Biochemical Pharmacology, Vol. 35, No. 9, pp. 1481–1489, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Press Ltd.

ROLE OF PROPIOLALDEHYDE AND OTHER METABOLITES IN THE PARGYLINE INHIBITION OF RAT LIVER ALDEHYDE DEHYDROGENASE

EUGENE G. DEMASTER*†, FRANCES N. SHIROTA* and HERBERT T. NAGASAWA*‡

* Medical Research Laboratories, VA Medical Center, Minneapolis, MN 55417;
and ‡ Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, U.S.A.

(*Received* 16 *July* 1985; *accepted* 10 *October* 1985)

Abstract—The metabolism of pargyline proceeds by way of three separate cytochrome P-450 catalyzed N-dealkylation reactions: N-depropargylation, N-demethylation and N-debenzylation. Propiolaldehyde, a product of N-depropargylation, is a potent inhibitor of aldehyde dehydrogenase (AlDH). The formation of pargyline-derived propiolaldehyde by isolated rat liver microsomes *in vitro* was confirmed using gas chromatographic/mass spectrometric techniques. The measured rates of propiolaldehyde formation for uninduced and phenobarbital-induced microsomes *in vitro* were 0.2 ± 0.03 and 0.9 ± 0.2 μmole/30 min/g wet weight liver respectively. However, these rates may have been artificially low due to competition between semicarbazide, the trapping agent, and microsomal proteins for the generated propiolaldehyde. CO significantly inhibited the microsome-catalyzed N-depropargylation reaction *in vitro*, whereas $CoCl_2$ pretreatment of rats partially blocked the pargyline-induced rise in blood acetaldehyde after ethanol. Inhibition of the low K_m liver mitochondrial AlDH by propiolaldehyde *in vitro* exhibited first-order kinetics, which is consistent with irreversible inhibition. Acetaldehyde did not attenuate the inhibition of AlDH by propiolaldehyde *in vitro* or by pargyline *in vivo*. Propargyl alcohol, a substance which is metabolized to propiolaldehyde by alcohol dehydrogenase, also inhibited AlDH *in vivo* and caused a quantitatively similar rise in blood acetaldehyde after ethanol as pargyline. Other putative metabolites of pargyline, namely benzylamine and propargylamine, inhibited AlDH *in vivo*, albeit to a lesser degree than pargyline, but neither of these amines inhibited AlDH directly. Monoamine oxidase was implicated in the conversion of benzylamine to an active inhibitory species, possibly an imine. From these studies, we conclude that propiolaldehyde was the primary metabolite responsible for the pargyline inhibition of AlDH *in vivo*; however, certain amine metabolites may have contributed to a lesser degree by conversion to yet unknown inhibitory forms.

The monoamine oxidase (MAO§) inhibitor, pargyline (Eutonyl), which is frequently used as an experimental drug to alter brain levels of biogenic amines, is also a potent inhibitor of aldehyde dehydrogenase (AlDH) *in vivo* [1–4]. Thus, the administration of pargyline to rodents followed by ethanol raises blood acetaldehyde (AcH) in a dose-dependent manner [1, 5]. This elevation of blood AcH by pargyline is accompanied by a commensurate and preferential decrease in activity of the low K_m mitochondrial AlDH [2], the isozyme known to catalyze in major part the hepatic oxidation of ethanol-derived AcH in the rat [6]. The high K_m AlDH isozymes are unaffected [2].

Unlike the classical AlDH inhibitor, disulfiram, pargyline does not inhibit AlDH *in vitro*, a result that implicates a metabolite of pargyline as the *in vivo* inhibitor of AcH oxidation [1, 3]. Our previous studies with pargyline indicated that the biotransformation of pargyline to an active inhibitory metabolite is catalyzed by the hepatic microsomal cytochrome P-450 system [3, 7–9]. Evaluation of a series of pargyline metabolites and their analogs for inhibitory activity toward AlDH, both *in vitro* and *in vivo*, suggested that the active metabolite of pargyline is propiolaldehyde (HC≡C—CHO), an α,β-unsaturated acetylenic aldehyde [3, 8]. This is further supported by (a) the isolation of pargyline-derived propiolaldehyde as its semicarbazone from an *in vitro* microsomal system [7], (b) the positive relationship of microsomal N-depropargylation of propargyl-containing compounds with their ability to inhibit AlDH *in vivo* [8], and (c) the inhibition of the low K_m mitochondrial AlDH by propiolaldehyde *in vitro* [3, 7].

In this paper, we further describe the microsomal cytochrome P-450 catalyzed conversion of pargyline to propiolaldehyde and provide additional evidence in support of propiolaldehyde as the pargyline metabolite responsible for the *in vivo* inhibition of AlDH. Since the overall pathway for pargyline metabolism has not been fully described, we have presented a metabolic scheme for pargyline based on known metabolites of pargyline [7, 10–15], the microsome-catalyzed N-dealkylation reactions [7, 8, 15, 16], and the results of our inhibitor studies [3, 8].

† Address correspondence to: Eugene G. DeMaster, Ph.D., Medical Research Laboratories (151B), VA Medical Center, 54th St. & 48th Ave. So., Minneapolis, MN 55417.

§ Abbreviations: MAO, monoamine oxidase; AlDH, aldehyde dehydrogenase; AcH, acetaldehyde; PB, phenobarbital; GC/MS, gas chromatography/mas spectrometry; and S9 fraction, 9000 *g* supernatant fraction.

MATERIALS AND METHODS

Animals and materials. Male Sprague–Dawley rats were purchased from Harlan Sprague–Dawley, Inc. (Madison, WI) and used when the animals reached a body weight of 200–250 g unless indicated otherwise. Pargyline·HCl, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from the Sigma Chemical Co. (St. Louis, MO). Propargyl alcohol, *N*-propargylbenzylamine, benzylamine and propargylamine were purchased from the Aldrich Chemical Co. (Milwaukee, WI) and were redistilled before use.

Propiolaldehyde was synthesized as previously described [8, 17]. The propiolaldehyde semicarbazone standard was prepared from synthetic propiolaldehyde and purified by recrystallization from absolute ethanol [7].

Mitochondrial AlDH assay. The activity of the low K_m AlDH isozyme was determined in intact rat liver mitochondria by measuring the disappearance of AcH in a closed system [3]. Liver mitochondria were isolated in 0.25 M sucrose–0.1 mM EDTA (pH 7.4) by differential centrifugation and were washed once with the above and twice with 0.25 M sucrose before use.

Intact mitochondria were preincubated for 5 min at 38° in a reaction mixture containing 0.25 M sucrose, 5 mM $MgSO_4$, 1.0 mM EDTA, 10 mM KCl and 10 mM sodium arsenate, pH 7.5, in a final volume of 1.0 ml. The reaction was initiated by the addition of 200 nmoles AcH and was allowed to proceed for 5 min unless indicated otherwise. The reaction was stopped with 0.1 ml of 5.5 N $HClO_4$, and the samples were immediately frozen on Dry Ice. The AcH remaining was determined by head space gas chromatography using the same conditions as previously described [3].

Proteins were determined by the method of Lowry *et al.* [18] using bovine serum albumin as a standard.

Microsomal N-*depropargylation* in vitro. The rats were maintained on a standard laboratory chow (Purina Ralston Co., St. Louis, MO) and water *ad lib*. The phenobarbital (PB)-treated animals were administered PB by replacing their drinking water with 0.1% sodium PB for 8 days [19]. Following an overnight fast, the animals were decapitated and livers were removed and homogenized in 3 vol. of 0.15 M KCl. The homogenate was centrifuged at 10,000 *g* for 10 min. The microsomes were isolated from the supernatant fraction by centrifugation at 100,000 *g* for 60 min. The pellet was washed once with 0.15 M KCl and resuspended to a concentration corresponding to 0.5 g wet weight liver/ml.

The microsomal reactions were carried out in incubation flasks containing 3 mM substrate (pargyline or *N*-propargylbenzylamine), 2.0 mM $NADP^+$, 2.5 mM glucose-6-phosphate, 40 units glucose-6-phosphate dehydrogenase, 16.5 mM KCl, 4.0 mM $MgCl_2$, 8.3 mM nicotinamide, 30 mM semicarbazide, 1.0 ml microsomal preparation isolated from untreated or PB-treated rats, and 83 mM sodium phosphate buffer, pH 7.4, in a final volume of 12 ml. The reaction was initiated by the addition of microsomes, allowed to proceed for 30 min at 37°, and stopped by dilution with 15 ml of cold 0.1 M phosphate buffer, pH 7.4. The contents of six identical incubation flasks were pooled.

Propiolaldehyde semicarbazone was extracted from the pooled samples with five 200-ml volumes of ethyl acetate. The combined ethyl acetate extracts were dried over Na_2SO_4, the solvent was removed *in vacuo*, and the residue was dissolved in 10 ml water. For gas chomatographic/mass spectrometric (GC/MS) analysis, 2-ml aliquots of the final aqueous samples or synthetic semicarbazone of propiolaldehyde were added to 20-ml serum bottles. The samples were frozen on Dry Ice and lyophilized to dryness. Following the addition of 0.5 ml of 5.5 N $HClO_4$ to generate the free aldehyde, the samples were heated at 55° for 10 min and a 5-ml aliquot of the head space was injected onto the column for GC/MS analysis (Tenax-GC, 35–60 mesh, 122 cm glass column; 100° isothermal; 290° ion source; LKB 9000 GC-mass spectrometer). For quantitation of propiolaldehyde formation by gas chromatography, 0.2-ml aliquots were added to the 20-ml serum bottles which were then capped and placed on Dry Ice. These frozen samples were stored at −20° until assayed. Propiolaldehyde was released by the addition of 0.5 ml of 5 N $HClO_4$ and measured by head-space gas chromatography as previously described [20]. Total propiolaldehyde formed in the combined set of six incubation flasks was calculated against a standard curve prepared using standard solutions of the synthetic propiolaldehyde semicarbazone. The data shown in Tables 1 and 2 represent the mean ± S.E.M. of three to five determinations.

Blood AcH and ethanol analyses. Blood for ethanol and AcH determinations was obtained by stunning the animals and quickly drawing blood by open chest cardiac puncture. Duplicate samples were prepared by addition of 0.2-ml aliquots of blood to 20-ml serum bottles containing 1.0 ml of 5 mM sodium azide and 20 μl of 40 mM *n*-propanol, internal standard. The sample bottles were immediately capped, frozen on Dry Ice and stored frozen at −20° until assayed for AcH and ethanol by head space gas chromatography [20]. In this procedure, artifactual formation of AcH from ethanol in blood is inhibited by sodium azide [21].

Statistical analysis. The results are expressed as mean ± S.E.M., and analyses of variance were calculated by Student's *t*-test. P values of <0.05 were accepted as significant.

RESULTS

Identification of propiolaldehyde as a metabolite of pargyline. In an earlier study [7], pargyline was incubated with a 9000 *g* supernatant (S9) liver fraction prepared from PB-treated rats. The pargyline-derived propiolaldehyde formed was trapped with semicarbazide and, after separation of the semicarbazone product from the liver protein by solvent extraction, the sequestered propiolaldehyde was released with acid for identification by GC/MS. The previous identification of propiolaldehyde as a metabolite of pargyline was verified using isolated microsomes. GC/MS data were obtained for acid-released propiolaldehyde trapped as its semicarba-

zone from pooled incubations containing microsomes, pargyline and NADPH, and for propiolaldehyde released from authentic semicarbazone of synthetic propiolaldehyde (Fig. 1). The electron ionization mass spectra of these samples were essentially identical.

Quantitation of microsome-catalyzed propiolaldehyde formation in vitro. Measurable propiolaldehyde was observed from pargyline, *N*-propargylbenzylamine and tripropargylamine when incubated with the 100,000 *g* rat liver microsomal fraction and an NADPH-generating system (Table 1). Microsomes isolated from PB-treated rats were at least four times more active in generating propiolaldehyde than microsomes isolated from control animals. This PB-induced increase in microsomal N-depropargylation agrees with previous *in vivo* studies which demonstrated that PB treatment enhances the effect of pargyline, *N*-propargylbenzylamine, and tripropargylamine in elevating blood AcH levels after ethanol [7, 8].

The rate of formation of pargyline-derived propiolaldehyde was 0.20 ± 0.03 μmole/30 min/g liver for uninduced microsomes, and 0.86 ± 0.17 μmoles/30 min/g liver for PB-induced microsomes (Table 1), which represent, respectively, a 0.1 and 0.4% conversion of pargyline to propiolaldehyde. Using the $V_{max(app)}$ for the microsome-catalyzed formation of pargyline-derived *N*-methylbenzylamine reported by Weli and Lindeke [16], we estimate that the measured amounts of propiolaldehyde found represent <20% of actual product formation. There are several possible reasons for these artifically low values. Although semicarbazide was present as the trapping agent during the incubation, the presence of microsomal protein created a competitive situation where only a fraction of the highly reactive propiolaldehyde formed was trapped by semicarbazide. This became apparent in preliminary experiments where simple acidification of the incubation mixture (containing microsomes) with perchloric acid did not yield detectable levels of free propiolaldehyde in the sample head space. Thus, at low pH, the propiolaldehyde released from its semicarbazone reacted with the microsomal proteins. Measurable levels of propiolaldehyde were observed only after the sequestered propiolaldehyde was separated from the proteins by solvent extraction. Also, the low reactivity of semicarbazide with propiolaldehyde at physiologic pH necessitated the use of high concentrations of this reagent (30 mM) which is known to inhibit microsomal demethylation reactions [22, 23] and, thus, possibly reducing the rate of the N-depropargylation reaction.

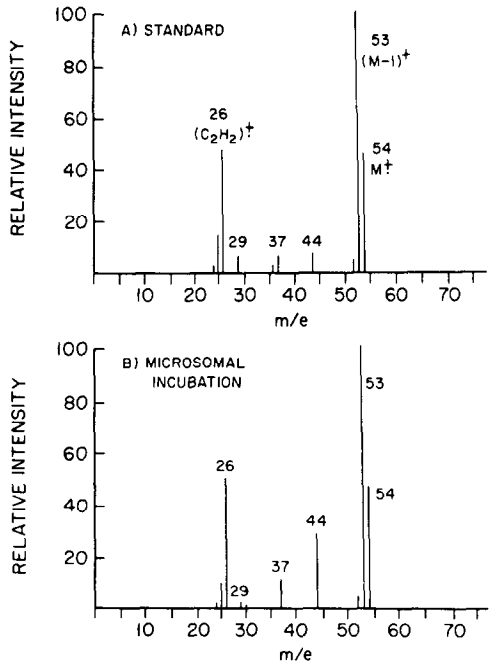

Fig. 1. GC/MS spectra at 70 eV of (A) propiolaldehyde standard and (B) pargyline-derived propiolaldehyde isolated from a microsomal incubation system. The experimental details are given under Materials and Methods.

Table 1. Propiolaldehyde formation *in vitro* from pargyline, *N*-propargylbenzylamine and tripropargylamine catalyzed by liver microsomes from control and PB-treated rats

Substrate (3 mM)	Propiolaldehyde formation (μmoles/30 min/g liver)
Pargyline	
Control	0.20 ± 0.03
PB-treated	0.86 ± 0.17
N-Propargylbenzylamine	
Control	ND*
PB-treated	0.39 ± 0.04
Tripropargylamine	
Control	0.080 ± 0.007
PB-treated	1.15 ± 0.08

The incubation system and the method of assay are described under Materials and Methods. Each value is the mean ± S.E.M.

* Nondetectable.

The low apparent rates of propiolaldehyde formation were also not due to poor recovery of propiolaldehyde semicarbazone from the incubation mixture, since a consistent 85% recovery was observed for the isolation of standard propiolaldehyde semicarbazone from complete microsomal systems without added pargyline.

Influence of cytochrome P-450 inhibitors on pargyline metabolism. In previous studies, we showed that the pargyline-induced elevation of blood AcH after ethanol administration could be blocked by SKF-525A [3] and enhanced by PB [7]. The data of Table 1 indicate that microsomes isolated from PB-treated rats showed greater rates of N-depropargylation than did uninduced microsomes. To further document the involvement of microsomal cytochrome P-450 in the metabolism of pargyline, the effects of inhibitors such as CO and $CoCl_2$ on the microsomal N-depropargylation reaction *in vitro* and on the pargyline-induced elevation of blood AcH *in vivo* were investigated.

Microsome-catalyzed propiolaldehyde formation from pargyline was assessed with and without CO, using a closed as well as an open incubation system (Table 2). In the closed system, the microsomes were exposed to CO or air, and the reaction vessels were

Table 2. Inhibition of the microsome-catalyzed N-depropargylation reaction by carbon monoxide*

Incubation system	Propiolaldehyde formation (μmoles/30 min/g liver)	% Inhibition
Expt. 1 (closed system)†		
Control	0.43 ± 0.07‡	
CO-treated	0.11 ± 0.07	74
Expt. 2 (open system)§		
N_2/O_2, 90/10	0.34 ± 0.02	
CO/O_2, 90/10	0.21 ± 0.01	37

* Microsomes were isolated from PB-treated rats.

† Incubated in stoppered flasks. CO treatment consisted of bubbling CO through the stock incubation mixture and the microsomes before starting the reaction. Control samples were exposed only to air.

‡ Mean ± S.E.M.

§ Metered CO/O_2 or N_2/O_2 was bubbled through the incubation mixtures throughout the 30-min incubation period.

sealed during the incubation period, whereas, in the open system, the reaction mixtures were exposed continuously to a stream of CO/O_2 or N_2/O_2 gas mixtures. As shown in Table 2, CO significantly inhibited the formation of propiolaldehyde from pargyline in either incubation system.

The effect of pretreatment with $CoCl_2$—an agent known to inhibit the biosynthesis of microsomal cytochrome P-450, thereby lowering the cytochrome P-450 mediated metabolism of drugs [24]—on pargyline metabolism was assessed indirectly by measuring blood AcH levels following acute ethanol administration (Table 3). Those animals administered $CoCl_2$ and pargyline had significantly lower blood AcH levels (50%, $P < 0.01$) than those given pargyline alone; however, $CoCl_2$ was not as effective as SKF-525A in this same system [3].

Time course for pargyline inhibition of hepatic AlDH in vivo. The inhibition and recovery of the low K_m hepatic mitochondrial AlDH was determined in male rats after acute pargyline treatment (Fig. 2). An 80% decrease in AlDH activity was observed within 2 hr, and maximum inhibition occurred between 5 and 10 hr post-drug treatment. Sixty-two hours were required for the AlDH activity to return to within 80% of control levels. The overall inhibition and recovery of AlDH activity after pargyline administration was similar to that reported for cyanamide on the same rat liver AlDH isozyme [25]. In contrast, *in vivo* inhibition of this isozyme by disulfiram [26] and coprine [25] resulted in a slower recovery of AlDH activity.

Propiolaldehyde—the pargyline metabolite responsible for the inhibition of mitochondrial AlDH. Although propiolaldehyde has been shown to be a potent inhibitor of the low K_m rat liver mitochondrial AlDH *in vitro* [3, 7], benzylamine, a putative amine metabolite of pargyline, has also been shown to inhibit this AlDH isozyme both *in vivo* and *in vitro* [27]. Another theoretically possible amine metabolite of pargyline which could contribute to the *in vivo* inhibition of mitochondrial AlDH is propargylamine. Accordingly, the inhibitory properties of benzylamine, proparglyamine and propiolaldehyde were compared along with pargyline, *N*-propargylbenzylamine and propargyl alcohol *in vivo* (Fig. 3) and *in vitro* (Fig. 4).

Propiolaldehyde was found to be toxic when administered *in vivo* and no animals survived a 1.25 mmole/kg dose. As noted earlier, propiolaldehyde is a highly reactive substance and, therefore, administered propiolaldehyde could bind ubiquitously to proteins and never reach the liver mitochondria. For these reasons, the effect of propiolaldehyde on AlDH activity and blood AcH level was examined indirectly by using compounds that can generate propiolaldehyde *in vivo*.

Administration of propargyl alcohol, pargyline and *N*-propargylbenzylamine to rats led to the inhibition of hepatic mitochondrial AlDH by more than 80%, whereas equimolar doses of benzylamine and propargylamine inhibited the enzyme by 65 and 45% respectively (Fig. 3). If benzylamine or propargylamine were the active metabolite of pargyline or its

Table 3. Partial blockade of the pargyline-induced elevation of blood AcH by $CoCl_2$

Treatment*	N	Blood AcH (μM)	Blood ethanol (mM)
Control	3	9.8 ± 1.3†	37.3 ± 8.1
Pargyline (0.625 mmole/kg)	3	110 ± 6.0	55.2 ± 2.3
$CoCl_2$ (60 mg/kg × 2)	3	11.4 ± 2.5	41.5 ± 1.9
$CoCl_2$, Pargyline	3	55.5 ± 7.8	48.2 ± 6.0

* $CoCl_2$ (s.c.) or saline was administered 48 and 24 hr before pargyline treatment. Pargyline (i.p.) or saline was given at zero time, ethanol (2 g/kg, i.p.) at 1 hr, and blood was taken at 2 hr post-pargyline treatment.

† Mean ± S.E.M.

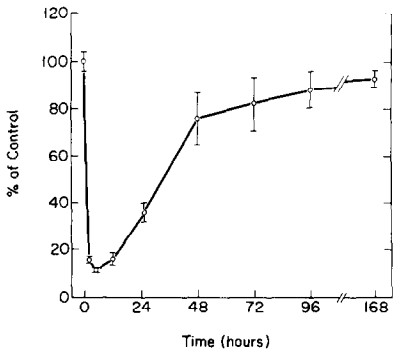

Fig. 2. Time course of inhibition and recovery of the low K_m rat liver mitochondrial AlDH activity after pargyline administration. Pargyline (0.625 mmole/kg in isotonic saline) or vehicle (control) was administered i.p. at zero time to nonfasted Sprague–Dawley male rats (200–300 g). AlDH activity in intact mitochondria was determined as described in Materials and Methods. Activity from control animals (100%) was 17.3 ± 0.6 nmoles AcH oxidized/min/mg protein. Each point represents a minimum of four animals; the vertical bars denote standard error of the means (S.E.M.)

immediate precursor, an equal or greater degree of inhibition would have been expected when compared to pargyline. The degrees of inhibition of AlDH following propargyl alcohol, pargyline and *N*-propargylbenzylamine were similar, as were the elevations of blood AcH levels.

The AlDH inhibitory activities *in vitro* of propargylamine and benzylamine were compared to propiolaldehyde using intact rat liver mitochondria (Fig. 4). Propiolaldehyde and benzylamine inhibited

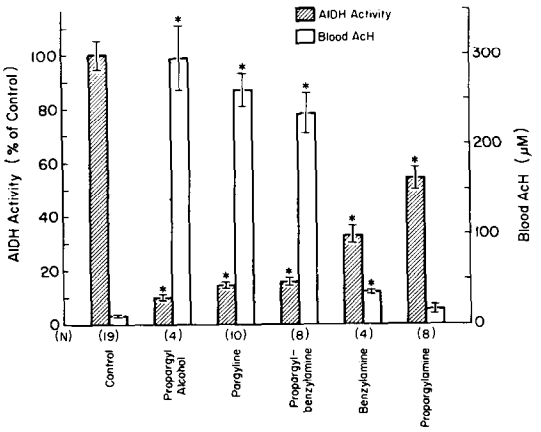

Fig. 3. Effect of pargyline, amine metabolites of pargyline, and propiolaldehyde precursors on blood AcH levels and the low K_m mitochondrial AlDH activity. Equimolar doses of the drugs (1.25 mmoles/kg) were administered (i.p.) to 24-hr fasted animals followed by ethanol (2 g/kg, i.p.) 1 hr later. One hour after ethanol, blood was taken for AcH determination, and the liver was removed for mitochondrial AlDH activity measurements. The mitochondrial fraction was prepared from each rat individually. AlDH activity for the control was 10.83 ± 0.61 nmoles AcH oxidized/min/mg protein. AlDH activity and blood AcH levels are given as the mean ± S.E.M. An asterisk (*) indicates $P < 0.001$ vs control.

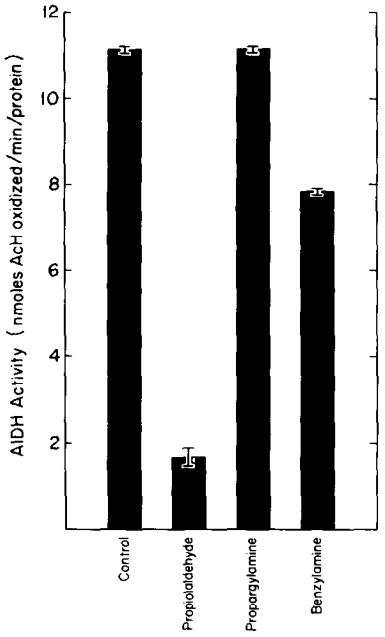

Fig. 4. Inhibition of mitochondrial AlDH *in vitro* by putative metabolites of pargyline. Intact rat liver mitochondria (1.77 mg protein) were preincubated for 5 min at 38° in the presence of equimolar concentrations of propiolaldehyde (200 nmoles), propargylamine, benzylamine or isotonic saline (control) in a final volume of 1.0 ml. Other experimental details were as described in Materials and Methods.

AlDH activity 85 and 30%, respectively, whereas propargylamine was without effect. The inhibition of AlDH by propargylamine *in vivo* (Fig. 3) but the lack of inhibition *in vitro* (Fig. 4) suggests that metabolism of propargylamine—perhaps to propiolaldehyde—is a requirement for inhibition.

The modest inhibition of AlDH *in vitro* by benzylamine (Fig. 4), a substrate for the pargyline-sensitive MAO isozyme [28], was blocked by pargyline (Fig. 5), indicating that benzylamine did not directly interact with the enzyme. These results suggest that a product of MAO oxidation, possibly benzalimine, is reponsible for this inhibition of AlDH by benzylamine *in vivo*.

Mechanistic aspects of the propiolaldehyde inhibition of AlDH. The inhibition of the low K_m mitochondrial AlDH by propiolaldehyde was found to be irreversible. The kinetics of inhibition presented in Fig. 6 show a linear (pseudo) first-order plot. The $T_{\frac{1}{2}}$ of the inactivation reaction calculated from the plot extrapolated to 100% enzyme activity was 2.72 min. The approximate 40% inhibition at zero time may indicate an initial rapid inactivation rate which is then followed by a slower rate of inactivation, providing a biphasic reaction curve. Alternatively, this initial activity may merely reflect inherent technical difficulties of adequately stopping the inactivation process due to the high reactivity of propiolaldehyde.

A possible protective effect by AcH on the propiolaldehyde inhibition of mitochondrial AlDH was also studied (Table 4). The AlDH activity in mitochondria in which propiolaldehyde was added *after*

Table 4. Inhibition of the low K_m AlDH by propiolaldehyde *in vitro* and the absence of a protective effect by AcH

Propiolaldehyde addition		AlDH activity	
Concn (μm)	Time	Specific activity* (nmoles AcH oxidized/ min/mg protein)	% Inhibition
Control		19.13 ± 0.03	
100	15 sec before AcH	2.81 ± 0.10	85
100	15 sec after AcH	3.25 ± 0.24	83
200	15 sec before AcH	1.28 ± 0.28	93
200	15 sec after AcH	1.55 ± 0.03	92
200	5 min before AcH	0.28 ± 0.04	99

Pooled, intact rat liver mitochondria from two, 18-hr fasted, 200 g male rats were assayed for the low K_m AlDH as described in Materials and Methods. Following a 5-min preincubation period of the mitochondria at 37°, the reaction was initiated by the addition of AcH (400 nmoles) and allowed to proceed for 10 min. Propiolaldehyde addition times were as indicated.

* Results represent the means ± S.E.M. of triplicate analyses.

AcH, as compared to *before*, demonstrated that AcH had no protective effect on the propiolaldehyde inhibition of this enzyme. Since the propiolaldehyde inhibition of AlDH was shown to be time dependent (Fig. 6), the small difference in enzyme activity due to order of addition can be attributed to differences in contact time (in seconds) between the enzyme and inhibitor.

The effect of pargyline inhibition of AlDH on ethanol-derived AcH *in vivo* was examined indirectly by the measurement of blood AcH (Table 5). In agreement with the above *in vitro* data (Table 4), the pargyline-induced elevation of blood AcH in animals administered ethanol 1 hr before (Group A) was not significantly different from animals given ethanol 1 hr after pargyline treatment (Group C). In the rat, blood AcH levels of this magnitude are accompanied by a 70–80% inhibition of the low K_m mitochondrial AlDH activity [8].

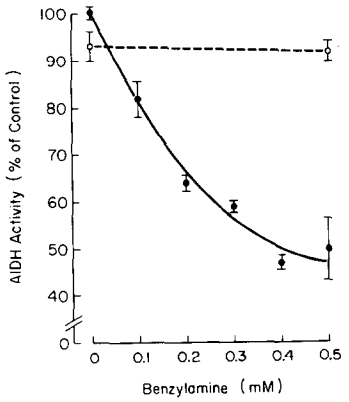

Fig. 5. Blockade of benzylamine inhibition of mitochondrial AlDH by pargyline *in vitro*. Pargyline (200 nmoles) was added to intact rat liver mitochondria (2.4 mg protein) at zero time, benzylamine at 5 min, and AcH (200 nmoles) at 10 min. All reactions were stopped by the addition of $HClO_4$, 5 min after AcH. The specific activity of control incubations (without benzylamine and pargyline) was 10.76 ± 0.52 nmoles AcH oxidized/min/mg protein. Each point represents the mean ± S.E.M. of a minimum of three determinations. Key: dashed line, with pargyline; solid line, without pargyline.

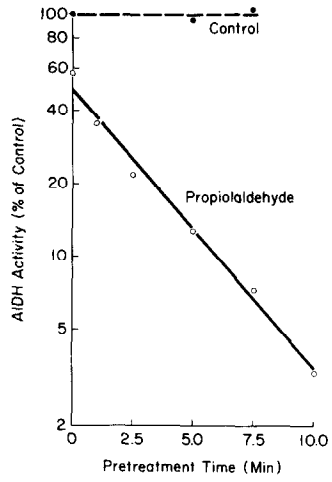

Fig. 6. Time-dependent irreversible inhibition of the low K_m rat liver mitochondrial AlDH by propiolaldehyde. Intact rat liver mitochondria (23.8 mg protein) were pretreated at 37° over the times indicated, with or without (control) 2.0 μmoles propiolaldehyde, in an incubation mixture containing 0.25 M sucrose, 1.5 mM $MgSO_4$, 0.3 mM EDTA and 3 mM arsenate, pH 7.5, in a final volume of 2.0 ml. Following pretreatment, the mitochondria were immediately chilled and washed three times with 0.25 M sucrose. Aliquots of the resuspended mitochondria were assayed for AlDH activity as described in Materials and Methods. The 100% AlDH activity corresponded to 9.98 ± 0.45 (S.E.M.) nmoles AcH oxidized/min/mg protein. No inhibition was observed for control samples (without added inhibitor) over this time period. Each point represents the mean of a minimum of triplicate analyses.

Table 5. Effect of order of administration of pargyline and ethanol on blood AcH in the rat

Group	Treatment (time before sacrifice) Pargyline (0.625 mmole/kg)	Ethanol (3 g/kg)	N	Blood AcH* (μM)	Blood ethanol† (mM)
A	2 hr	3 hr	6	78.5 ± 10.6	62.1 ± 1.0
B		3 hr	3	6.0 ± 1.0	59.0 ± 1.4
C	2 hr	1 hr	5	127.8 ± 34.2	74.6 ± 2.2
D		1 hr	3	3.8 ± 0.2	72.1 ± 2.3

Male rats (250–350 g, fasted overnight) were administered ethanol (i.p.) and pargyline or isotonic saline (i.p.) at the times indicated. Each value is the mean ± S.E.M.

* Blood AcH levels of groups A and C were not significantly different, $P > 0.05$.

† P values for the blood ethanol levels of groups A and B compared to C and D were < 0.001.

DISCUSSION

The inhibition of AlDH by pargyline requires the metabolic transformation of this drug to an active metabolite which, in turn, inhibits the enzyme. A causal relationship between metabolic depropargylation *in vitro* and inhibition of the low K_m mitochondrial AlDH *in vivo* was demonstrated previously using a number of compounds bearing a propargyl substituent on nitrogen or oxygen [8]. Only those compounds that released propiolaldehyde when incubated with rat liver microsomes *in vitro* significantly elevated blood AcH levels when administered *in vivo*. The relationship between metabolic depropargylation of pargyline and inhibition of AlDH *in vivo* was further supported by demonstrating that propiolaldehyde was not only a potent inhibitor of AlDH *in vitro* [3, 7, 29] but—using the S9 rat liver fraction—also a metabolite of pargyline [7]. The latter has now been confirmed with isolated microsomes (Fig. 1).

Further studies were conducted to determine whether propiolaldehyde was the only metabolite involved in the inhibition of mitochondrial AlDH by pargyline. Since inhibition of this AlDH isozyme *in vitro* has also been observed with the putative amine metabolites of pargyline, namely *N*-propargylbenzylamine, propargylamine and benzylamine [27], the effects of these metabolites on AlDH activity *in vivo* and *in vitro* were re-examined. *N*-Propargylbenzylamine and pargyline were found to be equally effective in inhibiting AlDH and in elevating blood AcH *in vivo* (Fig. 3), whereas neither agent inhibits AlDH *in vitro* [3]. Propargylamine and benzylamine also inhibited AlDH *in vivo* (Fig. 3) albeit to a lesser degree than did pargyline; however, only benzylamine elevated blood AcH significantly over control. Propargylamine, like *N*-propargylbenzylamine and pargyline, was without effect on AlDH *in vitro* (Fig. 4). Although benzylamine did inhibit this enzyme *in vitro* (Fig. 4), the inhibition was effectively blocked by pargyline (Fig. 5), suggesting that benzylamine did not interact with the enzyme directly. Benzylamine is a substrate for the pargyline-sensitive MAO but not for the pargyline-insensitive isozyme [28]. Thus, the oxidative deamination to an active metabolite—possibly an imine—by MAO is implicated in the inhibition of AlDH by benzylamine. The imine may cross the inner mitochondrial membrane in its hydrated form as a less reactive carbinolamine.

Propargyl alcohol and pargyline share a common metabolic product, propiolaldehyde. Accordingly, both were equally effective in elevating blood AcH at a dose of 1.25 mmoles/kg. Moreover, when the propargyl group of pargyline is substituted with the saturated n-propyl group, this analog does not elevate blood AcH following ethanol treatment [30]. Thus, propiolaldehyde alone can account for the observed *in vivo* inhibition of AlDH by pargyline (Fig. 3).

Three distinct N-dealkylation reactions can take place during pargyline metabolism [16]. Although a minor pathway [16], microsome-catalyzed N-debenzylation of pargyline would yield benzaldehyde as a product. Consequently, a secondary role for benzaldehyde as a competitive substrate in the *in vivo* inhibition of AcH metabolism by pargyline remains a possibility. Other possible inhibitory species include the imine or immonium ion forms of the dehydrated carbinolamine intermediates of the primary, secondary and tertiary amine metabolites.

A linear (pseudo) first-order plot for the inhibition of the low K_m mitochondrial AlDH by propiolaldehyde (Fig. 6) is indicative of irreversible inactivation. This agrees with the findings of Ferencz-Biro and Pietruszko [29] who showed, using purified human E_2 mitochondrial AlDH, that the original activity of the propiolaldehyde-inactivated enzyme could not be restored by exhaustive dialysis or treatment with 2-mercaptoethanol. Lebsack and Anderson [31] also reported that the pargyline inhibition of AlDH *in vivo* was irreversible.

The biphasic inactivation reaction observed (Fig. 6) could be due to the presence of hydrated and unhydrated species of propiolaldehyde. Although propiolaldehyde exists primarily in the hydrated form in solution (NMR data*), the initial burst of inactivation could result from free propiolaldehyde existing in solution. The subsequent slower rate of inactivation could be related to the rate of dehydration of propiolaldehyde hydrate. Such a phenomenon may also account for the findings of Ferencz-Biro and Pietruszko [29] with purified human E_1 and E_2 AlDH isozymes. They observed that nonlinear plots of log activity decrease over time with a total loss for E_2 of approximately 50% of control at 7°.

* Unpublished data.

Table 6. Summary of pargyline metabolites identified from various biological sources

Metabolites	Method of identification	Biological source	Species	References or source of data
Propiolaldehyde	GC/MS	Hepatic S-9 fraction	Rat	7
	GC/MS	Hepatic microsomes	Rat	Fig. 1
Formaldehyde	Nash reagent	Hepatic microsomes	Rat	13, UD*
Benzaldehyde	GC/MS	Hepatic microsomes	Rat	14, UD
Benzylamine	GC/MS	Liver slices	Rat	11
	GC/MS	Hepatic microsomes	Rat	12
	GC/MS	Hepatic microsomes	Human	12
N-Methylbenzylamine	GC/MS	Urine	Rat	10
	GC/MS	Hepatic microsomes	Rat	14, 12
	GC/MS	Hepatic microsomes	Human	12
	GC/MS	Urine	Human	12
	GC/MS	Plasma	Human	12
	GLC	Hepatic microsomes	Rat	15, 16
N-Propargylbenzylamine	GC/MS	Urine	Rat	10
	GC/MS	Hepatic microsomes	Rat	14
	TLC	Hepatic microsomes	Rat	13
	GLC	Hepatic microsomes	Rat	15
N-Methylpropargylamine	GLC	Hepatic microsomes	Rat	15, 16
Pargyline *N*-oxide	GLC	Hepatic microsomes	Rat	15, 16
N-Propargylbenzylamine (hydroxylated in the aromatic ring)	GC/MS	Urine	Rat	10

* UD, our own unpublished data.

At this low temperature it is possible that they were following the initial phase of inactivation of the enzyme by propiolaldehyde.

The conversion of pargyline to propiolaldehyde is catalyzed by the hepatic microsomal cytochrome P-450 enzymes. The suggested mechanism for the N-depropargylation reaction involves the initial hydroxylation of pargyline at a position *alpha* to the acetylenic bond to a carbinolamine intermediate, followed by dissociation of this carbinolamine [9]. The involvement of cytochrome P-450 in the microsomal N-depropargylation reaction was shown indirectly *in vitro* by (a) inhibition by CO (Table 2); (b) the NADPH dependence of this reaction [7]; and (c) the enhancement of this reaction with microsomes from PB-treated rats (Table 1). *In vivo* studies also support this mechanism, viz. (a) the blockade of pargyline-induced acetaldehydemia by the microsomal enzyme inhibitors, $CoCl_2$ (Table 3) and SKF-525A [3] and (b) the enhancement of pargyline-induced acetaldehydemia by PB pretreatment [7].

Besides hydroxylation at the position *alpha* to the

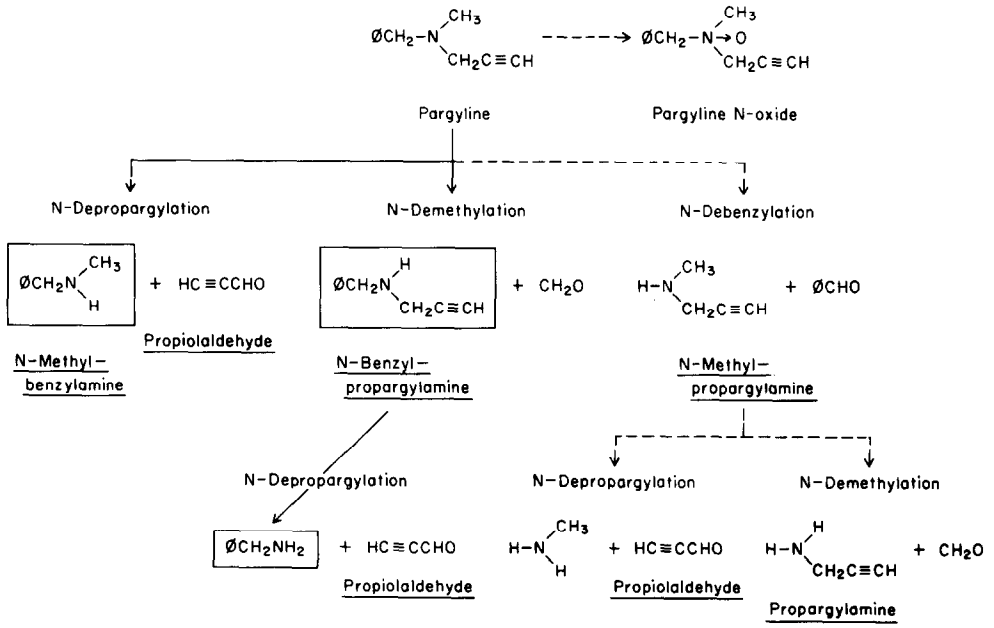

Fig. 7. Proposed pathways for pargyline metabolism. The solid lines denote major pathways, while the dashed lines indicate minor pathways. The major urinary metabolites are boxed. Possible metabolites that are hydroxylated in the aromatic ring are not included in the figure.

acetylenic bond of the propargyl group as observed in pargyline metabolism, oxidative biotransformation of the acetylenic functional group has been described in the metabolism of numerous other acetylene containing compounds [32–34]. This possibility was considered since both alpha-hydroxylation and oxidation of the acetylenic group are catalyzed by microsomal cytochrome P-450. However, oxidation of the acetylenic bond of pargyline does not appear to take place since the theoretically anticipated metabolites of this oxidative mechanism have not been observed (Table 6). In addition, oxidation of terminal acetylenic functional groups frequently results in inactivation of cytochrome P-450 [34–36]. White [37] has shown that pargyline does not inactivate cytochrome P-450 *in vitro* or *in vivo*.

An overall pathway for the metabolism of pargyline is shown in Fig. 7. This metabolic scheme is based (a) in part on our studies *in vivo* with AlDH inhibitors, i.e. pargyline and analogs thereof [3], and *in vitro* with a rat liver microsomal system which gave rise to pargyline-derived propiolaldehyde (Fig. 1) and other aldehyde products, namely benzaldehyde and formaldehyde, and (b) in part on the nature of the metabolites isolated and identified from plasma, urine and *in vitro* microsomal systems by others (Table 6). Three different N-dealkylation reactions are represented and, apart from the *N*-oxide, these reactions can account for all identified metabolites. Based on the relative amounts of the individual amine metabolites found in urine [15] and formed by microsomes *in vitro* [16], N-demethylation and N-depropargylation appear to be the major pathways of metabolism (Fig. 7).

In summary, (a) the identification of propiolaldehyde as a product of the microsome-catalyzed N-depropargylation reaction, (b) the irreversible inhibition of the low K_m liver mitochondrial AlDH by propiolaldehyde *in vitro*, (c) the quantitatively similar inhibition of AlDH and consequent elevation of blood AcH by metabolically derived propiolaldehyde from propargyl alcohol and pargyline *in vivo* and (d) the identification of N-depropargylation as a primary pathway for pargyline metabolism, all lead to the conclusion that propiolaldehyde is the major metabolite responsible for the pargyline inhibition of AlDH *in vivo*. The amine metabolites, propargylamine and benzylamine, may contribute to a lesser degree, but only after further metabolism to yet unidentified species.

Acknowledgements—This work was supported by the Veterans Administration. We are indebted to S. E. Redfern and J. A. Elberling for technical assistance and to T. P. Krick for the GC/MS analyses. The mass spectrometer facility is provided and maintained by the Minnesota Agricultural Experimental Station.

REFERENCES

1. D. Dembiec, D. MacNamee and G. Cohen, *J. Pharmac. exp. Ther.* **197**, 332 (1976).
2. M. E. Lebsack, D. R. Peterson, A. C. Collins and A. D. Anderson, *Biochem. Pharmac.* **26**, 1151 (1977).
3. E. G. DeMaster and H. T. Nagasawa, *Res. Commun. chem. Path. Pharmac.* **21**, 497 (1978).
4. S. Nakanishi, H. Yamazaki, K. Nishiguchi and R. Saladin, *Archs Toxic.* **46**, 241 (1980).
5. E. G. DeMaster and H. T. Nagasawa, *Res. Commun. Subst. Abuse* **3**, 211 (1982).
6. R. Parrilla, K. Ohkawa, K. O. Lindros, U. J. P. Zimmerman, K. K. Ashi and J. R. Williamson, *J. biol. Chem.* **249**, 4926 (1974).
7. F. N. Shirota, E. G. DeMaster and H. T. Nagasawa, *J. med. Chem.* **22**, 463 (1979).
8. F. N. Shirota, E. G. DeMaster, J. A. Elberling and H. T. Nagasawa, *J. med. Chem.* **23**, 669 (1980).
9. E. G. DeMaster, F. N. Shirota and H. T. Nagasawa, *Adv. exp. Med. Biol.* **132**, 219 (1980).
10. E. Diehl, S. Najm, R-E. Wolff and J-D. Ehrhardt, *J. Pharmac., Paris* **7**, 563 (1976).
11. D. A. Durden, S. R. Philips and A. A. Bouton, *Biochem. Pharmac.* **25**, 858 (1976).
12. R. Pirisino, G. B. Ciottoli, F. Buffoni, B. Anselmi and C. Curradi, *Br. J. clin. Pharmac.* **7**, 595 (1979).
13. D. M. Valerino, E. S. Vesell, J. T. Stevens and S. L. Rudnick, *Pharmacology* **17**, 113 (1978).
14. G. Hallström, B. Lindeke, A-H. Khuthier and M. A. Al-Iragi, *Chem. Biol. Interact.* **34**, 185 (1981).
15. A. M. Weli, N-O. Ahnfelt and B. Lindeke, *J. Pharm. Pharmac.* **34**, 771 (1982).
16. A. M. Weli and B. Lindeke, *Biochem. Pharmac.* **34**, 1993 (1985).
17. J. C. Sauer, in *Organic Synthesis* (Ed. N. Rabjohn), Vol. IV, pp. 813–5. John Wiley, New York (1963).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. J-K. Lin, D. A. Kennan, E. C. Miller and J. A. Miller, *Cancer Res.* **38**, 2424 (1978).
20. H. T. Nagasawa, D. J. W. Goon, E. G. DeMaster and C. S. Alexander, *Life Sci.* **20**, 187 (1977).
21. E. G. DeMaster, B. Redfern, E. K. Weir, G. L. Pierpont and L. J. Crouse, *Alcoholism: Clin. expl Res.* **7**, 436 (1983).
22. T. Matsubara, A. Touchi and Y. Tochino, *Jap. J. Pharmac.* **27**, 127 (1977).
23. E. M. Savenije-Chapel and J. Noordhoek, *Biochem. Pharmac.* **29**, 2023 (1980).
24. T. R. Tephly, C. Webb, P. Trussler, F. Kniffen, E. Hasegawa and W. Piper, *Drug Metab. Dispos.* **1**, 259 (1973).
25. H. Marchner and O. Tottmar, *Acta pharmac. tox.* **43**, 219 (1978).
26. R. A. Deitrich and V. G. Erwin, *Molec. Pharmac.* **7**, 301 (1971).
27. M. E. Lebsack and A. D. Anderson, *Curr. Alcoholism* **3**, 351 (1978).
28. D. S. Grosso and A. M. Gawienowski, *Biochem. Pharmac.* **25**, 957 (1976).
29. K. Ferencz-Biro and R. Pietruszko, *Alcoholism: Clin. expl Res.* **8**, 302 (1984).
30. H. T. Nagasawa, J. A. Elberling and E. G. DeMaster, *J. med. Chem.* **27**, 1335 (1984).
31. M. E. Lebsack and A. D. Anderson, *Res. Commun. Chem. Path. Pharmac.* **26**, 263 (1979).
32. A. Wade, A. M. Symons, L. Martin and D. V. Parke, *Biochem. J.* **184**, 509 (1979).
33. P. R. Ortiz de Montellano and K. L. Kunze, *Archs Biochem. Biophys.* **209**, 710 (1981).
34. P. R. Ortiz de Montellano and K. L. Kunze, *J. biol. Chem.* **255**, 5578 (1980).
35. I. N. H. White, *Biochem. J.* **174**, 853 (1978).
36. I. N. H. White, J. B. Campbell, P. B. Farmer, E. Bailey, N-H. Nam and D-O. Thang, *Biochem. J.* **220**, 85 (1984).
37. I. N. H. White, *Biochem. Pharmac.* **29**, 3253 (1980).