Toxicology, 62 (1990) 95—106
Elsevier Scientific Publishers Ireland Ltd.

Inhibition of rat heart mitochondrial electron transport in vitro: implications for the cardiotoxic action of allylamine or its primary metabolite, acrolein*,**,***

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(Received September 26th, 1989; accepted December 28th, 1989)

Summary

Allylamine (3-aminopropene) is a specific cardiac toxicant that causes aortic, valvular and myocardial lesions in many species. Myocardial necrosis can be observed 24 h after a single dose. Acute toxicity is believed to involve metabolism of allylamine to highly reactive acrolein (2-propenal). Allylamine has been shown to bind to mitochondria from aorta and heart, suggesting that the subcellular site of injury is at or near the mitochondrion. The present investigation compared the effect of allylamine and its primary metabolite, acrolein, on electron transport and oxidative phosphorylation in mitochondria isolated from rat heart (RHM). Both compounds weakly inhibited mitochondrial electron transport with either the combination of glutamate, malate, and malonate (GMM, NADH-linked) or succinate as substrate.

Comparisons of the slopes of concentration-effect regression (range of concentrations tested, 0.20—2.0 mM) lines showed acrolein to have significantly greater inhibitory effects than allylamine (range of concentrations tested, 0.22—6.4 mM) on GMM oxidation, while no significant difference in the abilities of the compounds to inhibit succinate oxidation were observed, indicating site preferences for inhibitory action. The addition of an uncoupling agent could not reverse inhibition with either substrate system. These results indicate that both the parent compound and its proposed metabolite primarily inhibit electron transport with little direct effect on the coupling mechanism. The State III

*Presented in part at the Annual Meeting of the Society of Toxicology, Dallas, TX, February, 1988.

**This research was supported in part by NIOSH Contract No. 87-11174 awarded to GWW.

***Mention of a trade name, proprietary product, or specific equipment does not constitute an endorsement by NIOSH.

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0300-483X/90/$03.50

Printed and Published in Ireland

EC_{50} (effective concentrations for 50% inhibition of control mitochondrial enzyme activities) for allylamine (2.29 mM with succinate as substrate and 1.22 mM with GMM) and acrolein (0.80 mM with succinate as substrate and 0.39 mM with GMM) are probably too great to invoke the direct action of either the parent compound or its oxidized metabolite on mitochondrial electron transport as a primary mechanism in the cardiotoxic action of allylamine.

Keywords: Allylamine; Acrolein; Rat Heart Mitochondria; Inhibition; Electron Transport

Introduction

Allylamine (3-aminopropene) is a primary alkylamine used in the synthesis of a variety of pharmaceuticals and other commercial products [1]. Although chronic intravenous administration of allylamine produces lesions in lung, liver and kidney [2,3], the cardiovascular system appears to be particularly sensitive (see extensive review by Boor and Hysmith, 1987 [4]). Because the sequelae of allylamine cardiotoxicity is believed to involve, at least in part, myocardial ischemia due to lesions in the cardiac vasculature [5], much attention has focused on its effects on the aorta and coronary arteries. Boor and coworkers [6,7], however, showed that myocardial lesions can precede vascular lesions following allylamine treatment. They also demonstrated that myocardial damage occurs within 24 h of a single exposure [8], and that it can be produced within 2 h of the onset of perfusion in the isolated heart [9]. Furthermore, studies with [^{14}C]allylamine indicate that there is considerably more covalent binding of allylamine or a metabolite to mitochondria in heart than in aorta [10,11]. This suggests that heart mitochondria may be a target site for the toxicity of allylamine or its metabolite(s).

It has been proposed that allylamine is metabolized to acrolein [8,12] and that acrolein is the reactive reagent responsible for producing cardiovascular damage. Acrolein is a highly reactive aldehyde that is considered to be the proximal liver toxicant resulting from hepatic metabolism of allyl alcohol by alcohol dehydrogenase [13,14]. The reactive acrolein is a potent inhibitor of rat liver mitochondrial glutamate transport, inorganic phosphate transport, and succinate dehydrogenase activity [15]. The specificity of the heart and aorta to allylamine toxicity appears to be due to the metabolism of allylamine to acrolein by monoamine or benzylamine oxidases [7,16,17]. The critical role of the mitochondria is suggested by the observation that phospholipases, which stimulate some monoamine oxidases, increase the [^{14}C] moiety of radiolabeled allylamine binding to mitochondria, and deprenyl, which inhibits monoamine oxidase, decreases ^{14}C binding [10]. Furthermore, mitochondrial malate dehydrogenase activity is inhibited by allylamine [18].

Previously, we presented preliminary findings [19] that indicated allylamine was a weak inhibitor of bovine heart mitochondrial respiration in vitro. Rigorous assignment of spectral absorbance changes caused by allylamine (reduced minus reduced spectra) showed an accumulation of cytochrome *b*, indicating terminal inhibition in Complex II (succinate-Coenzyme Q oxidoreductase) near the site where the classical inhibitor of mitochondrial electron transport, antimycin A, is active. The present investigation extends this work to include the effects of ally-

lamine and acrolein on electron transport and oxidative phosphorylation in mitochondria isolated from rat heart.

Materials and Methods

Animals

Heart mitochondria were isolated from male Sprague—Dawley rats (400—500 g) obtained from Charles River Breeding Laboratories (Wilmington, MA). The animals were fed standard laboratory chow (Ralston Purina Co., St. Louis, MO.) and water ad libitum. Food was removed 12 h prior to sacrifice.

Isolation of mitochondria

High quality mitochondria were obtained by modifications of a rapid isolation procedure previously described [20]. Five to eight rats were decapitated with a laboratory guillotine. The hearts were excised, trimmed of fat and connective tissue, rinsed free of blood and immediately placed into an ice cold buffer containing 0.25 M sucrose, 3 mM *N*-2-hydroxyethylpiperazine-*N*^{1}-2-ethanesulfonic acid (HEPES), 1.0 mM ethylene glycol bis(β-aminoethyl ether) *N,N,N*^{1},*N*^{1}-tetraacetic acid (EGTA), and 0.5% bovine serum albumin (Fraction V, essentially fatty acid free, Sigma Chemical Co., St. Louis, MO), pH 7.2. The washed hearts were finely minced with scissors, repeatedly washed to remove traces of blood and ground by hand with a tissue press. Ten ml of buffer/g of ground heart tissue was added to a small beaker and 0.1 mg/ml Nagarse (proteolytic enzyme, Enzyme Development Corp, New York, NY) was added per gram of heart. The suspension was allowed to incubate at 4 °C for 15 min with occasional stirring. The digested heart tissue was decanted from the enzyme solution and homogenized in a Potter-Elvehjem homogenizer (clearance between the pestle and the homogenizing vessel was 0.15 mm). The homogenized suspension was centrifuged at 600 × *g* for 10 min and the supernatant decanted. The decanted supernatant was centrifuged at 10 000 × *g* for 10 min and the supernatant again decanted. Damaged mitochondria, identified as light colored fringes around the main pellet at the bottom of the tube, were removed by careful agitation with a glass rod. The resulting mitochondrial pellet was resuspended in approximately 30 ml of buffer and centrifuged at 8000 × *g* for 10 min. Damaged mitochondria were removed as above and the resulting pellet resuspended in a small volume of buffer. The final mitochondrial suspension (20—30 mg protein/ml) was stored as a stock suspension on ice. All biochemical measures were performed within 2—3 h of the initial isolation of the mitochondria. Protein was measured using the method according to Lowry [27] using bovine serum albumin as the standard.

Respiration/phosphorylation analysis

Isolated mitochondria were analysed for their respiratory and phosphorylating activity by polarimetry [20—22] at 25 °C in a closed water-jacketed reaction vessel equipped with a Clark oxygen electrode (Gilson 5/6 pH oxygraph, Gilson Medical Electronics, Madison, WI). The respiration medium contained 0.25 M sucrose, 5 mM K-HEPES, 2 mM $MgCl_2$, 3 mM K-phosphate (pH 7.2) and 1—2

mg mitochondrial protein in a 1.7 ml total volume. A mixture of glutamate (5 mM), 1 mM malate and 1 mM malonate (GMM) or 10 mM succinate were used as substrates in individual experiments. The oxygraph system was calibrated by the addition of a few crystals of sodium dithionite to cause anaerobiosis, in accordance with the manufacturer's instructions. Respiration rates in the absence (State IV) and presence (State III) of phosphate acceptor (ADP, adenosine diphosphate, Tris salt, 150—300 nmol, Sigma Chemical Co., St. Louis, MO) were measured in the presence of these substrates. From these measurements the respiratory control ratio (RCR = State III oxygen consumption rate/State IV oxygen consumption rate) was calculated [23]. Respiration was uncoupled from phosphorylation using carbonyl cyanide 4-(trifluromethoxy) phenylhydrazone (FCCP, Aldrich Chemical Co., Milwaukee, WI) at a final concentration of 10 μM.

Allylamine and acrolein

Allylamine and acrolein were obtained from Aldrich Chemical Co. (Milwaukee, WI). Acrolein was distilled at 57 °C to remove it from the hydroquinone added to commercial acrolein preparations to prevent polymerization. The distilled acrolein solution had a UV_{max} of 207 nm (log molar extinction coefficient [ε], 4.03). Acrolein and allylamine were diluted in absolute ethanol to appropriate concentrations just prior to an experiment and were added immediately after the addition of substrate for a specific experiment.

Statistical analysis

Orthogonal least square regression lines were calculated for mitochodrial respiratory activity verses allylamine or acrolein concentration. Computations were performed using commercially available graphics analysis software (Sigmaplot, Jandel Inc., Corte Madera, CA) and a statistical analysis package (Number Cruncher Statistical System, Kaysville, UT), both operated on a microcomputer. Concentration dependent responses were identified as significant changes in slope vs. the alternative of the slope equalling zero (null hypothesis, H_0: $\beta = 0$). Differences between the effects of allylamine and acrolein on a specific respiratory activity were identified by slope comparison (H_0: $\beta_{acrolein} = \beta_{allylamine}$) [24], and by comparison of corresponding concentrations causing 50% inhibition of the control mitochondrial enzyme activities (EC_{50}). EC_{50}s were determined by interpolation from the best fit line.

Results

Respiratory control ratios, ADP/oxygen ratios, and enzymatic activities using both succinate and GMM as substrates are reported in Table I. The values for these activities indicate the the RHM preparations used in these studies were well coupled and had suffered little or no damage from the isolation procedures. Control experiments using 10—20 μl aliquots of ethanol showed no effects on any of the mitochondrial activities measured.

Both acrolein and allylamine inhibited State III (Fig. 1, Panel A), State IV

TABLE I

CONTROL VALUES FOR MITOCHONDRIAL PREPARATIONS[a]

Tissue source	Substrate	Uncoupler				
		State III	State IV	Stimulated	RCR[b]	ADP:O[c]
Rat heart	Succinate	154.0 ± 9.1	38.1 ± 2.7	192.8 ± 14.7	4.1 ± 0.2	1.9 ± 0.08
Rat heart	GMM[d]	122.5 ± 5.5	17.4 ± 1.9	148.8 ± 11.0	7.4 ± 0.4	2.8 ± 0.09

[a]State III, State IV and Uncoupler Stimulated activities are given in ngatoms/min/mg protein (N = at least 3 trials performed in replicate).
[b]Respiratory control ratio.
[c]ADP: oxygen ratio.
[d]Glutamate, malate and maleate.

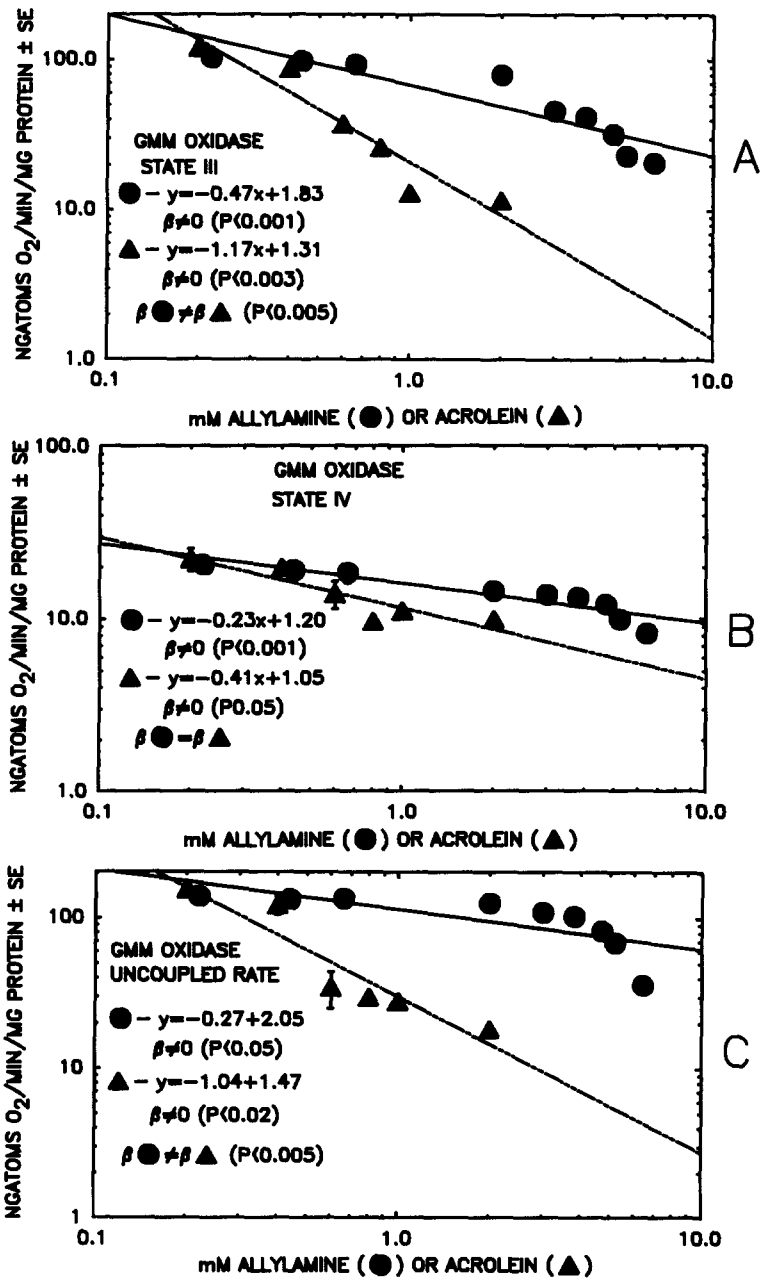

Fig. 1. The mitochondrial effects of increasing concentrations of allylamine and acrolein on GMM oxidase state III (Panel A), state IV (Panel B) and uncoupler-stimulated (Panel C) respiratory activity (ng atoms/min/mg protein ± S.E., each point is the mean of 3 trials done in replicate). Respective linear formulae as well as the results of hypotheses tests H_0: $\beta = 0$ are given. The test for a difference in slope between concentration-effect of allylamine and acrolein are represented by β (filled circle) = β (filled triangle). An inequality indicates rejection of the null hypothesis.

(Fig. 1, Panel B), and uncoupler stimulated (Fig. 1, Panel C) respiration in a concentration-dependent fashion when added to mitochondria respiring on GMM as substrate. Although the concentration-dependent inhibitory effect was statistically greater for acrolein than allylamine for State III and uncoupler stimulated oxygen uptake, the concentrations necessary to produce these effects were in the mM range for both compounds. The EC_{50} values calculated for allylamine and acrolein toward State III and State IV respiration with GMM as substrate are shown in Table II.

Allylamine and acrolein also displayed concentration-dependent effects (again, in the mM concentration range) on respiratory enzyme activities in mitochondria actively respiring on succinate as substrate. State III (Fig. 2, Panel A), State IV (Fig. 2, Panel B), and uncoupler stimulated (Fig. 2, Panel C) oxygen uptake were significantly inhibited by increasing concentrations of allylamine or acrolein. In contrast to the results obtained with GMM, with succinate as substrate, no significant differences between allylamine and acrolein in concentration-effect slopes were observed for State III, State IV and uncoupler stimulated activities (Fig. 2, Panels A, B and C), indicating site selectivity between the two compounds. The EC_{50} values calculated for allylamine and acrolein toward State III and State IV respiration with succinate as substrate are shown in Table II.

Respiratory control ratios were significantly decreased by allylamine and acrolein with GMM as substrate (Fig. 3, Panel A). When succinate was used as substrate, the respiratory control ratio was significantly reduced only by acrolein (Fig. 3, Panel B), again suggesting the site selective nature of this compound.

Discussion

The present investigation reports the first finding of the in vitro effects of allylamine and its proposed metabolite, acrolein, on electron transport and oxidative

TABLE II

EC_{50}[a] VALUES FOR ALLYLAMINE AND ACROLEIN IN RAT HEART MITOCHONDRIAL PREPARATIONS

Respiratory state	Substrate	Compound	EC_{50} mM
III	Succinate	Allylamine	2.29
IV	Succinate	Allylamine	3.47
III	Succinate	Acrolein	0.80
IV	Succinate	Acrolein	>2.0[b]
III	GMM[c]	Allylamine	1.22
IV	GMM	Allylamine	>6.4[b]
III	GMM	Acrolein	0.39
IV	GMM	Acrolein	1.85

[a]Effective concentration for 50% inhibition of control mitochondrial enzyme activity.
[b]EC_{50} value was greater than the highest concentration tested.
[c]Glutamate, malate and maleate.

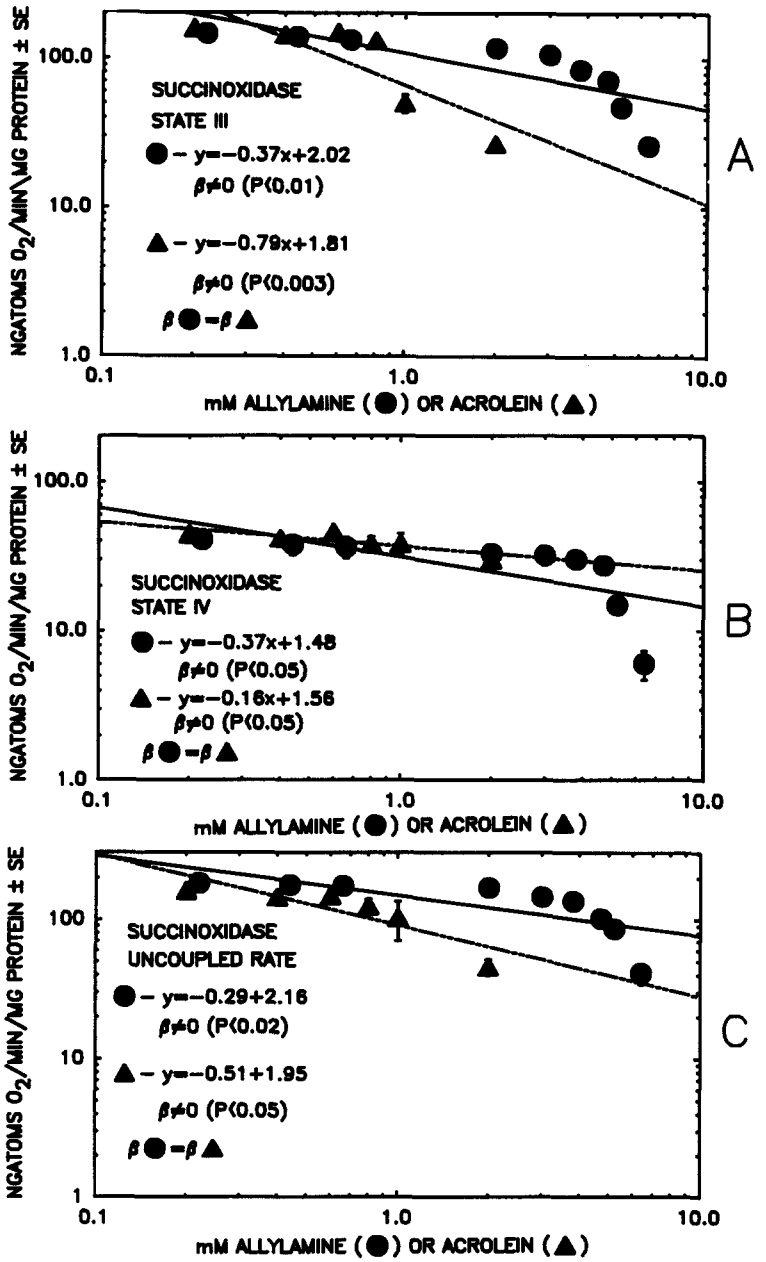

Fig. 2. The mitochondrial effects of increasing concentrations of allylamine and acrolein on succinoxidase state III (Panel A), state IV (Panel B) and uncoupler-stimulated (Panel C) respiratory activity (ng atoms/min/mg protein ± S.E., each point is the mean of 3 trials done in replicate). The best fit regression line for each compound is given on each panel with the solid line representing allylamine's regression and the dot-dot-dash-dot-dot line representing the regression line for acrolein. Respective linear formulae as well as the results of hypotheses tests H_0: $\beta = 0$ are given. The test for a difference in slope between concentration-effect of allylamine and acrolein are represented by β (filled circle) = β (filled triangle). An inequality indicates rejection of the null hypothesis.

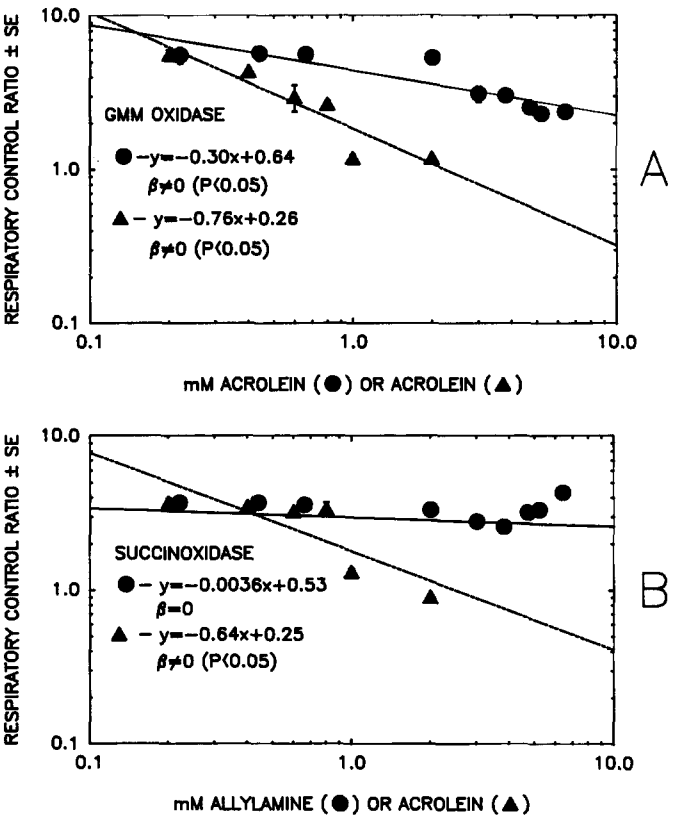

Fig. 3. The effect of allylamine and acrolein on GMM (Panel A) and succinoxidase (Panel B) respiratory control ratios. The best fit regression line for each compound is given on each panel with the solid line representing allylamine's regression and the dot-dot-dash-dot-dot line representing the regression line for acrolein. The results of the hypotheses H_0: $\beta = 0$ are given below each formulae. The test for a difference in slope between concentration-effect of allylamine and acrolein are represented by β (filled circle) = β (filled triangle). An inequality indicates rejection of the null hypothesis.

phosphorylation of coupled mitochondria isolated from rat heart. Both allylamine and acrolein were shown to be statistically significant inhibitors of electron transport in isolated RHM with either succinate or GMM as substrate. The inhibition of mitochondrial function by these compounds is concentration-dependent, albeit, over the range of 0.2—2.0 and 0.2—6.4 mM for acrolein and allylamine, respectively. The concomitant inhibition of ADP-stimulated and uncoupler stimulated respiration (i.e., the inability of uncoupler to stimulate oxygen consumption over inhibited State III values) suggests that both compounds are acting primarily as inhibitors of the electron transport chain with little direct effect, if any, on the phosphorylation of ADP to ATP. Both allylamine and acrolein are statistically significant inhibitors of mitochondrial oxygen consumption with either GMM or succinate as substrate (indicating inhibition both in Complex I [NADH-Coenzyme

Q oxidoreductase], and Complex II [succinate-Coenzyme Q oxidoreductase]. Post-treatment of acrolein- or allyl amine-inhibited mitochondria with FCCP (a potent uncoupler of oxidative phosphorylation) did not reverse the inhibition in either substrate system. These data are consistent with allylamine and acrolein acting primarily as inhibitors of electron transport with little direct effect on the energy coupling mechanism. When the individual toxicities of allylamine and acrolein were compared, it was found that acrolein was a significantly greater inhibitor (when compared to allylamine) of State III and uncoupler-stimulated respiratory activity when GMM was used as substrate. No significant differences in slope were observed when analysis of comparative inhibition with succinate as substrate was performed. These site specific inhibitions agree well with results reported for inhibition of bovine heart submitochondrial particles by allylamine [19] and the effect of acrolein on rat liver mitochondria [15]. The generalized decrement in respiratory control ratios with increasing concentrations of allylamine and acrolein are due to a relatively greater inhibition of State III activity in comparison to inhibition of State IV activity leading to a resultant decrease in the State III/State IV ratio (RCR). The apparent increase in succinate respiratory control ratios at high concentrations of allylamine are due to the marked relative decrease in State IV activity in relation to State III inhibition. This may be a non-specific effect due to massive disruption of membranes and a perturbation of protein structure at these higher allylamine concentrations [19] or due to conversion of allylamine to acrolein while simultaneously inhibiting electron transport.

In the present investigation, the primary toxicant, allylamine, is a weak inhibitor of electron transport in its own right in Complex I and Complex II; its proposed metabolite, acrolein, is also an inhibitor at Complex I and Complex II, with comparatively greater (compared to allylamine) inhibition at Complex I; however, the EC50 for acrolein is still in the mM range. It has been suggested that allylamine is biotransformed to acrolein [8,12] in vivo and in vitro. Benzylamine oxidase, monoamine oxidases, or mixed function oxygenases have been implicated as enzymes responsible for this conversion [7,8,12,25]. Rat heart mitochondria contain both MAO-A and MAO-B in their outer membranes [26] which is consistent with this organelle having the potential of converting allylamine to acrolein. The data of the present investigation demonstrating significantly greater concentration-effect slopes for acrolein when compared to allylamine for both state III and uncoupler stimulated respiratory activity supports this hypothesis.

The sole urinary metabolite of allylamine metabolism has been identified as 3-hydroxypropylmercapturic acid [26]. Interestingly, the capacity of the heart to conjugate acrolein appears to be only a fraction of that of the liver [26], which might explain the relatively high sensitivity of heart, as compared to liver, of allylamine in vivo. Although both organs would possess the ability to metabolize allylamine, the heart appears to have only a limited capacity to detoxify reactive metabolites by glutathione conjugation [4]. In this regard it is plausible that local concentrations could be relatively high, possibly approaching the mM range. Taken at face value, our in vitro data suggests that allylamine and acrolein are in vitro inhibitors of electron transport in rat heart mitochondria, with effects that occur instantaneously. The inhibition observed for acrolein was significantly

greater than that observed for allylamine. In neither case, however, was the degree of inhibition potent enough to invoke a direct effect of either chemical on the in vivo cardiac sequelae of allylamine treatment, as it is unlikely that the mM concentrations needed for inhibition could be reached in tissues of intact animals or humans. Whether concentrations approaching the mM range could in fact be achieved as a result of low cardiac mercapturic acid formation (as outlined above) cannot be rigorously excluded without further experimentation.

Acknowledgment

The writers wish to thank Randall Moreadith of the late Albert Lenninger's laboratory at Johns Hopkins University for discussions on the isolation of high quality RHM. In addition, the writers wish to thank Svetlana Strom of The Ohio State University for technical assistance.

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