PURIFICATION AND SOME PROPERTIES OF A HIGHLY DICUMAROL-SENSITIVE LIVER DIAPHORASE *

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Previous communications from this laboratory (Ernster, 1958; Ernster and Navazio, 1958) reported the occurrence in the soluble fraction of rat liver homogenates of an enzyme catalyzing the reduction of 2,6-dichlorophenolindophenol (DCPIP) by reduced di- and triphosphopyridine nucleotide (DPNH and TPNH). This enzyme, hereafter called DT diaphorase, has now been partially purified and some of its properties have been investigated. Among the striking properties of the enzyme are that it reacts with a great variety of electron acceptors, including vitamin K and coenzyme Q, that it is highly sensitive to dicumarol, and that it, although water-soluble, is markedly activated by certain dispersing agents. Data relating to these and some other properties of DT diaphorase are presented below.

Purification. The purification involved the following steps: (1) precipitation of the final supernatant (105,000 g x 60 min.) of a 0.25 M sucrose homogenate of rat liver with 9 volumes of acetone at -12°C; (2) suspension of the dried acetone-precipitate in water and dialysis against running water to remove sucrose; (3) fractionation of the extract, cleared by centrifugation, with neutral ammonium sulfate at 40, 55 and 65 per cent saturation; (4) chromatography of the precipitate obtained between 55 and 65 per cent saturation, resuspended in water and dialysed free of ammonium sulfate, on DEAE cellulose column with a 0.01-0.3 M phosphate gradient at pH 6.4. When starting from 50 grams of liver the most act-

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ive chromatographic fraction showed a slight yellow coloration and corresponded to a purification on a protein basis of about 450-fold as compared with the initial liver supernatant.

Activators. Bovine serum albumin, polyvinylpyrrolidone (PVP), Tween-20 and Tween-60 were found to activate the enzyme in a reversible manner. The maximum activation obtained was equal with these compounds but varied between 3- and 10-fold from one enzyme preparation to another. The concentration (mg/ml) required for maximal activation was 0.7 for albumin, 10 for PVP and 2 for the Tweens. The activating effect of albumin could not be removed by pretreatment of the albumin with isooctane (cf. Deul et al., 1958). Although the only common denominator for these compounds seems to be that they are dispersing agents, not all detergents were found to give an activation. Deoxycholate, for example, gave no activation, and at a concentration of 0.26 per cent it even abolished the activation given by albumin without inhibiting the non-activated enzyme. The albumin-activation could also be lowered by increasing the ionic strength of the incubation medium. From kinetic data the activation seems to consist of an increase of the turnover number of the enzyme as well as of its affinity to the substrates.

Kinetic data. Although no accurate determination of the flavin content of the enzyme has yet been made preliminary estimates indicate that the turnover number is of the order of 10^7. V_{max} is equal for DPNH and TPNH, both in the presence and in the absence of activator. The Michaelis constants on the other hand are slightly different for DPNH and TPNH, 0.08 and 0.04 mM, respectively, with albumin, and 0.18 and 0.13 mM without albumin. At saturating concentrations, the reaction rates with DPNH and TPNH are not additive.

Electron acceptors. Besides DCPIP, the following compounds have been found to act as electron acceptors (the values in parentheses give the maximum relative activities, taking that obtained with 0.04 mM DCPIP as 1, followed by the mM concentrations of acceptors at which these were obtained, in all cases in the presence of albumin as activator): p-benzoquinone (4.0; 0.44), coenzyme Q_0 (2.4; 0.08), coenzyme Q_2 (0.5; 0.13), 1,4-naphthoquinone (1.9; 0.017), vitamin K_3 (1.8; 0.067), ferricyanide (0.8; 0.667), methylene blue (0.5; 0.18). The reactions with

ferricyanide and methylene blue were not activated by albumin. Vitamin K_1 (in Tween) and cytochrome c did react with the enzyme but the reaction rates were of the order of 1/10,000 of those obtained with the above acceptors. However, cytochrome c was active as an acceptor provided that a catalytic amount of vitamin K_3 or coenzyme Q_0 was added. Essentially negative results were also obtained with blue tetrazolium (cf. Williams et al., 1959) and with cytochrome b_5. The enzyme exhibits no pyridine nucleotide transhydrogenase activity.

Inhibitors. The enzyme is inhibited by the flavin antagonists, atebrin (2.5×10^{-3}) and chlorpromazine (1.5×10^{-3}), as well as by flavin adenine nucleotide (5×10^{-4}), whereas flavin mononucleotide has only a slight effect (30 per cent inhibition at 2.5×10^{-3}). In parentheses are given the molar concentrations required for half-inhibition, unless otherwise indicated. Marked inhibition of the enzyme is obtained also with the sulfhydryl-inhibitors, p-chloromercuribenzoate (1×10^{-4}) and o-iodosobenzoate (4×10^{-4}), and furthermore with the thyroid-active compounds, thyroxine (6×10^{-5}), triiodothyronine (6×10^{-5}) and tetraiodothyronoacetate (2.2×10^{-5}). All these data refer to measurements made with DCPIP as electron acceptor and albumin as activator.

Dicumarol, which is the most potent inhibitor of DT diaphorase found so far, inhibits the enzyme to 50 per cent at a concentration of 10^{-8}M without albumin, and 10^{-7}M with albumin. This effect of albumin is not shared by Tween, indicating that it is probably not related to the activation of the enzyme. The dicumarol inhibition is independent of the nature of the electron acceptor, the same degree of inhibition being obtained when DCPIP is replaced by p-benzoquinone, vitamin K_3, or ferricyanide. The extent of inhibition obtained with a given concentration of dicumarol is independent of the amount of enzyme.

Occurrence in mitochondria and microsomes. Although DT diaphorase is concentrated in the soluble fraction of the cytoplasm, its occurrence has been established as well in both mitochondria and microsomes from which it could be selectively extracted after mechanical disruption with a Turrax blender. It accounts for the entire TPNH diaphorase activity of the mitochondria (cf. Stein and Kap-

lan, 1958) and for about one-half of the TPNH diaphorase activity of the microsomes, In addition, both mitochondria and microsomes contain a specific DPNH diaphorase (D diaphorase) and microsomes also a TPNH diaphorase (T diaphorase), which is probably specific for TPNH and which can be clearly separated from the DT diaphorase. These diaphorases are not extracted in the treatment indicated above. The quantitative cytoplasmic distribution of the three types of diaphorase is the following (approximate mean values of several determinations with no activator added):

Cytoplasmic fraction	Protein mg/g liver	Diaphorase activity (μmoles DCPIP reduced/min./g liver) Total		Accounted for by:		
		DPNH	TPNH	D diaph.	DT diaph.	T diaph.
Mitochondrial	20	4	1	3	1	0
Microsomal	30	10	2	9	1	1
Soluble	90	30	30	0	30	0

For details of tissue fractionation, see Ernster and Navazio, 1958. The test system contained 0.04 mM DCPIP, 0.1 mM DPNH or TPNH, 0.33 mM KCN and 0.05 M potassium phosphate, pH 7.5.

Reactivity with vitamin K and dicumarol-sensitivity. While all three types of diaphorase were found to react with vitamin K (strongly with K_3 and weakly with K_1) only the DT diaphorase proved to be sensitive to dicumarol. This fact, together with the finding that the DT diaphorase is dicumarol-sensitive even with electron acceptors other than vitamin K, indicates that dicumarol-sensitivity and reactivity with vitamin K are not intrinsically correlated properties.

The present enzyme is similar to the vitamin K reductase described by Martius and Märki (Martius and Märki, 1957; Martius, 1959) in several respects. These are its reactivity with vitamin K, its reactivity with both DPNH and TPNH, and its sensitivity to dicumarol and thyroxine. However, it markedly differs from Martius enzyme in that it reacts only very slowly with vitamin K_1 (data concerning other electron acceptors have not been reported by Martius), that it is inhibited by p-chloromercuribenzoate, and that its inhibition by dicumarol, which is reported to be competitive in the case of vitamin K reductase, is independent of the concentration of vitamin K and even of the nature of the electron

acceptor used.

Function. The cellular function of DT diaphorase is sought along three lines: (1) its abundant occurrence in the soluble cytoplasm suggests a possible function as a catalytic link between cytoplasmic reduced pyridine nucleotides and the mitochondrial respiratory chain (cf. Conover and Ernster, this issue); (2) being the major TPNH diaphorase principle of the liver cell, it may constitute a strategic point in the regulation of TPN-linked oxidations, both mitochondrial and cytoplasmic; (3) in view of its distinctive sensitivity to dicumarol, a general uncoupler of mitochondrial electron transport-coupled phosphorylation, it may have a key function in the coupling of mitochondrial electron transport to phosphorylation.

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