MITOCHONDRIAL OXIDATION OF EXTRAMITOCHONDRIAL TPNH[1] MEDIATED BY PURIFIED DT DIAPHORASE

Thomas E. Conover[2] and Lars Ernster

Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden

Received December 30, 1959

The recognition (Ernster, 1958; Ernster and Navazio, 1958) and purification (Ernster et al., this issue) of a soluble-cytoplasmic, pyridine nucleotide-unspecific diaphorase (DT diaphorase) capable of reacting with a number of electron acceptors, including vitamin K_3, raises the question of the natural acceptor for this abundant enzyme and its possible function in vivo. Since reduced vitamin K_3 has been reported to interact with the mitochondrial cytochrome system (Colpa-Boonstra and Slater, 1958) it was thought possible that by the mediation of such a compound, the cytoplasmic DT diaphorase might act as an electron transmitter between extramitochondrial reduced pyridine nucleotides and the mitochondrial respiratory chain. Support for this view is presented below by showing that added purified DT diaphorase in the presence of vitamin K_3 markedly enhances the mitochondrial oxidation of extramitochondrial TPNH generated by way of the glucose-6-phosphate dehydrogenase system.

Mitochondria freshly prepared from rat liver as previously described (Ernster and Low, 1955) were incubated in an isotonic, buffered medium containing TPN, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, phosphate, Mg^{++}, adenosine triphosphate, hexokinase and glucose. As shown in Fig. 1, the extramitochondrial TPNH was not oxidized under these conditions to any appreciable extent by the mitochondria, in agreement with the findings of Pullman and Rac-

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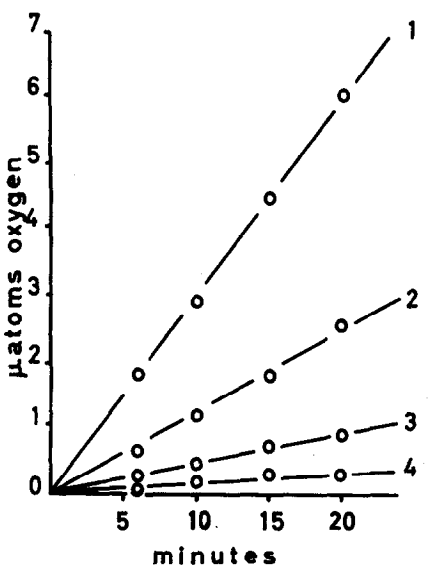
[1] Abbreviations: TPNH, reduced triphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

[2] Fellow of The National Foundation, New York, N.Y.

ker (1956). The addition of vitamin K_3 in a concentration of 10^{-5}M produced a 2- to 3-fold stimulation of the total respiration. Addition of purified cytoplasmic DT diaphorase (Ernster et al., this issue) to the vitamin K_3-stimulated system gave a further 2- to 3-fold stimulation of the respiration. It might be pointed out that this rate of respiration was close to that obtained maximally with succinate or glutamate and may represent the limit of the cytochrome system to react with oxygen rather than that of TPNH to react with the cytochrome system. DT diaphorase in the absence of vitamin K_3 had no effect on the respiration. The concentration of 10^{-5}M vitamin K_3 was not ascertained to be the minimal concentration required; however, 10^{-6}M vitamin K_3 gave but very little stimulation of respiration either in the presence or absence of added DT diaphorase.

Fig. 1. Mitochondrial oxidation of extramitochondrial TPNH, mediated by DT diaphorase and vitamin K_3



The complete system contained per Warburg vessel: 0.5 µmoles TPN, 20 µmoles glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase (Sigma), 20 µmoles Tris buffer (pH 7.4), 12 µmoles orthophosphate (pH 7.4), 4 µmoles $MgCl_2$, 2 µmoles adenosine triphosphate, 24 µmoles glucose, an excess of yeast hexokinase, 50 µmoles sucrose, mitochondria from 200 mg rat liver, 0.01 µmole vitamin K_3, and an amount of 450-fold purified DT diaphorase (together with 1 mg serum albumin) capable of reducing 1 µmole vitamin K_3 per minute. Final volume, 1.0 ml. Temp., 30°C. Reading begun after 6 min. thermoequilibration.
1. Complete system.
2. No DT diaphorase.
3. No DT diaphorase, no vitamin K_3.
4. No mitochondria.

The point of entry of electrons into the respiratory chain has been investigated with a number of inhibitors as shown in Table I. While antimycin A and cyanide gave strong inhibition of the DT diaphorase-vitamin K_3-mediated respiration, amytal showed no inhibition and actually a slight and possibly significant stimulation of the respiration was observed. These results are in

TABLE I

Effect of some inhibitors on the DT diaphorase-vitamin K_3-mediated oxidation of extramitochondrial TPNH

Conditions as in Fig. 1.

Additions (+) or omissions (-)	μatoms oxygen
Complete system	5.56
" " + 2 x 10^{-3}M amytal	6.59
" " + 1 μg antimycin A	1.14
" " + 10^{-3}M cyanide	2.33
" " + 10^{-6}M dicumarol	1.31
" " + 10^{-4}M tetraiodothyronoacetate	6.63
" " - DT diaphorase, - vitamin K_3	1.12

agreement with the report of Colpa-Boonstra and Slater (1958) on the oxidation of reduced vitamin K_3 by mitochondria. It is clearly indicated, then, that electrons from TPNH transferred by the DT diaphorase and vitamin K_3 enter the respiratory chain between the amytal-sensitive site and cytochrome c. The most likely point of entry would be at cytochrome b.

Of further interest are the effects of two inhibitors of the DT diaphorase. Dicumarol at a concentration of 10^{-6}M is a highly selective inhibitor for the DT diaphorase (Ernster et al., this issue). The results in Table I show a nearly complete inhibition of the DT diaphorase-vitamin K_3-mediated respiration by this concentration of dicumarol. It is worth noting that the respiration induced by vitamin K_3 alone appears to have been inhibited as well as that observed with the addition of DT diaphorase. This suggests that the respiration observed with vitamin K_3 alone may utilize the dicumarol-sensitive DT diaphorase in the mitochondria (Ernster et al., this issue). Tetraiodothyronoacetate, another inhibitor of the enzyme, was without effect on the respiration.

In accordance with the findings of Colpa-Boonstra and Slater (1958) with reduced vitamin K_3, the DT diaphorase-vitamin K_3-mediated oxidation of extra-

TABLE II

Esterification of phosphate accompanying the DT diaphorase-vitamin K_3-mediated oxidation of extramitochondrial TPNH

Conditions as in Fig. 1. Phosphate uptake was estimated according to the modified Martin and Doty procedure (Lindberg and Ernster, 1955).

Additions (+) or omissions (-)	μatoms oxygen	μmoles phosphate
Complete system	3.49	3.30
" " + 10^{-4}M 2,4-dinitrophenol	3.28	0.30
" " - DT diaphorase, - vitamin K_3	0.19	0.44

mitochondrial TPNH was accompanied by an esterification of phosphate which was suppressed by 10^{-4}M dinitrophenol. Preliminary data are shown in Table II.

The present results would explain the findings recently reported by Wenner (1959) that vitamin K_3 stimulated the hexose monophosphate shunt in intact ascites tumor cells and that this stimulation was highly dicumarol-sensitive. Wenner concluded from these data that the limiting step for the function of the hexose monophosphate shunt is the oxidation of TPNH by cytochrome c. From the present data it would seem possible to further localize this step, at least for the case of liver, to the reoxidation of the reduced cytoplasmic DT diaphorase by the mitochondrial cytochrome b.

Since the DT diaphorase is non-specific with regard to pyridine nucleotide it is obvious that DPNH may act as the electron source as well as TPNH. It would seem therefore that this pathway might represent a general way in which oxidation of extramitochondrial reduced pyridine nucleotides by the mitochondrial respiratory system may be explained without involving the necessity for extramitochondrial cytochrome c. It should be emphasized that vitamin K_3 is not the only electron acceptor for the DT diaphorase and the authors are by no means suggesting a role for vitamin K. It is entirely possible that coenzyme Q, or other naturally occurring quinones, may act in such a manner. Further work is at present in progress and will be reported at a later date.

This work has been supported by a grant from the Swedish Cancer Society.

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