Energetics of Proline Racemase: Transition-State Fractionation Factors for the Two Protons Involved in the Catalytic Steps†

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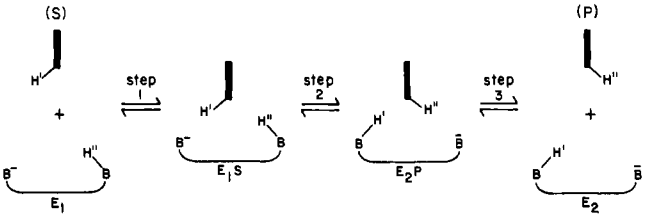
ABSTRACT: The isotope effects for the interconversion of L-proline and D-proline, catalyzed by proline racemase, have been determined in the saturated region with both [2-^{2}H]proline and [2-^{3}H]proline. The deuterium fractionation factors for each of the protons in flight have been obtained from two kinds of experiment: (i) by measuring the *rate of racemization* of one [2-^{2}H]proline enantiomer as it racemizes into an equilibrated pool of unlabeled proline and (ii) by measuring the *deuterium content* of a proline sample at the optical rotation maximum that occurs when an equimolar mixture of one deuterium-labeled enantiomer and the other unlabeled enantiomer runs to equilibrium. The tritium fractionation factors for each of the protons in flight have been determined from measurements of the rate of loss of tritium to the solvent as one [2-^{3}H]proline enantiomer runs to equilibrium. Good agreement is found among the fractionation factors determined by each method. The deuterium fractionation factors for the two protons are not identical: that for the proton derived from L-proline is 0.375 and that for the proton derived from D-proline is 0.44. This difference has been confirmed by a double-competition experiment in which the optical rotation of a mixture of DL-[2-^{2}H]proline and unlabeled DL-proline is followed with time. The rotation (initially zero) passes through a maximum, from which the ratio of the two fractionation factors (0.86) is obtained. These data, coupled with the equilibrium fractionation factor for the 2-position of proline (which has been determined to be 1.17), provide the transition-state factors for each of the in-flight protons, and delineate the nature of the transition state(s) for the enzyme-catalyzed racemization.

The reaction catalyzed by proline racemase is known from the work of Abeles and his group (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975) to involve the direct abstraction and delivery of protons from and to the α-carbon atom of proline. The enzyme does not require cofactors or metal ions for activity, and it appears that the racemization reaction is achieved by the action of a pair of general acid–general base catalysts at the active site. When [2-^{2}H]proline is used as substrate, the enzyme-catalyzed reaction proceeds more slowly, and Cardinale and Abeles (1968) showed that the primary kinetic isotope effect is about 2.5 when the initial reaction rate of L-[2-^{2}H]proline is studied. Furthermore, these authors demonstrated that when the enzyme-catalyzed racemization of L-proline in ^2H_2O is followed polarimetrically, there is an "overshoot": the optical rotation is initially negative, becomes zero, then positive, and finally again approaches zero. This result is readily accommodated by the two-base mechanism proposed for the enzyme, as follows. Initially, unlabeled L-proline is converted into deuterated D-proline product, the conversion of which back to (deuterated) L-proline is slowed by the existence of the primary deuterium isotope effect. The rate constant for the formation of labeled D-proline from unlabeled L substrate therefore initially exceeds the rate constant for consumption of the labeled D enantiomer, and the observed net optical rotation changes sign. At long times, of course, isotopic exchange becomes complete, the two (now fully labeled) enantiomers come to equilibrium, and the rotation falls to zero.

Scheme I: Interconversion of L-Proline (S) and D-Proline (P) by Proline Racemase[a]



[a] The different protonic sites are indicated by primes. The proline ring is shown edge-on as a heavy bar. B^- and B–H are enzyme catalytic groups.

In this paper we explore further the involvement of the hydrogen at C-2 on both L-proline and D-proline through studies of the kinetic isotope effect using both deuterium and tritium. The reaction is studied in the saturated region, where the rate-limiting transition states all involve enzyme bound to proline (Fisher et al., 1986a,b). As is clear from Scheme I, there are *two* protonic sites, designated by a single and a double prime. While the equilibrium fractionation factors for S and P must be identical (S and P are enantiomers), those for E_1 and E_2 need not be the same, since E_1 and E_2 are not enantiomers. Thus, it is possible that the isotope effects for the forward and backward reactions of Scheme I could be different and that the reactions of deuterated L-proline and of deuterated D-proline proceed at different rates. This difference is, indeed, observed. In this paper, we analyze the results from four different types of isotopic experiment, to find values for the mixed fractionation factors $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$ for each of the two protons[1] involved in the substrate–product

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interconversion steps of the reaction.

The most direct method of finding the primary deuterium kinetic isotope effect for the racemase-catalyzed reaction is to measure the velocity of the reaction starting with either L-[2-^{2}H]proline (S′) or D-[2-^{2}H]proline (P″) and to compare this velocity with that of unlabeled proline. The absence of usable coupling enzyme systems for proline racemase means, however, that such experiments have to be performed under reversible conditions where the time course of the equilibration is followed. Under these circumstances, the kinetic analysis is much simplified if the deuterated proline (S′ or P″) is allowed to react in the presence of an equilibrated pool of the unlabeled prolines, S and P. This is a *clamped deuterium washout experiment*, in which the deuterated proline isomerizes with concomitant loss of its 2-^{2}H label and joins the equilibrated pool of unlabeled material.

Values for the fractionation factors $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$ can also be found by studying the *deuterium content* of the substrate as a function of the progress of the reaction as the isotope is washed out into the solvent. If one starts with equal concentrations of S′ and P, P reacts to form S more rapidly than the (deuterated) S′ reacts to form P, leading initially to a greater concentration of S + S′ than of P. Since the deuterium label is constantly being lost to the solvent, all of S′ is eventually converted into S, and one ends up with equal concentrations of S and P. The optical rotation therefore starts at zero ($s_0' = p_0$), then passes through a maximum or a minimum (depending upon the sign of the rotation of S), and ultimately returns to zero. This is called a *competitive deuterium washout experiment*.

Third, in the racemase-catalyzed reaction, the C-2 proton of the substrate is released into the solvent, and the tritium isotope effect on the interconversion of the 2-^{3}H-labeled substrates can be found by comparing the rate of tritium release (which measures the rate for the ^{3}H species) with the extent of racemization (which measures the rate for the ^{1}H species). In these experiments, the tritium content of the substrate is followed as a function of the conversion of the substrate to a racemic mixture, as monitored by optical rotation. This is called a *tritium washout experiment*.

Finally, the *ratio* of the fractionation factors $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$ can be measured by carrying out a double competition in which the optical rotation of a racemic mixture of deuterated proline ($s' = p'$) is measured under clamped conditions (i.e., in the presence of an equilibrium mixture of unlabeled substrates: $s = p$). Since $\phi_S = \phi_P$ (S and P are enantiomers), if $\phi_{1,2,3}' = \phi_{1,2,3}''$, then no perturbation in the optical rotation from its initial value of zero will be observed. On the other hand, if $\phi_{1,2,3}'$ differs from $\phi_{1,2,3}''$, a perturbation in the optical rotation will be observed. The ratio of the fractionation factors ($\phi_{1,2,3}'/\phi_{1,2,3}''$) can then be calculated from the size of the perturbation. As shown below, when such a *double-competitive deuterium washout experiment* is done with proline racemase, a perturbation is indeed seen, confirming that there is a real difference between $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$.

Experimental Procedures

Materials

In addition to the materials described in Fisher et al. (1986a,b), L-(+)-tartaric acid, D-(−)-tartaric acid, flavin adenine dinucleotide (FAD),[2] *N,N*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane, catalase (bovine liver), and D-amino acid oxidase (pig kidney) were obtained from Sigma. ^2H_2O (99.8%) was from Bio-Rad, and ^3H_2O (5 Ci mL^{-1}) was from Amersham/Searle. Bio-Rex 40 (100–200 mesh) analytical grade cation exchange resin was from BioRad. Dowex 1 (8% cross-linked, 200–400 mesh) was from Sigma.

DL-[*2-^{3}H*]*Proline* was prepared by incubating L-proline (2.3 g) in 200 mM sodium pyrophosphate buffer, pH 8.2 (1.80 mL), containing tritiated water (~1.1 Ci), 2-mercaptoethanol (20 mM), and proline racemase (500 units) at 37 °C, according to the method of Rudnick and Abeles (1975). After 24 h, a further 500 units of enzyme and 2-mercaptoethanol (5 μL) was added, and the incubation continued for 36 h more. The solvent was then removed by bulb-to-bulb distillation in vacuo, the residue was dissolved in hot methanol (4 mL), and the solution was filtered. Proline was precipitated from the filtrate by the addition of acetone and recrystallized from methanol–acetone. The isolated DL-[2-^{3}H]proline had a specific radioactivity of 3.03×10^5 cpm μmol^{-1}. All the radioactivity could be released into the solvent by incubation with proline racemase.

Resolution of DL-[*2-^{3}H*]*Proline*. DL-[2-^{3}H]proline was resolved by a modification of the methods of Yamada et al. (1977). To a stirred solution of DL-[2-^{3}H]proline (0.4 g) and L-tartaric acid (0.26 g) in water (350 μL) was added dropwise absolute ethanol (~3 mL) until a permanent turbidity appeared. The solution was seeded with a few crystals of the L-proline–L-tartaric acid complex, and the mixture was stirred for 20–30 min before the remaining ethanol (1.1 mL) was added. The suspension was stirred for 45 min and then left overnight at 5 °C. The solid complex was filtered, washed with a small amount of ice-cold 94% (v/v) ethanol, and dried in air. [The mother liquor was retained for use in the isolation of D-[2-^{3}H]proline (see below).] The solid complex was then recrystallized from ethanol–water to yield the L-[2-^{3}H]proline–L-tartaric acid complex (220 mg).

L-[2-^{3}H]Proline was recovered from the tartaric acid complex by dissolving the complex in water and applying the solution to a column (3 mL) of Bio-Rex 40 (H^+ form). The column was washed with water (~20 mL) to remove L-tartaric acid, and the proline was then eluted with 2 N ammonium hydroxide (~15 mL). The solution was evaporated to dryness to yield L-[2-^{3}H]proline (72 mg, 36%). A portion of the material was converted to the methyl ester and then allowed to react with *N*-(trifluoroacetyl)-L-prolyl chloride as described by Fisher et al. (1986b). The resulting dipeptides were analyzed by gas chromatography, which showed that the L-[2-^{3}H]proline contained <1.6% of the D enantiomer.

[2-^{3}H]Proline enriched in D-[2-^{3}H]proline was obtained from the mother liquor from the above resolution involving L-tartaric acid. The [^{3}H]proline was recovered free from L-tartaric acid by ion-exchange chromatography as described above, and the complex between D-[2-^{3}H]proline and D-tartaric

[1] The two protonic sites are specified by a single or a double prime (see Scheme I). The deuterium fractionation factor of the 2-proton of free L-proline is ϕ_S and that of the substrate proton where L-proline is bound to the enzyme is ϕ_{ES}'. Analogously, the 2-proton of D-proline has the fractionation factor for the free substrate of ϕ_P and for the bound substrate of ϕ_{EP}''. The enzyme's catalytic site in E_2 that bears the proton is the singly primed site and in E_1 is the doubly primed site. The lower case letter, ϕ, is used for deuterium fractionation factors, and the upper case letter, Φ, is used for tritium fractionation factors. Mixed fractionation factors are discussed in Albery and Knowles (1986). These factors are related to the kinetic isotope effect as, for example, $k_{1,2,3}^H/k_{1,2,3}^D = \phi_S/\phi_{1,2,3}'$.

[2] Abbreviations: FAD, flavin adenine dinucleotide; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

acid was prepared as follows. [2-^{3}H]Proline (180 mg, enriched in the D enantiomer) and D-tartaric acid (250 mg) were dissolved in water (330 μL) by stirring at 50 °C. Absolute ethanol (1.17 mL) was added, and the solution was maintained at 50 °C until all the solid had dissolved. The solution was then seeded with a few crystals of the D–D complex and allowed to cool (over 4 h) to room temperature. The solid complex was isolated by filtration, washed with 95% ethanol, and dried in air.

D-[2-^{3}H]Proline (75 mg, 38%) was recovered from the D-[2-^{3}H]proline–D-tartaric acid complex by ion-exchange chromatography as described above. Gas chromatographic analysis of the *N*-(trifluoroacetyl)-L-prolyl-D-proline methyl ester derivative showed that the D-[2-^{3}H]proline contained <0.8% of the L enantiomer.

Seed crystals of the L–L and the D–D complexes used in the resolution described above were made as follows. L-Proline (185 mg) and L-tartaric acid (125 mg) were dissolved in water (160 μL), and the solution was left at room temperature for 30 min. The solid complex was isolated by filtration and dried in air: yield 76 mg; mp 152–154 °C [Yamada et al. (1977) give 153–154 °C]. The complex between D-proline and D-tartaric acid was prepared analogously.

DL-[*2-^{2}H*]*Proline.* Sodium pyrophosphate buffer (20 mM), pH 8.2 (25 mL), was lyophilized, dissolved in D_2O (5 mL), and lyophilized again. L-Proline [5 g, that had been lyophilized from solution in D_2O (6 mL)], 2-mercaptoethanol (175 μL), D_2O (30 mL), and proline racemase [1200 units, which had been lyophilized and redissolved in D_2O (5 mL)] were then added. The solution was incubated with gentle shaking at 37 °C for 24 h. More proline racemase (800 units, in D_2O) was then added, and the solution was left at 37 °C for a further 24 h. The progress of the exchange reaction was followed by ^{1}H NMR on a portion of the reaction mixture. The solution was evaporated to dryness and the residue treated with hot anhydrous methanol. Denatured protein was removed by filtration, and the solution was again taken to dryness. The residue was recrystallized from anhydrous methanol–acetone to yield DL-[2-^{2}H]proline (3.3 g). The deuterium content of the DL-[2-^{2}H]proline was determined by mass spectrometric analysis of the volatile bis(trimethylsilyl) derivative. DL-[2-^{2}H]Proline (1 mg) was incubated with *N*,*N*-bis(trimethylsilyl)trifluoroacetamide (100 μL) containing 1% trimethylchlorosilane, at 135 °C for 15 min. The solution was analyzed by gas chromatography–mass spectrometry. The glass column (6 ft × 2 mm) contained gc P coated with SE-30 (1%) maintained at an oven temperature of 200 °C. The ionizing voltage of the mass spectrometer was 70 eV. The molecular separator was kept at 150 °C. The deuterium content of the DL-[2-^{2}H]proline was determined from the ratio of m/z 143/142 ($M^+ - COOSiMe_3$) and found to be >97%.

DL-[2-^{2}H]Proline was resolved by using the method described above for DL-[2-^{3}H]proline, to yield L-[2-^{2}H]proline (185 mg, 32%, $[\alpha]^{23}_D = -84°$) and D-[2-^{2}H]proline (170 mg, 29%, $[\alpha]^{23}_D = +83.7°$). The enantiomeric excess of the samples was determined by gas chromatography of the *N*-(trifluoroacetyl)-L-prolylproline methyl ester derivatives. The L-[2-^{2}H]proline contained <0.9% of the D isomer, and D-[2-^{2}H]proline contained <0.5% of the L isomer.

Methods

Analytical and kinetic methods were as described earlier (Fisher et al., 1986a,b).

Tritium Transfer. (*a*) *Purification of* L-[*2-^{3}H*]*Proline.* L-[2-^{3}H]Proline (140 mg) was dissolved in 200 mM ammonium bicarbonate buffer, pH 8.4 (10 mL), containing FAD (34.6 μM) and catalase (3100 units). D-Amino acid oxidase (6 units) was added and the solution incubated in the dark at 37 °C. After 17 h, a further portion of oxidase (3 units) and of FAD (50 μg) was added, and the solution was incubated at 37 °C for another 5 h. Charcoal was then added to adsorb FAD, and the solution was filtered. The filtrate was acidified with HCl (2 N) and applied to a column (5.5 × 1.5 cm) of Bio-Rex 40 (H^+ form). The column was washed with water, and the washings were pooled and counted to determine the fraction of the radioactivity released by the oxidase treatment. The column was then eluted with NH_4OH (2 N) (~40 mL), and the L-[2-^{3}H]proline was recovered by lyophilizing the eluate. After recrystallization from methanol–acetone, the yield of L-[2-^{3}H]proline was 105 mg. The specific radioactivity of the product was 954 700 cpm μmol^{-1}. The oxidase treatment released <2.5% of the radioactivity originally contained in the L-[2-^{3}H]proline.

(*b*) *Racemase Reaction.* A solution of purified L-[2-^{3}H]-proline (210 mM) in 200 mM Tris-HCl buffer, pH 8.0 (1 mL), containing 2-mercaptoethanol (20 mM) and EDTA (8 mM) was incubated at 37 °C. Proline racemase (1.2 units, in 50 μL of buffer) was then added, and the progress of the racemization reaction was followed by monitoring the optical rotation at 365 nm and 37 °C. After a small extent of reaction (4.35% conversion to D-proline) the reaction mixture was quenched into 0.1 N NaOH (5 mL). This solution was applied to a column (8 × 1 cm) of Dowex-1 (OH^- form), and the column was thoroughly washed with water. The combined washings were counted to determine the fraction of the radioactivity released by the racemase. The column was washed with 2 N acetic acid (~40 mL), and the column effluent containing the [^{3}H]proline was then applied to a column (5 mL) of Bio-Rex 40 (H^+ form). The column was washed thoroughly with water to remove acetic acid and eluted with 2 N NH_4OH (~40 mL). The [^{3}H]proline was recovered by lyophilizing the column effluent. The recovery of [^{3}H]proline based on radioactivity was 84%. The racemase released approximately 1.37 × 10^6 cpm (i.e., 0.7% of the total radioactivity).

(*c*) *Analysis of the Tritium Content of the* D-*Proline Formed in the Racemase Reaction.* The reisolated [^{3}H]proline was dissolved in 50 mM sodium pyrophosphate buffer, pH 8.5 (3 mL). To a portion (1 mL) of this solution (containing L-[2-^{3}H]proline, 56.1 mM, and D-proline, 2.55 mM) were added FAD (30 μg) and catalase (312 units), and the solution was incubated at 37 °C in the dark. D-Amino acid oxidase (0.6 unit) was then added. Samples (100 μL) were withdrawn over a 30-min period and quenched in 2 M sodium benzoate (10 μL). Each quenched sample was applied to a column (2 × 0.6 cm) of Bio-Rex 40 (H^+ form), and the columns were eluted with water (2 mL). The column effluent was then counted for tritium. In a control experiment carried out under identical conditions but using L-proline (56.1 mM) and D-[2-^{3}H]proline (2.6 mM, 954 700 cpm μmol^{-1}), it was shown that all the tritium was released to the solvent after incubation for 15 min with the oxidase.

Clamped Deuterated Substrate Rates. The rates of racemization of the [2-^{2}H]proline were followed polarimetrically as described by Fisher et al. (1986a). The solutions contained equal concentrations of L- (or D-)[2-^{2}H]proline and unlabeled DL-proline in 200 mM Tris-HCl buffer, pH 8.0, containing 2-mercaptoethanol (20 mM) and EDTA (8 mM), at 37 °C. The reactions were initiated by the addition of proline racemase (6.0 units). The concentration of the [2-^{2}H]proline at the start was determined from the optical rotation of 365 nm,

and the reaction was followed by monitoring the optical rotation at this wavelength. [That the specific rotations of [2-^{2}H]- and [2-^{1}H]prolines are identical was evident from the work of Cardinale and Abeles (1968) and from our measurements of the (zero) optical rotation from equimolar mixtures of one [2-^{2}H]proline and its unlabeled enantiomer.]

Competitive Deuterium Washout. These experiments were done under the conditions used for the kinetic experiments described in Fisher et al. (1986a). The value of the optical rotation at the maximum perturbation from equilibrium and the deuterium content of the proline at that point were measured by the methods described in this and the preceding papers (Fisher et al., 1986a,b).

Tritium Washout. The racemase-catalyzed release of tritium from L- and D-[2-^{3}H]proline into the solvent was measured as follows. D-(or L-)proline was dissolved in 200 mM Tris-HCl buffer, pH 8.0, containing 2-mercaptoethanol (20 mM) and EDTA (8 mM). D-(or L-)[2-^{3}H]Proline (303 000 cpm μmol^{-1}) was added to give a solution containing $\sim$2.5 $\times$ 10^6 cpm. The exact concentration of the proline was determined from the optical rotation at 365 nm and 37 °C. Proline racemase (1.0 unit) was then added to the buffered solution (2 mL) containing [2-^{3}H]proline, at 37 °C. The progress of the reaction was followed by the optical rotation at 365 nm of a portion of the solution (1 mL). The remaining reaction mixture was sampled at suitable intervals by withdrawing portions (100 μL) which were quenched in 20% (w/v) trichloroacetic acid (25 μL). Each quenched sample was applied to a column (2 $\times$ 0.6 cm) of Bio-Rex 40 (H^+ form), which was eluted with water (2 mL). The effluent was collected in a scintillation vial containing the scintillation fluid (10 mL) of Bray (1960), and counted.

Double-competitive deuterium washout experiments were done under the conditions used for the kinetic experiments described in Fisher et al. (1986a).

Determination of ϕ_S and ϕ_P. The reactant fractionation factors for L- and D-proline (ϕ_S and ϕ_P) were determined by allowing a racemic mixture of proline to equilibrate at pH 7.5, 37 °C, in the presence of proline racemase, in an approximately equimolar mixture of H_2O and D_2O prepared precisely by weight. After a period long enough to ensure complete isotopic equilibration (as a control, an incubation was also maintained for twice as long), the proline was isolated, purified, and converted into the *N*-(trifluoroacetyl)-L-prolylproline methyl ester derivatives as described by Belasco et al. (1986a). The diastereoisomeric dipeptide derivatives were separated by preparative gas chromatography and subjected to mass spectrometric analysis (Belasco et al., 1986a).

Theory

Considering Scheme II and using composite rate constants (Fisher et al., 1986a), we can write the equations for the flux f from S to P and for the flux f' from the isotopically labeled substrate S′ to P:

$$f = k_{1,2,3}e_1{\cdot}s - k_{-3,2,1}e_2{\cdot}p \quad (1)$$

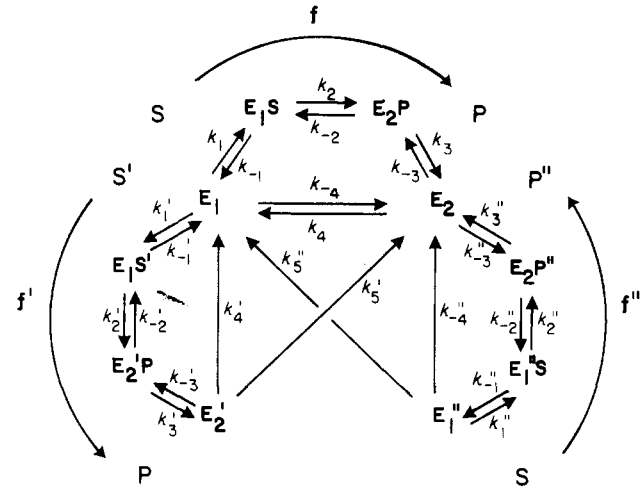
$$f' = k_{1,2,3}'e_1{\cdot}s' \quad (2)$$

Expressions describing the reaction of P″ rather than S′ (Scheme II) are obtained by making the appropriate substitutions in the equations for S′.

The experiments reported in this paper have been carried out in the saturated region [which for proline racemase is bound state saturated (Fisher et al., 1986b)] where the enzyme interconversion steps are not rate limiting, so we can write

$$e_1 = K_4e_2 \quad (3)$$

Scheme II: Pathways for the Reaction of Unlabeled L-Proline (S), L-[2-^{2}H or -^{3}H]Proline (S′), Unlabeled D-Proline (P), and D-[2-^{2}H or -^{3}H]Proline (P″) with Proline Racemase, in ^1H_2O[a]



[a] The fluxes of S, S′, and P″ are f, f', and f'', respectively. The rate constants k_4 and k_5 indicate the possible routes for the loss of enzyme-bound deuterium or tritium to the solvent.

Substituting eq 3 into eq 1 and using the fact that for a racemase $K_{1,2,3,4} = 1.0$, we obtain

$$f = k_{1,2,3}e_1(s - p) \quad (4)$$

In the bound-state saturated region, the significant enzyme forms are those where the enzyme is bound to substrate, E_1S, E_2P, E_1S', and $E_2'P$, and the total concentration, e_Σ, is

$$e_\Sigma = e_1s + e_2p + e_1s' + e_2'p \quad (5)$$

From the steady-state concentrations in the f' loop of Scheme II, we have

$$e_2'p = f'/k_3' \quad (6)$$

$$e_1s' = f'/k_2' + f'/k_3' \quad (7)$$

where we have used the fact that for proline racemase, $K_2 = 1$ (Fisher et al., 1986a). Similarly, for the f loop, we have

$$e_1s = K_1e_1{\cdot}s - f/k_{-1} \quad (8)$$

$$e_2p = e_1{\cdot}p/(K_3K_4) + f/k_3 \quad (9)$$

Substitution from eq 4 into eq 8 and eq 9, together with the fact that $K_2 = 1$, gives

$$e_1s + e_2p = \frac{K_1f}{k_{1,2,3}}\frac{(s+p)}{(s-p)} + \frac{f}{k_3} - \frac{f}{k_{-1}} \quad (10)$$

Clamped Deuterated Substrate Reactions. Now we design the experiment so that the first term on the right-hand side of eq 10 is larger than the other two terms. This is done by adding S′ to an equilibrated pool of S and P. In such a "clamped" experiment, $s \approx p$ throughout the experiment, which ensures that the right-hand side of eq 10 is dominated by the $(s - p)$ term.

From eq 2 and 4

$$\frac{f}{s-p} = \frac{f'}{s'}\frac{k_{1,2,3}}{k_{1,2,3}'} = \frac{\phi_S}{\phi_{1,2,3}'}\frac{f'}{s'} \quad (11)$$

where ϕ_S is the ground-state fractionation factor for the substrate S, and $\phi_{1,2,3}'$ is the mixed transition state fractionation factor for reaction through steps 1–3 (Scheme I).[1] Substitution of eq 11 into eq 10 (neglecting the last two terms on the right-hand side), together with eq 5–7, then gives

$$\frac{e_\Sigma}{f'} = \frac{\phi_S K_1}{\phi_{1,2,3}' k_{1,2,3}} \frac{(s+p)}{s'} + \frac{1}{k_2'} + \frac{2}{k_3'} \quad (12)$$

Now

$$s + p = c - s' \quad (13)$$

where, as before, c is the total concentration of substrates. Substitution of eq 13 into eq 12 and integration ($ds'/dt = -f'$) give

$$\ln \frac{s'}{s_0'} = -\frac{k_I' e_\Sigma t}{c} + A\frac{(s_0' - s')}{c} \quad (14)$$

where

$$k_I' = (\phi_{1,2,3}'/\phi_S)(k_{1,2,3}/K_1) \quad (15)$$

and

$$A = k_1'/k_2' + 2k_1'/k_3' - 1$$

Since in a clamped experiment $s \approx p$,[3] the concentration of S′ can be measured from the optical rotation, and eq 14 can be rearranged to give

$$\frac{\ln(\lambda/\lambda_0)}{\lambda_0 - \lambda} = -\frac{k_I' e_\Sigma}{c} \frac{t}{(\lambda_0 - \lambda)} + A \quad (16)$$

where $\lambda = (s + s' - p)/c \approx s'/c$ and $\lambda_0 = s_0'/c$.

From eq 8, 9, and 12 of Fisher et al. (1986a) we find that, in the bound-state saturated region (i.e., where k_4 and k_{-4} are too fast to limit k_{cat}) with $K_2 = 1$, the rate constant for the *un*labeled substrate, k_1, is given by

$$\frac{1}{k_I} \approx \frac{1}{4}\left(\frac{1}{k_{cat^+}} + \frac{1}{k_{cat^-}}\right) = \frac{1}{2}\left(\frac{1}{k_{-1}} + \frac{1}{k_2} + \frac{1}{k_3}\right) = \frac{K_1}{2k_{1,2,3}} \quad (17)$$

Substitution in eq 15 gives

$$\phi_{1,2,3}'/\phi_S = 2k_I'/k_I \quad (18)$$

The reader may be puzzled by the factor of 2 in this equation. It arises from the fact that the observed rate constant k_I' describes the irreversible washout of isotope, while the observed rate constant k_I describes the approach to equilibrium of unlabeled substrates. The rate constant describing an approach to equilibrium is the sum of the forward and backward rate constants which for a racemase are equal, and this gives rise to the factor of 2.

Our more general treatment (Albery & Knowles, 1986) gives the corrections for the fact that the system is not perfectly saturated and that there is some contribution from unsaturated and oversaturated terms. If the values of k_I and k_I' for the reaction of an unlabeled and a labeled substrate are compared (at the same values of c and e_Σ), we can write, from eq 7.8 of Albery and Knowles (1986)

$$\frac{k_I}{2k_I'} = \left[\frac{\phi_S}{\phi_{1,2,3}'} + \frac{c\phi_S x}{c_P \phi_4}\right](1 + c/c_P)^{-1} \quad (19)$$

where $x = k_4'/(k_4' + k_5')$, c_P is the peak-switch concentration (Fisher et al., 1986a), and x and ϕ_4 describe the washout of isotope that converts E_2' into E_1 and/or E_2. We shall later show (Belasco et al., 1986b), however, that $x \approx 1$ and that $\phi_4 \approx \phi_{1,2,3}'$, which means that the more general eq 19 reduces to the simpler eq 18. Second, instead of the simplified expression for k_I in eq 17, we should use the full equation (Fisher et al., 1986a):

$$\frac{1}{k_I} = \frac{1}{4}\left(\frac{1}{k_{cat^+}} + \frac{1}{k_{cat^-}}\right)\left(1 + \frac{c}{c_P} + \frac{c_D}{c}\right) \quad (20)$$

where c_D is the dip-switch concentration. This allows the appropriate corrections to be made.

Competitive Deuterium Washout Experiments. We next turn to the competitive deuterium washout experiments, where we start with equal concentrations of S′ and of P and measure the *size* of the maximum perturbation and the *isotopic content* of the substrates at the maximum.

We define

$$\sigma' = s'/s_0' \quad (21)$$

$$\sigma = (p - s)/s_0' \quad (22)$$

Then, remembering that $p + s + s'$ is constant, we can write

$$f = \frac{-ds}{dt} = \frac{1}{2}\frac{d}{dt}(p + s + s' - 2s) = \frac{s_0'}{2}\left(\frac{d\sigma}{dt} + \frac{d\sigma'}{dt}\right)$$

Equation 11 then gives

$$\frac{d\sigma}{d\sigma'} = \frac{R\sigma}{\sigma'} - 1 \quad (23)$$

where

$$R = 2\phi_S/\phi_{1,2,3}' = k_I/k_I' \quad (24)$$

At the start of the experiment, both σ and σ' are unity. Integrating eq 23 with this boundary condition gives

$$(R - 1)\sigma = (R - 2)(\sigma')^R + \sigma'$$

Now, the perturbation in the optical rotation is given by $s + s' - p$, which is proportional to $\sigma' - \sigma$:

$$\sigma' - \sigma = \frac{s + s' - p}{s_0'} = \frac{R - 2}{R - 1}[\sigma' - (\sigma')^R]$$

Differentiation gives the maximum when

$$(\sigma')_{max} = \left(\frac{s'}{s_0'}\right)_{max} = R^{1/(1-R)} \quad (25)$$

and the size of the maximum

$$(\sigma' - \sigma)_{max} = 2\lambda_{max} = (R - 2)R^{R/(1-R)} \quad (26)$$

where $\lambda = (s + s' - p)/c$. Equations 25 and 26 are special cases for the bound-state saturated region of the more general equations given by Albery and Knowles (1986). Use of these more complete equations that contain terms that dominate in the oversaturated region makes no significant difference to the analysis. We therefore use eq 25 and 26 to obtain values of R and then eq 24 to derive $\phi_{1,2,3}'/\phi_S$.

It may be noted that eq 26 has the same general form as that derived for the equilibrium perturbation method of Schimerlik et al. (1975), where the fractional perturbation can be written as

$$(\sigma' - \sigma)_{max} = (\alpha - 1)\alpha^{\alpha/(1-\alpha)}$$

where α is the isotope effect (equivalent to $R/2$ in eq 26). This equation is not identical with eq 26 because here the substrate isotope washes out irreversibly into the solvent as the reaction proceeds, whereas the equation of Schimerlik et al. relates to

[3] In practice, clamping is not perfect. Analysis shows, however, that after one half-life the imbalance in $(p - s)$ decays exponentially with the same rate constant ($k_I' e_\Sigma/c$) as that which governs the decay of λ in eq 16. This means that $d(\ln \lambda)/dt = d(\ln s')/dt$, so gradients derived from eq 16 need no correction for imperfect clamping.

an equilibration in which isotope is not lost from substrate or product.

Tritium Washout Experiments. In these experiments, the tritium content (s') of the substrate S is measured as a function of the conversion of S to P. The concentration of the tracer S′ is very much less than that of unlabeled S and P, and we may write

$$f = -\frac{1}{2}\mathrm{d}(s - p)/\mathrm{d}t$$

[Note that the factor of $^1/_2$ arises because p rises as s falls, so $(s - p)$ changes twice as fast as s.] Substitution in an equation analogous to eq 11 then gives

$$\mathrm{d}\ln s' = (\Phi_{1,2,3}/2\Phi_S)\mathrm{d}\ln(s - p)$$

where $\Phi_{1,2,3}$ and Φ_S are tritium fractionation factors. Integration gives

$$\ln(s'/s_0') = (\Phi_{1,2,3}/2\Phi_S)\ln\lambda \qquad (27)$$

where $\lambda = (s - p)/c$. This equation describes the tritium content of the substrate as a function of the extent of the reaction.

Double-Competitive Deuterium Washout Experiments. Finally, the ratio $\phi_{1,2,3}'/\phi_{1,2,3}''$ can be measured most accurately by carrying out a double-competitive deuterium washout experiment. This experiment is clamped in the sense that $s \approx p$ throughout the experiment. The starting conditions are $s_0' = p_0'' = s_0 = p_0$.

For the loss of P″ we write the equivalent of eq 2:

$$f'' = k_{3,2,1}''e_2{\cdot}p'' \qquad (28)$$

Equations 2, 3, and 28, together with the fact that $\phi_S = \phi_P$, give

$$\mathrm{d}\ln s' = \xi\mathrm{d}\ln p'' \qquad (29)$$

where

$$\xi = \phi_{1,2,3}'/\phi_{1,2,3}'' \qquad (30)$$

Integration of eq 29 yields

$$\frac{s' - p''}{s_0'} = \sigma' - (\sigma')^{1/\xi} \qquad (31)$$

Because S and P equilibrate more rapidly than the deuterium label washes out of either S′ or P″, because there is not much difference between $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$ so that S and P are formed from P″ and S′, respectively, at almost the same rate, and because the experiment is under clamped conditions, the concentration of S is approximately equal to that of P throughout the whole reaction. The perturbation in the optical rotation is caused by the small imbalance in the concentrations of S′ and P″ given by eq 31. Differentiation of this equation shows that the maximum value of the perturbation is given by

$$\left(\frac{s' - p''}{s_0'}\right)_{\max} = 4\lambda_{\max} = [1 - \xi]\xi^{\xi/(1-\xi)} \qquad (32)$$

where $\lambda = (s + s' - p - p')/c$. The factor of 4 in eq 32 derives from the fact that the starting conditions for this experiment were $s_0' = p_0'' = s_0 = p_0$.

Results and Discussion

Proton Transfer from Substrate to Product. In the isomerization of a deuterated or tritiated sample of proline, we need to know the fate of the isotopic label so as to be able to interpret both the rate measurements and isotopic discrimination experiments involving isotopically labeled substrates. For instance, in experiments described later in this paper, the rate of release of tritium from [2-^{3}H]proline into the solvent is followed, and we must know whether there is significant *transfer* of ^{3}H from one enzyme catalytic base to the other and how much of the isotopic label that starts in L-proline (H′ in S in Scheme I) ends up in the product D-proline (H″ in P in Scheme I). This can occur even for a two-base mechanism such as that of proline racemase if H′ in E_2 (which derived from substrate S) is transferred from the left-hand enzyme base (in E_2) to the right-hand enzyme base (to make E_1) and then is delivered to a new substrate molecule in a second turnover.

Two experiments from Abeles' group suggest that the extent of hydrogen isotope transfer between bases at the active site of proline racemase is small. First, Cardinale and Abeles (1968) showed that the enzyme-catalyzed racemization of L-proline and of D-proline in D_2O resulted in the *initial* incorporation of about one deuterium atom in the product. Had there been substantial *proton* transfer from one enzyme base to the other, the product enantiomer would have contained significantly less than one deuterium atom. Second, Rudnick and Abeles (1975) showed that the initial rate of racemase-catalyzed release of ^{3}H from L-[2-^{3}H]proline followed saturation kinetics as the concentration of substrate was increased. This implies that enzyme-bound tritium that derives from L-[2-^{3}H]proline cannot be captured by the reaction of a second molecule of L-proline. Neither of these experiments, however, provides an especially sensitive test of a small extent of proton transfer between the two catalytic bases of the enzyme, and this question was addressed directly as will be described.

To establish whether the product of the proline racemase reaction contains a proton derived *only* from the solvent pool, L-[2-^{3}H]proline was used as substrate for the enzyme in ^1H_2O. The L enantiomer was chosen both because it can be completely freed from trace contamination by D-[2-^{3}H]proline by treatment with D-amino acid oxidase and because the oxidase can be used specifically to analyze for tritium at C-2 of the product D-proline. The oxidase stereoselectively takes D-proline to Δ^1-pyrroline-2-carboxylate and releases the C-2 proton to the solvent. From the kinetic parameters of the oxidase (Marcotte & Walsh, 1978; Wellner & Scannone, 1964), one part of D-proline can be detected in the presence of 15000 parts of the L enantiomer. Provided, therefore, that the oxidase does not show a large isotopic discrimination against tritiated proline, this enzyme can be used to measure a small amount of D-[2-^{3}H]proline in the presence of a large excess of L-[2-^{3}H]proline. Independent experiments showed that D-amino acid oxidase indeed shows very little discrimination ($\sim$1.1-fold) against tritiated substrate, which ensures its suitability for this analysis. When L-[2-^{3}H]proline was used as substrate for the racemase, and the reaction was stopped after 4.3% conversion to D-proline, the amount of ^{3}H released into the solvent by D-amino acid oxidase was less than 0.1% of that expected if complete tritium transfer from L-proline to D-proline had occurred in the racemase-catalyzed reaction. These results are shown in Figure 1.

We therefore conclude that there is negligible incorporation of tritium from L-[2-^{3}H]proline into the product D-proline during the racemization catalyzed by proline racemase. A tritium label on substrate L-[2-^{3}H]proline is abstracted by the enzyme, and a solvent-derived proton is delivered to give the product D-proline. The enzyme-bound tritium that comes from L-[2-^{3}H]proline cannot be captured by another molecule of L-proline, even at 200 mM. While, as Rudnick and Abeles (1975) have shown, tritium relinquished by L-[2-^{3}H]proline

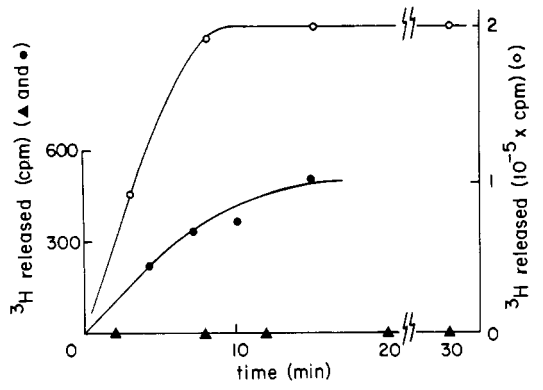


FIGURE 1: Analysis of the tritium content of D-proline in samples of [2-^{3}H]proline. Radioactivity at C-2 of D-proline was released into solvent by D-amino acid oxidase in the presence of FAD and catalase. Samples (100 μL) of the reaction mixture (1 mL) were quenched in aqueous sodium benzoate, from which the solvent was isolated for counting. (▲) Tritium released from proline (56.1 mM L-[2-^{3}H]-proline; 2.55 mM D-proline) isolated after partial racemization of L-[2-^{3}H]proline by proline racemase. (○) Tritium released from an artificial mixture of D-[2-^{3}H]proline (2.55 mM, ~10^6 cpm μmol^{-1}) and L-proline (56.1 mM). (●) Tritium released from an artificial mixture prepared from the real sample (56.1 mM L-[2-^{3}H]proline; 2.55 mM D-proline) to which D-[2-^{3}H]proline (~5400 cpm) was added to simulate a 1% transfer of tritium from L-proline to D-proline.

Table I: Reactant Deuterium Fractionation Factors[a] for C-2 of Proline[b]

m_{D_2O}[c]	ϕ_S	ϕ_P
0.49_8	1.18 ± 0.06	1.14 ± 0.02
0.49_3	1.23 ± 0.05	1.12 ± 0.02
0.49_2	1.25 ± 0.01	1.12 ± 0.02
mean value $\phi_S = \phi_P = 1.17 \pm 0.02$		

[a] Following the practice recommended earlier (Albery & Davies, 1969), fractionation factors are defined relative to HDO as solvent [see Albery (1975)]. On this basis, the values are about 3% lower than if H_2O had been used as the standard. [b] The error limits are precision estimates only. [c] m_{D_2O} is the mole fraction of deuterium in the solvent.

can be captured by D-proline at high concentrations (regenerating L-[2-^{3}H]proline), *this tritium is never transferred to the other base at the active site.* The substrate-derived proton therefore dissociates from the enzyme (after the product proline has departed) into free solution, rather than finding the other enzyme base. This fact demands two forms of the free enzyme that must differ minimally in their state of protonation.

Reactant Fractionation Factors. The reactant fractionation factors for L- and D-proline (ϕ_S and ϕ_P) were determined by allowing a racemic mixture of proline to equilibrate in an approximately equimolar mixture of H_2O and D_2O. The deuterium content of the proline, m_D, was measured, and the fractionation factors were calculated (Gold, 1960) from

$$\phi_S = \phi_P = \frac{m_D}{1 - m_D} \frac{(1 - m_{D_2O})^{1/2}}{(m_{D_2O})^{1/2}} \quad (33)$$

where m_{D_2O} is the mole fraction of deuterium in the solvent. The results are reported in Table I.

Clamped Deuterium Washout Experiments. In these experiments, one deuterated proline enantiomer is racemized (with the concomitant loss of its 2-^{2}H label) in the presence of a pool of equilibrated unlabeled proline. Measurements of the optical rotation as a function of time provide the normalized parameter λ, where

$$\lambda = (s + s' - p)/c \quad (34)$$

Figure 2 shows typical results obtained for the last half of

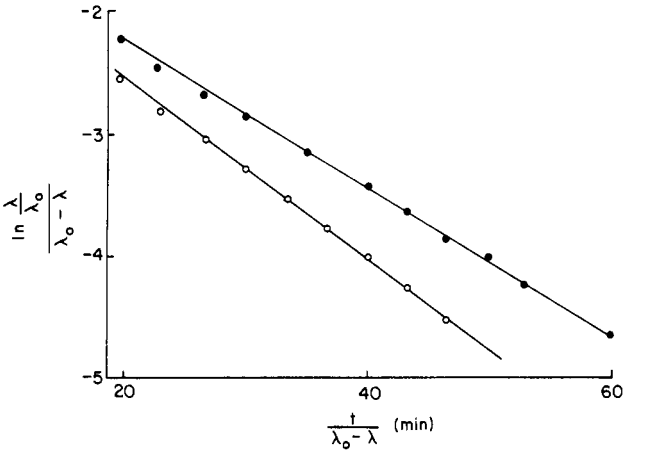


FIGURE 2: Plots of $\ln (\lambda/\lambda_0)/(\lambda_0 - \lambda)$ vs. $t/(\lambda_0 - \lambda)$ for the enzyme-catalyzed isomerization of L- or D-[2-^{2}H]proline in the presence of DL-proline. L-[2-^{2}H]proline (16.2 mM) and DL-proline (16.2 mM) (●); D-[2-^{2}H]proline (16.5 mM) and DL-proline (16.5 mM) (○). All reactions were run in 200 mM Tris-HCl buffer, pH 8.0, at 37 °C. The enzyme concentration was 5.03×10^{-8} M.

Table II: Results from Clamped Deuterium Washout Experiments

	labeled substrate	
	L-[2-^{2}H]proline (S′)	D-[2-^{2}H]proline (P″)
e_Σ (nM)	50.3	50.3
s_0 (mM)	8.1	8.25
p_0 (mM)	8.1	8.25
s_0' (mM)	16.2	
p_0'' (mM)		16.5
c (mM)	32.3	32.9
k_I'[a] (s^{-1})	650	805
k_I[b] (s^{-1})	3800	3800
$\phi_{1,2,3}'/\phi_S = 0.34$;[c] $\phi_{1,2,3}''/\phi_P = 0.38$[c]		

[a] From the gradients of plots of eq 16 (see Figure 2). [b] Calculated from eq 20 and 34. [c] Calculated from eq 18.

clamped experiments starting with L-[2-^{2}H]proline or with D-[2-^{2}H]proline, plotted according to eq 16. Good straight lines are obtained, confirming the theoretical treatment and the effectiveness of clamping, particularly toward the end of the reaction. Values of k_I' can be found from the gradients of the lines in Figure 2. From the data in Table III of Fisher et al. (1986a) we can calculate the corresponding values of k_I, using eq 20 and the values of $c_D = 2.9$ mM and $c_P = 125$ mM [from Fisher et al. (1986b)]. Use of eq 18 then yields $\phi_{1,2,3}'/\phi_S$ and the equivalent parameter in the reverse direction, $\phi_{1,2,3}''/\phi_P$. These results are collected in Table II.

Competitive Deuterium Washout Experiments. In these experiments, we start with equal concentrations of the two enantiomers of proline, *one* of which is deuterated. As described above, the existence of a deuterium isotope effect causes a transient perturbation in the optical rotation. Values of the optical rotation from such progress curves are converted into values of λ, and typical plots of 2λ vs. time for both the S′ → P and P″ → S reactions are shown in Figure 3.

Values of R can be derived both from the size of the maximum perturbation (using eq 26) and from the isotopic content of the substrates at the maximum (using eq 25). These data are reported in Table III, and it is gratifying that very similar values for R are found from the two different experiments. [The results from the size of the maximum are likely to be more reliable than those from the isotopic content at the maximum, since the precise timing of the latter measurement is not easy.] From the parameter R, values of $\phi_{1,2,3}'/\phi_S$ and $\phi_{1,2,3}''/\phi_P$ can be obtained (eq 24), and these results compare well with those in Table II. It should be emphasized that these

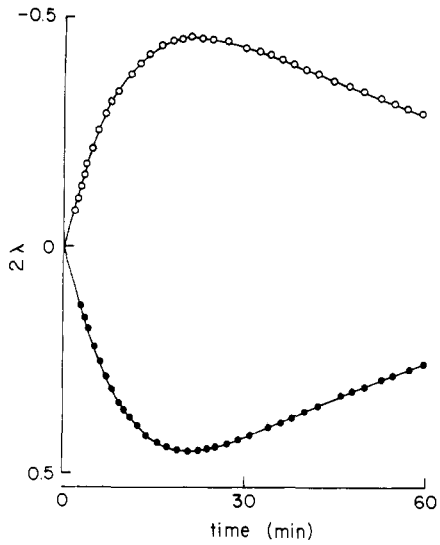


FIGURE 3: Perturbation experiments using L- or D-[2-^{2}H]proline in the presence of an equimolar amount of the unlabeled proline enantiomer. Two times the normalized optical rotation, λ, is plotted vs. time. Lower curve (●), L-proline (20 mM) + D-[2-^{2}H]proline (20 mM); upper curve (○), D-proline (20 mM) + L-[2-^{2}H]proline (20 mM). The reactions were run in 200 mM Tris-HCl buffer, pH 8.0, at 37 °C. The enzyme concentration was 1.82×10^{-8} M.

Table III: Results from Competitive Deuterium Washout Experiments

	L-[2-^{2}H]-proline (S′) + D-proline (P)		D-[2-^{2}H]-proline (P″) + L-proline (S)
p_0 (mM)	20	s_0 (mM)	20
s_0' (mM)	20	p_0'' (mM)	20
$(s'/s_0')_{max}$[a]	0.69	$(p''/p_0'')_{max}$[a]	0.70
R[b]	5.7	R	5.9
$[(s + s' - p)/s_0']_{max}$[c]	0.46	$[(p + p'' - s)/p_0'']_{max}$	0.45
R[d]	5.9	R	5.7
$\phi_{1,2,3}'/\phi_S$[e]	0.34	$\phi_{1,2,3}''/\phi_P$	0.35

[a] Deuterium content of S or P at the optical rotation maximum. [b] Calculated from eq 25. [c] Normalized size of the optical rotation maximum (see Figure 3). [d] Calculated from eq 26. [e] Calculated from eq 24 using the results for R from the optical rotation maximum.

Table IV: Results from Tritium Washout Experiments

	L-[2-^{3}H]proline		D-[2-^{3}H]proline
s_0 (mM)	30	p_0 (mM)	32
$\Phi_{1,2,3}'/\Phi_S$[a]	0.182	$\Phi_{1,2,3}''/\Phi_P$[a]	0.202
	0.184		0.205
mean value	0.183	mean value	0.204
$\phi_{1,2,3}'/\phi_S$[b]	0.31	$\phi_{1,2,3}''/\phi_P$[b]	0.33

[a] From gradients of plots of eq 27; see Figure 4. [b] Calculated from Swain–Schaad relation (Swain et al., 1958).

two sets of results derive from quite different kinds of experiments. Those in Table II are from velocity measurements of deuterated substrates under clamped conditions, and those in Table III are from deuterium content measurements at the optical rotation maxima.

Tritium Washout Experiments. In these experiments, the tritium content of [2-^{3}H]proline is followed as one labeled enantiomer is racemized. Typical results for tritium washout experiments in each direction are plotted according to eq 27 in Figure 4. Good straight lines are found, and from the gradients we obtain the values of $\Phi_{1,2,3}'/\Phi_S$ and $\Phi_{1,2,3}''/\Phi_P$ collected in Table IV. We have applied the Swain–Schaad relation (Swain et al., 1958) to the tritium fractionation factors

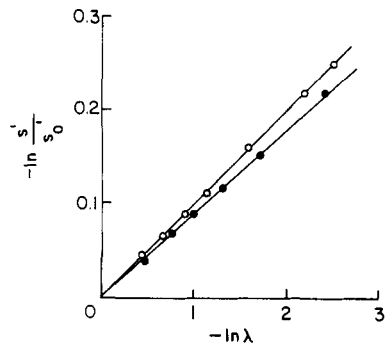


FIGURE 4: Tritium washout from L-[2-^{3}H]proline (●) and from D-[2-^{3}H]proline (○) catalyzed by proline racemase, in 200 mM Tris-HCl buffer, pH 8.0. The experimental data are plotted according to equation 27. The initial substrate concentrations were the following: L-[2-^{3}H]proline, 36 mM; D-[2-^{3}H]proline, 32 mM.

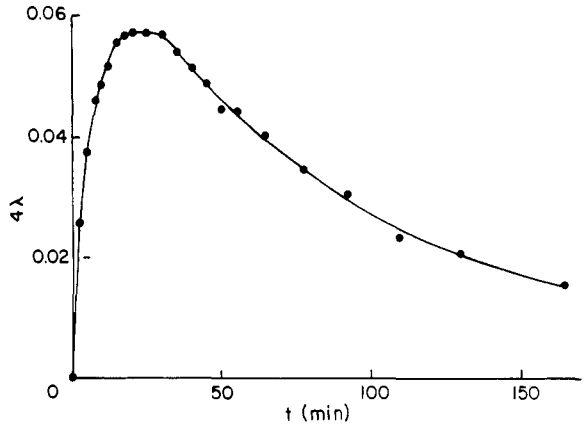


FIGURE 5: Double-competition experiment, $s_0 = p_0 = s_0' = p_0''$. The starting concentrations of L-proline, D-proline, L-[2-^{2}H]proline, and D-[2-^{2}H]proline were each 23.85 mM. Four times the normalized optical rotation, λ, is plotted vs. time. The ratio $\phi_{1,2,3}'/\phi_{1,2,3}''$ is then obtained from the value of $4\lambda_{max}$ (eq 32).

Table V: Values for $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$

type of experiment	data from Table	$\phi_{1,2,3}'$	$\phi_{1,2,3}''$
clamped ^{2}H washout	II	0.40	0.49
competitive ^{2}H washout	III	0.40	0.41
^{3}H washout	IV	0.36	0.39
preferred values		0.37_5	0.44

so that they may be compared [*pace* any small deviation arising from the fact that these are mixed factors (Northrop, 1975; Albery & Knowles, 1977)] with the results in Tables II and III using the deuterated substrates. Reasonable agreement is found.

Double-Competitive Deuterium Washout Experiments. The results presented so far in Tables II–IV suggest that $\phi_{1,2,3}'$ is smaller than $\phi_{1,2,3}''$. That is, the isotope effects for the reaction of the diastereoisomeric complexes E_1S' and E_2P'' (to $E_2'P$ and $E_1''S$, respectively) are slightly different. To determine the ratio $\phi_{1,2,3}'/\phi_{1,2,3}''$ precisely, the reaction of an equimolar mixture of each of the two [2-^{2}H]prolines is followed. The slightly different isotope effects cause a transient perturbation in the optical rotation. A typical result for substrate concentrations in the saturated region is shown in Figure 5. From eq 32, we find that

$$\xi = \phi_{1,2,3}'/\phi_{1,2,3}'' = 0.86 \qquad (35)$$

Fractionation Factors $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$. In Table V we collect together the results for the fractionation factors $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$ obtained from the different types of experiment described in this paper. We have used the value of $\phi_S = \phi_P$

from Table I to remove the reactant fractionation. Good agreement is found for the results from the different types of experiment. Values from competitive deuterium washout experiments are more precise than those from the other experiments, since competitive experiments are unaffected by such variables as enzyme activity. For example, the values obtained from the clamped deuterium velocity measurements depend on the enzyme activity being precisely the same as in the all-hydrogen experiments (Fisher et al., 1986a) and this is harder to ensure. The tritium washout experiments are competitive, but measurement of tritium content is in this case less reliable than that of deuterium. Furthermore, the tritium fractionation factors have to be scaled by using the Swain–Schaad relation, and this may not hold exactly if $\phi_{1,2,3}$ is a mixed fractionation factor (Albery & Knowles, 1976; Kresge et al., 1977). On the other hand, the ratio of the fractionation factors is accurately measured by the double-competitive deuterium washout experiment. The preferred values of $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$ given in Table V are in accord with this ratio. It is interesting that $\phi_{1,2,3}'$ and $\phi_{1,2,3}''$ are not equal. This is, of course, possible despite the obvious symmetry of the reaction, because the two enzyme–substrate complexes are diastereoisomeric, and the two proton sites are not identical in these complexes.[4]

The size of both the fractionation factors shows that there is significant protonic motion at each site in at least one kinetically significant transition state. The transition state that involves the protonation and deprotonation of proline (the transition state of step 2, in Scheme I) must be at least partially rate limiting and, if it is a single transition state, must involve proton motion at both sites. In this case the two proton transfers (of H′ and H″) would be concerted. Another possibility, however, is that the proton transfers occur in a stepwise manner and that step 2 (Scheme I) really describes two sequential transfers. In this case, even if steps 1 and 3 are faster than step 2 and $\phi_{1,2,3} = \phi_2$, ϕ_2' and ϕ_2'' would each be mixed fractionation factors describing the proton in flight (in one transition state, 2A) and the same proton bound to the enzyme (in the other transition state, 2B). This could explain why the observed fractionation factors are somewhat large for protons in flight. Experimental approaches to defining the nature of step 2 are presented in the next two papers (Belasco et al., 1986a,b).

Finally, from the fractionation factors derived in this paper, we have the primary kinetic isotope effect (k_H/k_D) for the reaction of D-[2-^{2}H]proline, which is $\phi_P/\phi_{1,2,3}'' = 2.66$ (Tables I and V). This can be compared with the isotope effect of 2.6 derived by Cleland (1977) from the original results of Cardinale and Abeles (1968) for the reaction of L-proline in D_2O. This is, however, an inexact comparison, both because that reaction was run in D_2O not H_2O and, more seriously, because the total concentration of substrate used (67 mM) will have meant a more substantial contribution from oversaturation [i.e., from the transition state(s) for free enzyme interconversion: see Figure 11 of Fisher et al. (1986a)].

[4] Professor W. W. Cleland has pointed out that a possible source of this nonidentity is a difference in the steric constraint on the torsional motion of the protons on the two active site bases of the enzyme.

Registry No. $^2H_2$, 7782-39-0; $^3H_2$, 10028-17-8; proline racemase, 9024-09-3.

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