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DIFFERENCES IN THE DURATION OF THE ENHANCEMENT OF LIVER MIXED-FUNCTION OXIDASE ACTIVITIES IN ETHANOL-FED RATS AFTER WITHDRAWAL

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Abstract—Liver microsomal mixed-function oxidase activities were determined in female Sprague–Dawley rats after 3 weeks of ethanol feeding and for up to 10 days after withdrawal. Ethanol (36% of total calories) was administered in a high fat liquid diet and was replaced isocalorically by carbohydrates in controls. Chronic ethanol feeding similarly enhanced both microsomal cytochrome P-450 content and benzphetamine *N*-demethylase activity, per mg of protein, and resulted in a disproportionate increase in both aniline hydroxylase and 7-ethoxycoumarin *O*-deethylase activities. A 6- to 7-day withdrawal period was apparently necessary for the overall disappearance of these effects of ethanol. Marked differences, however, were seen in the time courses of return of these variables to control levels, as also indicated by changes, during this period and specially during the first 24 hr after withdrawal, in the apparent molar activity of the microsomal fraction with the three substrates tested. The results were interpreted as indicating that the distinct ethanol-inducible cytochrome P-450 isozyme, with a high specific activity toward aniline, undergoes a very rapid turnover in liver microsomes. Induction of another form of cytochrome P-450, differing from the former by its slower turnover rate, would explain the induction by ethanol of 7-ethoxycoumarin *O*-deethylase activity. The withdrawal of ethanol was followed by a rapid but transient increase in benzphetamine *N*-demethylase activity above the ethanol-induced level, at a time when other activities were rapidly declining. This could suggest that the microsomal content of other cytochrome P-450 isozyme(s), with high specific activity toward this substrate, would also be temporarily altered during ethanol withdrawal. Important alterations in microsomal cytochrome P-450-dependent mixed-function oxidase activities occurred during the initial 24-hr period of withdrawal, even in the absence of a change in microsomal cytochrome P-450 content, indicating that the effects of chronic ethanol ingestion on hepatic drug-metabolizing enzyme activities may also be highly dependent on the proximity of ethanol intake.

The effects of ethanol administration on the liver microsomal mixed-function oxidase system in the rat have been the subject of numerous studies. Chronic ethanol administration results in a proliferation of the smooth endoplasmic reticulum [1–5] and in an increase in the cytochrome P-450 content of the liver microsomal fraction [2–15]. Ethanol also increases microsomal aniline hydroxylase activity, a well documented phenomenon [2, 3, 6–12, 14] postulated to result, in the rat, from the induction of a cytochrome P-450 isozyme which is distinct from other known inducible forms [16, 17], and the existence of which has been clearly established recently in the rabbit [18, 19]. This ethanol-inducible cytochrome P-450 isozyme, for which aniline is a preferential substrate, is also characterized by its relatively high activity in the oxidation of ethanol and of other alcohols, as suggested from studies done in the rat [20, 21] and as demonstrated in the rabbit [19]. A partially purified cytochrome P-450 preparation from ethanol-fed rats was also shown to be highly active in the *O*-deethylation of 7-ethoxycoumarin [22], and chronic ethanol consumption was also found to greatly enhance the rate of microsomal biotransformation of several aromatic and halogenated hydrocarbons [13].

Conflicting results, however, have been reported by various laboratories regarding the effects of ethanol on the metabolism of other substrates [5, 7–10, 12, 14, 16, 23] that are apparently not, at least in the case of benzphetamine, aminopyrine, ethylmorphine and benzo[*a*]pyrene, specific substrates for the ethanol-inducible form(s) of cytochrome P-450 [16, 19]. It is also apparent, from these studies, that sex-related differences in the response of certain mixed-function oxidase activities to ethanol may explain, at least in part, some of the observed discrepancies, and that the degree of enhancement of microsomal mixed-function oxidases after ethanol is also influenced by the dietary models used for chronic ethanol administration [6, 8–10, 12, 23]. Dietary carbohydrates have been shown to play a major role in this regard, a low carbohydrate intake markedly augmenting the effect of ethanol consumption on the activity of liver mixed-function oxidases [15, 24].

Certain reports suggest that the elapsed time between the last exposure to ethanol and the isolation of liver microsomes may significantly affect the level of activity that will be observed for various mixed-function oxidases after chronic ethanol consumption. The withdrawal of ethanol for only 1 day, after chronic administration, was thus shown to

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markedly alter the effect of ethanol on microsomal pentobarbital hydroxylase and benzphetamine *N*-demethylase activities [7]. This withdrawal period could also be sufficient, according to others, to practically abolish the increases in microsomal cytochrome P-450 content and aniline hydroxylase activity, as well as the increase in the rates of biotransformation of several aromatic and halogenated hydrocarbons [13, 14].

The latter observations [13, 14], however, are not in agreement with other studies showing that liver cytochrome P-450 content [25] and microsomal aniline hydroxylase activity [8] remain elevated for at least 1 day after ethanol withdrawal. An earlier detailed report on this subject had, moreover, shown that a 7-day withdrawal period, after chronic ethanol consumption, was apparently necessary for the return to control values of the enhanced microsomal cytochrome P-450 content and of both aniline hydroxylase and ethanol-oxidizing activities [3]. Such reported differences between the results of various laboratories suggest that the experimental model may thus have a significant influence on the persistence of the effects of chronic ethanol consumption after its withdrawal. This, moreover, could markedly affect the results of studies looking at the effect of ethanol consumption on the liver metabolism of various xenobiotics *in vivo*, since the duration of the ethanol withdrawal period that usually precedes the administration of the tested drug, in such studies, may differ from one experimental model to another.

This prompted us to examine the duration of the induction of liver mixed-function oxidases, after withdrawal from ethanol, in the experimental model of chronic ethanol consumption used in our laboratory. The latter involves feeding ethanol to female rats as part of a frequently used liquid diet, developed by DeCarli and Lieber [26], which apparently maximizes the inductive effects of ethanol on this enzyme system in the rat [24]. The present study was designed to examine the time course of the decrease in microsomal cytochrome P-450 content after withdrawal of ethanol and, by relating it with changes in the rates of biotransformation of aniline, 7-ethoxycoumarin and benzphetamine, which apparently are preferential substrates for different cytochrome P-450 isozymes [27–29], to look for indications of possible differences in their respective turnover rates.

MATERIALS AND METHODS

Materials. Benzphetamine was supplied by the Upjohn Co. (Kalamazoo, MI). Aniline hydrochloride was purchased from the Eastman Kodak Co. (Rochester, NY), and 7-ethoxycoumarin and 7-hydroxycoumarin from the Aldrich Chemical Co. (Milwaukee, WI). Enzymes, coenzymes and biochemicals were from Boehringer Mannhein Biochemicals (St-Laurent, Quebec, Canada), and all other reagent grade chemicals were purchased from Fisher Scientific (Montreal, Quebec, Canada).

Animals and diets. Female Sprague–Dawley rats, purchased in groups of weanling littermates, were fed laboratory chow (Purina rat chow, No. 5012) and water until they reached a weight of 140–160 g. They were then housed individually and pair-fed a liquid diet [25] containing 18, 35 and 11% of total calories as protein, fat and carbohydrates, respectively, and 36% of total calories as ethanol, which was replaced isocalorically by carbohydrates in controls. The average daily intake of ethanol was 13–15 g/kg.

The effect of chronic ethanol consumption on liver mixed-function oxidases was determined in pairs of rats fed the ethanol-containing or control diet for 3 weeks and having had access to their respective diet up to the time of death.

The effect of withdrawal from ethanol was also studied after the same 3-week feeding period. Rats fed ethanol chronically were then given the control liquid diet, along with their pair-fed partner, and groups of rats were killed after 1-10 days of this regimen.

Preparation of liver microsomes. 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 [9], resuspended in 1.15% KCl, and used on the same day.

Assays. The cytochrome P-450 content of microsomal preparations was measured by the method of Omura and Sato [30], at an optimal protein concentration of 3 mg/ml [31], using an Aminco DW-2 UV/VIS spectrophotometer in the split-beam mode. Protein was determined according to Lowry *et al.* [32]. Microsomal aniline hydroxylase and benzphetamine *N*-demethylase activities were determined by measuring the rate of formation of

Table 1. Effect of ethanol administration for 3 weeks on liver microsomal cytochrome P-450-dependent mixed-function oxidases in female rats*

	Cytochrome P-450†	Aniline hydroxylase‡	Benzphetamine *N*-demethylase§	7-Ethoxycoumarin *O*-deethylase‖
Control	0.81 ± 0.03	1.00 ± 0.06	3.50 ± 0.31	1.76 ± 0.18
Ethanol	1.42 ± 0.05	3.50 ± 0.20	6.05 ± 0.73	5.34 ± 0.46
Ethanol / Control	1.75 ($P < 0.001$)	3.50 ($P < 0.001$)	1.73 ($P < 0.01$)	3.03 ($P < 0.001$)

* Results are means ± S.E. of nine pairs of animals.
† Expressed as nmoles/mg microsomal protein.
‡ Activity is expressed as nmoles *p*-aminophenol/mg protein/min.
§ Activity is expressed as nmoles HCHO/mg protein/min.
‖ Activity is expressed as nmoles 7-hydroxycoumarin/mg protein/min.

p-aminophenol and formaldehyde, respectively, as described previously [9]. The microsomal 7-ethoxycoumarin *O*-deethylase activity was assayed by the fluorometric determination of the rate of production of 7-hydroxycoumarin [33]. Under the assay conditions used, the initial rates of metabolite production were a linear function of time and protein concentration.

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

RESULTS

Female rats fed the ethanol-containing liquid diet for 3 weeks, and having had access to ethanol up to the time they were killed, had a 75% higher liver microsomal cytochrome P-450 content, per mg of protein, than pair-fed controls (Table 1). Microsomal benzphetamine *N*-demethylase activity was also increased significantly, and in the same proportion, after chronic ethanol consumption. The observed increases in both aniline hydroxylase and 7-ethoxycoumarin *O*-deethylase activities, by contrast, were much greater than the associated change in cytochrome P-450 content, indicating that these two mixed-function oxidase activities are preferentially induced by ethanol in these animals.

The return of these variables to control levels following the withdrawal of ethanol is illustrated in Fig. 1. The degree of enhancement of liver microsomal cytochrome P-450 content, per mg of protein, seen after chronic ethanol consumption was apparently unaffected by the withdrawal of ethanol for 1 day, while a sharp decline then occurred during the subsequent 24- to 48-hr period. Microsomal cytochrome P-450 content was still elevated significantly ($P < 0.005$) after a 5-day withdrawal period but not thereafter. The variability in the observed ethanol-withdrawn over control ratios [117.0 ± 7.8% (mean ± S.E.) after 3 days vs 126.8 ± 5.6% after 5 days (P:NS)] was probably responsible for the apparent rebound in this variable after day 5 of withdrawal from ethanol.

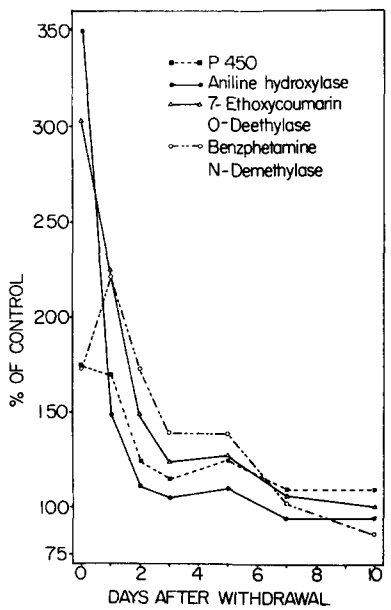

Fig. 1. Changes in cytochrome P-450 content and mixed-function oxidase activities in the liver, per mg of microsomal protein, of rats withdrawn from ethanol after chronic ethanol consumption for 3 weeks. Results are in percent of controls and represent the mean of nine to twelve determinations for each time point.

The three mixed-function oxidases studied were each affected in a distinct way by the withdrawal of ethanol. As seen in Fig. 1, microsomal aniline hydroxylase activity, which is greatly enhanced after chronic ethanol consumption, was strikingly decreased after withdrawal of ethanol for only 1 day, even in the absence of a significant change in microsomal cytochrome P-450 content. This activity, while still elevated significantly ($P < 0.001$) on day 1, had moreover returned to control level on the following day. Microsomal 7-ethoxycoumarin *O*-deethylase activity, which is also increased in a greater proportion than cytochrome P-450 content after ethanol consumption, also exhibited an initial phase of rapid decline after withdrawal of ethanol. The latter, however, differed in duration from that seen in the case of aniline hydroxylase activity and was extended over the first 2 days after removal of ethanol from the diet. Microsomal 7-ethoxycoumarin *O*-deethylase activity, like cytochrome P-450 content, still remained significantly higher ($P < 0.02$) in ethanol-pretreated rats than in controls after 5 days without ethanol, as was also observed at that time in the case of benzphetamine *N*-demethylase activity ($P < 0.005$). The withdrawal of ethanol, however, had a distinct influence on the latter which, unlike the two other activities studied, was first further increased by another 50% ($P < 0.001$) above its ethanol-induced level after 1 day without ethanol. This initial increase in benzphetamine *N*-demethylase activity also occurred in the absence of significant quantitative alterations in the microsomal cytochrome P-450 content.

It is thus apparent, from these observations, that only the later phase of the return of mixed-function oxidase activities to control levels roughly parallels the normalization of microsomal cytochrome P-450 content. The early phase of ethanol withdrawal, on the other hand, is associated with rapid and distinct alterations in the activities tested and these are best illustrated by changes in the apparent molar activities (nmoles of product formed per/min/per nmole of cytochrome P-450) of liver microsomes with the three substrates used.

As seen in Fig. 2, chronic ethanol consumption was associated with a 100% increase ($P < 0.001$) in the microsomal apparent molar activity for aniline hydroxylation. This increase was, however, completely abolished during the first 24-hr period of withdrawal from ethanol, after which aniline hydroxylase molar activity was already decreased below control level. The latter also apparently remained depressed for at least 7 days after withdrawal of ethanol. The relatively small depressions noted were, however statistically significant only on days 1 ($P < 0.05$) and 7 ($P < 0.01$).

This, on the other hand, sharply contrasts with the

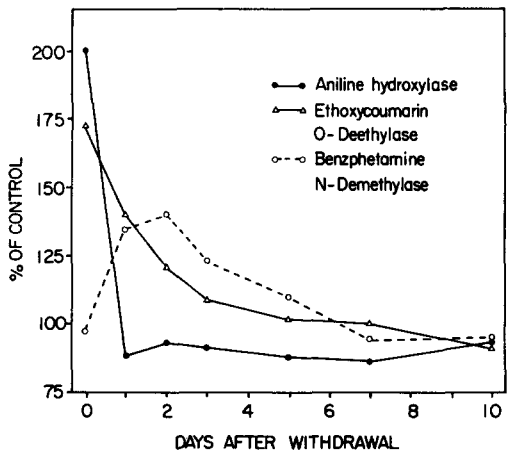

Fig. 2. Changes in the apparent molar activity (nmoles of product formed/per min/per nmole of cytochrome P-450) of microsomal mixed-function oxidases in liver microsomes of rats withdrawn from ethanol after chronic ethanol consumption for 3 weeks. Results are in percent of control and represent the mean of nine to twelve determinations for each time point.

slower, more gradual decline in the apparent molar activity for 7-ethoxycoumarin *O*-deethylation seen in the same microsomal preparations (Fig. 2). The observed values in this case remained significantly elevated ($P < 0.01$) for up to 2 days after cessation of ethanol consumption and did not subsequently drop below control levels.

The microsomal apparent molar activity for benzphetamine *N*-demethylation, unlike the other two, was not altered by chronic ethanol consumption. The withdrawal of ethanol for 1 day was, however, associated with a rapid and significant ($P < 0.001$) elevation in this variable, which also persisted on day 2 ($P < 0.01$). The molar activity for benzphetamine *N*-demethylation also remained higher than in controls for the longest period, still being significantly elevated ($P < 0.001$) for at least 3 days after withdrawal.

DISCUSSION

The dietary model used in the present study [25] apparently maximizes the influence of ethanol on liver microsomal cytochrome P-450 content and mixed-function oxidase activities [9, 15, 23]. This, along with the use of female rats [8], may account for the relatively large differences observed at this level between ethanol-fed and control animals, when compared to those reported in other studies [8, 10, 12, 14].

Chronic ethanol administration greatly enhances the apparent molar activity of liver microsomes for aniline hydroxylation and, to a slightly lesser degree, for 7-ethoxycoumarin *O*-deethylation. Preliminary studies from this laboratory indicate that aniline and 7-ethoxycoumarin are preferential substrates for chromatographically distinct cytochrome P-450 isozymes in microsomes from ethanol-fed rats [34], as also shown in ethanol-fed rabbits [19]. These observations, along with the distinct effects of ethanol withdrawal on these activities, suggest that their enhancement after chronic ethanol consumption results from the induction of cytochrome P-450 isozymes of different substrate specificity and rate of turnover.

The induction of aniline hydroxylase and 7-ethoxycoumarin *O*-deethylase activities by ethanol is not sex-related [8, 35]. Ethanol consumption, however, decreases the rate of microsomal N-demethylation of benzphetamine or of substrates such as ethylmorphine and aminopyrine in male rats [8, 12, 14] and, as also seen in the present study, mildly increases this activity in female rats [5, 9, 36]. This, along with the particular effect of ethanol withdrawal on benzphetamine *N*-demethylase activity, suggests that ethanol consumption also affects the microsomal content of cytochrome P-450 isozyme(s) that may be distinct from those responsible for the enhancement of the other two activities tested.

The time required for the return of the microsomal cytochrome P-450 content to control level after ethanol withdrawal, in the present study, is in keeping with the 5- to 7-day period reported to be necessary after chronic ethanol consumption [3] or after phenobarbital treatment [37, 38]. Our data, however, indirectly suggest that changes in the microsomal content of individual cytochrome P-450 isozymes during this period may not necessarily parallel the change in this relatively non-specific variable.

Our observations in this regard may indicate that the ethanol-inducible cytochrome P-450 isozyme for which aniline is a preferential substrate has a particularly high turnover rate in rat liver microsomes, when compared to that of the isozyme responsible for the enhancement of 7-ethoxycoumarin *O*-deethylase activity. This, interestingly, could also constitute an alternate explanation for the rapid and transient "stimulatory" effect of acute ethanol administration on the rate of microsomal biotransformation of aniline, ethanol and of other potential substrates of this cytochrome P-450 isozyme [6, 39–45], an effect of ethanol which, like the early phase of ethanol withdrawal, may apparently involve only a minimal alteration in the overall microsomal cytochrome P-450 content [6, 44, 45]. Ethanol withdrawal, in female rats, may also result in a temporary increase in the microsomal content of cytochrome P-450 isozyme(s) for which benzphetamine is a preferential substrate. Such an increase could counterbalance the decline in the microsomal content of other cytochrome P-450 isozymes, for at least 24 hr after ethanol withdrawal, thus possibly accounting for the fact that the major effects of this initial withdrawal period on the activities tested occurred in the absence of a significant alteration in the overall cytochrome P-450 content of the microsomal fraction.

This, however, remains to be confirmed by direct immunochemical quantitation of the cytochrome P-450 isozymes involved, and our observations, for instance, still do not rule out the possibility that the increase in benzphetamine *N*-demethylase activity after ethanol withdrawal could also result from the disappearance of an inhibitory effect of ethanol consumption on certain cytochrome P-450 isozyme(s).

The latter, however, would imply the relatively selective influence of a tightly bound inhibitor, not removable either by the washing step included in the preparation of the microsomal fractions used, or, as suggested from earlier studies [16, 17], by the solubilization and partial purification of microsomal cytochromes P-450. Recent *in vitro* observations [46] nevertheless indicate that more needs to be known about the extent, selectivity and potential consequences of acetaldehyde covalent binding to microsomal proteins *in vivo*.

The major influence of ethanol withdrawal for 1 day on various liver mixed-function oxidase activities, as reported both here and in previous studies [13, 14, 23, 44], finally indicates that the results of studies looking at the effects of chronic ethanol consumption on the hepatic cytochrome P-450-dependent metabolism of xenobiotics *in vivo* may be greatly affected by the duration of the withdrawal period used to avoid interference by ethanol at this level [47, 48]. Such considerations, in fact, may be very important for our understanding of the role played by individual cytochrome P-450 isozymes in the acceleration of the *in vivo* metabolism and, in some cases, of the bioactivation of various drugs [47, 49] and hydrocarbons [12] after chronic ethanol consumption and withdrawal. The importance of the ethanol-inducible isozyme that is highly active in the biotransformation of aniline remains to be determined in this regard, particularly in light of its very fast apparent rate of turnover and of the fact that, at least in female rats, ethanol withdrawal is associated with a rapid increase in the microsomal N-demethylation of benzphetamine, which is also known to be a preferential substrate for the main phenobarbital-inducible cytochrome P-450 isozyme [16, 17, 29].

In conclusion, the present study clearly indicates that the magnitude of the influence of chronic ethanol consumption on liver mixed-function oxidase activities may be greatly affected by short-term ethanol withdrawal. Major changes in some activities rapidly took place after cessation of ethanol intake, even preceding the start of a decline in the overall microsomal cytochrome P-450 content, and their occurrence should be taken into consideration in the design and interpretation of the results of *in vivo* ethanol–drug interaction studies.

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