Chiral [^{16}O,^{17}O,^{18}O]Phosphate Esters

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Received May 19, 1982 (Revised Manuscript Received November 3, 1982)

Stereochemical analysis has long been recognized as a powerful mechanistic tool in chemistry and enzymology. However, despite the widespread occurrence of phosphate esters and anhydrides, and their central importance in all living systems, the methodology for investigating the stereochemical course of chemical and enzyme-catalyzed phosphoryl-transfer reactions has been developed only recently. Characteristically the approach has clarified mechanistic ambiguities, and some important generalizations have emerged.

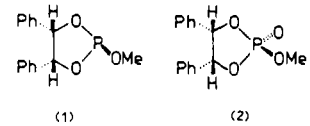
In order to determine the stereochemical course of a chemical or enzyme-catalyzed phosphoryl-transfer reaction, the *pro-pro*-chiral phosphate ester or anhydride must be made chiral, ideally by isotopic substitution. Although chiral phosphorothioates have received considerable attention, enzyme-catalyzed thiophosphoryl transfer invariably occurs more slowly than phosphoryl transfer and in some cases not at all.[1] Moreover, until the advent of stereochemical studies with chiral [^{16}O,^{17}O,^{18}O]phosphate esters, doubts arose as to the relevance of the stereochemical conclusions for the natural substrate from studies undertaken with chiral thiophosphate esters.

Synthesis of Chiral [^{16}O,^{17}O,^{18}O]Phosphate Esters

Oxygen is the lightest element to exist naturally as three stable isotopes, namely, ^{16}O, ^{17}O, and ^{18}O. By the mid 1970s ^{17}O and ^{18}O were available as water and dioxygen at enrichments that made it feasible to synthesize chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters. Currently ^{17}O is available at about 50 atom % ^{17}O and ^{18}O in excess of 99 atom % ^{18}O as water.

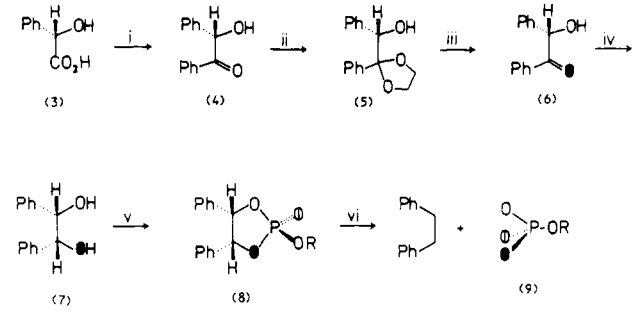
As so often happens in science, two groups became interested independently in the synthesis and analysis of chiral [^{16}O,^{17}O,^{18}O]phosphate esters with a view to investigating the stereochemistry of chemical and enzyme-catalyzed phosphoryl-transfer reactions. In 1978 the two groups reported almost simultaneously synthetic routes to chiral [^{16}O,^{17}O,^{18}O]phosphate esters.[2,3]

The Oxford synthesis was based on a strategy that was constrained by the following considerations. First, the route should be general so that any chiral [^{16}O,^{17}O,^{18}O]phosphate monoester could be made. Secondly, the route should enable ^{17}O and ^{18}O to be incorporated from isotopically labeled water. Thirdly, the absolute configuration should follow from the method of synthesis. These considerations led to the adoption of the 2-substituted 2-oxo-4,5-diphenyl-1,3,2-dixoaphospholans as the molecular framework into which the three oxygen isotopes were to be incorporated, since catalytic hydrogenolysis of the benzylic oxygen bonds should release the chiral [^{16}O,^{17}O,^{18}O]-phosphate monoester without perturbing any of the phosphorus–oxygen bonds. Newton and Campbell had shown that transesterification of *meso*-hydrobenzoin with trimethyl phosphite gave a single crystalline cyclic phosphite triester **1**, which on oxidation with ozone gave a single crystalline cyclic phosphate triester **2**; the cyclic



phosphite and phosphate triesters were shown to be the trans-diastereoisomers **1** and **2** by X-ray analysis.[4] Their method of synthesis, however, did not meet all our requirements since ^{17}O and ^{18}O could not both be incorporated from isotopically enriched water. However, Ukita et al. had shown that when *meso*-hydrobenzoin was treated with phosphorus oxychloride in pyridine, a single crystalline 2-chloro-2-oxo-4,5-diphenyl-1,3,2-dioxaphospholan was formed, which on treatment with methanol gave 2-methoxy-4,5-diphenyl-1,3,2-dioxaphospholan.[5] Although initially this product was considered to be the cis diastereoisomer,[2] we were able to demonstrate conclusively that it was, in fact, identical with the trans diastereoisomer ob-

Scheme I[a]
The Oxford Route to Chiral [(*S*)-^{16}O,^{17}O,^{18}O]Phosphate Esters or Anhydrides



[a] ⓪ = ^{17}O, ● = ^{18}O. Reagents: (i) PhLi, (ii) $HOCH_2CH_2OH$, *p*-$MeC_6H_4SO_3H$, (iii) $H_2{}^{18}O$, dioxan, *p*-$MeC_6H_4SO_3H$, (iv) $LiAlH_4$ or $NaBH_4$, (v) $P^{17}OCl_3$, C_5H_5N and ROH, C_5H_5N, (vi) H_2, Pd-C or Na, liquid NH_3.

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(1) F. Eckstein, *Angew. Chem., Int. Ed. Engl.*, **14**, 160 (1975); *Acc. Chem. Res.*, **12**, 204 (1979); J. R. Knowles, *Annu. Rev. Biochem.* **49**, 877 (1980); P. A. Frey, *Tetrahedron*, *38*, 1541 (1982); P. A. Frey, "New Comprehensive Biochemistry", A. Neuberger and L. L. M. Van Deenen, Eds., Elsevier Biomedical, Amsterdam, 1982, Vol. 3, p 201.

(2) P. M. Cullis and G. Lowe, *J. Chem. Soc., Chem. Commun.*, 512 (1978).

(3) S. J. Abbott, S. R. Jones, S. A. Weinman, and J. R. Knowles, *J. Am. Chem. Soc.*, **100**, 2560 (1978).

(4) M. G. Newton and B. S. Campbell, *J. Am. Chem. Soc.*, **96**, 7790 (1974).

(5) T. Ukita, A. Hamada, and A. Kobata, *Chem. Pharm. Bull.* **9**, 363 (1961); T. Ukita, U.S. Patent 3 006 911 (1961); *Chem. Abstr.*, **57**, 11103f (1962); Japanese Patent 24058 (1961); *Chem. Abstr.*, **57**, 16488i (1962).

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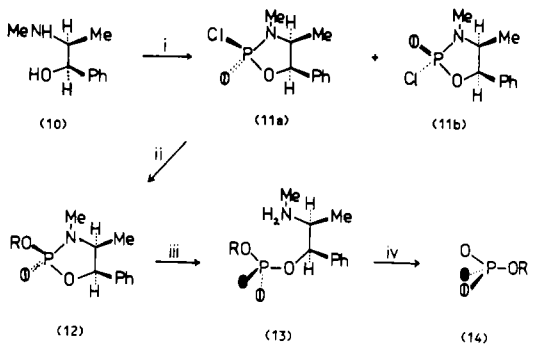
Scheme II[a]
The Harvard Route to Chiral [^{16}O,^{17}O,^{18}O]Phosphate Esters



[a] ⓪ = ^{17}O, ● = ^{18}O. Reagents: (i) $P^{17}OCl_3$, NEt_3, (ii) ROH, NEt_3, (iii) $H_2{}^{18}O$, CF_3CO_2H, (iv) H_2, Pd-C.

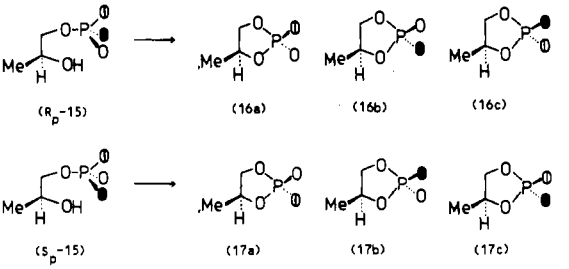
tained by the method of Newton and Campbell.[4,6] Since it was evident that ^{17}O could be incorporated efficiently as phosphorus [^{17}O]oxychloride (from PCl_5 and [^{17}O]-water), this route was adopted for the synthesis of chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters. The problem to be solved was the synthesis of "*meso*-hydrobenzoin", which was chiral by virtue of isotopic substitution. The route developed is outlined in Scheme I.[2,7] The absolute configuration of the [^{16}O,^{17}O,^{18}O]phosphate monoester follows from the absolute configuration of (*S*)-mandelic acid (**3**) and the relative configuration of the 2-substituted 2-[^{17}O]oxo-[1-^{18}O]-1,3,2-dioxaphospholan (**8**). Only the trans-diastereoisomer **8** is formed owing to the thermodynamic control in pyridine solution;[7] hence the route is stereospecific.

The Harvard synthesis is based on the studies of Inch et al.[8] and is outlined in Scheme II.[3,9] (−)-Ephedrine (**10**) is treated with phosphorus [^{17}O]oxychloride and the diastereoisomeric 2-chloro-[2-^{17}O]oxo-1,3,2-oxazaphospholine-2-ones (**11a** and **11b**) separated. Although both diastereoisomers were occasionally used and the diastereoisomeric products separated, in general the major diastereoisomer **11a** was used for the subsequent displacement by an alcohol to give a 2-alkoxy-1,3,2-oxazaphospholin-2-[^{17}O]one (**12**). The ^{18}O was introduced by hydrolyzing the P–N bond in [^{18}O]water under acidic conditions to give the acyclic phosphate diester **13** and the chiral [^{16}O,^{17}O,^{18}O]phosphate monoester **14** was released by catalytic hydrogenolysis of the benzylic bond. The absolute configuration of the chiral [^{16}O,^{17}O,^{18}O]phosphate monoester was determined by independent analysis.

Stereochemical Analysis of Chiral [^{16}O,^{17}O,^{18}O]Phosphate Esters

Three methods have so far been used to investigate the chirality of [^{16}O,^{17}O,^{18}O]phosphate esters, namely, chiroptical methods, mass spectrometry, and ^{31}P NMR spectrometry. Undoubtedly the simplest and most direct method for analyzing the chirality of [^{16}O,^{17}O,^{18}O]phosphate monoesters would be to use a chiroptical method. Methyl [(*S*)-^{16}O,^{17}O,^{18}O]phosphate was found to possess a measurable circular dichroic spectrum,[2,6] but the ellipticity was too small for it to be more than of theoretical interest. Raman optical activity, however, is a more promising technique since this measures optical activity in the vibrational spectrum and is particularly well-suited for measuring chirality due to isotopic substitution.[10] Thus it should be possible to observe the optical activity of a chiral [^{16}O,^{17}O,^{18}O]phosphate monoester even when attached to a chiral alcohol such as a sugar or nucleoside. Although encouraging results were obtained in 1978, the method at that time was rather insensitive, and large amounts of sample and long exposure in the high-power laser beam were necessary, which led to a build up of fluorescence while measurements were being made.[11] However, a new generation of instruments using multichannel detection methods are now being built that could make it feasible to measure directly the absolute configuration of a chiral [^{16}O,^{17}O,^{18}O]phosphate monoester, even when associated with a chiral alcohol.

Scheme III[a]



[a] The isotopomeric cyclic phosphate diesters are shown that would be formed if the cyclization of the (R_p)- and (S_p)-1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol proceeded with inversion of configuration at phosphorus.

Mass Spectrometric Method

When 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol (**15**) is treated with (diphenylphosphoryl)imidazole, three isotopomeric cyclic diesters (**16a–c** or **17a–c**) are formed. Since this cyclization reaction involves a displacement at phosphorus, it could occur with inversion or retention of configuration at phosphorus. It was therefore necessary to assume the stereochemical course of this cyclization reaction since no sample was available of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol of proven absolute configuration at phosphorus. The Harvard group argued that the cyclization would proceed with inversion of configuration at phosphorus, and hence [1-(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol would give **16a–c**, whereas [1(*S*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol would give **17a–c** (Scheme III).[3,9] In order to distinguish between the isotopomers **16a–c** and the isotopomers **17a–c** they were methylated with diazomethane to give a mixture of isotopomeric syn and anti cyclic triesters, which were separated by high-pressure liquid chromatography. The mass spectrum of the isotopomeric syn and anti cyclic triesters had parent ions of 153, 154, and 155 and daughter ions at 122, 123, and 124 (corresponding to loss of [^{16}O]-, [^{17}O]-, and [^{18}O]formaldehyde) irrespective of whether they

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(7) P. M. Cullis and G. Lowe, *J. Chem. Soc., Perkin Trans. 1*, 2317 (1981).
(8) D. B. Cooper, C. R. Hall, J. M. Harrison, and T. D. Inch, *J. Chem. Soc., Perkin Trans. 1*, 1969 (1977).
(9) S. J. Abbott, S. R Jones, S. A. Weinman, F. M. Bockhoff, F. W. McLafferty, and J. R. Knowles, *J. Am. Chem. Soc.*, **101**, 4323 (1979).
(10) L. D. Barron, *Acc. Chem. Res.*, **13**, 90 (1980).
(11) L. D. Barron, P. M. Cullis, and G. Lowe, unpublished results; P. M. Cullis, D. Phil. Thesis, Oxford University, Oxford, England, 1978.

were derived from **16a–c** or **17a–c**. In order to distinguish their origin it was necessary to relate parent with daughter ions. Unfortunately, no suitable metastable fragmentation could be found; moreover, extensive scrambling of isotope occurred in the mass spectrometer. However, ring opening of the syn and anti cyclic triesters with methanol gave 1- and 2-(dimethylphosphoryl)-(*S*)-propane-1,2-diol, which fragments to $[(MeO)_3PO]^{+\cdot}$ in the mass spectrometer and then loses formaldehyde in a strong metastable transition. By relating the intensities of the isotopically labeled formaldehyde derived from each isotopically labeled $[(MeO)_3P{=}O]^{+\cdot}$ species in a linked-scan metastable ion mass spectrometer, it was possible to establish the relative proportions of the isotopomeric syn (and anti) cyclic triesters and hence the chirality at phosphorus of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol. However, for a number of reasons the method is technically difficult to perform.

^{31}P NMR Spectrometric Method

^{17}O has a nuclear spin quantum number of 5/2 and therefore possesses a nuclear electric quadrupole moment. Consequently, when ^{17}O is directly bonded to phosphorus, the ^{31}P resonance is broadened and is not observable generally in the ^{31}P NMR spectrum.[12] ^{18}O has a nuclear spin quantum number of zero and does not effect the relaxation of the ^{31}P resonance. It does, however, cause a small but measurable isotope shift of the ^{31}P resonance to higher field when directly bonded to phosphorus.[14] The crucial observation that was the key to the application of ^{31}P NMR spectrometry for the analysis of chiral [^{16}O,^{17}O,^{18}O]phosphate esters was the recognition that the magnitude of the ^{18}O isotope effect is dependent on the nature of the phosphorus to oxygen bond: the higher the bond order, the greater the isotope shift.[12,15] This observation has been exploited also for the analysis of the chirality of [^{18}O]phosphate diesters.[16]

The analysis depends on the stereospecific cyclization of the chiral [^{16}O,^{17}O,^{18}O]phosphate monoester of a chiral diol into a cyclic phosphate diester, which is then esterified (without perturbing the phosphorus–oxygen bonds) to give the diastereoisomeric cyclic phosphate triesters. In the cyclization step any one of the peripheral oxygen isotopes will be lost with equal probability (the kinetic isotope effect being negligible) and the residual oxygen isotopes will occupy diastereotopic sites. In order to establish the method and to determine the stereochemical course and stereospecificity of the cyclization reaction, D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate and adenosine 5′-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate were synthesized and a stereospecific method developed for cyclization to the isotopomers of D-glucose 4,6-cyclic phosphate and adenosine 3′,5′-cyclic phosphate, respectively. In Scheme IV the isotopomers of the diastereoisomeric methyl D-glucose 4,6-cyclic phosphates are shown that would be formed if the cyclization of D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate occurred with retention of configuration (**18a–c** and **19a–c**) or inversion of configuration (**20a–c** and **21a–c**) at phosphorus. Since those species containing ^{17}O directly bonded to phosphorus will not be observed in the ^{31}P NMR spectrum, only **18a** and **19a** or **20a** and **21a** will be observed. Since the axial triester **18a** contains ^{18}O in a phosphorus–oxygen single bond, whereas the axial triester **20a** contains ^{18}O in a phosphorus–oxygen double bond, they can be distinguished by the isotope shift. Likewise the equatorial triesters **19a** and **21a** can be distinguished by the magnitude of the isotope shift. The analysis as depicted in Scheme IV, however, is based on the assumption that all the labeled sites are fully enriched. In practice the sites labeled as ^{18}O will be about 99 atom % ^{18}O, but since ^{17}O is currently available at only about 50 atom % enrichment, species will also be present in which sites labeled as ^{17}O in Scheme IV will be ^{16}O and ^{18}O. This means that four axial and four equatorial triesters will be observed in the ^{31}P NMR spectrum, viz., [$^{16}O_2$], [$^{16}O_{ax}$,$^{18}O_{eq}$], [$^{18}O_{ax}$,$^{16}O_{eq}$], and [$^{18}O_2$] axial and equatorial triesters. This, however, is an advantage since the [$^{16}O_2$] and [$^{18}O_2$] triesters provide the necessary internal reference signals for determining the isotope shifts, and the ratio of the [$^{16}O_{ax}$,$^{18}O_{eq}$] and [$^{18}O_{ax}$,$^{16}O_{eq}$] triesters allow the stereospecificity of the reaction to be determined; thus **18a** and **19a** or **20a** and **21a** will predominate in the ^{31}P NMR spectrum but will not be the exclusive resonances.

The cyclization of D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate was achieved with diphenyl phosphorochloridate followed by potassium *tert*-butoxide and the potassium salt of the isotopomers of D-glucose 4,6-cyclic phosphate were alkylated with methyl iodide. The diastereoisomeric six-membered cyclic phosphate triesters are perfectly stable; moreover, since they resonate at chemical shifts that differ by about 2 ppm in the ^{31}P NMR spectrum, they do not need to be physically separated. This is clearly a major advantage of the ^{31}P NMR spectrometric method. The ^{31}P NMR spectrum is shown in Figure 1. As expected, the isotopomeric axial and equatorial triesters of D-glucose 4,6-cyclic phosphate exist as anomeric pairs, and consequently eight resonances are observed for the axial triesters and eight resonances are observed for the equatorial triesters. From the spectrum it is clear that [$^{16}O_{ax}$,$^{18}O_{eq}$] axial and equatorial triesters are more intense than the [$^{18}O_{ax}$,$^{16}O_{eq}$] axial and equatorial triesters, and hence cyclization has occurred with inversion of configuration. By comparing the ratio of the [$^{16}O_{ax}$,$^{18}O_{eq}$] and [$^{18}O_{ax}$,$^{16}O_{eq}$] triesters for both the axial and equatorial diastereoisomers with the ratio calculated from the known isotopic composition of the reagents used in the synthesis, it was possible to conclude that the cyclization occurs with inversion of configuration at phosphorus with a stereoselectivity in excess of 94%.[17]

In the same way adenosine 5′-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate was cyclized and methylated to give the isotopomers of the axial and equatorial methyl *N*-methyladenosine 3′,5′-cyclic phosphate. The ^{31}P NMR spectrum (Figure 2) shows the expected four lines for the axial and four lines for the equatorial triesters. The ratio of the [$^{16}O_{ax}$,$^{18}O_{eq}$] and [$^{18}O_{ax}$,$^{16}O_{eq}$] axial and

(12) G. Lowe, B. V. L. Potter, B. S. Sproat, and W. E. Hull, *J. Chem. Soc., Chem. Commun.*, 733 (1979).
(13) M.-D. Tsai, *Biochemistry*, **18**, 1468 (1979); M.-D. Tsai, S. L. Huang, J. F. Kozlowski, and C. C. Chang, *ibid.*, **19**, 3531 (1980).
(14) M. Cohn and A. Hu, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 200 (1978); G. Lowe and B. S. Sproat, *J. Chem. Soc., Chem. Commun.*, 565 (1978); O. Lutz, A. Nolle, and D. Staschewski, *Z. Naturforsch. A*, **33**, 380 (1978).
(15) R. L. Jarvest and G. Lowe, *J. Chem. Soc., Chem. Commun.*, 364 (1979); M. Cohn and A. Hu, *J. Am. Chem. Soc.*, **102**, 913 (1980).
(16) J. A. Gerlt and J. A. Coderre, *J. Am. Chem. Soc.*, **102**, 4531 (1980).
(17) R. L. Jarvest, G. Lowe, and B. V. L. Potter, *J. Chem. Soc., Perkin Trans. 1*, 3186 (1981); *J. Chem. Soc., Chem. Commun.*, 1142 (1980).

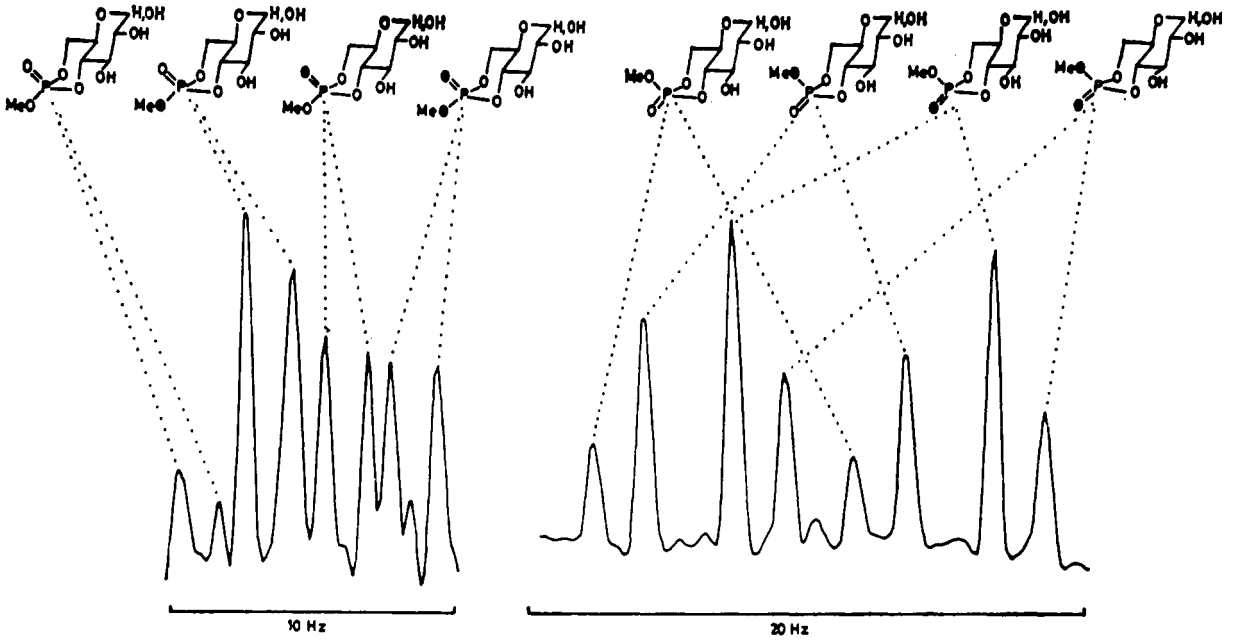

Figure 1. The ^{31}P NMR spectrum (121.5 MHz) of the axial and equatorial triesters obtained by cyclization and esterification of D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate (● = ^{18}O).

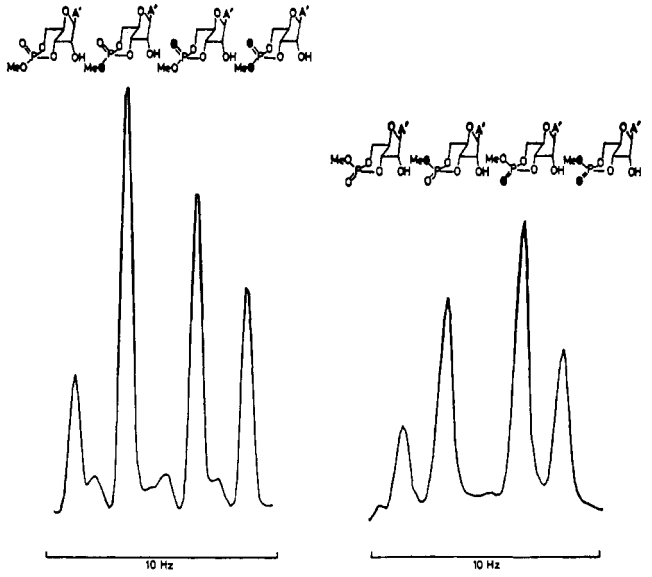

Figure 2. The ^{31}P NMR spectrum (121.5 MHz) of the axial and equatorial triesters obtained by cyclization and methylation of adenosine 5′-[(*S*)-^{16}O,^{17}O,^{18}O]phopshate (● = ^{18}O, A′ = *N*-methyladenine.

equatorial triesters established that the cyclization of adenosine 5′-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate occurs with inversion of configuration at phosphorus with a stereoselectivity in excess of 94%.[17]

The Harvard group have shown independently that the ^{31}P NMR method can be used to analyze the chirality of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol by the cyclization and esterification procedure developed for their mass spectrometric analysis.[18] It would now seem that the ^{31}P NMR spectrometric method is currently accepted as the analytical method of choice.

Phosphokinases

The Harvard group has determined the stereochemical course of [^{16}O,^{17}O,^{18}O]phosphoryl transfer catalyzed by glycerol kinase, acetate kinase,[19] and creatine kinase[20] (Table I). In order to do so it was necessary to develop a method for transferring the chiral [^{16}O,^{17}O,^{18}O]phosphoryl group from the product of the enzymic reaction to (*S*)-propane-1,2-diol. Alkaline phosphatase was selected for this purpose since it transfers phosphoryl groups to alcohols as well as to water; it was found to catalyze phosphoryl transfer with retention of configuration at phosphorus.[21] Although this method has the merit of generality, since the majority of the chiral [^{16}O,^{17}O,^{18}O]phosphoryl group is transferred to water, and that which is transferred to (*S*)-propane-1,2-diol (generally between 10% and 15%) comprises a mixture of 1- and 2-[^{16}O,^{17}O,^{18}O]phosphopropane-1,2-diol that needs to be separated, the enzymic study needs to be conducted on a considerably larger scale (more than tenfold) than when the analysis is performed on glucose 6-[^{16}O,^{17}O,^{18}O]phosphate or adenosine 5′-[^{16}O,^{17}O,^{18}O]phosphate by ^{31}P NMR spectroscopy.

By synthesizing [γ(*S*)-^{16}O,^{17}O,^{18}O]ATP the Oxford group was able to determine the stereochemical course

Table I
Stereochemical Course of Chiral [^{16}O,^{17}O,^{18}O]Phosphoryl Transfer Catalyzed by Phosphokinases

enzyme	stereochemical course	ref
glycerol kinase (*E. coli*)	inversion	19
acetate kinase (*E. coli*)	inversion	19
creatine kinase (rabbit muscle)	inversion	20
hexokinase (yeast)	inversion	22
glucokinase (liver)	inversion	23
polynucleotide kinase (T_4-infected *E. coli*)	inversion	24
phosphofructokinase (*B. stearothermophilus*)	inversion	25
phosphofructokinase (rabbit muscle)	inversion	25
pyruvate kinase (rabbit muscle)	inversion	26

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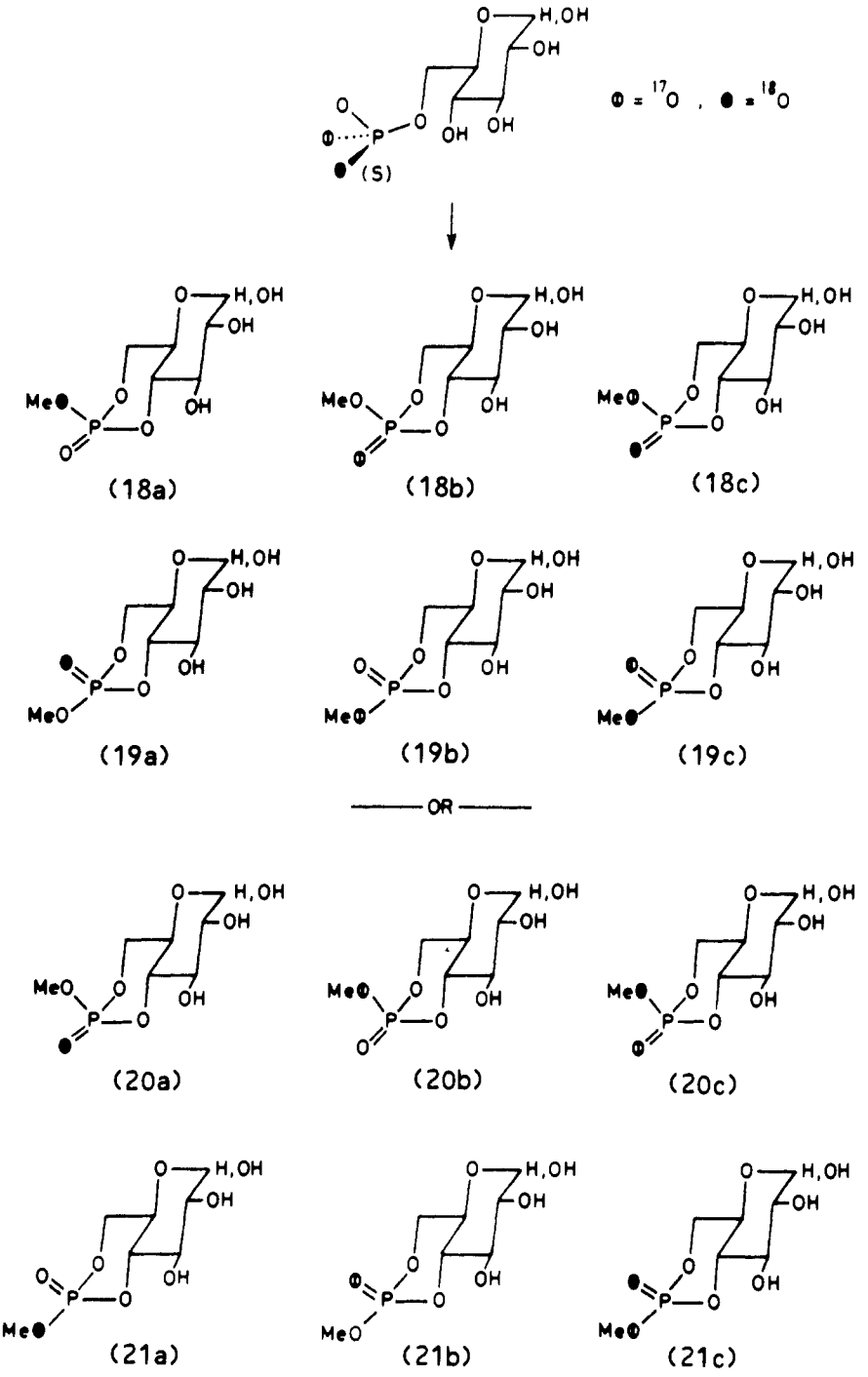
(19) W. A. Blättler and J. R. Knowles, *Biochemistry*, 18, 3927 (1979).
(20) D. E. Hansen and J. R Knowles, *J. Biol. Chem.*, 256, 5967 (1981).
(21) S. R. Jones, L. A. Kindman, and J. R. Knowles, *Nature (London)*, 275, 564 (1978).

Scheme IV[a]



[a] D-Glucose 6-[(*S*)-^{16}O, ^{17}O, ^{18}O]phosphate, after cyclization and methylation, would give species 18a-c and 19a-c or 20a-c and 21a-c, depending on whether the cyclization occurs with retention or inversion of configuration at phosphorus.

of phosphoryl transfer catalyzed by hexokinase,[22] glucokinase,[23] and polynucleotide kinase (T_4-infected *Eschericia coli*)[24] by the ^{31}P NMR analysis of glucose 6-[^{16}O,^{17}O,^{18}O]phosphate or adenosine 5′-[^{16}O,^{17}O,^{18}O]-phosphate formed. Moreover, since ATP is a common substrate for all phosphokinases, it was then possible to analyze the stereochemical course of any phosphokinase by synthesizing the appropriate chiral [(*S*)-^{16}O,^{17}O,^{18}O]phosphate ester and incubating it with MgADP and the phosphokinase to give [γ-^{16}O,^{17}O,^{18}O]ATP whose chirality at P_γ was determined after transfer to glucose with hexokinase and analyzing the chirality of the glucose 6-[^{16}O,^{17}O,^{18}O]phosphate by ^{31}P NMR spectroscopy. In this way the stereochemical course of phosphofructokinase[25] and pyruvate kinase[26] was also determined (Table I).

The stereochemical course of all the phosphokinases that have so far been investigated with chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters is shown in Table I. The impressive stereochemical conservatism suggests

(22) G. Lowe and B. V. L. Potter, *Biochem. J.*, **199**, 227 (1981).
(23) D. Pollard-Knight, B. V. L. Potter, P. M. Cullis, G. Lowe, and A. Cornish-Bowden, *Biochem. J.*, **201**, 421 (1982).
(24) R. L. Jarvest and G. Lowe, *Biochem. J.*, **199**, 273 (1981).
(25) R. L. Jarvest, G. Lowe, and B. V. L. Potter, *Biochem. J.*, **199**, 427 (1981).
(26) G. Lowe, P. M. Cullis, R. L. Jarvest, B. V. L. Potter, and B. S. Sproat, *Philos. Trans. R. Soc. London, Ser. B*, **293**, 75 (1981).

Table II
Stereochemical Course of Chiral [$^{16}O,^{17}O,^{18}O$]Phosphoryl Transfer Catalyzed by Phosphomutases

enzyme	stereochemical course	ref
phosphoglycerate mutase (rabbit muscle)	retention	28
phosphoglycerate mutase (wheat germ)	retention	28
phosphoglucomutase (rabbit muscle)	retention	29

Table III
Stereochemical Course of Chiral [$^{16}O,^{17}O,^{18}O$]Phosphoryl Transfer Catalyzed by Phosphatases

enzyme	stereochemical course	ref
alkaline phosphatase (*E. coli*)	retention	21
acid phosphatase (liver)	retention	33
glucose 6-phosphatase (liver)	retention	34

Table IV
Stereochemical Course of Hydrolysis Catalyzed by Phosphodiesterases

enzyme	stereochemical course	ref
5'-nucleotide phosphodiesterase (*Crotalus adamanteus*)	retention	35
5'-nucleotide phosphodiesterase (*Crotalus durissus terrificus*)	retention	36
3'-nucleotide phosphodiesterase (bovine spleen)	retention	37
staphylococcal nuclease	inversion	38
cAMP phosphodiesterase (bovine heart)	inversion	39
cAMP phosphodiesterase (yeast)	inversion	39

that all of these enzymes follow the same chemical mechanism that is most simply interpreted as a direct "in line" transfer of the phosphoryl group between the substrates in the enzyme–substrate ternary complexes. However, since the stereochemical observations are consistent with any odd number of phosphoryl-transfer steps each occurring with inversion of configuration, it has been suggested by those who are convinced of the general importance of covalent intermediates in enzymic reactions that three rather than one phosphoryl-transfer steps may be involved with the requirement for two phosphoryl enzyme intermediates.[27] This escalation in mechanistic complexity is not justified by the evidence currently available.

Phosphomutases

Phosphoglucomutase and phosphoglycerate mutase from rabbit muscle are both cofactor-dependent enzymes, the cofactors being D-glucose 1,6-diphosphate and D-glycerate 2,3-diphosphate, respectively. Moreover both enzymes have a phosphoenzyme intermediate on their reaction pathways that has been thoroughly characterized. Kinetic evidence suggests that the cofactor-independent phosphoglycerate mutase from wheat germ also has a phosphoenzyme intermediate on the reaction pathway, but this species has not been directly observed or characterized. As shown in Table II all three phosphomutases catalyze [$^{16}O,^{17}O,^{18}O$]-phosphoryl transfer with retention of configuration.[28,29] This is the stereochemical course to be expected if single phosphoryl-transfer steps take place with inversion of configuration as was found for the phosphokinases. Thus retention of configuration is consistent with the involvement of a single covalent phosphoryl-enzyme intermediate, although that intermediate may undergo a conformational change in accord with the kinetic evidence.[30] It should be added that although we consider it less likely, the evidence is in accord with both phosphoryl-transfer steps occurring with retention of configuration, as proposed from the cyrstallographic work on the (nonproductive) phosphoglycerate mutase–3-phosphoglycerate complex.[31]

The question naturally arises as to why the phosphokinases have evolved a catalytic mechanism with "in line" phosphoryl transfer between substrates in the ternary complexes, whereas the phosphomutases have evolved a double-displacement mechanism involving a phosphoryl–enzyme intermediate. A possible explanation is provided by the fact that the two substrates for a phosphokinase are generally grossly different (an exception is nucleoside diphosphate kinase, which follows ping-pong kinetics and catalyzes thiophosphoryl transfer with retention of configuration[32]), so that the enzyme must provide a distinctive binding site for each substrate. By requiring both substrates to be present simultaneously at the active site, the "in line" mechanism can be utilized. By contrast the two substrates (and cofactor) for the mutases possess the same carbon–oxygen skeleton, so that a single binding site could suffice to bind both substrates but not simultaneously. The phosphoryl group to be transferred must therefore become temporarily bound to the enzyme while substrate exchange takes place. Since the phosphoryl-transfer potential of the substrates that the mutases transform are low, the intervention of a covalent phosphoryl–enzyme intermediate could also have a catalytic advantage. So by providing a refuge for the phosphoryl group on the enzyme, the overall activation energy for phosphoryl transfer should be lowered and the need to evolve more than one substrate binding site unnecessary.

Phosphatases

Alkaline phosphatase, acid phosphatase, and glucose 6-phosphatase all have the ability to catalyze phosphoryl transfer to alcohols as well as to water. In this way it has been possible to preserve the chiral integrity of the [$^{16}O,^{17}O,^{18}O$]phosphate ester and so determine the stereochemical course of these enzyme-catalyzed reactions.

Independent evidence had been provided for the involvement of a phosphoryl–enzyme intermediate in all three enzymes. The observed retention of configuration of the [$^{16}O,^{17}O,^{18}O$]phosphoryl group transferred by alkaline phosphatase,[21] acid phosphatase,[33] and glucose 6-phosphatase[34] (Table III) is in accord with the growing

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evidence that single phosphoryl-transfer steps occur with inversion of configuration.

Phosphodiesterases

The stereochemical course of six phosphodiesterases have now been established, and it is clear that this class of enzymes does not follow a uniform stereochemical course (Table IV). Thus the 5′-nucleotide phosphodiesterases (snake venom)[35,36] and 3′-nucleotide phosphodiesterase (bovine spleen)[37] catalyze the hydrolysis of 5′-nucleotide esters and 3′-nucleotide esters with retention of configuration, whereas staphylococcal nuclease,[38] and cAMP phosphodiesterases (Mg^{2+} dependent from bovine heart and Zn^{2+} dependent from yeast)[39] catalyze the hydrolysis of phosphate diesters with inversion of configuration at phosphorus. There was no prior evidence for a nucleotidyl-enzyme intermediate in either the venom or the spleen phosphodiesterases, although this is the clear implication from the stereochemical course of the reaction. Some evidence for a nucleotidyl-enzyme intermediate had, however, been obtained for a 5′-nucleotide phosphodiesterase from bovine intestine.[40]

Nucleotidyl Transferases

This large and important class of enzymes has not as yet been very much explored. Adenylate cyclase, which converts ATP into cyclic AMP, has been studied in the reverse direction and shown to occur with inversion of configuration.[41] ATP sulfurylase, the enzyme used by microorganisms for the activation of sulfate, has been shown to proceed with inversion of configuration and hence by an "in line" mechanism,[42] which effectively eliminates the mechanism proposed from kinetic studies involving an adenylyl–enzyme intermediate.[43] However, the recent development of three closely related methods using cyanogen bromide,[44] *N*-bromosuccinimide,[45] and bromine[46] in [^{17}O]- or [^{18}O]water for converting S_p and R_p diastereoisomers of nucleoside 5′-[1-thio]diphosphates (the cyanogen bromide method requires the use of the 2-cyanoethyl ester[44]) stereoselectively into R_p and S_p nucleoside 5′-[1-^{17}O or -^{18}O]diphosphates, respectively, and thence to R_p and S_p nucleoside 5′-[1-^{17}O or -^{18}O]triphosphates, has provided access to suitable substrates for the exploration of the stereochemical course of a much wider range of enzymes of this class. Using adenosine 5′-[(*R*)α-^{17}O]triphosphate and the appropriate [$^{18}O_2$]amino acid, we have shown that isoleucyl-tRNA synthetase from *Escherichia coli*,[47] methionyl-tRNA synthetase from *E. coli*,[48] and tyrosyl-tRNA synthetase from *B. stearothermophilus*[49] catalyze nucleotidyl transfer to form the amino acyl adenylate with inversion of configuration at phosphorus; the synthesis of NAD^+ from nicotinamide mononucleotide and adenosine 5′-[(*R*)α-^{17}O]triphosphate catalyzed by NAD^+ pyrophosphorylase also occurs with inversion of configuration.[50] The stereochemical results obtained with nucleotidyl transferases are summarized in Table V.

Table V
Stereochemical Course of Some Nucleotidyl Transfer Reactions

enzyme	stereochemical course	ref
adenylate cyclase (*Brevibacterium liquefaciens*)	inversion	41
ATP sulfurylase (yeast)	inversion	42
Ile-tRNA synthetase (*E. coli*)	inversion	47
Met-tRNA synthetase (*E. coli*)	inversion	48
Tyr-tRNA synthetase (*B. stearothermophilus*)	inversion	49
NAD^+-pyrophosphorylase (hog liver)	inversion	50

Stereochemical Course of Chemical Reactions

The cyclization of D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate and adenosine 5′-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate by activation with diphenyl phosphorochloridate followed by treatment with potassium *tert*-butoxide was shown to occur stereospecifically with inversion of configuration at phosphorus.[17] These reactions are used in all our stereochemical analyses and have been discussed already in this connection.

The intramolecular acid-catalyzed migration of the [^{16}O,^{17}O,^{18}O]phosphoryl group in 2-[(*R*)-^{16}O,^{17}O,^{18}O]-phosphopropane-1,2-diol to the 1-position has been found to occur with retention of configuration in accord with the expected adjacent attack of the 1-hydroxy group to form a pentacoordinate intermediate that must undergo pseudorotation to allow the leaving group to depart from an apical position.[51]

The solvolysis of both phenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate in 50% aqueous methanol at pH 4.7 and 2,4-dinitrophenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate in 50% aqueous methanol at pH 10.2 was found to proceed with complete inversion of configuration at phosphorus.[52] These stereochemical observations are not incompatible with the extensive mechanistic information that suggests that the phenyl phosphate monoanion and the 2,4-dinitrophenyl phosphate dianion solvolyze by a dissociative mechanism, since if the lifetime of the metaphosphate ion is short compared with the rate of diffusion of solvent, it must either collapse back to starting material with unchanged configuration or react with preassociated solvent to give product with inversion of configuration.[53] Stereochemical evidence therefore

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cannot distinguish between preassociative stepwise and concerted mechanisms.

Conclusions

Chiral [^{16}O,^{17}O,^{18}O]phosphate esters have provided a welcome clarification of a substantial number of mechanistic ambiguities in both chemical and enzyme-catalyzed phosphoryl-transfer reactions. All the phosphokinases that have been investigated with chiral [^{16}O,^{17}O,^{18}O]phosphate esters or anhydrides obey sequential kinetics and catalyze phosphoryl transfer with inversion of configuration. This has led to the confident assertion that single enzyme-catalyzed phosphoryl-transfer steps occur with inversion of configuration. By contrast the phosphomutases and phosphatases that have been studied all catalyze phosphoryl transfer with retention of configuration and all are known to have phosphoenzyme intermediates on their reaction pathway. The stereochemical course found for these three classes of phosphotransferases provided the necessary evidence for the more disparate stereochemical events observed with phosphodiesters to be interpreted with confidence.

Although the extensive use of chiral [^{18}O]-phosphorothioates for studying the stereochemical course of phosphoryl and nucleotidyl transferases have not been reviewed here, all the enzymes that have been studied with both chiral [^{18}O]phosphorothioate and chiral [^{16}O,^{17}O,^{18}O]phosphate esters (viz., glycerokinase, hexokinase, pyruvate kinase, polynucleotide kinase, snake venom phosphodiesterase, cAMP phosphodiesterase, adenylyl cyclase, methionyl-tRNA synthetase and tyrosyl-tRA synthetase)[54] have been found to follow the same stereochemical course. This gratifying result means that the stereochemical studies with chiral thiophosphate esters and anhydrides can now be confidently accepted as revealing the same stereochemical course as that followed by the natural substrate. This is particularly important for 5′-nucleotidase[55] and the myosin, mitochondrial, and sarcoplasmic reticulum ATPases[56–58] that cannot be investigated by other means.

It is a pleasure to acknowledge the collaboration of R. Bicknell, P. M. Cullis, R. L. Jarvest, B. V. L. Potter, and G. Tansley. Our work was supported by the Science and Engineering Research Council.

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Micelles of Nonionic Detergents and Mixed Micelles with Phospholipids

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Received May 4, 1982 (Revised Manuscript Received December 6, 1982)

Nonionic surfactants are widely employed as detergents, solubilizers, and emulsifiers and are particularly effective in the solubilization of the protein and phospholipid components of biological membranes. Most of the commercial surfactants are polydisperse preparations with a distribution of molecular species. Only recently have synthetic, monodisperse compounds become readily available, such as dodecyl octaoxyethylene ether and octyl glucoside.

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A comprehensive treatise on nonionic surfactants edited by Schick[1a] in 1967 includes an abundance of information on the organic chemistry, physical chemistry, analytical chemistry, and biology of nonionic surfactants. This volume contains chapters on micelle formation,[1b] on thermodynamics of micelle formation,[1c] on solubilization,[1d] and on synthesis.[1e] Since 1967, another comprehensive volume on the preparation, chemistry, and industrial applications of poly(oxyethylene)-containing surfactants has appeared.[2] A volume on poly(oxyethylene)[3] has also been published, and sections of many monographs, review articles, and

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