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In Vitro and *in Vivo* Effects of Dimethylnitrosamine on Mouse Liver Mitochondrial Function[1] (39710)

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Summary. The *in vivo* and *in vitro* effects of the carcinogen dimethylnitrosamine (DMN) on the metabolic activity of mitochondria were studied. Oxygen consumption by mitochondria in state 3 and state 4 was determined polarographically following the addition of succinate or glutamate as substrates. The control of respiration by ADP is indicated by the ratio of state 3 to state 4 oxygen utilization (RCR), and is indicative of the tightness of coupling of the oxidative phosphorylation and electron transport systems. DMN in concentrations of 37.8 μM and higher significantly decreased state 3 oxidation of glutamate. State 4 oxidation of glutamate was inhibited significantly only at DMN concentrations of 75, 151, and 226 μM (5, 10, and 15 μg/ml). The RCR was decreased corresponding to the decrease in state 3 oxidation. Similar results, but to a lesser magnitude, were obtained using succinate as substrate. In mitochondria from mice treated with DMN (25 mg/kg ip) state 3 oxygen consumption was decreased and the RCR was significantly different from control with either substrate. The results indicate that DMN inhibits oxidative phosphorylation both *in vivo* and *in vitro*.

Introduction. Our laboratory has been involved in studies on the relationship between environmental compounds influencing mixed function oxidase activity and dimethylnitrosamine (DMN) metabolism. The basis for supposition of this interrelationship is the presence of dimethylnitrosamine demethylase activity in liver microsomes. Magee and Barnes (1) in their classic review on nitrosamines emphasized this relationship. More recently, Zeiger (2) reviewed the relationships among DMN demethylase activity, factors influencing its levels, and mutagenic activity of dimethylnitrosamine. Our laboratory, in studying antagonistic activity of alkylnitrosamines and methylenedioxybenzene derivatives with dimethylnitrosamine, has generally supported Zeiger's contentions (3). However, one clear toxicological parameter which measures significance of this enzyme is mortality (LD_{50}) due to the ease of interpretation and irreversibility of the response. Inhibitors of murine DMN demethylase, however, generally have not raised the DMN LD_{50} which is 20 mg/kg in mice (4). In order to explain this paradox, toxic effects of DMN were investigated. We report here that DMN is an inhibitor of oxidative phosphorylation *in vitro* and that DMN also inhibits oxidative phosphorylation *in vivo*. The assay system employed measured oxygen consumption in metabolic state 4[2] and state 3[3] of mitochondria as defined by Chance and Williams (5).

Methods and Materials. Male Swiss (ICR) mice were housed in shoe-box type cages and fed Purina chow and water *ad libitum*. They were sacrificed by cervical dislocation, and the livers were removed and homogenized in ice-cold 0.25 M sucrose with 5×10^{-4} M EDTA. Mitochondria were isolated by the method of Schneider and Hogeboom (6, 7) and used immediately.

Oxygen consumption was measured polarographically with a Clark fixed voltage polarizing probe (7). Rate of oxygen utilization was measured both in the presence and absence of ADP, and is expressed as nanomoles of atomic oxygen (nanogram atom equivalents of oxygen) consumed per milli-

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[2] State 4, resting state respiration (velocity after ADP exhaustion).

[3] State 3, active state respiration (rate of substrate oxidation in presence of phosphate acceptor, ADP).

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gram of mitochondrial protein per minute. The respiratory control ratio (RCR)[4] is the ratio of state 3 to state 4 respiratory velocities (5). In addition to 2 mg of mitochondrial protein the reaction mixture contained 0.33 *M* mannitol, 0.33 m*M* EDTA, 3.5 m*M* KCl, 3.5 m*M* potassium phosphate, and 2.4 mg of bovine serum albumin, in a total volume of 1.8 ml (7). All determinations were made at 30°.

Substrates were titrated pH 7.3 and added during incubation to concentrations of 1.4 m*M* succinate (pH 7.4) or 1.4 m*M* glutamate (pH 7.4), and 0.14 m*M* ADP (7). For the *in vitro* studies, DMN diluted in distilled water was added to the reaction chamber and preincubated with the mitochondria for 3 min before the determination was started. Animals used in the *in vivo* studies were injected with 25 mg/kg ip of DMN, 30 min prior to sacrifice. The mitochondria were isolated and handled in the same manner as those for the *in vitro* studies. Each experiment was repeated on at least three separate occasions. Mitochondrial protein was solubilized with 0.75 *M* NaOH and determined by a biuret method.

Results. The results of incubating DMN with murine liver mitochondria are shown in Fig. 1. With glutamate as the substrate, the respiratory control ratio (RCR) was 3.82. Additions of DMN induced a marked decrease in the RCR. At concentrations of 37.8 μM DMN the RCR was 85% of controls. Similar suppression of state 3 oxygen consumption took place with a 20% inhibition at 37.8 μM and a 60% inhibition at 226 μM concentration. State 4 oxidation was decreased at the three higher doses by 29, 25, and 22% at 75, 151, and 226 μM DMN, respectively. Each of these three points is statistically different from control.

Succinate oxidation does not appear as sensitive as glutamate oxidation (see Fig. 2). Addition of ADP to those mitochondria yielded an RCR of 2.63. Only at 75 and 226 μM DMN were the RCRs statistically different from controls. At higher concentrations of DMN (75, 151, and 226 μM) the RCR was suppressed by 7, 9, and 29%, respectively. State 4 oxidation was slightly below controls throughout the experiment reaching inhibitory levels of 22% at the two highest concentrations. These were the only

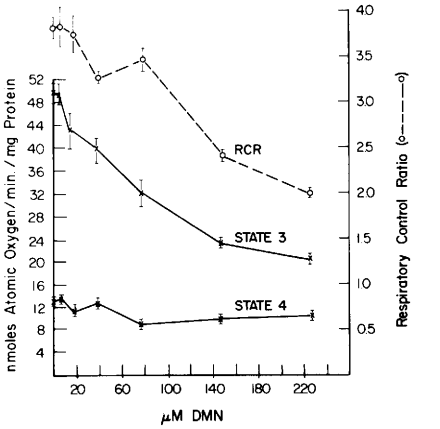

FIG. 1. *In vitro* effect of dimethylnitrosamine on mitochondrial oxidation of glutamate. Oxygen consumption was measured polarographically in the presence of ADP (state 3) and after exhaustion of ADP (state 4) using L-glutamate as substrate. Mitochondria were isolated from livers of male Swiss mice. Distilled water or DMN was added to the reaction mixture (total volume, 1.8 ml) and preincubated with the mitochondria for 3 min.

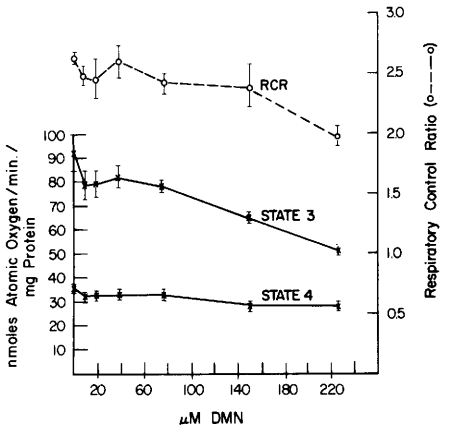

FIG. 2. *In vitro* effect of dimethylnitrosamine on mitochondrial oxidation of succinate. Oxygen consumption was measured polarographically in the presence of ADP (state 3) and after exhaustion of ADP (state 4) using succinate as substrate. Mitochondria were isolated from livers of male Swiss mice. Distilled water or DMN was added to the reaction mixture (total volume, 1.8 ml) and preincubated with the mitochondria for 3 min.

[4] RCR, respiratory control ratio (ratio of active state to resting state velocities), a measure of the control of respiration by phosphate acceptor.

TABLE I. EFFECT OF *in Vivo* ADMINISTRATION OF DMN ON MITOCHONDRIAL RESPIRATION.[a]

Treatment	Number of mice	Substrate	RCR	Oxygen consumption, state 3	(Mean ± SE),[b] state 4
Control	4	Succinate	2.63 ± 0.07	92 ± 5	35.0 ± 2.2
DMN	3	Succinate	1.97 ± 0.20*	80 ± 4	40.7 ± 4.5
Control	6	Glutamate	3.45 ± 0.14	42.0 ± 3.8	12.1 ± 0.78
DMN	5	Glutamate	2.57 ± 0.13*	32.9 ± 3.8**	12.8 ± 1.17

[a] Male Swiss mice were treated with 25 mg/kg, i.p. DMN, and killed 30 minutes later. Mitochondria were isolated from the liver, and oxygen consumption measured polarographically.

[b] Exressed as nanomoles of atomic oxygen per milligram of mitochondrial protein per minute.

* Denotes statistically different from control ($p < 0.01$).

** Denotes statistically different from control ($p < 0.05$).

determinations which were statistically different from controls. At 7.5 and 18.9 μM DMN, state 3 oxidation was suppressed 18 and 15%, respectively. Since state 3 was always affected to a greater extent than state 4, the RCRs were also always below controls. However, as we stated earlier, only the high doses were statistically significant.

Oxygen consumption by mitochondria of mice treated with DMN is shown in Table I. In the case of succinate oxidation, although depression of the state 3 velocity was not significant, the RCR was decreased 24%. As was the case *in vitro* glutamate oxidation was more sensitive to DMN than was succinate. There was no change in state 4 oxidation while state 3 oxidation was inhibited 22%. Therefore, the RCR was suppressed by 25%.

Purified mitochondria were tested for DMN demethylase activity. At no time did mitochondrial preparations produce any measurable formaldehyde when incubated with DMN and several cofactors. Microsomal preparations, under similar conditions, produce significant and easily detectable amounts of formaldehyde.

Discussion. There has been a long history of observations leading to the conclusion that enzymatic activation of DMN was essential for its biological activity. As early as 1956, Dutton and Heath (8) followed the rapid production of $^{14}CO_2$ from [^{14}C]DMN and concluded that DMN had to be enzymatically activated. This conclusion was further emphasized in Magee and Barnes' (1) review on carcinogenic nitroso compounds. They pointed to the absence of mutatgenic activity in microorganisms lacking the ability to enzymatically activate DMN, and to the finding that T_4 phage was unaffected by exposure to a 25% solution of DMN (9). Magee and Hultin (10) had shown that DMN alkylates biological materials. McLean and Vershuuren (11) showed that dietary protein deprivation affected tumor incidence and DMN demethylase activity in the same manner. Heath (12) stated definitively that DMN must be metabolically activated to cause its toxic effects and that the unmetabolized molecule was inactive.

With this wealth of congruous information, it was very surprising to find that a variety of inhibitors of DMN demethylase such as piperonyl butoxide, nitrososarcosine, dibutylnitrosamine, and diethylnitrosamine had no suppressing effect on the DMN LD_{50} (4). Therefore, a different mechanism of action was hypothesized for the acute toxicity—one which, perhaps, did not involve enzymatic activation. Doherty and Campbell (13) have shown that incubation of rat liver mitochondria with aflatoxin B_1 results in inhibition of electron transport. Therefore, similar experiments were undertaken to determine whether DMN mimics aflatoxin in this sense. The *in vitro* data presented here show that DMN, probably in its unmetabolized form (there is no way to be absolutely conclusive about this), inhibits oxidative phosphorylation with glutamate as the substrate and, to some extent, with succinate. However, alternate metabolic pathways in mitochondria not involving formaldehyde production still remain a possibility.

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