PURIFICATION AND SOME PROPERTIES OF A BRAIN DIAPHORASE

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During the course of our studies of the oxidation of reduced pyridine nucleotides by brain tissue (Englard and Strecker, 1956; Giuditta and Strecker, 1958; 1959) we have extracted two enzymes from ox cerebral cortex which catalyzed the oxidation of both DPNH and TPNH by various electron acceptors (Levine et al, 1960). One of these enzymes has been highly purified and appears to differ in some characteristics from other enzymes of animal origin with diaphorase activity. In view of recent reports by Martius (1959) and Ernster et al (1960) on somewhat similar enzymes found in rat and beef liver, we are reporting at this time some of the properties of the brain enzyme.

Occurrence - The enzyme has been found in the supernatant fraction obtained by high speed centrifugation of either 0.25 M or 0.88 M sucrose homogenates of either rat or ox cerebral cortex and is also present, although at lower concentrations in human, rabbit and pigeon brain.

Purification - The enzyme was readily extracted by homogenizing the tissue with water. The aqueous extract was adjusted to pH 5.0 and centrifuged. The supernatant fluid was fractionated between 52 and 72 per cent saturation with ammonium sulfate. The precipitate thus obtained was dissolved in H_2O and dialyzed. Enzymic activity was adsorbed onto and eluted

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from calcium phosphate gel. Ammonium sulfate was added to the gel eluate to 80 per cent saturation. The precipitate contained the enzyme purified more than 200 fold relative to the initial aqueous extract.

Requirements for Activity - The purified enzyme contained flavin adenine dinucleotide which was dissociated from the apoprotein by treatment with acid. An increase of activity at all stages of purification was obtained by the additions of either FAD (10^{-6}M) or FMN (10^{-5}M). The flavin moiety appeared to be partially removed during the purification procedure and the final preparation was stimulated about six fold by the addition of FAD whereas the first extract was stimulated about 50 per cent. Bovine serum albumin (55μg/ml) increased the activity at all steps of purification about 70 per cent. Our routine assay used menadione (2-methyl-1,4-naphthoquinone) as acceptor at a concentration of $1x10^{-5}$M. Higher concentrations were inhibitory.

Electron Donor Specificity - DPNH and TPNH were both oxidized. Under the conditions of assay with menadione as acceptor, our most active preparations had a specific activity with DPNH of 140 μmoles per min per mg of protein and with TPNH about 180 μmoles per min/mg. At pH 7.3, maximal velocities at non-inhibitory concentrations of menadione were calculated to be about 380 μmoles per min per mg with an apparent Km of $1.2x10^{-4}$M for DPNH. For TPNH, the corresponding values were 220 μmoles per min per mg and $1.2x10^{-5}$M.

Electron Acceptor Specificity - Vitamins K_1 and K_3 (menadione), methylene blue, ferricyanide and 2,6-dichlorophenol-indophenol were quite active with either DPNH or TPNH. Table I compares the maximal velocities at pH 7.3 as calculated from a plot of reciprocal velocity vs. reciprocal acceptor concentration for the first four acceptors, using a concentration of $1x10^{-4}$M pyridine nucleotide which is not saturating for DPNH. The velocity with 2,6-dichlorophenol-indophenol although greater than with menadione varied according to the source of the dye, even after further purification of the compound. The brain enzyme was essentially inactive

with cytochrome c, coenzyme Q_{10}, lipoic acid, liver microsomal cytochrome, Janus green and various tetrazolium dyes.

Table I

Activity of brain diaphorase with various electron acceptors

Electron Acceptor	DPNH	TPNH
	μmoles/min/mg	μmoles/min/mg
Menadione	175	687
Vitamin K_1	69	64
Methylene blue	33	41
Potassium ferricyanide	290	176

The incubation mixture contained 150 μmoles of potassium phosphate pH 7.3, 0.3 μmole of reduced pyridine nucleotide, 0.74×10^{-6}M FAD, 500 μg of bovine serum albumin, 0.05-0.2 μg of enzyme and varying concentrations of electron acceptor, in a total volume of 3.0 ml.

Inhibitors - As previously reported (Englard and Strecker, 1956) and in common with the liver enzyme, the brain diaphorase was highly sensitive to dicumarol. The inhibition is not competitive with DPNH. In addition inibition was obtained with relatively low concentrations of 2,4-dinitrophenol, p-hydroxymercuribenzoate, o-phenanthroline, N-ethyl maleimide and amytal. Table II compares the concentrations required for 50 per cent inhibition of the rate of oxidation of DPNH with menadione as acceptor. It is of interest that for maximum effect by o-phenanthroline, preincubation of the enzyme with reduced pyridine nucleotide is required. Menadione as noted previously, is also inhibitory at concentrations above 1×10^{-5}M when reduced pyridine nucleotide was at 1×10^{-4}M. A plot of reciprocal velocity vs. reciprocal pyridine nucleotide concentrations indicated the inhibition to be competitive. Vitamin K_1 was not inhibitory at concentrations up to 1.5×10^{-4}M. Antimycin A was also not inhibitory.

Table II

Inhibitors of brain diaphorase

Inhibitor	Concentrations required for 50% inhibition
	M
Dicumarol	$3x10^{-8}$
p-Hydroxymercuribenzoate	$3x10^{-5}$
Dinitrophenol	$5.6x10^{-5}$
o-Phenanthroline	$5.7x10^{-5}$
N-Ethyl Maleimide	$1x10^{-4}$
Amytal	$6x10^{-3}$

DISCUSSION

The DT diaphorase of Ernster et al (1960) has been reported to be present in the soluble fraction of rat liver homogenates, whereas the vitamin K reductase of Martius (1959) was reportedly obtained from the mitochondrial fraction. We have found that two enzymes with diaphorase activity can be extracted from brain (Levine et al 1960) and are presently purifying a second DPNH and TPNH diaphorase. Preliminary experiments indicate this second diaphorase to have a similar spectrum of electron acceptor specificity as the enzyme described in this communication. This second diaphorase however seems to be associated with the particulate fraction of brain homogenates. One of the aims of experiments now in progress is to elucidate the relationship of these two enzymes to each other and compare them with the liver enzymes.

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