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ALLYLAMINE CARDIOTOXICITY—IV

METABOLISM TO ACROLEIN BY CARDIOVASCULAR TISSUES

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Abstract—Acrolein was detected in homogenates of rat aorta, lung, skeletal muscle, and heart incubated with allylamine. Semicarbazide and hydralazine, which protect against allylamine-induced myocardial injury *in vivo* in the rat, inhibited acrolein formation. Hydrogen peroxide, a product of oxidative deamination, was generated during allylamine oxidation. Acrolein was also produced from allylamine by bovine plasma amine oxidase and porcine kidney diamine oxidase but not by rat liver or brain homogenates. Allylamine competitively inhibited benzylamine oxidation in rat aorta, but pargyline-sensitive monoamine oxidase was not involved in acrolein production. The high activity in aorta, the competition with benzylamine, and the sensitivity to benzylamine oxidase inhibitors indicate that benzylamine oxidase is the active enzyme in oxidizing allylamine. The formation of acrolein may be the basis of the cardiotoxic action of allylamine.

Allylamine is a relatively specific cardiovascular toxin which causes vascular and myocardial lesions in several species. When administered orally to rats, allylamine causes subendocardial myocardial necrosis and fibrosis similar in some respects to the lesions produced by sympathomimetic amines [1, 2]. In previous experiments in this laboratory [3, 4] and elsewhere [5], it was found that these allylamine-induced myocardial lesions were markedly diminished in severity when hydralazine, an anti-hypertensive agent with monoamine oxidase (MAO) inhibitor activity [6], or semicarbazide, an inhibitor of pyridoxal-type enzymes including plasma amine oxidase [7], was fed simultaneously with allylamine.

Based on these findings, we hypothesized that allylamine is converted by an amine oxidase into acrolein (CH_2=CH—CHO), in a manner analagous to the conversion of allyl alcohol into acrolein by hepatic alcohol dehydrogenase [8]. Acrolein, a highly reactive aldehyde with potent cytotoxicity [9], has been identified as an *in vitro* metabolite of allylamine in bovine serum [10], but not in rat liver fractions [8]. However, the confinement of allylamine-induced injury to the heart and blood vessels suggested that cardiovascular tissue might be active while liver is inactive in transforming allylamine into acrolein.

We have recently demonstrated a marked histochemical and biochemical derangement of cardiac MAO in rats fed allylamine [2, 4], indicating some role for a cardiac amine oxidase in allylamine toxicity. In this paper we report the conversion of allylamine into acrolein by homogenates of rat aorta, heart and several other tissues. We further characterize the enzymatic oxidation of allylamine using specific enzyme inhibitors and inhibition kinetics.

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MATERIALS AND METHODS

Materials. The following compounds were received as donations: clorgyline (May & Baker Ltd., Dagenham, U.K.), procarbazine HCl (Roche Products Ltd., Welwyn Garden City, U.K.), and (−)-deprenyl (J. Knoll, Budapest, Hungary).

Purified bovine plasma amine oxidase (sp. act. 29 units/g) was purchased from Miles Laboratories, Elkhart, IN. Porcine kidney diamine oxidase was obtained from the Sigma Chemical Co., St. Louis, MO. Allylamine HCl (ICN, Plainview, NY), semicarbazide HCl (Sigma), benzylamine, tyramine HCl, pargyline and tranylcypromine HCl (Aldrich Chemical Co., Milwaukee, WI) were all reagent grade quality. Benzylamine was converted to benzylamine sulfate and recrystallized from methanol–water (1:1). [1-^{14}C]Tyramine was purchased from the New England Nuclear Corp. (Boston, MA).

The purity of our allylamine was established by gas chromatography using head-space analysis over a solution of the free base. No propargylamine could be detected in the allylamine solutions.

Acrolein semicarbazone was prepared as described by Shriner *et al.* [11]. The product had a melting point (sealed capillary tube method) of 168° (literature value 171°).

Tissue homogenates. Male Sprague–Dawley rats weighing 400–500 g were anesthetized with ether, and blood was withdrawn from the inferior vena cava using a heparinized syringe. Heart, liver, aorta, pectoralis muscle, lung and brain were excised and blotted; the heart was trimmed at the base to prevent contamination with aorta. Aortas were scraped along the adventitia to remove adipose tissue and blood. Plasma was diluted 1:5 in distilled water and stored at 4°. Other tissues were homogenized immediately after collection in 9 vol. of ice-cold 0.25 M sucrose using a Sorvall Omni-Mixer and stored at −20° until used. For experiments involving acrolein production, the homogenates and plasma were dialyzed at 4°

against 0.1 M phosphate buffer (pH 7.4) for 24 hr, centrifuged at 600 *g* for 10 min, and stored at −20° until used.

Acrolein determination. Aliquots (0.5 ml) of dialyzed tissue homogenate or enzyme solution were put into 20 ml glass-stoppered tubes containing 0.5 ml of 0.3 M sodium phosphate buffer (pH 7.4) and 0.5 ml of allylamine solution. The tubes were bubbled with oxygen, stoppered, and incubated at 37° for 6 hr. This 6-hr incubation was necessary to ensure the formation of sufficient levels of product for detection of acrolein in tissues with low activity. Incubation was terminated by the addition of 0.5 ml of 30% trichloroacetic acid (TCA) and freezing to condense any vapor, followed by thawing and centrifugation (600 *g*, 10 min). Controls were treated identically except that the allylamine was added after the incubation instead of before.

The TCA supernatant fractions were then transferred to a distillation apparatus consisting of a 1.8 mm (i.d.) × 100 cm piece of glass tubing in an ice water jacket, connected via a 45° bend to a 25-ml Erlenmeyer flask. In preliminary experiments, this distillation apparatus was found to be necessary to prevent loss of trace amounts of acrolein. The supernatant fractions were rapidly distilled, the distillate was brought to a volume of 2 ml, and acrolein was measured fluorometrically by reaction with *m*-aminophenol [12].

Recovery of acrolein solutions of 0.1 to 10 μg/ml in the distillation procedure alone was 80–85%. Recovery of acrolein added to tissue homogenate and incubated for 6 hr and distilled as above averaged 50–60% overall. Recoveries were similar for all tissues except plasma, from which 25% overall recovery was obtained. Acrolein values were corrected for percent efficiency of recovery from homogenate. The minimum detectable amount of acrolein incubated for 6 hr in the presence of homogenate was about 1 nmole.

The concentrations of acrolein present were too low for any significant polymerization to occur; standards of acrolein incubated alone for 6 hr showed no noticeable deterioration. The major loss of acrolein during distillation was thought to be due to incomplete condensation and handling. Recoveries of replicate distillations varied by no more than ± 5%.

The effect of MAO inhibitors on acrolein production was assessed by preincubation of tissue homogenates at 37° for 20 min with three different concentrations of inhibitor before the addition of allylamine. Control samples were preincubated for the same period.

Identification of acrolein. Incubations for spectroscopic identification of acrolein as its semicarbazone were performed using 10 ml of centrifuged rat muscle homogenate or 3.5 ml of centrifuged rat aorta homogenate with 10 ml of 0.9 mM allylamine HCl and 10 ml of 0.3 M sodium phosphate buffer (pH 7.4) in 250-ml glass stoppered flasks. The solutions were bubbled with oxygen, a 6-hr incubation was performed as before, and the reaction was stopped by adding 5 ml of conc. HCl. Tubes were centrifuged (600 *g*, 20 min) and acrolein was collected by bubbling air through the supernatant fraction at 20° at a rate of 150 cm^3/min for 10 min, into a cold trap surrounded by crushed ice. With 1 ml of distilled water in the cold trap, 10% of the acrolein was recovered at a concentration of about 4 times over the original solution. Larger volumes in the cold trap greatly increased the efficiency but lowered the final concentration. Ultraviolet spectroscopy was done on the cold trap solutions with a Gilford 250 split-beam spectrophotometer, using as a reference either distilled water or a solution obtained by substituting 30 ml of distilled water for HCl supernatant fluid in the cold trap apparatus. Acrolein solutions >1 μg/ml exhibited an absorption maximum at 209 nm. Then 0.1 ml of 0.175 M semicarbazide HCl was added, the spectrum was taken again, and the height of the acrolein semicarbazone peak at 257 nm was determined.

Hydrogen peroxide production. Initial rates of hydrogen peroxide production as an indicator of oxidative deamination were measured fluorometrically using the homovanillic acid peroxidase assay of Snyder and Hendley [13]. Continuous tracings of activity were obtained from incubation mixtures reacted in a temperature-controlled cuvette holder at 35°, using 0.05 ml of either fresh undialyzed homogenate or homogenate dialyzed as above as the enzyme source. Approximately 60% of the original activity was retained in the homogenate after 24-hr dialysis and centrifugation. Fluorescence changes in control reaction mixtures lacking substrate were subtracted from the final readings; only in cases where the enzyme activity was present in trace amounts was this effect a major portion of the fluorescence changes observed.

Monoamine oxidase activity. MAO activity was measured in whole tissue homogenates by a standard method [14] using radioactive tyramine as substrate and 0.05 ml of homogenate as the enzyme source. Activity was determined at 37° over the initial 1.0 min after substrate addition. All inhibitor and substrate solutions were made up in 0.067 M phosphate buffer (pH 7.4). Best-fit lines for initial-velocity experiments were drawn using kinetic parameters calculated by a computer program similar to that of Cleland [15].

Benzylamine oxidase activity. BzAO activity was measured in homogenates by the method of Lewinsohn *et al.* [7] using radioactive benzylamine as substrate and 0.05 ml of homogenate as the enzyme source, in the presence of 0.40 mM deprenyl and 10^{-7} M clorgyline. Activity was determined at 37° over the initial 5 min after addition of substrate.

Protein was measured by the method of Lowry *et al.* [16] using bovine serum albumin as a standard.

RESULTS

Conversion of allylamine to acrolein. Acrolein was found in the distillates of rat aorta, lung, muscle and heart homogenates incubated with allylamine (Table 1). The optimal allylamine concentration (0.1 to 0.3 mM) appeared to be the same for all tissues. Aorta produced the largest amount of acrolein under these conditions, converting at least 80% of the allylamine into acrolein. Acrolein was not detected in rat liver or brain homogenates or rat plasma

Table 1. Conversion of allylamine into acrolein and generation of hydrogen peroxide by rat tissue homogenates and pure enzymes*

Sample	Activity: Acrolein (% conversion)	Activity: H_2O_2 [nmoles · min^{-1} · (mg protein)$^{-1}$]
Allylamine only†	0	0
Rat aorta	80	0.148
Rat lung	22	0.077
Rat skeletal muscle	8	0.068
Rat heart	5	0.009
Rat heart + chloral hydrate (1 mM)	4	
Rat liver	0	0.003
Rat liver + chloral hydrate (1 mM)	0	
Rat brain	0	0.002
Rat plasma	0	
Bovine plasma amine oxidase‡	92	
Porcine kidney diamine oxidase§	7	

* Values are means of duplicate determinations.
† Allylamine concn = 0.3 mM.
‡ One-tenth milligram of enzyme was used.
§ Five milligrams of enzyme was used.

incubated with allylamine at concentrations ranging from 0.05 to 20 mM. Commercially obtained bovine plasma amine oxidase converted allylamine to acrolein actively, while comparatively slight activity was seen with commercially obtained porcine kidney diamine oxidase. No acrolein could be detected in any sample if allylamine was added after the incubation or if allylamine was incubated alone.

Hydrogen peroxide was also generated from allylamine by rat aorta, lung, muscle and heart homogenates (Table 1), with the highest rates occurring in aorta and lung homogenates; traces were also produced by liver and brain homogenates. The data shown in Table 1 were obtained using dialyzed homogenates. Omission of the dialysis step did not affect the relative rates of H_2O_2 production in different tissues.

To determine whether the allylamine-oxidizing activity was stable over the 6-hr period used to measure acrolein production, aliquots of non-dialyzed rat aorta and heart homogenate were preincubated without substrate from 0 to 6 hr, after which allylamine was added and the rates of H_2O_2 production were monitored over the initial 1 min. The results indicated that allylamine-oxidizing activity was relatively stable in these tissues, decreasing by approximately 30–35% after 6 hr at 37°.

Since the H_2O_2 production was measured under conditions in which H_2O_2 does not accumulate, and since in the assay system the horseradish peroxidase concentration was high enough to vitiate any effect caused by endogenous catalase or peroxidase, it can be assumed that these numbers represent the actual relative rates of allylamine oxidation to acrolein in these tissue homogenates. Further metabolism of acrolein by alcohol dehydrogenase or aldehyde dehydrogenase can occur [17], although the alcohol dehydrogenase reaction favors the reverse reaction of acrolein formation from allyl alcohol [8]. Addition of chloral hydrate, an aldehyde dehydrogenase inhibitor, to reaction mixtures containing allylamine and heart or liver homogenate, did not increase the amount of acrolein detected (Table 1). Since aldehyde dehydrogenase does not produce H_2O_2, further metabolism of acrolein would probably not affect the initial rates of peroxide formation. Aldehyde dehydrogenase is not present in lung tissue [17].

The production of acrolein by heart homogenate was inhibited by a 20-min aerobic preincubation with 1 μM phenelzine, procarbazine or hydralazine or 50 μM semicarbazide, but not by 50 μM clorgyline,

Table 2. Effect of various inhibitors on acrolein formation in rat heart homogenate*

Inhibitor	% Inhibition: 1 μM	50 μM	1 mM
Clorgyline	0	0	70
Pargyline	0	13	19
Deprenyl	0	11	26
Tranylcypromine	0	77	75
Semicarbazide	0	92	89
Hydralazine	71	100	100
Procarbazine	74	100	100
Phenelzine	100	100	100

* Reaction mixtures were aerobically preincubated in duplicate for 20 min at 37° with the indicated concentration of inhibitor. Following preincubation, allylamine was added, and the solution was bubbled with oxygen and incubated for 6 hr at 37° prior to acrolein determination. (Allylamine concentration = 0.3 mM; control activity = 5.0% conversion into product.)

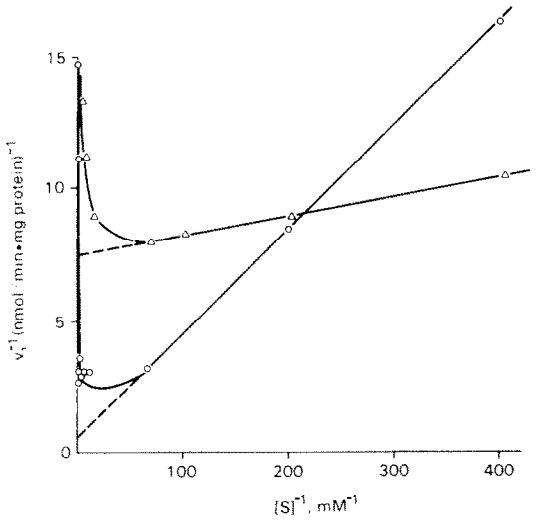

Fig. 1. Lineweaver–Burk plots of allylamine and benzylamine oxidation in rat aorta. Data points represent initial rates of H_2O_2 production from 0.05 ml of non-dialyzed rat aorta homogenate (see Materials and Methods) from allylamine (○), and from benzylamine (△).

and only slightly by 50 μM pargyline or deprenyl (Table 2). Tranylcypromine (50 μM) produced partial inhibition. The higher concentrations of hydralazine and semicarbazide were also found to react directly with acrolein, creating uncertainty as to whether their effect was due to enzyme inhibition or to interference in the assay. However, semicarbazide and hydralazine also inhibited H_2O_2 production, indicating that their mode of activity is enzyme inhibition and not simply direct reaction with acrolein. Other inhibitors did not react with acrolein at the concentrations used in this experiment.

Initial rates of allylamine oxidation, as measured by H_2O_2 production, were inhibited by high concentrations of allylamine (Fig. 1). Benzylamine oxidation was similarly substrate-inhibited. Substrate inhibition was also observed in experiments measuring acrolein production, although this effect was difficult to distinguish from a direct reaction between acrolein and allylamine which reduced the recovery of acrolein at allylamine concentrations >5 mM.

Identification of the reaction product. To verify the identity of the reaction product formed from allylamine, the *m*-aminophenol-reacted distillates of heart homogenate containing allylamine added before or after incubation were neutralized and extracted into ether, following the procedure suggested by Alarcon and Meienhofer [18]. The ether extract was evaporated and redissolved in 2 ml of water. The fluorescence excitation and emission spectra of allylamine-incubated samples were identical to the spectra produced by authentic acrolein.

Several compounds were tested for possible fluorescence interference in the acrolein assay. Ammonia, formic acid, formaldehyde, acetic acid, allyl alcohol and acrylic acid did not interfere. Allyl bromide produced a fluorescent product associated with a dark yellow color which was not observed in any allylamine-incubated samples. Crotonaldehyde also gave a fluorescent product but was eliminated as a possible metabolite because of its different fluorescence spectrum [12].

The u.v. spectra of cold trap solutions obtained from rat aorta or muscle homogenate incubated with allylamine exhibited peaks at acrolein's absorbance maximum of 209 nm. After addition of 0.1 ml of 0.175 M semicarbazide HCl, a peak appeared at 257 nm which was absent when semicarbazide was added to distilled water or to control samples to which allylamine had been added after the incubation. The absorbance peak at 257 nm coincided with that of synthetic acrolein semicarbazone.

To test for the possibility that acrolein was being produced by contaminating bacteria, which contain an enzyme similar to bovine plasma amine oxidase [19], additional rat hearts were collected under sterile conditions and the experiment was repeated using sterile filtered solutions and sterilized glassware. The rate of acrolein production was found to be unchanged.

Kinetic studies. To determine the enzyme responsible for acrolein production, we studied inhibition of pargyline-sensitive monoamine oxidase by allylamine in rat heart homogenate using tyramine as substrate. Benzylamine oxidase in rat aorta was studied using the substrate benzylamine. Competitive inhibition would be expected if allylamine were a substrate for either of these enzymes, since this type of inhibition would indicate an affinity of allylamine for the active site.

Preincubation of rat heart homogenate with allylamine for increasing times reduced MAO activity for tyramine to a final level which was related to allylamine concentration (Fig. 2). The concentration required to give 50% inhibition after 20 min of incubation was about 0.5 mM. Lineweaver–Burk plots of tyramine oxidation in heart homogenate

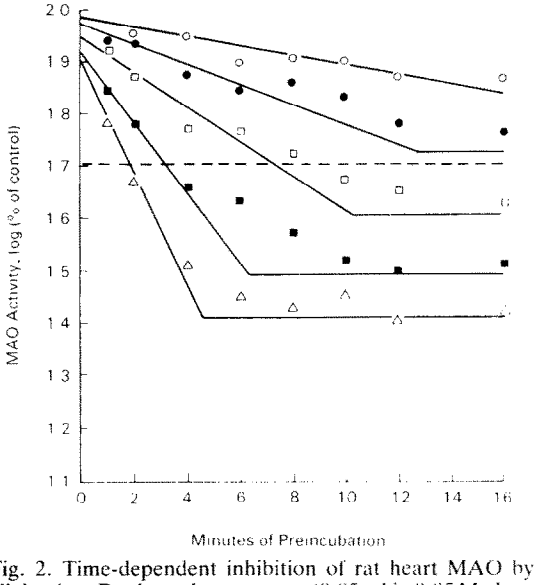

Fig. 2. Time-dependent inhibition of rat heart MAO by allylamine. Rat heart homogenate (0.05 ml in 0.05 M phosphate buffer, pH 7.4) was preincubated for various periods at 37° with 0.2 mM (○), 0.5 mM (●), 1 mM (□), 2 mM (■), or 4 mM (△) allylamine HCl. Radioactive tyramine was then added to a concentration of 1 mM and the reaction stopped 1.0 min later by addition of 0.1 ml of 3 M HCl. (Dashed line = 50% inhibition).

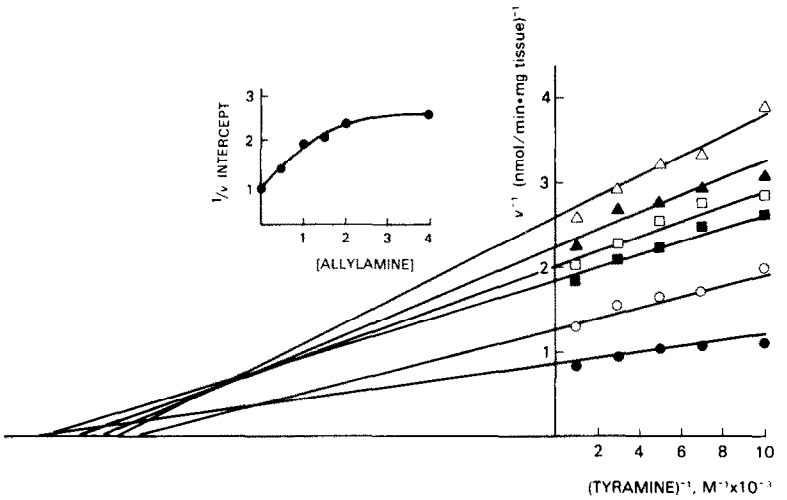

Fig. 3. Lineweaver–Burk plots of time-dependent inhibition of rat heart MAO by allylamine. Preincubation reaction mixtures, containing 5 mg of homogenized rat heart tissue in 0.35 ml of 0.05 M sodium phosphate buffer (pH 7.4) with 2 mM NaCl (no inhibitor) (●), 0.5 mM allylamine (○), 1.0 mM allylamine (■), 1.5 mM allylamine (□), 2.0 mM allylamine (▲) or 4.0 mM allylamine (△), were maintained at 37° for 20 min. Following preincubation, 0.05 ml of radioactive tyramine in buffer was added and MAO activity was measured over the initial 1.0 min. (Inset: replot of intercept vs allylamine concentration.)

after a 20-min preincubation with allylamine took the form of a family of lines intersecting near the 1/*S* axis (Fig. 3). When the preincubation step was omitted, only a slight inhibition was observed. This time-dependent, non-competitive inhibition, which closely resembles the type of inhibition described for allylamine in the liver [20], would not be expected if allylamine and tyramine were common substrates.

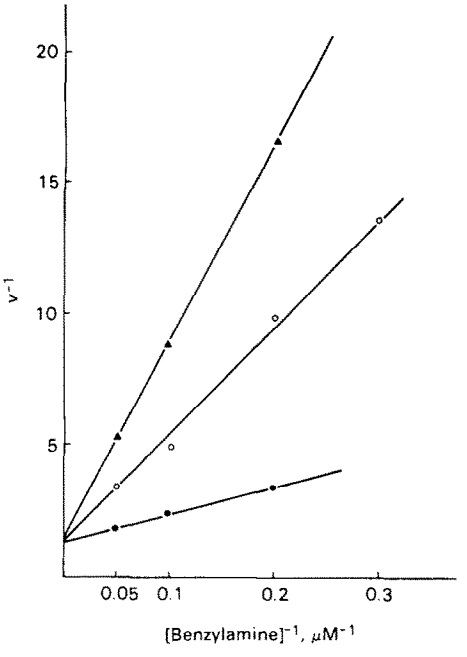

Fig. 4. Lineweaver–Burk plots of inhibition of rat aorta benzylamine oxidase by allylamine. Product formation from radioactive benzylamine was measured in reaction mixtures containing 5 mg of homogenized rat aorta tissue in 0.40 ml of 0.05 M phosphate buffer (pH 7.4), after a 5-min incubation at 37° with no inhibitor (●), 0.3 mM allylamine (○), or 0.9 mM allylamine (▲).

Benzylamine oxidation in rat aorta, on the other hand, was strongly inhibited by allylamine without the requirement for preincubation. Lineweaver–Burk plots of benzylamine oxidase activity in the presence of allylamine indicated that allylamine is a competitive inhibitor (Fig. 4). No time-dependent inhibition by 1 mM allylamine was observed in rat aorta homogenate when benzylamine was used as a substrate. Benzylamine-deaminating activity in rat heart was not high enough to determine whether it is competitively inhibited by allylamine.

DISCUSSION

In this study we have demonstrated that homogenates of several rat tissues convert allylamine to acrolein. The relative rates of hydrogen peroxide production during allylamine oxidation in different rat tissues correspond with the reported distribution of the enzyme benzylamine oxidase, which is highest in aorta, lung and the gastrointestinal system [7]. Semicarbazide and procarbazine, selective inhibitors of benzylamine oxidase [7, 21], and the hydrazine derivatives hydralazine and phenelzine, potent inhibitors of amine oxidases [6, 22, 23], all inhibited acrolein formation, whereas clorgyline and deprenyl, preferential type "A" and "B" MAO inhibitors [24], were relatively ineffective. The competitive inhibition observed between allylamine and benzylamine is further evidence that the active enzyme is benzylamine oxidase. The inability to detect acrolein in liver, brain or plasma is consistent with the low levels of this enzyme in these tissues [7].

Previous *in vivo* experiments in this laboratory [4] have demonstrated a 50% inhibition of MAO activity in rat heart, liver and brain tissues after substituting 11 mM allylamine for drinking water. In the present study, we found a similar degree of MAO inhibition *in vitro* in rat heart homogenate prein-

cubated with an allylamine concentration of 0.5 mM, suggesting that the *in vivo* tissue concentration of allylamine in the rat is probably in the same range. This concentration would be nearly optimal for production of acrolein.

Acrolein itself reacts non-enzymatically with proteins with such vigor that it has been used as a fixative for electron microscopy [25]. It is also reactive toward sulfhydryl groups [26], and may bind to nucleic acids or disrupt nucleic acid synthesis [9]. These toxic properties would cause denaturation and inactivation of enzymes and interference with cellular functions. In addition, acrolein reportedly has a direct pressor effect on the heart following inhalation or intravenous administration [27].

The high levels of acrolein produced by aorta compared with other organs would be expected to result in a specific cardiovascular toxicity. The reported toxic effects of allylamine, including hyalinized aortic plaques [5], cartilagenous metaplasia of the aortic arch [5], medial hypertrophy of coronary arteries [28], myocarditis [29], and myocardial fibrosis culminating in a transmural aneurysmal scar [1], may be explained by localized production of acrolein in vessels, including small vessels of the heart. The present study, however, demonstrates that allylamine oxidation also occurs in lung and skeletal muscle tissues, which are not morphologically injured during allylamine intoxication. Lung might be expected to rapidly exhale volatile substances such as acrolein. Skeletal muscle is subject to extreme alterations in blood flow, and may be spared injury by allylamine or its metabolite, acrolein, due to reduced blood flow during allylamine intoxication. *In vivo* allylamine distribution and excretion studies would be helpful in resolving these considerations.

In conclusion, the present series of experiments suggests that allylamine is metabolized by cardiovascular benzylamine oxidase to acrolein. The high reactivity of this conjugated aldehyde and the apparent tissue specificity of its site of formation may be the basis for the toxic effects of allylamine on cardiovascular tissue.

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