# The Rates of Oxidation of Some Deuterio Isomers of Succinate by Succinic Dehydrogenase*

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ABSTRACT: The effect of substitution of deuterium for hydrogen in succinate on the rates of the enzymic oxidation has been determined for four isotopic isomers: tetradeuteriosuccinate, $\alpha,\alpha$-dideuteriosuccinate, (*R*)-monodeuteriosuccinate, and (*S*)-monodeuteriosuccinate. The rates of the reactions relative to normal succinate defined as *R* differ somewhat for enzymes prepared from *Escherichia coli*, *Claviceps purpurea*, and beef heart. *R* is independent of pH from 6.8 to 8.0. It decreases with ionic strength, temperature, and the concentration of electron acceptor. Malonate is a stronger inhibitor for tetradeuteriosuccinate than for normal succinate, suggesting that the binding of deuteriosuccinate to the enzyme is weaker than that of normal succinate. A deuterated succinic dehydrogenase from *E. coli* grown in $D_2O$ required a higher malonate concentration for 50% inhibition of the oxidation of normal succinate than the nondeuterated enzyme. Comparison of the rates of oxidation of the four isotopic isomers suggests that the rate-limiting step is the removal of two hydrogen atoms from the substrate. Our data are not consistent with a mechanism involving the removal of a hydride ion in the rate-determining step.

The oxidation of succinate to fumarate by succinic dehydrogenase is one of the most studied enzyme reactions. With the publication of the paper of Ogston (1948) the question of the stereochemistry of this reaction arose. Progressive substitution of deuterium for hydrogen atoms yields first the (*S*) and (*R*) isomers[1] of monodeuteriosuccinic acid, then three isomers of $\alpha,\alpha'$-dideuteriosuccinic acid, one isomer of $\alpha,\alpha$-dideuteriosuccinic acid, two isomers of trideuteriosuccinic acid (*R* and *S*), and finally tetradeuteriosuccinic acid.

The rate of oxidation by succinic dehydrogenase of these various isotopic isomers should be affected by the replacement of the H atoms by D since the energy required to break the CD bond is greater than for the CH bond. Such a primary isotope effect on the kinetics exists only if the breaking of the CD bond is a rate-determining step of the reaction. In 1936, Erlenmeyer *et al.* found that the rate of oxidation of tetradeuteriosuccinate with succinic dehydrogenase from beef heart was from $^1/_2$ to $^1/_3$ as fast as the oxidation of the protio analog. Subsequently, Thorn (1951) and Thomson and Klipfel (1960) confirmed these results and showed that the slowing of the rate of oxidation was proportional to the deuterium content of the succinate. Their succinate was a mixture of different isomers.

Chen and van Milligan (1960) prepared *meso*-dideuteriosuccinic acid by the catalytic reduction of maleic acid with deuterium and the racemic mixture, (*RS*)-dideuteriosuccinic acid, by the catalytic reduction of furmaric acid. With these compounds they demonstrated that the enzymic oxidation of succinate is *trans* (in the Newman projection, Figure 1). As has been clearly indicated by both Hirschmann (1960) and Englard and Colowick (1956), the configurations of the four hydrogen atoms of succinate are not identical. The hydrogen atoms $H^1$ and $H^4$ (see Figure 1) are stereochemically indistinguishable (as are $H^2$ and $H^3$) but can readily be distinguished from $H^2$ and $H^3$. It is clear that there are two pairs of hydrogen atoms whose *trans* removal will yield fumarate: $H^1$ and $H^3$ or $H^2$ and $H^4$. The enzyme–substrate complex can form in two ways. In half the collisions of the enzyme and the succinate, the configuration of the enzyme–substrate complex is such as to initiate the attack on $H^1$,$H^3$. Alternatively the complex can form so that the attack will be on the pair $H^2$,$H^4$. To go from one configuration to the other requires a rotation of the molecule of succinate by 180°. If $H^1$ is deuterium then removal of $H^1$,$H^3$ will result in the loss of deuterium while the removal of the equivalent pair $H^2$,$H^4$ will not. The rate of reaction in which monodeuteriosuccinate is oxidized to normal fumarate involves the breaking of a CD bond and would be expected to be slower than the reaction in which monodeuteriosuccinate is oxidized to monodeuteriofumarate. A second cause for a difference in the rate of oxidation of monodeuteriosuccinate would depend

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[1] The nomenclature of Cahn *et al.* (1956) is used to denote the configuration of asymmetric molecules.

on whether the rate of the reaction is determined by the rate of the breaking of only one of the CH (or CD) bonds or by two CH bonds involved in the reaction. Both possibilities exist since the mechanism of the reaction may involve the removal of two hydrogen atoms or of a proton and a hydride ion.

In order to obtain further information on the enzymic oxidation of succinate we have studied the oxidation of four deuterio-substituted succinates: succinate-$d_4$,[2] the unsymmetrical succinate-$d_2$, and the (*R*)(−) and (*S*)(+) isomers of succinate-$d_1$ by succinic dehydrogenases from *Escherichia coli*, *Claviceps purpurea*, and beef heart.

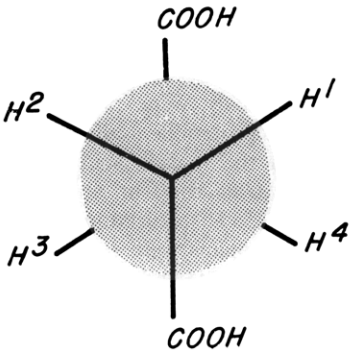


FIGURE 1.

## Experimental Section

*Compounds.* A. SUCCINATE-$d_4$. Succinate-$d_4$ was made by the catalytic reduction with 99% $d_2$ of the dimethyl ester of acetylenedicarboxylic acid by the method described by Williams and Ronzio (1958). The final product contained 95 atom % excess deuterium. The free acid was converted to its anhydride by treatment with acetyl chloride. The crystalline anhydride was removed by filtration and washed with ether. The anhydride was analyzed in a Hitachi mass spectrometer. The intensities of the ion beams at *m/e* 56, 57, 58, 59, and 60 were employed to calculate the abundance of the five isotopic isomers. The succinate was an isotopic mixture of 80% succinate-$d_4$, 18% $d_3$, 2% $d_2$, and a trace of $d_1$.

B. $\alpha,\alpha$-SUCCINATE-$d_2$. The sodium salt of $\alpha$-carboxyethyl-$\gamma$-butyrolactone was prepared according to Traube and Lehmann (1901). It was dissolved in $D_2O$ and acidified with DCl. Water was removed by distillation at atmospheric pressure. During this procedure, the ester was hydrolyzed and decarboxylated. The solution was made alkaline with NaOD and oxidized by addition of $KMnO_4$. The reaction mixture was centrifuged, and the supernatant was acidified with $H_2SO_4$ and extracted continuously with ether. The ether extract was dried and evaporated, and the residue was crystallized from tetrahydrofuran–benzene. The melting point (in a capillary tube) was 185–186°, which is the same as a sample of authentic succinic acid in our apparatus. Mass spectrometric analysis of the anhydride showed it to contain 94% $d_2$, 4% $d_1$, and 1% $d_0$.

C. (*R*)-(−)-MONODEUTERIOSUCCINATE. This monodeuteriosuccinate was made according to the method of Cornforth *et al.* (1962) as modified by D. Arigoni (1964, personal communication). Fumarate dissolved in $D_2O$ was hydrated by the action of fumarase to *erythro*-L-malate-$d_1$. L-Malic acid was esterified with ethanol and was converted to the ethyl ester of chlorosuccinic acid-$d_1$ which was catalytically reduced with normal hydrogen (Zn–Cu couple in acetic acid) and subsequently hydrolyzed to succinic acid-$d_1$. Mass spectrometric analysis showed it to contain 1.4% $d_2$, 91.4% $d_1$, and 7.2% $d_0$. Optical rotation (in methanol) using a Bendix–Ericson polaramatic spectropolarimeter gave $\alpha_{250}$ −23.0°. It is, therefore, (*R*)-(−)-succinate-$d_1$ slightly contaminated with succinate-$d_2$ and $d_0$.

D. (*S*)-(+)-MONODEUTERIOSUCCINATE. This compound was given to us by Dr. S. Englard who prepared it by an asymmetric synthesis from isocitric acid (Englard and Listowsky, 1963). Analysis by mass spectrometer showed it to contain 2.7% $d_2$, 90.3% $d_1$, and 7% $d_0$.[3] Using these data we calculated that the succinate contained 23.9 atom % excess D; theory 25.0. The optical rotation was $\alpha_{250}$ +21°. The melting points of all the isotopic isomers of succinic acid were the same as normal succinic acid.

*Enzyme Preparation.* A crude particulate enzyme fraction was prepared from *E. coli* ML 308 and *E. coli* B. These bacteria were grown aerobically on a modified synthetic medium of Gray and Tatum (1944), with 0.2% sodium acetate and 0.04% disodium succinate as carbon sources. The cells were harvested after 18–20 hr when the culture had reached an optical density of 150 Klett units. The pH was maintained at 7.0 by addition of acetic acid to the growing culture. The cells were washed and disrupted by sonic vibrations, the debris was removed by centrifugation at 25,000*g* for 20 min, and the supernatant was centrifuged 1 hr at 150,000*g*. The sediment was resuspended in phosphate buffer, diluted to approximately the same activity, and used as the source of the enzyme.

A partially purified, soluble succinic dehydrogenase was obtained from *C. purpurea* grown according to McDonald *et al.* (1960) in his glucose–urea medium to which we added 0.4% sodium succinate·$6H_2O$. Germinated spores served as an inoculum. After five days the mycelia were harvested. The purification of the enzyme followed the procedure of McDonald

[2] We designate tetradeuteriosuccinate as succinate-$d_4$, dideuterio as succinate-$d_2$, the two monodeuteriosuccinates as (*R*)-succinate-$d_1$ and (*S*)-succinate $d_1$, and normal succinate as succinate-$d_0$. We designate the various kinetic constants by a numerical superscript which denotes the number of deuterium atoms in the succinate, *i.e.*, $K_m^4$, $K_4^0$, and $V_m^2$ represent the Michaelis–Menton constants for succinate-$d_4$, succinate-$d_0$, and the value of $V_m$ for succinate-$d_2$. Abbreviations used: PMS, phenazine methosulfate; DCPIP, sodium 2,6-dichlorophenolindophenol.

[3] We are indebted to Professor V. Prelog for the mass spectromatic analysis of this compound.

*et al.* (1963). The mycelia were suspended in 0.05 M Tris buffer (pH 8.0) and disintegrated in the Vir-Tis blendor. Cell walls were removed by centrifugation and the nucleic acids were precipitated from the supernatant with protamine sulfate at pH 7.5. The supernatant from the nucleic acid precipitation was centrifuged at 150,000*g* for 1 hr, and this supernatant was brought to 55% saturation with $(NH_4)_2SO_4$. The precipitated proteins were redissolved in 0.005 M phosphate buffer (pH 7.5) and dialyzed for 3 hr against 0.005 M phosphate buffer and 2 hr against 0.002 M phosphate buffer (pH 7.5). The solution contained 15 mg of protein/ml and was used as the enzyme source. A soluble enzyme was prepared from beef hearts by the procedure of Bernath and Singer (1962).

*Determination of Reaction Rates.* The enzyme reactions were carried out in a thermostated cuvet and the reaction was followed by measurements of optical density at a suitable wavelength by a Cary recording spectrophotometer. With the enzyme preparations from *E. coli*, $K_3Fe(CN)_6$ was used as the electron acceptor. The reaction mixture contained 2.0 ml of 0.1 M phosphate buffer and 0.1 ml of 0.15 M KCN in a total volume of 2.8 ml. The concentration of succinate was $3.5 \times 10^{-3}$ M, this being an order of magnitude greater than $K_m$ (see below). The amount of enzyme was chosen to give a change of optical density at 400 mμ of approximately 0.12/min at pH 7.35 and at 30°. The system was incubated for 5 min at 30° before addition of $K_3Fe(CN)_6$. The change of optical density was constant during the first 10 min.

With the soluble enzyme from ergot fungus, the phenzine methosulfate method described by Arrigoni and Singer (1962) was used. The reaction mixture (3 ml) was made up to have a concentration of $3.3 \times 10^{-3}$ M in succinate, $3.3 \times 10^{-4}$ M in KCN, $1.7 \times 10^{-2}$ M in phosphate, and $9 \times 10^{-5}$ M in DCPIP. From 0.25 to 1 mg of PMS and 1.5 mg of active protein were added. The reaction mixture without dyes was incubated for 5 min at 30° before the start of the reaction (pH 7.6, $t = 30°$). The change in absorption at 600 mμ was constant for the first 2 min. In this system also the concentration of succinate was much greater than $K_m$ for the system.

The beef heart preparation was assayed using the same method as with *C. purpurea*. The reaction mixture (total volume 3.0 ml) contained 2.5 ml of 0.035 M phosphate buffer (pH 7.6) and 0.1 ml of 0.01 M KCN. The final concentration of succinate was $3.3 \times 10^{-3}$ M, of DCPIP $9 \times 10^{-5}$ M, and of PMS $5.4 \times 10^{-4}$ M. A suitable amount of the enzyme (30–40 μl) was added. Any changes for a particular experiment are indicated.

## Results

*Succinate-$d_4$.* We find the reaction rate to be proportional to the enzyme concentration. With each enzyme preparation and with all conditions tested, the rate of oxidation with succinate-$d_4$ was always less than that with succinate-$d_0$. In Table I are given the values of $R$ defined as the ratios of the rate of oxidation of the deuterio compound relative to that of the normal succinate. These values vary from one enzyme preparation to another. Since these variations were small, we have averaged the results of our determinations of $R$ performed under common conditions. The effect of pH on $R$ is small (see Table II).

TABLE I: Reaction Rate of Succinate-$d_4$ Relative to Succinate-$d_0$.[a]

| Enzyme Source | $R$ = Rate with Succinate-$d_4$/ Rate with Succinate-$d_0$ |
|---|---|
| *E. coli* B | 0.47 ± 0.05 (6) |
| *E. coli* ML 308 | 0.59 ± 0.04 (8) |
| *C. purpurea* | 0.48 ± 0.04 (5) |
| Beef heart | 0.42 ± 0.02 (18) |

[a] The figures in parentheses in column 2 are the number of determinations averaged. The experiments with *E. coli* were done at pH 7.4 to 7.8. The concentration of $K_3Fe(CN)_6$ was $3.5 \times 10^{-3}$ M. With *C. purpurea* and beef heart the pH was 7.6. The mixture was incubated for 5 min before addition of the electron acceptors. The final concentration of PMS was $5.4 \times 10^{-4}$ M and of DCPIP $9 \times 10^{-5}$ M. In all experiments the concentration of succinate was in the range where small changes did not alter the rate of the reaction.

TABLE II: Effect of pH on Relative Reaction Rate of Succinate-$d_4$ with the Enzyme from *E. coli*.[a]

| pH | $R$ Values | |
|---|---|---|
| | *E. coli* B | *E. coli* ML 308 |
| 6.8 | — | 0.61 |
| 7.2 | 0.46 | 0.62 |
| 7.6 | 0.45 | 0.61 |
| 7.8 | 0.41 | 0.63 |
| 8.0 | 0.41 | 0.63 |

[a] Conditions: 0.07 M phosphate buffer, $t = 30°$, succinate $3.5 \times 10^{-3}$ M, $K_3Fe(CN)_6$ $3.2 \times 10^{-3}$ M.

The rates of oxidation of mixtures of succinate-$d_0$ and succinate-$d_4$ by beef heart dehydrogenase are shown in Figure 2. Thorn (1951) found the curve representing the rate of the reaction is not a linear function of the concentration of succinate-$d_4$ but is concave toward the origin. We confirm his observation. This nonlinear curve results from the fact that $K_m^4$ is larger than $K_m^0$.

The effect of increasing concentrations of the oxidant, $K_3Fe(CN)_6$, with the enzyme from *E. coli* ML 308 is shown in Figure 3. Increasing concentrations of $K_3Fe(CN)_6$ increase the rate of the reaction. The value of $R$ for the enzymes from the four sources decreases steadily with increasing concentrations of the oxidant (see Tables III and IV).

TABLE III: Effect of Concentration of Electron Acceptor on Relative Reaction Rate of Succinate-$d_4$ with Enzymes from *E. coli*.[a]

| Electron Acceptor ($K_3Fe(CN)_6$) (mM) | $R$ Values | |
|---|---|---|
| | *E. coli* B | *E. coli* ML 308 |
| 1.07 | 0.47 | — |
| 1.61 | 0.47 | 0.70 |
| 2.14 | 0.46 | 0.66 |
| 2.68 | 0.44 | 0.66 |
| 3.21 | 0.41 | 0.60 |
| 4.28 | 0.40 | 0.55 |
| 4.82 | 0.39 | 0.51 |
| ∞ (extrapolated) | 0.38 | 0.37 |

[a] Conditions: 0.07 M phosphate buffer, pH 7.8, for *E. coli* B and pH 7.2 for *E. coli* ML 308, $t = 30°$, and succinate concentration $3.5 \times 10^{-3}$ M.

The inhibition by malonate is given in Table V. With the dehydrogenases from *E. coli* and from beef heart the concentration of malonate required to inhibit the rate by 50% at a fixed concentration of succinate is smaller for succinate-$d_4$ than for succinate-$d_0$.

In Table VI are given the results of the inhibition of the succinic dehydrogenase from *E. coli* B and ML 308 grown in a medium in which $D_2O$ replaced $H_2O$. The enzyme activity was determined in normal $H_2O$ so that all exchangeable hydrogen atoms of the enzyme were hydrogen rather than deuterium. Replacement of hydrogen by deuterium in the enzyme appears to weaken the relative binding of malonate to succinate to the enzyme.

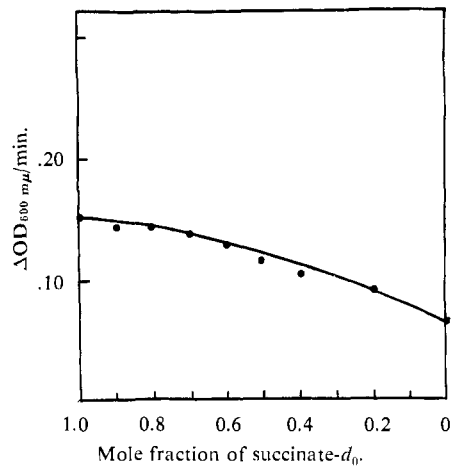


FIGURE 2.

Increasing the ionic strength of the phosphate buffer from 0.1 to 0.2 with tne beef heart dehydrogenase at pH 7.6 decreases the rate of the reaction by 14% for succinate-$d_0$ and 31% for succinate-$d_4$, the value of $R$ for succinate-$d_4$ in these experiments being 0.40 and 0.32, respectively.

The rate of the reaction for beef heart dehydrogenase with succinate-$d_4$ and succinate-$d_0$ from 10 to 45° was determined and from these values $R$ was calculated (see Table VII). The values of $R$ for (*R*)-succinate-$d_1$, (*S*)-succinate-$d_1$, $\alpha,\alpha$-succinate-$d_2$, and succinate-$d_4$ for the four sources of succinic dehydrogenases are given in Table VIII.

TABLE IV: Effect of Concentration of Electron Acceptor on Relative Reaction Rate, $R$, of the Deuterated Succinates.[a]

| Concn of PMS (mM × 10) | Enzyme Source | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *C. purpurea* Succinate | | | | Beef Heat Succinate | | | |
| | $d_4$ | $d_2$ | (*R*)-$d_1$ | (*S*)-$d_1$ | $d_4$ | $d_2$ | (*R*)-$d_1$ | (*S*)-$d_1$ |
| 2.7 | 0.48 | 0.71 | 0.83 | 0.86 | — | — | — | — |
| 3.8 | 0.46 | 0.70 | 0.81 | 0.84 | 0.44 | 0.60 | 0.88 | 0.94 |
| 5.4 | 0.44 | 0.67 | 0.81 | 0.84 | 0.41 | 0.57 | 0.85 | 0.88 |
| 7.1 | 0.41 | 0.66 | 0.80 | 0.84 | 0.39 | 0.57 | 0.78 | 0.88 |
| 10.9 | 0.41 | 0.65 | 0.82 | 0.86 | 0.36 | 0.51 | 0.75 | 0.84 |
| 13.6 | 0.40 | 0.65 | — | — | — | — | — | — |
| ∞ (extrapolated) | 0.36 | 0.59 | 0.78 | 0.84 | 0.30 | 0.47 | 0.63 | 0.74 |

[a] Conditions: phosphate buffer 0.029 M, pH 7.6, $t = 30°$, and succinate $3.3 \times 10^{-3}$ M.

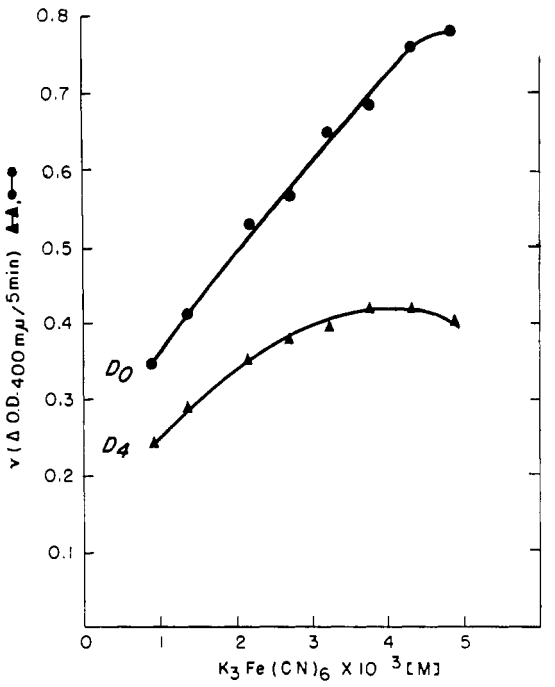


FIGURE 3.

## Discussion

As previous investigators have reported, we find that the rate of oxidation of succinate-$d_4$ is considerably less than that of succinate-$d_0$ (see Table I). This is to be expected if the breaking of a CH or CD bond were a rate-limiting step since the zero-point energy of the CH bond is greater than that of the CD bond. The kinetic data follow Michaelis–Menton kinetics (see Figure 4).

The effect of pH on $R$ for the enzymes from *E. coli* is small (see Table II). Since at pH above 7 succinic acid is completely dissociated to the dianion the changes of reaction rate must be due to changes induced in the enzyme or cofactor. Whatever these changes may be, they are identical for experiments with succinate-$d_0$ and succinate-$d_4$.

TABLE V: Ratio of Malonate to Succinate Concentrations Required for 50% Inhibition.[a]

| Enzyme | Succinate-$d_0$ | Succinate-$d_4$ |
|---|---|---|
| *E. coli* B | 0.025 (7) | 0.020 (1) |
| *E. coli* ML 308 | 0.054 (7) | 0.020 (2) |
| Beef heart | 0.027 (2) | 0.010 (2) |

[a] The figures in parentheses in columns 2 and 3 are the number of determinations averaged. Concentration of succinate $3.3 \times 10^{-3}$ M. In one experiment the inhibitory effect of malonate on succinate-$d_2$ and (*R*)-succinate-$d_1$ was found to be intermediate between the values for succinate-$d_0$ and succinate-$d_4$.

TABLE VI: Ratio of Malonate to Succinate-$d_0$ Required to give 50% Inhibition of Succinic Dehydrogenase of *E. coli*.[a]

| Enzyme Source | Growth Medium | |
|---|---|---|
| | $H_2O$ | $D_2O$ |
| *E. coli* B | 0.022 | 0.049 |
| *E. coli* ML308 | 0.055 | 0.074 |

[a] The organisms grown on medium containing $D_2O$ in place of $H_2O$ were harvested and treated exactly as those grown in a $H_2O$ medium. The phosphate buffer for the growth medium was made using the ratio of the monobasic to dibasic salts which gives a pH of 7.0 in water. For the determination of the reaction rates: $t$ = 30°, succinate $3.5 \times 10^{-3}$ M, $K_3Fe(CN)_6$ $3.5 \times 10^{-3}$ M, pH 7.2, and solvent $H_2O$.

The detailed steps of the mechanism for the action of succinic dehydrogenase are not completely understood. For our discussion we assume the following simplified mechanism. The enzyme contains a tightly bound flavin which can be oxidized or reduced. We denote by $E_o$ and $E_r$ the oxidized and reduced forms of this complex. In the oxidation, electrons from succinate pass through the flavin system bound to the enzyme to the electron acceptor.

*Kinetics.* We postulate the following sequence in which S and F represent succinate and fumarate and $A_o$ and $A_r$ the oxidized and reduced forms of the electron acceptor (either $K_3Fe(CN)_6$ or PMS).

$$E_o + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_oS \xrightarrow{k_2} E_r + F$$

$$E_r + A_o \xrightarrow{k_3} E_o + A_r$$

TABLE VII: Effect of Temperature on $R$ for Succinate-$d_4$ for Beef Heart Dehydrogenase.[a]

| $T$, °C | $R$ |
|---|---|
| 10 | 0.47 (1) |
| 22.5 | 0.42 (1) |
| 25 | 0.44 (2) |
| 30 | 0.41 (3) |
| 35 | 0.40 (3) |
| 45 | 0.36 (4) |

[a] Conditions: phosphate buffer, pH 7.6; the figures in parentheses in column 2 are the number of experiments averaged. Concentration of succinate $3.3 \times 10^{-3}$ M, and PMS $5.4 \times 10^{-4}$ M.

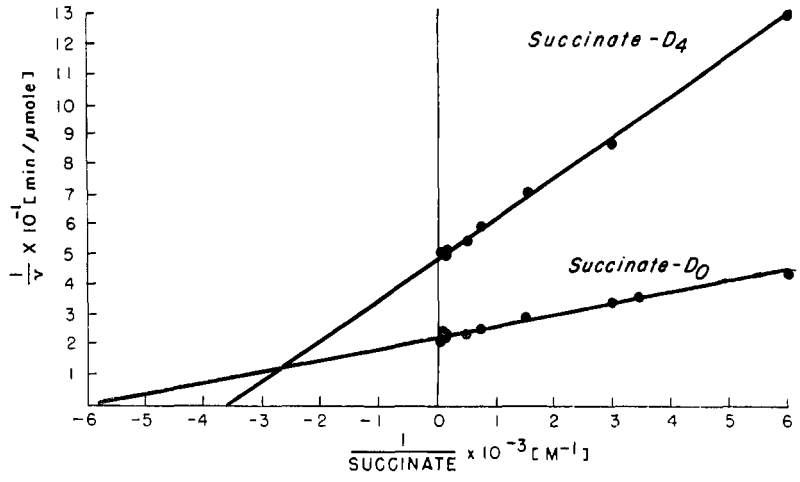


FIGURE 4.

The complex $E_oS$ is one in which no primary valence bonds of succinate have been broken and is earlier in the reaction path than the free-radical complex of the enzyme and the substrate studied by Kearney *et al.* (1955), Singer and Kearney (1954), Hollocher and Commoner (1961), and Griffin and Hollocher (1966). As we consider only the initial reaction rate when F and $A_r$ are small we neglect the reverse of $k_2$ and $k_3$. Substitution of D for H in succinate could effect $k_1$, $k_{-1}$, $k_2$, and $k_3$.

The velocity of the reaction, $v$, for the system postulated is

$$v = \frac{k_2 ES \Big/ 1 + \frac{k_2}{k_3 A_o}}{S + \frac{k_{-1} + k_2}{k_1} \Big/ 1 + \frac{k_2}{k_3 A_o}} \qquad (1)$$

and values of $K_m$ and $V_m$ are

$$V_m = k_2 E \Big/ 1 + \frac{k_2}{k_3 A_o} \qquad (2)$$

$$K_m = \frac{k_{-1} + k_2}{k_1} \Big/ 1 + \frac{k_2}{k_3 A_o} \qquad (3)$$

$V_m$ and $K_m$ are both functions of $A_o$.

Equation 1 in the reciprocal form is

$$\frac{1}{v} = \frac{1}{k_2 E}\left[1 + \frac{k_2}{k_3 A_o} + \frac{k_{-1} + k_2}{k_1 S}\right] \qquad (4)$$

At constant value of $A_o$, the data for $1/v$ *vs.* $1/S$ should yield a straight line. In Figure 4 are graphed the data for a typical experiment for the enzyme from *C. purpurea* in which the concentration of $A_o$ (in this case PMS) was held constant at $5.4 \times 10^{-4}$ M. From this reciprocal plot the values of the constants of the Michaelis–Menton equation, $K_m$ and $V_m$, were calculated to be $K_m^0 = 1.7 \times 10^{-4}$ M, $K_m^4 = 2.8 \times 10^{-4}$ M, $V_m^0 = 4.6 \times 10^{-2}$ mole/min, and $V_m^4 = 2.1 \times 10^{-2}$ mole/min. These values of $V_m$ were determined at high, but not saturating concentration of electron acceptor. From similar experiments with the enzyme from beef heart, we calculate that $K_m^0$ is $2.7 \times 10^{-4}$ M and $K_m^4$ is $3.5 \times 10^{-4}$ M.

With $S$ constant, the data for $1/v$ *vs.* $1/A_o$ should also yield a straight line. In Figure 5 we plot the data from a typical experiment for the enzyme from *C. purpurea* at a constant concentration of succinate

TABLE VIII: Relative Reaction Rates, $R$, for the Oxidation of Deuteriosuccinates.[a]

| Succinic Acid | $R$ for the Dehydrogenase of | | | |
|---|---|---|---|---|
| | *E. coli* B | *E. coli* ML 308 | *C. purpurea* | Beef Heart |
| $d_4$ | 0.48 (5) | 0.62 (7) | 0.45 (16) | 0.41 (17) |
| $d_2$ | 0.72 (3) | 0.80 (3) | 0.70 (16) | 0.58 (13) |
| (*R*)-$d_1$ | 0.81 (5) | 0.88 (3) | 0.85 (16) | 0.81 (8) |
| (*S*)-$d_1$ | 0.87 (1) | 0.95 (4) | 0.89 (15) | 0.89 (7) |
| $d_0$ | 1.00 | 1.00 | 1.00 | 1.00 |

[a] The determinations with *Claviceps* and beef heart enzyme were carried out at pH 7.6 and with the bacterial enzyme at pH 7.4 to 7.9. Since pH has little effect on $R$ in this range we have averaged the values ignoring variations of pH. For the *E. coli*, the electron acceptor was $K_3Fe(CN)_6$; for *C. purpurea* and beef heart, PMS. The temperature was 30° and the succinate concentration $3.5 \times 10^{-3}$ M for *E. coli* and $3.3 \times 10^{-3}$ M for the others. The figure in the parentheses denotes the number of values used for the average given.

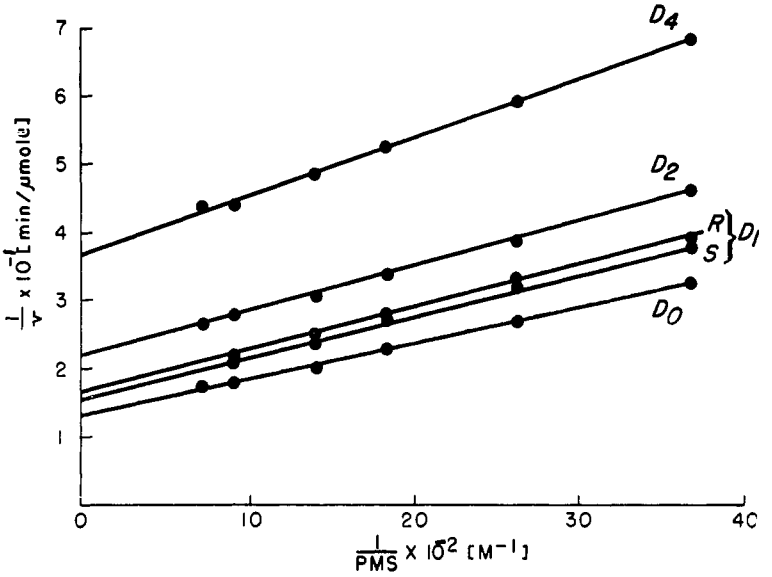


FIGURE 5.

($3.3 \times 10^{-3}$ M) for the various isotopic isomers. By extrapolating the curves to intersect the ordinate we determined the velocity of the reactions when the concentration of PMS is infinite. From these rates we have calculated the values of $R$ for the various isotopic isomers. These values for infinite concentration of oxidant are given in Table IV. Similar treatment of the data for the dehydrogenases of beef heart and of *E. coli* yields values of $R$ for infinite concentration of oxidant (see Tables III and IV). They are lower than the values in Table I since those are determined where neither the concentrations of succinate nor of the oxidant are infinite.

We can proceed further in our analysis of the kinetic data by making two approximations. (1) $k_2$ is negligible compared to $k_{-1}$. This is tantamount to assuming that the complex $E_oS$ is in equilibrium with $E_o$ and S. This seems reasonable for a reaction as slow as is the oxidation of succinate. This assumption is supported by the results of Hollocher and Commoner (1961). These authors measured the concentration of the free radical which forms on the addition of succinate to succinic dehydrogenase. This free radical according to our simple mechanism is derived from the complex $E_oS$ presumably by the breaking of at least one CH bond of the succinate. In the absence of an oxidant, $E_o$ and S are in equilibrium with the free-radical intermediate and consequently with $E_oS$. When an oxidant is added to the equilibrium system perturbing this state, the concentration of the free radical does not change appreciably, supporting our assumption that $E_o$ and S are in equilibrium with $E_oS$. (2) The ratio $k_{-1}/k_1$ is not affected by isotopic substitution in the succinate. This ratio is the equilibrium constant for the formation of the complex $E_oS$ in which valence bonds between the succinate and the enzyme are neither formed nor broken. In general, equilibrium constants, even of equilibria involving the formation of new valence bonds, are not greatly changed by isotopic substitution.

With these approximations $k_2^4/k_2^0$ can be evaluated.

$$K_m = \frac{k_{-1}}{k_1} \Big/ 1 + \frac{k_2}{k_3 A_o} \tag{5}$$

$$V_m/K_m = \frac{k_2 E_o}{k_{-1}/k_1} \tag{6}$$

$$\frac{V_m^4}{V_m^0} \Big/ \frac{K_m^4}{K_m^0} = \frac{k_2^4}{k_2^0} \tag{7}$$

Using the values $K_m^4$, $K_m^0$, $V_m^4$, and $V_m^0$ for *C. purpurea* in eq 7, we calculate $k_2^4/k_2^0$ to be 0.28. Since the reaction described by $k_2$ involves the transfer of H (or D) atoms from S to $E_o$, an isotope effect of this size is quite reasonable.

When $S$ is constant and $A_o$ is varied, eq 4 requires that the slopes of the curves in Figure 5, obtained by plotting $1/v$ *vs.* $1/A_o$, are proportional to $1/k_3$. The ratio of the slopes of the curves for succinate-$d_0$ to succinate-$d_4$ is $k_3^4/k_3^0$ and has the numerical value of 0.62. In the same manner $k_3^2/k_3^0$ and $k_3^1/k_3^0$ for the (*R*) isomer, and $k_3^1/k_3^0$ for the (*S*) isomer are 0.79, 0.84, and 0.87, respectively. Since there is an isotope effect for this reaction which involves the transfer of hydrogen or deuterium atoms from the reduced enzyme to the oxidant it demonstrates that the hydrogen (or deuterium) atoms on the N-1 and N-10 of the reduced flavin of succinic dehydrogenase do not instantly exchange with the water of the environment. Indeed the rate of the exchange reaction must be of the same order of magnitude as $k_3$.

From the experimentally determined ratio, $K_m^4/K_m^0$

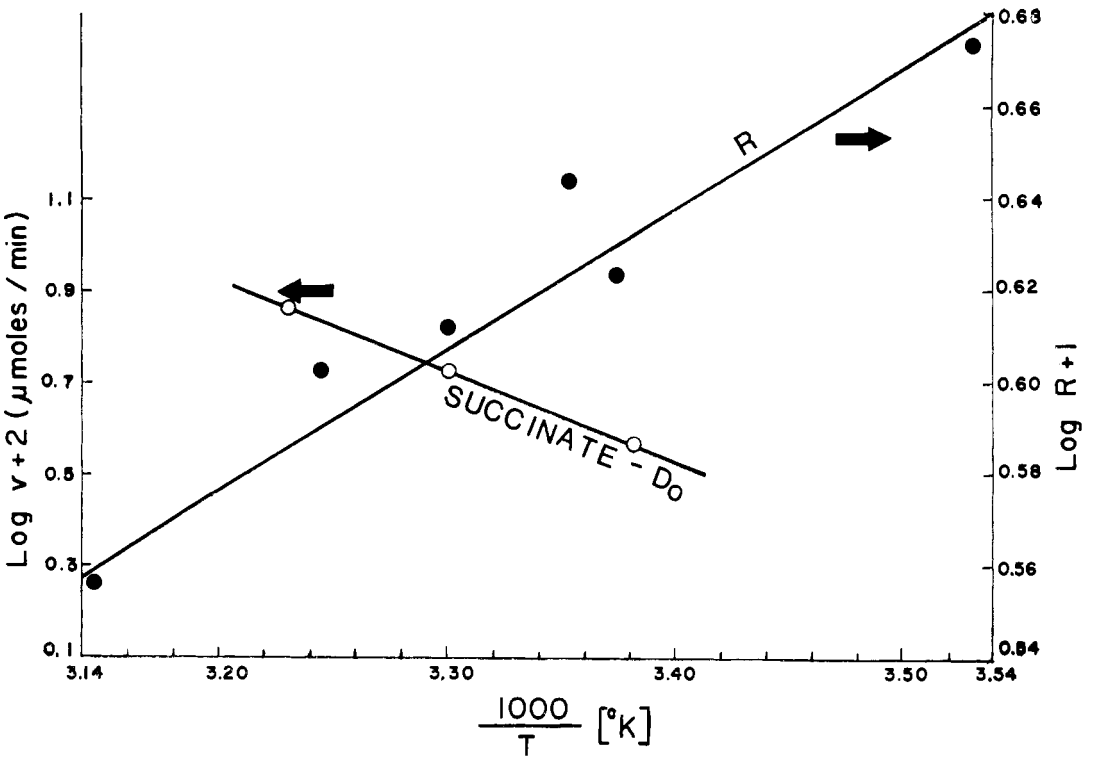


FIGURE 6.

(see eq 5), for the enzyme from *C. purpurea* and the calculated values of $k_2^4/k_2^0$ and $k_3^4/k_3^0$ we find $k_2^0/k_4^0A_0 = 2.5$ and $k_2^4/k_3^4A_0 = 1.1$. While the kinetic constants are independent of $A_0$, $K_m^0$ and $K_m^4$ are functions of $A_0$. Introducing the value of $A_0$ ($5.4 \times 10^{-4}$ M) used to evaluate $K_m^4$ and $K_m^0$, we find $k_2^0/k_3^0 = 14 \times 10^{-4}$ and $k_2^4/k_3^4 = 6.0 \times 10^{-4}$. Introducing the values of $K_m^0$ and $k_2^0/k_3^0A_0$ in eq 5, the value of $k_{-1}/k_1$ is calculated to be $6.0 \times 10^{-4}$.

With the enzymes from all four sources and with all isotopic isomers tested, the value of $R$ decreases for increasing concentrations of the electron acceptor (see Tables III and IV). Inspection of Figure 3 shows that increasing concentrations of oxidant accelerate the reaction rate for succinate-$d_0$ more than for succinate-$d_4$. Under these conditions where the concentration of succinate is large ($S > K_m$), eq 1 predicts that increasing concentrations of the oxidant will have just that effect on the slopes of the curves shown in Figure 3. Very high concentrations of $K_3Fe(CN)_6$ inhibit the rate of dehydrogenation of succinate.

Since $K_m$ in this system is a kinetic constant being determined largely by $k_1$, $k_{-1}$, $k_2$, and $k_3$, one cannot use it to determine the binding of succinate to the enzyme. The finding (see Table V) that the ratio of malonate to succinate required to give 50% inhibition is larger for succinate-$d_0$ than for succinate-$d_4$ confirms that $K_m^4$ is greater than $K_m^0$.

Of interest in this regard is the effect of substitution of D for H in the enzyme obtained from *E. coli* grown in $D_2O$ (see Table VI). Here malonate is a poorer inhibitor of the oxidation of succinate-$d_0$ by the deuterio enzyme than the protio enzyme. Similar results have been reported earlier by Rittenberg and Borek (1963). It seems plausible that in the deuterio enzyme the three-dimensional structure around the active site is different from that for the normal enzyme.

Our finding that increasing ionic strength of buffer decreases the rate of the enzymic reaction and lowers the value of $R$ for succinate-$d_4$ indicates that coulombic forces are involved in the total enzymic reaction. This effect could exert its influence at a step of the reaction other than the formation of the $E_0S$ complex. If the thermodynamics activity of $A_0$ were affected by the salt concentration it could account for the results we observe.

In Figure 6 we have plotted the logarithm of reaction rate *vs.* $1/T$ for succinate-$d_0$ with the enzyme from beef heart and calculated the energy of activation, $E^0$, from the slope of the curve. It is 8130 cal/mole. In Figure 6 we also plot the logarithm of the values of $R$ in Table VII against $1/T$. The slope of this curve is equal to $(E^4 - E^0)/2.3R$. From this slope we calculate $E^4 - E^0$ to be $-1380$ cal/mole. Under the conditions of our experiments the energy of activation for succinate-$d_4$ is $8130 - 1380 = 6750$ cal/mole. These energies of activation are those for the over-all reactions and not for any individual step.

*The Mechanism of the Reaction.* We shall assume for our discussion of the mechanism of the reaction that the substrate is bound to the active site of the enzyme with at least one carboxylate group and two

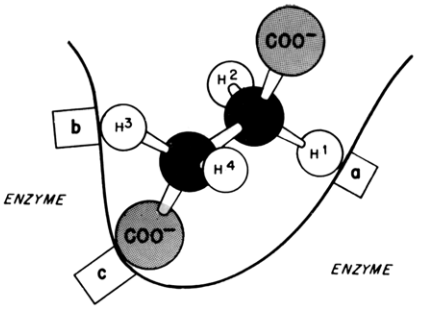


FIGURE 7.

hydrogen atoms of the adjacent methylene groups. Since it is known that the pair of hydrogen atoms removed in the oxidation are in the *erythro* configuration (Chen and van Milligan, 1960), it is reasonable that this pair is in contact with the enzyme. We illustrate in Figure 7 the interaction at three points of the enzyme with succinate in the *trans* configuration. We designate the site of interaction on the enzyme of the carboxylate group by the letter c and the sites for the methylene hydrogens by a and b. The four methylene hydrogen atoms are differentiated by superscripts $H^1$ to $H^4$. The conclusions from our arguments below would not change if the succinate were to interact in the gauche or eclipsed conformations. Since succinate is formed from two identical halves ($(CH_2\text{-}COOH)_2$), it can interact with the enzyme in two ways depending on which carboxylate group binds to the appropriate site of the enzyme. In one case $H^1$ and $H^3$ (see Figure 7) bind to the enzyme, in the other $H^2$ and $H^4$. As was indicated by Englard and Colowick (1956), the enzyme cannot distinguish the pair $H^1$,$H^3$ from $H^2$,$H^4$.

There are two plausible mechanisms for the reaction. (A) Only one CH bond is activated with the removal of a hydride ion and subsequently a proton is eliminated. The rate-determining step is the removal of the hydride ion. (B) Both CH bonds are activated with the removal of two protons with their electrons. We shall consider which of the two reaction mechanisms is consistent with our data on the isotope effects for the oxidation of the isotopic isomers of succinate by the enzyme from *C. purpurea* (see Table VIII).

For mechanism A the value of $R$ for succinate-$d_4$ is 0.45. Since the rate-determining step involves the breaking of one CD bond the isotope effect is equal to $R$ for succinate-$d_4$.

The unsymmetrical succinate-$d_2$ in which we designate the two deuterium atoms as $H^1$ and $H^2$ could interact with the enzyme in two configurations, though our argument would not differ if they were assumed to be at $H^3$ and $H^4$. (1) In one configuration of succinate-$d_2$, deuterium atom $H^1$ is in contact with the enzyme at site a. (2) In the other configuration, hydrogen atom $H^4$ is in contact with the enzyme at the same site. We assume that the breaking of the CD or CH bond is determined by the interaction with site a and that interaction at site b is not rate determining. In this case, therefore, one configuration will give an isotope effect of 0.45 and the other of 1. Since either configuration is equally probable, the over-all isotope effect will be $(1.00 + 0.45)/2 = 0.73$. This is close to what we observe, 0.70 (see Table VIII).

With the two succinates-$d_1$, this mechanism predicts again two configurations for the enzyme–substrate complex for each isomer. For (*S*)-succinate-$d_1$ the deuterium atom is $H^1$. In one way of interacting with the enzyme $H^1$ would occupy site a and $H^3$ would be at site b. Since the catalytically active site is a, there will be an isotope effect of 0.45 in the oxidation to fumarate. In the other configuration $H^4$ will be at site a and $H^2$ at site b. Since $H^4$ is a hydrogen atom there will be no isotope effect. This isomer would have an isotope effect of $(1 + 0.45)/2 = 0.73$. For (*R*)-succinate-$d_1$ the deuterium atom is $H^2$ and there is no manner of interacting with the enzyme by which $H^2$ could be at the catalytically active site a. There should be no isotope effect observed with this isomer. This does not agree with our data since (*R*)-succinate-$d_1$ and (*S*)-succinate both show an isotope effect. This suggests that mechanism A is not correct.

For mechanism B in which two hydrogen atoms with their electrons are removed from the succinate, *i.e.*, two CH (or two CD or a CH and a CD) bonds are broken and are both rate determining, similar calculations can be made. For the two configurations of the enzyme–succinate-$d_4$ complex either the CD bonds at $H^1$ and $H^3$ or $H^2$ and $H^4$ must be broken. Since the isotope effect of the whole reaction is 0.45 the isotope effect in breaking one CD bond will be $\sqrt{0.45} = 0.67$.

For succinate-$d_2$ we assume as before that the two deuterium atoms are at $H^1$ and $H^2$. In one way for succinate to combine with the enzyme, $H^1$ (a deuterium atom) is at site a and $H^3$ (a hydrogen atom) is at site b. The isotope effect at site a is 0.67 and at site b is 1. The over-all isotope effect will be the product of these two values or 0.67. For the other manner of interacting with the enzyme, $H^4$ (a hydrogen atom) will be at site a and $H^2$ (a deuterium atom) will be at site b. Here also the isotope effect will be 0.67. For both interactions of succinate-$d_2$ with the enzyme, the isotope effect will be 0.67. This is in good agreement with our data.

In the case of the monodeuteriosuccinates, (*S*)-succinate-$d_1$ (D atom at $H^1$) can combine with the enzyme so that $H^1$ is at site a and $H^3$ at site b to give an isotope effect of 0.67. In the other configuration, $H^4$ is at site a and $H^2$ at site b. In this case, since $H^2$ and $H^4$ are hydrogen atoms, the isotope effect is 1. The over-all isotope effect for (*S*)-succinate-$d_1$ is $(1.00 + 0.67)/2 = 0.84$. (*R*)-Succinate-$d_1$ (deuterium atom at $H^2$) can interact with the enzyme with $H^4$ at site a and $H^2$ at site b or with $H^1$ at site a and $H^3$ at site b. As with (*S*)-succinate-$d_1$ one configuration will have an isotope effect of 0.67 and the other of 1.00 with an

over-all isotope effect of (1.00 + 0.67)/2 = 0.84. These calculated isotope effects for both isomers agree well with our data. Similar considerations for the results with the enzymes from the other sources lead to the same conclusions as those from the enzyme from *C. purpurea;* mechanism A is not in accord with our data and mechanism B is.

In each case, however, the value of $R$ for the ($S$) isomer is greater than for the ($R$) isomer. Both our ($R$) and ($S$) isomers are contaminated with about 7% of succinate-$d_0$ and the ($R$) isomer has a greater absolute optical activity than the ($S$) isomer. Part or possibly all the difference in the kinetics of the oxidation of the ($R$) and ($S$) isomers may be due to partial racemization during the preparation of these compounds.

Whether the oxidation of succinate is stereochemically specific resolves itself into a semantic problem. From one viewpoint the oxidation is stereochemically specific since only one pair ($H^1$,$H^3$) or the other pair ($H^2$,$H^4$) can be removed and $H^1$,$H^2$, or $H^3$,$H^4$ cannot be removed as pairs. On the other hand, each hydrogen atom has an equal probability of being removed during the oxidation to fumarate.

In the above discussions we have ignored secondary isotope effects. These in general are quite small compared to the primary isotope effect and would in any case affect the oxidations of ($R$)- and ($S$)-succinate-$d_1$ identically.

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