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Brain NADH-tetraazolium reductase activity, lipoamide dehydrogenase and activating lipids

During the past several years a number of enzymes catalyzing the oxidation of nicotinamide nucleotides have been identified in brain[1-5]. In the course of these investigations tetraazolium salts and Janus Green B were tested as electron acceptors. These compounds have been widely used, especially by cytochemists, to determine the intracellular location of nicotinamide nucleotide-linked oxidative enzymes. In using these dyes it was assumed that they were reduced by components of the electron-transfer chain in mitochondria or microsomes[6-8].

With brain homogenates, active acceptors for NADH and NADPH oxidation were found to include tetraazolium violet, tetraazolium blue, neotetraazolium chloride, triphenyltetraazolium chloride, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetraazolium chloride and Janus Green B. NADH was oxidized more rapidly than NADPH. In attempting to solubilize the enzyme or enzymes catalyzing these reactions, extracts of acetone-dried brain were made which reduced INT and Janus Green B with NADH and much more slowly with NADPH. The other tetraazolium compounds showed little or no activity with this soluble extract. Experiments aimed at finding optimal conditions for retention of enzymic activity during storage revealed considerable stimulation of NADH–INT reductase activity by Mephyton, a commercial suspension of Vitamin K_1 and lecithin (Table I, Line 1). Part of this

TABLE I

EFFECT OF LECITHIN AND OF MEPHYTON ON NADH–INT REDUCTASE ACTIVITY

NADH–INT reductase activity was measured spectrophotometrically at 500 mμ at 28–30°, in a total volume of 3.0 ml, containing 0.05 M potassium phosphate buffer (pH 8.2), $3.0 \cdot 10^{-4}$ M NADH and 0.53 mg/ml of INT; lecithin, 0.33 mg/ml; Mephyton equivalent to 0.4 mg Vitamin K_1 and 0.08 mg of lecithin.

Preparation	*NADH oxidized (μmoles/min/ml)*		
	No addition	*+ lecithin*	*+ mephyton*
Acetone-powder extract	0.016	0.096	0.667
Brain NAD(P)H oxidoreductase	0.096	0.483	25.8
DEAE-cellulose eluate	0.012	0.035	0.035

stimulation was found to be due to the reduction of Vitamin K_1, catalyzed by the dicumarol-sensitive NAD(P)H oxidoreductases* (ref. 2–5) present in the extract, followed by the non-enzymic reduction of INT by the reduced naphthoquinone. This was verified by experiments with purified brain enzyme[4] (specific activity = 188 μmoles NADH/min/mg protein) and vitamin K_1. Nevertheless, stimulation with both the acetone-powder extract and with purified enzyme was also obtained with lecithin alone in the absence of a quinone carrier (Table I, Line 2).

Abbreviation: INT, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetraazolium chloride.

* These enzymes have been referred to as diaphorases in previous publications in order to maintain historical continuity with the original observations of ADLER *et al.*[9]. They are listed as menadione reductase (EC 1.6.5.2) by the Enzyme Commission.

To obtain an NADH–INT reductase activity free of the brain NAD(P)H oxidoreductases, the acetone-powder extract was fractionated with ammonium sulfate and then chromatographed on a DEAE-cellulose column. An eluate devoid of the latter activity as determined with menadione as acceptor, was still stimulated by lecithin but did not show any additional effect of Mephyton (Table I, Line 3).

In addition to lecithin, stimulation of this partially purified enzymic activity was obtained with lipid fractions from ox brain prepared by treatment with chloroform–methanol[10] followed by passage through a silicic acid column[11]. Active fractions included the total lipid extract, as well as one containing chiefly neutral lipids and lecithin, and one containing chiefly cerebrosides. Stimulation was also obtained with detergents such as Triton X-100 and Tween 20 and 80.

With this fraction, other active acceptors included 2,6-dichlorophenolindophenol, $K_3Fe(CN)_6$, methylene blue, Janus Green B and lipoamide. Inactive acceptors included oxygen, benzoquinones, naphthoquinones, cytochrome *c* and coenzyme Q_{10}. Stimulation with Triton X-100 was only obtained with INT (Table II).

TABLE II

NADH DEHYDROGENASE ACTIVITY OF THE DEAE-CELLULOSE ELUATE* WITH VARIOUS ACCEPTORS

Conditions as described in Table I, and in previous papers[1,2] for INT, 2,6-dichlorophenolindophenol and methylene blue. Activity with Janus Green B and lipoamide was measured spectrophotometrically at 340 mμ. The concentrations of Janus Green B and DL-lipoamide were $3 \cdot 10^{-5}$ M and $8 \cdot 10^{-4}$ M, respectively. 0.1 ml of a 1:10 dilution of Triton X-100 was used per 3 ml total volume.

Acceptor	DPNH oxidized (μmole/min/ml)	
	without Triton X-100	with Triton X-100
INT	0.30	3.60
2,6-Dichlorophenolindophenol	0.23	0.11
Potassium ferricyanide	0.54	0.64
Janus Green B	1.26	0.20
Methylene Blue	0.40	0.20
Lipoamide	2.16	1.80

* Concentrated by ultrafiltration.

Because of the high activity of the acetone-powder extract with lipoamide, the solution was treated by the same procedure as that used by MASSEY for the lipoamide dehydrogenase (EC 1.6.4.3) of heart[12], *i.e.* precipitation by 50% ammonium sulfate followed by passage through a calcium phosphate gel–cellulose column. Two active fractions were obtained. Fraction A was not adsorbed by the column and was not active with lipoamide but did catalyze the reduction of INT by NADH. Fraction C, like heart lipoamide dehydrogenase, was adsorbed by the gel and eluted with 4% ammonium sulfate and was active with lipoamide. As shown in Table III the activity of both fractions with INT as acceptor was stimulated by Triton X-100. Fraction C was inhibited by EDTA which also inhibits heart lipoamide dehydrogenase. To confirm the reactivity of INT and detergents with lipoamide dehydrogenase, a sample of pure heart lipoamide dehydrogenase was tested and found to behave like

TABLE III

COMPARISON OF CALCIUM PHOSPHATE-GEL FRACTIONS WITH LIPOAMIDE DEHYDROGENASE

Conditions were as in previous tables. 1 mM EDTA.

Component	DPNH oxidized (μmoles/min/ml)		
	Fraction A	Fraction C	Lipoamide dehydrogenase
INT alone	0.145	0.064	0.152
INT + Triton	0.380	2.510	0.565
INT + Triton + EDTA	0.380	0.325	0.120
Lipoamide	0.005	2.950	1.030

Fraction C (Table III), and catalyzed the reduction of both INT and Janus Green B.

These results demonstrate that a large part of the NADH–INT reductase activity in easily soluble form in brain is due to lipoamide dehydrogenase. The slight activity of brain NAD(P)H oxidoreductase with INT as acceptor is enhanced by lipids and detergents. The basis of the major activity with INT which remains in the residual brain suspension as well as the activity in Fraction A remains to be explored.

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