Mandle, R., Jr., Colman, R. W., & Kaplan, A. P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4179–4183.
McConnell, D. J. (1972) *J. Clin. Invest. 51*, 1611–1623.
Meier, H. L., Pierce, J. V., Colman, R. W., & Kaplan, A. P. (1977) *J. Clin. Invest. 60*, 18–31.
Ouchterlony, O., & Nilsson, L. A. (1978) in *Handbook of Experimental Immunology* (Weir, D. M., Ed.) 3rd ed., pp 19.1–19.44, Blackwell Scientific, Oxford.
Pierce, J. V., & Guimaraes, J. A. (1976) in *Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease* (Pisano, J. J., & Austen, K. F., Eds.) pp 121–127, DHEW Publication No. (NIH) 76-791, U.S. Government Printing Office, Washington, DC.
Ratnoff, O. D., Pensky, J., Ogston, D., & Naff, G. B. (1969) *J. Exp. Med. 129*, 315–331.
Reboul, A., Arlaud, G. J., Sim, R. B., & Colomb, M. G. (1977) *FEBS Lett. 79*, 45–50.
Rosen, F. S., Charache, P., Pensky, J., & Donaldson, V. (1965) *Science (Washington, D.C.) 148*, 957–958.
Saito, H. (1977) *J. Clin. Invest. 60*, 584–594.
Saito, H., Ratnoff, O. D., Waldman, R., & Abraham, J. P. (1975) *J. Clin. Invest. 55*, 1082–1089.
Scott, C. F., & Colman, R. W. (1980) *J. Clin. Invest. 65*, 413–421.
Scott, C. F., Liu, C. Y., & Colman, R. W. (1979) *Eur. J. Biochem. 100*, 77–83.
Sealey, J. E., Atlas, S. A., Laragh, J. H., Silverberg, M., & Kaplan, A. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5914–5918.
Silverberg, M., Thompson, R., Miller, G., & Kaplan, A. P. (1980) in *Regulation of Coagulation* (Mann, K., & Taylor, F., Eds.) pp 531–541, Elsevier/North-Holland, New York.
Thompson, R. E., Mandle, R., Jr., & Kaplan, A. P. (1977) *J. Clin. Invest. 60*, 1376–1380.
Thompson, R. E., Mandle, R., Jr., & Kaplan, A. P. (1978) *J. Exp. Med. 147*, 488–499.
Thompson, R. E., Mandle, R., Jr., & Kaplan, A. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4862–4866.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wickerhauser, M., Hao, Y. L., & Mercer, J. (1978) *Joint Congress of the International Societies of Hematology and Blood Transfusion*, Abstr., p 483.
Wiggins, R. C., Bouma, B. N., Cochrane, C. G., & Griffin, J. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4636–4640.
Wuepper, K. D., Miller, D. R., & Lacombe, M. J. (1975) *J. Clin. Invest. 56*, 1663–1672.

Medium Effects in Enzyme-Catalyzed Decarboxylations†

Marion H. O'Leary* and George J. Piazza

ABSTRACT: Carbon isotope effects and steady-state kinetic parameters have been measured for the decarboxylation of arginine and homoarginine by the pyridoxal 5′-phosphate dependent arginine decarboxylase from *Escherichia coli*. In water at pH 5.25, 5 °C, homoarginine shows an isotope effect $k^{12}/k^{13} = 1.061$, indicating that the decarboxylation step is entirely rate determining. In the presence of 16 mol % ethylene glycol under otherwise identical conditions, the decarboxylation rate is increased 3-fold, and the carbon isotope effect is 1.044, indicating that the rate of the decarboxylation step is increased by the presence of the less polar solvent. The decarboxylation of arginine under the same conditions shows a similar trend: in water, the isotope effect is 1.027, decreasing to 1.003 in 16% ethylene glycol, with little change in the steady-state rate. Again, the rate of the decarboxylation step is substantially increased by the presence of the nonpolar solvent. Thus, pyridoxal phosphate dependent enzymatic decarboxylations show a medium effect similar to that observed in a number of nonenzymatic decarboxylations. This suggests that these enzymes may accelerate the decarboxylation step by providing a nonpolar environment. Evidence is also presented that desolvation of the substrate carboxyl group may contribute to catalysis.

Most amino acid decarboxylases require pyridoxal 5′-phosphate (pyridoxal-P)[1] for activity. The decarboxylation mechanism (Scheme I) was suggested nearly simultaneously by Metzler et al. (1954) and by Westheimer (Mandeles et al., 1954). Subsequent studies of enzymes and models have confirmed most features of this mechanism (Boeker & Snell, 1972).

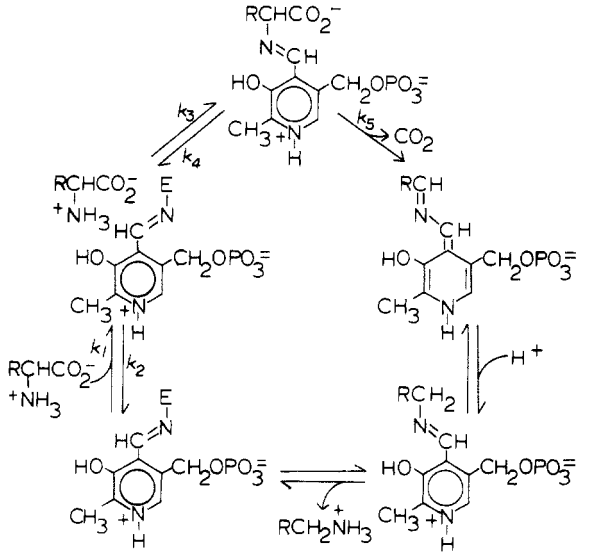
Considerable progress has been made in understanding the underlying mechanisms of catalysis of the various reaction steps. Initial substrate binding involves particularly the distal functional groups of the substrate (Boeker & Snell, 1972). In model reactions, the Schiff base interchange step is catalyzed by the 3-hydroxyl group of the coenzyme (Bruice & Benkovic, 1966), and similar catalysis probably also occurs in enzymatic decarboxylations. Catalysis of the decarboxylation step is still somewhat enigmatic (O'Leary, 1977). At a first level the coenzyme functions as an "electron sink", obviating the necessity for forming a highly unstable carbanion. This function has been demonstrated in numerous model studies with pyridoxal analogues (Bruice & Benkovic, 1966; Kalyankar & Snell, 1962). However, the rates of enzymatic decarboxylations exceed those of model reactions by many orders of magnitude, and other factors must also contribute to the catalysis. It is clear, following the suggestion of Dunathan (1966), that stereochemical factors also play an important role. Decarboxylases must control the conformation of the enzyme-bound pyridoxal-P–amino acid Schiff base in order to prevent the kinetically more favorable transamination. The

† From the Department of Chemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received May 27, 1980.* This work was supported by Grant NS-07657 from the National Institutes of Health and by Grant PCM77-00812 from the National Science Foundation.

[1] Abbreviation used: pyridoxal-P, pyridoxal 5′-phosphate.

0006-2960/81/0420-2743$01.25/0

Scheme I



pyridoxal-P-dependent α-decarboxylases which have been studied show a complete retention of configuration during the decarboxylation (Yamada & O'Leary, 1978; Belleau & Burba, 1960; Leistner & Spenser, 1975). The high substrate specificity of decarboxylases is related to steric control of the decarboxylation step (O'Leary & Piazza, 1978). However, the degree of rate acceleration which can be achieved by steric control is undoubtedly modest, as it is related to the proportion of Schiff base forms which might otherwise be in improper conformations.

Another factor which may be important in enzymatic catalysis of decarboxylation is the medium effect. Crosby et al. (1970) have shown that in the case of thiamine pyrophosphate dependent decarboxylations the rate of the model reaction is very substantially affected by the polarity of the environment; decreasing medium polarity causes a greatly increased rate. The explanation for this phenomenon is that the starting state for the decarboxylation is a zwitterion with a very large charge separation. In the transition state for the decarboxylation, the dipolar character is largely dissipated. A similar phenomenon has been observed in the decarboxylation of benzisoxazole-3-carboxylic acids by Kemp & Paul (1975) and Kemp et al. (1975) and in the decarboxylation of 4-pyridylacetic acids by Button & Taylor (1973). The same polarity effect might be expected to operate in pyridoxal-P-dependent decarboxylations. The starting state for the decarboxylation is a zwitterion having a carboxylate anion and a pyridinium cation a great distance apart. During the decarboxylation step, these charges are dissipated, forming a neutral quinoid intermediate. To date, this medium effect has not been demonstrated for pyridoxal-P-dependent decarboxylations either in model systems or in enzyme systems. In this paper, we use isotope effects to study the medium effect on the decarboxylation rate for the pyridoxal-P-dependent arginine decarboxylase of *Escherichia coli*. The decarboxylation step becomes faster as the medium becomes less polar, suggesting that a medium effect may also be important in enzymatic decarboxylations.

Experimental Procedures

Materials

L-Arginine hydrochloride and pyridoxal-P were purchased from Sigma. L-Homoarginine hydrochloride (gold label) was purchased from Aldrich. The amino acid substrates were dried under high vacuum until their weights were constant. Ethylene glycol was fractionally distilled under vacuum from sodium hydroxide pellets. Sodium acetate buffer was prepared by titrating a solution of glacial acetic acid with concentrated sodium hydroxide. Water was purified by a Millipore Super-Q filtration system. All other materials were of the highest available purity. Arginine decarboxylase (EC 4.1.1.19) from *E. coli* B was purified to homogeneity by the procedure of Boeker et al. (1969).

Methods

pH Measurements. pH was measured on a Radiometer 26 pH meter calibrated by the two-buffer method. pH changes with temperature in acetate buffers were found to be insignificant. The pH* in various concentrations of ethylene glycol was adjusted to 5.25 by interpolation of the data obtained by Douzou (1977). These data consist of tables of pH values at 20 °C of acetate buffers, together with the resulting pH* values at various temperatures when the acetate buffer is mixed with 50% (by volume) ethylene glycol. The interpolation was based on the assumption that the difference between pH and pH* is directly proportional to the mole fraction of ethylene glycol.

Enzyme Assay. Steady-state kinetic data were obtained from the rate of carbon dioxide evolution monitored by using a Gilson all-glass differential respirometer and were corrected by the method of Gregory & Winter (1965). The solubility of all forms of carbon dioxide at pH* 5.25 in various concentrations of ethylene glycol was determined empirically by measuring the amount of carbon dioxide evolved when adding 0.5 mL of a potassium bicarbonate solution of known concentration to the assay buffer. This small amount of bicarbonate did not change the pH significantly. The kinetic assays were linear for at least 45 min at high substrate concentration. However, an initial lag was observed whose length increased at lower temperatures and increased as the solution volume increased. Since the solubility of carbon dioxide increases at lower temperatures, it is likely that the lag is due to the retention of carbon dioxide in the solution phase. At 5 °C with 20 mL of assay solution, the lag typically lasted for about 15–20 min. At very low substrate concentrations, a significant fraction of substrate was used during the lag period. The total amount of substrate consumed was estimated from the rate obtained from the linear portion of the assay curve, and the substrate concentration was adjusted accordingly when analyzing the data. All of the data were statistically analyzed by using the computer programs written by Cleland (1967). All of the data were correlated to the rate obtained at 37 °C with 25 mM arginine. The reported errors therefore reflect not only the errors given by the statistical analysis but also the errors due to the referencing procedure.

Carboxyl Carbon Isotope Effects. The general procedure has been published (O'Leary, 1980) and will not be repeated here except for presenting specific details relating to the experiments discussed in this study.

Variation in Ethylene Glycol Concentration. The buffer was mixed with the appropriate amount of ethylene glycol by volume. The final pH* was always 5.25 under the conditions of the experiment. The concentration of acetate buffer after mixing was always 0.2 M. The final concentrations of arginine or homoarginine were 25 mM, and the final concentration of pyridoxal-P was 10^{-2} mM. The enzyme was degassed on Sephadex G-25 by using aqueous buffer. Before the enzyme was added to the decarboxylation solution, it was mixed with the appropriate amount of ethylene glycol. The ethylene glycol had been freed of extraneous carbon dioxide by bubbling carbon dioxide free nitrogen through it for about 1 h. Care

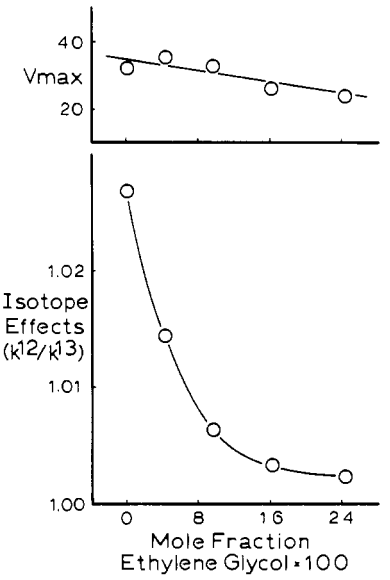


FIGURE 1: Variation of V_{max} and carbon isotope effect with ethylene glycol concentration in the decarboxylation of arginine by arginine decarboxylase at 5 °C, pH* 5.25.

was taken to make sure that the temperatures of the enzyme solution and ethylene glycol were below 5 °C before mixing. Arginine decarboxylase is stable at high temperatures in up to 50% (by volume) ethylene glycol, but lower temperatures during the mixing process are beneficial probably because a large amount of heat is liberated when ethylene gycol is mixed with water.

Extra care had to be taken in isolating carbon dioxide because carbon dioxide is quite soluble in ethylene glycol. All of the reactions that were run to completion contained the appropriate amount of ethylene glycol, but were run at room temperature. The isotope ratio of carbon dioxide from the reaction that was allowed to run to completion was the same as that from a reaction in purely aqueous solution. This is a good indication that ethylene glycol had no detrimental effects upon the isolation or analysis procedure.

Arginine decarboxylase was stable for at least several hours at the highest concentration of ethylene glycol used, and the effects of ethylene glycol on both rates and isotope effects could be fully reversed by returning the enzyme to aqueous buffer free of cosolvent.

Results

In Figure 1 are shown carbon isotope effects and maximum velocities for the decarboxylation of arginine by arginine decarboxylase in various water–ethylene glycol mixtures. Kinetic measurements are maximum velocities obtained from reciprocal plots. Isotope effects are the result of 3–5 independent determinations and are generally reproducible to ±0.0002.

In Table I are kinetic parameters and carbon isotope effects for the decarboxylation of arginine in water and in 16% ethylene glycol at temperatures from 5 to 50 °C. At higher temperatures, the addition of ethylene glycol causes a more pronounced decrease in V_{max} than is seen at 5 °C. This increased sensitivity is not due to enzyme denaturation since at high substrate concentration the rate of reaction is constant for more than 30 min. The rate change observed on addition of ethylene glycol is not due to pH/pK changes because V_{max} is independent of pH in the range pH 4–6 (data not shown). The carbon isotope effect is also independent of pH in this same range (Figure 2).

A similar decrease in the isotope effect was observed when dimethyl sulfoxide rather than ethylene glycol was used as a cosolvent. In the presence of 25% dimethyl sulfoxide (v/v) at 5 °C, k^{12}/k^{13} = 1.0024.

Table I: Steady-State Kinetic Constants and Carboxyl Carbon Isotope Effects for the Decarboxylation of Arginine at Various Temperatures in Water and in 16.2 mol % Ethylene Glycol

temp (°C)	mol fraction of ethylene glycol	V_{max}[a] (s^{-1})	V/K (s^{-1} mM^{-1})	k^{12}/k^{13}[b]
5	0	32.1 ± 0.9	40.4 ± 3.6	1.0277 ± 0.0005
25	0	243 ± 10.7	395 ± 18	1.0147 ± 0.0004
37	0	560 ± 27	560 ± 27	1.0122 ± 0.0002
50	0	958 ± 44	998 ± 46	1.0126 ± 0.0001
5	0.162	26.0 ± 0.6	18.1 ± 1.0	1.0033 ± 0.0002
25	0.162	142 ± 6	128 ± 12	
37	0.162	353 ± 17	269 ± 15	1.0022 ± 0.0001

[a] Steady-state kinetic measurements were performed manometrically. The assays in water contained 0.2 M sodium acetate buffer, pH 5.25, and 2.0 × 10^{-5} M pyridoxal-P. The assays in ethylene glycol–water contained 0.2 M sodium acetate buffer, pH* 5.25, and 2.5 × 10^{-5} M pyridoxal-P. [b] Carbon isotope effects were measured at pH* 5.25 in 0.2 M sodium acetate buffer containing 1 × 10^{-5} M pyridoxal-P.

Table II: Temperature and Solvent Effects on Catalytic Constants and Carbon Isotope Effects for the Decarboxylation of Homoarginine at pH 5.25

mol fraction of ethylene glycol	temp (°C)	V_{max}[a] (s^{-1})	V/K (s^{-1} mM^{-1})	k^{12}/k^{13}[b]
0	5	0.038 ± 0.006	0.038 ± 0.008	1.0608 ± 0.0003
0	25	1.5 ± 0.1	0.82 ± 0.01	1.0566 ± 0.0003
0	37	6.2 ± 0.3	6.2 ± 1.3	
0.162	5	0.108 ± 0.018	0.154 ± 0.043	1.0442 ± 0.0003

[a] Steady-state kinetic measurements were performed manometrically at pH 5.25 in 0.2 M sodium acetate buffer containing 2.5 × 10^{-5} M pyridoxal-P. [b] Carbon isotope effects were measured at pH (or pH*) 5.25 in 0.2 M sodium acetate buffer containing 1 × 10^{-5} M pyridoxal-P.

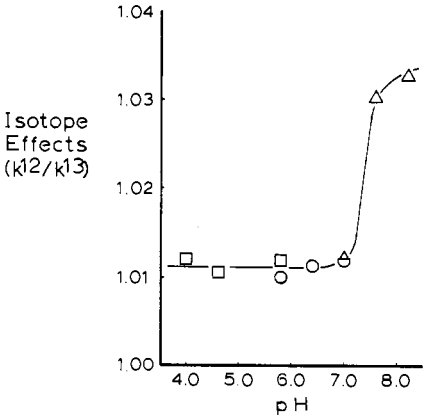


FIGURE 2: pH dependence of carbon isotope effects for the decarboxylation of arginine by arginine decarboxylase at 25 °C in (□) 0.2 M acetate buffer, (○) 0.05 M sodium 2-(*N*-morpholino)ethanesulfonate, and (△) 0.05 M sodium *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonate.

Table II shows the results of steady-state and isotope effect studies at various temperatures with homoarginine as the substrate. Also shown are the results of these studies at 5 °C in an ethylene glycol–water mixture. The change in the isotope effects in going from 25 to 5 °C is about one-third as large as that observed with arginine as the substrate. At 5 °C, the isotope effects decrease when ethylene glycol is added, but the V_{max} increases by about a factor of 2.8.

Discussion

The mechanism of action of arginine decarboxylase is shown in Scheme I. With the assumption, as usual (O'Leary, 1977), that only the decarboxylation step shows an isotope effect (although we will later modify this assumption slightly) and that the decarboxylation step is irreversible under our reaction conditions, the observed isotope effect $[k^{12}/k^{13}(\text{obsd})]$ is related to the rate constants of Scheme I and to the isotope effect on the decarboxylation step (k_5^{12}/k_5^{13}, called the "intrinsic isotope effect") by eq 1 (O'Leary, 1978). According to this equation,

$$k^{12}/k^{13}(\text{obsd}) = \frac{k_5^{12}/k_5^{13} + (k_5/k_4)(1 + k_3/k_2)}{1 + (k_5/k_4)(1 + k_3/k_2)} \quad (1)$$

the observed isotope effect is smaller than the intrinsic isotope effect by an amount related to $(k_5/k_4)(1 + k_3/k_2)$. Unless this factor is small, the observed isotope effect will be much smaller than the intrinsic isotope effect. Under many conditions, substrate binding should be relatively near equilibrium. When this is so, $k_3/k_2 \ll 1$, and eq 1 can be simplified to

$$k^{12}/k^{13}(\text{obsd}) = \frac{k_5^{12}/k_5^{13} + k_5/k_4}{1 + k_5/k_4} \quad (2)$$

The maximum velocity in this case is given by

$$V_{max} = \frac{k_3 k_5}{k_3 + k_4 + k_5} \quad (3)$$

assuming all steps subsequent to decarboxylation are fast.

Decarboxylation of Homoarginine. Arginine decarboxylase acts on homoarginine at a rate which is reduced about 100× compared to arginine, but with little change in K_m. Because of this reduced rate, it is safe to conclude that substrate binding and dissociation are not slow for homoarginine and $k_3/k_2 \approx 0$. Thus, the carbon isotope effect on the decarboxylation of homoarginine can be discussed in terms of eq 2, rather than eq 1. This carbon isotope effect is the largest ever seen for an enzymatic decarboxylation (O'Leary, 1977) and is in the range of isotope effects observed in nonenzymatic reactions in which the decarboxylation step is entirely rate determining (Dunn, 1977). Thus, we conclude qualitatively that decarboxylation is entirely rate determining or nearly so for homoarginine ($k_5/k_4 \approx 0$).

The temperature dependence of the maximum velocity reinforces the conclusion regarding the rate-determining step. Unlike the decarboxylation of arginine (see below), the decarboxylation of homoarginine gives a linear Arrhenius plot, as expected for a reaction with a single rate-determining step.

The temperature dependence of the carbon isotope effect on the decarboxylation of homoarginine is small and is of the direction and approximate magnitude expected for an intrinsic isotope effect (Bigeleisen & Wolfsberg, 1958). This is different from the case of arginine, for which there is a very large temperature effect.

When the kinetics and carbon isotope effects are measured in solvents containing ethylene glycol, a significant increase in the decarboxylation rate is observed, along with a decrease in the decarboxylation isotope effect. These changes are not due to pH/pK effects because both the isotope effect and the V_{max} are independent of pH in water for at least 1 pH unit around the pH at which the experiments were done.

Since decarboxylation is rate determining in the case of homoarginine, the rate increase observed in the mixed solvent indicates that the rate of the decarboxylation step is *increasing* as the medium becomes less polar.

The other change which occurs as the medium becomes less polar is that the observed isotope effect decreases. This might mean either that the intrinsic isotope effect decreases or that decarboxylation is no longer entirely rate determining $[(k_5/k_4)(1 + k_3/k_2)$ in eq 1 is no longer $\approx 0]$.

We do not believe that the change in the isotope effect is due to changes in the intrinsic isotope effect. This explanation would require that there be a very significant change in the transition-state structure with solvent. In an extensive study of solvent and substituent effects on the decarboxylation of benzisoxazole-3-carboxylic acids, Kemp & Paul (1975) and Kemp et al. (1975) showed that substituent effects on the decarboxylation were substantially independent of the solvent, even though the decarboxylation rate varied over many orders of magnitude. This indicates that the transition-state structure in decarboxylations is largely unaffected by solvent.

It is more likely that the change in the isotope effect for homoarginine occurs because in the mixed solvent decarboxylation is no longer entirely rate determining. In water, the rate of the Schiff base interchange is sufficiently faster than the rate of the decarboxylation that k_5/k_4 is approximately zero, and the observed isotope effect is nearly equal to the intrinsic isotope effect. However, as the medium becomes less polar, k_5 increases. Simultaneously, k_4 decreases somewhat, with the result that in 16.2% ethylene glycol, k_5/k_4 has increased from a value of ~ 0 to about 0.3. The solvent dependence of k_4 can be more clearly seen in the case of arginine (see below).

Intrinsic Isotope Effects in Decarboxylations. The magnitude of the decarboxylation isotope effect for homoarginine finally dispels one of the difficult questions which has been posed with respect to isotope effects on enzymatic decarboxylations (O'Leary, 1977, 1978): Is the intrinsic isotope effect in an enzyme-catalyzed decarboxylation of approximately the same magnitude as those observed in nonenzymatic decarboxylations, or does the enzyme operate to reduce significantly the size of the intrinsic isotope effect? The magnitude of the isotope effect for homoarginine indicates that the enzyme does not operate in this way and that isotope effects in models provide a proper reference for isotope effects in enzymatic decarboxylations. However, it should be noted that this conclusion applies with certainty *only* to the case of enzymatic decarboxylations, and the answer may be different for reactions of other types.

We must now ask whether we expect the same intrinsic isotope effect for arginine as for homoarginine. We believe that the two intrinsic effects will be equal or nearly so for several reasons: First, electronically, the two substrates are so similar that on purely chemical grounds we would expect very similar transition states and, therefore, very similar isotope effects. Further, even though there is a nearly 100× difference in rate between the two substrates, they are two similar substrates reacting by the same mechanism at the same enzyme active site. Decarboxylation transition states are in general quite independent of structure and environment (indeed, this is the basis for the fact that intrinsic isotope effects for a wide variety of model decarboxylations fall in a fairly narrow range), and that independence should extend to the present case. It

is hard to see what the enzyme could do to change the size of the intrinsic isotope effect. The ground states are the free amino acids (not the enzyme-bound amino acids), so there is no important potential for change there. For the enzyme to affect the structure of the transition state, it would have to somehow affect the carbon–carbon bond which is in the process of being cleaved by changing either a stretching or a bending mode of this bond. In enzymes where a group is being transferred between a donor and an acceptor (for example, in hydride transfer reactions), it is easy to see how the enzyme might manipulate transition-state structure (for example, by manipulating donor and acceptor geometries), but it is much harder to imagine a mechanism by which this same effect might be achieved in decarboxylations. It is conceivable in the decarboxylation case that the enzyme might provide some limited steric compression, leading to a change in bending vibrations, but even if such an effect were to occur, we do not think it would be significantly different for arginine and homoarginine.

Thus, we conclude that intrinsic isotope effects on the decarboxylation of arginine and homoarginine are very similar. It follows that enzymatic intrinsic isotope effects for decarboxylations of both good and bad substrates are similar in magnitude to the corresponding nonenzymatic values.

Decarboxylation of Arginine. The case of arginine is more complex than that of homoarginine. Carbon isotope effects for arginine in water are 1.012–1.028, depending on the temperature. With the assumption the intrinsic isotope effect is near 1.06, this indicates that the ratio $(k_5/k_4)(1 + k_3/k_2)$ (cf. eq 1) is slightly larger than unity. This, of course, means that decarboxylation is not entirely rate limiting: prior steps contribute significantly to the rate. Temperature effects on the rates and isotope effects for decarboxylation of arginine are consistent with this interpretation. Arrhenius plots of the temperature dependences of V_{max} and V_{max}/K_m (Table I) are significantly curved, as expected for a reaction in which two steps contribute approximately equally to the rate (Laidler & Bunting, 1973). This is different from the case of homoarginine, in which a single step is rate determining and the Arrhenius plot is linear. The temperature dependence of the carbon isotope effect is much larger than in the case of homoarginine, in which a single step is rate determining, and we conclude that the ratio $(k_5/k_4)(1 + k_3/k_2)$ for arginine is very temperature dependent.

The effect of ethylene glycol on the rates and isotope effects for arginine is even more dramatic than for homoarginine. Addition of ethylene glycol to the solvent at 5 °C causes only a very slight decrease in the rate (12% decrease in 16% ethylene glycol) but causes at the same time a very large decrease in the isotope effect. There is a smooth decrease in the isotope effect (Figure 1) from 1.0269 in pure water to 1.0023 in 24% ethylene glycol, and it appears that the isotope effect will reach a final value slightly greater than 1.000—probably in the range 1.001–1.002. The trend in both isotope effects and kinetics is the same at 37 °C as at 5 °C.

Again, these observations are not the result of pH/pK effects, because both the isotope effect and the V_{max} are independent of pH for more than 1 unit on either side of the pH of the experiments. In addition, it is clear that we are not dealing with a uniquely low-temperature property of the enzyme.

The interpretation of the solvent dependence of the isotope effect begins with the observation that in pure water at 5 °C $(k_5/k_4)(1 + k_3/k_2)$ is approximately 1.2. As ethylene glycol is added, the rate of the decarboxylation step increases just as the rate of the decarboxylation step increases for homoarginine. If this were the only effect occurring, then we would expect to see an increase in V_{max} as the concentration of ethylene glycol increases. The fact that such an increase is not seen must indicate that there is a compensating decrease in k_2 and/or k_3 and k_4, with the result that V_{max} stays approximately constant (we assume that the equilibrium constant for Schiff base interchange is independent of solvent, so that these changes are reflected in both k_3 and k_4). In 25% ethylene glycol, $(k_5/k_4)(1 + k_3/k_2)$ has increased to a value of at least 25; thus, in this solvent, decarboxylation is not the rate-determining step. The same solvent dependence is observed at 37 °C. In water, $(k_5/k_4)(1 + k_3/k_2)$ is approximately 3.5, whereas in 16% ethylene glycol, the value is at least 25.

Thus, decarboxylations catalyzed by arginine decarboxylase show large medium effects. The rate of the Schiff base interchange step is decreased by the inclusion of ethylene glycol or dimethyl sulfoxide in the solvent. The rate of the decarboxylation step, on the other hand, is significantly increased in the mixed solvent.

Role of Solvation in Decarboxylation. Recent studies of the decarboxylation of benzisoxazole-3-carboxylic acids (Kemp & Paul, 1975; Kemp et al., 1975; Shirai & Smid, 1980) indicate that desolvation of the carboxyl group is an important factor in determining rates of nonenzymatic decarboxylations. Decarboxylation in a non-hydrogen-bonding medium is significantly faster than in a hydrogen-bonding medium, and decarboxylation can be catalyzed by extraction from a hydrogen-bonding into a non-hydrogen-bonding medium. Might enzymes also catalyze decarboxylation by removing the carboxyl group from contact with the solvent?

It appears that the limiting value of the carbon isotope effect on the enzymatic decarboxylation of arginine at 5 °C in solvents containing ethylene glycol is not 1.000, but a value near 1.002 (cf. Figure 1 and Table I). Isotope effects in water–ethylene glycol mixtures at 37 °C and in water–dimethyl sulfoxide mixtures at 5 °C are less extensive but appear to be approaching the same limit. We do not believed that this is simply due to our inability to attain conditions in which Schiff base interchange is fully rate determining. Instead, we believe that this residual isotope effect represents a real isotope effect on some step prior to decarboxylation.

A carbon isotope effect in the range of 1.001–1.002 is of the proper magnitude and direction to be associated with desolvation of the substrate carboxyl group during substrate binding. Appropriate models for such an effect are limited, but include the carbon isotope effect on the solubility of CO_2 in water [1.0009, with ^{13}C concentrating in the solution phase; see Mook et al. (1974)] and the carbon isotope effect on the acid dissociation constant of benzoic acid [1.0013, with ^{13}C concentrating in the protonated state; see Bayles et al. (1976)].

Conclusion

This study provides evidence for two mechanisms by which enzymes may accelerate decarboxylation reactions, a medium effect and a desolvation effect.

A combination of steady-state kinetics and carbon isotope effects indicates that for the decarboxylation of arginine and homoarginine a decrease in medium polarity leads to an increase in the rate of the decarboxylation step. Because of the complexities of solvent effects associated with Schiff base interchange, this effect is not necessarily obvious in the steady-state kinetics alone. This medium effect is as expected for a step in which charges at opposite ends of a long conjugated system are neutralized and is consistent with the idea that decarboxylases may provide a nonpolar environment

which favors the decarboxylation step. This is the first demonstration of this medium effect for a decarboxylase.

Because of uncertainties connected with any carbon isotope effect on the initial steps of the reaction, it is not possible to estimate with certainty the magnitude of the medium effect on the decarboxylation step, but it appears that the medium effect is substantially smaller than that in model reactions. This is presumably because the enzyme itself provides a large fraction of the requisite medium effect and in addition shields the substrate from effects of the solvent.

This study also provides evidence that desolvation of the carboxyl group of the substrate may provide a significant driving force toward decarboxylation. It is not currently possible to estimate the magnitude of this effect, but studies in model systems indicate that it may be substantial.

Thus, enzymatic catalysis of decarboxylation can be explained in terms of a small number of components: (1) the "electron sink" concept; (2) stereochemical control; (3) the medium effect; (4) desolvation of the carboxyl group. Although it is not possible to make a quantitative appraisal of the contributions of these various effects, it seems possible that these four components represent a relatively complete list.

References

Bayles, J. W., Bron, J., & Paul, S. O. (1976) *J. Chem. Soc., Faraday Trans. 1 72*, 1546.

Belleau, B., & Burba, J. (1960) *J. Am. Chem. Soc. 82*, 5751.

Bigeleisen, J., & Wolfsberg, M. (1958) *Adv. Chem. Phys. 1*, 15.

Boeker, E. A., & Snell, E. E. (1972) *Enzymes, 3rd Ed. 6*, 217.

Boeker, E. A., Fischer, E. E., & Snell, E. E. (1969) *J. Biol. Chem. 244*, 5239.

Bruice, T. C., & Benkovic, S. J. (1966) *Bioorg. Mech. 2*, 181.

Button, R. G., & Taylor, P. J. (1973) *J. Chem. Soc., Perkin Trans. 2*, 557.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1.

Crosby, J., Stone, R., & Lienhard, G. E. (1970) *J. Am. Chem. Soc. 92*, 7312.

Douzou, P. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 157.

Dunathan, H. C. (1966) *Proc. Natl. Acad. Sci. U.S.A. 55*, 712.

Dunn, G. E. (1977) *Isot. Org. Chem. 3*, 1.

Gregory, K. F., & Winter, H. C. (1965) *Anal. Biochem. 11*, 519.

Kalyankar, G. D., & Snell, E. E. (1962) *Biochemistry 1*, 594.

Kemp, D. S., & Paul, K. G. (1975) *J. Am. Chem. Soc. 97*, 7305.

Kemp, D. S., Cox, D. D., & Paul, K. G. (1975) *J. Am. Chem. Soc. 97*, 7312.

Laidler, K. J., & Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, p 196, Oxford University Press, New York.

Leistner, E., & Spenser, I. E. (1975) *J. Chem. Soc., Chem. Commun.*, 378.

Mandeles, S., Koppelman, R., & Hanke, M. E. (1954) *J. Biol. Chem. 209*, 327.

Metzler, D. E., Ikawa, M., & Snell, E. E. (1954) *J. Am. Chem. Soc. 76*, 648.

Mook, W. G., Bommerson, J. C., & Staverman, W. H. (1974) *Earth Planet. Sci. Lett. 2*, 357.

O'Leary, M. H. (1977) *Bioorg. Chem. 1*, 259.

O'Leary, M. H. (1978) in *Transition States of Biochemical Processes* (Gandour, R., & Schowen, R. L., Eds.) p 285, Plenum Press, New York.

O'Leary, M. H. (1980) *Methods Enzymol. 64B*, 83.

O'Leary, M. H., & Piazza, G. J. (1978) *J. Am. Chem. Soc. 100*, 632.

Shirai, M., & Smid, J. (1980) *J. Am. Chem. Soc. 102*, 2863.

Yamada, H., & O'Leary, M. H. (1978) *Biochemistry 17*, 669.