Biochemical Pharmacology, Vol. 36, No. 24, pp. 4347–4353, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

IN VIVO METABOLISM OF THE CARDIOVASCULAR TOXIN, ALLYLAMINE

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(*Received* 17 *February* 1987; *accepted* 28 *May* 1987)

Abstract—Previous evidence from this laboratory demonstrated that allylamine, a known cardiovascular toxin, is metabolized *in vitro* to acrolein, which has been hypothesized to act as a distal toxin. In this study, 3-hydroxypropylmercapturic acid was isolated and identified by MS, NMR, and 2D-NMR spectroscopy as the sole urinary metabolite of allylamine metabolism *in vivo*. Parallel experiments showed reduced glutathione (GSH) depletion in several organs (most marked in aorta, blood, and lung), which is consistent with GSH conjugation of the proposed acrolein intermediate. These findings indicate that allylamine was metabolized *in vivo* to a highly reactive aldehyde which was converted to a mercapturic acid through a GSH conjugation pathway; the exact mechanisms of cellular damage remain unclear.

Allylamine (3-aminopropene) is a unique cardiovascular toxin which causes extensive and progressive myocardial and vascular lesions in several animal species when administered by a variety of routes [1–3]. We previously reported the *in vitro* metabolism of allylamine to acrolein by rat [4] and human [5] cardiovascular tissues; at that time we hypothesized that acrolein acts as the distal toxin responsible for the cellular damage caused by allylamine intoxication.

The toxic effects of several diverse compounds have been related to their ability to form acrolein, an extremely reactive and toxic aldehyde (for review of acrolein toxicity, see Ref. 6). The antitumor agents cyclophosphamide, ifosfamide, trofosfamide and cytoxyl alcohol [6, 8], and several allylic compounds including allyl alcohol and allyl formate [9], are all presumably metabolized to acrolein. Extensive study of the metabolism of these anti-tumor agents and allylic compounds indicates that the acrolein intermediate is conjugated with reduced glutathione (GSH) to form a mercapturic acid (MCA) as the urinary excretion product. The measurement of mercapturic acids in urine has become increasingly popular, because the presence of these compounds implicates an underlying mechanism of glutathione conjugation *in vivo* [10].

Earlier experiments in this laboratory showed that, in rats given [^{14}C]allylamine, the majority of radiolabel is excreted in the urine by 24 hr [11]. In the present work, we isolate and identify a solitary MCA as the excretory metabolite in urine. We also demonstrate systemic alterations in GSH during acute allylamine intoxication. The results indicate that allylamine was metabolized *in vivo* to acrolein, which was conjugated with glutathione and ultimately excreted in the urine as a mercapturic acid.

MATERIALS AND METHODS

Chemicals

[^{14}C]Allylamine was custom synthesized (California Bionuclear) with ^{14}C at the C-1 position (sp. act. 0.5 mCi/mmol). Purity was established at 98% by thin-layer chromatography. The purity of unlabeled allylamine (ICN Pharmaceuticals) was established at 98% by gas chromatography using head space analysis over a solution of the free base. Allylamine was given to rats as the HCl salt dissolved in double-distilled water. All other chemicals used were of reagent grade. The solvents used for chromatography were of HPLC grade.

General methods

Melting point was determined by the capillary tube method and was uncorrected. Thin-layer chromatography was performed on silica gel F_{254} pre-coated chromoplates (Merck, Darmstadt); the plates were developed in *n*-butanol–acetic acid–H_2O (4:1:1, by vol.) and were sprayed with chloroplatinic acid reagent, ninhydrin (1% in acetone) and universal reagent [12]. Autoradiography was used to detect radiocarbon on the plates, while non-radioactive standards were located by spraying the plates with universal reagent. Quantitation of the radioactivity was accomplished tor TLC plates and HPLC fractions by scintillation counting.

Mass spectra were recorded on a Finnigan 3300 GC-MS. NMR were obtained on a Nicolet NT-300 in D_2O or $CDCl_3$ (TMS or *p*-dioxane was used as an internal reference).

Animal treatment

Male Sprague–Dawley rats were obtained from a commercial breeder (Harlan, Houston, TX) and

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were housed in hanging cages and maintained on standard laboratory rat chow and water *ad lib.* All rats were acclimatized in our animal care facility for 1 week prior to treatment. In the study of urinary metabolites, four rats (200–230 g) in groups of two were given [^{14}C]allylamine (150 mg/kg, 2.0 μCi/kg) by gavage as previously described [11]. Rats were placed in metabolic cages with free access to food and water, and total urine was collected at 24 and 48 hr. Four rats were treated similarly by gavage with water only to obtain control urine. Histopathologic examination of hearts from experimental rats showed myocardial lesions typically seen at 24 or 48 hr after this dose of allylamine [3, 13].

In the separate glutathione studies, groups of four to eight rats were similarly given 150 mg/kg of allylamine by gavage; rats were treated at 12:00 and were killed concomitantly with controls (three to four rats) at 1, 2, 4, 6, 12 and 24 hr after administration. Rats were anesthetized with ether, whole blood was collected from inferior vena cava, and rats were killed by removing the heart. Portions of ventricular myocardium, liver, kidney, lung and aorta (scraped free of connective tissue) were frozen on dry ice and stored at −80° for later biochemical analysis.

Isolation and identification of urinary metabolite

Urine of rats treated with allylamine and control urine were counted for radioactivity using standard scintillation methods. The maximum radioactivity was found in the 24-hr urine from allylamine-treated rats; 62% of the given dose was recovered at this time. The 24-hr samples were pooled and lyophilized. The solid residue was washed repeatedly with 20% aqueous methanol containing 0.1% acetic acid on a C_{18} Sep-Pak column until no radioactivity was detected in the washing (10 ml × 3). The combined wash was then concentrated under nitrogen to approximately 1 ml, which was used for further purification by HPLC.

HPLC was performed at room temperature (21°) on a Beckman Isocratic Liquid Chromatograph, model 330, equipped with a 10 cm RP_{18} cartridge in a radial compression zeta module (Water Associates, Milford, MA). H_2O–MeOH–AcOH (86:14:0.1, by vol) was used as solvent at a flow rate of 1 ml/min. The column effluent was monitored at 254 nm. One hundred microliters (40,500 dpm) was injected in each case. Sixty fractions of 1 ml were collected. The radioactive fractions 8–10 were combined after repeated injections, concentrated, and rechromatographed over an ultrasphere ODS column (5 μm, 25 cm × 4.6 mm i.d.), under the conditions described above. Virtually all radioactivity was observed in fractions 10 and 11 (combined dpm was 34,960, 86.3% recovery).

Synthesis of reference compounds

The following procedure was used to synthesize the expected MCA [14].

S-(3-Hydroxypropyl)-L-cysteine (I). L-Cysteine hydrochloride hydrate (5 g) was dissolved in 50 ml of water, and KOH pellets were added to bring the solution to pH 11. Three millilitres of 3-chloro-1-hydroxypropane was added; after 6 hr at room temperature the solution was acidified to pH 4 with 6 N HCl and reduced in volume to about 20 ml with a stream of nitrogen; solid was precipitated by adding EtOH (25 ml) dropwise. The precipitate was filtered and then washed with acetone and ether (10 ml). The filtrate formed white crystals, which were filtered off after 2 hr. Acetone and ether (10 ml) were again added to the filtrate; after 4 days at 5°, colorless crystals, which were filtered and dried, were obtained: m.p., 210° (decomp.) (R_f 0.44), yield 4.2 g. ^{1}H-NMR (300 MHz; δ; D_2O): 1.52 (2H, m), 2.35 (2H, dd, J = 11.9, 7.2 Hz), 2.75 (2H, dt*, J = 11.9, 5.8 Hz), 3.35 (2H, m), 3.61 (1H, dt, J = 11.9, 5.8 Hz). MS (70 eV, *m/z*; % abundance): 179 (M^+, 18), 162 (31), 161 (9.5), 157 (11), 141 (9.2), 134 (38), 121 (18), 117 (89), 75 (50), and 61 (100).

S-(3-Hydroxypropyl)-N-acetylcysteine (3-OH Pr MCA, II). Acetylation of *S*-(3-hydroxypropyl)-cysteine (I) with acetic anhydride at room tem-

* dt indicates triplet distorted.

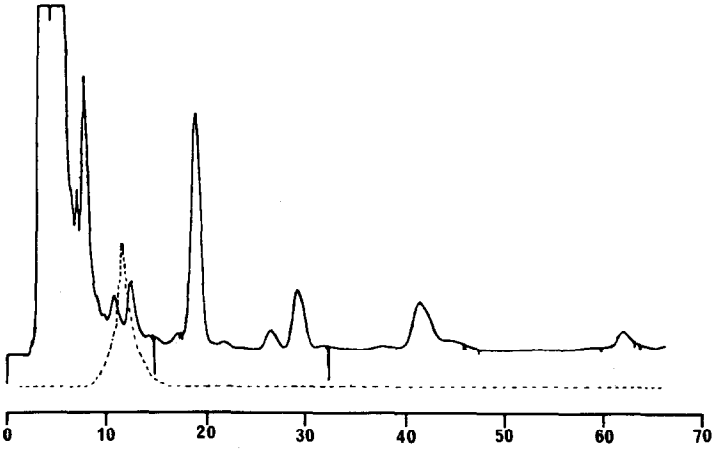

Fig. 1. HPLC profiles of urine extract (solid line) from rats given allylamine (24-hr collection) and purified urinary metabolite, 3-OH Pr MCA (dotted line).

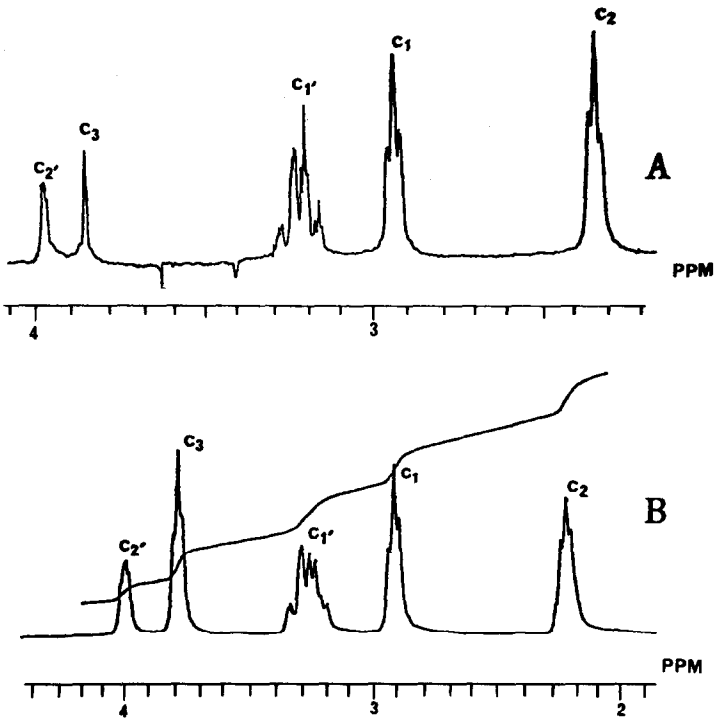

Fig. 2. Proton NMR spectra of (A) 3-OH Pr MCA isolated from urine of allylamine-treated rats and (B) synthesized 3-OH Pr MCA.

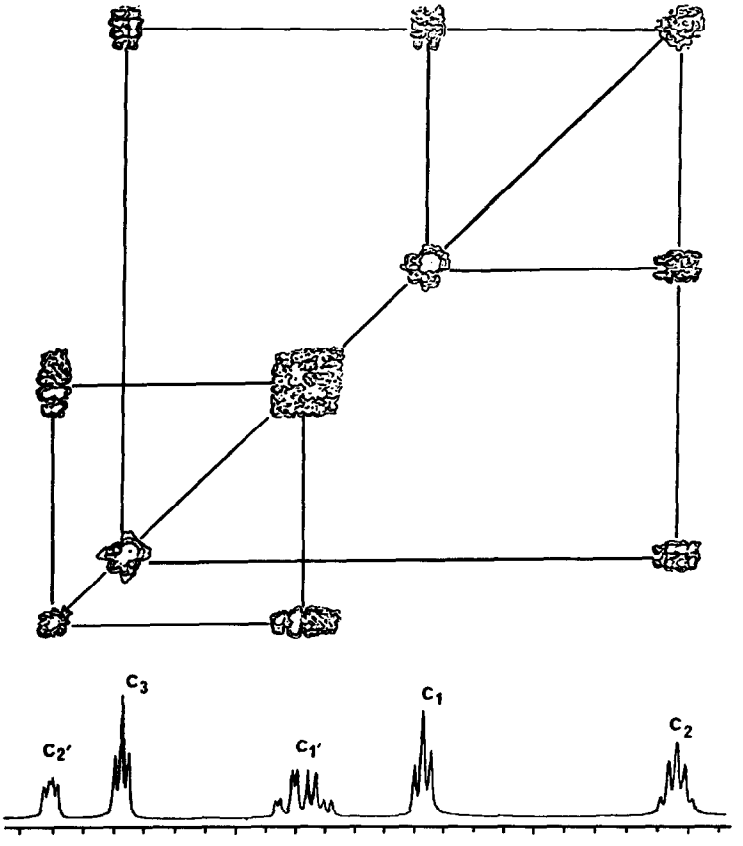

Fig. 3. Contour plot showing the two-dimensional NMR (COSY) spectrum of 3-OH Pr MCA recorded at 300.068 MHz in deuterium oxide.

perature for 24 hr gave the expected mercapturic acid (II) as a brown oil. This was purified by column chromatography over RPC_{18} silica gel (0.6 × 30 cm), eluted with 10% water in methanol, and purified further by preparative TLC, giving a colorless thick oily compound (R_f 0.67). ^{1}H-NMR (δ; D_2O): 1.89 (3H, S, —$COCH_3$)*, 2.23 (2H, m), 2.86 (2H, dt), 3.35 (2H, m), 3.89 (2H, dt), 4.05 (1H, t). MS (70 eV; *m/z*; % abundance): 221 (M^+, 11.5), 179 (18.0), 162 (28), 134 (26), 117 (82), 105 (17), 75 (48), and 61 (100).

Glutathione analysis

Tissues were minced and homogenized in 0.1 M phosphate buffer containing 5 mM EDTA (1/5 or 1/10, w/v), and glutathione (GSH) was assayed fluorometrically with *o*-phthalaldehyde according to the method of Hissin and Hilf [15]. Values of GSH are given in μg/g for all wet tissues except whole blood, which is given in μg/ml. Experimental values were compared to time-matched control values (three to four rats) by ANOVA because of the well-known diurnal variation of GSH in several organs; control values varied 10–30% in different organs over the 24-hr period (data not shown).

RESULTS

Isolated metabolites

The radioactive fractions purified from the urine of experimental animals gave a single spot on TLC (R_f 0.67) and a single major peak on HPLC having retention time between 10 and 11 min (Fig 1). Synthetic 3-OH Pr MCA had the same retention time as the purified fraction from experimental urine when co-chromatographed by TLC under the same conditions. Control urine samples showed no similar TLC spot or HPLC peak when compared to the experimental sample. The NMR and mass spectral fragmentation pattern [16] (Figs. 2, 4 and 5) of the isolated metabolite support the structure as 3-OH Pr MCA. The proton NMR spectra (Fig. 2) gave the following: (300 MHz; δ, D_2O): 1.88 (3H, S, —$COCH_3$)*, 2.31 (2H, m, C_2-H), 2.90 (2H, dt, J = 11.9, 6.8 Hz; C_1-H), 3.34 (2H, m, $C_{1'}$-H), 3.89 (2H, dt, J = 11.9, 6.9 Hz; $C_{3'}$-H), 4.05 (1H, t, J = 11.1, 5.9 Hz; $C_{2'}$-H).

The NMR assignment was further confirmed by the proton connectivities in COSY 2D-NMR spectrum [17] (Fig. 3).

The electron-impact mass spectrum (Fig. 4) of the isolated metabolite gave the molecular ion peak at *m/z* 221 (M^+, 12.2%), corresponding to the molecular formula as $C_8H_{15}NO_4S$. The fragmentation patterns are shown in Fig. 5.

GSH studies

Aorta (Fig. 6A) showed rapid, profound decreases in GSH (25% of control) at 1 and 2 hr after allylamine administration. Recovery of GSH occurred from 4 to 12 hr, with GSH reaching control levels by 12 hr. Lung GSH (Fig. 6A) was decreased profoundly at all times examined (7–30% of control at 1–12 hr). Blood GSH (Fig. 6A) also decreased significantly and remained low at all times examined (20–40% of control). GSH content of heart (Fig. 6B) dropped slightly (90% of control) at early times (1, 2, and 4 hr) and continued to be decreased (approximately 80% of control) throughout the experiment.

Kidney (Fig. 6B) showed less pronounced alterations in GSH than other organs, different from control only at 4 and 6 hr; hepatic GSH (Fig. 6B) slowly decreased from 2 to 12 hr (52% of control at 12 hr) and remained lower than time-matched control at 24 hr.

DISCUSSION

Allylamine (3-aminopropene) is a relatively specific cardiovascular toxin in a variety of species [1–3, 18, 19]. Given acutely by gavage at the dose utilized in this study, allylamine causes acute myocardial necrosis which is apparent by 24 hr [13].

* Acetyl peak with water peak.

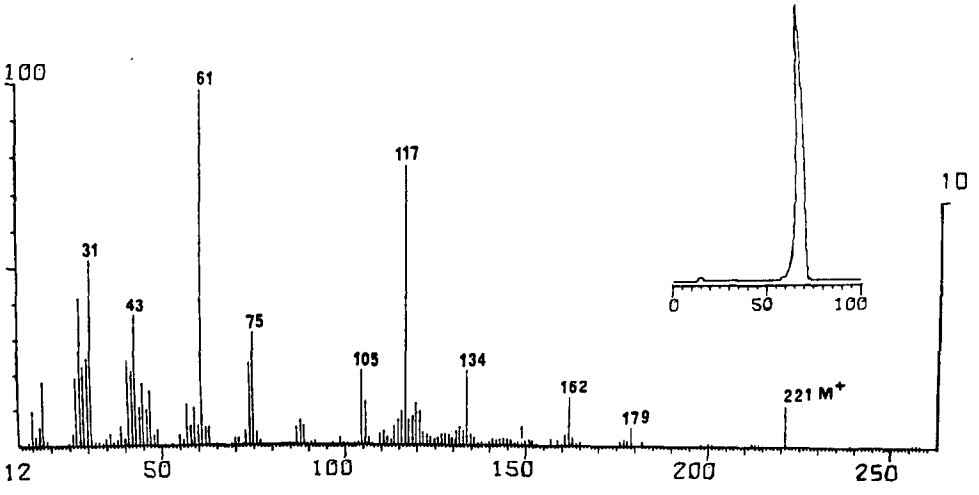

Fig. 4. EI (electron-impact mass spectrum) of 3-OH Pr MCA isolated from urine of rats given allylamine (24-hr collection).

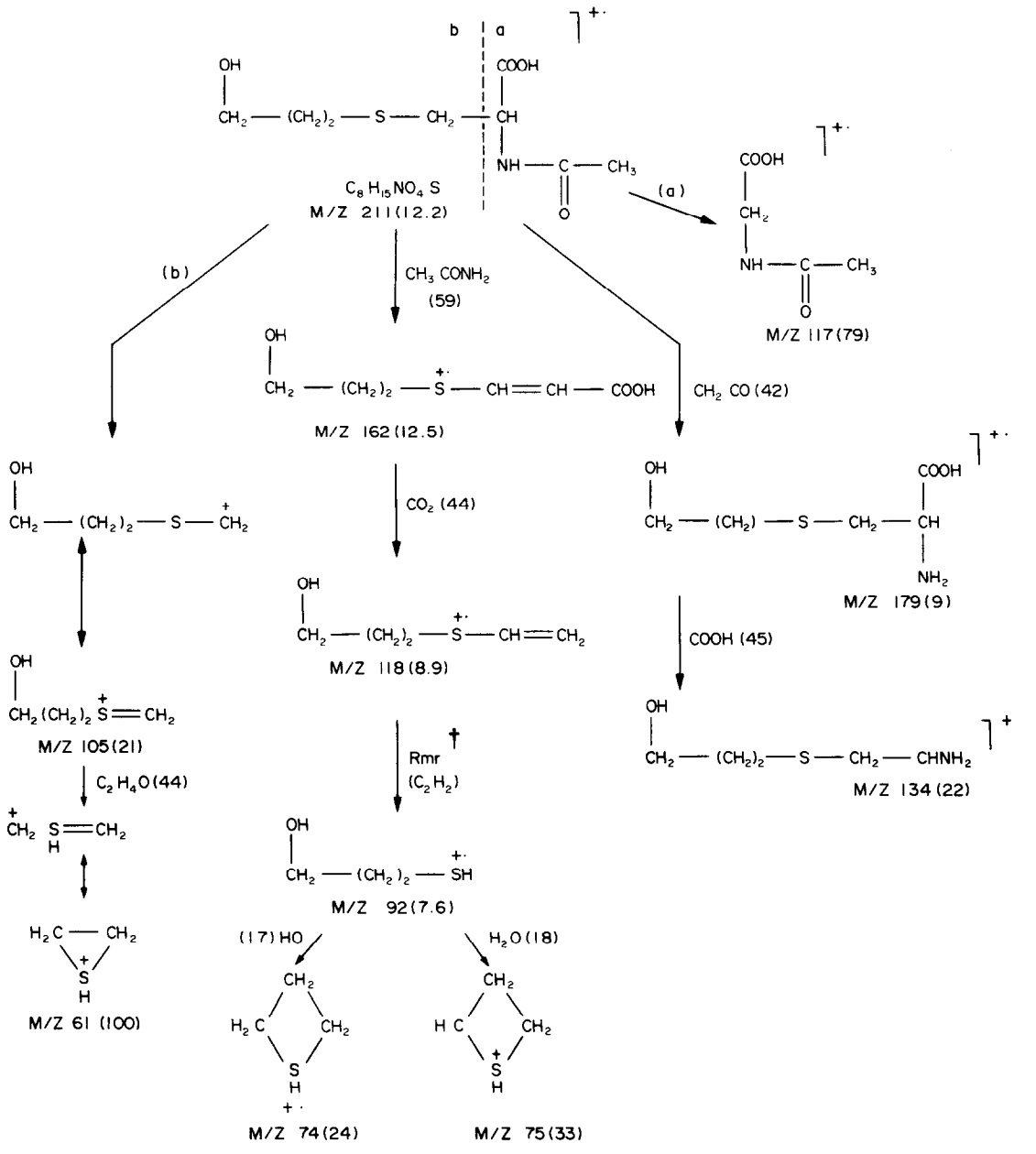

Fig. 5. Proposed mass spectral fragmentation of 3-OH Pr MCA.

Although the myocardium is the major target organ of acute toxic injury, previous investigators have demonstrated that chronic allylamine consumption results in systemic vascular lesions. These lesions include intimal fibrosis and cartilagenous metaplasia of mesenteric, pancreatic, and testicular arteries [20] and aorta [21], and intimal smooth muscle cell proliferation in coronary arteries [22]. These severe myocardial and vascular lesions occur in the absence of pathologic changes in the parenchyma of extracardiac organs.

Previous studies from this laboratory indicated that 60% of radiolabel in rats given [^{14}C]allylamine was excreted in the urine by 24 hr [11]. In this study, we isolated and identified a solitary urinary MCA as the excretory end-product of allylamine metabolism. This specific end-product, 3-OH Pr MCA, has been identified previously in the urine of animals given a variety of allyl compounds which presumably form an acrolein intermediate; such compounds include allyl halides [23], allyl esters [24], and allyl alcohol [24, 25]. 3-OH Pr MCA has also been found in the urine of rats given acrolein itself [7, 25]. More complex antitumor compounds which form acrolein as a metabolic product *in vitro* also are excreted as 3-OH propyl MCA; in fact, this MCA has been suggested as a possible quantitative indicator of the degree of activation of these antitumor agents [7]. Further-

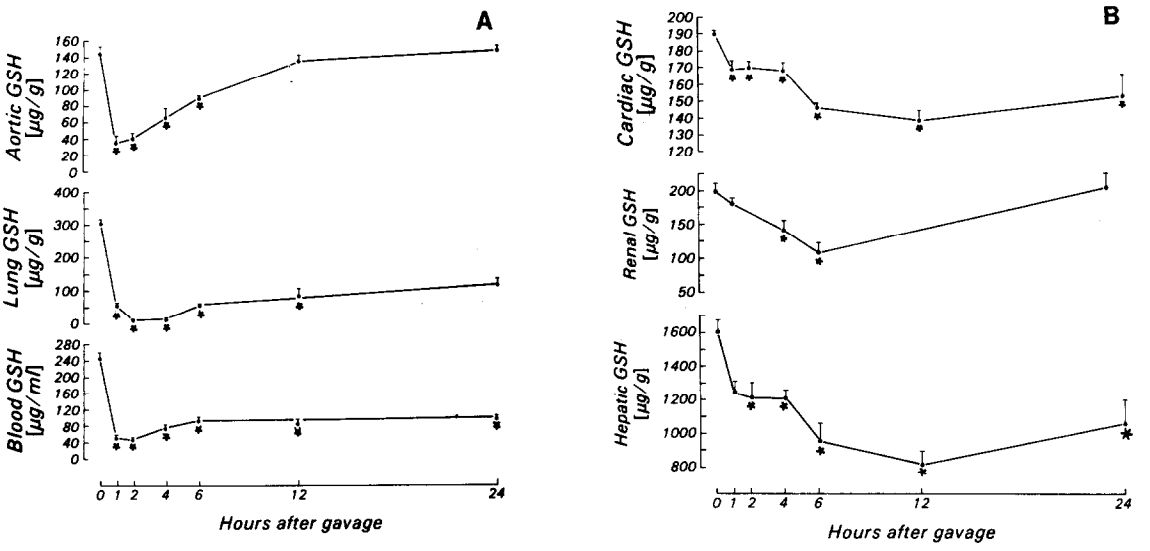

Fig. 6 (A) Glutathione (GSH) content in aorta, lung, and blood of rats given allylamine (150 mg/kg) by gavage. (B) glutathione (GSH) content in heart, kidney, and liver or rats given allylamine (150 mg/kg) by gavage. Each point represents the mean of four to eight rats; error bars represent the SEM. The asterisk indicates $P < 0.05$ when compared to the time-matched control group; only the control group at time 0 is shown.

more, efforts to attentuate the specific uro-toxicity of one of these clinically significant antitumor agents, cyclophosphamide, have concentrated on utilizing thiol compounds such as mesna and *N*-acetylcysteine to detoxify acrolein produced during the metabolic activation of this drug [8]. In view of these many experimental and clinical examples of the metabolism of allylic compounds and antitumor agents and our previous demonstration of acrolein generation from allylamine *in vitro* [4, 5], we conclude that the excretion of allylamine as 3-OH Pr MCA is due to the generation of acrolein *in vivo*. Acrolein would be expected to form a GSH conjugate which is metabolized through the well-described MCA pathway (see Fig. 7).

Because GSH conjugation is a required step in the *in vivo* conversion of acrolein to 3-OH Pr MCA, it is not surprising that the present study has demonstrated marked systemic depletion of GSH during acute allylamine intoxication. These alterations again support the supposition that acrolein is the major reactive intermediate of allylamine metabolism; however, the degree and organ distribution of these GSH alterations are somewhat perplexing.

Although GSH depletion occurs in the two prime target organs of allylamine toxicity—heart and aorta—the degree of depletion in the heart is not as marked as that seen in aorta, blood, or lung. Kidney and liver, which are not target organs of toxicity, show transient or mild decreases. Thus, there does not appear to be a perfect correlation between the extent of GSH depletion and the organ in which toxic damage occurs, but rather profound GSH depletion occurs in target and non-target organs.

Our hypothesis, based partially on specific inhibitor studies of acrolein formation *in vitro* [4], is that the metabolism of allylamine to acrolein occurs through the action of benzylamine oxidase, a form of amine oxidase with high activity in vascular tissue (especially aorta). Recent studies from this [26] and other laboratories [27] indicate that this enzyme is

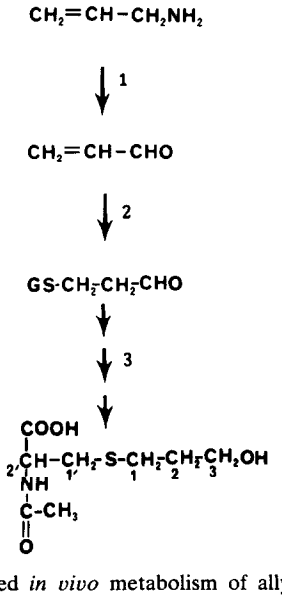

Fig. 7 Proposed *in vivo* metabolism of allylamine. The metabolism involves oxidation to the aldehyde, possibly by action of a form of amine oxidase (1), followed by conjugation with GSH (2) and subsequent formation [probably through several reactions (3)] of the mercapturic acid, 3-OH Pr MCA, from the glutatione conjugate.

localized to vascular smooth muscle cells and may be identical in some species to plasma amine oxidase. If this enzyme is involved in acrolein formation, the drastic depletion of GSH in aorta and blood could be easily explained by this localization of the enzyme. Further evidence from our laboratory [11] supporting this concept is the marked sequestration of radiolabeled allylamine (or a metabolite) in aorta and large arteries during allylamine intoxication, a finding which could indicate active metabolism and/or cellular binding in the vascular wall.

Similarly, the decrease of GSH in the lung—in view of the absence of demonstrated pulmonary toxicity of allylamine—could also be related to involvement of the pulmonary vascular component in allylamine metabolism. Lalich in his study of chronic allylamine intoxication in rats [20] noted pulmonary artery medial hypertrophy, suggesting that allylamine may have some effect on vascular smooth muscle in lung. It is of interest that, in our previous studies of the metabolism of allylamine by homogenates of several organs [4, 5], one of the most metabolically active was the lung. Only aorta was more active. Lung is an extremely vascular organ, and this vascular component may be responsible for the metabolism of allylamine, as suggested by our previous experiments indicating that the heart's capacity to metabolize allylamine is largely concentrated in larger coronary arteries [5]. Lung parenchyma may be protected from toxic metabolites, however, because extremely volatile substances such as acrolein might be rapidly exhaled.

It is evident, therefore, that the exact mechanisms by which allylamine injures heart and vascular tissue remain obscure. Acrolein would be expected to be an extremely reactive and deleterious intermediate at its subcellular site of formation, and presumably the conjugation of this reactive intermediate with GSH would serve as a detoxification mechanism. The toxicologic implications of the reduced glutathione conjugation pathway, however, are not yet completely defined in this or other forms of toxic injury to cells [28].

Thus, in the present study we provide *in vivo* evidence that allylamine is metabolized to the highly reactive aldehyde, acrolein, which is converted to a sole excretory metabolite, 3-OH Pr MCA, through a glutathione conjugation pathway, as reflected by marked systemic depletion of GSH in vascular tissues and blood. The exact mechanisms by which acrolein or other intermediates cause cellular damage remain to be elucidated.

Acknowledgements—We gratefully acknowledge the expert word processing assistance of Ms. Avis Morgan. This work was supported by Grants HL-26189 and DK-27135 from the NIH, Grant OH-02149 from NIOSH, and Research Career Development Award HL-9929 (Dr. Boor).

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