(8) Anal: Calcd for $C_{28}H_{34}O_6S$: C, 67.45; H, 6.87; S, 6.43. Found C, 67.68; H, 6.93; S, 6.34. IR (Nujol mull): 1709 cm^{-1} (s). ^1H NMR (pyridine-d_5, 220 MHz): δ 8.342 and 8.098 (d of d, 4, J_{AB} = 1.3 Hz, aromatic C*H*), 1.799 (s, 6, OCCH_3), 1.782 (s, 6, OCCH_3), 1.318 (s, 18, *t*-BuCH_3). The chirality of **1** was evidenced by the addition of optically active 2,2,2-trifluoro-1-phenylethanol, purchased from Burdick and Jackson Laboratories, Inc., to a CD_2Cl_2 solution of **1** (−30 °C) in the 220-MHz NMR spectrum. The methyl singlet at δ 1.782 and the *tert*-butyl singlet were each resolved into two singlets. For a discussion of the use of this chiral alcohol in NMR spectroscopy of racemic mixtures, see W. H. Pirkle, *J. Am. Chem. Soc.*, **88**, 1837 (1966) and W. H. Pirkle and S. D. Beare, *ibid.*, **90**, 6250 (1968).

(9) Anal. Calcd for $C_{28}H_{34}O_6S$: C, 67.45; H, 6.87; S, 6.43. Found C, 67.18; H, 6.78; S, 6.63. IR (Nujol mull): 3400 (m), 1710 (s), 1320 (s) and 1165 cm^{-1} (s). ^1H NMR (pyridine-d_5): δ 7.805 and 7.340 (d of d, 4, J_{AB} = 2.1 Hz, aromatic C*H*), 5.277 (br s, 2, olefinic C*H*), 4.818 (br s, 2, olefinic C*H*), 1.939 (s, 6, CH_3 at olefinic carbon), 1.316 (s, 18, *t*-BuCH_3).

(10) J. C. Martin and L. J. Adzima, preceding communication.

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Received November 22, 1976

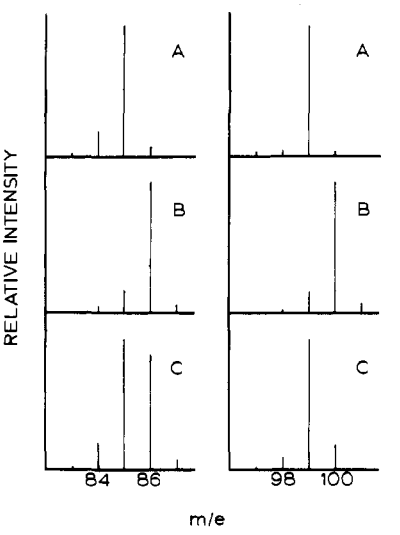


Figure 1. (left panel) Mass spectrometer scans of the molecular-weight region of the γ-butyrolactam formed by enzymatic decarboxylation of glutamic acid at pL 4.6, 22 °C (A) in H_2O; (b) in D_2O; (C) in 50:50 H_2O:D_2O. (right panel) Same, for the γ-valerolactam formed by enzymatic decarboxylation of α-methylglutamic acid at pL 4.6, 22 °C.

A Solvent Isotope Effect Probe for Enzyme-Mediated Proton Transfers

Sir:

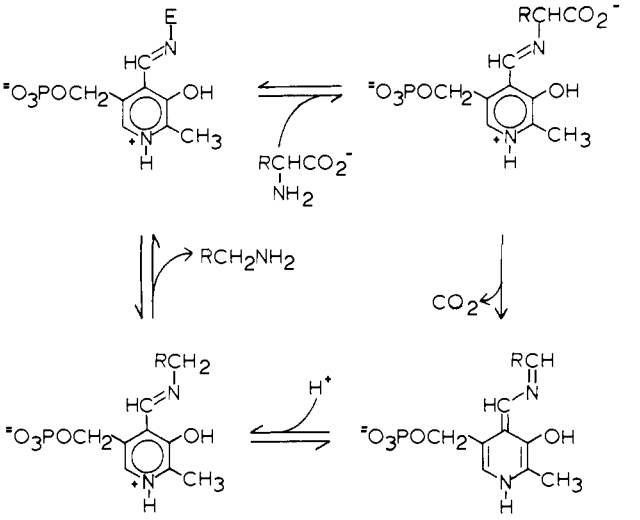
A crucial step in a number of enzyme-catalyzed reactions is the transfer of a proton from the solvent to a carbon atom of the substrate. Examples of such reactions include decarboxylations and other carbon-carbon bond cleavage reactions, some hydration and dehydration reactions, and a variety of others. The proton transfer may occur either directly from the solvent or else through the mediation of some catalytic group of the enzyme. We show here that a distinction between these two possibilities can sometimes be made by measuring the hydrogen isotope discrimination which occurs when the reaction is conducted in 50:50 H_2O:D_2O.

Proton transfers to and from carbon are ordinarily subject to hydrogen isotope effects[1,2] in the range k^H/k^D = 2-10. If a proton transfer from the solvent to a carbon atom of the substrate occurs during the course of an enzymatic reaction, then such an isotope effect is expected to occur in that proton transfer step. If the reaction is conducted in 50:50 H_2O:D_2O the isotopic composition of the product will reflect this isotope effect, provided that (1) the proton transfer occurs either directly from the solvent or from a catalytic group of the enzyme which undergoes rapid hydrogen exchange with the solvent and (2) the enzyme does not catalyze hydrogen exchange between solvent and product under the conditions of the experiment.

On the other hand, this isotope discrimination may not be observed if the proton transfer occurs through the mediation of a monoprotic[3] catalytic group which is shielded from hydrogen exchange with the solvent. Under such conditions the only proton available for transfer to the intermediate is the hydrogen attached to that catalytic group and the isotopic content of the product will reflect only the equilibrium isotope fractionation between the solvent and the catalytic group. For imidazole and carboxyl groups, this equilibrium fractionation is near unity,[4] and no hydrogen isotope discrimination in the product is expected.[5] Thus, the absence of hydrogen isotope discrimination in the protonation of an enzyme-substrate complex may serve as evidence for mediation of the proton transfer by a monoprotic catalytic group of the enzyme which is shielded from proton exchange with the solvent during the lifetime of the appropriate intermediate.[6]

The pyridoxal 5′-phosphate dependent amino acid decarboxylases function by a mechanism involving an enzyme-bound Schiff base between the coenzyme and the substrate[7] (Scheme I). Decarboxylation produces a quinoid intermediate which

Scheme I



is protonated almost exclusively on the terminal carbon of the conjugated system.[8] No evidence has been provided for the existence of an enzyme catalytic group which mediates this protonation.

L-Glutamic acid (0.03 M in 3 ml of 0.2 M pyridinium chloride buffer, pH 4.6, containing 1.7×10^{-6} M dithiothreitol, 1.7×10^{-6} M pyridoxal 5′-phosphate, and 0.2 M total chloride ion) was decarboxylated completely by treatment for 18 h with 320 units of purified glutamate decarboxylase from *E. coli*.[9] Similar experiments were conducted at the same pL in D_2O and in 50:50 H_2O:D_2O. The product γ-aminobutyric acid formed in each case was isolated, washed repeatedly with water, and converted by pyrolysis to γ-butyrolactam.[10] Mass spectra of the lactam samples so obtained are shown in Figure 1. When the decarboxylation was conducted in D_2O, 1.00 deuterium atom was incorporated into the product.[11] The sample from the mixed solvent contained 0.48 deuterium atom. Six repetitions of this procedure gave 1.00 ± 0.02 deuterium for experiments conducted in D_2O and 0.48 ± 0.03 deuterium in the mixed solvent. The results were independent of acidity over the range pL 4.1-5.1. Because glutamate decarboxylase does not catalyze hydrogen exchange in γ-aminobutyric acid[12,13] the results of the isotope discrimination experiments

can be used to calculate a deuterium isotope effect of 1.1 ± 0.1 on the protonation of the quinoid intermediate.

Glutamate decarboxylase catalyzes a very slow decarboxylation of α-methylglutamic acid.[14] Decarboxylation of 6.2 ml of 0.02 M D,L-α-methylglutamic acid in the buffer described above but containing 4×10^{-4} M pyridoxal 5′-phosphate for 10 days with 1000 units of enzyme resulted in approximately 20% decarboxylation. The product γ-aminovaleric acid was isolated, washed repeatedly with water, and pyrolyzed to the corresponding lactam. Mass spectra of the lactam are shown in Figure 1. In D_2O the product contained 0.9 deuterium atom,[15] whereas the product from the mixed solvent contained only 0.14 ± 0.01 deuterium. The hydrogen isotope effect calculated from these isotopic composition measurements is $k^H/k^D = 6.2 \pm 0.4$.

The lack of appreciable hydrogen isotope discrimination in the decarboxylation of glutamic acid indicates that the proton source for protonation of the quinoid intermediate is a monoprotic catalytic group of the enzyme which is sufficiently shielded from the solvent that hydrogen exchange between this group and the solvent does not occur during the lifetime of the quinoid intermediate. Transfer of the proton from the solvent to the catalytic group probably occurs prior to the decarboxylation step. Enzymatic decarboxylations of amino acids occur with retention of configuration at the α-carbon atom,[16] and it is possible that the catalytic group involved in protonation is the group that binds the α-carboxyl group of the substrate prior to decarboxylation. It is possible that the decarboxylation of glutamic acid is "ordered", with protonation of this catalytic group necessarily occurring prior to substrate binding.

The large hydrogen isotope effect observed in the decarboxylation of α-methylglutamic acid is in striking contrast to the lack of an effect in the decarboxylation of glutamic acid. There are two possible reasons for the presence of a large isotope discrimination in this case: Protonation of the quinoid intermediate might occur from a different proton source—either directly from the solvent or from an exposed catalytic group. Alternatively, protonation might occur from the same catalytic group as before, but the lifetime of the quinoid intermediate might be significantly longer and the conformation of the enzyme might be such as to allow hydrogen exchange between the catalytic group and the solvent.

Hydrogen isotope discrimination experiments of the type discussed here may be useful for studying a variety of enzymatic reactions involving proton transfer to carbon. Only if hydrogen discrimination is absent is it possible to make any statement about the route of the proton from solvent to substrate. Even in the absence of hydrogen discrimination, several factors must be considered: It must be shown that the lack of discrimination is not the result of readily reversible proton transfer; if such transfer takes place and the catalytic group of the enzyme is in contact with the solvent, then the enzyme will catalyze facile hydrogen exchange between solvent and product. It is possible at least in principle that the transition state for the proton transfer might be very asymmetric and that the absence of a hydrogen isotope discrimination might be the result of this asymmetry. However, such asymmetric transition states generally give rise to small, though measurable, isotope effects, and it is unlikely that an isotope effect of 1.0 would result from such a circumstance. This technique is not capable of detecting the presence of catalytic sulfhydryl groups, because such groups can give rise to appreciable isotope fractionation even if the catalytic group is shielded from the solvent.

Acknowledgment. This research was supported by Grant NS-07657 from the National Institute of Neurological Diseases and Stroke and by Grant PCM75-15315 from the National Science Foundation.

References and Notes

(1) (a) R. P. Bell, "The Proton in Chemistry", 2nd ed, Cornell University Press, Ithaca, N.Y., 1973, Chapter 12; (b) R. A. More O'Ferrall in "Proton-Transfer Reactions," E. Caldin and V. Gold, Ed., Halsted Press, New York, N.Y., 1975, p 201.
(2) It is possible at least in theory that the transition state for a proton transfer such as being discussed here might be very asymmetric and thus give rise to a very small isotope effect. Such situations in proton transfers to and from carbon are seldom, if ever, observed. Alternatively, small hydrogen isotope effects are observed in diffusion-controlled proton transfers. However, diffusion-controlled proton transfer to carbon occurs only when little or no electronic rearrangement of the carbon substrate takes place on protonation (see J. E. Crooks, ref 1b, p 153). Most proton transfers to carbon of interest in enzymology are accompanied by extensive electronic rearrangement, so are not expected to be diffusion controlled.
(3) If this same situation obtains except that the enzyme functional group is a lysine ammonium group, a large hydrogen isotope discrimination will be observed as a result of intramolecular competition among the various hydrogens and deuteriums of the ammonium group.
(4) Reference 1b, p 216.
(5) The fractionation factor for the sulfhydryl group is appreciably different from unity,[4] so hydrogen isotope discrimination may be observed in this case even if a shielded catalytic group is present.
(6) A similar method has occasionally been used for studying hydrogen isotope effects in organic reactions. See, for example, M. M. Kreevoy and R. A. Kretchmer, *J. Am. Chem. Soc.*, **86**, 2435 (1964); V. Gold and M. A. Kessick, *Pure Appl. Chem.*, **8**, 273 (1964); V. Gold and M. A. Kessick, *Proc. Chem. Soc., London*, 295 (1964).
(7) E. A. Boeker and E. E. Snell, "The Enzymes", 3rd ed, Vol. 6, 1972, p 217.
(8) However, see M. H. O'Leary and R. L. Baughn, *Nature (London)*, **253**, 52 (1975), and B. S. Sukhareva and A. E. Braunstein, *Mol. Biol.*, **5**, 302 (1971).
(9) The enzyme was prepared as described by M. H. O'Leary, *Biochemistry*, **8**, 1117 (1969). A unit of enzyme is the amount needed to form 1 μL of CO_2 in 100 s in 0.1 M pyridinium chloride buffer containing 0.025 M L-glutamic acid, 10^{-5} M pyridoxal 5′-phosphate, and 10^{-4} M dithiothreitol at pH 4.9, 37 °C.
(10) S. Gabriel, *Chem. Ber.*, **22**, 3335 (1889).
(11) This is consistent with previous studies of S. Mandeles, R. Koppelman, and M. E. Hanke, *J. Biol. Chem.*, **209**, 327 (1954).
(12) Specifically deuterated γ-aminobutyric acid was prepared by decarboxylation of L-glutamic acid in D_2O with glutamate decarboxylase. Three milliliters of 0.2 M pyridinium chloride buffer, pH 4.5, containing 1.7×10^{-6} M dithiothreitol, 1.7×10^{-6} M pyridoxal 5′-phosphate, and 0.03 M γ-aminobutyric acid-γ-D was incubated with 820 units of glutamate decarboxylase at 22 °C for 120 h. Proton-decoupled ^{13}C NMR spectroscopy was used to look for enzyme-catalyzed hydrogen exchange (such exchange would cause the γ-carbon resonance of γ-aminobutyric acid-γ-D to change from a triplet to a singlet). No exchange was observed.
(13) A previous report[11] that glutamate decarboxylase catalyzes stereospecific hydrogen exchange of γ-aminobutyric acid is apparently in error. The enzyme used in those studies was actually unfractionated bacterial acetone powder, and we assume that significant amounts of γ-aminobutyric acid transaminase were present.
(14) T. Huntley and D. E. Metzler, Abstracts, 154th National Meeting of the American Chemical Society, Chicago, Ill., 1967, p 201c.
(15) Because of the need to use large amounts of enzyme in these experiments, the D_2O solvent contained more H_2O than was present in the glutamic acid experiments. Because of the large isotope effect on the protonation this led to incorporation of less than a full equivalent of deuterium into the product.
(16) For tyrosine decarboxylase: B. Belleau and J. Burba, *J. Am. Chem. Soc.*, **82**, 5751 (1960). For lysine decarboxylase: E. Leistner and I. D. Spenser, *J. Chem. Soc., Chem. Commun.*, 378 (1975). For glutamate decarboxylase: H. Yamada and M. H. O'Leary, unpublished.

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Received August 19, 1976

cis-Dimethyldiazene

Sir:

Although the properties of the trans isomers of the simple diazenes, HN=NH,[1] CH_3N=NH,[1c,2] and CH_3N=NCH_3,[3] are rather well known, the only cis isomer that has been reported is that of dimethyldiazene (C). Hutton and Steel obtained small amounts of C by direct photoisomerization of the solid trans at liquid nitrogen temperature but did not secure enough material for a full characterization.[4] Nelsen prepared a mixture of cis and trans isomers by the pyrolysis of 1,2,3,6-tetrahydropyridazine.[5]

We report here the isolation of millimole quantities of pure