

COMPARISON OF SIX RABBIT LIVER CYTOCHROME P-450 ISOZYMES  
IN FORMATION OF A REACTIVE METABOLITE OF ACETAMINOPHEN

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**Summary:** This laboratory has recently reported the isolation of an ethanol-inducible form of rabbit liver microsomal cytochrome P-450, designated isozyme 3a. 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, all of which are present in significant amounts in the liver microsomes from rabbits chronically administered 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<sub>5</sub>. The results obtained thus indicate that induction by ethanol of P-450 isozyme 3a (or a homologous enzyme in other species) may contribute to the toxicity of acetaminophen but that other cytochromes also play a significant role.

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Acetaminophen (Tylenol; *p*-hydroxyacetanilide), a widely used antipyretic and analgesic drug, is normally nontoxic but in large doses produces acute hepatic necrosis (1-3). 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*-benzoquinoneimine (4-6). This intermediate is conjugated with glutathione (GSH)<sup>1</sup> and excreted under normal conditions, but covalent binding to proteins occurs when cellular GSH is depleted (7,8).

Pretreatment of animals with compounds which alter the mixed function oxidase system has a profound effect on the metabolism of acetaminophen (3,4, 7-9). Increased hepatotoxicity of acetaminophen has been reported in human alcoholics (1,10-13) and in ethanol-treated rats and mice (14,15) with a concomitant increase in the hepatic metabolism of the drug and covalent bind-

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<sup>1</sup>The abbreviations used are: P-450<sub>LM</sub>, liver microsomal cytochrome P-450; GSH, reduced glutathione; GPC, glyceryl-3-phosphorylcholine, and HPLC, high performance liquid chromatography. The isozymes of P-450<sub>LM</sub> are numbered according to their relative electrophoretic mobilities in accord with the recommendation of the Commission on Biochemical Nomenclature.

ing of the metabolites to microsomal proteins (1,14,15). We have recently reported the purification and characterization of a unique form of P-450 from ethanol-treated rabbits, designated as isozyme 3a on the basis of its electrophoretic behavior (16,17). Evidence is given in the present paper that this cytochrome catalyzes the metabolism of acetaminophen in the reconstituted system but that other isozymes of rabbit P-450<sub>LM</sub> also contribute to varying extents to the activation of this drug.

#### MATERIALS AND METHODS

The isozymes of P-450<sub>LM</sub> were purified to electrophoretic homogeneity by procedures previously described (16,18,19). Isozyme 6 was isolated from microsomes of ethanol-treated rabbits according to the general procedure described for isozymes 3a, 3b, and 3c (16,19). The specific contents of the preparations of isozymes 2 (induced by phenobarbital), 3a (induced by ethanol), 3b, 3c, 4 (induced by isosafrole), and 6 were 16.4, 15.0, 17.6, 16.5, 18.1, and 14.0 nmol of P-450 per mg of protein, respectively. Rabbit NADPH-cytochrome P-450 reductase was purified as described by French and Coon (20) and cytochrome b<sub>5</sub> was purified to a specific content of 59 nmol of heme per mg protein essentially by the method of Strittmatter *et al.* (21).

The P-450-dependent formation of glutathione-acetaminophen conjugates was determined by HPLC as described by Moldèus (8) using an IBM LC/9533 liquid chromatograph equipped with an IBM LC/9523 variable UV detector, an IBM LC/9540 chromatography Data Integrator, and an IBM octadecyl column (4.2 mm  $\times$  250 mm). The glutathione conjugate was quantified using acetaminophen as a standard since the reported extinctions of the conjugate and acetaminophen are essentially the same (8). In some experiments [<sup>3</sup>H(G)]acetaminophen (New England Nuclear) or L-[glycine-2-<sup>3</sup>H]glutathione (reduced form, New England Nuclear) were utilized as described in the figure legend. Glutathione (reduced form) was obtained from Sigma and acetaminophen from Aldrich. Other materials were obtained as described previously (16).

#### RESULTS AND DISCUSSION

HPLC analysis of incubation mixtures which contained purified isozymes of P-450, phosphatidylcholine, NADPH-cytochrome P-450 reductase, glutathione, acetaminophen, and NADPH revealed the time-dependent appearance of a single metabolite peak absorbing at 250 nm and having a retention time of 15.5 min. A representative profile is shown in Fig. 1. The metabolite peak corresponds well with the glutathione-conjugate metabolite with a retention time of 15 to 16 min observed by Moldèus (8) after incubation of acetaminophen with isolated hepatocytes or hepatic microsomes in the presence of reduced glutathione. In experiments not shown, it was established that the concentration of GSH used in these studies (0.5 mM) did not significantly inhibit the activities of the isozymes with other substrates. Higher concentrations of GSH inhibited some reactions, however. The formation of the GSH-acetaminophen conjugate displayed an absolute requirement for P-450, the reductase, and NADPH, and was linear with respect to time up to 10 min at 30 °C.

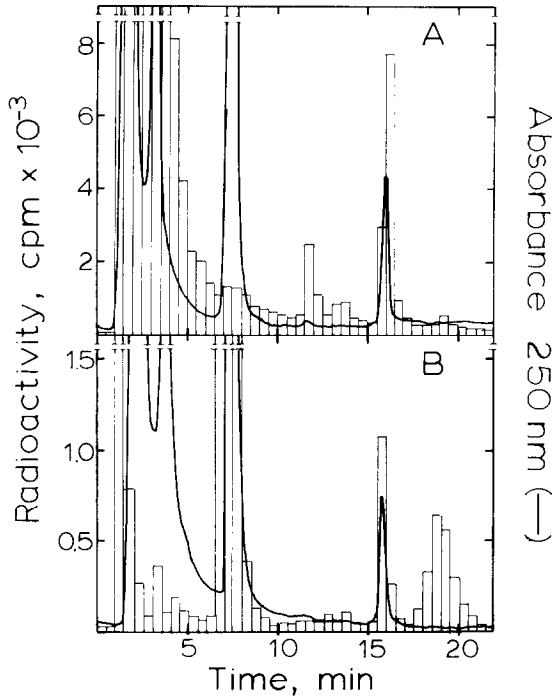


Fig. 1. Separation of substrate and products by HPLC. Reaction mixtures contained 0.2  $\mu$ M P-450 isozyme 4, 0.6  $\mu$ M reductase, 30  $\mu$ g of di-lauroyl-GPC, 2.0 mM acetaminophen, 0.5 mM GSH, 1.0 mM NADPH, and 50 mM potassium phosphate buffer, pH 7.6, in a final volume of 1.0 ml. Radioactive acetaminophen or GSH was included in the incubations as follows: A, 5  $\mu$ Ci of L-[glycine-2-<sup>3</sup>H]GSH; or B, 20  $\mu$ Ci of [<sup>3</sup>H(G)]acetaminophen. After incubation of the mixtures at 30 °C for 60 min, the reactions were quenched with 0.5 ml of ice-cold 3.0 N perchloric acid. A 0.1-ml aliquot was analyzed by HPLC as described by Mold us (8). The absorbance of the column eluate was monitored continuously at 250 nm, and is shown in this figure on an arbitrary scale. In addition, fractions were collected every 30 s and examined for radioactivity. [<sup>3</sup>H(G)]acetaminophen was purified by thin layer chromatography with ethyl acetate as the solvent immediately before use.

In order to confirm that the metabolite peak observed at 15.5 min in the HPLC assay was the GSH conjugate of acetaminophen, experiments were run in which unlabeled GSH or acetaminophen was replaced by the radioactive compound. As shown in Fig. 1, when radioactive GSH (Expt. A) or acetaminophen (Expt. B) was present the product peak absorbing at 250 nm with a retention time of about 15.5 min contained the radioactive label. These results confirm the presence of both GSH and acetaminophen in the metabolite peak. Other radioactive peaks are seen in the chromatograms shown in Fig. 1A and B, but, since they did not contain the label from both GSH and acetaminophen and lacked absorption at 250 nm, no attempt was made to identify these compounds.

The most active cytochrome in the generation of the GSH-acetaminophen conjugate in the reconstituted system was isozyme 4 (Table I). Isozymes 3a

TABLE I  
Activities of P-450 Isozymes in the Formation  
of GSH-Acetaminophen Conjugate

System	Activity of isozyme					
	(nmol 2	GSH conjugate 3a	formed/min/nmol 3b	P-450) 3c	4	6
Complete	<0.04	2.16	0.14	0.22	3.67	1.44
Complete + cytochrome <u>b<sub>5</sub></u>	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

The complete system contained 0.2  $\mu$ M P-450<sub>LM</sub>, 0.6  $\mu$ M NADPH-cytochrome P-450 reductase, 30  $\mu$ g of dilauroyl-GPC, 0.5 mM GSH, 12 mM acetaminophen, 50 mM potassium phosphate buffer, pH 7.6, and 1 mM NADPH in a final volume of 1.0 ml. When present, cytochrome b<sub>5</sub> or Tris-chloride buffer, pH 7.6, were at concentrations of 0.2  $\mu$ M 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 Moldèu (8). Values less than 0.04 are below the limits of accurate determination under the conditions of this assay.

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<sub>5</sub> was tested in view of the previous finding that it is required for prostaglandin hydroxylation by isozyme 2 (22) and the report of Miki *et al.* (23) that P-450<sub>LM</sub>, an isozyme apparently identical to isozyme 3c (19), requires cytochrome b<sub>5</sub> for the metabolism of some substrates. In the presence of cytochrome b<sub>5</sub>, 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<sub>5</sub>. The activities 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<sub>5</sub> 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.

The formation of the GSH-acetaminophen conjugate displayed saturable kinetics when the concentration of acetaminophen was varied in incubations with isozymes 3a, 4, or 6. Double reciprocal plots of the initial rates were linear over the 50-fold substrate concentration range examined. The apparent Michaelis constants of acetaminophen and the maximal velocities for the three cytochromes are given in Table II. From the results presented it is evident that the relative participation of each isozyme in acetaminophen

TABLE II  
Kinetic Parameters of P-450-Catalyzed  
Metabolism of Acetaminophen

P-450 isozyme	$K_m$ (mM)	$V_{max}$ (nmol/min/nmol P-450)
3a	5.9	3.42
4	1.3	3.92
6	0.7	1.65

Reaction mixtures were the same as described for the complete system in Table I, except the concentration of acetaminophen was varied from 0.1 to 5 mM. The apparent  $K_m$  and  $V_{max}$  values were obtained by extrapolation of double reciprocal plots of the initial rate data.

activation in the hepatic endoplasmic reticulum is dependent not only on the level of that cytochrome but also on the concentration of the drug.

The metabolic profile of liver microsomes would be expected to be a composite of the activities of the individual isozymes of P-450 present. The results presented indicate that the increase in acetaminophen toxicity upon chronic exposure to ethanol may be accounted for, at least in part, by the induction of a specific form of liver cytochrome P-450 (identified as isozyme 3a in the rabbit). The possibility should be considered that isozymes 4 and 6, which are also quite effective in acetaminophen activation in the reconstituted system and known to be at increased levels in the hepatic endoplasmic reticulum following the administration of a variety of foreign compounds, may also be induced to some extent by ethanol. Preliminary experiments have shown an increase in isozyme 6 following chronic alcohol administration, but an increase in isozyme 4 was not apparent under the conditions employed.

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#### REFERENCES

1. Proudfoot, A. T., and Wright, N. (1970) *Brit. Med. J.* 3, 557-558.
2. Prescott, L. F., Wright, N., Roscoe, P., and Brown, S. S. (1971) *Lancet* 1, 519-522.
3. Mitchell, J. R., Jollaw, D. J., Potter, W. Z., David, D. C., Gillette, J. R., and Brodie, B. B. (1973) *J. Pharmacol. Exp. Ther.* 187, 185-194.
4. Potter, W. Z., Davis, D. C., Mitchell, J. R., Jollaw, D. J., Gillette, J. R., and Brodie, B. B. (1973) *J. Pharmacol. Exp. Ther.* 187, 203-210.
5. Corcoran, G. B., Mitchell, J. R., Vaishav, Y. N., and Horning, E. C. (1980) *Mol. Pharmacol.* 18, 536-542.

6. Nelson, S. D., Forte, A. J., and Dahlin, D. C. (1980) *Biochem. Pharmacol.* 29, 1617-1620.
7. Mitchell, J. R., Jollaw, D. J., Potter, W. Z., Gillette, J. R., and Brodie, B. B. (1973) *J. Pharmacol. Exp. Ther.* 187, 211-217.
8. Moldéus, P. (1978) *Biochem. Pharmacol.* 27, 2859-2863.
9. Jollaw, D. J., Mitchell, J. R., Potter, W. Z., Davis, D. C., Gillette, J. R., and Brodie, B. B. (1973) *J. Pharmacol. Exp. Ther.* 187, 195-202.
10. Wright, N., and Prescott, L. F. (1973) *Scot. Med. J.* 18, 56-58.
11. Emby, D. J., and Fraser, B. N. (1977) *S. Afr. Med. J.* 51, 208-209.
12. Goldfinger, R., Ahmed, K. S., Pitchumani, C. S., and Wesely, S. A. (1978) *Am. J. Gastroenterol.* 70, 385-388.
13. McClain, C. J., Kromhout, J. P., Peterson, F. J., and Holtzman, J. L. (1980) *J. Amer. Med. Assoc.* 244, 251-253.
14. Peterson, F. J., Holloway, D. E., Erickson, R. R., Duquette, P. H., McClain, C. J., and Holtzman, J. L. (1980) *Life Sci.* 27, 1705-1711.
15. Sato, C., Matsuda, Y., and Lieber, C. S. (1981) *Gastroenterology* 80, 140-148.
16. Koop, D. R., Morgan, E. T., Tarr, G. E., and Coon, M. J. (1982) *J. Biol. Chem.* 257, 8472-8480.
17. Morgan, E. T., Koop, D. R., and Coon, M. J. (1982) *J. Biol. Chem.* 257, 13951-13957.
18. Coon, M. J., van der Hoeven, T. A., Dahl, S. B., and Haugen, D. A. (1978) *Methods Enzymol.* 52, 109-117.
19. Koop, D. R., Persson, A. V., and Coon, M. J. (1981) *J. Biol. Chem.* 256, 10704-10709.
20. French, J. S., and Coon, M. J. (1979) *Arch. Biochem. Biophys.* 195, 565-577.
21. Strittmatter, P., Fleming, P., Connors, M., and Corcoran, D. (1978) *Methods Enzymol.* 52, 97-101.
22. Vatsis, K. P., Theoharides, A. D., Kupfer, D., and Coon, M. J. (1982) *J. Biol. Chem.* 257, 11221-11229.
23. Miki, N., Sugiyama, T., and Yamano, T. (1980) *J. Biochem. (Tokyo)* 88, 307-316.