propane-1,2-diols (in a 4.0:1 ratio) for stereochemical analysis.

Results

Isotopically labeled [γ-(*S*)-^{16}O,^{17}O,^{18}O]ATP, **4**, was synthesized by a modification of our published route to chiral phosphate monoesters (Scheme I) (Abbott et al., 1978, 1979). Since the conversion of **1** to **2** is known to proceed with retention of the configuration at phosphorus (Cooper et al., 1974, 1977) and the ring opening of **2** to **3** involves "in-line" attack at phosphorus (Cooper et al., 1977), the product **4** has the *S* configuration at the γ-phosphorus. This was proved by independent stereochemical analysis of **4** by transfer of the γ-phosphoryl group with retention (Jones et al., 1978) to (*S*)-propane-1,2-diol using alkaline phosphatase, followed by analysis of the absolute configuration at phosphorus (Abbott et al., 1978, 1979). The results in Table I show that the

Table II: Results from Linked-Scan Metastable Ion Mass Spectrometry of the Isotopically Labeled Trimethyl Phosphate Ions Derived from the Samples of [^{16}O,^{17}O,^{18}O]Phospho-(*S*)-propane-1,2-diol[a]

	ratio of granddaughter ion intensities at *m/z* 111 and 113[b]		percentage of (*R*)-phospho compound derived from the ratio of granddaughter ion intensities		
	anti[c]	syn[c]	anti[c]	syn[c]	av[d]
ideal case for					
(*R*)-phosphopropanediol	0	0.5	100	100	100
racemic mixture	0.25	0.25	50	50	50
(*S*)-phosphopropanediol	0.5	0	0	0	0
observed for phosphopropanediol from					
glycerol kinase sequence	0.173	0.320	65.5	64.0	64.8[e]
acetate kinase sequence	0.163	0.353	67.5	70.5	69.0[f]
predicted[g] for (*R*)-phosphopropanediol from					
glycerol kinase sequence	0.135	0.353	73.1	70.6	71.8
acetate kinase sequence	0.135	0.366	73.1	73.1	73.1

[a] See footnote *a* of Table I. [b] See footnote *b* of Table I. [c] See footnote *c* of Table I. [d] See footnote *d* of Table I. [e] This value should be compared with the predicted value of 71.8% and shows that the phosphopropanediol from the glycerol kinase sequence is 90.2% *R* at phosphorus. [f] This value should be compared with the predicted value of 73.1% and shows that the phosphopropanediol from the acetate kinase sequence is 94.4% *R* at phosphorus. [g] See footnote *f* of Table I.

γ-phosphoryl group of **4** indeed has the *S* configuration, in 99 ± 10% enantiomer excess.

The labeled ATP, **4**, was used as the substrate for the reactions catalyzed by two kinases: glycerol kinase and acetate kinase. The configuration of the phosphoryl group in each of the products (*sn*-glycerol 3-phosphate and acetyl phosphate) was determined by phosphoryl-group transfer to (*S*)-propane-1,2-diol with retention of configuration effected by alkaline phosphatase (Jones et al., 1978) and analysis of the absolute configuration of the phosphoryl group in the resulting samples of phosphopropanediol (Abbott et al., 1978, 1979).

From the transfer reaction of the phosphoryl group from *sn*-glycerol 3-phosphate to (*S*)-propane-1,2-diol, the phosphate monoesters [*sn*-glycerol 3-phosphate and 1- and 2-phospho-(*S*)-propane-1,2-diol] were isolated together from an ion-exchange column. The glycerol phosphate in this mixture was then completely oxidized with periodate, and the mixture of 1- and 2-phospho-(*S*)-propane-1,2-diols was isolated for analysis.

For the transfer of the phosphoryl group from acetyl phosphate to (*S*)-propane-1,2-diol, the conditions of the alkaline phosphatase incubation had to be modified to minimize the *non*-enzyme-catalyzed alcoholysis of acetyl phosphate (Di Sabato & Jencks, 1961), the stereochemical course of which is as yet unknown. It was shown that, under the conditions used, less than 2% of the recovered phosphopropanediol was derived from *non*-enzyme-catalyzed alcoholysis of acetyl phosphate.

Stereochemical analysis (Abbott et al., 1978, 1979) of the samples of phosphopropanediol showed that, in the sample produced from *sn*-glycerol 3-phosphate, the configuration was *R* (90 ± 8%) (Blättler & Knowles, 1979) and the sample from acetyl phosphate was also *R* (94 ± 8%) (see Table II).

Discussion

The stereochemical course of glycerol kinase and of acetate kinase evidently involves *inversion* of the phosphoryl group that is transferred: when [γ-(*S*)-^{16}O,^{17}O,^{18}O]ATP is used as the substrate, the products *sn*-glycerol 3-phosphate and acetyl phosphate each have the *R* configuration at phosphorus. Since we have earlier shown, using a γ-[γ-^{18}O]phosphorothioate of ATP of one configuration at the γ-phosphorus, that glycerol kinase, hexokinase, and pyruvate kinase all proceed with the same stereochemical course (Orr et al., 1978), it is clear that *all of these four phosphokinases go with inversion at phosphorus*. The simplest interpretation of these results is, of course, that in each case the phosphoryl group is transferred directly between the two enzyme-bound substrates with in-line geometry. But let us first consider the evidence from other kinds of experiments that bear upon the nature of these phosphoryl-transfer processes. Since the quality, the extent, and the nature of the evidence varies depending on the enzyme, each system is considered separately.

Hexokinase. The kinetic pathway followed by hexokinase has been studied by a variety of techniques, which are in broad agreement that the reaction is sequential rather than ping pong (i.e., that the chemical reaction(s) occur within ternary complexes) and that substrate addition is mostly ordered, with glucose leading. This evidence comes from investigations of steady-state kinetics, isotope exchange rates at equilibrium, preequilibrium isotope exchanges, and flux measurements [for a summary, see Colowick (1973)]. These data require that both substrates bind to hexokinase before the phosphoryl-group transfer, though the hints of partial exchange reactions (Kaji & Colowick, 1965; Solomon & Rose, 1971; Walsh & Spector, 1971) and ATPase activity (Kaji & Colowick, 1965; De la Fuente et al., 1970) have led to suggestions that the actual group-transfer process does occur via the enzyme. The alternative is that these hints are merely from side reactions and are not relevant to the normal catalytic pathway.

The knowledge that hexokinase proceeds with inversion of the configuration at phosphorus persuades us to the view that the catalytic reaction involves a simple in-line displacement, in which the 6-hydroxyl group of bound glucose attacks the γ-phosphorus of bound ATP. For hexokinase, therefore, our stereochemical result is in gratifying agreement with the consensus from kinetic experiments and provides further detail of the events that occur within the ternary complex of enzyme and substrates.

Glycerol Kinase. The steady-state kinetics of glycerol kinase, including studies of product inhibition and dead-end inhibition, show an ordered sequential pathway with glycerol leading (Janson & Cleland, 1974a) and do not support a ping-pong pathway involving a phosphoryl–enzyme. The finding that the phosphoryl group is transferred with inversion is thus consistent with a single in-line displacement mechanism for this enzyme.

Pyruvate Kinase. The kinetic behavior of pyruvate kinase is more complicated than the two previous enzymes discussed (Kayne, 1973), by virtue of its role in the control of glycolysis

and gluconeogenesis. However, the steady-state kinetics suggest a random sequential mechanism [Janson & Cleland, 1974b; see also MacFarlane & Ainsworth (1972, 1974), Ainsworth & MacFarlane (1973), and Dann & Britton (1978)], which agrees with the earlier view (Boyer, 1962) that, on the basis that the sites for ATP and phosphoenolpyruvate overlap, phosphoryl transfer occurs within ternary complexes. One is then left with "the primary question concerning the mechanism ... whether the enzyme facilitates the phosphoryl transfer ... by forming intermediate covalent complexes with the phosphoryl group, or supplies the proper electronic effects to facilitate the direct transfer" (Kayne, 1973). Our finding that the phosphoryl group is transferred (by the muscle enzyme, at least) with inversion most obviously supports the direct transfer pathway.

Acetate Kinase. Of all the phosphokinases except nucleoside diphosphokinase (which in terms of the present discussion is a special case, see below), the most complete and persuasive arguments for the mechanistic importance of a phosphoryl–enzyme have been presented for acetate kinase. Thus, Spector and his group (Anthony & Spector, 1971, 1972) reported that acetate kinase catalyzed both of the "partial" reactions [ATP–ADP exchange in the absence of added acetate and acetyl phosphate–acetate exchange in the absence of added nucleoside oligophosphate (Anthony & Spector, 1971)] and isolated a phosphorylated enzyme from incubations of partially purified enzyme with either [γ-^{32}P]ATP or acetyl [^{32}P]-phosphate. This phosphoryl–enzyme appeared to be chemically competent [in the sense that the phosphoryl group could be transferred either to ADP or to acetate (Anthony & Spector, 1972)]. The phosphoryl–enzyme intermediate was not kinetically competent, in that although the ATP–ADP exchange was 7 times faster than enzyme turnover in the overall reaction, the acetyl phosphate–acetate exchange was only 1.5% of the overall reaction rate (Anthony & Spector, 1971). "Substrate synergism" (Bridger et al., 1968) would have to be invoked to accommodate this slow reaction into a basic ping-pong scheme. Further, the first kinetic studies of Purich & Fromm (1972) suggested that acetate kinase followed a simple ping-pong pathway, consistent with the necessary formation of a free phosphoryl–enzyme in the reaction. Subsequently, Purich and co-workers, while noting that the maximal extent of phosphorylation of acetate kinase that could be achieved was very variable (Webb et al., 1976), showed that the phosphoryl group was attached to a glutamic acid residue in the protein (Todhunter & Purich, 1974). The above evidence did not explain, however, why Rose et al. (1954) had *not* detected the "partial" isotopic exchange reactions in an earlier study nor did the simple ping-pong mechanism accommodate the results of subsequent experiments from the laboratories of Cleland, Purich, and Silverstein. In 1974, Janson & Cleland (1974b) reported intersecting patterns in the double-reciprocal plots for acetate kinase, which requires a *sequential* mechanism for the majority of the enzyme-catalyzed flux, and these workers proposed that the observed ADP–ATP exchange reaction could be accommodated by a side reaction of the ping-pong type (Janson & Cleland, 1974b). Todhunter et al. (1976) then showed that this "nucleoside diphosphate kinase activity of acetate kinase" did indeed follow ping-pong kinetics and demonstrated that there was a concomitant loss of acetate kinase and nucleoside diphosphate kinase activity when the phosphoryl–enzyme was treated with hydroxylamine. These data are consistent with the intermediacy of a phosphoryl–enzyme for both types of catalytic activity. Finally, Skarstedt & Silverstein (1976) looked at the isotope exchange reactions at equilibrium and modified some of the earlier findings of Spector's group. It was shown, for instance, that whereas acetate does not affect the ATP–ADP exchange reaction, the second partial reaction (between acetyl phosphate and acetate) *could not be observed* in the absence of nucleotide. These workers concluded that there could be no phosphoryl–enzyme formation in the absence of nucleoside oligophosphate.[1]

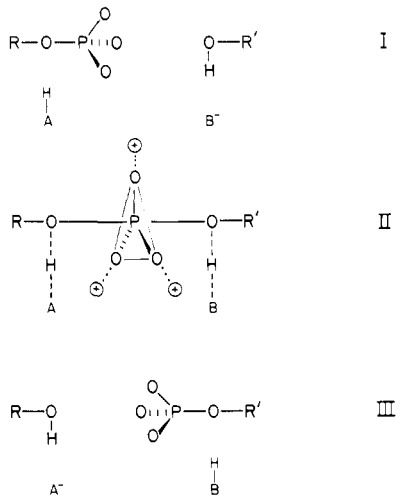
Even so, the possibility that acetate kinase formed a mechanistically obligatory phosphoryl–enzyme intermediate remained real. This enzyme was therefore a strong candidate for stereochemical analysis. Since it is extremely unlikely that (even in the commercially available enzyme) more than one protein is catalyzing the phosphorylation of acetate by ATP, the danger of artifacts[1] is negligible. It is therefore interesting that acetate kinase proceeds with inversion of the configuration at phosphorus, just like the other three kinases for which the kinetic evidence had already suggested a direct phosphoryl-group transfer reaction between enzyme-bound substrates. Undoubtedly the simple interpretation of this result is that acetate kinase is not a special case and catalyzes the in-line transfer of the γ-phosphoryl group of ATP to acetate. It is tempting to propose[2] that the phosphoryl–enzyme formed from MgATP (but not from acetyl phosphate in the complete absence of nucleotide) results from the reversible phosphorylation of one of the enzyme's own carboxyl groups when acetate is not present as an acceptor. Proof of this hypothesis must await the isolation of functionally pure enzyme.

General. The stereochemical course of phosphoryl transfer catalyzed by seven enzymes is now known. In this paper, we have shown, using [^{16}O,^{17}O,^{18}O]phosphoryl groups, that hexokinase, glycerol kinase, pyruvate kinase, and acetate kinase all proceed with inversion. In addition, we recently demonstrated that alkaline phosphatase, an enzyme for which a phosphoryl–enzyme intermediate has been unambiguously demonstrated by many different kinds of experiment, catalyzes transphosphorylation with overall retention (Jones et al., 1978). Whether the phosphoryl group suffers two inversions or two retentions in this reaction remains to be proved, but for the reasons elaborated below, we currently prefer the former. Frey and his group, using [^{18}O]phosphorothioates, have recently shown that the (thio)phosphoryl group that is transferred in the adenylate kinase reaction also suffers inversion (Richard & Frey, 1978) and that nucleoside diphosphate kinase [which is the only phosphokinase for which there is no evidence other than that supporting a phosphoryl–enzyme intermediate (Parks & Agarwal, 1973)] proceeds with retention (P. Frey, private communication). Overall, in-line displacements appear to be the preferred mode of transfer for all intermolecular phos-

[1] In most of this recent work, efforts have been made to accommodate all the results, both kinetic and other, into global schemes of increasing complexity. Yet for many of these studies relatively crude commercial enzyme was used, and efforts to ensure the absence of substrate (or substrate analogue) contaminants were often not reported. The phosphokinase field is bedeviled by problems arising from the presence of small amounts of contaminating kinases (it is rare, for instance, to find a commercial kinase preparation that will *not* support ATP–ADP exchange) or from the presence of small amounts of contaminating substrate [see, e.g., Johnson et al. (1976) and Switzer & Simcox (1974)]. The effective consequence of most such contamination is to yield results that suggest the kinetic importance of a phosphoryl–enzyme intermediate. We should therefore be wary of such suggestions, unless proper efforts have been made carefully to exclude the possibility of contaminant-induced artifacts. It should be stressed, however, that experiments of the kind reported here (where the product of a kinase reaction is isolated and analyzed) are free from the problems of contaminating enzymes that kinetic studies are so prone to.

[2] We are grateful to Professor W. W. Cleland for this suggestion.

Scheme II: Associative Displacement at a Phosphate Monoester with In-Line Geometry[a]



[a] (I) Starting materials (HA is a general acid, and B^- is general base); (II) pentacoordinate transition state or intermediate (⊕ represents positive charges or hydrogen-bond donors on the enzyme); (III) products.

phoryl-group transfers. This conclusion is supported by the apparent universality of the inversion path for displacements at phosphate *di*esters. Thus, by use of phosphorothioates, it has been shown that the following 13 enzyme-catalyzed displacements at phosphorus all go with inversion: ribonuclease A (both steps) (Usher et al., 1970, 1972); ribonuclease T_1 (one step) (Eckstein et al., 1972); uridinediphosphoglucose pyrophosphorylase (Sheu & Frey, 1978); galactose-1-phosphate uridylyltransferase (two steps) (P. Frey, private communication); phosphoribosylpyrophosphate synthetase (Li et al., 1978); DNA-dependent RNA polymerase, DNA-dependent DNA polymerase, tRNA nucleotidyltransferase, and polynucleotide phosphorylase (F. Eckstein, private communication); acetyl-CoA synthetase (Midelfort & Sarton-Miller, 1978); the phosphohydrolase from *Enterobacter aerogenes* (J. A. Gerlt and W. H. Y. Wan, private communication).

Is it reasonable that "in-line" displacements should be preferred over "adjacent" displacements? From what is known of the physical–organic chemistry of reactions at phosphate esters, it does seem likely that in-line S_N2-type reactions could be more readily catalyzed than adjacent displacements (Benkovic & Schray, 1971; Guthrie, 1977). To catalyze a displacement at phosphorus, the enzyme presumably needs a general base (B) to deprotonate the attacking group (R'OH), a general acid (HA) to assist the departure of the leaving group (ROH), and some constellation of functions to stabilize the transition state for the displacement. For an in-line displacement, the transition state will have the trigonal bipyramidal structure II (Scheme II), in which there are partial negative charges on each of the equatorial oxygens. Provided that the enzyme can provide three electron-deficient sites (positive charges or hydrogen-bond donors) positioned so as to stabilize the trigonal bipyramidal arrangement II rather than the tetrahedral disposition of oxygen atoms in either the starting material I or the product III (Scheme II), then catalysis will result. In contrast, the positioning of stabilizing enzymic groups and the convolutions of these groups required to catalyze an adjacent displacement [that necessarily involves at least one pseudorotation (Westheimer, 1968)] are clearly much more demanding. Mechanistic economy, in the absence of any facts that suggest otherwise, favors the in-line path. [In passing, it should be emphasized that the scope for the catalysis of dissociative mechanisms [see, e.g., Lowe & Sproat (1978)] involving monomeric metaphosphate seems limited to the general acid–base catalysis that aids the attacking group and the leaving group. The limitations to enzymic catalysis of dissociative mechanisms have been pointed out by Benkovic & Schray (1971) and fully discussed by Guthrie (1977).]

Finally, one caveat must be made. Any reaction that proceeds with overall inversion may in principle involve many steps, with any odd number of inversions and any number of retentions. It would therefore be consistent with our results that the four kinases we have studied each involve phosphoryl–enzymes that mediate the phosphoryl-group transfer within ternary complexes whose components never separate. In this case, however, one would minimally require one adjacent displacement ("to" the phosphoryl–enzyme) and one in-line displacement ("from" the phosphoryl–enzyme). With the accumulation of evidence cited here, the phosphoryl–enzyme postulate is an unattractive alternative to the simple elegance of direct in-line transfer between the two participating substrates. The stereochemical restrictions reported herein, combined with the large number of high-resolution crystal structures of phosphokinases that are now emerging, will provide the more precise mechanistic definition of these important enzymes.

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Kinetics of Carboxymethylation of Histidine Hydantoin†

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ABSTRACT: The reaction of the imidazole group of histidine hydantoin with bromoacetate was studied as a model for carboxymethylation of histidine residues in proteins. pK values of 6.4 and 9.1 (25 °C) and apparent heats of ionization of 7.8 and 8.7 kcal/mol were determined for the imidazole and hydantoin rings, respectively. At pH values corresponding to the isoelectric points for histidine hydantoin, the rates of carboxymethylation at 12, 25, 37, and 50 °C were determined; the modified hydantoins were hydrolyzed to the corresponding histidine derivatives for quantitative amino acid analysis. At pH 7.72 and 25 °C, the imidazole *tele*-N was alkylated (k = 3.9×10^{-5} M^{-1} s^{-1}) twice as fast as the *pros*-N. The monocarboxymethyl derivatives were carboxymethylated at the same rate at the *pros*-N ($k = 2.1 \times 10^{-5}$ M^{-1} s^{-1}) but 3 times faster at the *tele*-N ($k = 11 \times 10^{-5}$ M^{-1} s^{-1}). The enthalpies of activation determined for carboxymethylation of the imidazole ring and its monocarboxymethyl derivatives were similar (15.9 ± 0.7 kcal/mol). $\Delta S^{\ddagger}$ for the four carboxymethylations was −25 ± 2 eu. The electrostatic component of $\Delta S^{\ddagger}$ ($\Delta S^{\ddagger}_{es}$) was calculated from the influence of the dielectric constant on the reaction rate at 25 °C. $\Delta S^{\ddagger}_{es}$ was slightly negative (−4 ± 1 eu) for mono- or dicarboxymethylations, indicating some charge separation in the transition state. The nonelectrostatic entropy of activation was −21 ± 2 eu for all four carboxymethylations.

Carboxymethylation with haloacetates is commonly used to study protein structure and function (Gurd, 1967; Stark, 1970; Cohen, 1970). Histidine residues in several enzymes, such as human carbonic anhydrase (Bradbury, 1969), swine heart fumarase (Bradshaw et al., 1969), and bovine pancreatic ribonuclease (Gundlach et al., 1959), are unusually reactive toward alkylation, compared with denatured protein or lower molecular weight imidazole compounds. Carboxymethylation of free histidine (Heinrikson et al., 1965), N^{α}-acetylhistidine (Crestfield et al., 1963b), poly-L-histidine (Goren & Barnard, 1971), and the copper(II) complex of histidine (Wieghardt & Goren, 1975) have all been used as models for the spontaneous, or unfacilitated, reaction of haloacetates with histidine residues. However, the α-ammonium and carboxylate groups of free histidine can interact with the negatively charged haloacetate; N-acetylation can eliminate one ionic interaction, but the bulky substituent might interfere sterically with the reaction (Crestfield et al., 1963b). The copper(II)–histidine chelate may have several structures existing simultaneously

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