THE STEREOCHEMISTRY OF THE SUCCINATE D_2O EXCHANGE AS CATALYSED BY SUCCINIC DEHYDROGENASE

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The enzyme succinic dehydrogenase in an anaerobic environment catalyses an exchange reaction between succinate and D_2O (Weinman et al, 1947). This reaction is accelerated by the addition of fumarate to the system (Englard and Colowick, 1956). The exchange reaction is slow compared to the rate of oxidation of succinate in the presence of an oxidizing agent. It is reported that the deuteriosuccinate formed in this reaction is the optically active form, L (+) monodeuteriosuccinic acid (Gawron et al.,1964), and that a hydride ion is involved in the exchange. A recent study from this laboratory on the kinetics of the enzymic oxidation of the two isomers of monodeuteriosuccinate was interpreted as ruling out a mechanism involving a hydride ion (Vitale and Rittenberg, 1967). We have therefore repeated the exchange reaction as reported by Gawron et al (1964) and find the exchanged deuteriosuccinate to be optically inactive.

Succinic dehydrogenase was prepared from fresh beef hearts by a modification of Slater's method as described by Gawron (1962). The preparation was assayed spectrophotometrically, measuring at 600 mμ the reduction of dichlorophenolindophenol. The assay consisted of 3.3×10^{-3}M sodium succinate, 3.3×10^{-4}M potassium cyanide, 1.6×10^{-2} phosphate buffer pH 7.6, 9.0×10^{-5} dichlorophenolindophenol, 1.0 mg phenazine methosulfate and 0.2ml diluted enzyme preparation in a volume of 3.3 ml. The mixture was incubated for 5 minutes at room temperature before the addition of the dye. The activity of the enzyme preparation was 2.42×10^{-2} moles succinate oxidized /min/ml enzyme preparation.

The exchange reaction of succinate was carried out in a mixture containing 0.0635 M succinate, 0.0548 M fumarate, 0.045 M phosphate buffer (pH 7.3) and 40 ml of the enzyme in a total volume of 400 ml. The deuterium concentration of the final medium was 50 atom percent. After the 5 hours the reaction was stopped by the addition of 6N H_2SO_4 to bring the pH to one. The proteins were coagulated by heating for 5 minutes at 70° and removed by filtration. The succinic and fumaric acids were isolated from the filtrate by continuous ether extraction and separated on a silica gel column (Varner 1957). The fractions containing succinic acid, contaminated with some fumaric acid, were pooled, taken to dryness and purified by sublimation at 100° in vacuo. Sublimation partly converts succinic acid to its anhydride as indicated by the low melting point of the product (175°C); authentic succinic acid 186°. After crystallization from water the

melting point rose to 186°. Pure succinic anhydride for deuterium analysis was prepared by dissolving it in excess acetyl chloride and refluxing for one hour. The acetic acid and excess acetyl chloride were removed in vacuo and the residue dried in vacuo over KOH pellets. The melting point of the anhydride was 120°; authentic anhydride 120°. The succinic anhydride was analyzed in a direct inlet Hitachu mass spectrometer with an inlet temperature of 56° and an electron accelerating voltage of 75. The parent peak (M=100) had a reasonable intensity. Percentages of mono and dideuteriosuccinates were calculated from the ratio of the intensity of the parent ion (m/e = 100) to that of the ions at m/e = 101 and 102. These latter were corrected for the normal abundance of C^{13}. The exchanged succinic acid contained 37% monodeuteriosuccinate and 1.6% dideuteriosuccinate.

The optical rotation of a solution of the exchanged succinic acid at a concentration of 3.3 mg/ml was determined on a Bendix-Ericson recording spectropolarimeter. At this concentration of L (+) succinic acid, optical rotations to be expected at 270.3 and 300.3 mμ according to the data of Englard (1963) are 13.2 and 7.1 millidegrees. Our measurements determined at maximum sensitivity gave zero rotation $\pm$ 1 millidegree at all wavelength above 250 mμ . We are indebted to P. Kahn who measured the circular dichroism of the exchanged succinate and found a value of zero.

The same results were obtained in a second experiment.

Our mass spectrometric data confirm the results of Gawron (1964) that the exchange leads primarily to the formation of a monodeuteriosuccinate. The mechanism involved in the exchange reaction is obscure. It is possible that the enzyme leads to the formation of the enol of succinate $^{-}O_2C\ CH_2CH{=}\ C\langle^{OH}_{O^-}$. The enolic hydrogen would be expected to exchange rapidly with the medium and the reversal of the enolization would result in the formation of a monodeuteriosuccinate. A ketonic group in the enzyme could initiate the formation of the enol of succinate in a manner resembling the first step of the Stobbe reaction (1893).

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