

The Effect of Ethanol on Drug Oxidations *In Vitro* and the Significance of Ethanol-Cytochrome P-450 Interaction

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The effect of ethanol on *N*-demethylation of aminopyrine in rat liver slices and in the microsomal fraction and on microsomal hydroxylation of pentobarbital and aniline was studied. With liver slices *N*-demethylation of aminopyrine was stimulated by 35-40% at low ethanol concentrations (2mM), whereas no stimulation occurred at high concentrations (100mM). With the liver microsomal fraction, an inhibitory effect was observed only at high ethanol concentrations (100mM). This was also observed with the other drugs studied. In agreement with these results, only at a high concentration did ethanol interfere with the binding of drug substrates to cytochrome P-450. Further, as previously reported, ethanol produced a reverse type I spectral change when added to the liver microsomal fraction. Evidence that this spectral change is due to removal of substrate, endogenously bound to cytochrome P-450, is reported. A dual effect of ethanol is assumed to explain the present findings; in liver slices, at a low ethanol concentration, the enhanced rate of drug oxidation is the result of an increased NADH concentration, whereas the inhibitory effect observed with the microsomal fraction at high ethanol concentration is due to the interference by ethanol with the binding of drug substrates to cytochrome P-450.

Orme-Johnson & Ziegler (1965) reported the oxidation of both methanol and ethanol *in vitro* by hepatic microsomal fraction in the presence of NADPH and molecular O₂. The oxidation of ethanol to acetaldehyde by the microsomal fraction was confirmed and extended by Lieber & DeCarli (1968) who, in contrast to Orme-Johnson & Ziegler (1965), observed inhibition (60-70%) of microsomal ethanol metabolism by CO. The requirement for NADPH and O₂ and the sensitivity to CO are also characteristic of the microsomal cytochrome P-450-containing mono-oxygenase system. Further, the experimental administration of ethanol to human volunteers, both alcoholics and non-alcoholics, and to male rats resulted in a proliferation of the smooth endoplasmic reticulum of the hepatocytes (Iseri *et al.*, 1966; Lane & Lieber, 1966), a finding similar to that produced by many drugs (see Conney, 1967, for review). These observations led Lieber & DeCarli (1968) to postulate the presence of an ethanol-oxidizing system in the microsomal fraction which is distinct from hepatic alcohol dehydrogenase, an enzyme present in the soluble fraction (see Jacobsen, 1952, for review).

Rubin *et al.* (1970) reported that microsomal ethanol-oxidizing-system activity is significantly inhibited by drug substrates of the mono-oxygenase system and in addition that ethanol inhibits the hydroxylation of pentobarbital and aniline *in vitro* by the

microsomal fraction; with aniline hydroxylation, the inhibition was shown to be competitive (Rubin & Lieber, 1968). These findings suggested that the microsomal metabolism of various drugs and ethanol involves common enzyme components. Further, the addition of high concentrations of ethanol to a microsomal suspension induced a reverse type I (modified type II) spectral change (Schenkman *et al.*, 1967), suggesting an interaction between cytochrome P-450 and ethanol (Rubin *et al.*, 1971).

However, based on other investigations, not only the role of the microsomal ethanol-oxidizing system *in vivo* but also its actual existence have been questioned. For example, inhibitors and inducers of the hepatic microsomal mono-oxygenase system did not affect metabolism of ethanol *in vivo* (Klaassen, 1969; Tephly *et al.*, 1969; Khanna & Kalant, 1970; Khanna *et al.*, 1972). Moreover, Hassinen & Ylikahri (1972), using isolated perfused livers from normal and phenobarbital-treated rats, reported that addition of ethanol to the perfusion medium did not cause any redox changes of cytochrome P-450, concluding that the role of the microsomal ethanol-oxidizing system in ethanol oxidation in intact liver is insignificant. Aminotriazole, an inhibitor of the biosynthesis of catalase (Tschudy & Collins, 1959) was found to decrease the microsomal production of acetaldehyde from ethanol when added *in vitro* or when injected *in vivo* (Roach *et al.*, 1969; Isselbacher & Carter, 1970), implicating catalase in the oxidation of ethanol. Thurman *et al.* (1972) presented strong

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evidence that the microsomal ethanol-oxidizing system is due to contaminating catalase plus H_2O_2 generated by microsomal NADPH oxidase in the presence of NADPH and O_2 (Gillette *et al.*, 1957). If the microsomal ethanol-oxidizing system is indeed a non-entity as suggested by the results of Thurman *et al.* (1972), then what is the explanation for the inhibition of microsomal drug metabolism by ethanol? Secondly, what is the significance of the reverse type I spectral change observed on addition of ethanol to the liver microsomal fraction? Is this spectral change due to a binding of ethanol to cytochrome *P*-450? The aim of the present study is directed toward answering these questions, in the hope of elucidating the role of ethanol in drug metabolism.

Materials and Methods

NADP⁺, NADPH, NADH, trisodium isocitrate and isocitrate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); aminopyrine was obtained from Matheson, Coleman and Bell (Norwood, Ohio, U.S.A.); hexobarbital (sodium salt) was obtained from Bayer A.G. (Leverkusen, Germany); acetaldehyde was purchased from BDH Chemicals Ltd. (Poole, Dorset, U.K.); 4-methylpyrazole was obtained as the hydrochloride through the courtesy of Dr. B. Sjöberg, Astra AB (Södertälje, Sweden). Pentobarbital was generously provided by May and Baker Ltd. (Dagenham, Essex, U.K.), as was 5-allyl-5-isopropylbarbituric acid (aprobarbital) by ACO Läkemedels AB (Solna, Sweden). All other chemicals and reagents were purchased from local commercial sources and were used without further purification.

Untreated male Sprague-Dawley rats (250–300 g) were obtained commercially from Eklund's Animal farm (Täby, Sweden), and fed on a standard laboratory diet. Livers were removed and treated as described previously (Cinti & Schenkman, 1972). The slices (approx. 0.5 mm thick) were prepared by using a Stadie-Riggs microtome. Livers were homogenized in 5 vol. of cold 0.25 M-sucrose with a glass-Teflon homogenizer and microsomal fractions were prepared either by differential ultracentrifugation as described by Ernster *et al.* (1962) or by the Ca^{2+} sedimentation procedure as described by Schenkman & Cinti (1972). Identical results were obtained with either method of preparation of the microsomal fraction. Protein concentrations were determined by the method of Lowry *et al.* (1951).

The rate of demethylation of aminopyrine in the presence and absence of 4-methylpyrazole and ethanol was measured in a system containing 50 mM-Tris-HCl buffer, pH 7.5, 5 mM-MgCl₂, 1 mM-NADP⁺, 5 mM-DL-isocitrate and 0.4 unit of pig heart isocitrate dehydrogenase, in a final volume of 2 ml. When pre-

sent, the final concentration of 4-methylpyrazole was 2 mM. The concentrations of aminopyrine and ethanol used varied with each experiment and are indicated in the figure legends or the tables. The reaction was started by addition of either microsomal fraction (1.0 mg of protein/ml) or liver slices (200–250 mg). Incubations were performed in a Dubnoff shaker for 10 min at 37°C in an atmosphere of air. Formaldehyde formed was measured by the procedure of Nash (1953).

The hydroxylation of aniline was measured with the same incubation medium used for aminopyrine *N*-demethylation. The reaction was started with 1.5 mg of microsomal protein/ml. The incubation time in the Dubnoff shaker was 20 min at 37°C in air. Aniline hydroxylase activity was determined by measuring the formation of *p*-aminophenol (Schenkman *et al.*, 1967).

For the determination of pentobarbital metabolism, incubations were performed as for aniline, but with 0.1 mM-sodium pentobarbital and 1.5 mg of microsomal protein per incubation mixture. At the end of 20 min, the reaction was stopped by freezing the tubes in a solid CO₂-ethanol mixture; 0.5 g of NaCl and known concentrations of aprobarbital were then added to each tube and the mixture was extracted with 10 ml of chloroform by shaking for 5 min. After centrifugation to separate the phases, the chloroform phase was evaporated and the residue dissolved in 0.5–1 ml of chloroform, of which portions (0.5–1 μl) were injected into a gas chromatograph. G.l.c. was performed with a Varian 1440 gas chromatograph equipped with a H₂ flame ionization detector. Flash heater and detector were kept at 240°C and column oven at 210°C. N₂ at 30 ml/min was used as the carrier gas. The silicone-treated coiled glass columns (2 m × 2 mm internal diam.) were packed with 3% Hi-EFF-3A (neopentyl glycol adipate) on Gas-chrom Q. The peak areas were measured by cutting and weighing the chromatograms, and pentobarbital concentrations were calculated by using aprobarbital as an internal standard.

Cytochrome *P*-450 was measured with an Aminco-Chance split-beam dual-wavelength recording spectrophotometer as described by Omura & Sato (1964) by using the extinction coefficient of 91 mm⁻¹·cm⁻¹. Spectral changes produced by addition of substances to suspensions of liver microsomal fractions were also recorded with the same spectrophotometer. Spectral dissociation constants (K_s) were obtained from double-reciprocal plots (Schenkman, 1970).

Washing of the microsomal fraction was performed as follows. Microsomal fractions were resuspended in 0.15 M-KCl to a final concentration of 10–15 mg of protein/ml; a sample was removed for measurement of cytochrome *P*-450 and for titration of the spectral changes with hexobarbital and ethanol. To the remaining microsomal suspension was added 0.15 M-

KCl containing 100mM-ethanol (final concn.). The mixture was gently stirred for 2 min at 4°C, followed by centrifugation at 105000g for 45 min (Ca²⁺-sedimented microsomal fractions were centrifuged at 20000g for 10 min); the pellets were then washed three times with 10 vol. of 0.15M-KCl to remove the ethanol, resuspended in 0.15M-KCl to a final protein concentration of 10–15 mg/ml and a sample was taken for cytochrome *P*-450 measurement and titration of hexobarbital- and ethanol-produced spectral changes (washed fraction 1). The remaining microsomal suspension was washed again as described above, yielding washed fraction 2. The control microsomal fractions were also washed twice with 0.15M-KCl containing no ethanol.

Kinetic plots were determined by linear regression analyses; all correlation coefficients were greater than 0.9.

Results

Effect of ethanol on N-demethylation of aminopyrine in liver slices and microsomal fractions

In the light of the discrepancies mentioned in the introduction we studied the effect of various concentrations of ethanol on the *N*-demethylation of aminopyrine in both liver slices and the microsomal fraction.

As shown in Table 1, 2mM- and 20mM-ethanol increased the rate of *N*-demethylation of aminopyrine in rat liver slices by about 35–40%. However, at a much higher ethanol concentration the stimulation was completely abolished (503 compared with 493 nmol of formaldehyde/10 min per g of liver), suggesting a dual or more complex effect of ethanol. To determine whether the observed increase in oxidation of aminopyrine was due, at least in part, to an increased concentration of NADH resulting from the metabolism of ethanol, 2mM-4-methylpyrazole, a potent inhibitor of alcohol dehydrogenase (Theorell *et al.*, 1969), was added to the incubation medium (Table 2). The presence of 4-methylpyrazole alone did not affect the rate of *N*-demethylation of aminopyrine (493 compared with 498 nmol of formaldehyde/10 min per g of liver). However, in the presence of 2mM-ethanol the 35–40% increase in demethylation was diminished by 50% (669 compared with 595 nmol of formaldehyde/10 min per g of liver), although the rate was still significantly higher than that observed in the controls. At higher concentrations of ethanol, the observed increase was removed, and in fact a 15–20% decrease in the rate of *N*-demethylation was often observed. It thus appears that at least two ethanol effects exist: (1) the oxidation of ethanol by alcohol dehydrogenase resulting in an increased NADH/

Table 1. *Rate of aminopyrine N-demethylation in liver slices in the presence of various concentrations of ethanol*

The concentration of aminopyrine in the incubation medium was 8.0mM; the reaction was initiated by the addition of the liver slices. Values for demethylation are means \pm s.e.m. with number of observations in parentheses, and *P* values are a comparison between the presence and absence of ethanol. N.S., not significant.

Additions	Aminopyrine <i>N</i> -demethylation (nmol of formaldehyde/10 min per g of liver)	Change (%)	<i>P</i>
Control	493 \pm 29 (29)	—	—
Ethanol (2mM)	669 \pm 90 (10)	36	<0.025
Ethanol (20mM)	664 \pm 72 (10)	35	<0.025
Ethanol (200mM)	503 \pm 66 (10)	2	N.S.

Table 2. *Rate of aminopyrine N-demethylation in liver slices in the presence of 2.0mM-4-methylpyrazole and various concentrations of ethanol*

Methods are as described in Table 1. Values for demethylation are means \pm s.e.m. with number of observations in parentheses, and *P* values are a comparison between the presence and absence of ethanol. N.S., not significant.

Additions	Aminopyrine <i>N</i> -demethylation (nmol of formaldehyde/10 min per g of liver)	Change (%)	<i>P</i>
Control (+ 4-methylpyrazole)	498 \pm 39 (29)	—	—
Ethanol (2mM)	595 \pm 62 (10)	20	<0.05
Ethanol (20mM)	419 \pm 50 (10)	16	N.S.
Ethanol (200mM)	422 \pm 53 (10)	15	N.S.

NAD⁺ ratio, which could conceivably support drug metabolism, and (2) an inhibitory effect by high ethanol concentrations on the microsomal mono-oxygenase system, directly or indirectly via a non-specific effect on the membrane.

In contrast to the oxidation rate in liver slices, microsomal *N*-demethylation of aminopyrine was not affected by ethanol when the concentrations in the incubation medium ranged between 1 and 50 mM; an inhibitory effect, possibly competitive, was observed, however, with 100 mM-ethanol (Fig. 1). The lack of stimulation of aminopyrine *N*-demethylation by the microsomal fraction in the presence of low concentrations of ethanol was not unexpected, however, since microsomal oxidation of ethanol, if indeed any occurred, would not result in the generation of NADH.

To be certain that our observations were real and not artifacts attributed to interference by acetaldehyde, which is a product of ethanol oxidation, increasing concentrations of acetaldehyde ranging

from 10 μ M to 10 mM were added to the incubation medium during analysis of aminopyrine demethylation in both liver slices and microsomal fractions. The amount of formaldehyde formed remained constant in the presence of increasing concentrations of acetaldehyde.

Effect of ethanol on pentobarbital metabolism by rat liver microsomal fraction

Contrary to a previous report (Rubin & Lieber, 1968), pentobarbital hydroxylation by the microsomal fraction was not significantly affected by ethanol concentrations in the range 2–50 mM (Fig. 2); at 100 mM-ethanol, however, there was a decrease, an effect similar to that observed with aminopyrine.

Effect of ethanol on aniline hydroxylation by rat liver microsomal fraction

Rubin *et al.* (1970) reported that in the presence of 3 mM- and 8 mM-ethanol, microsomal aniline hydroxy-

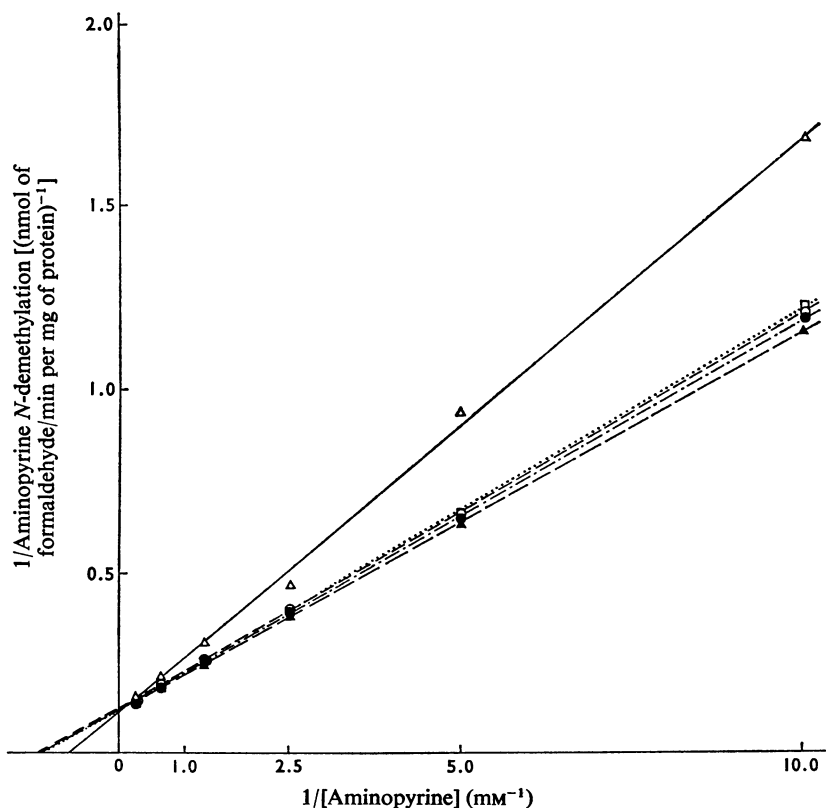


Fig. 1. Kinetic plots of the effect of ethanol on the *N*-demethylation of aminopyrine by rat liver microsomal fraction

The mixtures containing 1 mg of microsomal protein/ml were incubated for 10 min at 37°C in air. \blacktriangle , No ethanol added; \bullet , 1.0 mM-ethanol; \circ , 10 mM-ethanol; \square , 50 mM-ethanol; \triangle , 100 mM-ethanol.

lase activity was competitively inhibited. Using similar conditions, we observed no effect with 3mM-

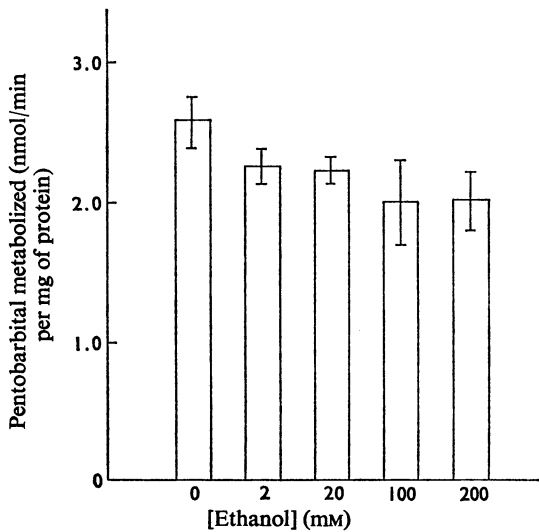


Fig. 2. Effect of ethanol on the microsomal hydroxylation of pentobarbital

The microsomal protein concentration in the incubation mixture was 0.75mg/ml. Incubations were for 20min at 37°C in air; rate of pentobarbital disappearance was determined by g.l.c. Bars indicate means and variations of three experiments.

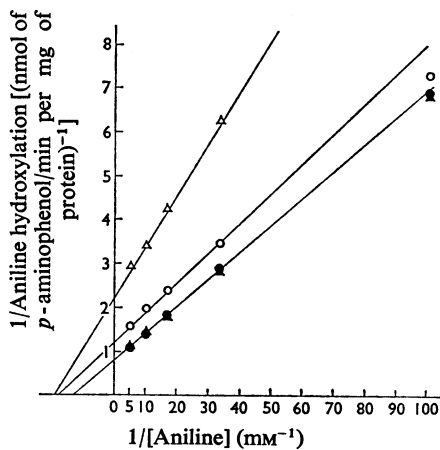


Fig. 3. Lineweaver-Burk plots of the effects of ethanol on microsomal hydroxylation of aniline

The incubation mixture contained 1.5mg of microsomal protein/ml and the reaction was run for 15min at 37°C in air. ▲, No ethanol added; ●, 3mM-ethanol; ○, 8mM-ethanol; △, 100mM-ethanol.

ethanol, but with 8mM- and 100mM-ethanol a complex mixed-type inhibition was seen, in that both the K_m and V_{max} were altered (Fig. 3).

Effect of ethanol on the binding of hexobarbital to rat liver microsomal fraction

It is well known that the addition of hexobarbital to a microsomal suspension results in a type I spectral change, the magnitude of which is proportional to the amount of haemoprotein available for interaction with the substrate (Schenkman *et al.*, 1967). As shown in Fig. 4, the presence of ethanol in both cuvettes caused a decrease in the magnitude of the hexobarbital-induced type I spectral change. The spectral dissociation constant (K_s) tended to increase (from 0.11 to 0.15mM), suggesting a competitive type of inhibition, but only in the presence of high concentrations of ethanol.

In the presence of hexobarbital in the sample cuvette containing a microsomal suspension, addition of 100mM-ethanol to the reference cuvette resulted in a greater hexobarbital-induced type I spectral change (Fig. 5, curves A and B). When ethanol was present in

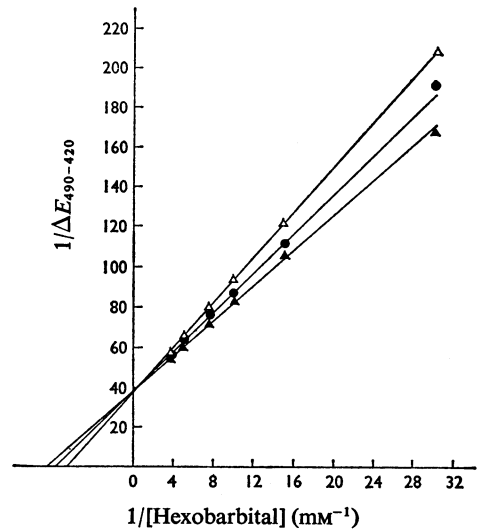


Fig. 4. Lineweaver-Burk plots of hexobarbital binding to rat liver microsomal fraction in the presence of ethanol

A microsomal suspension (6ml) containing 0.05M-Tris-HCl buffer, pH 7.5, and 2mg of protein/ml was divided equally between two cuvettes. Increasing concentrations of hexobarbital were added to the sample cuvette and corresponding amounts of buffer to the reference. ▲, No ethanol added; ●, 100mM- and △, 200mM-ethanol present in both cuvettes.

both cuvettes, the type I spectral change was decreased (Fig. 4, and Fig. 5, curve C). Addition of ethanol to a microsomal suspension resulted in a reverse type I spectral change (Fig. 5, curve D) as reported by Rubin *et al.* (1971). Further, adding hexobarbital to both cuvettes increased the magnitude of the ethanol-induced reverse type I spectral change (Fig. 5, curve E). These effects can be explained in two ways. (1) Ethanol physically binds to the apoprotein of cytochrome *P*-450 and in doing so produces the reverse type I spectral change. This would explain the increase in the magnitude of the hexobarbital-induced type I spectral change on addition of ethanol to the reference cuvette, which would be due to an algebraic summation of the type I spectral change in the sample cuvette and the reverse type I spectral change in the reference cuvette (curve B). The decrease in the hexobarbital-produced type I spectral change when ethanol was present in both cuvettes (curve C) and the increase in the ethanol-induced reverse type I spectral change when hexobarbital was present in both cuvettes (curve E) could in turn be due to a type I component of ethanol binding (cf. Schenkman, 1970). (2) Ethanol does not bind to cytochrome *P*-450 but induces a reverse type I spectral change by its ability to remove endogenously bound substrate from cytochrome *P*-450; this would adequately explain curves B, C, D and E in Fig. 5.

Effect of ethanol washing of the microsomal fraction on the hexobarbital- and ethanol-induced spectral changes

To separate these two apparently valid explanations of the ethanol effect, microsomal fractions were washed with 100mM-ethanol, based on the belief that if indeed ethanol was removing endogenously bound substrate, washing with ethanol would release substrate from cytochrome *P*-450, making more haemoprotein available for interaction with hexobarbital; in addition the magnitude of the ethanol-induced reverse type I spectral change would be decreased. If, however, ethanol did bind to cytochrome *P*-450, then washing of microsomal fraction would not affect either the type I or reverse type I spectral change. As shown in Fig. 6(a) microsomal fraction washed twice with 100mM-ethanol (see the Materials and Methods section) produced a greater type I spectral change on addition of increasing concentrations of hexobarbital; further, the reverse type I spectral change induced by ethanol was diminished (Fig. 6b). To substantiate the ethanol effect, microsomal fractions were washed twice with 0.15M-KCl only; these washed microsomal fractions produced hexobarbital- and ethanol-induced spectral changes identical with those observed with control microsomal fraction. Moreover, to ensure that washed microsomal fractions did not contain residual

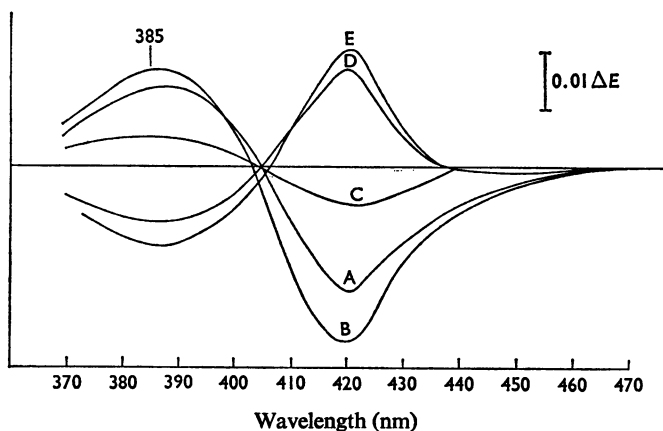


Fig. 5. Optical difference spectra of substrate binding to rat liver microsomal fraction

A microsomal suspension containing 1.6 mg of protein/ml in 0.05M-Tris-HCl buffer, pH 7.5, was divided equally between two cuvettes. Curve A, 0.33mM-hexobarbital (final concn.) was added to sample cuvette and a corresponding volume of buffer was added to the reference cuvette; curve B, 100mM-ethanol was added to the reference cuvette (18 μ l of ethanol); curve C, new microsomal suspension also containing 1.6 mg of protein/ml and 240mM-ethanol was divided between two cuvettes and 0.33mM-hexobarbital was added to the sample cuvette; curve D, addition of 250mM-ethanol to the sample cuvette containing 2.0 mg of microsomal protein/ml, followed by addition of 0.5mM-hexobarbital to both cuvettes (curve E). For further details see the text.

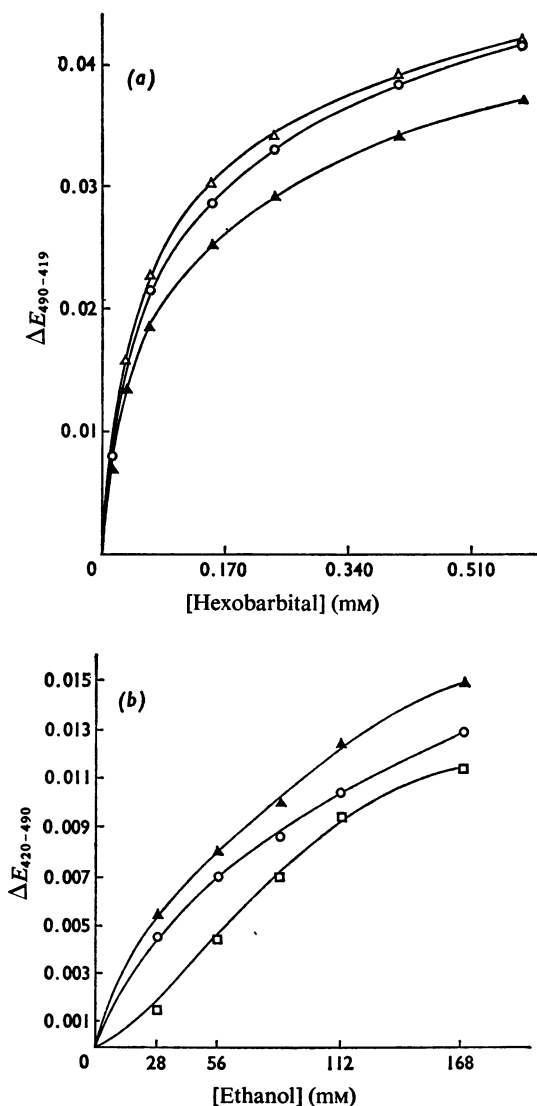


Fig. 6. Effect of washing of the microsomal fraction with 100 mM-ethanol on the spectral changes produced by hexobarbital and ethanol

(a) Hexobarbital-induced spectral change: ▲, control; ○, first wash; △, second wash. (b) Ethanol-induced spectral change: ▲, control; ○, first wash; □, second wash. See the Materials and Methods section for details.

amounts of ethanol, the pellets were rinsed three times with 0.15M-KCl. In addition, had ethanol remained bound to the microsomal fraction after washing, then the magnitude of the hexobarbital type I spectral change would have been even lower than

that obtained with the control microsomal fraction (see Fig. 5, curve C).

Discussion

Whereas the role of the microsomal cytochrome-P-450-containing mono-oxygenase system in liver has recently been questioned with regard to its involvement in the oxidation of ethanol both *in vivo* and *in vitro*, there is general agreement that various drug oxidations are affected by high concentrations of ethanol. Based on our observations with liver slices and microsomal fractions, we propose at least two diametrically opposed effects of ethanol; at low concentrations of ethanol, drug oxidation is stimulated, possibly by an increase in the cytoplasmic NADH/NAD⁺ ratio resulting from alcohol dehydrogenase-dependent oxidation of ethanol; Rawat (1972) has shown that rat liver slices utilize more than 40 μ mol of ethanol/h per g wet wt. of liver, which results in the production of a stoichiometrically equivalent amount of NADH. Since Cohen & Estabrook (1971), Hildebrandt & Estabrook (1971) and Cinti *et al.* (1972) have shown that drug oxidations are enhanced in the presence of both NADPH and NADH, the increased NADH/NAD⁺ ratio would explain such a stimulatory effect of ethanol in liver slices, where the concentration of reduced nicotinamide nucleotides may be more critical for optimum drug oxidation rates than *in vivo*. That the stimulatory effect of ethanol was in fact due to NADH production is strongly suggested by our experiments with 4-methylpyrazole. Moreover, this effect was not obtained with microsomal fractions, which was expected since alcohol dehydrogenase is present only in the soluble fractions.

At high ethanol concentrations (100 mM or greater), however, an inhibitory effect on drug oxidation was observed. This inhibition has been attributed to a direct effect by ethanol on the mono-oxygenase system by interacting with the terminal oxidase, cytochrome P-450 (Imai & Sato, 1967; Rubin *et al.*, 1971). Evidence for this assertion has been based on (a) inhibition of microsomal ethanol-oxidizing system activity by CO, (b) the reverse type I spectral change obtained when ethanol is added to a liver microsomal suspension, (c) inhibition of microsomal ethanol-oxidizing system activity by drugs, and (d) inhibition *in vitro* of pentobarbital and aniline hydroxylase activities by ethanol. Our studies also showed inhibition *in vitro* of microsomal aminopyrine N-demethylation, but only when high ethanol concentrations were used (see Fig. 1). Similarly, pentobarbital metabolism was inhibited only in the presence of high concentrations of ethanol. Moreover, contrary to the report of Rubin *et al.* (1970), who found a competitive inhibition of aniline hydroxylase activity in the presence of 3 mM-ethanol, we observed no change in either the K_m or V_{max} for aniline hydroxylation

with the same concentration of ethanol (see Fig. 3). Although higher concentrations of ethanol did inhibit hydroxylation of aniline, the inhibition was complex, in that both V_{max} and K_m were changed.

Since various drug oxidations were affected only in the presence of high concentrations of ethanol and since the inhibition observed was not usually competitive, it seems unreasonable to assert that ethanol is acting as an alternative substrate of the mono-oxygenase system. Therefore, another explanation for the inhibition of drug oxidations by high ethanol concentrations is required. We propose that the ethanol-induced inhibition is due to interference by ethanol with the binding of substrate by cytochrome P-450. This is based on the decrease in the magnitude of the hexobarbital-induced type I spectral change in the presence of high concentrations of ethanol, and the increased magnitude of the hexobarbital-induced spectral change, coupled with the decreased magnitude of the ethanol-induced reverse type I spectral change, after ethanol washing of the microsomal fraction. This would in turn imply that the reverse type I spectral change is caused by removal of added or endogenously bound substrate from cytochrome P-450. Evidence for the presence of endogenously bound substrate of the mono-oxygenase system, even after washing of the microsomal fraction, has been reported by Diehl *et al.* (1970) and Orrenius *et al.* (1973).

Hence, ethanol does not appear to bind to cytochrome P-450, as has been previously suggested by

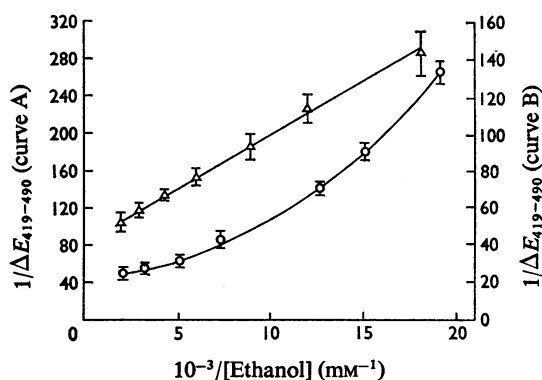


Fig. 7. Lineweaver-Burk plots of ethanol interaction with rat liver microsomal fraction

Nine different microsomal fractions were prepared as described in the Materials and Methods section. The microsomal suspensions contained 0.05M-Tris-HCl buffer, pH7.5, and 1.9–2.6mg of protein/ml. Curve A, non-linear curve, obtained with five preparations. Curve B, linear curve obtained with four preparations.

Rubin *et al.* (1971), who based their conclusion in part on the linearity of the double-reciprocal plots of spectral change versus ethanol concentration. As shown in Fig. 7, we also obtained linearity, but only in four of nine different microsomal preparations (curve B); the other five preparations produced a non-linear curve, which was concave upward (curve A). This difference could be attributed to differences in amounts of endogenous substrate bound to cytochrome P-450 at the time of preparation of the microsomal fraction. Therefore, the shape of the curve as well as the magnitude of the reverse type I spectral change would be dependent on the amount of substrate-bound cytochrome P-450 present. This interpretation would also be compatible with the observations of an increased magnitude of the ethanol-produced reverse type I spectral change in microsomal fractions from ethanol-treated rats (Rubin *et al.*, 1971), since chronic ethanol administration is known to increase the content of liver cytochrome P-450 (Rubin *et al.*, 1968) and thus probably also of endogenously substrate-bound cytochrome P-450.

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