(freshly distilled from sodium). The reduction was carried out by dipping a 1-ml Mohr pipet containing sodium into the solution until the blue color spread throughout the entire solution and persisted for 20–30 sec. (If the blue color persists beyond this time a few milligrams of ammonium chloride is added to oxidize the excess sodium.) The solution was concentrated to about 20 ml under reduced pressure at the water pump and then lyophilized. The residue was dissolved in 600 ml of 0.03% trifluoroacetic acid, and the pH of the solution was adjusted to 8.0 with 1% ammonium hydroxide. An excess (3.0 ml) of a 0.2 *N* solution of potassium ferricyanide was added and the solution was stirred for 15 min. AG 3-X4 resin (Bio-Rad Laboratories, Richmond, Calif.) in the trifluoroacetate form was added and stirring was continued for 15 min. The resin was removed by filtration and the solution was lyophilized. A column of Sephadex G-25 (100–200 mesh) (2.82 × 65.3 cm) in 0.2 *N* acetic acid was prepared and equilibrated with the lower phase of the solvent system 1-butanol–benzene–3.5% acetic acid in 1.5% aqueous pyridine (1:2:3). The lyophilized powder was dissolved in 12 ml of the upper phase of this solvent system, applied to the column, and eluted with the upper phase. Fractions of 400 drops (9.9 ml) were collected. The chromatogram obtained by plotting the Folin–Lowry color values of alternate fractions indicated a major peak with R_f 0.25. The contents of fractions 32–44 corresponding to this peak were pooled, concentrated under reduced pressure, and lyophilized. The lyophilized powder (50 mg) was dissolved in 10 ml of 0.2 *N* acetic acid and subjected to gel filtration on a Sephadex G-25 (200–270 mesh) column (2.82 × 62.0 cm) that had been equilibrated with 0.2 *N* acetic acid. Fractions of 9.0 ml were collected. Folin–Lowry color values of the fractions showed a single peak with a maximum at fraction 36. The fractions corresponding to this peak were pooled and lyophilized; wt 46 mg, $[\alpha]^{22}D$ +30.2° (*c* 0.7, 1 *N* acetic acid).

Anal. Calcd for $C_{45}H_{69}N_{11}O_{12}S_2$: C, 53.0; H, 6.81; N, 15.1. Found: C, 53.0; H, 6.70; N, 15.0.

A sample was hydrolyzed in 6 *N* HCl at 110° for 22 hr and analyzed[20] in the 50° system of the Beckman-Spinco amino acid analyzer (Model 116). The following molar ratios were obtained: aspartic acid, 1.02; glutamic acid, 1.04; proline, 0.97, glycine, 1.00; half-cystine, 0.46; mixed disulfide of cysteine and δ-mercaptovaleric acid, 0.58; isoleucine, 1.01; leucine, 1.01; tyrosine, 0.92; and ammonia, 2.83. Thin layer chromatography on Silica Gel G in the system 1-butanol-glacial acetic acid-water (4:1:1) showed one spot (R_f 0.36) with the chlorine–tolidine reagent.

Mixed Disulfide of δ-Mercaptovaleric Acid and Cysteine. S-Benzyl-δ-mercaptovaleric acid (1.12 g) and cysteine hydrochloride monohydrate (2.64 g) were suspended in about 150 ml of stirred, boiling ammonia and treated with sodium as described earlier. The ammonia was allowed to evaporate under reduced pressure, and the resulting residue was dissolved in 200 ml of water. Potassium ferricyanide (1 *N*) was added while the pH was maintained near 7 with 1% ammonium hydroxide. When 22 ml of the ferricyanide solution had been added, the white precipitate of cystine was collected on a filter. The filtrate was concentrated to about 70 ml, acidified with 2 *N* HCl until the pH reached 1.0, and allowed to stand in the refrigerator overnight. The white precipitate was filtered off, washed with two 2-ml portions of water, and dried *in vacuo;* wt 151 mg, mp 194–195° dec. This compound was crystallized from 25 ml of boiling water; wt 112 mg, mp 194–195° dec, $[\alpha]^{21}D$ −101.5° (*c* 1, dimethylformamide).

Anal. Calcd for $C_8H_{15}NO_4S_2$: C, 37.9; H, 5.97; N, 5.53. Found: C, 38.2; H, 6.08; N, 5.68.

A sample was analyzed in the 50° system of the Beckman-Spinco amino acid analyzer. The compound was chromatographically pure as shown by the presence of only a single peak with a position between those of leucine and tyrosine. Thin layer chromatography on Silica Gel G in the solvent system 1-butanol–acetic acid–water (4:1:1) showed only one spot (R_f 0.35) with ninhydrin or with the KCN–sodium nitroprusside reagent.

Acknowledgments. The authors are indebted to Miss Paula Glose for the bioassays done under the direction of Dr. L. Nangeroni, New York State Veterinary College, Cornell University.

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Rate-Determining Steps in the Oxidation of Succinate Catalyzed by Succinic Dehydrogenase[1]

Thomas C. Hollocher, Kwan-sa You, and Mike Conjalka

Contribution No. 699 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received April 10, 1969*

Abstract: The magnitude of the kinetic isotope effect observed in the oxidation of tetradeuteriosuccinate depends on the concentration of oxidant and not on the concentration of succinate. Fumarate and dideuteriofumarate are identically effective as competitive inhibitors of succinic dehydrogenase against both succinate and tetradeuteriosuccinate We conclude (1) that the isotope effect occurs at a step prior to the attack of oxidant, presumably upon the reduction of enzyme by succinate, and (2) that this step is rate determining when the step involving attack by oxidant is not rate determining.

The oxidation of succinate catalyzed by succinic dehydrogenase proceeds by the attack of an oxidant on an enzyme–substrate complex, which can be represented probably as a complex between reduced enzyme and fumarate, rather than by attack on reduced enzyme after the dissociation of fumarate.[2,7–10] The kinetics

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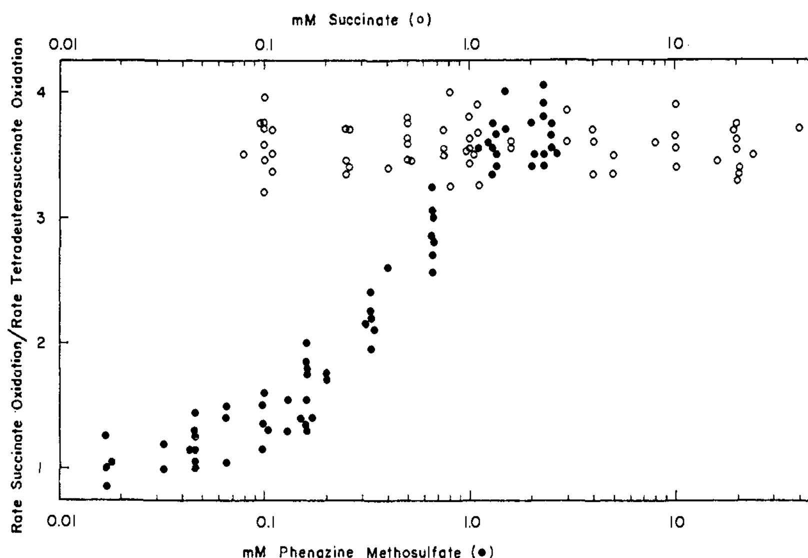
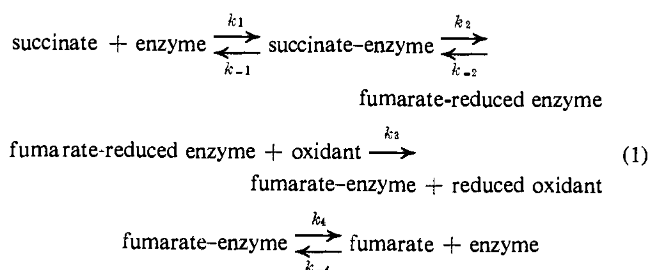


Figure 1. Kinetic isotope effect in the oxidation of succinate and tetradeuteriosuccinate at 22°: succinate concentrations from 4 to 40 mM and phenazine methosulfate concentrations as illustrated, ●; phenazine methosulfate concentrations from 2 to 2.5 mM and succinate concentrations as illustrated, ○. Those experiments in which oxidant was variable (●) involved about a 20-fold range of rates and necessitated a 4–5-fold increase in the concentration of succinic dehydrogenase at the lowest absolute rates. Those experiments in which succinate was variable (○) involved a 4–5-fold range of rates and did not require a change in the enzyme concentration. Five different enzyme preparations were used in the set of experiments illustrated. In some cases enzyme was activated prior to use; in others, it was not. K_m (succinate and tetradeuteriosuccinate) = 0.2 ± 0.05 mM at 2–2.5 mM phenazine methosulfate. K_m (phenazine methosulfate) ≅ 0.6 mM at succinate concentrations above about 4 mM; lower values apply for tetradeuteriosuccinate, as a consequence of the isotope effect.

require nevertheless, that an effectively irreversible step involving the enzyme and succinate precedes the attack by oxidant, since apparent ping-pong kinetics are observed normally in the absence of competitive inhibitors.[11,12] The ability of structurally dissimilar compounds, such as phenazine methosulfate and ferricyanide, to serve as oxidants makes it unlikely that soluble succinic dehydrogenase has a specific binding site for oxidants. Thus, the reaction with oxidant probably is second order in the range of concentrations usually used. In addition, stopped-flow kinetics require a step leading to enzyme reduction which is independent of succinate concentration.[10] These and related considerations suggest the following as a minimal reaction scheme.[13]



$$\text{succinate} + \text{enzyme} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{succinate–enzyme} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{fumarate-reduced enzyme}$$

$$\text{fumarate-reduced enzyme} + \text{oxidant} \xrightarrow{k_3} \text{fumarate–enzyme} + \text{reduced oxidant} \quad (1)$$

$$\text{fumarate–enzyme} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{fumarate} + \text{enzyme}$$

Step 3 is considered to be thermodynamically irreversible with high-potential oxidants and, in the absence of fumarate, step 4 is effectively irreversible. The rate may be limited through the fractional saturation of the enzyme by decreasing the concentration of succinate. At low concentrations of succinate, either step 1 or step 2 may be viewed as rate limiting, depending on the magnitude of the rate constants associated with these steps.[14] Step 3 may be made rate limiting by decreasing the concentration of oxidant.

Uncertainty exists regarding whether step 2 or 4 becomes rate limiting when both succinate and oxidant are present in high concentrations. Two very effective oxidants, phenazine methosulfate and ferricyanide, were observed to give the same extrapolated maximum velocity of reaction;[8–10,12] and it was concluded that step 4 was then rate limiting.[8,9] The conclusion, however, could apply equally well to step 2. Kinetic isotope effects as high as 6 have been reported in the oxidation of deuterated succinates[15–17] and somewhat greater than 2 with deuterated L-chlorosuccinates.[18] These isotope effects and data from freeze-quenching experiments[19] and from stopped-flow studies[10] suggest that step 2 should be rate limiting at high concentrations of succinate and oxidant. An isotope effect could occur at step 4 only if deuterated fumarate were to dissociate more slowly from succinic dehydrogenase than fumarate and if step 4 were rate determining. Such an effect of deuterium seems unlikely in principal and unsupportable experimentally. We find that fumarate and dideuteriofumarate have identical properties as competitive inhibitors of succinic dehydrogenase in the oxidation of both succinate and tetradeuteriosuccinate. Furthermore, isotope effects of 2 or more have been reported during the oxidation of both β-*threo*-deuterio-L-chlorosuccinate and α,β-*threo*-dideuterio-L-chlorosuccinate relative to L-chlorosuccinate or α-deuterio-L-chlorosuc-

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(13) Step 3 surely occurs in two steps when ferricyanide (a one-electron acceptor) is the oxidant and may occur in two steps when phenazine methosulfate is the oxidant. The relevant kinetic parameter remains the oxidant concentration experimentally and not the square of the concentration. This implies that one rate constant is much larger than the other if or when step 3 occurs in two sequential steps.
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cinate;[18] but the resulting chlorofumarate is free of deuterium in all cases.

The data summarized in Figure 1 directly support the ideas that step 2 can become rate limiting and that the isotope effects occur at step 2 rather than step 3. The magnitude of the isotope effect for tetradeuterated succinate decreases and approaches one with decreasing oxidant concentration.[20] The same isotope effect is observed with either phenazine methosulfate or ferricyanide as oxidant, providing the oxidant concentrations are adjusted to give similar turnover numbers. On the other hand, the isotope effects are independent of succinate concentration and absolute rate at high concentrations of oxidant. In addition, we find that deuteration of succinate has little or no effect on K_m.[15,17] It follows that step 1 does not become rate limiting and that a prior equilibrium must occur at step 1.

Since step 2 can become rate limiting, k_2 approximates the maximum enzyme turnover which is 3500–4000 min^{-1} at 22° (flavin basis) for the preparations used in this study.[21] Thus k_4 must exceed about 2×10^4 min^{-1} and, from the inhibition constant for fumarate, k_{-4} must exceed about 10^7 M^{-1} min^{-1}. At high concentrations of oxidant, K_m approaches $(k_{-1} + k_2)/k_1$. Since k_2 decreases in the case of deuterated succinates while K_m does not change significantly, $k_{-1} > k_2$. For the same reasons developed above, it follows that k_1 also probably exceeds 10^7 M^{-1} min^{-1}. Thus, the association–dissociation step for both succinate and fumarate (steps 1 and 4) would appear to be comparatively fast steps.

Competition experiments among normal, deuterated, and tritiated succinates show that the dissociation constant, K_D, also is unaffected by hydrogen isotope substitution.[7] Since $k_2 > k_{-2}$, it is clear that K_D will reflect

$$K_D = \frac{k_{-1}}{k_1(1 + k_2/k_{-2})} \quad (2)$$

whether k_2 and k_{-2} are affected in a similar or dissimilar manner by deuterium substitution. The negative result implies that k_2/k_{-2} is essentially constant and that the same isotope effects applies in both directions in step 2. This is consistent, at least, with the observation that the exchange of hydrogen between water and the methylene positions of succinate is very slow sompared to the oxidation of succinate.[2,7] It is interesting that step 3 exhibits little or no isotope effect, since the oxidant must attack what may be considered a deuterated enzyme when deuterated succinate serves as substrate. Calculations based on Figure 1 and related data suggest that k_3 equals $1.0 \pm 0.2 \times 10^7$ M^{-1} min^{-1} and that the maximum (extrapolated) isotope effect obtainable with our systems is 4.0 ± 0.2. Two observations indicate that step 3 is second order, as written, and does not involve the formation of reactive Michaelis complexes in kinetically significant concentrations: a single value for k_3 fits our data on isotope effects over a 100-fold range of concentrations of phenazine methosulfate; both the K_M for phenazine methosulfate and the maximum rate of succinate oxidation show very similar isotope effects.

Our results agree with those of Vitale and Rittenberg,[17] who observed small changes in isotope effects in the oxidation of deuterated succinates in response to changes of five-fold or less in the concentration of phenazine methosulfate or ferricyanide. We disagree with these workers, however, with regard to reaction scheme.

It seems clear, at least with preferred oxidants such as phenazine methosulfate and ferricyanide, that the breaking of C–H bonds in succinate precedes the involvement of oxidant. The existence of isotope effects in the oxidation of succinate by the succinoxidase system of mitochondria suggests that the same reaction scheme applies *in vivo* where the primary physiological oxidant is as yet unknown and where its concentration cannot be varied relative to the concentration of succinic dehydrogenase.

Experimental Section

Materials. Fumarate, succinate, and tetradeuteriosuccinate were obtained commercially; other deuterated derivatives of succinate were prepared as described by Hüfner and Hollocher;[2] and dideuteriofumarate was prepared by the method of Hoberman and D'Adamo.[3] Deuterium analyses were carried out by infrared observations of KBr pellets and commercially by the falling drop method. All other reagents used in the purification of succinic dehydrogenase and in the subsequent kinetic studies were obtained commercially. Soluble succinic dehydrogenase was prepared from beef heart by a method very similar to that of Singer, Kearney, and Bernath[4] and carried through the second ammonium sulfate precipitation step. Certain preparations were further purified by gel filtration through Sephadex G-150. All solutions contained 1 mM ethylenediaminetetraacetic acid.

Kinetic Systems. Steady-state kinetics were based on the spectrophotometric method of Arrigoni and Singer,[5] which employs phenazine methosulfate and 2,6-dichlorophenolindophenol as primary and terminal oxidants, respectively, and by the method of Keilin and King,[6] which uses the single oxidant, ferricyanide. The concentration of 2,6-dichlorophenolindophenol used in the former method was 50–70 μM. This concentration is sufficient to assure that the reaction between phenazine methosulfate and 2,6-dichlorophenolindophenol does not become rate limiting over the period of about 30 sec required to establish the initial reaction rate, providing the rates of succinate oxidation remain below 15 $\mu M \times min^{-1}$. Kinetic observations were considered unreliable at very low concentrations of succinate or phenazine methosulfate. The concentration of succinate changes significantly during the reaction when its initial concentration is 50 μM or less, and, in addition, even a relatively low concentration of the oxidation product, fumarate, then causes significant competitive inhibition. At phenazine methosulfate concentrations less than 15 μM, the oxidative turnover number falls to the point at which the rate of hydrogen exchange between water and the methylene positions of succinate becomes significant compared to the rate of oxidation of succinate.[2,7] It becomes uncertain then whether the enzyme catalyzes the oxidation of the original compound or a compound which has already lost deuterium by exchange. Similar but more severe restrictions apply in the ferricyanide systems, because of a ten-fold lower sensitivity.

All kinetic systems contained 50 mM potassium phosphate buffer, pH 7.6, 3 mM ethylenediaminetetraacetic acid, and, in some cases, 1–1.3 mg/ml of bovine serum albumin. While albumin may offer some protection against inactivation, its presence increases total protein and causes an increase in the succinate-independent reduc-

(20) Parallel studies with *rac*-2,3-dideuteriosuccinate and *meso*-2,3-dideuteriosuccinate confirm previous observations[16,17] that the magnitude of the isotope effects are very nearly proportional to the gross deuterium content and unrelated to the stereochemistry of deuterium substitution. The results with these compounds are similar to those illustrated in Figure 1 except that the maximum isotope effect observed is only about 2.3.

(21) The maximum turnover number of our succinic dehydrogenase preparations was estimated by rate extrapolation to infinite concentrations of succinate and phenazine methosulfate, and it applies to activated enzyme which shows an activity 2–4 times greater than unactivated enzyme. See E. B. Kearney, *J. Biol. Chem.*, **229**, 363 (1957), and T. Kimura, J. Hauber, and T. P. Singer, *ibid.*, **242**, 4987 (1967), for discussions of the process of activation. The magnitude of the isotope effects observed in these studies seemed unrelated, however, to the state of activation.

tion of oxidant. The oxidants used were not observed to oxidize succinate nonenzymatically at 22°, but they do oxidize albumin and other proteins at low but measurable rates. All results were corrected for these (usually negligible) rates.

Reactions were initiated by the addition of enzyme followed immediately thereafter by oxidant(s). Readings were begun usually 5-10 sec after mixing. A Cary Model 14 recording spectrophotometer was used in most cases.

The Biosynthesis of the Aflatoxins

Michel Biollaz, G. Büchi, and George Milne[1]

Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.
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Abstract: Degradative studies on radioactive aflatoxin-B_1 prepared by fermentation of added [1-^{14}C]- and [2-^{14}C]-acetates and of methyl-^{14}C-methionine are described. The origin of thirteen of the seventeen carbon atoms present was determined and the results show that the carbon skeleton is derived entirely from acetic acid. The activity of the labeled carbon atoms was equal throughout the molecule suggesting the intermediacy of a single polyacetate chain. A hypothetical pathway for the biosynthesis of aflatoxins and related mold metabolites, consonant with the distribution of labels determined, is proposed.

The aflatoxins constitute a family of secondary metabolites produced by some *Aspergillus* species. Because of their pronounced toxicity and extreme carcinogenicity in many animal species they have become the subject of intense investigation. The chemistry of the various aflatoxins and their interrelationships are well in hand, but work on the mode of action is only in its beginning stages.[2,3] The biosynthesis of the aflatoxins has been a topic of much conflicting speculation.[4-7] An early incorporation study[8] implicated phenylalanine and tyrosine as precursors but more recent work has shown this to be erroneous.[9] To clarify the biogenetic origins of the toxins we have determined the distribution of labels in aflatoxin-B_1 (**1**) derived from methyl-^{14}C-methionine, [1-^{14}C]- and [2-^{14}C]-acetate.[10]

Administration of methyl-^{14}C-methionine yielded radioactive aflatoxin-B_1 (**1**)[8] which on Zeisel degradation gave methyl iodide, isolated as triethylmethyl ammonium iodide, containing 97.8% of the total activity.[11] In agreement with this finding, degradation of aflatoxin-B_1 derived from [1-^{14}C]- and [2-^{14}C]-acetate[9] yielded triethylmethylammonium iodide containing only 0.32 and 0.31% of the radioactivity, respectively. The methoxyl carbon atom thus arises from the C_1 pool and in particular from methionine as expected from analogy with other mold metabolites (*e.g.*, mycophenolic acid[12]).

The most notable feature in the structure of the aflatoxins is the bisdihydrofuran moiety and it became of much interest to compare the actual distribution of labels in this portion of the molecule with that predicted by the previously cited postulates. Treatment of tetrahydrodesoxoaflatoxin-B_1 (**2**), obtained by catalytic reduction of aflatoxin-B_1 (**1**), with aluminum chloride yielded the furocoumarin **3**. Tosylation to give the ester **4**, followed by reduction with lithium aluminum hydride and methylation afforded the dimethoxybenzofuran **5**. Modified Kuhn–Roth oxidation of the latter with 2 *N* chromic acid afforded a mixture of propionic and acetic acids. These were separated by preparative gas–liquid phase chromatography and the pure acids were degraded stepwise to give each carbon atom sequentially as carbon dioxide (Scheme I). Degradation of aflatoxin-B_1 (**1**) derived from [1-^{14}C]-acetate gave the distribution of isotope outlined in Table I.

Table I. [1-^{14}C]-Acetate Incorporation[a]

Carbon atoms	Estimated as	% of total radioactivity
C-14–C-16	*p*-Bromophenacyl-propionate	12.01
C-14	CO_2	1.35
C-15, C-16	*p*-Bromophenacyl-acetate	11.21
C-15	CO_2	8.61
C-16	CO_2	1.21

[a] Theoretical activity per carbon atom = 11.1% for 9 labels.

The results indicate that both propionic and acetic acids contain one label with the preponderance of the activity residing in the carboxyl carbon of the acetic acid. This labeling pattern, unfortunately, was not

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