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PATHWAYS OF REDUCED PYRIDINE NUCLEOTIDE OXIDATION IN RAT-BRAIN HOMOGENATE DEMONSTRATED BY A TETRAZOLIUM METHOD

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SUMMARY

In order to facilitate the development of satisfactory histochemical methods for reduced pyridine nucleotide oxidative enzymes, studies with sucrose homogenates of rat brain were undertaken. For this purpose either oxygen or an *N*-thiazol substituted monotetrazole were used as final electron acceptor. A quinone (menadione) was used as an intermediary carrier.

Comparable activities with both $NADH_2$ and $NADPH_2$ were obtained with the mitochondrial and cytoplasmic fractions.

Tetrazolium reduction by enzymically formed hydroquinone was faster than its autoxidation, independent of pH. Kinetic activation of menadione reduction was achieved by irreversible removal of hydrogen by formazan formation. An equimolar relationship was found between reduction of monotetrazole and oxidation of reduced pyridine nucleotide substrate.

Comparison of apparent K_m values at the optimum pH suggested a close relationship between mitochondrial and extramitochondrial menadione reductase. The kinetic data indicate that mitochondrial $NADH_2$–$NADPH_2$–tetrazolium reductase is not the same enzyme as menadione reductase.

It is suggested that tetrazolium methods can be used for parallel studies of the activity of alternative pathways of electron transport in tissue fractions and sections.

INTRODUCTION

Studies by GIUDITTA AND STRECKER[1] have indicated that alternative pathways of reduced pyridine nucleotide oxidation exist in cerebral tissue. These authors distinguished between an $NADH_2$-oxidative pathway in rat-brain mitochondria and an $NADH_2$/$NADPH_2$–cytochrome *c* reductase probably located in the endoplasmic reticulum. The mitochondrial enzyme system was sensitive to both Antimycin A and Amytal in contrast to the extra-mitochondrial reductase which was not inhibited by these agents. In subsequent investigations LEVINE *et al.*[2] succeeded in characterizing two additional types of particulate diaphorase, in water and phosphate buffer extracts

Abbreviation: MTT, 3-(4,5-dimethyl-thiazolyl-2-)-2,5-diphenyltetrazolium bromide.

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from beef-brain homogenate. These enzymes catalyzed the oxidation of both $NADH_2$ and $NADPH_2$ by a number of electron acceptors, menadione being the most active. A purified diaphorase preparation from water extracts of beef cerebral cortex oxidized $NADPH_2$ at higher rates than $NADH_2$ in the presence of menadione. FAD was identified as the prosthetic group of this enzyme[3].

In attempting to localize both particulate and soluble reductase activity in the central nervous system by a histochemical method we used an irreversible electron acceptor (tetrazolium) in order to obtain a highly coloured end-product (formazan). Tetrazolium reductase activity was observed to be present only in the mitochondrial fraction of brain homogenate prepared in 0.25 M sucrose. With frozen–thawed mitochondria both $NADH_2$ and $NADPH_2$ served as electron donors. With menadione as electron acceptor, quinone reductase activity was found in both the mitochondrial and extra-mitochondrial (cytoplasmic) portion of the homogenate.

Since enzymically produced hydroquinone is capable of reducing tetrazolium salt quantitatively[4], measurement of formazan can be used for the estimation of quinone reductase activity. In this paper we describe the kinetic properties of enzyme systems in which a reversible intermediate electron acceptor (menadione) and an irreversible final reduction indicator (tetrazole) were used together. Preliminary accounts of this work have been published[5,6].

Histochemical application of the methods reported below, demonstrated the main activity of non-mitochondrial menadione reductase in the white matter of the brain and spinal cord[7].

MATERIAL AND METHODS

Animals

Male and female rats of the Medical Research Council hooded strain were used (body weight 150–200 g). The animals were bled from the carotid arteries under light ether anaesthesia. The whole brain, including the cerebellum, was removed quickly, freed from meningeal tissue, chopped with scissors and weighed.

Enzyme preparation

Brain tissue was homogenized for 3 min at 0° in an all-glass homogenizer of the Potter-Elvehjem type in 10 parts (v/w) aq. 0.25 M sucrose containing 0.1 mM EDTA adjusted to pH 7.3 with NaOH. The homogenate was centrifuged twice at 1000 × *g* for 5 min to remove nuclei and cell debris and the resulting supernatant at 10000 × *g* for 15 min to yield mitochondria. These operations were carried out at 0° in an MSE-centrifuge using the high-speed attachment. The method for preparing the mitochondrial fraction is similar to that of ABOOD AND ALEXANDER[8] except that no detergent (Triton) was added. After resuspending twice in 0.25 M sucrose–EDTA (10 parts, v/v) the mitochondrial fraction was twice frozen to −70° and thawed to +2° and used for enzymic assay. The supernatant fluid, after removal of the mitochondria and the whitish "fluffy" layer, was considered to contain possible microsomal and soluble enzymes. This was used as the "cytoplasmic" fraction. It was stored at −70° before use.

Substrates

$NADH_2$ and $NADPH_2$ were obtained from C. F. Boehringer and Soehne, Mannheim. The concentrations were calculated from the absorbancy at 340 mμ using the molecular extinction coefficient, $\varepsilon = 6.22 \cdot 10^6 (cm^2/mole)$ (see ref. 9).

Electron acceptors

Menadione was obtained from L. Light and Co., Colnbrook, Bucks, and recrystallized twice from methanol. Fresh solutions (kept protected from light) were prepared from each experiment by dissolving 20 μmoles in 1 ml of acetone and diluting with water. Reduced menadione was prepared according to FIESER[10]. Both menadioneand reduced menadione yielded absorption spectra identical to those recorded by COLPA-BOONSTRA AND SLATER[11]. MTT was a gift from Prof. H. BEYER. MTT–form azan (BEYER AND PYL[12], mol. wt. 335.4) was extracted in ethyl acetate (AnalaR grade) and the concentration determined from the absorbancy at 560 mμ where maximum extinction was observed. A calibration curve, showing a linear relationship between formazan concentration and absorbancy is given in Fig. 1.

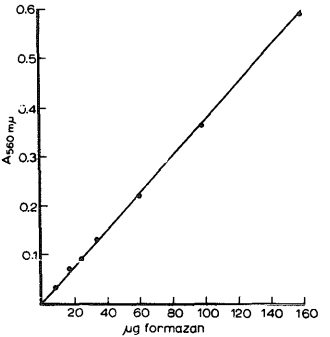

Fig. 1. Concentration curve of MTT–formazan dissolved in 4 ml ethylacetate. The formazan was prepared from the tetrazolium bromide by reduction with excess 0.1 M ascorbic acid.

Protein

Protein was determined by the biuret method[13].

Reductase assay

In the standard assay for menadione reductase the following were placed in a silica cell (light path 1 cm, final volume 3 ml, final concentration given): Na_2HPO_4–NaH_2PO_4 buffer (pH 7.4), 60 mM; menadione, 0.1 mM (with mitochondria) or 0.01 mM (with cytoplasmic fraction); $NADH_2$ or $NADPH_2$, 0.05 mM. Enzyme suspension (0.1–0.2 ml; 0.2–0.5 mg protein) was added and $E_{340\ m\mu}$ measured at 30-sec intervals for the first 5 min. When formazan production was measured, MTT (0.1 mM) was incorporated in the above medium, prepared in ouadruplicate, and formazan was extracted in 4 ml ethyl acetate at 5-min intervals.

For tetrazolium reductase assay (mitochondria) the MTT-supplemented medium was used without menadione.

All absorbancy measurements were made in a Unicam SP-500 spectrophotometer at 20°–22°.

Sources of other materials

Dicoumarol, California Corp. for Biochem. Research, Los Angeles (U.S.A.); Amytal (sodium 5-ethyl-5-isoamylbarbiturate), Savory and Moore, Ltd., London; 2-phenyl-1,3-indanedione, (phenindione) Evans, Dindevan; Antimycin A, FAD and FMN, Sigma Chem. Co., St. Louis, Mo. (U.S.A.); *p*-chloromercuribenzoate, L. Light and Co. Ltd., Colnbrook, Bucks; 2,4-dinitrophenol, British Drug Houses, Ltd., Poole, Dorset.

RESULTS

Oxidation of reduced coenzymes by brain fractions

The mitochondrial and the cytoplasmic fraction catalyzed the oxidation of both $NADH_2$ and $NADPH_2$ in presence of menadione. The reactions proceeded linearly with time for the first 12 min and, under the conditions used, the activities were proportional to enzyme concentration. Maximum velocities of the preparations with both substrates are given in Table II.

Using menadione as primary electron acceptor in the presence of O_2, plotting of initial velocities against pH revealed two distinct optima for the mitochondrial enzyme and one single optimum in the alkaline range for the cytoplasmic fraction (Fig. 2). Similar curves were obtained for both $NADH_2$ and $NADPH_2$. Below pH 6.24 the mitochondrial oxidation of pyridine nucleotide becomes dependent on the function of menadione oxidase[11], since the reduced quinone is only slowly oxidized by O_2 in the acid range. On the addition of sodium cyanide (0.01 M) or of sodium azide (0.01 M) the rate of menadione-mediated $NADH_2$ oxidation was reduced 80% and 70% respectively. Conversely, the quinone-dependent oxidation of $NADH_2$ by the cytoplasmic fraction (which lacks the oxidase system) required a pH higher than 6.6 to run

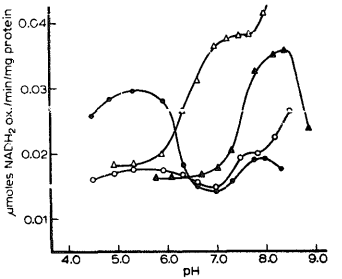

Fig. 2. Oxidation of $NADH_2$ in presence of menadione by brain fractions as a function of pH. The assay system given in the METHODS section was used, employing phosphate buffer at various pH levels. The incubation mixture (final volume 3 ml) contained 0.5 mg protein. MTT was used together with sodium azide (0.01 M) incorporated in the medium. Mitochondrial fraction with O_2 (●—●) or with MTT (○—○) as final acceptor; cytoplasmic fraction with O_2 (▲—▲) or with MTT (△—△).

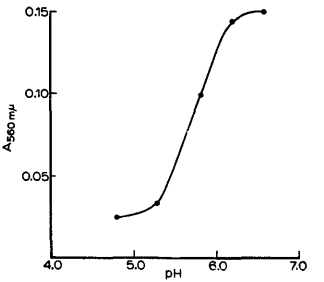

Fig. 3. pH-dependence of reduction of monotetrazole by reduced menadione. 0.2 μmole MTT was dissolved in 3 ml 0.06 M phosphate buffer containing 20% acetone to which was added 0.15 μmole reduced menadione. After 60 sec the formazan formed was extracted in 4 ml ethylacetate and measured at 560 mμ.

to completion in presence of O_2. At pH 6.24 the reaction ceased after 65 % of $NADH_2$ had been oxidized, although the initial reaction rate (first 10 min) was identical to that observed at pH 6.6. A similar effect was noted when, at pH 7.4, the reaction was performed under nitrogen. In this system it was shown that reduced menadione was inhibitory in a manner similar to that described by FRIMMER[14]. Conclusive kinetic data were not obtained, however, because at a concentration higher than 0.1 mM oxidized menadione was also inhibitory.

Influence of tetrazolium on menadione-mediated oxidations

In presence of menadione, formazan formation by oxidation of $NADH_2$ was linear with time for at least 20 min, with both mitochondrial and cytoplasmic enzymes. At both pH 6.24 and pH 7.4 the reaction led to complete oxidation of substrate. Similarly, tetrazolium was reduced linearly with both $NADH_2$ and $NADPH_2$ by brain mitochondria in the absence of menadione. However, the rate of this "tetrazolium-reductase" reaction was only 30–50 % of the rate of menadione-mediated formazan production.

Non-enzymic reduction of MTT by reduced menadione[4] was found to be stoichiometric. The velocity of this reaction was dependent upon pH as shown in Fig. 3. Independently of pH MTT reduction was always faster than autoxidation of reduced menadione in the presence of O_2 and identical amounts of formazan were produced by reduced menadione, whether the reaction was performed under oxygen or nitrogen.

The nature of the kinetic activation of enzymic menadione reduction by MTT acting as irreversible electron acceptor differed in the case of the mitochondrial and cytoplasmic enzymes. The effect was best shown at a low pH, where autoxidation of reduced menadione is slow and rate limiting (Figs. 4 and 5). Due to high activity of

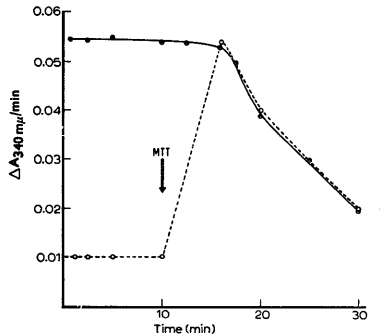

Fig. 4. $NADH_2$ oxidation by the mitochondrial fraction at pH 6.24 in presence of menadione. The reaction mixture (for composition see METHODS section) contained 0.4 mg enzyme protein. Final electron acceptor O_2 (●—●) (no inhibitor used); monotetrazole (MTT) (○—○) in presence of sodium cyanide, 0.01 M; 0.1 mg MTT in 0.1 ml 0.06 M phosphate buffer was added to both substrate and reference cuvette at arrow.

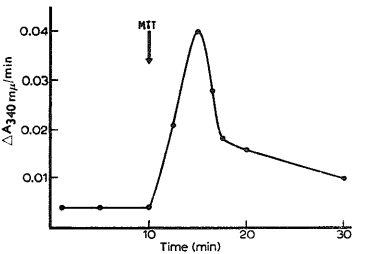

Fig. 5. $NADH_2$ oxidation by the cytoplasmic fraction at pH 6.24 in presence of menadione and sodium azide, 0.01 M. Conditions of experiment as in Fig. 4, except that the medium contained 0.2 mg protein.

reduced-menadione-oxidase system[11] brain mitochondria oxidized $NADH_2$ at a high rate, which could not be increased further by the addition of MTT. However, after inhibiting the oxidase by cyanide (0.01 M), MTT was able to trap electrons from reduced menadione efficiently (in competition with autoxidation) and the reaction rate was increased to its original, non-inhibited level (Fig. 4). At pH 6.6 and below, the cytoplasmic fraction catalyzed $NADH_2$ oxidation *via* menadione only in the presence of tetrazole, if contaminating oxidase was inhibited by cyanide or azide.

The different modes of competition by tetrazole, with reduced-menadione-oxidase on one hand and with O_2 on the other, could be expected to modify the influence of pH on the activity of menadione reduction in presence of the irreversible electron acceptor MTT. As shown in Fig. 2, MTT caused an activation of the cytoplasmic rate at neutral pH, a peak being reached at pH 7.4. Tetrazole interaction with reduced-menadione-oxidase seemed to be less complete at an acid pH; formazan production by the mitochondrial fraction within the region of pH 5–6 was less than could be expected from the rate of $NADH_2$-oxidation observed in presence of O_2 as sole H-acceptor. The rapidly increased tetrazolium reduction observed above pH 8.0 is non-enzymic in nature.

TABLE I

OXIDATION OF $NADH_2$ AND PRODUCTION OF MONOFORMAZAN BY BRAIN MITOCHONDRIAL (M) AND CYTOPLASMIC FRACTIONS (C) IN PRESENCE OF VARYING AMOUNTS OF MENADIONE

The initial oxidation rate for $NADH_2$ was measured at 340 mμ for 2 min in presence of 0.1 mg MTT per 3 ml incubation medium. For measurement of extractable formazan after 5 and 10 min, the medium contained 0.1 mg MTT per ml. Other conditions of the experiment as described in the METHODS section.

Fraction	*Menadione concentration (mM)*	*$NADH_2$ oxidation (μmoles/min/mg)*	*MTT–formazan (μmoles/min/mg)*
M		0.010	0.009
M	0.1	0.022	0.020
C	0.3	0.025	0.026
C	0.07	0.034	0.030
C	0.007	0.036	0.032

Stoichiometry of tetrazolium reduction

The stoichiometry of the reaction was studied by comparing $NADH_2$ oxidation in the presence of both menadione and MTT (initial reaction rate) with the amount of formazan extracted after 5 and 10 min. Table I indicates the values obtained with various concentrations of menadione as intermediate carrier. An equimolar relationship between substrate oxidation and formazan production was obtained independently of menadione concentration with both enzyme fractions tested. With the mitochondrial enzyme, $NADH_2$ oxidation and formazan formation were also equimolar in the absence of menadione, though the reaction rate was reduced considerably.

Kinetic constants

Table II shows the values of apparent K_m and the velocity at infinite substrate concentration (V_{max}) for $NADH_2$ and $NADPH_2$ and for mitochondrial and cytoplasmic enzyme, derived from double-reciprocal plots[15] of results obtained by measuring

TABLE II

K_m AND V_{max} FOR PYRIDINE NUCLEOTIDES FOR MITOCHONDRIAL (M) AND CYTOPLASMIC (C) FRACTIONS

The incubation mixtures and experimental conditions were as described in the METHODS section. In varying the substrate ($NADH_2$ or $NADPH_2$) concentration the reaction velocity was determined by either observing ΔA at 340 mμ (a) or by measuring formazan production (b) in parallel experiments. The reaction was initiated by adding 0.3 mg of enzyme protein. Values are given for menadione reductase and for mitochondrial tetrazolium reductase.

Enzyme fraction	*Substrate*	*Menadione (mM)*	*pH*	*V_{max} (calculated) (μmoles/min/mg)*	*K_m (moles/l) ($\times 10^4$)*	
					a	*b*
M	$NADH_2$	—	7.6	0.017	2	2
M	$NADH_2$	0.1	7.6	0.028	0.9	0.8
M	$NADPH_2$	—	7.6	0.30	2	1.9
M	$NADPH_2$	0.1	7.6	0.40	0.8	0.8
C	$NADH_2$	0.01	7.4	0.42	1.0	1.2
C	$NADPH_2$	0.01	7.4	0.048	1.1	1.0

either substrate oxidation or formazan production at varying substrate concentrations. K_m values obtained by both methods were in reasonable agreement.

From Table II it may be concluded that with menadione as intermediate carrier the oxidation of both $NADH_2$ and $NADPH_2$ is catalyzed by a similar enzyme system situated either in the mitochondrial or cytoplasmic fraction. The direct reduction of tetrazolium by brain mitochondria seems catalyzed by an enzyme system which, although specific for both $NADH_2$ and $NADPH_2$, is different from the menadione-

TABLE III

ACTION OF VARIOUS INHIBITORS ON TETRAZOLIUM REDUCTION BY MITOCHONDRIAL AND CYTOPLASMIC FRACTIONS

The enzyme suspensions (0.3–0.5 mg protein/3 ml) were pre-incubated in the assay medium (METHODS section) containing the inhibitor, at 22° for 3 min before the substrate, $NADH_2$, was added to initiate the reaction. Incubation was for 3 min at 22°. The concentration of inhibitor necessary for 50% inhibition of tetrazolium reduction is recorded.

Inhibitor	*Mitochondria*	*Mitochondria plus menadione (0.1 mM)*	*Cytoplasmic fraction plus menadione (0.01 mM)*
Dicoumarol	$6 \cdot 10^{-5}$	$8 \cdot 10^{-7}$	$2 \cdot 10^{-8}$
Phenyl-indanedione	$15 \cdot 10^{-4}$	$5 \cdot 10^{-5}$	$1 \cdot 10^{-4}$
Amytal	No inhibition	$2 \cdot 10^{-5}$	$2 \cdot 10^{-3}$
p-Chloro-mercuri-benzoate	$5 \cdot 10^{-5}$	$3 \cdot 10^{-6}$	$7 \cdot 10^{-6}$
2,4-Dinitro-phenol	$1 \cdot 10^{-2}$	$1 \cdot 10^{-2}$	$6 \cdot 10^{-5}$
Antimycin A	No inhibition up to 10 μg/ml		

mediated pathway. Determining either change in absorbancy of coenzyme, or formazan production at various substrate concentrations, yielded almost identical K_m-values for a given crude enzyme fraction.

Activators

Possible cofactor requirements were tested with mitochondrial and cytoplasmic fractions by adding either FAD (0.001 mM) or FMN (0.1–0.001 mM) to the standard assay systems at various pH values. Both FAD and FMN are without influence on the oxidation of $NADH_2$ and $NADPH_2$. Likewise, the addition of bovine albumin (1 mg to a total volume of 3.0 ml) did not alter the reaction rate.

Inhibitors

A number of inhibitors was used with both mitochondria and cytoplasmic fraction in the standard assay procedure with $NADH_2$ as substrate at pH 7.4, with or without menadione. The concentrations of inhibitors necessary for 50 % inhibition of tetrazolium reduction are given in Table III.

DISCUSSION

Using tetrazolium as terminal electron acceptor, at least three reductase systems have been distinguished in crude 0.25 M sucrose homogenate of rat brain. These enzymes are specific for both $NADH_2$ and $NADPH_2$ and may be classified as diaphorases. When tetrazole is used as sole H-acceptor, mitochondrial formazan production is insensitive to both Amytal and Antimycin A. The metabolic function of this mitochondrial tetrazolium reductase is possibly related to lipoyl dehydrogenase[16]. In keeping with this assumption is the present finding that, in absence of any added intermediate electron carrier, one mole of tetrazole is reduced per mole of reduced coenzyme oxidized. The active form of lipoyl dehydrogenase from pig heart has been demonstrated by MASSEY AND VEEGER[17] to be a half-reduced form in which both a flavin and a disulphide moiety are only partially reduced. The mitochondrial $NADH_2$, $NADPH_2$–tetrazolium reductase of rat brain is different from the succinate–tetrazolium reductase of the same preparation. We found that with frozen–thawed mitochondria and sodium succinate (0.04 M) as substrate in the present tetrazolium reductase assay system, inhibition of formazan production by Antimycin A amounted to 68 %. Some studies by NACHLAS, MARGULIES AND SELIGMAN[18] suggest that the degree of interference shown by this inhibitor, on tetrazolium reduction by the succinoxidase system, may be dependent on the nature of the tetrazolium salt used as electron acceptor.

High activity of a quinone (menadione) as electron acceptor used as intermediary carrier to tetrazole, was found in both frozen–thawed mitochondria and the remaining non-mitochondrial cytoplasmic fraction. Flavoprotein enzymes of the latter are inactive with tetrazolium salts as has been shown already by GUIDITTA AND STRECKER[3]. The equimolar relationship between $NADH_2$ oxidation and menadione-mediated formazan formation is explicable on the assumption of a two-step reaction as proposed by WOSILAIT AND NASON[19]: The first (enzymic) step leads to the formation of hydroquinone anion which, in the second step of the reaction, forms hydroquinone. Thus two hydrogens can be transferred to one molecule of monotetrazole from one

molecule of $NADH_2$. In the presence of excess tetrazole, the amount of quinone in the reaction mixture is not rate limiting. This mechanism is probably the only one concerned in the kinetic activation of menadione-mediated $NADH_2$ oxidation by tetrazole. It is similar in nature to the activation of menadione reduction by ferricyanide or ethyleneiminoquinones observed by FRIMMER[14] with an $NADH_2$ quinone reductase from pig liver.

Brain diaphorase exhibits an increasing requirement for exogenous FAD during purification[3]. The addition of FAD to brain homogenate did not influence the reaction, but its presence was necessary to obtain an optimal menadione–tetrazolium reductase reaction in sections from frozen tissue[6]. Apparently, cofactor requirements in histochemical preparations may differ from those observed in homogenates from the same tissue.

A common denominator of both mitochondrial and extra-mitochondrial menadione reductase is their sensitivity to dicoumarol, SH-inhibitors and Amytal. This is in many respects similar to the properties of purified brain diaphorase[3], purified liver diaphorase[10] and particulate $NADH_2$–coenzyme Q reductase[21]. Mitochondrial menadione reductase can be distinguished from cytoplasmic menadione reductase by the higher concentration of dicoumarol, Amytal and 2,4-dinitrophenol required for 50% inhibition of activity. Mitochondrial tetrazolium reductase, on the other hand, is insensitive to Amytal and is about 100-fold less sensitive to dicoumarol than mitochondrial menadione reductase. Neither of the activities tested were affected by Antimycin A. Unlike the other inhibitors 2,4-dinitrophenol acted mainly as an inhibitor of the extramitochondrial enzyme system.

The inhibitor studies presented do not allow us directly to compare the mitochondrial menadione–tetrazolium reductase and its cytoplasmic counterpart either to purified brain diaphorase I (extractable by either 0.25 M sucrose or water[3]) or to diaphorase II (solubilized by treatment with phosphate buffer[22]). The activities as measured by the tetrazolium method can be localized mainly in the white matter[7]. They may represent mixtures of enzymes differing in solubility but having a close spatial relationship along the axonal cytoplasm.

Menadione reductases from tissues other than brain, although similar in electron donor and acceptor specificity, differ in sensitivity to inhibitors known to affect cell respiration and oxidative phosphorylation. Thus, the liver DT-diaphorase (specific for both $NADH_2$ and $NADPH_2$) is highly sensitive to dicoumarol and insensitive to Amytal, while the D-diaphorase and the T-diaphorase are not[23]. The particulate $NADH_2$–coenzyme Q reductase of HATEFI, HAAVIK AND JURTSHUK[21] is sensitive to Amytal but relatively insensitive to dicoumarol.

The physiological intracellular electron acceptor for the brain enzymes tested is not known and their metabolic role therefore remains obscure. As suggested by CONOVER AND ERNSTER[23] it may be that systems of this sort link extramitochondrial reduced pyridine nucleotide to the mitochondrial respiratory chain. Tetrazolium methods are useful in that they allow direct comparison between the behaviour of tissue sections and homogenates.

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