Possible Mechanisms of Demethylation of 14α-Methyl Sterols in Cholesterol Biosynthesis[1]

Sir:

The precise mechanism(s) of the enzymatic removal of the 14α-methyl group (carbon atom 32) of lanosterol and other precursors of cholesterol has not been conclusively established. On the basis of analogy with the reactions involved in the removal of the 4α- and 4β-methyl groups of cholesterol precursors, the first reaction at carbon atom 32 has been presumed to be an oxygen-dependent hydroxylation to yield the corresponding 14α-hydroxymethyl sterol.[2-5] The results of studies of the metabolism of a number of 14α-methyl and 14α-hydroxymethyl sterol precursors of cholesterol specifically labeled at C-32 with either ^3H or ^{14}C have shown that rat liver microsomes catalyze the removal of C-32 to yield formic acid.[6-10] However, the identity of the initial sterol product formed upon removal of C-32, the cofactor and oxygen requirements for this process, and the nature of the intermediates involved have been the subject of considerable controversy. Fried et al.[11,12] reported the enzymatic conversion of lanost-7-ene-3β,32-diol and the corresponding 32-aldehyde to cholesterol upon incubation with rat liver homogenate preparations under aerobic conditions. They also reported that washed microsomes, supplemented with NAD, catalyzed the formation of the 32-aldehyde from the 14α-hydroxymethyl sterol under anaerobic conditions; under aerobic conditions, carbon atom 32 reportedly was removed to yield a $\Delta^{8(14)}$ monoene sterol, i.e., 4,4-dimethyl-5α-cholest-8(14)-en-3β-ol.[11-13] More recently, evidence has been presented which implied that both oxygen and NADPH were required for both the enzymatic oxidation of lanost-7-ene-3β,32-diol to the 32-aldehyde and the further metabolism of the aldehyde to give formic acid and a $\Delta^{7,14}$ diene sterol, i.e., 4,4-dimethyl-5α-cholesta-7,14-dien-3β-ol.[6,10] These authors reported the formation of only insignificant amounts of $\Delta^{8(14)}$ sterol products. Under similar conditions, lanost-8-ene-3β,32-diol was reported to be metabolized to formic acid and 4,4-dimethyl-5α-cholesta-8,14-dien-3β-ol.[9,14] However, the recent observation in our laboratory that 5α-cholest-8(14)-en-3β-ol was virtually the sole C_{27} monohydroxysterol product isolated after incubation of 14α-(hydroxymethyl)-5α-cholest-7-en-3β-ol with washed rat liver microsomes supplemented with NAD[15] led us to investigate the oxygen and cofactor requirements for the enzymatic removal of the steroidal C-32.

The purpose of this communication is to report that washed microsomes are able to catalyze the removal of carbon atom 32 of 14α-(hydroxymethyl)-5α-cholest-7-en-3β-ol (**1**) under anaerobic conditions. Moreover, 5α-cholest-8(14)-en-3β-ol (**2**) is essentially the sole C_{27} monohydroxysterol product, and oxidized pyridine nucleotides appear to be the only required source of oxidizing equivalents for this process.

Compound **1** has been prepared in a variety of specifically labeled forms in our laboratory: [3α-^{3}H]**1**,[7] [32-^{3}H]**1**,[7] and [2,4-^{3}H]**1**.[16] **1** has been shown to be efficiently converted to cholesterol upon incubation with rat liver homogenate preparations,[7] and C-32 was recovered in the form of formic acid upon incubation of **1** with washed microsomes supplemented with NADPH.[7] Because **1** lacks geminal methyl groups at position 4, its metabolism yields sterol products of a simpler distribution and type than the products derived from the metabolism of substrates possessing the lanostane carbon skeleton, for which oxidative demethylation at carbon 4 yields a variety of sterol products not directly pertinent to the study of the mechanism of the removal of C-32. Furthermore, powerful chromatographic methods have been developed for the separation and identification of C_{27} monohydroxysterols,[5,17,18] which permit the unambiguous analysis of the products of the enzymatic removal of C-32 of **1**.[19]

[2,4-^{3}H]**1** was incubated with washed rat liver microsomal preparations[16] under a variety of conditions. After extraction of the sterols from the saponified incubation mixtures,[20] the monohydroxysterols were separated from the polar sterols by silicic acid–Super Cel column chromatography.[16] The monohydroxysterols were acetylated, unlabeled steryl acetate standards[21] were added, and the mixtures were subjected to analysis by alumina–$AgNO_3$ medium-pressure liquid chromatography (MPLC).[17,20] Table I summarizes the results of these studies. Incubations of [2,4-^{3}H]**1** with washed microsomes supplemented with NAD and an NADPH-generating system under aerobic conditions yielded cholesterol and 5α-cholest-7-en-3β-ol as the principal C_{27} monohydroxysterol products. Surprisingly, under both aerobic and strictly anaerobic[16] conditions, incubations of [2,4-^{3}H]**1** with washed microsomes in the absence of any added cofactors yielded

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(2) Bloch, K. *Science (Washington, D.C.)* **1965**, *150*, 19.

(3) Clayton, R. B. *Q. Rev., Chem. Soc.* **1965**, *19*, 168.

(4) Frantz, I. D., Jr.; Schroepfer, G. J., Jr. *Annu. Rev. Biochem.* **1967**, *36*, 691.

(5) Schroepfer, G. J., Jr.; Lutsky, B. N.; Martin, J. A.; Huntoon, S.; Fourcans, B.; Lee, W.-H.; Vermilion, J. *Proc. R. Soc. London, Ser. B* **1972**, *180*, 125.

(6) Alexander, K.; Akhtar, M.; Boar, R. B.; McGuie, J. F.; Barton, D. H. R. *J. Chem. Soc., Chem. Commun.* **1972**, 383.

(7) Trowbridge, S.; Lu, Y. C.; Shaw, R.; Chan, J.; Spike, T. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1975**, *34*, 560.

(8) Mitropoulos, K. A.; Gibbons, G. F.; Reeves, B. E. A. *Steroids* **1976**, *27*, 821.

(9) Akhtar, M.; Freeman, C. W.; Wilton, D. C. *Bioorg. Chem.* **1977**, *6*, 473.

(10) Akhtar, M.; Alexander, K.; Boar, R. B.; McGuie, J. F.; Barton, D. H. R. *Biochem. J.* **1978**, *169*, 449.

(11) Fried, J.; Dudowitz, A. E.; Brown, J. W. *Biochem. Biophys. Res. Commun.* **1968**, *32*, 568.

(12) Dudowitz, A. E.; Fried, J. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1969**, *28*, 665.

(13) Unfortunately, these results were presented only in preliminary form, and characterization of the $\Delta^{8(14)}$ sterol was based solely upon the results of gas-liquid chromatographic analysis in a system which does not permit resolution of all potential metabolites of the incubated substrate.

(14) Aoyama and Yoshida have recently reported (Aoyama, Y.; Yoshida, Y. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 28–34), that incubation of lanosterol with cytochrome P-450 and NADPH-cytochrome P-450 reductase (from yeast microsomes) in the presence of oxygen and NADPH gave a product which on the basis of the results of GLC and GLC–MS analyses was assigned the structure 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol. Whether or not these results are applicable to the case of the rat liver microsomal system is not known. Evidence has recently been presented (Gibbons, G. F.; Pullinger, C. R.; Mitropoulos, K. A. *Biochem. J.* **1979**, *183*, 309) which suggests that cytochrome P-450 of rat liver microsomes is involved only in the initial hydroxylation of the 14α-methyl group of lanosterol.

(15) Chang, P.; Schroepfer, G. J., Jr. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1977**, *36*, 816.

(16) Pascal, R. A., Jr.; Schroepfer, G. J., Jr. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 932.

(17) Pascal, R. A., Jr.; Farris, C. L.; Schroepfer, G. J., Jr. *Anal. Biochem.* **1980**, *101*, 15.

(18) Thowsen, J. R.; Schroepfer, G. J., Jr. *J. Lipid Res.* **1979**, *20*, 681.

(19) The possibility that 14α-methylcholestenols might not represent suitable substrates for the studies of the mechanism of the removal of the 14α-methyl group in cholesterol biosynthesis has been considered in view of the common assumption that the enzymatic removal of the 14α-methyl group of lanosterol precedes the removal of the methyl groups at carbon atom 4. However, the reported isolations of a number of 4α,14α-dimethylcholestenols and 14α-methyl sterols from a variety of plant sources, yeast mutants, and from the feces and meconium of newborn infants (Djerassi, C.; Knight, J. C.; Wilkerson, D. I. *J. Am. Chem. Soc.* **1963**, *85*, 835; Goad, L. J.; Williams, B. L.; Goodwin, T. W. *Eur. J. Biochem.* **1967**, *3*, 232; Atallah, A. M.; Nicholas, H. J. *Steroids* **1971**, *17*, 611; Doyle, P. J.; Patterson, G. W.; Dutky, S. R.; Cohen, C. F. *Phytochemistry* **1971**, *10*, 2093; Ragsdale, N. N. *Biochim. Biophys. Acta* **1975**, *380*, 81; Trocha, P. J.; Jasne, S. J.; Sprinson, D. B. *Biochemistry* **1977**, *16*, 4721; Gustafsson, J.-A.; Eneroth, P. *Proc. R. Soc. London. Ser. B* **1972**, *180*, 179) suggest that the removal of the three extra methyl groups of lanosterol can be initiated by removal of either the 4α-methyl function (Rahman, R.; Sharpless, K. B.; Spencer, T. A.; Clayton, R. B. *J. Biol. Chem.* **1970**, *245*, 2667; Knapp, F. F., Jr.; Trowbridge, S. T.; Schroepfer, G. J., Jr. *J. Am. Chem. Soc.* **1975**, *97*, 3522, and references cited therein) or the 14α-methyl group. Nevertheless, the possibility remains that the mechanisms involved in the enzymatic removal, in rat liver preparations, of C-32 of 4,4,14α-trimethyl sterols differ from those for 14α-methyl sterols.

(20) Pascal, R. A., Jr.; Schroepfer, G. J., Jr. *J. Biol. Chem.* **1980**, *255*, 3565.

(21) 3β-Acetoxy-5α-cholest-8(14)-ene, 3β-acetoxy-5α-cholest-7-ene, cholesteryl acetate, 3β-actoxy-5α-cholesta-8,14-diene, and 3β-acetoxycholesta-5,7-diene.

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Table I. Distribution of the Radioactivity from Incubations of Tritium-Labeled 14α-(Hydroxymethyl)-5α-cholest-7-en-3β-ol (1)[a] in Rat Liver Microsomal Preparations

expt	incubation conditions[d]	treatment of microsomes	% conversion to monohydroxy sterols[b]	% composition of monohydroxy sterols[c]				
				cholestenols				all cholestadienols
				$\Delta^{8(14)}$	Δ^{7}	Δ^{5}	other	
1	aerobic + NAD[e] + NADPH generator[f]	washed	19.3[i]	1.2[i]	49.2[i]	36.0[i]	1.0[i]	12.6[i]
2	aerobic	washed	19.0[i]	90.3[i]	<0.1[i]	<0.1[i]	4.8[i]	5.0[i,j]
3	anaerobic	washed	21.5[i]	93.0[i]	<0.1[i]	<0.1[i]	4.2[i]	2.9[i,j]
4	aerobic	washed, boiled 2 min	<0.01[i]					
5	aerobic + NAD[g] + NADPH generator[h]	Triton-treated	11.2	28	24	2	<0.5	46
6	aerobic + NAD[g]	Triton-treated	8.5	97	<0.5	<0.5	2	1
7	aerobic	Triton-treated	0.4[i]					

[a] [2,4-^{3}H]1 was employed as the substrate in experiments 1-4; [3α-^{3}H]-1 was used in experiments 5-7. [b] Determined by silicic acid-Super Cel column chromatography. [c] Determined by alumina-silver nitrate MPLC in experiments 1-3; determined by sequential silicic acid-Super Cel-silver nitrate and alumina-Super Cel-silver nitrate column chromatography in experiments 5-6. [d] 3 h at 37 °C for experiments 1-4; 2 h at 37 °C for experiments 5-7. [e] 1.0 mM. [f] 1.0 mM NADP, 3.0 mM G-6-P, 0.3 units/mL G-6-P dehydrogenase. [g] 1.5 mM. [h] 1.2 mM NADP, 11.6 mM isocitrate, 0.5 mg/mL isocitrate dehydrogenase. [i] Average value from two separate experiments. A notation of "<0.1" indicates that the given metabolite was not observed at a level of detection of 0.1%. [j] Most of this radioactivity eluted immediately after changing the solvent from 9:1 to 6:4 hexane-toluene on the alumina-$AgNO_3$ MPLC system and probably does not represent a true cholestadienol.

amounts of total C_{27} monohydroxysterols comparable to those found in the cofactor-supplemented, aerobic incubations. Moreover, 5α-cholest-8(14)-en-3β-ol (**2**) was virtually the exclusive C_{27} monohydroxysterol formed.[22] This result clearly indicates the absence of an obligatory oxygen requirement for the removal of C-32 of **1**. However, any conclusion with regard to the cofactor requirements for this process from this data alone would have been premature due to the possible presence of tightly bound pyridine nucleotides in the washed microsomal preparations. Therefore, a series of aerobic incubations of [3α-^{3}H]**1** were conducted with rat liver microsomal preparations previously treated with the nonionic detergent Triton WR-1339.[23] This treatment of liver microsomes has been reported to provide preparations showing an absolute requirement for added NAD for the oxidative demethylation of 4,4-dimethyl-5α-cholest-7-en-3β-ol.[23] Our results with compound **1** (Table I) show that, in the absence of added cofactors, essentially no labeled C_{27} monohydroxysterols were observed. However, supplemental NAD or NAD plus an NADPH-generating system restored the ability of Triton-treated microsomes to catalyze the removal of the 14α-hydroxymethyl group of **1**. In the presence of only added NAD, 5α-cholest-8(14)-en-3β-ol (**2**) was virtually the sole monohydroxysterol product; however, in the presence of added NAD and an NADPH-generating system, 5α-cholest-7-en-3β-ol, cholesterol, and an unidentified diene sterol[24] were observed in addition to **2**.

These results indicate that NAD is the only source of oxidizing equivalents required for the enzymatic removal of C-32 of **1** under anaerobic conditions. Furthermore, a $\Delta^{8(14)}$ sterol, **2**, appears to be the initial C_{27} monohydroxysterol formed upon enzymatic removal of C-32 of **1** under these conditions. The metabolism of **2** to cholesterol and other C_{27} sterol precursors of cholesterol has been shown to have an absolute requirement for oxygen,[25-27] and on the basis of analogy with the closely related work of Fried et al.,[11,12] a requirement for NADPH is also indicated. Moreover, it is extremely unlikely that the $\Delta^{8(14)}$ sterol product could have

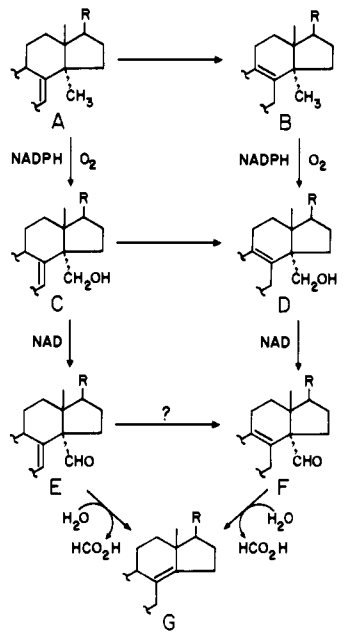

Figure 1. One possible reaction scheme for the enzymatic removal of carbon atom 32 of 14α-methyl and 14α-hydroxymethyl sterols.

resulted from the enzymatic reduction of a $\Delta^{7,14}$ or $\Delta^{8,14}$ diene sterol, since anaerobic incubation of 5α-cholesta-7,14-dien-3β-ol or 5α-cholesta-8,14-dien-3β-ol with cell-free rat liver preparations yields almost exclusively 5α-cholest-7-en-3β-ol (≥90% conversion), with no more than 1.4% conversion of the incubated substrate to 5α-cholest-8(14)-en-3β-ol.[28,29]

We therefore propose that the removal of C-32 of 14α-methyl sterols may proceed by the pathways depicted in Figure 1. We have recently established the existence of the isomerization reactions A → B and C → D,[16] thus linking the pathways for the removal of C-32 of 14α-methyl-substituted Δ^7 and Δ^8 sterols. After the initial oxygen-dependent hydroxylation of the 14α-methyl group (A → C and B → D), oxygen-independent processes suffice to remove C-32. The hydroxymethyl group could be oxidized to an aldehyde by an NAD-dependent dehydrogenase (C → E and D → F), and hydrolytic removal of C-32 as formic acid would be assisted by the presence of a β,γ-double bond at the Δ^7 or Δ^8 position (E → G and F → G). Further, metabolism of the product $\Delta^{8(14)}$ monoene sterol (G) would require oxygen

(22) The acetate derivative of labeled **2**, isolated by alumina-$AgNO_3$ MPLC, was diluted with unlabeled 3β-acetoxy-5α-cholest-8(14)-ene, and recrystallized twice from methanol and twice from acetone-water with no change in specific activity.

(23) Miller, W. L.; Kalafer, M. E.; Gaylor, J. L.; Delwiche, C. V. *Biochemistry* **1967**, *6*, 2673.

(24) The acetate derivative of this sterol diene did not cochromatograph with authentic standards of 3β-acetoxy-5α-cholest-7,14-diene, 3β-acetoxy-5α-cholesta-7,9(11)-diene, 3β-acetoxy-5α-cholest-8,14-diene, or 3β-acetoxy-cholesta-5,7-diene. Catalytic hydrogenation (Raney nickel W-2; 40 psi) of this material yielded 3β-acetoxy-5α-cholest-8(14)-ene.

(25) Lee, W.-H; Schroepfer, G. J., Jr. *Biochem. Biophys. Res. Commun.* **1968**, *34*, 635.

(26) Lee, W.-H.; Lutsky, B. N.; Schroepfer, G. J., Jr. *J. Biol. Chem.* **1969**, *244*, 5440.

(27) Lutsky, B. N.; Schroepfer, G. J., Jr. *Lipids* **1971**, *6*, 957.

(28) Lutsky, B. N.; Martin, J. A.; Schroepfer, G. J., Jr. *J. Biol. Chem.* **1971**, *246*, 6737.

(29) Lutsky, B. N.; Schroepfer, G. J., Jr. *J. Biol. Chem.* **1970**, *245*, 6449.

and NADPH. While the results of others cited above indicate that one pathway for the enzymatic removal of carbon atom 32 of 14α-alkyl and 14α-hydroxymethyl sterols requires molecular oxygen, our results clearly demonstrate that the enzymatic removal of a steroidal 14α-hydroxymethyl group can also proceed via oxygen-independent processes. Moreover, our findings provide a logical explanation for the occurrence of $\Delta^{8(14)}$ sterols in nature[30–34] and for the existence of enzymes in liver which catalyze the conversion of $\Delta^{8(14)}$ sterols to cholesterol.[11,25,26,35,36]

(30) Lutsky, B. N.; Schroepfer, G. J., Jr. *Biochem. Biophys. Res. Commun.* **1969**, *35*, 288.
(31) Zalkov, L. H.; Cabat, G. A.; Chetty, G. L.; Chosal, M.; Keen, G. *Tetrahedron Lett.* **1968**, 5727.
(32) Barton, D. H. R.; Harrison, D. M.; Moss, G. P.; Widdowson, D. A. *J. Chem. Soc. A* **1970**, 775.
(33) Bouvier, P.; Rohmer, M.; Benveniste, P.; Ourisson, G. *Biochem. J.* **1976**, *159*, 267.
(34) Withers, N. W.; Goad, L. J.; Goodwin, T. W. *Phytochemistry* **1979**, *18*, 899.
(35) Fiecchi, A.; Scala, A.; Cattabeni, F.; Grossi Paoletti, E. *Life Sci.* **1970**, *9*, 1201.
(36) Gibbons, G. F. *Biochem. J.* **1970**, *144*, 59.

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Determination of the Absolute Configuration of [^{16}O,^{17}O,^{18}O]Phosphate Monoesters by Using ^{31}P NMR

Sir:

We recently reported a general synthetic approach to chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters and a mass spectrometric method that allows the quantitative determination of the absolute configuration at phosphorus in such molecules.[1] These techniques have subsequently been used to determine the stereochemical course of enzymes that catalyze phosphoryl group transfer reactions, namely, phosphatases,[2] phosphokinases,[3] and phosphomutases.[4] Our original method for the determination of the absolute configuration involves linked-scan metastable ion mass spectrometry, and we have been concerned to devise a less circuitous approach to solve this problem. We report here an alternative solution that is simpler both conceptually and practically, and exploits the sensitivity of ^{31}P NMR signals to the nature of the attached oxygen isotopes.

To illustrate the method, we use the key compound of our earlier approach, 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol (**1**). When this material, *R* at phosphorus, is treated with (diphenylphosphoryl)imidazole,[1,5] three isomeric cyclic diesters (the 2,2-dioxo-1,3,2-dioxaphospholanes) are produced in equimolar ratios by "in-line" ring closure, as shown in Figure 1. Methylation of these species with diazomethane[1] results in the formation of the six cyclic triesters, comprising three "syn" isomers (**2**, **3**, and **4**) and three "anti" isomers (**5**, **6**, and **7**) (see Figure 1). While these two sets of diastereoisomers can be separated,[1] there is no need to do this in the present case, since the ^{31}P NMR signals for the syn and anti materials (for the all-^{16}O mixture) are separated by about 0.1 ppm. It has recently been shown that the quadrupolar

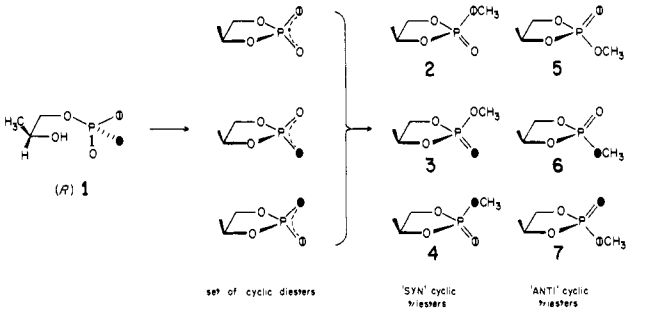

Figure 1. The three cyclic diesters and six cyclic triesters that are derived from 1-(*R*)-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol by "in-line" ring closure and methylation.[15]

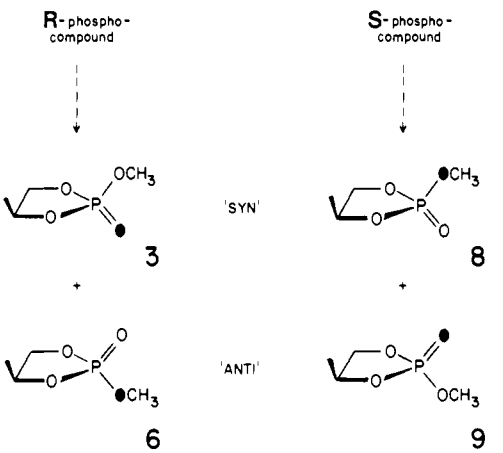

Figure 2. The two ^{18}O-labeled species that are derived from the "in-line" ring closure and methylation of labeled 1-phospho-(*S*)-propane-1,2-diols that are *R* or *S* at phosphorus.[15]

effect of ^{17}O causes such a broadening of the ^{31}P resonances of compounds containing ^{31}P–^{17}O bonds,[6,7] that the ^{31}P NMR spectrum of the mixture of compounds **2**–**7** should contain sharp peaks only for those species *not* containing any ^{17}O, i.e., **3** and **6**. If, therefore, the spectrum of a mixture of **3** and **6** (deriving from a phosphoryl group that was *R*) can be distinguished from the spectrum of a mixture of **8** and **9** (deriving from an *S* phosphoryl group), the absolute configuration at phosphorus can be determined (see Figure 2). The distinction between **3** + **6** and **8** + **9** is readily made, and depends upon the heavy oxygen isotope shift on the ^{31}P NMR signal.[8] The magnitude of the upfield chemical shift caused by ^{18}O directly bonded to ^{31}P depends on the nature of the ^{31}P–^{18}O bond,[7,9] the greater the double-bond character, the greater the shift. For the problem at hand, therefore, we can expect that the ^{31}P line[10] of the syn isomer will be at higher field for **3** than for **8**, and the resonance for the anti isomer will be further upfield for **9** than for **6**.

The solution is not quite so simple, however, since the isotopic content of the $H_2{}^{17}O$ used in the synthesis of **1** is no better than approximately 1:2:1 for ^{16}O/^{17}O/^{18}O.[11] This means that the ^{31}P NMR spectra will contain signals from the unlabeled ^{16}O,^{16}O compounds, the "incorrect" ^{16}O,^{18}O species, and the doubly labeled ^{18}O,^{18}O isomers, in addition to the "correct" ^{16}O,^{18}O materials. The relative proportions of all these species can easily be determined, of course, from the known isotopic content of **1** and the known isotopic composition of the $H_2{}^{17}O$ and $H_2{}^{18}O$ samples used

(1) Abbott, S. J.; Jones, S. R.; Weinman, S. A.; Knowles, J. R. *J. Am. Chem. Soc.* **1978**, *100*, 2558. Abbott, S. J.; Jones, S. R.; Weinman, S. A.; Bockhoff, F. M.; McLafferty, F. W.; Knowles, J. R. *Ibid.* **1979**, *101*, 4323.
(2) Jones, S. R.; Kindman, L. A.; Knowles, J. R. *Nature (London)* **1978**, *257*, 564.
(3) Blättler, W. A.; Knowles, J. R. *J. Am. Chem. Soc.* **1979**, *101*, 510; *Biochemistry* **1979**, *18*, 3927.
(4) Blättler, W. A.; Knowles, J. R. *Biochemistry* **1980**, *19*, 738.
(5) Konieczny, M.; Sosnovsky, G. *Z. Naturforsch., B: Anorg. Chem., Org. Chem.* **1978**, *33B*, 1033.
(6) Tsai, M-D. *Biochemistry* **1979**, *18*, 1468. Tsai, M-D.; Huang, S. L.; Kozlowski, J. F.; Chang, C. C. *Ibid.* **1980**, in press.
(7) Lowe, G.; Potter, B. V. L.; Sproat, B. S.; Hull, W. E. *J. Chem. Soc., Chem. Commun.* **1979**, 733.
(8) Cohn, M.; Hu, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 200. See also: Lowe, G.; Sproat, B. S. *J. Chem. Soc., Chem. Commun.* **1978**, 565.
(9) Cohn, M.; Hu, A. *J. Am. Chem. Soc.* **1980**, *102*, 913.
(10) Broad-band proton decoupling is used in all these experiments.
(11) The actual isotopic content of the peripheral oxygens of the *R* phospho compound is the following: ^{16}O, 44.1%; ^{17}O, 16.1%; ^{18}O, 39.8%. *S* phospho compound: ^{16}O, 44.7%; ^{17}O, 14.6%; ^{18}O, 40.7%.

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