STUDIES ON THE EXCHANGE OF HYDROGEN BETWEEN SUCCINATE AND WATER AS CATALYZED BY HEART MUSCLE SUCCINIC DEHYDROGENASE.*

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(Publication No. 515)

Received July 31, 1967

Summary. The exchange of tritium from meso-2,3-ditritiosuccinate and racemic-2-tritiosuccinate to water was studied using soluble and particulate preparations of succinic dehydrogenase from heart muscle. All the tritium exchanges. We conclude from kinetics that the enzyme fails to discriminate strongly among hydrogen atoms at the 2,3-methylene positions. A strongly differential retention of hydrogen, predicted by the work of Gawron and co-workers and attributed to enzyme asymmetry, is not observed. Complete exchange confirms the early observation of Weinmann et al. (1947). Since the exchange reaction does not show an obligatory requirement for fumaric acid, exchange can occur from the enzyme-succinate complex. The results support the view that exchange proceeds by the exchange of a trans pair of hydrogen atoms.

Experimental. Under equilibrium conditions, preparations of succinic dehydrogenase catalyze the exchange of hydrogen between the 2,3 positions of succinic acid or analogues and water (Weinmann et al., 1947; Englard and Colowick, 1956; Gawron et al., 1963, 1964, 1966; Kahn and Rittenberg, 1967). The mechanism of hydrogen exchange is of interest in connection with the overall process of succinate oxidation, which proceeds by the elimination of a trans pair of protons to produce fumaric acid (Gawron et al., 1961, 1962, 1963; Tchen and Van Milligan, 1960).

Gawron and co-workers (1963, 1964) conclude from their studies of the stereochemistry of hydrogen exchange in succinic and L-chlorosuccinic acids that one hydrogen of each trans pair in succinic acid is exchangeable with water protons, while the second hydrogen is non-exchangeable, or at least very slowly exchangeable. Exchange between succinate and DOH (50 atoms % D) is reported to yield S-(+)-monodeuterosuccinate (Gawron et al., 1964), but this result remains unconfirmed (Kahn and Rittenberg, 1967). Assuming the differential exchange according to Gawron et al.(1964), labeled succinates should lose none, half, or all of their label by exchange, depending on the extent and stereochemistry of labeling, i.e., S(+)-2-D_1, 100%; R(-)-2-D_1, 0%; racemic-2-D_1, 50%; SS(+)-2,3-D_2, 100%; RR(-)-2,3-D_2, 0%; racemic-2,3-D_2, 50%; meso-2,3-D_2, 50% 2,2-D_2, 50%; 2,2,3,3-D_4, 50%. We demonstrate in this communication that the four methylene hydrogens of succinate are, in fact, equivalent in the exchange reaction.

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* Supported by grants from the National Science Foundation.
** Fellow of the Deutscher Akademischer Austauschdienst.

Soluble succinic dehydrogenase was prepared from beef heart by the method of Singer et al. (1956) as modified by Griffin et al. (1967). Particulate preparations from horse heart were those of Slater (1949) as modified by Gawron et al. (1962). Tritiated succinates (New England Nuclear Corp.) were prepared by catalytic hydrogenation of maleic anhydride in ethyl acetate using carrier-free T_2 or HT obtained from the reaction of carrier-free T_2O vapor with lithium aluminum hydride. By analogy with deuteration of maleic acid derivatives, hydrolysis yields meso-2,3-ditritiosuccinate and racemic-2-tritiosuccinate, respectively. The products were adjudged to be chemically and radiochemically pure succinate by paper chromatography. Radiochemical purities of 95% and 98%, respectively, were computed on the basis of enzymatically non-exchangeable counts.

Fig. 1 illustrates the kinetics of tritium exchange from meso-2,3-ditritiosuccinate at 24°

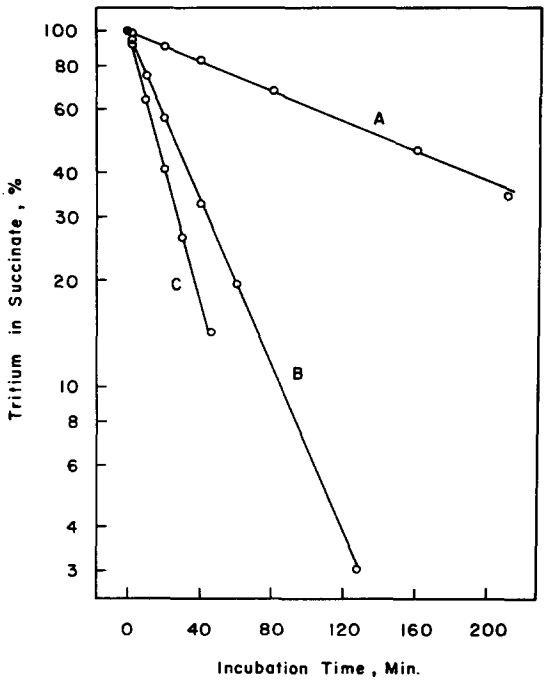

Fig. 1. Catalysis of tritium exchange from meso-2,3-ditritiosuccinate to water by soluble preparations of succinic dehydrogenase at 24°. A and C, under nitrogen; B, under air. Coordinates are semilogarithmic. For A and B, the reaction mixture contained 10 μM succinic dehydrogenase flavin, 2.0 mM succinate labeled with tritium, and 75 mM potassium phosphate buffer, final pH 6.9. For C, the reaction mixture contained 33 μM succinic dehydrogenase flavin, 1.2 mM succinate labeled with tritium, and 88 mM potassium phosphate buffer, final pH 6.8. The reaction was terminated by dilution into acetone. Water was removed by evaporation under vacuum, and tritiated succinate was assayed by standard scintillation techniques. The 100% level refers to control systems containing either water or boiled enzyme in place of enzyme. Initial specific activities were about 5 millicuries/millimole.

in the presence of soluble enzyme. The exchange approaches completion, and the linearity of the semilogarithmic curves suggests a simple first order process. No preferential retention of half the tritium is indicated. Parallel experiments with succinate-2,3-^{14}C show that succinate is conserved and that the difference in exchange rates between aerobic and anaerobic systems is not due to autoxidation of soluble succinic dehydrogenase to produce fumarate and other irreversible products (Griffin et al., in press). The effect of oxygen in enhancing the exchange rate is easily reversible and represents another expression of the ability of soluble succinic dehydrogenase to bind oxygen reversibly (Griffin and Hollocher, 1967; Griffin et al., in press).

The maximum oxidative turnover number for soluble succinic dehydrogenase in the kinetic system of Arrigoni and Singer (1962) is about 3000 moles/mole enzyme flavin/min. at 24°. At the same temperature and in the same units, the maximum turnover numbers for tritium exchange from meso-2,3-ditritiosuccinate and racemic-2-tritiosuccinate under aerobic conditions are about 5 and 6.5 respectively, and that for the transfer of label from succinate-2,3-^{14}C into fumarate and L-malate or from fumarate- and L-malate-2,3-^{14}C into succinate is about 0.2.

As shown in Fig. 2, the complete exchange of tritium from racemic-2-tritiosuccinate also approximates a simple first order process. There is no evidence that succinic dehydrogenase discriminates strongly between the two tritiated enantiomorphs. The loss of tritium from this compound is about 1.3 times fasterthan that from meso-2,3-ditritiosuccinate.

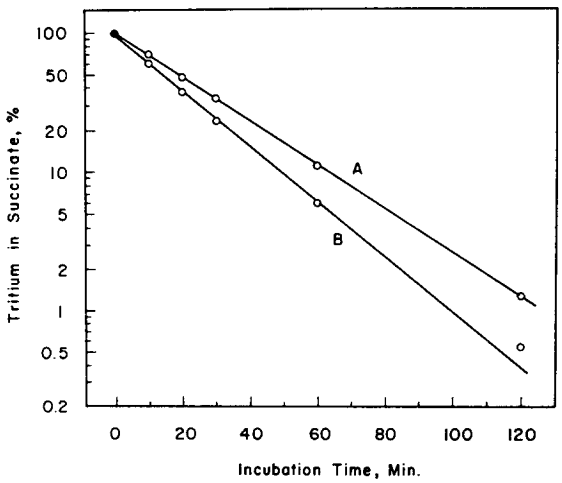

Fig. 2. Comparison of tritium exchange from meso-2,3-ditritiosuccinate and racemic-2-tritiosuccinate to water at 24°. The reaction mixtures contained 8.4 μM succinic dehydrogenase flavin (soluble enzyme), 1.08 mM succinate labeled with tritium, and 41 mM potassium phosphate buffer, final pH 6.8. Coordinates are semilogarithmic. A, meso-2,3-ditritiosuccinate; B, racemic-2-tritiosuccinate. Termination, counting, initial specific activities, and zero time controls were as described for Fig. 1. Gas phase was air.

The ability of succinic dehydrogenase to exchange hydrogen from all four methylene positions of succinate is not limited to soluble preparations. As illustrated in Fig. 3, strip counter recordings following the chromatographic separation of reaction products show that particulate preparations also catalyze the complete loss of tritium from meso-2,3-ditritiosuccinate and from traces of the tritiated oxidation products, fumarate and L-malate. The conservation of succinate-2,3-^{14}C (and traces of fumarate- and L-malate-2,3-^{14}C) in parallel experiments rules out compound destruction as a pathway for the loss of tritium.

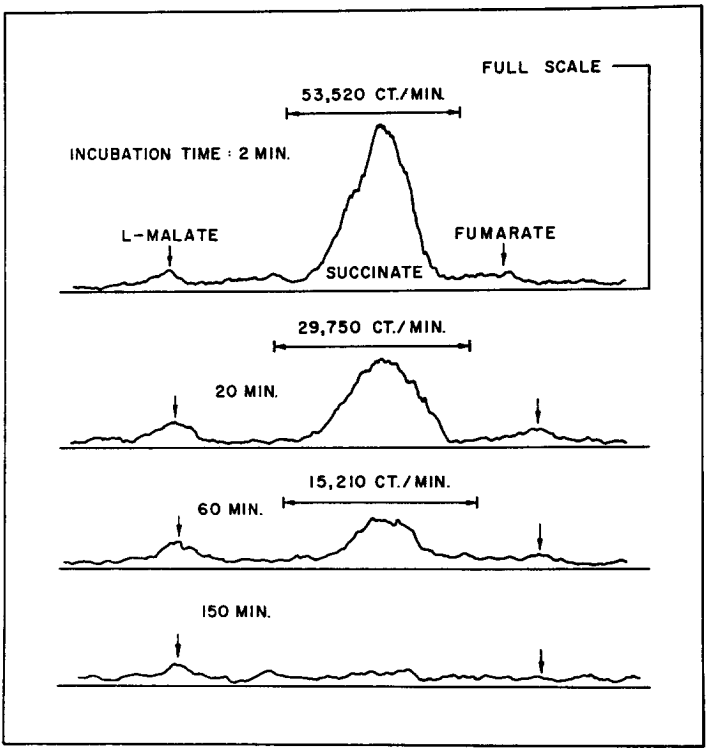

Fig. 3. Catalysis of tritium exchange from meso-2,3-ditritiosuccinate to water by a particulate preparation of succinic dehydrogenase at 24°. The figure shows strip counter recordings at constant gain of reaction products from equal aliquots after separation by ascending paper chromatography in water-saturated propanol: 90% formic acid: eucalyptol:: 50:20:50 v/v. Chromatographic development was from left to right. The reaction system contained 27 mg protein/ml, 8.5 mM succinate labeled with tritium, 1 mM KCN, and 60 mM potassium phosphate buffer, final pH 7.1. Gas phase was nitrogen. The reaction was terminated by dilution into acetone. The counts indicated were obtained by scintillation counting of the lengths of chromatographic strip between the markers.

In confirmation of complete exchange, infrared spectra† show that succinate is the final exchange product of 2,2,3,3-tetradeuterosuccinate and meso-2,3-dideuterosuccinate in H_2O and that 2,2,3,3-tetradeuterosuccinate is the final exchange product of succinate in D_2O.

Exchange at both the 2 and threo-3 positions (deuterium cis to chlorine) in L-chlorosuccinate (Gawron et al., 1966) is consistent with our results and contradicts the earlier conclusion of Gawron et al. (1964) that hydrogen at the 2-position of L-2-chlorosuccinate is non-exchangeable. Our findings suggest that exchange products of succinate should be largely or entirely optically inactive, and are in accord with the recent findings of Kahn and Rittenberg (1967). Our observation of complete exchange agrees with the studies of Weinmann et al. (1947) on the exchange of deuterium from dideuterosuccinate. It is of interest that the dideuterosuccinate used was prepared by the catalytic deuteration of fumarate ethyl ester using D_2, which yields racemic-2,3-dideuterosuccinate.

As indicated above for the soluble enzyme, the maximum rate of transfer of label from succinate-2,3-^{14}C to fumarate and L-malate is small compared to the rate of exchange of tritium from tritiated succinate to water. In addition, tritium exchange proceeds, as shown in Figs . 1 and 2, in systems lacking fumarate. For the soluble enzyme, at least, the reaction observed may be represented as

$$\text{Succinate}^* + \text{Enzyme} \rightleftharpoons \text{Complex}^*$$

$$\text{Complex}^* + H_2O \rightleftharpoons \text{Complex} + H_2^*O$$

That is, proton exchange from succinate appears to proceed directly from an enzyme-substrate complex.

The kinetic results of Fig. 2 suggest that 2-tritiosuccinate cannot be a major intermediate in the exchange of tritium from meso-2,3-ditritiosuccinate. The exchange reaction would appear, therefore, to proceed by the exchange of a trans pair of hydrogen atoms. Experiments using deuterium as label support this conclusion: Meso-2,3-dideuterosuccinate is the only major intermediate observed in the exchange of deuterium between succinate and D_2O and between 2,2,3,3-tetradeuterosuccinate and H_2O. Details of these and related studies will be published elsewhere.

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† David Portsmouth provided the following for infra red reference spectra: R(−)-2-monodeutero-succinate, RR(-)-2,3-didcuterosuccinate, and 2,3-dideuterosuccinate (NaHg reduction of maleic anhydride in D_2O). James Garb provided meso-2,3-dideuterosuccinate and racemic-2,3-di-deuterosuccinate. 2,2,3,3-tetradeuterosuccinate was obtained commercially.

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