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Alcohol Metabolism and Toxicity: Role of Cytochrome *P*-450[1]

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Alcohol Metabolism and Toxicity: Role of Cytochrome *P*-450. COON, M. J., KOOP, D. R., REEVE, L. E., AND CRUMP, B. L. (1984). *Fundam. Appl. Toxicol.* **4**, 134–143. A new isozyme of cytochrome *P*-450, designated form 3a on the basis of its relative electrophoretic mobility, has been purified to homogeneity from liver microsomes of rabbits treated chronically with ethanol. This cytochrome has the highest activity of the known rabbit *P*-450 isozymes in the oxidation of ethanol to acetaldehyde. In view of the reports of others that the hepatotoxicity of acetaminophen is increased in ethanol-treated animals and the human alcoholic, we have determined the activity of the six available *P*-450 isozymes in the activation of the drug to give an intermediate which forms a conjugate with reduced glutathione. Isozymes 3a, 4, and 6, the three major forms of cytochrome *P*-450 present in liver microsomes from rabbits chronically treated with ethanol, exhibited the highest activities in the reconstituted enzyme system, whereas isozymes 3b and 3c were 10- to 20-fold less effective and phenobarbital-inducible isozyme 2 was essentially inactive, even in the presence of cytochrome b_5. The results obtained thus indicate that induction by ethanol of *P*-450 isozyme 3a may contribute to the toxicity of acetaminophen but that other cytochromes also play a significant role.

Involvement of liver microsomal enzymes in ethanol oxidation and toxicity. In a brief historical review on the physiological combustion of alcohol, Theorell (1974) concluded some years ago that the main pathway is catalyzed by alcohol dehydrogenase but that alternate pathways exist, and stated that "alcohol and man is an old problem." Unfortunately, the very serious problem of alcoholism is growing instead of abating. This laboratory is presently studying a possible alternate pathway of alcohol oxidation involving cytochrome *P*-450 and the effect of ethanol on *P*-450-catalyzed reactions in which foreign compounds are sometimes detoxified and in other instances made more toxic, mutagenic, or carcinogenic. Such studies may provide basic information on alcohol metabolism and may also contribute to an understanding of the genetic factors (Deitrich and Collins, 1974) and various medical problems (Majchrowicz and Noble, 1979) associated with alcoholism.

Cytochrome *P*-450 represents a group of hemoproteins unique in having a sulfur atom ligated to the iron which forms CO complexes with a Soret band at about 450 nm. Liver microsomes are particularly rich in *P*-450 that oxygenates or otherwise modifies physiologically occurring lipids such as fatty acids, prostaglandins, and steroids as well as a host of foreign compounds, including drugs, petroleum products, anesthetics, insecticides, and carcinogens. No attempt will be made to discuss here the structure and function of *P*-450 in detail, but it should be noted that at least 10 distinct isozymes have been isolated from rabbit and from rat liver microsomes. There are hundreds, perhaps thousands of substrates, for which the isozymes exhibit only partial selectivity; the number of inducers of *P*-450 enzymes is also very great and includes drugs, carcinogens, halogenated substances, and so forth. The progress in this rapidly developing field has been described in recent reviews and

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proceedings of symposia, (Guengerich, 1979; White and Coon, 1980; Lu and West, 1980; Coon, 1981; Coon and Koop, 1983; Coon and White, 1980; Coon *et al.*, 1980a; Gustafsson *et al.*, 1980; Sato and Kato, 1982).

The catalase pathway for ethanol oxidation by H_2O_2 in peroxisomes (Keilin and Hartree, 1945; Chance, 1947; Lazarow, 1977) is apparently of minor significance in ethanol metabolism *in vivo* (Smith, 1961). The existence of a distinct microsomal ethanol-oxidizing system independent of contaminating catalase has been the subject of much controversy (Estabrook, 1974), with Lieber *et al.* (1974) as proponents and a number of other investigators (Oshino *et al.*, 1974; Thurman *et al.*, 1974; Isselbacher and Carter, 1974; Vatsis and Schulman, 1974) as skeptics armed with contrary data. Rubin *et al.* (1977) found that the proposed system was absent from a transplantable hepatocellular carcinoma owing to low activities of NADPH–cytochrome *c* reductase and the oxidase, but was apparently present in the well-differentiated tumor, thus indicating the complexity of the system. Our laboratory (Vatsis and Coon, 1977) reported some years ago that the phenobarbital- and benzoflavone-inducible forms of purified rabbit liver microsomal *P*-450 (designated as $P\text{-}450_{LM_2}$ and $P\text{-}450_{LM_4}$ or isozymes 2 and 4, respectively, on the basis of their relative electrophoretic mobilities) did not catalyze significant rates of ethanol oxidation, whereas Miwa *et al.* (1978) observed acetaldehyde formation with purified *P*-450 isozymes from several species. Other investigators (Werringloer *et al.*, 1977; Vatsis and Schulman, 1977; Vatsis *et al.*, 1977; Brentzel and Thurman, 1977) have provided evidence against the concept of a microsomal ethanol-oxidizing system.

The possible role of *P*-450 in the oxidation of ethanol and other aliphatic alcohols has been the subject of much debate. Alcohol oxidation in reconstituted enzyme systems by known inducible *P*-450 isozymes from several species has been studied with varying conclusions (Vatsis and Coon, 1977; Miwa *et al.*, 1978; Coon *et al.*, 1980b; Ingelman-Sundberg and Johansson, 1981). The effects of chronic ethanol administration to rats include proliferation of the hepatic smooth endoplasmic reticulum and an increase in the *P*-450 content and rate of hydroxylation of aniline and oxidation of alcohols in microsomes (Iseri *et al.*, 1966; Lieber and DeCarli, 1970; Ishii *et al.*, 1973; Villeneuve *et al.*, 1976; Ohnishi and Lieber, 1977a; Sharma *et al.*, 1979; Morgan *et al.*, 1981; Teschke *et al.*, 1981). An ethanol-mediated increase in *P*-450 in cultured chick embryo hepatocytes has also been reported (Sinclair *et al.*, 1981). In the rat both normal alcohol metabolism (Mezey *et al.*, 1973) and the enhanced rate after induction have been attributed to a hepatic microsomal system distinct from the known systems involving alcohol dehydrogenase and catalase (Lieber and DeCarli, 1970). The increased microsomal activity as a result of ethanol treatment was thought to be due to an enhanced content of one or more forms of *P*-450 (Villeneuve *et al.*, 1976; Ohnishi and Lieber, 1977b; Morgan *et al.*, 1981; Mungikar *et al.*, 1980). Comai and Gaylor (1973) first reported that pretreatment of rats with phenobarbital, 3-methylcholanthrene, and ethanol yielded cytochrome fractions with different affinities for cyanide. Several years later Ohnishi and Lieber (1977a) described an increase in two proteins following ethanol treatment and solubilized and reconstituted the microsomal ethanol-oxidizing activity, and Joly and co-workers (Villeneuve *et al.*, 1976; Mungikar *et al.*, 1980) reported the partial purification of an isozyme with high activity toward both ethanol and aniline, but presented no evidence for the isolation of a single isozyme. An electrophoretically homogeneous enzyme was obtained from liver microsomes of pigs treated with ethanol (Tsuji *et al.*, 1980), but the immunochemical cross-reactivity of this cytochrome with other forms indicated that it was apparently not a unique *P*-450; furthermore, the aniline hydroxylase activity was not high, and the ethanol-oxidizing activity was not reported.

Clearly, the isolation and characterization

of a highly purified *P*-450 isozyme that is enhanced by ethanol treatment of the animals and has significant ethanol-metabolizing activity, as recently achieved in this laboratory (Koop *et al.*, 1982; Morgan *et al.*, 1982; Coon *et al.*, 1983), were essential to progress in this controversial field. That the observed ethanol-oxidizing activity of our rabbit $P\text{-}450_{LM_{3a}}$ is not due to contaminating catalase or alcohol dehydrogenase was shown by several lines of evidence. LM_{3a} is isolated as an electrophoretically homogeneous preparation; the ethanol-oxidizing activity displays all the characteristics of a typical *P*-450-catalyzed reaction, including requirements for NADPH, reductase, and phospholipid. Catalatic activity in the preparation could not be detected, and ethanol oxidation was not inhibited by azide. Moreover, catalase has little activity toward two alcohols known to be substrates for $P\text{-}450_{LM_{3a}}$, propanol and butanol (Chance, 1947). Since oxidized pyridine nucleotides could not support the reaction, significant ADH activity can also be excluded.

As reviewed by Mezey (1976) and Lieber (1980), ethanol–drug interactions are quite complex; ethanol administration to animals or man may affect the normal metabolism or the toxicity or teratogenicity of other foreign substances (Randall *et al.*, 1977). These changes often involve the *P*-450 system, but much remains to be learned as to which isozymes are involved and whether induction of isozyme 3a (or a similar protein in species other than the rabbit) is responsible. Some examples of the effects of chronic alcohol consumption are increased hepatotoxicity of carbon tetrachloride (Hasumura *et al.*, 1974; Traiger and Plaa, 1974), acetaminophen (Sato *et al.*, 1981; McClain *et al.*, 1980), and cocaine (Smith *et al.*, 1981), and increased metabolism of methadone in rats (Borowsky and Lieber, 1978), hemotoxicity of inhaled benzene in peripheral blood, bone marrow, and spleen cells in mice (Baarson *et al.*, 1982), and variable effects on drug metabolism and toxicity in human alcoholics (Pelkonen and Sotaniemi, 1982). An interesting example of a change in mitochondrial reactions following alcohol administration is the inhibition of *p*-nitroanisole demethylation resulting from the decreased availability of NADPH for NADPH–cytochrome *P*-450 reductase (Reinke *et al.*, 1980).

INDUCTION OF CYTOCHROME *P*-450 BY ETHANOL AND OTHER AGENTS

As indicated above, the chronic administration of ethanol results in increased *P*-450 levels and increased rates of both ethanol and aniline oxidation in rat liver microsomes. Since enhancement of the activities of both of these substrates was also observed in hepatic microsomes following ethanol administration to rabbits (Koop *et al.*, 1982), we have made use of the more convenient assay for aniline *p*-hydroxylation in testing a series of possible inducers of *P*-450 isozyme 3a. The results, which are given in Table 1, show that not only ethanol, but also trichloroethylene, *m*-xylene, pyrazole, and imidazole bring about enhanced activity toward aniline, whereas with aniline administration to the animals the findings are equivocal. Such experiments are complicated, of course, by possible effects of the compounds tested on rates of protein synthesis and heme incorporation into the cytochromes, as well as the question of whether some *P*-450 isozymes are repressed while another, isozyme 3a, is induced. Experiments are in progress to obtain quantitative immunological evidence with $P\text{-}450_{LM_{3a}}$ antibodies on the effectiveness of the inducers. On the other hand, it appears clear that numerous compounds may serve as inducers of aniline hydroxylase and presumably, therefore, of alcohol oxidase activities associated with cytochrome *P*-450.

CATALYTIC ACTIVITIES OF CYTOCHROME *P*-450 ISOZYMES TOWARD ETHANOL AND OTHER SUBSTRATES

The substrate preference of isozyme 3a was compared with that of the other *P*-450 iso-

TABLE 1

ANILINE HYDROXYLASE ACTIVITY OF LIVER MICROSOMES FROM RABBITS TREATED WITH VARIOUS INDUCERS[a]

Compound administered	*P*-450 content (nmol/mg protein)	Aniline hydroxylase activity	
		nmol/min/nmol *P*-450	nmol/min/mg protein
None	2.5	0.13	0.38
5,6-Benzoflavone	3.4	0.10	0.34
Trichloroethylene	1.6	0.33	0.52
Benzene	3.2	0.10	0.32
m-Xylene	2.6	0.22	0.57
Pyrazole	3.6	0.30	1.08
Aniline	2.1	0.21	0.44
Imidazole	4.2	0.22	0.96
Ethanol	2.6	0.24	0.53

[a] Aniline hydroxylation was determined in reaction mixtures which contained 2.5 mM aniline, 1.0 mg of microsomal protein, 50 mM potassium phosphate buffer, pH 7.6, and 1.0 mM NADPH as the final addition. After a 30-min incubation at 30°C, the reaction was quenched by the addition of trichloroacetic acid, and the *p*-aminophenol was measured (Schenkman *et al.*, 1967). In control experiments all components were present but trichloroacetic acid was added prior to NADPH.

zymes purified in this laboratory, as shown in Table 2, in which the data are mostly taken from a previous report (Morgan *et al.*, 1982). *P*-450$_{LM_{3a}}$ had the highest activity of all isozymes examined toward aniline, ethanol, propanol, and butanol and was 2.5 times as active as the next most active isozyme, LM_4, with ethanol as substrate, and 5.5 times as active as LM_2 toward butanol. In contrast, only low activities of all of the isozymes were observed with methanol as substrate. Another isozyme purified in this laboratory which we believe to be identical to form 6, first described by Johnson and Muller-Eberhard (1977), had a turnover number of only 3.0 toward ethanol. The aforementioned ethanol-oxidizing activity of the reductase in the absence of *P*-450 may contribute to the observed rates of ethanol oxidation in the presence of the various isozymes, but whether the presence of *P*-450 has an effect on this intrinsic activity of the reductase is not known. If we assume no such effect and correct the *P*-450 activities for the reductase present, the ethanol-oxidizing activity of LM_{3a} relative to the other isozymes becomes 3-fold higher than that of LM_2 and 4.5-fold higher than that of LM_4. LM_{3b} and LM_{3c} do not have significantly higher activities than the reductase alone. LM_{3a} had no detectable activity toward ethylmorphine, whereas the other four isozymes all had similar but low activities with this substrate. The constitutive isozymes, 3b and 3c (Koop *et al.*,

TABLE 2

CATALYTIC ACTIVITY OF *P*-450$_{LM_{3a}}$ COMPARED TO OTHER ISOZYMES[a]

Substrate	Catalytic activity of isozymes (nmol product formed/min/nmol *P*-450)					
	2	3a	3b	3c	4	6
Methanol	1.3	1.7	1.1	1.1	1.9	
Ethanol	3.6	10.8	1.2	1.6	4.4	3.0
1-Propanol	3.1	12.6	1.3	0.9	1.6	
1-Butanol	2.5	14.1	0.7	0.4	1.9	
Aniline	3.0	12.6	2.1	0.8	1.7	4.0
Ethylmorphine	4.6	<0.2[b]	4.9	3.0	2.6	
Chlorcyclizine	1.5	0.4	6.4	5.0	1.0	
7-Ethoxycoumarin	14.2	1.2	2.7	0	1.4	2.5

[a] Incubation conditions for each substrate were optimized using the most active isozyme for that substrate. Alcohol concentrations were: methanol, 116 mM; ethanol, 80 mM; propanol, 40 mM; and butanol, 22 mM.

[b] This value is at the limit of detection in the assay used for ethylmorphine.

1981) were the most active toward chlorcyclizine, and 3a was the least active. LM_2 was about fivefold more active toward 7-ethoxycoumarin than LM_{3b}, the next most active isozyme, and LM_{3a} had only low but significant activity.

Liver microsomal *P*-450 isozymes are also known to metabolize a number of physiologically occurring compounds including medium chain length fatty acids, prostaglandins, and steroids. Probably the most extensively characterized of these reactions is the hydroxylation of testosterone. Isozyme 3b exhibits the highest activity for testosterone hydroxylation followed in order by isozymes 6, 4, 3c, and 3a. All these isozymes hydroxylate testosterone predominantly at the 6β position and to a lesser extent at the 16α position. Isozyme 2 hydroxylates testosterone slowly, but, in contrast to the other isozymes, shows a preference for the 16α position. In addition to these two sites of attack, all six isozymes oxidize the 17β-hydroxy substituent to the ketone to form androstenedione, a biologically active metabolite. Isozyme 3b exhibits the highest activity for this reaction followed in order by isozymes 4, 2, 3a, 6, and 3c. Similar activity has recently been reported for rat liver *P*-450 isozymes (Wood *et al.*, 1983). From these results it is evident that steroid metabolism may be altered significantly by the administration of ethanol or other compounds which induce the liver microsomal *P*-450 system. Furthermore, ethanol and testosterone would be expected to function as competitive substrates for the *P*-450 isozymes.

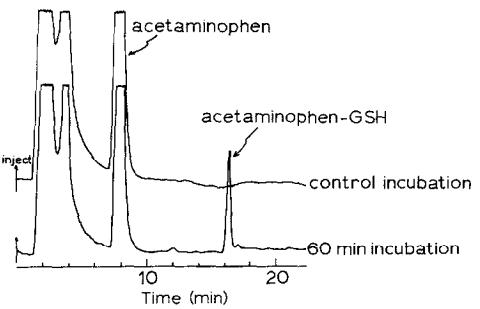

FIG. 1. Separation of glutathione conjugate from reactants by HPLC.

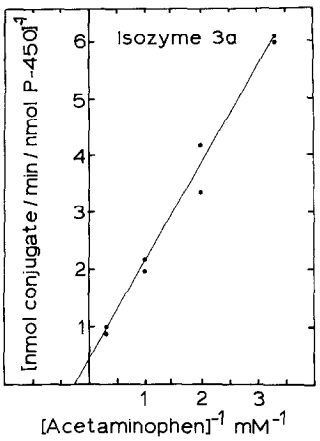

FIG. 2. Lineweaver–Burk plot, used to determine the K_m of acetaminophen in formation of the glutathione conjugate.

ALCOHOL ADMINISTRATION AND ACETAMINOPHEN TOXICITY: ROLE OF CYTOCHROME *P*-450

Our studies on the ethanol-inducible isozyme of liver microsomal cytochrome *P*-450 may also throw some light on toxicities of other foreign compounds as influenced by alcohol consumption. For example, in view of the reports of other investigators that the hepatotoxicity of acetaminophen is increased in ethanol-treated animals and the human alcoholic, we have determined the activity of the six available rabbit *P*-450 isozymes in the activation of the drug to give an intermediate which forms a conjugate with reduced glutathione (Morgan *et al.*, 1983). Acetaminophen (Tylenol; *p*-hydroxyacetanilide), a widely used antipyretic and analgesic drug, is normally nontoxic but in large doses produces acute hepatic necrosis (Proudfoot and Wright, 1970; Prescott *et al.*, 1971; Mitchell *et al.*, 1973a). Metabolism of the drug by the liver microsomal *P*-450-containing oxygenase system results in the formation of an arylating species, thought to be *N*-acetyl-*p*-benzoqui-

TABLE 3

ACTIVITIES OF *P*-450 ISOZYMES IN THE FORMATION OF THE GLUTATHIONE–ACETAMINOPHEN CONJUGATE[a]

System	Activity of isozymes (nmol conjugate formed/min/nmol *P*-450)					
	2	3a	3b	3c	4	6
Complete	<0.04	2.16	0.14	0.22	3.67	1.44
Complete + cytochrome b_5	0.07	2.09	0.14	0.40	5.50	0.94
Complete, Tris buffer substituted for phosphate	<0.04	4.17	0.22	0.14	4.86	0.90

[a] This table is taken from Morgan *et al.* (1983). The complete system contained 0.2 μM $P\text{-}450_{LM}$, 0.6 μM NADPH–cytochrome *P*-450 reductase, 30 μg of dilauroyl-GPC, 0.5 mM glutathione, 12 mM acetaminophen, 50 mM potassium phosphate buffer, pH 7.6, and 1.0 mM NADPH in a final volume of 1.0 ml. When present, cytochrome b_5 or Tris–chloride buffer, pH 7.6, were at concentrations of 0.2 μM and 50 mM, respectively. After incubation at 30°C for 10 min, the reactions were quenched by the addition of 0.5 ml of 3 N perchloric acid and analyzed by HPLC as described by Moldèus (1978). Values less than 0.04 are below the limits of accurate determination under the conditions of this assay.

noneimine (Potter *et al.*, 1973; Corcoran *et al.*, 1980; Nelson *et al.*, 1980). This intermediate is conjugated with glutathione and excreted under normal conditions, but covalent binding to proteins occurs when cellular glutathione is depleted (Mitchell *et al.*, 1973b; Moldèus, 1978). Pretreatment of animals with compounds which alter the mixed function oxidase system has a profound effect on the metabolism of acetaminophen (Mitchell *et al.*, 1973a,b; Potter *et al.*, 1973; Moldèus, 1978; Jollow *et al.*, 1973). Increased hepatotoxicity of acetaminophen has been reported in human alcoholics (Proudfoot and Wright, 1970; Wright and Prescott, 1973; Emby and Fraser, 1977; McClain *et al.*, 1980) and in ethanol-treated rats and mice with a concomitant increase in the hepatic metabolism of the drug and covalent binding of the metabolites to microsomal proteins (Proudfoot and Wright, 1970; Peterson *et al.*, 1980; Sato *et al.*, 1981).

The formation of the glutathione–acetaminophen conjugate was determined by HPLC as described by Moldèus (1978), with the results shown in Fig. 1. The product, a single metabolite peak absorbing at 250 nm, had a retention time of 15 to 16 min and was well separated from the reactants. Formation of the conjugate required the presence of *P*-450, NADPH–cytochrome *P*-450 reductase, and NADPH, and was linear with respect to time up to 10 min at 30°C. This assay was used to determine the effect of the acetaminophen concentration on the rate of the reaction. From the results presented as a Lineweaver-Burk plot in Fig. 2, the Km of acetaminophen was determined to be 5.9 mM.

The most active cytochrome in the generation of the glutathione-acetaminophen conjugate in the reconstituted system was isozyme 4, as shown in Table 3. Isozymes 3a and 6 were also quite active, while isozymes 3b and 3c were much less effective and isozyme 2 produced only trace amounts of the conjugate which were too small to be determined accurately. The effect of cytochrome b_5 was tested in view of the previous finding that it is required for prostaglandin hydroxylation by isozyme 2 (Vatsis *et al.*, 1982) and the report of Miki *et al.* (1980) that $P\text{-}450_{B1}$, an isozyme apparently identical to isozyme 3c (Koop *et al.*, 1981) requires cytochrome b_5 for the metabolism of some substrates. In the presence of cytochrome b_5, a measurable but still very low activity of isozyme 2 in acetaminophen conjugate formation was detected, and that of isozymes 3c and 4 was significantly increased; in contrast, the activity of isozyme 6 was inhibited, and that of 3a and 3b was not altered by the presence of cytochrome b_5. The activ-

ities of isozymes 3a, 3b, and 4 were substantially increased when Tris was substituted for phosphate buffer, whereas those of 3c and 6 were decreased. The reason for these effects is not known; despite the variable effects of both cytochrome b_5 and the buffer composition on the activities of the individual isozymes, the order of activities of the four most effective cytochromes was unchanged: 4 > 3a > 6 > 3b or 3c.

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