STUDIES ON THE ENZYMIC DEHYDROGENATION OF DEUTERATED SUCCINATE

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SUMMARY

1. Increasing the deuterium content of succinic acid decreased the rate of dehydrogenation by succinoxidase. In cell-free systems, completely deuterated succinate was dehydrogenated at about one-sixth the rate of ordinary succinate.

2. Solvent effects of D_2O were of less importance. In pure D_2O, the dehydrogenation of ordinary succinate was inhibited about 35 %; in 30 % D_2O, the inhibition was only 5 %.

3. Metabolism of succinate by liver slices showed the same effect of deuteration of the substrate that was observed with cell-free preparations, except that the isotope effect was somewhat less.

INTRODUCTION

In the study of the effects of heavy water on animals, homogenates of liver and kidney were assayed for a number of enzymes[1]. Only liver catalase and DPN–cytochrome *c* reductase were markedly decreased (65 % of control activity in moribund animals). Succinic dehydrogenase was diminished slightly in both liver and kidney.

However, these studies were carried out with substrates containing ordinary hydrogen. It was thus of interest to study the effects of substitution of partially and completely deuterated substrates in the assay systems. THORN[2] had previously shown that dideutero- and tetradeuterosuccinate (actually containing 42 and 77 atom % D in the methylene groups) were dehydrogenated significantly more slowly than ordinary succinate. We have extended THORN's observations, using both more and less completely deuterated succinates, and have also compared the dehydrogenation of these succinates in both H_2O and D_2O.

METHODS

Tetradeuterosuccinate was prepared by catalytic hydrogenation of acetylene dicarboxylic acid by deuterium gas. The details of the synthesis will be published elsewhere[3]. The deuterium content of tetradeuterosuccinic acid was 96.2 atom %; the minimum value for the methylene groups was thus 94.6 atom % D, considerably higher than the 77 atom % reported by THORN[2].

Dideuterosuccinate was prepared by reduction of either fumaric or maleic acid

by zinc in 5 N D_3PO_4. The acid was extracted with ether and recrystallized from D_2O. The deuterium content of several preparations ranged from 63.7 to 65.0 atom %, corresponding to a minimum of 46 to 48 % deuterium on the methylene carbons.

The reduction of fumaric acid by zinc in mixtures of D_3PO_4 and H_3PO_4 was used to prepare succinates containing smaller amounts of deuterium. From a mixture of phosphoric acids containing 60 atom % D, succinate with 15.6 atom % D in the methylene groups was obtained. It was necessary to use a mixture with 80 atom % D to prepare succinate with approximately 30 atom % D. There is thus an appreciable discrimination between H and D in this reaction.

Various sources of succinoxidase were employed: homogenized rat kidney, mitochondria prepared from rat liver[4], and a preparation from beef heart[5]. In one experiment, an attempt was made to obtain an enzyme preparation with an appreciable percentage of non-exchangeable deuterium: subtotal hepatectomy was performed on a rat with about 25 % of its body water replaced by D_2O; after operation, the rat was given 30 % D_2O to drink for two weeks; the rat was then killed, and mitochondria were prepared from the regenerated liver. Succinoxidase was usually assayed with cytochrome c as the acceptor[6]; methylene blue[5] and phenazine methasulfate[7] were used in some experiments. In most cases, the oxygen consumption occurring between 5 and 25 min after addition of the substrate was used as the basis of comparison.

For the study of oxidative phosphorylation with deuterosuccinates as the substrate, the system of LARDY AND WELLMAN was used[8].

Liver slices were cut with a Stadie-Riggs microtome. Oxygen was used as the gas phase, and the final concentration of succinate in Krebs–Ringer–phosphate was 0.03 M. Oxygen consumption was measured over a 40 min interval.

RESULTS

Oxidation of deuterosuccinates in D_2O

The oxidation of tetradeuterosuccinate was studied in a nearly completely deuterated system. The only hydrogen (*i.e.*, protium) present was in the acceptors (cytochrome c, methylene blue, or phenazine methosulfate) and in the enzyme preparation. For comparison, ordinary succinate in both D_2O- and H_2O-systems and tetradeuterosuccinate in H_2O were studied simultaneously. Two experiments were carried out, one with a preparation of mitochondria from a normal rat, the other with mitochondria prepared from regenerated liver of a rat drinking 30 % D_2O. In both experiments the mitochondria were washed and resuspended in 0.25 M sucrose in 100 D_2O. The results of these experiments are shown in Table I. No differences were observed when different acceptors were used; further, the mitochondrial preparation from the deuterated rat had the same Q_{O_2} succinate as did that from the normal animal. Hence all of the data have been pooled, so that each entry represents the average of six determinations, each of which was carried out in duplicate.

It is clear that in the case of tetradeuterosuccinate virtually the same degree of inhibition was observed in D_2O as in H_2O; the solvent effects of D_2O were almost completely masked by the primary isotope effect. The dehydrogenation of ordinary succinate was somewhat inhibited in D_2O, but the rate of dehydrogenation of this

TABLE I

RELATIVE RATES OF DEHYDROGENATION OF DEUTEROSUCCINATES IN H_2O AND D_2O

Atom % D		*No. determinations*	*Per cent control activity*			
			H-succinate		*D-succinate*	
Substrate	*Medium*		*In H_2O*	*In D_2O*	*In H_2O*	*In D_2O*
94.6	100	6	100	65.9	17.2	16.1
28.8	30	3	100	95.4	82.8	80.4
15.6	15	4	100	94.5	89.9	87.0

compound in D_2O was much faster than that of tetradeuterosuccinate in either H_2O or D_2O.

Table I also shows the results of similar experiments carried out with 30 and 15 % deuterosuccinates, in both H_2O and 30 or 15 % D_2O. The higher concentration was chosen because it represents the maximum amount of replacement of body water by D_2O which a rat can tolerate. The lower concentration produces very few effects in the living animal. In these experiments only cytochrome *c* was used as the acceptor; rat kidney homogenate was the source of the enzyme.

Both the 30 and 15 % deuterosuccinates were dehydrogenated more slowly than ordinary succinate in H_2O, the former about 20 %, and the latter about 10 %. D_2O in the concentrations employed had a very slight inhibitory action.

Dehydrogenation of deuterosuccinates in H_2O

In Fig. 1 are shown the relative rates of dehydrogenation of succinates containing 0, 15.6, 28.8, 47.5, and 94.6 atom % D. The data of THORN[2] are also shown for 44 and 72 atom % D. It is evident that there is a virtually linear relationship between activity and extent of replacement of hydrogen by deuterium.

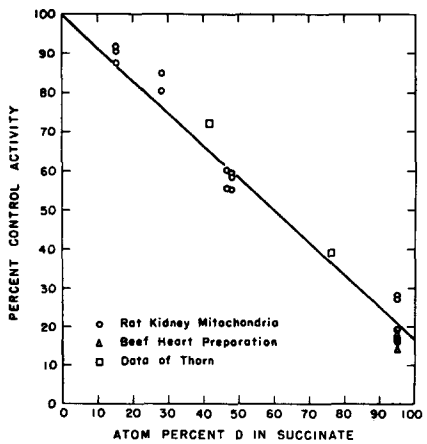

Fig. 1. Effect of deuteration of succinate on succinoxidase activity of rat kidney homogenate or beef heart preparation.

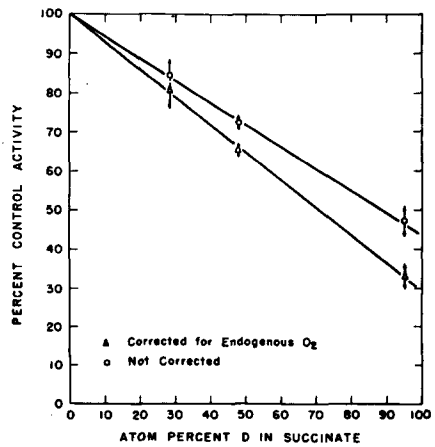

Fig. 2. Effect of deuteration of succinate on succinoxidase activity of slices of rat liver.

Dehydrogenation by liver slices

Fig. 2. shows the effect of deuteration of succinate on the rate of oxidation by

slices of rat liver. As with homogenates, the response was linear. However, the effect was not quite so marked, even when the data were corrected for oxygen consumption in the absence of succinate. Tetradeuterosucinate was oxidized at 33 % of the control rate by liver slices, but only 22 % by kidney homogenates or beef heart preparations. The reason for this discrepancy is doubtless attributable to the fact that the succinoxidase system was not acting at maximum potential in the slices; under optimum conditions, liver homogenates oxidize succinate at 4 to 5 times the rate observed in slices.

Oxidative phosphorylation

Table II shows that, under the conditions of assay for this system, only tetradeuterosuccinate was dehydrogenated more slowly than ordinary succinate. The esterification of inorganic phosphate was similarly reduced, but the fact that the P/O ratios did not differ significantly provides evidence that the efficiency of the system was not impaired.

TABLE II

OXIDATIVE PHOSPHORYLATION WITH DEUTEROSUCCINATES AS SUBSTRATES

Atom % D Succinate	*μMP/ mg N*	*μ atoms O/ mg N*	*P/O ratio*
0	26.0	13.9	1.9
28.8	24.0	14.2	1.7
47.9	26.5	15.0	1.8
94.6	18.1	9.5	1.9

DISCUSSION

On the basis of measurements of the Michaelis constants, THORN estimated that the affinity between succinoxidase and ordinary succinate was 1.45 times that between the enzyme and succinate containing 77 atom % D (see ref. 2). It is reasonable to believe that the affinities of the other deuterosuccinates would differ from that of ordinary succinate, probably in proportion to the deuterium content.

In these experiments, dideutero- and tetradeuterosuccinate may be considered as homogeneous compounds, for which meaningful Michaelis constants could be derived. However, the 15 and 30 % deuterosuccinates represent mixtures of succinic acid containing, in unknown proportions, 0, 1, and 2 atoms of deuterium. One would thus expect that the molecules containing no deuterium would be preferentially dehydrogenated. This is probably the reason why the dehydrogenation of 15 and 30 % deuterosuccinate was slightly faster than would be anticipated from the observations with dideutero- and tetradeuterosuccinates (Fig. 1).

The data presented in Table I show that the solvent effects of D_2O are much less important than the primary isotope effects in determining the rate at which succinate is oxidized. The rate of dehydrogenation of ordinary succinate in 100 % D_2O was reduced to about the same level (65 %) as was observed with dideuterosuccinate in H_2O. Levels of D_2O below 30 % had very little effect on the dehydro-

genation of succinate, normal or deuterated. Some years ago THUNBERG[7] observed that the "dehydrogenase" activity of an extract of peas was inhibited by D_2O; the effect was linear, and in pure D_2O the inhibition was 30 %, not much different from the 35 % noted for succinoxidase in these experiments. JACOBSOHN AND SOARES, on the other hand, reported that the malic dehydrogenase activity of a preparation of cucumber seeds was enhanced in pure D_2O (see ref. 10).

Since the rate of oxidation of tetradeuterosuccinate was uniformly slow in relation to that of ordinary succinate regardless of the acceptor—phenazine methosulfate, methylene blue, or cytochrome *c*—it is probable that the initial transfer of hydrogen is the rate-limiting step. If it were not, one would expect that a higher rate would be observed with phenazine or with methylene blue (or both) than with cytochrome *c*, since the three acceptors function at different levels of the electron transport mechanism.

The lack of effect of all but the completely deuterated succinate in the oxidative phosphorylation system indicates that the rate of oxygen consumption was determined by factors other than the rate of dehydrogenation of the substrate. Only when the deuterium content of succinate was increased to some value above 50 % of the normal did this reaction become the rate-limiting step. The presence of tetradeuterosuccinate did not affect the efficiency of phosphate esterification; in this system, the solvent effects of D_2O may be of greater importance, since 60 % D_2O decreased the P/O ratio by about 10 % (see ref. 11).

The influence of deuterated substrates on the development of the syndrome of heavy water intoxication in mammals is not easy to assess. All dehydrogenation reactions would probably be slowed somewhat, probably to about the same extent as succinate dehydrogenation, since the rate-limiting step seems to be the initial hydrogen transfer. Thus on the basis of the experiments with both liver slices and cell-free preparations, one would predict that in an animal with 30 % of his body water replaced by D_2O there would be a decrease in dehydrogenase activity to about 80 % of normal. Whether such a decrease actually takes place *in vivo* is open to question. BARBOUR reported that the metabolic rate of D_2O-treated mice was increased in the early stages of D_2O intoxication (10 to 20 % replacement) but fell to very low levels shortly before death[12]. Because of the profound adaptive changes which are known to occur in animals drinking D_2O (see ref. 1), these data are difficult to interpret. Possibly a study of the effects of D_2O on the metabolic rate of hypophysectomized animals would be useful.

One factor complicating the extension of results of *in vitro* experiments to the intact animal is the enzymic discrimination between deuterated and non-deuterated substrates[2]. Another is an enzyme-catalyzed exchange between the deuterium of the substrate (ordinarily non-exchangeable) and the hydrogen of the medium. The latter has been described by several authors; the magnitude of this exchange is uncertain, however. WEINMANN *et al.*[13] considered it to be large, while ENGLARD AND COLOWICK[14] found it to be very small in relation to succinate oxidation unless fumarate was present. In the short-term *in vitro* experiments reported here, the enzyme-catalyzed exchange was probably negligible; it might be appreciable in the intact animal. The effect of enzymic discrimination would be the accumulation of the more heavily deuterated succinates; however, the exchange reaction could probably re-establish the same equilibrium between substrate and medium.

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OXYDATION DU SULFITE EN SULFATE PAR LA RACINE D'AVOINE

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SUMMARY

Oxidation of sulphite to sulphate by wheat roots

Wheat roots contain an enzymic system catalysing the oxidation of sulphite to sulphate. The optimum pH of the non-purified system is 7.0 in a phosphate buffer. The metal-chelating agents used are shown to inhibit the oxidation.

INTRODUCTION

Au cours de l'étude de la réduction du sulfate en sulfite dans la feuille de tabac[1], il est apparu que le sulfite est simultanément réoxidé en sulfate. La rapidité de cette oxydation est telle qu'elle compromet une mesure exacte de la quantité de sulfite