ISOTOPE EFFECTS ON THE KINETICS OF THE SUCCINIC DEHYDROGENASE CATALYZED OXIDATION OF L-CHLOROSUCCINATE

Oscar Gawron, Andrew J. Glaid, III, Kishan Mahajan, Gerald Kananen and Marie Limetti

Department of Chemistry, Duquesne University
Pittsburgh, Pennsylvania 15219

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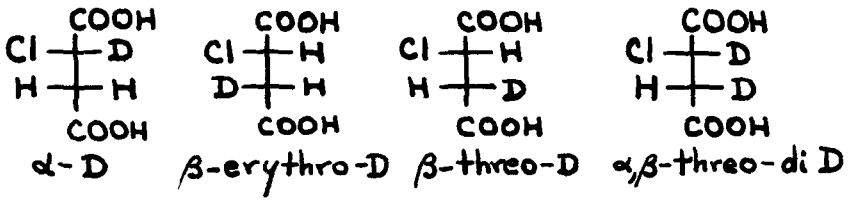
L-Chlorosuccinate, a recognized substrate for succinic dehydrogenase (Gawron et al., 1962; Dervartanian and Veeger, 1965), posesses one set of oxidizeable hydrogens while the natural substrate possesses two such sets (Gawron et al., 1962; Levy et al., 1962). In both instances enzyme catalyzed oxidation gives the unsaturated trans acid and assuming the same trans arrangement of carboxyl groups for the reactive conformation of the substrates, then the hydrogens removed are also trans (Gawron et al., 1962; Tchen and Van Milligan, 1960). L-Chlorosuccinate with one set of trans removeable hydrogens is, therefore, particularly well suited for a study of isotope effects on the enzyme catalyzed oxidation.

For this study, α-deuterio-L-chlorosuccinate, β-erythro-deuterio-L-chlorosuccinate, β-threo-deuterio-L-chlorosuccinate and α,β-threo-dideuterio-L-chlorosuccinate were synthesized by nitrosyl chloride treatment (with retention of configuration) of the corresponding L-aspartic acids, the deuterated L-aspartic acids being obtained by the proceedures of Tamiya and Oshima (1962). The routes to the several deuterated aspartates are as follows: α-deuterio-L-aspartate via transaminase catalyzed exchange of L-aspartate with deuterium oxide; β-erythro-deuterio-L-aspartate via aspartase catalyzed trans addition (Gawron and Fondy, 1959; Anet, 1960) of ammonia-d_3 to fumarate; β-threo-deuterio-L-aspartate via transaminase catalyzed exchange of α,β-threo-dideuterio-L-aspartate with protium

oxide; α,β-threo-dideuterio-L-aspartate via aspartase catalyzed addition of ammonia to dideuterio-fumarate, the dideuterio-fumarate being obtained by catalytic hydrogenation of ethyl acetylenedicarboxylate (Hoberman and D'Adamo, 1960).



Comparative rates of oxidation by ferricyanide of the several L-

Table I

Comparative Rates of Oxidation of

Deuterium Substituted L-Chlorosuccinates [a]

Compound	Deuterium[b] Atom/mole	Rate[c]	Ratio $Rate_H/Rate_D$
Normal		$8.57 \pm 0.71 \times 10^{-2}$	1.00
α-Deuterio	0.933	$6.42 \pm 0.25 \times 10^{-2}$	1.33
β-erythro-Deuterio	0.900	$6.93 \pm 0.35 \times 10^{-2}$	1.24
β-threo-Deuterio[d]	0.986	$3.57 \pm 0.09 \times 10^{-2}$	2.40
α,β-threo-Dideuterio	1.78	$3.03 \pm 0.11 \times 10^{-2}$	2.83

a. At 29.8°, pH 7.8 in a total volume of 3.00 ml. Reaction mixtures contained 0.1 M phosphate buffer, 0.001 M potassium cyanide, 3.0 mg. cryst. egg albumin, 0.006 M ferricyanide, 0.04 M substrate and 0.1 ml. enzyme (Slater, 1949)[6], equal to 8.0 mg. protein.

b. All deuterium analyses by J. Nemeth.

c. In optical density units per min. at 455 mμ. Average of three runs.

d. Not corrected for the presence of deuterium in the α-position. The compound was made from the α,β-threo-dideuterio compound (see text) and deuterium over 0.89 atom/mole may be atributed to deuterium on α-carbon which has not been washed out by transaminase exchange.

e. The assay conditions are those of Dervartanian (1965) for measuring enzyme activity. With succinate the specific activity at 29.8° was 0.204 umoles oxidized per min. per mg. protein.

chlorosuccinates are presented in Table I. It is immediately apparent that only the β-threo hydrogen of the trans removeable pair is important in the rate determining step, $Rate_H/Rate_{\beta\text{-threo-D}}$ being 2.40 while $Rate_H/Rate_{\alpha\text{-D}}$ is 1.33. This latter ratio is but slightly larger than $Rate_H/Rate_{\beta\text{-erythro-D}}$ and is, therefore, essentially a secondary isotope effect. It is to be noted that the determined value of $Rate_H/Rate_{\beta\text{-threo-D}}$ is not that for isotopically pure β-threo-deuterio-L-chlorosuccinate (see footnote d, Table I). Accordingly, the true value of $Rate_H/Rate_{\beta\text{-threo-D}}$ will be greater than 2.40, the increment being dependent on distribution of enzyme between unsubstituted substrate-enzyme complex and isotopic substrate-enzyme complex (Dixon and Webb, 1964).

Participation of only one of the trans removeable hydrogens in a rate determining step is consistent with a previously expressed hypothesis (Gawron et al., 1962; Gawron et al., 1963) that one of the hydrogens may be removed as a proton and the other as a "hydride" ion, a free radical process for removal of trans hydrogen atoms being hard to visualize. Kinetics of ferricyanide oxidation of succinate and L-chlorosuccinate, catalyzed by both particulate and soluble succinic dehydrogenase, are interpretable (Gawron et al., 1966) by a reaction scheme which provides for oxidation of substrate by an intramolecular process; the enzyme-substrate complex initially formed from oxidized enzyme and reduced substrate undergoing intramolecular transformation to reduced enzyme-product complex. It remains to be seen whether the isotopic rate effect obtained with β-threo-deuterio-L-chlorosuccinate is interpretable on the basis of this step in the reaction sequence.

Loss of isotope from residual substrate during oxidation was also experimentally considered. The results are presented in Table II. It is apparent that over the time required for 20% oxidation, no loss of deuterium occurs from residual β-threo-deuterio-L-chlorosuccinate and that residual α-deuterio-L-chlorosuccinate loses a small amount of deuterium. Assuming

Table II

Deuterium Loss from Residual Substrate on Oxidation [a]

Compound	Atom D/Mole	
	Initial	After 20% Oxid'n[b]
α-deuterio	0.933	0.893
β-threo-deuterio	0.986[c]	1.00

a. In a total volume of 40.0 ml. at pH 7.8, 29.8°. Reaction mixtures contained 0.1M phosphate, 0.001 M potassium cyanide, 40 mg. cryst. egg albumin, 0.006 M ferricyanide and 0.01 M substrate. For the α-deuterio run, 1.33 ml. of enzyme, specific activity 0.19, 57 mg. protein per ml., was employed and 20% oxidation required 12 mins. For the β-threo-deuterio run, 2.0 ml. of enzyme was employed for 15.5 mins. A 3.0 ml. aliquot of each run was monitored at 455 mμ. Carrier L-chlorosuccinate was used to facilitate isolation.

b. After correction for dilution.

c. See footnote d, Table I.

the experimental significance of this latter result, it would seem that, at best, attack on α-hydrogen is of little importance in initiating the reaction.

Loss of deuterium by anarobic "exchange" was also investigated (Table III), the experiments being carried out in the presence of fumarate to provide adequate rates. Both α-deuterio-L-chlorosuccinate and β-threo-deuterio-L-chlorosuccinate lose deuterium; the α-deuterio compound at a faster rate than the β-threo deuterio compound, the ratio of the exchange rates being 1.83. It is interesting to note that for oxidation, $Rate_{\alpha\text{-}D}/Rate_{\beta\text{-}D}$ is 1.80. Thus the two sets of experiments yield similar results despite the difference in experimental conditions. It would seem that both exchange and oxidation are proceeding by the same mechanism. Accordingly, fumarate is acting as an oxidant, possible as a hydrogen acceptor, as previously suggested,

Table III

Comparative Rate of Deuterium Loss on Anaerobic "Exchange" [a]

L-Chlorosuccinate	Deuterium (Atom/Mole)		
	Initial	Final	% Loss
α-Deuterio	0.933	0.385	58.8
β-threo-Deuterio	0.986[b]	0.645	32.1[c]

a. In a total volume of 20.0ml. at pH 7.5, 35° under nitrogen for 45 mins. Reaction mixtures contained 0.06 M phosphate, 0.018 M magnesium chloride, 0.005 M fumarate, 0.02 M deuterated L-chlorosuccinate and 3.0 ml. of enzyme preparation, specific activity 0.20, 80 mg. protein per ml. At the end of the incubation period, carried L-chlorosuccinate was added to facilitate isolation.

b. See footnote d, Table I.

c. Calculated on the basis of 0.89 atom β-D/mole and assumption of loss of 58.8% of α-D (0.096 α-D/mole, initially).

(Gawron et al., 1963), albeit the transferred hydrogen is capable of exchange.

Acknowledgements

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