some cases PIX can be induced with product inhibition by the nonlabeled product. In the present case, however, dead-end complexes are also formed, and conditions required to give sufficient turnover would require large volumes (2–3 L), making these experiments impractical.

There are two possible means of explaining the lack of positional isotope exchange:

(1) Once catalysis has taken place, the dissociation of the products is appreciably faster than the net rate of catalysis and product release in the reverse direction. This would require that either the chemical steps themselves or some step (e.g., conformational change) prior to the chemical step in the forward direction be rate determining. Either of these two would be consistent with a rapid equilibrium random mechanism.

(2) Catalysis may occur and the enzyme may not provide enough freedom to allow a free rotation about the O–P bond so that the oxygens in the intermediate are not torsionally symmetric. In other words, the oxygens of phosphate are tightly bound. This is rather unlikely since molecular orbital calculations have shown this barrier to rotation, at least free of enzyme, to be very low ($\Delta G^{\ddagger} \simeq 1$ kcal, $k_{rot} = 10^{12}$ s^{-1}) (Engelke, 1973). However, this possibility cannot be strictly ruled out, particularly since there is very likely interaction of both nucleophile and leaving group with Mg^{2+} (Herschlag & Jencks, 1987). In this case, the back-reaction would occur prior to release of products, but PIX would not be observed.

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Nitrogen Isotope Effects on Glutamate Decarboxylase from *Escherichia coli*†

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ABSTRACT: The nitrogen isotope effect on the decarboxylation of glutamic acid by glutamate decarboxylase from *Escherichia coli* has been measured by comparison of the isotopic composition of the amino nitrogen of the product γ-aminobutyric acid isolated after 10–20% reaction with that of the starting glutamic acid. At pH 4.7, 37 °C, the isotope effect is $k^{14}/k^{15} = 0.9855 \pm 0.0006$ when compared to unprotonated glutamic acid. Interpretation of this result requires knowledge of the equilibrium nitrogen isotope effect for Schiff base formation. This equilibrium isotope effect is $K^{14}/K^{15} = 0.9824$ for the formation of the unprotonated Schiff base between unprotonated valine and salicylaldehyde. Analysis of the nitrogen isotope effect on decarboxylation of glutamic acid and of the previously measured carbon isotope effect on this same reaction [O'Leary, M. H., Yamada, H., & Yapp, C. J. (1981) *Biochemistry 20*, 1476] shows that decarboxylation and Schiff base formation are jointly rate limiting. The enzyme-bound Schiff base between glutamate and pyridoxal 5′-phosphate partitions approximately 2:1 between decarboxylation and return to the starting state. The nitrogen isotope effect also reveals that the Schiff base nitrogen is protonated in this intermediate.

Most amino acid decarboxylases require pyridoxal 5′-phosphate (PLP)[1] as a cofactor. The generally accepted mechanism for this reaction is shown in Scheme I. The first step is substrate binding. The second step is Schiff base interchange, in which the nitrogen of the enzyme–PLP Schiff base is replaced by the amino nitrogen of the substrate. This is a multistep process involving numerous proton transfers. The third step is the actual decarboxylation step, in which the protonated pyridine nitrogen serves as an electron sink. Stereochemical and conformational control are principally manifested in this step (O'Leary & Piazza, 1978). All available evidence indicates that this step is irreversible (O'Leary et al., 1981). Steps corresponding to k_7 and k_9 involve Schiff base interchange and release of the decarboxylated product. Protonation of the decarboxylated intermediate usually occurs with retention of configuration at the α-carbon atom (Dunathan, 1971; Floss & Vederas, 1982). Steps following decarboxylation are presumably of little kinetic significance and are

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; GABA, γ-aminobutyric acid; P_i, inorganic phosphate.

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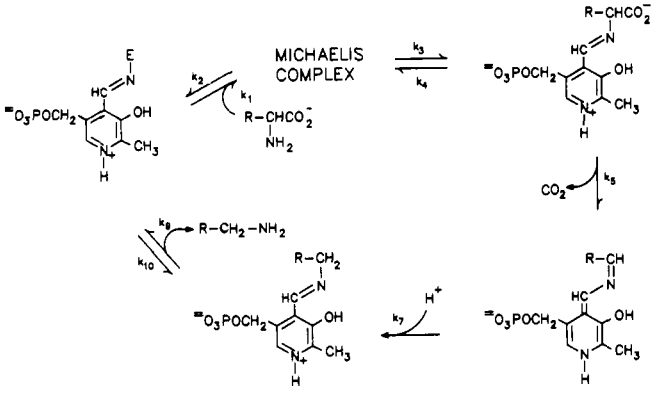
Scheme I



represented in less detail because they do not enter into the consideration of carbon and nitrogen isotope effects.

Heavy-atom isotope effects have become common as a probe of enzymatic reaction mechanisms (O'Leary, 1977a,b; Cleland, 1982, 1987), and carbon isotope effects have been extensively utilized to study the mechanisms of PLP-dependent decarboxylases (O'Leary et al., 1970, 1981; O'Leary & Piazza, 1981; Rosenberg & O'Leary, 1985). Under optimum conditions of pH, temperature, and substrate, most PLP-dependent decarboxylases exhibit carbon isotope effects in the range 1.010–1.018 (O'Leary et al., 1970, 1981; O'Leary & Piazza, 1981; Rosenberg & O'Leary, 1985). Both model studies and studies with alternate substrates indicate that the intrinsic isotope effect[2] should be 1.05–1.06. These smaller carbon isotope effects indicate that the decarboxylation step and some preceding step must occur at similar rates; that is, neither step is solely rate limiting.

To date, the step responsible for reducing the observed isotope effects has been assumed to be the Schiff base interchange step. However, the possibility remains that some other step preceding decarboxylation (e.g., substrate binding or an enzyme conformation change associated with substrate binding) might be partially rate determining. This possibility is particularly intriguing because of the prediction based on rates of model reactions that Schiff base interchange should be much faster than decarboxylation. In model studies, decarboxylation of amino acid–PLP Schiff bases occurs slowly at temperatures in excess of 125 °C (Kalyankar & Snell, 1962), whereas Schiff base formation and Schiff base interchange occur at 25 °C (Bruice & Benkovic, 1966). Thus, we would have expected decarboxylation to be orders of magnitude slower than Schiff base interchange. Nitrogen isotope effects on reactions catalyzed by PLP-dependent enzymes would be of particular interest because such effects principally reflect the chemistry of the Schiff base interchange step. An isotope effect different from unity would be evidence that this step is in fact the other slow step. Such nitrogen isotope effects should be sensitive to the acid–base chemistry and the detailed kinetics of Schiff base interchange. To our knowledge, no previous studies have been devoted to nitrogen isotope effects on PLP-dependent enzymatic reactions.

Nitrogen isotope effects on C–N bond-making and -breaking reactions have been studied in a number of enzymatic systems. Hermes et al. (1985) measured nitrogen isotope effects k^{14}/k^{15} = 1.0021 and 1.0047 for phenylalanine and 3-(1,4-cyclohexadienyl)alanine, respectively, with phenylalanine ammonia-lyase. From the reduction of the nitrogen isotope effect observed on deuteriation of the substrate, they concluded that the reaction proceeds by an Elcb mechanism, with C–H bond breaking being partially rate determining. Aspartase appears to react by a similar mechanism, but the nitrogen isotope effect is significantly larger (1.024; Nuiry et al., 1984). Adenosine deaminase gives a small nitrogen isotope effect for adenosine (1.0040) but a much larger isotope effect with the slower substrate 8-hydroxyadenosine (1.015). The variation in isotope effect apparently reflects the variable partition ratio of the putative tetrahedral intermediate; both substrates probably have intrinsic isotope effects near 1.03 (Weiss et al., 1987). Parkin and Schramm (1987) reported a nitrogen isotope effect k^{14}/k^{15} = 1.03 for the cleavage of AMP by AMP nucleosidase; in this case the C–N bond-breaking step is rate determining. Hydrolysis of *N*-benzoyl-L-argininamide by papain shows a nitrogen isotope effect of 1.024, which may be close to the intrinsic value for this reaction (O'Leary et al., 1974). Hydrolysis of *N*-acetyl-L-tryptophanamide by chymotrypsin shows a much smaller isotope effect (1.010 or less, depending on pH), presumably because C–N bond breaking is not rate determining (O'Leary & Kluetz, 1972). Thus, enzymatic reactions in which a carbon–nitrogen single bond is broken may show intrinsic isotope effects of 1.02–1.03.

Interpretation of the nitrogen isotope effect on glutamate decarboxylase requires knowledge of the ^{15}N equilibrium isotope effect on Schiff base formation and the ^{15}N isotope effect on the protonation of the amino nitrogen. We determined the equilibrium isotope effect on Schiff base formation for the reaction of valine with salicylaldehyde. For ammonia itself, the nitrogen isotope effect for the acid–base equilibrium defined in eq 1 is K^{14}/K^{15} = 1.0192, with ^{15}N concentrating

$$NH_4^+ \rightleftharpoons NH_3 + H^+ \qquad (1)$$

in the conjugate acid (Hermes et al., 1985). The corresponding number for phenylalanine is 1.0165 (Hermes et al., 1985). Values for other amines are not known, but they are presumably in the same range. We show in this paper that nitrogen isotope effects can be used not only to identify rate-limiting steps in the enzymatic reaction but also to deduce the protonation state of the glutamic acid–PLP Schiff base intermediate formed during the enzymatic decarboxylation of glutamic acid.

Experimental Procedures

Materials

Glutamate decarboxylase from *Escherichia coli* (ATCC 11246) was obtained from Sigma Chemical Co. as a purified powder that was 20–40 units/mg of protein and was used without further purification. Prior to use the enzyme was dialyzed against NH_3-free P_i buffer, pH 6.8, for 15 h at 4 °C.

(Hydroxypropyl)cellulose was obtained from Aldrich Chemical Co. Ion-exchange resins were cleaned prior to use by the method of Dunaway-Mariano and Cleland (1980). Water was purified with a Millipore Super-Q purification system. Aldrich salicylaldehyde was purified by distillation at reduced pressure.

Methods

Spectrophotometric assays were carried out on a Cary 118C spectrophotometer. Isotope ratios were measured in the laboratory of Dr. D. Kohl at Washington University, St. Louis, MO, by using a VG Micromass Model 602E isotope ratio mass

[2] An intrinsic isotope effect is an isotope effect on an individual step in a multistep reaction mechanism. The observed isotope effect is related to the intrinsic isotope effect(s) by an equation involving a collection of terms of the form k_n/k_{n-1}, in which the odd-numbered rate constants are for forward steps and the even-numbered rate constants are for reverse steps.

spectrometer. All isotope ratios are given as $\delta^{15}N$ values, defined by

$$\delta^{15}N = 1000(R_{sple}/R_{std} - 1) \quad (2)$$

where $R = {}^{15}N^{14}N/{}^{14}N_2$ and the standard is solid ammonium chloride that was standardized to air (Mariotti, 1983).

Valine concentrations were determined by Kjeldahl digestion (see below) followed by NH_3 assay with glutamate dehydrogenase. Salicylaldehyde concentration was determined from the absorbance at 264 nm. Glutamate decarboxylase was assayed manometrically at 37 °C by using a Gilson differential respirometer (O'Leary et al., 1970) in phosphate solutions containing 0.03 mg/mL (hydroxypropyl)cellulose. All solutions were tested for contamination with ammonia by use of Nessler's reagent (Sigma). Precise ammonia concentrations were obtained by using glutamate dehydrogenase (low-ammonia form) (Sigma).

Nitrogen Analyses. Kjeldahl digestions were conducted as described by Burris and Wilson (1957).

Isotopic analysis of NH_3 was accomplished by conversion to N_2 using hypobromite. For this conversion, a 1-mL ammonia sample (50–150 μmol) was placed in one side of a Y-tube, and 1 mL of a NaOBr solution (Bremner, 1965) was placed in the other side. The tube was placed on a vacuum manifold, and the contents were frozen in a dry ice–ethanol slurry. The sample was degassed by three freeze–thaw cycles. The sample was then removed from the vacuum manifold and thawed, after which the two solutions were mixed and allowed to react for 15 min. The tube was frozen in liquid nitrogen just prior to admission to the mass spectrometer. In control experiments using unreacted valine, no isotope fractionation was observed in the presence of $NaBH_4$, due to the ion-exchange chromatography, or during the Kjeldahl digestion procedure and isotopic analysis.

Equilibrium Isotope Effect on Schiff Base Formation. Valine (10 mM) and salicylaldehyde (20 mM) were incubated at 25 °C, pH 10.0, in 0.1 M PP_i buffer for 24 h. Excess $NaBH_4$ was added with vigorous stirring to reduce the Schiff base. In less than 1 min the yellow solution turned colorless. After 30 min, 1 mL of acetone was added to destroy any unreacted $NaBH_4$, and the solution was stirred for another 30 min. Two milliliters of 0.1 M PP_i buffer, pH 10, was added to the reaction mixture, and the solution was applied to a Dowex 1 column (1 × 20 cm, PP_i form) that had been equilibrated with 0.1 M PP_i buffer, pH 10. The column was eluted with the same PP_i buffer. The Dowex resin was used only twice before being discarded. The fractions containing valine were combined. The UV spectrum of the combined fractions demonstrated the absence of contamination by the reduced Schiff base. No decarboxylation of valine occurred during the equilibration or reduction. The valine was subjected to Kjeldahl digestion and isotope ratio analysis.

The ^{15}N equilibrium isotope effect was calculated by using the equation

$$\left(\frac{K^{15}}{K^{14}}\right)_{obsd} = \frac{R_{v0}}{R_v} + \frac{V}{S}\left[\frac{R_{v0}}{R_v} - 1\right] \quad (3)$$

where R_{v0} is the isotope ratio ($^{15}N/^{14}N$) of the initial valine, R_v is the same ratio at equilibrium, V is the valine concentration at equilibrium, and S is the Schiff base concentration at equilibrium. Note that unlike the usual convention the heavier isotope is in the numerator in eq 3.

The isotope effect thus obtained is the actual isotope effect at the pH in question and thus reflects the isotope fractionation between Schiff base and *total* amine present. The correct isotope effect referred to the unprotonated amine is given by

$$\frac{K^{14}}{K^{15}} = \left(\frac{K^{14}}{K^{15}}\right)_{obsd} \frac{(1 + 1.0165[H^+]/K_a)}{(1 + [H^+]/K_a)} \quad (4)$$

where $(K^{14}/K^{15})_{obsd}$ is the equilibrium isotope effect observed at the pH in question, $[H^+]$ is the hydrogen ion concentration, K^{14}/K^{15} is the equilibrium isotope effect on Schiff base formation starting with unprotonated amine, K_a is the acid dissociation constant of the conjugate acid of the amine, and 1.0165 is the equilibrium isotope effect on protonation of the amine (Hermes et al., 1985).

Characterization of the Reduced Schiff Base. The reduced Schiff base was eluted from the Dowex resin by washing batchwise with 95% ethanol. The resin was removed by filtration, and the filtrate was evaporated to dryness under vacuum. ^1H NMR: δ 7.2 (2 H, multiplet), 6.8 (2 H, multiplet), 4.2 (2 H, AB pattern, J_{AB} = 13.3 Hz), 3.18 (1 H, d, J = 5.56 Hz), 2.08 (1 H, multiplet), 1.0 (6 H, d, J = 6.95 Hz). UV spectrum in water: 290, 279, 235 nm.

Nitrogen Isotope Effect on Decarboxylation of Glutamic Acid. The nitrogen isotope effect was measured by comparing the isotopic composition of the nitrogen in the product GABA after 10–20% reaction with that of the starting glutamate. Reactions were carried out with 10 mL of 100 mM glutamate, pH 4.7, containing 0.03 mg/mL (hydroxypropyl)cellulose and 65 mM NaCl at 37 °C. Prior to reaction, the solution was demonstrated to be free of NH_3. The reaction was quenched at the appropriate time with 1 mL of 1 M H_2SO_4. The extent of reaction was determined from the amount of CO_2 isolated (O'Leary, 1980). Protein was removed from the reaction mixture by ultrafiltration with a small Amicon PM-10 filter, and the pH of the solution was adjusted to 7.0.

This solution was then applied to a Dowex 1 column (2.5 × 32 cm, acetate form) that had been equilibrated with 50 mM sodium acetate buffer at pH 7.0. the column was eluted with this buffer, and the GABA-containing fractions were combined and concentrated to a volume of 20–40 mL. The bound glutamate was eluted with 0.5 M acetic acid. This procedure was demonstrated not to fractionate nitrogen isotopes. Each resulting GABA solution was demonstrated to be free of ammonia by Nessler's assay and free of glutamate by assay with glutamate dehydrogenase. The GABA was subjected to Kjeldahl digestion and isotope ratio analysis. The isotope effect was calculated as described by O'Leary (1980).

Results

^{15}N Equilibrium Isotope Effect on Schiff Base Formation. For measurement of the nitrogen isotope effect on this process, valine and salicylaldehyde were incubated at pH 10.0 for 24 h at 25 °C, after which time $NaBH_4$ was added to reduce the Schiff base. Remaining valine was isolated by ion-exchange chromatography, and the isotope effect was calculated by comparison of the isotopic composition of valine prior to the start of the reaction with that of the remaining valine. This method is most accurate if more than half of the starting valine is converted to Schiff base, and that requirement was met in these studies by use of an excess of salicylaldehyde.

The equilibrium constant for Schiff base formation between valine and salicylaldehyde, defined by eq 5 and 6, was found

$$RNH_2 + R'CHO \rightleftharpoons RN{=}CHR' + H_2O \quad (5)$$

$$K = [RN{=}CHR']/[RNH_2][R'CHO] \quad (6)$$

from the amount of valine recovered after equilibration and reduction to be 288 M^{-1}, in good agreement with the value of Metzler et al. (1980). This result indicates that reduction with $NaBH_4$ did not perturb the equilibrium. The lifetime

Table I: Nitrogen Isotope Effects on Decarboxylation of Glutamic Acid by Glutamate Decarboxylase from *E. coli* at pH 4.7, 37 °C

$\delta^{15}N$ (‰)	% reaction	k^{14}/k^{15}
	Glutamic Acid	
−2.633 ± 0.043		
−2.188 ± 0.052		
−2.530 ± 0.051		
−3.296 ± 0.086		
−2.837 ± 0.058		
−1.736 ± 0.051		
−2.537 ± 0.536 (av)		
	GABA	
−4.415 ± 0.048	17.2	1.0021
−4.161 ± 0.041	12.7	1.0017
−3.624 ± 0.034	12.0	1.0012
−4.405 ± 0.062	12.0	1.0020
−4.615 ± 0.025	13.0	1.0022
−4.344 ± 0.044	12.4	1.0019
−3.487 ± 0.060	12.0	1.0010
−4.626 ± 0.062	14.0	1.0023
−4.519 ± 0.078	14.0	1.0020
		1.0018 ± 0.0002 (av)[a]
		0.9855 ± 0.0006 (corrected for protonation of glutamate)

[a] Isotope effect compared to that of protonated glutamic acid.

of $NaBH_4$ under the reduction conditions is so short that it is not expected to perturb the Schiff base equilibrium.

Eight determinations of the isotope effect at 70–75% conversion of Schiff base gave an apparent isotope effect at pH 10.0 of 0.9869 ± 0.0007. This isotope effect reflects the isotope fractionation associated with protonation of valine. The isotope effect for unprotonated valine (cf. eq 4) is 0.9824.

Nitrogen Isotope Effect on Decarboxylation of Glutamic Acid. This isotope effect was measured by comparing the isotopic composition of the amino nitrogen of glutamate prior to reaction with the isotopic composition of the nitrogen in GABA produced after 10–20% decarboxylation. Since each molecule only contains one nitrogen, analysis could be accomplished by Kjeldahl digestion and treatment of the resulting ammonia with hypobromite. No buffer other than glutamate itself was used in the isotope effect reactions. The second pK_a of the substrate glutamate provided sufficient pH control at pH 4.7. The reaction mixtures also contained 0.03 mg/mL (hydroxypropyl)cellulose in order to stabilize the enzyme and 65 mM NaCl to match the Cl^- concentration of previous studies of carbon isotope effects (O'Leary et al., 1970, 1981). The results are summarized in Table I. Because the experiments were conducted at pH 4.7, the observed isotope effect is that for protonated glutamate. This isotope effect was corrected for the ^{15}N equilibrium isotope effect on the protonation–deprotonation step by dividing by 1.0165, the magnitude of this equilibrium isotope effect.[3] The corrected isotope effect of $k^{14}/k^{15} = 0.9855 \pm 0.0006$ is thus the isotope effect for decarboxylation assuming unprotonated glutamate is the substrate.

Discussion

Nitrogen Isotope Effect on Schiff Base Formation. The salicylaldehyde/valine system is ideally suited for study of nitrogen isotope effects on Schiff base equilibria for three main reasons. First, although most Schiff base equilibria in water are quite unfavorable, this one is several orders of magnitude more favorable than most others. Second, unlike PLP and most of its analogues, salicylaldehyde is essentially unhydrated in aqueous solution, and thus the Schiff base equilibrium may be studied unhindered by any prior chemical equilibria associated with the dehydration of the carbonyl group. Third, nitrogen isotope effect measurements with this system are further simplified by the fact that there is only one type of nitrogen in this system, the amino nitrogen in valine.

At the same time, we believe that the nitrogen isotope effect on this Schiff base equilibrium is a good proxy for nitrogen isotope effects to be expected in reactions of amino acids with PLP. With the exception of proline, differences among amino acids should be small (the difference in isotope effects on equilibrium constants should be much smaller than the difference in equilibrium constants themselves) and salicylaldehyde, with its aromatic ring and ortho hydroxyl group, should be a reasonable model for PLP.

The equilibrium nitrogen isotope effect derived above, 0.9824, is that for unprotonated valine and unprotonated Schiff base (cf. eq 5). The fact that this value is less than unity indicates that at equilibrium ^{15}N concentrates in the Schiff base, as expected from the greater degree of bonding in the Schiff base compared to the amine. The equilibrium nitrogen isotope effect involving protonated amine and unprotonated Schiff base (eq 7) can be calculated to be 0.9981, and this is

$$RNH_3^+ + R'CH{=}O \rightleftharpoons RN{=}CHR' + H_2O + H^+ \quad (7)$$

the value that would be expected to be observed in Schiff base equilibria at low pH. The other value of interest is the equilibrium isotope effect involving unprotonated amine and protonated Schiff base (eq 8). This value is more difficult

$$RNH_2 + R'CH{=}O + H^+ \rightleftharpoons RNH^+{=}CHR' + H_2O \quad (8)$$

to estimate because the nitrogen isotope effect on protonation of the Schiff base has not been measured. However, to a first approximation we suggest that this value will be similar to the nitrogen isotope effect on the protonation of amino acids. The equilibrium isotope effect for eq 8 then becomes 0.9669.

Decarboxylation of Glutamic Acid. The enzymatic decarboxylation of glutamic acid shows a significant carbon isotope effect (O'Leary et al., 1970, 1981) and a significant nitrogen isotope effect. The fact the carbon isotope effect is smaller than the expected intrinsic isotope effect has been interpreted as being due to partially rate-determining Schiff base formation. The occurrence of a nitrogen isotope effect different from unity is consistent with this suggestion and is inconsistent with the alternate possibility that substrate binding or an associated conformation change is partially rate determining. A more quantitative analysis of the isotope effects is given below.

The mechanism of the enzymatic decarboxylation of glutamic acid is shown in Scheme I, and a number of isotope effects on this reaction are summarized in Table II. We

Table II: Summary of Isotope Effects on Glutamate Decarboxylase from *E. coli* at pH 4.7, 37 °C[a]

isotope effect	value
k^{12}/k^{13}	1.0182 ± 0.0004
k^{12}/k^{13} in D_2O	1.0090 ± 0.0002
$[V/K(H_2O)]/[V/K(D_2O)]$	2.6
$V_{max}(H_2O)/V_{max}(D_2O)$	5.0
k^{14}/k^{15}[b]	0.9855 ± 0.0006

[a] Values from O'Leary et al. (1981). [b] This work.

[3] The equilibrium isotope effect for protonation of the amino group of phenylalanine is 1.016, with ^{15}N concentrating in the protonated state. This value has not been measured for valine or glutamic acid, but we are confident that it would not be significantly different from the value for phenylalanine. Thus, we use the phenylalanine value to correct these results.

Table III: Calculated Rate Ratios k_5/k_4 and Intrinsic Nitrogen Isotope Effects for the Decarboxylation of Glutamic Acid at pH 4.7, 37 °C

assumed k_5^{12}/k_5^{13}	1.05		1.06	
calcd k_5/k_4	1.75		2.30	
assumed K^{14}/K^{15}	0.9824	0.9669	0.9824	0.9669
calcd k_3^{14}/k_3^{15}	0.9873	0.9961	0.9868	0.9936
calcd k_4^{14}/k_4^{15}	1.0050	1.0302	1.0045	1.0276

recognize that formation of the PLP–amino acid Schiff base is a multistep process, but available data do not permit us to say anything quantitative about the separate steps. For the present, we leave unanswered the question of number and placement of protons within the various intermediates. Within this mechanistic framework, and with the further assumption that substrate binding and dissociation are rapid compared to catalysis, the carbon isotope effect on the reaction is given by eq 9 and the nitrogen isotope effect by eq 10. In eq 9 we

$$k^{12}/k^{13} = \frac{k_5^{12}/k_5^{13} + k_5/k_4}{1 + k_5/k_4} \quad (9)$$

$$k^{14}/k^{15} = \frac{K_{eq}^{14}/K_{eq}^{15} + (k_3^{14}/k_3^{15})(k_5/k_4)}{1 + k_5/k_4} \quad (10)$$

have assumed that only k_5 shows a significant carbon isotope effect. Although previous studies show that in the case of arginine decarboxylase there is probably a small carbon isotope effect associated with substrate binding (O'Leary & Piazza, 1981), this effect is too small to be important in the present analysis. In eq 10 it is assumed that there is no nitrogen isotope effect on k_5. Although this assumption may not be precisely correct, the change in bonding to nitrogen in this step is sufficiently small that we expect the nitrogen isotope effect to be correspondingly small (unless, of course, a change in protonation state is associated with this step). The present analysis explicitly includes the acid–base chemistry of the substrate amino group that is associated with substrate binding. However, it is possible that there is also a nitrogen isotope effect associated with desolvation of the substrate amino group during substrate binding. We have no information on the size of such an effect, and so it is neglected for the present.

The present analysis of carbon isotope effects is consistent with and extends our previous analysis of this reaction (O'Leary et al., 1970, 1981). In our previous studies we have considered a wide range of values for the carbon isotope effect on k_5. This range can now be narrowed by use of the decarboxylation of 4-pyridylacetic acid as a model (Marlier & O'Leary, 1985); thus, we expect this isotope effect to be in the range 1.05–1.06. The difference between the observed isotope effect and the expected isotope effect on k_5 is due to the term k_5/k_4 in eq 10, and insofar as we know the intrinsic isotope effect, we can calculate k_5/k_4. Estimated values of this ratio are near 2 (Table III), indicating that neither Schiff base interchange nor decarboxylation is entirely rate limiting. Consistent with this conclusion, the large solvent isotope effect on the carbon isotope effect is due to a large solvent isotope effect on k_3 and k_4 (O'Leary et al., 1981).[4]

If the ratio k_5/k_4 is obtained from the carbon isotope effects, then the observed equilibrium isotope effect on Schiff base formation and the observed nitrogen isotope effect on decarboxylation of glutamic acid can be used to estimate values for the nitrogen isotope effects on k_3 and k_4. The equilibrium isotope effect reported above for the valine–salicylaldehyde Schiff base should be an adequate model for the Schiff base equilibrium involved here. Although the equilibrium isotope effect for Schiff base formation reported above refers to aldehyde as the starting state, the same equilibrium isotope effect will be obtained in the present case, where another Schiff base (i.e., one involving a lysine amino group of the enzyme) is the starting state.

The acid–base chemistry of the amino nitrogen of the substrate bears careful scrutiny. Both the experimental value derived above for the kinetic isotope effect (0.9855) and the theoretical values derived here are referred to *unprotonated* glutamate as the starting state because of the fact that Schiff base interchange must begin with unprotonated glutamate (this procedure is satisfactory even though the pH dependence of the kinetics of decarboxylation shows that it is actually protonated glutamate which binds to the enzyme).

However, two different values of the equilibrium isotope effect for Schiff base formation might be used, depending on whether the Schiff base nitrogen is unprotonated (cf. eq 7) or protonated (cf. eq 8). In Table III are derived two alternate sets of values for the intrinsic isotope effects on k_3 and k_4: One set assumes that the glutamate–PLP Schiff base is unprotonated and therefore uses an equilibrium isotope effect of 0.9824; the other assumes that the Schiff base is protonated and uses an equilibrium isotope effect of 0.9669. In both cases, the intrinsic isotope effect on k_3 is slightly inverse.

It is difficult to predict a range of intrinsic isotope effects on k_3. Because this is a bond-forming reaction, the isotope effect might be either normal or inverse depending on the relative contribution of zero-point energy and imaginary frequency factor terms to the Bigeleisen equation (Bigeleisen & Wolfsberg, 1958; Melander & Saunders, 1980). Although the few isotope effects known for nitrogen acting as a nucleophile in S_N2 reactions are in the range 1.002–1.004 (Ando et al., 1985) and a few calculations of intrinsic isotope effects exist (Yamataka et al., 1986), these data are not sufficient to provide unambiguous values. We can only conclude for the present that either set of calculated intrinsic isotope effects is consistent with our expectations from previous nitrogen isotope effects.

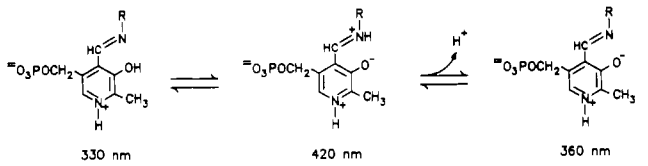
Fortunately, comparison of calculated and predicted isotope effects on k_4 is easier. k_4 represents a bond-breaking reaction, and both the zero-point energy term and the imaginary frequency factor will serve to make the isotope effect be normal. Comparison can be made with nitrogen isotope effects on a variety of reactions, including amide hydrolysis and a number of elimination reactions (see introduction). On the basis of these data, we expect an intrinsic nitrogen isotope effect on k_4 of 1.02–1.03.

Comparison of these values with the data in Table III reveals that the intrinsic isotope effects calculated by assuming that the substrate–PLP Schiff base is protonated are in the expected range, whereas those assuming that the Schiff base is unprotonated are not. Thus, we believe that the Schiff base is protonated.

The protonation state of this intermediate can be obtained only with difficulty from previous studies. However, the protonation state of the starting enzyme–PLP Schiff base is relatively well established from absorption spectra (Johnson & Metzler, 1970). Glutamate decarboxylase, like many (though not all) PLP enzymes, shows a weak absorption maximum at 420 nm, which is attributed to the ion-pair

[4] In the original analysis of the effect of D_2O on the reaction, we were forced to assume that the intrinsic isotope effect on the decarboxylation step would be unaffected by the substitution of D_2O for H_2O. Subsequent model studies have confirmed the correctness of this assumption (Marlier & O'Leary, 1986).

Scheme II



structure shown in Scheme II, in which the Schiff base nitrogen is protonated. The tautomer in which the hydrogen is on the phenolic oxygen absorbs near 330 nm. The structure in which that proton is missing absorbs near 360 nm. In the case of glutamate decarboxylase, the enzyme shows an absorption at high pH at 330 nm due to a *gem*-diamine structure (O'Leary & Brummund, 1974). Thus, if the glutamate–PLP Schiff base bound to glutamate decarboxylase has a structure analogous to the 420-nm-absorbing structure shown in Scheme II, it corresponds to our expectation that this Schiff base is protonated.

These nitrogen isotope effects indicate that Schiff base interchange is kinetically significant and that the PLP–substrate Schiff base is protonated. The X-ray crystal structure of aspartate aminotransferase (Kirsch et al., 1984) is consistent with the idea that the PLP–substrate Schiff base is protonated.

Thus, it appears that the rate of decarboxylation of glutamic acid is jointly limited by Schiff base interchange and by decarboxylation. The PLP–glutamate Schiff base partitions approximately 2:1 toward decarboxylation versus reverse Schiff base interchange. Thus, the heights of the two energy barriers involved differ by less than 1 kcal/mol, and glutamate decarboxylase fulfills the "perfection" criterion of Knowles and Albery (1977), at least for all the steps through decarboxylation.

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