THE CATALYTIC MECHANISM OF MANDELATE RACEMASE FROM PSEUDOMONAS PUTIDA

by

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B.S., The University of Connecticut, Storrs, Connecticut, 1979

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

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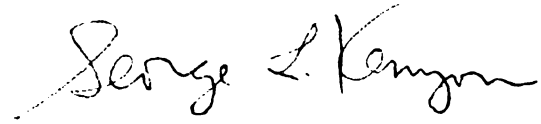
ABSTRACT

Mandelate Racemase (E.C.5.1.2.2) catalyzes the interconversion of R- or S-mandelate without the assistance of a co-factor. By using the circular dichroic properties of the substrate (CD bands at 262nm) as a direct assay for monitoring the kinetics of the racemase reaction, a kinetic symmetry has been found; that is, nearly equal kinetic parameters (K_M, V_{max}, deuterium isotope effects, and pH profiles) were measured in both directions.

R,S-α-Phenylglycidate is known to be an active-site-directed irreversible inhibitor of the enzyme. The R- and S-α-phenylglycidates were resolved via their diastereomeric octyl esters using low-pressure liquid chromatography. Assignments of their absolute stereochemistries have been made by $LiAlH_4$ reduction of the octyl esters to their respective optically active 2-hydroxy-2-phenylpropanols of known configuration. The assignments were confirmed by circular dichroic correlations. Hydrolysis of the octyl esters yielded the enantiomeric acids as their potassium salts.

Incubation of these enantiomers with the enzyme revealed the presence of an asymmetry at the active site not obvious in the processing of the natural substrate. The R-(+)-α-phenylglycidate was approximately 10-fold more efficient as an irreversible inhibitor of mandelate racemase than the S-(-)-isomer. These results raised the possibility that catalysis might proceed by a so-called two-base acceptor mechanism. A hallmark characteristic of such a two-base acceptor mechanism is the occurrence of an overshoot region during the time course of racemization in D_2O. Mandelate racemase did not exhibit this feature, however, a result consistent with a one-base rather than a two-base mechanism.

Constraint of the epoxide ring apparently prevents the oxygen of the S-isomer from being effectively protonated by a nearby amino acid residue which assists in ring-opening of the epoxide during nucleophilic attack of the affinity label. This side amino acid residue is presumed to be responsible for binding of the α-hydroxy group of mandelate in the normal mechanism.



ACKNOWLEDGEMENTS

I am grateful to Professor George L. Kenyon for his advice, encouragement, and seemingly unlimited patience during my five years at the University of California. I also thank him for allowing me to pursue this project at my own pace.

I am indebted to Professor George D. Hegeman (Indiana University, Bloomington, Indiana) for his advice on the microbiological aspects of this project.

I wish to thank Professor W.W. Cleland (University of Wisconsin, Madison, Wisconsin) for allowing me to spend two months in his laboratory in order to learn enzyme kinetics.

I also greatly appreciate the time and interest that Professor John C. Craig showed in this project. His help was invaluable.

I also am indebted to Professor Norman Oppenheimer for the many helpful discussions and keen insights.

During my five years at the University of California, many faculty, staff, post-docs, and fellow graduate students have helped me along. I am particularly indebted to Tom Marschner for his generous help with many of the technical aspects of this project.

Finally, I am grateful to the National Institutes of Health and to the Earle C. Anthony Trust Fund for financial support.

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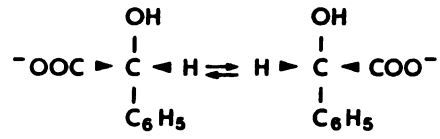
A. Mandelate Racemase

Mandelate racemase, isolated from *Pseudomonas putida* strain A.3.12, catalyzes the racemization of R- or S- mandelate as shown below.



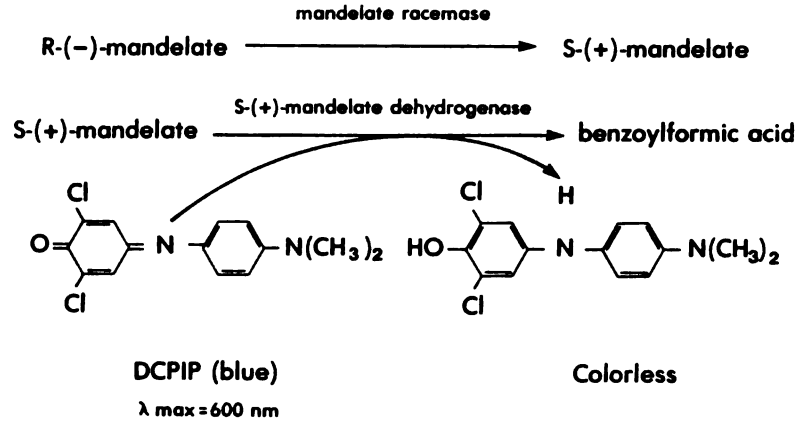
R-Mandelate S-Mandelate

The enzyme is the first member of an inducible series of five enzymes known collectively as the mandelate group, enabling strain A.3.12 to use either R- or S-mandelate as its sole source of carbon and energy. Hegeman has demonstrated that the enzymes making up the mandelate group behave as members of an operon[1-3]. After metabolism of the mandelate to benzoic acid by the mandelate group, a second set of enzymes, the β-keto-adipate group, further metabolizes the benzoic acid to succinic acid and acetyl coA.

Hegeman et al.[4] obtained the first highly purified preparation of the enzyme using a six-step procedure resulting in a 550 fold purification. The molecular weight of the protein has been estimated to be 280,000 as determined by cross-linking of the enzyme with dimethyl suberimidate followed by sodium dodecyl sulfate (SDS) gel electrophoresis. It is a tetramer consisting of four identical subunits.

Mandelate racemase does not require coenzymes such as pyridoxal phosphate (PLP), NAD^+, or FAD^+. The reaction does, however, require a divalent cation (e.g., Mg^{2+}) for activity as shown by Fee et al.[5]

The enzymatic reaction can be followed spectrophotometrically via a coupled assay developed by Hegeman.[1] The assay is described below.



The assay only allows one to follow racemization in the R(-) to S(+) direction. It has other drawbacks as discussed by Sharp et al.[6] Consequently, a circular dichroic assay, based on the strong and opposite circular dichroic ellipticities which R(-)- and S(+)-mandelate manifest at 262 nm, was developed.[6] This assay makes possible the measurement of the Michaelis-Menton kinetic parameters (K_M and V_{max}), deuterium isotope effects, and pH profiles, among other things, for both reactants and products.

Kenyon and Hegeman [7] considered and discussed in a recent review the most likely mechanisms for enzymatic racemization along with the experimental evidence inconsistent with certain other mechanisms. A preponderance of the evidence is consistent with racemization proceeding via a carbanion intermediate using the so-called single base acceptor mechanism.[8] In such a scenario , one can envision a flexible single base (such as the γ-carboxyl group of a glutamate residue) at the active site abstracting the α-proton and then "swinging around" to place the same proton, corrected for partial solvent exchange, back onto the other face of the planar intermediate.

The divalent cation acts as an electron sink to labilize the α-proton. It can either coordinate itself directly to the carboxyl oxygens of the substrate or to a water molecule between it and the carboxyl group of mandelate. Studies of Maggio et al. indicate the latter.[9]

There are two experiments that are consistent with the carbanion intermediate. Firstly, there is a large deuterium isotope effect in the overall enzymatic reaction (ca. 5) as measured by Kenyon et al.[10] Secondly, electron-withdrawing substituents in the para position on the aromatic ring tend to increase the V_{max}. The increase in V_{max} is presumably due to the ability of the benzene ring to stabilize the increasing carbanionic character at the α-position.

The most compelling argument for a single-base acceptor mechanism is the racemase-catalyzed tritium exchange experiment conducted by Kenyon and co-workers.[7] It was found that racemization of α-[^{3}H]-S(+)-mandelate yielded R(-) mandelate containing about 80% of the tritium, corresponding to about 20% exchange with water. This partial conservation of tritium suggests that there is a single base at the active site which, after abstracting the α-proton, exchanges with solvent one in five times while returning the same proton in a stereochemically random manner four out of five times.

A second experiment, conducted by Sharp et al.[11], is also consistent with the one-base acceptor mechanism. In this experiment an equimolar mixture of R,S-[α-^{2}H] mandelate and R,S-[α-^{13}C] mandelate was incubated with the enzyme. If two bases were present at the active site, then a doubly labeled mandelate, both α-^{2}H and α-^{13}C, and an unlabeled molecule would be the predicted result. However, a single base at the active site would not permit the enzymatic production of a doubly labeled molecule. The latter prediction was borne out experimentally.

These experiments do not rule out, however, a rapid direct proton transfer between two bases on the enzyme. In one such scenario, a second base could receive the proton abstracted by the first base, and put it back

onto the opposite face of the planar carbanion. Upon binding of the opposite enantiomer, this second base abstracts the proton, and transfers it to the first base which then returns the same proton to the presumably planar carbanion intermediate. Such a transfer could occur with only occasional solvent exchange. As of yet, there is no literature precedence for such a mechanism.

In an attempt to identify the nature of the nucleophilic base at the active site, Fee et al.[12] synthesized R,S-α-phenylglycidate as an active-site-directed irreversible inhibitor of mandelate racemase. These studies provided indirect evidence to suggest that a carboxyl group on an aspartate or glutamate residue may be the amino acid labeled by R,S-α-phenylglycidate.

The kinetic studies performed in this thesis suggest a symmetric active site for the binding and processing of the substrate by mandelate racemase. However, Wang and Walsh[13] have shown that a functional asymmetry exists for the active site of alanine racemase and , although this asymmetry is not obvious in the processing of the natural substrate, it manifests itself most dramatically in the processing of suicide inhibitors. Therefore, it was decided to probe further the active site of mandelate racemase for potential asymmetry through the use of enantiomeric inhibitors - both reversible and irre-

versible.

The R and S phenylglycerates were selected for the reversible inhibitors for two reasons. Firstly, Maggio et al.[9] had demonstrated that R,S-phenylglycerate was a potent competitive inhibitor of mandelate racemase. Secondly, its close structural resemblence to mandelate ensured that a relatively facile and quick method of optical resolution could either be found or readily adapted from plentiful literature precedents. The obvious candidates for the irreversible inhibitors were the R and S enantiomers of α-phenylglycidate. Not only had it been shown by Fee et al.[12] that the racemic mixture was an irreversible inhibitor, but preliminary work of Sharp et al.[14] had indicated that the α-phenylglycidates could be resolved as their diastereomeric octyl esters.

B. Other Racemases and Epimerases

1. Racemases

Enzymes interconverting the configuration at a single center of a substrate can be classified as racemases or epimerases. A racemase acts on substrates with a single asymmetric center while an epimerase acts on substrates with multiple asymmetric centers.

A number of racemases have been studied in detail. A review by Adams covers the literature to 1975.[15] Enzymatic racemization can proceed by a variety of mechanisms. In the following discussion these racemases fall into three categories: 1) those requiring the coenzyme pyridoxal phosphate ; 2) one racemase utilizing ATP; and, 3)the largest group of racemases, consisting of those enzymes that catalyze racemization without the aid of coenzymes.

The best characterized racemases are the pyridoxal phosphate requiring enzymes that are known for many of the common α-amino acids. The well-studied alanine and arginine racemases are representative of this group, along with a recently discovered α-amino-ε-caprolactam racemase.[16] The physiological role of these amino acid racemases, found thus far only in bacteria, is probably to supply R-amino acids as components of the cell wall.[17] The mechanistic details are discussed elsewhere.[17]

The second category consists of the only known ATP-requiring racemase, S-phenylalanine racemase. The racemase is used by *Bacillus brevis* to supply R-phenylalanine in its elaboration of the antibiotic gramicidin S. The mechanistic details are, again, discussed elsewhere.[18]

Finally, there are a number of racemases that act as catalysts without the benefit of PLP, ATP, or other coenzymes. These include mandelate racemase[7], lactate racemase[19], proline racemase[20], methylmalonyl CoA racemase[21], and most recently, an allantoin racemase[22].

The experimental evidence compiled for proline racemase suggests that it catalyzes racemization through a carbanion intermediate using the so-called two-base acceptor mechanism.[8] The bases are believed to be two cysteine residues.[20] Adams has compared the kinetic and mechanistic properties of the active site of this racemase to those for the hydroxyproline epimerase and has noted that there is a remarkable similarity, including the putative catalytic use of the two cysteine bases. The only exception is that each shows a marked specificity for their physiologically appropriate substrate.[15]

Lactate racemase is unique because it does not utilize NAD^+, and unlike other racemases in this category, it apparently does not exchange the abstracted proton with

solvent. It reportedly initiates racemization through the formation of an enzyme thiol ester intermediate followed by an internal hydride transfer and subsequent hydrolysis to release the transformed substrate.[19] The mechanism is somewhat similar to that proposed for glyoxylase.[15]

The nonenzymatic exchange of the acidic C-2 proton on methylmalonyl CoA has prevented reliable mechanistic studies.[17] At present, there are no mechanistic details available for allantoin racemase.[22]

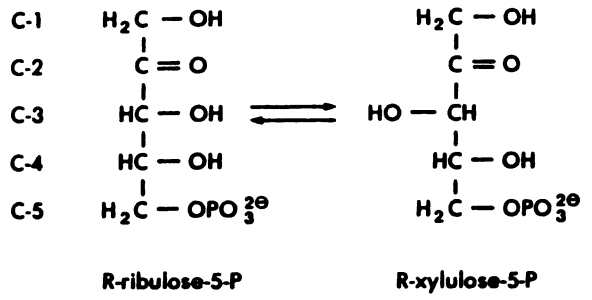
2. Epimerases

The number of known epimerases is considerably greater. The discussion will be limited to representative examples. Included will be UDP-glucose-UDP-galactose epimerase[23], ribulose-5-phosphate 3-epimerase[24], and ribulose-5-phosphate 4-epimerase[17,24] which, like lactate racemase, does not utilize NAD^+ and catalyzes epimerization without concurrent solvent exchange.

The lack of exchange with solvent protons for UDP-glucose epimerase suggested to Hogness and Wilson the possibility of NAD^+-dependent catalysis. After exhaustive efforts, a single NAD^+ per catalytic center (2 subunits), reversibly reduced by either UDP-glucose or UDP-galactose, was found.[23] Other evidence indicates that only one face of the NAD^+ is utilized for the shuttle of the abstracted proton between coenzyme and the C-4-keto intermediate. Although it has been the object of intense scrutiny, the question still remains as to how the UDP-glucose epimerase acts to accept a proton from either epimer and return it.[15] Currently, conformational changes, either to bind the substrate in a different conformation or to move the NAD^+, are used to explain the mechanism.[15]

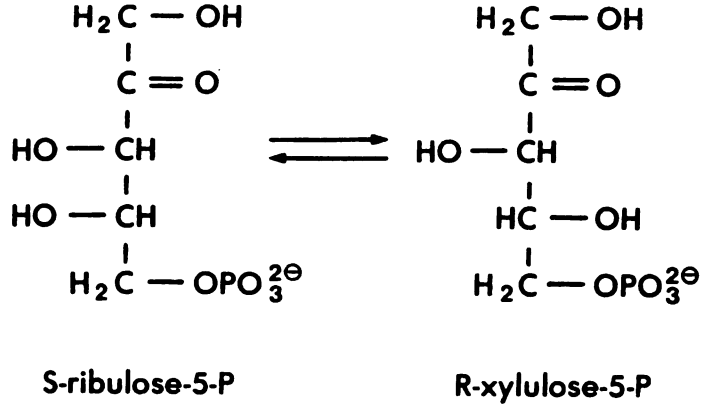
Ribulose-5-phosphate 3-epimerase[24], in contrast to UDP-glucose-UDP-galactose epimerase, incorporates one atom of solvent proton at C-3 of R-ribulose-5-phosphate

during epimerization.



The C-3 proton, labilized by its proximity to the carbonyl group, is most likely abstracted by a base on the enzyme to form a carbanion. The increasing carbanionic character at C-3 is stabilized by the formation of an enediolate anion.

On the other hand, S-ribulose-5-phosphate 4-epimerase does not catalyze epimerization with concurrent solvent exchange, and there is no known cofactor.[17,24] Also, unlike ribulose-5-phosphate 3-epimerase, there is no adjacent carbonyl group to stabilize increasing carbanionic character. There may be a group on the enzyme to stabilize a carbanion, if one does indeed form, but there is no literature precedence for it.[17]



Rose has invoked a one base acceptor mechanism to transfer a proton between the carbanion intermediate and enzyme, with the base being on the end of a flexible arm such as the ε-amino group of lysine or the γ-carboxyl group of glutamate and shielded from solvent.[8,25]

C. Symmetry of Kinetic Rate Constants

The notorious stereospecificity of enzyme catalysis is the "central dogma" of modern enzymology. Except in the case of the interconversion of stereoisomers, enzymes almost always use only one optical antipode as a substrate. This stereospecificity is not at all surprising since enzymes are composed only of S-amino acids and so are inherently asymmetric. Therefore, interaction between the enzyme's chiral active site and an enantiomeric pair of substrates will result in two diastereomeric transition states. These transition states will have different energies of activation, different reactivities, and therefore will partition differently between reactants and products.[17]

It is noteworthy to mention that even racemases, interconverting two enantiomers, still maintain the stereospecificity of the intermediate which, chemically, would rapidly and randomly equilibrate into racemic products.[25] For instance, proline racemase apparently generates an enzyme-bound carbanion intermediate which receives a proton from the enzyme in a stereospecific manner.[20] Such an enantiomeric carbanion generated in solution rapidly interconverts and the resulting racemic mixture is randomly protonated.

Enzymatic stereospecifity is controlled two ways

- through binding and/or catalysis. For example, an enzyme may bind one enantiomer, but not the other, or it may bind both enantiomers and only process a reaction with one. Kinetic parameters, such as the familiar Michaelis-Menten ones, K_M and V_{max}, and deuterium isotope effects, as broken down into an effect on binding(V_{max}/K_M) and an effect on catalysis(V_{max}), are a measure of the rate of binding and partitioning occurring during catalysis. Up till the time of this thesis, of these parameters only the K_M and V_{max} have been measured in both directions for any of the racemases. Table I summarizes the available data for racemases.

The Haldane relationship, below, relates the K_M and V_{max} of reactants and products to the equilibrium constant.[26]

$$K_{eq} = \frac{(V_{max})_f (K_m)_p}{(V_{max})_r (K_m)_s}$$

By definition, the equilibrium constant is unity for a racemization reaction. For the values measured there is good experimental agreement with the Haldane relationship.

Epimerases, on the other hand, do not catalyze symmetric reactions, as by definition, their substrates

Table 1

ENZYME	K_M (mM) S	K_M (mM) R	V_{max} S → R	V_{max} R → S
Allantoin Racemase[22]	-	4.4* 2.8§	-	-
α-Amino-ε-Caprolactam Racemase[16]	6	8	-	-
Phenylalanine Racemase[18]	0.06	0.13	-	-
Lactate Racemase[19]	8	8	-	-
Proline Racemase[20]	2.3	3.8	4.4×10^{-4} mM/mL	5.8×10^{-4} mM/mL
Alanine Racemase[13]	1.3	1.2	1124 s^{-1}	974 s^{-1}
Mandelate Racemase	0.26 ± 0.03	0.23 ± 0.02	0.75 mM/mL	0.83 mM/mL

* measured in 50 mM phosphate buffer pH 6.5.

§ measured in 300 mM acetate buffer pH 5.1.

are diastereomers. As a result, there is a difference between the energy levels of the reactant and product and the equilibrium constant is not unity. Therefore, the kinetic relationships of binding and catalysis do not readily and symmetrically simplify for epimerases as they do for racemases. For instance, the Haldane relationship sets the V_{max}/K_M ratios of the forward and reverse reactions of racemases equal to one another. This equality, in turn, mandates certain ratios among the various kinetic rate constants of the enzyme-catalyzed reaction. As a result of these interrelationships, one is able to make a number of predictions and generalizations about racemase-catalyzed reactions. In contrast, one cannot make a like simplification of the Haldane relationship for epimerases.

The following epimerases are illustrative. Glaser[24] and Adams[15] have tried to extrapolate from available data a possible asymmetry in the binding of the two substrates for UDP-glucose-UDP-galactose epimerase. Their reasoning resulted from the current catalytic picture in which a single NAD^+ is reversibly reduced by either substrate. They were, however, unable to make generalizations. For instance, the K_M was 15-fold greater for UDP-galactose for the E.coli enzyme while the K_M for UDP-glucose was 16-fold greater for the mammary gland enzyme.[15] Enzyme preparations from other sources, though, showed similar K_M values, although not identical. The K_M

ratios for the liver enzyme was 1.4 while it was 2 for the wheat germ enzyme. Here one cannot make predictions about the corresponding V_{max} ratios or other rate constants based on the K_M ratios without first considering the equilibrium constant.

A second example comes from the kinetic constants of hydroxyproline epimerase. Similar values were reported by Adams[15] for all four substrates, (S-cis-4-hydroxyproline $\rightleftharpoons$ R-trans-4-hydroxyproline and R-cis-4-hydroxyproline $\rightleftharpoons$ S-trans-4-hydroxyproline), though no other comments were made. Again, however, predictions about the kinetic properties of this epimerase cannot be made without taking into account the equilibrium constant. In this specific case, though, the equilibrium constant is probably near unity, accounting for the nearly identical Michaelis-Menten parameters.

D. Active-Site-Directed Irreversible Inhibitors

The past decade has witnessed an explosion in the use of active-site-directed irreversible inhibitors, so-called affinity labels, of enzymes. Reviews include those by Shaw,[27] Maycock and Abeles,[28] Rando,[29] and Walsh.[30] The architectural approach to the design of an affinity label is to synthesize a substrate analog bearing an electrophilic portion while still maintaining sufficient structural similarities to the natural substrate.

The structural similarities of the affinity label facilitate binding to the enzyme, enabling the electrophilic portion to be brought into close apposition to the various amino acid residues at the active site; and, in the best situation, labeling of the nucleophilic amino acid residue responsible for the enzyme's catalytic activity will occur. Upon degradation of the inactive protein (e.g., via either acid hydrolysis or exhaustive proteolytic digestion), the identity of the amino acid residue may be determined. By such an approach more will be learned either about the amino acid groups making up the active site or about the nature of the actual catalytic group.[30]

As previously mentioned, enzymes, being asymmetric, have chiral active sites and so will stereospecifically discriminate among different configura-

tions of substrates, coenzymes, and products. Therefore, the stereoselective affinity labeling of enzymes with asymmetric reagents can occur. Indeed, the literature is replete with such examples. In the following discussion examples of the use of asymmetric modifying reagents will be presented, followed by examples of enantiomeric affinity labels, and finally, by examples of chiral suicide substrates.

Early use of stereospecific reagents involved asymmetric chemical modifying reagents which are designed for broad specificity and so can be used to study several different enzymes. Such enzymes studied were ribonuclease,[31,32] papain,[33] yeast alcohol dehydrogenase,[34] and yeast glyceraldehyde phosphate dehydrogenase.[35] For each of these enzymes, a more rapid inactivation resulted using one of the two possible enantiomers. The most comprehensive work involved a series of optically active alkylating reagents for ribonuclease.[31,32] Incubation with the enzyme of a series of α-bromo acids ranging in chain length from two to six carbons revealed that each of the three amino acid residues implicated in the catalytic mechanism, His 12, His 119, and Lys 41, had different reactivities toward the chiral alkylating reagents. Those of the R configuration favored alkylation at His 12 while those of the S configuration favored reactions at His 119. There was no selectivity of the optical

isomers of a given reagent for alkylation for the lysine residue. The active site permits binding of the alkylating reagent in such configurations that with one enantiomer the electrophilic center is positioned within reacting distance of the His 12 while the other enantiomer is placed within reacting distance of the His 119.[31,32]

The use of enantiomeric active-site-directed irreversible inhibitors provides specific probes of the active site of the enzyme under study. These enantiomeric inhibitors can interact with the active site in one of two ways. Either both enantiomers can label the enzyme, but at significantly different rates of reaction, or only one of the enantiomers can label the enzyme with the other enantiomer remaining totally inactive. Examples of the first possibility include R- and S-glycidol phosphate (2,3-epoxypropanol phosphate) used to label muscle triose isomerase[36] and 6-diazo-5-oxo-R- and S-norleucine used to label γ-glutamyl transpeptidase.[37] β-Chloro-R- and S-alanine as affinity labels of S-aspartate-β-decarboxylase exemplify the second possible type of interaction.[38]

The racemic mixture of glycidol phosphate had been shown to be an active-site-directed irreversible inhibitor of muscle triose phosphate isomerase.[36] Therefore the separate enantiomers were synthesized and tested. The concentration for the half maximal rates of inactivation was found to be similar, 5.2 mM for the R-enantiomer

and 4mM for the S-enantiomer. The maximal velocity of inactivation, though, was measured to be 10-fold greater using the R-glycidol-phosphate (38% for the R-glycidol phosphate vs. 3.8% for the S enantiomer). Another experiment indicated that there was no variation in the rate of inactivation for either enantiomer over the pH range of 5.5 to 10.0. [36]

Mechanistically, these results are attributed to the unfavorable reorientation the S-glycidol phosphate must undergo in order for it to achieve alkylation while the R-glycidol phosphate binds in a favorable position. The picture of the active site put forth by Schray and coworkers has a proton- donating residue proximal to the oxirane oxygen to assist in catalysis while the nucleophilc residue, identified as glutamate, is so positioned to allow backside attack.[36]

Another explanation for differences in rates of reactions of enantiomeric affinity labels is provided by the results of the labeling of rat kidney γ-glutamyl transpeptidase by 6-diazo-5-oxo-R- and S- norleucine.[37] In this case the S antipode was found to inactivate at a rate 4-5 times greater than that for the R label. The rate difference was explained by the presence on the enzyme of two functionally distinct sites - an acceptor site for the amino acid or peptide and a donor site for the γ-glutamyl moiety of glutathione. The donor site, it was

suggested, is poor in stereoselectivity while the acceptor site exhibits strict specificity.[39,40] Therefore, the authors concluded, the S-label inactivates at the acceptor site while the R-label inactivates at the donor site.

Other active sites have more rigid stereo requirements for binding so that only one enantiomeric affinity label will bind and subsequently inactivate the enzyme. The inactivation of S-aspartate β-decarboxylase by β-chloro-S-alanine is such an example.[38] In this case there was no interaction of the enzyme with the β-chloro-R- alanine. Presumably, the R-enantiomer is not able to bind due to steric incompatibility of the chloro substituent with the active site in the R-configuration.

Finally, there is a most interesting example with implications for the affinity labeling of mandelate racemase. It involves the R- and S- enantiomers of β-chloro and β-flouro alanines as so-called suicide substrates of alanine racemase from *E. coli*. At first the kinetic data seemed to imply a symmetric active site for binding and processing of the R and S enantiomers of alanine because nearly identical kinetic parameters were measured[13,41] ($K_{M\ S\text{-}Ala}$ = 1.3mM $K_{M\ R\text{-}Ala}$ = 1.2mM, $V_{max\ S\rightarrow R}$ = 1124s^{-1} $V_{max\ R\rightarrow S}$ = 974s^{-1}). However, early work of Roze and Strominger[42] raised the possibility of some degree of active site asymmetry for alanine racemase. The subsequent work of Wang and Walsh systematically examined

this asymmetry in detail.[13,41] As a result, a markedly different tolerance at the active site for suicide substrates revealed the "functional asymmetry" which exists for the E. coli enzyme.

A summary of the results demonstrates this asymmetry. R-fluoroalanine, S-fluoroalanine, and R-chloroalanine all react at similar initial rates for halogen elimination, while the S-chloroalanine eliminates 50 -100 times more slowly. Evidently, the steric bulk of the β-chloro group in the S-isomer slows either the binding or the subsequent catalysis. The halogen elimination represents turnover which occurs before inactivation.[13]

Likewise, the first-order rate constants for inactivation show differences in that the R-fluoro and R-chloroalanines are more efficient as inactivators than the corresponding S-haloalanines. The R-fluoro compound is 14-fold more efficient in time (for inactivation) while the R-chloro compound exhibits a 100-fold difference.[13]

In subsequent experiments,[41] it was found again that the R-difluoroalanine is a more potent suicide inhibitor than the S-isomer. The R,S-trifluoroalanine resulted in a <u>decreased</u> inactivation with <u>increased</u> concentration - a result which Wang and Walsh attribute to protection against inactivation effected by the S-isomer.[41] The chloroalanines were not tested.

The systematic examination of these halo deriva-

tives revealed that alanine racemase shows a differential tolerance for β-substituents in the R- vs. S-isomers, "a reflection", Wang and Walsh concluded, "of the chiral structure of the enzymatic catalyst."[41]

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A. Assignment of the Absolute Stereochemistry of Potassium α-Phenylglycidate.

1. Introduction.

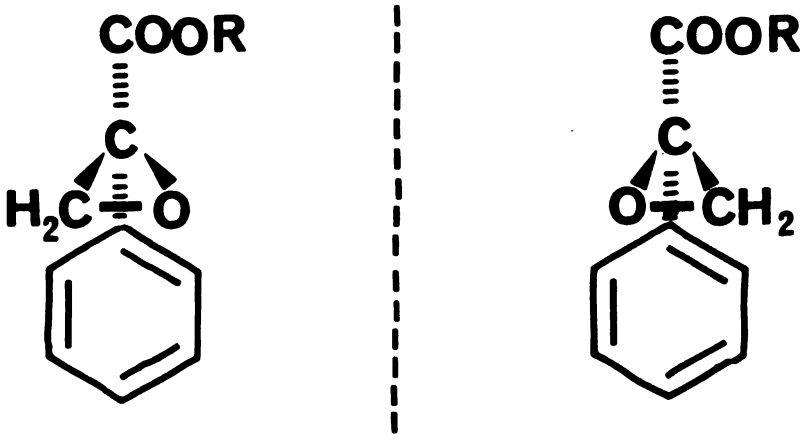
Numerous biologically active compounds, either as synthetic products or of natural origin, possess one or more asymmetric centers. Of the possible enantiomers or diastereomers, frequently only one occurs in nature, or in cases where a synthetic material has been resolved, only one isomer elicits a given biological response. Therefore, the determination of the absolute configuration of bioactive compounds is of considerable interest as well as of the utmost importance.

There are a number of physical and chemical methods available to determine the absolute configuration of the groups about an enantiomeric center.[1,2] Except for the method of anomalous X-ray scattering, it is necessary to relate the compound of unknown configuration to a compound of known configuration. However, one must exercise extreme caution when assigning configuration based solely on one method as each has its limitations, and as a result, wrong assignments have been made in the past.

The choice of a standard compound, too, becomes very important. Historically, the R(+)- and S(-)-glyceraldehydes have been used as the standard. A large number of other sugars and amino acids have been related to these molecules, and, in turn, an even larger number has been

related to these compounds. In recent years, the X-ray diffraction patterns have been used to confirm these chemical correlations. In order to avoid making a wrong assignment, one must examine carefully the methods used to establish the configuration of the compound chosen for the standard.

Two approaches can be used to relate these compounds. In one, a series of chemical reactions is used which do not involve bond-breaking or making at the asymmetric atom; or if bond-breaking or making is involved, then the stereochemical course of the reaction must be known with certainty. In the other method, the relationship between compounds is established by the comparison of physical data such as the Cotton effects seen in the Optical Rotatory Dispersion (ORD) spectra or the Circular Dichroic (CD) spectra. It was decided to establish the absolute stereochemistry of compound 1 and 2, (Figure 1) potassium S and R α-phenylglycidate, used in the biological studies in this thesis, by both chemical and physical methods. The first method involved a lithium aluminum hydride reduction of either the octyl S- or R-α-phenylglycidate molecule to a compound of known absolute configuration, whereas the second method correlated the Cotton effects associated with the CD spectra of molecules closely related to 1 and 2 to those Cotton effects exhibited in the CD spectra of compounds of known configuration.



1 R = K
3 R = (-)-2-octyl
5 R = CH_3

2 R = K
4 R = (-)-2-octyl
6 R = CH_3

Figure 1. Described in text.

2. Summary of the Synthetic Schemes.

a. Synthesis and Resolution of R,S-(-)-2-octyl α-Phenylglycidate.

Ethyl atropate was synthesized from ethyl phenylacetate and diethyl oxalate according to the procedure of Ames and Davey.[3] Saponification of the ester yielded the free acid which was esterified with excess R-(-)-2-octanol in the presence of a catalytic amount of sulfuric acid. After distillation the resulting octyl atropate was treated with a 50% excess of m-chloroperbenzoic acid to yield the diastereomeric mixture R-(-)-2-octyl R,S-α-phenylglycidate. Preliminary work had indicated that these racemic epoxides could be separated as their diastereomeric R-(-)-octyl esters using high or low pressure liquid chromatography.[4] Resolution using the so-called Pirkle column[5] failed, while resolution of the free acid using resolving agents such as brucine or ephedrine was not favored because of the instability of epoxides towards acids and bases. The mixture was therefore fractionated using low pressure liquid chromatography. The first compund to elute was unreacted octyl atropate, followed by octyl phenylacetate, which, in turn, was followed by the diastereomeric mixture of R-(-)-2-octyl S- and R-α-phenylglycidates (3 and 4 in Figure 1) as two overlapping peaks. Portions of the peaks were collected, concentrated, and reinjected onto the column. In this manner,

it was possible to obtain compound 3 as 78% optically pure while compound 4 was 75% optically pure. Optical purity was determined by capillary gas chromatography which separated the two diastereomers.

b. Saponification of the Resolved Octyl Esters.

The separated octyl α-phenylglycidates (3 and 4) were saponified with a 10% excess of potassium hydroxide to yield the desired enantiomeric potassium salts of α-phenylglycidic acid (1 and 2).

c. Methylation of the Potassium α-Phenylglycidates.

The enantiomeric salts (1 and 2) were separately esterified by complexing the salts with 18-crown-6 ether and dissolving the resulting complex in tetrahydrofuran. Addition of iodomethane yielded an oil which was subjected to "flash chromatography"[6] to afford the pure methyl α-phenylglycidate esters (5 and 6 in Figure 1). An attempt was made to acidify the salts at low temperature, to be followed by treatment with diazomethane, resulted only in an uncharacterized polymeric material.

The methyl esters 5 and 6 did not have a measurable rotation at the D line. However, in order to show that racemization had not occurred during the methylation procedure, the methyl α-phenylglycidate (5) was saponified back to its precursor potassium salt (1) which was found to have a rotation in excess of 96% of its original value.

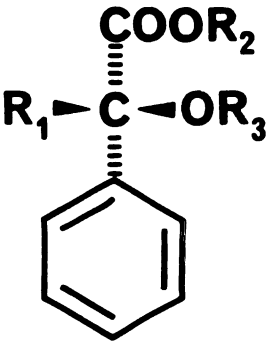
d. Synthesis and Resolution of R-and S-α-Phenylglyceric acid.

For configurational comparison 2,3-dihydroxy-2-phenylpropanoic acid (α-phenylglyceric acid)(7 in Figure 2). was required. Ethyl R,S-α-phenylglycidate, synthesized according to Fee et al.[7] and purified further by flash chromatography, was hydrolyzed in the presence of an excess of potassium hydroxide to yield potassium R,S-α-phenylglycerate which was acidified to yield the free R,S-α-phenylglyceric acid.

The structural similarities between mandelic acid (8 in Figure 2) and α-phenylglyceric acid suggested that (-)- and (+)-ephedrine, known to resolve racemic mandelic acid[8], could be used to resolve α-phenylglyceric acid. This proved to be the case, and (-)-ephedrine gave (after three crystallizations) a (-)-ephedrine (+)-α-phenylglycerate salt of constant melting point from which acid treatment produced (+)-α-phenylglyceric acid (7). In the same way (+)-ephedrine afforded an enantiomeric salt from which (-)-α-phenylglyceric acid was obtained.

e. Methylation of the Resolved α-Phenylglyceric Acids.

The resolved (+)- and (-)-α-phenylglyceric acids (e.g., 7) were treated with an excess of diazomethane to yield the optically active methyl esters (e.g., 9), which were, in turn, purified using flash chromatography.



7 $R_1 = CH_2OH, R_2 = R_3 = H$

8 $R_1 = R_2 = R_3 = H$

9 $R_1 = CH_2OH, R_2 = CH_3, R_3 = H$

10 $R_1 = R_2 = CH_3, R_3 = H$

11 $R_1 = CH_3, R_2 = R_3 = H$

12 $R_1 = R_2 = H, R_3 = CH_3$

Figure 2. Described in text.

f. Alternate Synthesis of Potassium α-Phenylglycerate.

The potassium salt of R-(+)-α-phenylglyceric acid (7) was also produced directly from the (-)-2-octyl R-α-phenylglycidate (4) by saponification in the presence of excess base. The NMR spectrum indicated that the material produced by this reaction was identical to potassium α-phenylglycerate obtained by the previously described reaction. (see d.)

3. Assignment of Configuration by Chemical Means.

a. 2-Phenyl-1,2-propanediol as the Standard.

Eliel and Freeman[9] established that the absolute configuration of (+)-2-phenyl-1,2-propanediol was S by a $LiAlH_4$ reduction of S(+)-atrolactic acid (11 indicates the R-isomer) of known optical purity and stereochemistry. It was highly unlikely that the configuration at the chiral carbon was disturbed by this chemical reaction. Since application of the sequence rules had not changed the order of the priorities of the substituents, the (+)-2-phenyl-1,2-propanediol was assigned the S configuration. The rotation values were measured in ether.

Mitsui and Imaizumi[10] reduced the R(-)- and S(+)-methyl and ethyl atrolactates, (e.g. 10 and 11) again of known optical purity and configuration, with $LiAlH_4$ to produce the individual 2-phenyl-1,2-propanediols. Again, since it was highly improbable that the configuration at

the chiral carbon changed, it was concluded that the (+)-2-phenyl-1,2-propanediol had the same configuration as (+)-methyl atrolactate, and therefore (+)-2-phenyl-1,2-propanediol was also designated S. The rotation values were measured in ethanol. In a like manner, the configuration of the (-)-2-phenyl-1,2-propanediol (13) was determined to be R.[9,10]

These two independent reports firmly establish that the (+)-2-phenyl-1,2-propanediol was of the S configuration while the (-)-2-phenyl-1,2-propanediol was of the R configuration. In both cases, it has been assumed that the configuration of atrolactic had been correctly assigned.

It is apparent that the absolute configuration of S(+)-atrolactic acid and R(-)-atrolactic acid (e.g. 11) and their methyl and ethyl esters (e.g., 10) has been unequivocally assigned based on several different lines of evidence. First, a series of chemical interconversions, summarized by Cram et al.[11], and devised by Cram[12], Brewster[13], and McKenzie et al.[14], related the enantiomers of atrolactic acid to mandelic acid. Support for the assignments based on these conclusions was provided by Prelog's studies of asymmetric reactions of methyl Grignard reagent with the phenylglyoxylates of optically active alcohols of known configuration (e.g., menthol).[15,16] Barth et al.[17] also confirmed these assignments by means of circular

dichroism. Finally, Eliel et al.[18] reported their asymmetric synthesis of S(+)-atrolactic acid methyl ether which again reinforced the earlier assignments.

b. The Chemical Reaction Relating the Octyl α-Phenylglycidates to the Standard.

It is well known that the reagent $LiAlH_4$ will reduce epoxides to replace the carbon oxygen bond with a carbon hydrogen bond.[19]. It also has been clearly established that the hydride will generally attack at the least hindered carbon atom. In this case, the β position will be the vulnerable site to attack. With the simultaneous reduction of the ester to the primary alcohol, the reduction of the octyl esters 3 and 4 could lead either to the optically active product 13 or to the achiral product 14 which are easily dinstinguishable by the respective NMR spectra. The reaction is shown in Figure 3.

An NMR spectrum indicated that the product consisted entirely of 2-phenyl-1,2-propanediol 13, with no evidence for the presence of 14. This conclusion is based on six different reductions of either the racemic mixture of the isolated diastereomeric octyl esters.

c. The Assignment.

Having firmly established both that both the absoulte configuration of 2-phenyl-1,2-propanediol has been correctly assigned and that the $LiAlH_4$ reduction of

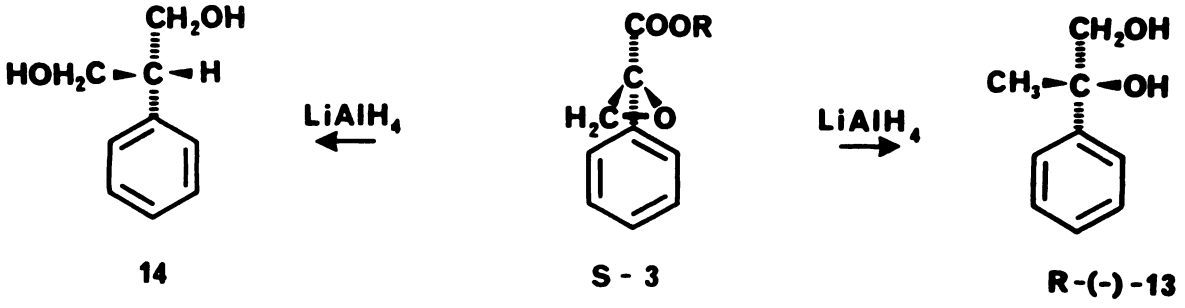


Figure 3. The $LiAlH_4$ reduction of compound 3.

the octyl α-phenylglycidate can lead only to 2-phenyl-1,2-propanediol, then we can assign the configurations of the groups about the unknown chiral center of octyl α-phenylglycidate relative to the diol. Superimpositioning of the chiral center of 3 onto that of 2-phenyl-1,2-propanediol (13) indicates that R-(-)- 2-octyl S-α-phenylglycidate has the same configuration as R-(-)-2-phenyl-1,2-propanediol. The configuration of 3 is S because of a change in priorities due to the sequence rules in the Cahn-Ingold-Prelog system. Likewise, superimpositioning of the enantiomers indicates that R-(-)-2-octyl R-α-phenylglycidate has the same configuration as S-(+)-2-phenyl-1,2-propanediol.

The subsequent hydrolysis of the octyl group leads to potassium R(+)-α-phenylglycidate (2) and potassium S(-)-α-phenylglycidate (1) - the two active-site-directed inhibitors used in the following biological studies. The hydrolysis does not disturb the asymmetric center as there is no bond-breaking to it . Therefore the correlations made for the octyl esters can be extended to the glycidates.

4. Circular Dichroic Correlations.

a. Assignment of Configuration by Circular Dichroic Correlations.

Circular dichroism (CD) is a common method used to assign the absolute stereochemistry of compounds. It has been demonstrated that the electronic transition associated with a chromophore (e.g. phenyl group, carbonyl group, etc.) will exhibit optical activity when substituent groups on it contain a chiral center. The signs and relative magnitues of the so-called Cotton effects associated with these transitions are presumably determined by conformational as well as configurational relationships which the substituent groups bear relative to the chromophore undergoing the transition. Consequently, a comparison of the Cotton effects exhibited in the CD spectra by a molecule of unknown configuration to those Cotton effects exhibited in the CD spectra by a molecule of known configuration enables one to assign the configuration of the former molecule.[20] In addition, one can obtain some information about the conformations of these same molecules.[21]

Since the signs and magnitudes of the Cotton effects are sensitive to the conformational as well as electronic state of a molecule,[20,21] the following modifications of the enantiomeric potassium α-phenylglycidate salts were necessary in order to assign correctly the configuration and conformation. Firstly, it was necessary

to make the methyl S- and R-α-phenylglycidates (5 and 6) in order to eliminate possible interfering electronic transitions resulting from the negative charge. Secondly, the enantiomeric acyclic compounds, methyl R- and S-α-phenylglycerates (e.g., 9) were synthesized in order to observe if potential conformational effects exist in the parent compounds (1 or 2) as a result of the strained epoxide moiety. These effects could lead to changes in the signs and magnitudes of the Cotton effects. Finally, R-(-)-2-octyl S- and R-α-phenylglycidate (3 and 4) were used in these correlations in order to observe the effects of the bulky octyl group on the conformation of the epoxide.

The CD spectra of all compounds were examined in 95% ethanol solution in the spectral region 200-300nm. Other conditions for measurement were discussed in the Experimental section. Table I summarizes the observable [α] values, the CD maxima at 220 and 240nm, and the ratio of the absorbance at 220nm to the absorbance at 240nm ($[\theta_{220}/\theta_{240}]$).

Before one can assign the configuration of a molecule based on CD correlations, though, one must ensure that the signs of the Cotton effects exhibited in the CD spectra are sensitive to the configuration in a consistent manner. Therefore, it is necessary to discuss the origin and nature of the CD bands in the region 200 - 300nm. Within this spectral region, aromatic compounds exhibit

	CD Maximum Values[a]					
	SPECIFIC ROTATION[a]	220nm BAND		240nm BAND		
	$[\alpha]_D$,(c)	λ_{max}(nm), λ_o(nm)[b]		λ_{max}(nm), λ_o(nm)[b]		$[\theta]_{220}$ / $[\theta]_{240}$, %
COMPOUND		[θ]		[θ]		
R-(-)-Mandelic acid (8)	-155° (0.23)	224 -34,200	208 248	--[c]		--
R-(-)-2-Methoxy-phenylacetic acid (12)	-149.4° (1.0)	224 -26,400	208 247	—		—
R-(-)-Atrolactic acid (11)	-37.6° (3.5)	222 -19,300	209 239	246 335	240 255	57.6/1, 98.3%
Methyl R-(-)-atrolactate (10)	-5.0° (4.9)	221 -4,000	206 236	240 295	236 252	13.5/1, 93.1%
(-)-Menthyl R-(-)-2-methoxy-2-phenylpropionate (17)[e]	—	228 -29,800[f]	210 246	—		--
Methyl S-α-phenylglycidate (5)	—	225 -11,900[f]	216 240	--[c]		—
R-(-)-2-Octyl S-α-phenylglycidate (3)	-22° (0.43)	225 -16,300[f]	212 243	--[d]		--
R-(-)-2-Octyl R-α-phenylglycidate (4)	-16° (0.4)	225 13,300[f]	215 240	243 -340	240 257	39.1/1, 97.5%
Methyl S-(-)-2,3-dihydroxy-2-phenylpropionate (9)	-12° (0.38)	222 -18,800	210 240	245 160	241[c] 254	117.5/1, 99.2%

a In 95% ethanol.

b At λ_o, [θ] = 0

c (+) Enantiomer shows mirror image CD spectrum.

d Above 240nm, shows only the ^1L_b band of C_6H_6 at 250 - 270 nm in agreement with the UV spectrum (250 -267nm, ε = 233).

e Mizuno, H. and Yamada, S., Chem. Pharm. Bull. (Japan), 23, 527 (1975).

f Corrected to optical purity.

two electronic absorption bands which are attributed to the $\pi \rightarrow \pi^*$ transition.[22] These transitions are known in the Platt notation[23] as the ^1L_b and ^1L_a bands, occurring at 250-275nm and 210-230nm, respectively. Aliphatic carboxylic acids exhibit an electronic absorption band near 220nm attributable to an $n \rightarrow \pi^*$ transition. Complications arise when these two chromophores are coupled, and not necessarily conjugated, within the same molecule. One such series of compounds, the α-phenylacetic acids, has evoked much controversy as a result of the coupled chromophores. This controversy results from attempts to identify the origin of certain bands found in the CD spectra for these coupled systems. Some researchers[24] have attributed these bands to transitions of the aromatic system while others have insisted these bands result from an enhancement of the $n \rightarrow \pi^*$ transition of the carboxyl functional group[25]. Barth et al.[17] clearly demonstrated that the two observable absorption bands in this region are due to an enhancement of the $n \rightarrow \pi^*$ transition of the carboxylate group as a result of its coupling to the aromatic system. These bands are found near 220nm (band 1) and in the 230-245nm region (band 2).

Furthermore, their results indicated that the CD spectra of the S-α- substituted phenylacetic acids exhibit a very strong positive Cotton effect near 220nm (band 1) as well as a very intense negative Cotton effect in the

200-215nm region[17]. (For reasons discussed by Barth et al. this latter band is not experimentally observable.)[17]

The CD spectra of these compounds also exhibit a smaller negative Cotton effect in the 230-245nm region (band 2). To date, chemical correlations and molecular orbital calculations continue to support these predictions.[26,27] Finally, it was apparent that the Cotton effects associated with the ^1L_b and ^1L_a bands of the α-phenylacetic acids and esters were of indeterminate sign and itensity. Therefore, accurate predictions concerning the configuration of such compounds cannot be based on these bands.[17]

Compound 5 and 6 were correlated (Figure 4) with R-(-)-atrolactic acid (11), (Figure 5) its methyl ester (10) (Figure 4) and R(-)-mandelic acid (8) while octyl esters (3 and 4) (Figure 4) were correlated to R(-)-menthyl 2-methoxy-2-methyl phenylacetate (17). (Figure 5) The absolute configuration of atrolactic acid and methyl atrolactate has been well established as discussed earlier. The absolute configuration of R(-)-mandelic acid has been unequivocally established by a series of chemical interconversions to that of R(-)-lactic acid. The reactions are summarized by Mislow[28]. CD and ORD correlations have confirmed these assignments.[29,30] The compound, (-)-menthyl R-(-)-2-methoxy-2-methyl phenylacetate was synthesized from R-(-)-atrolactic acid. The chiral center under

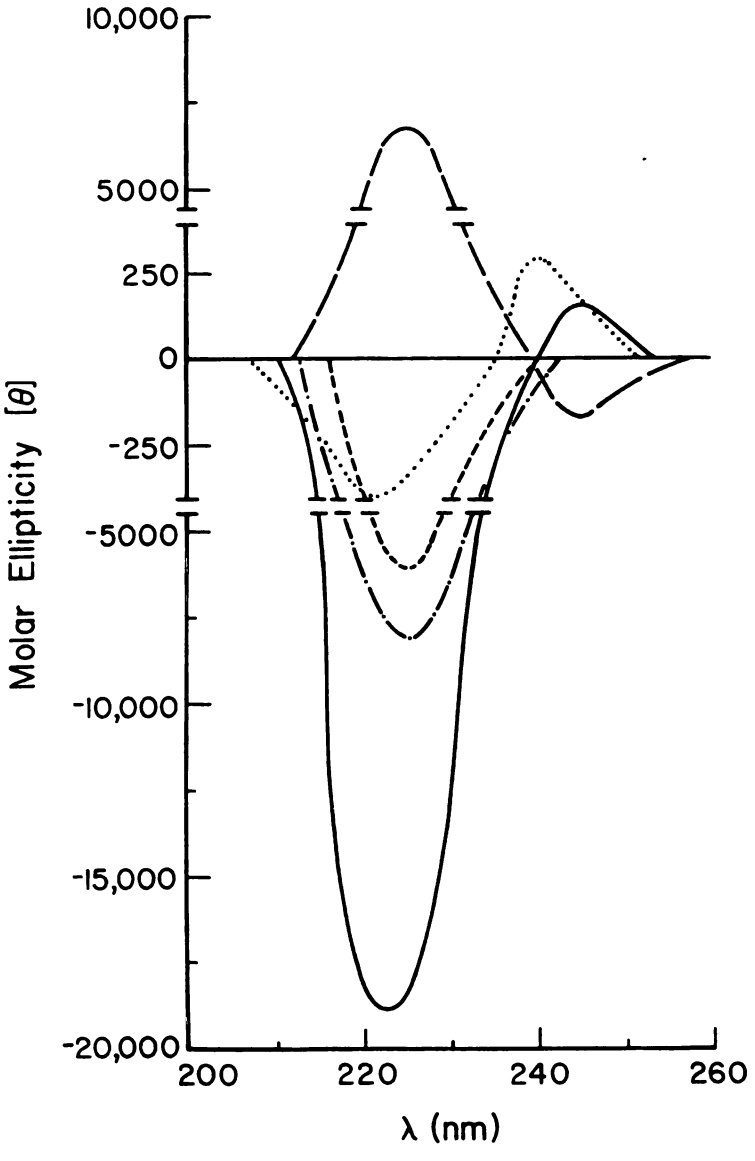


Figure 4. Circular Dichroic Spectra of Compounds 3 (-·-·-·-); 4 (— —); 5 (- - -); 9 (——); and 10 (·····).

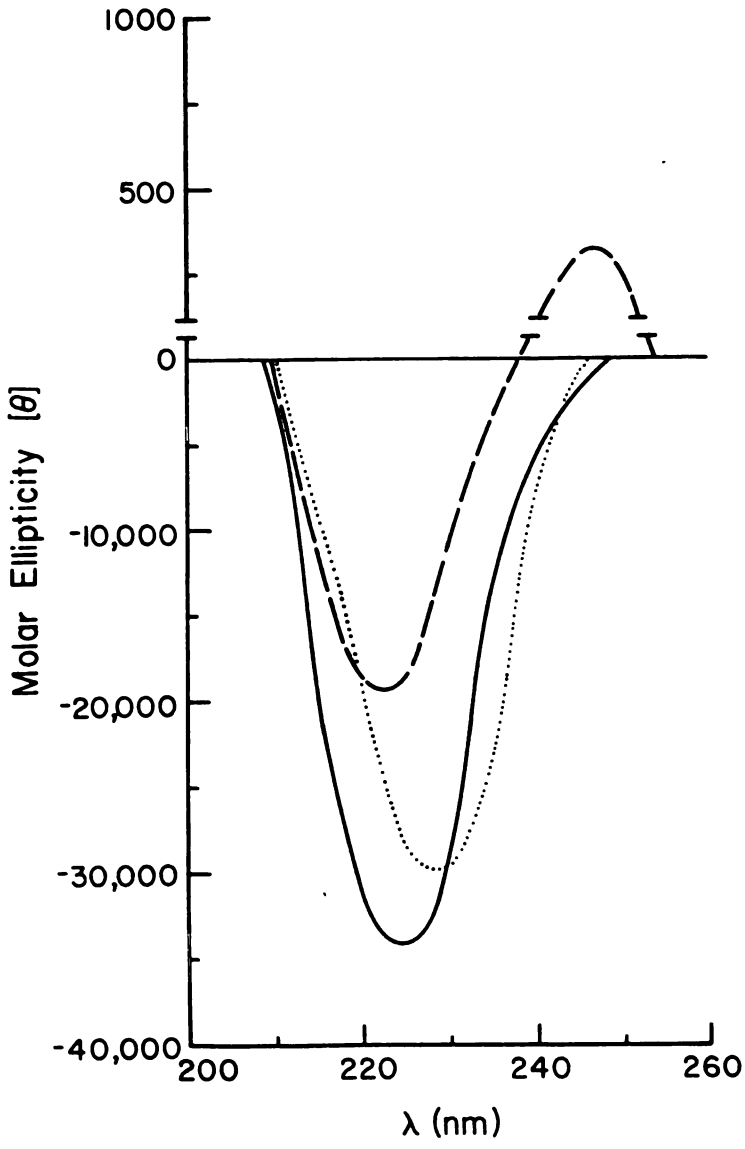


Figure 5. Circular Dichroic Spectra of compounds 8 (——); 11 (– –); and 17 (·····).

observation remains undisturbed by the chemical reaction involved in this interconversion. Therefore, its absolute configuration remains R. Accordingly, the stereoisomers of compounds 3, 5 and 9 exhibiting a large negative Cotton effect near 220nm and a small positive Cotton effect in the 240nm region can be superimposed at the chiral center onto a model of R(-)-atrolactic acid (or its methyl ester) and assigned the S configuration. The configuration is S because of a change in priorities due to sequence rules in the Cahn-Ingold-Prelog system. The enantiomers of these compounds do indeed yield a mirror image CD spectra. (Note: Compound 3 exists as diastereomers. Therefore these CD spectra were not mirror images.) The correlations made in this manner are in complete accord with the assignments made on the basis of the chemical transformations.

b. Conformations of α-Phenylglycidate and Related Compounds.

The CD spectra (See Figures 4 and 5) also enable one to make the following statements concerning the relative populations of conformers which exist for compounds 3, 4, 5, and 9. The $\theta_{220}/\theta_{240}$ ratio in Table 1 is an indication of the relative populations.

Conformational information can be obtained through the use of the octant rule.[1,21,31] The octant rule divides a carbonyl compound into octants by three perpendicular planes which meet at the carbonyl carbon. The three planes cut the molecule into eight sections. Substituents on the carbonyl carbon can make a positive or negative contribution to the observed Cotton effect depending upon which octant the substituent is positioned. Substituents falling on the plane, such as the carbonyl group, by definition, do not make a significant contribution to the observed Cotton effect. It has been an empirical observation that the magnitude of the contribution is a function of the bulk of a substiuent within a homologous grouping.[21,31] Only in recent years has it become increasingly possible to use quantum mechanical calculations in order to quantitate these contributions.[28,29]

For α-hydroxy phenylacetic acids, the Cotton effect at 220-225nm is assigned to the preferred conformation 6A which is energetically favored whereas the smaller

and oppositely-signed Cotton effect in the 240-245nm region is associated with the less populated conformation 6B where Figure 6 represents the octant projection of the carboxylic acids [17,32] In the case of mandelic acid (8) and its O-methyl ether (12) only the 224nm CD maximum is found, in agreement with the calculated preferred conformation 6A [26], while in compounds 11 and 10, the presence of a small percentage (2 to 7%) of the less populated rotamer 6B is indicated on the basis of the small Cotton effect at 240 -245nm.

The two methyl α-phenylglycidates (5 and 6) lack any observable Cotton effect in the 240nm region indicating the absence of the second conformer.

The CD spectra of the diastereomeric R-(-)-2-octyl esters 3 and 4 proved to be interesting. (See Figure 4) Compound 3 showed chiroptical properties superimposable on those of 5, i.e. a strong negative CD at 225nm and no CD maximum at 245nm, again suggesting the existence of a single conformer. Since the R-(-)-2-octanol chiral center has a transition in the $n \rightarrow \sigma^*$ region of the alcohol oxygen only (about 175nm),[21] it will not interfere with the appearance of a transition in the 200-250nm region.

The diastereomer 4 had a strong mirror image positive CD maximum at 225nm, and in addition showed a small negative CD at 243nm, pointing to the presence of a

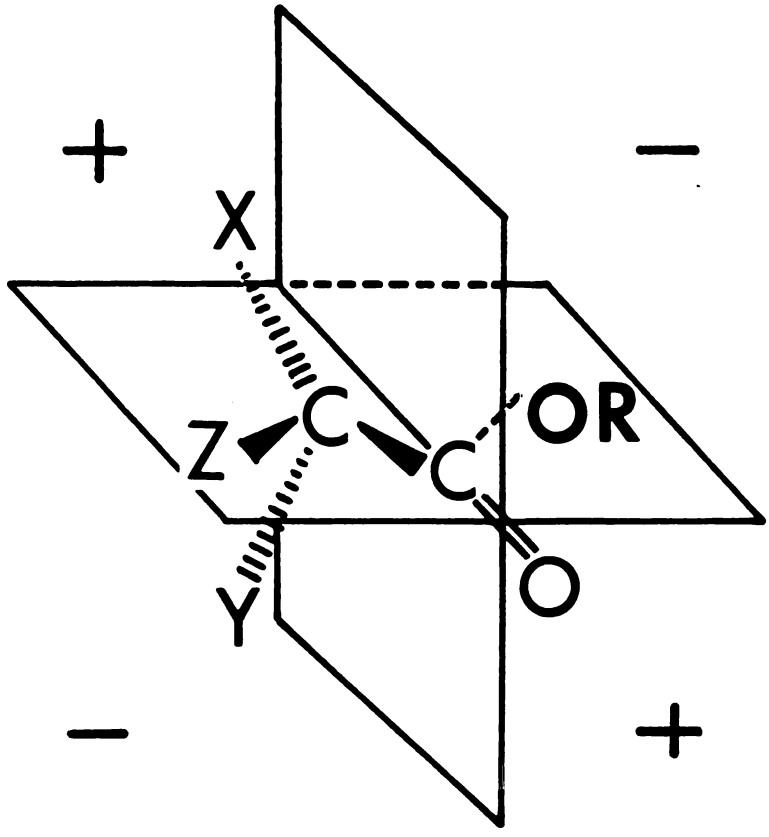


Figure 6. Octant Projection for S-α-hydroxy-phenylacetic acids.

COMPOUND	X	Y	Z	R
A	C_6H_5	H	OH	H
B	OH	C_6H_5	H	H

small (ca. 2.5%) percentage of a second conformer. Examination of molecular models suggests that this behavior of the two diastereomeric esters 3 and 4 is due to the conformational effect caused by the R-(-)-2-octanol moiety present in both compounds.

5. A Relationship Between the Chemical Transformation and the Circular Dichroic Correlations.

The assignments of the configurations of the epoxide compounds 1 and 2 made by the chemical transformation and the CD correlations were strengthened further by a relationship made between the R-(-)-2-octyl R-α-phenylglycidate and R(+)-α-phenylglycerate (4 and 15). Hydrolysis of 4 in the presence of two moles of potassium hydroxide yielded potassium α-phenylglycerate. The resulting basic hydrolysis of R-(-)-2-octyl R-α-phenylglycidate led to potassium R(+)-α-phenylglycerate. The CD correlation established that this latter compound belonged to the R series. Therefore, the octyl (+)-α-phenylglycidate can be assigned to the R series since the sequence about, and bonded to, the chiral center remain undisturbed by this hydrolysis.

B. Kinetic Constants

1. Comparison of the Michaelis-Menten Rate Constants.

The initial velocity patterns for mandelate racemase as determined by the circular dichroic assay of Sharp et al.[33], in both the R to S and S to R directions, are shown in Figure 7. The K_M for R-mandelate was 0.23 mM ± 0.02 and for S-mandelate was 0.26 mM ± 0.03. The $V_{max\ R\rightarrow S}$ was 0.75 ± 0.02 μmoles min^{-1} ml^{-1} and $V_{max\ S\rightarrow R}$ was 0.83 ± 0.03 μmoles min^{-1} ml^{-1}, under identical conditions at pH 7.5. For the chemically symmetrical reaction R-mandelate $\rightleftharpoons$ S-mandelate, the equilibrium constant must be unity and this has been validated with other racemases[34]. The Haldane relationship allows one to compute a K_{eq} from measured V_{max} and K_M values.[35]

$$K_{eq} = \frac{(V_{max}/K_M)_{R\text{-mandelate}}}{(V_{max}/K_M)_{S\text{-mandelate}}} = 0.99 \pm 0.09 \quad (1)$$

The consequences of this kinetic symmetry are interesting. Recently, Kenyon and Hegeman[36] put forth predictions about the individual kinetic rate constants of the racemase-catalyzed reaction based upon the assumption that the K_M values for R- and S-mandelate may be equal. Having demonstrated this equality, their predictions bear more substance. These predictions were based on the following assumptions. Mandelate racemase, being a one-substrate enzyme, obeys the classical Michaelis-Menten equa-

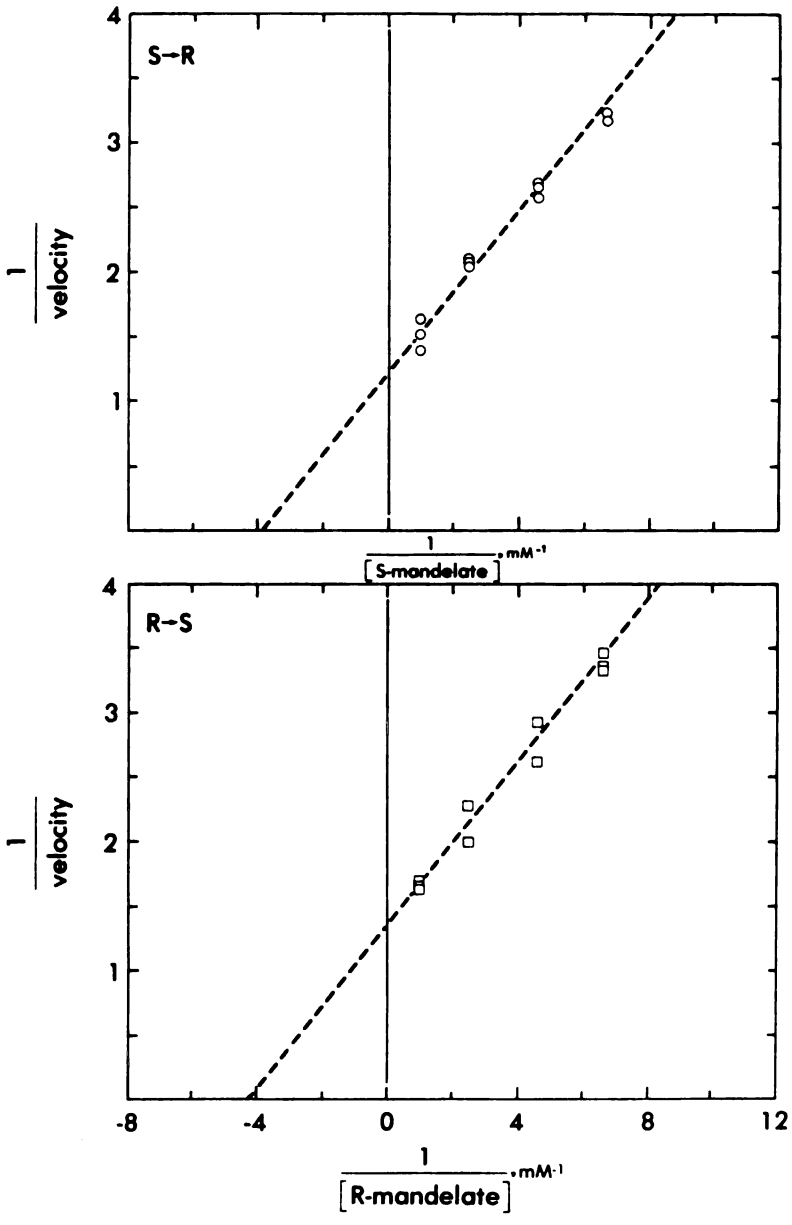


Figure 7. Comparison of initial velocities for mandelate racemase in the R to S direction and S to R direction at pH 7.5 and 25° C.

tion shown in equation 2, where E = enzyme.

$$E + \text{R-mandelate} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot\text{R-mandelate} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \qquad (2)$$

$$E\cdot\text{S-mandelate} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + \text{S-mandelate}$$

Earlier, Maggio et al.[37] had obtained a value (K_S) for the dissociation of the R,S-mandelate from a ternary complex of enzyme, Mn^{2+}, and substrate. The K_S value was nearly identical to the K_M value, suggesting that the K_M value probably represents a true dissociation constant.

On the basis of these two assumptions and knowing now that the K_M values are nearly identical in both directions, one is able to say the following:

$$K_M \simeq k_2/k_1 = k_5/k_6 \qquad (3)$$

At equilibrium:

$$K_{eq} = 1 = \frac{k_1\ k_3\ k_5}{k_2\ k_4\ k_6} \qquad (4)$$

Rearranging equation 4 one obtains

$$k_3/k_4 = \frac{k_2/k_1}{k_5/k_6} \qquad (5)$$

Substituting equation 3 into equation 5 results in equation 6.

$$k_3/k_4 = 1 \quad \text{or} \quad k_3 = k_4 \qquad (6)$$

One is able to conclude that the rate of the enzyme-catalyzed racemization step are approximately equal in both directions. Thus, not only does mandelate racemase bind either enantiomer with equal affinity, but it also processes each enantiomer at a nearly identical rate.

2. Deuterium Isotope Effects.

It is well known that if one replaces a hydrogen atom with deuterium or tritium, then the rate of cleavage of the C--D or C--T bond is slower than that of the C--H bond. This is the basis for isotope effects which, for deuterium, range from 5 to 15. For a chemical reaction, one is able to see the full isotope effect since the bond breaking step is generally rate limiting.[38] As a result, the interpretations are considerably simplified. Enzyme-catalyzed reactions, however, are not so readily interpreted. This is so because steps other than the catalytic one are often at least partially rate limiting. Therefore, the observed isotope effect is usually smaller than the true, or intrinsic, one.[39] Considerable caution must be exercised when extracting mechanistic data from these effects. Recent articles by Cleland[40] and Northrup[41] address these concerns.

Isotope effects can be expressed in the two independent kinetic parameters, V and V/K, resulting from the two limiting conditions of the Michaelis-Menten equation.[42] The degree to which the isotope effects is expressed in these parameters is an indication of where the rate-limiting steps are in the mechanism.[39] In order to determine deuterium isotope effects on V and V/K, one makes reciprocal plots with the deuterium- and hydrogen-containing molecules and takes the ratio of the slopes as

the V/K effect, and the ratio of the intercepts as the V effect.[39]

The reciprocal plots determined from the CD assay in the R to S and S to R directions are shown in Figure 8. The measured isotope effect in the R to S direction was 3.20 ± 0.11 while it was 3.56 ± 0.12 in the S to R direction. The V_{max} and V/K_M isotope effects were found to be equal.

The data suggest a number of things. Firstly, its magnitude confirms the results of Kenyon et al.[43] in that cleavage of a carbon-hydrogen bond is involved in the rate-limiting step of the racemization reaction. Also, the rate-limiting step is essentially the same in either direction.

One might expect a much larger isotope effect (ca. 6-8) as the cleavage involves a carbon hydrogen bond. However, as stated earlier, in an enzymatic reaction, there are other steps which are at least partially rate limiting. These other steps, such as conformational changes necessary for binding or catalysis, are apparently partially rate limiting in mandelate racemase catalysis.

Secondly, the equivalence of V and V/K provides insight into the relative rates of binding of the substrate, processing of the substrate, and the dissociation of the product. Under initial velocity conditions where $[P] \rightarrow 0$, the kinetic scheme for mandelate racemase (equa-

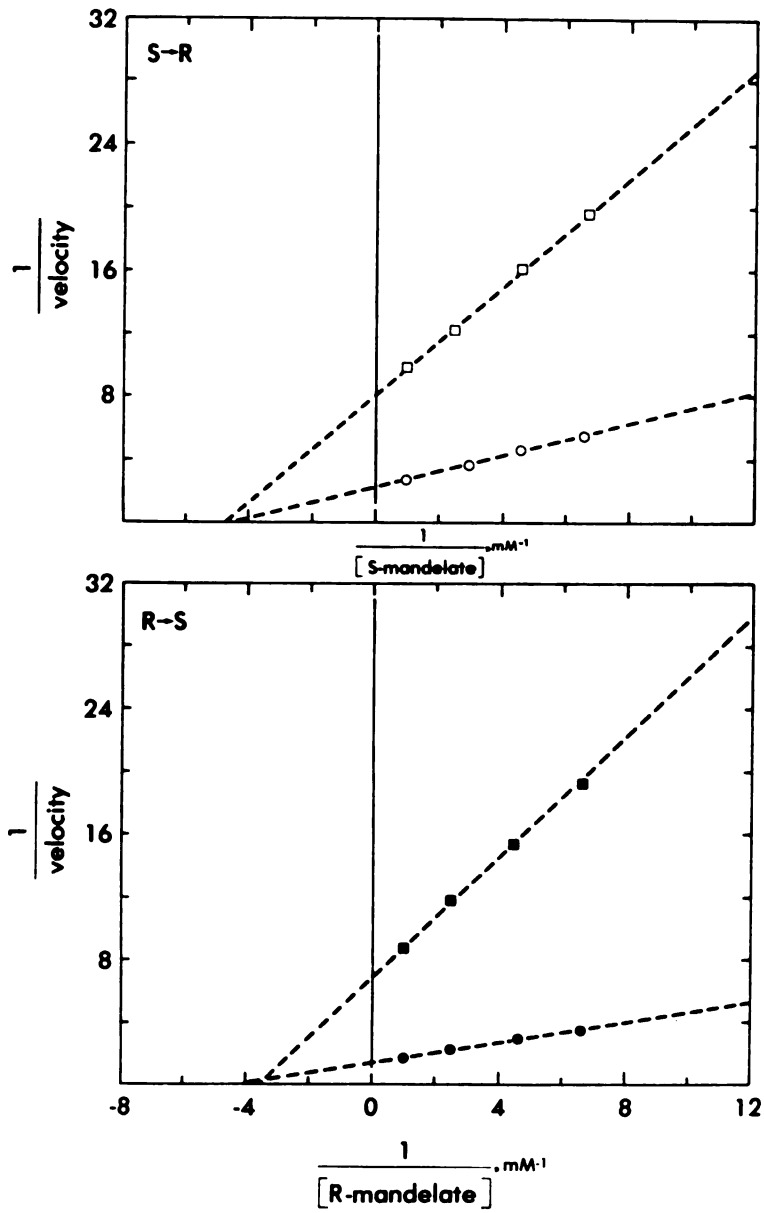


Figure 8. Comparison of the deuterium isotope effects from mandelate racemase in the R to S direction and S to R direction at pH 7.5 and 25° C.

tion 1) simplifies to equation 7

$$E + \text{R-mandelate} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot\text{R-mandelate} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\cdot\text{S-mandelate} \xrightarrow{k_5} E + \text{S-mandelate} \qquad (7)$$

where the kinetic constants k_3 and k_4 are the rates of the catalytic steps. Under these conditions, the V/K isotope effect depends on the ratio of k_3/k_2 - the ratio of the rate constant for the catalytic step to the off rate from the enzyme-R-mandelate complex. The V_{max} isotope effect, on the other hand, depends on the ratio of k_3/k_5 - the ratio of the rate constant for the chemical step to the rate constant for product release.[38]

The equality of the V_{max} and V/K_M isotope effects indicates that in both directions of the enzyme-catalyzed reaction, k_3 - the catalytic step - remains rate-limiting when compared to either dissociation of substrate from the ES complex or product release.[38]

A more rigorous treatment of the data follows.[38] The following equations can be derived from the mechanism outlined in equation 7 where the kinetic constants k_3 and k_4 are the isotopically sensitive steps.

$$D'_{(V/K)} = \frac{D_{k_3} + k_3/k_2 + k_4/k_5}{1 + k_3/k_2 + k_4/k_5} \qquad (8)$$

$$D_{(V)} = \frac{D_{k_3} + k_3/k_5 + k_4/k_5}{1 + k_3/k_5 + k_4/k_5} \qquad (9)$$

The equality of V and V/K necessitate that $k_2 = k_5$. This is a necessary corollary to the results of the inital velocity studies. One is also able to conclude from these equations that k_5 and k_2 are much greater than either k_3 or k_4. The catalytic step remains rate-limiting when compared to either dissociation of substrate from the ES complex or product release (i.e., the substrate is not "sticky").[40]

The results are consistent with the kinetically symmetrical racemase reaction.

3. pH Profiles.

The kinetic parameters of an enzyme can vary with the pH. This is not surprising in view of the fact that the active site of an enzyme contains several ionizable groups. Depending upon the state of protonation of these groups a reaction may or may not occur. There are several reasons for this. First, either the substrate or some catalytic group or groups on the enzyme may have to be in a given state of protonation (protonated or deprotonated) for either a reaction to occur or a substrate to bind.[39] Second, conformational changes may occur as a result of the state of protonation. These conformational changes could be vital to the placement of an amino acid residue in the correct position for either binding or the subsequent catalysis of the substrate.[39]

It is the pH variation of V/K and V_{max} that is of interest. The parameters are plotted as log log plots so that a change of 1 pH unit corresponds to a 10-fold drop in rate of reaction resulting from a 10-fold drop in the concentration of the correct form of enzyme or substrate for optimimal binding and/or catalysis. This will produce a pH profile with a region of maximal activity falling off with a slope of either -1 or +1 as the change in pH either increases or decreases the rate of reaction.[39]

Using the CD assay, pH profiles were generated

in both directions for mandelate racemase. As shown in Figure 9, the $V/K_{R\text{-mandelate}}$ profile decreases at low pH with pK_as of 6.2 ± 0.3 and 7.8 ± 0.2. The $V_{R\rightarrow S}$ profile does not vary over the pH range 5.5 -10.0. Likewise, it is shown in Figure 10, that $V/K_{S\text{-mandelate}}$ decreases at low pH with pK_as of 6.4 ± 0.3 and 7.6 ± 0.2. Again, there was no variation in the $V_{S\rightarrow R}$ profile.

Since the pK_as are not found in the log V_{max} profile, but are found in the log V/K profiles and do not correspond to any of the pK_a values for mandelate, they presumably represent pK_a values of amino acid residues on the enzyme necessary for the binding of the substrate. The pk_as of 6.2 and 6.4 indicate that this group must be deprotonated for optimal binding while the pK_as of 7.8 and 7.6 reflect groups which need to be protonated for optimal binding.

Without further experimentation, however, one cannot identify these groups solely from their pK_as as it is generally known that the pK_a of an amino acid residue in a protein can be greatly perturbed from that for an amino acid free in solution.[39] The state of solvation as well as the presence of other amino acid residues in the microenvironment of these amino acids will affect their pK_a value.

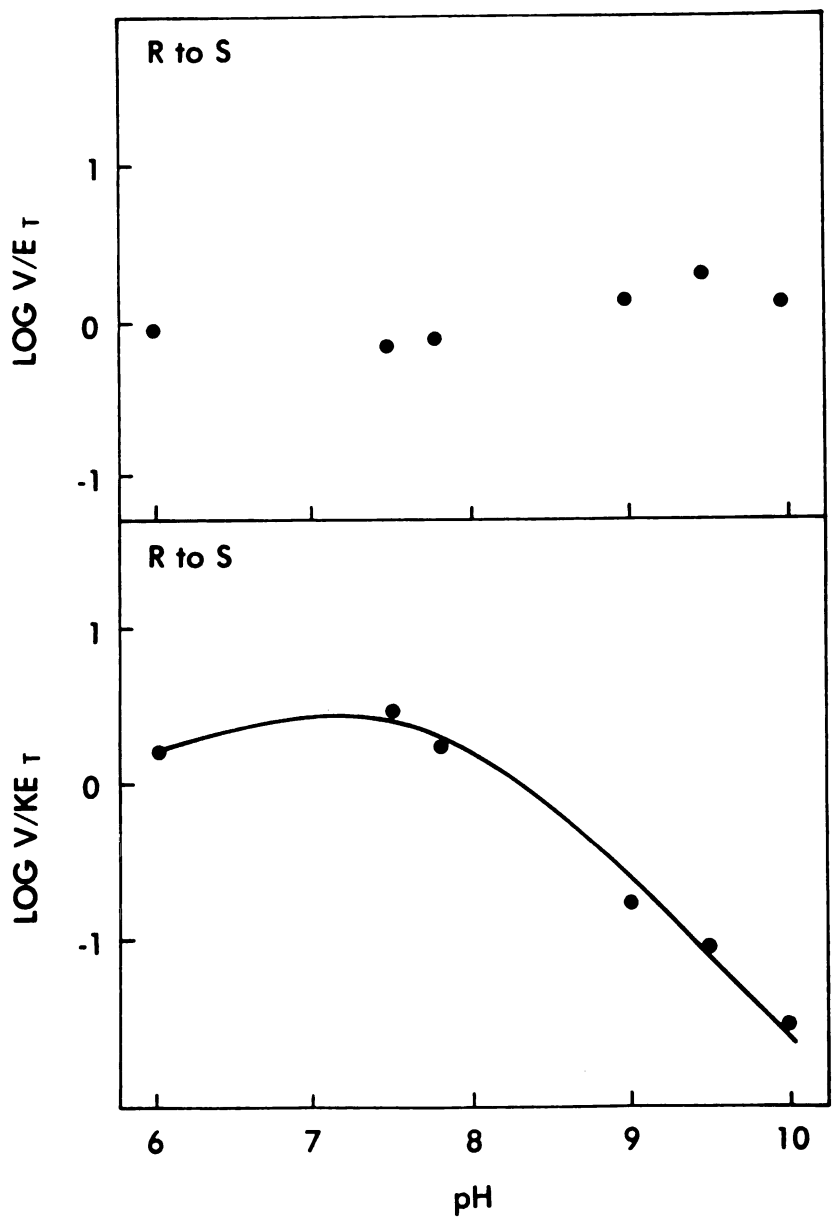


Figure 9. The pH dependence of $V/K_{R\text{-mandelate}}$ and $V_{R\text{-mandelate}}$ for mandelate racemase in the R to S direction.

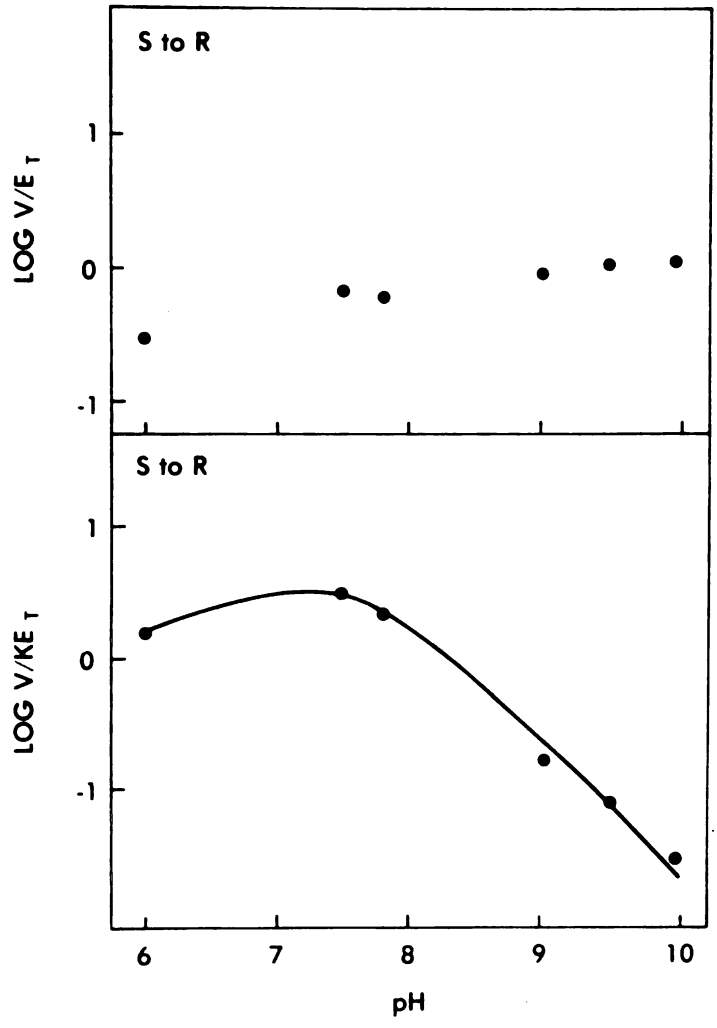


Figure 10. The pH dependence of $V/K_{S\text{-mandelate}}$ and $V_{S\text{-mandelate}}$ for mandelate racemase in the S to R direction.

C. Kinetics of Irreversible Inhibition.

1. R(+)- and S(-)-α-Phenylglycidate as Active-Site-Directed Irreversible Inhibtors of Mandelate Racemase.

Fee et al.[7] demonstrated that R,S-α-phenylglycidate was an active-site-directed irreversible inhibitor of mandelate racemase because it satisfied the following kinetic criteria. Firstly, it inhibited the enzyme completely and irreversibly. Secondly, the inactivation resulted from the formation of an enzyme-inhibitor complex, as the time-dependent loss of catalytic activity was first order and therefore the inactivation was said to obey saturation kinetics. Thirdly, the normal substrate protected the enzyme from inactivation in a competitive manner. Finally, the binding of the inhibitor was stoichiometric in that one inhibitor molecule was bound per active site. For reasons discussed earlier, it was of considerable interest to us to separate and test the R and S enantiomers of α-phenylglycidate. The results are discussed herein.

The half-times for inactivation of mandelate racemase were measured for several different concentrations of R(+)- and S(-)-α-phenylglycidate at 25° (pH 7.5) in the presence of 1.0mM $MgCl_2$. Figure 11 shows the lines obtained at six different inhibitor concentrations for R(+)-α-phenylglycidate while the lines obtained for five concentrations of S(-)-α-phenylglycidate are shown in

Figure 12. These plots do not show so much curvature as those of Fee et al.[7] for reasons discussed later.

The formation of a dissociable complex between enzyme and the affinity label at the active site prior to covalent bond formation - analogous to the formation of the enzyme-substrate complex in Michaelis-Menten kinetics - has testable kinetic consequences. This process can be represented by the equation 10.[44]

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot I \xrightarrow{k_{inact.}} E\text{-}I \qquad (10)$$

where E is the free enzyme and I is the inhibitor, E•I is the enzyme•inhibitor complex, and E-I is the inactivated enzyme. Using the steady-state assumption, Meloche[44] derived the following rate equation for inactivation:

$$t_{\frac{1}{2}} = \frac{[\ T_{\frac{1}{2}}\ K_{inact.}\ ]}{[I]} + T_{\frac{1}{2}} \qquad (11)$$

where $t_{\frac{1}{2}}$ is the inactivation half-time at a given inhibitor concentration [I], $T_{\frac{1}{2}}$ is the observed rate constant if all the enzyme were in the E•I complex, and the value K_{inact} is $(k_{-1} + k_2)/k_1$. Physically, K_{inact} is the concentration of the inhibitor that gives half-maximal inactivation rate and presumably half-saturates the enzyme.It provides some measure of the affinity of the inactivator for the enzyme analogous to the Michaelis constant.[44] A positive value $T_{\frac{1}{2}}$ indicates that the inactivator obeys

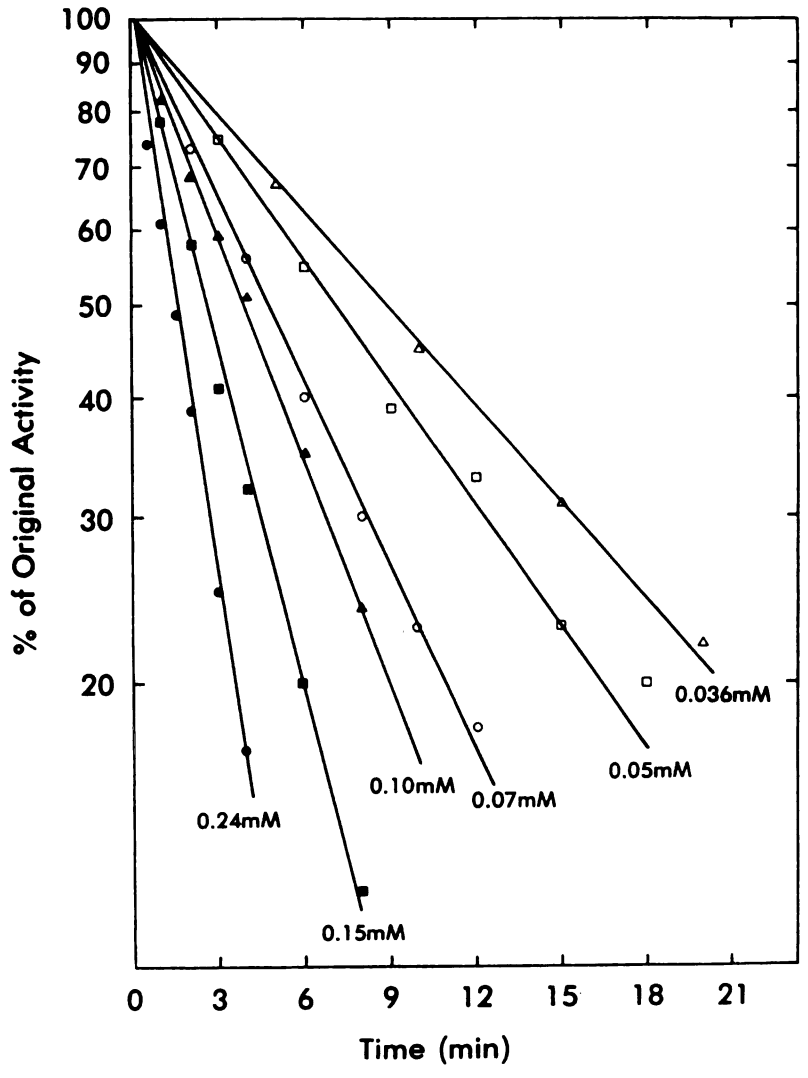


Figure 11. Irreversible Inhibition of Mandelate Racemase at Various Concentrations of R-(+)-α-Phenylglycidate.

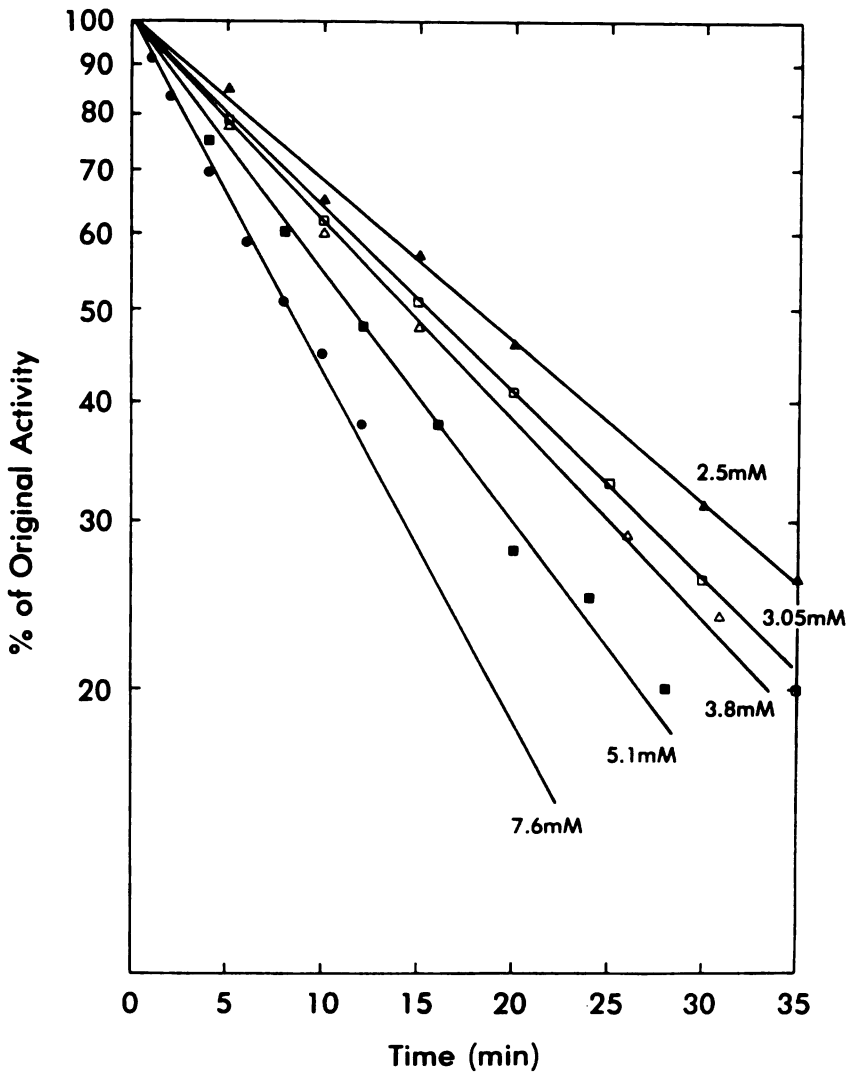


Figure 12. Irreversible Inhibition of Mandelate Racemase at Various Concentrations of S-(-)-α-Phenylglycidate.

saturation kinetics, and kinetically establishes that there is a dissociable E•I complex prior to covalent bond formation.

The $t_{\frac{1}{2}}$ values for R(+)- and S(-)-α-phenylglycidate were plotted against a series of inhibitor concentrations, according to equation 11, as shown in Figures 13 and 14, respectively. Both R(+)- and S(-)-α-phenylglycidate form complexes with the enzyme as indicated by the finite vertical intercepts (i.e., $T_{\frac{1}{2}} > 0$). For R(+)-α-phenylglycidate $T_{\frac{1}{2}} = 0.5$ min while it is 4.8 min for S(-)-α-phenylglycidate, a 9.6-fold difference. These values correspond to a first-order rate constant k of 2.31×10^{-2} sec^{-1} for R(+)-α-phenylglycidate and $k = 2.41 \times 10^{-3}$ sec^{-1} for S(-)-α-phenylglycidate. The value for K_{inact} for R(+)-α-phenylglycidate was 0.67 mM while it was 6.85 mM for S(-)-α-phenylglycidate, a 10.2-fold difference.

It is apparent that the R-isomer is the more potent affinity label of mandelate racemase in that the enzyme has a 10.2-fold higher affinity for it as well as a 9.6-fold faster rate of reaction. However, the R- and S-α-phenylglycidates were not enantiomerically pure as determined by gas chromatographic analysis. Separation of diastereomeric derivatives - the (-)-2-octyl R- and S-α-phenylglycidate - indicated that the R(+)-isomer was 73% optically pure whereas the S(-)-isomer was 76.2% optically pure. The partial resolution of these inhibitors raises

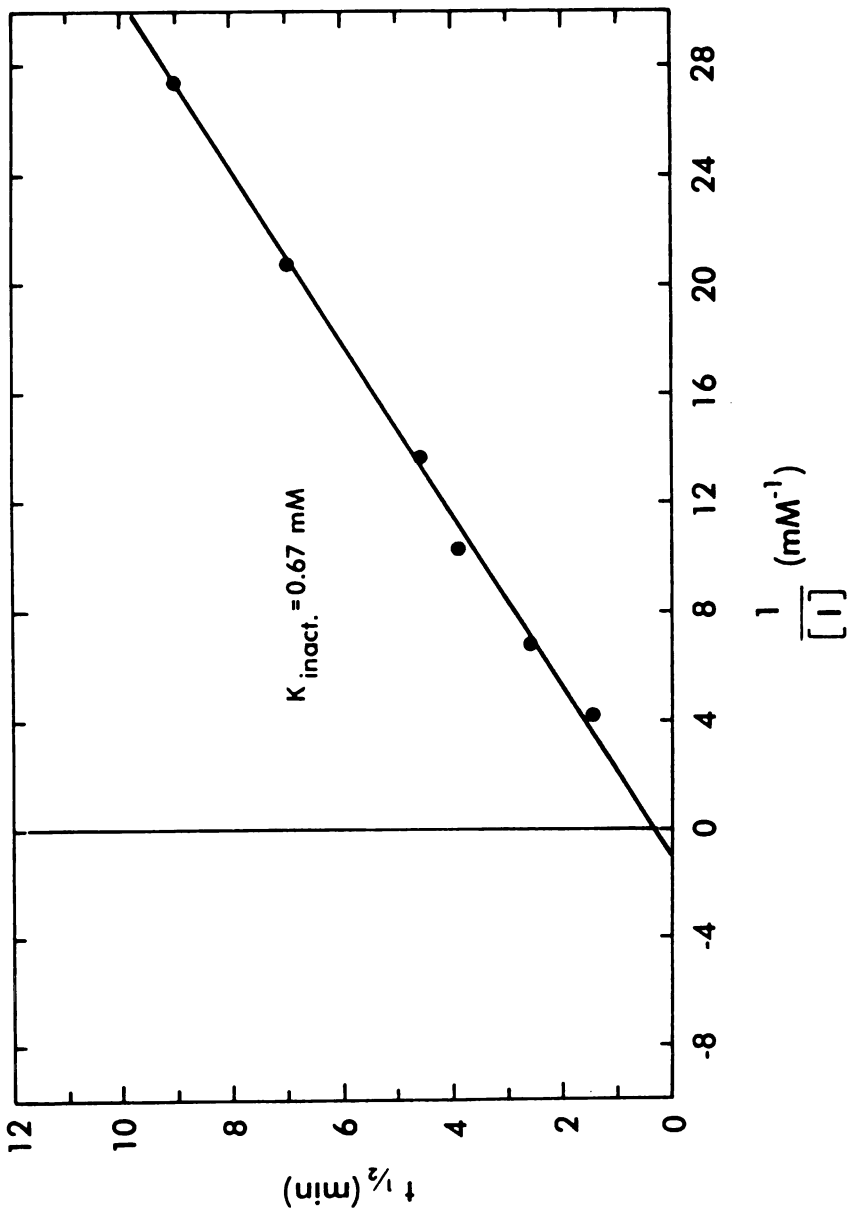


Figure 13. Inhibition of Mandelate Racemase ($t_{\frac{1}{2}}$ values versus $(I)^{-1}$) by R-(+)-α-Phenylglycidate.

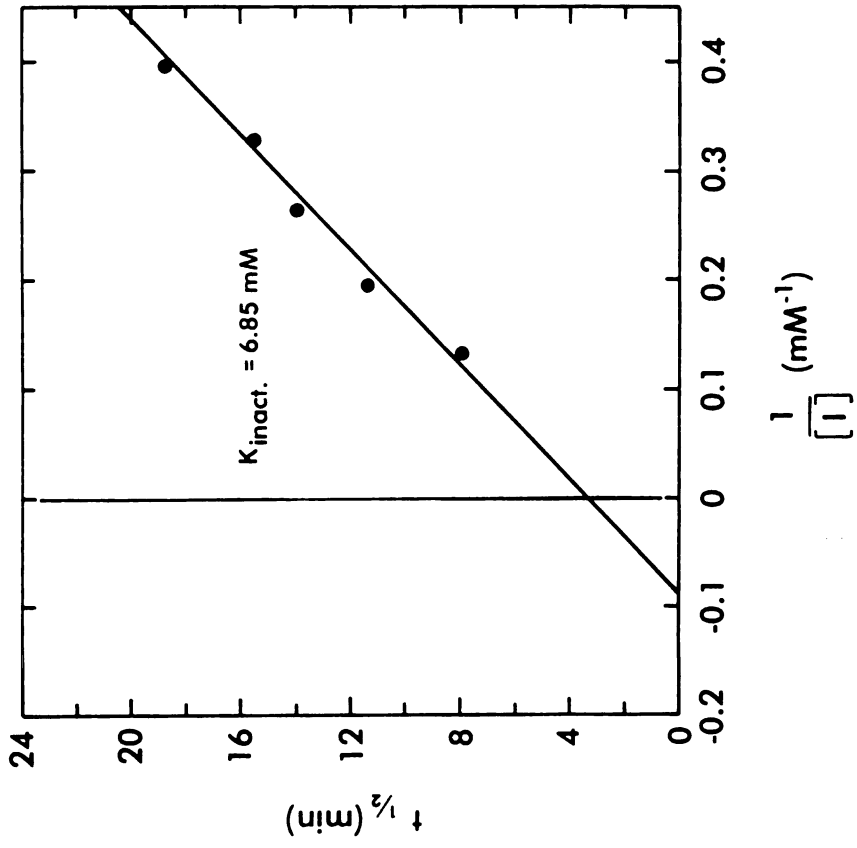


Figure 14. Inhibition of Mandelate Racemase ($t_{1/2}$ values versus $(I)^{-1}$) by S-(-)-α-Phenylglycidate.

some interesting possibilties. Because they are not enantiomerically pure, it is conceivable that the R(+)-isomer, once pure, could be even more potent as an irreversible inhibitor, and that the S(-)-isomer, once pure, could be entirely inactive as an irreversible inhibitor. In fact, it is likely that the inhibitory activity in experiments using the S(-)-isomer may be due entirely to the presence of the contaminating R(+)-isomer.

The data, though, do suggest another possible role for the S(-)-isomer. The S(-)-isomer could be acting to protect mandelate racemase from irreversible inhibition. The following two observations support this contention. Firstly, at the concentrations of the S(-)-isomer used in these experiments, there was more than an adequate concentration of the R(+)-isomer present to inhibit the enzyme irreversibly. In fact, it has been calculated that there was a 10-fold greater concentration of R(+)-isomer present in the S(-)-isomer solutions than is normally necessary to inhibit the enzyme irreversibly. Secondly, Fee et al.[7] had proposed that the less reactive enantiomer could be protecting against inhibition which would result in deviation from linearity in the % Original Activity vs time plots. Indeed, the plots for the nearly pure enantiomers showed less curvature thereby lending credence to the prediction of Fee et al.[7]

2. Competitive Inhibition Experiments.

Maggio et al.[37] demonstrated that the racemic mixture of potassium α-phenylglycerate acted as a competitive inhibitor of mandelate racemase with a K_I value equal to 1.5mM. Furthermore, Maggio et al.[37], using NMR techniques, followed the titration of the enzyme•Mn^{2+} complex with R,S-α-phenylglycerate, and found evidence for formation of a ternary complex. The dissociation constant (K_S) for R,S-α-phenylglycerate was measured to be 1.3mM in near agreement with the K_I value. Clearly, these two results show that R,S-α-phenylglycerate is able to bind to the same form of enzyme as the substrate.

It was of interest to us to resolve the racemic mixture of R,S-α-phenylglycerate and to test the separate enantiomers for competitive inhibition of the enzyme. The methods of resolution are discussed in the Experimental section. The compounds were judged to be of equal optical purity as determined by their equal and opposite $[\alpha]_D$ values. Using the spectrophotometric assay of Hegeman[45], the K_I values of R- and S-α-phenylglycerates were determined to be nearly identical. The K_I for R(+)-α-phenylglycerate was 1.25 ± 0.12mM and the K_I for S(-)-α-phenylglycerate was 1.29 ± 0.11mM. The double reciprocal plots for R- and S-α-phenylglycerate are shown in Figures 15 and 16, respectively. The K_I values were determined from the replots shown in Figures 17 and 18.

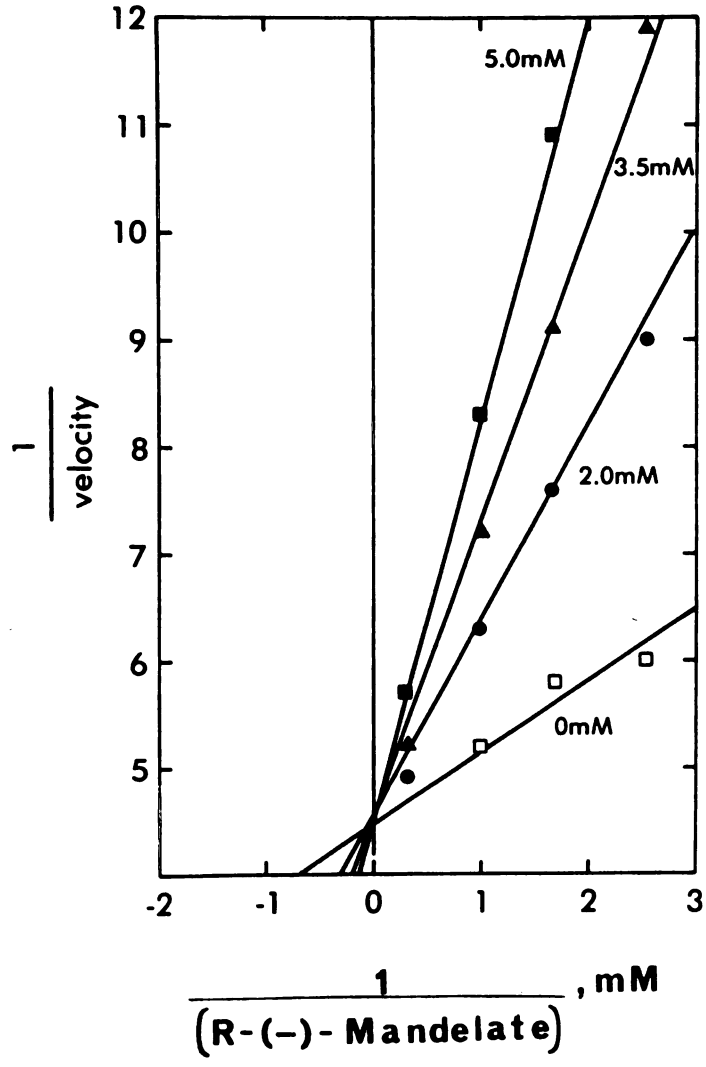


Figure 15. Competitive Inhibition of Mandelate Racemase by R-(+)-α-Phenylglycerate in the R to S direction.

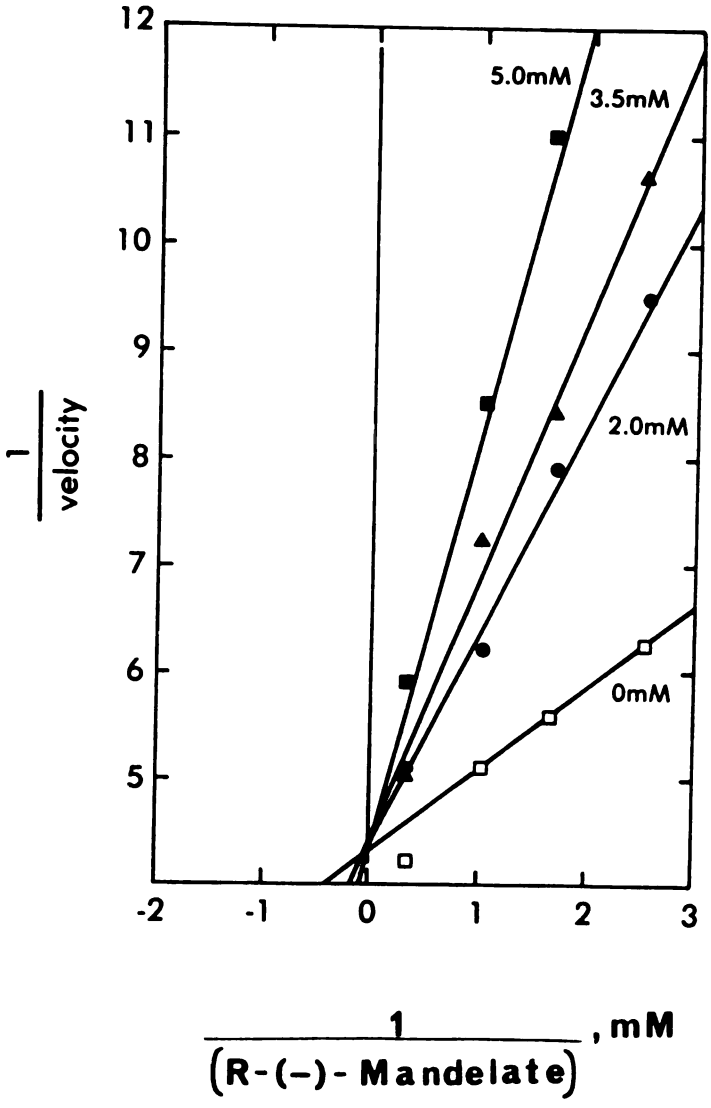


Figure 16. Competitive Inhibition of Mandelate Racemase by S-(-)-α-Phenylglycerate in the R to S direction.

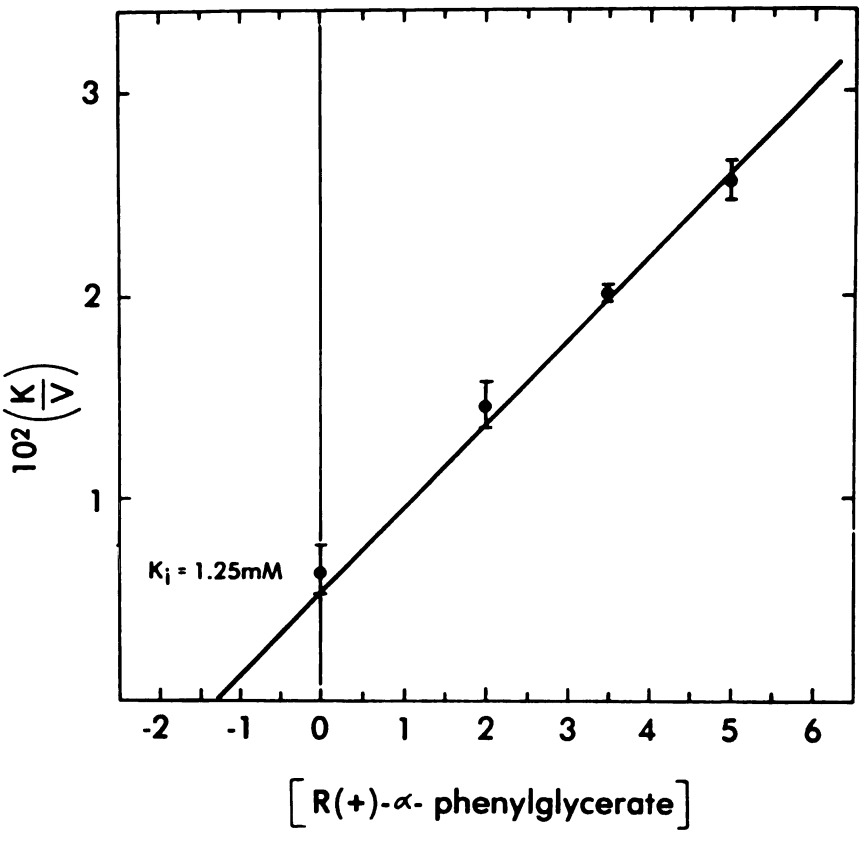


Figure 17. A replot of the K/V values from Figure 15 versus R-(+)-α-phenylglycerate concentation.

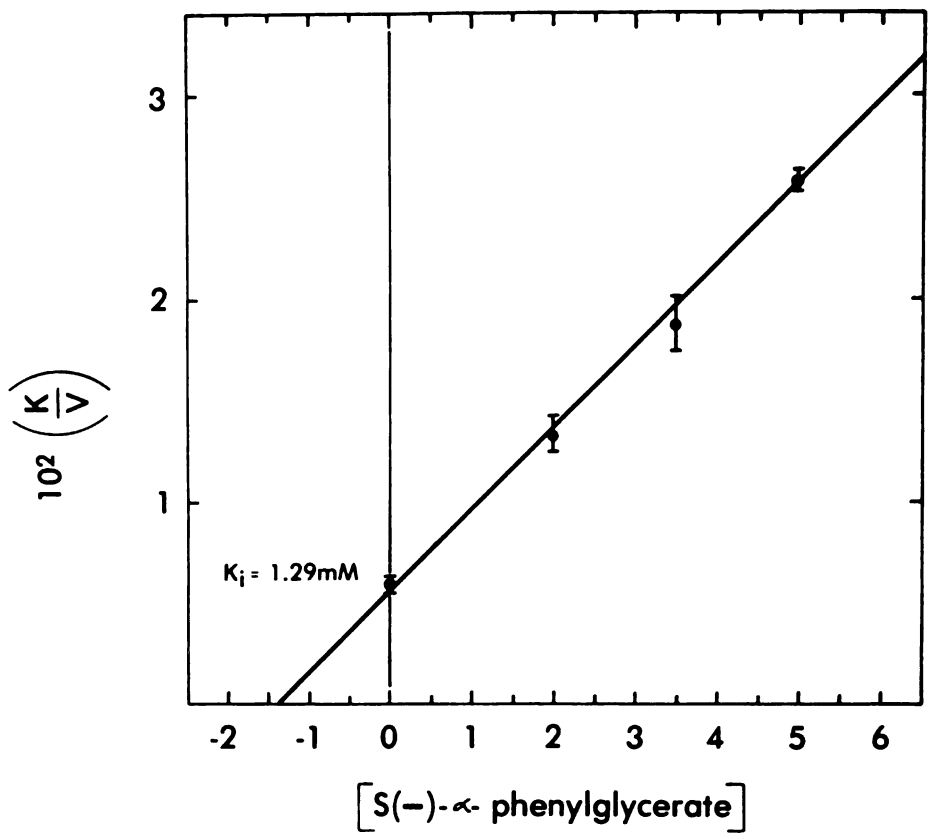


Figure 18. A replot of the K/V values from Figure 16 versus S-(-)-α-phenylglycerate concentration.

The purpose of these studies was two-fold. First, if enantiomeric substrates (in this case R- and S-mandelate) have equal affinities for the enzyme, then presumably either enantiomer of enantiomeric competitive inhibitors will have equal K_I values. This is because, by definition, competitive inhibition results from mutually exclusive binding of inhibitor and substrate to the same form of the free enzyme. The experimental results validate this prediction.

Secondly, with regards to the irreversible inhibition data, the structure of α-phenylglycerate is closely related to that of α-phenylglycidate - the irreversible inhibitor of mandelate racemase. Since two independent lines of evidence, the kinetic studies[7] and the NMR titration experiments[37], suggest that either R- or S-α-phenylglycerate can form a complex with the same form of the enzyme as the substrate, then one can infer that either R- or S-α-phenylglycidate can form a complex with the same form of free enzyme before covalent modification of it. This is in accord with the kinetic evidence suggesting formation of a complex between the irreversible inhibitor and enzyme before covalent modification of the enzyme as discussed in the last section.

These results allow one to infer that the S(-)-α-phenylglycidate, because of its structural similarities to the S(-)-α-phenylglycerate, can form a complex with the

enzyme; and, in a competitive manner, and it will protect against irreversible inhibition by the R(+)-α-phenylglycidate.

3. Differences in the Rate of Inactivation Resulting from Different Enzyme Preparations.

Preliminary experiments indicated that one preparation of mandelate racemase was inactivated by R,S-α-phenylglycidate only at concentrations 10-15-fold greater than those concentrations used by Fee et al.[46] This preparation, designated as "Prep. I", resulted from the loading of the first fractions eluting from a Sephadex G-200 column onto a DEAE-Sephadex A-50 column. Subsequent collection and pooling of the fractions from the DEAE column resulted in an enzyme preparation with high specific activity.

The preparation, designated "Prep. II", resulted from a pooling of the later fractions eluting from the Sephadex G-200 column. Prep. II was inhibited at concentrations of R,S-α-phenylglycidate similar to those required by Fee et al.[46]

One explanation for the difference in the reactivity toward the affinity label is that there is some proteolytic cleavage of portions of the whole enzyme during its purification. Prep I, eluting first because of its higher molecular weight, probably is intact or nearly intact enzyme. Prep. II, on the other hand, elutes later due to its lowered molecular weight, perhaps as a result of having had portions of it cleaved by proteases released during the purification procedure. Presumably, proteolytic

cleavage made the active site of the enzyme more accessible and consequently, more susceptible to irreversible inhibition by the α-phenylglycidate.

Fee et al.[46], on the other hand, pooled all fractions eluting off the G-200 column with mandelate racemase activity and loaded them onto the DEAE column. This preparation probably included both forms of enzyme and the reactivity of one overshadowed the inactivity of the other.

Both enzyme prepaprations had very nearly identical kinetic parameters (K_M, V_{max}, and turnover number). This raises a very interesting question of how much of the enzyme is actually necessary for the racemization process. In other words, how many amino acid residues can be cleaved from the whole enzyme and still leave a viable enzyme - one that will still catalyze a racemization reaction.

This question could probably be answered best using genetic engineering techniques. First, it would be necessary to characterize more fully the residues at the active site. The use of affinity labels is one step toward this goal. Next, it would be necessary to find the nucleic acid sequence on the gene for mandelate racemase which encodes for the active site. Having done this, then one could, theoretically, eliminate nucleotides on the gene which code for amino acids not found at the active site.

Eventually, these genetic manipulations would produce a new truncated form of the enzyme with, perhaps, intact catalytic activity.

D. Kinetics of the Racemization in D_2O and H_2O.

Cardinale and Abeles[47] first reported that the time course of proline racemization, as catalyzed by proline racemase and followed by the optical rotation of the solution, was normal in H_2O, but not in D_2O. Instead of decreasing initially and approaching zero asymptotically in D_2O, the optical rotation went through zero and became of opposite sign before finally returning to zero. The anomalous behavior has been named an overshoot and is now considered a hallmark observation consistent with a so-called two-base acceptor mechanism in enzymatic racemization reactions catalyzed without the assistance of a cofactor. Indeed, Finlay and Adams[48] later observed an overshoot region in the time course of hydroxyproline-2-epimerase epimerization.

The explanation for the overshoot is as follows.[47] Initially, in the absence of substrate, the protons on the two bases exchange with D_2O. Upon binding of the substrate (L-proline has been selected for this example), the first base abstracts the proton from L-proline while the second base replaces it with a deuteron to form D-proline. The D-isomer dissociates and the first base exchanges its substrate-derived proton with D_2O. After ca. 15-20% of racemization has occurred, the back reaction (D-proline $\rightarrow$ L-proline) becomes significant. It is only because for every molecule of the non-isotopical-

ly labeled substrate catalyzed there is one molecule of isotopically labeled product produced that this back reaction becomes significant. There are two competing reaction characterized by two rate constants k_1 and k_2 as shown in equation 12.

$$\begin{aligned} {}^{H}_{\text{(L-proline)}} &\xrightarrow{k_1} {}^{D}_{\text{(D-proline)}} \\ {}^{D}_{\text{(D-proline)}} &\xrightarrow{k_2} {}^{D}_{\text{(L-proline)}} \end{aligned} \qquad (12)$$

Because of the deuterium isotope effect k_1 will be greater than k_2 and an accumulation of D-proline results. Experimentally, one observes the optical rotation as it passes through zero to a positive value. The initial pass through zero results when the concentration of ^{D}L-proline and ^{H}L-proline are equal to the concentration of ^{D}D-proline. As each of the two reactions reach equilibrium, the optical rotation approaches and finally reaches zero.

Such a situation is inconsistent with a one-base acceptor mechanism for the following reasons. The single base is able to abstract the proton from one isomer, and after partial exchange with D_2O, it can replace the abstracted proton with either a proton or a deuteron. The result is a mixture of the isotopically and non-isotopically labeled product. The back reaction of the isotopically labeled isomer does not become a signficant factor in the overall process because of its lower concentration

and the presence of a non-isotopically labeled back reaction.

The course of the reaction as monitored by the change in ellipticity at 227nm is shown for S(+)-mandelate in Figure 19 both in H_2O and D_2O. In contrast to the results of Cardinale and Abeles[47] for proline racemase and Finlay and Adams[48] for hydroxyproline epimerase, it is seen that the approach to equilibrium in D_2O is asymptotic for mandelate racemase. That is, it is not characterized by an overshoot region followed by a return to equilibrium as was the case for these other two enzymes.

In Figure 20, the course of the reaction as monitored by the change in optical rotation at the D line of sodium is shown for R(-)-mandelate in both H_2O and D_2O. Again, it is not characterized by an overshoot region.

These two results are consistent with a one-base mechanism for mandelate racemase.

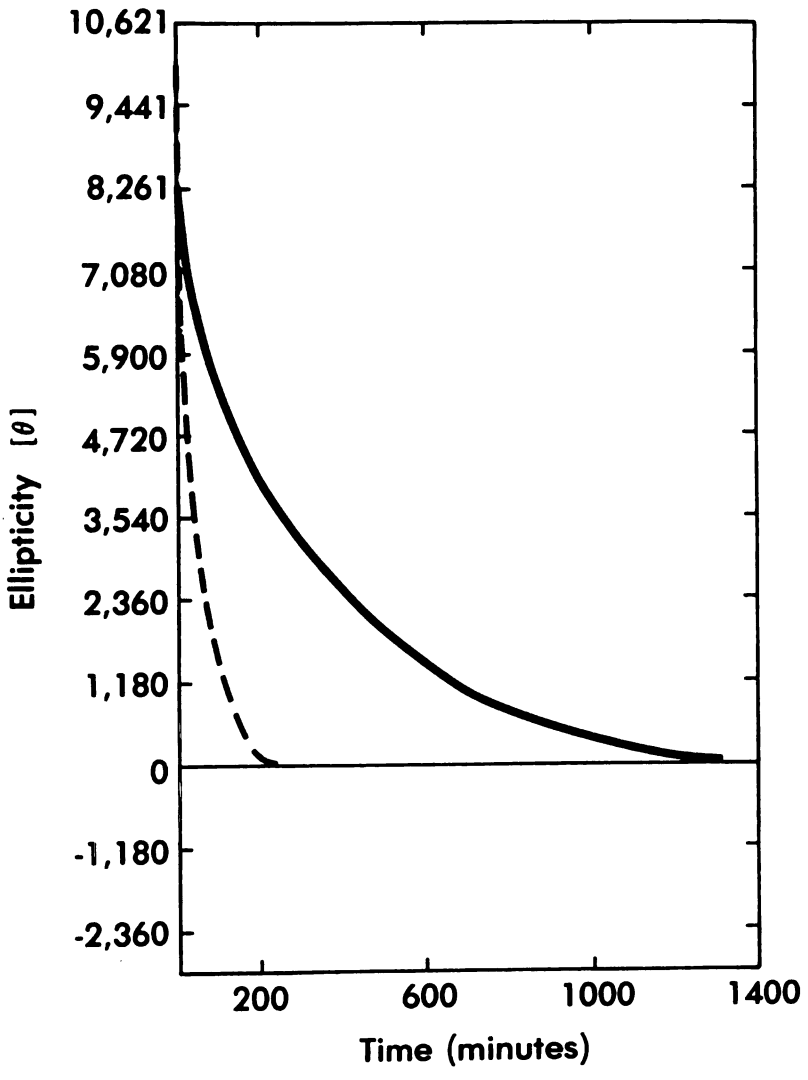


Figure 19. Racemization of S-(+)-Mandelate in H_2O (·····) and D_2O (——).

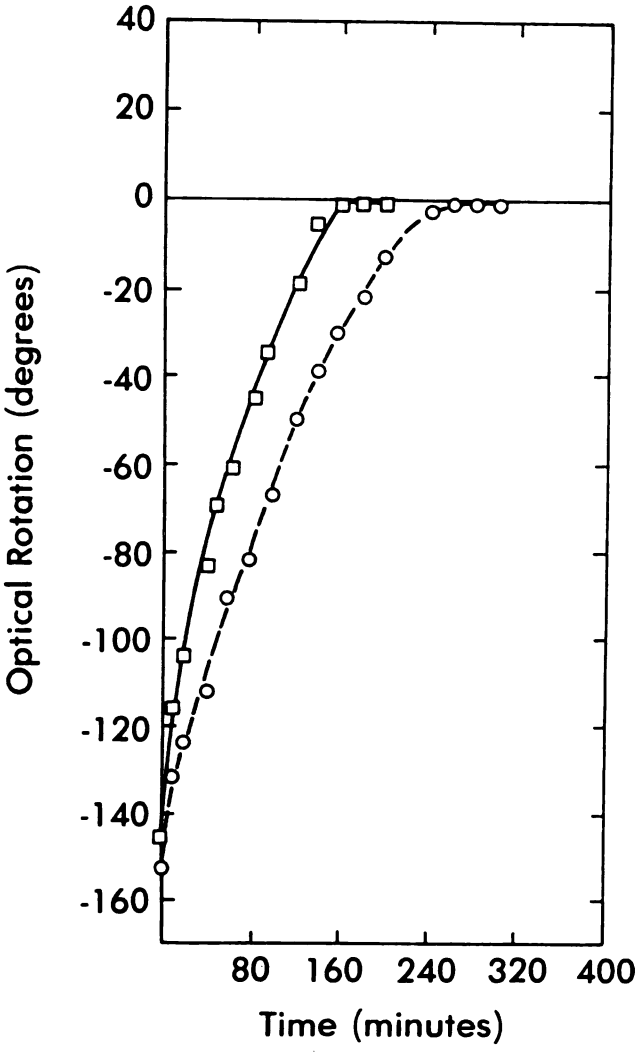


Figure 20. Racemization of R-(-)-Mandelate in H_2O (□ □) and D_2O (○ ○).

E. Equilibrium Perturbation Experiment.

It is seen in Figure 21 that upon addition of enzyme to an equimolar mixture of S(+)-α-[^{2}H]-mandelate and R(-)-α-[^{1}H]-mandelate, there is an equilibrium perturbation followed by an asymptotic return to equilibrium. The perturbation was monitored by observing the change in elliticity at 227nm in the CD. The maximum displacement was 35 mm which corresponded to a molecular ellipticity of 8365 degrees ml^{-1} cm^2. From this perturbation, one is able to calculate an isotope effect of 3.2 (using equation 5 in the Experimental Section) in excellent agreement with the values calculated from the comparison method.

An equilibrium perturbation results from the following kinetic situation.[38,49] There are two processes contributing to the observable ellipticity in the reaction mixture as shown in equation 13.

$$^{D}(\text{S-mandelate}) \xrightarrow{k_1} {}^{D}(\text{R-mandelate})$$

$$^{H}(\text{R-mandelate}) \xrightarrow{k_2} {}^{H}(\text{S-mandelate}) \qquad (13)$$

Each process is characterized by a kinetic rate constant k_1 and k_2. Because of the deuterium isotope effect, k_2 is greater than k_1. This results in an accumulation of the isotopically labeled species - the R-mandelate. Experimentally, one observes a perturbation. As each reaction reaches equilibrium, the ellipticity approaches and finally becomes zero.[49]

The equilibrium perturbation experiment was performed primarily to serve as a control for the racemization of mandelate in D_2O and H_2O. Cleland[38] has shown that the overshoot region seen in the time course of racemization of proline by proline racemase and the perturbation seen in the time course of the equilibrium perturbation exepriment result from the same phenomenon, i.e., the accumulation of one enantiomer that is isotopically labeled, due to an isotope effect. However, the origin of the isotopically labeled material is different for the two different types of experiments. In the racemization experiment, the enzyme synthesizes the isotopically labeled enantiomer as an inherent feature of its mechanism whereas in the equilibrium perturbation experiment, the isotopically labeled enantiomer is added to the reaction mixture by the experimenter. Because of this difference, one can use the equilibrium perturbation experiment as a control for the racemization experiment. If one does not observe an overshoot region upon enzymic racemization in D_2O, but does observe the equilibrium perturbation, then one is assured the the racemization experiment is valid, even if no overshoot region is observed, as is the case here with mandelate racemase. That is, if such an overshoot were possible as a result of an inherent feature of the mechanism, one should have been able to observe an overshoot region in the racemization experiment.

Two additonal controls were performed in order to ensure that the equilibrium perturbation experiment itself was not an artifact.[38] First, the same experiment was carried out without enzyme in order to observe the stability of the baseline. There was no drift. Second, the same experiment was run using non-isotopically labeled mandelate. No perturbation was observed in this experiment.

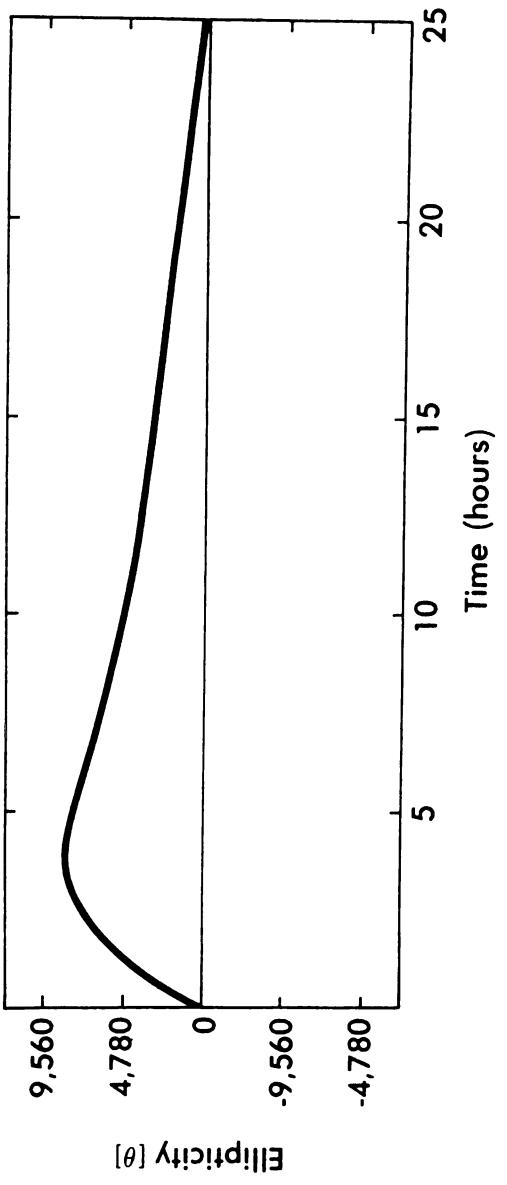


Figure 21. Equilibrium Perturbation with Mandelate Racemase added to an equimolar mixture of S-(+)-α-(^{2}H)-Mandelate and R-(-)-α-(^{1}H) - Mandelate.

F. The Active Site of Mandelate Racemase.

1. Introduction.

Asymmetry at the active site of mandelate racemase - a sine qua non for enzymes in general - is not apparent in either the binding or processing of the normal substrate. However, the asymmetry becomes apparent in the differential binding affinities of the potassium R- and S-α-phenylglycidates for the enzyme and their subsequent reactivities.

On the one hand, the results of the initial velocity studies, the competitive inhibition studies, the deuterium isotope effect experiments, and the pH studies all suggest that the active site of mandelate racemase, although inherently asymmetric, appears to bind and/or process the enantiomeric substrates and reversible inhibitors with a remarkable symmetry. The enzyme binds either R- or S-mandelate equally; it catalyzes the rates of reaction equally; it binds either R- or S-α-phenylglycerate with equal affinity; and finally, the same groups appear to be involved in the binding of the substrate. Thus, the results of these experiments pose a paradox. How are the groups so arranged at the active site to create such a high degree of symmetrical binding <u>and</u> catalysis within an inherently asymmetric environment? These results are consistent with the other experimental data suggesting a one-base-acceptor mechanism for mandelate catalysis as

discussed by Kenyon and Hegeman.[36]

On the other hand, the results of the experiments using the irreversible inhibitor of mandelate racemase indicated that the active site can differentiate between potassium R- and S-α-phenylglycidate. Moreover, these results are the first to suggest that a two-base acceptor mechanism maybe operating in mandelate racemase catalysis. In such a situation, when the enzyme binds R(+)-α-phenylglycidate (the potent irreversible inhibitor), one base, B_1, acting as an acid catalyst, protonates the oxygen of the epoxide moiety to assist the nucleophilic opening of it by the other base (B_2). Binding of the S-(-)-isomer, however does not result in inactivation of the enzyme because B_1 and B_2 now have different functions. B_1 is positioned in the role to act as the nucleophile while B_2 is responsible for the protonation of the oxygen of the epoxide moiety. One can explain the data by saying that either B_1 is not so good a nucleophile as B_2 or B_2 is unable to assist in catalysis by its inability to protonate the epoxide moiety.

The possibility of a two-base acceptor mechanism is eliminated, however, by the results of the racemization experiment. By two methods, polarimetry and circular dichroism, it was shown that there is no overshoot in the racemization of either R- or S-mandelate in D_2O. Furthermore, the equilibrium perturbation experiment serving as

the control demonstrated that such an overshoot would have been evident if it were possible as an inherent feature of the enzyme's mechanism.

Confronted with such results, it can stated unequivocally that mandelate racemase catalyzes racemization using a so-called one-base acceptor mechanism.

The following discussion proposes a mechanism consistent with the data by which mandelate racemase catalyzes racemization using the one-base acceptor mechanism.

2. The Mechanism.

First, it is assumed that the amino acid residue being covalently modified at the active site of mandelate racemase is the same residue responsible for the enzyme's catalytic reaction (i.e., removal and replacement of the α-hydrogen of mandelate). This is a reasonable assumption in that the structures of mandelate and α-phenylglycidate are very similar. Therefore, the electrophilic center of the epoxide will be bound in close apposition to the nucleophile responsible for catalysis. Second, it is assumed that R(+)-α-phenylglycidate, once enantiomerically pure, is the isomer entirely responsible for activity whereas the S(-)-isomer, once enantiomerically pure, is completely inactive as an irreversible inhibitor. Finally, the S(-)-isomer is binding at the same site as the substrate and the R(+)-isomer. Furthermore, the S(-)-isomer is protecting the enzyme against inhibition by the R(+)-isomer. The basis for this assumption has been discussed previously.

An ideal candidate for the abstraction and transfer of the α-hydrogen from either substrate is either the γ-carboxyl group of glutamate or the β-carboxyl group of aspartate, although thus far only circumstantial arguments and sparse data can be used to support this. The affinity labeling experiments of Fee et al.[7] provide the indirect evidence for this choice. It was shown that

hydrolysis of the affinity label-derived adduct released the diol α-phenylglyceric acid from the enzyme. Also, added hydroxylamine accelerated the rate of loss of the ^{14}C label consistent with the idea that an ester linkage is involved. Kenyon and Hegeman[36] speculated that within the asymmetric active site, the two oxygens of this putative carboxyl group become diastereotopic. One of the oxygens could deprotonate R-mandelate to allow formation of the carbanion intermediate. In the course of the oxygen's movement within the active site, it could then reprotonate the opposite side of the intermediate in order to form S-mandelate. This movement could not be random or a racemic mixture would result. Likewise the other oxygen could deprotonate S-mandelate and the above scenario could be repeated.[36]

The binding of the substrate at the active site is postulated to be as follows. It is most likely that the positioning of the carboxyl group of mandelate is rigidly controlled. The NMR studies of Maggio et al.[37] provided evidence for the existence of one tight binding site per subunit for Mn^{2+}. The tight binding affinity infers favorable interactions of binding of the carboxyl group with certain areas within the active site. It is likely that the carboxyl group of either R- or S-mandelate will bind in one of the coordination spheres of the metal ion in order

to retain the stabilization associated with this binding.

The binding position of the phenyl group will be set for either enantiomer in a region of hydrophobicity. Consequently, the binding position for the hydrogen and the hydroxyl group will interchange depending upon which enantiomer is binding (i.e. in one enantiomer, the hydroxyl group occupies one spatial position within the active site while in the other enantiomer the hydrogen will occupy this same position).

In order to accommodate this interchange it is possible that there is an amino acid residue present in this region with a plane of symmetry at these binding positions. Either the imidazoyl group of histidine or the carboxyl groups of aspartate or glutamate could satisfy this requirement. The hydrogen on one nitrogen of the imidazoyl group of histidine forms a hydrogen bond with the hydroxyl group for one enantiomer while the hydrogen on the other nitrogen could do likewise for the hydroxy group upon the binding of the opposite enantiomer. Tentatively, the pK_as measured from the pH profiles belong to a histidine residue as the measured pK_as are similar to those known for histidine. In a like manner, a hydrogen from a protonated carboxyl group of either an aspartate or a glutamate could also serve to act as binding points for the hydroxyl group.

Thus far, the data and this proposal are in

accord. It is necessary now to account for the results of the irreversible inhibition studies. Because of the structural similarities between the enantiomeric α-phenylglycidates and mandelate, it is likely that these molecules will bind to the enzyme in an analogous fashion. This places the epoxide-oxygen in the same region as that for the α-hydroxy group of R- or S-mandelate. The β-carbon, vulnerable to nucleophilic attack, would be left to bind in the area where the α-hydrogen of R- or S-mandelate normally binds.

In order to achieve covalent modification of the enzyme by an epoxide, it is believed that as well as having a nucleophilic amino acid residue placed adjacent to the β carbon there must be a relatively acidic proton from an amino acid residue placed in the vicinity of the oxygen of the epoxide moiety in order to assist in its opening. The acidic proton can come from either 1) a hydronium ion after protonation by a side chain on the enzyme acting as a general acid catalyst or 2) directly from the side chain (e.g. $-CO_2H$, $-NH_3^+$, etc.). It has been postulated, although not proved, that a general acid catalyst exists at the active site of other enzymes inactivated by epoxides in order to facilitate opening of the epoxide ring. For instance, Schray et al.[50] and Rose et al.[51] suggested that a properly situated general acid, by effectively protonating the epoxide-oxygen, was responsible for

the high rate of alkylation of the enzyme by the poor glutamate nucleophile. Similarly, Cassidy and Kahan[52] invoked such a mechanism to explain the inactivation of pyruvyl transferase by phosphonomycin. Likewise, Quaroni et al.[53] postulated that the presence of a general acid catalyst explained the inactivation of the sucrase-isomaltase complex by conduritol β-epoxide. Finally, Kupchan et al.[54] postulated the neighboring intramolecular catalysis was critical to the antileukemic properties of two alkaloids, triptolide and tripdiolide. It was suggested that a 14-hydroxy group assisted in the selective alkylations of thiols by a 9,11-epoxide within these alkaloids. A NMR spectrum indicated that there was hydrogen bonding between the 14-hydroxy and the 9,11-epoxide groups. A structurally similar compound, triptonide, differed only at the C-14 position at which a ketonic function occurred instead of the hydroxy group. An NMR spectrum did not indicate the presence of a hydrogen-bonded hydroxy-epoxide system. Triptonide showed no antileukemic properties and did not react with thiols in model systems.[54]

Strategically, this model has placed the β-carbon of either enantiomeric α-phenylglycidate within the correct distance for attack by the putative carboxyl group of either aspartate or glutamate. Again, such a flexible arm would be able to attack either enantiomer - one oxygen is able to attack one isomer while the other oxygen would be

able to attack the other isomer. There is some literature precedence to invoke opening of epoxide rings by a carboxylate-oxygen nucleophile. First, model studies indicate that carboxylate groups can react with epoxides in the presence of a general acid catalyst.[55] Also, there is direct evidence in the epoxide inactivation of triose phosphate isomerase[56] and indirect evidence in the epoxide inactivation of phosphoglucose isomerase[57] that oxygen ester formation results upon inactivation of the enzyme due to attack of a carboxylate group on the enzyme.

It is possible that instead of reacting directly as a nucleophile, the carboxyl group acts as a general base. In this role, it would abstract a proton from a water molecule to form an incipient hydroxide ion, which then would attack the epoxide ring. Proteolytic degradation and identification of the inactivator-derived adduct could differentiate between the possible roles for the carboxyl group.

The differences in the reactivities of the two enantiomeric affinity labels is explained as follows. If the imidazoyl moiety of a histidine is responsible for the binding of the α-hydroxy group of either R- or S-mandelate (as suggested by the pH-rate profile), and likewise responsible for the binding of the epoxide-oxygen of the enantiomeric α-phenylglycidates, then it is entirely possible that one of the enantiomers of mandelate or α-

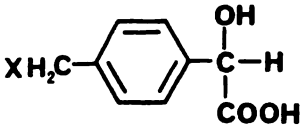
phenylglycidate is bound less tightly by the enzyme due to a lesser interaction between the α-hydroxy group or epoxide-oxygen and the imidazoyl group of histidine as a result of a greater intermolecular distance between them. It is postulated that the greater distance is a direct result of the inherent asymmetry at the active site. The flexibility of the α-hydroxy group on mandelate can probably compensate for the greater distance so that both enantiomers have nearly identical measureable kinetic properties. Conversely stated, if there were no interaction between the α-hydroxy group of one enantiomer and the enzyme, then some noticeable differences in the kinetic properties would be evident.

This asymmetry of spatial arrangement of the groups at the active site, however, becomes very evident upon the binding and attempted catalysis of the epoxide. Here, the epoxide-oxygen of the R(+)-isomer is within the necessary distance to form a hydrogen bond with the proton on the imidazoyl group of histidine, and, as a result, the epoxide ring opens. The epoxide-oxygen of the S(-)-isomer, however, is not within the necessary distance to the histidine, and, consequently, without the general acid catalyst assistance of this group, the epoxide ring cannot open. Furthermore, unlike the oxygen of the substrate, the epoxide-oxygen is rigidly constrained, and therefore cannot move into the correct position to form a hydrogen

bond.

Precedence for these dramatic differences in the processing of enantiomeric affinity labels as a result of the inherent asymmetry at the active site comes from the work of Wang and Walsh[58,59] on alanine racemase. Their work is discussed in the introduction. The results of the following experiments may test this proposal. Much information could be gained about the mechanism by the identification of the residue being labeled by the epoxide. This would involve making a radioactively labeled epoxide, incubating it with the enzyme, performing a proteolytic degradation of the labeled enzyme, separating the peptides and locating the labeled peptide. These experiments may show if the group being labeled is the carboxyl group.

Second, the proposal predicts that there should be no detectable differences in the ability of enantiomers of the following series of compounds to inactivate mandelate racemase. This assumes that there is not an asymmetrically placed group near the para position of the bound compounds.



X = Cl, Br, F

Currently, these compounds are being synthesized and tested for possible inactivation of mandelate racemase.[60]

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A. Microbiological Procedures

1. General.

Spectrophotometric assays were performed on a Hitachi 100-80 spectrophotometer equipped with an automatic six-cell changer and Peltier temperature controller. The cell compartment was thermostated at 25° C.

Bacterial cell cultures were incubated in either a New Brunswick Environmental Incubator Shaker, Model G25 (floor model) or Model G24 (bench model). Bacterial growth was followed by turbidity readings taken on a Klett-Summerson colorimeter using a No. 54 (520-580 nm) filter.

Low-speed centrifugation was carried out in a Sorvall RC-2 refrigerated centrifuge using either an SS34 (8 X 40ml capacity) or a GSA (6 X 250ml capacity) rotor. High-speed centrifugation was carried out in a Beckman-Spinco Model L2-65B refrigerated ultracentrifuge using a Beckman SW 28 rotor (6 X 35ml capacity).

Sonication of cells was carried out using a Heat Systems - Ultrasonics Inc. Sonicator.®

All reagents were prepared using doubly distilled-deionized water. Chemical reagents were purchased from either Aldrich Chemical Company, Sigma Chemical Company, or Mallinckrodt.

2. Maintenance of Stock Culture.

Stock cultures of *Pseudomonas putida* A.3.12 (ATCC 12633) and *Pseudomonas aeruginosa* (ATCC 15692) were maintained on yeast-agar-phosphate (YAP) slants as described elsewhere.[1] Preparation of the slants and transfer of the strains were carried out in accordance with the procedure of Halpin.[1]

3. Formulation of Mineral Base (Hutner's Concentrated Base).

Preparation of the Hutner's mineral base and the culture medium is described elsewhere.[1,2] In order to increase the yield of *P. putida*, the culture medium was modified as described below.

To 800mL of water were added:

40mL 1M $NaKHPO_4$ buffer, pH 6.8

10mL 10% $(NH_4)_2SO_4$

20mL Hutner's Concentrated Base

10mL 2M R,S Mandelate, NH_4^+ salt, pH 7.0

The pH of the solution was adjusted to pH 6.8 and diluted to 1 L with water.

The following solutions were made up to be used in the 200 liter fermenter.

8 L 1M $NaKHPO_4$ buffer pH 6.8

2 L 10% $(NH_4)_2SO_4$

4 L Hutner's Concentrated Base

2 L 2M R,S-Mandelate, NH_4^+ pH 7.0

These ingredients were transported to the University of California, Berkeley, on the day before a run. Preparation and sterilization of the culture medium were performed by the staff at U.C., Berkeley. The following morning, the pH was tested with litmus paper and adjusted to pH 6.8, if necessary.

4. Procedure for the Growth of *P. putida*.

P. putida is the source for both the enzymes mandelate racemase and benzoylformate decarboxylase. The total amount of time required to prepare approximately 1 kg of bacterial cells using the 200 liter fermenter is six days. The generation time is 60 min. The schedule for growth is described below.

(a) Initially, a YAP slant was removed from the refrigerator and allowed to grow at room temperature (25°) overnight. Then a fresh YAP slant was streaked using standard microbiological technique and allowed to grow at 25° C. for 16-20 h (the time can be shortened by incubating the slant at 30° for 6-10 h).

(b) The culture medium was prepared and autoclaved as described by Halpin[1]. The culture medium was distributed as follows:

(1) 500mL to 6-2800mL Fernbach flasks (the preculture for the 200 liter fermenter).

(2) 100mL to 2-500mL nephelometer flasks (the preculture for the 6 Fernbach flasks).

(3) 20mL to 1-125mL nephelometer flask (the preculture for the 500mL nephelometer flasks).

(4) 20mL to 1-125mL nephelometer flask (blank).

The 125mL nephelometer flask was innoculated with 2 or 3 loopfuls of cells from the YAP slant with a heat-sterilized nickel loop using standard microbiological technique. The nephelometer flask was incubated at 30° C. with the shaker speed set at 350 rpm for 12-18 h. The turbidity reading was usually 400 klett units at the end of the growth period.

(c) To each of the 2-500mL nephelometer was added 1mL of preculture. These flasks were incubated under the conditions described in step (b) for 12-18 h. After this period, the turbidity reading was generally 300 klett units.

(d) Then 5mL of preculture from the 500mL nephelometer flasks was used to innoculate the 6-2800mL Fernbach flasks. These were incubated at 30° C. while shaking at 325 rpm for 12-18 h, producing 3 liters of preculture.

(e) The 3 liters of preculture was used to innoculate the

culture medium in a 200 liter fermenter located in the Department of Biochemistry , University of California, Berkeley, under the direction of Robert Kuderna. The aeration on the fermenter was set on full and the temperature was maintained at 30° C. Aliquots were removed and turbidity readings were made every hour. The following table shows the progress of the growth.

TIME (hr.)	READING (klett units)
0	0
1	10
2	29
3	54
4	125
5	242
6	300

After six hours, the cells were harvested using an unrefrigerated air-driven Sharples centrifuge. The cells were weighed, frozen, and stored at -20° C. until ready for use.

5. Procedure for the Growth of *P. aeruginosa*.

P. aeruginosa is the source for S(+)-mandelate dehydrogenase. The procedure for its growth is similar to that for *P. putida*. However, *P. aeruginosa* lacks mandelate

racemase necessitating the following modifications. The generation time is 4 hours. The cells are harvested after six generations (24 h).

(a) Culture Medium

The protocol described for the growth of *P. putida* was followed, substituting a 4M R,S mandelate, NH_4^+ salt, pH 7.0 solution in place of the 2M mandelate solution.

(b) Distribution of Culture Medium

The distribution of the culture medium was carried out as described for *P. putida*.

(c) Schedule for the Growth of *P. aeruginosa*

The total amount of time required to prepare approximately 800g of bacterial cells using the 200 liter fermenter was nine days.

Incubation of the flasks and Fernbachs was always at 30° C. and the shaker speed was set at 350 rpm for the bench model and 325 rpm for the floor model. The schedule is summarized in Table I.

TABLE I

GROWTH VESSEL	GROWTH PERIOD	INNOCULANT QUANTITY	FINAL TURBIDITY READING
YAP slant	16-20 h	-	-
125 mL nephelometer	3 days	2-3 ringfuls	325 klett units
500 mL nephelometer	2 days	5 mL	290 klett units
2800 mL nephelometer	2 days	10 mL	-
200 L fermenter	24 h	3 L	-

B. Enzyme Purification

1. General.

Assays for enzyme activity were performed as described in Section C. Protein determinations were made using the Biorad biuret method with crystalline bovine serum albumin containing 97.5% dry bovine serum albumin by weight. Enzyme solutions were concentrated using Lyphogel®. Sodium dodecylsulfate (SDS) gel electrophoresis was performed according to the method of Weber and Osborn[3] as modified by Laemmli.[4]

2. Mandelate racemase.

The purification procedure for mandelate racemase has been developed by Hegeman[2,5]. The procedure was followed with the modifications listed below.

Typically, 30g of frozen cells was suspended in 150mL of 0.05M $NaKHPO_4$ buffer (pH 6.8) in a 600ml beaker. The beakers were chilled in an ice-ethanol bath. The cells were then sonicated for 8 - 3 min bursts every 15 min. After the first 3 min burst, 50µmoles of phenylmethanesulfonyl fluoride was added to the solution to inhibit protease activity. The "output control" on the Sonicator® was set between 9 and 10 and the "duty cycle" was set at 90%. The Sonicator® was set for continuous cycle.

The heat treatment step described by Hegeman[5] was not performed in any of the preparations. Typically, the enzyme preparation was used after the Sephadex G-200 step.

3. S(+)-Mandelate Dehydrogenase.

The cells were disrupted in the quantity and manner as described above.

The pellets resulting from the 100,000 x g centrifigation step were collected and washed with the 0.05M $NaKHPO_4$ buffer. These pellets consisted of the broken cell membranes and contained S(+)-mandelate dehydrogenase activity. The pellets were resuspended in approximately 20mL of the 0.05M $NaKHPO_4$ buffer at pH 6.8 made 40% (v/v) in ethylene glycol, and then forced by a syringe through a 20 gauge blunt-ended needle into a collection vessel set in ice. The S(+) mandelate dehydrogenase solution used in these experiments was made from 25mL of this crude preparation diluted to 1 liter with the same phosphate buffer.

The S(+)-mandelate dehydrogenase is stored frozen at -20° C.

4. Benzoylformate Decarboxylase.

This enzyme was purified as described by Hegeman[2,6].

C. Enzyme Assays

1. General.

All reagents were purchased from either Aldrich Chemical Company or Sigma Chemical Company. Abbreviations used: Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; DCPIP, 2,6-dichlorophenolindophenol; MDH, S(+)-mandelate dehydrogenase-containing vesicles.

2. Mandelate Racemase.

There are two assays for monitoring mandelate racemase activity - the coupled assay developed by Hegeman[2,5] and the circular dichroic assay developed by Sharp et al.[7]

a. The Spectrophotometric Assay.

The coupled assay of Hegeman[5] was used, with the modifications below, for measurement of initial velocity studies, the activity of fractions during various stages of purification , reversible inhibition, and irreversible inhibition. Measurements using this assay can only be made in the R to S direction.

(1) In order to quantitate mandelate racemase activity at each stage of its purification, the following assay was used:

SAMPLE	REFERENCE	SOLUTION
2.267mL	2.28mL	100mM K^+Hepes buffer pH 7.5
0.013mL	-	100mM K^+ R(-)-mandelate pH 7.5
0.01mL	0.01mL	100mM DCPIP
0.1mL	0.1mL	100mM KCN
0.01mL	0.01mL	300mM $MgCl_2$
0.6mL	0.6mL	MDH solution
3.0mL	3.0mL	Total Volume

The solutions were incubated at room temperature for 70 minutes. The assay was initiated by the addition of the enzyme.

(2) The following protocol was used for the initial velocity experiments and the competitive inhibition studies:

SAMPLE	REFERENCE	SOLUTION
A sufficient quantity to make a final vol. of 3mL.	2.28mL	100mM K^+Hepes buffer pH 7.5
The desired concentration	-	100mM K^+ R(-)-mandelate pH 7.5
The desired concentration	-	R- or S-α-phenylglycerate pH 7.5
0.1mL	0.1mL	100mM KCN
0.01mL	0.01mL	300mM $MgCl_2$
0.01mL	0.01mL	100mM DCPIP
3.0mL	3.0mL	Total Volume

The solutions were left to stand at room temperature for 70 minutes. Then 10-20μL racemase was added to

the sample cuvette to begin the assay.

(3) The following protocol was used for the irreversible inhibition experiments:

A stock solution was made up daily with the following quantities multiplied by the number of assays to be done that day. This stock solution was kept on ice while not in use.

2.267mL	100mM K^+Hepes buffer pH 7.5
0.1mL	100mM KCN
0.01mL	300mM $MgCl_2$
0.01mL	100mM DCPIP

Into each sample cuvette was pipetted 2.387mL of the stock solution.

Into the reference cell was pipetted 2.4mL of the stock solution.

To both the sample and reference cells was added 0.6mL of the MDH solution. The cuvettes were incubated at room temperature for 40 minutes. Normally, there was a 70 minute incubation period after the addition of the MDH solution and before the initiation of an assay. However, the protocol for the irreversible inhibition kinetics[8] called for a 30 minute incubation period making a total incubation period of 70 minutes. The assay was started by the addition of 0.013mL of 2M K^+ R(-)-mandelate, pH 7.5, to the sample cuvettes.

(4) Assay Notes

(a) The DCPIP Dye.

The DCPIP dye did not readily go into solution. The necessary quantity was weighed out in a 25mL volumetric flask. Approximately 15mL of water was added and the volumetric was sealed with parafilm and shaken vigorously by hand for 2-3 min. It was placed in a dark cupboard overnight. The next morning, it was shaken for another 2-3 min and then filtered by vacuum through a fine sintered glass funnel. The residue on the filter was discarded. The solution was stable for 2 weeks if stored at room temperature in a dark cupboard.

(b) The Incubation Period.

The 70 minute incubation period was incorporated into the assay because preliminary work had indicated that there was a finite, non-enzymatic rate of reaction. The non-enzymatic rate, indicated by the reduction of DCPIP, occurred when either KCN or the MDH solution was added to a solution containing only buffer, $MgCl_2$, and DCPIP. There was no mandelate in the solution. Two factors were believed responsible for this non-enzymatic conversion. Firstly, DCPIP is a pH indicator. Addition of the KCN and/or coupling enzyme will change the pH enough to prevent consistency in the measurement. Secondly, the S(+)-mandelate dehydrogenase-containing vesicles aggregate and disaggregate as a result of the difference in the ionic

strengths between the assay solution and the buffer in which the vesicles are stored. This process results in light scattering which gives rise to the apparent non-enzymatic rate.[9] The incubation period most likely gave both the vesicles and the solution time to come to a new equilibrium[9]. After this 70 min incubation period, there was a flat baseline.

The incubation period did not appear to harm the coupling enzyme. The following results confirmed this. Firstly, the Michaelis-Menten parameters measured using this assay and those measured using the circular dichroic assay were nearly identical. Secondly, the addition of more similarly incubated coupling enzyme did not increase the rate.

(c) The Reference Cell.

The reference cell was used for two reasons. Firstly, its presence reduced the effects of the light-scattering due to the S(+)-mandelate dehydrogenase-containing vesicles. Secondly, it cancelled a rate of reaction due to S(+)-mandelate dehydrogenase activity on the contaminating S(+)-isomer present in the R(-)-mandelate solutions.

All assay solutions were made up in 3mL glass spectrophotometric cells. After addition of either the enzyme or substrate the solutions were mixed by placing a

piece of parafilm on top of the cell and inverting the cell 2 or 3 times.

(5) Spectrophotometric parameters.

The wavelength was set at 600nm. The full scale was set at 0.05 optical density (OD) units. The chart speed was adjusted to give a 45-60° line (generally 10sec/div, 20 sec/div, 1 min/div, or 2 min/div depending upon the assay). The sample and the reference cells were placed in their respective cell holders. The "auto zero" button was depressed in order to zero the spectrophotometer, and then the "start" button was depressed.

The initial period (0.000 - 0.025 OD units) was too noisy and curved for reproducible measurements. This was due to the lag period in the assay as discussed by Halpin[1] and Hegeman[2,5]. The line straightened out from 0.025 -0.05 OD units; the spectrophotometer could again be set to 0 by depressing the "auto zero" button, and the line produced from 0.050 - 0.075 OD units was generally linear with a hint of curvature. Measurement of this line, though, will give reproducible results. In order to obtain the velocity in μmoles/min the following calculations were done. The slope, measured in vertical blocks/cm, was multiplied by the full scale value, measured in OD units/vertical blocks. The chart speed was recorded in min/div. Multiplication of this value by 1 div/2 cm translated the chart speed into min/div. The

reciprocal of this value multiplied by the product of the slope X the full scale value yielded the velocity in OD units/min. Hegeman reported a value of 6.72 OD units/μmoles of mandelate.[2] Division of the velocity in OD units/min by this value gave the velocity in μmoles/min. This was defined as a unit of enzyme activity[1].

The specific activity was obtained by dividing the units by the amount of enzyme used in the assay (units/ml enzyme). Division of this value by the mg protein/ml gave a value for specific activity in units/mg protein.

b. Circular Dichroic Assay.

The circular dichroic assay (CD assay) was used to measure the Michaelis-Menten parameters (V_{max} and K_M), the deuterium isotope effects, and the pH profiles. All measurements were made in both directions (R→S and S→R).

The solutions were made up in disposable test tubes (13 x 100mm) and transferred to the CD cell using a pipette. To each test tube was added the appropriate amount of substrate with a sufficient quantity of buffer to make a 3mL solution. The buffers were all made 1.0 mM in $MgCl_2$. Enzyme (generally 20μL) was added to the cells and mixed in by the inversion method.

The cell was placed in the CD instrument . The wavelength of the instrument was set at 262nm. The chart speed and sensitivity were set to produce a 45-60° line.

In all cases the lines curved off. In order to obtain reproducible results, these lines were fitted to a French curve; a straight edge was then placed against the curve at the initial point. A straight line was drawn. The slope of this line, when substituted into the formula below, measured the velocity of the enzyme-catalyzed reaction in μmoles x min^{-1} x ml^{-1} or mM/min/mg protein.

$$v = \frac{\text{(slope) (chart speed) (full scale) (vol. of CD cell)}}{\text{(path length of cell) (quantity of enzyme added)}}$$

3. S(+)-Mandelate Dehydrogenase.

Assay of S(+)-mandelate dehydrogenase activity was measured as described by Hegeman[2] with the following modifications.

SAMPLE	REFERENCE	SOLUTION
2.03mL	2.28mL	100mM K^+Hepes buffer pH 7.5
0.25mL	-	100mM K^+ S(+)-mandelate pH 7.5
0.1mL	0.1mL	100mM KCN
0.01mL	0.01mL	300mM $MgCl_2$
0.01mL	0.01ml	100mM DCPIP
2.4mL	2.4ml	Total Volume

These solutions were incubated at room temperature for 30-40 min. To each cuvette was added 0.6ml of the S(+)-mandelate dehydrogenase. The solutions were mixed by

the inversion method. The lack of S(+)-mandelate in the reference cell enabled one to negate the nonenzymatic rate of reaction described previously.

4. Benzoylformate Decarboxylase.

Activity was measured using the assay developed by Hegeman[2].

D. Kinetic Studies

1. General.

Abbreviations used: Mes, 2-(N-morpholino)ethanesulfonic acid; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); Hepes, N-(2-hydroxyethyl)piper-azine-N'-2-ethanesulfonic acid; Taps, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; Ches, (2-[N-cyclohexylamino]ethanesulfonic acid); Caps, (3-[cyclohexylamino]-1-propanesulfonic acid).

2. Competitive Inhibition Studies.

Competitive inhibition studies were carried out using the spectrophotometric assay of Hegeman[2].

A solution of either S(-)- or R(+)-α-phenylglycerate was made up as an aqueous solution and adjusted to pH 7.5 on the day of the experiment. The solution was kept on ice while not in use. Initial velocity studies were performed at four different concentrations of inhibitor (0, 2.0mM, 3.5mM, and 5.0mM).

3. Kinetics of Irreversible Inhibition.

The irreversible inhibition of mandelate racemase was monitored using the spectrophotometric assay.[2]

The kinetic experiments were carried out according to the protocol of Fee et al.[8] with the following modifications. K^+Hepes buffer at pH 7.5 was used in place of the phosphate buffer. The assay was initiated by

the addition of substrate.

Depending upon the preparation of enzyme used in these experiments, there was a significant difference in the kinetics of inactivation. Routinely, the fractions with the highest specific activity eluting from Sephadex G-200 were pooled and loaded onto a DEAE Sephadex A-50 column and collected in the usual manner[2,5]. This preparation was designated as "Prep. I". Prep I excluded fractions eluting later which had lower specific activity, but nonetheless, still significant racemase activities. These later fractions, which were not loaded onto a DEAE Sephadex A-50 column, were designated "Prep. II".

Subsequent kinetic studies with the R- and S-α-phenylglycidate revealed that although the kinetic parameters of the two preparations were nearly identical (similar K_M, V_{max}, and turnover number), there was a markedly different inhibition by the enantiomeric α-phenylglycidates. The enzyme used in the irreversible inhibition experiments was that designated Prep. II.

4. Initial Velocity Studies.

Initial velocity studies were carried out using the circular dichroic (CD) assay of Sharp et al.[7] on the Jasco Circular Dichroic instrument monitoring the change in ellipticity at 262nm. Solutions were made up as previously described.

5. Deuterium Isotope Effects.

Deuterium isotope effects were carried out using the CD assay of Sharp et al.[7] There was no detectable differences in the observed molar ellipticities between the isotopically and non-isotopically labeled mandelate.

Determination of the V/K_M and V_{max} isotope effects was made from the reciprocal plots generated from initial velocity studies using the R or S mandelate. The substrate was labeled in the α position with either hydrogen or deuterium. The ratios of the intercepts from a Lineweaver-Burke plot of the data from separate initial velocity studies using either the hydrogen-containing mandelate or the deuterium-containing mandelate yielded the V_{max} effect while the ratios of the slopes of these same graphs yielded the V/K_M effect.

6. pH Studies.

pH studies were carried out using the CD assay of Sharp et al.[7]

Determination of the V and V/K for the R- and S-mandelate as a function of pH was made by performing initial velocity studies at the indicated pH. Buffers (100mM, made 1.0mM in $MgCl_2$) were used over the following pH ranges Mes, 6.0 - 6.5; Pipes, 6.5 - 7.5; Hepes, 7.5 - 7.8; Taps, 7.8 - 9.0; Ches, 9.0 - 9.5; and Caps, 9.5 - 10.0. All buffers were adjusted to pH with KOH. In all

cases, sufficient overlaps were obtained between buffers either to rule out entirely or to permit minor corrections for buffer effects.

7. Kinetics of R(-)-Mandelate in H_2O and D_2O.

To 2 10 mL volumetric flasks were added 85 µmoles of R(-)-mandelate, 1000 µmoles of Hepes buffer, and 10 µmoles of $MgCl_2$. The contents of each flask were dissolved in either 10 mL of H_2O or D_2O. The solutions were then taken to dryness *in vacuo*. This process was repeated three further times in order to ensure complete exchange of all labile protons. The total volumes were made up to 10 mL by the addition of either H_2O or D_2O. The final pH was adjusted to pH 7.0 with either NaOH or NaOD. Racemase solution in H_2O (0.5 mL containing 60µg of protein, 3.5 units/mL/mg protein) was added making the final D_2O solution 95% D_2O. Aliquots (1.0 mL) were removed from the solution to a polarimeter tube (1 dm) at the recorded intervals and the optical rotation was measured at the D line of sodium using a Perkin-Elmer 141 polarimeter. The aliquots were returned to the volumetric flask after the reading.

8. Kinetics of S(+)-Mandelate in H_2O and D_2O.

To two 2 mL volumetric flasks were added 12 µmoles of S(+)- mandelate, 177 µmoles of Hepes buffer, and 2 µmoles of $MgCl_2$. The contents of each flask were

dissolved in either 2 mL of H_2O or D_2O. The solutions were then taken to dryness *in vacuo*. This process was repeated three further times in order to ensure complete exchange of all labile protons. The total volumes were made up to 2 mL by addition of either H_2O or D_2O. The final pH was adjusted to pH 7.0 with either NaOH or NaOD. Racemase solution in H_2O (0.1 mL containing 60 µg of protein, 3.5 units/mL/mg protein) was added making the final D_2O solution 95% D_2O. The contents of each were immediately transferred to a capped 1 mm CD cell. The change in ellipticity was monitored at 227 nm on a Roussel-Jouan Mark II dichrograph. The chart speed was set at 9 mm/min. the sensitivity was set at 1×10^{-5} optical density units/mm.

9. Equilibrium Perturbation Experiment.

The experimental procedure employs the so-called "two-pot" method as described by Cleland.[10] To 1-10 mL volumetric flask was added 112 µmoles of R(-)-α-[^{1}H]-mandelate, 1000 µmoles of Hepes buffer, 10 µmoles of $MgCl_2$ while to another 10 mL volumetric flask was added 113 µmoles of S(+)-α-[^{2}H]-mandelate, 1000 µmoles of Hepes buffer, and 10 µmoles of $MgCl_2$. The contents were dissolved in 10 mL of H_2O and the pH was adjusted to 7.0 with KOH. A mixture of 0.85mL of the R(-)-α-[^{1}H]-mandelate solution and 0.87 mL of the S(+)-α-[^{2}H]-mandelate

solution resulted in a null at 227nm indicative of the presence of an equilibrium mixture. Racemase solution (0.1 mL containing 60 µg of protein, 3.5 units/mL/mg protein) was added to the mixture to make a final volume of 1.82 mL. The perturbation was monitored at 227 nm with the chart speed set at 9 mm/min and the sensitivity set at 1×10^{-5} optical density units/mm.

E. Data Processing

Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear. The data were fitted to the appropriate equation with the Fortran programs of Cleland.[11,12] All initial velocity studies including the individual saturation curves used to obtain pH profiles and competitive inhibition plots were fitted to equation 1

$$v = VA/K + A \qquad (1)$$

where A is the substrate concentration.

Data for the competitive inhibitor studies were fitted to equation 2

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \qquad (2)$$

where K_{is} is the inhibition constant and I is the concentration of inhibitor.

Data for pH profiles which showed a decrease in log V/K at both low and high pH were fitted to equation 2 where H was $[H^+]$ and c is the pH-independent value of

$$\log y = \log[C/(1 + H/K_1 + K_2/H)] \qquad (3)$$

the parameter y. The parameters K_1 and K_2 represent the dissociation constants for groups on the enzyme.

Because the deuterium isotope effects appeared

equal on V and V/K, the data were fitted to equation 4 where F_i is the fraction of deuterium label and VI is the isotope effect - 1.

$$y = \frac{VA}{(K + A)\,[1 + (F_i)(VI)]} \qquad (4)$$

The data obtained from the irrevesible inhibition experiments were fitted to the best straight line using linear regression analysis.

The isotope effect from the equilibrium perturbation experiment was calculated using equation 5 as derived by Cleland

$$R_{min} = \frac{\alpha}{2-\alpha}(\alpha - 1)^{-2/\alpha - 2} - \frac{2(\alpha - 1)}{2-\alpha}(\alpha - 1)^{-\alpha/\alpha - 2} \qquad (5)$$

where R_{min} is the amount of perturbation observed and α is the isotope effect.[12]

F. Chemical Syntheses

General. All reagents except (+)-ephedrine were purchased from Aldrich Chemical Co. and were used without further purification. (+)-Ephedrine was obtained from Fluka AG Chemische Fabrik, Switzerland. Nuclear magnetic resonanace spectra were determined on either a Varian FT-80 spectrometer or a 240 MHz widebore spectrometer equipped with Nicolet 1180 Data System, Cryomagnet Systems for magnet and probes, and custom built electronics. NMR spectra were expressed on the δ scale in parts per million downfield from an internal tetramethylsilane standard. The α rotation values were measured at the D line of sodium on a Perkin-Elmer 141 Polarimeter in a 1 dm polarimeter tube. The reported concentrations were expressed in g/100ml. Circular dichroic measurements were carried out on a Roussel-Jouan Mark II dichrograph at room temperature and were recorded in molar ellipticity units [θ] (degrees mol^{-1} cm^2). Enantiomers gave essentially ($\pm$ 5%) mirror image curves. Gas Chromatography was performed by Thomas Everhart using a Varian 1200 Gas Chromatograph modified with Grob injector enabling the instrument to accept a fused silica capillary column containing DB 1701 silicone. The column's dimensions were 0.32 mm (i.d.) X 15 m. It was obtained from J & W Scientific Inc., Rancho Cordova, Calif. All melting points were uncorrected. Microanalyses were carried out by the Microanalytical Laboratory,

Department of Chemistry, University of California, Berkeley.

(-)-2-Octyl atropate. Ethyl atropate was synthesized according to the procedure of Ames and Davey.[13] The resulting ester was saponified to tropic acid and recrystallized; m.p. 103-105°(lit. 106-107°)[14] The NMR spectrum showed contaminating ethyl phenylacetate. To 11.2 g (75.7 mmol) was added 76.9 g (591.3 mmol) of (-)-2-octanol. The mixture was heated at reflux for 44 hr. The desired ester distilled over a temperature range of 88-116° at 0.05 mm pressure. The NMR spectrum indicated that the product was contaminated with the octyl and ethyl phenylacetates. No further attempts were made at purification because it was possible to remove the contaminants in the following step in the reaction sequence. NMR ($CDCl_3$) δ: 0.7-1.6 (brd m, 16H, octyl group), 3.55 (s, 2H, CH_2 of octyl and ethyl phenylacetates), 4.65-5.1 (brd m, 1H, CH), 5.8 (brd s, 1H, CH_2), 7.15-7.35 (brd m, 5H, Ph).

(-)-2-Octyl α-phenylglycidate (3 and 4). A solution of 6.7 g (25.8 mmol) of (-)-2-octyl atropate in 50 ml of CH_2Cl_2 was added dropwise to a rapidly stirring solution of 0.7 g (38.7 mmol) of 85% m-chloroperbenzoic acid in 70 ml of CH_2Cl_2 at room temperature over 40 min. The solution was then heated at reflux for 38 hr. The mixture was cooled to room temperature , transferred to a separatory funnel, extracted with $NaHSO_3$ (1 X 100 ml of a 10% aq.

soln.), followed by repeated washings with 10% $NaHCO_3$. The CH_2Cl_2 was dried over $MgSO_4$, filtered, and evaporated to dryness to yield a translucent, brown oil. The oil was dissolved in a mixture of 90% hexanes and 10% ethyl acetate and allowed to stand in a separatory funnel overnight. The mixture then separated into two layers of which the top was collected, dried over $MgSO_4$, filtered, and evaporated to dryness to yield a clear bronze oil.

The oil was made up in 10% (w/w) portions with 90% hexanes, 10% chloroform and injected in 1 ml aliquots onto a silica gel LoBar® Size B column. This low pressure liquid chromatography yielded first the (-)-2-octyl atropate with a retention time of 42-52.4 min, followed by octyl phenylacetate at 52.4-64 min, which, in turn, was followed by the diastereomeric mixture of the (-)-2-octyl α-phenylglycidates as 2 overlapping peaks at 78-112 min. The (-)-2-octyl R-α-phenylglycidate (4) was collected during the first 6 min (78-84 min) while the (-)-2-octyl S-α-phenylglycidate (3) was collected during the last 16 min (96-112 min) The 2 diastereomers were typically collected in round-bottom flasks and evaporated to dryness to yield oils which were made up as 10% (w/w) solutions with 90% hexanes, 10% chloroform and then reinjected onto the LoBar® column. Upon reinjection 4 eluted as a broad peak between 68.1-96.3 min while 3 eluted between 100.5-140.7

min. Typically, the fraction eluting between 68.1-89.7 min was saved as 4 and the fraction eluting between 100.7-140.7 min was saved as 3. The optical purity of the oils was determined by GC analysis. Resolution of the diastereomers was obtained by using the fused silica capillary column starting at 120° and increasing the temperature by 4°/min. Compound 4 consisted of 75 % R,R and 25 % R,S while compound 3 was determined to be 78 % R,S and 22 % R,R. The helium carrier gas flow was maintained at 0.75 bar. Compound (4) eluted at 167.5° while (3) eluted at 168.5°. NMR ($CDCL_3$): δ 0.7-1.7 (brd m, 16H, octyl group), 2.87 (d, 1H, CH_2), 3.32 (d, 1H, CH_2), 4.7-5.1 (brd m, 1H, CH), 7.05-7.6 (brd m, 5H, Ph). (Found: C, 73.97; H, 8.49. $C_{17}H_{24}O_3$ requires: C, 73.87; H, 8.77%). (4): [α] = -16° (3): [α] = -22°. (c = 0.40 ; 0.43, ethanol)

Still higher chemical purity of the octyl esters as determined by NMR analysis can be obtained by distillation of the oil in a micromolecular still. A clear oil distills at 0.1 mm pressure and bath temperature ca. 150°C (Found: C, 73.84; H, 8.84. $C_{17}H_{24}O_3$ requires: C, 73.87; H, 8.77%).

Potassium R(+)-α-phenylglycidate (2) was prepared by the saponification of 0.38 g (1.39 mmol) of 4 in a solution of 0.086 g (1.5mmol) of KOH in 25 ml of 95% ethanol heated at reflux for 1.5 hr. The solution was evaporated to dryness to yield a gummy solid which solidified after being dried under mechanical vacuum for 3 hr. After cry-

stallization from EtOH ether the hygroscopic crystals were collected in a sintered glass funnel and quickly transferred to a tared container and dried under vacuum in a desiccator. The crystals were stored below 0° C. in a desiccator to prevent decomposition (93.4 mg, 33%). The NMR (D_2O) corresponded to that of Fee et al.[8]: δ 3.3 (brd s, 2H, CH_2), 7.45 (brd s, 5H, Ph). [α] = +41° (c = 0.112, water).

Potassium S(-)-α-phenylglycidate (1) was synthesized by the hydrolysis of 0.52 g (1.88 mmol) of 3 in a solution of 0.12 g (2.13 mmol) of KOH in 30 ml of 95% ethanol by heating at reflux for 2 hr. The reaction yielded 85.7mg (22.5%) of product after work-up according to the procedure used for the R(+)-isomer. The crystals were stored in a desiccator below 0° C. The NMR spectrum (D_2O) corresponded to that for the racemic sodium salt of Fee et al.[8] $[\alpha]_D$ = -28° (c = 0.116, water).

Methyl R,S-α-phenylglycidate. A mixture of 0.50 g (2.48 mmol) of the R,S potassium salt synthesized according to Fee et al.[8] and 0.65 g (2.46 mmol) of 18-crown-6 ether was dissolved in 1 ml of water. The solution was evaporated to dryness to yield a crystalline glass. Most of the glass dissolved in 50 ml of tetrahydrofuran (freshly distilled after reflux over $LiAlH_4$), leaving a fine precipitate which dissolved upon the addition of 5.3 g (37.3 mmol) of iodomethane (freshly distilled after reflux

over copper turnings). Immediately after addition, the solution turned yellow, followed by the precipitation of a solid. The reaction mixture was left to stir at room temperature overnight. It was then evaporated to dryness to yield both a solid and an oil. The mixture was dissolved in 90% hexanes, 10% ethyl acetate and filtered to yield an orange solution which was dried over $MgSO_4$, filtered, and evaporated to dryness to yield an oil. The oily residue was subjected to "flash chromatography"[15] (90% hexanes, 10% ethyl acetate) on 50 g of silica gel to yield 0.32 g (71%) of pure ester. NMR:($CDCl_3$) δ 2.87 (d, 1H, CH_2), 3.37 (d, 1H, CH_2), 3.75 (s, 3H, CH_3), 7.2-7.55 (brd m, 5H, Ph). (Found: C, 67.58; H, 5.76. $C_{10}H_{10}O_3$ requires: C, 67.40; H, 5.67%).

Methyl R-α-phenylglycidate (6) was synthesized from 2 exactly according to the procedure used to prepare the racemic mixture. The NMR spectrum ($CDCl_3$) revealed approximately 15% impurity in the δ 1.05-1.5 region. No further attempt was made at purification. The reaction yielded 24.6 mg (21%). An α value was not measurable.

Methyl S-α-phenylglycidate (5) was synthesized from 1 according to the procedure used to prepare the racemic mixture. The reaction yielded 45.3 mg (60%) of a clear oil after flash chromatography. An α value was not measurable. Consequently, in order to ensure that significant racemization had not occurred during the reaction, 22.5 mg (0.13

mmol) of 5 was heated at reflux for 1.5 h in an ethanolic solution of 5.8 mg (0.15 mmol) of KOH. After rotary evaporation of the solution, crystals were obtained. $[\alpha]_D$ = -27° (c = 0.23 , water) (starting material $[\alpha]_D$ = -28°).

Methyl R(+) 2,3-dihydroxy-2-phenyl propanoate (15). Ethyl atropate was prepared according to Ames and Davey[13] and then flash chromatographed (90% hexanes, 10% ethyl acetate) on 50 g of silica gel in 1.0 g portions to remove the ethyl phenylacetate. NMR ($CDCl_3$): δ 1.2 (t, 3H, CH_3), 2.87 (d, 1H, CH_2), 3.32 (d, 1H, CH_2), 4.2 (q, 2H, CH_2), 7.2-7.5 (brd m, 5H, Ph). The pure oil was treated with m-chloroperbenzoic acid as described above and saponified to yield potassium α-phenylglycidate which was, in turn, hydrolyzed to yield potassium α-phenylglycerate. NMR (D_2O): δ 4.05 (q, 2H, CH_2), 7.25-7.6 (brd m, 5H, Ph). Potassium phenylglycerate (1.8 g 8.2 mmol) was dissolved in 9 ml of water, acidified to pH 1 with conc. HCl, extracted with ether (8 X 15 ml), dried over $MgSO_4$, filtered, and evaporated to dryness to yield racemic α-phenylglyceric acid, m.p. 146-148°(lit. 148-150°)[16]. The free acid (1.3 g, 7.14 mmol) and (-)-ephedrine (1.3 g, -7.87 mmol) were dissolved in 10 ml of 100% ethanol. After 3 crystallizations, 0.3 g of the (-)-ephedrine (+)-α-phenylglycerate salt was recovered by filtration, m.p. 96-97°. The salt was dissolved in water, acidified with conc.

HCl, and extracted with ether to yield 122 mg of the (+)-acid (7) (9.4% yield). $[\alpha]_D$ = + 22° (c = 0.25 , ethanol)

Diazomethane was generated from Diazald®, collected as an ethereal solution, and added in portions to a chilled solution of 122 mg of phenylglyceric acid in 25 ml of ether until the yellow color persisted. The reaction mixture was left to stir at room temperature overnight. Then it was extracted with saturated $NaHCO_3$ (1X100 ml), dried over $MgSO_4$, filtered, and evaporated to dryness to yield an oil. The oily residue was then flash chromatographed (75% hexanes, 25% ethyl acetate) on 20 g of silica gel to yield 56 mg (43%) of white crystals, m.p. 63-65°. NMR ($CDCl_3$): δ 2.55-3.0 (brd s, 1H, OH), 3.63 (s, 1H, OH), 3.75 (s, 3H, CH_3), 4.05-4.3 (brd s, 2H, CH_2), 7.2-7.6 (brd m, 5H, Ph). (Found: C, 61.41; H, 6.08. $C_{10}H_{12}O_4$ requires: C, 61.22; H, 6.18%). $[\alpha]_D$ = + 14° (c = 0.40 , ethanol).

Methyl S(-)-2,3-dihydroxy-2-phenylpropanoate (9). S(-)-α-phenylglyceric acid (16) was obtained from the racemic acid after three crystallizations with (+)-ephedrine (m.p. (+)-ephedrine (-)-α-phenylglycerate 96-102°).The free (-)-acid of the salt formed [83.6 mg, 0.46 mmol [α] = - 22° (c = 0.29 , ethanol)] was esterified with diazomethane and flash chromatographed (75% hexanes, 25% ethyl acetate) on 25 g of silica gel to yield 77.4 mg (86%) of white crystals, m.p. 61-63°. The NMR spectrum ($CDCl_3$) was identical to that of the R(+) isomer. (Found: C, 61.13; H, 6.15.

$C_{10}H_{12}O_4$ requires C, 61.22; H, 6.18%). [α] = - 12° (c = 0.38 , ethanol).

R,S-1,2-Dihydroxy-2-phenylpropane. To a chilled solution of 146.6 mg (3.86 mmol) of $LiAlH_4$ in 200 ml of tetrahydrofuran (freshly distilled after reflux over $LiAlH_4$) was added a solution of 266.1 mg (0.96 mmol) of (-)-2-octyl R,S α-phenylglycidate in 30 ml of the tetrahydrofuran over a 20 minute period. The solution was heated at reflux for 4 hr. The reaction vessel was then immersed in an ice bath, and the excess $LiAlH_4$ was destroyed by the addition of 0.15 ml water, 0.15 ml of a 15% aq. NaOH solution, and 0.45 ml water from a syringe. The solution was filtered and evaporated to dryness to yield an oily residue. The residue was flash chromatographed (50% hexanes, 50% ethyl acetate) on 50 g of silica gel to yield 63.8 mg (44%) of an oil which solidified upon standing. NMR ($CDCl_3$): δ 1.4 (s, 3H, CH_3), 2.5-3.0 (brd s, 1H, OH, collapses upon the addition of 1 drop of D_2O), 3.1-3.7 (brd s, 3H, CH_2OH), 7.1-7.4 (brd m, 5H, Ph). (Found: C, 70.73; H, 7.82. $C_9H_{12}O_2$ requires: C, 71.02; H, 7.96%).

S(+)-1,2-dihydroxy-2-phenylpropane was prepared by reducing 4 resulting in 77.7 mg (56%) of product after flash chromatography. The NMR spectrum ($CDCl_3$) was similar to that of the racemic mixture except that there were some impurities in the δ 1.85-2.2 region. Two rotations mea-

sured in two different solvents showed the compound to be dextrorotatory corresponding to the S configuration as determined by Eliel and Freeman[17] and by Mitsui and Imaizumi[18]. $[\alpha]_D$ = 3.0° (c = 1.55 , ether) (Lit. $[\alpha]_D$ = 8.42 , c = 6.8)[17]; $[\alpha]_D$ = 3.25° (c = 1.24 , ethanol) (Lit. $[\alpha]_D$ = 5.4, c = 8.9)[18].

R(-)-1,2-dihydroxy-2-phenylpropane (13) was prepared by reducing 3 to yield 33.2 mg (25%) of 13 after flash chromatography. The NMR spectrum ($CDCl_3$) was similar to that for the racemic mixture with a small amount of impurity in the δ 2.4 region. $[\alpha]_D$ = -4° (c = 0.66 , ether).

Fotassium R(+)-2,3-dihydroxy-2-phenyl propanoate. (17) was prepared by the base hydrolysis of 4. Typically, 0.51 g (1.85 mmol) of 4 was hydrolyzed in a solution of 0.21 g (3.74 mmol) of KOH in 10ml of 95% ethanol heated at reflux for 1.5 hrs. After cooling to room temperature, the solution was evaporated to dryness to yield a solid. Recrystallization from ether/EtOH yielded 0.05 g (14%) of compound 17. The NMR spectrum (D_2O) was identical to that reported for the R,S mixture used in the preparation of 15. $[\alpha]_D$ = 26° (c = 0.1 , water).

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