PATHWAY OF HYDROGEN TRANSFER FROM SUCCINATE TO DPN IN MITOCHONDRIA*

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The present communication was prompted by a recent report that oxidation of tritiated succinate by a Keilin-Hartree preparation resulted in direct transfer of tritium to DPN (1). During the past year, using mitochondrial systems, we also have attempted to find evidence of direct transfer of tritium from tritiated succinate to DPN. In contrast with the data of Gawron et al., (1) our results show that, under conditions giving reduction of DPN with incorporation of tritium into DPNH, tritiated malate is a major product of oxidation of the labeled succinate, while under conditions which result in a relatively small accumulation of tritiated malate, reduction of DPN by tritiated succinate takes place without appreciable incorporation of tritium into DPNH.

Experiments were carried out with succinate-2,2'-^{3}H using beef heart and rat liver mitochondria, providing conditions for the trapping of any tritium in position 4 of DPNH by exchange into lactate, in the case of heart mitochondria, and by net oxidation of the reduced coenzyme with acetoacetate, in the case of liver mitochondria. Reaction products, which were separated by chromatography using

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Dowex-1-formate columns, were continuously monitored for radioactivity in the column effluent by passage of the latter through an anthracene-containing cell located in the well of a scintillation spectrometer. Final assessment of the amount of radioactivity in the collected fractions was made by liquid scintillation counting.

We observed that beef heart mitochondria prepared according to the directions of Singer, et al., (2) when frozen and thawed once or twice were capable of bringing about incorporation of tritium from succinate-2,2'-^{3}H into L-lactate when lactate dehydrogenase was added to the system. The reaction was observed with or without addition of ATP, while in the presence of Amytal a marked increase in the yield of radioactive lactate was seen. The results of chromatographic analyses of a typical experiment are shown in Table I and demonstrate that tritiated fumarate and malate were products of the reaction and were formed in especially large amounts in the presence of Amytal.

Table I

Distribution of tritium in products formed during incubation of beef heart mitochondria with tritiated succinate and normal L-lactate

	Per cent of dpm in extract		
	Complete system*	+2 mM Amytal	+2 mM Amytal - LDH
H_2O	1.5	17.2	20.0
Unknown	2.9	2.1	2.9
Lactate	1.9	9.2	0
Succinate	74.6	15.5	15.8
Malate	11.1	40.1	44.2
Fumarate	1.9	15.8	17.1

*The complete system contained: 1 mM succinate-2,2'-^{3}H, specific activity 1.05 x 106 dpm/μmole; 5.0 mM L-lactate; 0.25 mM DPN; 0.1 mM ATP; 80 mM glycyl-glycine; 0.5 mg Worthington beef heart lactate dehydrogenase (LDH); 10 mg mitochondria. Total volume 2.0 ml; pH 7.4. Incubation was carried out at 38° for 20 minutes.

It should be emphasized that this agent, a potent inhibitor of the succinate-linked reduction of DPN (3) and of DPNH oxidation via the respiratory chain (4) did not inhibit, but, in fact, increased the extent of incorporation of tritium into lactate.

Thus even though reduction of DPN by succinate was blocked, tritium found its way to lactate; malate, it must be concluded, not succinate, supplied tritium for the labeling of lactate. The results also demonstrate that transfer of hydrogen from malate to lactate does not require net oxidation of malate but can take place by exchange.

It is interesting to note the marked stimulation by Amytal of succinate oxidation, an effect of this agent previously observed by Chance and Hollunger (5).

The foregoing results are in contrast with those obtained from studies of the succinate-linked reduction of acetoacetate catalysed by rat liver mitochondria. As will be shown, reduction of acetoacetate by tritiated succinate gave relatively little incorporation of isotope into β-OHbutyrate. Indeed, the observed labeling can be attributed to reduction of acetoacetate by tritiated malate formed by oxidation of the tritiated succinate.

Rat liver mitochondria were prepared as described by Ernster and Löw (6). The conditions of the experiments were in general similar to those described by Azzone et al., (3). In the present studies the use of acetoacetate-3-^{14}C made possible a comparison of β-OHbutyrate production, as assessed by the extent of incorporation of ^{14}C into that product, and acetoacetate removal, as estimated by the method of Walker (7). In those experiments in which ^{3}H and ^{14}C were present together, the radioactivity of each isotope was estimated by the method of simultaneous equations (8). The results of a typical series of experiments is shown in Table II.

It will be seen from the data given by Experiments I and 3 that the correlation between β-OHbutyrate formation and acetoacetate removal is good. The results of Experiment 2 show that the incorporation of tritium into β-OHbutyrate, observed in Experiment 3, was not an artifact of the counting method.

Table II

Isotope incorporation into β-OHbutyrate, β-OHbutyrate production and acetoacetate removal in succinate-linked reduction of acetoacetate.

Exp. #		Isotope Incorporation Into β-OHbutyrate			β-OHbutyrate Formed	Acetoacetate Removed
		^{14}C dpm x 10^4	^{3}H dpm x 10^4	^{3}H dpm/μmole x 10^4	μmoles	μmoles
1	acetoacetate-3-^{14}C + normal succinate	7.2	---	---	2.9	2.9
2	normal acetoacetate + succinate-2,2'-^{3}H	---	2.2	1.1		2.1
3	acetoacetate-3-^{14}C + succinate-2,2'-^{3}H	8.4	1.6	0.5	3.4	3.3

The system contained 20 mM glycylglycine, 8 mM $MgCl_2$, 50 mM KCl, 50 mM sucrose, 9.5 mg mitochondrial protein and additions as follows:
Exp. 1 - 7.0 mM acetoacetate-3-^{14}C and 10 mM normal succinate.
Exp. 2 - 8.0 mM normal acetoacetate and 10 mM succinate-2,2'-^{3}H.
Exp. 3 - 7.9 mM acetoacetate-3-^{14}C and 10 mM succinate-2,2'-^{3}H.
The s.a. of the acetoacetate was 2.48 x 10^4 dpm/μmole and of the succinate, 3.83 x 10^5 dpm/μmole. Total volume was 2 ml, pH 7.5 and incubation was for 20' at 30°.

In the absence of an isotope effect* the reduction of acetoacetate by direct transfer of hydrogen from succinate would be expected to give β-OHbutyrate with a specific activity equal to one-fourth that of the succinate, i.e., about 10^5 dpm/μmole. This value is from 10 to 20 times greater than the specific activities of β-OHbutyrate shown in Table II, i.e., from 90 to 95% of the hydrogen utilized for the reduction of acetoacetate was in equilibrium with the hydrogen atoms of water.

*By counting the tritiated products remaining in the reaction mixtures afforded by Experiments 2 and 3, it was ascertained that about 6 μmoles of succinate had been oxidized. In the absence of an isotope effect half the tritium initially present in this succinate would be expected to appear as tritiated water, i.e., 1.1 x 10^6 dpm. In fact only 0.7 x 10^6 dpm were found in water, indicating that the removal of two atoms of ^{1}H was favored over removal of one atom of ^{1}H and one of ^{3}H by a factor of 1.6.

The reaction mixtures of Experiments 2 and 3 contained tritiated malate equivalent, in terms of radioactivity, to about 13% of the tritium initially added to the system as succinate. It would seem likely that this malate served as the source of tritium for the labeling of β-OHbutyrate.

The foregoing results demonstrate that the transfer of hydrogen from succinate to DPN is indirect. It is interesting to note that the data are in accord with the concept of reduction of DPN by reversal of oxidative phosphorylation, as postulated by Chance and Hollunger (9).

The conclusions of Gawron et al., are inconsistent with recent studies of the steric specificity of the succinate-linked reduction of DPN (10). Using submitochondrial particles prepared from beef heart, the reduction of DPN-4-^{3}H by succinate yielded DPNH-4A-^{3}H, i.e., the steric specificity is of the B type. When DPNH-4B-^{3}H was incubated with the particles, the nucleotide was rapidly and completely detritiated. It is thus apparent that the labeling of DPN observed by Gawron et al., could not have been due to direct transfer of tritium from succinate for even if tritium were introduced into the 4B position of DPNH, its removal would be brought about by action of the B-specific oxidase. The results of Gawron et al., would seem most likely to be due to formation of tritiated fumarate, thence tritiated malate, by oxidation of labeled succinate. Although the preparation used by these investigators was said not to oxidize fumarate, introduction of tritium into DPNH could have taken place by exchange between DPNH and tritiated malate. Since the steric specificity of malate dehydrogenase is of the A type (11), DPNH, 4-A-^{3}H would be the product of the reaction. This, on oxidation by the B-specific DPNH oxidase system, would result in the observed retention of tritium in oxidized DPN.

References

1. Gawron, O., Glaid, A.J., III, Nobel, S., and Gan, M., Biochem. Biophys. Res. Comm., 16, 432 (1964).

2. Singer, T.P., Kearney, E.B., and Bernath, P., J. Biol. Chem., 223, 599 (1956).

3. Azzone, G.F., Ernster, L., and Weinbach, E.C., J. Biol. Chem., 238, 1825 (1963).

4. Ernster, L., Jalling, O., Löw, H., Lindberg, O., Exptl. Cell Research, Suppl. 3, 124 (1955).

5. Chance, B., and Hollunger, G., J. Biol. Chem., 238, 418 (1963).

6. Ernster, L., and Löw, H., Exptl. Cell Research, Suppl. 3, 133 (1955).

7. Walker, P.G., Biochem. J., 58, 699 (1954).

8. Okita, G.T., Kabara, J.J., Richardson, F., and LeRoy, G.V., Nucleonics, 15, 111 (1957).

9. Chance, B., and Hollunger, G., Nature, 185, 666 (1960).

10. Lee, C.P., Duquesne, N., Hoberman, H.D., and Ernster, L., to be reported.

11. Loewus, F.A., Tchen, T.T., and Vennesland, B., J. Biol. Chem., 212, 787 (1955).