Deuterium Solvent Isotope Effects in Reactions Catalyzed by Isocitrate Dehydrogenase*

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Summary. The result of substituting deuterium oxide for water on the rates of the overall and partial reactions catalyzed by the pig heart TPN-dependent isocitrate dehydrogenase has been studied. Although the overall oxidative decarboxylation of isocitrate proceeds 4.9 times as fast in H_2O as in D_2O, the rate of the separately measured oxalosuccinate decarboxylation reaction is only 1.4-1.7 times greater in water. In contrast, the oxalosuccinate reduction reaction proceeds 5.0 times faster in H_2O. These results suggest that dehydrogenation is the slow reaction in the oxidative decarboxylation of isocitrate and that a proton transfer is involved in this rate determining step.

Deuterium oxide solvent isotope effects have been widely used in physical organic chemistry to implicate proton transfer in the rate determining step of reactions as well as to distinguish between nucleophilic and general base catalysis (Wiberg, 1955; Bender et al, 1962a). Application to enzymatic systems has been more limited and interpretation of results must be made cautiously because of possible effects of deuterium oxide on the affinities of substrates as well as on the structure of the protein itself (Calvin et al, 1959; Hermans and Scheraga, 1959). Nevertheless, the study of solvent isotope effects has aided in the formulation of mechanisms for oxaloacetate decarboxylase (Seltzer et al, 1959), the condensing enzyme (Kosicki and Srere, 1961), chymotrypsin (Caplow and Jencks, 1962), carbonic anhydrase (Pocker and Stone, 1968) and glyceraldehyde-3-phosphate dehydrogenase (Lindquist and Cordes, 1968).

Isocitrate dehydrogenase is a bifunctional enzyme catalyzing both the

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pyridine-nucleotide dependent dehydrogenation of isocitrate and the decarboxylation of oxalosuccinate. The data of Siebert et al (1957) and Moyle (1956) on the rates of the overall and partial reactions of the enzyme suggest that the slow step in the conversion of isocitrate to α-keto glutarate (forward direction) is probably the initial dehydrogenation reaction; whereas the rate-determining step in the reverse reaction is the carboxylation of α-keto glutarate. A study of the differential loss of dehydrogenase as compared to decarboxylase activity upon alkylation of isocitrate dehydrogenase was consistent with these rate determining steps (Colman, 1968). In the present paper, the influence of deuterium oxide on the rates of reactions catalyzed by the enzyme has been scrutinized to elucidate the nature of the rate determining step.

Materials and Methods. Pig heart TPN-dependent isocitrate dehydrogenase was obtained from Boehringer Mannheim Corporation and purified to homogeneity as described previously (Colman, 1968). Deuterium oxide (99.8%) was obtained from Biorad.

Isocitrate dehydrogenase activity was measured at 25° in imidazole chloride and sodium acetate buffers (.03 M in anion) with $1x10^{-4}$M, $4x10^{-3}$M and $2x10^{-3}$M TPN, DL-isocitrate and manganous sulfate, respectively, in a total volume of 1.0 ml. Initial velocities were determined spectrophotometrically at 340 mμ with an expanded scale recorder (0.1 absorbance full scale) and the pH of the reaction mixture was measured immediately thereafter. For deuterium oxide experiments, all substrate and buffer solutions were prepared fresh in D_2O although the time of incubation of substrates in this solvent did not influence the enzymatic reaction rate. The enzyme (.03 ml) was always added in a water buffer in order to minimize the exchange of deuterium into the protein and thus the deuterium oxide reaction mixtures contained 3% H_2O. The deuteron content was calculated from the relationship, $pD = pH_{measured} + 0.40$ (Glasoe and Long (1960); Srere et al (1961)).

Oxalosuccinate reductase activity also was measured spectrophotometrically at 340 mμ with $1x10^{-4}$M TPNH, $2x10^{-4}$M $MnSO_4$ and $2.3x10^{-3}$M oxalosuccinate, and the buffers which were used for the isocitrate dehydrogenase experiments. The enzymatic reaction was initiated by the addition of oxalosuccinate.

Oxalosuccinate decarboxylase activity was followed by the rate at which protons or deuterons are taken up following loss of carbon dioxide. A Radiometer automatic titrator equipped with a syringe burette (0.5 ml capacity) and a titrigraph were used. A total volume of 2.0 ml contained $1.34x10^{-1}$M KCl, $6x10^{-4}$M $MnSO_4$ and 2.3 or $4.6x10^{-3}$M oxalosuccinate in an unbuffered solution adjusted to the pH or pD indicated at room temperature. The volume of uptake of .01 M HCl or DCl was recorded as a function of time and corrections were made for the rate of non-enzymatic decarboxylation. The Michaelis constant for oxalosuccinate was measured by following the decarboxylase reaction spectrophotometrically at 240 mμ and constant slit width in accordance with Grafflin and Ochoa (1950). A total volume of 1.0 ml contained $1.34x10^{-1}$M KCl and $2.4x10^{-4}$M manganous sulfate in 0.2 M sodium acetate buffer, pH 5.49.

Results and Discussion. The rate of dehydrogenation of isocitrate by the pig heart enzyme is profoundly affected by changing the solvent from water to deuterium oxide as shown in Fig. 1. The relative rates in the two solvents were examined from pH or pD 5.1 to 7.4 in order to distinguish between an effect of D_2O on the pK of an essential enzyme group and an influence on the intrinsic pH-independent maximum velocity ($Vmax_i$). The effects of pH on enzymatic activity were shown to be reversible throughout this range and, since the substrate concentrations used were high relative to their Michaelis constants (Colman, 1968, 1969), the enzyme remained saturated with substrate. In H_2O, the observed maximum velocity ($Vmax_o$) depends on a basic group with a pK of 5.66 in the enzyme-substrate complex. This observed pK lies between values generally thought to be characteristic of carboxyl and imidazolium groups in a protein (Cohn and Edsall, 1943); however, on the basis of a striking increase in the pK of the enzymic group when measured in 20% ethanol, the essential

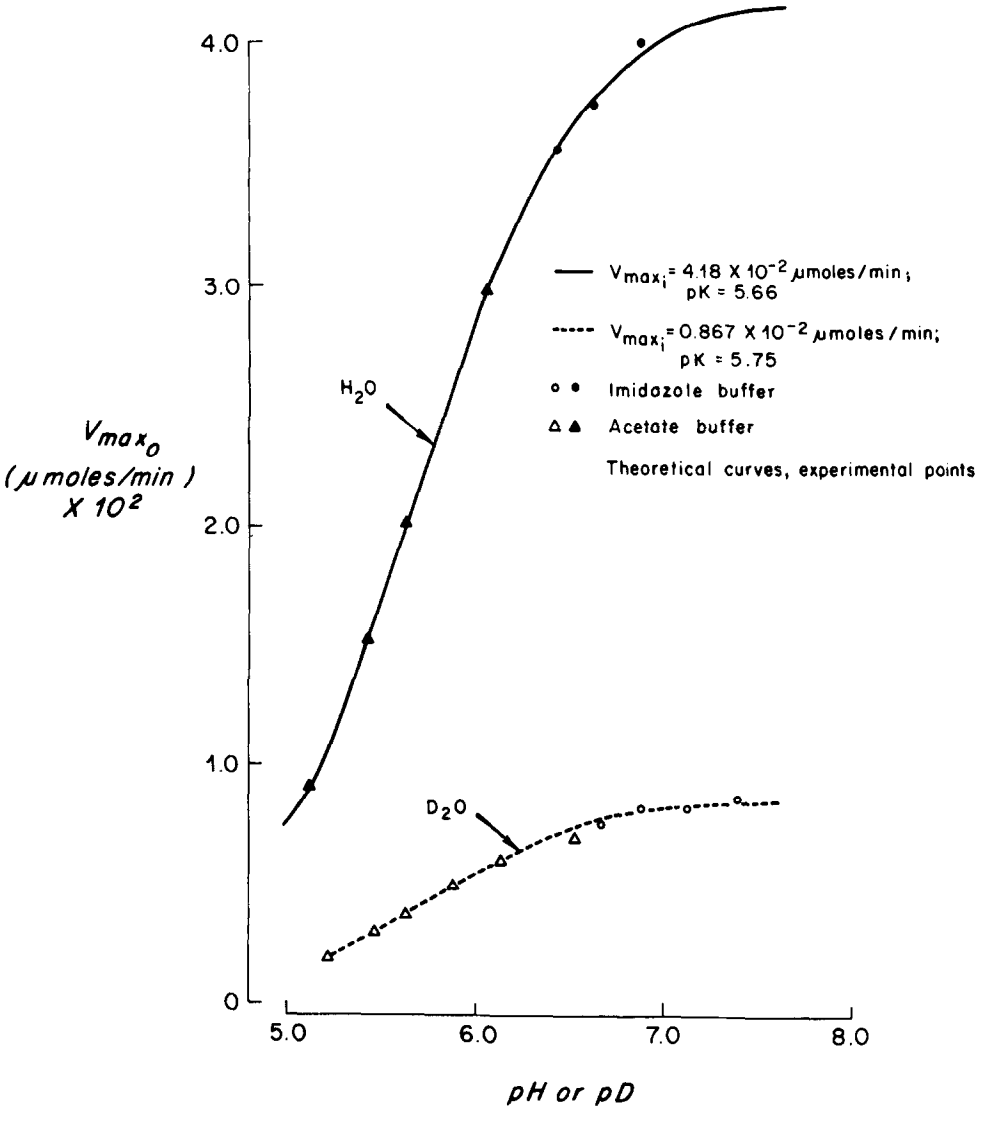

Figure 1 - pH dependence of Vmax for the overall isocitrate dehydrogenase reaction in H_2O and D_2O. Velocities were measured spectrophotometrically as described in Materials and Methods. The theoretical curve was calculated from the equation $Vmax_o = \frac{Vmax_i}{1 + (H^+/K_{aes})}$ where $Vmax_o$ = observed maximum velocity at a given (H^+); $Vmax_i$ = intrinsic maximum velocity; and K_{aes} = dissociation constant of activity-dependent ionizable group in the enzyme-substrate complex.

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group has tentatively been identified as a carboxylate ion (Colman, 1968). In D_2O, the experimental points are best described in terms of a dissociable group of pK 5.75. This small shift in pK cannot account for the magnitude of

the solvent deuterium isotope effect; it is apparent that the rate of dehydrogenation of isocitrate is significantly lower in D_2O than H_2O over the entire range in pH. The intrinsic maximum velocity in H_2O is 4.9 times that of D_2O, suggesting that a proton transfer is involved in the rate-determining step of this reaction.

The isocitrate dehydrogenase assay measures the slow step in the overall oxidative decarboxylation of isocitrate. The direct effect of deuterium oxide on the oxalosuccinate decarboxylase reaction was examined for the purpose of determining which of the two steps was rate limiting. In attempting to evaluate the effect of a solvent on Vmax it is essential to work at a substrate concentration considerably greater than the Michaelis constant. Disparate values of Km for oxalosuccinate exist in the literature. Ochoa and Weisz-Tabori (1948) calculated 2.6×10^{-2}M by an insensitive manometric determination of the decarboxylase reaction; whereas Moyle (1956) has reported a value of 5.6×10^{-4}M determined by a spectrophotometric measurement of the oxalosuccinate reduction reaction. The Michaelis constant has now been re-evaluated by means of the sensitive spectrophotometric assay of the oxalosuccinate decarboxylase reaction yielding a value of 2.33×10^{-4}M as shown in Figure 2. Support for a relatively low value of this Michaelis constant is given by the failure to observe a significant increase in the rates of decarboxylation of oxalosuccinate when measured at 4.6 as compared to 2.3×10^{-4}M substrate (Fig. 3).

In contrast to the striking decrease in the overall rate of oxidative decarboxylation of isocitrate in D_2O, the rate of enzymatic decarboxylation of oxalosuccinate is only modestly reduced in deuterium oxide over the pH (pD) range from 5.0 to 6.2. Figure 3 indicates that the rate in H_2O is only 1.4-1.7 times as great as that in D_2O. This small effect is of the magnitude to be expected from a secondary solvent isotope such as that due to a differential solvation of participating ions, but it is too small to be ascribed to general base catalysis or proton transfer (Bender and Hamilton, 1962b). These results imply that it is the initial dehydrogenation rather than the subsequent

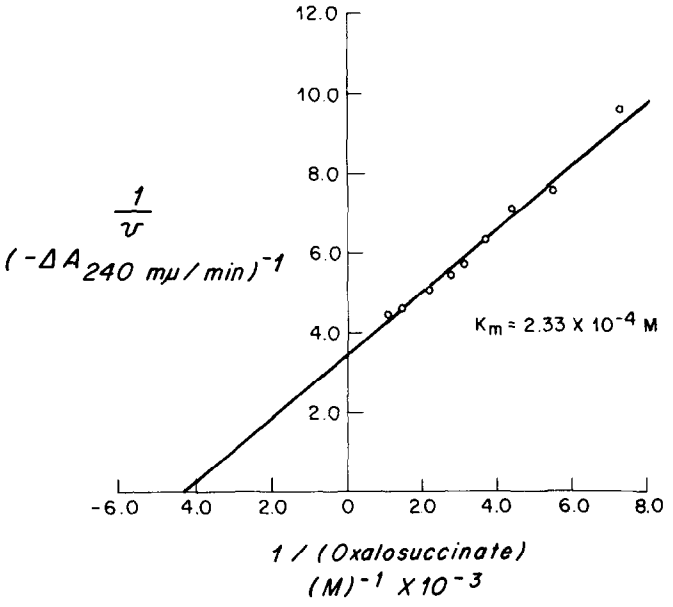

Figure 2 - Determination of Km for oxalosuccinate. The rate of decarboxylation of oxalosuccinate was measured spectrophotometrically at pH 5.49 as described in Materials and Methods.

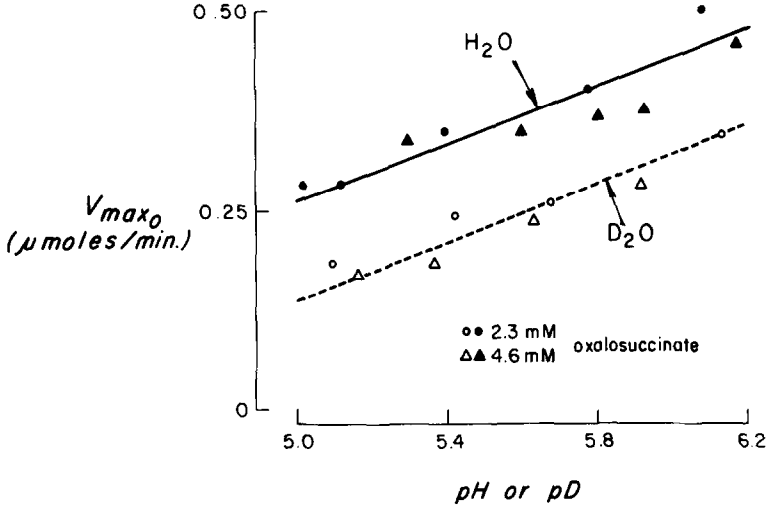

Figure 3 - pH dependence of Vmax for the oxalosuccinate decarboxylase reaction in H_2O and D_2O. Velocities were measured titrimetrically as described in Materials and Methods.

decarboxylation step that is rate limiting in the oxidative decarboxylation of isocitrate.

That the solvent isotope effect illustrated in Fig. 1 reflects an effect on the oxidation-reduction step has been confirmed by direct measurement of the influence of solvent on the oxalosuccinate reductase activity of the enzyme. The pH-Vmax profile for this enzymatic function from pH 5.4 to 7.4 is very similar to that of the isocitrate dehydrogenase activity shown in Fig. 1. The pK of the essential basic group is 5.64 in water. In the plateau region, at pH (pD) 7.4, the rate is 5.0 times greater in H_2O than D_2O.

Analysis of the influence of solvent on the rates of enzymatic reactions is subject to the restriction that the conformation of the protein may be altered and thereby cause a change in the kinetic parameters. In the initial velocity measurements, enzyme was exposed to deuterium oxide for only 1-2 minutes. Separate experiments demonstrated that there was no significant change in the specific activity of the enzyme after a 2 minute incubation in this solvent, indicating that irreversible inactivation cannot account for the low rates in D_2O. Optical rotatory dispersion spectra of isocitrate dehydrogenase dissolved in water or deuterium oxide at pH (pD) 5.9 were superimposable as measured from 320 to 220 mμ within 30 minutes after preparation of the solutions. No gross structural changes appear to accompany short exposures to D_2O.

The data is most readily interpreted by invoking the transfer of a proton in the rate-determining isocitrate oxidation step. General base catalysis by an enzyme functional group may be involved in the abstraction of a proton from the α-hydroxyl group of isocitrate.

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