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EFFECTS OF CHRONIC ACETONE ADMINISTRATION ON ETHANOL-INDUCIBLE MONOOXYGENASE ACTIVITIES IN THE RAT

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Abstract—Liver microsomal monooxygenase activities known to be ethanol-inducible were determined in female Sprague–Dawley rats after 2-week treatment with 1% (v/v) acetone. Daily acetone intake was in the order of 1.2 g/kg. The final body weight, liver weight and microsomal protein content of acetone-treated rats were identical to those of untreated controls. Microsomal NADPH–cytochrome *c* reductase activity was also unaffected, while cytochrome P-450 content was only increased 12–18%. Ethanol-inducible *p*-nitrophenol hydroxylation, aniline hydroxylation and 7-ethoxycoumarin O-deethylation activities were enhanced 5.3-, 4.4- and 2.6-fold, respectively, by chronic acetone treatment. The sex-dependent inducing effect of ethanol on benzphetamine N-demethylation activity in female rats was not observed however, after acetone. Addition of acetone *in vitro* had a stimulatory effect on aniline hydroxylation by microsomes from control and acetone-induced rats. Acetone, however, was found to be a competitive inhibitor of *p*-nitrophenol hydroxylation activity (apparent $K_i = 1.8$ mM), an observation suggesting that *p*-nitrophenol is a more selective substrate than aniline for rat liver ethanol- and acetone-inducible cytochrome P-450j. Interruption of the chronic acetone treatment for 24 hr resulted in the almost complete disappearance of its inducing affects, this treatment apparently reproducing only the rapidly reversible preferential inducing effects of chronic ethanol administration. This experimental model of induction by acetone in the rat, when compared to chronic ethanol administration, would thus permit a more selective look at the consequences of these common inducing effects in particular, with respect to drug metabolism and toxicity *in vivo*, and this, in the absence of the hepatotoxic effects of ethanol itself.

The alcohol-oxidizing cytochrome P-450 isozyme 3a of rabbit liver microsomes is the main ethanol-inducible isozyme in these animals [1, 2]. It shows high *in vitro* activity towards aniline hydroxylation [1–3], low K_m for *N*-nitrosodimethylamine demethylation [4], and conversion of acetaminophen to a reactive intermediate [5]. It is also inducible by several other agents [6]. Considerable indirect evidence had been accumulated suggesting the inducibility, by ethanol and by some inducers of rabbit liver isozyme 3a, of a rat liver isozyme having similar catalytic properties [7]. Cytochrome P-450j, an isozyme homologous to rabbit liver isozyme 3a, is inducible by isoniazid [7, 8] and ethanol [8] in the rat.

The chronic administration of ethanol to rats, using the liquid diet model of De Carli and Lieber [9], apparently maximizes the inducing effect of ethanol on cytochrome P-450j [10–12]. However, it is also a complex experimental model to study the *in vivo* consequences of its induction [12]. While this treatment increases the microsomal aniline hydroxylation activity 3- to 4-fold in the rat, it may also enhance their microsomal cytochrome P-450 specific content by as much as 75%, as seen in female animals [12]. A similar increase in this activity, however, may, be achieved in isoniazid-treated rats or in ethanol-treated rabbits, in the absence of an increase in microsomal cytochrome P-450 content [6, 7]. This suggests that factors inherent to the administration of the liquid diets to rats may contribute to the induction of additional isozymes.

Chronic treatment with more potent or selective inducers of cytochrome P-450j may provide a simpler experimental model for *in vivo* studies of this phenomenon in the rat. A number of observations suggested that acetone could be a particularly interesting alternative to ethanol for such studies. Acetone administered chronically is a potent inducer of isozyme 3a in the rabbit [6]. It is also a highly selective and possibly a physiological substrate of this isozyme and of an immunochemically homologous acetone-inducible rat liver isozyme [13, 14]. The induction of cytochrome P-450j by acetone could also explain why its administration, like the administration of ethanol, enhances activities such as aniline hydroxylation [15, 16] and low K_m *N*-nitrosodimethylamine demethylation [17, 18] in the rat. A recent review [19] of the results of comparative studies of the influence of short-term treatment with these two inducers on the hepatotoxicity of various xenobiotics has discussed many similarities, but also potentially interesting differences, between their effects under *in vivo* conditions.

To test this hypothesis, we examined the effects of chronic acetone administration, and of its withdrawal, on liver microsomal cytochrome P-450 content and on ethanol-inducible monooxygenase

activities in female rats. Substrates that are preferentially metabolized by different cytochrome P-450 isozymes in the rat [20–22], such as aniline, benzphetamine and 7-ethoxycoumarin, were used. We also measured *p*-nitrophenol hydroxylation activity, recently shown to be highly inducible by ethanol in the rat [23, 24] and to be catalyzed almost exclusively by isozyme 3a in the rabbit [25].

MATERIALS AND METHODS

Materials. Benzphetamine was supplied by the Upjohn Co. (Kalamazoo, MI). Aniline hydrochloride and 4-aminophenol were purchased from the Eastman Kodak Co. (Rochester, NY), and 7-ethoxycoumarin, 7-hydroxycoumarin, 4-nitrophenol and 4-nitrocatechol, from the Aldrich Chemical Co. (Milwaukee, WI). Enzymes, coenzymes and biochemicals were from Boehringer Mannheim Biochemicals (St-Laurent, Québec, Canada). Acetone as well as all other reagent grade chemicals were from Fisher Scientific (Montréal, Québec, Canada).

Animals. Female Sprague–Dawley rats (Charles River Canada Inc., St.Constant, Québec, Canada) weighing 170–180 g were given either drinking water or, in the case of acetone-treated animals, drinking water containing 1% (v/v) acetone with free access to Purina rat chow (No. 5012) for 2 weeks.

Preparation of liver microsomes and assays. Animals were anesthetized with ether, and the liver was quickly perfused *in situ* with ice-cold 1.15% KCl solution and excised. Liver microsomes were prepared and washed as described previously [26], resuspended in 1.15% KCl, and used on the same day.

Cytochrome P-450 was measured in microsomal suspensions according to Omura and Sato [27], and in liver homogenates by the method of Greim [28], using an Aminco DW-2 UV/VIS spectrophotometer in the split-beam mode. Values of microsomal protein per g of liver were corrected for losses incurred during the isolation of this fraction, as described previously [26]. Protein was determined according to Lowry *et al.* [29]. NADPH-Cytochrome *c* reductase activity was determined according to Masters *et al.* [30]. Microsomal aniline hydroxylation and benzphetamine *N*-demethylation activities were determined by measuring the rate of formation of *p*-aminophenol and formaldehyde, respectively, as described previously [26]. The microsomal 7-ethoxycoumarin O-deethylase activity was assayed by the fluorometric determination of the rate of production of 7-hydroxycoumarin [31]. Microsomal *p*-nitrophenol hydroxylase activity was assayed by the determination of the rate of production of *p*-nitrocatechol according to the method of Reinke and Moyer [24], with slight modifications. Incubations were carried out in 0.05 M potassium phosphate buffer, pH 7.4, and a final concentration of *p*-nitrophenol of 0.1 mM was used since slight substrate inhibition was observed in the presence of microsomes (0.5 mg protein/ml) from acetone-treated rats at the 0.2 mM *p*-nitrophenol concentration used by these authors.

Statistical analyses. Statistical significance with respect to controls was assessed using Student's *t*-test for unpaired data.

RESULTS

The average consumption of acetone was 1.20 ± 0.05 g (20.6 mmol) of acetone/kg/day. As seen in Table 1, acetone had no effect on body weight, liver weight, or liver microsomal protein content. It was associated however, with a modest (19%) but significant ($P < 0.001$) increase in liver cytochrome P-450 content per g of tissue as measured in whole homogenates. This was accounted for by the increase in the cytochrome P-450 specific content of liver microsomes. This was associated with a "red shift" in the CO-difference spectrum of the reduced microsomal sample. The absorption maximum was at 450.0 nm in control microsomes and at 450.8 nm in microsomes of acetone-treated rats (spectra not shown).

The small increase in microsomal cytochrome P-

Table 1. Effects of chronic acetone administration for 2 weeks on liver microsomal mixed-function oxidases in female rats*

	Control	Acetone	Acetone Control
Body weight (g)	215.3 ± 3.3	216.7 ± 4.5	1.01
Liver weight (g/100 g body wt)	3.97 ± 0.07	3.95 ± 0.09	0.99
Microsomal protein (mg/g liver)	40.7 ± 1.6	40.9 ± 2.2	1.00
Cytochrome P-450 (nmol/mg protein)	0.76 ± 0.04	0.90 ± 0.03	1.18†
(nmol/g liver)	30.4 ± 1.1	36.2 ± 0.7	1.19‡
NADPH-cytochrome *c* reductase (nmol/min/mg protein)	125.0 ± 7.1	125.1 ± 5.8	1.00
p-Nitrophenol hydroxylase (nmol/min/mg protein)	0.96 ± 0.04	5.15 ± 0.19	5.36‡
Aniline hydroxylase (nmol/min/mg protein)	0.48 ± 0.02	2.15 ± 0.03	4.48‡
7-Ethoxycoumarin *O*-deethylase (nmol/min/mg protein)	1.19 ± 0.06	3.14 ± 0.11	2.64‡
Benzphetamine *N*-demethylase (nmol/min/mg protein)	4.25 ± 0.26	3.64 ± 0.22	0.86

* Results are means ± SE of ten animals in each group.
† $P < 0.01$.
‡ $P < 0.001$.

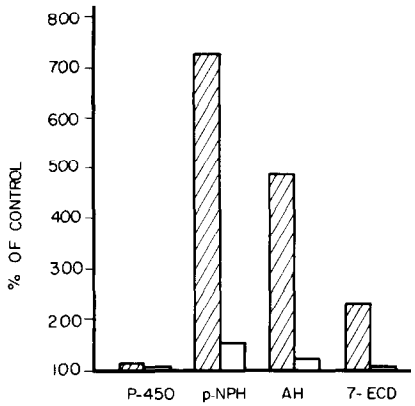

Fig. 1. Rat liver microsomal cytochrome P-450 content and *p*-nitrophenol hydroxylation (p-NPH), aniline hydroxylation (AH) and 7-ethoxycoumarin O-deethylation (7-ECD) activities after chronic treatment with acetone for 2 weeks (▨) and after withdrawal of acetone 24 hr prior to the time of death (□). Results represent the mean of values obtained from six animals in each group and are presented as the percent of corresponding values in untreated controls (mean ± SE for P-450, 0.59 ± 0.02 nmol/mg protein and for activities tested, in nmol of product formed/min/mg protein, p-NPH, 0.72 ± 0.02; AH, 0.38 ± 0.02; and 7-ECD, 0.77 ± 0.04).

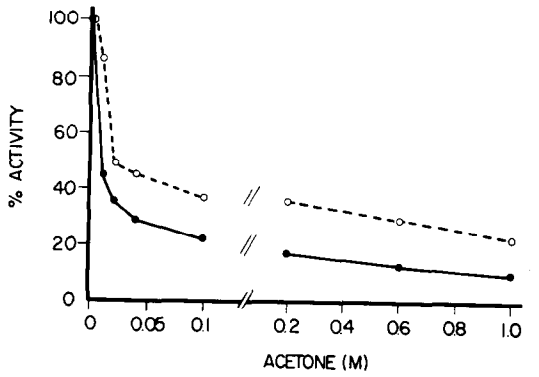

Fig. 3. Inhibition of *p*-nitrophenol hydroxylation activity by acetone *in vitro*. The incubation mixture contained microsomes from control rats (2 mg protein/ml)(○—○) or from rats pretreated with acetone for 2 weeks (0.5 mg protein/ml) (●—●) and 0.1 mM *p*-nitrophenol. The rates of *p*-nitrocatechol formation in the absence of acetone were 0.87 and 3.85 nmol/min/mg protein, respectively, for control and acetone-induced microsomes.

450 content seen after acetone treatment, in the absence of change in microsomal NADPH–cytochrome *c* reductase (Table 1), was associated, however, with marked enhancement of three of the four mixed-function oxidase activities measured (Table 1). The 5.4-fold increase in microsomal *p*-nitrophenol hydroxylation was higher than the 4.5- and 2.6- fold increases in aniline hydroxylation and 7-ethoxycoumarin O-deethylation respectively. Acetone slightly decreased microsomal benzphetamine N-demethylation activity. Cessation of acetone administration for 24 hr practically abolished the increases in *p*-nitrophenol and aniline hydroxylation and the increase in 7-ethoxycoumarin O-deethylation (Fig. 1). Only *p*-nitrophenol hyroxylation, enhanced 7-fold by acetone in this experiment, remained slightly (50%) but significantly elevated ($P < 0.01$) after acetone withdrawal.

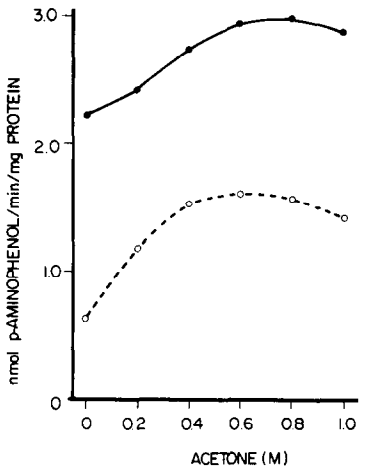

Fig. 2. Enhancement of aniline hydroxylation activity by acetone *in vitro*. The incubation mixture contained 2.5 mM aniline and microsomes (1.2 mg protein/ml) from control rats (○—○) or from rats pretreated with acetone for 2 weeks (●—●).

Since acetone, aniline and *p*-nitrophenol appear to be preferential substrates for the same cytochrome P-450 isozyme [13, 25], we measured the effect of the *in vitro* addition of acetone on microsomal aniline and *p*-nitrophenol hydroxylation activities. As seen in Fig. 2, acetone had a stimulatory effect on aniline hydroxylation activity of control and acetone-induced microsomes, increasing the rate of *p*-aminophenol formation by 0.98 and 0.77 nmol/min/mg protein, respectively, in these microsomal preparations. Maximal stimulation of this activity was achieved at 0.6 M acetone in control microsomes and at 0.8 M in acetone-induced microsomes. The slight decline in activity seen with both preparations at 1.0 M acetone, on the other hand, could be due to a possible inactivation of a small portion of the microsomes by a temporary high local concentration of acetone upon its addition.

In contrast, acetone inhibited *p*-nitrophenol hydroxylation, assayed at a concentration of 0.1 mM *p*-nitrophenol (Fig. 3). With microsomes from acetone-pretreated rats, double-reciprocal analysis gave an apparent K_m value of 0.026 ± 0.005 mM (mean ± SE of four microsomal preparations) for *p*-nitrophenol in the absence of acetone. At and below 3 mM acetone, competitive inhibition of *p*-nitrophenol hydroxylation was observed (Fig. 4). The *p*-nitrophenol hydroxylation activity of the microsomal preparation used in this experiment had a K_m for *p*-nitrophenol of 0.041 mM and a V_{max} of 4.38 nmol/min/mg protein, and the K_i value for acetone was 1.828 ± 0.180 mM (mean ± SE of three determinations).

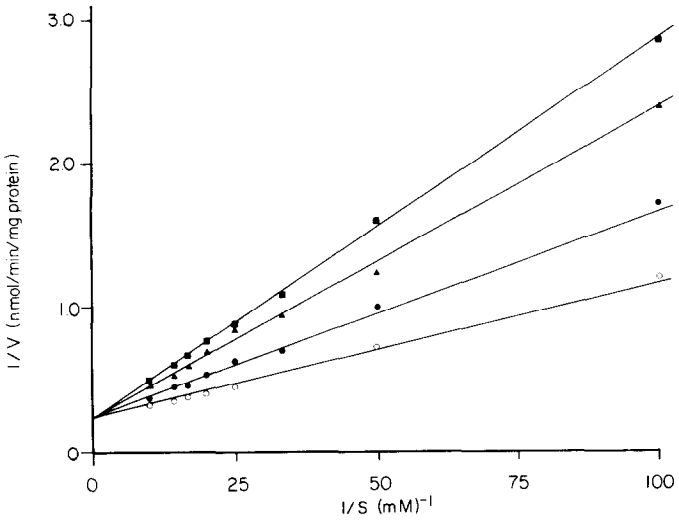

Fig. 4. Inhibition of *p*-nitrophenol hydroxylation activity by acetone. Microsomes (0.5 mg protein/ml) from rats chronically pretreated with acetone were incubated in the absence of acetone (○) and in the presence of acetone at concentrations of 1 mM (●), 2 mM (▲) and 3 mM (■). In the absence of inhibitor, the apparent K_m value was 0.041 mM and the V_{max} was 4.38 nmol *p*-nitrocatechol formed/min/mg protein. Acetone was a competitive inhibitor with a K_i of 1.828 ± 0.180 mM (mean ± SE of three determinations).

DISCUSSION

In previous studies, we have shown that chronic ethanol administration in a liquid diet to female rats increases liver microsomal cytochrome P-450 content up to 75–80% [12, 32]. This treatment increases the rate of 7-ethoxycoumarin O-deethylation 3-fold, of aniline hydroxylation 3.5-fold [12, 32], and of *p*-nitrophenol hydroxylation 6-fold [24]. It also mildly increases the rate of microsomal N-demethylation of benzphetamine and of other substrates such as aminopyrine or ethylmorphine in female rats, while depressing their microsomal metabolism in males [12].

The three enzyme activities markedly enhanced by ethanol were similarly increased by chronic acetone administration in spite of a much smaller increase in liver microsomal cytochrome P-450 content. Acetone administration in drinking water appears a more selective model of induction since it also does not show the sex-dependent enhancing effect of ethanol on benzphetamine N-demethylation activity in the female rats used in this study.

Similar increases in aniline hydroxylation and 7-ethoxycoumarin O-deethylation activities have also been reported after the acute administration of relatively large doses of acetone [16, 18]. Acetone, under these conditions, enhances microsomal NADPH–cytochrome *c* reductase activity [16–18], a phenomenon also observed after chronic ethanol administration [26, 33]. By contrast, this activity is unaffected by chronic exposure to a low daily dose of acetone, and its influence in this case is apparently restricted to the cytochrome P-450 component of the monooxygenase system.

Induction by acetone is associated with a shift of the maximum of the reduced CO-complex toward a longer wavelength [18]. The observation of a similar phenomenon after chronic administration of ethanol [34, 35] or isoniazid [36] has been explained by the spectral characteristics of cytochrome P-450j, the isozyme preferentially induced by these two agents [7, 8]. Induction by acetone, accordingly, is best explained by a similar inducing effect rather than by the induction of cytochrome P-450d, the only other rat liver isozyme known for having a high activity toward aniline hydroxylation but different spectral characteristics [7].

Acetone is a highly selective substrate of the acetone- an ethanol-inducible isozyme 3a in the rabbit and of an immunochemically homologous rat isozyme [13, 14] recently identified as cytochrome P-450j [8]. It has been reported to inhibit isozyme 3a-catalyzed aniline hydroxylation, but also to stimulate the same reaction with other rabbit liver isozymes [13]. Accordingly, the occurrence of similar opposite effects of acetone on rat liver isozymes would imply that inhibition of cytochrome P-450j-dependent aniline hydroxylation activity, in the presence of acetone, could be more than compensated for by stimulation of other isozymes. This, however, still leaves unexplained the large difference, between control and acetone-induced microsomes, in maximal acetone-stimulated aniline hydroxylation activities.

Acetone *in vitro* is a relatively weak inhibitor of low K_m *N*-nitrosodimethylamine demethylation [18]. Its acute administration inhibits the formation of the reactive metabolite of acetaminophen *in vivo* in the rat [37]. In keeping with these observations, our data show that acetone *in vitro* is a potent competitive inhibitor of *p*-nitrophenol hydroxylation activity. The K_i for this inhibitory effect of acetone is similar to the apparent K_m for acetone in the acetone oxidation reaction catalyzed by microsomes from

control, ethanol- and acetone-pretreated rats [14]. When compared to aniline hydroxylation, microsomal *p*-nitrophenol hydroxylation appears to be a more sensitive index of exposure to ethanol [23, 24] and to acetone. *p*-Nitrophenol is a more selective substrate than aniline for rabbit liver isozyme 3a [25, 38] and apparently also for cytochrome P-450j in rat liver [38], and this could explain the absence of any stimulatory effect of acetone on *p*-nitrophenol hydroxylation activity.

Our data also suggest that studies of the effects of acetone on the *in vivo* metabolism of xenobiotics must take into consideration both the need to avoid the presence of circulating acetone, and the rapid disappearance of its inducing effects, even after chronic exposure. Indeed, a 24-hr withdrawal period was sufficient to abolish almost completely its enhancing effects on the microsomal metabolism of aniline, *p*-nitrophenol and 7-ethoxycoumarin. A similar rapid disappearance of the inducing effect of ethanol on aniline hydroxylation activity has already been demonstrated [12, 39]. This may indicate that cytochrome P-450j has a high turnover rate. The same characteristic would also apply to the isozyme responsible for the enhancing effect of acetone on 7-ethoxycoumarin O-deethylation activity, an isozyme probably distinct from cytochrome P-450j as suggested by the low activity of the latter toward this substrate [7]. The inducing effect of chronic ethanol administration in a liquid diet on 7-ethoxycoumarin O-deethylation activity, on the other hand, was found to persist for 24 hr more than in the same female rats submitted to acetone treatment in the present study [12]. The significance of this difference, however, is still unclear, given the low selectivity of this substrate [40] and the relatively slow and complex changes, including apparently a transient increase in isozyme(s), that occur after the withdrawal of ethanol in female animals [12].

The present observations point to the need for better characterization of the cytochrome P-450 isozyme having a high 7-ethoxycoumarin O-deethylation activity that is apparently preferentially induced along with cytochrome P-450j by acetone and ethanol. The induction of these isozymes by chronic acetone administration, on the other hand, can be achieved under conditions where the body weight, the liver weight and the liver microsomal protein content of the animals are unaffected, while their total liver cytochrome P-450 content is only minimally increased. This, along with the limited influence of this treatment on microsomal cytochrome P-450 specific content, offers the opportunity to study the *in vivo* implications of these preferential inducing effects of acetone and ethanol in the rat in a much simpler content than after the chronic administration of an ethanol-containing liquid diet [12]. Of particular interest, in this regard, may be the possibility of looking at their potentially important contribution to the potentiation of the hepatotoxicity of certain xenobiotics in the absence of the hepatotoxic effects of ethanol [19]. The identification of non-toxic selective substrates of these isozymes, suitable for *in vivo* use in humans, is moreover clearly needed to determine whether the rapid disappearance of these particular inducing effects in the rat has its counterpart in alcoholics or in humans exposed to acetone in their working environment.

Finally, recent observations suggest that fasting and diabetic keto-acidosis could also result in the induction of cytochrome P-450j in the rat [7]. Such conditions, on the other hand, are associated at least in humans, with plasma concentrations of acetone in the range of 1–10 mM [41, 42]. Our data on the inhibitory effect of acetone on microsomal *p*-nitrophenol hydroxylation suggest that significant competitive inhibition by acetone of the metabolism of certain xenobiotics could occur *in vivo* under these conditions and this even at the lower acetone concentrations reported. This factor should thus be taken into consideration in the planning of *in vivo* studies of the influence of fasting or of diabetes on the metabolism of xenobiotics.

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