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Mechanism of Action of Aspartase. A Kinetic Study and Isotope Rate Effects with ^2H†

Thomas B. Dougherty, Virginia R. Williams,‡ and Ezzat S. Younathan*

ABSTRACT: The mechanism of the aspartase-catalyzed reaction has been investigated at pH 7.0 in the presence of 1 mM Mg^{2+} by kinetic analysis and isotope rate studies with ^2H. Product inhibition and initial velocity patterns for the forward and reverse reactions, respectively, are consistent with a random mechanism in which all steps prior to the interconversion of the central complexes are in rapid equilibrium. The Michaelis, dissociation, and inhibition constants for fumarate and NH_4^+, as well as the Michaelis constant for 2*S*-aspartate, have been determined. No primary isotope effect was observed when the initial rates of deamination of 2*S*,3*R*-[3-^2H]aspartate and unlabeled 2*S*-aspartate were compared. However, a secondary isotope rate effect of 1.11 ± 0.02 was obtained with 2*S*-[2-^2H]aspartate. Provided that the release of products is not rate limiting, these findings suggest that the rate-determining step of the reaction could be C–N bond breakage and imply that, as the amino group leaves, the resulting intermediate could be accompanied by considerable carbonium-ion development at carbon 2. The possibility that the release of one of the products could be rate limiting is discussed.

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the conversion of 2*S*-aspartate (equivalent to L-aspartate) to fumarate and NH_4^+ in the following reversible reaction

$$^-OOCCH_2\underset{\displaystyle NH_3^+}{\underset{|}{C}}HCOO^- \rightleftharpoons {}^-OOCCH{=}CHCOO^- + NH_4^+$$

In the direction of fumarate amination, NH_4^+ can be replaced by hydroxylamine as an alternate substrate (Emery, 1963).

Aspartase has long been regarded as a catabolic enzyme in the amino acid metabolic schemes of various kinds of bacteria and plants. However, unlike most catabolic reactions, the aspartase reaction is readily reversible, and its equilibrium constant actually favors aspartate formation (Bada and Miller, 1968). This, together with the allosteric properties of the enzyme (Williams and Lartigue, 1967, 1969), suggests that aspartase may be a regulatory enzyme that could function synthetically, particularly under conditions in which aspartate was removed. In fact, Chibata *et al.* (1970) have patented a method for synthesizing 2*S*-aspartic acid with 83% yield by passing ammonium fumarate through a TEAE-cellulose column to which aspartase has been bound.

Englard (1958) and Krasna (1958) have shown independently that the addition of NH_4^+ to fumarate and its elimination from 2*S*-aspartate are stereospecific. Using the trans nature of the fumarate–hydratase reaction as a model, Gawron and Fondy (1959) have concluded from the data of Krasna

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‡ Deceased June 16, 1970.

(1958) that the steric course of the aspartase reaction is trans. However, the chemical mechanism of this reaction is uncertain. Furthermore, a kinetic scheme for the reaction has not been suggested in the literature. In this report, a kinetic mechanism is proposed based upon initial velocity studies of fumarate amination and product inhibition studies of 2*S*-aspartate deamination. The rate-limiting step of the reaction sequence has been investigated with 2*S*-aspartate stereospecifically labeled with deuterium at carbons-2 or -3, and certain organic mechanistic implications of the results are discussed.

Experimental Procedure

Reagents and Materials. 2*S*-Aspartic and fumaric acids were obtained from K and K Laboratories and Eastman Organic Chemicals, respectively. These acids were converted to their potassium salts, pH 7.0, for use in the various kinetic studies. ^2H_2O (99.8 atom % ^2H) was a product of International Chemical and Nuclear Corp. Glutamate–oxaloacetate transaminase with bound pyridoxal phosphate and with a specific activity of 10^5 units/mg of protein was purchased from Worthington Biochemical Corp.

Sephadex G-200 and DEAE-Sephadex A-50 (capacity 3.5 ± 0.5 mequiv/g) were obtained from Pharmacia Fine Chemicals, Inc. Dowex 50W-X10 (H^+ form) and Bio-Gel hydroxylapatite were from Bio-Rad Laboratories. Dowex 1-X8 (Cl^- form) of reagent grade (J. T. Baker Chemical Co.) was converted to the acetate form with 3 M potassium acetate by the procedure of Hirs *et al.* (1954).

All other chemicals were reagent grade.

Aspartase Assay. During its purification, aspartase was routinely assayed by the method of Williams and Lartigue (1967). Unless otherwise indicated, the initial velocity was determined by the change in absorbance at 240 nm and 29° in a Beckman Model DB spectrophotometer.

For all kinetic studies, the reaction mixture contained in a total volume of 3.0 ml: 50 mM Tris-HCl buffer, pH 7.0; 1 mM $MgSO_4$; 0.1 mM EDTA; and appropriate concentrations of substrate(s) and, when tested, of product. Magnesium ion was present at a concentration approximately 60 times its K_m[1] as determined for aspartase from *Escherichia freundii* by Sekijo *et al.* (1965). Stock solutions of substrates or products were freshly prepared prior to each study.

In the product inhibition studies of the deamination reaction, K^+ concentration was maintained at 50 mM. The initial rates of deamination in the presence of potassium fumarate were obtained with a Cary Model 14 recording spectrophotometer. Cuvets containing the standard assay mixture with fumarate at concentrations corresponding to those in the sample cells but without enzyme were used as references. On the other hand, inhibition by NH_4^+ in the direction of aspartate deamination was observed with a Gilford Model 2000.

As 2*S*-aspartic acid could be prepared with nearly complete ^2H incorporation at stereoselective locations in the molecule, kinetic isotope effects were determined from a comparison of the initial deamination rates ($v_{^2H}$) of 2*S*,3*R*-[3-^{2}H]aspartate and 2*S*-[2-^{2}H]aspartate with those (v_H) of unlabeled 2*S*-aspartate. An isotope effect for a particular labeled substrate was considered to exist if the corresponding value of $v_H/v_{^2H}$ was significantly greater than unity. The composition of the reaction mixture was identical with the standard assay except that the concentration of the substrates was varied over a predetermined range.

Protein concentration was determined by the procedure of Lowry *et al.* (1951) or the method of Exton (1925). Both methods were standardized with bovine serum albumin.

Preparation of Aspartase. Aspartase was prepared according to the scheme of Williams and Lartigue (1967) from *Enterobacter aerogenes*, subspecies *alvei*. However, several changes were incorporated in the present procedure which should be mentioned.

The harvested cells were suspended in 3 volumes of cold 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM mercaptoethanol, and were subjected to sonication for 5 min at maximum amperage. All potassium phosphate buffers used in subsequent steps were also 1 mM in mercaptoethanol.

During pH fractionation, the supernatant retained from the previous protamine sulfate precipitation was brought to pH 4.6 by the rapid addition of 2 M acetic acid. The resulting suspension was centrifuged at 27,000*g* for 30 min. The precipitate was resuspended in a solution of 0.05 M phosphate buffer, pH 7.0, containing 10 μM $MgSO_4$. The volume used was one-third of that of the supernatant remaining after precipitation with protamine sulfate.

The above suspension was centrifuged at the same speed for 20 min, and the supernatant was decanted and brought to 35% saturation with $(NH_4)_2SO_4$. After being stirred for 15 min, the suspension was centrifuged for 30 min and the precipitate discarded. The supernatant was brought to 55% saturation with $(NH_4)_2SO_4$, stirred, and centrifuged to collect the precipitate.

For further purification of the enzyme, columns of the following materials and sizes were used: Sephadex G-200 (jacketed, 3 × 33 cm), hydroxylapatite (1.0 × 10 cm), and DEAE-Sephadex A-50 (1.5 × 65 cm). Each column was equilibrated with 0.05 M phosphate buffer, pH 7.0, before use.

The $(NH_4)_2SO_4$ precipitate was dissolved in 3–5 ml of 0.05 M phosphate buffer, pH 7.0, and placed on the column of Sephadex G-200. Elution was achieved with the same buffer solution using a flow rate of 5–10 ml/hr. The eluent was collected in 5-ml fractions. Those fractions containing aspartase activity were combined and placed on the column of hydroxylapatite. The column was eluted successively at pH 7.0 with 25-ml volumes of 0.1, 0.2, and 0.3 M phosphate buffer solutions and with a 50-ml volume of a 0.5 M phosphate buffer solution. The recovered aspartase was dialyzed overnight in 0.05 M phosphate buffer, pH 7.0. The preparation was then placed on the DEAE-Sephadex column. This column was washed successively with 100-ml volumes of 0.05 M phosphate buffer solutions, pH 7.0, containing 0.0, 0.15, and 0.25 M KCl. The enzyme was eluted with a 0.05 M phosphate buffer solution, pH 7.0, containing 0.3 M KCl.

The aspartase preparations used in the kinetic studies were essentially fumarase free and had a specific activity of approximately 60 μmoles of fumarate formed/min per mg of protein.

Synthesis of Deuterium-Labeled 2S-Aspartic Acid. 2*S*-[2-^{2}H]Aspartic acid was prepared by a variation of the procedure of Tamiya and Oshima (1962). In a typical preparation, 200 mg of 2*S*-aspartic acid was dissolved in approximately 7 ml of distilled water with the addition of 5 N KOH to obtain the potassium salt. The pH of the solution was adjusted to 7.6 with 0.1 N KOH. After 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.6, was added, the solution was lyophilized to dryness. The residue was dissolved in about 10 ml of ^2H_2O;

[1] Abbreviations used are: K, K_d, and K_i, Michaelis, dissociation, and inhibition constants, respectively; A, 2*S*-aspartate; P, NH_4^+; Q, fumarate; V_2 and v_2, the maximal and initial velocities of the aspartase reaction, respectively, in the reverse direction; E, the free enzyme saturated with Mg^{2+}.

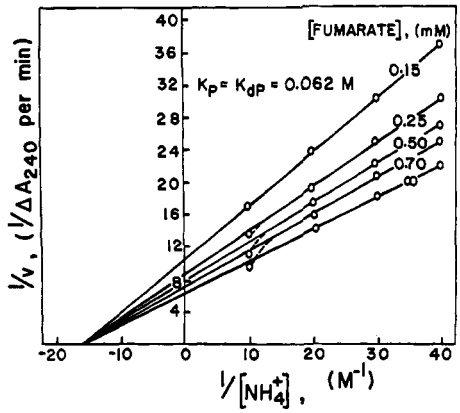


FIGURE 1: Double-receiprocal plots of the initial rate of fumarate amination as a function of NH_4^+ concentration at the indicated concentrations of fumarate. The curve for infinite fumarate concentration is a replot of the extrapolated intercepts from the pattern in Figure 2.

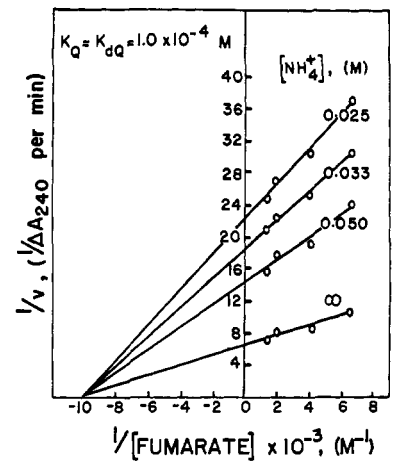


FIGURE 2: Double-receiprocal plots of the initial rate of fumarate amination as a function of fumarate concentration at the indicated concentrations of NH_4^+. The curve for infinite NH_4^+ concentration is a replot of the extrapolated intercepts from the pattern in Figure 1.

the lyophilization was repeated, and the residue was redissolved in ^2H_2O and brought to a final volume of 10 ml. Glutamate–oxaloacetate transaminase (1 mg) and a drop of toluene were added to the solution.

After the solution was incubated at room temperature for 12 hr, the reaction was stopped by heating the solution to 100° for 5 min. Activated charcoal (25 mg) was added, and the suspension was filtered. The aspartate was isolated and purified as the acid by passage first through a column of Dowex 50W-X10 (H^+ form) and then through a column of Dowex 1-X8 (acetate form). The acid was subsequently recrystallized from ethanol and water. The nuclear magnetic resonance (nmr) spectrum of the 2*S*-[2-^{2}H]aspartic acid in ^2H_2O containing 2 N trifluoroacetic acid (Smith and Ihrig, 1969) indicated that exchange had occurred at the α carbon to an extent greater than 90%.

2*S*,3*R*-[3-^{2}H]Aspartic acid was synthesized by a reversal of the aspartase reaction. $MgSO_4$ (0.7 ml, 30 mM), 0.7 ml of 3 mM EDTA, 270 mg of NH_4Cl, and 6.7 ml of 0.15 M Tris-HCl buffer (pH 7.0) were added to 10 ml of 0.4 M dipotassium fumarate (pH 7.0). The solution was lyophilyzed to dryness. The residue was dissolved in approximately 20 ml of ^2H_2O; the lyophilization was repeated, and the resulting residue was redissolved in ^2H_2O. About 70 units of aspartase, previously dialyzed in a ^2H_2O solution of 0.01 M potassium phosphate, pH 7.0, was then added. The volume of the reaction solution was brought to 20 ml with ^2H_2O.

After an incubation period of 9 hr, an additional 30 units of the aspartase preparation was added, and the incubation was continued for 4 hr. The reaction was stopped by lowering the pH of the mixture to 4.6 with HCl and heating the solution to 100° until protein began to precipitate. Following filtration of the precipitated protein, the 2*S*,3*R*-[3-^{2}H]aspartate was recovered and purified as the acid by ion-exchange chromatography and recrystallized from ethanol and water. The proton-to-deuteron ratio at carbon-3 of the acid was shown to be 1:1 by nmr.

Treatment of Data. Typical double-reciprocal plots of initial velocity *vs.* substrate concentration have been shown to be hyperbolic (curved downward) at high substrate concentrations (Williams and Lartigue, 1967). Nevertheless, as discussed later, the likely cause of this hyperbolicity should not affect conclusions drawn concerning the kinetic scheme of aspartase by restricting the analysis to the linear portions of such plots. Consequently, data from the linear part of each double-reciprocal plot were routinely fitted to a linear equation by a regression program.

The kinetic constants were calculated from replots of the slopes against the inhibitor concentration (from product inhibition patterns) and from replots of the intercepts *vs.* the reciprocal of the nonvaried substrate (from initial velocity patterns of the amination reaction). When applicable, some kinetic constants were determined directly from the appropriate primary plots.

Results

Initial Velocity Studies of the Amination Reaction. The initial velocity pattern obtained for varying NH_4^+ concentration in the presence of fixed levels of fumarate is shown in Figure 1. The data from this pattern were replotted in Figure 2 to show the initial velocity pattern for varying fumarate in the presence of fixed levels of NH_4^+. In each case, the family of linear plots intersected on the abscissa to the left of the ordinate suggesting that the apparent K_d and K were equivalent for each substrate. In addition, the extrapolated intercepts of Figure 2, which represent the apparent maximal velocities at infinite fumarate concentration, were replotted against the reciprocal of NH_4^+ concentration in Figure 1 (∞ line). Likewise, the intercepts of Figure 1 were replotted *vs.* the reciprocal of fumarate concentration in Figure 2 (∞ line). The data appear to fit eq 1 for a sequential mechanism in which $K_P \simeq K_{dP}$ and $K_Q \simeq K_{dQ}$.

$$\frac{V_2}{v_2} = \left(\frac{K_P}{P} + 1\right)\left(\frac{K_Q}{Q} + 1\right) \quad (1)$$

Product Inhibition Studies of the Deamination Reaction. As illustrated in Figure 3, NH_4^+ was found to inhibit competitively the deamination of 2*S*-aspartate. The linearity of the slope replot given in the inset of Figure 3 suggested that the inhibition was linear within the concentration range of NH_4Cl used. Although not shown here, the K_i for NH_4^+ obtained from the intersection of linear Dixon plots (Dixon and Webb, 1964) of $1/v$ against NH_4^+ concentration agreed with the K_i calculated from the slope replot.

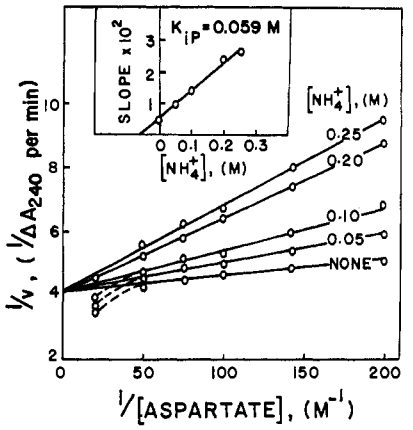


FIGURE 3: Competitive inhibition of the deamination of 2*S*-aspartate by NH_4^+. Inset: Relationship of the slopes of the primary plots to NH_4^+ concentration. Initial velocities were determined at 28°.

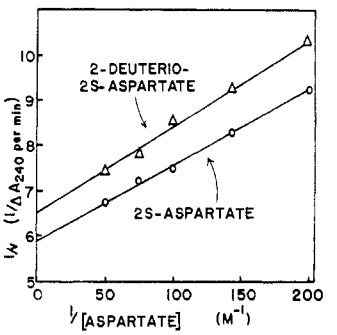


FIGURE 5: Double-reciprocal plots of the initial rate of fumarate production as a function of 2*S*-aspartate and 2*S*-[2-²H]aspartate concentrations. Initial velocities were determined at 28°. $v_H/v_{^2H}$ = 1.11 ± 0.02.

As shown in Figure 4, potassium fumarate, within the concentration range used, also gave competitive inhibition with 2*S*-aspartate as substrate. This is in agreement with the earlier data of Sekijo *et al.* (1965). The linearity of this inhibition was ascertained from the linear slope replot given in the inset of Figure 4. The K_i for fumarate determined from the slope replot and from the intersection of corresponding Dixon plots were in agreement.

Kinetic Constants. The following values were obtained for the kinetic constants of the aspartase reaction at pH 7.0: K_A = 2.0 ± 0.9 mM; $K_P \simeq K_{dP}$ = 62.4 ± 1.3 mM; K_{iP} = 56.9 ± 2.2 mM; $K_Q \simeq K_{dQ}$ = 0.1 ± 0.003 mM; K_{iQ} = 0.089 ± 0.005 mM. The variations are given in terms of one standard deviation. The K's and K_d's for NH_4^+ and fumarate are averages obtained from initial velocity studies in the direction of fumarate amination. The K for 2*S*-aspartate represents the average calculated from the controls in the product inhibition studies. The K_i's are averages obtained from slope replots and Dixon plots derived from the product inhibition patterns.

Kinetic Isotope Effects. The initial deamination rates of 2*S*,3*R*-[3-²H]aspartate were not significantly less than the corresponding rates for unlabeled 2*S*-aspartate. However, as shown by the double-reciprocal plots of Figure 5, significantly slower rates were obtained with the 2*S*-[2-²H]aspartate than with the unlabeled form. This rate difference was interpreted as an apparent secondary isotope effect of 1.11 ± 0.02.

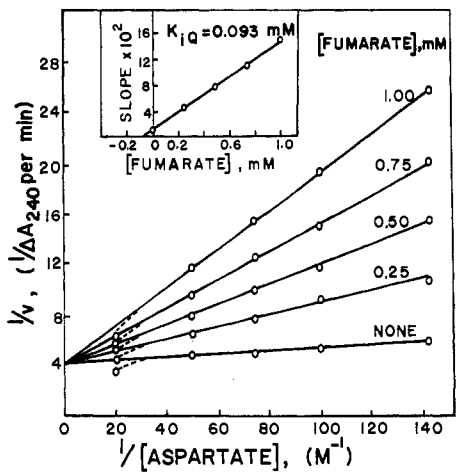


FIGURE 4: Competitive inhibition of the deamination of 2*S*-aspartate by fumarate. Inset: Relationship of the slopes of the primary plots to fumarate concentration. Initial velocities were determined at 26°.

Discussion

As mentioned previously, typical plots of $1/S$ *vs.* $1/v$ for the aspartase reaction, pH 7.0, are 2/1 functions, concaving downward, at high substrate concentrations. Several alternatives may be offered to explain this hyperbolic behavior (Cleland, 1970).

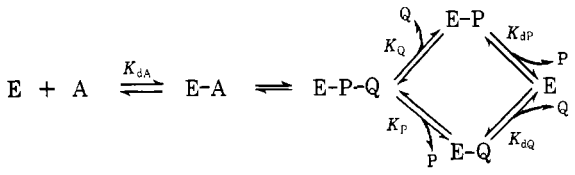
First, one could assume that two enzymes are present which catalyze the same deamination reaction but with different apparent K values. Such an assumption would give nonlinear reciprocal plots which concave downward. But if this were the case, both enzymes would have to possess very similar physical and ionic properties because Lartigue (1965) was unable to achieve separation of aspartase into two fraction with different kinetic parameters.

Since aspartase activity is enhanced by certain divalent metal ions (Wilkinson and Williams, 1961; Depue and Moat, 1961), a second alternative suggests that 2*S*-aspartate and Mg^{2+} bind to the enzyme by a random bireactant mechanism in which the rapid-equilibrium assumption does not hold. However, all kinetic studies were conducted with saturating concentrations of Mg^{2+}. As a result, the reaction should be pseudounireactant wherein all forms of the enzyme are complexed with Mg^{2+}. Moreover, computer-simulated studies (Cleland, 1970) have shown that for realistic values of rate constants, random mechanisms tend to give initial velocity patterns resembling rapid-equilibrium types even if the interconversion of central complexes is not solely rate limiting. For this reason, the tendency for the double-reciprocal plots given in Figure 1 to curve downward may be unreliable as an indicator of the invalidity of the rapid-equilibrium assumption as it applies to fumarate amination.

A third possibility is that aspartase displays negative cooperativity at high substrate concentrations resulting in an increase in both K and V_{max}. This alternative appears most attractive since aspartase has been shown to be composed of four subunits, to exhibit homotropic substrate interactions which are pH dependent, and to be activated by certain mononucleotides under special conditions (Williams and Lartigue, 1967; Williams and Scott, 1968).

Recently, Rudolph and Fromm (1971) reported the purification of an aspartase from *Escherichia coli* which is quite similar in properties to the enzyme from *Enterobacter aero-*

SCHEME I



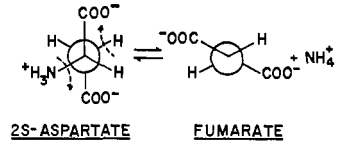


FIGURE 6: The trans nature of the reversible reaction catalyzed by aspartase.

genes used routinely in this laboratory. Their preparation, in contrast to the latter, is very stable and exhibits an almost absolute dependence on the presence of a divalent metal ion for catalysis. This implies that the failure of Williams and Lartigue (1967) to prepare an absolutely Mg^{2+}-dependent aspartase may be the result of a tightly bound metal–enzyme complex, rather than the lack of Mg^{2+} dependence.

Because the stability constant for the complex between Mg^{2+} and 2*S*-aspartate is relatively small (Bright, 1965) and assuming that the 1:1 complex forms with ease only if the α-amino group is uncharged, then at pH 7.0, an insignificant amount of substrate should be complexed by Mg^{2+} (Bada and Miller, 1968). As a consequence, a complex of Mg^{2+} and 2*S*-aspartate probably does not function as a substrate in the present kinetic studies which have been conducted in the presence of 1 mM Mg^{2+}. Bright (1965) has presented evidence that a complex of Mg^{2+} and β-methylaspartate is not a substrate in the deamination reaction catalyzed by β-methylaspartase.

The initial velocity patterns shown in Figures 1 and 2 suggest a sequential mechanism for the aspartase reaction in the direction of fumarate amination. Since the double-reciprocal plots converge on the abscissa in both cases, the attachment of NH_4^+ is independent of the concentration of fumarate, and the attachment of fumarate is independent of the concentration of NH_4^+. In other words, the *K* for each substrate may actually be equivalent to a dissociation constant in which the additional rate constants contained in the *K* are negligibly small. Therefore, in the reverse reaction, NH_4^+ and fumarate could be in rapid equilibrium with the enzyme forms prior to the E–P–Q complex. This would further imply that in the direction of 2*S*-aspartate deamination, the departure of products from the E–P–Q complex may not be rate limiting. Caution should be exercised, however, when using kinetic data of this type alone to establish unequivocally the location of the rate-determining step of a sequential mechanism. Nevertheless, from these studies, a kinetic mechanism in which there is a rapid-equilibrium ordered addition of substrates may be eliminated. For such a mechanism, the appropriate rate equation predicts that varying the second substrate in the presence of fixed levels of the first yields double-reciprocal plots that intersect on the ordinate (Cleland, 1970). In neither pattern of Figures 1 and 2 did an ordinate intersection occur.

From product inhibition studies of aspartate deamination, the competitive patterns illustrated in Figures 3 and 4 indicate that both fumarate and NH_4^+ compete with 2*S*-aspartate for the free Mg^{2+}–enzyme complex. Moreover, Emery (1963) has shown that 2*S*-aspartate is a competitive inhibitor of the aspartase-catalyzed addition of hydroxylamine to fumarate giving *N*-hydroxyaspartate. To explain the above experimental results, a random kinetic scheme rather than an ordered sequential mechanism is proposed for the aspartase reaction (Scheme I). The kinetic constants in this scheme are defined as indicated.

The problem of locating the rate-limiting step of the overall reaction has been investigated with forms of 2*S*-aspartate stereospecifically labeled with deuterium at either the 2*S* or the 3*R* position. As shown by the projection formula for 2*S*-aspartate in Figure 6, the proton removed from carbon-3 during enzymatic deamination is trans to the amino group. When 2*S*,3*R*-[3-^{2}H]aspartate is the substrate, the label is lost during conversion of E–A to E–P–Q. Failure to observe the accompanying primary isotope effect excludes C–^{2}H bond breakage as the rate-limiting step. Likewise, removal of the corresponding proton from 2*S*-aspartate cannot be rate limiting. On the other hand, during the conversion of 2*S*-[2-^{2}H]aspartate to [^{2}H]fumarate, the C–^{2}H bond is not broken but undergoes an sp^3-to-sp^2 hybridization. Observation of an apparent secondary isotope rate effect of 1.11 ± 0.02 is consistent with the reaction at carbon 2 being rate determining. Bright (1964, 1965) demonstrated that the β-methylaspartase-catalyzed deamination of β-methylaspartate follows a random mechanism in which the rate-limiting step is breakage of the C–N bond during conversion of the central complexes.

Since a carbon in the carbonium-ion stage is considered to have achieved sp^2 hybridization, secondary isotope effects have been useful in studying reactions involving carbonium-ion intermediates (Richards, 1970). For example, in solvolytic reactions involving SN1 processes, secondary isotope effects ($k_H/k_{^2H}$) that result from single deuterium substitutions commonly range around 1.12–1.20 (Schmidt *et al.*, 1969). In the enzymatic case, if the release of products can be shown to be rapid relative to the conversion of the central complexes, the observed isotope effect with 2*S*-[2-^{2}H]aspartate would indicate the possible development of some carbonium character at the α carbon of the aspartate. The location of its value at the lower end of the range commonly observed for model systems could be due to the fact that the reaction at carbon-2 is not entirely rate limiting.

The somewhat reduced isotope effect could also be explained by the participation of a carboxylate group from the substrate itself or some nucleophile from the enzyme during or immediately following breakage of the C–N bond (Richards, 1970). Pertinently, the nonenzymatic deamination of an amino acid with nitrous acid is thought to proceed through the formation of a transient α-lactone intermediate leading to retention of configuration in the α-hydroxy acid produced (Neuberger, 1948; Brewster *et al.*, 1950).

From mechanistic studies of the aspartase reaction in ^2H_2O, Englard (1958) found that the rate of incorporation of ^{2}H at carbon-3 of 2*S*-aspartate was in agreement with that expected if the sole route of ^{2}H incorporation into 2*S*-aspartate was through the amination of fumarate. He suggested that, very likely, the reaction proceeded *via* a carbonium-ion intermediate. A similar finding has been obtained in this laboratory from preliminary studies of the exchange of the β proton of 2*S*-aspartate in tritiated water during the initial forward reaction. If the proton removed from 2*S*-aspartate during deamination is not bound to the enzyme in such a manner as to prevent its exchange with solvent, then C–N bond breakage could occur as the rate-limiting step prior to proton abstrac-

tion. This would lead to considerable carbonium-ion development or allow the formation of a transient α-lactone or some other enzyme-bound intermediate.

The absence of a primary β-isotope effect is also consistent with a mechanism in which the release of one of the products is rate limiting with all prior steps in rapid equilibrium. In this mechanism the lack of a primary isotope effect is expected since the product involved in the rate-determining step does not contain the isotope. Accordingly, the observed secondary isotope effect may be an equilibrium effect. This alternative mechanism for aspartase would be analagous to that for fumarate hydratase (Hansen *et al.*, 1969; Rose, 1970). One approach to distinguish between the two mechanisms suggested here is to measure the comparative rates of exchange between fumarate and 2*S*-aspartate as well as between NH_4^+ and 2*S*-aspartate under equilibrium conditions. If the release of one of the products is rate limiting, these two rates of exchange will not be equal. Alternatively, if C–N bond breakage is rate determining, the two exchange rates should differ only by the nitrogen or carbon isotope effects which would be very small. Such an experiment is currently under way in our laboratory, and its results and other pertinent data will be reported elsewhere.

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