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EFFECTS OF ETHANOL AND INHIBITORS ON THE BINDING AND METABOLISM OF ACETAMINOPHEN AND *N*-ACETYL-*p*-BENZOQUINONE IMINE BY HEPATIC MICROSOMES FROM CONTROL AND ETHANOL-TREATED RATS

JAGARLAPUDI SIVA PRASAD,*† NENGQAIN CHEN,* YUXIU LIU,* DAVID J. W. GOON‡ and JORDAN L. HOLTZMAN*†§‖

Departments of * Pharmacology and † Medicine, University of Minnesota, Minneapolis, MN 55455; and ‡ Research and § Medical Services, Veterans Administration Medical Center, Minneapolis, 55417, U.S.A.

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Abstract—Acetaminophen is metabolized by cytochrome P450 to *N*-acetyl-*p*-benzoquinone imine (NABQI). This metabolite reacts with critical cellular macromolecules to give toxicity. The administration of 10% ethanol in the drinking water to 100 g male rats for 6 weeks markedly increases the toxicity of acetaminophen. This increase was associated with a 71% increase in microsomal protein binding of acetaminophen [4.8 pmol/min/mg protein in control microsomes versus 8.2 pmol/min/mg protein in ethanol microsomes ($P < 0.01$)] and a 131% increase in aniline hydroxylase [0.52 nmol/min/mg protein in control microsomes versus 1.20 nmol/min/mg protein in ethanol microsomes ($P < 0.001$)]. On the other hand, cysteine conjugation of acetaminophen showed an increase of only 12% [2.8 nmol/min/mg protein in control microsomes versus 3.1 nmol/min/mg protein in ethanol microsomes ($P < 0.05$)]. Ethylmorphine- and benzphetamine *N*-demethylases did not increase. In microsomes from both control and ethanol animals, imidazole (1 mM) inhibited the two *N*-demethylases, aniline hydroxylation and acetaminophen binding by 85–95% but inhibited the cysteine conjugation by only 50%. For control and ethanol animals, both 80% CO/20% O_2 and SKF-525A (1 mM) totally inhibited cysteine conjugation but only inhibited the other activities by about 36–60%. KCN (1 mM) had no effect on any of the activities except protein binding (60–67% inhibition). Scavengers of reactive oxygen [mannitol (1 mM), dimethyl sulfoxide (1 mM), superoxide dismutase (15 μg/mL) and catalase (65 μg/mL)] had no effect on any of the reactions. Of all these treatments only CO/O_2 decreased the protein binding and cysteine conjugation of NABQI in the presence of either $NADP^+$ or NADPH. The data from the inhibitor studies and the effect of ethanol on acetaminophen and NABQI metabolism would suggest that protein binding and cysteine conjugation are catalyzed by different isozymes of cytochrome P450. Finally, the current results indicate that the increased toxicity of acetaminophen observed with ethanol more closely parallels the increase in protein binding activity rather than cysteine conjugation.

Previous studies from our laboratory, and from a number of other groups, have suggested that the chronic administration of ethanol increases the acute toxicity of acetaminophen [1–4]. On the other hand, the acute administration of ethanol decreases this toxicity [5, 6]. It is currently thought that these two phenomena are related to changes in the rate of the cytochrome P450 catalyzed formation of the ultimate toxin, *N*-acetyl-*p*-benzoquinone imine (NABQI). These observations would suggest that the chronic administration of ethanol leads to the induction of cytochrome P450j [7, 8], and possibly other isozymes of cytochrome P450, which rapidly metabolize acetaminophen to form NABQI. On the other hand, the acute administration of ethanol leads to concentrations of this drug which inhibit formation of the toxic metabolite.

There has been some concern that the usual assay which has been employed to determine the metabolism of acetaminophen to the toxic metabolite, the covalent binding of the drug to the microsomal proteins, is too non-specific to give meaningful biochemical information. Hence we, and other groups, have sought to develop a more specific assay technique for the *in vitro* determination of acetaminophen metabolism [6]. Since reduced glutathione totally inhibits protein binding, a logical approach to the development of a new assay would be to determine the *in vitro* rate of formation of the cysteine conjugate of acetaminophen under oxidative conditions. In line with this concept, we recently implemented an HPLC assay for the determination of the rate of formation of the cysteine conjugate in incubation mixtures. Our initial observations [9, 10] suggested that the protein binding assay and the cysteine conjugate assays do not measure the same metabolic activity. In particular, in the same study preparations, there was only a small, although statistically significant, increase in the rate of formation of the cysteine conjugate with chronic ethanol feeding, whereas there was a much more marked increase in the *in vitro* protein binding of acetaminophen. This latter increase paralleled the previously noted increase in the acute toxicity of this drug with chronic ethanol feeding [1, 2].

‖ Correspondence: Dr. Jordan L. Holtzman, Departments of Pharmacology and Medicine, Veterans Administration Medical Center, One Veterans Drive, Minneapolis, MN 55417.

A major problem in interpreting these studies is that in either assay the bulk of the NABQI formed by the metabolism of acetaminophen is reduced back to acetaminophen, either by an NADPH-dependent reaction catalyzed by NADPH-cytochrome P450 reductase [11] or by direct reduction by thiols [12]. Hence, the observed rates in both of the binding assays are determined by the balance between the rate of formation of NABQI and the branching ratio between the binding of the NABQI and its reduction back to acetaminophen. The inhibitors and ethanol treatment, therefore, could affect the apparent rate of acetaminophen metabolism by either altering the rate of formation of the NABQI or its rate of reduction. To ascertain whether the changes in binding observed with the various manipulations are due to changes in the rate of formation of the NABQI or to changes in the branching ratio for the disposition of the NABQI, it is necessary to directly examine the effect of these various experimental conditions on the metabolism of NABQI.

In view of these considerations, we have, in the current study, expanded our investigations of these two assay techniques by examining the effect of both ethanol treatment and a number of inhibitors of the mixed-function oxidases on both the protein binding and cysteine conjugation of acetaminophen and the metabolism of NABQI. Our current studies support our previous observations and suggest that the cysteine conjugation and protein binding of acetaminophen are measuring two different metabolic activities. In view of our previous results in correlating the protein binding assay with the increased toxicity of acetaminophen observed in animals chronically receiving ethanol in their drinking water, we feel that this assay is a better measure of the biochemical phenomena related to acetaminophen toxicity.

MATERIALS AND METHODS

Male, 100 g, Sprague–Dawley rats were obtained from Charles River (Wilmington, MA). They were housed in individual cages and given a standard laboratory chow and water *ad lib*. Ethanol-treated animals received 10% ethanol (v/v) in their drinking water. Previous studies from our laboratory have indicated that these animals consume 17% of their calories as ethanol [9, 10]. Further, after an initial decrease in food consumption for about 2–3 days, the ethanol-treated animals show weight gains comparable to those observed in the control animals [9, 10]. Hence, the observed effects would not be due to the effect of starvation.

The animals were killed by decapitation and the livers perfused with ice-cold KCl–Tris–HCl (150 mM–50 mM; pH 7.5, 0–4°). The livers were minced and suspended in the KCl–Tris buffer (3 mL/g liver) and then homogenized with a Brinkman Polytron at 0–4°. The homogenates were centrifuged at 10,000 *g* for 15 min in a Sorvall RC-2B with an SS-34 rotor. The supernatants were centrifuged at 105,000 *g* for 60 min in a Beckman LS 8 with a 35 Ti rotor. The microsomal pellets were resuspended in KCl–Tris.

NABQI was synthesized by a modification of the procedure of Dahlin and Nelson [13]. A mixture of acetaminophen (1.0 g, 6.6 mmol), freshly prepared silver oxide (3.0 g, 13 mmol) and dry chloroform (50 mL) was stirred at room temperature for 1 hr. The solids were removed by suction filtration through a medium-pore, fritted disc funnel into a 100-mL, round bottom flask containing butylated hydroxytoluene (1 mg). The filtrate was concentrated to 5 mL on a rotary evaporator, and the residue was eluted through a 2.2 × 10 cm column of Florisil* with anhydrous ethyl ether to give a bright yellow band. The ether was removed on a rotary evaporator and the residue was immediately sublimed (50–52° at 0.015 torr) to give 0.24 g (24% yield) of yellow crystals, m.p. 68–73° (corrected) (lit. [13] m.p. 73–74°). Crushing of the residue followed by sublimation gave an additional 0.18 g (18%), m.p. 73–77° (corrected). The solids were sublimed again just before use.

[Ring-U-^{14}C]NABQI was synthesized by the method of Nelson.† In this synthesis acetaminophen [ring-U-^{14}C] (250 μCi, 7.3 mCi/mmol) (Sigma Chemical Co., St. Louis, MO) was suspended in dry chloroform (10 mL); freshly prepared silver oxide (0.21 g) was added; and the mixture was stirred at room temperature for 1 hr. The solids were removed by suction filtration through a medium-pore, fritted disc funnel and the filtrate was concentrated to 2 mL on a rotary evaporator. The residue was transferred to a microsublimator containing unlabeled NABQI (17 mg) and the solvent was evaporated under nitrogen gas. The residue was immediately sublimed (50–55° at 0.030 torr) to give 9.4 mg of yellow crystals. The [^{14}C]NABQI was stored in the dark at −20° and used within 48 hr of preparation.

The L-cysteine conjugate of acetaminophen [*S*-(5-acetylamino)-2-hydroxyphenyl)-L-cysteine] was prepared by modifications of the methods of Nelson.† In this method a solution of NABQI (48 mg, 0.32 mmol) in chloroform (10 mL) was stirred with a magnetic stirrer at 36° under nitrogen. A solution of L-cysteine (0.24 g, 1.9 mmol) and tetrabutylammonium bisulfate (18 mg) in 0.5 M NaOH (10 mL) was added. The mixture was stirred for 35 min at 35–41°. The yellow color moved from the organic phase to the aqueous phase within the first 5 min. The aqueous phase was separated, washed with ethyl ether (3 × 10 mL), neutralized (pH 6–7) with 2 M HCl, and washed again with ether (2 × 10 mL). After standing overnight in a cold room, the solids that had formed were removed by suction filtration, and the yellow filtrate was eluted through a 1.8 × 23 cm column of Sephadex LH-20 with water. The effluent was monitored at 280 nm and the fractions containing the conjugate were combined and lyophilized to give 0.46 g of buff-colored solids. Methanol (15 mL) was added and the precipitate removed by suction filtration. The filtrate was concentrated to 5 mL and applied to two 1000 μm silica gel GF TLC plates (Analtech, Inc.) under a nitrogen atmosphere. The plates were developed in a nitrogen atmosphere with ethyl acetate:methanol:acetic acid:water

* Harvison PJ and Nelson SD, personal communication, cited with permission.

† Nelson SD, personal communication, cited with permission.

Table 1. Effects of various metabolic inhibitors on the activity of aniline hydroxylase in microsomes from control rats and rats chronically fed 10% ethanol in their drinking water for 6 weeks*

Inhibitor	Aniline hydroxylase (nmol *p*-aminophenol/min/mg protein)	
	Control	Ethanol
None	0.52 (100)	1.20† (100)
Imidazole (1 mM)	0.025 (5)	0.072 (6)
80% CO/20% O_2	0.158 (30)	0.462 (39)
KCN (1 mM)	0.57 (110)	1.38 (115)
SKF-525A (1 mM)	0.307 (59)	0.540 (45)

* All incubations were run in triplicate with a coefficient of variation of less than 5%. Values in parentheses are the percentages with no inhibitor.
† Significantly different from control ($P < 0.001$).

(60:30:1:9). The main fluorescent quenching spot was removed with methanol. The methanol was removed on a rotary evaporator to give 0.15 g of while solids. The solids were dissolved in water (2 mL) and eluted through a 1.8 × 23 cm column of Sephadex LH-20 with water. The effluent was monitored at 280 nm. The fractions containing the conjugate were combined and lyophilized to give 45 mg of white, fluffy solids, m.p. 159–161° (corrected) (lit. [14] m.p. 192–193°).

The metabolic activities were determined in an incubation medium consisting of KCl–Tris buffer (pH 7.4 at 37°) containing: microsomes (1–2 mg protein/mL), substrate (2 mM), $MgCl_2$ (5 mM), $NADP^+$ (0.39 mM) and glucose-6-phosphate (5 mM) in a final volume of 3.0 mL. The reaction was initiated by the addition of microsomes. In incubations containing NADPH, 2 units of glucose-6-phosphate dehydrogenase were included in the incubation mixture. The samples were incubated for 10 min at 37° in a shaking water bath.

Ethylmorphine- and benzphetamine *N*-demethylase activities were determined by the formation of formaldehyde by the method of Nash [15] as previously described [16]. Aniline hydroxylase activity was determined by the formation of *p*-aminophenol as previously described [16]. The protein binding of acetaminophen was determined by the addition of [^{3}H(G)]acetaminophen (1 μCi) (New England Nuclear, Boston, MA) which had been purified just prior to use by thin-layer chromatography on silica gel GF and developed with ethyl acetate [2]. The activity was taken as the difference between the binding in the presence and absence of NADPH [2]. The cysteine conjugation of acetaminophen was determined by incubating the acetaminophen (2 mM) with cysteine (2 mM). The reaction was terminated by the addition of 30% trichloroacetic acid (0.5 mL), and the samples were centrifuged at 1500 *g* for 10 min. The supernatant (1 mL) was neutralized with NaOH (0.5 M; 0.6 mL) and 20 μL was injected directly onto a HPLC system consisting of an Altex 110a pump, a Rheodyne 7125 injection valve, an ODS-C18 column (Regis, Chicago, IL) and a Kratos 770 spectrophotometer set to 256 nm. The mobile phase consisted of methanol (7.0%) and glacial acetic acid (0.75%) in 0.1 M KH_2PO_4 [17]. Unlike the original method described by Wilson *et al.* [17], we found that the addition of the electrochemical detector did not improve the determination of the conjugate.

In incubations examining the effects of various manipulations on NABQI metabolism, [^{14}C]NABQI (50 μM) was added to the incubation mixture in the presence of acetaminophen (2 mM) and in the presence and absence of NADPH. The radioactivity of the protein was determined as above. The radioactivity of the cysteine conjugate and the acetaminophen was determined after collection of the HPLC peaks in the above assay. The activities in this portion of the study are given as the total amount of product formed during the incubation rather than a rate per unit time, since the binding of the NABQI is essentially complete in 1–2 min [11].

Protein was determined by the method of Lowry *et al.* [18].

RESULTS

As in our previous studies, we found that the chronic consumption of ethanol significantly increased the activities of aniline hydroxylase (Table 1) and acetaminophen protein binding (Table 2), but had little effect on the other activities (Table 2). Interestingly, the cysteine conjugation of acetaminophen consistently showed increases with ethanol feeding which, although statistically significant ($P < 0.05$) were much less than those observed with aniline hydroxylation or protein binding (Tables 1 and 2). This has been a consistent observation in all of our studies.

Imidazole had a profound inhibitory effect on all of the assays (Tables 1 and 2), but this effect was much less for the cysteine conjugation than for the others (Table 2). On the other hand, CO and SKF-525A had much more profound effects on cysteine conjugation than they had on the other activities (Tables 1 and 2). The effects of CO and imidazole suggest that all of these activities are catalyzed by metalloenzymes, such as various isozymes of cytochrome P450, while the differences in inhibition with CO, imidazole and SKF-525A would suggest that these activities are probably catalyzed by different

Table 2. Effects of various metabolic inhibitors on the activity of ethylmorphine *N*-demethylase, benzphetamine *N*-demethylase, cysteine conjugation of acetaminophen and protein binding in microsomes from control rats and rats chronically fed 10% ethanol in their drinking water for 6 weeks*

	Ethylmorphine *N*-demethylase (nmol HCHO/min/mg protein)		Benzphetamine *N*-demethylase (nmol HCHO/min/mg protein)		Cysteine-acetaminophen (nmol/min/mg protein)		Protein binding (pmol/min/mg protein)	
Inhibitor	Control	Ethanol	Control	Ethanol	Control	Ethanol	Control	Ethanol
None	6.40 (100)	7.21 (100)	4.93 (100)	5.12 (100)	2.80 (100)	3.10† (100)	4.80 (100)	8.20‡ (100)
Imidazole (1 mM)	0.53 (8)	0.87 (12)	0.74 (15)	0.51 (10)	1.30 (48)	1.60 (50)	0.00 (0)	0.00 (0)
80% CO/20% O_2	1.66 (26)	2.88 (40)	1.77 (36)	1.95 (38)	0.03 (1)	0.03 (1)	1.90 (40)	2.40 (30)
Metyrapone (1 mM)	1.84 (29)	2.31 (32)	1.97 (40)	2.56 (50)	ND§	ND	2.70 (57)	4.90 (60)
KCN (1 mM)	6.40 (100)	7.20 (100)	4.85 (98)	5.24 (102)	3.05 (109)	3.10 (100)	1.60 (33)	3.30 (40)
SKF-525A (1 mM)	2.56 (40)	2.74 (38)	1.48 (30)	2.30 (45)	0.28 (10)	0.31 (10)	3.10 (64)	3.20 (39)

* All incubations were run in triplicate with a coefficient of variation of less than 5%. Values in parentheses are the percentage of control.
†‡ Significantly different from control: † $P < 0.05$, and ‡ $P < 0.01$.
§ ND, not determined.

Table 3. Effects of various inhibitors of reduced oxygen species on the activity of ethylmorphine *N*-demethylase, benzphetamine *N*-demethylase, cysteine conjugation of acetaminophen and protein binding in microsomes from control rats and rats chronically fed 10% ethanol in their drinking water for 6 weeks*

	Ethylmorphine *N*-demethylase (nmol HCHO/min/mg protein)		Benzphetamine *N*-demethylase (nmol HCHO/min/mg protein)		Cysteine-acetaminophen (nmol/min/mg protein)		Protein binding (pmol/min/mg protein)	
Inhibitor	Control	Ethanol	Control	Ethanol	Control	Ethanol	Control	Ethanol
None	6.40 (100)	7.21 (100)	4.93 (100)	5.12 (100)	2.80 (100)	3.10 (100)	4.80 (100)	8.20† (100)
D-Mannitol (1 mM)	6.20 (97)	6.34 (88)	4.73 (96)	5.12 (100)	3.90 (114)	3.10 (100)	4.80 (100)	8.40 (102)
DMSO (1 mM)	6.40 (100)	7.57 (105)	5.22 (106)	5.38 (105)	3.90 (114)	3.10 (100)	4.30 (90)	ND
SOD (15 μg/mL)	6.40 (100)	6.49 (90)	5.02 (102)	5.02 (98)	2.80 (100)	3.10 (100)	4.80 (100)	8.00 (98)
Catalase (65 μg/mL)	6.78 (106)	7.43 (103)	4.93 (100)	5.02 (98)	3.10 (109)	3.40 (111)	4.80 (100)	8.00 (98)

* All incubations were run in triplicate with a coefficient of variation of less than 5%. Values in parentheses are the percentage of control. Abbreviations: DMSO, dimethyl sulfoxide; SOD, bovine erythrocyte superoxide dismutase; and ND, not determined.
† Significantly different from control ($P < 0.01$).

isozymes of cytochrome P450, each of which has a different sensitivity to these various inhibitors.

Interestingly, only the protein binding assay shows any inhibition with KCN (Tables 1 and 2). This sensitivity is extremely unusual for a mixed-function oxidase activity catalyzed by a cytochrome P450. The other microsomal system which is inhibited significantly by KCN is the fatty acid desaturase system. This system has been shown to receive electrons through the cytochrome b_5 electron chain and to have a cyanide sensitive factor [19]. The overall pattern of inhibition which was observed is not consistent with this activity being catalyzed by the desaturase system. The only studies that we are aware of which show an interaction between KCN and a cytochrome P450-dependent system, are those of Correia and Mannering [20, 21]. They have reported that the cytochrome b_5 mediated, NADH stimulation of ethylmorphine N-demethylation is enhanced by KCN. They suggested that this enhancement was due to a switching of the electron flux from the desaturase pathway to the cytochrome P450 system. These results would not appear to be relevant to our current studies since NADH was not included in the incubations and we found that KCN inhibited rather than stimulated the protein binding.

Previous studies from our laboratory have suggested that in the mouse a portion of the protein binding could be related to the production of reactive oxygen species [22]. Yet, in the current study, we have found no effect of any of the common scavengers of reactive oxygen species on the *N*-demethylases, the protein binding or the cysteine conjugation in either control or ethanol-treated animals (Table 3). Our current data suggest that in the rat, unlike the mouse, the activation of acetaminophen by the mixed-function oxidases is not mediated by the production and release into the medium of reactive oxygen species.

In a final set of studies, we examined the effects of these various manipulations on the metabolism of NABQI (Tables 4 and 5). First, it should be noted that we were able to account for about 20–25 nmol of NABQI/mg protein as either bound metabolite or acetaminophen (Tables 4 and 5). Since we included 2 mg of protein/mL of incubate, this would suggest that in all of our studies we were able to account for, as the sum of these two products, essentailly all of the 50 nmol of NABQI which we added to the incubations. Further, of all the metabolic inhibitors, only CO had even a modest effect on either the protein or cysteine conjugation of NABQI (Tables 4 and 5). This decrease in binding was probably due to a direct reduction of NABQI by the CO. In line with this, there was a commensurate increase in the acetaminophen formed from the labeled NABQI, even in the presence of $NADP^+$ (Tables 4 and 5). Since this reduction was seen with both assays, the differences in the inhibition of the cysteine conjugation and the protein binding of acetaminophen by imidazole and CO cannot be explained by changes in the branching ratio in the metabolism of NABQI (Tables 4 and 5). Similarly, the inhibition of the protein binding by KCN appears to result from an inhibition of formation of NABQI rather than changes in its disposition (Table 4). It is

Table 4. Effects of various metabolic inhibitors on the protein binding of *N*-acetyl-*p*-benzoquinone imine to microsomal proteins and its reduction to acetaminophen in microsomes from control rats and rats chronically fed 10% ethanol in their drinking water for 6 weeks*

Inhibitor	With NADP†				With NADPH			
	NABQI bound (nmol/min/mg protein)		Acetaminophen formed (nmol/min/mg protein)		NABQI bound (nmol/min/mg protein)		Acetaminophen formed (pmol/min/mg protein)	
	Control	Ethanol	Control	Ethanol	Control	Ethanol	Control	Ethanol
None	11.6 (100)	11.5 (100)	13.3 (100)	11.9 (100)	4.20 (100)	3.82 (100)	24.6 (100)	24.8 (100)
Imidazole (1 mM)	11.7 (101)	11.8 (102)	9.7 (73)	13.4 (113)	3.85 (92)	3.72 (97)	21.7 (88)	25.3 (102)
80% CO/20% O_2	9.4 (81)	10.7 (93)	16.9 (127)	18.9 (159)	3.15 (75)	3.53 (93)	26.7 (109)	29.0 (117)
Metyrapone (1 mM)	11.5 (99)	11.3 (98)	12.3 (92)	12.6 (106)	3.55 (85)	3.66 (96)	24.2 (98)	24.1 (97)
KCN (1 mM)	11.4 (98)	11.5 (100)	10.3 (91)	13.5 (113)	3.75 (89)	3.67 (96)	23.6 (96)	24.0 (97)
SKF-525A (1 mM)	11.9 (103)	11.3 (98)	11.1 (83)	13.6 (114)	3.55 (85)	3.83 (100)	21.9 (89)	25.2 (102)

* All incubations were run in triplicate with a coefficient of variation of less than 5%. Values in parentheses are the percentage of control.

Table 5. Effects of various metabolic inhibitors on the formation of the cysteine adduct of *N*-acetyl-*p*-benzoquinone imine and the reduction of *N*-acetyl-*p*-benzoquinone imine to acetaminophen by microsomes from control rats and rats chronically fed 10% ethanol in their drinking water for 6 weeks*

	With NADP+				With NADPH			
	Cysteine-acetaminophen (nmol/min/mg protein)		Acetaminophen formed (nmol/min/mg protein)		Cysteine-acetaminophen (nmol/min/mg protein)		Acetaminophen formed (pmol/min/mg protein)	
Inhibitor	Control	Ethanol	Control	Ethanol	Control	Ethanol	Control	Ethanol
None	12.0 (100)	10.9 (100)	13.2 (100)	13.2 (100)	11.1 (100)	11.3 (100)	12.1 (100)	12.5 (100)
Imidazole (1 mM)	11.7 (98)	11.4 (105)	12.2 (92)	12.8 (97)	12.2 (110)	10.6 (94)	13.0 (107)	12.8 (102)
80% CO/20% O_2	8.0 (67)	7.5 (69)	14.6 (111)	15.5 (117)	7.9 (71)	7.5 (66)	14.9 (123)	15.3 (122)
Metyrapone (1 mM)	11.2 (93)	10.9 (100)	12.4 (94)	12.5 (95)	10.6 (95)	11.0 (97)	13.0 (107)	13.0 (104)
KCN (1 mM)	11.5 (95)	11.2 (103)	12.4 (94)	12.5 (95)	11.6 (105)	11.0 (97)	13.2 (109)	12.9 (103)
SKF-525A (1 mM)	11.7 (98)	10.8 (99)	13.0 (98)	12.9 (98)	11.3 (102)	10.6 (94)	13.1 (108)	12.8 (102)

* All incubations were run in triplicate with a coefficient of variation of less than 5%. Values in parentheses are the percentage of control.

of interest that protein sulfhydryls reduced approximately half the NABQI to acetaminophen in the absence of NADPH and this reduction increased to 84% of the total NABQI when NADPH was added (Table 4). On the other hand, NADPH had no effect on the proportion of NABQI reduced in the presence of cysteine (Table 5).

DISCUSSION

Our current studies indicate that the *N*-demethylase activities we have examined could be catalyzed by the same isozymes of cytochrome P450, since the activities were not induced by ethanol feeding and showed the same response to the various inhibitors we have examined. Since these studies were performed in control male rats, these two activities are most likely catalyzed by cytochromes P450g and P450h [23]. On the other hand, the other three activities showed distinct features which suggest that each is primarily catalyzed by a different isozyme. In line with our previous results [2, 9, 10], only aniline hydroxylation and protein binding showed marked increases with chronic ethanol feeding. Hence, one might conclude, as has been shown for other enzyme systems, that these two activities are catalyzed by a previously described ethanol-induced cytochrome P450, cytochrome P450j [7, 8], and that some other isozyme of cytochrome P450 catalyzes the cysteine conjugation. While the inductive effects of ethanol support this model, the sensitivity of the protein binding to KCN and the lack of effect of KCN on aniline hydroxylase would suggest that even these two activities may be catalyzed by two separate isozymes of cytochrome P450.

If there were a single enzymatic pathway catalyzing the production of NABQI, it would be difficult to explain the observation that the rate of formation of the cysteine conjugate is three orders of magnitude greater than the rate of protein binding. There are two possible explanations for this marked difference in rates. First, it is possible that this difference could occur if the proteins bind less than 1% of the NABQI which is formed and if cysteine binding were essentially quantitative. Hence the apparent differences in the rate of binding could be due to differences in the percentage of the NABQI which binds, rather than in its rate of formation. Yet such is not the case, since the percentage of NABQI which bound to the cysteine was only 2- and 3-fold greater than the binding to protein. Second, a more likely explanation for the marked difference in the two metabolic rates is that the cysteine binding is catalyzed both by the enzyme catalyzing the protein binding, as cytochrome P450j, and another isozyme(s) of cytochrome P450 which is more active in this pathway. These data would suggest that this second isozyme is also induced by ethanol, since even though the isozyme catalyzing the protein binding shows a doubling of activity, this increase in activity is too small to account for the increase in the cysteine conjugation. To date no other isozymes of cytochrome P450 have been reported to be induced in rats by ethanol, although Khani *et al.* [24] have reported, on the basis of RNA and DNA studies, that there is a second ethanol-induced isozyme in the

rabbit. They have not as yet reported the isolation of the gene product. Finally, the differences we observed between the protein binding and cysteine conjugation would also suggest that these two cytochromes P450 produce different pools of NABQI. The NABQI in one pool can bind to both protein and nonprotein thiols, while that in the second binds only to nonprotein thiols.

Another concern is that the increase in protein binding, which we observed with ethanol feeding, could be due to either an increase in the rate of formation of NABQI or an increase in the number of binding sites on the protein. It may well be that there are relatively few protein thiols exposed to the medium and that the maximal rate is limited by the concentration of these sites. If such were the case, then the increased protein binding seen after chronic ethanol treatment could be due to increases in the concentration of microsomal, protein thiols exposed to the aqueous phase and not to differences in the rate of formation of NABQI. Our data do not support this concept since neither the protein binding nor the cysteine conjugation of NABQI was affected by ethanol. Hence, the increased protein binding seen after ethanol treatment appears to be due to an increased rate of formation of NABQI rather than changes in the concentration of microsomal thiol groups exposed to the medium.

Irrespective of the reason for the differences between these two assays for the oxidative metabolism of acetaminophen, our results clearly indicate that during any purification procedure, it will be necessary to follow both activities to determine whether the two are catalyzed by the same enzyme system.

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