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Multiple Isotope Effect Probes of Glutamate Decarboxylase†

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ABSTRACT: The enzymatic decarboxylation of glutamic acid shows a carbon isotope effect $k^{12}/k^{13} = 1.018$ at 37 °C, pH 4.7. In D_2O under otherwise identical conditions, $k^{12}/k^{13} = 1.009$. Under the same conditions solvent isotope effects are $V_{max}^{H_2O}/V_{max}^{D_2O} = 5.0$ and $(V_{max}/K_m)^{H_2O}/(V_{max}/K_m)^{D_2O} = 2.6$. With the assumption that the carbon isotope effect on the decarboxylation step is in the usual range (1.05–1.07), it is possible to derive relative rates and solvent isotope effects for all steps in the enzyme mechanism. Substrate binding is ~2-fold weaker in H_2O than in D_2O, probably because of the desolvation which accompanies binding of the substrate to the enzyme. A proton inventory analysis of the reaction shows that the Schiff base interchange has a large solvent isotope effect composed of relatively small contributions from at least four separate sites. A conformation change probably accompanies this step. The decarboxylation step shows a solvent isotope effect of ~2. Schiff base interchange and decarboxylation are both partially rate determining. The pH dependence of the isotope effects indicates that the initial step in the reaction can occur by way of two different pathways.

Isotope effects are finding increasing use in studies of enzyme reaction mechanism (Cleland et al., 1977; Gandour & Schowen, 1978; Klinman, 1978). In most cases the observed isotope effect reflects some combination of the isotope effects on individual steps (called *intrinsic isotope effects*). The weighting factors which define this combination, called *partition factors*, are not isotopically sensitive.

Currently, the principal problem in applying isotope effects to problems in enzymology is the difficulty of extracting the intrinsic isotope effects and the partition factors from the observed effects. Several procedures for making this separation are currently in use. The important discovery by Northrop (1975, 1977) that deuterium and tritium isotope effects provide, by use of the Swain relationship (Swain et al., 1958), a separation of variables has given new impetus to studies of hydrogen isotope effects. However, the procedure is experimentally quite difficult and is not applicable in all cases (Albery & Knowles, 1977). In some cases it is possible to use pre-steady-state kinetics or partial reactions to determine isotope effects on individual reaction steps. This procedure has been used, among others, for aspartate aminotransferase (Jenkins & Harruff, 1979) and for serine *trans*-hydroxymethylase (Ulevitch & Kallen, 1977).

Manipulation of reaction conditions can sometimes provide the key to the separation. Use of abnormal substrates (Klinman, 1978; O'Leary & Piazza, 1978) or extreme pH

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values (Blanchard & Cleland, 1980a) may cause a single step to become rate determining, thus providing one intrinsic effect directly. This effect may be used to interpret isotope effects obtained under other conditions. Multiple isotope effect probes may also provide a useful approach, as in the case of isocitrate dehydrogenase (O'Leary & Limburg, 1977) and formate dehydrogenase (Blanchard & Cleland, 1980b).

Carbon isotope effects in enzyme-catalyzed decarboxylation reactions have proved fruitful for isotope effect studies because only a single step in the reaction sequence (the decarboxylation step) has an intrinsic isotope effect different from unity (O'Leary, 1977, 1978), and available evidence suggests that this intrinsic effect will fall within a narrow range (k^{12}/k^{13} = 1.05–1.07) for a wide variety of reactions. In this situation, partition factors can be readily calculated.

Solvent isotope effects have only recently begun to provide significant information regarding enzyme reaction mechanisms (Schowen, 1977, 1978). The "proton inventory" technique provides a method for separating isotope effects for hydrogens actually involved in the reaction sequence from generalized solvation isotope effects (Schowen, 1977, 1978).

We report here a new approach to dissecting isotope effects on multistep enzymatic reactions. The method depends upon the fact that observed primary isotope effects may differ in D_2O from the corresponding effects in H_2O. This change in primary isotope effect with solvent isotopic composition may at first seem surprising, because the rule of the geometric mean (Bigeleisen, 1955) states that isotope effects at separate sites are independent and should not affect each other. However, the rule of the geometric mean applies only to a single transition state. The usefulness of this approach to enzymatic reactions results from the fact that the solvent change may affect partition factors and thus change the observed isotope effect without changing intrinsic isotope effects. In this paper we apply this method to glutamate decarboxylase and show that we can obtain a relatively unique dissection of isotope effects and relative rates for various steps. Proton inventory studies provide further details regarding the natures of the separate steps.

Glutamate decarboxylase (EC 4.1.1.15) is one of the most studied of the bacterial amino acid decarboxylases (Boeker & Snell, 1972). The mechanism of action of the enzyme (Scheme I) is well established. The pH optimum of the enzyme is near 4.5, and the activity gradually decreases above pH 5 (O'Leary et al., 1970; Fonda, 1972). Carboxyl carbon isotope effects on the decarboxylation are significantly smaller than the expected intrinsic isotope effect on the decarboxylation (O'Leary et al., 1970), indicating that some step other than decarboxylation is partially rate determining. The isotope effects vary with pH.

Materials and Methods

Glutamate decarboxylase was isolated from *Escherichia coli* (ATCC 11246) by the method of O'Leary (1969). L-Glutamic acid was obtained from Sigma Chemical Co. H_2O was purified by means of a Millipore Super Q filtration system. D_2O was obtained from Aldrich Chemical Co. and was used without further purification. The pL values were obtained from pH meter readings as described by Schowen (1978).

Carbon isotope effects on the decarboxylation of glutamic acid were measured by the competitive method as described by O'Leary (1980). D_2O contains ^{17}O in excess of natural abundance, and this material might potentially interfere with $^{13}C/^{12}C$ isotope ratio measurements. For alleviation of this problem, CO_2 samples from decarboxylations in D_2O were exchanged with H_2O before isotopic compositions were measured. Steady-state kinetic parameters for the decarboxylation of glutamic acid were measured manometrically and corrected as described by Gregory & Winter (1965).

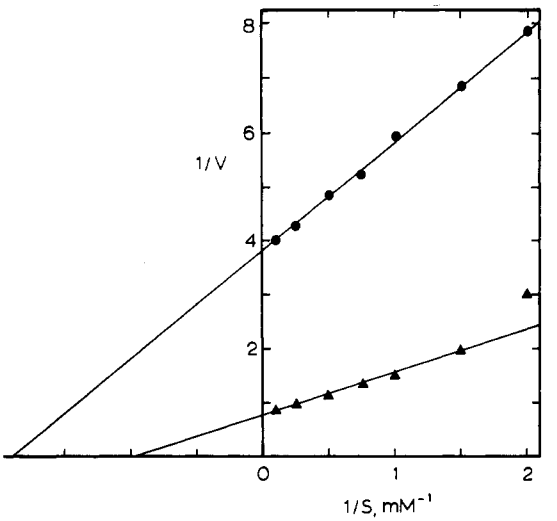


FIGURE 1: Reciprocal plots for decarboxylation of glutamic acid at pL 4.5 in 0.2 M pyridinium chloride buffer containing 0.2 M total chloride at 37.0 °C in H_2O (▲) and in D_2O (●). The same amount of enzyme was used in both experiments.

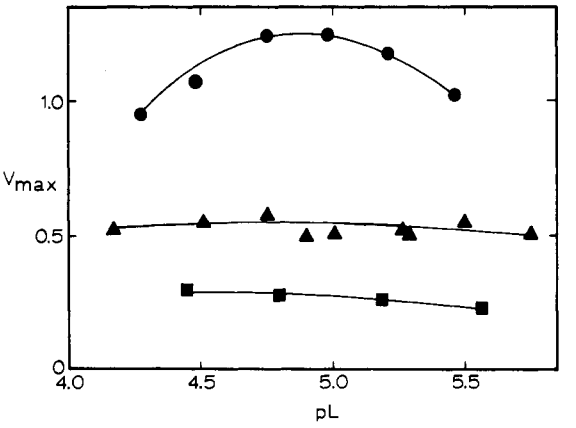


FIGURE 2: pH dependences of V_{max} for the decarboxylation of glutamic acid at 37 °C in H_2O (●), 50% D_2O (▲), and 100% D_2O (■). Each point represents the average of at least triplicate determinations.

Results

Carboxyl carbon isotope effects have been determined for the enzymatic decarboxylation of glutamic acid at pL 4.7, 37 °C, in 0.1 M pyridinium chloride buffer containing 0.02 M L-glutamic acid. In H_2O, k^{12}/k^{13} = 1.0182 ± 0.0004, in satisfactory agreement with the value of 1.0166 ± 0.0011 measured a number of years ago in our laboratory (O'Leary et al., 1970). In D_2O under otherwise identical conditions k^{12}/k^{13} = 1.0090 ± 0.0002.

Steady-state kinetic parameters for the decarboxylation of glutamic acid at pL 4.5 in 0.2 M pyridinium chloride buffer containing 0.2 M total chloride in H_2O and in D_2O (Figure 1) give $V_{max}^{H_2O}/V_{max}^{D_2O}$ = 5.0 and $(V_{max}/K_m)^{H_2O}/(V_{max}/K_m)^{D_2O}$ = 2.6. The pH dependence of V_{max} in H_2O and D_2O is shown in Figure 2.

A proton inventory study of the decarboxylation of glutamic acid under conditions of saturating substrate at pL 5.0 is shown in Figure 3. At pL 5.45 the solvent isotope effect is 4.0, and the proton inventory plot is less curved than at pL 5.0.

Discussion

In what follows we will first set out the mathematical analysis of the isotope effects. This will enable us to derive

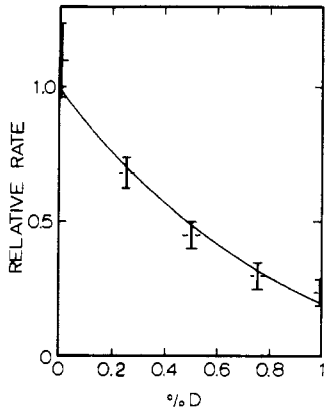
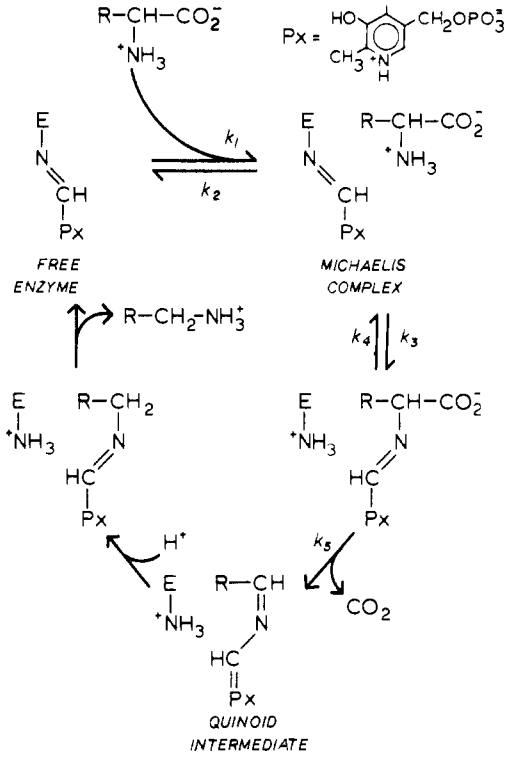


FIGURE 3: Proton inventory study of decarboxylation of glutamic acid at pL 5.0, 37 °C, in 0.2 M pyridinium chloride buffer. Errors given are one standard deviation. The curve is a theoretical curve for four protons with equal fractionation factors in each step in a two-step mechanism.

Scheme I



separate carbon and solvent isotope effects for each step in the enzymatic reaction. We will then consider the proton inventory analysis and the pH dependence of the kinetics and the isotope effects. Finally, we will combine all of this information into a detailed description of the mechanism of enzymatic decarboxylation of glutamic acid.

Treatment of Isotope Effects. Before analyzing our data in detail, we will set out the assumptions needed for the analysis: (1) The mechanism shown in Scheme I is basically correct. This does not exclude the possibility that the Schiff base interchange may involve more than a single step, nor does it exclude the possibility that a conformation change may occur at some point in the mechanism. The precise number of protons in each intermediate is also left unspecified for the present. (2) Steps subsequent to decarboxylation do not influence the observed isotope effects. The decarboxylation of glutamic acid is virtually irreversible (Koppelman et al., 1958; W. S. Caldwell and M. H. O'Leary, unpublished experiments), and the quinoid intermediate cannot be intercepted by high concentrations of CO_2 (W. S. Caldwell and M. H. O'Leary, unpublished experiments). (3) Only k_5 will have a significant isotope effect, and this effect will be in the range k_5^{12}/k_5^{13} = 1.05–1.07 (O'Leary, 1977, 1978; O'Leary & Piazza, 1978; Dunn, 1977). (4) The carbon isotope effect on k_5 is the same in H_2O as in D_2O. This is the correct way to apply the rule of the geometric mean (Bigeleisen, 1955). (5) There is no equilibrium solvent isotope effect on the Schiff base interchange step. This step is a Schiff base interchange involving two primary amino groups of similar basicity. In the absence of a solvent isotope effect on any conformation change which might be associated with Schiff base interchange, no equilibrium isotope effect is expected. (6) The substrate binding step is at equilibrium. The Michaelis constant for the substrate is near 1 mM, and the magnitudes of the isotope effects indicate that substrate dissociation from the Michaelis complex is not slow. The important result of this assumption is that partitioning the factor k_3/k_2 is quite small. We have arbitrarily assumed a value of 0.1 in our calculations, although the exact magnitude of this factor is unimportant.

Table I: Derived Kinetic Parameters for Enzymatic Decarboxylation of Glutamic Acid

parameter	calcd values 1.05[a]	1.06[a]	1.07[a]
k_5/k_4 in H_2O	1.6	2.1	2.6
k_5/k_4 in D_2O	4.1	5.1	6.1
$(k_4^H/k_4^D)/(k_5^H/k_5^D)$	2.56	2.42	2.34
k_5^H/k_5^D	2.7	2.9	3.0
k_4/k_3	2.5	1.0	0.7
K_m^H/K_M^D	2	2	2

[a] Assumed values of k_5^{12}/k_5^{13}. Other values assumed are k_3/k_2 = 0.1 and $k_3^H/k_3^D = k_4^H/k_4^D = 7$.

With these assumptions we can write equations for carbon and solvent isotope effects on the decarboxylation of glutamic acid (eq 1–3) in which the superscripts refer to the appropriate

$$V_{max}^H/V_{max}^D = (k_3^H/k_3^D)(k_5^H/k_5^D)\{[k_3^D/k_3^H + (k_4/k_3)[k_4^D/k_4^H + (k_5/k_4)(k_5^D/k_5^H)]]/[1 + (k_4/k_3)(1 + k_5/k_4)]\} \quad (1)$$

$$(V_{max}/K_m)^H/(V_{max}/K_m)^D = (k_1^H/k_1^D)\{[(k_3^H/k_3^D) \times (k_5^H/k_5^D)/[(k_2^H/k_2^D)(k_4^H/k_4^D)] + (k_5/k_4)(k_3^H/k_3^D)/(k_2^H/k_2^D) + (k_5/k_4)(k_3/k_2)]/[1 + k_5/k_4 + (k_5/k_4)(k_3/k_2)]\} \quad (2)$$

$$k^{12}/k^{13} = [k_5^{12}/k_5^{13} + k_5/k_4 + (k_5/k_4) \times (k_3/k_2)]/[1 + k_5/k_4 + (k_5/k_4)(k_3/k_2)] \quad (3)$$

isotopic species and unsuperscripted rate constants are for carbon-12 in H_2O. In the following discussion we will use the assumptions made above together with eq 1–3 to derive various kinetic parameters for glutamate decarboxylase. Table I summarizes this discussion.

We begin our analysis by taking the assumed range of values for k_5^{12}/k_5^{13} (the three columns of Table I). For each assumed value, we can calculate a value of the partition ratio k_5/k_4 in H_2O and in D_2O. These values immediately give us the relationship between the solvent isotope effect on k_5 and that on k_4: the latter is 2.3–2.5 times the former.

With this information, the solvent isotope effect on V_{max} can be used to derive individual solvent isotope effects on k_3, k_4, and k_5. The assumption made above that there is no equilibrium solvent isotope effect on the Schiff base interchange step requires that solvent isotope effects on k_3 and k_4 be equal. We can thus rewrite eq 1 as

$$V_{max}^{H}/V_{max}^{D} = [(k_5^{H}/k_5^{D})(1 + k_4/k_3) + (k_4^{H}/k_4^{D}) \times (k_5/k_4)(k_4/k_3)]/[1 + (k_4/k_3) + (k_5/k_4)(k_4/k_3)] \quad (4)$$

which is an equation of the form

$$V_{max}^{H}/V_{max}^{D} = x(k_5^{H}/k_5^{D}) + (1 - x)(k_4^{H}/k_4^{D}) \quad (5)$$

in which x is less than unity and depends only on rate constants in H_2O. Thus, the solvent isotope effect on V_{max} is a weighted average of the isotope effects on k_4, and k_5 is smaller than the larger of the two individual isotope effects.

It is next necessary to assume a value for the solvent isotope effect on one of the reaction steps. The smallest value for the solvent isotope effect on k_4 which fits the experimental data is ~7. This assumption then provides values for other solvent isotope effects and for k_4/k_3, the equilibrium constant for Schiff base interchange (Table I). It appears that this equilibrium constant is near unity.

It is, of course, possible to assume still larger values for the solvent isotope effect on k_4. This would make k_4/k_3 somewhat smaller.

Solvent Isotope Effect on Substrate Binding. The difference between the solvent isotope effects on V_{max} and on V_{max}/K_m enables us to derive a solvent isotope effect $K_m^{H}/K_m^{D} = 2$ for substrate binding; that is, glutamic acid binds to the enzyme more tightly in D_2O than in H_2O.

Proton Inventory. The proton inventory theory (Schowen, 1977, 1978) provides a way of estimating the contributions of individual proton-transfer events to the overall solvent isotope effect. The analysis derives from a plot of solvent isotope effect vs. the fraction of deuterium in the solvent (Figure 3). The important variable in the analysis is the curvature of this plot. In our case, the plot is significantly concave upward. This concavity indicates that the solvent isotope effect is primarily governed by transition-state contributions, rather than by ground-state contributions. This is not surprising, since the only type of acid–base functional group that is expected to make an appreciable ground-state contribution is the sulfhydryl group (Schowen, 1978). Thus, we will assume that there are only transition-state contributions to the solvent isotope effect, and the proton inventory equation for a single-step reaction becomes

$$k_n/k_0 = \prod_i (1 - n + n\phi_i) \quad (6)$$

where n is the atom fraction of deuterium in the solvent, k_n is the rate observed in that solvent, k_0 is the rate in pure water, and ϕ_i is the fractionation factor (the reciprocal of the isotope effect) for the ith site.

In the present case the reaction rate is actually governed by two steps, both of which are partially rate determining and which have different solvent isotope effects. Both steps contribute to the curvature of the proton inventory plot. Rather than simply using eq 6, we must substitute two expressions of the form of eq 6 into eq 5, giving eq 7

$$V_{max}^{H}/V_{max}^{D} = x/\prod_i (1 - n + n\phi_i) + (1 - x)/\prod_j (1 - n + n\phi_j) \quad (7)$$

in which the first product is for k_5 and the second is for k_4. In the present case, the data in Figure 3 should be fitted to eq 7 with the constraints given in Table I. Curvature of the proton inventory plot might arise either intrinsically as a result of the form of eq 7 or as a result of multiproton contributions ($i > 1$ and/or $j > 1$) in one or both steps.

We have carried out numerical modelling studies in an attempt to fit the data shown in Figure 3. For a single proton mechanism ($i = j = 1$), the proton inventory plot is bowed upward, whereas the corresponding plot for a single-step mechanism is linear. The effect of a two-step mechanism for any number of protons is to make the proton inventory plot more concave upward than is the plot for a corresponding single-step mechanism. Mechanisms involving only one or two protons in each step do not provide a satisfactory fit to the experimental data. One or both steps must involve a large number of protons, probably four or more, in order to reproduce the shape of the proton inventory plot. Because the overall isotope effect on k_4 is ~2.5 times as large as that on k_5, it is logical to assume that k_4 is the principal contributor to the curvature. The theoretical curve in Figure 1 is for four protons in each step with equal fractionation factors within each group of four.

pH Dependence. Two separate studies (O'Leary et al., 1970; Fonda, 1972) are in good agreement concerning the pH dependence of the steady-state kinetic parameters for glutamate decarboxylase, although O'Leary et al. (1970) failed to take proper account of the effect of anions on the rate. The pH dependence of V_{max}/K_m is bell shaped, with a maximum around pH 4.5. The pK_a on the low side is ~4.2 and has been attributed to the γ-carboxyl group of the substrate, which must be ionized for binding to the enzyme. The pK_a on the high side of the profile is very anion dependent and is associated with a conformation change of the free enzyme which occurs above pH 5 (O'Leary & Brummund, 1974).

One additional fact concerning the pH dependence of substrate binding appears to have been missed in previous investigations. In the range of maximum activity of the enzyme, the coenzyme chromophore shows a visible absorption band at 415 nm (Shukuya & Schwert, 1960) due to the protonated enzyme–pyridoxal P Schiff base (Scheme I). The spectral data do not enable us to determine whether the proton on the pyridine nitrogen is present in this form, although we will later discuss this point in connection with the pH dependence of the isotope effects. The plot of V_{max}/K_m vs. pH requires that it be the substrate form having a net charge of −1 which binds to the enzyme; thus, the amino group of the substrate is protonated and both carboxyl groups are ionized (we do not believe that the minor form in which both the amino group and one carboxyl group are neutral is a viable alternative). Before Schiff base interchange can occur, this extra proton on the amino group must be disposed of in some way. The fate of this proton is unknown. It might simply be stored at some site on the enzyme during the course of the reaction. Alternatively, this proton might be transferred to the group which later in the mechanism is used to protonate the quinoid intermediate (Yamada & O'Leary, 1977). A third alternative, which is perhaps the most attractive, is that the Schiff base interchange steps occur by way of intermediates having one more proton than usually assumed. Following the interchange, the extra proton could be stored on the lysine amino group which earlier was used to form the Schiff base to pyridoxal P. Evidence from model studies (Cordes & Jencks, 1962) is inconclusive with regard to whether Schiff base interchange might occur by way of this more highly protonated state. It is possible to draw a plausible mechanism for such an interchange.

The plot of V_{max} vs. pH for glutamate decarboxylase is also bell shaped. The pK_a on the low side is near 3.5 and might be attributed to protonation of one of the carboxyl groups of the enzyme-bound substrate, but there is no evidence for such an explanation. The pH optimum of the enzyme is near 4.5, and the activity of the enzyme gradually decreases above this

pH. The pK_a for this transition is near 5.7.

Carbon isotope effects on the enzymatic decarboxylation are independent of pH from pH 3.6 to 4.5 ($k^{12}/k^{13} = 1.015$). Above this pH, the isotope effect increases; at pH 5.5, $k^{12}/k^{13} = 1.022$ (O'Leary et al., 1970). A pH-dependent carbon isotope effect will not result from ionizations of enzyme, substrate, or enzyme–substrate complexes (O'Leary, 1978). Rather, such a pH dependence implies the existence of parallel reaction pathways having different numbers of protons.

The pH dependence of the kinetics and the isotope effects for glutamate decarboxylase probably arises as a result of loss of the proton from the pyridine nitrogen of pyridoxal P. At and below the pH optimum the nitrogen is protonated. The carbon isotope effect is ~1.015 and k_5/k_4 is ~3. The solvent isotope effect principally reflects the Schiff base interchange step. As the pH is increased, the pyridine nitrogen becomes deprotonated. Substrate binding and Schiff base interchange can still occur in the deprotonated state (though not necessarily at the same rate as in the protonated state) but decarboxylation cannot. Decarboxylation thus becomes more nearly rate limiting at higher pH, and the carbon isotope effect increases. For the same reason the solvent isotope effect becomes smaller, and the proton inventory plot becomes less curved. V_{max} decreases, but the pK_a may not correlate with that derived from the isotope effects. The expanded mechanism is

$$\begin{array}{ccccccccc} HE & + & S & \rightleftharpoons & HES & \rightleftharpoons & HES' & \longrightarrow & Q & \longrightarrow & HE + P \\ \uparrow\downarrow & & & & \uparrow\downarrow & & \uparrow\downarrow & & & & \\ E & + & S & \rightleftharpoons & ES & \rightleftharpoons & ES' & & & & \end{array}$$

in which the proton shown is the one on the pyridine nitrogen. HES and ES are Michaelis complexes, HES′ and ES′ are the glutamate Schiff bases, and Q is the quinoid intermediate.

Summary of Mechanism. The first step in the decarboxylation, substrate binding, appears to be at equilibrium. Binding is ~2-fold tighter in D_2O than in H_2O. This effect is probably derived principally from the desolvation of substrate and active site which occur on binding (O'Leary & Piazza, 1981). Most ionic substances are more soluble in H_2O than in D_2O (Arnett & McKelvey, 1969). If the desolvation which accompanies precipitation of salts is similar to the desolvation which accompanies substrate binding, then this effect is in the right direction. However, we cannot eliminate the possiblity that protein conformational factors are also involved in the solvent isotope effect on substrate binding.

The second step (Schiff base interchange) is partially rate determining. The solvent isotope effect on this step is ~7, and the proton inventory study shows that several protons show appreciable fractionation factors in the transition state. For two reasons we think that this step involves a conformation change along with the Schiff base interchange. In the first place, solvent isotope effects on model Schiff base interchanges are substantially smaller than the effect measured here (Fischer et al., 1980). Secondly, the proton inventory results are hard to explain on the basis of a simple Schiff base interchange. Although a number of protons change places in the course of the Schiff base interchange, the proton transfers presumably occur one at a time, and the aggregate effect of such a sequence of proton transfers does *not* produce a multicomponent proton inventory of the type observed here.

The more likely explanation is that this step is associated both with Schiff base interchange and with a conformational change at the active site (Ivanov & Karpeisky, 1969). In this mechanism, the Schiff base interchange is accompanied by rotation of the coenzyme, using the phosphate group as a pivot. A multiproton solvent isotope effect could easily arise in such a process. Stereochemical (Bertola, 1973) and X-ray crystallographic (Ford et al., 1980) studies of aspartate aminotransferase are consistent with the occurrence of a conformation change in this step.

Decarboxylation is at least partially rate determining for glutamate decarboxylase under all conditions studied. The specificity of the reaction is manifested primarily in this step (O'Leary & Piazza, 1978). The solvent isotope effect of ~3 is surprising, considering that no protons should be in transit during this step. We have previously argued, on the basis of medium effects and other data (O'Leary & Piazza, 1981), that amino acid decarboxylases operate by providing a favorable nonpolar medium in which the decarboxylation step can occur. Studies of the effect of ethylene glycol on the rate of the decarboxylation step (O'Leary & Piazza, 1981) indicate that the enzyme-bound pyridoxal P–substrate Schiff base is not entirely isolated from the effects of the medium. The solvent isotope effect observed in the decarboxylation step for glutamate decarboxylase may be related to this same phenomenon: a change in the solvent structure adjacent to the active site occurring as a result of the charge dispersal in the decarboxylation step.

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Mechanism of Action of Glutaryl-CoA and Butyryl-CoA Dehydrogenases. Purification of Glutaryl-CoA Dehydrogenase†

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ABSTRACT: Glutaryl-CoA dehydrogenase, a flavoprotein, catalyzes the reaction $^{-}OOCCH_2CH_2—CH_2COSR$ (FAD → $FADH_2$) → $CH_3CH{=}CHCOSR + CO_2$ (SR = CoA or pantetheine). With the isolated enzyme, a dye serves as the final electron acceptor. The enzyme from *Pseudomonas fluorescens* (ATCC 11250) has been purified to homogeneity. It was established with appropriate isotopic substitutions that the proton which is added to the γ position of the product, subsequent to decarboxylation, is not derived from the solvent but is derived from the α position of the substrate. Under conditions where no net conversion of substrate occurs, i.e., in the absence of electron acceptor, the enzyme catalyzes the exchange of the β hydrogen of the substrate with solvent protons. Butyryl-CoA dehydrogenase (*M. elsedenii*), which catalyzes an analogous reaction, catalyzes the exchange of both the α and β hydrogens with solvent protons in the absence of electron acceptor. Glutaryl-CoA dehydrogenase and butyryl-CoA dehydrogenase are irreversibly inactivated by the substrate analogues 3-butynoylpantetheine and 3-pentynoylpantetheine. These inactivators do not form an adduct with the flavin and probably react with a nucleophile at the active site. Upon inactivation, the spectrum of the enzyme-bound flavin is essentially unchanged, and the flavin can be reduced by $Na_2S_2O_4$. We suggest that inactivation involves intermediate allene formation. We proposed that these results support an oxidation mechanism for glutaryl-CoA dehydrogenase and butyryl-CoA dehydrogenase which is initiated by proton abstraction. With glutaryl-CoA dehydrogenase, the base, which abstracts the substrate α proton, is shielded from the solvent and is then used to protonate the carbanion (CH_2⁻⁻CH⁻⁻CHCOSCoA) formed after oxidation and decarboxylation.

Several different experimental approaches have led to the proposal that the initial step in reactions catalyzed by some flavoproteins is the abstraction of the substrate α hydrogen as a proton (Bright & Porter, 1975). The resulting carbanion then transfers electrons to the flavin by a currently unknown mechanism. Enzymes for which evidence for a carbanion mechanism has been obtained are the following: D- and L-amino acid oxidase (Walsh et al., 1971, 1973; Massey et al., 1976; Voet et al., 1972; Porter et al., 1972), L-lactic acid oxidase (Ghisla et al., 1976; Schonbrunn et al., 1976), and D-lactic acid dehydrogenase (Massey et al., 1979). The extensive model studies carried out by Bruice and his collaborators establish that in nonenzymatic reactions oxidations involving flavins can involve carbanionic intermediates when substrates are involved which form stable carbanions (Bruice, 1980). It should be noted that the above-mentioned enzymes act on substrates which are carboxylic acids; i.e., the hydrogen which is removed is α to a carboxyl group, and hence a mechanism for carbanion stabilization exists.

Several flavin dehydrogenases catalyze oxidations which lead to the formation of carbon–carbon double bonds. Examples of enzymes in this category are acyl-CoA dehydrogenases, glutaryl-CoA dehydrogenase, and succinic dehydrogenase. One of the hydrogens removed in these reactions is α to a carboxyl group or to a thioester group, and therefore the possible involvement of a carbanionic intermediate should be considered. The chemical mechanism of these reactions has not been extensively studied. It has been established that succinic dehydrogenase can catalyze the elmination of HF from difluorosuccinate (Tober et al., 1970). This observation is consistent with a carbanion mechanism. The reaction catalyzed by glutaryl-CoA dehydrogenase (eq 1) (Numa et

$$^{-}OOCCH_2CH_2—COSCoA \xrightarrow{Fl} {}_{Fl_{red}} CH_3CH{=}CHCOSCoA + CO_2 \quad (1)$$

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