INTERACTION OF ETHANOL AND PYRAZOLE WITH HEPATIC MICROSOMES

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SUMMARY

Binding of ethanol to hepatic microsomes is inhibited by compounds which produce a type 2 binding spectrum, whereas it is unaffected by those whose binding yields a type 1 spectrum. Pyrazole inhibits hepatic microsomal drug metabolizing enzymes and binds to the hemoprotein, resulting in a type 2 spectrum (trough 394, peak 430 nm). Both ethanol and pyrazole inhibit the reduction of cytochrome P450. The data indicate that ethanol shares certain characteristics with other agents which produce a type 2 spectrum. The interaction of pyrazole with hepatic microsomes should be noted when it is used to study in vivo metabolism of ethanol.

Hepatic ethanol oxidation has for some time been attributed solely to hepatic alcohol dehydrogenase, an enzyme of the supernatant fraction. The recent demonstration of a hepatic microsomal ethanol oxidizing system (1), has led to increased interest in the interaction of ethanol and hepatic microsomes. We have recently shown that ethanol binds to hepatic microsomal hemoprotein, and that this binding is enhanced by pretreatment with ethanol (2). Pyrazole, a 5-membered heterocyclic ring, with 2 nitrogen atoms in 1,2 position, forms a complex with alcohol dehydrogenase (ADH) (3), and has been considered a specific inhibitor of that enzyme. It has therefore been used in vivo to assess the role of alcohol dehydrogenase in ethanol metabolism (4,5). In this paper we present further evidence for the interaction of ethanol with microsomal hemoprotein. We further demonstrate that pyrazole also interacts with hepatic microsomes, and is a potent inhibitor of certain microsomal functions, including interference with ethanol binding.

MATERIALS AND METHODS

Hepatic microsomes were obtained from 150-200 g male, Sprague-Dawley rats by homogenizing the liver in 0.25 M sucrose, and centrifuging the 9000 x g supernatant at 105,000 x g for one hour. The activities of aniline hydroxylase (6) and pentobarbital hydroxylase (7) were measured, and the kinetics of the reactions determined. Nitroreductase activity was assayed according to Kato, et al (8). Microsomes were washed in 1.19 percent KCl to remove hemoglobin and diluted with tris-KCl buffer, pH 7.4, to a concentration of 2.5 mg protein per ml, determined according to Lowry (9). The difference spectrum produced by the binding of pyrazole to these microsomes, and to those obtained from rats fed ethanol for 24 days (10), was determined in an Aminco-Chance dual wave length spectrophotometer, in the split beam mode. Ethanol was added to the microsomal suspension and the spectral change recorded. Pyrazole was added to 6 ml of this microsomal suspension; 3 ml were then placed in both reference and sample cuvettes, after which ethanol was added to the sample cuvette and the spectral change recorded. For comparison, the effects of hexobarbital and aniline upon the magnitude of the spectral changes induced by ethanol binding were determined in the same manner. The rate of reduction of cytochrome P450 by NADPH was determined according to Gigon, et al (11), using the Aminco-Chance dual wave length spectrophotometer. In these studies, the reaction was recorded for six seconds, and only the initial rate was considered, since it is this rate which is thought to be a valid measure of cytochrome P450 reductase activity (11).

RESULTS

Addition of ethanol to hepatic microsomes resulted in a modified type 2 spectral change, characterized by a peak at 420 and a trough at 394 nm (Fig. 1). Prior exposure of the microsomes to hexobarbital had no effect on the magnitude of this spectral change, whereas prior addition of pyrazole and aniline conspicuously diminished it (Fig. 1).

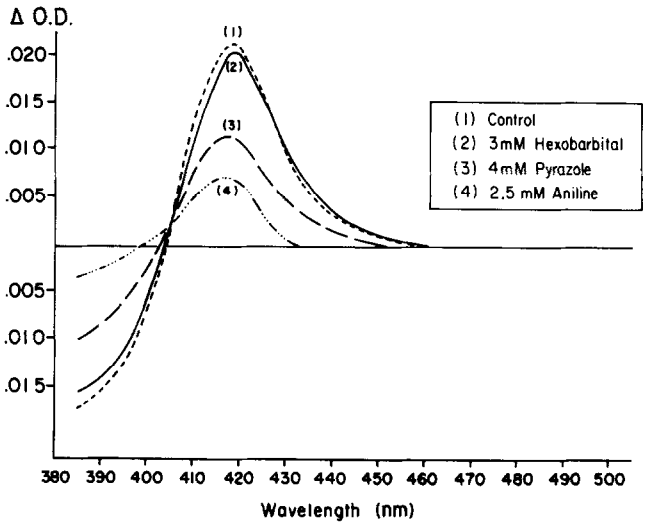

Fig. 1. Effect of the binding of other substrates on the binding of ethanol to hepatic microsomal hemoprotein. Pyrazole, aniline and hexobarbital were added to suspensions of washed hepatic microsomes. Suspensions were poured into reference and sample cuvettes, and 100 mM ethanol added to the sample cuvette, after which the difference spectrum was recorded. Ethanol alone was added to the control microsomes.

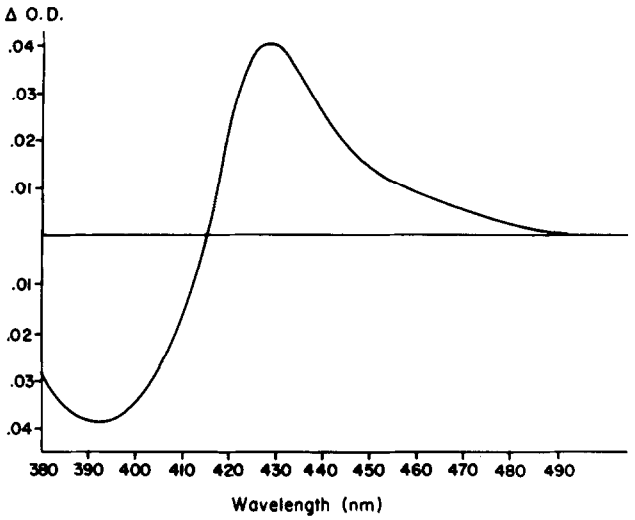

Fig. 2. Difference spectrum produced by the addition of 2 mM pyrazole to hepatic microsomes.

Pyrazole bound to hepatic microsomes, producing a typical type 2 spectral change (peak 430, trough 394 nm) (Fig. 2). In six pairs of rats fed

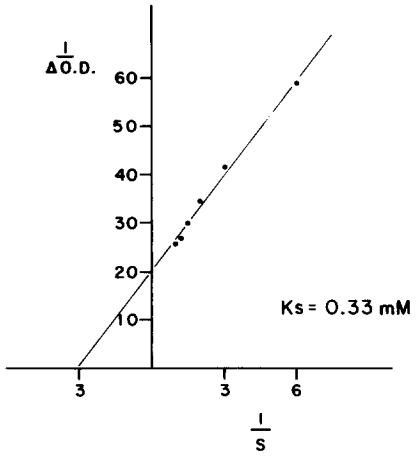

Fig. 3. Double reciprocal plot of pyrazole concentration versus magnitude of the spectral peak (430-500 nm). K_S - Dissociation constant. S - millimolar concentration of pyrazole.

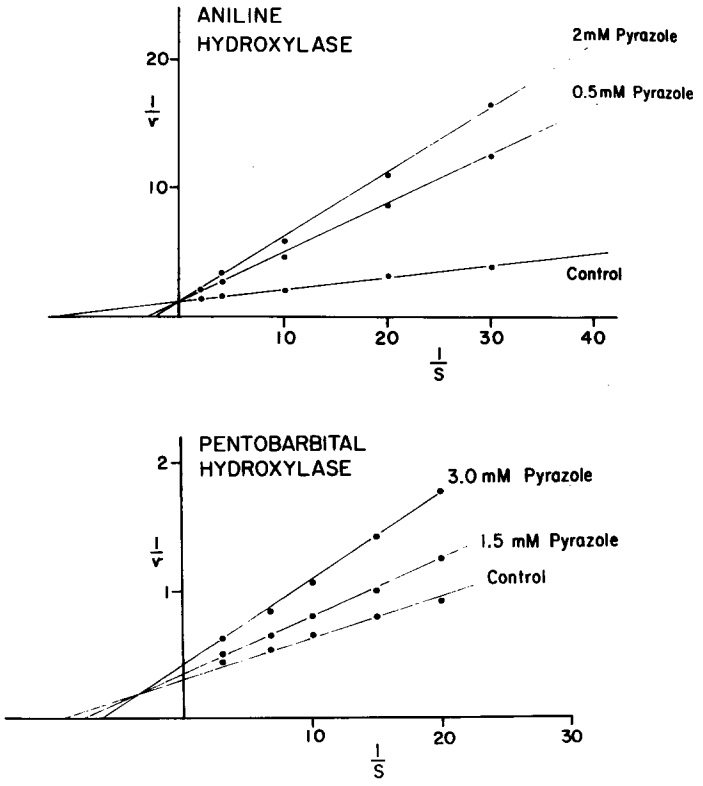

Fig. 4. Lineweaver-Burk plots showing inhibition of the activities of microsomal aniline and pentobarbital hydroxylases by pyrazole. V, aniline hydroxylase - nMoles para-aminophenol formed per minute per mg microsomal protein.
V, pentobarbital hydroxylase - nMoles pentobarbital metabolized per minute per mg microsomal protein.
S - millimolar substrate concentration.

the control or ethanol containing diet, for 24 days, the magnitude of this spectral change (per mg microsomal protein) was doubled by ethanol consumption ($p < .05$). A double reciprocal plot of pyrazole concentration versus the magnitude of the spectral peak (430-500 nm) showed the dissociation constant (K_s) to be 0.33 mM (Fig. 3). Pyrazole inhibited the activity of aniline hydroxylase competitively and that of pentobarbital hydroxylase in a mixed fashion (Fig. 4). The calculated K_i for the inhibition of aniline hydroxylase by pyrazole is 0.25 mM. Nitroreductase activity, using a concentration

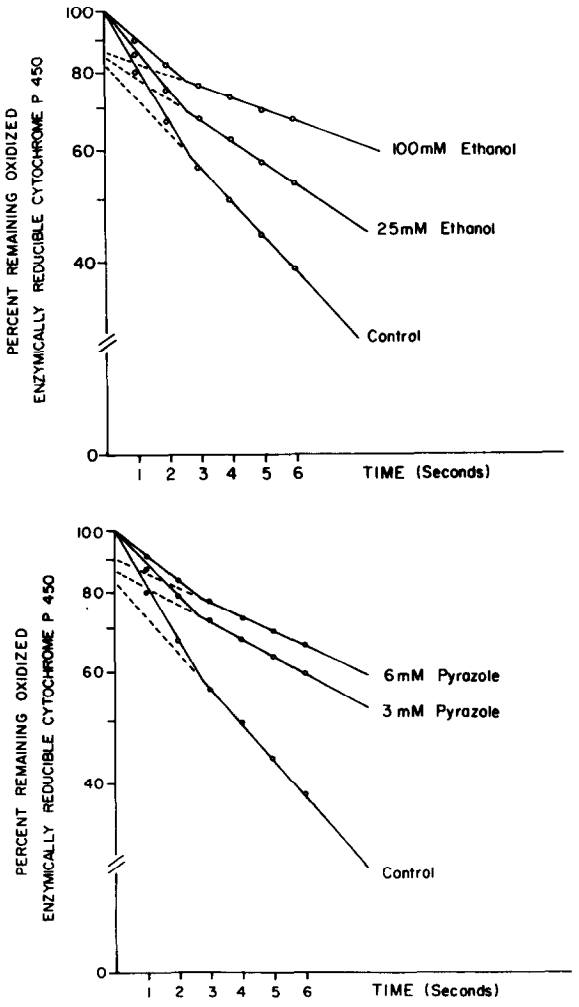

Fig. 5. Effect of ethanol on the rate of reduction of microsomal cytochrome P450. Concentration of microsomal protein - 3 mg/ml.

of 0.5 mM p-nitrobenzoic acid, was inhibited 23 percent by 4 mM pyrazole (control value, 2.46 nMoles p-aminobenzoic acid formed per minute per mg protein). 1 mM hexobarbital had no effect on nitroreductase activity.

In each of four experiments, both ethanol and pyrazole inhibited the rate of reduction of cytochrome P450, as shown by plotting the percent of remaining enzymically reducible cytochrome P450 against time. A representative experiment is illustrated in Fig. 5.

DISCUSSION

In this study we have shown that pyrazole binds to hepatic microsomal hemoprotein and produces a type 2 spectral change. Ethanol also binds to microsomal hemoprotein, leading to a modified type 2 spectral change (2). Ethanol probably binds at the same site as type 2 binders, or one closely related to it, since type 2 binders inhibited ethanol binding, while a type 1 binder had no effect. Furthermore, ethanol inhibits the reduction of cytochrome P450, a characteristic which it shares with other agents which produce a type 2 spectral change (11). By contrast, compounds whose binding results in a type 1 spectral change, facilitate electron transfer to cytochrome P450 (11). Chronic ethanol administration to rats enhances binding of ethanol to hepatic microsomes (2) and leads to increased activities of a variety of microsomal drug metabolizing enzymes (12,13), while *in vitro*, ethanol inhibits the activities of drug metabolizing enzymes (13,14). These data suggest an interaction of ethanol and hepatic microsomes

Pyrazole is not a specific inhibitor of ADH, since it also inhibits the *in vitro* activities of microsomal drug metabolizing enzymes. In this respect it is similar to many drugs oxidized by microsomes (including ethanol) which mutually inhibit each other's metabolism (15). It resembles other drugs in its ability to induce a variety of microsomal enzyme systems *in vivo*: witness the induction of aniline and benzpyrene hydroxylases 24 hours after acute administration of pyrazole to rats (16). Pyrazole binds to microsomal hemoprotein, producing a type 2 spectral pattern, similar to that produced by

aniline (17). Since pyrazole is a type 2 binder, it is not surprising that its inhibition of the metabolism of pentobarbital, a type 1 binder (17), is of a mixed type. This pattern of enzyme inhibition is similar to that produced by ethanol (13). Moreover, pyrazole, in contrast to hexobarbital, inhibits nitroreductase activity. This is consistent with the finding of Sasame and Gillette (18) that type 2 binders inhibit nitroreductase activity, whereas type 1 do not. Furthermore, as is the case with other type 2 binders, pyrazole impedes electron transfer to cytochrome P450. Pyrazole is similar to the other type 2 binder, aniline, in that it interferes with the binding of ethanol to microsomes. It is possible, therefore, that the inhibition of microsomal detoxifying systems by both pyrazole and ethanol may be related, at least in part, to interference with the binding of substrates to, and the rate of reduction of, the microsomal hemoprotein.

This study further substantiates the similarity of the microsomal effects of ethanol and compounds which exhibit a type 2 binding spectrum. Our data also demonstrate that pyrazole cannot be accepted as a specific inhibitor of alcohol dehydrogenase, a fact which should be taken into account when pyrazole is used for in vivo studies of ethanol metabolism.

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