A 2,4-DNP derivative of the product was made in the usual manner, mp 196°, undepressed on admixture with an authentic sample of **14**.

2α-Isopropyl-4aβ-methyl-1β-toluenesulfonyloxy-1,2,3,4,4a,7,8,-8aβ-octahydronaphthalen-5(6H)-one (16). The ketol **15** (460 mg, 2.04 mmol) was dissolved in 7 ml of dry pyridine under nitrogen. Toluenesulfonyl chloride (1.0 g, 5 mmol) was added and the solution let stand for 3 days at room temperature. Water (10 ml) was added to hydrolyze the excess tosyl chloride, and the solution was placed in the refrigerator for crystallization; 775 mg (100%) of the desired keto tosylate **16**, mp 145.5–146°, was collected.

3-Isopropyl-6-methyltricyclo[4.4.0.0^{2,8}]decan-7-one (17). A 0.50 *M* solution of dimethylsulfinyl carbanion in DMSO was prepared according to the procedure of Corey.[17] This solution (5 ml) was added to a solution of keto tosylate **16** (775 mg, 0.00204 mol) in 5 ml of DMSO under nitrogen, and the resulting solution was heated at 60° for 2 hr. After this period, the reaction was cooled, diluted with water, and extracted several times with ether. The ether extracts were washed with water and with brine, were dried ($MgSO_4$), filtered, and evaporated. The residue was distilled to yield 370 mg (90%) of the desired tricyclic product, homogeneous by vpc. An analytical sample was prepared by preparative vpc on a 10-ft 15% Carbowax 20M on Chromosorb W column: ir (neat) 1745 cm^{-1} (C=O); nmr (CCl_4) τ 9.01 (s, 3 H) and 9.02 (d, 6 H, J = 5 Hz).

Anal. Calcd for $C_{14}H_{22}O$: C, 81.49; H, 10.75. Found: C, 81.10; H, 10.80.

***dl*-Sativene (1).** The tricyclic ketone **17** (60 mg, 0.30 mmol) was dissolved in 5 ml of dry ether and added to 20 ml of a 5% solution of methyllithium in ether. The solution was refluxed for 2 days under nitrogen, then quenched with water. The ether layer was drawn off, and the aqueous layer was further extracted with ether. The organic layers were combined, washed with brine, dried ($MgSO_4$), filtered, and evaporated. Ir showed complete absence of carbonyl absorption, and the presence of hydroxyl absorption at 3430 cm^{-1}. This crude alcohol was dissolved in 4 ml of dry pyridine under nitrogen at 0°. Thionyl chloride (0.1 ml) was added, and the reaction was stirred 30 min at 0°. Water was added and the mixture extracted several times with ether. The ether extracts were washed with cold 6 *N* hydrochloric acid and with saturated sodium bicarbonate, then were dried ($MgSO_4$), filtered, and evaporated. The clear oily residue was distilled to yield 50 mg of *dl*-sativene. The analytical sample was prepared by preparative vpc on a 10-ft 15% Carbowax 20M on Chromosorb W column: ir (CCl_4) 3060, 1660, and 885 cm^{-1}; nmr (CCl_4) τ 5.60 and 5.28 (singlets, 2 H), 8.95 (s, 3 H), 9.10 (d, 3 H, J = 5 Hz), and 9.13 (d 3 H, J = Hz); mass spectrum P$^+$ *m/e* 204.

Anal. Calcd for $C_{15}H_{24}$: C, 88.16; H, 11.84. Found: C, 87.88; H, 11.83.

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Isotope Effects on the Succinate Dehydrogenase–L-Chlorosuccinate System[1,2]

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Abstract: Relative rates, $rate_H/rate_D$, at pH 7.8, 30°, of succinate dehydrogenase, soluble and particulate-bound, catalyzed ferricyanide oxidation of several deuterated L-chlorosuccinates are, α-deuterio-, 1.1; β-*erythro*-deuterio-, 1.0; α,β-*threo*-dideuterio-, 2.1; β-*threo*-deuterio-, 2.0. A kinetic isotope effect is thereby established for breaking the β-*threo*-carbon–hydrogen bond of substrate and analysis of steady-state kinetic data obtained with normal substrate and α,β-*threo*-dideuterio-L-chlorosuccinate suggests that this isotope effect occurs at reduction of enzyme by substrate. Interpretation of steady-state data obtained with soluble enzyme is made on the basis of a previously proposed kinetic mechanism involving reoxidation of reduced enzyme before and after dissociation of product. Steady-state data obtained with particulate-bound enzyme require a mechanism involving reoxidation of reduced enzyme after dissociation of product and, in addition, competitive inhibition by ferricyanide. Experiments regarding application of an enol–hydride mechanism to the succinate dehydrogenase catalyzed oxidation of L-chlorosuccinate indicate little or no exchange prior to oxidation.

Succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1), a nonheme iron- and flavin-containing protein,[3] is of considerable interest with respect to its mechanism of action.[4] The enzyme catalyzes the dehydrogenation of L-chlorosuccinate[5,6] as well as the dehydrogenation of the natural substrate, succinate, and in both instances the unsaturated *trans* acid is obtained. Assuming the same *trans* arrangement of carboxyl groups for the reactive conformation of the substrates, then the hydrogens removed are also *trans*.[7,8] L-Chlorosuccinate thus possesses one set of oxidizable hydrogens while the natural substrate possesses two such sets.[7,9] L-Chlorosuccinate with one set of *trans*-oxidizable hydrogens is, then, particularly

(1) Supported by grant GM-06245 from the General Medical Sciences Division, National Institutes of Health, U. S. Public Health Service.

(2) A preliminary account of this work has appeared: O. Gawron, A. J. Glaid, III, K. Mahajan, G. Kananen, and M. Limetti, *Biochem. Biophys. Res. Commun.*, **25**, 518 (1966).

(3) T. P. Singer in "Comprehensive Biochemistry," Vol. 14, M. Florkin and E. H. Stotz, Ed., Elsevier Publishing Co., Amsterdam, 1966, Chapter 3.

(4) D. V. DerVartanian, W. P. Zeylemaker, and C. Veeger in "Flavins and Flavoproteins," E. C. Slater, Ed., Elsevier Publishing Co., Amsterdam, 1966, p 183.

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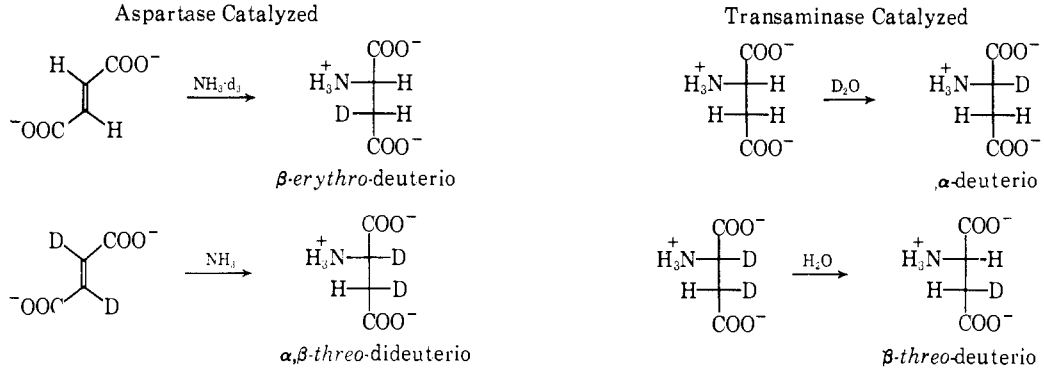

Figure 1. Enzyme-catalyzed routes to the several deuterated L-aspartates.

well suited for a study of isotope effects on the enzyme-catalyzed oxidation. In this report we are concerned with the effects of stereospecific substitution of deuterium for substrate hydrogen on steady-state kinetics and other aspects of the enzyme-catalyzed reaction.

For the study α-deuterio-L-chlorosuccinate, β-*erythro*-deuterio-L-chlorosuccinate, β-*threo*-deuterio-L-chlorosuccinate, and α,β-*threo*-dideuterio-L-chlorosuccinate were utilized, the compounds being synthesized with retention of configuration by nitrosyl chloride treatment of the corresponding L-aspartic acids, the deuterated L-aspartic acids being obtained by the procedures (Figure 1) of Tamiya and Oshima.[10] In the course of the study, particulate-bound enzyme as well as soluble enzyme was employed, the preparations showing, as previously observed,[11] different behavior with respect to the oxidant, ferricyanide.

Experimental Section[12]

Deuterated L-Aspartic Acids. The several deuterated L-aspartic acids were obtained by the enzyme-catalyzed reactions depicted in Figure 1, the procedures of Tamiya and Oshima[10] being followed in detail. For reactions involving transaminase-catalyzed exchange, glutamic-oxaloacetic transaminase suspension, Sigma Chemical Co., was employed. For reactions involving aspartase, a partially purified preparation, made from *P. vulgaris* by the method of Williams and McIntyre,[13] was utilized. The aspartase-catalyzed additions were based on the method of Krasna,[14] the aspartase-catalyzed addition being *trans*.[15,16] α,α′-Dideuteriofumaric acid, required for the synthesis of α,β-*threo*-dideuterio-L-aspartic acid, was prepared by catalytic hydrogenation of dimethylacetylene dicarboxylate with deuterium (Matheson Coleman and Bell) according to the procedure of Hoberman and D'Adamo.[17] Deuterium oxide, 99.77%, was obtained from Bio-Rad Laboratories.

Deuterated L-Chlorosuccinic Acids. The deuterated L-aspartic acids were converted to the corresponding deuterated L-chlorosuccinic acids with nitrosyl chloride by published procedures.[7] Typical data are: α-deuterio-L-chlorosuccinic acid, 18.65 atom % deuterium, 0.933 atom of deuterium/mol; α-deuterio-L-aspartic acid, 13.75 atom % deuterium, 0.964 atom of deuterium/mol; β-*erythro*-deuterio-L-chlorosuccinic acid, 18.00 atom % deuterium, 0.900 atom of deuterium/mol; β-*erythro*-deuterio-L-aspartic acid, 12.75 atom % deuterium, 0.895 atom of deuterium/mol; β-*threo*-deuterio-L-chlorosuccinic acid, 19.75 atom % deuterium, 0.988 atom of deuterium/mol; β-*threo*-deuterio-L-aspartic acid, 13.63 atom % deuterium, 0.956 atom of deuterium/mol; α,β-*threo*-dideuterio-L-chlorosuccinic acid, 35.65 atom % deuterium, 1.78 atom of deuterium/mol; α,β-*threo*-dideuterio-L-aspartic acid, 24.67 atom % deuterium, 1.73 atom of deuterium/mol.

Enzyme Preparations. The particulate-bound enzyme preparation employed was a cytochrome *c* deficient Keilin-Hartree muscle preparation prepared from beef heart by the procedure of Tsou[18] with overnight extraction by phosphate buffer. The soluble enzyme was prepared directly from the particulate preparation by the method of Wang, *et al.*,[19] with modification providing for a nitrogen atmosphere.[20] The soluble dehydrogenase was carried through calcium phosphate gel adsorption and one ammonium sulfate precipitation.

Assay. Initial rates at 30° were followed at 455 mμ in 1-cm cuvettes in the thermostated compartment of a Beckman DB spectrophotometer with attached recorder (Photovolt), the assay being conducted according to the conditions of DerVartanian and Veeger.[20] The standard assay reaction mixture, 3.0 ml total volume, contained 0.1 *M* phosphate, pH 7.8, 0.001 *M* potassium cyanide (preneutralized), 3 mg of bovine serum albumin, 0.04 *M* succinate, enzyme, and, unless otherwise noted, 0.006 *M* ferricyanide. The reference blank lacked succinate but contained 0.003 *M* ferricyanide. Under these conditions, an extinction coefficient of 95 M^{-1} cm^{-1} was obtained from the slope at 0.006 *M* ferricyanide of an optical density *vs.* concentration curve.[21]

Kinetic Runs. Initial rates were determined as above, utilizing the 420-mμ absorption band of ferricyanide. For kinetic runs employing 0.002 and 0.001 *M* ferricyanide, blanks contained 0.0015 and 0.0005 *M* ferricyanide, respectively, to keep absorbances below 1.0, the linear absorbance range of the recorder being 0–1.0. For these two situations respective extinction coefficients 814 and 1005 M^{-1} cm^{-1} were obtained while at lower concentrations of ferricyanide, in the absence of ferricyanide in the blank, the extinction coefficient was 1040 M^{-1} cm^{-1}.[22] Reaction mixtures were identical with those utilized in the standard assay except that substrate and ferricyanide concentrations were varied. For the runs (in triplicate) with soluble enzyme, enzyme of specific activity from 0.4 to 0.8 μmol of succinate oxidized/min/mg of protein was used at a level of 0.15–0.33 mg of protein/ml of reaction mixture. The unstable enzyme was monitored during each run, and the data were normalized to an enzyme content giving an initial rate of 0.267 μmol ml^{-1} min^{-1} in the standard assay. For the runs with particulate enzyme, preparations with specific activity from 0.49 to 0.58 μmol of succinate oxidized/min/mg of protein were used at a level of 0.27–0.40 mg of protein/ml of reaction mixture. Successive runs were normalized to an enzyme content giving an initial rate of 0.179 μmol ml^{-1} min^{-1} in the standard assay. The data for nonisotopic substrate was previously reported.[11] α,β-*threo*-Dideuterio-L-chlorosuccinate, 1.78 atoms of deuterium/mol, was utilized for detailed steady-state kinetic studies.

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(21) Previously reported[11] assays require correction by the factor, 1.9.
(22) Previously reported[11] kinetic runs at 0.002 and 0.001 *M* ferricyanide were carried out in a similar manner but not corrected for the change in extinction coefficient.

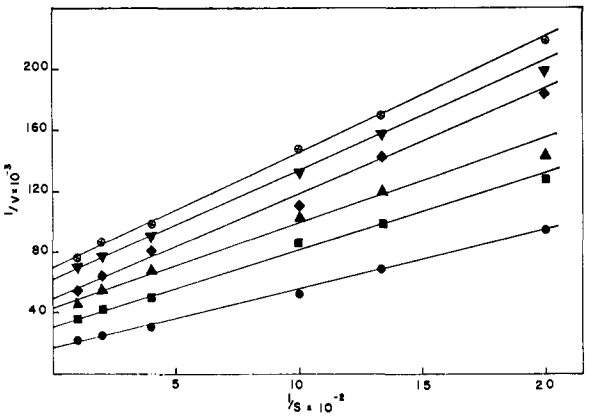


Figure 2. Reciprocal initial velocity, mol^{-1} l. min, *vs.* reciprocal substrate concentration at several ferricyanide concentrations for the soluble succinate dehydrogenase catalyzed oxidation of α,β-*threo*-dideuterio-L-chlorosuccinate, 35.65 atom % deuterium, 1.78 atoms of deuterium/mol. From top to bottom, 0.2, 0.25, 0.4, 0.6, 1.0, and 2.0 m*M* ferricyanide, respectively.

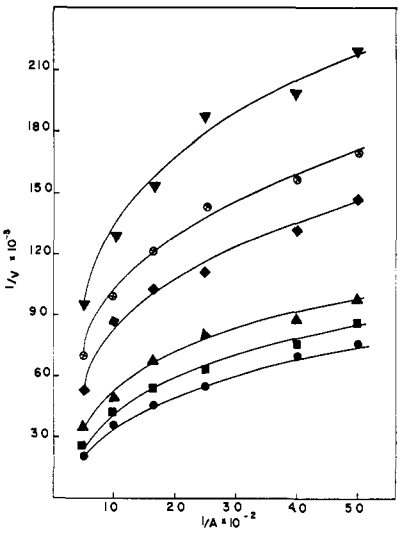


Figure 3. Reciprocal initial velocity, mol^{-1} l. min, *vs.* reciprocal ferricyanide concentration, [A], at several substrate concentrations for oxidation of α,β-*threo*-dideuterio-L-chlorosuccinate, 1.78 atoms of deuterium/mol, catalyzed by soluble succinate dehydrogenase. From top to bottom, 0.5, 0.75, 1.0, 2.5, 5.0, and 10.0 m*M* substrate, respectively.

Results and Discussion

Figures 2 and 3 are plots of primary rate data obtained with labeled substrate, α,β-*threo*-dideuterio-L-chlorosuccinate, using soluble succinate dehydrogenase. Figure 2 presents $1/v$ *vs.* 1/[S] plots for isotopic substrate at several ferricyanide concentrations. The corresponding $1/v$ *vs.* 1/[ferricyanide] plots are presented in Figure 3. In a similar way primary rate data obtained with particulate-bound enzyme are presented in Figures 6 and 7 for isotopic substrate.

The deuterated L-chlorosuccinate, while of relatively high deuterium content, is a mixture of normal compound with deuterated compound and, as such, steady-state kinetic data must be considered first in terms of a two-substrate, one-enzyme system. For such a system at a given ferricyanide concentration, eq 1 with subscript

$$V = \frac{\frac{V[S]}{K} + \frac{V_D[S]_D}{K_D}}{1 + \frac{[S]}{K} + \frac{[S]_D}{K_D}} \qquad (1)$$

D indicating isotopic substrate and its kinetic constants, is applied.[23] For a given concentration ratio, r, of normal compound to isotopic compound, eq 1 becomes, in double reciprocal, form, eq 2.

$$\frac{1}{V} = \frac{rK_D + K}{rVK_D + V_DK} + \frac{KK_D}{[S]_D[rVK_D + V_DK]} \qquad (2)$$

Using the theoretical slope to intercept ratio of eq 2, $KK_D/(rK_D + K)$, K_D may be calculated from experimental data and then utilized to calculate V_D from the data and the intercept function, $(rK_D + K)/(rVK_D + V_DK)$, of eq 2. The ratio, V_D/K_D, then gives the slope of $1/v$ *vs.* $1/[S]_D$ plots that can be constructed from the calculated K_D and V_D values at the several ferricyanide concentrations. In this manner, using $r \cong 0.1$, kinetic constants for 100% α,β-*threo*-dideuterio-L-chlorosuccinate were calculated from the experimental data presented in Figure 2 and the data for normal substrate.[11] These constants were then used for the plots of Figures 4 and 5.

(23) M. Dixon and E. C. Webb, "The Enzymes," 2nd ed, Academic Press, New York, N. Y., 1964, p 84.

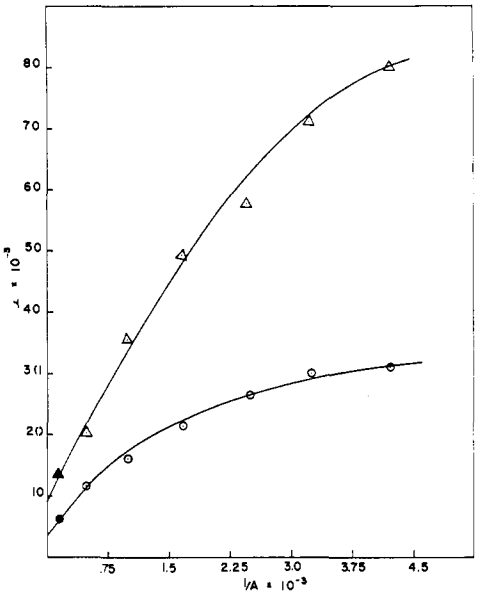


Figure 4. Intercepts, i, mol^{-1} l. min, of $1/v$ *vs.* 1/[S] plots plotted against reciprocal ferricyanide concentration, [A]. Intercepts for L-chlorosuccinate taken from Figure 1 of ref 11 after correction of rates at 0.001 and 0.002 *M* ferricyanide. Intercepts for α,β-*threo*-dideuterio-L-chlorosuccinate are corrected for the presence of nonisotopic substrates (10%), the data of Figure 2 and previous data (Figure 1, ref 11) being used for the calculation as presented in the text. The points at 166 M^{-1} ferricyanide are taken from Table I, 0.040 *M* substrate being saturating. Top curve for α,β-*threo*-dideuterio-L-chlorosuccinate, bottom curve for normal substrate.

Figure 4 presents a plot of $1/V$ values, for normal substrate and of $1/V_D$ values, calculated as indicated above, against the reciprocal of the ferricyanide concentration. Figure 5 presents a plot of the slopes calculated for $1/v$ *vs.* $1/[S]_D$ plots and the slopes of $1/v$ *vs.* 1/[S] plots for normal substrate against the reciprocal of the ferricyanide concentration.

The experimental data and derived plots presented in Figures 2–5 may be rationalized on the basis of the

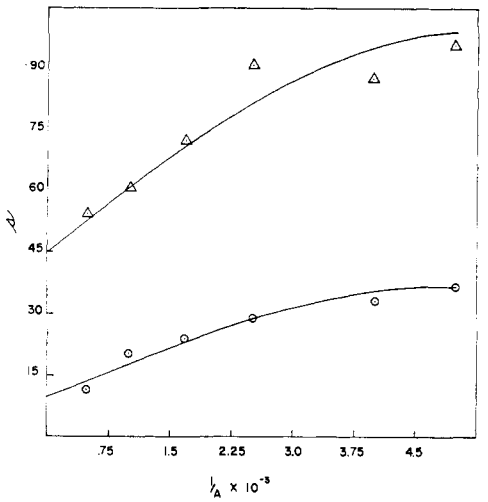


Figure 5. Slopes, s, of $1/v$ *vs.* 1/[S] plots plotted against reciprocal ferricyanide concentration, [A]. Slopes for L-chlorosuccinate (bottom curve) taken from Figure 1 of ref 11. Slopes for α,β-dideuterio-L-chlorosuccinate (top curve) are corrected for the presence of nonisotopic substrate (10%), the data of Figure 2 and the previous data (Figure 1, ref 11) being used for the calculation according to the text.

kinetic mechanism previously described[11] for the ferricyanide oxidation, catalyzed by soluble succinic dehydrogenase, of L-chlorosuccinate and succinate. In terms of this mechanism substrate is oxidized by enzyme and reduced enzyme (E′′) is reoxidized with ferricyanide (A), the reoxidation of reduced enzyme occurring either before or after dissociation of product from reduced-enzyme–product complex.[24] The mechanism is described by reactions 3–8 and leads to eq 9 for the total steady-state rate.

$$E + S \underset{2}{\overset{1}{\rightleftharpoons}} ES \tag{3}$$

$$ES \underset{4}{\overset{3}{\rightleftharpoons}} E''P \tag{4}$$

$$E''P \xrightarrow{5} E'' + P \tag{5}$$

$$E''P + A \xrightarrow{7} EP + A_{red} \tag{6}$$

$$E'' + A \xrightarrow{9} E + A_{red} \tag{7}$$

$$EP \xrightarrow{11} E + P \tag{8}$$

$$\frac{1}{V} = \frac{\frac{k_1k_3k_5k_{11}[S]}{[A]^2} + \frac{k_9k_{11}}{[A]}\{k_1[S](k_4 + k_5) + k_1k_3[S] + k_2(k_4 + k_5) + k_3k_5\} + k_7k_9\{k_1k_{11}[S] + k_1k_3[S] + k_2k_{11} + k_3k_{11}\}}{\frac{k_1k_3k_5k_9k_{11}E_0[S]}{[A]} + k_1k_3k_7k_9k_{11}E_0[S]} \tag{9}$$

Equation 9, linear in 1/[S], accounts for the curvilinear form of $1/v$ *vs.* 1/[A] plots. The intercepts ($1/V$) of $1/v$ *vs.* 1/[S] plots as a function of 1/[A] are, according to eq 9

$$i = \frac{\frac{k_3k_5k_{11}}{[A]^2} + \frac{k_9k_{11}}{[A]}(k_4 + k_5 + k_3) + k_7k_9(k_{11} + k_3)}{\frac{k_3k_5k_9k_{11}E_0}{[A]} + k_3k_7k_9k_{11}E_0} \tag{10}$$

Equation 10 for its intercept at 1/[A] equal to zero gives the velocity for infinite substrate and oxidant concentrations.

$$\frac{1}{V_{max}} = \frac{k_3 + k_{11}}{k_3k_{11}E_0}$$

Comparison of the intercepts of $1/V$ *vs.* 1/[A] plots, Figure 4 for normal L-chlorosuccinate and for α,β-*threo*-dideuterio-L-chlorosuccinate gives a $V_{max\,H}/V_{max\,D}$ ratio of 2. This decrease in V_{max} on deuterium substitution represents an isotope effect on k_3 since k_{11}, the rate constant for dissociation of reduced-enzyme–product complex, is necessarily the same for the normal and isotopic compound, both deuteriums of the isotopic compound being removed since they constitute the *trans*-removable pair. In the absence of kinetic data relating directly to the magnitudes of k_3 and k_{11}, it can not be concluded, however, that this isotope effect on V_{max} is directly proportional to the isotope effect on k_3. It is quite possible that the isotope effect on k_3 is larger than that noted for V_{max}. Considering slopes of $1/v$ *vs.* 1/[S] plots, eq 9 predicts for a plot, Figure 5, of these slopes *vs.* 1/[A] an intercept equal to $(k_2 + k_3)/k_1k_3E_0$. From Figure 5 it may be noted that the isotope effect on the intercept gives an $\text{intercept}_D/\text{intercept}_H$ ratio of approximately 4. If the isotope effect on k_2 and k_1 is negligible, as one might expect for formation and dissociation of the enzyme–substrate complex, then the change in intercept value must reflect an isotope effect on k_3. In this connection it may be noted[25] that the flavoprotein–substrate system, glucose oxidase, D-glucose-1-^{1}H, shows $k_3 \gg k_2$ so that the ratio $(k_2 + k_3)/k_1k_3E_0$ is essentially equal to $1/k_1E_0$ while for glucose oxidase, D-glucose-1-^{2}H, $k_3 \cong k_2$ and the ratio $(k_2 + k_3)/k_1k_3E_0$ can not be reduced to simpler terms. Comparison of the $(k_2 + k_3)/k_1k_3E_0$ ratios gives a glucose-1-^{2}H:glucose-1-^{1}H ratio of 2.3 while the actual isotope effect is calculated to be 15. It may also be remarked that no isotope effect is noted for k_1 and k_2 in the glucose oxidase system.

Of further interest in connection with the isotope effects noted with α,β-*threo*-dideuterio-L-chlorosuccinate is the fact that substitution of β-*threo*-H by deuterium is responsible for the effect. Reference to the relative rates of Table I discloses that no isotope effect is noted if α-H or β-*erythro*-H is substituted by deuterium while substitution of β-*threo*-H by deuterium gives rise to an isotope effect equal in magnitude to that seen with α,β-*threo*-dideuterio-L-chlorosuccinate. Since the concentrations of substrate and ferricyanide

(24) J. D. W. Van Voerst, C. Veeger, and D. V. Dervartanian, *Biochim. Biophys. Acta*, **146**, 367 (1967), subsequently have suggested that in the absence of a rapidly reacting electron acceptor product may dissociate from reduced enzyme.

(25) H. J. Bright and Q. H. Gibson, *J. Biol. Chem.*, **242**, 994 (1967).

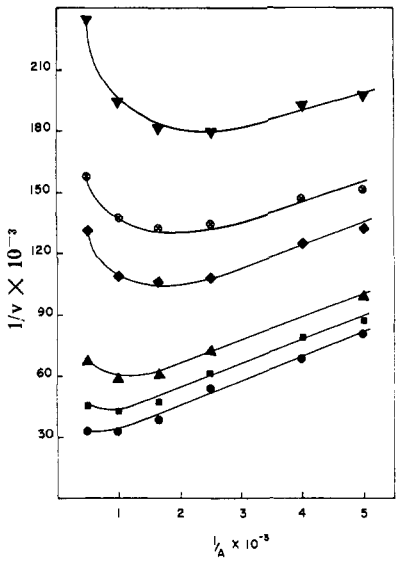


Figure 6. Reciprocal initial velocity, $1/v$, mol^{-1} l. min, *vs.* reciprocal ferricyanide concentration, [A], at several substrate concentrations for particulate-bound succinate dehydrogenase catalyzed oxidation of α,β-*threo*-dideuterio-L-chlorosuccinate, 35.65 atom % deuterium, 1.78 atoms of deuterium/mol. From top to bottom, 0.5, 0.75, 1.0, 2.5, 5.0, and 15.0 m*M* substrate.

employed, 0.04 and 0.006 *M*, respectively, are saturating, the observed rates are close to maximal and the calculated ratios are therefore comparable to $V_{\max H}/V_{\max D}$ ratios. These results demonstrate that only one of the two hydrogens of the *trans*-removable pair from L-chlorosuccinate is participating in the rate-determining step and that this rate-determining step is most likely the breaking of the β-carbon–*threo*-H bond.

Table I. Relative Rates of Oxidation of Deuterated L-Chlorosuccinates

Compound	Atoms of D/mol	Rate$_H$/rate$_D$[a] Soluble[b]	Particulate[c]
H		1.0	1.0
α-D	0.92	1.06	1.15 (1.33)
β-*threo*-D	0.875[d]	2.0	2.04 (2.40)
β-*erythro*-D	0.90	1.0	0.94 (1.24)
α,β-*threo*-D	1.78	2.08	2.13 (2.83)

[a] Without correction for the presence of normal compound. An estimated correction yields ≅2.4 for rate$_H$/rate$_{\alpha D,\beta D}$, for soluble enzyme. Average of relative rates obtained from two separate experiments. [b] Under the usual conditions with a substrate concentration of 0.04 *M* and a ferricyanide concentration of 0.006 *M*. [c] With 0.04 *M* substrate but 0.002 *M* ferricyanide to avoid ferricyanide inhibition. The results in parentheses were previously reported[2] and were obtained at substrate and ferricyanide concentrations of 0.04 and 0.006 *M*, respectively. [d] Not corrected for the presence of deuterium in the α position. The compound was made from α,β-*threo*-dideuterio-L-aspartic acid, 1.57 atoms of deuterium/mol, and deuterium over 0.79 atom/mol may be attributed to α-deuterium not removed by transaminase exchange.

The above isotopic effects are consistent with results of rapid-rate studies on the reduction of succinate dehydrogenase by succinate and reoxidation by ferricyanide. DerVartanian, *et al.*,[26] have shown that succinate reduction of enzyme, producing kinetically identifiable nonheme iron and flavin intermediates,

(26) D. V. DerVartanian, C. Veeger, W. H. Orme-Johnson, and H. Beinert, *Federation Proc.*, **26**, 732 (1967).

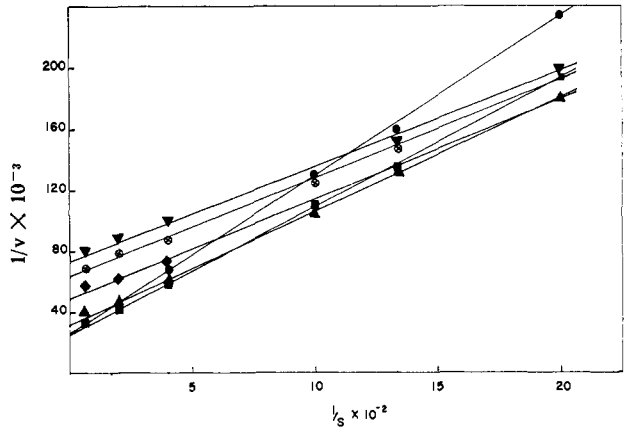


Figure 7. Reciprocal initial velocity, $1/v$, mol^{-1} l. min, *vs.* reciprocal substrate concentration at several ferricyanide concentrations for particulate-bound succinate dehydrogenase catalyzed oxidation of α,β-*threo*-dideuterio-L-chlorosuccinate, 35.65 atom % deuterium, 1.78 atoms of deuterium/mol: ▼, 0.2 m*M*; ⊗, 0.25 m*M*; ◆, 0.4 m*M*; ▲, 0.6 m*M*; ■, 1.0 m*M*; ●, 2.0 m*M* ferricyanide.

proceeds with a half-life of ≅20 msec while reoxidation by ferricyanide proceeds with a half-life of ≦10 msec. Accordingly, steady-state kinetic studies may reveal a rate-determining step involving reduction of enzyme by substrate, provided, of course, that dissociation of product from enzyme is not rate limiting. Apparently this is the situation with the L-chlorosuccinate–succinate dehydrogenase–ferricyanide system, and substitution of β-*threo*-H by deuterium reveals an isotope effect.

The foregoing isotopic effects obtained with soluble enzyme were also obtained with a particulate-bound succinate dehydrogenase preparation, albeit the steady-state behavior of the particulate succinate dehydrogenase system differs from that of the soluble enzyme in that inhibition by ferricyanide is noted with particulate enzyme. This inhibition, noted directly in the $1/v$ *vs.* 1/[A] plots of Figure 6 and indirectly in the increasing slopes of the $1/v$ *vs.* 1/[S] plots of Figure 7, coupled with the observed parallel segments of Figure 6 suggests that ferricyanide is acting as a competitive inhibitor and, in contrast to soluble enzyme, that oxidation of reduced enzyme only occurs after product dissociation. On this basis, the system may be described by the following sequence of reactions.

$$EA \underset{k_{14}}{\overset{k_{13}[A]}{\rightleftharpoons}} E \underset{k_2}{\overset{k_1[S]}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E''P \underset{k_6[P]}{\overset{k_5}{\rightleftharpoons}} E'' \xrightarrow{k_9[A]} E \qquad (11)$$

A similar set of reactions, including not only competitive inhibition by oxidant (lipoate) but also competitive inhibition by substrate (thiosulfate), has been kinetically demonstrated for the rhodanese-catalyzed thiosulfate–lipoate reaction.[27] It may also be noted that Vitale and Rittenberg[28,29] have observed ferricyanide inhibition of succinate oxidation when a particulate enzyme preparation from *E. coli* ML 308 was employed. The kinetic scheme for beef heart particulate-bound enzyme then differs from that for soluble enzyme derived from

(27) M. Volini and J. Westley, *J. Biol. Chem.*, **241**, 5168 (1966).
(28) L. Vitale and D. Rittenberg, *Biochemistry*, **6**, 690 (1967).
(29) Ferricyanide, 5 m*M*, was required to note inhibition with apparently, 3.5 m*M* succinate.[28] T. E. King, *J. Biol. Chem.*, **238**, 4032 (1963), has noted some inhibition of soluble enzyme with ferricyanide concentrations greater than 5 m*M*.

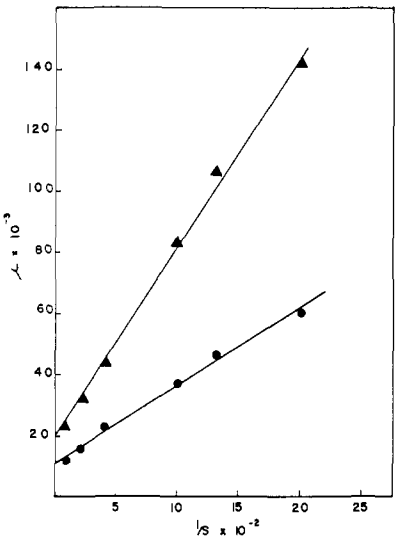


Figure 8. Intercepts, i', obtained by extrapolation of the straight line segments of $1/v$ *vs.* 1/[A] plots plotted against 1/[S]. Top curve, α,β-*threo*-dideuterio-L-chlorosuccinate; bottom curve, normal substrate. For isotopic substrate straight line segments of Figure 6 were utilized; for normal substrate data from Figure 4 of ref 11 were utilized.

particulate-bound enzyme in inhibition by ferricyanide at relatively low concentrations and the requirement for dissociation of product before oxidation of reduced enzyme, the scheme for soluble enzyme permitting oxidation of reduced enzyme before and after product dissociation. These differences may be rationalized if it is considered that particulate-bound enzyme has a site for ferricyanide interaction which is also common to substrate. On this basis, reoxidation by ferricyanide could only occur after dissociation of product and ferricyanide would be a competitive inhibitor of substrate. Whatever the cause for this behavior of particulate-bound enzyme, organization in the particulate or a molecular basis, solubilization of the enzyme removes inhibition by ferricyanide at relatively low concentrations and permits oxidation of reduced enzyme in the presence of product. Consequently, it is possible that reduced soluble enzyme has more than one component for ferricyanide oxidation and that this results from disorganization of the particulate or from a molecular change on alkaline solubilization. Since soluble enzyme has at least two redox functions (nonheme iron and flavin), with both participating in the catalytic process,[26] it seems likely that substrate or product is not able to mask both functions from interaction with ferricyanide.

Steady-state treatment of sequence 11 yields the initial rate eq 12 predicting the observed dependence on ferricyanide concentration of slopes of $1/v$ *vs.* $1/v$ *vs.* 1/[S] plots and the curvilinear $1/v$ *vs.* 1/[A] plots with parallel linear portions at low ferricyanide concentration. The data, Figures 6 and 7, for α,β-*threo*-dideuterio-L-chlorosuccinate and that for L-chlorosuccinate[11] are in agreement with eq 12.

$$\frac{1}{v} = \frac{k_3 + k_4 + k_5}{k_3k_5E_0} + \frac{1}{k_9E_0[A]} + \frac{[(k_2k_4 + k_2k_5 + k_3k_5)](k_{14} + k_{13}[A])}{k_1k_3k_5k_{14}E_0[S]} \quad (12)$$

Figure 8 presents a plot of the intercepts of the extrapolated linear segments of Figure 6, the intercepts being plotted against reciprocal substrate concentration. These plots correspond to eq 13, and the intercepts of

$$i' = \frac{k_3 + k_4 + k_5}{k_3k_5E_0} + \frac{k_2k_4 + k_2k_5 + k_3k_5}{k_1k_3k_5E_0[S]} \quad (13)$$

Figure 8 are then equal to reciprocal maximum rates $(k_3 + k_4 + k_5)/k_3k_5E_0$, in the absence of the inhibitory effect of ferricyanide. Consideration of Figure 8 gives a value 1.9 for the ratio intercept α_D,β_D/intercept$_H$ and accordingly, $V_H/V_{\alpha_D,\beta_D} \cong 1.9$. Similarly, slopes of Figure 8 show an isotope effect, slope$_H$/slope$_{\alpha_D,\beta_D}$ $\cong 2.4$. These isotope effects observed with particulate-bound enzyme are similar to those observed with soluble enzyme and are also attributable (Table I) to substitution by deuterium of the β-*threo*-H of L-chlorosuccinate. Analysis of the isotope effects on the kinetic constants obtained with particulate-bound enzyme leads to the same general conclusions obtained with soluble enzyme. Since enzymatic oxidation removes both deuteriums from the dideuterio substrate, k_5, the rate constant for dissociation of reduced-enzyme–product complex, is necessarily the same for the isotopic and normal compound. Accordingly the observed isotope effects on the maximum rate and on the slope must be due to changes in the other rate constants with k_3 the most likely rate constant to be affected.

While the results reported herein demonstrate a rate-determining step involving only one of the two *trans*-removable hydrogens of L-chlorosuccinate, Vitale and Rittenberg[28] have concluded on the basis of steady-state studies with deuterated succinates that both removable hydrogens of a *trans*-removable pair participate in the rate-determining step. These studies utilized phenazine methosulfate as the oxidant and a kinetic scheme similar to that of reaction scheme 11 of this paper except that inhibition by oxidant was not noted and not included in the scheme. In analyzing their data, Vitale and Rittenberg concluded that the rate-determining step was at enzymatic dehydrogenation of substrate and on the basis of probability considerations also concluded that the rate ratios (rate$_D$/rate$_H$)—tetradeuteriosuccinate, 0.45; α,α-dideuteriosuccinate, 0.70; (*R*)-monodeuteriosuccinate, 0.85; (*S*)-monodeuteriosuccinate, 0.89—observed with soluble enzyme from *C. purpurea*, for example,[30] were best explained by postulating bond breaking at both C–H bonds in the rate-determining step. This is a somewhat unexpected conclusion in view of the commonly accepted[31] principle that *trans* processes proceed by ionic steps and in view of the previously referred to demonstration by Bright and Gibson[25] that in the absence of knowledge of individual rate constants a precise interpretation of isotope effects on maximum rates is difficult to make.

Pertinent to the results obtained with deuterated L-chlorosuccinates and deuterated succinates are steady-state deuterium effects noted for *trans*-diaxial elimination of 1-α,2β-hydrogen atoms from 3-keto steroids, the reaction being catalyzed by cell-free preparations of *Bacillus sphaericus*.[32] In the case of 5α,3-keto steroids

(30) Similar data were obtained by Vitale and Rittenberg[28] with succinic dehydrogenase from other sources.

(31) E. M. Kosower, "Molecular Biochemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p 227.

(32) R. Jerussi and H. J. Ringold, *Biochemistry*, **4**, 2113 (1965).

Table II. Deuterium Loss from Residual Substrate after 20% Oxidation[a]

Compd	Atom of deuterium/mol	
	Initial	20% oxidation[b]
α-D	0.923	0.893
β-*threo*-D	0.986[c]	1.00

[a] With particulate-bound enzyme at pH 7.8, 29.8°, the reaction mixtures, 40 ml total volume, containing 0.1 *M* phosphate, 0.001 *M* potassium cyanide, 40 mg of serum albumin, 0.006 *M* ferricyanide, and 0.01 *M* substrate. For the α-deuterio run, 76 mg of enzyme protein, specific activity 0.19, was employed, 20% oxidation requiring 12 min. For the β-*threo*-deuterio run, 114 mg of enzyme protein was employed, 20% oxidation requiring 15.5 min. After adjustment of pH to 1.5 with formic acid, carrier L-chlorosuccinate, 4 times the amount of the residual substrate was added, and reaction mixtures were concentrated to 15 ml by lyophylization. L-Chlorosuccinate acid was then isolated by continuous ether extraction and purified by recrystallization from ethyl acetate. [b] After correction for dilution by carrier. [c] Not corrected for the presence of deuterium in the α position. The compound was made from α,β-*threo*-dideuterio-L-aspartic acid and deuterium over 0.89 atom/mol may be deuterium on α-carbon not removed by transaminase-catalyzed exchange.

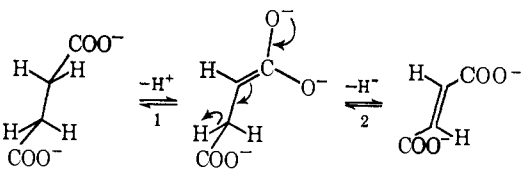
be noted during oxidation. The data presented in Tables II and III bear on this point. The data of Table II, concerning loss of deuterium from residual substrate during 20% oxidation by ferricyanide, indicates no loss of deuterium from the β position, as expected, and an insignificant or, at best, a very small loss of deuterium from the α position. This preliminary data suggests that enolization, if the enol–hydride mechanism is applicable, of β-*threo*-H is rate limiting. From Table III it may be noted that deuterium is lost from both the α position and the β-*threo* position on anaerobic exchange, similar results being obtained with both soluble and particulate bound enzyme. The ratio, $\text{loss}_{\alpha\text{-D}}/\text{loss}_{\beta\text{-threo-D}}$, is 1.87 for soluble enzyme and 1.61 for particulate-bound enzyme. These ratios are comparable to the velocity ratios obtainable from Table I, the latter, $\text{rate}_{\alpha\text{-D}}/\text{rate}_{\beta\text{-threo-D}}$, being 1.89 and 1.77, respectively, for soluble and particulate-bound enzyme. It would then seem that loss of deuterium from the α and β positions is under the same rate-controlling step as is

Table III. Deuterium Loss on Exchange[a]

Compound	Atoms of deuterium/mol								
	Soluble enzyme						Particulate-bound enzyme		
	Control			Run					
	Initial	Final	Loss	Initial	Final	Net loss	Initial	Final	Loss[b]
α-D	0.910	0.715	0.195	0.910	0.415	0.300	0.933	0.385	0.548
β-*threo*-D	0.820	0.630	0.190	0.820	0.47	0.16	0.986	0.645	0.341
α,β-*threo*-di-D	1.73	1.55	0.18	1.73	1.21	0.34			

[a] In a total volume of 20.0 ml at pH 7.5 for 45 min under nitrogen. Reaction mixtures contained 0.06 *M* phosphate, 0.018 *M* magnesium chloride and 0.02 *M* isotopic compound. For the runs with soluble enzyme, 0.004 *M* chlorofumarate and 25.6 mg of enzyme protein were used, the run being conducted at 37°. For the runs with particulate enzyme, 0.005 *M* chlorofumarate and 240 mg of enzyme protein were present, the run being conducted at 35°. Isolation of L-chlorosuccinic acid was carried out in the manner described in Table II, footnote *a*. [b] Controls were not conducted in these runs since the experiments of Table II, conducted at the same time, indicated no loss of deuterium.

a significant kinetic isotope effect was found for deuterium at the 1-α but not the 2-β position while for Δ^4-3-keto steroids, deuterium at either position affects V_{max}. For the latter compound the conclusion was reached[32] that the isotope effects observed at the 1-α position and the 2-α position cannot be true isotope effects for single rate constants but are, instead, composite results of a complex situation. The similarity of these observations with the two steroid substrates to those obtained with L-chlorosuccinate and succinate is immediate and it is also instructive to consider a proposed[32] enol hydride mechanism with respect to the succinate dehydrogenase system. Without considering enzyme interactions the reaction may be written as shown below to yield a proton and a hydride ion.



For L-chlorosuccinate, breaking of the β-*threo* carbon–hydrogen bond is rate determining and, *a priori*, cannot be assigned to either step 1 or 2. If breaking of the β-C–H bond involves step 1, enolization, then no prior exchange would be noted during oxidation. On the other hand, if breaking of the β-C–H bond involves step 2 then exchange of α-H should the oxidation. This tentative conclusion is seemingly substantiated by deuterium loss from the α,β-*threo*-dideuterio compound noted in Table III. The net loss from this compound, 0.34 atom of deuterium/mol, is very close to twice the net loss from the β-*threo*-compound. This is expected if the exchange rate at the α-H position is controlled by bond breaking at the β-carbon–*threo*-hydrogen bond. A net loss of 0.436 atom of deuterium/mol would be expected if exchange at the α-H position preceeded the rate-determining step. If the enol hydride mechanism is applicable to succinic dehydrogenase then the data again suggest that the rate-determining step is step 1.

The above succinate dehydrogenase catalyzed exchange experiments involving replacement of substrate deuterium by protons of the medium are in agreement with previous experiments[33] in which substrate hydrogens were replaced by deuterium from a 50% deuterium oxide medium. In the latter experiments the proportion of monodeuterated L-chlorosuccinate molecules was found to be considerably greater than dideuterated L-chlorosuccinate molecules and it was suggested that the exchangeable hydrogens of L-chlorosuccinate exchange at different rates. Since exchange of β-*threo*-deuterium is slower than exchange of α-deuterium and since exchange of β-*threo*-deuterium appears rate determining for exchange of α-deuterium, it would seem

(33) O. Gawron, A. J. Glaid, III, J. Francisco, and T. P. Fondy, *Nature*, **197**, 1270 (1963).

on the basis of microscopic reversibility that the monodeuterated L-chlorosuccinate obtained by exchange replacement of H by deuterium has deuterium in the α position, a conclusion opposite to the previous provisional conclusion[34] reached on the basis of tenuous rotation data. This new conclusion, of course, requires corroboration by direct demonstration of the stereochemistry of monodeuterio-L-chlorosuccinate obtained by exchange. It may, however, be noted that the new evidence[35] for the stereochemistry of replacement of succinate hydrogen by deuterium from the medium (100% deuterium oxide) shows that, initially, an H_R atom is replaced, the product being (−)-(*R*)-succinic-d_1 acid and this stereochemistry is the same as that suggested above.

Investigations into succinate dehydrogenase catalyzed exchange of succinate hydrogens for deuterium of the medium have yielded varying results. Retey, *et al.*,[35] in a definitive set of experiments, referred to above, utilizing soluble succinate dehydrogenase and 100% deuterium oxide as the medium report that initial exchange yields both succinate-d_1 and succinate-d_2, the former, as previously mentioned, (*R*)-succinate-d_1 and the latter *meso*-succinate-d_2, the two deuterated species being formed simultaneously. Further exchange yields succinate-d_3 and succinate-d_4 species. Kahn and Rittenberg[36] using a particulate preparation and a 50% deuterium oxide medium found essentially only succinate-d_1 in the exchange succinate, the succinate-d_1 being optically inactive. A similar experiment[34] utilizing particulate-bound enzyme and 50% deuterium oxide also gave predominantly succinate-d_1 in the exchanged succinate.[37] It would seem that rate discrimination effects between H^1 and H^2 are sufficiently great so that in the 1:1 deuterium oxide–protium oxide experiments essentially only one substrate H^1 is replaced by H^2 from the medium while in 100% deuterium oxide two substrate H^1 atoms may be replaced simultaneously, concurrently with replacement of one substrate H^1, as might be expected on the basis of the previous results.

(34) O. Gawron, A. J. Glaid, III, and J. Francisco, *Biochem. Biophys. Res. Commun.*, **16**, 156 (1964).

(35) J. Retey, J. Seibl, D. Arigoni, J. W. Cornforth, G. Ryback, W. P. Zeylemaker, and C. Veeger, *Nature*, **216**, 1320 (1967).

(36) J. Kahn and D. Rittenberg, *Biochem. Biophys. Res. Commun.*, **27**, 484 (1967).

(37) On the basis of reported rotation data, it was concluded[34] that the succinate-d_1 was (*S*)-succinate-d_1. In view of the results of Retey, *et al.*, this report needs corroboration.

Of further interest are the exchange experiments, involving rates of loss of isotopic hydrogen from labeled succinates, reported by Hufner, *et al.*[38] From these experiments it was concluded that all four methylene hydrogen atoms of succinate are sterically equivalent and that two *trans* hydrogens are exchanged. These conclusions are in apparent contradiction to those of Retey, *et al.*[35] Whatever the cause for this seeming contradiction, the results of Hufner, *et al.*,[38,39] in a practical sense suggest the equivalence of the four hydrogens in processes in which they are removed and it may be noted that the kinetic data of Vitale and Rittenberg[28] may be equally well explained on the basis of equivalence of the four hydrogens in conjunction with the breaking of a single C–H bond as rate determining as on the suggested basis of a *trans* process involving rate-determining breaking of two C–H bonds.

In connection with a hydride ion concept of succinate oxidation it is of interest to note that with mitochondrial multienzyme preparations a small amount of hydrogen from succinate is directly transferred to NAD^+[40,41] despite exchange at the succinate dehydrogenase level and at the level of the β-specific enzyme,[42] presumably the β-specific NADH dehydrogenase, concerned with NAD^+ reduction.[43] Hydrogen from succinate is also utilized, again in small amounts, for reductive biosynthesis of fatty acids[44] and for reduction of acetoacetate,[45] albeit it is suggested[45] that malate arising from succinate is the hydrogen donor in transfer to NAD^+ and acetoacetate.[46]

Acknowledgment. The technical assistance of Mr. David Ford is gratefully acknowledged.

(38) M. Hufner, L. M. Buckley, and T. C. Hollocher, *Biochem. Biophys. Res. Commun.*, **28**, 791 (1967).

(39) These experiments were carried out in the absence of fumarate whereas other exchange experiments[33-36] were carried out in the presence of fumarate, replacement of substrate H by deuterium from the medium being faster in the presence of fumarate (S. England and S. P. Colowick, *J. Biol. Chem.*, **221**, 1019 (1956)).

(40) O. Gawron, A. J. Glaid, III., S. Nobel, and M. Gan, *Biochem. Biophys. Res. Commun.*, **16**, 432 (1964).

(41) D. E. Griffiths and H. J. MacNiece, *Biochem. J.*, **97**, 17P (1965).

(42) D. E. Griffiths and A. M. Roborton, *ibid.*, **94**, 30P (1965).

(43) NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively.

(44) A. F. Whereat, *Proc. Soc. Exptl. Biol. Med.*, **118**, 888 (1965).

(45) H. D. Hoberman, L. Prosky, P. G. Hempstead, and H. W. Arnip, *Biochem. Biophys. Res. Commun.*, **17**, 490 (1964).

(46) NOTE ADDED IN PROOF. Recent experiments of Walker, *et al.* (W. H. Walker, P. Hemmerich, and V. Massey, *Helv. Chim. Acta*, 50, 2269 (1967)), demonstrating the addition of a benzyl residue (generated from phenylacetate anion) and H across the 4a,5 double bond of flavins suggest the possibility of a similar mechanism being operative with succinate dehydrogenase. If such an intermediate were formed from succinate, subsequently ionization of H^+ and transfer of electrons to flavin would lead to fumarate and fully reduced flavin. It is clear that such a mechanism provides for individual removal of *trans* hydrogens. It may be noted that attack at position 4a of flavin was previously suggested[38] to account for preferential exchange of one hydrogen succinate and L-chlorosuccinate.