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INHIBITION OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION BY 2-METHYL-4-DIMETHYLAMINOAZOBENZENE

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The azodye 2-methyl-4-dimethylaminoazobenzene inhibited oxidation and phosphorylation in tightly coupled rat liver mitochondria. Phosphorylation was more sensitive to the inhibitory action of the azodye than was the oxidation of succinate or ascorbate. The oxidation of NAD^+-linked substrate was severely inhibited by the compound. In submitochondrial particles, only NADH oxidation was sensitive. The site of inhibition has been identified to lie between the dehydrogenase flavoprotein and ubiquinone.

Azodyes have enjoyed wide-spread use as colour additives in the cosmetic, pharmaceutical, food and textile industry. The potential health hazard in their unrestricted use was spotlighted by the discovery that some substituted azobenzenes like DAB and 3′-Me-DAB are potent hepatocarcinogens [1,2]. These compounds on prolonged dietary administration not only produced tumors but also decreased hepatic mitochondrial content [3–6]. In contrast, the isomer 2-Me-DAB which was generally non-carcinogenic but exhibited weak carcinogenic activity under specific conditions [7,8] increased the content of hepatic mitochondria [5–7]. In this paper we report that 2-Me-DAB is a selective and potent inhibitor of electron transport in the NADH-ubiquinone segment of the respiratory chain.

In rat liver mitochondria both substrate oxidation and coupled phosphorylation were progressively inhibited by increase in the concentration of 2-Me-DAB in the reaction system until more than 80% of the activity was inhibited. From linear regression equations correlating activity (oxygen uptake and phosphate disappearance) with dye concentration, the amount required for 50% inhibition ($\phi_{1/2}$) was determined.

The values of $\phi_{1/2}$ provide a measure of the relative inhibitor potency for different substrates. The data in Table I reveal that the oxidation of NAD^+-linked substrates (glutamate + malate) was most sensitive to the inhibitory action of the dye. Succinate oxidation was only half as sensitive as NADH oxidation. Cytochrome oxidase activity was least sensitive. The inhibition of phosphorylation appeared to be independent of the substrate used. This would imply that the dye was a more potent inhibitor, of phosphorylation than of electron transport at the last two coupling sites. In agreement with this, 2-Me-DAB stimulated state-4 respiration (ADP exhausted; see Ref. 11), abolished respiratory control, decreased ADP/O ratio and inhibited state 3 (ADP present) respiration in tightly-coupled mitochondria (data not shown).

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Abbreviations: DAB, 4-dimethylaminoazobenzene; 3′-Me-DAB; 3′-methyl-4-dimethylaminoazobenzene; 2-Me-DAB, 2-methyl-4-dimethylaminoazobenzene; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; 2,2′,5,5′-tetraphenyl-3,3′-(4,4 biphenylene)-ditetrazolium chloride.

TABLE I

INHIBITION OF OXIDATIVE PHOSPHORYLATION BY 2-ME-DAB IN RAT LIVER MITOCHONDRIA

Oxidative phosphorylation was measured by manometry using freshly prepared mitochondria [9,10]. The values of $\phi_{1/2}$ (2-Me-DAB concentration for 50% inhibition) given are calculated from the regression equations ($\gamma = 0.88–1.00$) and are the mean ± S.D. of four independent estimations. The rate of oxygen uptake with glutamate + malate, succinate and ascorbate + TMPD were 97, 198 and 120 ng atom O/min per mg protein, respectively.

Substrate	Reaction	$\phi_{1/2}$ (μM)
Glutamate + malate	oxidation	11.7 ± 1.6
	phosphorylation	10.7 ± 0.7
Succinate	oxidation	20.6 ± 2.5
	phosphorylation	13.6 ± 1.0 *
Ascorbate + TMPD	oxidation	124.7 ± 34.6
	phosphorylation	19.3 ± 1.1 *

* $p < 0.01$ (oxidation vs. phosphorylation).

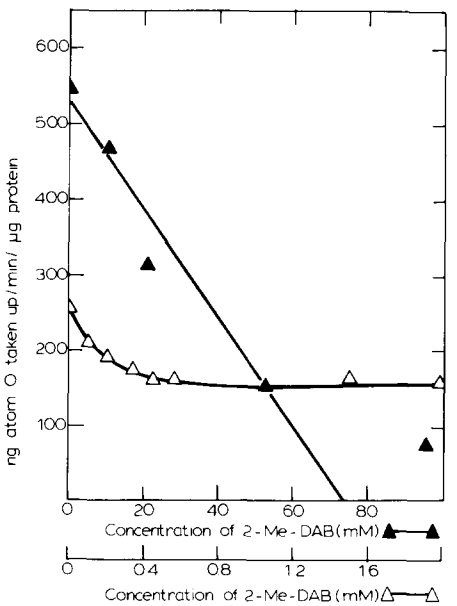

Fig. 1. Effect of 2-Me-DAB on NADH and succinate oxidation in sonic submitochondrial particles. Sonic particles were made according to Graven et al. [12]. Oxidation of NADH (▲) and succinate (△) were measured by polarography in a reaction system containing 1 mg of particle protein in 1.4 ml.

To rule out the possibility that the inhibitory action of 2-Me-DAB is caused primarily by interference with translocation of NAD^+-linked substrates (inhibition of adenine nucleotide translocation may be ruled out by the lower potency in the inhibition of succinate oxidation), the effect of the compound on substrate oxidation by sonic submitochondrial particles was tested. In these particles succinate oxidation appeared refractory to inhibition by the azodye, 0.6 mM being required to achieve 35% inhibition. Further increase up to 3 mM failed to produce any further inhibition. Measured under similar conditions, oxygen uptake with NADH as substrate was inhibited by 85% at 100 μM dye concentration ($\phi_{1/2} = 35.8$ μM) (Fig. 1). The NADH-dehydrogenase activity of sonic particles (2.7 μmol of NADH oxidized/min per mg protein) was not inhibited by 2-Me-DAB even at a concentration of 1.5 mM; so also was the succinate dehydrogenase activity.

The onset of anaerobiosis on the addition of NADH to a reaction system containing sonic submitochondrial particles was delayed by more than 3-fold in the presence of 2-Me-DAB. The rate of reduction of cytochrome *b* under these conditions showed a substantial decrease (compare experiments 1 and 2 in Fig. 2). In contrast, when succinate was used as the electron donor, neither the time taken by the system to reach anaerobiosis nor the rate of reduction of cytochrome *b* was altered

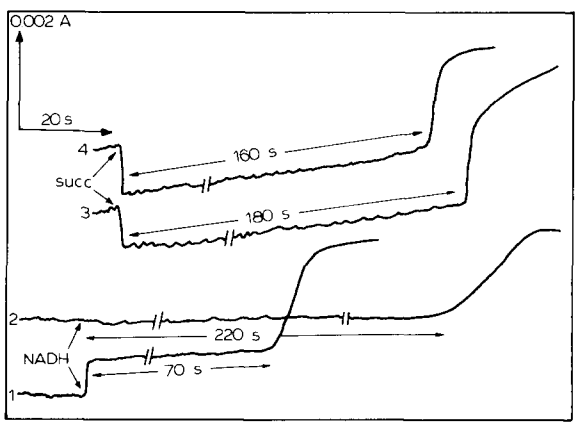

Fig. 2. Effect of 2-Me-DAB on reduction of cytochrome *b* in liver submitochondrial particles. Substrate (NADH in 1 and 2 and succinate in 3 and 4) was added as indicated and the reduction of cytochrome *b* was recorded at 562–577 nm in a Aminco DW2 dual wavelength spectrophotometer [13]. The time for the onset of anaerobiosis and reduction of the cytochrome is indicated. The azo-dye 120 μM was added (samples 2 and 4) at least 1 min before addition of substrate.

TABLE II

EFFECT OF 2-Me-DAB ON NEOTETRAZOLIUM REDUCTION BY SONIC SUBMITOCHONDRIAL PARTICLES

The reduction of neotetrazolium was determined spectrophotometrically in a reaction system (1 ml) containing 200 μg sonic particle protein, after extraction of the formazan into ethyl acetate. Other additions were made as indicated. The values are the mean of two independent determinations. Ubiquinone-9 was added as emulsion in water.

Substrate	Addition	Concn.	Activity (μmol formazan formed per min per mg protein)
NADH	None		1.9
	2-Me-DAB	60 μM	2.3
	Ubiquinone	0.5 mM	3.5
	Ubiquinone	0.5 mM	
	+2-Me-DAB	60 μM	2.3
	Rotenone	25 μM	1.8
	Ubiquinone	0.5 mM	
	+Rotenone	25 μM	1.6
Succinate	None		0.3
	2-Me-DAB	450 μM	0.5
	Ubiquinone	0.5 mM	2.5
	Ubiquinone	0.5 mM	
	+2-Me-DAB	450 μM	3.4

by the presence of the azodye (experiments 3 and 4 in Fig. 2).

The results presented in Fig. 2 indicated that the azodye interfered with the transfer of electrons to cytochrome *b* from NADH. The observation that the primary dehydrogenase was not inhibited by the compound suggested that the transfer of electrons from the primary dehydrogenase to ubiquinone or from reduced ubiquinone to cytochrome *b* or both could be susceptible to inhibition by the azo-dye. Because of inherent difficulty in the direct measurement of the degree of reduction of endogenous ubiquinone [14], we resorted to the indirect method of measurement of the reduction of neotetrazolium in the presence of antimycin A. The reduction of neotetrazolium to the formazan is stimulated by exogenous ubiquinone [15,16]. Exogenous ubiquinone is known to accept electrons from its endogenous counterpart [17]. The degree of stimulation of neotetrazolium reduction by exogenous ubiquinone should be a measure of the passage of electrons through ubiquinone. The effect of 2-Me-DAB on neotetrazolium reduction was therefore measured.

When NADH was used as the electron donor for the reduction of neotetrazolium, the activity was not amenable to inhibition either by 2-Me-DAB or by rotenone (Table II). However, the activity stimulated by exogenous ubiquinone was inhibited by 2-Me-DAB as well as by rotenone. When succinate was used as the electron donor, almost 8-fold higher concentration of 2-Me-DAB had no adverse effect on ubiquinone-stimulated tetrazolium reduction (Table II).

The results presented in this report show unequivocally that 2-Me-DAB is a potent inhibitor of mitochondrial oxidative phosphorylation. Apart from effectively interfering with the process of coupled phosphorylation, the azodye acts as a strong inhibitor of electron transport in the complex I region of the respiratory chain inhibiting transfer of electrons from the NADH-dehydrogenase flavoprotein to ubiquinone. Since substantial portions of the ingested azodyes are retained in the liver [18], this study highlights the toxic nature of 2-Me-DAB despite its being only a weak carcinogen.

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