Studies on the Mechanism of Hydrogen Exchange between Succinate and Water Catalyzed by the Soluble Succinic Dehydrogenase of Singer from Bovine Heart*

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SUMMARY

The equilibrium exchange of hydrogen between the 2,3-methylene positions of succinate and water was studied under aerobic conditions with specifically tritiated and deuterated succinates and soluble succinic dehydrogenase prepared by the method of Singer from bovine heart.

The exchange of tritium from racemic 2-tritiosuccinate (racemic-T1), racemic 2,3-ditritiosuccinate (racemic-T2), and meso-2,3-ditritiosuccinate (meso-Ts) proceeds to completion and exhibits simple, first order kinetics. The rates of exchange of tritium from racemic-T1 and racemic-T2 are identical and 1.35 times faster than that from meso-Ts. The enantiomorphs of racemic-T1 and racemic-T2 are not distinguished kinetically.

Hydrogen exchange products of deuterated succinates were identified by their infrared spectra. In the exchange of deuterium from 2,2,3,3-tetradeuterosuccinate (D4) to water, meso-2,3-dideuterosuccinate (meso-Db) is the only major intermediate detected between D4 and the final product, succinate. 2-Deuterosuccinate is detected as an intermediate during the exchange of deuterium from racemic 2,3-dideuterosuccinate, but not from meso-D4. In the exchange of deuterium from D2O to succinate, meso-D4 is again the major intermediate detected between succinate and the final exchange product, D4. The exchange of deuterium from succinate to water goes to completion regardless of the extent and stereochemistry of deuteration.

Hydrogen exchange proceeds mainly through the exchange of trans (RS) pairs of methylene hydrogens, and is analogous to the trans elimination of a proton pair during the oxidation of succinate to fumarate. Kinetic isotope effects appear to relate only to the nature of trans hydrogen pairs and to be unrelated to the stereochemical differences between labeled enantiomorphs. Activation of succinate seems to involve both hydrogens of a trans pair symmetrically and thus all 4 methylene hydrogen atoms are very nearly equivalent kinetically.

METHODS

Reagents—Tritiated succinates (New England Nuclear) were prepared by the catalytic hydrogenation of suitable olefins in ethyl acetate: racemic 2-tritiosuccinate (racemic-T1) from maleic anhydride and TH gas (produced by the reaction of carrier-free T2O with lithium aluminum hydride); racemic 2,3-ditritiosuccinate (racemic-T2) from carrier-free tritium gas and fumaric dimethyl ester; meso 2,3-ditritiosuccinate (meso-Ts) from carrier-free tritium gas and maleic anhydride. The compounds were received at specific activities of 154, 85, and 107

1 The abbreviations used for succinates labeled in the methylene positions have the following pattern. A prefix refers to a definite position or stereochemistry, or both, of labeling; T and D refer to tritium and deuterium, respectively; and the subscript refers to the number of tritium or deuterium atoms in each labeled molecule.
mC per mmole, respectively, and were routinely diluted 10- or 20-fold further with cold succinate for exchange experiments. On the basis of paper chromatography and residual tritium in compounds not susceptible to attack by succinic dehydrogenase, we estimate the radiochemical purities to be: racemic-T1, 98%; racemic-T2, 98%; meso-T3, 95%. Exchange kinetics (see below) failed to reveal a cross-contamination between racemic-T1 or racemic-T2 and meso-T3.

Racemic 2,3-dideuterosuccinate (racemic-D3) and meso 2,3-dideuterosuccinate (meso-D3) were prepared with deuterium gas (98 atom % D) in reactions similar to those described for racemic-T1 and meso-T3 above. The reactions were performed at room temperature in ethyl acetate over 10% palladium-charcoal catalyst. These deuterated succinates were subjected to a permanganate oxidation step (2, 3) prior to purification. Deuterium analysis: racemic D3, 32.7 atom % excess; meso-D3, 31.3 atom % excess. Expected excess was 33.3 atom %. Isotope purities correspond to 98 and 94%, respectively. Infrared spectra indicate an isotope purity and a stereospecific purity of labeling of about 95% for both compounds. Melting point was 191–192° for both compounds.

David Portsmouth supplied 2(R) (-)-deuterosuccinate, 2(2),3(R) (-)-dideuterosuccinate, and a mixture of 2,3-dideuterosuccinates. The optically active forms were prepared enzymatically (4, 5). The mixture of dideuterosuccinates was prepared by the reduction of maleic anhydride by sodium amalgam in D2O and represented a mixture of racemic-D3 and meso-D3. 2,2,3,3-Tetradeuterosuccinate (D4) was obtained from Volk. Deuterium analysis: 66.7 atom % excess; expected, 66.7 atom % excess.

A mixture containing 2,2-dideuterosuccinate (2,2-D2), 2,2,3-trideuterosuccinate (D3), and D4 was prepared by a modification of the method of Popjak et al. (6). The triethyl ester of 1,1,2-trideuteroxycarbonylate was incubated in D2O for 24 hours at 170° under nitrogen, evaporated to dryness, and saponified at 100° in D2O, NaOD. The product was acidified in water, extracted into diethyl ether, and purified by crystallizations. Deuterium analysis: 46 atom % excess; expected, 50 atom % excess. Infrared spectra indicated that the major species was D3. Melting point was 183–184°.

All deuterium analyses were carried out commercially by the falling drop method.

Enzyme Preparation—The preparations of soluble succinic dehydrogenase used were those described in the preceding paper (7).

Tritium Exchange—The exchange of tritium from tritiated succinates to water under aerobic conditions was studied with the method described in the preceding paper (7).

Deuterium Exchange—Reaction systems consisted, typically, of 0.03 μmole of succinic dehydrogenase flavin, 180 μmole of potassium phosphate, and 60 μmole of succinate in a total volume of 1.5 ml. Incubation temperature was 24°. The composition of reaction mixtures is specified in terms of concentrations in the figure legends. The gas phase was air.

Deuterated succinates or D2O served as the source of deuterium. At intervals during incubation 1.5-ml aliquots were removed, heated to 100° for 5 min, diluted to 2.5 ml, filtered, and finally lyophilized. The dry residues were extracted twice with a ml of warm chloroform, acidified with 0.3 ml of HCl, and evaporated to dryness. Succinic acid was extracted into and crystallized at least twice from anhydrous diethyl ether.

![Figure 1](http://www.jbc.org)  
**FIG. 1.** Catalysis of tritium exchange from tritiated succinates to water by soluble succinic dehydrogenase at 24°. The reaction mixtures contained 8.1 μmole succinic dehydrogenase flavin, 1.1 mmole succinate labeled with tritium, and 41 mmole potassium phosphate buffer, final pH 6.8. ○, meso 2,3-ditrifluorosuccinate (5.4 mC per mmole); ●, racemic 2-trifluorosuccinate (7.7 mC per mmole); X, racemic 2,3-ditrifluorosuccinate (8.5 mC per mmole). Gas phase was air.

An additional solubilization step involving water was used when the solvent in the exchange system was D2O.

Infrared Spectra—A few small crystals of succinic acid were ground with about 100 parts of spectra purity KBr, and 0.5-mm diameter pellets were pressed in a micro-pellet apparatus. Spectra were obtained with a grating infrared spectrophotometer, Perkin Elmer model 621 A, fitted with a beam condenser. The frequency sweep program was set on scale change, and an appropriate recording paper was used.

**RESULTS**

**Tritium Exchange**

Fig. 1 shows the relative rates of transfer of tritium from tritiated succinates to water. The points have been corrected for traces of nonexchangeable radiochemical impurities. Two aspects of Fig. 1 are of interest. First, the three curves indicate simple, first order kinetics throughout. The slopes of the semilog curves do not decrease after 50% exchange as would be expected if the tritiated enantiomers of racemic-T1 and racemic-T2 were kinetically nonequivalent or if, in the case of meso-T3, the trans methylene hydrogens were positionally nonequivalent by virtue of an asymmetrical activation or a differential exchange rate. Second, a small kinetic isotope effect of 1.35 is observed between meso-T2 on the one hand and racemic-T1 and racemic-T2 on the other. Since neither the latter two compounds nor their enantiomers are distinguished kinetically, the determining factor in the isotope effect would appear to be the nature of the trans hydrogen pair undergoing exchange.

1 The terms cis and trans apply to hydrogens at the methylene positions of succinate and have the usual meanings with respect to the Newman (axial) projection (8). Specifically, when the carboxyl groups of succinate are positioned 180° apart, the cis hydrogen pair is trans if the hydrogens are 180° apart as viewed axially and cis if they are 60° apart, that is, if they both lie on the same side of the plane containing the 4 carbon atoms. The T-T pair in meso-T3 is a trans pair in the Newman projection or an E,S pair in the rectus (R)-sinister (S) convention of Cahn, Ingold, and Prelog (9). Racemic-T1 is composed of the 2(R)-, 3(R) (-)- and 2(S),3(S) (+)-enantiomers of succinate in which the T-T pairs are cis pairs. The same considerations apply to deuterated succinates.
Fig. 2. Infrared reference spectra of succinic acid and deuterated succinic acids in micro KBr pellets.
FIG. 2B

- Meso 2,3-Dideutero Succinic Acid
- R(-)-2-Deutero Succinic Acid
- Mixture A + B
FIG. 3. Infrared spectra of succinic acid recovered during the exchange of deuterium from 2,2,3,3-tetradeterosuccinate to water. The indicated time refers to the time of incubation at 24°. The reaction mixture contained 6 μM succinic dehydrogenase flavin, 13 mM 2,2,3,3-tetradeterosuccinate, and 39 mM potassium phosphate buffer, final pH 7.0. Gas phase was air.
exchange: T-T pair in meso-T₂; T-H pair in racemic-T₁ and racemic-T₂. The nature of the other trans pair, H-H, H-H and T-H, respectively, does not seem to influence the kinetics. The fact that the slope of the exchange curve for meso-T₂ does not increase and approach that of racemic-T₁ suggests that T₁ is not an exchange intermediate between meso-T₂ and succinate.

The rate ratio for exchange, racemic-T₁:meso-T₂ or racemic-T₁:meso-T₂, of 1.35 is independent of pH at least from pH 6.3 to 7.3, which includes the maximum (pH 6.9) of the pH rate profile for exchange (7). The rate ratios differ from 1.35, however, in systems containing deuterium in the solvent. For example, in a system containing 8% atom % deuterium in water, a rate ratio of 1.25 was observed at an apparent pH 6.7 which very nearly corresponds to the maximum in the apparent pH rate profile at the particular deuterium content used (7).

Deuterium Exchange

Fig. 2 shows infrared spectra of deuterated succinic acids and a known mixture which served, together with other spectra, a reference function in our exchange studies. The spectra of different deuterated succinic acids are distinctive (10) and certain bands are useful for purposes of identification.

Succinic Acid—Strong bands at 1200, 800, and 580 cm⁻¹ and a weak band at 885 cm⁻¹.

2(R) (-)-Deuterosuccinic Acid—A pair of strong bands at 1240 and 1215 cm⁻¹ of nearly equal intensity, and weak bands at 1100, 1000 (split to 990 and 1008), and 750 cm⁻¹. The band at 1280 cm⁻¹ is definitive for D₁, in the absence or virtual absence of racemic-D₂. The base of the broad 920 cm⁻¹ band is dimpled.

Meso 2, 3-Dideuterosuccinic Acid—A strong band at 1270 cm⁻¹ and a pair of sharp bands at 990 and 977 cm⁻¹ are distinctive for meso-D₁. The sample shown in Fig. 2 contains a small amount of D₁ as indicated by the weak bands at 1240 and 1215 cm⁻¹ and by other features of the spectrum. Meso-D₁ exhibits a pair of very weak bands at 1240 and 1222 cm⁻¹ which, in general, can be neglected. A weak pair of bands appear at 725 cm⁻¹.

Racemic 2, 3-Dideuterosuccinic Acid—Strong bands at 1280, 1292, and 1000 cm⁻¹. The band at 1000 cm⁻¹ is the most useful band for characterization. The bands at 1197 and 1180 cm⁻¹, while not characteristic, are weak compared to their intensities in D₁ and meso-D₁. Infrared spectra cannot distinguish between the deuterated enantiomorphs, and the spectrum of racemic-D₁ is identical with that of 2(R) (-)-Deuterosuccinic Acid.

2, 2, 3, 3-Tetradideuterosuccinic Acid—The strong band found at 1302 to 1310 cm⁻¹ in the above compounds is found at 1285 cm⁻¹ in D₁. In addition there are strong bands at 1055, 1012, 945, 843, and 525 cm⁻¹. The weak split band at 1200 cm⁻¹ is not due to a deuterated succinic acid and represents an unknown contaminant.

Extremely sharp, strong bands at 1197 and 1180 cm⁻¹ are characteristic of D₁ and meso-D₂, and the band at 1280 cm⁻¹ is shown only by D₁ and racemic-D₂. D₁ exhibits strong bands at 1155 and 1237 cm⁻¹ and a somewhat weaker one at 1226 cm⁻¹.

From the tritium exchange results, above, it was particularly important to distinguish between meso-D₂ and D₁. Several bands, particularly the strong bands at 1270 and 1280 cm⁻¹, respectively, serve this purpose.

By converting measured transmittance ratios (base line to peak) to extinctions and by making extinction comparison with reference spectra and with an internal band unaffected by methylene deuterium atoms, such as the broad carboxyl band at 3000 cm⁻¹, it was possible to arrive at semiquantitative estimates of the mole fraction of deuterated succinates in mixtures.

If 1 hydrogen were to exchange without stereochemical specificity at each successful event, then D₁ would yield D₂, 2,2-D₂, all possible 3,3-D₂ species, and D₃ as intermediates. If 2 hydrogens were to exchange from 2,3-positions at each successful event, then D₁ would yield 2,3 D₃ as an intermediate: one or both cis² forms if exchange were cis; the trans (meso-) form if exchange were trans. Fig. 3 shows infrared spectra of succinic acid recovered during the exchange of deuterium from D₁ to water. At an early time, bands at 1197, 1180, 990, and 977 cm⁻¹ appear. These bands strengthen and a weak pair at 1240 and 1222 cm⁻¹, also characteristic of meso-D₂, become evident. At the same time a pair appears at 795 cm⁻¹. The 1970 cm⁻¹ band appears first as a shoulder and later intensifies into a distinct band. Finally, bands characteristic of succinic acid appear and intensify at 800 and 580 cm⁻¹. Bands at 1055, 1012, 945, 843, and 525 cm⁻¹ due to D₄ weaken as the reaction progresses.

Trace amounts of D₂ are suggested by the weak band at 1155 cm⁻¹. The appearance of trace amounts of D₃ late in the reaction is suggested by the very weak 1100 cm⁻¹ band. Aside from these minor constituents, the components identified are D₁, meso-D₂, and succinate.

Fig. 4 illustrates the time course of the reaction and shows the progressive decrease in D₁ (1012 cm⁻¹) and the appearance of meso-D₂ (977 cm⁻¹) and succinate (800 cm⁻¹). D₂ and meso-D₂ are reciprocally related initially, and the sum of the mole fractions of the three major species is about 1 throughout the reaction. The kinetics suggest that meso-D₂ has the requirements of an intermediate.

Fig. 5 illustrates the changes observed in the exchange of deuterium from D₂O to succinate. On the basis of those considerations discussed in connection with Fig. 3, meso-D₂ appears to be the major intermediate between succinate and the final product, D₄. Traces of D₃ could be detected early in the reaction, and substantial amounts of D₄ were found late in the reac-

![Fig. 4. Kinetics of exchange of deuterium from 2,2,3,3-tetradideuterosuccinate to water. The curves were obtained from the spectra of Fig. 3.](http://www.jbc.org/)

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FIG. 5. Infrared spectra of succinic acid recovered during the exchange of deuterium from D₂O to succinate. The indicated time refers to the time of incubation at 24°. The reaction mixture contained 8 μM succinic dehydrogenase flavin, 7 mM succinate, and 40 mM potassium phosphate buffer. Final pH meter reading was 7.0; pD was 7.4. Final deuterium content of solvent was about 98 atom %. Gas phase was air. The first spectrum is a reference spectrum of succinic acid.
FIG. 6. Infrared spectrum of succinic acid recovered during the exchange of deuterium from meso-2,3-dideuterosuccinate to water. The indicated time refers to the time of incubation at 24°. The reaction mixture contained 8.3 μM succinic dehydrogenase flavin, 11 mM meso-2,3-dideuterosuccinate, and 50 mM potassium phosphate buffer, final pH 7.0. Gas phase was air. The first spectrum is a reference spectrum of meso-2,3-dideuterosuccinic acid.

tion. After an incubation of 8 hours, for example, meso-D₃ (1270, 1197, 1180, and 977 cm⁻¹) has nearly disappeared, succinate is no longer detected, and the predominant species is D₄. However, bands at 1237 and 1155 cm⁻¹ (expanded scale) indicate D₄ quite clearly, and the sharp, weak band at 1000 cm⁻¹ probably indicates one or both of the cis-D₂ enantiomorphs. The amount of D₄ is estimated to be 20 ± 5%. Since the D₂O contained about 2 atom % H, we would expect the succinate to contain 7 to 8% D₂ at isotopic equilibrium. The amount of D₄ observed is probably in excess of that expected on statistical grounds in this and other experiments.

The disparity may be due to an isotope selection effect or to the operation of an exchange mechanism involving 1 hydrogen atom per event. In an attempt to decide between these possibilities, experiments were carried out in D₂O (50 atom % D). During the initial phase of the exchange reaction, the major species detected were meso-D₃, D₃, and succinate, and the relative amounts of the deuterated species were about that expected from statistical considerations. A sample incubated for 4 hours in one experiment was found to contain about 45% D₃ and 15% meso-D₃ with the remainder being succinate and traces of D₄ and dideuterated species other than meso-D₃. The mole ratio, D₃:meso-D₃, was about 3; expected ratio for initial exchange conditions, 2; expected ratio at isotopic equilibrium, 2. Ratios between 2 and 3 were obtained in these experiments. Since the error in estimating the mole fractions of D₃ and meso-D₃ was about 5%, we cannot consider the mole ratio, D₃:meso-D₃, to differ significantly from 2. Any isotope section effect would appear to be small. While the results are not conclusive, they favor, nevertheless, the idea that the exchange of 1 hydrogen per event occurs at significant rates in systems containing D₂O as solvent. We have not determined whether optical activity develops from deuterium incorporation in these systems because of a possible stereochemical selectivity at R or S positions.

Numerous kinetic experiments have been carried out on the exchange of tritium from tritiated derivatives of succinate to water, D₂O, and mixtures of water and D₂O (7). In all cases, the loss of tritium exhibited simple first order kinetics throughout. The results indicate that any kinetic selectivity toward tritiated enantiomorphs must be of a low order, even when D₂O is solvent.
Fig. 7. Infrared spectrum of succinic acid recovered during the exchange of deuterium from racemic 2,3-dideutosuccinate to water. The indicated time refers to the time of incubation at 24°C. The reaction mixture contained 7.5 μM succinic dehydrogenase flavin, 10 mM racemic 2,3-dideutosuccinate, and 38 mM potassium phosphate buffer, final pH 7.0. Gas phase was air. The first spectrum is a reference spectrum of racemic 2,3-dideutosuccinate.

By analogy, we would not expect large specific rotations to occur among the deuterated succinates formed in deuterium exchange experiments. The exchange of deuterium from succinate to water was studied with the use of deuterated succinates other than D₄.

Fig. 6 shows a spectrum of succinic acid recovered after 2.5 hours from an exchange system containing meso-D₂ initially. In addition to meso-D₂ and the end product, succinate, no deuterated intermediate was detected. Note that the 1280 cm⁻¹ band of D₁ is missing. The 800 and 580 cm⁻¹ bands of succinic acid appear promptly, and the D₁ impurity initially in the meso-D₂, represented by the weak band at 1215 cm⁻¹, decreases with time rather than increases. It is clear that D₁ cannot be an intermediate in the exchange of deuterium from meso-D₂.

However, as illustrated in Fig. 7, D₁ is easily detected during the exchange of deuterium from racemic-D₆, in which the D-D pairs are cis pairs.² The strong bands at 1222 and 1000 cm⁻¹, characteristic of cis deuterium pairs, decrease and bands due to D₁ appear and intensify at 1240, 1215, and 750 cm⁻¹. Succinic acid bands at 800 and 580 cm⁻¹ are slow to appear. After an incubation period of 4 hours, the 1000 cm⁻¹ band has almost disappeared, and the 800 cm⁻¹ band of succinate is apparent. The rather strong band at 1240 cm⁻¹ can be used to characterize D₁ since meso-D₂ and D₄ are absent. The band at 1280 cm⁻¹ is less characteristic of D₁ in this case because of a contribution by racemic-D₆. However, from the low intensity of the 1000 cm⁻¹ band, we estimate that the 1280 cm⁻¹ band is due almost entirely to D₁.

We believe that kinetic isotope effects involving labeled succinates are small. In addition to the rate comparisons among tritiated succinates in Fig. 1, comparisons of exchange rates have been made between tritiated and deuterated succinates. For example, the rate ratio, D₁ : meso-T₂, was about 3 and compared two D-D trans pairs with one T-T trans pair in each labeled molecule. The result is biased with a statistical factor of 2 toward a large ratio. Considering trans hydrogen pairs, we estimate the relative exchange rates to be roughly 1.7, 1.5, and 1 for H-H, D-D, and T-T, respectively, exchanging with H₂O.

In the deuterium exchange studies discussed above, infrared spectra were compared with reference spectra of fumaric and
malic acids in an attempt to detect autoxidation. In no case could either compound be detected unambiguously in material recovered from exchange systems. This negative result was to be expected from the exceedingly low rates of autoxidation observed for soluble succinic dehydrogenase (7, 11).

**DISCUSSION**

Our studies indicate that the exchange of hydrogen between succinate and water, as catalyzed by the soluble succinic dehydrogenase of Singer from bovine heart, occurs mainly with trans (RS) specificity and involves 2 hydrogen atoms per event. These characteristics, plus the enzyme’s inability to distinguish kinetically between labeled enantiomorphs, make the 1-methylene hydrogens of succinate very nearly equivalent kinetically. The principal reaction observed, therefore, is analogous to the trans elimination of a proton pair during the oxidation of succinate to fumarate (8, 12–14).

Our results differ somewhat from the findings of others. While Weinmann, Morehouse, and Winzler (2) observed complete exchange of deuterium from racemic-D2, the reported isolation of 2(S) (-)-D1 from exchange systems containing succinate, DOH (50 atom % D), and heart muscle particles was interpreted by Gawron, Glaid, and Francisco (15), as a positional nonequivalence among the methylene positions imposed by succinic dehydrogenase. Specifically, a trans proton-hydride elimination was envisioned in which the proton resulting from succinate, DOH (50 atom % D), and heart muscle particles was retained at a nonexchangeable (or slowly exchangeable) position on the enzyme. Thus, two nonequivalent cis pairs of hydrogen were required, and the mechanism would predict the formation of 3(S) (--)D2 as end product from succinate in D2O and 2(R), 3(R) (--)D3 as end product from D1 in H2O. However, Kahn and Rittenberg (16), in repeating the experiment of Gawron et al. (15), were unable to find optical activity in the D1 obtained by exchange of succinate in DOH (50 atom % D). This finding requires that all 4 methylene hydrogen atoms are equally exchangeable. Analysis of exchange products by mass spectra indicated a predominance of D1 over D2 species (37 and 1.6%, respectively), and led to the conclusion that exchange involves 1 hydrogen per event. The significance of these distribution data depends, however, on possible isotope selection effects in DOH and on the accuracy of the mass spectrometric analysis, neither of which was discussed. Our results with a soluble preparation of succinic dehydrogenase indicate a quite different situation and suggest that isotope selection effects involving solvent are small.

Monodeuterated L-2-chlorosuccinate has been obtained from the exchange of L-2-chlorosuccinate in mixtures of D2O and water (14, 15) and the product was deduced to be the D(-)-2-deuterated derivative on the basis of the 2-deuterosuccinate obtained after reductive depolymerization (15). It was concluded that hydrogen at position 2 of L-2-chlorosuccinate is nonexchangeable and represents the hydride ion in a trans activation process. This result is not consistent, however, with the later observation (17) that the deuterium in 2-deutero-L-2-chlorosuccinate exchanges with water about 1.8 times faster than that in 3-deutero-L-2-chlorosuccinate. L-2-Chlorosuccinate is an asymmetric compound, and the differential exchange rate may represent a differential isotope effect between the two monodeuterated derivatives; that is, the trans pairs, 2 D-threo-3 H and 2 H-threo-3 D, may not be kinetically equivalent. The rate difference, therefore, provides no information about the number of hydrogens involved in each exchange event, and does not rule out an activation involving both hydrogen atoms. The former question could be decided by studying the exchange of deuterium from 2-threo-3-dideuterot-L-2-chlorosuccinate to water or from D2O to L-2-chlorosuccinate.

In the papers just discussed, the conclusion that exchange involves 1 hydrogen per event was based principally on experiments carried out in H2O-D2O mixtures and terminated long before reaching isotopic equilibrium. The interpretative difficulties associated with such situations are well known. An exchange reaction involving 1 hydrogen per event was demonstrated unambiguously in a recent report by Rétey et al. (18). With soluble succinic dehydrogenase prepared by the method of Dervartanian and Veeger (19) from pig heart, Rétey and co-workers observed that the transfer of deuterium from D2O to succinate under anaerobic conditions resulted in the formation initially of more D1 than D2. The initial mole ratio, D1:D2, was about 2. The D1 and D2 were found to be mainly 2(R) (--)-D1 and meso-D2 from optical rotation measurements. As the reaction progressed, the specific levo rotation increased and D1 appeared in significant amounts. It would appear that 2(R), 3(R) (--)-D2 is formed from 2(R), 3(R) (--)-D1 and that 2(R), 2(S), 3(R) (--)-D3 accumulates at the expense of optically inactive meso-D2. Some (S) (+)-deuterated succinic acids were reported to arise during the transfer of deuterium from D1 to water. In this situation, however, the isotope species to increase most rapidly initially were D2 (presumably meso-D2) and succinate. Note that the stereochemical selectivity deduced is toward R positions and not toward S positions, as reported by Gawron et al. (15).

In the systems described by Rétey et al., the course of the exchange reaction appears superficially to depend on the nature of the solvent. An exchange reaction involving 1 hydrogen per event with R specificity occurs at a rate twice as great as one involving 2 hydrogens per event with trans or RS specificity when D2O is solvent. The latter reaction predominates, however, when water is solvent. In our systems, exchange with trans specificity predominates in both D2O and water. A minor process involving 1 hydrogen per event is detectable, however, and appears to have greater importance in D2O than in water. We cannot account for the differences between our results and those of Rétey et al. on the basis of pH, succinate concentration, deuterium content of solvent, or the presence or absence of oxygen. The kinetics of tritium exchange from our titiated succinates remain first order under all circumstances and fail to indicate a strong discrimination between R and S positions. We suggest that the differences may relate to the enzyme preparations used.

It is possible that kinetic effects other than those imposed by solvent affect the course of the exchange reaction. Our results and those of Rétey et al. together suggest that the kinetic selectivity for R positions may decrease in the order H, D, T. The results may be explained, at least in part, by a kinetic selectivity for R positions combined with a counteracting isotope effect. A kinetic preference for the R position would be apparent when H exchanges from succinate, less apparent when D exchanges, and, at least in our systems, not evident at all when T exchanges.
In this view our results with tritiated succinates may be considered a fortuitous result of two minor but opposing kinetic effects. It would be of interest to compare the relative rates of exchange of \((R)\) \((-\)-)D1, \((S)\) \((+\)-)D1, and \(\text{meso}\)-D2, just as we have done in this report with tritiated succinates.

Hydride ion and hydrogen atom mechanisms have been proposed to account for the catalytic activities of succinic dehydrogenase (13–15, 20). The results presented here cannot be used to decide between these possibilities, nor to support necessarily a mechanism in which a pair of hydrogens leaves succinate as protons. The results would appear to rule out the enol mechanism proposed by Kahn and Rittenberg (16), since the mechanism as written would require the exchange of only one hydrogen per event.

Our results show that a pair of hydrogens from trans positions is transferred to water at nearly every successful exchange event and that activation appears to be nearly symmetrical. This follows from the inability of the enzyme to discriminate between tritiated enantiomorphs of racemic-T1 and racemic-T2, and would seem to rule out activation by a single group on the enzyme.

Since we have been unable to detect an interconversion of \(\text{meso}\)-D2 (trans D-D pairs) and D2 with cis configuration, it would appear that neither internal return nor exchange can proceed with retention of configuration at 1 methylene carbon and inversion at the other. Both events must proceed either with retention or inversion at both positions. Whether retention or inversion applies with succinate is unknown at present and is currently under investigation. Since exchange of the trans hydrogen pair of \(L\)-2-chlorosuccinate is not associated apparently with the formation of \(D\)-2-chlorosuccinate nor with the transfer of deuterium between the \(3\text{-threo}\) and \(3\text{-erythro}\) positions and since exchange does not involve the \(3\text{-erythro}\) position (17), retention of configuration seems more likely than inversion.

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