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MICROSOMAL METABOLISM OF ACETONITRILE TO CYANIDE

EFFECTS OF ACETONE AND OTHER COMPOUNDS

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Abstract—Oral acetone exposure delays and potentiates acetonitrile toxicity in rats. Results of previous pharmacokinetic studies suggested that acetone exerted a biphasic effect on the metabolism of acetonitrile to cyanide; the presence of acetone *in vivo* appeared to inhibit the metabolism of acetonitrile to cyanide, whereas the disappearance of acetone from serum was followed by stimulation of acetonitrile metabolism. The current experiments were designed to characterize further the metabolism of acetonitrile to cyanide and the effects of acetone and other compounds upon this metabolism. Liver microsomes were isolated and pooled 24 hr after oral pretreatment of female Sprague–Dawley rats (180–250 g) with acetone (1960 mg/kg) or water. Microsomal metabolism of acetonitrile to cyanide was found to be oxygen and NADPH dependent, and heat-inactivated tissue was unable to catalyze the reaction. NADH antagonized the NADPH-dependent metabolism of acetonitrile. The metabolism of acetonitrile to cyanide was linear with protein concentrations of 0–8 mg per incubation. Following a characteristic lag period of 10 min, the reaction was linear from 15 to 30 min. This metabolism was inhibited by carbon monoxide, metyrapone and SKF 525-A. Acetone pretreatment (−24 hr) *in vivo* increased the apparent V_{max} for acetonitrile metabolism without affecting the apparent K_m. When added *in vitro*, acetone competitively inhibited the metabolism of acetonitrile, with a K_I of 0.41 mM. Dimethyl sulfoxide (K_I = 0.51 mM) and ethanol (K_I = 0.11 mM) were also competitive inhibitors of acetonitrile metabolism, and aniline HCl (K_I = 4.77 μM) appeared to be a mixed inhibitor. These data are consistent with the hypothesis that the metabolism of acetonitrile to cyanide is mediated by a specific acetone-inducible isozyme of cytochrome P-450.

Acetonitrile, a two-carbon aliphatic nitrile, is a commercially useful solvent, and its toxicology has been summarized [1]. The toxicity of acetonitrile, and other saturated aliphatic nitriles, has been attributed to the metabolic release of cyanide [2, 3]. This metabolism has been hypothesized to result from a mixed-function oxidase-dependent oxidation at the alpha-carbon, and subsequent degradation of a cyanohydrin intermediate [4].

Pozzani *et al.* [5] and Smyth *et al.* [6] reported that acetone enhances the toxicity of acetonitrile in rats exposed to these solvents either orally or by inhalation. Results of studies conducted in this laboratory indicated that acetone both potentiates and delays the onset of acute acetonitrile toxicity in rats [7]. Pharmacokinetic data suggested that acetone had a biphasic effect on the metabolism of acetonitrile to cyanide [7]. It appeared that elevated serum acetone concentrations inhibited the metabolism of acetonitrile to cyanide, and the disappearance of serum acetone to control concentrations resulted in a stimulation of this metabolism [7]. Since the changes in the time course of toxicity correlated with the changes in blood cyanide concentrations, it was hypothesized that acetone potentiated acute acetonitrile toxicity by increasing the metabolism of acetonitrile to cyanide [7].

The present studies were conducted with hepatic microsomes to study further the effects of acetone upon the metabolism of acetonitrile to cyanide. The effects of other compounds on the metabolism of acetonitrile to cyanide were also studied in order to identify other chemicals that modify the biotransformation of this solvent and thus further characterize the metabolism of acetonitrile.

MATERIALS AND METHODS

Chemicals. Acetonitrile (99+%) and acetone (99+%) were purchased from the Aldrich Chemical Co., Milwaukee, WI. Aniline HCl, aminopyrine, glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD), NADP, NADPH, and NADH were purchased from the Sigma Chemical Co., St. Louis, MO. All other chemicals were of the highest purity commercially available. Aniline HCl was recrystallized from isopropanol and washed with acetone prior to use.

Animals and treatments. Female Sprague–Dawley derived rats (180–252 g) were purchased from the Charles River Breeding Laboratories (Lakeview

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Facility, Newfield, NJ). All rats were housed two per cage and were given Purina Lab Chow and tap water *ad lib*. Animals were given a single p.o. dose (10.0 ml/kg) of water (control) or a 25% (v/v) aqueous solution of acetone (1960 mg/kg = 2.5 ml/kg), and rats were killed 24 hr later after an overnight fast. A single administration of this dose of acetone results in no overt signs of toxicity. Based on previous studies this dose is estimated to be less than an LD_{01} [7]. This same dose of acetone has been demonstrated previously to potentiate the acute toxicity of acetonitrile in rats and to result in increased cyanide liberation from acetonitrile *in vivo* [7], and to enhance the metabolism and/or toxicity of other xenobiotics, including halogenated hydrocarbons [8, 9], and dimethylnitrosamine [10].

Tissue preparation and assay conditions. The buffer used throughout these experiments was a 0.1 M sodium phosphate buffer with 0.15 M KCl, pH 7.4. Microsomes were isolated from pooled, homogenized livers by differential centrifugation, washed once with 0.15 M KCl, and resuspended in buffer. Assays were performed at 37° and pH 7.4 in tightly capped (acetonitrile vapor pressure = 88.8 torr at 25°) 16 × 100 mm test tubes (with shaking) in a final volume of buffer of 2.0 ml. Unless otherwise noted, the reaction mixtures contained 2.0 mg protein, substrate, and an NADPH-generating system consisting of 0.5 mM NADP, 4.0 mM G6P, 2.5 units G6PD, and 25.0 mM $MgCl_2$. In most experiments, the reaction tubes were preincubated at 37° for 3 min, initiated with NADP + G6P, incubated for 20 min, and terminated by addition to microdiffusion cells containing 0.4 ml of 100% trichloroacetic acid. Product (cyanide) formation was monitored in all experiments. Cyanide recovery from microsomal tissue was determined in each experiment and was typically 60–70%. All data were corrected for cyanide recovery. Phosphate buffer, KCl, and water were passed through Chelex-100 resin prior to use.

Microsomal metabolism studies. All experiments were conducted at least twice, with most repeated three or more times. The microsomal metabolism of acetonitrile to cyanide was characterized by time (0–30 min) and dependence upon NADPH, oxygen, microsomal protein (0–8.0 mg/reaction) and substrate concentration (0–160 mM). In some experiments, the NADPH-generating system was replaced by NADPH (1.0 mM) and/or NADH (1.0 mM). Heat inactivation of microsomal protein was performed at 100° for 5 min. Oxygen depletion was accomplished by gentle bubbling with nitrogen for 1 min prior to the addition of substrate to the reaction mixture. The effects of acetone (250–1000 μM), ethanol (125–500 μM), DMSO (0.5–2.0 mM), aniline HCl (6.25–25.0 μM), metyrapone (2.0–16.0 mM), SKF 525-A (125–1000 μM) and carbon monoxide were studied in control microsomes. With the exception of carbon monoxide, each of these chemicals was preincubated in the reaction mixtures at 37° for 10 min. Substrate was then added, and 5 min later the reactions were initiated. Carbon monoxide inhibition was studied in the dark using a gas mixture containing approximately 4.4% oxygen and 5.6% carbon monoxide (v/v) with nitrogen as the inert carrier gas (a carbon monoxide/oxygen ratio of 1.25). Control reactions were exposed to a similar gas mixture which did not contain carbon monoxide. These gas mixtures were gently bubbled through the reaction mixtures for 1 min prior to the addition of substrate.

The amounts of cytochromes P-450 and b_5, and NADPH–cytochrome *c* reductase activity were measured in microsomes from acetone-pretreated rats and compared to the results obtained in control microsomes.

Analytical methods. Cyanide was recovered by microdiffusion [11] and determined colorimetrically according to Blanke [12]. This procedure had a detection limit of 0.04 nmol. Formaldehyde was determined by the method of Nash [13] as modified by Kleeberg and Klinger [14]. Cytochromes b_5 and P-450 were determined by the method of Omura and Sato [15] and NADPH–cytochrome *c* reductase activity was determined by the method of Mazel [16]. Protein content was determined according to Lowry *et al.* [17].

Data analysis. All data referred to in this manu-

Table 1. Requirements for the microsomal metabolism of acetonitrile to cyanide

Assay conditions*	Cyanide production† (nmol/mg protein/20 min)
NADPH-generating system‡	2.74 ± 0.12§
NADPH (1.0 mM)	2.61 ± 0.02‖
NADH (1.0 mM)	0.22 ± 0.02¶
NADPH + NADH (each at 1.0 mM)	1.90 ± 0.06**
No NADP	0.05 ± 0.06††
Heat-inactivated tissue‡‡	0.02 ± 0.02††

* All reactions were performed for 20 min at 37° in 0.1 M sodium phosphate buffer containing 0.15 M KCl, pH 7.4. All reaction mixtures contained 160 mM acetonitrile and 1.0 mg protein/ml. Following preincubation for 3 min, reactions were initiated with NADP, NADPH or NADH.

† Cyanide values are expressed as mean ± SD (N = 4).

‡ The NADPH-generating system was omitted when NADPH and NADH (both at final concentrations of 1.0 mM) were used.

§–†† Groups with a different superscript are significantly different ($P < 0.05$) from each other.

‡‡ Heat-inactivation of tissue was performed at 100° for 5 min.

script are mean values ± SD. Reaction kinetics were analyzed graphically using double-reciprocal plots. Each individual data plot was determined by least squares linear regression. Regression analysis was used to determine IC_{50} values. Results from two experimental groups were compared by Student's *t*-test, and an SAS program for analysis of variance was utilized to compare data from several experimental groups.

RESULTS

Cofactor requirements. The microsomal metabolism of acetonitrile to cyanide required NADPH, as a cofactor: the addition of either NADPH or an NADPH-generating system effectively supported the reaction (Table 1). However, addition of NADH to the reaction mixture resulted in only about 8% of the cyanide production that was observed after NADPH addition and, when both NADPH and NADH were added to the reactions, 27% less cyanide was produced than with NADPH alone (Table 1). Cyanide was not produced in the absence of microsomes or in the presence of heat-inactivated microsomes (Table 1). Acetonitrile metabolism also required oxygen: flushing of the reaction mixture with nitrogen for 1 min inhibited acetonitrile metabolism by 67%. Longer flushing with nitrogen did not result in further inhibition.

Time course and effect of protein concentration. The time course of acetonitrile metabolism was determined at an acetonitrile concentration of 160 mM. Following a typical lag period of approximately 10 min, the reaction was linear between 15 and 30 min (Fig. 1A). This lag period was independent of the method of initiating the reaction since it was observed following reaction initiation with either acetonitrile (as shown), NADP plus G6P, NADPH, or microsomes. Longer preincubation periods (i.e. 15 min, which approximated the time of the lag period) did not eliminate the lag phase in acetonitrile metabolism (data not shown). Using this same microsomal system, the time course of aminopyrine *N*-demethylation was linear (data not shown). Due to the time lag in acetonitrile metabolism, all further investigations of the microsomal metabolism of acetonitrile were performed for 20 min, and the data are expressed as product formation per 20-min incubation period.

Acetonitrile metabolism to cyanide was linear with respect to protein contents of 1.0 to 8.0 mg (data not shown). These data were used to establish the standard protein concentration (1.0 mg/ml) used in the microsomal experiments.

Effects of carbon monoxide, SKF 525-A and metyrapone. It has been hypothesized that saturated aliphatic nitriles are metabolized by a cytochrome P-450-dependent mixed-function oxidase [4]. To investigate this hypothesis, and to characterize further acetonitrile metabolism, the effects of known inhibitors of cytochrome P-450-dependent metabolic pathways were studied *in vitro*. Both carbon monoxide and metyrapone were effective inhibitors of the metabolism of acetonitrile to cyanide. The amount of cyanide liberated in the presence of carbon monoxide was 0.34 ± 0.08 nmol/mg protein/20 min, a

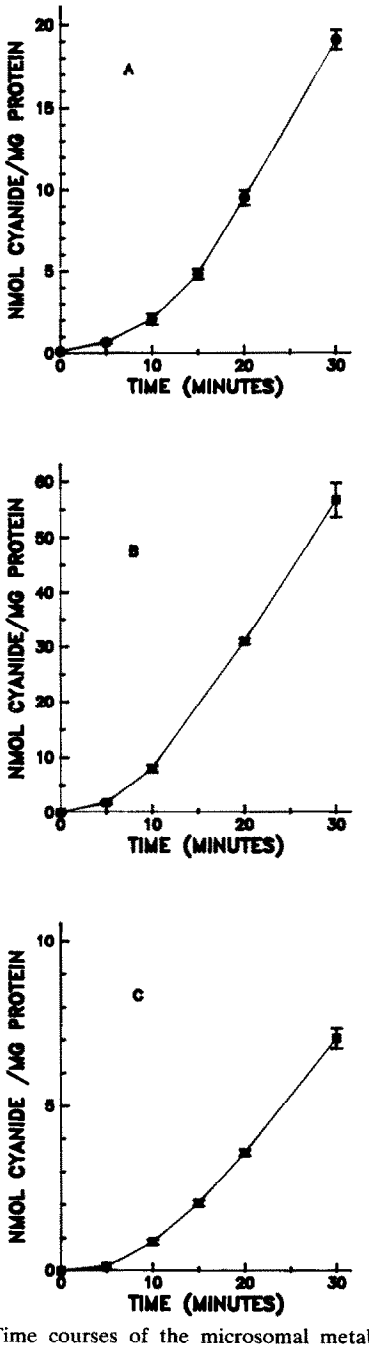

Fig. 1. Time courses of the microsomal metabolism of acetonitrile to cyanide. The reaction mixtures contained 160 mM acetonitrile, 1.0 mg/ml microsomal protein and an NADPH-generating system. The reactions were initiated with either acetonitrile (A) and (B) or NADP + G6P (C). (A) control microsomes, (B) microsomes isolated from acetone-pretreated (1960 mg/kg, 24 hr) rats, and (C) control microsomes in the presence of 3 mM acetone. Results are expressed as the mean ± SD of quadruplicate determinations.

91% and highly significant ($P < 0.0005$) reduction from the control value (3.61 ± 0.12). An IC_{50} of 5.9 mM was determined for metyrapone. SKF 525-A was less effective at inhibiting acetonitrile metab-

olism. Although four concentrations of SKF 525-A ranging from 125 to 1000 μM were tested, the magnitudes of inhibition were not different: 28 and 32% inhibition were observed at 125 and 1000 μM respectively.

Effects of acetone. The results of pharmacokinetic studies [7] suggested that the effects of acetone upon acetonitrile metabolism may be responsible for the effects of acetone upon acetonitrile toxicity. The following experiments were performed to investigate this hypothesis further.

A lag period in the metabolism of acetonitrile, similar to that observed in control microsomes, was observed in microsomes isolated from acetone-pretreated rats (Fig. 1B). However, cyanide production was greater following pretreatment with acetone. This finding suggests that acetone pretreatment results in an increase in the rate of metabolism without affecting the lag period. The metabolism of acetonitrile to cyanide also appeared to fit classical Michaelis–Menten kinetics (Fig. 2). In microsomes isolated from acetone-pretreated rats, the apparent V_{max} (38.7 ± 10.2 nmol cyanide/mg protein/20 min) was significantly greater ($P < 0.005$) than the corresponding control value (7.9 ± 1.8). The apparent K_m value in microsomes from acetone-pretreated rats (20.5 ± 2.4 mM) was similar to control (21.3 ± 4.2 mM).

The inhibitory effects of acetone upon acetonitrile metabolism to cyanide were characterized with control microsomes. Addition of 3.0 mM acetone to the microsomal system inhibited the metabolism of acetonitrile to cyanide without a major effect on the lag period (Fig. 1C), suggesting that acetone inhibited the rate of metabolism. Upon further characterization of this inhibition, acetone was found to fit a competitive model of inhibition (Fig. 3). The K_I of this inhibition was 0.41 ± 0.05 mM (three experiments).

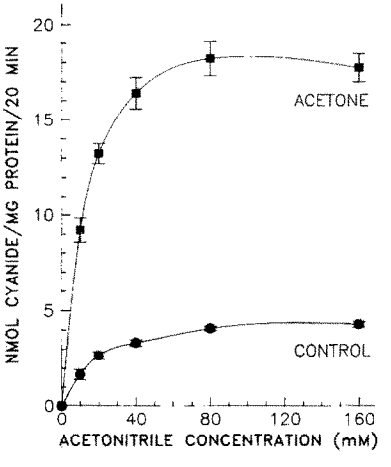

Fig. 2. Effect of pretreatment of rats with acetone upon the kinetics of the microsomal metabolism of acetonitrile to cyanide. Rats were administered acetone (1960 mg/kg, p.o.) or water (10.0 ml/kg, p.o.) 24 hr prior to preparation of microsomes. The assay system contained 10–160 mM acetonitrile, 1.0 mg/ml microsomal protein, and an NADPH-generating system. The reactions were initiated with NADP + G6P. All results are expressed as the mean ± SD of quadruplicate cyanide determinations.

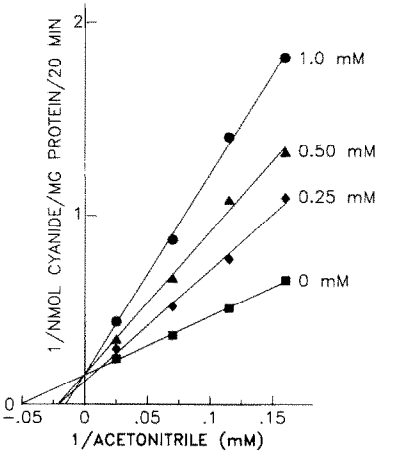

Fig. 3. Effects of acetone addition *in vitro* on the metabolism of acetonitrile to cyanide. The reaction mixtures contained 6.25–40 mM acetonitrile, 0–1.0 mM acetone, 1.0 mg/ml microsomal protein, and an NADPH-generating system, and were initiated with NADP + G6P. The results are representative of three experiments and are expressed as the average of duplicate cyanide determinations.

Since acetone increases acetonitrile metabolism to cyanide and this metabolism may be mediated by a mixed-function oxidase, the effects of acetone pretreatment on the mixed-function oxidase system were investigated. No statistically significant changes in the concentrations of cytochrome P-450 or cytochrome b_5, or in the activities of NADPH-cytochrome *c* reductase or aminopyrine *N*-demethylase were detected (data not shown).

Effects of ethanol, dimethyl sulfoxide and aniline HCl. Recent evidence suggests that acetone both induces and is metabolized by a cytochrome P-450 enzyme (isozyme LM3a or LMeb) that is also inducible by ethanol [18, 19]. Dimethyl sulfoxide inhibits cytochrome P-450 LM3a-mediated ethanol metabolism, and aniline is a good substrate for this enzyme [20]. Since the present studies indicate that acetonitrile metabolism is affected by acetone, and since other studies have showed that ethanol altered the metabolism and toxicity of saturated nitriles [21, 22], it was of interest to examine the effects of these substrates and inhibitors of cytochrome P-450 LM3a upon acetonitrile metabolism. Ethanol (125–500 μM), dimethyl sulfoxide (0.5–2.0 mM) or aniline HCl (6.25–25 μM) were preincubated with microsomes for 10 min. Acetonitrile (6.25–40 mM) was added to the mixtures, the reactions were initiated, and cyanide production was measured 20 min later. The results in Fig. 4 show that each of these chemicals inhibited the metabolism of acetonitrile to cyanide. Ethanol and dimethyl sulfoxide appeared to be competitive inhibitors, with K_I values of 0.11 ± 0.01 and 0.51 ± 0.06 mM respectively (three experiments each). Inhibition by aniline HCl appeared to be mixed (K_I = 4.77 ± 0.50 μM, three experiments).

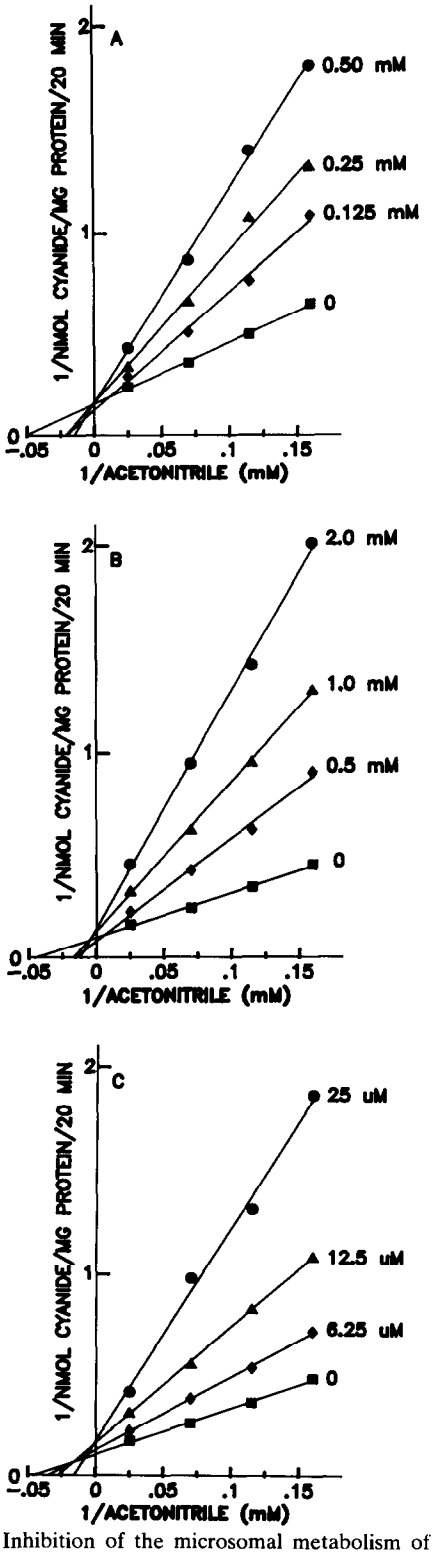

Fig. 4. Inhibition of the microsomal metabolism of acetonitrile by ethanol (A), dimethyl sulfoxide (B) and aniline HCl (C). The reaction mixtures contained ethanol (0–500 μM), or dimethyl sulfoxide (0–2.0 mM) or aniline HCl (6.25–25 μM) and acetonitrile (6.25–40 mM), 1.0 mg/ml microsomal protein and an NADPH-generating system. The results are representative of three experiments each and are expressed as the average of duplicate cyanide determinations.

DISCUSSION

The occurrence of a toxicological interaction in rats between acetone and acetonitrile was first reported by Pozzani *et al.* [5]. Research in our laboratory indicated that acetone both potentiates and delays the onset of acute acetonitrile toxicity, and it was hypothesized that the effects of acetone upon acetonitrile toxicity resulted from alterations in the metabolism of acetonitrile to cyanide [7]. In support of this hypothesis, the results of pharmacokinetic studies in rats suggested that acetone had a biphasic effect on the metabolism of acetonitrile to cyanide, that is, an initial inhibition followed by a stimulation of this metabolism upon the elimination of acetone [7]. The results of the present studies provide further support for this hypothesis: the pretreatment of rats with acetone markedly increased the microsomal metabolism of acetonitrile to cyanide, and acetone inhibited this metabolism *in vitro*. Also, acetone did not affect the lag period but altered the rate of metabolism of acetonitrile.

The metabolism of certain nitriles (i.e. benzyl cyanides) has been hypothesized to occur by a mixed-function oxidase-dependent oxidation at the alpha-carbon, yielding a cyanohydrin which may be unstable and breakdown spontaneously to hydrogen cyanide and an aldehyde [4]. The metabolism of aliphatic nitriles, including acetonitrile, has generally been assumed to occur in a similar manner. It has been reported that NADPH-fortified mouse or hamster hepatic microsomes catalyzed the release of cyanide from two other aliphatic nitriles, *n*-butyronitrile and succinonitrile [2], and from acetonitrile [23, 24]. The data reported here for rat liver microsomes confirm that NADPH is an absolute requirement for the metabolism of acetonitrile to cyanide. Oxygen was also shown to be required for the metabolism of acetonitrile to cyanide. In addition, the metabolism of acetonitrile was inhibited by carbon monoxide, metyrapone and SKF 525-A. SKF 525-A also inhibits the metabolism of *n*-butyronitrile and succinonitrile [2]. These data are consistent with a cytochrome P-450-mediated pathway of metabolism for acetonitrile and possibly other saturated nitriles as well.

If the metabolism of acetonitrile to cyanide involves cytochrome P-450, then the effects of pretreatment with acetone upon the metabolism of acetonitrile may be attributable to enzyme induction. It is now known that acetone as well as ethanol induces hepatic cytochrome P-450 LM3a (LMeb) in rabbits. This isozyme of cytochrome P-450 has high catalytic activity for aniline, carbon tetrachloride and dimethylnitrosamine and is inhibited competitively by dimethyl sulfoxide [18, 19, 25–27]. Patten *et al.* [28] recently purified an acetone-inducible cytochrome P-450 from the hepatic microsomes of rats. This isozyme is apparently similar to cytochrome P-450j, which has been isolated from the hepatic microsomes of isoniazid-treated rats by Ryan *et al.* [29]. Ethanol, fasting, and diabetes may induce the same isozyme [30–32]. Induction of this enzyme by acetone often occurs in the absence of changes in total cytochrome P-450 [10, 33]. Acetone-induced changes in the isoenzyme profile of cytochrome P-

450 have been demonstrated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis [28, 30], and cycloheximide prevents this induction of specific isoenzymes [30]. In those studies, a single dose of acetone was sufficient to induce cytochrome P-450j [28, 30].

The metabolism of acetonitrile as well as these other xenobiotics is increased in animals pretreated with acetone and, therefore, it is likely that acetonitrile is metabolized by this same isozyme of cytochrome P-450, cytochrome P-450j (LM3a, LMeb). Several findings support this hypothesis. Acetone pretreatment *in vivo* increased the apparent V_{max} without affecting the apparent K_m of acetonitrile metabolism in microsomes. Agents (acetone, ethanol, dimethyl sulfoxide and aniline) that are substrates for, or inhibitors of, this isozyme inhibited the metabolism of acetonitrile, but SKF 525-A produced only limited inhibition. Cobaltic protoporphyrin 9-chloride, which has been demonstrated to deplete hepatic cytochrome P-450 content, markedly decreases the metabolism of acetonitrile to cyanide in isolated rat hepatocytes [34]. Tanii and Hashimoto [24] reported that pretreatment of mice with ethanol resulted in an increased metabolism of acetonitrile to cyanide, and that ethanol inhibited this metabolism *in vitro*. In addition, Koop and Casazza [18] have reported recently that acetone is metabolized to acetol and methylglyoxal by cytochrome P-450 LM3a (LMeb). Therefore, it is probable that acetone and acetonitrile are substrates for the same isozyme of cytochrome P-450. It is noteworthy that the effects of acetone upon the metabolism of acetonitrile (an initial inhibition followed by a later induction) are consistent with the known biphasic effects of enzyme inducers [35].

The unusual time course of acetonitrile metabolism, with its initial lag phase, raises questions about the mechanism of acetonitrile metabolism. This time lag is not unique to the microsomal system nor to acetonitrile because a similar lag period has been observed during the metabolism of acetonitrile by isolated rat hepatocytes [34] and during the metabolism of propionitrile to cyanide by rat liver microsomes*. Tanii and Hashimoto [36] reported, however, that acetonitrile metabolism in mouse liver microsomes is linear for 40 min. This finding, which is in contrast to the results of the time–course studies described here, could represent a species difference in nitrile metabolism or a difference in experimental protocol, e.g. differences in times at which measurements were made. It is also noteworthy that, in rat liver microsomes, the metabolism of glycolonitrile, the cyanohydrin (and possible metabolite) of acetonitrile, is linear with time [37]: the metabolism of acetonitrile to cyanide might require two enzymatic steps.

Willhite and Smith [2] reported that death in animals poisoned with acetonitrile is delayed by several hours. It is probable that the 10-min lag period in the microsomal metabolism of acetonitrile is not a significant factor in this delay. Rather, it is more likely that the slow overall rate of metabolism of acetonitrile is the cause for the long delay before lethal quantities of cyanide accumulate in animals poisoned with this solvent.

Another unusual but reproducible finding in these studies was the antagonism by NADH of the NADPH-supported metabolism of acetonitrile. Cytochrome b_5 is believed to participate in the transfer of reducing equivalents from NADH to cytochrome P-450, resulting in an increased metabolism of certain substrates. The present data suggest that, if the metabolism of acetonitrile occurs via mono-oxygenation, then cytochrome b_5 may not play an important role in this metabolism. However, NADH also reduces the NADPH-oxidase activity of cytochrome P-450 [38] which generates active oxygen species and plays a role in the metabolism of some compounds. Although not conclusive, the finding that NADH antagonized the NADPH-dependent metabolism of acetonitrile to cyanide, suggests that cytochrome P-450-dependent NADPH-oxidase activity may be involved in the metabolism of acetonitrile. We have shown previously that a hydroxyl radical generating system reacts with succinonitrile and other saturated nitriles, resulting in the production of cyanide [39].

In summary, the metabolism of acetonitrile to cyanide is hypothesized to be mediated by a specific acetone-inducible isozyme of cytochrome P-450, and the effects of acetone upon the acute toxicity and metabolism of acetonitrile appear to be related to the inhibition and induction of this enzyme. The observations concerning the lag period and the antagonism of NADPH-supported acetonitrile metabolism by NADH are interesting. These observations suggest that, in addition to mono-oxygenation, other mechanisms may be involved in acetonitrile metabolism.

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* J. J. Freeman and E. P. Hayes, unpublished data.

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