Smithies, O. (1959), *Biochem. J. 71*, 585.
Stanier, R. Y., Gunsalus, I. C., and Gunsalus, C. F. (1953), *J. Bacteriol. 66*, 543.
Stanier, R. Y., Palleroni, N. J., and Doudoroff, M. (1966), *J. Gen. Microbiol. 43*, 159.
Streitwieser, Jr., A., and Koch, H. F. (1964), *J. Amer. Chem. Soc. 86*, 404.
Streitwieser, Jr., A., and Pudjaatmaka, A. H. (1965), reported by Streitwieser, Jr., A., and Hammons, J. H., *Progr. Phys. Org. Chem. 3*, 41.
Weichselbaum, T. E. (1946), *Amer. J. Clin. Pathol. 16*, 40.
Weil-Malherbe, H. (1966), *Biochem. J. 101*, 169.

Mandelic Acid Racemase from *Pseudomonas putida*. Evidence Favoring a Carbanion Intermediate in the Mechanism of Action*

George L. Kenyon and George D. Hegeman

ABSTRACT: Substantial deuterium exchange occurs in the α-carbon position of mandelic acid during the racemization of unlabeled D-(−)-mandelic acid by mandelic acid racemase (EC 5.1.2.2) in 96% D_2O, or when the racemization of D-(−)-α-deuteriomandelic acid is catalyzed by the enzyme in H_2O. Under similar conditions no significant amount of ^{18}O was found to be incorporated into the mandelic acid when D-(−)-mandelic acid was enzymatically racemized in ^{18}O-enriched water. Benzoylformic acid was excluded as a reaction intermediate since, in the presence of the enzyme, [^{14}C]benzoylformic acid did not give rise to [^{14}C]mandelic acid and [^{14}C]mandelic acid did not yield [^{14}C]benzoylformic acid.

The enzyme catalyzed with approximately equal efficiency the incorporation of radioactivity into the D-(−) and L-(+) enantiomers during partial racemization of the D-(−)-mandelic acid in tritiated water. The V_{max} for the enzyme-catalyzed conversion of D-(−)-α-deuteriomandelic acid to the L-(+) enantiomer was approximately five times less than that observed for the normal protonated substrate. A mechanism involving a carbanion intermediate is consistent with these and other findings.

The apparent lack of pyridine nucleotide or flavin cofactors necessary for activity of mandelic acid racemase and the increase in rate accompanying substitution of the substrate in the 4 position by electron-withdrawing substituents (Hegeman *et al.*, 1970) suggest the occurrence of certain possible intermediates in the course of racemization mediated by the enzyme. In order to determine which intermediate or intermediates may be involved in the racemization process we have used various isotopes to investigate the possible occurrence of enzyme-catalyzed exchange between (1) the substrate and the solvent and (2) the substrate and the potential keto intermediate, benzoylformic acid.

Materials and Methods

Analytical and preparative gas–liquid chromatography was performed with an F&M Model 700 instrument using 0.125 or 0.25 in. × 6 ft columns packed with 10% diethylene glycol succinate on Chromosorb W (Applied Sciences Laboratories, Inc.). The chromatograph was fitted with a 1:1 stream splitter and flame ionization detector. Helium was employed as carrier gas (flow rate 40–60 ml/min) and the oven temperature was maintained at 150°. Samples were collected using glass U-tubes cooled in a Dry Ice–acetone bath.

Peak areas were integrated by weighing peaks excised from xerographic copies of the original chromatographic tracings.

Radioactivity was determined using a Nuclear Chicago, Inc. Model Mark I scintillation spectrometer. A toluene scintillation fluid containing 15.1 g of 2,5-diphenyloxazole and 0.189 g of *p*-bis[2-(5-phenyloxazolyl)]benzene per 3.78 l. of toluene was used.

A Varian Instruments Model T-60 spectrometer was used to determine nmr spectra.

Consolidated Electrodynamics Corp. Model 21-110B double-focusing high resolution mass spectrometer was used to determine mass spectra and make mass measurements.

D-(−)-Mandelic acid, DL-mandelic acid, and benzoylformic acid were obtained from the Aldrich Chemical Co. Diazomethane was generated using Diazald from the same company.

DL-[^{14}C]Mandelic acid, labeled in the carboxylate position, was prepared from benzaldehyde and [^{14}C]NaCN by the procedure of Fieser (1957) except that the sodium cyanide was used as the limiting reagent. Also, the crystals of the sodium bisulfite addition complex with benzaldehyde were isolated and purified by filtering and washing with dilute $NaHSO_3$ solution. The DL-[^{14}C]mandelic acid was purified

* From the Departments of Chemistry, and Bacteriology and Immunology, University of California, Berkeley, California 94720. *Received May 19, 1970.* This work was supported by U. S. Public Health Service Grants AM-13529 from the National Institute of Arthritis and Metabolic Diseases and HD-02448 from the National Institute of Child Health and Human Development. Experiments entailing use of the mass spectrometer were partially supported by NSF Grant GP-5323. This is the second publication of a series, the first of which also appears in this volume (Hegeman *et al.*, 1970).

by microsublimation and had a specific activity of 5.07 mCi per mmole.

D-(−)-[^{14}C]Mandelic acid and [^{14}C]benzoylformic acid, both labeled in the carboxylate position, were prepared simultaneously by subjecting the DL-[^{14}C]mandelic acid prepared above (20 mg) to the action of the racemase-free preparation of L-(+)-mandelic acid dehydrogenase (Hegeman *et al.*, 1970). A 10-mg portion of the product mixture was methylated with diazomethane in ether, and the ester products were separated and purified twice by preparative gas–liquid chromatography. The potassium salts of both the [^{14}C]-benzoylformic acid and the D-(−)-[^{14}C]mandelic acid were each prepared by saponifying solutions of the purified methyl esters in 1.5 ml of 2 N KOH for 18 hr at room temperature. These solutions were stored at 4° until used in the experiments with the enzyme.

DL-α-Deuteriomandelic acid was prepared as follows. Ethyl benzoylformate (3.6 g, 20 mmoles, Eastman Organic Chemicals) was dissolved in 20 ml of diethylene glycol dimethyl ether which had been distilled from $LiAlH_4$ and stored over molecular sieves. $NaBD_4$ (1.0 g, 28 mmoles, Merck, Sharp and Dohme, Ltd.) was added and the slurry was stirred overnight at room temperature. After treatment with 5 ml of D_2O (Bio-Rad Laboratories, 99.88 atom % deuterium), the solvents were removed *in vacuo*. A small amount of D_2O was added, and the crude ethyl α-deuteriomandelate was extracted with three 10-ml portions of ether. The ether extracts were dried over anhydrous Na_2SO_4. After filtering and removing the ether, the ester, which according to the nmr spectrum was contaminated with some unreacted ethyl benzoylformate, was saponified by the addition of 20 ml of D_2O and 2.0 g of anhydrous K_2CO_3 and refluxing for 1 hr.[1] After acidification (pH 2) with dilute HCl solution, the product was extracted with three 10-ml portions of ether. After drying the ether extracts over anhydrous Na_2SO_4, the solution was filtered, and the ether removed on a steam bath. The product was recrystallized from benzene to yield 450 mg, mp 116–118°; lit. (Fieser, 1957) 118.5°. The ultraviolet spectrum (H_2O, pH 7.0) was superimposable on that of authentic DL-mandelic acid. The nmr spectrum (D_2O) showed a small singlet at δ 5.10 and a large multiplet at δ 7.20. By integration of this spectrum the deuterium content in the α position was determined to be 79 ± 5%. A deuterium analysis[2] gave a value of 80.00%.

D-(−)-α-Deuteriomandelic acid was prepared from DL-α-deuteriomandelic acid by the enzyme-catalyzed stereospecific oxidation of the L-(+) enantiomer to benzoylformic acid. Thus 95 mg of the DL-α-deuteriomandelic acid was treated with the racemase-free preparation of L-(+)-mandelic acid dehydrogenase in 10 ml of 0.1 M phosphate buffer (pH 6.8) for 4 hr at 37°. The reaction was stopped by removal of the enzyme in the ultracentrifuge (105,000*g* for 60 min) followed by addition of concentrated HCl to pH 2, and the products were extracted with five 10-ml portions of ether. The ether extracts were combined, dried over anhydrous $MgSO_4$, and taken to dryness by a stream of dry N_2 to leave 76 mg of a mixture of the D-(−)-α-deuteriomandelic acid and benzoylformic acid. This mixture was then purified by microsublimation, first at 80° (45 mm) and then at 110° (47 mm). The former conditions gave a small amount of material rich in benzoylformic acid and which was discarded. The latter conditions gave 25 mg of product. A 4-mg portion of this material was converted into the mixture of methyl esters by reaction with diazomethane in ether. After removal of the ether, the product was dissolved in $CHCl_3$ and subjected to gas–liquid chromatography. The analysis indicated that the mixture was 56% methyl D-(−)-α-deuteriomandelate and 44% methyl benzoylformate.

The remaining 20 mg of the mixture of the two free acids was neutralized with 0.065 ml of 2 N KOH, 0.2 ml of 1 M phosphate buffer was added, and the solution was diluted to a final volume of 2.0 ml (pH 6.8) for use in the enzyme studies. An enzymatic assay procedure using the racemase-free preparation of L-(+)-mandelic acid dehydrogenase confirmed the complete absence of the L-(+) enantiomer in the product mixture.

Experimental Procedures and Results

Comparison of the Racemase-Catalyzed Rates of Conversion of D-(−)-α-*Deuteriomandelic Acid and* D-(−)-*Mandelic Acid to the* L-(+) *Enantiomer.* D-(−)-Mandelic acid and D-(−)-α-deuteriomandelic acid were directly compared as substrates for mandelic acid racemase by use of the standard L-(+)-mandelic acid dehydrogenase coupled assay (Hegeman *et al.*, 1970). Rates were measured at several substrate concentrations (10- and 30-fold greater than the K_m of the enzyme) and at several enzyme concentrations. There is some substrate inhibition by mandelic acid, so that the averages of several determinations at equivalent enzyme and substrate concentrations were compared with each other. At 1×10^{-3} M substrate concentration the $V(H)/V(D)$ was calculated to be 5.1; at 3×10^{-3} M a value of $V(H)/V(D)$ of 5.6 was obtained. It should be noted that within experimental error the K_m for D-(−)-α-deuteriomandelic acid was the same as that for D-(−)-mandelic acid.

Base-Catalyzed Exchange of Deuterium into DL-*Mandelic Acid.* DL-Mandelic acid (0.05 g), previously exchanged several times in D_2O, was dissolved in 1.0 ml of 2 M KOD solution in D_2O. The solution was sealed in a standard nuclear magnetic resonance tube (0.5 × 19 cm) and was heated at 100° for several hours. At regular intervals the tube was cooled and the nuclear magnetic resonance spectrum taken. After 1.5 hr, the α-hydrogen had exchanged to the extent of 50 ± 5% as determined by electronic integration using the aromatic hydrogens as the internal standard. Under these conditions the total area under the HOD peak and the α-hydrogen peak remained a constant ratio of the aromatic hydrogens.

Racemase-Catalyzed Partial Racemization of D-(−)-*Mandelic Acid in the Presence of Tritiated* H_2O. D-(−)-Mandelic acid (15.2 mg) was dissolved in 9.6 ml of H_2O to which 0.1 ml of 1 M phosphate buffer (pH 6.8) and 0.2 ml of tritium-enriched H_2O (Bio-Rad Laboratories, specific activity 25 mCi/ml) had been added. Racemase solution (0.1 ml, 8.6 units/ml) was added and the racemization was allowed to proceed for 15 min at room temperature. After addition of concentrated HCl to pH 2 to stop the reaction, the solution

[1] Under these conditions no detectable amount of deuterium was incorporated into the benzene ring of mandelic acid as determined by nmr spectroscopy.

[2] Deuterium analyses were performed by Mr. Josef Nemeth, Urbana, Ill. 61801.

TABLE I: Relative Extents of Incorporation of Radioactivity from Tritiated Water into D-(−)- and L-(+)-Mandelate during Partial Racemization of D-(−)-Mandelate Catalyzed by Mandelate Racemase.

Sample No.	Amount of Methyl Ester Recovered (μg)[a]		Radioactivity in Methyl Mandelate[a] Fraction (cpm)	% Conversion Benzoylformate × 100/ (Benzoylformate + Mandelate)	Specific Activity of Methyl Mandelate (cpm/μg)	Specific Activity (%)
	Benzoylformate	Mandelate				
I	0	685	1550	0	2.26 (D,L)	(100) (D,L)
II	146	389	517	27.2	1.33 (D)	58.8 (D)
III	116	370	480	23.9	1.30 (D)	57.6 (D)

[a] The average of two or more determinations (±5%). These values are corrected for cross-contamination in the chromatographic separations (see footnote to Table III). Samples of 5 μl were injected.

was extracted with three 5-ml portions of ether. The combined ether layers were dried over anhydrous $MgSO_4$ and the ether was removed under a stream of N_2. The mandelic acid recovered was then exhaustively exchanged with several portions of H_2O which were removed *in vacuo*. The exchanged mandelic acid (now tritium labeled only on the α-carbon position) was then dissolved in 15 ml of dry ether. A 5-ml portion (sample I) was treated with excess diazomethane, the excess diazomethane and ether were removed, and the sample was dissolved in a known amount of $CHCl_3$ and stored at 4° until analyzed. The ether was removed from the 10-ml portion of mandelic acid remaining, and the L-(+) enantiomer was selectively oxidized to benzoylformic acid using an excess of a racemase-free preparation of L-(+)-mandelic acid dehydrogenase in 10 ml of 0.1 M phosphate buffer (pH 6.8). After 30 min of incubation with shaking at 30°, a 5-ml portion of this reaction mixture was removed and stored at 0° (sample II). The remaining 5-ml portion was incubated for an additional 30 min (sample III). The tubes containing samples II and III were then subjected to ultracentrifugation (105,000*g*, 45 min) to remove the enzyme. The supernatant fluids from the 30- and 60-min oxidations were separately acidified with 0.5 ml of concentrated HCl and each was extracted three times with 5-ml portions of ether. The ether layers were dried over anhydrous $MgSO_4$ and treated with excess diazomethane. The excess diazomethane and ether were removed in a stream of dry N_2. The methyl esters of the mandelic acid and benzoylformic acid that remained after 30 min of specific oxidation (sample II) and 60 min of specific oxidation (sample III) were dissolved in a known amount of $CHCl_3$ and, together with sample I, simultaneously analyzed and purified by gas–liquid chromatography. Integration of the methyl mandelate peak from sample I and the methyl benzoylformate and methyl mandelate peaks in samples II and III gave the relative amounts of these materials recovered by the extraction procedure. The amounts of radioactivity in the methyl mandelate eluents, collected by means of a stream splitter fitted to the gas chromatograph, were measured in a liquid scintillation counter.

The amounts of methyl mandelate and methyl benzoylformate recovered and the specific activity of the mandelate both before and after the enzymatic oxidation of the L-(+) isomer to benzoylformate are presented in Table I. The results show that after 25.5% conversion into the L-(+) enantiomer the D-(−)-mandelic acid remaining had 58% of the specific activity of the partially racemized DL mixture. This is somewhat lower than the value calculated for the indiscriminate labeling of the D-(−) and L-(+) enantiomers (67%).[3] Considering the possible margin of error in the determinations of the areas beneath the methyl mandelate and methyl benzoylformate peaks in the gas–liquid chromatographic tracings, the percentage of specific activity of the D-(−)-mandelic acid could be substantially higher. Alternatively, of course, the D-(−) and L-(+) enantiomers exchanged at somewhat different rates (see Discussion).

Racemase-Catalyzed Exchange of Deuterium into Mandelic Acid. D-(−)-Mandelic acid (152 mg) was dissolved in 50 ml of 0.1 M phosphate buffer (pH 6.8). This solution was taken to dryness *in vacuo* and exhaustively exchanged with portions of D_2O. The total volume of the solution was then made up to 50 ml by the addition of more D_2O. Racemase solution in H_2O (0.17 ml containing 50 μg of protein, 9.5 units) was added, which made the water in the final solution 96% D_2O. Change in optical rotation was followed polarimetrically (Hegeman *et al.*, 1970) as a function of time. After the last reading had been taken, the solution was acidified and the mandelic acid was extracted with three 30-ml portions of ether. After removing the ether, the sample was repeatedly dissolved in water and the water removed under reduced pressure to remove readily exchangeable deuterium. The acid was then purified by vacuum microsublimation and a

[3] Assuming indiscriminate labeling had occurred, then 50% of the counts per minute would have been contributed by the D-(−) enantiomer and 50% by the L-(+) enantiomer in the partially racemized DL mixture. Therefore, after 25.5% conversion into the L-(+) enantiomer (the average of 27.2 and 23.9 from Table I) the 74.5% of the partially racemized mandelic acid which is the D-(−) enantiomer would have had 50% of the total counts per minute. The expected per cent specific activity contribution of the pure D-(−) enantiomer is thus 0.5 of the total cpm/0.745 of the total mandelic acid × 100 = 67% of that of the partially racemized DL mixture. In fact the results indicate that the D-(−) enantiomer contributed 44% of the counts per minute to the partially racemized mixture whereas the L-(+) enantiomer contributed 56%.

TABLE II: Racemase-Catalyzed Exchange of the α-Hydrogen of Mandelic Acid with the Medium.[a]

Substrate	Deuterium Initially in α Position in the Substrate (%)	Solvent for Reaction	Enzyme Concn (μg/ml)	Reaction Time (hr)	Racemization (%)	Deuterium Finally in α Position in the Substrate (%)	Exchange in α Position (%)
D-(−)-Mandelic acid	0	96% D_2O	1.0 (0.19 unit)	7.0	82	29.23	29
D-(−)-α-Deuteriomandelic acid	80.00	H_2O	3.0 (0.57 unit)	7.5	70	74.82	5.2[b]
DL-α-Deuteriomandelic acid	78.08	H_2O	3.0 (0.57 unit) (inactivated by boiling)	7.5		77.92	0.2[c]

[a] Reactions were carried out at 30° in 0.1 M phosphate buffer (pH 6.8) containing 10^{-3} M $MgCl_2$ and 152 mg of substrate in a final volume of 50 ml. A drop of $CHCl_3$ was added as a preservative. Reaction time was measured from the time of addition of the enzyme until the reaction was halted by the addition of concentrated HCl prior to extraction. [b] Because of the deuterium isotope effect, unlabeled mandelate reacts much more rapidly than the labeled compound. Making the simplifying assumption that the initial 20% racemization was unaccompanied by exchange since it involved only the unlabeled compound, only 70 − 20 = 50% racemization was accompanied by 80 − 74.8 = 5.2% exchange. [c] This amount of exchange is considered insignificant. In the absence of active mandelic acid racemase, exchange of the α-hydrogen with the medium occurs only under extreme conditions.

30-mg portion was submitted for deuterium analysis.[2] The result is shown in Table II. The nmr spectrum revealed that only the α proton had exchanged.

Racemase-Catalyzed Loss of Deuterium from D-(−)-α-*Deuteriomandelic Acid.* The reaction mixture contained in a total volume of 50 ml of 0.1 M phosphate buffer (pH 6.8) 152 mg of D-(−)-α-deuteriomandelic acid (80.00% deuterated in the α position) and 0.5 ml of racemase solution (0.3 mg of protein/ml; 190 units/mg of protein). Change in optical rotation was followed polarimetrically. After isolation the mandelic acid was submitted for deuterium analysis and the amount of deuterium exchange in the α position was determined by nuclear magnetic resonance spectroscopy.

In a control experiment DL-α-deuteriomandelic acid (78.08% deuterium in the α position) was incubated for 7.5 hr at 30° under similar conditions to those described above with racemase which had been rendered inactive by boiling. After extraction and isolation in the usual manner a deuterium analysis was again performed and the nuclear magnetic resonance spectrum taken. The results of this and the previous exchange experiment are shown in Table II.

That it was solely the hydrogen on the α-carbon which exchanged was confirmed in a separate experiment in which L-(+)-mandelic acid was racemized by the enzyme under the same conditions as the preceding reaction conditions, except that the reaction was run for 24 hr in 90% D_2O. The extracted, purified sample gave a deuterium analysis consistent with 74.3% deuterium in the α position. By nuclear magnetic resonance spectroscopy the extent of exchange in the α position was determined to be 74 ± 5% deuterium (82% exchange).

If we assume that the D-(−) and L-(+) enantiomers become labeled with deuterium (or tritium) with equal efficiency throughout the racemization, then potential differences in the specific rotations of unlabeled and deuterated substrate need not be considered in using polarimetry to measure the extent of racemization. Using this assumption we may calculate that the deuterium-exchange experiment in 96% D_2O (Table II) gave 29% deuterium exchange with 82% racemization. Similarly, the experiment with deuterated substrate in H_2O indicated that 5.2% deuterium exchange had occurred with 70% racemization (Table II). Even if the D-(−) and L-(+) enantiomers acquire the deuterium label at somewhat different rates, the above values are probably good estimates of the relative rates of exchange and racemization since the pure enantiomers of the α-deuteriomandelic acids are not likely to have specific rotations which are very different from the corresponding unlabeled mandelic acids.

Racemization of D-(−)-*Mandelic Acid in* ^{18}O-*Enriched* H_2O. D-(−)-Mandelic acid (152 mg) was dissolved in 9 ml of H_2O and neutralized with KOH solution to pH 7.0. The total volume was then adjusted to 10.0 ml. Into each of two tubes was placed 1.32 ml of this stock solution along with 25 μl of 1.0 M phosphate buffer (pH 7.0). The final pH of each solution was 7.0. The water was removed from these tubes by lyophilization and 0.25 g of ^{18}O-enriched H_2O (Bio-Rad Laboratories, 60 atom % ^{18}O, 13.2 atom % deuterium) was added to each. To one tube was added 25 μl of racemase (0.3 mg of enzyme/ml, 36 units of activity per ml) and to the other was added 25 μl of racemase which had been rendered inactive by boiling. The water in the solutions in each case was therefore 55 atom % ^{18}O. A drop of chloroform, previously shown to be harmless to the enzyme, was added to each tube as a preservative. The tubes were incubated at 31° for 25 hr. The reactions were stopped by the addition of 20 μl of concentrated HCl and the mandelic acid was recov-

TABLE III: Lack of Racemase-Catalyzed ^{14}C Exchange between Benzoylformic Acid and Mandelic Acid.

Expt No.	^{14}C-Labeled Acid Added	Unlabeled Acid Added	Radioactivity in Recovered Methyl Esters (cpm)		Cpm Recovered in Unlabeled Acid (%)	Net Exchange (%)
			Benzoylformate	Mandelate		
1a	Benzoylformate	D-(−)-Mandelate	49,800	3,150	6.33	+2.27
b (control, boiled enzyme)	Benzoylformate	D-(−)-Mandelate	49,800	2,020	4.06	
2a	D-(−)-Mandelate	Benzoylformate	26,900	983,000	2.74	−0.31
b (control, boiled enzyme)	D-(−)-Mandelate	Benzoylformate	30,700	1,265,800	2.43	
1a[a]	Benzoylformate	D-(−)-Mandelate	56,828	2,369	4.17	+0.55
b (control, boiled enzyme)	Benzoylformate	D-(−)-Mandelate	62,296	2,253	3.62	

[a] This experiment is a replica of expt 1 in which the order of sample injection was reversed during gas–liquid chromatographic analysis. This experiment showed that the chromatographic separation is not perfectly complete.

ered from the reaction mixtures by extraction with three 5-ml portions of ether. In each case the yield of mandelic acid recovered after removal of the ether was nearly quantitative. A portion of each sample was analyzed using the double-focusing, high-resolution mass spectrometer. The two samples, which displayed large parent ion peaks,[4] showed no detectable difference in the ratio of the parent ion to the parent ion +2 peak (*m/e* 154). In each case the intensity of the peak at *m/e* 154 was 4.5% of the intensity of the peak at *m/e* 152.[5] The peaks at *m/e* 156 and *m/e* 158 were negligibly small. Moreover, inspection of the base peak in the spectrum at *m/e* 107, presumably arising from the $[C_6H_5CHOH]^+$ ion,[6] and the peak at *m/e* 109 showed that they also had a constant ratio in the two samples. In both cases the intensity of the peak at *m/e* 109 was 2.1% of that of the peak at *m/e* 107.[5] In another experiment the mass spectrum of DL-mandelic acid which had not been dissolved in ^{18}O-enriched water was measured. The spectrum was identical with the spectra obtained from the other two samples, except that the peak at *m/e* 154 had an intensity of 2.0% of that of the parent ion and the peak at *m/e* 109 had an intensity of 1.3% of that of the peak at *m/e* 107. Thus the peaks in both samples at *m/e* 154 were slightly enriched when compared with mandelic acid which had never been dissolved in the ^{18}O-enriched H_2O, but this was not unexpected and, since it was observed even in the presence of inactivated enzyme, is not related to the racemase mechanism. The small observed increase in intensity of the *m/e* 109 peak in those samples exposed to ^{18}O-enriched water with both active and inactive enzyme is within experimental error and is probably not significant.

Lack of Racemase-Catalyzed ^{14}C Exchange between Benzoylformic Acid and Mandelic Acid. Into each of four numbered vessels was placed 0.5 ml of mandelic acid racemase solution (3.33 units of activity per ml). The solutions in vessels 2 and 3 were boiled for 10 min to inactivate the enzyme. Phosphate buffer (0.1 ml, 1.0 M) was added to each to give solutions buffered at pH 6.8. Toluene (0.1 ml) was also added to each as a preservative. Into vessels 1 and 2 were each placed 1.0 ml (0.036 μmole) of potassium [^{14}C]benzoylformate (specific activity 5 mCi/mmole) and 0.1 ml of 0.1 M potassium D-(−)-mandelic acid. Into vessels 3 and 4 were each placed 1.0 ml (0.735 μmole) of potassium D-(−)-[^{14}C]-mandelate (specific activity 5 mCi/mmole) and 0.1 ml of 0.1 M potassium benzoylformate. All four tubes were incubated at 37° for 16 hr and then 0.5 ml of fresh racemase solution (3.33 units per ml; boiled enzyme again placed in vessels 2 and 3) was added. The tubes were then incubated at 37° for an additional 8 hr. The reactions were stopped by the addition of concentrated HCl to pH 2, and each of the solutions was extracted with three 5-ml portions of ether. The ether layers were separated and, after drying over anhydrous $MgSO_4$, the solutions were treated with freshly prepared diazomethane. The ether and excess diazomethane were removed by a stream of dry N_2, and the solutions were taken up in a 50:50 mixture of methyl benzoylformate and methyl mandelate (1 mg) in 25 μl of $CHCl_3$. In each case the esters were separated by preparative gas–liquid chromatography and the radioactivity was determined in the scintillation counter. The results (Table III) show that although the separation of the methyl esters of mandelic acid and benzoylformic acid on gas–liquid chromatography was good, there was always some cross-contamination

[4] Accurate mass measurement of the parent ion confirmed its composition. Calcd for $C_8H_8O_3$, 152.0473; found, 152.0469.

[5] A variety of inlet conditions did not alter these ratios significantly.

[6] This formula was confirmed by accurate mass measurement. Calcd for C_7H_7O, 107.0497; found, 107.0497.

presumably due to trailing of peaks on the column. Accordingly, several experiments were performed to test for possible racemase catalysis of the ^{14}C exchange. In each case the tube containing boiled, inactivated racemase served as an important control. The order of sample injection into the gas–liquid chromatograph was found to be a factor, substantiating the idea that trailing of peaks was occurring. For example, when ^{14}C-labeled benzoylformic acid was incubated with unlabeled mandelic acid in the presence of the enzyme or boiled enzyme, the net exchange was found to be 2.3% if the product isolated from the reaction with active racemase was injected first but only 0.55% if the product from the control reaction was injected first. This indicates that these small differences in the observed exchange in the presence or absence of the active enzyme are not significant. Also, when [^{14}C]mandelic acid was incubated with unlabeled benzoylformic acid, the boiled enzyme control showed a small (0.31%) apparent *enhancement* of labeling of the benzoylformic acid compared with the same reaction in which active enzyme was used. Within the experimental error and considering the prolonged time of incubation and large amount of enzyme used, the enzyme does not catalyze the exchange.

Discussion

That the bond between the α-carbon of the mandelic acid and the hydrogen on that carbon is broken in the transition state of the racemase-catalyzed racemization is shown by the demonstration that the V_{max} for the conversion of D-(−)-α-deuteriomandelic acid to the L-(+) enantiomer is five times slower than the V_{max} for the corresponding conversion of unlabeled D-(−)-mandelic acid. A deuterium isotope effect of this magnitude is too large to be a secondary effect or even several accumulating secondary effects (Melander, 1960). In fact it compares favorably with known primary isotope effects. Some of the ambiguities and difficulties in interpreting deuterium isotope effects in enzyme reactions (Jencks, 1969) do not apply to our observations since the deuterium substitution affected the V_{max} while not affecting the K_m and since the hydrogen on the α-carbon of mandelic acid does not exchange with the solvent in the absence of the enzyme, except under extreme conditions.

Substantial deuterium exchange with the aqueous medium was observed to accompany racemization in the racemase reaction. Exchange occurred both when D-(−)-mandelic acid was racemized with the enzyme in 96% D_2O and when D-(−)-α-deuteriomandelic acid was racemized with the enzyme in H_2O. These results confirm the hypothesis that carbon–hydrogen bond cleavage is involved in the racemization mechanism which was indicated by the observation of the deuterium isotope effect of approximately 5. In both deuterium-exchange experiments the rate of exchange was slower than the rate of racemization. It is always possible to postulate in these circumstances that the active site of the enzyme is buried and that only partial exchange with the medium is permitted. A more likely explanation for the observed partial deuterium exchange accompaning racemization has been discussed recently by Jencks (1969). If the turnover number for an enzyme-catalyzed reaction is large enough, the solvent evidently cannot compete favorably with the proton acceptor at the active site of the enzyme. This is apparently the case for the enzyme Δ^5-3-ketosteroid isomerase (Wang *et al.*, 1963) which has a turnover number of 2.8×10^5 sec^{-1} and in which practically no deuterium exchange with the solvent is observed. When the turnover number of an enzyme-catalyzed reaction is of a similar order of magnitude to that for proton transfer to solvent, then partial exchange might be expected. Partial exchange is observed with the glucose phosphate isomerases (Baich *et al.*, 1960) which have turnover numbers in the range $2.5–6.0 \times 10^2$ sec^{-1}. Mandelic acid racemase has a similar turnover number to these latter enzymes (17.2×10^2 sec^{-1}, Hegeman *et al.*, 1970) and so the observed partial exchange is not unreasonable.

Two possible mechanisms should be considered *a priori* in a racemization process which is accompanied by exchange with the medium. Starting, for example, with the D enantiomer, they are

$$\text{D} \longrightarrow \text{L (labeled)} \qquad (1)$$

$$\text{D} \longrightarrow \text{L (labeled)} + \text{D (labeled)} \qquad (2)$$

If the pure D enantiomer is allowed to undergo partial racemization, then either the L enantiomer will be exclusively labeled initially (eq 1) or the D and L enantiomers will be labeled with equal efficiency throughout the racemization (eq 2). Our results with exchange of mandelic acid using mandelic acid racemase in tritiated water clearly rule out the exclusive operation of the mechanism described by eq 1 since after 26% conversion into the L-(+)-mandelic acid only a small percentage of the tritium label should have been found in the D-(−)-mandelic acid; instead, our results indicated that nearly as much radioactivity had been incorporated into the D enantiomer as into the L enantiomer. The D enantiomer was somewhat less radioactive than calculated for the exclusive operation of eq 2 and, although this discrepancy could be due to a fortuitous accumulation of experimental errors, it is also possible that slightly different rates of exchange with the solvent may have occurred on the *si* and *re* faces (Hanson, 1966) of the intermediate postulated below. Cardinale (1965) states that this is the case for the enzyme proline racemase with which he performed a similar partial racemization–deuterium-exchange experiment and found a much larger differential rate of exchange. He also discusses the kinetic consequences of this differential exchange in some detail.

In contrast to the findings with deuterium and tritium exchange we found no evidence for enzyme-catalyzed ^{18}O exchange into mandelic acid when the racemase reaction was carried out in 55% ^{18}O-enriched H_2O. In addition to ruling out mechanisms involving C–O bond cleavage followed by hydration, this eliminates an active ester or thioester intermediate of the type presented as a possibility by Dennis and Shapiro (1965) for the lactic acid racemase reaction as it would be expected that regeneration of the free mandelic acid from the ester intermediate would lead to the incorporation of a mole of ^{18}O-enriched H_2O as observed in the chymotrypsin reaction (Bender and Kemp, 1957). One could argue that the active site is buried and inaccessible to exchange with the ^{18}O-enriched water in the medium. Our results with deuterium exchange, however, demonstrate that the active site is at least partially accessible to exchange with the medium.

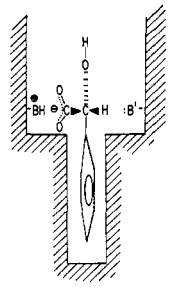
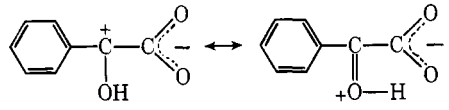


FIGURE 1: Hypothetical enzyme-D-(−)-mandelic acid complex showing aromatic binding site and specific placement of basic side chain groups B and B′. In the case of binding the L-(+) enantiomer the roles played by the B and B′ groups would presumably be reversed.

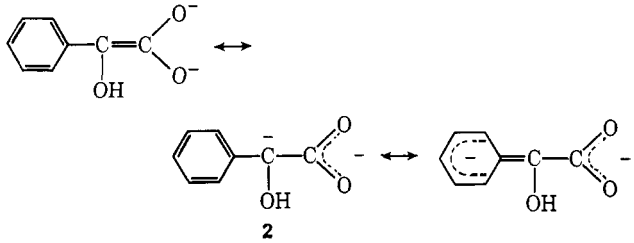
The findings that [^{14}C]benzoylformic acid did not give rise to [^{14}C]mandelic acid and that [^{14}C]mandelic acid did not yield [^{14}C]benzoylformic acid in the mandelic acid racemase reaction provide further evidence against a mechanism similar to that postulated for the UDP glucose–UDP galactose epimerase reaction involving the NAD cofactor (Maxwell, 1957; Wilson and Hogness, 1964) in which a keto intermediate presumably would be involved. It should also be noted that a carbonium ion intermediate of the type **1** is



1

also excluded since as shown it is equivalent to an unstable tautomeric form of benzoylformic acid itself.

A direct hydride transfer similar to that proposed by Dennis and Shapiro (1965) for lactic acid racemase should also be considered as a possible mechanism. If the direct hydride transfer mechanism were operating in the mandelic acid racemase system, however, one would be forced to propose that the postulated hydride donor–acceptor could exchange to some extent with protons in the medium. Furthermore, the hydride transfer mechanism as well as a mechanism involving a carbonium ion intermediate are inconsistent with our findings (Hegeman *et al.*, 1970) that electron-withdrawing substituents in the para position of mandelic acid enhance the V_{max} of the racemization reaction. These results suggest that stabilization of negative charge on the α-carbon of the mandelic acid in the transition state favors the enzyme-catalyzed racemization and indicate that a carbanion intermediate (**2**) may be involved. The observed deuterium isotope effect of 5, the apparent lack of cofactor requirement (Hegeman *et al.*, 1970), the lack of exchange with externally supplied benzoylformic acid, the observed deuterium and tritium exchange, and the lack of ^{18}O exchange with H_2O in the medium all are consistent with the occurrence of intermediate **2**. Such a carbanion would evidently be a strongly basic, highly unstable species. For example, our results show that in order to observe deuterium exchange in the α-hydrogen position in the absence of the enzyme very vigorous conditions are required.



2

An epimerase and some racemases which apparently act without pyridine nucleotide or flavin and show at least partial deuterium exchange with the medium at the racemizing carbon position include D-ribulose 5-phosphate 3-epimerase (McDonough and Wood, 1960), methylmalonyl CoA racemase (Overath *et al.*, 1962), hydroxyproline-2-racemase (Adams and Norton, 1964), proline racemase (Cardinale, 1965), and glutamic acid racemase (Rose, 1966). The probability of involvement of carbanion intermediates in several of these cases has been discussed by Rose (1966).

In light of our results which demonstrate that the mandelic acid racemase catalyzed with approximately equal efficiency the incorporation of radioactivity into the D-(−) and L-(+) enantiomers during partial racemization of D-(−)-mandelic acid in tritiated water, it is reasonable to suggest that the mandelic acid racemase may contain a single binding site for the D-(−) and L-(+) enantiomers, shown in Figure 1 for the D-(−) enantiomer, in which the postulated roles for the B and B′ groups could be simply reversed in the case of binding the L-(+) isomer. This possibility is being further investigated.

Acknowledgements

We wish to thank Mrs. Emiko Rosenberg for her skillful technical assistance, Dr. C. W. Koch for his help with the mass spectrometry experiments, and Professor D. S. Noyce for helpful discussions.

References

Adams, E., and Norton, I. L. (1964), *J. Biol. Chem. 239*, 1525.
Baich, A., Wolfe, R. G., and Reithel, F. J. (1960), *J. Biol. Chem. 235*, 3130.
Bender, M., and Kemp, K. C. (1957), *J. Amer. Chem. Soc. 79*, 116.
Cardinale, G. J. (1965), Dissertation, The Ohio State University, *Diss. Abstr. B 34*, 13045c.
Dennis, D., and Shapiro, S. S. (1965), *Biochemistry 4*, 2283.
Fieser, L. F. (1957), *in* Experiments in Organic Chemistry, Boston, Mass., Heath, D. C., and Co., 3rd ed revised, p 97.
Hanson, K. R. (1966), *J. Amer. Chem. Soc. 88*, 2731.
Hegeman, G. D., Rosenberg, E. Y., and Kenyon, G. L. (1970), *Biochemistry 9*, 4029.
Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill.
Maxwell, E. S. (1957), *J. Biol. Chem. 229*, 139.
McDonough, M. W., and Wood, W. A. (1960), *J. Biol. Chem. 236*, 1220.
Melander, L. (1960), Isotope Effects on Reaction Rates, New York, N. Y., The Ronald Press.
Overath, P., Kellerman, G. M., Lynen, F., Fritz, H. P., and

Keller, H. J. (1962), *Biochem. Z. 335*, 500.
Rose, I. A. (1966), *Annu. Rev. Biochem. 35*, 23.
Wang, S.-F., Kawahara, F. S., and Talalay, P. (1963), *J. Biol. Chem. 238*, 576.
Wilson, D. B., and Hogness, D. S. (1964), *J. Biol. Chem. 239*, 2469.

Kinetic Studies of Tryptophan Synthetase. Interaction of Substrates with the B Subunit*

Edward J. Faeder† and Gordon G. Hammes‡

ABSTRACT: The synthesis of L-tryptophan from indole and L-serine, as catalyzed by the B subunit of *Escherichia coli* tryptophan synthetase, has been studied with steady-state and rapid-reaction kinetic techniques. Initial velocity measurements of the reaction have been made utilizing the absorption difference between indole and tryptophan at 289 nm. The results were consistent with both a compulsory sequence of substrate addition and with a random, rapid equilibration between enzyme and substrates. Dissociation constants and/or Michaelis constants for serine and indole were obtained. Temperature-jump, stopped-flow, and combined stopped-flow–temperature-jump measurements made on solutions of B protein combined with indole, L-serine, L-tryptophan, and indole plus L-serine reveal the following mechanistic features. (1) The enzyme exists in two rapidly interconvertible forms; (2) the binding of L-tryptophan can be described as a simple bimolecular reaction; (3) the L-serine binds rapidly and the complex formed undergoes an additional, relatively slow, isomerization which is independent of the absence or presence of indole; (4) only a single first-order (rate-limiting) process can be detected on interaction of the enzyme–serine complex with indole. The mechanistic implications of these findings are considered.

Tryptophan synthetase isolated from *Escherichia coli* is a multisubunit enzyme with two types of subunits, commonly designated as α and β (Crawford and Yanofsky, 1958). The fully associated enzyme complex has the composition $\alpha_2\beta_2$ (Goldberg *et al.*, 1966) and can be readily dissociated into two α subunits (A protein) and a β_2 dimer (B protein) (Wilson and Crawford, 1964; Hathaway *et al.*, 1969). The overall reaction catalyzed by tryptophan synthetase is

$$\text{InGP} + \text{Ser} \rightleftharpoons \text{Trp} + \text{GP} \quad (1)$$

where InGP is indole-3-glycerol phosphate, Ser is L-serine, Trp is L-tryptophan, and GP is glyceraldehyde 3-phosphate. Neither the A nor B protein will catalyze this reaction (*cf.* Crawford and Yanofsky, 1958). Instead the A protein catalyzes the reaction

$$\text{InGP} \rightleftharpoons \text{In} + \text{GP} \quad (2)$$

and the B protein catalyzes the reaction

$$\text{In} + \text{Ser} \rightleftharpoons \text{Trp} \quad (3)$$

with pyridoxal 5′-phosphate being a required cofactor for the reaction given in eq 3. The sum of eq 2 and 3 is eq 1; therefore tryptophan synthetase is a simple example of an organized enzyme system. Moreover addition of A protein to B protein enhances the catalysis of eq 3 by a factor of 30–40 (Wilson and Crawford, 1965), and the addition of B to A enhances the catalysis of eq 2 by a factor of 50–100 (Hatanaka *et al.*, 1962). An understanding of the catalytic mechanism for tryptophan synthetase and of the role the subunit interactions play in the catalysis may provide a basis for consideration of the many complex organized enzymes found in biological systems.

This work reports a detailed kinetic study, using steady-state and rapid-reaction techniques, of the synthesis of L-tryptophan from L-serine and indole (eq 3) by the B protein. The results obtained establish the following mechanistic features. (1) The enzyme exists in two rapidly interconvertible conformational states. (2) The binding of L-tryptophan can be described as a simple bimolecular reaction. (3) The bimolecular reaction of enzyme and serine is too fast to study; however the enzyme–serine complex can isomerize in a manner similar to that of the free enzyme, and the bimolecular reaction is followed by another, relatively slow, isomerization of the enzyme–serine complex. (4) Only a single first-order (rate-limiting) process can be detected on interaction of the enzyme–serine complex with indole. The mechanistic implications of these findings are discussed.

Experimental Section

Materials. The B protein of tryptophan synthetase was purified from a mutant of *E. coli* K-12 (A2/F′ A2), kindly

* From the Department of Chemistry, Cornell University, Ithaca, New York. This work was supported by a grant from the National Institutes of Health (GM 13292). *Received May 18, 1970.*
† National Institutes of Health Predoctoral Fellow 1967–1970.
‡ To whom correspondence should be addressed.