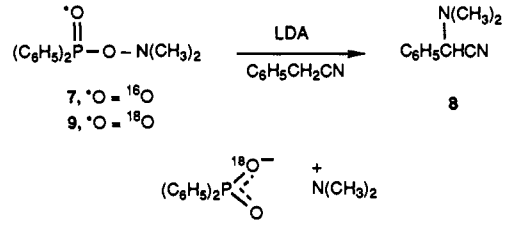
Table I. Isotropic Ratios for the Conversion of a Mixture of **1** and **3** to **2**, **4**, **5**, and **6**

mixture	M[a,b]	M + 1[a]	M + 2[a]
1 and **3**	60	4	37
2, **4**, **5** and **6**	40	47	14
intermolecular reactn[c]	38	48	15
intramolecular reactn[c]	60	4	37

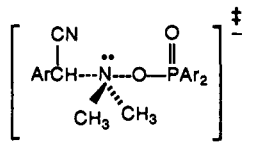
[a] Determined by FIMS (±5%). [b] Molecular ion for ^{12}C, ^{1}H compound. [c] Calculated on the basis of the mixture of **1** and **3**.

If the reaction were to involve an ion pair, the intermolecular result could be consistent with nucleophilic addition occurring on the side of the ion pair opposite the phosphinate leaving group. The second-order kinetics observed by Boche for similar reactions would require the ion pair to be formed reversibly.[3a] The work of Goering suggests that such an ion pair that reacts with an external nucleophile would be symmetrical by the criterion of scrambling of the oxygen atoms.[8] We have evaluated the possible intermediacy of a symmetrical ion pair from **7** in its reaction with benzyl cyanide and LDA to give **8** in 65% yield by the use of the ^{18}-O-labeled derivative **9**. If the reaction involved a reversibly formed symmetrical ion pair **10**, we would expect the ^{18}O label in **9** to be scrambled between the two oxygens of recovered reactant. However, we find that recovered **9** from a reaction that has proceeded to 80% completion has the ^{18}O label unscrambled. The position and extent of the label were established by reaction of **9** with phenyllithium, a substitution at phosphorus that gives triphenylphosphine oxide, which was found to have the same level of ^{18}O from both initial and recovered **9**. If scrambling had occurred, the triphenylphosphine oxide from recovered **9** would have been one-half of that from the initial **9**, in contrast to our observation.



10

The present results show that the displacement at the nitrogen of a dimethylamino group of an oxygen of a phosphinate by an anionic carbon cannot be achieved within the endocyclic confines of a six-membered ring and that the reaction does not involve internal return from a symmetrical ion pair. We suggest that nitrogen transfer occurs in a concerted reaction with the transition-structure geometry of a trigonal bipyramid shown for **11**. In this transition structure the entering and leaving groups are at a large angle analogous to that of a classic S_N2 displacement and consistent with the mechanism proposed by Boche for this reaction.[3a] This provides, to the best of our knowledge, the first experimental information about the geometry of nucleophilic displacement at a formally neutral nitrogen atom.[5] We also note that this work further illustrates the value of the endocyclic restriction test for an experimental evaluation of transition-structure geometry to allow a choice between alternative reaction mechanisms.[9]



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Acknowledgment. We are grateful to the National Science Foundation and National Institutes of Health for support of this work.

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Bile Salt Modulated Stereoselection in the Cholesterol Esterase Catalyzed Hydrolysis of α-Tocopheryl Acetates[1]

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A major characteristic of catalysis by enzymes is stereoselectivity[3] which can be modified by changing the precise structure of the active site. In principle, this can be achieved by exotic procedures such as "protein engineering",[4] or by the simple expedient of changing the reaction medium,[5] or by unfolding and then refolding the enzyme.[6] The potential impact of an altered enzymic stereoselectivity on organic syntheses and mechanistic biochemistry has greatly encouraged research on this topic. While attempting to reconcile differences between the in vivo[7] and in vitro[8] rates of the carboxylic ester hydrolase (EC 3.1.1.13) catalyzed hydrolysis of (2*R*,4′*R*,8′*R*)- and (2*S*,4′*R*,8′*R*)-α-tocopheryl acetates (*RRR*- and *SRR*-α-TAc)[9] in the presence of the obligatory 3α,7α,12α-trihydroxy bile salts,[10] cholate, taurocholate, and glycocholate, we made the novel discovery that *the structure of the bile salt also has a profound effect on the stereoselectivity of this reaction.*

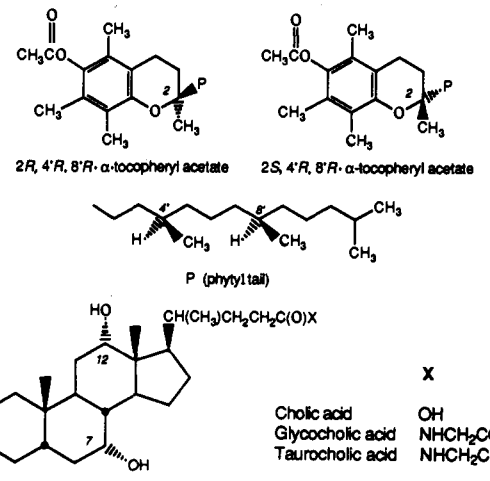

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Table I. Comparison of the Ratios of the Observed Pseudo-First-Order Rate Constants, k_{obsd}, Obtained for the Noncompetitive and Competitive (in Parentheses) Hydrolyses of (*RRR*)- and (*SRR*)-α-Tocopheryl Acetates in Bile Salt/DMPC Mixed Micelles by Bovine and Porcine Cholesterol Esterases[a]

	$k_{obsd}(RRR)/k_{obsd}(SRR)$		
bile salt	crude BCE	crude PCE	pure PCE[b]
cholate	0.28 (–)[c]	0.19 (0.21)	0.30 (0.26)
glycocholate	1.2 (0.18)	1.1 (0.18)	1.4 (0.32)
taurocholate	35 (3.0)	27 (3.0)	19 (4.2)

[a] Specific enzyme activity was determined by measuring the rate of hydrolysis of cholesteryl-3-glutaric acid-resorufin diester (a specific substrate for cholesterol esterase obtained from Boehringer Mannheim Biochemica, West Germany). One unit is the amount of enzyme required to hydrolyze 1 mmol of substrate in 1 min at 37 °C. Values for crude BCE, crude PCE, and pure PCE were 2.68×10^{-4}, 0.887×10^{-4}, and 3.43×10^{-4} unit/mg, respectively. In order to have a uniform reactivity of enzyme, the amount of each preparation used was adjusted according to the initial rate of hydrolysis of a sample of *SRR*-α-TAc (0.094 mM) in sodium cholate (40 mM)/DMPC (2.0 mM) mixed micelles in Tris buffer (50 mM; pH 8), the absorbance being monitored at 302 nm ($\epsilon_{\alpha\text{-T}} - \epsilon_{\alpha\text{-TAc}} = 2300$ L mol^{-1} cm^{-1}). Thus adjusted, the initial rates of hydrolyses by crude BCE, crude PCE, and pure PCE of *SRR*-α-TAc in this set of runs were 4.85×10^{-8}, 4.59×10^{-8}, and 4.90×10^{-8} M s^{-1}, respectively. All runs were performed under conditions identical with those given above. Individual substrate concentrations in the competitive runs were the same as in the noncompetitive runs. [b] Pseudo-first-order rate constants ($\times 10^5$, s^{-1}) for *RRR*-α-TAc/*SRR*-α-TAc obtained for noncompetitive (competitive) runs were 8.7/27 (5.4/21), 3.3/2.3 (0.57/1.8), and 7.0/0.37 (1.3/0.31) for cholate, glycocholate, and taurocholate, respectively. [c] Not measured for this particular batch of crude BCE under these specific conditions. However, measurement with an earlier batch of enzyme showed *RRR*/*SRR* < 1.

In our in vivo study,[7] rats were fed a diet containing an equimolar mixture of deuterium-labeled α-TAc's, d_6-*RRR*- and d_3-*SRR*-α-TAc. The *RRR*-α-TAc/*SRR*-α-TAc ratio of intestinal fecal material was ca. 0.2 while the corresponding ratio of the phenolic products of hydrolysis was ca. 1.3. Clearly, *RRR*-α-TAc was more extensively and presumably more rapidly hydrolyzed than *SRR*-α-TAc. Our in vitro model system[8] included sodium cholate (40 mM) as the obligatory bile salt,[10] one of the two α-TAc stereoisomers (0.04–0.20 mM), and either *dl*- or *l*-dimyristoylphosphatidylcholine (*dl*- or *l*-DMPC, 2.0 mM). At 37 °C commercial bovine cholesterol esterase (BCE) hydrolyzed the *RRR* stereoisomer *less rapidly* than the *SRR* stereoisomer. This result was confirmed by a *competitive* kinetic study involving the hydrolysis of an equimolar mixture of unlabeled (d_0) *RRR*-α-TAc and d_3-*SRR*-α-TAc in which the yields of the two free phenols were monitored over time by GC/MS.[8]

The failure of our in vitro model to duplicate the in vivo results was traced to the rat's bile (secreted from liver into the gut) rather than to the rat's esterase enzyme(s) (secreted from pancreas into the gut) via the following two *competitive* kinetic experiments, which employed equimolar mixtures of d_0-*RRR*- and d_3-*SRR*-α-TAc's: (i) rat bile[16] + rat pancreatic juice;[16] (ii) rat bile + BCE. In *both* experiments *RRR*-α-TAc was hydrolyzed *more rapidly* than *SSR*-α-TAc, proving that the stereoselectivities of BCE and of the carboxylic ester hydrolase(s) secreted by the rat are similar.

In the rat, cholic acid is not present in detectable amounts as free acid but rather as its taurine and glycine conjugates.[17] Noncompetitive kinetic experiments involving the BCE-catalyzed hydrolysis of *RRR*-α-TAc and *SRR*-α-TAc were therefore carried out at 37 °C under conditions generally similar to those previously employed[8] using the sodium salts of cholic, taurocholic, and glycocholic acids. Initial rates, measured both spectrophotometrically[8] and by GC/MS (see Table I), confirmed that with cholate *SRR*-α-TAc was hydrolyzed more rapidly than *RRR*-α-TAc. However, with taurocholate *SRR*-α-TAc was hydrolyzed much less rapidly than *RRR*-α-TAc, and with glycocholate *SRR*-α-TAc was hydrolyzed slightly less rapidly than *RRR*-α-TAc (Table I). *Competitive* experiments using d_0-*RRR*- and d_3-*SRR*-α-TAc yielded the same reactivity order with cholate (*SRR* > *RRR*) and with taurocholate (*SRR* < *RRR*). However, with glycocholate the order was reversed, i.e., in the competitive experiment *SRR*-α-TAc was hydrolyzed more rapidly than *RRR*-α-TAc.[18]

Commercial BCE is quite impure,[19] being contaminated with proteases which rapidly deactivate it. Despite extensive efforts, we were only able to achieve a partial purification of the BCE, obtaining a protease-free preparation by affinity chromatography [Affi-gel blue (Bio-Rad); elution with 50 mM Tris·HCl, pH 8, at 5 °C]. The protease-free enzyme's selectivity in hydrolyzing the α-TAc stereoisomers was modulated by each of the three bile salts in *both* the noncompetitive and competitive experiments in *exactly* the same way as was found with the less pure, commercial BCE (data not shown).

Commercial (Sigma) porcine cholesterol esterase (PCE) contains less protease than commercial BCE. Protease-free PCE, obtained as described for BCE, was further purified by ion-exchange chromatography [DEAE Affi-gel blue (Bio-Rad); elution with 50 mM Tris·HCl (pH 8)/0.5 M NaCl] to a single band on SDS polyacrylamide gel electrophoresis (estimated reduced molecular weight by SDS PAGE was 58 kDa).

The stereoselectivities of commercial BCE, commercial PCE, protease-free PCE (data not shown), and pure PCE in hydrolyzing the two α-TAc stereoisomers were modulated by each of the three bile salts in *exactly* the same way in *both* the noncompetitive and competitive experiments (Table I). This consistent behavior, independent of source and purity of enzyme preparation, strongly suggests that the order of the bile salt modulated selectivity in

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the hydrolysis of α-TAc stereoisomers is an *intrinsic* property of cholesterol esterases. The data for pure PCE (Table I, footnote *b*) show as much as a 60-fold and 14-fold range in *RRR*/*SRR* selectivity in the noncompetitive and competitive experiments, respectively. Interestingly, in the competitive experiments the rate of hydrolysis of *RRR*-α-TAc is significantly affected but that of *SRR*-α-TAc is little affected relative to the corresponding rates measured in the noncompetitive experiments.[18]

At 40 mM the bile salts will form mixed micelles[20] with the DMPC (2 mM) and α-TAc (0.1–0.2 mM). Although the initial rates of hydrolysis can be described by using classical Michaelis–Menten (MM) kinetics,[8] we are not, of course, dealing with a molecular solution of α-TAc but with micellized α-TAc which presumably reacts with the water-soluble BCE or PCE at the micelle–water interface.[21] The effect of the (chiral) bile salts on the relative (and absolute) rates of hydrolysis of *RRR*- and *SRR*-α-TAc could result from diastereoisomeric influences upon the epimeric acetates induced within the mixed micelle itself or, alternatively, may arise from direct bile salt–protein interactions.[19] The degree of chiral recognition that is achieved is genuinely dramatic in view of the fact that *the chiral center is separated by six bonds from the bond that is cleaved by the enzyme.*[23]

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(21) Such two-dimensional systems can appear to follow MM kinetics (even including product inhibition), but the derived kinetic parameters have more complicated meanings than for homogeneous enzyme reactions.[19,22]

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(23) It was recently reported[6] that a lipase treated with an organic solvent and deoxycholate is converted to a form that is more enantioselective toward hydrolysis of a variety of (±)-arylpropionic and (±)-phenoxypropionic esters. In these cases, however, the chiral center is separated by only a single bond from the bond that is cleaved.

Exogenous Ligand Binding to the $[Fe_4S_4]$ Cluster in *Pyrococcus furiosus* Ferredoxin

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Cubane-type $[Fe_4S_4]$ clusters functionalized by non-cysteinyl and/or unique ligation of a specific Fe or by replacement of one Fe by another transition metal (e.g., Mo, V, or Ni) have been proposed as components of the active sites of a range of metalloenzymes. These include aconitase,[1] several (de)hydratases,[1] sulfite reductase,[2] nitrogenase,[3] hydrogenase,[4] and CO dehydrogenase.[5] Synthetic strategies to investigate subsite-specific ligand binding to such clusters in aprotic media have recently focused on $[Fe_4S_4]^{2+}$ clusters ligated by a semirigid trithiolate cavitand ligand.[6] Simple ferredoxins containing $[Fe_4S_4]$ clusters offer the potential for investigating such ligand-binding interactions in a more physiologically relevant environment. However, of the more than 30 ferredoxins for which sequences are available,[7] only five appear to contain a $[Fe_4S_4]$ cluster with incomplete cysteinyl ligation.[8] Four of these are 8Fe-ferredoxins in which only one of the two $[Fe_4S_4]$ clusters has partial non-cysteinyl coordination. This leaves the ferredoxin (Fd) from the hyperthermophilic archaebacterium *Pyrococcus furiosus*[8] as the only example of a 4Fe-Fd with non-sulfur ligation to a specific Fe atom. This protein therefore provides an opportunity to study the subsite-specific ligand binding of a $[Fe_4S_4]$ cluster in a biological environment. We report here the binding of cyanide, an inhibitor for numerous Fe–S containing enzymes and a substrate for nitrogenase, to the $[Fe_4S_4]^{1+}$ cluster in dithionite-reduced *P. furiosus* Fd. This is the first report of cyanide binding to a biological $[Fe_4S_4]$ cluster, as well as the first example of exogenous ligand binding to a $[Fe_4S_4]^{1+}$ cluster in a bacterial ferredoxin.

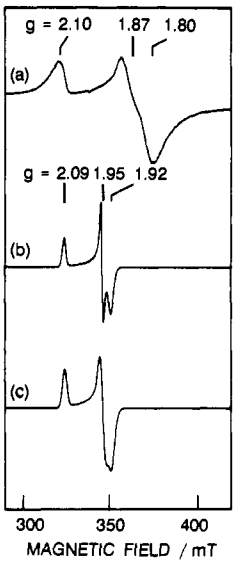

Figure 1. X-band EPR spectra of dithionite-reduced *P. furiosus* Fd, native (a), cyanide-treated (b), and ^{57}Fe-reconstituted cyanide-treated (c): (a) 0.85 mM Fd in 100 mM Tris·HCl buffer, pH 7.8; microwave power, 1 mW; temperature, 8 K; (b,c) 0.21 mM Fd in 100 mM Tris·HCl buffer with a 250-fold excess of potassium cyanide, pH 8.5; microwave power, 1 mW; temperature, 20 K. All spectra were recorded at 9.44 GHz with 0.63 mT modulation amplitude.

P. furiosus Fd, M_r = 7500, is a monomeric protein containing a single $[Fe_4S_4]$ cluster.[8a] The arrangement of cysteine residues in the amino acid sequence is analogous to that of other bacterial ferredoxins except that the second cysteine in the traditional sequence is replaced by an aspartate residue. Non-cysteinyl coordination of a specific Fe is manifest by novel spectroscopic

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