**Acid Equilibration of Rhyncophylline.**—Rhyncophylline (25 mg.) was refluxed overnight in pyridine (5 ml.) and 10% aqueous acetic acid, respectively, and assayed by thin layer chromatography (ethyl acetate–chloroform, 9:1, being the developing agent). Under the conditions used, rhyncophylline traveled 5.0 cm. whereas the iso derivative ran 11.5 cm.

| Equilibration conditions | Acidic | Basic |
|---|---|---|
| Rhyncophylline/ Isorhyncophylline | 2.0 | 0.5 |

**Chlorotetrahydroalstonine.**—*tert*-Butyl hypochlorite (5.3 ml. of 0.975 *M*) was added dropwise over 30 minutes to tetrahydroalstonine (250 mg.) in methylene chloride (15 ml.) containing triethylamine (0.1 ml.). After an additional 30 minutes, the solution was washed with water, taken to dryness and the crystalline residue was recrystallized from methanol to give the chloro derivative, m.p. 194–196° dec.; $\nu_{max}$ 1698, 1630, 1596 cm.$^{-1}$; $[\alpha]D$ +81°; $\lambda_{max}$ 225–227 mμ (log ε 4.45), 242 mμ (log ε 4.11).

*Anal.* Calcd. for $C_{21}H_{23}N_2O_3Cl$: C, 65.16; H, 5.99; N, 7.24; Cl, 9.17. Found: C, 64.91; H, 6.22; N, 7.11; Cl, 9.35.

The chloro compound was recovered in 85% yield after attempted methanolysis under conditions used successfully elsewhere in this paper.

**Examination of the Alkaloidal Mother Liquors from Mitragyna stipulosa.**—The mother liquors from which rhyncophylline had first been crystallized[27] were examined by paper chromatography using the solvent system benzene–chloroform, 1:1, plus 2% pyridine. Among other spots ($R_f$'s 0.0, 0.08, 0.13, 0.20, 0.25, 0.35), rhyncophylline ($R_f$ 0.55) and isorhyncophylline ($R_f$ 0.85) were present, but no spot corresponding to corynoxeine ($R_f$ 0.70) was seen.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, DUQUESNE UNIVERSITY, PITTSBURGH, PENNSYLVANIA]

# New Substrates, New Inhibitors and the Stereochemistry of the Succinic Dehydrogenase System[1]

BY OSCAR GAWRON, ANDREW J. GLAID, III, THOMAS P. FONDY[2] AND MARY M. BECHTOLD

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L-Chlorosuccinate and L-methylsuccinate are substrates for succinic dehydrogenase, chlorofumarate and mesaconate being produced, respectively, by enzyme catalyzed dehydrogenation. The respective enantiomorphs, D-chlorosuccinate and L-methylsuccinate, are inhibitors of the enzyme and the stereospecificity of succinic dehydrogenase is thereby established. The enzyme exhibits a *trans* stereoselectivity, dehydrogenating *erythro*-3-deuterio-L-chlorosuccinate to 3-deuterio-chlorofumarate. From the above facts the stereochemistry of the behavior of succinic acid in the enzyme substrate complex is deduced. $K_M$ values and $K_I$ values for the new substrates under specified conditions are presented and $E_0'$ values at pH 7.3, 34° for the chlorosuccinate/chlorofumarate and methylsuccinate/mesaconate half-cells are 0.0462 volt and −0.0018 volt, respectively.

In a preliminary communication[3] we have shown that succinic dehydrogenase acts in a stereospecific manner by demonstrating that L-chlorosuccinate and L-methylsuccinate are substrates for the enzyme whereas the corresponding D-enantiomorphs are inhibitors. The purposes of the present paper are to elaborate on the details of the above and related experiments, to report oxidation-reduction potentials for the new substrates and to demonstrate that the dehydrogenation effected by succinic dehydrogenase proceeds in a *trans* manner.[4] The nature, *cis* or *trans*, of the dehydrogenation was investigated with synthetic erythro-deuterio-L-chlorosuccinate as a substrate. This stereospecifically labelled substrate on submission to the action of succinic dehydrogenase yielded chlorofumaric acid containing deuterium, thus demonstrating the *trans* nature of the dehydrogenation.

The work reported herein is also an extension of previous studies[5] on the stereochemistry of Krebs cycle reactions and in this connection was undertaken to explore the stereochemistry of the succinic dehydrogenase system, it being conjectured for several reasons that this system would exhibit both stereospecificity and stereoselectivity. Our reasons for so conjecturing were the facts, that, in general, enzymes do exhibit stereospecificity and stereoselectivity, that succinic dehydrogenase exhibited a very limited substrate range[6] and that retention of deuterium in fumarate on oxidation of deuteriated succinate, the deuteriated succinate having been obtained by anaerobic exchange,[9] could be rationalized on the basis of a *trans* stereoselectivity.[10]

The α-substituted succinic acids were chosen for investigation to constrain the enzyme to demonstrate stereospecificity (in the D and L sense) for the α-hydrogen and because enzyme stereoselectivity (in the *cis* and *trans* sense) could be investigated readily with either a *threo* or *erythro*-3-monodeuterio-α-substituted succinic acid.

## Experimental

DL-Chlorosuccinic Acid.—This compound was synthesized from DL-malic acid by treatment with phosphorus pentachloride according to the procedure of Walden.[11] After recrystallization from acetic acid and then from acetone-benzene, the product melted at 151–153°, lit.,[12] 151.5–152°.

D-(+)-Chlorosuccinic Acid.—This compound was prepared from L-malic acid by the above procedure. The recrystallized acid melted 170–173° and its optical rotation

(1) Abstracted in part from the Ph.D. Thesis, August, 1961 of T. P. Fondy and the Master's Thesis, August, 1961, of M. M. Bechtold.

(2) National Science Foundation Coöperative Graduate Fellow.

(3) O. Gawron, A. J. Glaid, III, T. P. Fondy and M. M. Bechtold, *Nature*, **189**, 1004 (1961).

(4) During the course of our work, T. T. Chen and H. van Milligan, *J. Am. Chem. Soc.*, **82**, 4115 (1960), showed that succinate is dehydrogenated in a trans fashion by succinic dehydrogenase.

(5) O. Gawron, A. J. Glaid, III, and T. P. Fondy, *J. Am. Chem. Soc.*, **83**, 3634 (1961).

(6) At the start of this investigation, it was known that racemic methylsuccinic acid slowly decolorized methylene blue in the presence of a heart preparation,[7,8] albeit the product of the reaction, methylfumaric acid, had not been identified.

(7) T. Thunberg, *Ber.*, **258**, 48 (1933).

(8) W. Franke and D. Siewardt, *Z. physiol. Chem.*, **280**, 76 (1944).

(9) S. Englard and S. P. Colowick, *J. Biol. Chem.*, **221**, 1019 (1956).

(10) For an extensive discussion on this point see H. R. Levy, P. Talalay and B. Vennesland, "Progress in Stereochemistry," in press.

(11) P. Walden, *Ber.*, **26**, 214 (1893).

(12) R. Anschütz and C. Bennert, *ibid.*, **15**, 642 (1882).

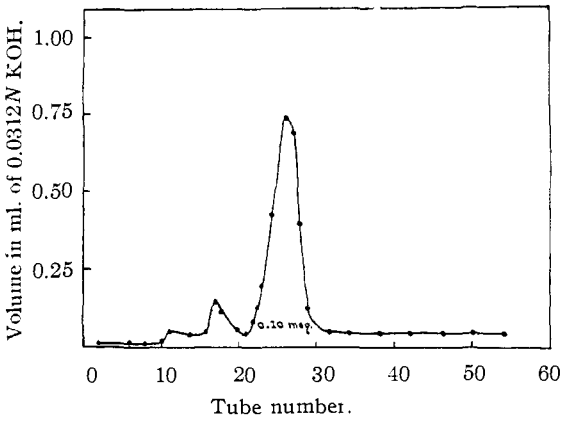



Fig. 1.—Analytical chromatograph of 17.1 mg. of the product of succinic dehydrogenase action on L-chlorosuccinic acid; 0.10 meq. of chlorofumaric acid found. The stationary phase consisted of 2.5 ml. of 0.5 *N* hydrochloric acid on 5.0 g. of Celite 535, column diameter 1.0 cm., and the eluent was a 72:25:3 mixture by volume of chloroform, benzene and ethanol. Chlorofumaric acid peak at tube 27. Under the same conditions chlorosuccinic acid emerges between tubes 30 and 40 with a sharp peak at tube 37 and fumaric acid and chloromaleic acid as broad ill-defined peaks over tubes 40–60.

was satisfactory, $[\alpha]^{25}D$ +19.6° (*c*, 6.2% in water); the corresponding literature values are 176° and $[\alpha]^{30}D$ +20.8°.

**L-(−)-Chlorosuccinic Acid.**—The method of Walden[13] as modified by Tilden and co-workers[14,15] was used to prepare this compound from L-(+)-aspartic acid by treatment with nitrosyl chloride. After recrystallization from acetone-benzene, the product melted at 177–178° and its specific rotation was $[\alpha]^{25}D$ −22.1° (*c*, 4.2% in water). The corresponding literature values are[14] 176° and $[\alpha]^{20}D$ −19.7°. Further purification with attendant loss of yield could be achieved by recrystallization from ethyl acetate. The product so purified melts at 180–181° and has a specific rotation $[\alpha]^{25}D$ −26°.

***erythro*-3-Deuterio-L-chlorosuccinic Acid.**[16]—*erythro*-3-Deuterio-L-aspartic acid,[17,18] obtained by the aspartase catalyzed *trans*[19] addition of $ND_3$ to fumaric acid, was used for the preparation of *erythro*-3-deuterio-L-chlorosuccinic acid by the above nitrosyl chloride method. Four hundred and eighty-five mg. (3.63 mmoles) of *erythro*-3-deuterio-L-aspartic acid from the aspartase reaction was diluted with 1.515 g. (11.4 mmoles) of unlabelled L-aspartic acid, and the mixture was submitted to the nitrosyl chloride reaction. The yield of recrystallized material (acetone–benzene) was 860 mg. (37.5%), melting at 173–175°. The deuterium content of this preparation was[20] 4.65 atom per cent. excess, the calculated value being 4.9 atom per cent. excess.

**Resolution of DL-Methylsuccinic Acid.**—The resolution was effected by literature procedures.[21–23] Sixty-six grams of DL-methylsuccinic acid yielded 40 g. of twice recrystallized (hot water) strychnine salt of the D-enantiomorph, melting point 180–182°, literature,[21] 186°. The purified strychnine salt yielded on decomposition 10.1 g. of D-methylsuccinic acid, melting point 111.5–112.5°, $[\alpha]^{25}D$ +9.28° (*c*, 6.0% in water). The corresponding literature values are[24] melting point 112° and $[\alpha]^{20}D$ +9.8°. The L-enantiomorph was obtained by decomposition of the soluble, impure strychnine salt of the L-modification present in the original mother liquid from the crystallization of the strychnine salt of the D-form. The acid, 26 g., thus isolated was optically impure, $[\alpha]^{25}D$ −5.1°, and was purified by conversion to the anhydride with acetic anhydride and recrystallization of the anhydride from benzene. The purified anhydride melted at 69.0–69.5°, literature value[23] 69–70°, and on solution in water gave $[\alpha]^{25}D$ −9.35° (*c*, 6% in water), literature[23] $[\alpha]^{20}D$ −9.87°.

**Chromatographic Procedures.**—Analytical and preparative chromatographic runs were carried out by a previously described procedure[25] with modifications in the solvent system. These modifications are described in the appropriate sections.

**Chlorofumaric Acid Assay.**—Chlorofumaric acid was assayed spectrophotometrically using a procedure similar to that of Wachsman[26] for mesaconic acid. A one ml. aliquot of a reaction mixture was diluted with 9.0 ml. of acid alcohol (absolute ethyl alcohol, water and concentrated sulfuric acid in the ratio of 95:4:1.4 by volume), filtered and optical density of the filtrate at 234 mμ was determined in a Beckman DU spectrophotometer in a one cm. cuvette against a reagent blank including enzyme. The readings were converted to concentration utilizing the molar extinction coefficient, $7.9 \times 10^3$, Beer's law being adhered to over the concentration range, $1 \times 10^{-5}$ to $25 \times 10^{-5}$ *M*.

**Succinic Dehydrogenase Preparation.**—Horse heart obtained within 30 min. after slaughtering and kept on ice for no more than 60 min. was cut into 100 g. pieces and stored at −20° until used. In a typical preparation by slight modification of the method of Slater,[27] 300 g. of tissue was passed through an electrical meat grinder. The resulting mince was then repetitively (six times) washed by gentle stirring with 10 l. of ice-cold tap water until the supernatant was colorless. The washed mince was collected on cheesecloth and after excess adhering water was removed by squeezing, the mince was suspended in 300 ml. of ice-cold 0.02 *M* phosphate buffer, *p*H 7.3, and the suspension was ground for 45 min. with sand in a chilled mortar. The resulting fine suspension was diluted with 400 ml. of the ice-cold buffer, stirred for 10 minutes and then centrifuged at 2000 r.p.m. at 0° for 10 minutes. The cloudy supernatant solution was then treated as per Slater's procedure.

**Identification of Products of Enzymatic Action.**—Chlorofumaric acid was identified as the product of succinic dehydrogenase attack on L-chlorosuccinic acid in the following way. A reaction mixture[28] 0.023 *M* in L-chlorosuccinic acid (neutralized with the equivalent amount of sodium bicarbonate), *p*H 7.3 and containing 30 ml. of succinic dehydrogenase suspension in a total volume of 215 ml. was shaken at 35° while a slow current of oxygen was passed through. After 4 hr., the reaction was terminated by heating to 75° for 10 min.[29] After cooling, coagulated protein was removed by centrifugation. The cloudy supernatant was filtered and the filtrate after adjustment to pH 1 with 18 *N* sulfuric acid was continuously extracted with ether for 36 hr. The ether extract was dried with anhydrous sodium sulfate and then evaporated *in vacuo* at room temperature to give 0.570 g. of material. This residue, analyzed by chromatography (Fig. 1), contained 43.9% of chlorofumaric acid so that 250 mg. of chlorofumaric acid (1.66 mmoles) was obtained. On the basis of oxygen uptake in the Warburg some 1.9 mmoles of chlorofumaric acid was expected.

Mesaconic acid (methylfumaric acid) was identified as the product of succinic dehydrogenase catalyzed dehydrogenation of methylsuccinic acid by a procedure similar to the above. A reaction mixture $1.77 \times 10^{-2}$ *M* in DL-methylsuccinic acid (neutralized to *p*H 7.0 with dilute base), $4.5 \times 10^{-3}$ *M* in potassium cyanide, $6.6 \times 10^{-4}$ *M* in methyl-

(13) P. Walden, *ibid.*, **29**, 133 (1896).
(14) W. A. Tilden and M. O. Forster, *J. Chem. Soc.*, **67**, 492 (1895).
(15) W. A. Tilden and B. W. C. Marshall, *ibid.*, **67**, 494 (1895).
(16) We wish to thank Mr. Donald J. Parker for the preparation of *erythro*-3-deuterio-L-aspartic acid.
(17) A. I. Krasna, *J. Biol. Chem.*, **233**, 1010 (1958).
(18) S. Englard, *ibid.*, **233**, 1003 (1958).
(19) O. Gawron and T. P. Fondy, *J. Am. Chem. Soc.*, **81**, 6333 (1959).
(20) Analysis by Dr. J. Nemeth.
(21) A. Ladenburg, *Ber.*, **28**, 1170 (1895).
(22) A. Ladenburg, *ibid.*, **29**, 1254 (1896).
(23) E. Berner and R. Leonardson, *Ann.*, **538**, 1 (1939).
(24) J. von Braun and F. Jostes, *Ber.*, **59**, 1447 (1926).
(25) O. Gawron, A. J. Glaid, III, A. LoMonte and S. Gary, *J. Am. Chem. Soc.*, **80**, 5856 (1958).
(26) J. T. Wachsman, *J. Biol. Chem.*, **223**, 19 (1956).
(27) E. C. Slater, *Biochem. J.*, **45**, 1 (1949).
(28) Cyanide was omitted in this run.
(29) This treatment causes disappearance of residual chlorosuccinic acid, presumably by hydrolysis and possibly by other displacements of the chloro group. Other reactions were terminated by the addition of sufficient 18 *N* sulfuric acid to bring the pH to 1.0. Unreacted chlorosuccinic acid could then be isolated by ether extraction and chromatography.

ene blue, 0.098 $M$ in phosphate buffer, pH 7.2 and containing 50 ml. of enzyme suspension, total volume[30] 150 ml., was shaken for 24 hr. at 37° while a slow current of oxygen was passed through the reaction mixture. At the expiration of the incubation period, the reaction mixture was treated as above. Analytical chromatography of the residue, 380 mg., showed (Fig. 2) 12.3% methylfumaric acid and 58.5% methylsuccinic acid. In subsequent runs the analytical yield of methylfumaric acid was of the order of 5%. In each case authentic methylfumaric acid, melting at 200–202°,[31] was obtained in approximately 3% yield by preparative chromatography and recrystallization. Blank runs containing all components of the reaction mixture but substrate were also performed. Chromatography of the residue from the ether extract of these runs gave no indication of the presence of any organic acid (to tube 120), confirming the expected absence of chlorosuccinic acid and methylsuccinic acid and their dehydrogenation products in the enzyme preparation. It might also be mentioned that both chlorofumaric acid and methylfumaric acid were stable to further attack by the enzyme preparation as evidenced by lack of oxygen uptake in the Warburg when these compounds were incubated with the enzyme preparation under the above conditions. Chlorofumaric acid was also found to resist the action of fumarase.

**Balance Studies.**—The ratio, μmoles chlorofumaric acid produced to μmoles oxygen consumed, was experimentally determined to verify the stoichiometry of the succinic dehydrogenase catalyzed dehydrogenation of L-chlorosuccinic acid. Oxygen consumption at 35° and pH 7.2 of reaction mixtures $2.2 \times 10^{-2}$ $M$ in L-chlorosuccinate (the acid was neutralized with sodium bicarbonate), $8 \times 10^{-2}$ $M$ in phosphate buffer, $6.7 \times 10^{-4}$ $M$ in methylene blue and containing 0.6 ml. of enzyme suspension, total volume 4.5 ml., was measured by the conventional Warburg technique. After 140 min. oxygen uptake was maximal and a one ml. aliquot was removed for determination of chlorofumaric acid by the previously described procedure. A run in triplicate yielded a molar ratio of 1.9:1.0; 22.2 ± 2.2 μmoles chlorofumaric acid produced to 11.7 ± 0.2 μmoles oxygen consumed. In the presence of $4.4 \times 10^{-3}$ $M$ cyanide the experimental ratio was 1.36:1.0; 24.8 ± 1.5 μmoles chlorofumaric acid produced to 18.3 ± 0.5 μmoles oxygen consumed.

**Determination of $K_M$ and $K_I$ Values.**—These values were determined at 34.2° and pH 7.3 by the spectrophotometric technique of Slater and Bonner[32] utilizing ferricyanide[33] as the electron acceptor. Reaction mixtures were $5.4 \times 10^{-2}$ $M$ in phosphate buffer, $7.5 \times 10^{-3}$ $M$ in cyanide, $3.7 \times 10^{-4}$ $M$ in potassium ferricyanide and contained one ml. of diluted enzyme suspension and the appropriate substrate concentration in a total volume of 4.00 ml. The reaction was followed in one cm. cuvettes in the thermostatted compartment of a Beckman DU spectrophotometer, optical density readings being taken at 50 second intervals against a blank containing distilled water and enzyme. Inhibition constants were determined as above, measuring initial rates of reaction as a function of substrate concentration in the presence of a constant concentration of inhibitor.

**Oxidation–Reduction Potentials.**—$E_0'$ values at pH 7.3, 34.0° for the L-chlorosuccinate-chlorofumarate half cell and for the L-methylsuccinate-methylfumarate half-cell were determined by the potentiometric technique. An "H" cell with saturated potassium chloride in 4% agar as the salt bridge was employed with the Beckman fiber type calomel electrode as a reference half-cell and the Beckman Model G pH meter as a potentiometer. In use one limb of the "H" cell was filled with saturated potassium chloride solution and the calomel electrode immersed in this solution. Solutions in the other limb were $7.5 \times 10^{-3}$ $M$ in cyanide, $7.5 \times 10^{-5}$ in methylene blue, $8.6 \times 10^{-2}$ $M$ in phosphate buffer and contained appropriate concentration of oxidant and reductant, and 4.0 ml. of diluted enzyme suspension, total volume 16.0 ml. All solutions were gassed for 20 min. with wet nitrogen prior to mixing, and wet nitrogen was slowly bubbled through the test solutions until equilibrium was attained. Voltages were measured at oxidant to reductant ratios of 10:1, 1:1 and 1:10, the concentrations being at the ten to one ratio $5 \times 10^{-3}$ $M$ and $5 \times 10^{-4}$ $M$, at the one to one ratio $5 \times 10^{-3}$ $M$ and at the one to ten ratio $5 \times 10^{-4}$ $M$ and $5 \times 10^{-3}$ $M$. For the L-chlorosuccinate-chlorofumarate half-cell measurements one-day old enzyme suspension was diluted two and one-half fold with 0.1 $M$, pH 7.3, phosphate buffer. For the L-methylsuccinate-methylfumarate half-cell measurements freshly prepared enzyme suspension was diluted four-fold with the phosphate buffer. The calomel electrode was standardized by measuring potentials of succinate–fumarate half-cells against the electrode, using the calculated value,[34] −0.0012 volt, for $E_0'$ of the succinate-fumarate system at pH 7.3 and 34°. Oxidant to reductant ratios for the succinate-fumarate half-cell measurements were also 10:1 ($5 \times 10^{-3}$ $M$ and $5.0 \times 10^{-4}$ $M$) 1:1 ($5 \times 10^{-3}$ $M$) and 1:10 ($5 \times 10^{-4}$ and $5 \times 10^{-3}$ $M$). A seven day old enzyme suspension was diluted two and one-half fold for the succinate–fumarate half-cell runs and concentrations of the other components and procedure were as previously described. In each case $E_0'$ was taken from a plot $E$ *vs.* log [Ox]/[Red] and in each case the slope of the line differed but slightly from the theoretical slope, 0.0305, at 34.0° for a two electron change.

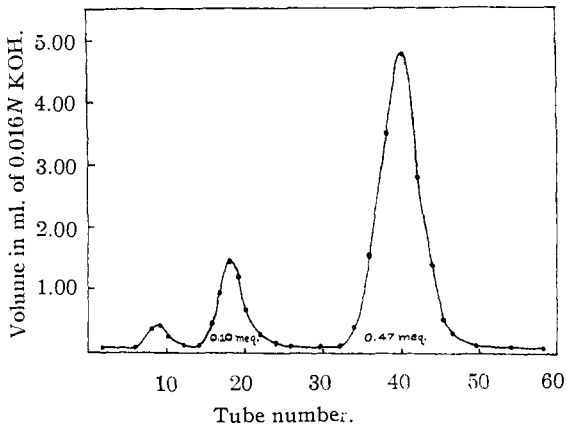



Fig. 2.—Analytical chromatograph of 53 mg. of the product from succinic dehydrogenase action on DL-methylsuccinic acid; 0.10 meq. of methylfumaric acid and 0.47 meq. of methylsuccinic acid found, the chromatographic conditions being those described in Fig. 1. Under these conditions citraconic acid (methylmaleic acid) emerges as an ill-defined broad band over tubes 40–60.

**Removal (*trans*) of H, H by Enzymatic Dehydrogenation of *erythro*-3-Deuterio-L-chlorosuccinic Acid.**—A reaction mixture 0.08 $M$ in pH 7.3 phosphate buffer, $1.33 \times 10^{-3}$ $M$ in methylene blue, containing 750 mg. of *erythro*-3-deuterio-L-chlorosuccinic acid (4.7 atom per cent excess D, neutralized with potassium hydroxide) and 25 ml. of enzyme suspension, was gently shaken at 37° while oxygen was bubbled through. After 2 hr. the reaction was terminated by heating to 75° for 10 minutes. The reaction mixture then was cooled, denatured protein removed by centrifugation and the supernatant adjusted to pH 1 with 18 $N$ sulfuric acid. Continuous ether extraction for 15 hr. of the acidified supernatant yielded 300 mg. of residue from which 100 mg. of recrystallized chlorofumaric acid melting[35] at 185–187° was obtained by recrystallization from acetone–benzene. Deuterium analysis[20] showed 6.23 atom per cent excess D.

Control studies to qualitatively indicate extent of exchange of hydrogens of chlorofumarate and L-chlorosuccinate with protons of the media were also performed. For estimating exchange in the over-all reaction the identical experiment to that above was performed with the exception that unlabelled L-chlorosuccinate and 15% by volume $D_2O$ were used. One hundred fifty milligrams of recrystallized

(30) Three ml. of 1% boric acid solution was added to minimize bacterial contamination.

(31) Literature melting point, 202–204°. R. P. Linstead and J. T. W. Mann, *J. Chem. Soc.*, 726 (1951).

(32) E. C. Slater and W. D. Bonner, *Biochem. J.*, **52**, 185 (1952).

(33) A molar extinction coefficient of $9.03 \times 10^2$ l. mole$^{-1}$ cm.$^{-1}$ at 400 mμ was employed.

(34) W. M. Clark, "Oxidation-Reduction Potentials of Organic Systems," William and Wilkins Co., Baltimore, Md., 1960, p. 507.

(35) Lit. melting point, 191–192°. K. V. Auwers and L. Harres, *Ber.*, **62**, 1885 (1929).

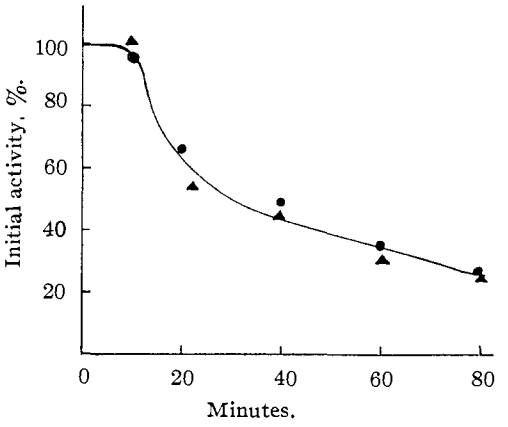



Fig. 3.—Heat denaturation studies at 42°. The enzyme solution incubated consisted of 0.4 ml. of a three day old enzyme suspension plus 30 ml. of 0.1 $M$ phosphate buffer, pH 7.3. At appropriate intervals one ml. aliquots were removed and activity immediately measured toward succinate (●) and L-chlorosuccinate (▲) by the ferricyanide technique at 34° Reaction mixtures for the activity measurements were $7.5 \times 10^{-3}$ $M$ in cyanide, $3.7 \times 10^{-4}$ $M$ in ferricyanide, $5.4 \times 10^{-2}$ $M$ in pH 7.3 phosphate buffer and $5 \times 10^{-3}$ in substrate, the total volume being 4.00 ml.

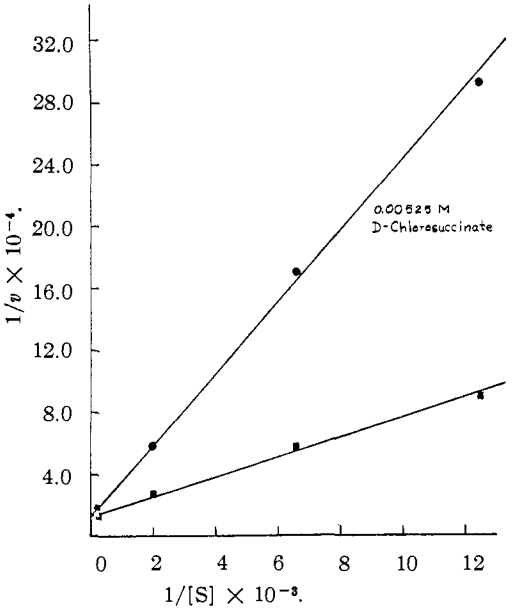



Fig. 4.—Competitive inhibition of succinic dehydrogenase by D-chlorosuccinate with succinate as substrate at 34.2° and pH 7.3. For this experiment the enzyme suspension was diluted 15 times with the phosphate buffer. The constants obtained from this plot are $V_{max}$ $8.55 \times 10^{-5}$ mole/l./min., $K'_M$ $4.4 \times 10^{-4}$ mole/l. and $K_I$ $1.5 \times 10^{-3}$ mole/l.

chlorofumaric acid, melting 186–187°, was obtained. Deuterium analysis showed little incorporation, 0.16 atom per cent. excess D being found.[20]

## Results and Discussions

**Stoichiometry.**—After identification of methylfumaric acid (mesaconic acid) and chlorofumaric acid as the enzyme-catalyzed dehydrogenation products of L-methylsuccinic acid and L-chlorosuccinic acid, respectively, the stoichiometry of the dehydrogenation was checked with L-chlorosuccinate as a substrate, as described under experimental. The experimental ratio, in the absence of cyanide, of 1.9

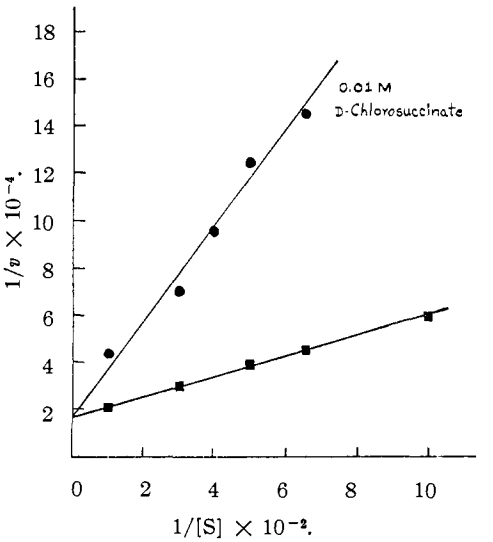



Fig. 5.—Competitive inhibition of succinic dehydrogenase by D-chlorosuccinate with L-chlorosuccinate as substrate at pH 7.3 and 34.2°. For this experiment the enzyme suspension was diluted 12 times with the phosphate buffer. The constants obtained from this plot are $V_{max}$ $6.14 \times 10^{-5}$ mole/l./min., $K'_M$ $2.64 \times 10^{-3}$ mole/l. and $K_I$ $2.8 \times 10^{-3}$ mole/l.

moles chlorofumarate:1.0 mole oxygen is in keeping with the 2:1 ratio expected for a preparation containing catalase and for a reaction utilizing methylene blue as the electron acceptor.[32]

The reactions may be summarized as

$$2 \text{ chlorosuccinate} + 2O_2 \longrightarrow 2 \text{ chlorofumarate} + 2H_2O_2 \quad (1)$$

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad (2)$$

$$2 \text{ chlorosuccinate} + O_2 \longrightarrow 2\text{-chlorofumarate} + 2H_2O$$

In the presence of cyanide which inhibits catalase,[11] the experimental ratio was reduced, as expected, the values being 1.36:1.0. That the ratio is not reduced to the theoretical 1.0:1.0 is most likely due to incomplete inhibition of catalase, relatively large amounts of the enzyme preparation being used in the experiment.

**Heat Denaturation Studies.**—Fig. 3 presents a plot of the stability at 42° of enzyme activity toward succinate and L-chlorosuccinate. The activity decays in a similar fashion toward both substrates indicating the absence of a chlorosuccinic dehydrogenase and that one enzyme, succinic dehydrogenase, is operative for both substrates.

**$K_M$ and $K_I$ Values.**—Figures 4 and 5 present typical Lineweaver–Burk plots for the competitive inhibition of succinic dehydrogenase by D-chlorosuccinic acid, and Table I summarizes the $K_M$ and $K_I$ data obtained by the ferricyanic method of Slater and Bonner[32] for the various substrates and inhibitors. The $K_M'$ values found for the various substrates can be corrected for the competitive inhibition provided by phosphate,[32] 0.054 $M$ in these experiments, utilizing the Salter and Bonner[32] value 0.1 for $K_I$ of phosphate at pH 7.3, 25° and the equation

$$K_M' = K_M\left(1 + \left(\frac{[\text{Phosphate}]}{K_I}\right)\right)$$

The corrected value of $K_M$, $3.4 \times 10^{-4}$, for succinate is in reasonable agreement with the value $2.3 \times 10^{-4}$ found by Slater and Bonner[32] at pH 7.3 and 25°, utilizing a histidine buffer and the ferricyanide technique. The corrected values of $K_M$ for L-chlorosuccinate and L-methylsuccinate are $2.1 \times 10^{-3}$ and $1.8 \times 10^{-2}$, respectively, and are 16 and 190 times greater than the $K_M$ of succinate. If for these substrates $k_2$ is much smaller than $k_3$, as it is for succinate,[32] then $K_M$ is a measure of the ratio $k_3/k_1$

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

and it is not unlikely that $k_1$ values for these substrates are smaller than that for succinate because the formation of the enzyme–substrate complex is sterically inhibited. However, the exact meaning of $K_M$ remains to be determined, particularly so since the reasonable correspondence between the $K_I$ value, $3.6 \times 10^{-3}$ $M$, for D-chlorosuccinate and the $K_M$ value for L-chlorosuccinate raises the possibility that the latter might also be an equilibrium constant. In this connection, L-bromosuccinate albeit possessing the correct configuration is an extremely poor substrate,[36] and presumably this is due to steric interference from the bromo group.

Of further interest is the fact that $K_I$ values found for D-chlorosuccinate are the same whether succinate or L-chlorosuccinate is used as the substrate, again indicating that succinic dehydrogenase is operative against both substrates. The $K_I$ values for the various inhibitors are not spectacular, being of the same order of magnitude as that for fumarate, $1.9 \times 10^{-3}$, and considerably larger than those for malonate, monoethyloxaloacetate and oxaloacetate, $3.3 \times 10^{-4}$, $3.8 \times 10^{-4}$ and $1.1 \times 10^{-5}$, respectively, the latter also being measured with ferricyanide as the electron acceptor.[37]

Since $V_{max}$ values for succinic dehydrogenase are limited by both the nature and concentration of the electron-acceptor used and in each case reach a limiting value at infinite acceptor concentration, the $V_{max}$ values reported herein are not limiting values (maximum rates at infinite acceptor concentration and infinite substrate concentration) but rather maximum rates obtained by saturating the enzyme with succinate in the presence of a fixed electron-acceptor concentration. Similarly, $K_M$ values reported herein were obtained at the same fixed concentration of ferricyanide. For internal comparison purposes, the $V_{max}$ values are given in Table I, and it is to be noted that the rate for succinate is some 2.5 times greater than that for L-chlorosuccinate and some 16 times greater than that for L-methylsuccinate.

TABLE I

$K_M$, $K_I$ AND $V_{max}$ VALUES[a]

| Substrate or inhibitor | $K'_M$[b] or $K_I$,[b] $\times 10^3$ | $K_M$[b] $\times 10^3$ | $V_{max}$,[c] $\times 10^8$ |
|---|---|---|---|
| Succinate | 0.53 ± 0.09 | 0.34 | 1.4 |
| L-Chlorosuccinate | 3.3 ± 0.6 | 2.1 | 0.67 |
| L-Methylsuccinate | 28 | 18 | 0.088 |
| D-Methylsuccinate | 14[d] | .. | ... |
| D-Chlorosuccinate | 3.6 ± 2[e] | .. | ... |
| D-Chlorosuccinate | 3.4 ± 0.7[f] | .. | ... |
| D-Bromosuccinate | 2.3 | .. | ... |

[a] By the ferricyanide technique at pH 7.3, 34.2°. [b] In mole/liter. [c] In mole/liter/min. [d] At 35.5° against succinate. [e] Against succinate. [f] Against L-chlorosuccinate.

TABLE II

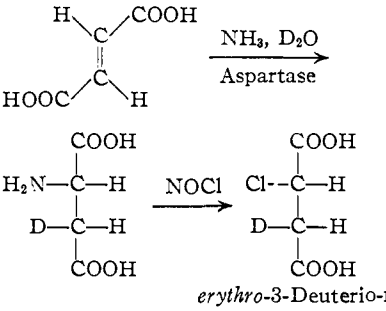
$E_0'$ VALUES AT pH 7.3, 34°

| Half-cell | $E_0'$ (volts) |
|---|---|
| L-Methylsuccinate–methylfumarate | −0.0018 |
| L-Chlorosuccinate–chlorofumarate | .0462 |
| Succinate–fumarate | − .0012[a] |

[a] Calculated from lit. value[27] at 25°, pH 7.0 and used to standardize the calomel electrode.

**Oxidation–Reduction Potentials.**—$E_0'$ values at pH 7.3, 34° are reported in Table II, the values being 0.0462 and −0.0018 volt, respectively, for the L-chlorosuccinate and L-methylsuccinate half-cells as against −0.0012 volt for the succinate half-cell. The more positive value for the chlorosuccinate-chlorofumarate system is expected since the chloro group is an electron withdrawing substituent and the somewhat more negative value for the methylsuccinate-methylfumarate system might also be expected since the methyl group is electron donating. These effects of substituents on $E_0'$ values are encountered in other oxidation–reduction systems, the benzoquinone–hydroquinone system,[38] for example.

**Stereochemistry.**—The assignment of L-configuration to the levorotatory isomers of α-halosuccinic acids and α-alkylsuccinic acids has been well established,[39] and the stereochemistry of the reaction sequence leading to the synthesis of *erythro*-3-deuterio-L-chlorosuccinic acid has been previously established.[17-19,40]




*erythro*-3-Deuterio-L-chlorosuccinic Acid

Utilizing *erythro*-3-deuterio-L-chlorosuccinic acid containing 4.65 atom per cent excess D as a substrate, aerobic oxidation catalyzed by succinic dehydrogenase yielded chlorofumaric acid containing 6.23 atom per cent excess D.[41] Assuming complete steric purity of substrate and process, *trans* dehydrogenation would be expected to yield chlorofumaric acid containing $4.65 \times 5/3$ or 7.75 atom per cent excess D, and the reaction seemingly proceeds by a *trans* mechanism to the extent of 80%. This result, however, plus the results of Chen and van Milligan[4] with *meso*-dideuterio-succinic acid and with DL-dideuterio-succinic acid establish the *trans* nature of the dehydrogenation effected by succinic dehydrogenase. Possible explanations for the failure to observe complete *trans* dehydrogenation may be found in the steric purity of the substrate and in fractionation by kinetic isotope effects. The substrate used in this experiment was some 85% sterically pure, and while the presence of the D-enantiomorph, a non-substrate does not influence the experimental results, it does suggest that racemization may have occurred also at the 3 position. In this event some *threo*-3-deuterio-L-chlorosuccinic would be present in the substrate and this of course would lose deuterium by a *trans* dehydrogenation.[42]

It is also likely that *erythro*-3-deuterio-L-chlorosuccinic acid is dehydrogenated by succinic dehydrogenase at a slower rate (secondary isotope effect) than is normal L-chlorosuccinic acid. Such a kinetic differentiation would lead to a fractionation effect and the ensuing chlorofumaric acid would contain less than the expected amount of deuterium. In the experiment described, the L-chlorosuccinic acid was not completely utilized, 100 mg. of chlorofumaric acid having been obtained from 750 mg. of L-chlorosuccinic acid, and a fractionation effect is thus possible. It is also of interest to note that if each step leading to deuterio-chlorofumaric acid, aspartase catalyzed addition, replace-

(36) Unpublished observations.

(37) L. Hellerman, O. K. Reiss, S. J. Parmar, J. Wein and N. L. Lasser, *J. Biol. Chem.*, **235**, 2468 (1961).

(38) Ref. 34, page 372.

(39) J. A. Mills and W. Klyne, *Progress in Stereochemistry*, 1, 177 (1954).

(40) Replacement of the amino group proceeds with retention of configuration.

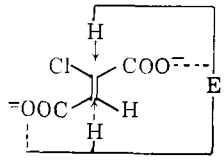
(41) This value might be slightly higher since the control experiment demonstrated a small amount of exchange, although not necessarily from the position occupied by the deuterium atom in *erythro*-3-deuterio-L-chlorosuccinic acid.

(42) In this connection *erythro*-3-deuterio-L-aspartic acid preparations prepared by the aspartase reaction sometimes contain more than one atom of deuterium per molecule (refs. 17 and 43). This extra deuterium must be found on either the α-carbon or the β-carbon or on both carbon atoms, and the possibility is thereby raised that seemingly pure (one atom of deuterium per molecule) *erythro*-3-deuterio-L-aspartic acid also contains some deuterium attached to the α-carbon atom and/or to the β-carbon atom in the threo position. In any event, *erythro*-L-chlorosuccinic acid losing some deuterium on *trans* dehydrogenation would result.

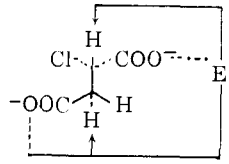
(43) O. Gawron, A. J. Glaid, T. P. Fondy and J. Francisco, unpublished observations.

ment of amino group with the chloro group and *trans* dehydrogenation, proceeds with 93% steric purity at the 3 position an 80% *trans* dehydrogenation would be expected.

The steric requirements for succinic dehydrogenase substrates and the *trans* dehydrogenation effected by succinic dehydrogenase can be rationalized as follows. Since carboxylate groups of substrates and inhibitors for succinic dehydrogenase serve as definitive points of attachment from substrate or inhibitor to enzyme,[37,44] fumaric acid or a substituted fumaric acid must "sit" in the enzyme in a particular way, *i.e.*, the two carboxylate groups occupy definite sites. If Structure I in the plane of the paper represents

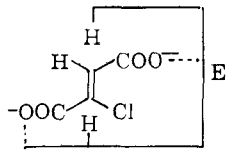


I



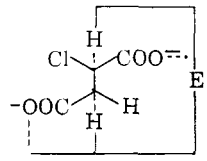
II

chlorofumarate in the enzyme-substrate complex then in order for L-chlorosuccinate (II) to be produced by succinic dehydrogenase catalyzed *trans* hydrogenation, H must add from the front to the $\alpha$-carbon atom and another H must add from the rear to the $\beta$- or 3-carbon atom. The reverse mode of addition, H from the rear at the $\alpha$-carbon and H from the front at the $\beta$-carbon, cannot occur since D-chlorosuccinate would result and this compound is not a substrate. It is also possible on paper for a chlorofumarate enzyme–substrate complex to form as indicated in structure Ia.



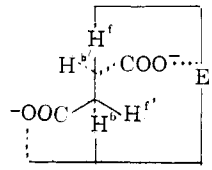
Ia

However, this structure cannot be a "real one" since addition of the H from the front to the top carbon atom of the doubly-bonded pair and addition of H from the rear to the bottom carbon atom would result in D-chlorosuccinic acid.[45] Now D-chlorosuccinate while not a substrate is an inhibitor and the enzyme-inhibitor complex may be diagrammed as III, and by comparison with II it is seen that one of the H atoms involved in the dehydrogenation is not in the correct spatial position for the enzymatic removal of H, and thus succinic dehydrogenase is stereospecific, *i.e.*, only one of the two possible H atoms is attacked at each of two carbon atoms. Extending this interpretation to succinic acid, by analogy with the L-chlorosuccinate enzyme–substrate complex the succinate enzyme–substrate complex would be

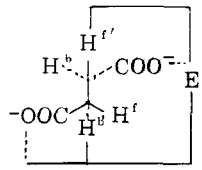


III

portrayed as



or



where the pairs $H^f$, $H^b$ or $H^{f'}$, $H^{b'}$ are the hydrogens removed in a *trans* fashion from the enzyme–substrate complex. It is to be noted that $H^f$ or $H^{f'}$ can only be removed (from the front) when the carbon atoms to which they are attached occupy the $\alpha$-carbon position in the enzyme–substrate complex, and $H^b$ or $H^{b'}$ can only be removed (from the back) when their attached carbon atoms occupy the $\beta$-carbon position in the enzyme–substrate complex. The hydrogens of each removable pair, $H^f$ $H^b$, have, of course, a *trans* relationship when the carboxyl groups are *trans* and a *cis* (or *gauche*) relationship when the carboxyl groups are *cis* (or *gauche*). It is further to be noted that with respect to the $H^f$ and $H^{f'}$, the corresponding carbon atoms are configurationally D and with respect to $H^b$ and $H^{b'}$, the corresponding carbon atoms are L.[46] While we have depicted enzymic attack on both $H^f$ and $H^b$ or both $H^{f'}$ and $H^{b'}$ in the above structures, it is not necessary for the enzyme to attack both H atoms of a removable pair and, in view of the *trans* nature of the reaction, it is not unlikely that one of the H atoms is initially removed by the enzyme. Such an enzymic initiation of the reaction might be removal of the hydrogen atom as a hydride ion. The resulting carbonium ion would then lose a proton, possibly to the enzyme, and the unsaturated product would result. It is of interest to note that succinic dehydrogenase and the hydrases, *cis*-aconitase and fumarase of the Krebs cycle, all act in a *trans fashion*[5] and that the stereochemistry of the Krebs cycle can be completely depicted.[5] It is also of interest to note that enzymes (aspartase and $\beta$-methylaspartase) which add H and $NH_2$ to a double bond do so in a *trans* fashion.[5]

**Acknowledgments.**—Support of this work by Research Grant RG-6245 from the Division of General Medical Sciences, National Institutes of Health, Public Health Service is gratefully acknowledged. We also wish to acknowledge the generosity and courtesy of the O. Weinman Co. in providing fresh horse hearts.

(44) For a comprehensive review of succinic dehydrogenase see T. P. Singer, E. B. Kearny and V. Massey, *Adv. in Enzymol.*, **19**, 65 (1957).

(45) Unless the mode of addition of the two H atoms changes in going from structure I to structure Ia, *i.e.*, L-chlorosucinnate could result from structure Ia if now H added from the rear at the top carbon and from the front at the bottom carbon atom of the doubly-bonded pair.

(46) In the Cahn, Ingold and Prelog designation[47] with the priority $H^f$ or $H^{f'}$, > $H^b$ or $H^{b'}$, R and S, respectively.

(47) R. S. Cahn, C. K. Ingold and V. Prelog, *Experientia*, **12**, 81 (1956).