Evidence of a Dissociative $S_N1(P)$ Mechanism of Phosphoryl Transfer by Rabbit Muscle Pyruvate Kinase

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Adenosine 5′-[αβ-^{18}O,β-$^{18}O_2$]triphosphate has been prepared and used to investigate the mechanism of action of rabbit muscle pyruvate kinase. In the presence of pyruvate kinase and pyruvate, randomisation of the β-$^{18}O_2$ labels with the βγ-O bridge occurs as expected. However randomisation also occurs in the presence of the potent inhibitor oxalate, an analogue of enol pyruvate, and also in the absence of pyruvate or a pyruvate analogue. Since there is evidence against the involvement of a phosphoenzyme intermediate in the reaction pathway of pyruvate kinase, the results indicate that phosphoryl transfer in pyruvate kinase occurs by a dissociative $S_N1(P)$ mechanism, involving the transient high energy but tightly enzyme bound metaphosphate ion intermediate. The rate constants for the pyruvate kinase catalysed ^{18}O-redistribution in adenosine 5′-[αβ-^{18}O,β-$^{18}O_2$]triphosphate alone and in the presence of oxalate and pyruvate are 1.1×10^2 min^{-1}, 2.0×10^2 min^{-1}, and 2.0×10^3 min^{-1} respectively.

PHOSPHORYL residues are transferred between ATP (adenosine 5′-triphosphate) and cellular constituents by a group of enzymes known as kinases. Several of these enzymes have now been studied by *X*-ray crystallography to high resolution, so that three-dimensional structural information is available for hexokinase,[1] phosphoglycerate kinase,[2] pyruvate kinase,[3] and adenylate kinase.[4] Kinetic and mechanistic studies however have been confined to establishing the nature of substrate binding (*i.e.* sequential or ping pong; random or ordered) and whether a phosphoenzyme intermediate is involved in the phosphoryl transfer process.[5]

The mechanism of phosphoryl transfer by a kinase is likely to fall into one of four types. The simplest of these is the associative ' in line ' transfer of the group, the $S_N2(P)$ mechanism (Figure 1a). The dissociative ' in line ' phosphoryl transfer, the $S_N1(P)$ mechanism, involves the formation of the metaphosphate ion (PO_3^-) as an intermediate (Figure 1b). The ' in line ' course of this reaction would be assured by the necessity for the enzyme to bind tightly such a highly reactive intermediate. The third mechanism involves ' adjacent ' attack on the phosphoryl residue which must be accompanied by pseudorotation of the pentacovalent intermediate for phosphoryl transfer to occur (Figure 1c), and the fourth mechanism involves the formation of a phosphoenzyme intermediate (which may occur by either an associative or dissociative mechanism, but which are not distinguished here; Figure 1d).

Although it is always possible to postulate further intermediates in a reaction pathway, it is difficult to conceive that further migration of the phosphoryl residue within the confines of the enzyme active site would be advantageous or possible. It seems reasonable to accept therefore that only these mechanisms are likely.

In principle two experiments will distinguish between these four mechanisms. The first two mechanisms (Figures 1a and b) would lead to inversion of configuration of the phosphoryl residue, whereas the latter two mechanisms (Figures 1c and d) would lead to retention of configuration. If the phosphoryl group was chirally labelled with oxygen isotopes so that its configuration in the substrate and product could be established, information would be provided which would eliminate two of the four mechanisms.

The second experiment necessary to identify the mechanism used by a particular enzyme (*i.e.* to distinguish mechanisms a and c from b and d, in Figure 1), requires a distinction to be made as to whether in the absence of the second substrate or in the presence of a suitable substrate analogue (*i.e.* inhibitor), the P_γ–OP_β bond in ATP is broken. If it is then provided the terminal phosphoryl residue of the ADP (adenosine 5′-diphosphate) so generated is not torsionally restricted by the enzyme, rotation about the P_β–OP_α bond would lead to randomisation of ^{18}O between the P_γ–O–P_β bridge and P_β–O_2, on collapse of the intermediate back to ATP. If no intermediate was formed then no randomisation of label would occur.

The exclusive ^{18}O labelling of either the P_γ–O–P_β bridge or the β-phosphoryl oxygen atoms of ATP is a formidable and unnecessary requirement. By treating ADP activated at its β-phosphate residue with [$^{18}O_4$]-P_i, ATP should be obtained with ^{18}O in the P_γ–O–P_β bridge and in the P_γ phosphoryl residue. Alternatively reaction of an activated AMP (adenosine 5′-phosphate) derivative with [$^{18}O_4$]-P_i followed by phosphorylation of the resulting [$^{18}O_4$]-ADP should give ATP labelled in the β-phosphoryl residue and the P_β–O–P_α bridge, but not in the P_γ–O–P_β bridge.

Recently a mechanistic study of glutamine synthetase using [$^{18}O_4$]-ATP prepared by the former approach was reported.[6] In spite of the attractiveness of this route,

[1] T. A. Steitz, R. J. Fletterick, W. F. Anderson, and C. M. Anderson, *J. Mol. Biol.*, 1976, **104**, 197.
[2] C. C. F. Blake and P. R. Evans, *J. Mol. Biol.*, 1974, **84**, 585; T. N. Bryant, H. C. Watson, and P. C. Wendall, *Nature*, 1974, **247**, 14.
[3] D. K. Stammers and H. Muirhead, *J. Mol. Biol.*, 1977, **112**, 309.
[4] G. E. Schulz, M. Elzinga, F. Marx, and R. H. Schirmer, *Nature*, 1974, **250**, 120.
[5] ' The Enzymes,' ed. by P. D. Boyer, H. Lardy, and K. Myrbäck, Academic Press, New York, 2nd edn., 1962, vol. 6; 3rd edn., 1973, vol. 8.
[6] C. F. Midelfort and I. A. Rose, *J. Biol. Chem.*, 1976, **251**, 5881.

the latter route was adopted because of our preferred analytical method.

The distribution of ^{18}O in a labelled compound has conventionally been determined by mass-spectrometric analysis. Although direct analysis is often possible with many compounds, this is not so with ATP even by

between the resonant nucleus and the isotopic substitution.[7] Although the effect of ^{18}O substitution on ^{31}P n.m.r. spectra does not appear to have been reported, it seemed likely that ^{18}O directly bonded to phosphorus would give rise to a significant change in the ^{31}P chemical shift.* A preliminary experiment showed this to be so,

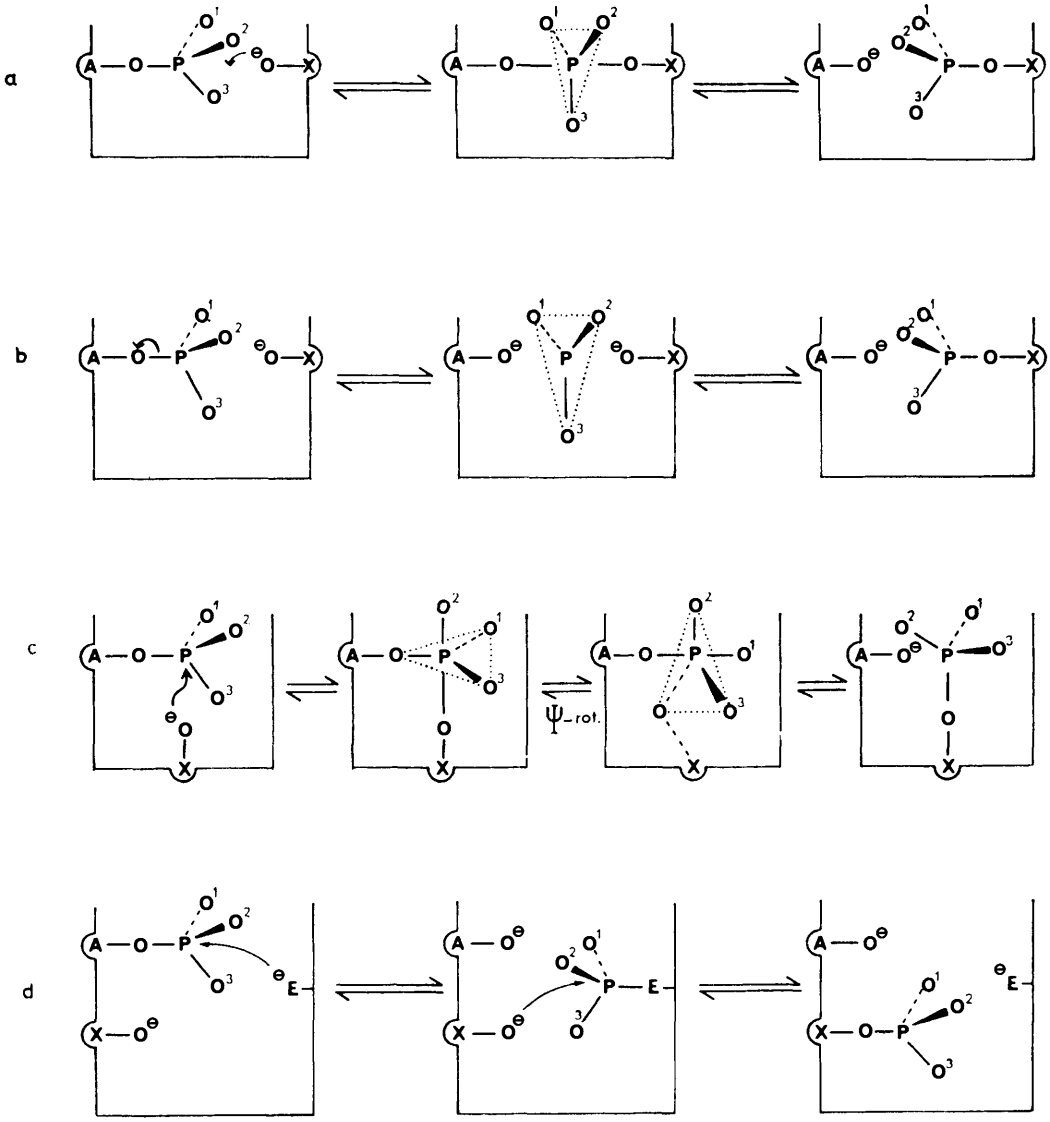


FIGURE 1 Possible mechanisms of enzyme-catalysed phosphoryl transfer: (a) the associative ' in line ' mechanism – $S_N2(P)$, (b) the dissociative ' in line ' mechanism – $S_N1(P)$, (c) the adjacent mechanism with pseudorotation, and (d) the double displacement mechanism *via* a phosphoenzyme intermediate

chemical ionisation or field desorption mass-spectrometry (unpublished results). It is, of course, known that isotopic substitution gives rise to a change in chemical shift (almost invariably to higher field) of a resonance in the n.m.r. spectrum of the compound containing the heavier isotope; the effect is essentially additive and attenuates rapidly as the number of bonds increase

a sample of inorganic phosphate obtained by hydrolysing phosphorus pentachloride with [^{18}O]water (50 atom % ^{18}O) showed the isotope shift to be additive and 0.74 ± 0.05 Hz to higher field (at 36.43 MHz and pH 9) for each atom of ^{18}O incorporated.[8] The magnitude of this isotope shift made it likely that ^{18}O would not give rise to an observable isotope shift of a ^{31}P nucleus to which it was

* *Note added in proof:* ^{18}O Isotope shifts on ^{31}P resonances have been reported recently, M. Cohn and A. Hu, *Proc. Nat. Acad. Sci. U.S.A.*, 1978, **75**, 200.

[7] H. Batiz-Hernandez and R. A. Bernheim, *Progr. in NMR Spectroscopy*, 1967, **3**, 63.

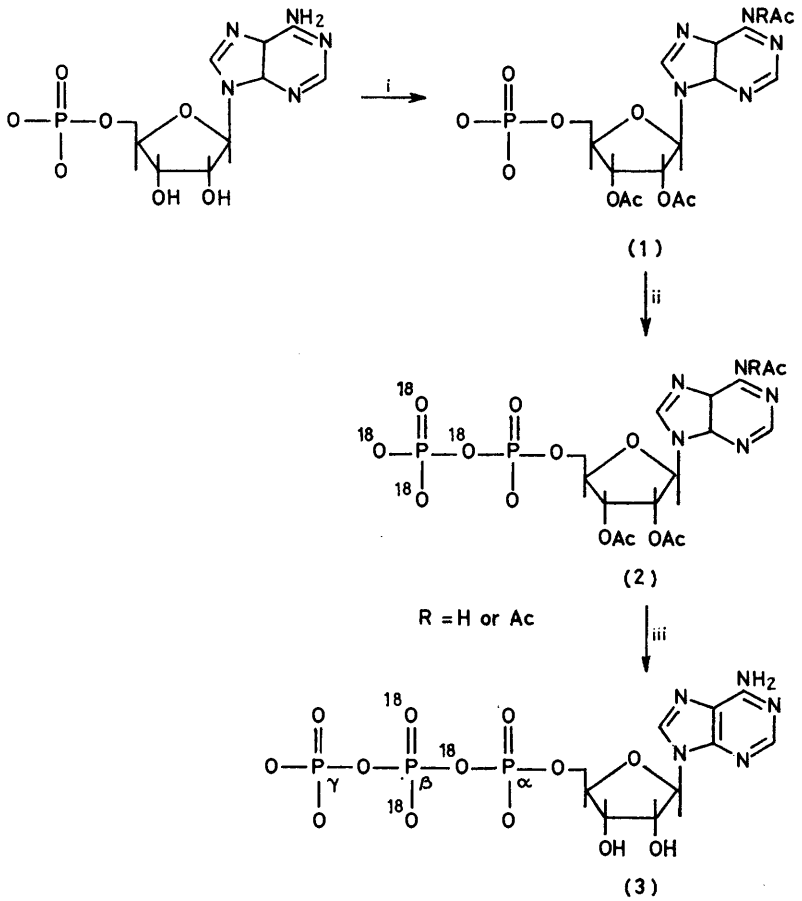
[8] G. Lowe and B. S. Sproat, *J.C.S. Chem. Comm.*, 1978, 565.

not directly bonded. ^{31}P N.m.r. spectroscopy offered, therefore, a simple and direct method of determining the distribution of ^{18}O in ATP.

If it is assumed that ^{18}O will give rise to the same isotope shift on a ^{31}P nucleus to which it is directly bonded irrespective of whether it is in a bridging or phosphoryl position, no change in the chemical shift of the $^{31}P_\beta$ resonance can be expected on randomisation of ^{18}O between P_γ–O–P_β and P_β–O_2. The $^{31}P_\gamma$ resonance, however, would allow such randomisation to be detected.

ation led to the adoption of the second synthetic approach which is outlined in Scheme.

The acetylation of AMP under conditions said to give $N^6,O^{2'},O^{3'}$-triacetyl-AMP,[9] was shown by ^1H n.m.r. spectroscopy to be a mixture of approximately equal amounts of $N^6,O^{2'},O^{3'}$-triacetyl-AMP (1; R = H) and $N^6,N^6,O^{2'},O^{3'}$-tetra-acetyl-AMP (1; R = Ac). They could not be separated by t.l.c. The mixture was used, therefore, to prepare the [$^{18}O_4$]-ADP derivatives (2; R = H and R = Ac) by the Michelson procedure.[10] It



SCHEME Reagents: i, Ac_2O, C_5H_5N; ii, $(PhO)_2PO{\cdot}Cl$, NBu^n_3; [$^{18}O_4$]$P_i{\cdot}H\overset{+}{N}Bu^n_3$, C_5H_5N; iii, $Bu^n_2PS{\cdot}Br$, C_5H_5N; $P_i{\cdot}H\overset{+}{N}Bu^n_3$, $AgNO_3$, C_5H_5N; NH_3

Since complete labelling is not possible because the [^{18}O]water used is less than 100 atom % ^{18}O and because of possible isotopic exchange during the synthesis of the [^{18}O]-ATP, the former synthetic route would give rise to [^{18}O]-ATP in which the $^{31}P_\gamma$ resonance was initially a composite of resonance arising from molecules with different isotopic content. The latter synthetic route, however, would give an unperturbed doublet for $^{31}P_\gamma$. If randomisation occurred a second doublet would be expected 0.7—0.8 Hz to higher field. This considerwas necessary to use a chemical method to convert the [$^{18}O_4$]-ADP derivatives into [$^{18}O_3$]-ATP since the enzymic conversion of [$^{18}O_4$]-ADP would have given [$^{18}O_4$]-ATP, *i.e.* with ^{18}O in the P_γ–O–P_β bridge. Although several chemical methods have been reported for this conversion, each have limitations. In our hands, activation of the [$^{18}O_4$]-ADP derivatives with di-n-butylphosphinothioyl bromide, a reagent recently introduced into nucleotide synthesis methodology,[11] but not hitherto used for this conversion, proved to be satisfactory. The [$^{18}O_3$]-ATP derivatives so prepared were

[9] S. N. Mikhailov, A. M. Kritsyn, L. I. Kolubushkina, and V. L. Florent'ev, *Izvest. Akad. Nauk S.S.S.R., Ser. Khim.*, 1974, 2588; *Bull. Acad. Sci., U.S.S.R. Chem. Sci.*, 1974, **23**, 2494; D. H. Rammler and H. G. Khorana, *J. Amer. Chem. Soc.*, 1962, **84**, 3112.

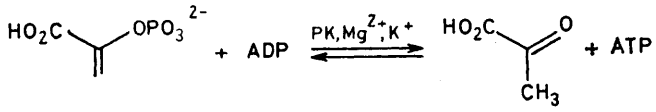
[10] A. M. Michelson, *Biochim. Biophys. Acta*, 1964, **91**, 1.

[11] K. Furusawa, M. Sekine, and T. Hata, *J.C.S. Perkin I*, 1976, 1711.

deprotected with methanolic ammonia to give [αβ-^{18}O,β-$^{18}O_2$]-ATP (3), which has been used to investigate the mechanism of action of rabbit muscle pyruvate kinase.

RESULTS AND DISCUSSION

Pyruvate kinase (PK) catalyses phosphoryl transfer between phosphoenol pyruvate (PEP) and ADP to give pyruvate and ATP and has a requirement for both divalent and monovalent cations.[12] The equilibrium is in favour of pyruvate and ATP (K_{eq} 6.45 × 10^3 at pH 7.4).[13]



The exchange of the methyl protons of pyruvate with solvent is catalysed by pyruvate kinase and accelerated by nucleotide analogues and activators, demonstrating that the enzyme also catalyses the keto–enol tautomerisation of pyruvate.[14] Several pieces of evidence indicate that a phosphoenzyme intermediate is not involved in the pyruvate kinase reaction pathway. Thus exchange of ^{14}C into phosphoenolpyruvate from [^{14}C]pyruvate takes place only in the presence of *all* the reactants; in the absence of ADP, no ^{14}C is transferred.[15] It is of course conceivable that binding of ADP is associated with a conformational change of the enzyme necessary for phosphorylation to occur and a recent kinetic investigation has shown that although substrate binding is random, the presence of one enzyme bound substrate influences the binding constant of its co-substrate [16] (contrary to earlier conclusions [17]), which could be due to a substrate induced conformational change of the enzyme. Pyruvate kinase however is not labelled with ^{32}P by [$\gamma^{32}P$]-ATP, nor is $^{32}P_i$ formed, but perhaps the most convincing evidence against the involvement of a phosphoenzyme intermediate is that phosphoenol pyruvate in the presence of a high concentration of pyruvate kinase, NADH and lactate dehydrogenase gave no u.v. change indicative of NAD formation.[18] Thus, even if the equilibrium to the putative phosphoenzyme intermediate was unfavourable, the removal of pyruvate (which binds relatively weakly to pyruvate kinase) by lactate dehydrogenase would pull the reaction towards the phosphoenzyme intermediate. Thus the involvement of a phosphoenzyme intermediate (*i.e.* mechanism d in Figure 1) is highly improbable.

Three possible mechanisms remain. Direct phosphoryl transfer by an associative mechanism, $S_N2(P)$, phosphoryl transfer by a dissociative mechanism, $S_N1(P)$ and an adjacent mechanism involving pseudorotation. The first and last of these three mechanisms (Figure 1a and c) would not lead to any redistribution of ^{18}O in adenosine 5′-[αβ-^{18}O,β-$^{18}O_2$]triphosphate, in the absence of the second substrate, whereas the dissociative mechanism (Figure 1b) should lead to incorporation of ^{18}O into the P_γ–O–P_β bridge provided that torsional symmetrisation of the β-phosphoryl residue of the enzyme bound ADP is possible.

The ^{31}P n.m.r. spectrum of ATP with proton noise decoupling is shown in Figure 2a, and the spectrum of a mixture of ATP and adenosine 5′-[αβ-^{18}O,β-$^{18}O_2$]triphosphate (3) is shown in Figure 2b.* From these spectra it is seen that the chemical shift of P_γ is unaffected by the ^{18}O in adenosine 5-[αβ-^{18}O,β-$^{18}O_2$]-triphosphate,† but P_α exhibits an upfield isotope shift of 0.9 ± 0.2 Hz and P_β experiences upfield isotope shifts of a similar magnitude for each ^{18}O directly bonded to it. The more complex spectrum for P_β in the [^{18}O]-ATP arises from the fact that the [^{18}O]-P_i used in its synthesis was not fully labelled. Resynthesis of adenosine 5′-[αβ-^{18}O,β-$^{18}O_2$]triphosphate using virtually completely labelled [^{18}O]-P_i showed P_β (as a double doublet) 2.5 Hz upfield from P_β in ATP.

Adenosine 5′-[αβ-^{18}O,β-$^{18}O_2$]triphosphate (3) was incubated with pyruvate kinase in the presence of pyruvate and sufficient Ap_5A to inhibit possible traces of adenylate kinase.[19] The ^{31}P n.m.r. spectrum of the [$^{18}O_3$]-ATP isolated from the experiment shows two sets of doublets separated by 0.8 Hz in the region of P_γ (Figure 3a).‡ Since the incubation period (80 min) allowed complete randomisation to occur, the departure of the ratio of the

* Although 99.5 atom % ^{18}O water was used for the preparation of the [^{18}O]phosphate used in the synthesis of adenosine 5′-[αβ-^{18}O,β-$^{18}O_2$]triphosphate, insufficient excess was used in the first preparation, so that in effect the % ^{18}O actually incorporated was significantly lower. It is for this reason that P_α of the unlabelled ATP has gained intensity from unlabelled ATP present in the labelled sample but also from the P_α of adenosine 5′-[β-^{18}O]-triphosphate and adenosine 5′-[β-$^{18}O_2$]triphosphate. For the same reason P_β will be a composite of resonances arising from [$^{18}O_3$]-, [$^{18}O_2$]-, [^{18}O]-ATP as well as ATP itself.

† Actually P_γ in adenosine 5′-[αβ-^{18}O,β-$^{18}O_2$]triphosphate is observably affected relative to P_γ of unlabelled ATP when 16 K data points are used in the Fourier transform. An isotopic shift of *ca.* 0.2 Hz can be detected which no doubt arises from the cumulative effect of the three ^{18}O atoms, from which it can be estimated that the isotopic shift of each ^{18}O is *ca.* 0.07 Hz. When 8 K data points and a large number of transients are used in the Fourier transform, the resolution is insufficient to detect this effect.

‡ Incomplete labelling explains why complete randomisation of the P_β–O_2 labels with P_γ–OP_β does not lead to a 2 : 1 ratio in favour of the higher field signal of P_γ. However the incomplete labelling of adenosine 5′-[αβ-^{18}O,β-$^{18}O_2$]triphosphate in no way effects the interpretation of the ^{18}O-randomisation experiments as this only depends on observation of the P_γ resonance. It has the very real advantage that because both P_γ resonances have similar intensity, peak separation is more readily discerned.

[12] F. J. Kayne in ' The Enzymes,' ed. by P. D. Boyer, Academic Press, New York, 3rd edn., 1973, vol. 8, p. 353.
[13] J. T. McQuate and M. F. Utter, *J. Biol. Chem.*, 1959, **234**, 2151.
[14] I. A. Rose, *J. Biol. Chem.*, 1960, **235**, 1170.
[15] W. H. Harrison, P. D. Boyer, and A. B. Falconer, *J. Biol. Chem.*, 1955, **215**, 303.
[16] L. G. Dann and H. G. Britton, *Biochem. J.*, 1978, **169**, 39.
[17] A. M. Reynard, L. F. Hass, D. D. Jacobsen, and P. D. Boyer, *J. Biol. Chem.*, 1961, **236**, 2277; S. Ainsworth and N. MacFarlane, *Biochem. J.*, 1973, **131**, 223.
[18] L. F. Hass, P. D. Boyer, and A. M. Reynard, *J. Biol. Chem.*, 1961, **236**, 2284.
[19] G. E. Lienhard and I. I. Secemski, *J. Biol. Chem.*, 1973, **248**, 1121.

two P_γ doublets from 1 : 2 is due to the incomplete labelling of P_β–O_2 in the [$^{18}O_3$]-ATP.*

The second experiment was conducted in the same way as the first except that pyruvate was omitted and the potent inhibitor, oxalate,[20] was added. The ^{31}P n.m.r. spectrum of the [$^{18}O_3$]-ATP isolated after incubation as before, is shown in Figure 3b. Again two closely spaced doublets appear for the P_γ resonance separated by 0.8 Hz, indicating that again ^{18}O originally present in P_β–O_2 has been randomly distributed between P_β–O_2 and P_γ–O–P_β.

The third experiment was conducted in exactly the same way as the first except that pyruvate was omitted. The ^{31}P n.m.r. spectrum of the [$^{18}O_3$]-ATP isolated together with lactate dehydrogenase and NADH. The phosphoenol pyruvate should prevent the [$^{18}O_3$]-ATP from binding to pyruvate kinase, but not to another enzyme which may be present as a trace impurity. The lactate dehydrogenase and NADH were added in order to rapidly remove any pyruvate that might arise adventitiously. Under these conditions no redistribution of ^{18}O occurred in the [$^{18}O_3$]-ATP as can be seen from the ^{31}P n.m.r. spectrum of the isolated sample (Figure 3d).

In order to rule out the possibility that randomisation of the ^{18}O was due to a trace of unknown but highly efficient enzyme impurity, the following control experiment was performed. The incubation mixture contained all the ingredients of the third experiment, but in addition a saturating concentration of phosphoenol pyruvate, (Figure 3c) shows that again ^{18}O label initially present in P_β–O_2 has been randomly distributed between P_β–O_2 and P_γ–O–P_β.

These experiments demonstrate that pyruvate kinase catalyses P_γ–OP_β bond cleavage of ATP in the presence of pyruvate as expected, in the presence of oxalate,

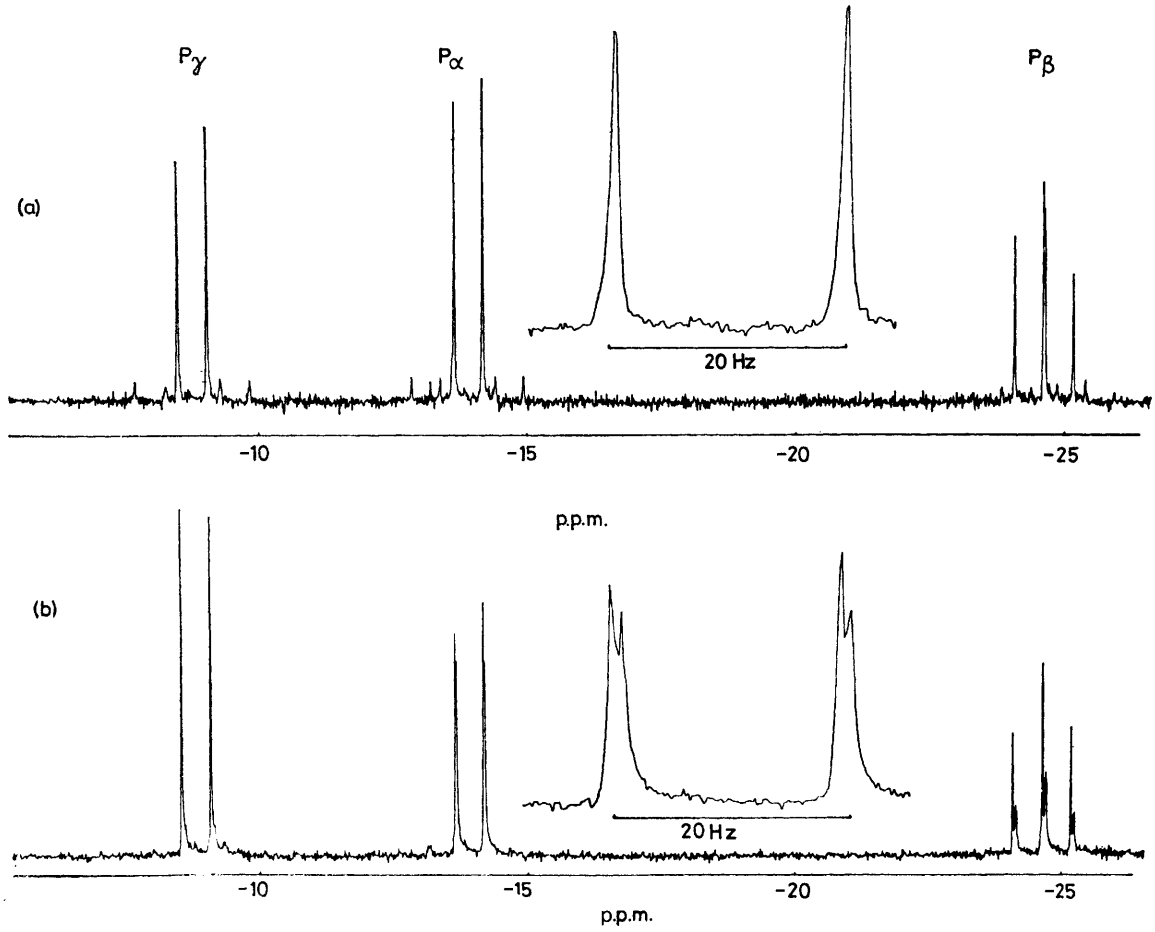


FIGURE 2 The ^{31}P n.m.r. spectra (pulse repetition rate, 5.12 s) of (a) ATP and (b) ATP and [$\alpha\beta$-^{18}O,β-$^{18}O_2$]-ATP in 100 mM-diethanolamine hydrochloride (pH 9) containing 5 mM-EDTA; see footnote * on page 1625. The inset is that of the expanded P_α resonance in each case. The isotope shift on P_α is 0.9 ± 0.2 Hz

* The enzyme and adenosine 5′-[$\alpha\beta$-^{18}O,β-$^{18}O_2$]triphosphate concentrations were such that the labelled ATP would be converted into [$^{18}O_3$]-ADP (free) and back to [$^{18}O_3$]-ATP with a half life of *ca.* 4 min. During the reisolation of [$^{18}O_3$]-ATP the expected equilibrium amount of ADP was obtained. Since the ADP in free solution can clearly achieve torsional symmetrisation of the β-phosphoryl group, the spectrum in Figure 3a represents the result of complete ^{18}O randomisation between $P\beta$–O_2 and P_γ–O–$P\beta$.

G. H. Reed and S. D. Morgan, *Biochemistry*, 1974, **13**, 3537.

a good substrate analogue of the enolate form of pyruvate, and in the absence of the second substrate or an analogue of it. It is, of course, conceivable that oxalate could become transiently phosphorylated by ATP in the presence of pyruvate kinase, with the equilibrium favouring overwhelmingly oxalate and ATP, but the fact that the P_γ–OP_β bond of ATP is cleaved in the absence of the second substrate or an analogue of it, indicates that the dissociative mechanism (Figure 1b) must operate.

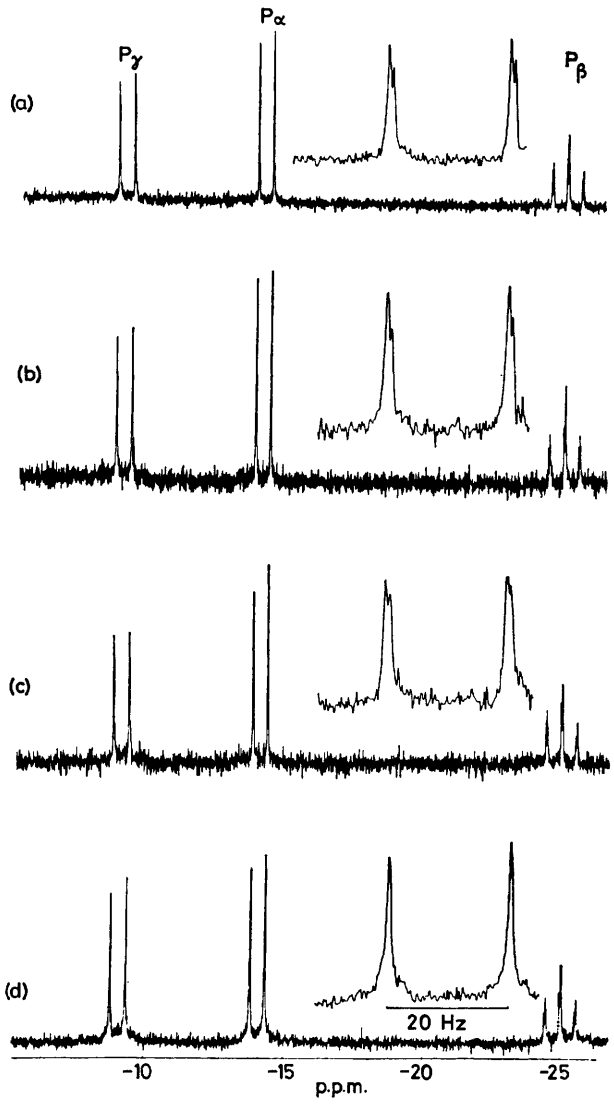


FIGURE 3 The ^{31}P n.m.r. spectra of [$^{18}O_3$]-ATP in diethanolamine hydrochloride buffer (pH 9) containing EDTA after incubation with pyruvate kinase in triethanolamine hydrochloride buffer containing KCl and $Mg(OAc)_2$ and (a) pyruvate, (b) oxalate, (c) nothing further, (d) phosphoenol pyruvate NADH and lactate dehydrogenase, for 80 min; see footnotes ‡ on p. 1625, * on p. 1626. See the Experimental section and Table for details of the conditions of incubation and n.m.r. spectral accumulation. The inset is that of the expanded P_γ resonance in each case. The isotope shift on P_γ is *ca.* 0.8 Hz in spectra (a), (b), and (c)

It is, of course, possible that in the presence of pyruvate, nucleophilic attack (by the enolate ion) is concerted with breaking of the P_γ–OP_β bond of ATP, but in its absence an $S_N1(P)$ mechanism operates. In order to investigate this possibility, experiments were performed such that only partial ^{18}O-redistribution occurred. The results are shown in the Table.* The concentration of [$^{18}O_3$]-ATP used in these experiments is such that the enzyme is virtually saturated, but the first-order rate constants (k) were corrected where appropriate using the known K_m values.[16] From the results it is evident that pyruvate kinase catalysed ^{18}O-redistribution occurs about 18-fold faster in the presence of pyruvate and about twice as fast in the presence of oxalate, than when [$^{18}O_3$]-ATP is present alone.

The rate of ^{18}O-redistribution in [$^{18}O_3$]-ATP catalysed by pyruvate kinase in the absence of the second substrate or inhibitor could be determined by one or a combination of three possible processes, *viz.* (1) an enzyme conformational change prior to the P_γ–OP_β cleavage of ATP, (2) the P_γ–OP_β cleavage of ATP itself, or (3) the torsional symmetrisation of P_β of the enzyme bound ADP. The release of ATP from the enzyme is known to be not rate limiting.[16] Rate-limiting torsional symmetrisation of P_β of enzyme-bound ADP is rendered unlikely however, since in the presence of pyruvate the rate constant for ^{18}O-redistribution is 18-fold faster than when [$^{18}O_3$]-ATP is present alone, yet ATP is bound more tightly; ADP is also bound more tightly in the presence of PEP.[16]

Enzyme conformational changes associated with binding of substrates have been extensively studied, but rate-limiting conformational changes associated with the interconversion of the ternary complexes or subsequent to the binding to ATP have not been detected.[21] Thus the rate constant for ^{18}O-redistribution in [$^{18}O_3$]-ATP can be associated tentatively with the pyruvate kinase catalysed P_γ–OP_β cleavage of ATP.

The increase in the rate constants for ^{18}O-redistribution in [$^{18}O_3$]-ATP catalysed by pyruvate kinase in the presence of pyruvate and oxalate are probably caused by conformational changes known to occur on bonding these molecules and not by a change to an associative mechanism. This conclusion is supported by the fact that the fluorokinase activity of pyruvate kinase, *i.e.* the formation of fluorophosphate and ADP from fluoride ion and ATP in the presence of hydrogen carbonate ion, is 7—13 times slower than pyruvate kinase activity.[22]

* For experiments (v)—(x) inclusive a second batch of adenosine 5′-[αβ-^{18}O,β-$^{18}O_2$]triphosphate was used which had 99 atom % ^{18}O in the sites specified. This had the advantage that after two half-lives for ^{18}O redistribution the peak intensity of the two P_γ signals were of equal intensity and clearly resolved.

[21] F. J. Kayne and C. H. Suelter, *J. Amer. Chem. Soc.*, 1965, **87**, 897; C. H. Suelter, R. Singleton, jun., F. J. Kayne, S. Arrington, J. Glass, and A. S. Mildvan, *Biochem.*, 1966, **5**, 131; F. J. Kayne and C. H. Suelter, *ibid.*, 1968, **7**, 1678; J. Reuben and F. J. Kayne, *J. Biol. Chem.*, 1971, **246**, 6227; N. C. Price, *FEBS Letters*, 1972, **24**, 21; G. H. Reed and M. Cohn, *J. Biol. Chem.*, 1973, **248**, 6436; C.-Y. Kwan, K. Erhard, and R. C. Davis, *ibid.*, 1975, **250**, 5951.

[22] A. Tietz and S. Ochoa, *Arch. Biochem. Biophys.*, 1958, **78**, 477.

Thus the fluorokinase activity is similar to or identical with the rate of ^{18}O-redistribution in [$^{18}O_3$]-ATP by pyruvate kinase in the presence of oxalate. This suggests that the two processes are controlled by a common step which is most probably the enzyme-catalysed dissociation of ATP into enzyme-bound ADP and metaphosphate ion. Moreover, the rate constant for the pyruvate kinase catalysed ^{18}O-redistribution in [$^{18}O_3$]-ATP in the presence of pyruvate (2.0×10^3 min^{-1}) is slower than the conversion of the pyruvate kinase-PEP-ADP complex into the pyruvate kinase-pyruvate-ATP complex (1.1×10^4 min^{-1} under similar conditions) [16] and must represent a step in the reverse process, since release of pyruvate and ATP from the enzyme is not rate limiting. The lower limit for this reverse process has been set at 8.4×10^2 min^{-1},[16] thus the rate constant of 2.0×10^3 min^{-1} controlling the ^{18}O-redistribution in [$^{18}O_3$]-ATP in the presence of pyruvate almost certainly represents the rate-limiting step in the overall interconversion of the ternary complexes.

Evidence that the hydrolysis of phosphate monoesters [23] and anhydrides [24] occur by way of the reactive monomeric metaphosphate ion (PO_3^-) has been reported. Such a mechanism has also been recognised as a possibility for enzyme-catalysed phosphoryl transfer reactions,[25] the enzyme providing the required microenvironment to ensure that the metaphosphate ion does not react with water. The presence of an enzyme bound divalent metal ion (Mg^{2+} or Mn^{2+}) in pyruvate kinase, in close proximity to the terminal phosphoryl group of enzyme bound MgATP, led however to the associative mechanism being favoured, since co-ordination of the γ-phosphoryl group with metal ions or protons are expected to retard the dissociative $S_N1(P)$ mechanism.[26]

It remains to be seen how general the $S_N1(P)$ mechanism is amongst kinases, but the fact that phosphate monoesters and anhydrides appear to adopt this mechanism suggests that it could be the favoured enzymic mechanism also. Recent *ab initio* SCF molecular orbital calculations on the metaphosphate ion (PO_3^-) have shown that it has comparable thermodynamic stability to that of the nitrate ion (NO_3^-) and that its high reactivity arises from the near degeneracy of the unoccupied π^* and σ^* molecular orbitals.[27] This allows efficient mixing of these orbitals under the influence of the symmetry-breaking perturbation of the incoming nucleophile. This mixing of the π^* and σ^* orbitals allows for a much wider angle of approach of the incoming nucleophile, which may be a further advantage of the $S_N1(P)$ mechanism in enzyme-catalysed phosphoryl-transfer reactions.

^{18}O-Redistribution in adenosine 5′-[$\alpha\beta$-^{18}O,β-$^{18}O_2$]triphosphate (6mM) catalysed by pyruvate kinase in the presence and absence of pyruvate, oxalate, and phosphoenol pyruvate. All solutions contained triethanolamine hydrochloride (100mM; pH 7.6), potassium chloride (100mM), magnesium acetate (15mM), EDTA (1mM), Ap_5A 400μM), and BSA (5 mg) and were incubated at 37 °C. k is the first-order rate constant for ^{18}O-redistribution under conditions of enzyme saturation (*i.e.* maximum velocity)

Second substrate or inhibitor	Expt. no	Enzyme conc. (μM)	Substrate or inhibitor conc. (mM)	Incubation period (min)	^{18}O Redistribution (%)	k / min^{-1}
Pyruvate	(i)	5.1	50	80	100	
	(vi)	0.17	50	27	75	2.0×10^3
	(v)	0.017	50	21	11	
Oxalate	(ii)	5.1	5	80	100	
	(viii)	0.17	5	27	11	2.0×10^2
	(iii)	5.1		80	100	
	(ix)	0.89		200	88	
	(x)	0.21		130	29	1.1×10^2
	(vii)	0.17		27	6	
PEP	(iv)	5.1	50	80	0	

EXPERIMENTAL

AMP, disodium ATP, and trilithium P^1,P^5-di(adenosine 5′-)pentaphosphate (Ap_5A) were purchased from Boehringer Corporation (London). Lyophilised salt-free rabbit muscle pyruvate kinase, specific activity 405 units mg^{-1} at pH 7.6 and 37 °C, rabbit muscle lactate dehydrogenase, specific activity approximately 970 units mg^{-1} at pH 7.5 and 37 °C, bovine serum albumin (BSA) disodium NADH, tricyclohexylammonium phosphoenolpyruvate, and Dowex-50W 50 × 4-200R resin were purchased from Sigma London. Water (99.5 atom % ^{18}O) was purchased from Prochem B.O.C. Ltd. and water (99.8 atom % ^2H) was purchased from Ryvan Chemical Company. DEAE-Sephadex A-25 was purchased from Pharmacia. Pyridine, methanol, tri-n-octylamine, tri-n-butylamine, triethylamine, dioxan, dimethylformamide, diphenyl phosphorochloridate, and dimethoxyethane were purified before use by conventional methods. All other chemicals were of AnalaR grade or the highest purity available commercially. Di-n-butylphosphinothioyl bromide was prepared from tetra-n-butyldiphosphine disulphide [28] using the procedure of Harwood and Pollart.[29]

T.l.c. of nucleotides was performed on PEI-cellulose sheets

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(Polygram CEL 300 polyethylenimine, u.v.; Macherey-Nagel and Co.) in 0.3M-trisodium citrate, pH 7.0. Optical densities were measured on a Pye–Unicam SP 1800 u.v. spectrophotometer. High-grade deionised water was obtained with a Milli-Q3 water purification system (Millipore Corporation). pH Values were measured on a Radiometer pH-meter.

^{31}P N.m.r. spectra were recorded at 36.43 MHz on a Bruker WH90 spectrometer in the Fourier transform mode, using broad-band proton noise decoupling. Signal averaging was performed on a Bruker-Data system B-NC12 computer interfaced with the spectrometer. The solution temperature was controlled with a Bruker variable-temperature controller. Aqueous solutions contained at least 10% ^2H_2O to provide a field frequency lock, and were placed in 10-mm outside-diameter tubes. Samples in other solvents were placed in 8-mm outside-diameter tubes which were fitted inside 10-mm outside-diameter tubes containing ^2H_2O. Chemical shifts (δ) were measured with reference to external trimethyl phosphate, and signals downfield from the reference were assigned positive chemical shifts. ^1H N.m.r. spectra were determined on a Perkin-Elmer R32 (90 MHz) spectrometer.

Nucleotides were isolated by column chromatography on DEAE-Sephadex A-25, HCO_3^- form, with a linear gradient from 100—600mM aqueous triethylammonium hydrogen carbonate (TEAB) pH 7.6; ATP is eluted at *ca.* 500mM. The pooled fractions containing nucleotide were evaporated to dryness under reduced pressure (*ca.* 0.2 mmHg) at room temperature. Residual TEAB was removed by repeated evaporation after addition of dry methanol (generally 4 × 20 ml). The concentration gradient was applied to the column by an LKB 11300 Ultrograd gradient mixer and LKB Perpex peristaltic pump and the effluent monitored by an LKB Uvicord II continuous-flow detector set at 254 nm.

Mono(tri-n-butylammonium) [$^{18}O_4$]*Phosphate.*— Phosphorus pentachloride (1.25 g, 6 mmol) was suspended in dry dioxan (10 ml) and a solution of $H_2{}^{18}O$ (0.5 g, 25 mmol) in dioxan (10 ml) was added dropwise with stirring during 0.5 h. The solution was stirred for a further 12 h at room temperature. Solvent was removed under reduced pressure and the residue dried by addition and evaporation of dry dimethoxyethane (2 × 15 ml). Pyridine (15 ml), dimethoxyethane (15 ml), and tri-n-butylamine (1.112 g, 6 mmol) were added to the residue and the mixture stirred until dissolution was complete. Solvent was then removed under reduced pressure and the residual syrup was dried by addition and evaporation of dry pyridine (2 × 10 ml).

N^6,$O^{2'}$,$O^{3'}$-*Triacetyladenosine 5'-Phosphate.*— Adenosine 5'-phosphate monohydrate (1.826 g, 5 mmol) was converted into its pyridinium salt and then treated with pyridine (17 ml) and acetic anhydride (25 ml) under anhydrous conditions.[9] Removal of the solvent under reduced pressure and of final traces of pyridine by addition and evaporation of dimethylformamide and toluene (30 ml; 5 : 1 v/v) gave product (virtually quantitative) with δ_P ($CDCl_3$) — 1.82(s). By ^1H n.m.r. spectroscopy the product was seen to be a mixture (in approximately equal amounts) of N^6,$O^{2'}$,$O^{3'}$-triacetyladenosine 5'-phosphate (1; R = H) pyridinium salt, δ_H ($CDCl_3$) 1.94 (s, 3 H, *O*^{2}- or *O*$^{3'}$-Ac), 2.06 (s, 3 H, *O*$^{3'}$- or *O*$^{2'}$-OAc), 2.50 (s, 3 H, *N*-Ac), 4.30 (m, 2 H, H$^{5'}$), 4.43 (m, 1 H, H$^{4'}$), 5.60 (m, 1 H, H$^{3'}$), 5.79 (m, 1 H, H$^{2'}$), 6.36 (d, *J* 7 Hz, 1 H, H$^{1'}$), 7.62 (m, 2 H, pyridinium H$_\beta$), 8.07 (t, 1 H, pyridinium H$_\gamma$), 8.64 (s, 1 H, H^2 or H^8), 8.76 (m, 2 H, pyridinium H$_\alpha$), 8.92 (s, 1 H, H^8 or H^2), 11.14 (bs, NH and pyridinium H$^+$), and N^6,N^6,$O^{2'}$,$O^{3'}$-tetra-acetyladenosine 5'-phosphate (1; R = Ac) pyridinium salt δ_H ($CDCl_3$) 1.92 (s, 3 H, $O^{2'}$- or $O^{3'}$-Ac), 2.05 (s, 3 H, $O^{3'}$- or $O^{2'}$-Ac) 2.30 (s 6 H NAc_2), 4.30 (m, 2 H, H$^{5'}$), 4.43 (m, 1 H, H$^{4'}$), 5.60 (m, 1 H, H$^{3'}$), 5.79 (m, 1 H, H$^{2'}$), 6.44 (d, *J* 7 Hz, 1 H, H$^{1'}$), 7.62 (m, 2 H, pyridinium H$_\beta$), 8.07 (t, 1 H, pyridinium H$_\gamma$), 8.76 (m, 2 H, pyridinium H$_\alpha$), and 9.01 (s, 2 H, H^2 and H^8).

Since the product ran as a single spot on PEI-cellulose t.l.c. sheets, no attempt to separate them preparatively was made. The product was used as though it were the triacetyl-derivative. Tri-n-octylamine (1.768 g, 5 mmol) was added to the pyridine salt of the protected AMP (5 mmol) in dioxan–dimethylformamide (30 ml; 2 : 1 v/v) and the solvent removed under reduced pressure to give the mono-tri-n-octylammonium salt.

N^6,$O^{2'}$,$O^{3'}$-*Triacetyladenosine* 5'-[$\alpha\beta$-^{18}O,β-$^{18}O_3$]*Diphosphate.*—The protected adenosine 5'-phosphate was converted into protected adenosine 5'-diphosphate in a dry glove-box under N_2, by the Michelson procedure.[10] The protected AMP tri-n-octylammonium salt (5 mmol) in dioxan (20 ml) was stirred with diphenyl phosphorochloridate (1.3432 g, 5 mmol) and tri-n-butylamine (0.927 0 g, 5 mmol) for 3 h at room temperature. The solvent was removed under reduced pressure and mono(tri-n-butylammonium) [$^{18}O_4$]phosphate (6 mmol) in pyridine (15 ml) was added with stirring to the residual syrup. Solvent was removed under reduced pressure after 40 min and the residue partitioned between 100 mM TEAB buffer (pH 7.6) and ether (400 ml; 1 : 1 v/v). The aqueous layer was separated and the ether layer extracted with 100mM-TEAB buffer (2 × 50 ml). The combined aqueous layers were chromatographed on a column (42 × 5 cm) of DEAE-Sephadex A-25, HCO_3^- form, with a linear gradient of TEAB buffer (0.1—0.6M over 48 h; total volume 7.5 l). The protected [$^{18}O_4$]-ADP was eluted at a buffer concentratian of approximately 0.38M. Removal of the buffer under reduced pressure (as above) gave the dry tristriethylammonium salt (0.98 g, 1.1 mmol) as a creamy white glass; δ_P (100mM-diethanolamine hydrochloride, 5mM-EDTA, pH 9 in 50% ^2H_2O) (*a*) triacetyl derivative (2; R = H), —13.60 (d, $^2J_{PP}$ = 22 Hz, P$_\alpha$), —8.98 (d, $^2J_{PP}$ = 22 Hz, P$_\beta$); (b) tetra-acetyl derivative (2; R = Ac) —13.72 (d, $^2J_{PP}$ = 22 Hz, P$_\alpha$), and —9.12 (d, $^2J_{PP}$ = 22 Hz, P$_\beta$).

Adenosine 5'-[$\alpha\beta$-^{18}O,β-$^{18}O_2$]*Triphosphate* (3).—The protected [$^{18}O_4$]-ADP was converted into the protected [$^{18}O_3$]-ATP by the procedure introduced by Furasawa *et al.*[11] The tristriethylammonium salt of the protected [$^{18}O_4$]-ADP (0.98 g, 1.1 mmol) was converted into the pyridinium salt with the pyridinium form of Dowex-50W 50 × 4-200R. The thoroughly dried salt was treated in a dry glove-box with di-n-butylphosphinothioyl bromide (0.282 9 g, 1.1 mmol) in dry pyridine (10 ml). The solution was stirred for 2 h and then added with stirring to tri-n-butylammonium phosphate (5.5 mmol). Silver nitrate (0.748 g, 4.4 mmol) in pyridine (5 ml) was added to the solution immediately. After 2 h the reaction was stopped by addition of water (0.5 ml) (prolonged exposure of ATP to pyridine leads to dismutation [30]). Hydrogen sulphide was passed through the solution and the silver sulphide filtered off through a Celite bed. The filtrate was evaporated to dryness under reduced pressure and the last traces of pyridine were

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removed by addition and evaporation of methanol–toluene (30 ml; 2 : 1 v/v). The residue was dissolved in dry methanol (15 ml) and to the solution at 0 °C was added, with stirring, a solution of methanol (15 ml) saturated with ammonia at 0 °C. An immediate precipitate was produced, but the mixture was stirred for 15 h at room temperature in order to ensure complete de-protection. Solvent was removed under reduced pressure and the white residual solid was chromatographed on a column (38 × 5 cm) of DEAE-Sephadex A-25 HCO_3^- form with a linear gradient of TEAB buffer (0.2—0.6M during 48 h; total volume 7.5 l). The [$^{18}O_3$]-ATP (A_{259} *ca.* 1 850, 12%) was eluted when the buffer concentration reached about 0.5M, as its tetratriethylammonium salt. It was converted into its tetrasodium salt by the method of Haley and Yount.[31] The tetrasodium salt (78 mg, 12%) was a free flowing white powder and was stored in a desiccator at −20 °C. It had $\lambda_{max.}$ 259 nm at pH 7.6 and $A_{280}/A_{259} = 0.15$. On PEI cellulose plates it was eluted as a single spot (R_F 0.19) with 300mM-citrate buffer, pH 7.0. ^{31}P N.m.r. spectrum of [$^{18}O_3$]-ATPNa_4 (10.5 mg) admixed with ATPNa_4 (8.1 mg) dissolved in diethanolamine hydrochloride (1.6 ml; 100mM, pH 9, 50% ^2H_2O) containing EDTA (5mM) is shown in Figure 2b and that of ATPNa_4 itself in Figure 2a. δ_P(ATP) −8.79 (d, $^2J_{PP}$ 20.1 Hz, P_γ), −13.89 (d, $^2J_{PP}$ 19.7 Hz, P_α), −24.61 (dd, P_β); δ_P([$^{18}O_3$]-ATP) −8.79 (d, $^2J_{PP}$ 20.1 Hz, P_γ), −13.92 (d, $^2J_{PP}$ 19.7 Hz, P_α), −24.61, −24.66, −24.68 (each dd, P_β). The line width of P_γ is *ca.* 0.7 Hz. The isotope shift observed on P_α is 0.9 ± 0.2 Hz.

The Effect of Pyruvate Kinase on [αβ-^{18}O,β-$^{18}O_2$]-ATP *in the Presence of Activators, Substrates, and Inhibitors.*—The following ten experiments were performed. (i) The incubation solution consisted of aqueous triethanolamine hydrochloride buffer (2.5 ml; 100mM, pH 7.6), potassium chloride (100mM), magnesium acetate (15mM), EDTA (1mM), [αβ-^{18}O,β-$^{18}O_2$]-ATP (6mM), sodium pyruvate (50mM), Ap_5A (400μM), BSA (5 mg), and pyruvate kinase (3 mg).

(ii) The incubation solution was as in (i) except that sodium pyruvate was replaced by oxalic acid (5mM).

(iii) The incubation solution was as in (i) except that no sodium pyruvate was present.

(iv) The incubation solution was as in (iii) but with PEP (50mM), NADH (4mM), and lactate dehydrogenase (0.5 mg) added. The lactate dehydrogenase had been dialysed exhaustively in order to remove sulphate ions.

(v) The incubation solution was as in (i) except that 10 μg of pyruvate kinase was used.

(vi) The incubation solution was as in (i) except that 100 μg of pyruvate kinase was used.

(vii) The incubation solution was as in (vi) except that sodium pyruvate was omitted.

(viii) The incubation solution was as in (vi) except that sodium pyruvate was replaced by oxalic acid (5mM).

(ix) The incubation solution was as in (i) except that 525 μg of pyruvate kinase was used.

(x) The incubation solution was as in (i) except that 125 μg of pyruvate kinase was used.

The solutions were incubated (for times see Table) at 37 °C and then EDTA (18.6 mg, 50 μmol) in aqueous triethanolamine hydrochloride (2.5 ml, 100mM, pH 7.6) was added to complex all the magnesium ions. Chloroform (3 ml) was added and the protein denatured by vigorous agitation (Cyclomixer) for several minutes. The aqueous phase was separated and filtered, the organic phase extracted with aqueous triethanolamine hydrochloride buffer (3 × 5 ml), and the aqueous phases combined. The [$^{18}O_3$]-ATP from each experiment was isolated by chromatography on a column (28 × 1.5 cm) of DEAE-Sephadex A-25 HCO_3^- form with a linear gradient of triethylammonium hydrogencarbonate (0.2—0.6M during 24 h; total volume 700 ml). ATP was eluted when the buffer concentration reached *ca.* 0.5M. The tetratriethylammonium salt was obtained as before.

The ^{31}P n.m.r. spectra of the [$^{18}O_3$]-ATP samples from the first four experiments are shown in Figure 3. The samples were dissolved in diethanolamine hydrochloride buffer (1.6 ml; 100mM, 1 : 1 H_2O–^2H_2O v/v, pH 9) containing EDTA (5 mM). 10 000 Accumulations were obtained with an offset of 1 950 Hz, a bandwidth of 800 Hz, a memory of 8 K, a pulse repetition rate of 5.12 s [except the sample from experiment (iv), Figure 3d, which was 2.56 s], a pulse width of 15 μs (flip angle *ca.* 70°) with broad band proton noise decoupling at 311 K with zero-line broadening.

The first-order rate constants (k in the Table) were derived from the rate of ^{18}O-redistribution given by the equation $-\ln(1 - F) = Vt/[ATP]$ where F is the fraction of ^{18}O-redistribution (to complete randomisation), V is the rate, and t the time, *i.e.* the equation for isotope exchange at equilibrium. k is calculated from $V = k[E]$ or the full Michaelis equation.

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