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CHARACTERIZATION OF A MITOCHONDRIAL NADH-DEPENDENT NITRO REDUCTASE FROM RAT BRAIN

HEINZ W. KÖCHLI, BENDICHT WERMUTH * and JEAN-PIERRE VON WARTBURG

Medizinisch-chemisches Institut der Universität, Bühlstrasse 28, CH-3012 Bern 9 (Switzerland)

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Summary

Rat brain mitochondria contain an NADH-linked nitro reductase that converts various aromatic nitro compounds, including the anti-schistosomal agent niridazole, into the hydroxylamine metabolites. The enzyme is tightly bound to the inner membrane and its activity is measurable only after disrupting the mitochondria. Triton X-100 (1%) and sonication partially solubilize the enzyme. The molecular weight determined by gel filtration is approx. 200 000. The temperature optima for the membrane-bound and for the solubilized enzyme are at 35 and 30°C, respectively. The pH optimum for the membrane-bound enzyme is 9.2. NAD^+ and 4-hydroxymercuribenzoate decrease the enzyme activity. Oxygen, carbon monoxide, cyanide, rotenone, barbiturates, chlorpromazine, dicumarol and chelating agents have no effect on the activity. The subcellular localization, substrate specificity and sensitivity to inhibitors distinguish the mitochondrial nitro reductase from the corresponding microsomal and cytosolic enzymes.

Introduction

While studying the distribution of aldehyde reductases (alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.2), in rat brain, we observed that the oxidation of NADH by mitochondrial preparations was related to the presence of compounds carrying a nitro rather than an aldehyde group. The reduction of nitro compounds is known to occur in mammalian tissues. Fouts and Brodie [1] reported the presence of enzyme systems in the liver and other tissues which convert 4-nitrobenzoate and chloramphenicol to the corresponding amines. Present evidence

* To whom correspondence should be addressed.

suggests that there are distinct NADH- and NADPH-dependent nitro reductase systems [2—5]. The NADPH-linked reduction occurs mainly in the endoplasmic reticulum and the NADH-dependent reaction takes place predominantly in the cytosol [2]. Several enzymes, such as cytochrome *P*-450 [6] and NADPH-cytochrome *c* reductase (NADPH:ferricytochrome oxidoreductase, EC 1.5.2.4) [7,8] in the endoplasmic reticulum, and xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2) [9] and aldehyde oxidase (aldehyde:oxygen oxidoreductase, EC 1.2.3.1) [10] in the cytosol have been shown capable of functioning as nitro reductases. However, the exact nature of all nitro reductase systems has not yet been elucidated.

In the present study we have investigated the nitro reductase activity of rat brain mitochondrial preparations. An NADH-linked, membrane-bound enzyme that reduces various aromatic nitro compounds is described.

Experimental

Materials

Pyridine nucleotide coenzymes were obtained from Boehringer, Mannheim, F.R.G. Niridazole (1-[5-nitro-2-thiazolyl]-2-imidazolidinone) was a gift from Ciba-Geigy AG, Basel. Barbiturates, chlorpromazine and imipramine were obtained from Siegfried, Zofingen, Switzerland, and pargyline from Saber, Morton Grove, U.S.A. All other chemicals were of the highest commercially available grade and used without further purification. Deionized water was used for all solutions. Substances only slightly soluble in water were dissolved in methanol before adding to the assay system. Pure methanol in this concentration had no effect on the activity of nitro reductase.

Preparation of subcellular fractions

Sprague-Dawley rats (200—300 g) were killed by decapitation. The brains were removed immediately and homogenized in 9 parts ice-cold 0.32 M sucrose by means of a glass-Teflon Potter-Elvehjem type homogenizer. The homogenate was separated into crude nuclear, crude mitochondrial, microsomal and supernatant fractions as described by Gray and Whittacker [11]. The crude mitochondrial fraction was further separated into myelin, synaptosomes and mitochondrial fractions by centrifugation through a discontinuous density gradient consisting of equal layers of 0.8 M and 1.2 M sucrose at 100 000 × *g* for 1 h.

The purified mitochondria were lysed either by the addition of 0.05—0.1% Triton X-100 or by sonication for twice 30 s using an MSE 150 W sonifier (20 kHz). Mitochondrial membranes were collected by centrifugation at 100 000 × *g* for 1 h. The separated subcellular fractions were dialysed against 5 mM sodium phosphate, pH 7.0 and analyzed for lactate dehydrogenase [12], NADPH-dependent cytochrome *c* reductase [13], acetylcholinesterase [14], monoamine oxidase [15] and glutamate dehydrogenase [16] as marker enzymes for the cytosolic, microsomal, synaptosomal, mitochondrial membrane and mitochondrial matrix fractions, respectively.

Enzyme assay

Nitro reductase activity was measured spectrophotometrically by following

the decrease in absorbance of NADH at 340 nm. The standard assay mixture in a total volume of 1 ml contained: 60 mM sodium pyrophosphate, pH 9.0; 0.08 mM NADH; 0.5 mM nitro compound and 2—4 mU of nitro reductase activity (1,3-dinitrobenzene as substrate) corresponding to 0.1—0.2 mg protein. Triton X-100 (0.05%) was added when using detergent lysed mitochondria, and 2 μM rotenone was added, if the mitochondria had been sonicated. 1 enzyme unit is defined as the decrease in absorbance at 340 nm/min corresponding to the oxidation of 1 μmol NADH.

Protein determination

The concentration of protein was estimated by the method of Lowry et al. [17] using crystalline bovine serum albumin as a standard.

Product analysis

1,4-Dinitrobenzene (0.1 mM) was reduced enzymatically by 0.32 mM NADH in the presence of rat brain mitochondrial membranes and chemically by 0.32 mM ascorbate [18]. The reactions were carried out in 60 mM sodium pyrophosphate pH 9.0, containing 0.05% Triton X-100. The reaction mixtures were extracted with twice the volume ethyl acetate. The organic phase was separated and the solvent evaporated with nitrogen. Equal portions of the yellow residue were taken up in 10 mM HCl and 10 mM NaOH for spectral analysis. The rest of the residue was dissolved in diethyl ether and analyzed by TLC on aluminium oxide (Merck) using the solvent systems ethanol/chloroform (1 : 2) and ethanol/benzene (1 : 4), respectively.

Results

Product analysis

In 1936 Kuhn and Weygand [18] showed that chemical reduction of 1,4-dinitrobenzene by ascorbate yields 4-nitrophenylhydroxylamine which in aqueous solution is yellow at acid pH, red at neutral and slightly alkaline pH and again yellow at pH values above 11. The changes in colour were explained by differently ionized forms of the 4-nitrophenylhydroxylamine molecule. In our experiments, the chemical and enzymatic reduction of 1,4-dinitrobenzene gave products with the same pH-dependent spectral characteristics as described by Kuhn and Weygand. At acid and alkaline pH values the product was stable, but at neutral pH a fading of the original red colour was observed. TLC of the chemically and enzymatically reduced 1,4-dinitrobenzene gave one major spot with the same R_F value. Minor spots were obtained with both reduction procedures, probably due to reaction intermediates and condensation products. No spots were detectable on the chromatograms at the position corresponding to 4-nitroaniline. The formation of NAD^+ during the enzymatic reduction of 1,4-dinitrobenzene by NADH could be demonstrated by the addition of ethanol and alcohol dehydrogenase at the end of the reaction. A rapid increase in absorbance at 340 nm due to the production of NADH was ascertained. Alcohol dehydrogenase or ethanol alone showed no such effect. Theoretically four reduction equivalents are used for the production of 1 molecule 4-nitrophenylhydroxylamine. However, the high instability of the hydroxylamine

TABLE I

SUBCELLULAR LOCALIZATION OF RAT BRAIN NITRO REDUCTASE

Rat brain homogenate was separated into subcellular fractions by differential centrifugation according to Gray and Whittacker [11]. Enzyme activities are quoted as total activities per rat brain. Nitro reductase uses 1,3-dinitrobenzene as substrate and monoamine oxidase uses tyramine as substrate.

	Nitro reductase		Monoamine oxidase		NADPH-cytochrome *c* reductase		Lactate dehydrogenase		Protein	
	mU	%	mU	%	mU	%	U	%	mg	%
1st supernatant	154	100	299	100	99	100	18.5	100	60	100
Crude mitochondria	116	75	174	57	31	31	4.9	26	25	42
Microsomes	2	1.3	54	18	33	33	3.3	18	14	23
Cytosol	0	0	0	0	38	38	8.6	46	17	28

metabolite, even in degassed solutions, prevented the experimental determination of the stoichiometry.

Subcellular localization

Each subcellular fraction, with the exception of the homogenate and the nuclear pellet which were too turbid to be analyzed, was assayed for nitro reductase activity. Using 1,3-dinitrobenzene as substrate, an average of 154 ± 52 mU (mean ± S.D., $n = 8$) per brain was calculated from the activities recovered in the first supernatant fraction. This amount of activity is comparable to the activities of the mitochondrial and microsomal marker enzymes, monoamine oxidase and NADPH-cytochrome *c* reductase, respectively (Table I). The distribution in the remaining fractions showed a predominantly mitochondrial localization for nitro reductase. Less than 2% of the original nitro reductase activity was found in the microsomal and cytosol fractions, whereas the recovery of the marker enzymes NADPH-cytochrome *c* reductase and lactate dehydrogenase was 33 and 46%, respectively. In the myelin, synap-

TABLE II

LOCALIZATION OF RAT BRAIN NITRO REDUCTASE IN THE CRUDE MITOCHONDRIAL FRACTION

The crude mitochondrial fraction was separated into myelin, synaptosomes and mitochondria. The enzyme activities in the crude mitochondrial fraction were set as 100%. Relative activities in the other fractions are actual yields. Nitro reductase uses 1,3-dinitrobenzene as substrate. Monoamine oxidase uses tyramine as substrate.

Fraction	Nitro reductase %	Monoamine oxidase %	Glutamate dehydrogenase %	Acetyl cholinesterase %
Crude mitochondria	100	100	100	100
Myelin	0	1	1	12
Synaptosomes	31	34	19	60
Mitochondria	54	50	69	8

tosomal and mitochondrial fractions, obtained from the crude mitochondrial pellet, nitro reductase activity paralleled the activities of the mitochondrial marker enzymes monoamine oxidase and glutamate dehydrogenase (Table II). About two-thirds of the nitro reductase activity was recovered in the purified mitochondrial fraction and about one-third in the synaptosomal fraction. No activity was associated with the myelin. Acetylcholinesterase as marker enzyme for synaptosomal membranes was present almost exclusively in the synaptosomal fraction.

Submitochondrial localization

Whole mitochondria showed no nitro reductase activity in the absence of detergent. Activity became measurable only after lysis of the mitochondria by detergents or by sonication, indicating that the catalytic site of the enzyme is inaccessible from the outside. Since the outer mitochondrial membrane is permeable for low molecular weight compounds, this suggests a localization of nitro reductase either on the inner side of the inner membrane or in the matrix. The highest activity was measured after lysis by 0.1% Triton X-100. At lower detergent concentrations the measurable activity decreased, probably due to incomplete lysis of the mitochondria. Higher concentrations reversibly inhibited the enzymatic activity. 1% Triton X-100 inhibited the enzyme activity by 35% compared to the value obtained in the presence of 0.1% detergent. Tween 80, (0.05%) or sodium deoxycholate (0.1%) gave 66 and 71%, respectively, of the activity measured in the presence of 0.1% Triton X-100.

Sonication of the mitochondria for twice 30 s yielded a preparation with about 60% of the activity of detergent-lysed mitochondria. However, rapid oxidation of NADH by freshly sonicated mitochondria, even in the absence of nitro compounds, prevented accurate measurements of nitro reductase activity. This spontaneous oxidation was inhibited by the addition of 0.05% Triton X-100, 2 μM rotenone or storage of the disrupted mitochondria for 48 h at 4°C. The nitro reductase activity remained unchanged for at least 4 days if the membranes were stored in the refrigerator. Also, a linear relationship between enzyme activity and amount of membrane protein was established.

When mitochondria were lysed either by 0.05% Triton X-100 or by sonication and centrifuged for 1 h at 100 000 $\times g$, 80% of the nitro reductase activity was recovered in the pellet. On the other hand, at least 60% of the matrix marker enzyme, glutamate dehydrogenase, was found in the supernatant. These studies show that nitro reductase is tightly bound to the inner mitochondrial membrane and may even constitute one of its integral proteins.

Solubilization of nitro reductase

Several methods for solubilizing nitro reductase, such as addition of high concentrations of detergents and chaotropic salts, limited proteolysis or extended sonication were tested. Incubation with papain caused a complete loss of activity. Chaotropic ions such as ClO_4^- also decreased the enzyme activity and appeared to have little effect in solubilizing the enzyme protein. Both the addition of Triton X-100 and sonication partially detached the nitro reductase from the membranes. About 30% of the activity was recovered in the 100 000 $\times g$ supernatant after treating the membranes with 1% Triton X-100,

and 10—15% of the activity was solubilized by sonication for four times 30 s. Longer periods of sonication inactivated the enzyme. The highest yield of solubilization, approx. 40%, was obtained by suspending the membranes in 1% Triton X-100 followed by sonication for twice 30 s.

The solubilized enzyme, when applied to a column (50 × 1 cm) packed with Sephadex G-200, eluted in a broad peak together with the marker enzyme bovine liver catalase. Calibration of the column with other marker enzymes yielded a molecular weight between 200 000 and 250 000.

Substrate specificity

The mitochondrial nitro reductase specifically used NADH as coenzyme. Less than 10% of the NADH-linked activity was obtained in the presence of NADPH.

The specificity of the enzyme for a variety of nitro compounds is shown in Table III. The membrane-bound and the solubilized enzyme exhibited the same relative rates of reduction, indicating that solubilization of the enzyme does not alter its substrate specificity. The best substrates were aromatic compounds carrying a neutral, electron-attracting substituent. However, no correlation between the rates of reduction and the degree of the electrophilic force of the substituents could be established. The negatively charged 4-nitrobenzoate anion, a model substrate for the microsomal and cytosolic nitro reductases, was not metabolized by the mitochondrial enzyme. It also did not reduce the carbonyl function of several aromatic aldehydes. Apparent Michaelis constants were determined for 1,3- and 1,4-dinitrobenzene, 4-nitrobenzaldehyde and the anti-schistosomal agent niridazole (Fig. 1). Graphical extrapolation from the

TABLE III

SUBSTRATE SPECIFICITY OF NITRO REDUCTASE FROM RAT BRAIN MITOCHONDRIA

The activities were calculated from the decrease in absorbance at 340 nm and are not corrected for contributions from the substrates due to different extinction coefficients of the oxidized and reduced form. The instability of most hydroxylamine metabolites prevented the determination of accurate correction factors. The greatest substrate contributions were obtained with 1,4-dinitrobenzene and niridazole. The V values for these substrates can be corrected by multiplying with a correction factor of about 2 and 0.5, respectively. The value obtained with 1,3-dinitrobenzene (which does not contribute to the decrease in absorbance at 340 nm) was set as 100%. The absolute specific activity was 21 mU/mg protein.

Substrate (mM)	Relative activity
1,3-Dinitrobenzene (0.5)	100
1,4-Dinitrobenzene (0.5)	226
Niridazole (0.1)	198
4-Nitrobenzaldehyde (0.5)	110
2-Nitrobenzaldehyde (0.5)	70
3-Nitrobenzaldehyde (0.5)	10
4-Nitrobenzylalcohol (0.5)	37
2,4-Dichloronitrobenzene (0.5)	27
4-Nitrobenzylchloride (0.5)	21
4-Nitroacetophenone (0.5)	16
Nitrobenzene (0.5)	8
4-Nitrobenzoate (0.5, 1.0)	0
3-Nitrophenol (0.5)	0
N,N'-Dimethyl-3-nitroaniline (0.5)	0
1-Nitropropane (0.25)	0

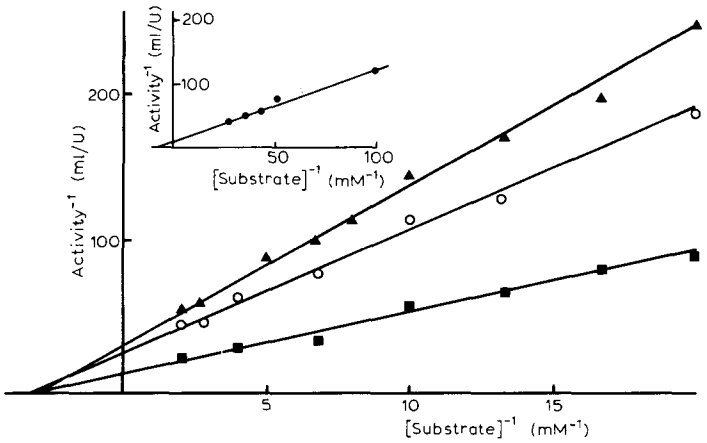

Fig. 1. Reduction of nitro compounds by mitochondrial nitro reductase. Mitochondrial membranes were assayed for nitro reductase activity in the presence of 0.05% Triton X-100. The results are presented as double-reciprocal plots of initial velocity vs. substrate concentration. ▲———▲, 1,3-dinitrobenzene; ○———○, 4-nitrobenzaldehyde; ■———■, 1,4-dinitrobenzene. The inset shows the Lineweaver-Burk plot for niridazole which was not soluble at the concentrations used for the other substrates.

Lineweaver-Burk plots yielded similar values between 0.3 and 0.4 mM for all four substrates.

Enzyme inhibition

Inhibition of the membrane-bound as well as the solubilized enzyme was observed in the presence of NAD^+. The decrease in activity was 70% at 0.5 and 90% at 2.5 mM NAD^+, respectively. $NADP^+$ at the same concentrations was not inhibitory. Inactivation of nitro reductase occurred in the presence of the SH-group blocking reagent 4-hydroxymercuribenzoate. At 0.5 mM the loss of activity after 10 min incubation was 50%.

In addition several known inhibitors of mitochondrial enzymes were tested as inhibitors of nitro reductase. Rotenone, carbon monoxide and cyanide which are powerful inhibitors of the respiratory chain, did not affect the enzymatic activity. Oxygen, whether present or absent, had no apparent influence on the reduction of nitro compounds. Moreover, no oxygen consumption could be detected during catalysis. Drugs, such as pheno- and allobarbital, chlorpromazine, imipramine, dicumarol, pargyline and disulfiram or chelating agents, such as EDTA, *o*-phenanthroline and 8-hydroxyquinoline did not influence the enzyme activity. Similarly, lactate and oxaloacetate inhibitors of β-hydroxybutyrate dehydrogenase and succinate dehydrogenase, respectively, had no influence on nitro reductase activity.

Effect of pH and temperature

Fig. 2 shows the pH dependence of nitro reductase activity. Similar curves with a maximum at pH 9.2 were obtained with mitochondria disrupted by sonication or by treatment with detergent.

The influence of temperature on the enzyme activity is shown in Fig. 3. The membrane-bound enzyme was most active at 35°C and the solubilized enzyme

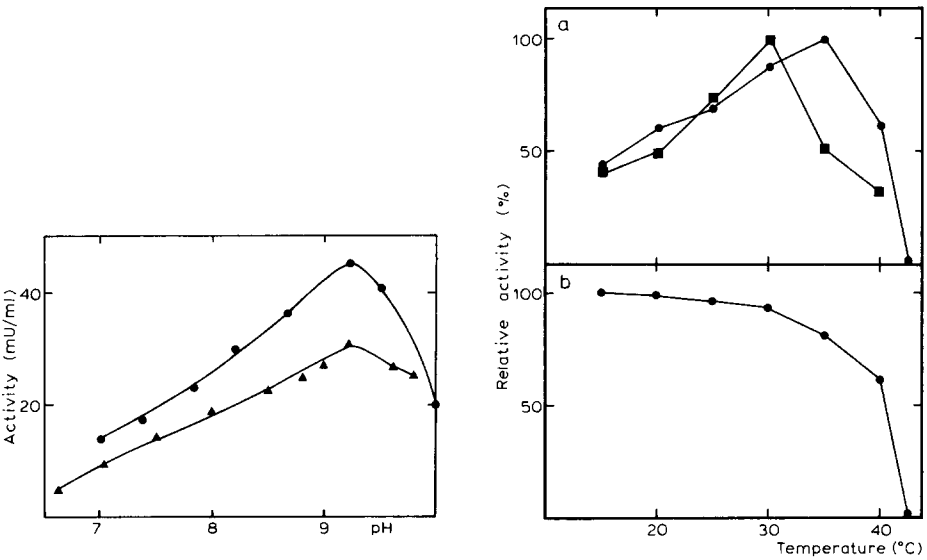

Fig. 2. pH dependence of nitro reductase. The enzyme was assayed in the presence of 0.5 mM 1,3-dinitrobenzene. The membranes of mitochondria which had been lysed by Triton X-100 (●——●) and by sonication (▲——▲) were used as the enzyme source. The buffers for the following pH ranges were: pH 6.4—7.8, sodium phosphate; pH 7.2—8.9, triethanolamine-Cl; pH 8.5—10, sodium pyrophosphate. The pH was measured before and after each assay.

Fig. 3. Temperature dependence of nitro reductase. The enzyme was assayed in the presence of 0.5 mM 1,3-dinitrobenzene and 0.05% Triton X-100. Panel A. The enzyme activity was measured at the indicated temperature. ●——●, membrane-bound enzyme; ■——■, solubilized enzyme. Panel B. The enzyme (detergent-lysed mitochondria) was incubated at the indicated temperature for 10 min and then assayed at 25°C.

at 30°C. Rapid inactivation of the enzyme took place above 35°C. The inactivation was also observed, if the enzyme was incubated for 10 min at temperatures above 35°C and then assayed at 25°C. (Fig. 3B). Mitochondria lysed either by sonication or by Triton X-100 exhibited the same temperature dependence. Comparable results were obtained using 1,4-dinitrobenzene, 1,3-dinitrobenzene or 4-nitrobenzaldehyde as substrate.

Discussion

The existence of several mammalian enzyme systems capable of reducing nitro groups has been reported. Some of them have been investigated in detail. Significant differences between these enzyme systems and the one reported in this paper were observed in respect to the subcellular localization, substrate specificity and sensitivity towards inhibitors.

Other nitro reductases that had been investigated occurred predominantly in liver and kidney [1], where they were located either in the endoplasmic reticulum [2,6—8] or in the cytoplasm of the cell [2,8,9]. The enzyme described here was found in liver (unpublished observation) as well as in brain and is located in the mitochondria.

Both the microsomal and the mitochondrial nitro reductase are membrane-

bound, but the enzyme system of the endoplasmic reticulum chiefly utilizes NADPH as an electron donor for the reduction of nitro compounds to the hydroxylamine metabolites [2], whereas the mitochondrial nitro reductase requires NADH. In this respect the enzyme resembles the soluble nitro reductases which also use NADH.

The microsomal and cytosolic nitro reductase systems are characterized by their ability to reduce 4-nitrobenzoic acid [1—7]. This compound is not reduced by the mitochondrial enzyme. On the other hand, 4-nitrobenzaldehyde and 4-nitrobenzylalcohol which are good substrates for the mitochondrial enzyme were not metabolized by liver slices in the presence of NADPH [1].

Oxygen and carbon monoxide are potent inhibitors of the microsomal nitro reductase system [2,6], while cyanide inhibits the nitro reduction in the cytosol [19]. None of these compounds inhibits the mitochondrial enzyme, and with the exception of NAD^+ and 4-hydroxymercuribenzoate no other inhibitor for the mitochondrial enzyme was found.

Several enzymes involved in other metabolic processes have been identified as nitro reductases. For instance, NADPH-cytochrome *c* reductase in the endoplasmic reticulum [8] and xanthine oxidase in the cytosol [9] metabolize the drug niridazole, and cytochrome *P*-450 has been shown to reduce 4-nitrobenzoic acid as well as other nitro compounds [6]. The enzyme that reduces 4-nitrobenzoic acid in the cytosol has not yet been identified. However, one has to consider that physiological substances such as $FADH_2$ and ascorbate are able to reduce nitro compounds non-enzymatically, therefore, enzymes using any one of these substances as a cofactor could function as nitro reductase. For example such a mechanism has been proposed for NADPH-cytochrome *c* reductase [4,5], this enzyme initially reduces FAD which is then capable of reducing 4-nitrobenzoic acid non-enzymatically. A similar mechanism could be involved in the reduction of nitro compounds by the mitochondrial enzyme. Several flavoproteins are integral parts of the inner mitochondrial membrane and could provide the hydrogen equivalents for the reduction of the nitro group. Our results unfortunately do not allow an assignment of the nitro reductase activity to a known mitochondrial enzyme.

The relevance of mitochondrial nitro reductase in brain and other tissues to the metabolism of xenobiotic nitro compounds is not known. Brain tissue contains 50—100 mU/g of mitochondrial niridazole reductase activity, which is 15—30% of the microsomal and cytosolic niridazole reductase activity, respectively, in 1 g liver tissue [8]. Hence, rat brain (approx. 2 g) is able to metabolize approx. 2 mg niridazole or dinitrobenzene per h. Since the reduction does not proceed to the amine as in the endoplasmic reticulum, considerable amounts of highly reactive nitroso and hydroxylamino metabolites may accumulate and eventually affect the metabolism of the mitochondrion.

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