Figure 1. Brønsted plot for general base catalysis of the iodine oxidation of methionine; aqueous solution, 25 °C, ionic strength 1.0 with KCl. The buffers shown are H_2O, CF_3COO^-, $(CH_3)_2AsOOH$, $H_2PO_4^-$, CH_3SOO^-, $(CH_3)_2AsOO^-$, and HPO_4^{2-}. The arrow indicates the upper limit for catalysis by HO^-. Values of $K_1 k_B$ were calculated from the nonlinear buffer plots using the method described by H. F. Gilbert and W. P. Jencks, *J. Am. Chem. Soc.*, **99**, 7931 (1977).
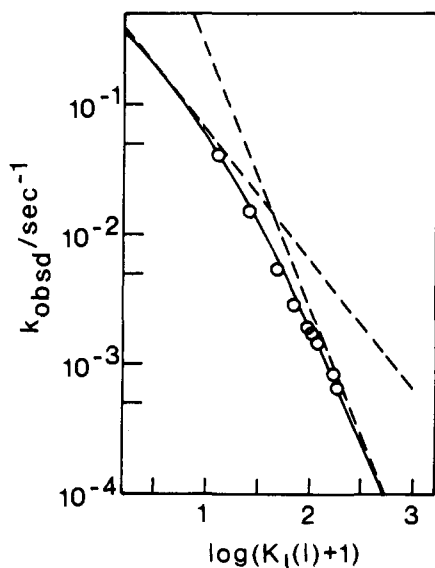

Figure 2. Dependence of log k_{obsd} for iodine oxidation of methionine on the quantity log $(K_1[I] + 1)$ where K_1 is the equilibrium constant for the formation of triiodide ion; aqueous solution, 25 °C, ionic strength 1.0 with KCl, pH 4.85, acetic acid buffer at a total concentration of 0.7 M. The solid line was calculated for a break from a slope of -1 to -2.

Brønsted plot diagnostic of a concerted mechanism; however, they are *most* consistent with the curved Brønsted plot expected for the stepwise-preassociation mechanism (Figure 1).

At high concentrations of buffer, the rates of reaction no longer show a simple inverse dependence on iodide concentration and approach a curve of slope -2.0 (Figure 2). This means that the breakdown of an intermediate coming *after* the buffer-mediated step has become rate limiting. This intermediate must contain the elements of dehydromethionine and iodide ion. Since the reaction goes to completion rather than to an equilibrium, the simplest explanation is that a tetracoordinate sulfurane is involved as an intermediate and that the breakdown of this sulfurane has become rate limiting. This is the first kinetic evidence that requires a sulfurane as an obligatory intermediate in a nucleophilic substitution reaction of this type.[5]

If a sulfurane intermediate is involved, then the proton

transfer in the buffer-mediated step must be transfer to and from this sulfurane. If the assignment of a stepwise mechanism is correct, then the break in the Brønsted plot at about $pK_a = 2$ reflects an upper limit for the pK_a of this species. The driving force for the catalysis that is observed is the generation of an intermediate with a lifetime sufficiently short so that it is not at proton or diffusional equilibrium with the solvent. This is consistent with the rules defined for "enforced" mechanisms of catalysis as described by Jencks[4] and this work represents the first extension of these rules to systems outside of the framework of carbonyl addition–elimination reactions, and as such supports the generality of the concept.

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(5) Kice has obtained kinetic evidence that an intermediate may be involved in nucleophilic substitution at dicoordinate sulfenyl compounds: J. L. Kice and T. E. Rogers, *J. Org. Chem.*, **41**, 225 (1976).

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Cyclonerodiol Biosynthesis and the Stereochemistry of the Conversion of Farnesyl to Nerolidyl Pyrophosphate

Sir:

Allylic pyrophosphates play a central role in the biosynthesis of isoprenoid metabolites. These substances may undergo a variety of transformations (Scheme I), including direct displacements (S_N2 type, pathway a), allylic displacements (S_N2' type, pathway b), and allylic transpositions (allylic rearrangement, pathway c). The class of direct displacements has been the most thoroughly studied, and is represented by the prenyl transferase catalyzed chain elongation reactions whereby successive units of isopentenyl pyrophosphate are added to the primary allylic pyrophosphates dimethallyl, geranyl, or farnesyl pyrophosphate.[1] These processes have been shown to involve inversion of configuration at C-1 of the allylic
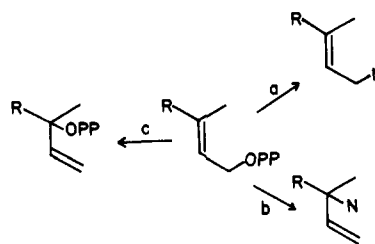
Scheme I

**Table I.** Conversion of Labeled Substrates to Chiral Acetates

substrate	acetate[a]	malate[b,c] from malate synthase	malate[d] after fumarase	tritium retention, %
		³H/¹⁴C		
[1,2-²H₂,1-³H]-(E)-nerolidyl-OPP	4.30	3.38	1.00	29.6
[5-²H,³H]-(3RS,5R)-mevalonate	3.38	2.70	1.86	68.9
[5-²H,³H]-(3RS,5S)-mevalonate	3.88	3.54	1.09	30.8
([2-²H,³H]-(2R)-acetate)[e]	3.95	3.41	2.60	76.2
([2-²H,³H]-(2S)-acetate)[e]	4.10	3.87	0.95	24.5

[a] ³H/¹⁴C ratio based on p-bromophenacyl ester of mixture of [2-³H]- and [2-¹⁴C]acetates. [b] Recrystallized from acetone–hexane after addition of inactive carrier and chromatography on Dowex-1 (formate). [c] The observed retention of tritium in going from acetate to malate is a function of both intra- and intermolecular isotope effects, as well as the extent of conversion of acetyl CoA to malate. On the other hand the retention of tritium after fumarase exchange depends only on an intramolecular isotope effect in the malate synthase reaction, k_H/k_D ≃ 3.5. Cf. ref 21, as well as H. Lenz, W. Buckel, P. Wunderwald, G. Biedermann, V. Buschmeier, H. Eggerer, J. W. Cornforth, J. W. Redmond, and R. Mallaby, *Eur. J. Biochem.*, **24**, 207 (1971). [d] Minimum 4-h incubation with fumarase followed by precipitation of barium phosphate, Dowex-50W-X8 chromatography, and recrystallization from acetone–hexane. ³H/¹⁴C ratio is unchanged upon further fumarase treatment. [e] Authentic (R)- and (S)-acetates were used as reference samples.

Scheme II

Scheme III
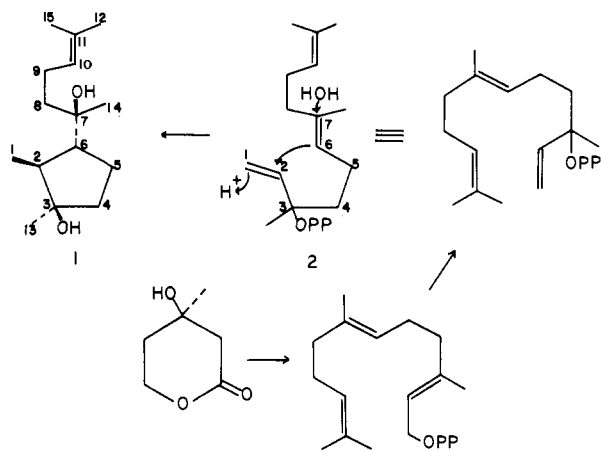
pyrophosphate.[2] Less well studied are the allylic displacements. Using ²H NMR spectroscopy, we have recently demonstrated that in the biosynthesis of the diterpene rosenonolactone, the biological S_N2' process which generates ring C with displacement of pyrophosphate takes place with overall anti (antarafacial) stereochemistry.[3] Independently, Arigoni and his collaborators have concluded that a related S_N2' process in the biosynthesis of a second diterpene, pleuromutilin, also occurs with exclusive anti stereochemistry.[4] The category of allylic transpositions, represented by the isomerization of geranyl to linalyl pyrophosphate and farnesyl to nerolidyl pyrophosphate, has been the least well studied in spite of the fact that these transformations have figured prominently in biogenetic speculations and related chemical model studies.[5] Our recent finding that a cell-free system prepared from the fungus *Gibberella fujikuroi* will convert both farnesyl and nerolidyl pyrophosphates to the cyclopentanoid sesquiterpene cyclonerodiol (**1**) has now provided a suitable experimental system for studying the stereochemistry of these allylic transposition reactions.[6] We report below the results of a study demonstrating that the conversion of *trans,trans*-farnesyl pyrophosphate to nerolidyl pyrophosphate takes place with syn (supraficial) stereochemistry.

Incorporations of labeled mevalonates[7] as well as the above-mentioned cell-free studies have provided evidence for a biosynthetic pathway to cyclonerodiol in which a molecule of water adds across the central double bond and the vinyl group of nerolidyl pyrophosphate, itself formed from mevalonate by isomerization of farnesyl pyrophosphate. Evidence was also presented indicating that cyclization precedes pyrophosphate hydrolysis[6] (Scheme II). Our experimental plan was to determine first the stereochemistry of cyclization of the
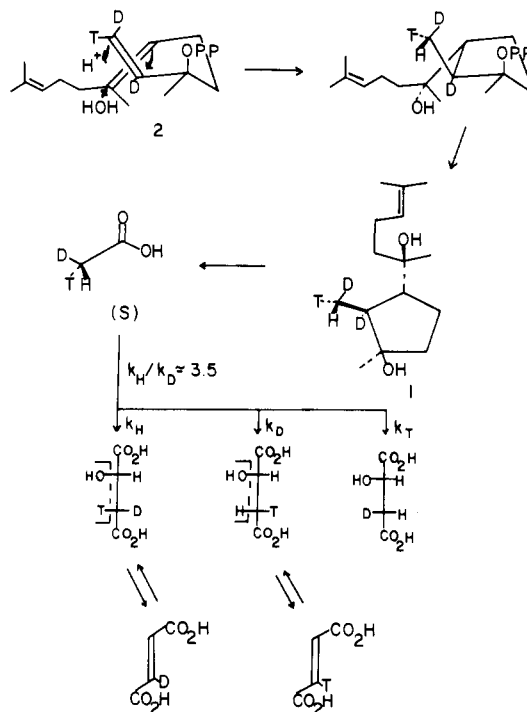
intermediate nerolidyl pyrophosphate. With the results of such a study in hand, we would then be in a position to examine the farnesyl–nerolidyl rearrangement. The stereochemistry of folding of the nerolidyl pyrophosphate, as well as the direction of attachment of the water oxygen at the side chain, is apparent from the established absolute configuration of cyclonerodiol[8] (Scheme III). Furthermore, if it is assumed that hydrolysis of the pyrophosphate ester occurs with P–O bond cleavage,[9] the intermediate nerolidyl pyrophosphate would then have the 3R configuration. Protonation of the terminal vinyl carbon of nerolidyl pyrophosphate generates the C-1 methyl group of cyclonerodiol. In order to determine the stereochemistry of this latter protonation, we required a sample of **2** stereospecifically labeled with both deuterium and tritium at C-1. Protonation at C-1 would then generate a methyl group with a chirality dependent on the stereochemistry of proton addition. The requisite sample of [1,2-²H₂,1-³H]-(E)-nerolidol was prepared by reduction of [1-³H]dehydronerolidol with lithium aluminum deuteride in the presence of sodium methoxide followed by quenching with deuterated water.[10–12] Mass spectrometry showed the resultant nerolidol to be 94.8% d_2, 3.4% d_1, and 1.8% d_0,[13] and examination of the ¹H NMR spectrum re-

Scheme IV
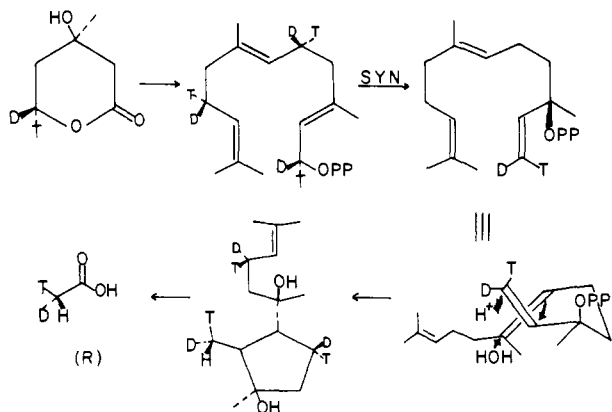
corded in the presence of 25 mol % Eu(DPM)₃ confirmed that the reduction of the propargyl alcohol had occurred with the expected trans stereochemistry. After conversion to the corresponding pyrophosphate ester as previously described,[6,14] the sample of [1,2-²H₂,1-³H]-(E)-2 was incubated with a cell-free extract from 4-day-old cultures of *G. fujikuroi.*[15] The resultant labeled cyclonerodiol was isolated and purified[6] and then diluted with inactive carrier (15 mg).[16] Labeled 1 was subjected to Kuhn–Roth oxidation,[18] giving rise to ~12 mg of potassium acetate. This sample of acetate consisted of chirally labeled acetate derived from C-1 and C-2 of cyclonerodiol, diluted with unlabeled acetate originating from the C-13, C-14, and either the C-12 or C-15 methyls of 1.[19] The chirality of this sample of acetate was determined using the procedure developed by Arigoni and Cornforth.[21] Thus, a mixture of the [2-³H]acetate and added [2-¹⁴C]acetate (³H/¹⁴C, 4.30)[22] was converted to acetyl-CoA and the resultant sample incubated with sodium glyoxylate and freshly purified yeast malate synthase.[23] After addition of carrier L-malate and extraction with ethyl acetate, the derived malate was isolated by ion-exchange chromatography and recrystallized to give ³H/¹⁴C of 3.38. Incubation of this latter malate sample with fumarase gave, after isolation and recrystallization, malate ³H/¹⁴C of 1.00[24] (Table 1). The observed retention of 29.6% of the tritium activity after fumarase incubation corresponds to [2-²H,³H]-(2S)-acetate.[21] Since Kuhn–Roth oxidation does not affect the chirality obtained,[19] the C-1 methyl of cyclonerodiol derived from [1,2-²H₂,1-³H]-(E)-nerolidyl pyrophosphate must also have S chirality and the protonation at C-1 of nerolidyl pyrophosphate therefore must have occurred on the *re* face. The overall stereochemistry of the addition of water across the vinyl and central double bonds is thus completely trans, as illustrated in Scheme III.

The above determination of the stereochemistry of the enzymatic conversion of nerolidyl pyrophosphate to cyclonerodiol provides a means of analyzing samples of labeled nerolidyl pyrophosphate of unknown stereochemistry at C-1. Thus isomerization of [1-²H,³H]-(1R)-farnesyl pyrophosphate will give either [1-²H,³H]-(E)- or -(Z)-nerolidyl pyrophosphate whose stereochemistry may be determined by conversion to cyclonerodiol and determination of the chirality of the resultant C-1 methyl. In actual practice, it was found most convenient to utilize [²H,³H]-(5R)- and -(5S)-mevalonates, which were prepared from the corresponding [1-²H,³H]-(1R)- and -(1S)-isopentenols[25] and fed separately to cultures of *G. fujikuroi.* The derived cyclonerodiol was then isolated and purified in the usual manner and subjected to Kunh–Roth oxidation. Although the mevalonates utilized label both C-5 and C-9 of 1 as well as C-1, only the latter carbon gives rise to chiral acetate upon oxidation. Analysis of the chirality of each of the resulting acetate samples by the sequence of malate syn-

thase/fumarase incubation described above established that incorporation of [5-²H,³H]-(5R)-mevalonate gave rise to [2-²H,³H]-(2R)-acetate, whereas [5-²H,³H]-(5S)-mevalonate resulted in [2-²H,³H]-(2S)-acetate. The results imply that the conversion of farnesyl pyrophosphate to nerolidyl pyrophosphate is a net suprafacial process. Thus [5-²H,³H]-(5R)-mevalonate gives rise to [1-²H,³H]-(1R)-farnesyl pyrophosphate, which is isomerized to (3R)-nerolidyl pyrophosphate. Suprafacial isomerization will give the [1-²H,³H]-(1Z)-nerolidyl stereoisomer which, upon cyclization and pyrophosphate ester hydrolysis with P–O bond cleavage, will yield [1-²H,³H]-(1R)-cyclonerodiol and therefore the corresponding [2-²H,³H]-(2R)-acetate upon oxidation, as observed (Scheme IV). The results with [5-²H,³H]-(5S)-mevalonate are entirely complementary and fully support the above arguments.

Recently Arigoni and Suga have examined the stereochemistry of linalool biosynthesis in *Cinnamomum camphora* and have concluded that the geranyl pyrophosphate–linalool isomerization takes place with syn stereochemistry, again assuming P–O bond cleavage.[28] These results parallel our own for the homologous sesquiterpene alcohols. A number of plausible mechanisms may be advanced to account for the observed syn rearrangement of the allylic pyrophosphate and studies utilizing ¹⁸O-labeled substrates are in progress to distinguish among the several possibilities.

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(6) D. E. Cane and R. Iyengar, *J. Am. Chem. Soc.*, **100**, 3256 (1978).

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(9) S. S. Stein and D. E. Koshland, *Arch. Biochem. Biophys.*, **39**, 229 (1952); H. N. Fernley in "The Enzymes", Vol. 4, 3rd ed., P. D. Boyer, Ed., Academic Press, New York, 1971, p 417; H.-L. Ngan and G. Popjak, *Bio-organic Chem.*, **4**, 166 (1975); H. Mackie and K. H. Overton, *Eur. J. Biochem.*, **77**, 101 (1977). An exception is the observed inversion at C-1 in the conversion of geranyl pyrophosphate to geraniol by *Menyanthes trifoliata;* cf. D. Arigoni, *Pure Appl. Chem.*, **41**, 219 (1975), and S. Escher, Dissertation, ETH, Zurich, No. 4887, 1972. In the absence of the normal cosubstrate isopentenyl pyrophosphate, prenyl transferase catalyzes the hydrolysis of geranyl pyrophosphate with inversion and introduction of water at C-1; cf. C. D. Poulter and H. C. Rilling, *Biochemistry*, **15**, 1079 (1976). The latter process is probably an artefact of the normal mode of action of the enzyme. The origin of the oxygen atoms of cyclonerodiol is currently under investigation.

(10) E. J. Corey, J. A. Katzenellenbogen, and G. H. Posner, *J. Am. Chem. Soc.*, **89**, 4245 (1967).

(11) *trans*-Dehydronerolidol was prepared by addition of ethynylmagnesium bromide to *trans*-geranylacetone in methylene chloride. The requisite

R = H
R = T
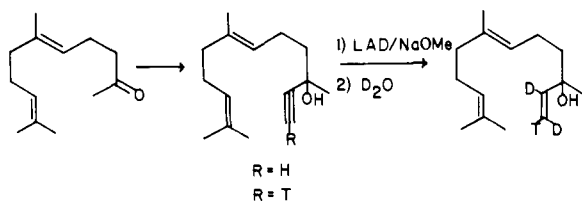
[1-³H]dehydronerolidol was then obtained by treatment of dehydronerolidol (0.37 g, 1.68 mmol) in tetrahydrofuran with *n*-butyllithium (2.01 mmol), followed by quenching with 0.20 mL (20 mCi) of [³H]water. The [1-³H]-propargyl alcohol (276 mg, 1.25 mmol) was treated with 158 mg (3.76 mmol) of lithium aluminum deuteride and 406 mg (7.51 mmol) of freshly prepared sodium methoxide in 2.0 mL of tetrahydrofuran at reflux overnight. The reaction was quenched by successive addition of 0.16 mL of D_2O, 0.16 mL of 5% NaOD, and 0.48 mL of D_2O. Ether was added and the products were isolated by filtration through a pad of Celite and evaporation. Purification by PLC (hexane–ether, two developments, 4:1) gave 159 mg of nerolidol, R_f 0.38, 3.58 × 10⁷ dpm/mg.

(12) The sequence of deuteride reduction–deuterated water quench was employed in order to ensure essentially 100% deuteration at C-1, as required by the analytical procedure for chiral methyl.[21] The presence of the extra deuterium at C-2 in no way affects the outcome of subsequent reactions.

(13) Based on the (M − H_2O) fragment.

(14) W. W. Epstein and H. C. Rilling, *J. Biol. Chem.*, **245**, 4597 (1970); S. S. Sofer and H. C. Rilling, *J. Lipid Res.*, **10**, 183 (1969); A. A. Kandutsch, H. Paulus, E. Levin, and K. Bloch, *J. Biol. Chem.*, **239**, 2507 (1964); F. Cramer and W. Boehm, *Angew. Chem.*, **71**, 755 (1959); P. W. Holloway and G. Popjak, *Biochem. J.*, **104**, 57 (1967); G. Popjak, J. W. Cornforth, R. H. Cornforth, R. Ryhage, and D. J. Goodman, *J. Biol. Chem.*, **237**, 56 (1962).

(15) the cell-free extract from 1.3 L of *G. fujikuroi* was prepared as previously described⁶ except that 25% glycerol (Mallinckrodt, A. R.) was added to improve enzyme stability. The S_{27} fraction was incubated with 1.5 mg (3.17 × 10⁷ dpm) of [1,2-²H₂,1-³H]-(1*E*)-nerolidyl pyrophosphate for 4 h at 26 °C.

(16) A portion of the labeled cyclonerodiol was converted to the bis(dinitrobenzoate),[7,17] which was recrystallized to constant activity, indicating an overall 1.1% conversion of nerolidyl pyrophosphate to 1.

(17) B. E. Cross, R. E. Markwell, and J. C. Stewart, *Tetrahedron*, **27**, 1663 (1971).

(18) E. Wiesenberger, *Mikrochimica Acta*, **33**, 51 (1948).

(19) A control experiment in which unlabeled 1 was subjected to Kuhn–Roth oxidation in the presence of 5 mCi of [³H]water, followed by conversion of the derived acetate to the crystalline *p*-bromophenacyl ester, indicated that oxidation is accompanied by as much as 26% exchange of the methyl hydrogens. Such small amounts of exchange have been observed before²⁰ and do not present a serious problem. For example, assuming that exchange takes place during oxidation and that the C-1, C-13, C-14, and C-12 (or C-15) methyls contribute eqully to the formation of acetate, that there is no isotope effect for exchange, and that each methyl exchanges only once, 26% exchange would correspond to at most 26% racemization of the chiral acetate. This would change the observed retention of tritium in the fumarase assay by only 6%. Furthermore an isotope effect would tend to protect chirally deuterated and tritiated methyl against exchange, with a consequent reduction in the observed extent of racemization.

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(21) J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Nature (London)*, **221**, 1212 (1969); *Eur. J. Biochem.*, **14**, 1 (1970); J. Luthy, J. Retey, and D. Arigoni, *Nature (London)*, **221**, 1213 (1969).

(22) Determined from the *p*-bromophenacyl ester.

(23) Malate synthase was purified from bakers' yeast by the procedure of Dixon and Kornberg (cf. G. H. Dixon, H. L. Kornberg, and P. Lund, *Biochim. Biophys. Acta*, **41**, 217 (1960); G. H. Dixon and H. L. Kornberg, *Methods Enzmol.*, **5**, 633 (1962)) or using an improved procedure developed by Professor H. Eggerer.

(24) The procedure followed was based on that of Arigoni.[21] Experimental details will be given in the full paper.

(25) J. W. Cornforth, F. P. Ross, and C. Wakselman, *J. Chem. Soc., Perkin Trans. 1*, 429 (1974); J. W. Cornforth and F. P. Ross, *Chem. Commun.*, 1395 (1970); The requisite [1-²H]- and [1-³H]-isopentenals were obtained by pyridinium chlorochromate oxidation²⁶ of the corresponding alcohols. [1-²H,³H]-(1*R*)-isopentenol was prepared by horse liver alcohol dehydrogenase (HLADH) catalyzed reduction of [1-²H]-isopentenal using 1 equiv of [4-³H]-NADH. The sample of [1-²H,³H]-(1*S*)-isopentenol was obtained from [1-³H]-isopentenal using a catalytic amount of NAD⁺ and a 70-fold excess of perdeuterated ethanol in the presence of HLADH.²⁷

(26) E. J. Corey and J. W. Suggs, *Tetrahedron Lett.*, 2647 (1975).

(27) B. Zagalak, P. A. Frey, G. L. Karabatsos, and R. H. Abeles, *J. Biol. Chem.*, **241**, 3028 (1966).

(28) D. Arigoni, S. Gotfredsen, and T. Suga, unpublished results. Cf. T. Suga, T. Shishibori, and M. Bukeo, *Bull. Chem. Soc. Jpn.*, **45**, 1480 (1972).

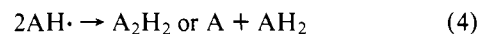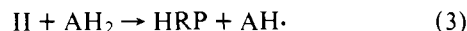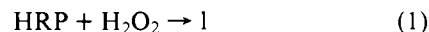**David E. Cane,* Radha Iyengar, Ming-Shi Shiao**

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Nuclear Magnetic Resonance Characterization of Compounds I and II of Horseradish Peroxidase

Sir:

Horseradish peroxidase (HRP), which contains iron(III) protoporphyrin, catalyzes the oxidation of wide variety of phenols and aromatic amines by H_2O_2. The widely accepted mechanism depicts¹ the enzymic cycle as

$$HRP + H_2O_2 \rightarrow I \qquad (1)$$

$$I + AH_2 \rightarrow II + AH\cdot \qquad (2)$$

$$II + AH_2 \rightarrow HRP + AH\cdot \qquad (3)$$

$$2AH\cdot \rightarrow A_2H_2 \text{ or } A + AH_2 \qquad (4)$$

This mechanism implies the binding of both H_2O_2 and the electron donor, AH_2, to the enzyme. The nature of the reaction intermediates, compounds I and II, which are respectively two and one oxidizing equivalents above the native enzyme, has been subject of intensive study for many years.² I and II are stable enough to have been isolated and characterized by several physical techniques. Magnetic susceptibility,³ Mössbauer,⁴ and resonance Raman⁵ studies on II showed it to be in a low-spin ferryl, Fe(IV) state. The Mössbauer spectrum yielded the same iron isomer shifts for both compounds, indicating that I is also in ferryl Fe(IV) state. Electronic absorption spectrum of II is a normal porphyrin type, but that of I resembles the spectrum of a porphyrin π-cation radical.⁶ Recently a weak ESR signal at $g = 2.005$ was detected for I to suggest that a free radical is located close to the paramagnetic iron.⁷

In spite of these extensive investigations, the structures of I and II have not been totally resolved. Particularly, it is uncertain whether the additional oxidizing equivalent on I is stored as a radical on the porphyrin ring or on a protein moiety.

We have studied the ¹H NMR spectra of these intermediates which allows us to suggest that I and II are, respectively, in high- and low-spin Fe(IV) iron states with different sixth iron ligands and that the free radical may be contained on an amino acid residue close to the heme iron rather than on the porphyrin ring.

In our NMR study, HRP purchased from Toyobo Co. as a liophylized sample ($RZ = 3.4$, isoenzyme c) was used. I (green solution) was generated by adding an equimolar amount of H_2O_2 to 3.0 mM HRP solution in 0.1 M citric acid–0.2 M phosphate buffer at pH 7.0. II (red solution) was prepared from the I solution at pH 9.2⁸ in 25 mM borate buffer by the addition of a stoichiometric amount of *p*-cresol. ¹H NMR spectra were recorded at various time intervals after the addition of H_2O_2, at various temperatures (5–40 °C) and at different pH's (4–12) with a Varian HR-220/Nicolet TT-100 in a pulsed Fourier transform mode.⁹

¹H NMR spectra in the hyperfine shifted region for native ferric HRP, I, and II are compared in Figure 1. The native HRP spectrum, which has previously been reported,¹⁰ is replaced by an entirely different well-resolved spectrum when H_2O_2 was added. The signals at 76.1, 72.1, 59.1, and 50.1 ppm in Figure 1B assigned to the four heme peripheral methyl proton peaks of I decreased in intensity in time with a concomitant increase in intensity of a proton peak at 14.1 ppm, and then the spectrum of the enzyme decayed back to the native enzyme spectrum. Upon immediate addition of *p*-cresol to the I green solution, the spectrum of I disappeared and a rather broad signal at 14.1 ppm grew in, accompanied by reappearance of the native HRP spectrum. Thus we assigned the signal at 14.1 ppm in Figure 1B and 1C to the two of four heme ring methyl proton peaks of II from the integrated intensity. This spectrum of II is also reduced in time, and the