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Stereochemistry of Biological Reactions at Proprochiral Centers

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ABBREVIATIONS USED

9-BBN, 9-borabicyclo[3.3.1]nonane; PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvate; ADH, alcohol dehydrogenase; CoA, coenzyme A; NAD, adenine nicotinamide dinucleotide; LDH, lactate dehydrogenase; TDP, thymine diphosphate; SAM, *S*-adenosylmethionine; COMT, catechol-*O*-methyltransferase; P_i, inorganic phosphate; Ps_i, inorganic thiophosphate; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; AMPS, adenosine 5′-thiophosphate; ADPαS, adenosine 5′-(1-thiodiphosphate); ADPβS, adenosine 5′-(2-thiodiphosphate); ATPαS, adenosine 5′-(1-thiotriphosphate); ATPβS, adenosine 5′-(2-thiotriphosphate); ATPγS, adenosine 5′-(3-thiotriphosphate); tRNA, transfer ribonucleic acid; TpNP, thymidine 3′-[(4-nitrophenyl)phosphate]; TMP, thymine monophosphate; U>pS, uridine 2′,3′-cyclic phosphorothioate; UMPS, uridine 5′-thiophosphate; DPPE, dipalmitoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; EDTA, ethylenediaminetetraacetate.

I. INTRODUCTION

Enzymes have a remarkable ability to recognize the stereochemical properties of chiral or prochiral substrates and to catalyze chemical transformations with a high degree of stereospecificity. This stereospecificity is exerted not only in cases where it may serve a utilitarian purpose, that is, in situations in which there is a functional advantage to operating with only one enantiomer, as in the distinction between D and L amino acids. It is also seen in instances where there

seems to be no particular advantage to carrying out a reaction stereospecifically, for example, in many reactions involving discrimination between two heterotopic* ligands at a prochiral center, such as the dehydrogenation of a primary alcohol to an aldehyde. This, of course, is because enzymes, in order to achieve high substrate specificity and significant reaction-rate enhancements, must orient substrates rigorously in their macromolecular matrix. Since the matrix is chiral, the reactions catalyzed are almost inescapably stereospecific.

The steric course of a reaction, for example, a substitution at an sp^3 carbon atom, can only be observed if the reaction leads from a chiral center to another chiral center. The majority of biochemical reactions, however, take place at centers that are not chiral, hence their steric course is not evident from an inspection of the substrates and products. Such reactions have been called "stereochemically cryptic" (1); to unravel their steric course requires labeling of the center with either isotopes or heteroatoms to convert it into a chiral center of observable stereochemical behavior. In the isotopic labeling approach a chiral center is generated by replacing stereoheterotopic or homotopic groups with groups containing or consisting of a different isotope of the same element, such as replacing ^1H with ^2H or ^3H, ^{12}C with ^{13}C, or ^{16}O with ^{17}O or ^{18}O. Since the size differences between the isotopes are very small, even in the extreme case of replacement of protium with tritium, the isotopically chiral species will bind to the enzyme in the same way as the nonlabeled version, and except for rate changes due to isotope effects, it will react in the same way. In the heteroatom labeling approach, one introduces chirality by replacing stereoheterotopic or homotopic groups with different elements, such as replacing hydrogen by CH_3, as in a study of the stereochemistry of the pyruvate kinase reaction (2), or replacing oxygen by sulfur, as in many of the studies on the stereochemistry of reactions at phosphorus. The advantage of this approach is that it requires, in general, less elaborate analytical methodology. Unlike the isotopic substitution, such a heteroatomic replacement in a substrate may, of course, be recognized by the enzyme, potentially resulting in a different orientation, binding of only one of the stereoisomers, or simply nonacceptance or nonreactivity of the substrate analog. For the latter reason, the utility of this approach is limited by the usu-

*The following terminology is used: **Homomorphic** groups are groups that are identical when detached from their environment. They can be **homotopic**, that is, occupy equivalent positions in space, or **heterotopic**, occupy nonequivalent positions. **Stereoheterotopic** groups occupy stereochemically nonequivalent positions in space; they may be **enantiotopic** if replacement of either one or the other (but not both) by a different achiral ligand gives rise to a pair of enantiomers, or they may be **diastereotopic** if their separate replacement gives rise to a pair of diastereomers. In general, stereoheterotopic groups in a molecule will be enantiotopic if the molecule itself is achiral and contains no chiral centers; they will be diastereotopic if the molecule is chiral or contains chiral centers. (For a more detailed discussion, see: E. L. Eliel, "Prostereoisomerism (Prochirality)," *Topics in Current Chemistry*, Vol. 105, Springer Verlag, Heidelberg, 1982.)

ally high substrate specificity of enzymes. It has, however, served extremely well in studies on biological reactions at phosphorus, where chiral phosphorothioates have been very useful tools. Because of the potential problem that heteroatomic substitution can be recognized by the enzyme and can result in different binding and reactivity, the results obtained by this approach are, strictly speaking, not as rigorous as those obtained by the isotopic substitution approach. Nevertheless, for example, in all the studies on enzymatic reactions at phosphorus there is no case in which the two approaches have given different stereochemical results.

Ever since the appearance of Ogston's pioneering article in 1948 (3), which pointed out the ability of an enzyme to distinguish what we now call enantiotopic groups in a substrate, the stereochemistry of many reactions at prochiral centers has been determined (for reviews, see refs. 1, 4–18). However, the stereospecificity of enzyme reactions, as pointed out before, is not limited to chiral and prochiral systems. This was first demonstrated experimentally in 1969, when the groups associated with Cornforth and Eggerer (19) and Arigoni and Rétey (20) synthesized (*R*)- and (*S*)-[2-D,T]acetic acid and used this chiral variety of an achiral center of the *Xaaab* type to determine the steric course of the reaction catalyzed by the enzyme malate synthase. Subsequently, chiral versions of another biologically important center of the *Xaaab* type, a phosphate monoester, were synthesized and used for stereochemical studies (21–23). Two other stereochemically interesting and biologically important achiral systems are malonic acid (substitution type *Xaabb*) and inorganic phosphate (*Xaaaa*). While no synthesis of chiral malonic acid has been reported as yet, two groups have recently prepared a chiral version of inorganic phosphate (24, 25). In general the conceptual difficulty in developing methods for studying the steric course of reactions at centers of the *Xaaab* and *Xaaaa* type resided not so much in the synthesis of chiral versions of such centers as in the design of analytical methodology to determine the chirality of samples of unknown configuration. This is due to the different properties of these systems compared with prochiral centers. However, in the malonic acid example of the *Xaabb* substitution type, even the synthesis is a formidable problem because the chiral integrity of this system is jeopardized by the exchangeability of the α-hydrogens.

The term "prochiral" was coined in 1966 by Hanson (26) to describe elements (centers, planes, axes) in a molecule giving rise to a pair of features (ligands, faces) that can be distinguished only by reference to an external or internal chiral environment. The term originates from the fact that one substitution step is required to transform a prochiral center *Xaabc* into a chiral center *Xabcd*; hence studies on the stereochemistry of reactions at prochiral centers require one isotopic or heteroatomic substitution. A prochiral center *Xaabc* has certain properties, in particular the attribute that the two stereoheterotopic groups *a* are distinct because they are seen, and behave, differently in a chiral environment. Hence an enzyme can differentiate between the two groups *a* by

virtue of the fact that the protein represents a chiral matrix. This is not true with systems of the *Xaaab* or *Xaabb* type. In these systems the homomorphic ligands are intrinsically identical, because even in a chiral environment they can be superimposed by internal or external rotations. The systems *Xaaab* and *Xaabb* are one substitution step removed from a prochiral center and two substitution steps removed from a chiral center. Therefore, two isotopic or heteroatomic substitutions are required to study the steric course of reactions at such centers. Based on this formalism we call the systems *Xaaab* and *Xaabb* proprochiral centers. This provides a convenient classification of the status of these relative to other achiral systems, although it must be recognized that proprochirality is not an observable property. In an enzyme reaction at a proprochiral center the replacement of a homotopic ligand will occur at random; apart from rate differences due to isotope effects this will be true also for the isotopically chiral species. However, a proprochiral center labeled with one heteroatom will assume the properties of a prochiral center—the remaining two homomorphic ligands are now stereoheterotopic and therefore distinguishable by the usual methods.

In a system *Xaaaa* the *a* groups are, of course, also homotopic and thus indistinguishable. By the same formalism used above such a center can be called *pro*proprochiral, because it is *three* substitution steps removed from a chiral center. Hence, three isotopic and/or heteroatomic replacements are required in order to study the steric course of reactions at such a center. The only chiral version of a proproprochiral center synthesized to date is an inorganic phosphate molecule carrying one heteroatomic and two isotopic replacements, (*R*)- and (*S*)-[^{16}O,^{17}O,^{18}O]phosphorothioate. The presence of the sulfur heteroatom, of course, gives this chirally labeled species the properties of a proprochiral center with the concomitant simplification in the analytical methodology required to distinguish an *R* from an *S* isomer.

In the following sections we discuss the methods that have been developed to study the steric course of reactions at proprochiral and proproprochiral centers on carbon and phosphorus, and some of the biochemical applications of these methods. Specifically, we consider chiral methyl groups, chiral malonic acid, chiral phosphate monoesters, and chiral inorganic phosphate.

II. CHIRAL METHYL GROUPS

A. General

The existence of enantiomers of a chiral methyl group C(R,H,D,T) was predicted as early as 1962 by Levy, Talalay, and Vennesland (27). The authors stated, "It is certain that two enantiomorphs of this molecule will exist, although no satisfactory methods are as yet available for their separation." While the

separation of the two enantiomers of such a molecule is indeed for all practical purposes impossible, their separate syntheses present no particular problem. However, the development of methods to distinguish a methyl group of *R* configuration from its *S* enantiomer required conceptually new approaches. With such methodology at hand it became possible to examine the steric course of reactions in which methyl groups are generated from methylene groups ($R—CH_2—X \rightarrow R—CH_3$) or are converted into methylene groups ($R—CH_3 \rightarrow R—CH_2—X$) and of processes in which methyl groups are transferred from one ligand to another ($R—CH_3 \rightarrow R'—CH_3$). This methodology and its application have been reviewed before (28–30).

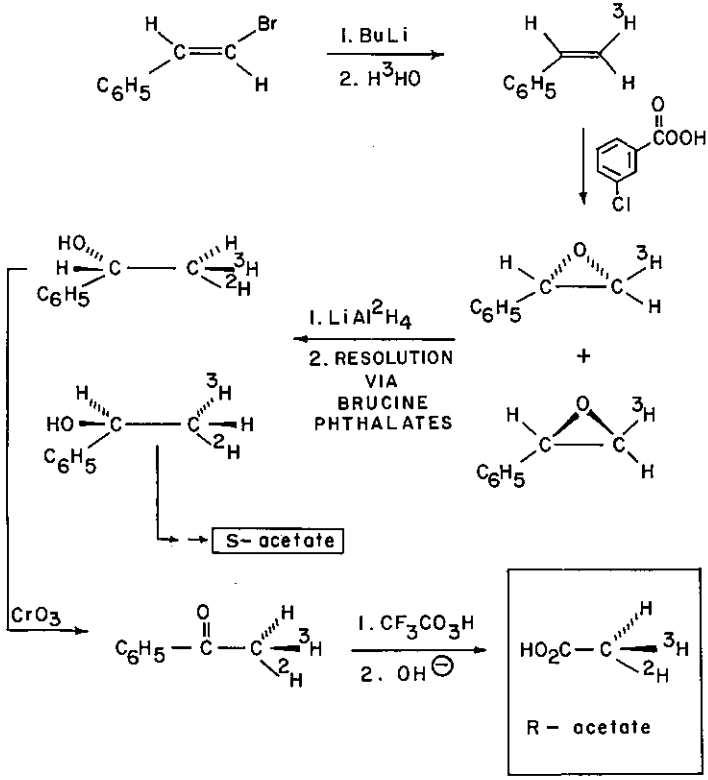
B. Synthesis of Chiral Methyl Groups

Most of the syntheses of chiral methyl groups reported to date involve the use of purely chemical methods, purely enzymic methods, or various combinations of the two. Most of these routes produce acetic acid, pyruvic acid, or lactic acid, which can then be converted into more complex molecules.

1. Chemical Methods

The synthesis reported by Cornforth et al. (31) is one of the original syntheses of chiral acetate and the first entirely chemical approach. The original method, which relied on the introduction of tritium with $[^3H]LiAlH_4$, proved unsatisfactory and was subsequently modified (32) to introduce the tritium via $[^3H]H_2O$ as shown in Scheme 1. The *trans*-2-tritio-1-phenylethylene was epoxidized in a stereospecific syn reaction to give only one racemic diastereomer. The mixture of epoxides was subjected to stereospecific reductive ring opening with $LiAl^2H_4$, a process known to proceed with inversion of configuration. The (1*S*,2*S*)- and (1*R*,2*R*)-2-deuterio-2-tritio-1-phenylethanol mixture thus obtained was then resolved into the enantiomers using classical resolution techniques since the benzylic carbon is a normal chiral center. The enantiomers, once resolved, contained methyl groups which had opposite configurations. The remainder of the synthesis involved the conversion of the phenylethanol isomers to acetate with retention of the configuration of the chiral methyl center. The analysis of the products revealed them to be of high chiral purity [(*R*)-acetate 93% e.e. (enantiomer excess), and (*S*)-acetate 86% e.e.]. This modified procedure allowed the synthesis of ~3 mCi of chiral acetate in an overall yield of 7.7% based on the starting styrene.

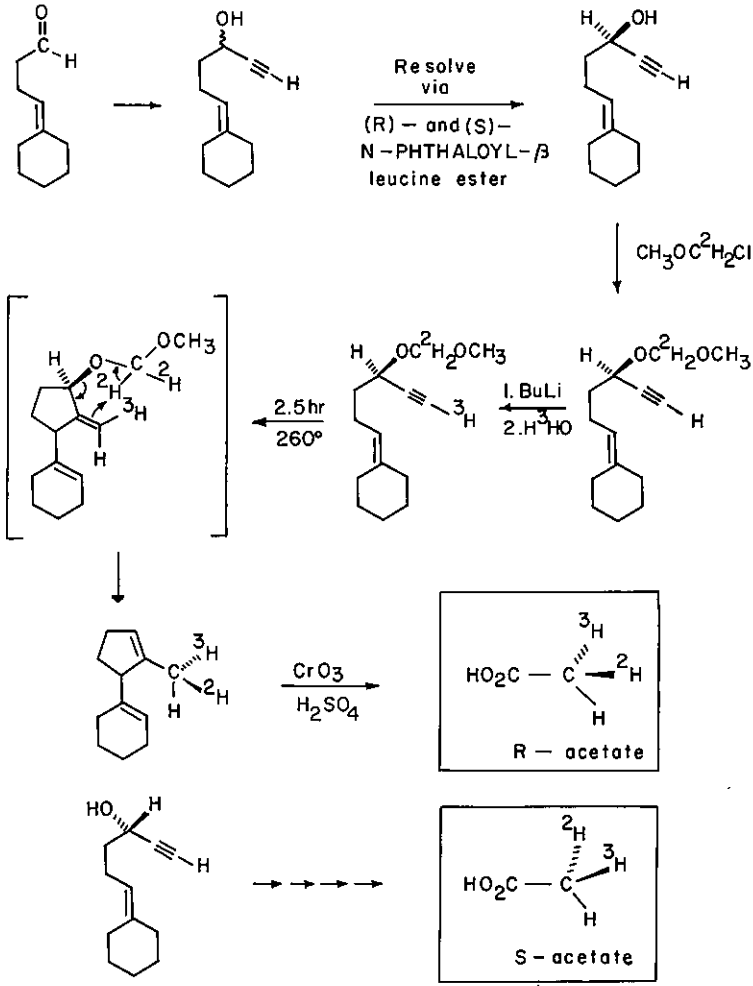
Clearly the most elegant chemical synthesis of chiral acetate is the route reported by Townsend et al. (33). The method, outlined in Scheme 2, has the advantage that tritium is introduced at a late stage in the synthesis, and both of the remaining steps, pyrolysis and Kuhn-Roth oxidation, proceed in high



Scheme 1

chemical yield. The pyrolysis of the deuterated ether brings together the three different isotopes of hydrogen in succession from three different positions in the molecule in a two-step process. The first reaction is an "ene" reaction in which the geometry of the system dictates syn addition to the triple bond. In the reductive elimination of methyl formate, the geometry of the addition of deuterium to the double bond is controlled by the stereochemistry of the alcohol function. The (*R*)-acetate was obtained from the ether of the (*R*)-alcohol in high chiral purity (93% e.e.) and the (*S*)-ether gave the corresponding (*S*)-acetate in equally high chiral purity.

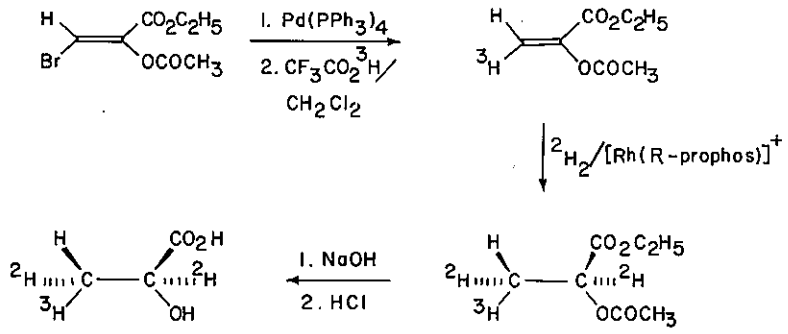
More recently, Bosnich and co-workers (34) have published a method for the synthesis of (*S*)-lactic acid containing a chiral methyl group using asymmetric catalytic hydrogenation. The (*Z*)-ethyl 2-acetoxy-3-bromoacrylate shown in Scheme 3 was converted into the isomerically pure (*Z*)-ethyl 2-acetoxy-3-tritio-acrylate by cleavage of the $Pd(PPh_3)_4$ adduct with $CF_3CO_2{}^3H$. The olefin was then hydrogenated with deuterium gas in the presence of $[Rh(R\text{-prophos})]^+$ to



Scheme 2

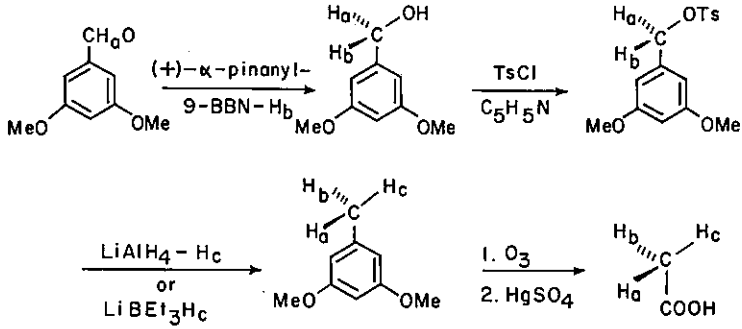
give the lactic acid shown. However, the validity of this method has not been verified by chirality analysis of the methyl group.

Two other purely chemical routes to chiral acetic acid have been pursued in our laboratory. In one of these, outlined in Scheme 4, stereospecifically α-deuterated or -tritiated 3,5-dimethoxybenzyl alcohol is prepared by reduction of the aldehyde with Midland's reagent (*B*-3-pinanyl-9-borabicyclo[3.3.1]-nonane, α-pinanyl-9-BBN) (35), followed by conversion to the tosylate and reductive displacement with lithium aluminum hydride or superhydride (lithium

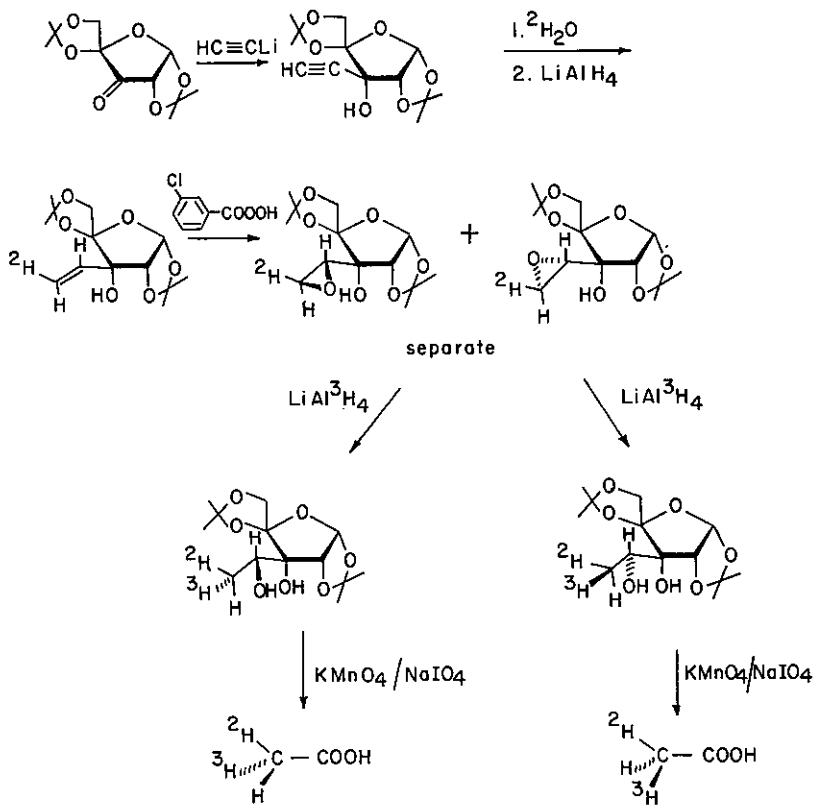


Scheme 3

triethylborohydride) (36). Various combinations of deuterated and tritiated reagents and substrates can be used to introduce the three hydrogen isotopes in the desired configuration. For example, deuterated aldehyde, prepared via the the 1,3-dithiane derivative, was reduced with unlabeled (+)- and (−)-α-pinanyl-9-BBN followed by displacement of the tosylate with [^{3}H]$LiAlH_4$ or, in better yield, [^{3}H]superhydride. The latter can be readily prepared from [^{3}H]LiH and triethylborane (37). The critical step in the overall sequence is the oxidation of the 3,5-dimethoxytoluene to acetic acid. Kuhn-Roth oxidation produced material of low chiral purity (~25-35% e.e.), but ozonolysis followed by afteroxidation with $HgSO_4$ gives consistently good yields (80-85%) of acetic acid of high chiral purity (>90% e.e.). Under these conditions the limiting factor for the chiral purity of the acetic acid is the optical purity of the α-pinene used. Another version of this synthesis involved reducing the unlabeled aldehyde with [^{3}H]$NaBH_4$, one of the cheapest sources of tritium, reoxidation to the tritiated aldehyde, followed by reduction with unlabeled (+)- and (−)-pinanyl-9-BBN and displacement of the tosylate with superdeuteride. Additional permuta-



Scheme 4



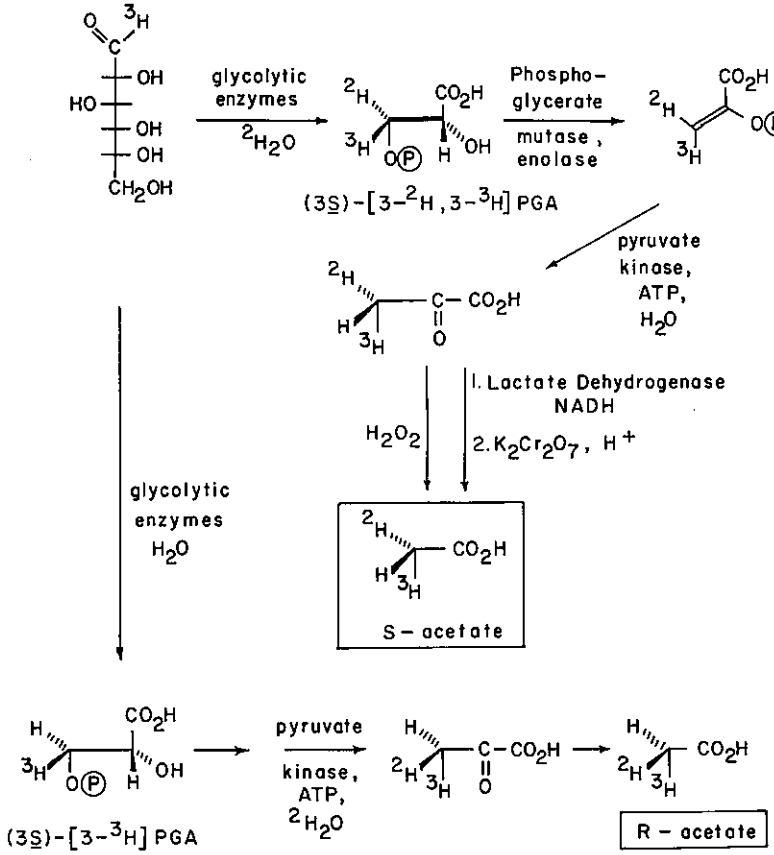
Scheme 5

tions are obvious; which one is most suitable for a given laboratory depends on the relative availability of different reagents and facilities.

Another chemical route is based on the work of Kakinuma et al. (38), who synthesized the two chiral, deuterated epoxides shown in Scheme 5. Separation of the two diastereomers and reductive epoxide ring opening with tritiated $LiAlH_4$ followed by permanganate/periodate oxidation gave acetic acid which within the limits of detection was chirally pure (100 ± 7% e.e.) (39).

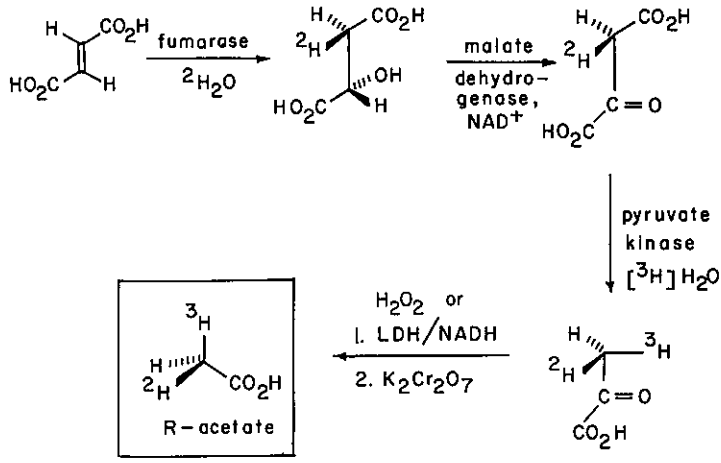
2. *Enzymatic Methods*

One of the most efficient methods relying on enzymatic reactions is that reported by our laboratory (40, 41), a modification of the original procedure published by Rose (42) for the synthesis of chiral pyruvate and acetate. The route, as shown in Scheme 6, utilizes the commercially available purified glycolytic



Scheme 6

enzymes to convert [1-^{3}H]glucose to (3*S*)-[3-^{3}H]phosphoglyceric acid (PGA) in one single incubation. The (3*S*)-[3-^{3}H]PGA was converted to (3*R*)-lactate in a second incubation by a series of enzymatic reactions carried out in deuterated water, the key step being the pyruvate kinase reaction (43) which stereospecifically protonates phosphoenolpyruvate from the *Si* face to give pyruvate. The chiral lactate was oxidized with dichromate to give (*R*)-acetate of 72% e.e. The lactate of opposite configuration was obtained by converting [1-^{3}H]glucose to (3*S*)-[3-^{2}H,3-^{3}H]PGA with the same enzymes previously described but in ^2H_2O instead of H_2O. The incubation with pyruvate kinase, in H_2O, gave (3*S*)-lactate, which upon oxidation produced (*S*)-acetate of 65% e.e. The overall yield based on [1-^{3}H]glucose was 20%. The low chiral purity of the acetate samples is due to the known slow deprotonation of pyruvate by pyruvate kinase before its release from the enzyme's active site (44).



Scheme 7

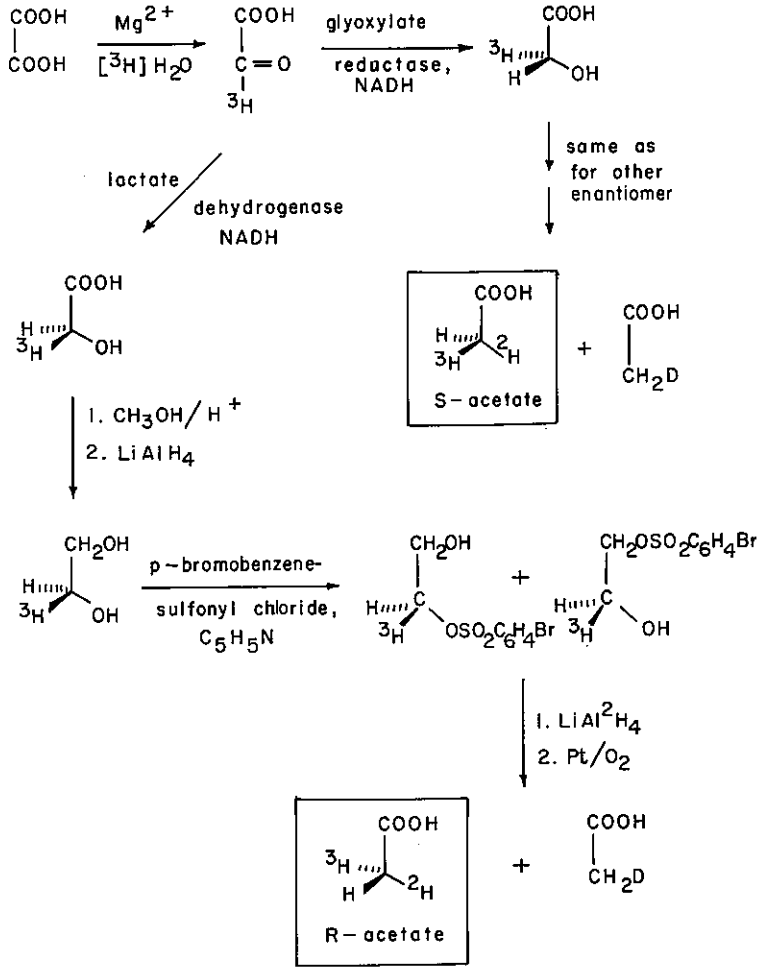
Creighton and Rose (45) have explored the route shown in Scheme 7 to synthesize chiral pyruvate. The method takes advantage of the fact that pyruvate kinase decarboxylates oxalacetate with retention of configuration. The requisite labeled oxalacetates were prepared as shown from the appropriately labeled L-malates, available from equilibration with fumarase in either deuterated or tritiated water. This method is convenient for the preparation of small amounts of chiral acetate but suffers from the low oxalacetate decarboxylase activity of pyruvate kinase.

Simon and co-workers (46) reported the quantitative conversion of (3*R*)-L-[3-^{2}H] malate to (3*S*)-[3-^{2}H,3-^{3}H] lactate by the organism *Leuconostoc mesenteroides* in tritiated water. The (3*R*)-lactate was obtained by fermentation of the tritiated malate in ^2H_2O; however, the chiral purity of both acetates, obtained by oxidation of the lactate samples with dichromate in dilute H_2SO_4, was lower than expected (34–52% e.e.). The specific activity of the acetates was somewhat lower than that of the starting lactates indicating possible racemization during oxidation. However, in our laboratory this oxidation of lactate has usually produced acetate without loss of chiral purity.

The Stickland reaction (47) has received much attention as a possible route to chiral acetate due to the availability of chiral glycine (48). In the Stickland reaction two moles of glycine and one mole of D-alanine are converted quantitatively into three moles of acetate, three moles of ammonia, and one mole of CO_2 by the organism *Clostridium sticklandii*. The presence of amino acid transaminase in the intact organisms leads to extensive hydrogen exchange although in the purified enzyme the replacement of NH_2 by H occurs stereospecifically with inversion (49, 50). Unfortunately, the rates of conversion with the purified enzyme are too low to be synthetically useful.

3. *Combinations of Chemical and Enzymatic Methods*

The second original route for the synthesis of chiral acetate as reported (20) by the Zürich group, outlined in Scheme 8, involved a combination of enzymatic and chemical reactions. [2-^{3}H]Glyoxylic acid was produced from oxalic acid and tritiated water and was reduced to (*S*)-[2-^{3}H]glycolic acid by lactate dehydrogenase (51) and to (*R*)-[2-^{3}H]glycolic acid with glyoxylate reductase from spinach leaves (52). The acids were converted to the methyl esters, which were reduced to ethylene glycol. The latter were converted to their monobrosylates and

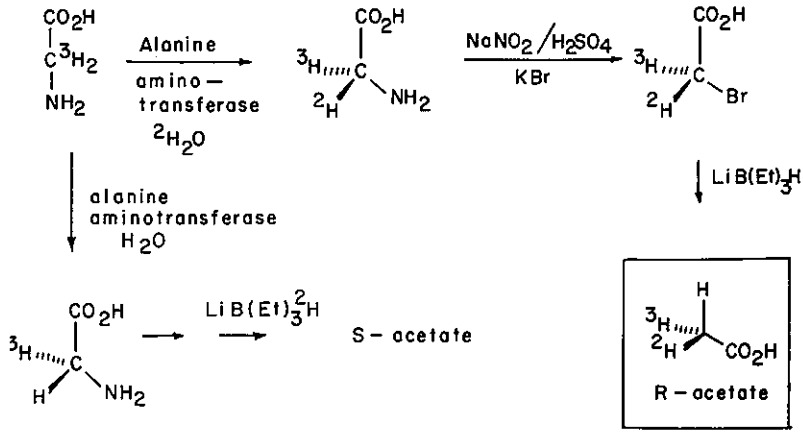


Scheme 8

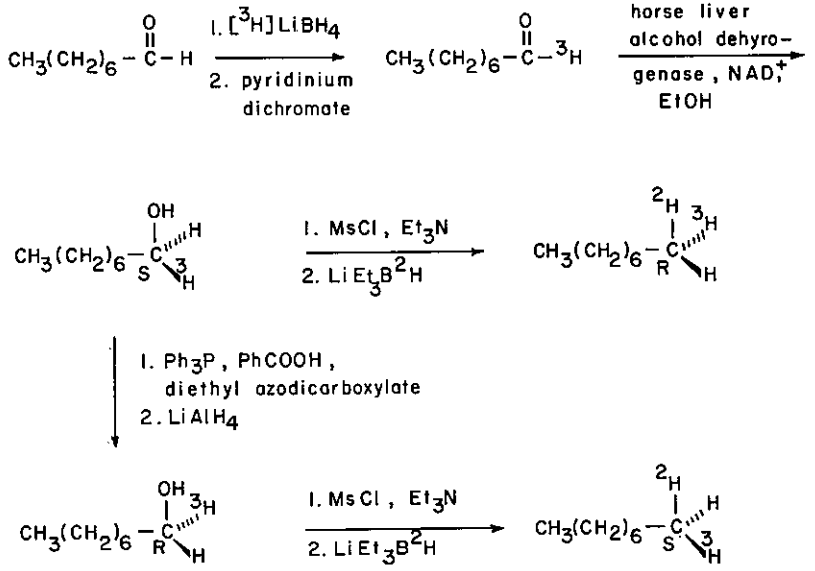
displaced with $LiAl^2H_4$, a reaction known to occur with inversion of configuration, to give the corresponding chiral-methyl ethanols, which were then oxidized with O_2 in the presence of Pt to their respective acetates. One of the problems with this approach is the generation of the symmetrical intermediate ethylene glycol. Half the tritiated molecules are converted to $CH_2{}^2H\text{-}CO_2H$, thereby lowering the specific activity by half. The overall yield is 2% based on oxalic acid.

Kajiwara et al. (53) reported a synthesis of chiral acetic acid based on the successive conversion of (*R*)-[2-^{2}H]glycine into bromoacetic acid and then, with tritiated $LiAlH_4$, into ethanol, which was oxidized with chromic acid to chiral acetic acid of *R* configuration (92% e.e.). The chiral glycine in their work was prepared from benzaldehyde in a seven-step sequence; however, it can also be obtained in other ways, such as enzymatic exchange of glycine with alanine transaminase (54). A simplified version of this synthesis, worked out in our laboratory, is outlined in Scheme 9. Chiral [2-^{2}H,2-^{3}H]glycine is generated by enzymatic exchange and the reductive displacement of the bromine in bromoacetic acid is carried out with superhydride to give directly acetic acid (55).

Caspi and co-workers (56) have reported the synthesis of *n*-octane containing a chiral methyl group by the displacement of the mesylate of (*R*)- and (*S*)-1-[1-^{3}H]octanol with superdeuteride. A unique feature of their approach (56, 57) was the chemical conversion of the more readily available tritiated 1*S* alcohol, obtained from the reduction of [1-^{3}H]octanal by horse liver alcohol dehydrogenase, to the more difficult-to-obtain 1*R* alcohol by the Mitsunobu reaction. Using the conditions shown in Scheme 10, the (1*S*)-alcohol was converted to the (1*R*)-benzoate which gave the (1*R*)-alcohol by reductive cleavage with $LiAlH_4$.



Scheme 9

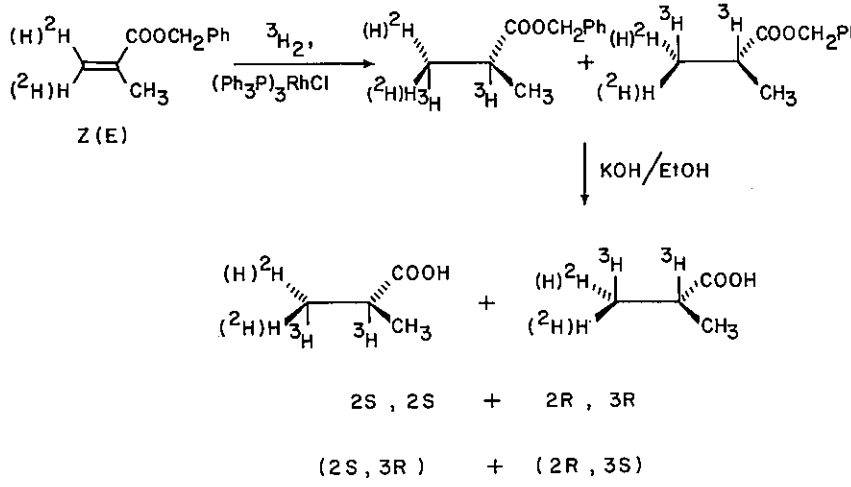


Scheme 10

This interconversion was carried out in 88% yield with complete inversion of configuration as judged, for example, by incubation with yeast alcohol dehydrogenase. A similar general route, reductive displacement of a sulfonate ester, was used to prepare chiral-methyl labeled decane (58). In neither case was the chiral purity of the product determined by analysis, but further work by Caspi's group (56, 59) indicated that their material must have had good chiral purity.

4. *Generation of Chiral Methyl Groups in More Complex Molecules*

In most of the examples discussed so far the synthetic target was chiral-methyl acetic acid either used per se as substrate or serving as starting material for the synthesis of more complex molecules, for example, (*3'R*)- and (*3'S*)-[3'-^{2}H,^{3}H]mevalonolactone (60) or (*methyl-R*)- and (*methyl-S*)-[methyl-^{2}H,^{3}H]methionine (40, 41, 61). In a number of other instances, chiral methyl groups were generated directly in more complex molecules required for a particular experiment. Chiral-methyl octane and decane, mentioned above, are examples, as is the synthesis of squalene carrying a chiral methyl group at carbon 6, which was published by Altman and his group (62). Two recent papers deal with the synthesis of compounds in which a chiral methyl group is part of an isopropyl group: systems containing two chiral centers which both owe their chirality only to isotopic substitution. In a synthesis by Aberhart and Tann (63), reduction of (*Z*)- and (*E*)-[3-^{2}H]methacrylate with tritium gas over chlorotris-

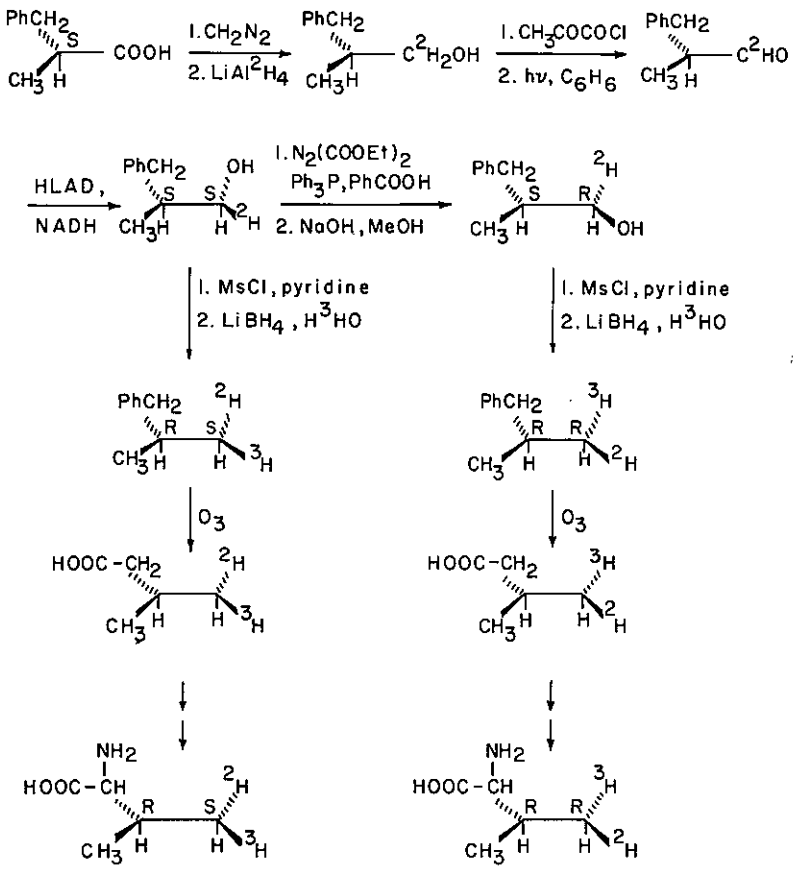


Scheme 11

(triphenylphosphine)rhodium catalyst gave isobutyrate as the 2*S*,3*R* + 2*R*,3*R* and the 2*S*,3*R* + 2*R*,3*S* racemic mixtures, respectively (Scheme 11). The problem of controlling the stereochemistry at the two isotopically chiral centers separately was addressed in a synthesis by Townsend and co-workers (64) of valine carrying a chiral methyl group in the pro-*R* position at C-3 (Scheme 12). The synthesis starts with (*S*)-α-methyldihydrocinnamic acid, predetermining the correct absolute configuration of the chiral center which is to become C-3 of valine. The chiral center which is to become the chiral methyl group is built up by alcohol dehydrogenase reduction of a deuterated aldehyde. Inversion of the configuration of the resulting (*S*)-alcohol by the Mitsunobu reaction allows independent elaboration of the methyl group in both configurations while keeping the configuration of the other chiral center fixed.

III. ANALYSIS OF CHIRAL METHYL GROUPS

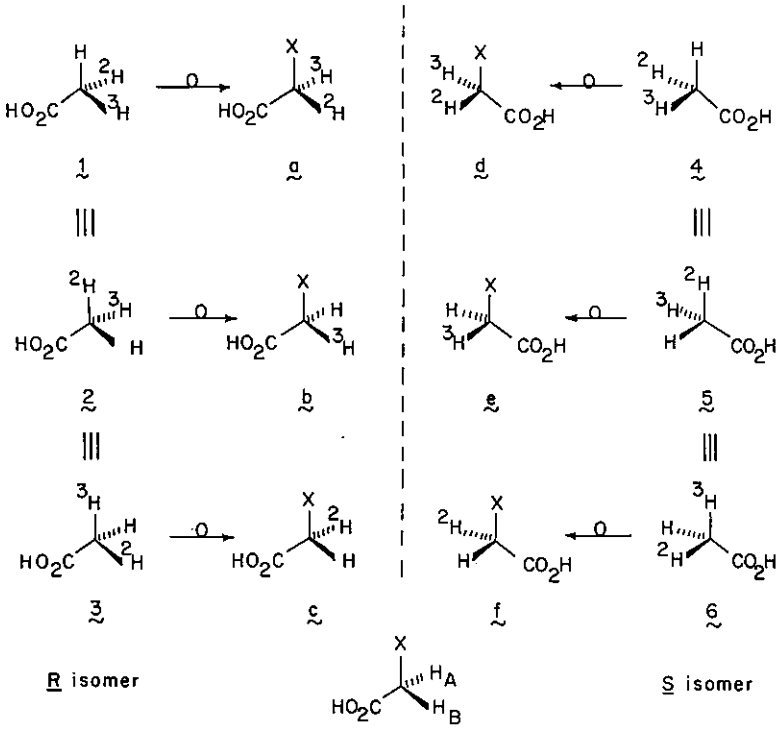
The standard method of distinguishing enantiomers by their ability to rotate the plane of polarized light is not applicable in the case of chiral methyl groups. Compounds of the type $R_1CH^2HR_2$ have specific rotations on the order of 1 to 2° (65), rarely greater than 5°, which are one or two orders of magnitude smaller than those of the corresponding $R_2CHR_2R_3$ type compounds. It would be expected that replacement of another "large" (R_1 or R_2) group by ^3H would again lower the specific rotation one or two orders of magnitude. However, unlike deuterium, tritium is present only at tracer levels, and therefore only a small



Scheme 12

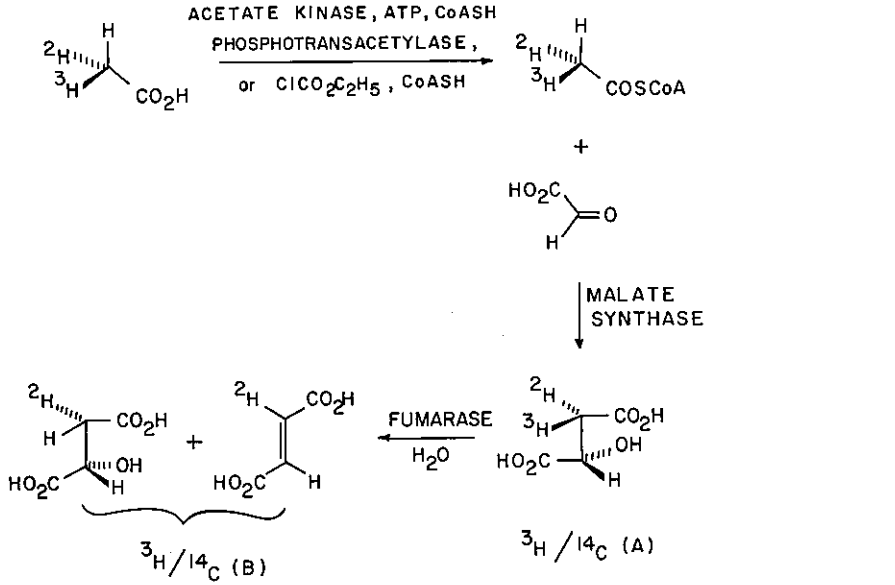
number of methyl groups actually contain all three isotopes of hydrogen and are thus chiral. The majority of methyl groups contain one deuterium and two hydrogens and are prochiral. Since only about one in 10^7 molecules contain tritium, at the levels of specific radioactivities handled in most laboratories, the specific rotation would be well below detectable levels. If any spectroscopic method has the potential to distinguish a chiral methyl group of *S* configuration from its *R* isomers, tritium NMR spectroscopy (66) is probably the best candidate. This would require several experimental conditions to be met; one is slowing the rotation of the methyl group down to below the NMR time scale of resolution in a chiral environment, and another is the availability of relatively large amounts of tritiated sample (0.1 to 1.0 mCi per analysis). The feasibility of this approach has yet to be established.

All practical methods of analysis are based on carrying out a reaction in which one of the methyl hydrogens is replaced in a stereospecific, irreversible reaction by some other ligand, thereby generating a chiral methylene group. Each enantiomer of the methyl group gives rise to a set of three products. This process is illustrated in Scheme 13 for a process in which a methyl hydrogen of acetic acid is replaced with inversion by a group X ($X \neq -CO_2H$). This replacement leaves the remaining two hydrogens stereoheterotopic and thus distinguishable. The product set **a, b, c**, arising from the *R* isomer (**1, 2, 3**), is clearly different from set **d, e, f**, produced by the *S* isomer (**4, 5, 6**); the two product sets are mirror images of each other. Since tritium is present only in tracer amounts, products (**c**) and (**f**) are useless for analysis because they are formed mostly from the carrier species $CH_2{}^2H\text{-}CO_2H$. Therefore, the analysis requires a distinction between the sets (**a**) + (**b**) and (**d**) + (**e**). This can be done, for example, by determining whether the species tritiated at H_A (see bottom of Scheme 13) has arisen from the replacement of a normal hydrogen or from the replacement of deuterium. This determination may be made by converting **1, 2, 3** into **a, b, c** and **4, 5, 6** into **d, e, f** by a reaction that exhibits a significant primary kinetic



Scheme 13

isotope effect in the carbon-hydrogen bond cleavage. If the isotope effect were infinitely high, rotamer (1) would react infinitely faster than (2) or (3) and thus only (a) (all tritium located in the H_A position) would be produced, whereas in the absence of an isotope effect equal amounts of (a) and (b) (tritium equally distributed between the H_A and H_B position) would be formed. By the same logic the opposite enantiomer would give rise to only (d), where all the tritium is located in the H_B position, if the isotope effect were infinitely large. It is therefore possible to distinguish between set (a, b, c) and set (d, e, f) by generating them in a reaction which exhibits a primary kinetic isotope effect and determining the tritium distribution between H_A and H_B. Four factors control the ratio of tritium in H_A to tritium in H_B: (i) the chiral purity of the methyl group; (ii) the configuration of the methyl group; (iii) the sign and magnitude of the isotope effect; (iv) the steric course of the reaction. Since the steric course and the isotope effect k_H/k_D are constant for a given enzyme, once the system has been calibrated with chiral methyl samples of known absolute configuration and chiral purity, the analysis of the configuration and chiral purity of unknown samples can be accomplished. The first (19, 20) and most widely employed method for chiral methyl group analysis, the malate synthase/fumarase assay, makes use of this principle. Scheme 14 summarizes the essential features of this method. The chiral acetic acid is mixed with a known amount of [^{14}C]acetic acid to bring the $^3H/^{14}C$ ratio to approximately 4 and is converted into the coenzyme A ester either enzymatically (31) or chemically via the anhydride (50, 67). The acetyl CoA is then condensed with glyoxylate using malate synthase (68) from yeast to give malate. The malate synthase reaction is known to involve condensation on the *Si* face of the glyoxylate aldehyde carbon to form (*S*)-malate. The purified malate is incubated with fumarase. In the reversible anti dehydration-hydration of (*S*)-malate and fumarate, respectively, catalyzed by fumarase, the *pro-3R* hydrogen of malate is stereospecifically removed or equilibrated with solvent protons. Therefore, an equilibrium mixture of malate and fumarate is formed in which tritium from the pro-3*S* position of the original malate remains carbon bound whereas tritium from the pro-3*R* position is released into the water. After lyophilization to remove the water, the $^3H/^{14}C$ ratio of the residue is measured and compared with that of the starting malate in order to establish the tritium distribution between the two diastereotopic hydrogen positions of malate. The tritium content of the water is also routinely analyzed. The percentage tritium retention in the fumarase reaction observed in the chiral acetate assay is referred to as the F value (61) where F = $^3H/^{14}C$ ratio of the residue of the fumarase reaction $\times$ 100 $\div$ $^3H/^{14}C$ ratio of the malate. Based on the primary kinetic deuterium isotope effect in the malate synthase reaction (k_H/k_D = 3.7-3.8) and the steric course of the reaction (inversion) established in the development of the assay (19, 20), a sample of chirally pure (*R*)-[$^2H_1$,$^3H_1$]acetate gives an F value of 79. Conversely, a chirally pure *S*-acetate



$$F = \frac{^{3}H/^{14}C \ B}{^{3}H/^{14}C \ A} \times 100$$

F = 50: RACEMIC
F > 50: R (Max 79)
F < 50: S (Max 21) [ie 50 ± 29]

$$\frac{|50 - F|}{29} \times 100 = \% \text{ enantiomeric excess}$$

Scheme 14

will give an F value of 21. These numbers for chirally pure samples are based on the work of Eggerer and co-workers (69, 70) who have shown that the amplitude of the configurational assay with the malate synthase/fumarase system is 50 ± 29. The reproducibility of the assay depends somewhat on the amount of radioactivity available; the F values are usually accurate to about ±1–2 unless only very small amounts of radioactivity (<5000 dpm ^{3}H) are available. The experimental details for the procedure have been described (29–31, 50).

Other enzymes that exhibit a primary kinetic isotope effect in the irreversible removal of one methyl hydrogen can also be used in the chirality analysis of methyl groups. Based on the work of Rose (42), pyruvate carrying a chiral methyl group was incubated by Walsh and Cheung (71) with CO_2 and pyruvate carboxylase in the presence of malate dehydrogenase to give malate. The malate was then incubated with fumarase as previously described to determine the distribution of tritium between the heterotopic hydrogens at C-4.

The two sets of products (**a**, **b**, **c**) and (**d**, **e**, **f**) of Scheme 13 can be distinguished based on a second principle. This involves determining whether the methylene species carrying tritium in the H_A position contains a normal hydrogen as in (**e**) or a deuterium as in (**a**) in the H_B position and conversely whether ^2H or H is present in the H_A position of the H_B-tritiated species. This distinction can be made by tritium NMR spectroscopy. Separate signals for the methylene hydrogens may be observed in the tritium NMR spectrum if they are diastereotopic or are analyzed in the presence of a chiral shift reagent. Analysis of the two coupling constants would immediately indicate which of the tritium atoms is coupled to deuterium and which is coupled to ^1H. This approach allows discrimination between the two enantiomeric starting materials (**1**, **2**, **3**) and (**4**, **5**, **6**) even if the conversion CH_3CO_2H to XCH_2CO_2H does not involve an isotope effect. This type of approach was used by Altman (62) to study cycloartenol biosynthesis in which the C-6α chiral methyl group is converted to the methylene carbon of a cyclopropane. The steric course of the reaction was determined by tritium NMR to be retention of configuration. More recently Aberhart and Tann have applied this methodology to study the dehydrogenation of isobutyryl-CoA by *Pseudomonas putida* (63).

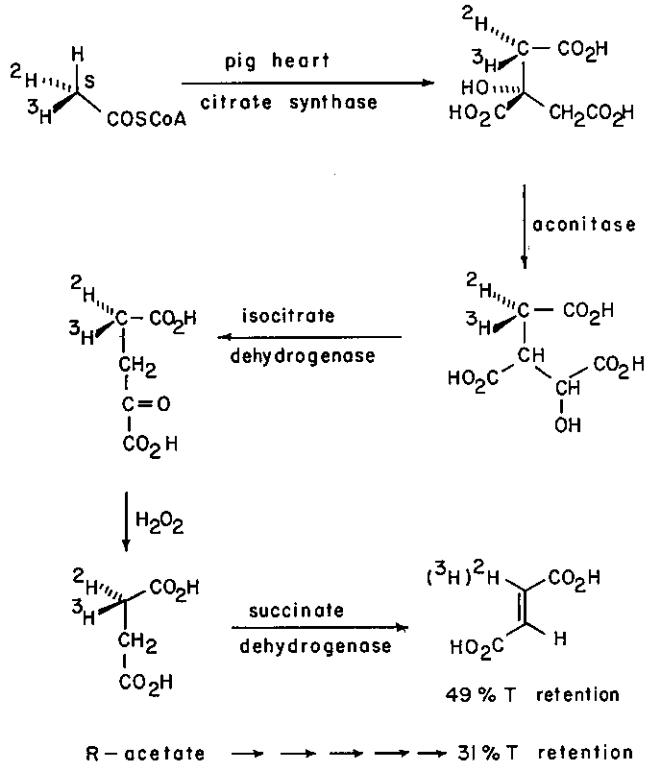
Rétey et al. (72) used this same principle in their work on the stereochemistry of citrate formation from chiral acetyl-CoA with *Si*-citrate synthase (Scheme 15). The chiral methyl group was converted into one of the methylene groups of succinate and the distinction between sets (**1**, **2**, **3**) and (**4**, **5**, **6**) was then based on the known different isotope effects for the removal of pro-*R* (k_H/k_D = 5.3 $\approx k_H/k_T$ = 12) vs. pro-*S* (k_H/k_D = 1.35 $\approx k_H/k_T$ = 1.5) hydrogens of succinate in the succinate dehydrogenase reaction (73). However, the malate synthase/fumarase procedure is clearly the most commonly used method to analyze the configuration and chiral purity of chiral methyl groups.

D. Biochemical Applications of Chiral Methyl Groups

Chiral methyl group methodology has been used to analyze about 70 different stereochemical questions, which can be categorized into three groups according to the type of conversion examined: (i) conversion of a methylene into a methyl group; (ii) conversion of a methyl into a methylene group; (iii) transfer of a methyl group. A detailed discussion of each of these applications is beyond the scope of this chapter. (For comprehensive reviews, see refs. 5, 29, 30.) However, a few examples within each class will be presented to demonstrate the principle involved in that type of conversion.

1. Conversion of a Methylene into a Methyl Group

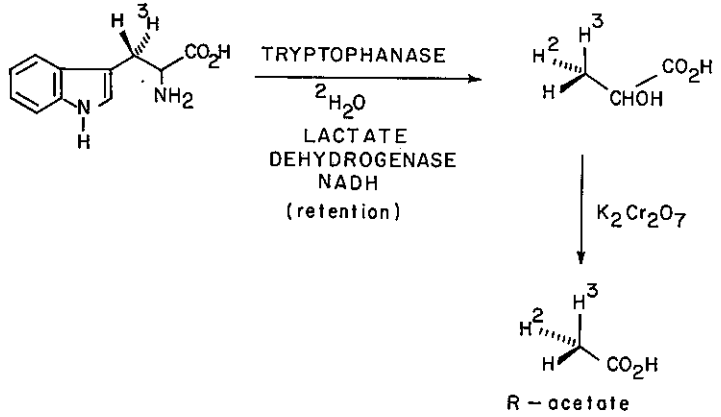
Almost two-thirds of the reported uses of the chiral methyl group methodology fall into this category. The chiral methyl group may be formed from either



Scheme 15

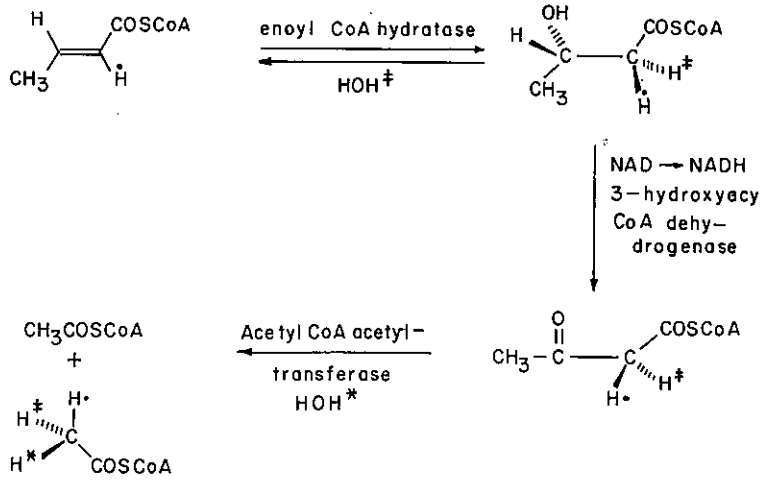
a saturated methylene (sp^3) group in such reactions as retro-Claisen condensations, decarboxylations, pyridoxal phosphate-catalyzed eliminations (both α, β, and β, γ) and others, or an unsaturated (sp^2) group in such reactions as those catalyzed by kinases and double-bond reductases. In order to analyze these reactions and the reactions of the type $CH_3 \rightarrow CH_2$, it is essential that the formation of the methyl or methylene group either be irreversible or be made irreversible by trapping the product, since repeated back reaction would lead to racemization.

If the steric course of the reaction that converts a methylene to a methyl group is known, then the analysis of the chiral methyl group could be used to determine the initial configuration at a stereospecifically labeled methylene group. This approach has been used in our laboratory to analyze the configuration of the methylene group of a stereospecifically tritiated tryptophan (74). The method outlined in Scheme 16 involved conversion of the tryptophan into lactate by incubation with tryptophanase/lactate dehydrogenase in D_2O. This methodology has been applied by Arigoni et al. (see 29) to determine the configuration of a mevalonate-C-5-derived methylene group in linalool.



Scheme 16

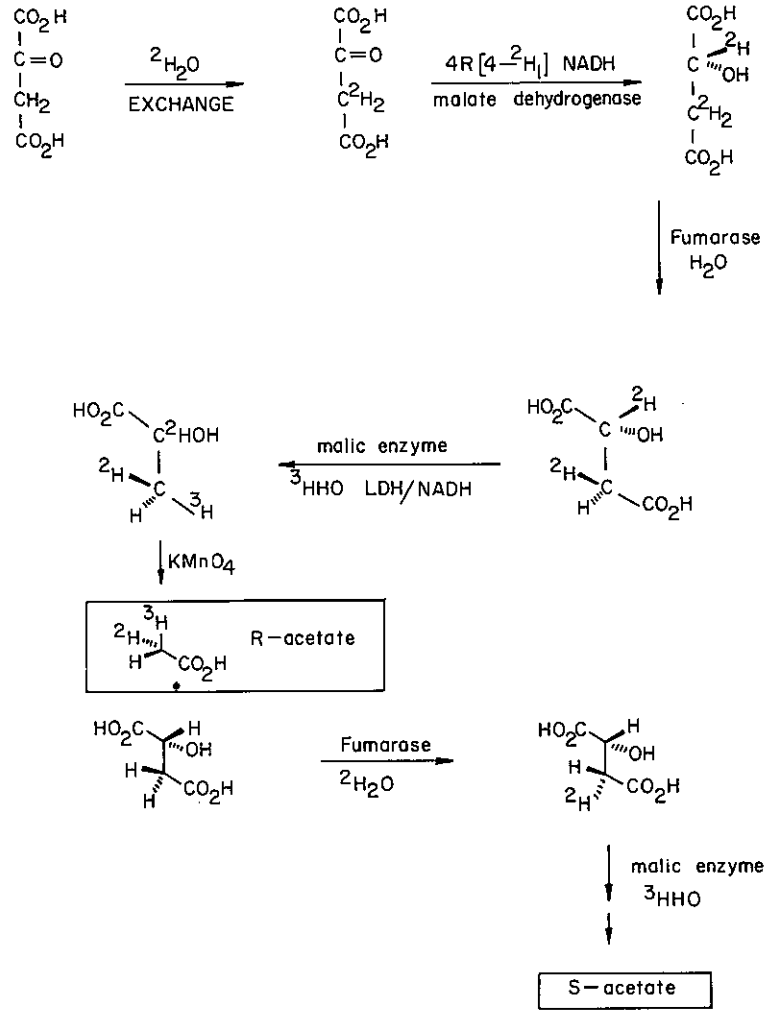
Willadsen and Eggerer (75) have studied the stereochemistry of the enzyme acetyl CoA acetyltransferase, a key enzyme in both the terminal step in C-3 oxidation of fatty acids and the initial step in the biosynthesis of terpenes and steroids. The enzyme, when incubated separately with (2*S*)-[2-$^2H_1$,2-$^3H_1$]acetoacetyl CoA and the (2*R*) isomer gave two moles of acetyl CoA as depicted in Scheme 17. Eggerer et al. (76) utilized the enzyme enoyl CoA hydratase to convert properly labeled crotonyl CoA, via syn addition, to the doubly isotopically labeled 3-hydroxyacyl CoA derivatives needed in this study. A discussion of this unique type of hydration has been presented by Rose (9). The labeled



Scheme 17

3-hydroxyacyl CoA samples were oxidized to the 3-keto acyl derivatives with NAD and 3-hydroxyacyl CoA dehydrogenase. The acetyl CoA samples obtained in the reverse Claisen condensation were then subjected to chirality analysis. This reaction occurs with inversion of configuration as do all the other Claisen reactions thus far studied.

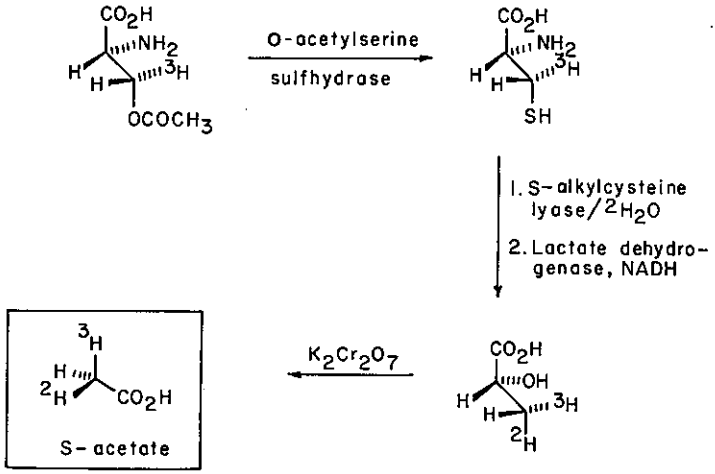
The stereochemistry of the malic enzyme from chicken liver (77), which catalyzes the conversion of malate to pyruvate (Scheme 18) has been studied by Cornforth's group. The two malic acids needed for the study were obtained



Scheme 18

by (i) incubation of fumaric acid in ^2H_2O with fumarase to give (2*S*,3*R*)-[3-$^2H_1$]malic acid, and (ii) by incubation of (2*S*)-[2,3,3-$^2H_3$]malic acid, available by reduction of dideuterated oxaloacetate with malate dehydrogenase and (4*R*)-[4-$^2H_1$]NADH, in H_2O with fumarase, to give (2*S*,3*S*)-[2,3-$^2H_1$]malic acid. The malic acids were incubated in separate experiments with the malic enzyme in ^3HHO and the pyruvate formed captured as lactate by LDH and NADH. The lactate samples were converted by oxidation with $KMnO_4$ to acetate for chirality analysis. The acetate samples (F values 70.5% and 43%) were slightly racemized but the results indicated a net retention of configuration in the decarboxylation reaction, which is in agreement with the finding of Rose on the enzymes from both *E. coli* and pigeon liver (42).

Our laboratory has studied the stereochemistry of methyl group formation in a number of α, β elimination reactions of amino acids catalyzed by pyridoxal phosphate enzymes. The reactions include the conversions of L-serine to pyruvate with tryptophan synthase β_2 protein (78) and tryptophanase (79), of L-serine and L-tyrosine with tyrosine phenol-lyase (80), and L-cystine with *S*-alkylcysteine lyase (81). In the latter study, the stereospecific isotopically labeled L-cystines were obtained enzymatically by incubation of L-serines appropriately labeled in the 3-position with the enzyme *O*-acetyl serine sulfhydrase (82). The serines tritiated in the 3-position were prepared enzymatically starting from [1-^{3}H]glucose and [1-^{3}H]mannose by a sequence of reactions of known stereochemistry (81). The cysteines were then incubated with *S*-alkylcysteine lyase in ^2H_2O as outlined in Scheme 19. The pyruvate was trapped as lactate, which was oxidized with $K_2Cr_2O_7$ to acetate for analysis. Similarly, Cheung and Walsh (71) examined the conversion of D-serine to pyruvate with

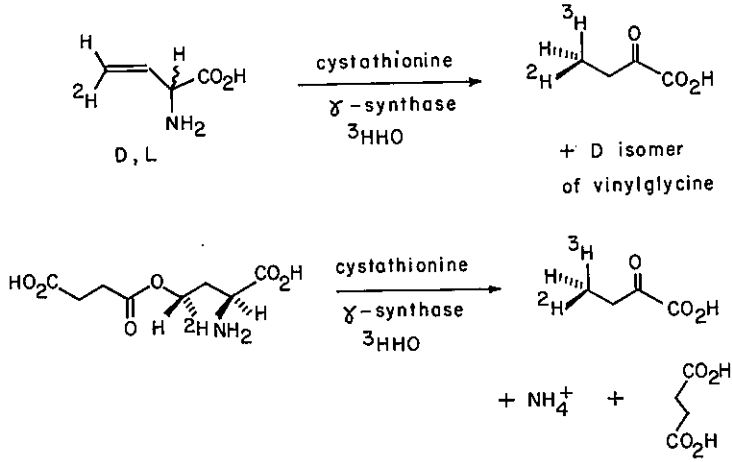


Scheme 19

D-serine dehydratase. This reaction as well as the other α, β elimination reactions of this type studied to date occur with retention of configuration.

Walsh and Chang (83) have investigated the stereochemistry of methyl group formation in β, γ elimination reactions of amino acids catalyzed by pyridoxal phosphate enzymes. They have solved the stereochemistry for both the γ replacement mode (vinylglycine or *O*-succinylhomoserine to cystathionine) and γ elimination mode (vinylglycine or *O*-succinylhomoserine to α-ketobutyrate) of the reaction catalyzed by the bacterial enzyme cystathionine γ-synthase. By using (*Z*)-D,L-[4-^{2}H]vinylglycine and (*E*)-D,L-[4-^{2}H]vinylglycine, one enters the reaction sequence at the halfway point and can solve the stereochemistry of the second half-reactions (either for the elimination mode or the replacement mode). If the reaction is carried out in ^{3}HHO in the absence of L-cysteine, α-ketobutyric acid is formed, which can be converted into acetate for chirality analysis. Likewise, the incubation of either (4*R*)- or (4*S*)-*O*-succinyl-L-[4-^{2}H]homoserines with cystathionine γ-synthase in the absence of cysteine in ^{3}HHO, gives α-ketobutyric acid chiral at C-4. Analysis of the chirality of this methyl group gives the overall stereochemistry of both the elimination and replacement reaction. Therefore, the steric course of the elimination half-reaction may be deduced. The results are shown in Scheme 20, (*Z*)- and (*E*)-[4-^{2}H]vinylglycine are converted to (4*S*)- and (4*R*)-[4-^{2}H,^{3}H]-2-ketobutyrate, respectively, while (4*R*)-*O*-succinyl-L-[4-^{2}H]homoserine yields (4*R*)-[4-^{2}H]cystathionine. The overall γ replacement reaction occurs with retention of configuration at C_4, the γ carbon undergoing substitution.

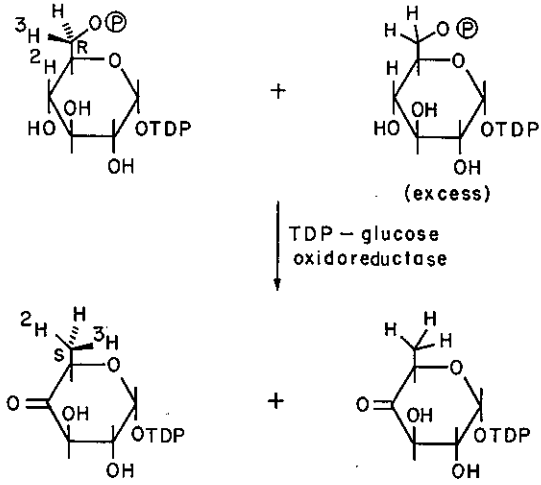
In situations where the third hydrogen required to convert a methylene into a methyl group comes from the same substrate, it is possible to determine by the chiral methyl group methodology whether this hydrogen is transferred inter-



Scheme 20

or intramolecularly. This is based on the fact that a methyl group is chiral only if ^1H, ^2H, and ^3H are present in the same molecule. We used this approach in a study on the steric course of the α,β-elimination of L-serine catalyzed by tryptophan synthase B_2 protein (78) and in some work on the stereochemistry of the enzyme TDP-glucose oxidoreductase. In the latter case (Scheme 21) conversion of (6*S*)- and (6*R*)-TDP-[4-^{2}H,6-^{3}H]glucose into TDP-4-keto-6-deoxyglucose in the presence of a 100-fold excess of unlabeled substrate gave product containing an *R* and an *S* methyl group, respectively, of high chiral purity (84), proving that the hydrogen transfer from C-4 to C-6 is strictly intramolecular. Had it been intermolecular, 99% of the tritiated methyl groups formed would have contained two normal hydrogens and would, hence, have been achiral.

In some recent work Markler and Rétey (85) established the steric course of the phosphoketolase reaction in which carbon atoms 1 and 2 of fructose-6-phosphate are converted to acetyl phosphate. The hydroxy group at C-1 of the sugar is replaced by a solvent hydrogen with inversion of configuration. Two groups have examined the decarboxylation of the acetic acid residues of uroporphyrinogen-III to the methyl groups of coproporphyrinogen-III, one in chicken erythrocytes as part of the heme biosynthetic pathway (86) and the other in *Rhodopseudomonas spheroides* in the formation of bacteriochlorophyll a (87). The latter work also examined the steric course of formation of the ethyl groups from propionic acid residues. All these reactions were found to occur with retention. Several reactions have been found in which a racemic or achiral methyl group is formed. For example, ethanol-amine ammonia-lyase (88) and propanediol dehydrase (89) convert (*R*)- and (*S*)-[2-^{2}H,^{3}H]ethanolamine and (*R*)- and



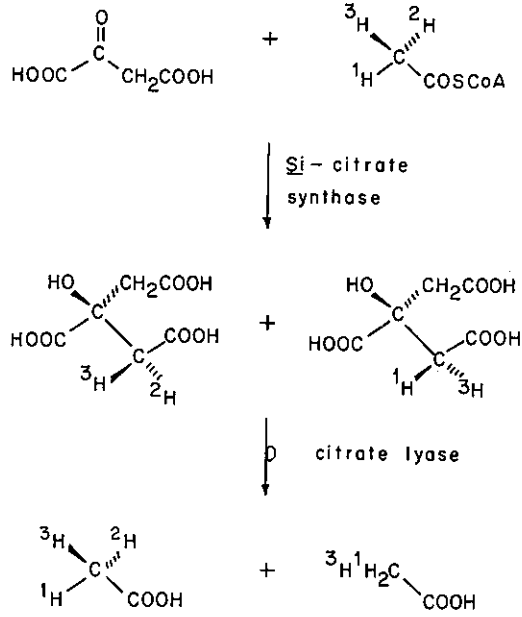
Scheme 21

(*S*)-[1-^{2}H,^{3}H]ethylene glycol, respectively, to racemic acetaldehyde. Racemic acetaldehyde is also formed in the cleavage of 2-deoxyribose 5-phosphate by a specific aldolase (90), and the incubation of stereospecifically labeled dihydroxyacetone phosphate with methylglyoxal synthetase gives racemic methylglyoxal (pyruvic aldehyde) (91).

2. *Conversions of Methyl into Methylene Groups*

The first efforts to utilize the chiral methyl group methodology were studies of the conversion of methyl groups to methylene groups in Claisen-type condensations, such as the conversions of acetate into malate or citrate. The stereochemical composition of the resulting labeled methylene group is then analyzed based on the principles discussed in Sect. II-C, Analysis of Chiral Methyl Groups. In the simplest case, when the conversion involves a substantial primary kinetic deuterium isotope effect, this requires only that one hydrogen of the methylene group is removed or replaced stereospecifically. Numerous studies have made use of this approach; these include several enzymatic Claisen and aldol condensations and carboxylation reactions (see 29, 30). All the Claisen condensations studied proceed with inversion, whereas all the aldol condensations and carboxylations proceed with retention of configuration. In some recent work the same analytical approach was employed by Caspi and co-workers (56, 59) to establish the steric course of hydroxylations at the chiral methyl group of octane. Analysis of the resulting 1-octanol for the configuration at C-1 by oxidation with horse liver alcohol dehydrogenase was complicated by further oxidation of the octanal formed to octanoic acid with a large isotope effect. The authors therefore developed an alternative procedure based on the differential exchange of the (1-pro-*R*-)- and the (1-pro-*S*)-hydrogen of 1-octanol with horse liver ADH and diaphorase (92).

In cases where the abstraction of a hydrogen from the methyl group is not accompanied by a substantial isotope effect one has to rely on methods, like tritium NMR, that can determine whether tritium in a given heterotopic position has deuterium or protium as a neighbor. The studies of Altman and coworkers (62) on cycloartenol formation and by Aberhart and Tann (63) on the dehydrogenation of isobutyryl coenzyme A are pertinent examples. Alternatively, one can convert the chiral methylene group back into a methyl group by a reaction or series of reactions of known steric course, followed by the normal configurational assay of this methyl group. The methylene group will contain two tritiated species, corresponding to **(a + b)** or **(d + e)** in Scheme 13. Reconversion into a methyl group, for example, by stereospecific replacement of X by ^{1}H, will generate a chiral methyl group from **a** and from **d**, mixed with an achiral $CH_2{}^3H$ group formed from **b** or **e**. Barring an isotope effect in the $CH_3 \rightarrow CH_2$ conversion, the regenerated methyl group will have half the chiral purity of the starting methyl group and either the same or opposite configura-



Scheme 22

tion, depending on whether the two reactions occurred with the same or opposite stereochemistry. Hence, if one knows the stereochemistry of one reaction that of the other can be determined. Cornforth, Eggerer, and their colleagues (32) first used this principle in their analysis of the stereochemistry of the *Si*-citrate synthase reaction (Scheme 22). The analytical reaction, catalyzed by citrate lyase, had been shown to proceed with inversion (93), and since the configuration of the chiral acetate eventually obtained was the same as that of the original starting material, the *Si*-citrate synthase reaction involves inversion also.

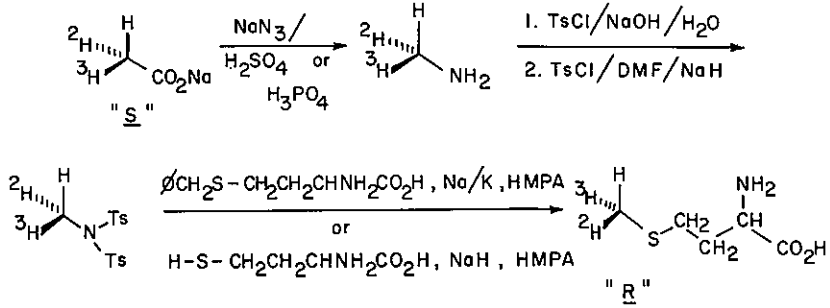
3. *Transfer of Methyl Groups*

Applications of this type are more recent. Elucidation of the steric course of methyl group transfers requires the incorporation of a chiral methyl group of known configuration into the transferring species. Once the methyl group has been transferred to the acceptor species, it is then necessary to convert the chiral methyl group into an analyzable form such as acetate or pyruvate in a series of reactions of known stereochemical consequences. It should be appreciated that both the activation of the chiral methyl group into a transferable moiety and the conversion of the transferred chiral methyl group into acetate or pyruvate frequently require manipulations at the chiral center that may lead to racemization.

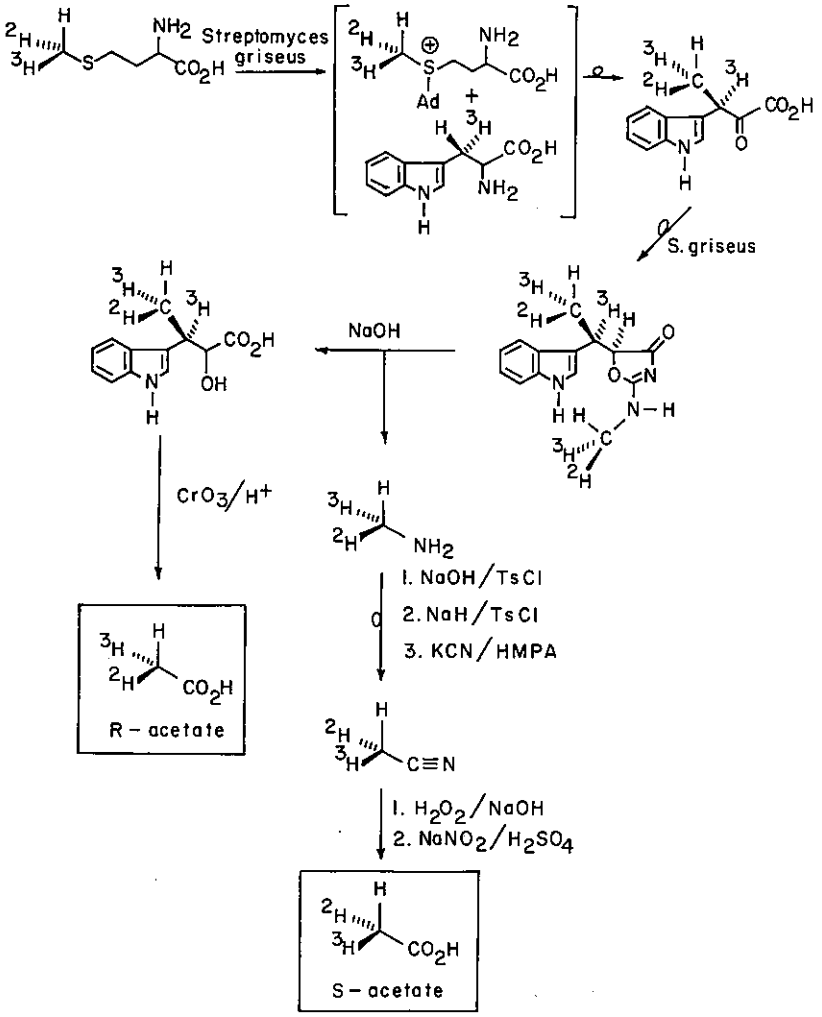
The first account of the transfer of an intact methyl group was reported by Phillips and Clifford (60). Their study determined the steric course of the methyl group rearrangement from C-14 to C-13 in lanosterol biosynthesis. This enzymatic carbonium ion rearrangement occurred with retention of configuration of the migrating chiral methyl group.

Both our laboratory and that of Arigoni have studied a variety of reactions in which the methyl group of *S*-adenosylmethionine (SAM) is transferred to nucleophilic substrates by methyltransferases. The requisite samples of methionine carrying a chiral methyl group were synthesized as shown in Scheme 23 (41, 61). The last step, alkylation of the *S*-anion of homocysteine by methyl-*N*, *N*-ditosylimine, proceeds with inversion at the methyl group, as we deduced indirectly and Arigoni's group demonstrated directly (61).

Our laboratory has utilized these chiral methionine samples to study both the *C*-methylation and *N*-methylation involved in the biosynthesis of the antibiotic indolmycin (41). They were fed to cultures of *Streptomyces griseus* and the isolated indolmycin was degraded as shown in Scheme 24, which shows the steric course of all the reactions involved. We have also examined the stereochemistry of the catechol-*O*-methyltransferase (COMT) reaction (94). For this study, the chiral methionine samples were enzymatically activated to the corresponding *S*-adenosylmethionines, which were then incubated with liver COMT and epinephrine or 3,4-dihydroxybenzoic acid as the acceptor substrate. These reactions as well as the degradation route of the methylated catechols are shown in Scheme 25 (p. 284). All three reactions as well as a fourth studied in our laboratory, the methylation of the carboxyl groups of polygalacturonic acid in pectin formation (95), were found to proceed with inversion of configuration of the methyl group. The same stereochemical result has been obtained for all the methyl transferases studied in Arigoni's laboratory (61), which catalyze methylations as diverse as those of homocysteine, loganic acid, and the corrin ring system of vitamin B_{12}. These stereochemical results, together with evidence from isotope effect studies (96), suggest that in all these cases the methyl group under-



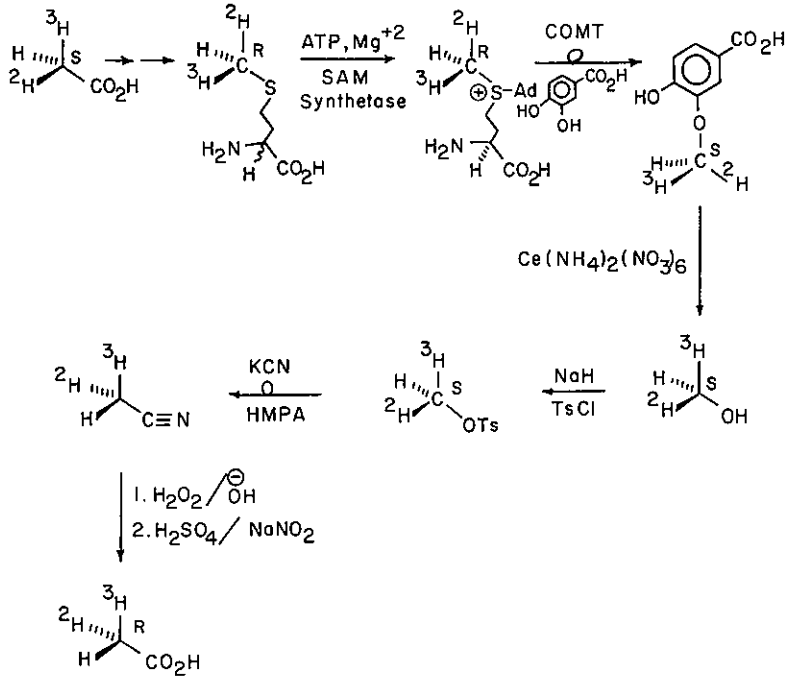
Scheme 23



Scheme 24

goes a single S_N2 type of transfer directly from the sulfur atom of SAM to the nucleophilic acceptor site in the second substrate.

A significantly more complicated situation exists in the case of the side-chain methylation leading to ergosterol, where the initial transfer of the methyl group is followed by a hydride shift from the acceptor carbon and proton loss from the methyl group to give a methylene intermediate (**I**), which is then reduced stereospecifically. The stereochemistry of each of these reaction steps has been determined by Arigoni and co-workers (61, 97), with the outcome summarized in Scheme 26 (p. 285).

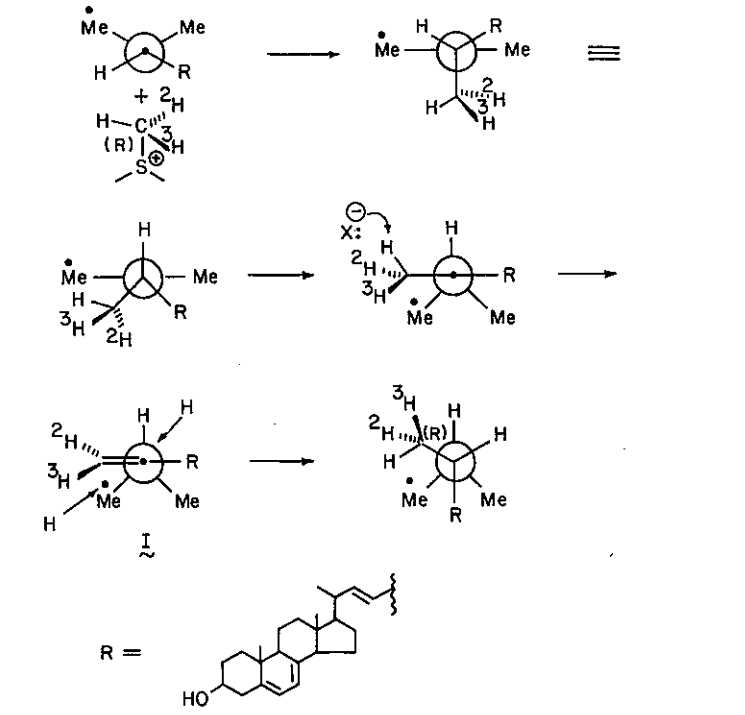


Scheme 25

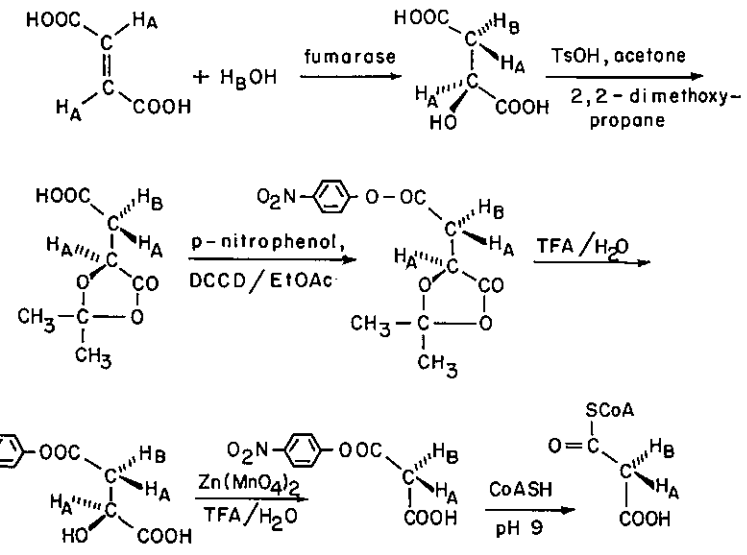
III. CHIRAL MALONATE

As mentioned earlier, no synthesis of chiral malonic acid, one of the simplest and biologically most relevant Caabb systems, has yet been reported. However, Sedgwick et al. (98) have executed a synthesis of (2*R*)- and (2*S*)-[2-^{3}H]malonyl-coenzyme A, the biological activation product of malonate, as shown in Scheme 27. The major obstacle in the preparation and use of this compound is the readily occurring hydrogen exchange at the methylene group which, of course, leads to racemization. Malonic acid itself in D_2O at 35°C shows a $t_{1/2}$ for exchange of the methylene hydrogens of about 90 min; thioesters of malonic acid exchange substantially faster (98). The conditions for the synthesis therefore had to be chosen very carefully. Under the conditions used, only the final transesterification step seems to involve some tritium exchange with the solvent.

The chiral samples of malonyl-CoA were then used to probe the steric course of fatty acid biosynthesis. Again, the experiment was complicated by tritium exchange during the incubation, both before and after the Claisen condensation step, resulting in 51% tritium retention from the *S* isomer and 23% tritium retention from the *R* isomer. Fatty acid biosynthesis involves Claisen condensation of malonyl-CoA with an acyl-CoA ester of $2n$ carbon chain length to



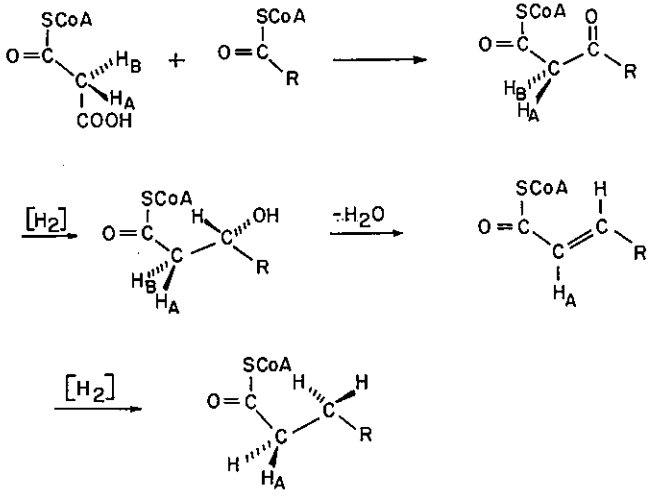
Scheme 26



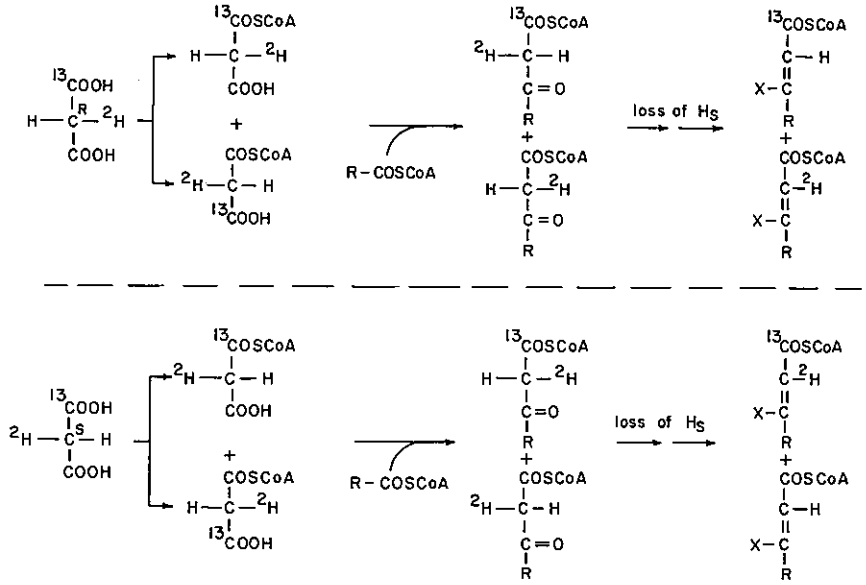
Scheme 27

give a 3-ketoacyl-CoA ester of chain length $2(n+1)$, which is reduced to a β-hydroxyacyl-CoA ester of 3-*R* configuration. Syn elimination of water (99) followed by reduction of the resulting *E* double bond completes the reaction cycle. Since the elimination of water involves removal of H_R from C-2, it follows that the 3-ketoacyl-CoA from (2*S*)-[2-^{3}H]malonyl-CoA carried tritium predominantly in the pro-*S* position, and that from the 2*R* isomer was tritiated predominantly in the pro-*R* position. These results allowed the conclusion that the Claisen condensation constituting the chain elongation step occurs with net inversion at the methylene group of the malonate unit (Scheme 28).

As a result of this work the necessary stereochemical information is now at hand to determine whether a chirally labeled malonic acid sample of unknown configuration ($HO_2C^{\bullet}CHH^{*}CO_2H$) represents the *R* or the *S* enantiomer. There remains, however, the problem of a suitable choice of isotopic labels and of analytical methodology to deduce the stereochemical identity of a given chiral malonate sample from its fate in a metabolic reaction sequence. One approach, outlined by one of us some time ago (100), is shown in Scheme 29. The malonic acid would be chirally labeled by carrying ^{13}C in one carboxyl group and deuterium in one methylene hydrogen. Activation to the coenzyme A ester would occur with equal probability at the labeled and the unlabeled carboxyl group, producing a mixture of two species of malonyl-CoA, which are epimeric at C-2 and which, in addition, differ in the location of the ^{13}C. Conversion of these malonyl-CoA species into fatty acids with yeast fatty acid synthase would ultimately produce from the (*R*)-malonate a fatty acid in which each chain extension unit carries one isotopic label, half being ^{13}C and the other half deuterium. The (*S*)-malonate, on the other hand, would produce fatty acids in which half



Scheme 28



Scheme 29

the chain extension units contain two labels, ^{13}C and deuterium, and the other half are devoid of label. These two sets of products can be distinguished by mass spectrometry or by NMR spectroscopy. Of course, if the configuration of the starting malonates is known, this approach can be used to elucidate the steric course of other biochemical processes utilizing malonate, for example, the biosynthesis of polyketides. The experimental feasibility of this general approach is under investigation.

IV. CHIRAL PHOSPHATE

A. Biological Reactions Involving a Proprochiral Phosphorus Center

1. Types of Reaction

The enzyme-catalyzed reactions involving a proprochiral phosphorus center can be categorized into the following types based on the stereochemistry involved:

(a) $ROPO_2\text{-}OR'$ (prochiral) $\rightleftarrows$ $ROPO_3$ (proprochiral)*

(b) $RO\text{-}PO_3$ (proprochiral) $\rightleftarrows$ $R'O\text{-}PO_3$ (proprochiral)*

(c) $RO\text{-}PO_3$ (proprochiral) $\rightleftarrows$ PO_4 (proproprochiral)*

*Here and in several of the schemes the negative charge (or delocalized charges) on phosphate is omitted.

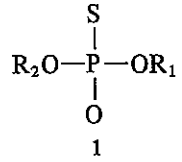
The recent development in the stereochemical study of these reactions has already been covered by several reviews (16, 17, 101–108). The aim of this survey is to provide a general view of the synthesis and configurational analysis of chiral phosphates, and of the significance of stereochemical results in enzyme mechanisms.

Enzymes which catalyze the reaction type (a) include phosphodiesterases, phospholipases (C and D), nucleotidyl transferases, nucleases, and pyrophosphokinases. The type (b) reaction involves mainly phosphokinases and phosphomutases. The hydrolysis of phosphomonoesters (reaction type c) is catalyzed by phosphatases, nucleotidases, ATPases, and so on. Most phosphatases also catalyze the phosphoryl transfer reaction, type (b), if an alcohol is used as an acceptor.

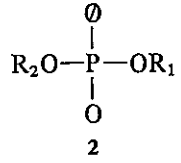
2. *General Approaches to the Elucidation of Reaction Stereochemistry*

The general approach in elucidating the stereochemical course of an enzyme-catalyzed reaction involves the following steps: (i) synthesis of substrates chirally labeled (with ^{17}O, ^{18}O, or S) at phosphorus; (ii) use of chirally labeled substrates to perform the reaction and isolate the product; and (iii) determination of the absolute or relative configuration of the substrate and the product.

A phosphodiester $ROPO_2OR'$ can be made chiral by substituting an oxygen with S (**1**), ^{17}O (**2**), or ^{18}O (**3**). A phosphomonoester $ROPO_3$ can be made chiral by labeling with ^{17}O and S (**4**), ^{18}O and S (**5**), or ^{17}O and ^{18}O (**6**). The inorganic phosphate P_i can be made chiral by labeling with ^{17}O, ^{18}O and S (**7**). (^{18}O is commonly represented by ● and ^{17}O by Ø).



1



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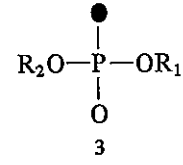

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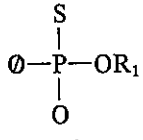

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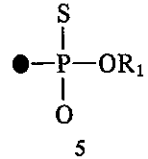

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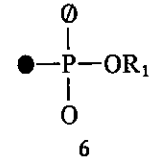

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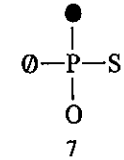


7

The sulfur atom, of course, representing a heteroatomic substitution, is considerably different from oxygen in chemical and physical properties. However, the stereochemical study of biological reactions at phosphorus began with the use of phosphorothioates (21). For the reactions involving a proproprochiral phosphorus center (i.e., inorganic phosphate, P_i), the use of sulfur is unavoidable since there are only three oxygen isotopes ^{16}O, ^{17}O and ^{18}O available (other isotopes of oxygen have very short lifetimes). For other reactions, both

chiral phosphates and chiral phosphorothioates have been employed for stereochemical study. In general, the use of chiral phosphates requires more sophisticated synthetic and analytical procedures, and gives more authentic results. Use of chiral phosphorothioates results in a decrease in reaction rates to $\lesssim$10% (in many cases $\lesssim$1%). Although it has been questioned whether the stereochemical course elucidated with phosphorothioates reflects the real mechanism of a particular enzyme, for all the enzymes that have been investigated by both chiral phosphates and chiral phosphorothioates (see tables in later sections), the stereochemical outcome is the same without exception. In addition, the use of phosphorothioates offers some advantages. It reduces a prochiral center to a chiral center, thus generating two separable diastereomers for most biphosphates. It also reduces a proprochiral center to a prochiral center and thus allows stereospecific phosphorylations at one of the two heterotopic oxygens. These stereoisomers of phosphorothioates are useful as stereochemical probes for the mechanism of enzyme catalysis.

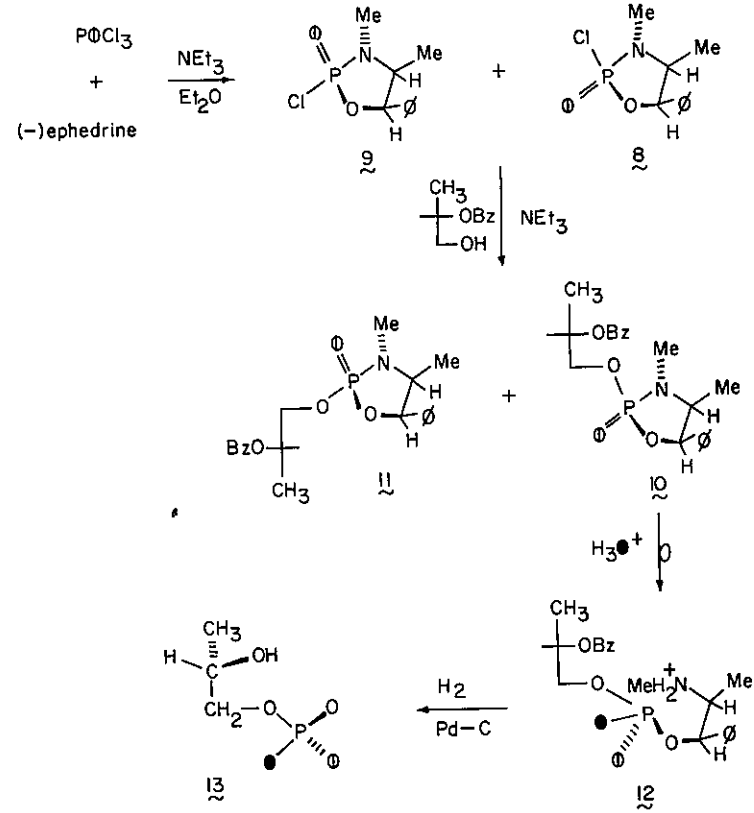
B. Synthesis of Chiral Phosphates

Description of the synthesis of phosphodiesters (prochiral phosphorus center) is beyond the scope of this chapter. Only the chemical synthesis of chiral phosphomonoesters (**4–6**) and chiral inorganic phosphates (**7**) are discussed. The chiral phosphates or chiral phosphorothioates obtained from enzyme reactions are not described in this section.

1. Synthesis of Chiral [^{16}O,^{17}O,^{18}O]Phosphomonoesters

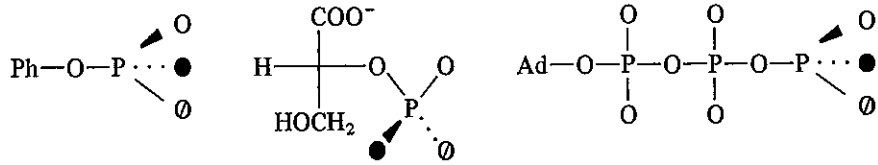
Two general synthetic procedures for chiral [^{16}O,^{17}O,^{18}O]phosphomonoesters have been reported, one by Knowles' group (22, 109) and the other by Lowe's group (23, 110).

The Knowles procedure is outlined in Scheme 30. Reaction of $P^{17}OCl_3$ with (-)-ephedrine yielded two diastereomeric chloro adducts **8** and **9** (approximately 9/1), which were then converted to **10** and **11** by reaction with 2-benzyl-(*S*)-propane-1,2-diol. The alcoholysis is known to proceed with retention of configuration (111). Chromatographic separation gave separate diastereomers **10** and **11** in 65% and 7% yields, respectively. Acid-catalyzed ring opening of **10** ($H_2{}^{18}O/CF_3COOH$) gave **12**. Hydrogenolysis of **12** yielded [^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol (**13**) in 72% yield from **10**. On the basis of the work of Inch and co-workers (111), **13** should have the *R* configuration at phosphorus, and this has been unequivocally established by the stereochemical analysis discussed in Sect. IV-C. By inverting the order in which ^{17}O and ^{18}O are introduced, the opposite isomer, [1(*S*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol can be synthesized. In addition, phenyl [^{16}O,^{17}O,^{18}O]phosphate (**14**) (112) and 2-[^{16}O,^{17}O,^{18}O]phospho-D-glycerate (**15**) (113) have been synthesized by this



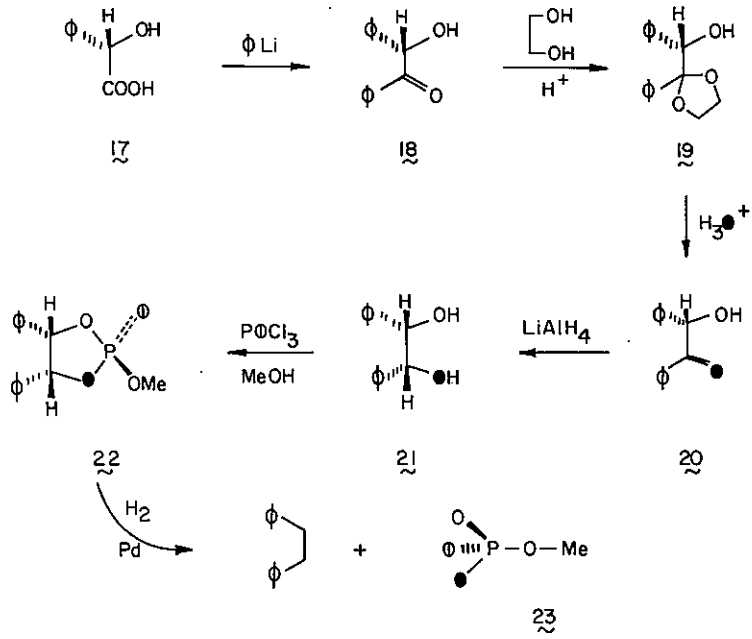
Scheme 30

route, and adenosine [γ-^{16}O,^{17}O,^{18}O]triphosphate (**16**) has been synthesized by a slight modification of Scheme 30 (114).



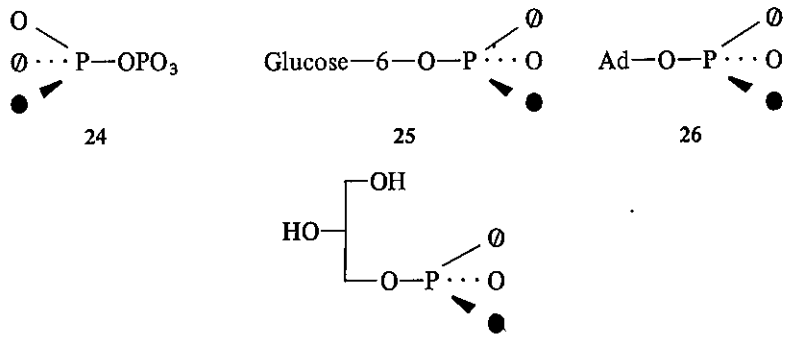
14 **15** **16**

The procedure of Lowe (110) is outlined in Scheme 31. Reaction of (*S*)-mandelic acid (**17**) with phenyllithium yielded (*S*)-benzoin (**18**). Acid-catalyzed ketalization of **18** with ethylene glycol gave **19**, which was converted back to [(*S*)-^{18}O]benzoin (**20**) by acidic hydrolysis in $H_2{}^{18}O$. Reduction of **20** with $LiAlH_4$ at 0°C (115) gave exclusively (1*R*,2*S*)-1,2-[1-^{18}O]dihydroxy-1,2-diphenylethane (**21**), according to Cram's (chelate) Rule. Treatment with



Scheme 31

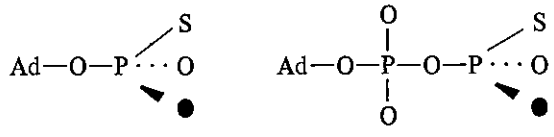
$P^{17}OCl_3$ followed by methanolysis in pyridine gave **22** as a single crystalline isomer. Catalytic hydrogenolysis of **22** gave methyl [(*S*)-^{16}O,^{17}O,^{18}O]phosphate (**23**). By use of other alcohols to replace methanol, the following chiral [^{16}O, ^{17}O,^{18}O]phosphate monoesters have also been synthesized (110): inorganic [^{16}O,^{17}O,^{18}O]pyrophosphate (**24**), glucose 6-[^{16}O,^{17}O,^{18}O]phosphate (**25**), adenosine 5′-[^{16}O,^{17}O,^{18}O]phosphate (**26**), adenosine 5′-[γ-^{16}O,^{17}O,^{18}O]triphosphate (**16**), *sn*-glycerol-3-[^{16}O,^{17}O,^{18}O]phosphate (**27**), and 2-[^{16}O,^{17}O, ^{18}O]phospho-(*R*)-glycerate (**15**). The isomers of opposite configuration at phosphorus can be obtained by inverting the order in which ^{17}O and ^{18}O are introduced.



In the preliminary report of the synthesis (23) **22** was erroneously assigned the opposite configuration at phosphorus, which resulted in the assignment of *R* configuration to **23** and other chiral phosphomonoesters. The authors have corrected the assignment recently (116), and the configurations reported in the full paper (110) and in Scheme 31 are the correct ones.

2. *Synthesis of Chiral Phosphorothioates*

A number of chiral [^{18}O]phosphorothioate monoesters (compound **5**) have been synthesized, and these syntheses have been described in detail in several reviews (102, 103, 108). In this section only the most commonly used compounds, chiral [α-^{18}O]AMPS (**28**), chiral [β-^{18}O]ADPβS (**29**), and chiral [γ-^{18}O]ATPγS (**30**) are discussed.



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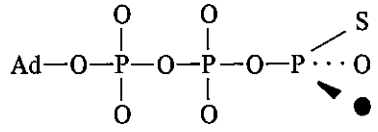

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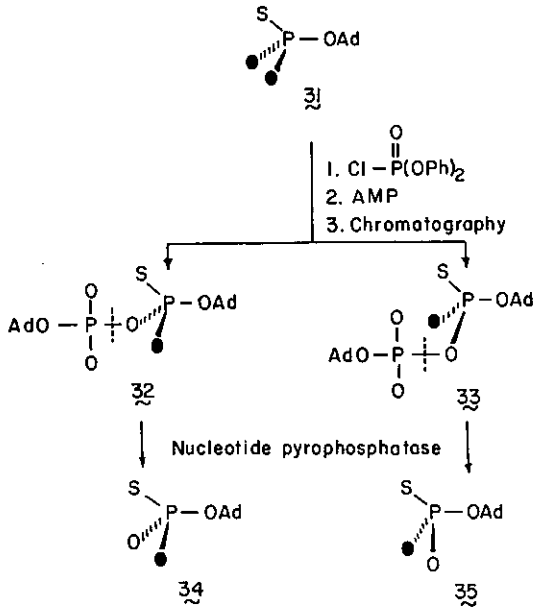
(*R*)- and (*S*)-[α-^{18}O]AMPS have been prepared by Lowe's group by a chemical synthetic procedure analogous to that shown in Scheme 31 (117). However, two simpler procedures based on a combination of chemical and biochemical reactions have been reported by Frey and co-workers (118) (Scheme 32) and by Tsai (25, 119) (Scheme 33). In Scheme 32, [α-$^{18}O_2$]AMPS (**31**), prepared from adenosine, $PSCl_3$, and $H_2{}^{18}O$ (120), was condensed with AMP by the procedure of Michelson (121) to give the two diastereomeric dinucleotides **32** and **33**, which were separated by chromatography. Hydrolysis of **32** and **33** by *C. adamanteus* nucleotide pyrophosphatase gave the two isomers of [α-^{18}O]AMPS **34** and **35**, respectively. The configuration of **34** and **35** at Pα can be determined by procedures to be discussed in Sect. IV-C. In an alternative synthesis (Scheme 33) (25, 119), [α-$^{18}O_2$]**31** was chemically phosphorylated to give a diastereomeric mixture of [α-^{18}O]ADPαS (**36**). Incubation of **36** with pyruvate kinase followed by chromatographic separation (122) gave (*S*)-[α-^{18}O]ATPαS (**37**) and unreacted (*R*)-[α-^{18}O]ADPαS (**38**). Hydrolysis of **37** and **38** with calf intestine alkaline phosphatase produced (*S*)-[α-^{18}O]AMPS (**39**) and (*R*)-[α-^{18}O]AMPS (**40**), respectively, with known configuration at phosphorus. The hydrolysis was easily



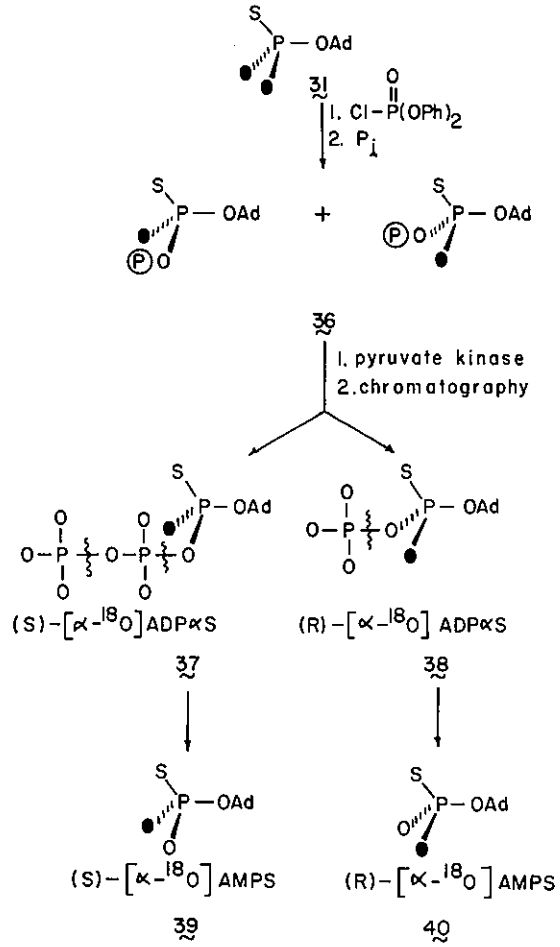
Scheme 32

arrested at the AMPS stage since most phosphorothioate monoesters are poor substrates for alkaline phosphatase.

(*R*)- and (*S*)-[β-^{18}O]ADPβS were synthesized by Richard et al. (123) according to Scheme 34. Condensation of [α-$^{18}O_2$]AMPS (**31**) with 2′,3′-methoxymethylidene-AMP (**41**) by the Michelson procedure gave two separable diastereomeric dinucleotides **42** and **43**. The configurations at the chiral phosphorus centers of **42** and **43** were assigned by chemically degrading each isomer to the corresponding [α-^{18}O]ADPαS and determining its Pα configuration by ^{31}P NMR. The diastereomers **42** and **43** of known configuration were then converted to (*R*)-[β-^{18}O]ADPβS (**44**) and (*S*)-[β-^{18}O]ADPβS (**45**), respectively, by chemical removal of the unblocked adenosyl group and deblocking of the protected nucleoside.

Chiral [γ-^{18}O]ATPγS was first synthesized by Richard and Frey (124) as shown in Scheme 35. The (*S*)-[α-^{18}O,$\alpha\beta$-^{18}O]ADPαS* (**46**), prepared from enzyme-catalyzed stereospecific phosphorylation of **31**, was condensed with 2′,3′-methoxymethylidene-AMP (**41**) to give the dinucleotide **47**. Chemical re-

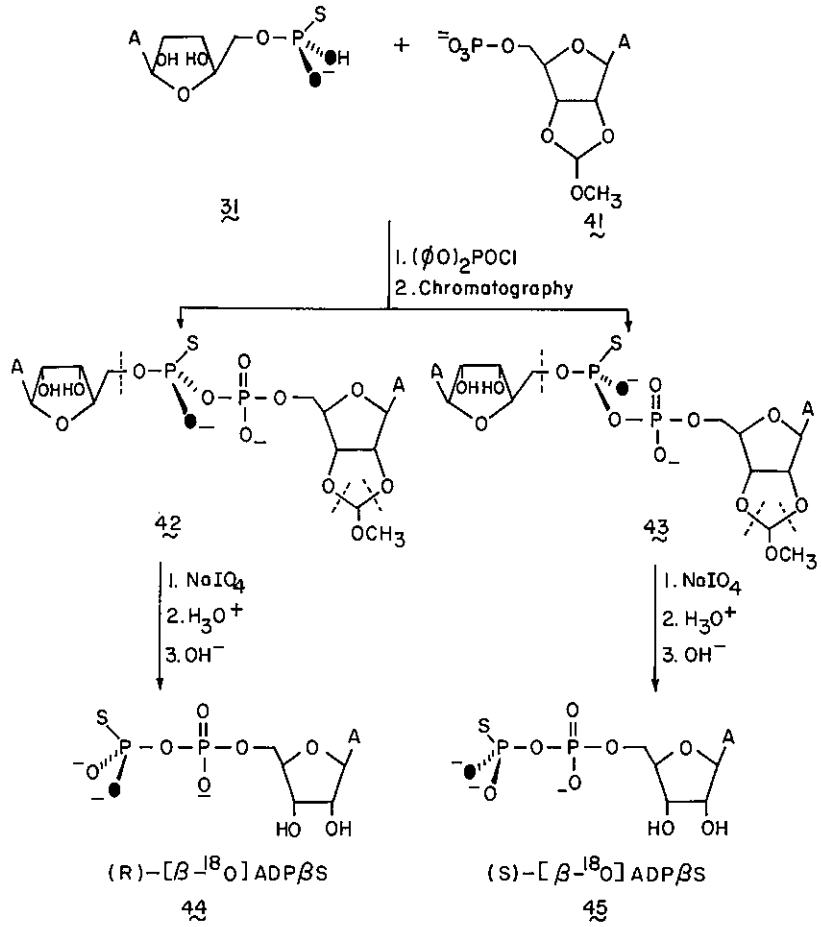
*Oxygen atoms bonded to only one phosphorus atom, i.e., nonbridged oxygens, are indicated by the Greek letter identifying the phosphorus atom to which they are attached. Similarly, bridged oxygens are designated by the two Greek letters identifying the attached phosphorus atoms.



Scheme 33

moval of the unblocked adenosyl group and the methoxymethylidene group gave (*R*)-[γ-^{18}O,$\beta\gamma$-^{18}O]ATPγS (**48**).

Synthesis of chiral inorganic [^{16}O,^{17}O,^{18}O]phosphorothioates (Ps_i) of known configuration was first reported by Webb and Trentham (24) according to Scheme 36. The two intermediates **49** and **50** were obtained analogously to **37** and **38**, respectively, in Scheme 33, except that ^{17}O-labeled P_i was used. After transferring its γ-phosphoryl group to glucose (catalyzed by hexokinase), **49** was treated with IO_4^- followed by OH^- to give the pyrophosphate **51**. The same treatment of **50** gave the enantiomer **52**. Hydrolysis of **51** and **52** by inor-



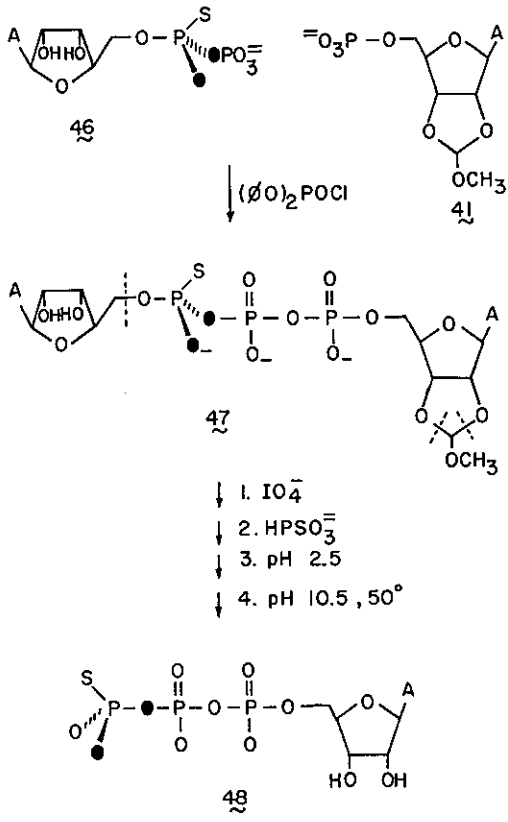
Scheme 34

ganic pyrophosphatase gave (*S*)-[^{16}O,^{17}O,^{18}O]Ps$_i$ (**53**) and (*R*)-[^{16}O,^{17}O,^{18}O]-Ps$_i$ (**54**), respectively.

C. Configurational Analysis of Chiral Phosphates

1. ^{18}O Isotope Shift and ^{17}O Quadrupolar Effect in ^{31}P NMR

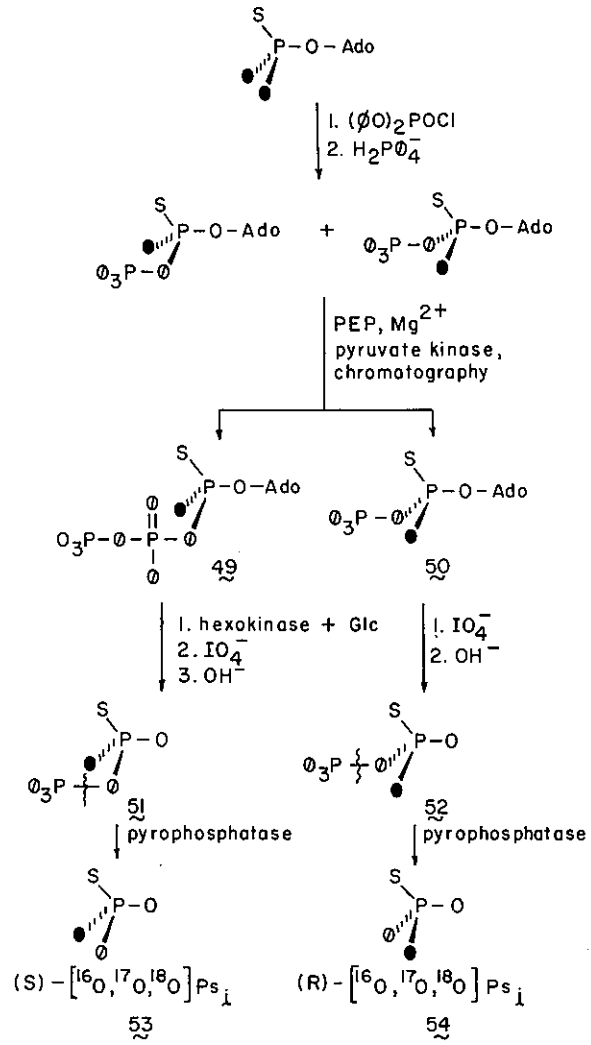
While the configurational analysis of chiral methyl groups is based on the radioactivity of ^{3}H and the kinetic isotope effect k_H/k_D, neither factor is applicable to a chiral phosphoryl group. Both ^{17}O and ^{18}O are stable isotopes, and



Scheme 35

the kinetic isotope effect is much smaller than 5%. In the early stages (ca. before 1979), the main tool in the configurational analysis of chiral phosphate was mass spectrometry, which requires relatively elaborate derivatization and degradation of the compound. Fortunately, ^{31}P NMR methods based on the ^{18}O isotope effect (125) and the ^{17}O quadrupolar effect (126) have been developed, which have now largely, if not completely, replaced the mass spectral method.

The ^{18}O isotope shift and the ^{17}O quadrupolar effect in ^{31}P NMR and their application in phosphorus stereochemistry have been reviewed recently by Cohn (106) and Tsai (105, 127). Figure 1 shows the ^{31}P NMR spectrum of $H_3P^{17}O_4$ (40 atom % ^{17}O). The spectrum consists of a "broad" signal due to the $^{31}P—^{17}O$ species and a "sharp" signal due to the residual non-^{17}O-labeled species. Since the ^{17}O-enriched water always contains some ^{18}O ($^{18}O/^{17}O = 0.67$ in this case), the "sharp" signal contains both ^{16}O and ^{18}O species, as shown by the expanded



Scheme 36

spectrum in the inset. In this compound, the ^{31}P NMR signal of the ^{18}O-labeled species is shifted upfield by 0.020 ppm per ^{18}O atom. The magnitude of the ^{18}O isotope shift in ^{31}P NMR is defined as the "S" value (127). The S value for a P=O double bond is 0.038–0.044 ppm, whereas that for a P—O single bond is 0.015–0.025 ppm. Bonds with a partial double bond character have S values between these two extremes. The correlation between the S value and the bond

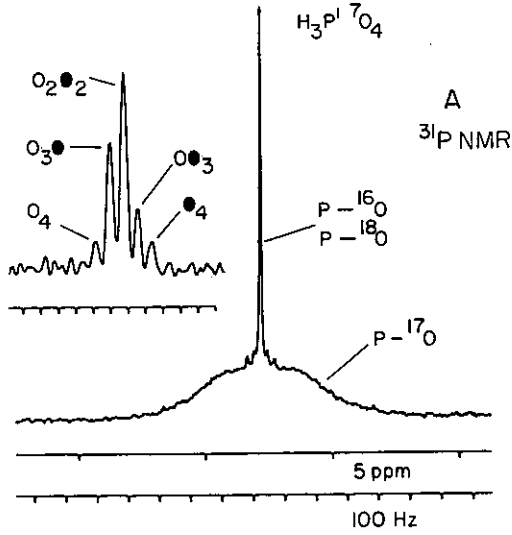


Figure 1. (A) ^{31}P NMR spectrum of 50 mM $H_3P^{17}O_4$ (40 atom % ^{17}O) in D_2O, pD = 1.8, at 81.0 MHz. Spectral parameters: Acquisition time 4.1 sec, delay time 1.0 sec, spectral width 2 KHz, 70° pulse, line broadening 2.0 Hz, 1600 scans. The insets show the expanded spectrum of the sharp peak, processed with Gaussian multiplication (LB = −2, GB = 0.2). Chemical shift: 0.09 ppm downfield from 1 *M* H_3PO_4.

order provides a convenient way to distinguish P—O—X (X = P, C, Si, etc.) bridging ^{18}O from a P=O (or P ⋯ O) nonbridging ^{18}O.

The "broadening" of the ^{31}P NMR signal by directly bonded ^{17}O nuclei is due to "scalar relaxation of the second kind," as termed by Abragam (128). Although the ^{31}P—^{17}O interaction depends greatly upon the quadrupolar relaxation time of ^{17}O (105, 127), it has been shown that the "line broadening effect" is present for most small biophosphate molecules in solution (129). The "broad signal" due to ^{31}P—^{17}O species is not always observable. However, the bonding of ^{31}P to ^{17}O can always be detected by a decrease in the apparent intensity of the "sharp" ^{31}P NMR signal (which represents the residual non-^{17}O species). In other words, the net effect of ^{17}O is that it "quenches" the ^{31}P NMR signal of all ^{31}P—^{17}O species, an important property upon which the configuration analysis of chiral phosphates is based.

2. *Configurational Analysis of Chiral Phosphates and Phosphorothioates*

Since most biophosphate compounds contain other chiral centers, the stereoisomers of type **1** (p. 288) can be distinguished simply based on ^{31}P chemical shifts.

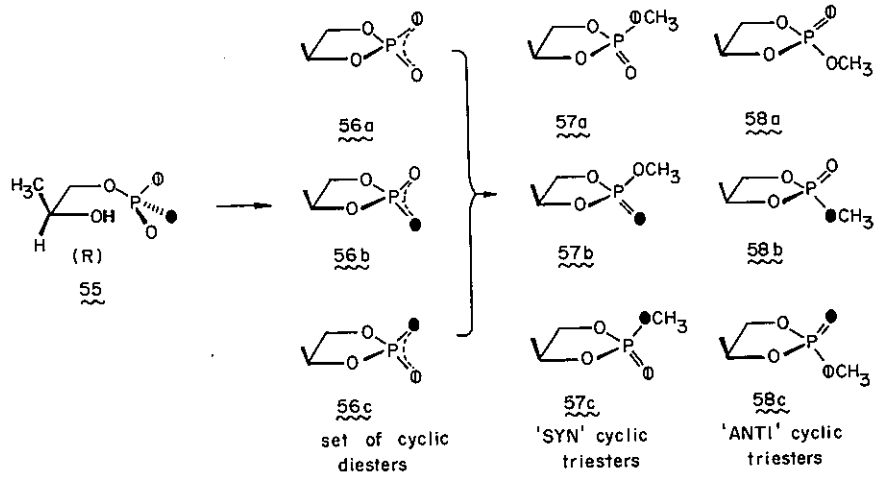
The configurational analysis of compounds **2** to **5** involves determining whether the labeled oxygen (^{17}O in **2** and **4**, ^{18}O in **3** and **5**) occupies the pro-*R* or pro-*S* position. In most cases, the compound is first stereoselectively derivatized at one of the two oxygens. The position of ^{17}O or ^{18}O is then determined by one of three methods: (i) stereoselective derivatization and degradation followed by mass spectral analysis; (ii) isotope shift effect in ^{31}P NMR; or (iii) ^{17}O quadrupolar effect in ^{31}P NMR. Since these methods are straightforward, no further discussion is provided here. Specific examples will be presented in Section IV, E.

The chiral [^{16}O,^{17}O,^{18}O]phosphomonoester **6** and the chiral [^{16}O,^{17}O, ^{18}O]Ps_i **7** both have a chiral phosphorus center due to three oxygen isotopes. Three techniques have been used to characterize the configuration at phosphorus: circular dichroism (CD) (23), mass spectrometry (109), and ^{31}P NMR (24, 25, 130).

Unlike a chiral methyl group, in which the tritium is isotopically dilute, a chiral phosphoryl group can be obtained with >50% purity ("purity" is defined as the percentage of the chirally labeled species, i.e., the M + 3 species) and with >95% chirality ("chirality" is defined as the optical purity of the chirally labeled species) (119). Therefore it is not impractical to ask whether the chiroptical properties of a chiral phosphoryl group can be observed. Cullis and Lowe (23) have reported a CD curve of methyl [(*S*)-^{16}O,^{17}O,^{18}O]phosphate with a maximum at 208 nm ($\Delta\epsilon = 2.7 \times 10^{-3}$). Although this is a direct way to measure the chirality of a phosphoryl group, it would be useful only in compounds which do not contain any other chiral centers. Furthermore, the CD curve of only one enantiomer of the above compound has been reported. Results based on CD should be evaluated with reservation until they can be confirmed with the opposite isomer.

Knowles and co-workers first developed a mass spectral analysis to determine the configuration of a chiral [^{16}O,^{17}O,^{18}O]phosphomonoester (22, 101, 109). As shown in Scheme 37, [1(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1, 2-diol (**55**) is first "cyclized", with inversion of configuration, to the 1,2-cyclic phosphate, which consists of an equimolar mixture of three isotopically different species **56a**, **56b**, and **56c**. Methylation of the cyclic phosphate **56** occurs on either of the exocyclic oxygens and gives two sets ("syn" and "anti") of diastereomeric phosphotriesters **57** and **58**, respectively. **57** and **58** are then separated chromatographically and analyzed by metastable ion mass spectrometry. Since this method is not now generally used in configurational analysis, it is not discussed in detail here. Interested readers are referred to the full description of the method by Buchwald et al. (101). In summary, the two sets of syn isomers (**57**, and the corresponding set from the opposite isomer of **55**) are differentiated based on the relationships between individual daughter ions and their parents.

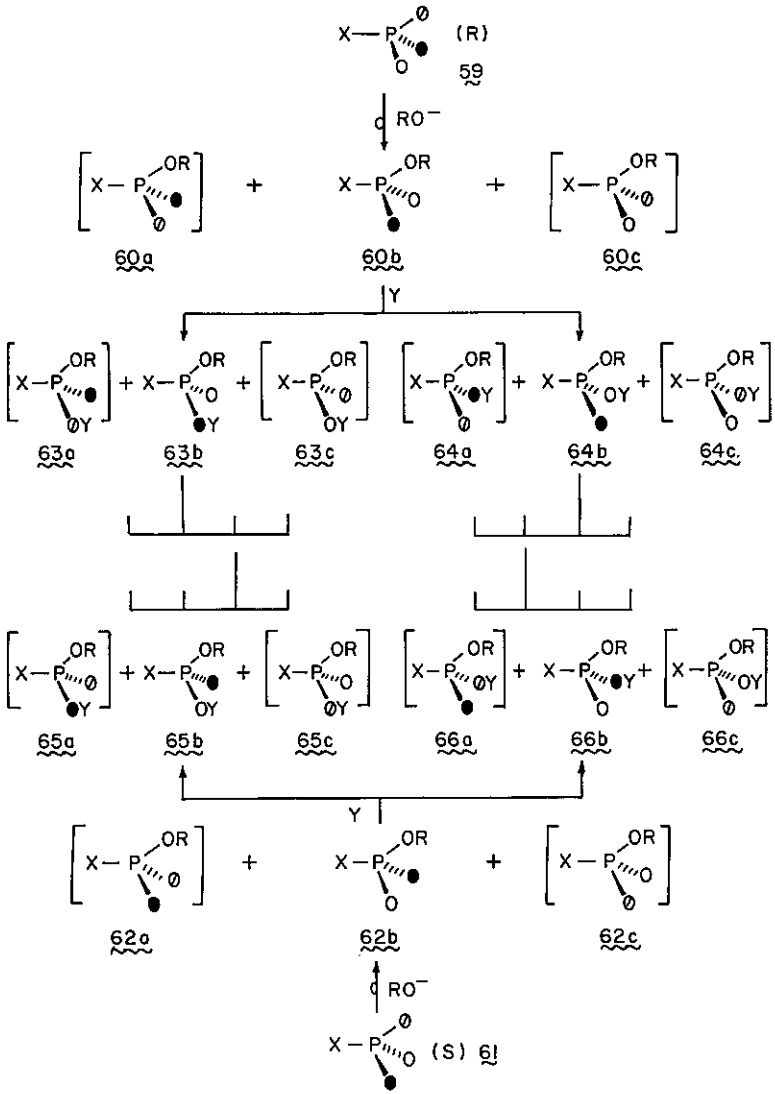
As the $^{31}P(^{18}O)$ and $^{31}P(^{17}O)$ NMR techniques became available later, it was



Scheme 37

obvious to a number of researchers that the two sets of triesters **57** and **58** can be distinguished by ^{31}P NMR. Each set of isomers consists of two species bearing a ^{17}O and a third species which contains only ^{16}O and ^{18}O. Since ^{17}O is expected to "quench" the ^{31}P NMR signal, only the species without ^{17}O (**57b** and **58b**) will show sharp ^{31}P NMR signals. In the syn isomer **57b**, the ^{18}O is nonbridging ($P=^{18}O$), thus causes a larger isotope shift (S = 0.043 ppm). In the anti isomer **58b**, the ^{18}O is located at the P—O—C bridging position, thus causes a smaller isotope shift (S = 0.018 ppm). An opposite pattern should be observed for the opposite isomer of **55**. In addition, the two diastereomers **57** and **58** show different chemical shifts, which allows a direct analysis of the mixture **57** and **58** without chromatographic separation. In reporting the ^{31}P NMR analysis of the configuration of **55** (130), the authors stated that the NMR method is "simpler both conceptually and practically".

The ^{31}P NMR method has now become a standard method of configurational analysis of chiral phosphomonoesters including [^{16}O,^{17}O,^{18}O]phospho(*S*)-propane-1,2-diol (**55**), adenosine 5′-[^{16}O,^{17}O,^{18}O]phosphate (AMP) (131), 3′-deoxyadenosine 5′-[^{16}O,^{17}O,^{18}O]phosphate (dAMP) (132), thymidine 3′-[^{16}O,^{17}O,^{18}O]phosphate (3′-TMP) (133), glucose 6-[^{16}O,^{17}O,^{18}O]phosphate (25) (134), dipalmitoyl [^{16}O,^{17}O,^{18}O]phosphatidic acid (135), and [^{16}O,^{17}O,^{18}O]-thiophosphate (Ps_i) (24, 25, 129). The general approach for the ^{31}P NMR analysis of a chiral phosphoryl group is illustrated in Scheme 38. The first required step is the displacement of one of the three oxygen isotopes by a process of known stereochemistry. Starting with **59**, (in which X = OR or S, with *R* configuration), and assuming the displacement by RO^- proceeds with inversion, a mixture of three inseparable, isotopically different species (**60a**, **60b**, **60c**) is



Scheme 38

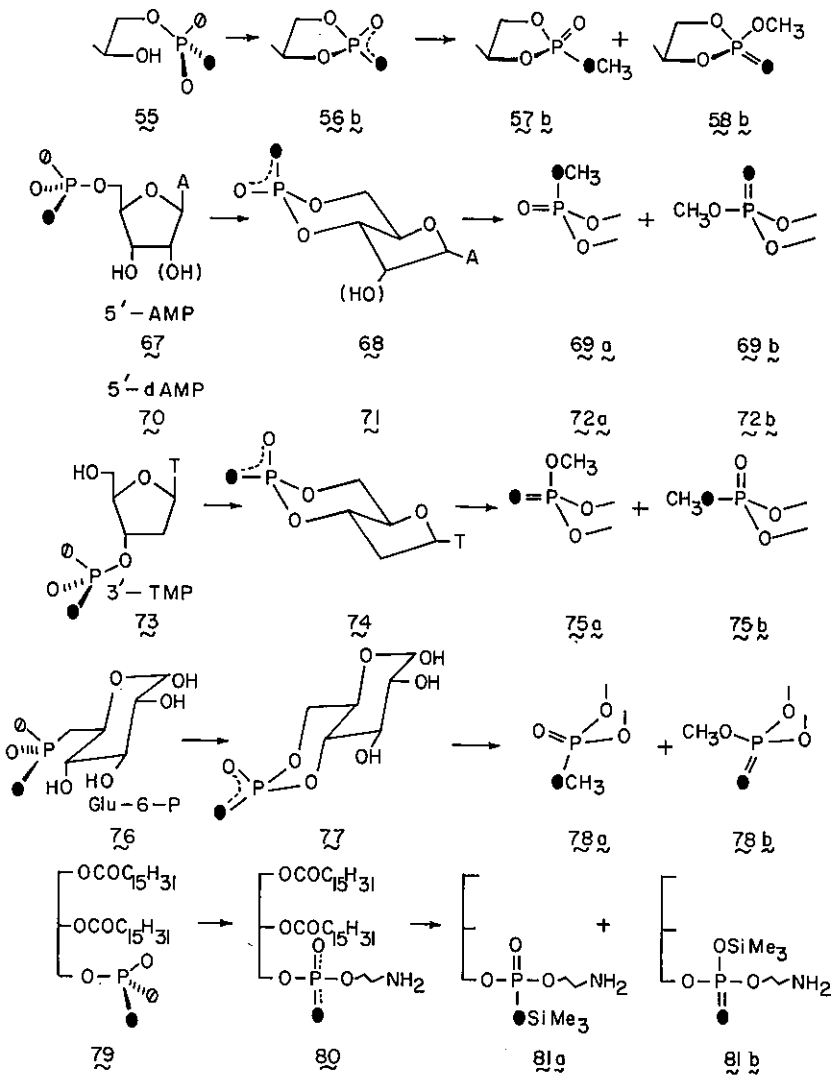
obtained. Among them, two (those shown in brackets) contain a ^{17}O isotope, which should quench the corresponding ^{31}P NMR signals. Only **60b**, which contains ^{16}O and ^{18}O, with ^{18}O at the pro-*R* position, should give a sharp, unquenched ^{31}P NMR signal. Analogously, the *S* isomer **61** should produce a non-^{17}O-containing species **62b** in which ^{18}O is located at the pro-*S* position (assuming X has highest sequence rule priority).

Thus, determination of whether ^{18}O is located at the pro-*R* and pro-*S* position would reveal the configuration of chiral Ps_i or chiral phosphate monoesters. A general way to achieve this is to derivatize the pro-*R* or pro-*S* oxygen stereospecifically. However, a nonstereospecific derivatization (by a group Y) is sufficient if a chiral center is present in either X or R. Under these conditions two sets of diastereomers **63** and **64** will be generated from **60**. Since diastereomers **63** and **64** most likely will have different ^{31}P chemical shifts, they can be analyzed directly without separation. Among the three species of **63**, only **63b** contains no ^{17}O and is observable by ^{31}P NMR. Since the ^{18}O isotope in **63b** is located at the P—O—Y bridging position, it is expected to cause a smaller isotope shift (0.015–0.025 ppm). On the other hand, the ^{18}O isotope in **64b** is located at the P=O nonbridging position, which should cause a larger isotope shift (0.038–0.044 ppm), as shown in the bar graphs of the expected spectra. In real experiments, however, a chiral phosphoryl group is not 100% pure. The observed spectra generally consist of 4 peaks for **63** and 4 peaks for **64**, due to unlabeled species, species with a bridging ^{18}O, species with a nonbridging ^{18}O, and species with both bridging and nonbridging ^{18}O. The opposite ^{31}P NMR pattern should be observed for the opposite isomer **61**.

Scheme 39 shows the procedures of chemical derivatization of the chiral phosphomonoesters analyzed by the ^{31}P NMR method. The procedures for phosphopropane-1,2-diol (**55**), 5′-AMP (**67**), 5′-dAMP (**70**), 3′-TMP (**73**), and glucose-6-phosphate (**76**) all involve a cyclization step (with inversion of configuration at phosphorus), followed by methylation or ethylation. The dipalmitoylphosphatidic acid (**79**) was first converted to dipalmitoylphosphatidylethanolamine (135), which was then silylated and analyzed by ^{31}P NMR (136).

For the chiral Ps_i, the two main steps required were already available separately in the literature (123, 124, 137), as shown in Scheme 40. The stereochemical course of each step in Scheme 40 had been elucidated earlier (123, 124), except that of phosphoglycerate kinase, which was established by Webb and Trentham by use of synthetic chiral Ps_i of known configuration (24) on the basis of the same NMR analysis discussed below.

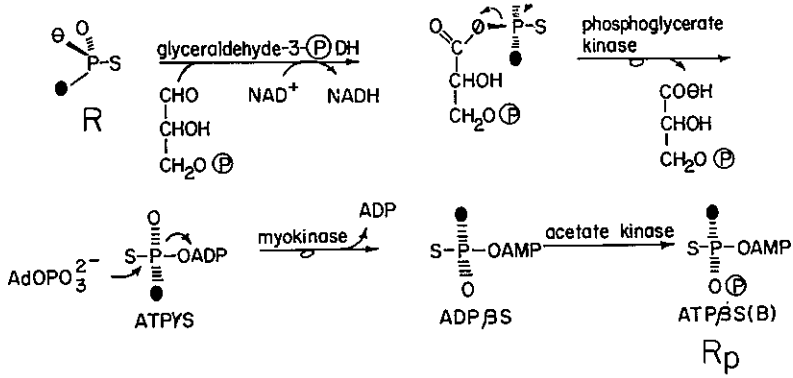
According to Scheme 40, the *R*-chiral Ps_i should give ATPαS(B) (isomer B has *R* configuration at P_β) with ^{18}O located specifically at the β-nonbridging position. The *S* enantiomer should give ATPβS(B) with ^{18}O at the $\beta\gamma$-bridging position. It must be noted that Scheme 40 only shows the species which will give an unquenched ^{31}P NMR signal. In reality, each chiral Ps_i species should give a mixture of three ATPβS(B) species (I/1, I/2 and I/3 in Scheme 41). In addition, it is impossible to have a chiral Ps_i of 100% purity. A chiral Ps_i sample actually contains up to six isotopic species, as shown in the left column of Scheme 41 (two of them, III and V, are identical species), each of them, reacting with net retention as shown in Scheme 40, gives three ATPβS(B) species. Fortunately, a careful examination of Scheme 41 reveals that there are only four different



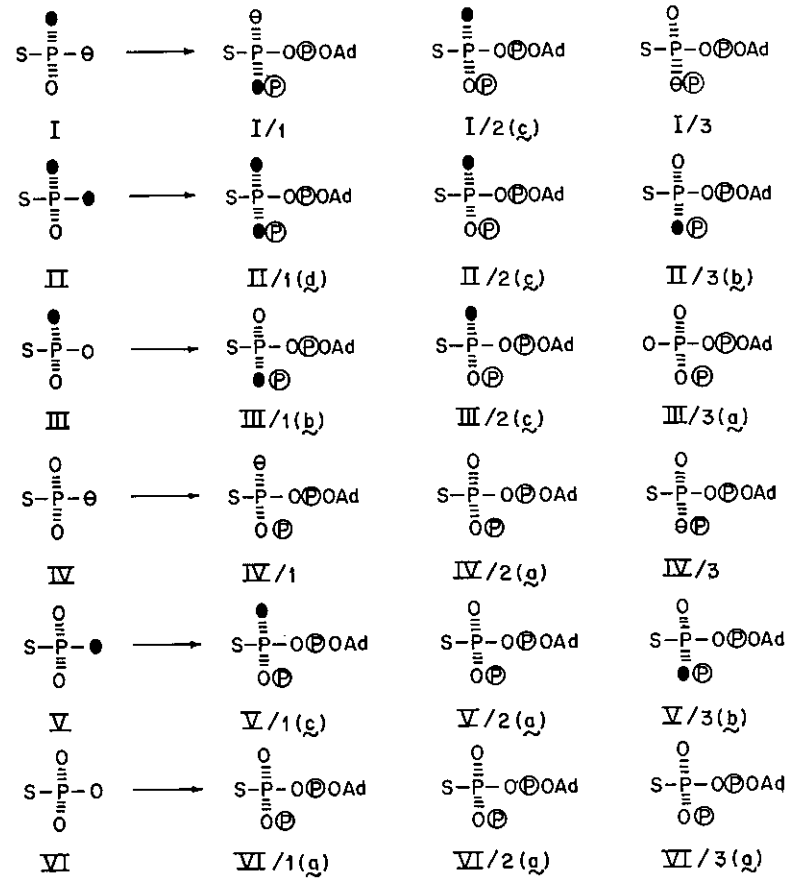
Scheme 39

non-^{17}O-containing species, **a**, **b**, **c** and **d**, and that all the non-chirally labeled Ps_i species contribute **equally** to species **b** and **c**. Only the [^{16}O,^{17}O,^{18}O]Ps_i species gives rise specifically to **b** or **c**, depending on whether the configuration is *S* or *R*, respectively. The amounts of species **a** and **d** depend only on isotopic enrichments but not on configuration.

Figure 2 shows the P_β signal of the ATPβS(B) obtained from $PS^{18}O_3^{3-}$



Scheme 40



Scheme 41

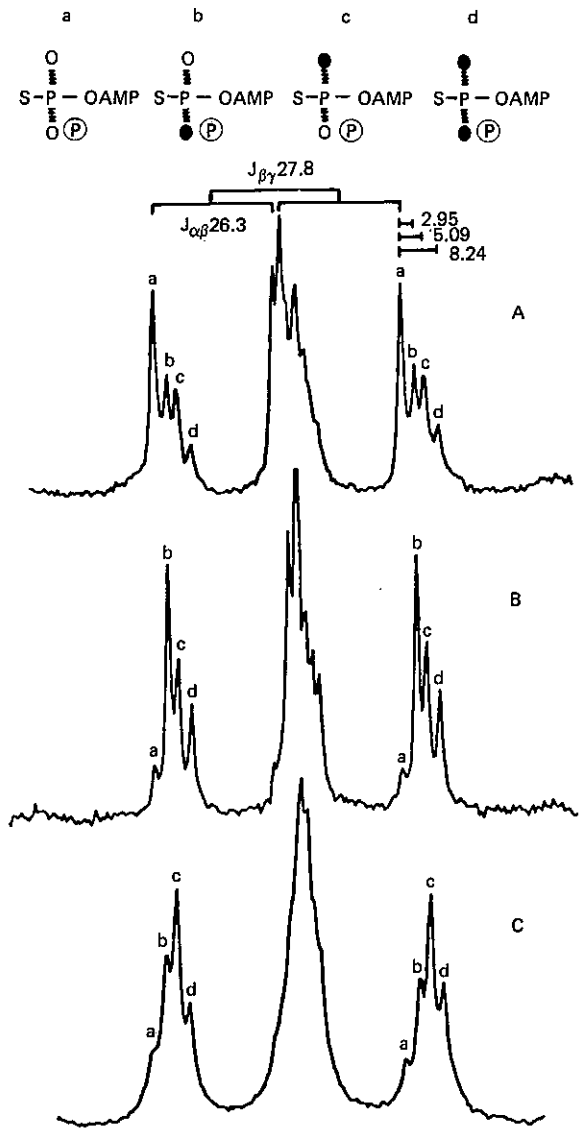


Figure 2. The P_β signals of the ^{31}P NMR spectra of the ATPβS(B) obtained from [$^{18}O_3$]-P_{si}(A) and from the two chiral P_{si}(B, C). The sample (30 μmol) was dissolved in 2.5 mL of D_2O containing 10 mM EDTA and the spectra were recorded at 145.7 MHz at ambient temperature. The coupling constants and isotope shifts are expressed in Hertz. The chemical shift of the P_β signal is 29.8 ppm downfield from H_3PO_4. From M.-D. Tsai (119).

and the two chiral Ps_i enantiomers. The signal contains two overlapping doublets due to $^{31}P—^{31}P$ coupling. Each half of a doublet (cf. the outer peaks) contains four lines due to the four species. The results are summarized in Table 1, where the "F" value is defined as the ratio **b/c**.

An issue remaining to be addressed is a unified way to represent the "chirality" of a chiral phosphoryl group. It is obvious from Scheme 41 that a pure *R* isomer of chiral Ps_i (Species I) should give exclusively species **c** (and a pure *S* iomer should give exclusively species **b**). It is the contaminating ^{16}O in the ^{18}O position and the contaminating ^{16}O and ^{18}O in the ^{17}O position (a typical commercial $H_2{}^{17}O$ contains ca. 50–55% ^{17}O and 30–40% ^{18}O) that give rise to other species. Tsai (119) has suggested separating "purity" from "chirality" in defining the quality of a chiral phosphoryl group. The highest possible purity of a chiral phosphoryl group is ca. 55% due to the limit in ^{17}O enrichment. A chiral phosphoryl group may have lower purity if there is further isotopic dilution during synthesis, but it could have a 100% "chirality" if all steps involved are 100% stereospecific. The "purity" of a chiral phosphoryl group can be determined from the isotopic enrichments at both the ^{17}O and the ^{18}O positions. The theoretical F values can then be calculated based on the known purity, assuming 100% chirality. If the observed F values deviate from the theoretical F values, it suggests that the chirality is <100%. Different values of chirality can then be assumed to calculate the F values until they fit the experimentally observed ones.

In a simplified representation, Knowles and co-workers (101) have suggested use of the Quality Index, Q, as a measure of the quality of both the experimental design and its execution. Assuming the ^{18}O enrichment in the "^{18}O-position" is very close to 100%, the maximum ratio of peak intensities **b/c** in Fig. 2B (and **c/b** in Fig. 2C) is 1/x, where x = fractional ^{16}O and ^{18}O contents of the ^{17}O used. They define Q_{max} as 1-x, the fractional ^{17}O content of the ^{17}O used. The experimental results provide Q_{obs}, which is the fractional difference

Table 1
^{31}P NMR Analysis of the ATPβS(B) Derived from Chiral Ps_i

Ps_i Samples	Intensity[a] (%)				F Value	Configuration
	a	b	c	d		
A	41.3 ± 1.2	24.6 ± 0.1	22.1 ± 0.0	11.8 ± 1.2	1.11	racemic
B	8.8 ± 0.5	42.8 ± 0.6	28.1 ± 0.5	20.3 ± 0.5	1.52	*S*
C	12.2 ± 0.5	26.5 ± 1.6	38.8 ± 0.1	22.4 ± 2.0	0.68	*R*

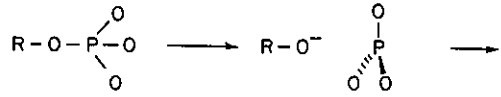
[a]Obtained from peak height measurements for the P_β signal of ATPβS. The errors represent deviations between the two nonoverlapping halves of the two doublets.

between peaks **b** and **c**. A comparison of Q_{obs} with Q_{max} provides a measure of the overall quality of the experimental procedures and the stereochemical integrity of the transformation. A comparison between the Q_{obs} of the substrate and that of the product in a biochemical transformation gives the extent of stereospecificity in the reaction. The best quality experiment reported is in the determination of the stereochemistry of cyclic phosphodiesterase (132), in which the Q_{max} was 0.51 and the Q_{obs} was 0.49 and 0.52.

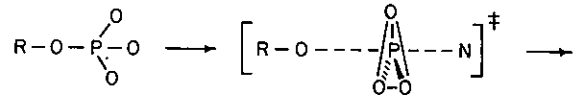
D. Mechanistic Significance of Stereochemistry

The initial interest in the field has been in trying to unravel the mechanism of enzyme catalysis: by which route they proceed among the four mechanistic extremes depicted by physical organic chemists (Scheme 42) (17, 138, 139). The dissociative pathway (mechanism A) involves the formation of the highly reactive metaphosphate intermediate, which is then captured by the acceptor group. If this species is free and symmetrically solvated, *racemization* at phosphorus can be predicted. Pathway B is analogous to a S_N2 reaction at carbon, and thus predicts *inversion* of configuration. Mechanism C is similar to B, except that it involves a stable pentacovalent intermediate which may undergo pseudorotation.

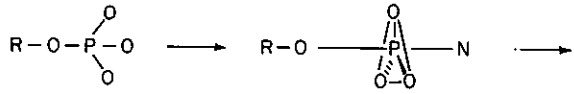
A. Dissociative (via monomeric metaphosphate):



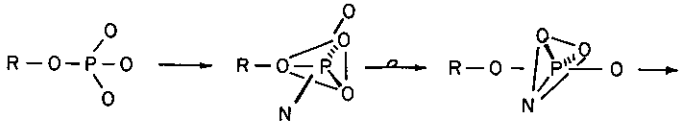
B. In-line associative (via a pentacoordinate transition state):



C. In-line associative (via a pentacoordinate intermedinat):



D. Adjacent associative (via a pentacoordinate intermediate and pseudorotation):



Scheme 42

In the "adjacent associative" pathway (mechanism D), a pseudorotation is *required* in order for the leaving group to leave from an apical position; it thus predicts *retention* of configuration. There is enough physical organic evidence to support the existence of these four pathways under different conditions (137, 138, 140–142).

However, the stereochemical results on enzymatic reactions have not led to identifying one of the four possibilities as the general mechanism in enzyme catalysis. First, formation of a metaphosphate intermediate (mechanism A) may not necessarily result in racemization since in the enzyme active site it may not be free to rotate before it is trapped by the acceptor. Racemization did not even occur in the chemical methanolysis of some phosphomonoesters under dissociative conditions (143). Therefore an observed inversion does not rule out pathway A. Second, the two in-line associative pathways B and C may not be distinguishable in enzyme catalysis and may both proceed with inversion. Lastly, stereochemical results can not differentiate between mechanism D and a double displacement mechanism in which each displacement occurs with inversion.

For all the enzymes investigated to date, *inversion* of configuration has always been observed for reactions known to be single-step processes. For reactions known to involve two steps (via the formation of a covalent enzyme-substrate intermediate), the stereochemical result is always *retention*. These results suggest that the general mechanism of enzyme-catalyzed phosphoryl transfer reactions always involves inversion, which rules out the adjacent pathway D but does not differentiate the dissociative pathway and the in-line associative pathway.

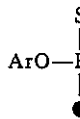
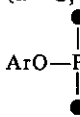
Although other approaches have been used to determine whether the dissociative mechanism or the in-line associative mechanism is the preferred mechanism in enzyme catalysis, the stereochemical results are now generally used to differentiate a double-displacement mechanism from a single-displacement mechanism. The stereochemical approach becomes especially powerful and valuable when there is an ambiguity in the mechanism proposed based on kinetic studies. However, caution in the interpretation of stereochemical results for 1,2-phosphoryl group migrations is advisable. Since mechanism D has been demonstrated in a chemical 1,2-phosphoryl migration (142), the observed retention in the reaction catalyzed by phosphoglycerate mutase (113) does not distinguish between a double-displacement mechanism and a single displacement via pathway D.

E. Stereochemistry of Specific Enzymes

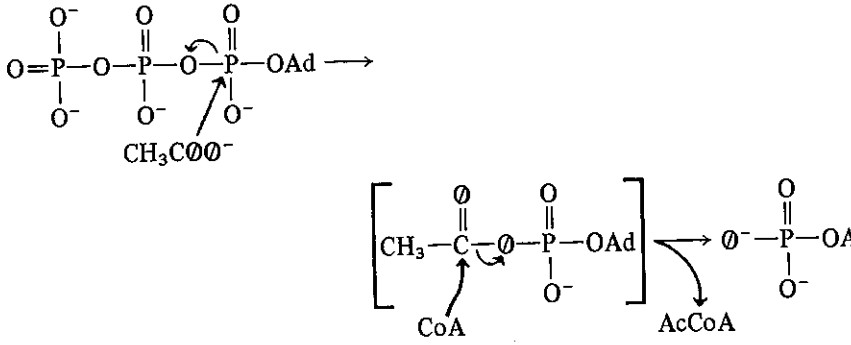
1. *Prochiral Substrate ⇄ Proprochiral Product*

The stereochemical results on enzymes catalyzing type (a) reactions are summarized in Table 2. There are two main approaches: use of sulfur and an oxygen isotope, and use of oxygen isotopes only. The substrate was in most cases syn-

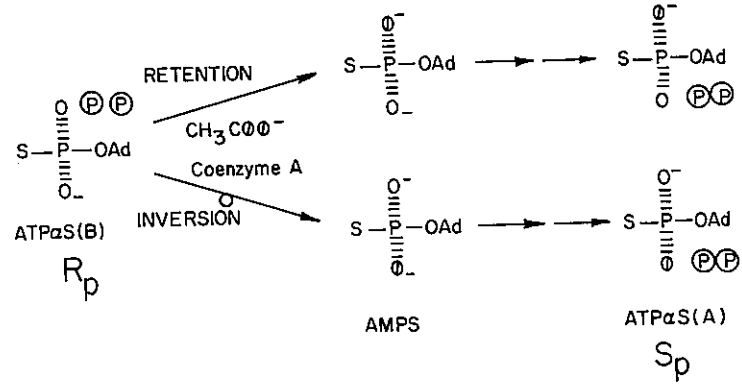
Table 2
Summary of Stereochemical Results for Type a Reactions: $ROPO_2—OR' \rightleftarrows ROPO_3$

Enzyme	Substrate	Product	Final Derivative	Analytical Method	Result	Ref.
Acetyl CoA synthetase	(*R*)-ATPαS (α-^{17}O)	AMPS (α-^{17}O)	(*S*)-ATPαS (α-^{17}O)	$^{31}P(^{17}O)$	Inv.	126
Aminoacyl tRNA synthetases	ATPαS (α-^{18}O)	AMPS (α-^{18}O)	ATPαS (α-^{18}O)	$^{31}P(^{18}O)$	Inv.	149, 150
cAMP phosphodiesterase	cyclic dAMP (^{17}O,^{18}O)	dAMP (α-^{16}O,^{17}O,^{18}O)	71	$^{31}P(^{17}O,^{18}O)$	Inv.	132
Exonuclease (bovine spleen)	TpNP (^{17}O,^{18}O)	3′-TMP (^{16}O,^{17}O,^{18}O)	74	$^{31}P(^{17}O,^{18}O)$	Ret.	133
Nucleotide pyrophosphatase	ATP (γ-CNEt) (α-^{17}O,^{18}O)	AMP (α-^{16}O,^{17}O,^{18}O)	Cyclization + ethylation	$^{31}P(^{17}O,^{18}O)$	Ret.	151
Nonspecific phosphohydrolase	*endo*-U > pS	UMPS (^{18}O)	*exo*-U > pS (^{18}O)	$^{31}P(^{18}O)$	Inv.	152
Phosphodiesterase (snake venom)		AMPS (α-^{18}O)	(*S*)-ATPαS (α-^{18}O)	MS	Ret.	153
Phosphodiesterase (snake venom)	(*R*)-ATPαS	AMPS (α-^{18}O)	(*S*)-ATPαS (α-^{18}O)	MS	Ret.	154
Phosphodiesterase (snake venom)	ATP (α-^{16}O,^{17}O,^{18}O)	AMP (α-^{16}O,^{17}O,^{18}O)	68	$^{31}P(^{17}O,^{18}O)$	Ret.	131
Nuclease (*Staphylococcal*)		ArO-P(^{16}O,^{17}O,^{18}O)	57 + 58	$^{31}P(^{17}O,^{18}O)$	Inv.	155
Ribonuclease A, T_1, T_2	(Two-step reaction)				Inv.	21, 156–158
Phospholipase D (cabbage)	DPPC (^{18}O)	79	81	$^{31}P(^{17}O,^{18}O)$	Inv. Ret.	135

thesized with known configuration. The product was often derivatized and analyzed by the mass spectral method or by ^{31}P NMR methods based on ^{17}O or ^{18}O effects or both. An example from Tsai's laboratory (126) is described in detail as follows. Acetyl-CoA synthetase catalyzes the following reaction:



It was found that the enzyme is specific for (*R*)-ATPαS but does not react with (*S*)-ATPαS. As shown in Scheme 43, when (*R*)-ATPαS and ^{17}O-acetate are used as substrates, the ^{17}O from acetate will be incorporated into the pro-*S* position of AMPS if the reaction proceeds with retention of configuration or into the pro-*R* position if inversion occurs. To determine the configuration of the ^{17}O-labeled AMPS (compound type 4), it is converted to (*S*)-ATPαS by stereospecific phosphorylation at the pro-*R* oxygen catalyzed by adenylate kinase, followed by a second phosphorylation catalyzed by pyruvate kinase (144, 145). By such a conversion, ^{17}O should be incorporated into the nonbridging position of (*S*)-ATPαS if the step of acetate activation proceeds with retention of configuration. On the other hand, ^{17}O should be located at the P—O—P bridging



Scheme 43

position if inversion occurs. A nonbridging ^{17}O at P_α should cause the P_α signal in the ^{31}P NMR to broaden and decrease, whereas a bridging ^{17}O should quench both the P_α and P_β signals.

Figure 3 shows ^{31}P NMR spectra of unlabeled (*S*)-ATPαS (A), the synthetic (*S*)-[α-^{17}O,αβ-^{17}O] ATPαS (B), and the (*S*)-ATPαS obtained from the enzyme reaction (C). The ^{17}O isotope used was 20% enriched and the enrichment of ^{17}O-acetate was determined as 19%. In Figure 3B, the P_α signal decreases to 67 ± 1% and the P_β signal to 83 ± 4%. In Figure 3C, the P_α signal decreases to 80 ± 4% and the P_β signal to 82 ± 5%. Since the signals for both P_α and P_β have decreased in Figure 3C, the results indicate that ^{17}O must be located at the bridging position, and the reaction catalyzed by acetyl-CoA synthetase must proceed with inversion of configuration (126).

The example presented above is a typical approach to a typical problem. However, there are several alternative analytical methods in differentiating a bridging oxygen from a nonbridging oxygen. The ^{17}O NMR signal of a bridging ^{17}O in adenine nucleotides was not observed at a low magnetic field (129). However, it has been observed, and shown to be distinguishable from the signal of a nonbridging ^{17}O, at higher magnetic field and higher temperature (105, 146, 147). If ^{18}O instead of ^{17}O is used, the position of ^{18}O can be located by the mass spectral method developed by Frey and co-workers (123, 124) or by the ^{31}P NMR method based on the magnitude of ^{18}O isotope shift. Midelfort and Sarton-Miller (148) have independently elucidated the steric course of acetyl-CoA synthetase by converting the product [^{18}O]AMPS to (*S*)-ATPαS as shown in Scheme 43, followed by degrading the (*S*)-[$^{18}O_1$]ATPαS with lysyl-tRNA synthetase and analyzing the resulting AMPS and PP_i by mass spectrometry.

2. *Proprochiral Substrate ⇄ Pro-prochiral Product*

The stereochemical studies in this category, the transfer of a phosphoryl group between two phosphomonoesters, are summarized in Table 3 for those using a thiophosphoryl group and in Table 4 for those using a [^{16}O,^{17}O,^{18}O]-phosphoryl group.

The approach by use of a thiophosphoryl group was mainly developed in Frey's and Knowles' laboratories. Various biophosphates carrying an [^{18}O]-thiophosphoryl group were synthesized and subjected to enzymatic reactions. The products were derivatized and analyzed mainly by the mass spectral method, since most work was done earlier, before the widespread use of ^{31}P NMR methods.

The use of a chiral [^{16}O,^{17}O,^{18}O]phosphoryl group to study the steric course of phosphoryl transfer reactions was developed mainly in two laboratories, initially in Knowles' group and more recently in Lowe's group. The key step in Knowles' approach is the reaction catalyzed by *E. coli* alkaline phosphatase. This

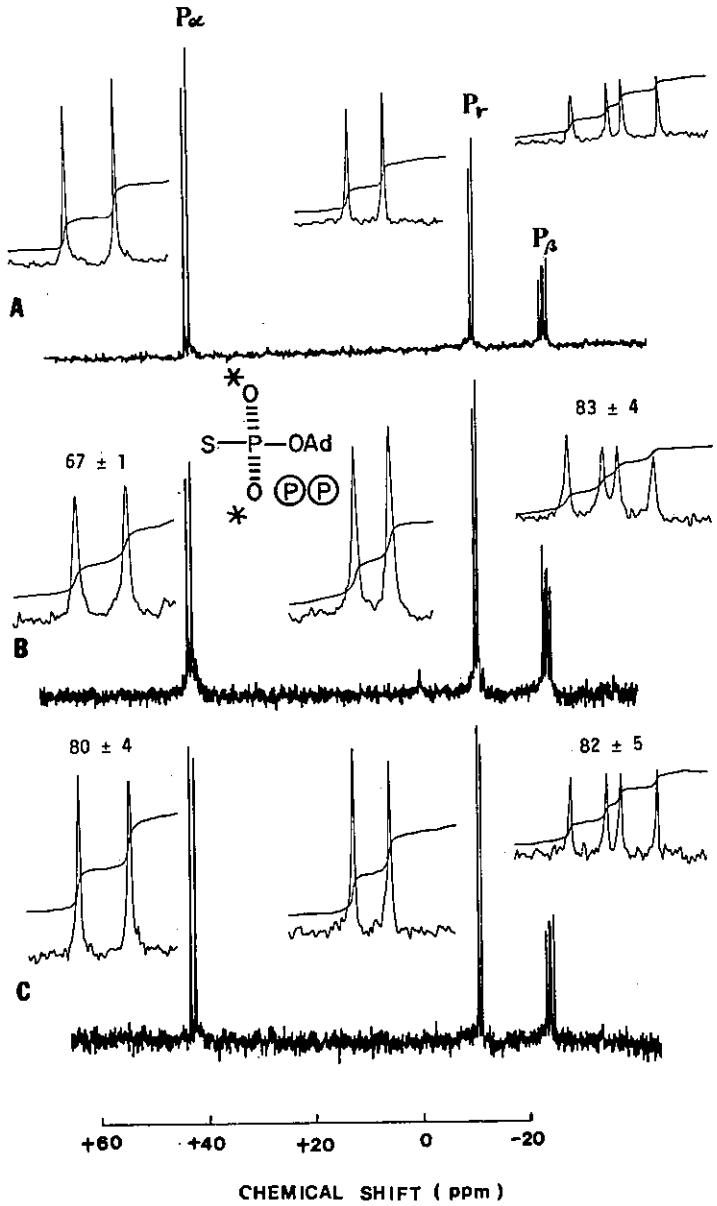


Figure 3. ^{31}P NMR spectra (at 32.2 MHz) showing the results on acetyl CoA synthetase. (A). Unlabeled (*S*)-ATPαS; (B). Synthesized (*S*)-[α-^{17}O,-αβ^{17}O] ATPαS; (C). The (*S*)-ATPαS from [$^{17}O_2$] acetate. The insets represent the integrations of the corresponding signals. From M.-D. Tsai (126).

Table 3
Summary of Stereochemical Results for Phosphoryl Transfer Reactions (Type b) by Use of a Thiophosphoryl Group

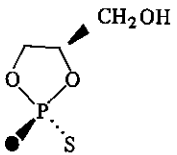
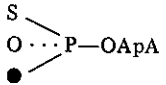
Enzyme	Substrate	Product	Final Derivative	Analytical Method	Result	Ref.
Phosphoglycerate kinase	Glycerate-1,3-diP (1-^{17}O,^{18}O,S)	ATPγS (γ-^{17}O,^{18}O)	(*S*)-ATPβS	$^{31}P(^{17}O,^{18}O)$	Inv.	24
Adenylate kinase	ATPγS (γ-^{18}O)	ADPβS (β-^{18}O)	ATPβS	MS	Inv.	124
Glycerol kinase	ATPγS (γ-^{18}O)	Glycerol-3-P (^{18}O,S)		MS X-ray	Inv.	159, 160
Pyruvate kinase	Phosphoenol-pyruvate (S,^{18}O)	ATPγS (γ-^{18}O)	Same as above		Inv.	159, 160
Hexokinase	ATPγS (γ-^{18}O)	Glucose-6-P (^{18}O,S)	Same as above		Inv.	159, 160
Adenosine kinase	ATPγS (γ-^{18}O)	AMPS (α-^{18}O)	(*S*)-ATPαS	MS	Inv.	161
Nucleoside phosphotransferase	AMPS (α-^{18}O)	AMPS (α-^{18}O)	(*S*)-ATPαS	MS	Ret.	122
Nucleoside diphosphate kinase	ATPγS (γ-^{18}O)	ATPγS (γ-^{18}O)	(*R*)-ATPβS	MS	Ret.	162
Polynucleotide kinase	ATPγS (γ-^{18}O)		(*S*)-ATPαS	MS	Inv.	163

Table 4
Summary of Stereochemical Results for Phosphoryl Transfer Reactions (Type b) by Use of a Chiral [$^{16}O,^{17}O,^{18}O$] Phosphoryl Group

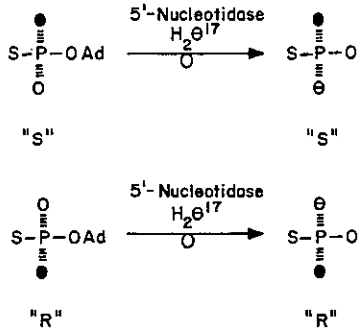
Enzyme	Substrate	Product	Final Derivative	Analytical Method	Result	Ref.
Alkaline phosphatase (*E. coli*)	Phenyl-P ($^{16}O,^{17}O,^{18}O$)	**55**	**57 + 58**	MS	Ret.	112
Glycerol kinase	ATP (γ-$^{16}O,^{17}O,^{18}O$)	Glycerol-P ($^{16}O,^{17}O,^{18}O$)	**57 + 58**	MS	Inv.	164
Acetate kinase	ATP (γ-$^{16}O,^{17}O,^{18}O$)	Acetate-P ($^{16}O,^{17}O,^{18}O$)	**57 + 58**	MS	Inv.	164
Phosphoglycerate mutase (rabbit muscle and wheat germ)	Glycerate-2-P	Glycerate-3-P	**57 + 58**	MS	Ret.	113
Creatine kinase	ATP (γ-$^{16}O,^{17}O,^{18}O$)	Creatine-P ($^{16}O,^{17}O,^{18}O$)	**57 + 58**	$^{31}P(^{17}O,^{18}O)$	Inv.	165
Acid phosphatase	Phenyl-P ($^{16}O,^{17}O,^{18}O$)	**55**	**57 + 58**	$^{31}P(^{17}O,^{18}O)$	Ret.	166
Hexokinase	ATP (γ-$^{16}O,^{17}O,^{18}O$)	Glucose-6-P ($^{16}O,^{17}O,^{18}O$)	**78**	$^{31}P(^{17}O,^{18}O)$	Inv.	167
Pyruvate kinase	Phosphoenolpyruvate ($^{16}O,^{17}O,^{18}O$)	ATP (γ-$^{16}O,^{17}O,^{18}O$)	**78**	$^{31}P(^{17}O,^{18}O)$	Inv.	168
Phosphofructokinase	Fructose-1,6-diP (1-$^{16}O,^{17}O,^{18}O$)	ATP (γ-$^{16}O,^{17}O,^{18}O$)	**78**	$^{31}P(^{17}O,^{18}O)$	Inv.	169
Phosphoglucomutase	Glucose-1-P ($^{16}O,^{17}O,^{18}O$)	Glucose-6-P ($^{16}O,^{17}O,^{18}O$)	**78**	$^{31}P(^{17}O,^{18}O)$	Ret.	170
Polynucleotide kinase	ATP (γ-$^{16}O,^{17}O,^{18}O$)	3′,5′-ADP (5′-$^{16}O,^{17}O,^{18}O$)	**78**	$^{31}P(^{17}O,^{18}O)$	Inv.	171

alkaline phosphatase is a nonspecific phosphate monoesterase which also catalyzes the transfer of phosphoryl groups to acceptors other than water, such as alcohols. By use of phenyl[(*R*)-^{16}O,^{17}O,^{18}O]phosphate as a substrate and (*S*)-propane-1,2-diol as an acceptor, the chiral phosphoryl group was transferred by alkaline phosphatase to form 1-[^{16}O,^{17}O,^{18}O]phosphopropane-1,2-diol, and its configuration was then analyzed as described in Scheme 37. The results indicate *retention* of configuration (112). Since alkaline phosphatase has a low substrate specificity, other phosphomonoesters can be analyzed by transferring their chiral phosphoryl group to (*S*)-propane-1,2-diol followed by configurational analysis.

The key compound in Lowe's approach is glucose 6-[^{16}O,^{17}O,^{18}O]phosphate (25), which was synthesized with known configuration according to Scheme 31 (p. 291). The configuration can be characterized by ^{31}P NMR analysis after cyclization of 25 followed by methylation (134). The steric course of hexokinase and phosphoglucomutase can be readily elucidated since glucose-6-phosphate is the product in these reactions. The steric course of other kinases can be determined by converting [γ-^{16}O,^{17}O,^{18}O]ATP (as a product) to glucose-6-[^{16}O,^{17}O,^{18}O]phosphate with hexokinase.

3. *Proprochiral Substrate ⇄ Proproprochiral Product*

The enzymes in this category inlcude phosphatases, nucleotidases, ATPases, and so on. Except for the phosphatases, which also catalyse the "transfer" reaction in addition to the "hydrolysis" reaction (112, 166), the stereochemistry has to be studied by use of a chiral inorganic phosphate (P_i, a proproprochiral center). To make a P_i chiral, it is necessary to use ^{16}O,^{17}O,^{18}O and sulfur. The synthesis and configurational analysis of chiral [^{16}O,^{17}O,^{18}O]thiophosphate (Ps_i) have been described in previous sections; the first application of chiral [^{16}O,^{17}O,^{18}O]Ps_i in the reaction type c is the elucidation of the stereochemistry



Scheme 44

Table 5
Summary of Stereochemical Results for Type c Reactions: $RO—PO_3 \rightleftarrows PO_4$

Enzyme	Substrate	Product	Final Derivative	Analytical Method	Result	Ref.
5′-Nucleotidase	AMPS (α-^{18}O)	Psi (^{16}O,^{17}O,^{18}O)	(*R*)-ATPβS	$^{31}P(^{17}O,^{18}O)$	Inv.	25, 119
Myosin ATPase	ATPγS (γ-^{18}O)	Psi (^{16}O,^{17}O,^{18}O)	(*S*)-ATPβS	$^{31}P(^{17}O,^{18}O)$	Inv.	172
Mitochondrial ATPase	ATPγS (γ-^{18}O)	Psi (^{16}O,^{17}O,^{18}O)	(*S*)-ATPβS	$^{31}P(^{17}O,^{18}O)$	Inv.	173
Sarcoplasmic reticulum ATPase	ATPγS (γ-^{17}O,^{18}O)	Psi (^{16}O,^{17}O,^{18}O)	(*S*)-ATPβS	$^{31}P(^{17}O,^{18}O)$	Ret.	174
Ribosome-dependent GTPase	GTPγS (γ-^{17}O,^{18}O)	Psi (^{16}O,^{17}O,^{18}O)	(*S*)-ATPβS	$^{31}P(^{17}O,^{18}O)$	Inv.	175

of hydrolysis of AMP to adenosine and P_i catalyzed by 5′-nucleotidase. As shown in Scheme 44, hydrolysis of (*R*)- and (*S*)-[α-$^{18}O_1$]AMPS in $H_2{}^{17}O$ gave (*R*)- and (*S*)-[^{16}O,^{17}O,^{18}O]Ps_i, respectively, indicating an "inversion" of configuration (25, 119). Webb and co-workers (172–175) have elucidated the steric course of several ATPases and GTPases. The results are summarized in Table 5.

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