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OXIDATION OF PYRAZOLE TO 4-HYDROXYPYRAZOLE BY INTACT RAT HEPATOCYTES

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Abstract—4-Hydroxypyrazole has been identified as a major metabolite found in the urine of rats and mice after *in vivo* administration of pyrazole, a potent inhibitor of alcohol dehydrogenase and of ethanol metabolism. The locus and the enzyme systems responsible for the oxidation of pyrazole have not been identified. In the current report, isolated hepatocytes from fed rats were shown to oxidize pyrazole to 4-hydroxypyrazole. An HPLC procedure employing UV and electrochemical detection was utilized to separate and quantify the 4-hydroxypyrazole. The apparent K_m for pyrazole by intact hepatocytes was about 2 mM, whereas the apparent V_{max} was about 0.06 nmol 4-hydroxypyrazole per min per mg liver cell protein. The production of 4-hydroxypyrazole was inhibited by carbon monoxide and metyrapone, as well as by competitive drug substrates such as aniline or aminopyrine. These results implicate a role for cytochrome P-450 in the oxidation of pyrazole by the hepatocytes. Ethanol was an effective inhibitor of pyrazole oxidation. Hepatocytes were also isolated from rats treated with acetone and 4-methylpyrazole, to attempt to evaluate whether pyrazole oxidation is induced. The rate of 4-hydroxypyrazole production by hepatocytes after acetone and 4-methylpyrazole treatment was actually lower than that of controls. Kinetic assays suggested the presence of an endogenous inhibitor (perhaps the inducer itself) in the induced hepatocytes. In contrast, hepatocytes isolated from rats fasted for 48 hr showed a 2-fold increase in the oxidation of pyrazole to 4-hydroxypyrazole. The K_m for pyrazole was the same in hepatocytes from fasted and fed rats, whereas V_{max} was increased after fasting. The locus and enzyme system responsible for the oxidation of pyrazole to 4-hydroxypyrazole, and the site of sensitivity to ethanol, appears to be the cytochrome P-450 system of the hepatocyte.

Pyrazole and its 4-substituted derivatives are potent inhibitors of alcohol dehydrogenase and are widely used to block the metabolism of ethanol *in vivo* and *in vitro* [1–4]. Pyrazole elimination *in vivo* was found to be a first-order process with a half-life for pyrazole in plasma of about 14 hr [4, 5]. Clay *et al.* [6] observed several hydroxylated pyrazole metabolites, as well as conjugated derivatives, in the urine of rats after administration of ^{14}C-labeled pyrazole. 4-Hydroxypyrazole is a major hydroxylated metabolite produced from *in vivo* metabolism of pyrazole [6, 7]. *In vivo* administration of ethanol causes a decrease in the production of 4-hydroxypyrazole [7], and ethanol increases the half-life of pyrazole in plasma [5]. The metabolic enzyme systems responsible for the oxidation of pyrazole to 4-hydroxypyrazole have not been determined.

An HPLC procedure employing UV and electrochemical detection which produced a sensitive and rapid method for the separation and quantification of 4-hydroxypyrazole was developed recently [8, 9]. Utilizing this methodology, pyrazole was found to be oxidized to 4-hydroxypyrazole by rat liver microsomes in an NADPH-dependent manner [8], as well as by hydroxyl radicals generated by a variety of iron-dependent, model chemical and enzymatic systems [9]. To date, there are no reports on pyrazole metabolism by intact cells. The current study was carried out to extend the above observations to intact hepatocytes, to evaluate the role of the cytochrome P-450 mixed-function oxidase system in pyrazole oxidation, and to assess the interactions between ethanol and pyrazole. Since pyrazole induces a cytochrome P-450 isozyme which appears to be similar to the isozyme induced by ethanol, isoniazid, acetone and fasting [10–16], the oxidation of pyrazole by intact hepatocytes isolated from rats treated with inducers of this cytochrome P-450 isozyme was also determined.

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MATERIALS AND METHODS

Hepatocytes were isolated from male, Sprague–Dawley rats weighing about 250–300 g as described previously [17]. Hepatocytes were isolated from fed rats, rats fasted for 48 hr, rats treated with pyrazole (200 mg/kg body wt, i.p.) for 2 days and then starved overnight, or rats allowed to consume a 1% (v/v) solution of acetone for about 10 days and then fasted overnight. All rats were allowed access to water until they were killed. Viability, assessed by trypan blue exclusion, was routinely greater than 90% for all preparations. The hepatocytes were suspended in Krebs–Ringer–bicarbonate buffer, pH 7.4, supplemented with 10 mM potassium phosphate, pH 7.4, and 1.25% (w/v) fatty acid-free bovine serum albumin. The buffer and hepatocytes were saturated with 95% O_2:5% CO_2.

The basic reaction system to evaluate pyrazole oxidation to 4-hydroxypyrazole consisted of the

above buffer, 10 mM pyrazole, and about 20 mg hepatocyte protein (equivalent to about 10×10^6 cells) in a final volume of 3 ml. Reactions were conducted at 37° and were initiated by the addition of hepatocytes. The reactions were terminated usually after 10 min by the addition of perchloric acid [3.5% (w/v) final concentration], and the samples were centrifuged for 5 min in a clinical centrifuge. The supernatant fraction was neutralized with 11 N KOH, and the potassium perchlorate was removed by centrifugation.

An HPLC procedure employing UV and electrochemical detection was utilized to separate 4-hydroxypyrazole from the other components of the reaction system and to quantify this metabolite. Full details and validation of the method are presented elsewhere [8, 9]. Briefly, 1 ml of the clear, neutral supernatant fraction was loaded onto a Sep-Pak C18 cartridge (Waters Associates, Milford, MA). The Sep-Pak was activated previously by washing with 6 ml of methanol followed by 10 ml of water. One milliliter of water (HPLC grade) was added to elute the metabolite 4-hydroxypyrazole off the Sep-Pak, and two 0.5-ml fractions were collected. The second fraction was evaporated to dryness using a Savant Speed Vac Concentrator and resuspended in 200 μl of HPLC grade water.

A 10-μl aliquot of the sample prepared as described above was injected into a Microsorb C18 column (4.6 mm × 25 cm, Rainin Instruments, Woburn, MA) using a Waters universal injector. The 4-hydroxypyrazole was separated from the remaining components of the reaction mixture isocratically, using a mobile phase of 10% acetonitrile plus 1% glacial acetic acid plus 5 mM octanesulfonic acid in H_2O. Under these conditions, the 4-hydroxypyrazole was detected at 254 nm using a Waters model 400 detector or a BAS electrochemical detector with a retention time of 5.9 min. Standard 4-hydroxypyrazole was a gift from the Eli Lilly Co. (Indianapolis, IN). All values were corrected for "zero-time" controls in which the perchloric acid was added to the reaction system prior to the addition of the hepatocytes. All values represent the mean of two or three experiments, each carried out in duplicate. Variability between experiments did not exceed 10% and, where indicated, results refer to mean ± SEM.

RESULTS

Oxidation of pyrazole to 4-hydroxypyrazole. Hepatocytes isolated from fed, untreated rats, were incubated with 10 mM pyrazole, and the production of 4-hydroxypyrazole was determined. The production of 4-hydroxypyrazole was linear with time over a 20-min reaction period and was proportional to the amount of hepatocyte protein added to the reaction system (Fig. 1). There was no "*production*" of 4-hydroxypyrazole in the absence of either pyrazole or hepatocytes, or in zero-time controls. Under these conditions, pyrazole was oxidized to 4-hydroxypyrazole by the heptocytes at a rate of about 0.05 to 0.07 nmole per min per mg cellular protein (equivalent to a rate of about 12 nmol per min per g liver, wet wt).

A concentration curve for the oxidation of pyrazole is shown in Fig. 2. The production of 4-hydroxypyrazole increased as the concentration of pyrazole was elevated over the range of 1–10 mM. Kinetic constants were calculated from a regression analysis of a Lineweaver–Burk plot of these results (Fig. 2, insert). The apparent K_m for pyrazole was about 2 mM, whereas the apparent V_{max} was about 0.06 nmol 4-hydroxypyrazole per min per mg liver cell protein, with a correlation coefficient of 0.96.

Role of cytochrome P-450 in pyrazole oxidation. To assess the possible role of a cytochrome P-450-

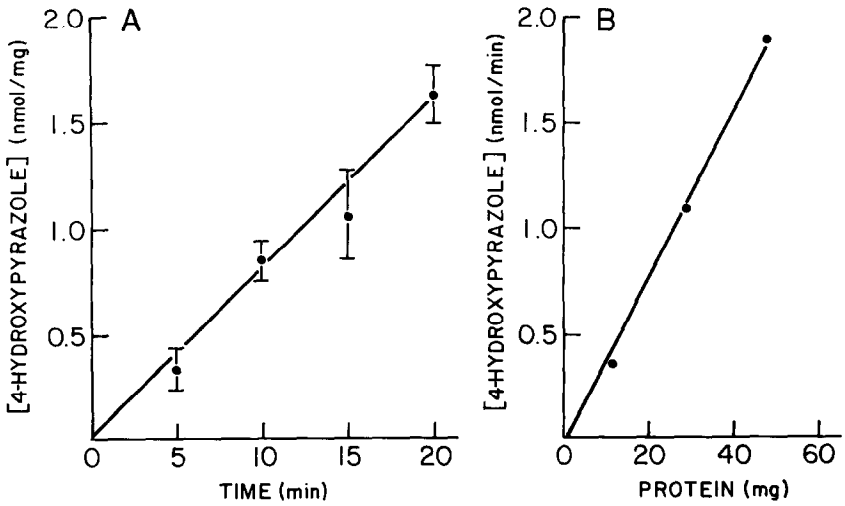

Fig. 1. Time course and protein concentration curve for the oxidation of pyrazole by hepatocytes isolated from fed rats. The production of 4-hydroxypyrazole from 10 mM pyrazole was determined using HPLC (see Materials and Methods). For experiment A, the concentration of protein ranged between 20 and 30 mg; for experiment B, the reaction period was 10 min. Results are from either three (mean ± SEM) (A) or two (B) experiments.

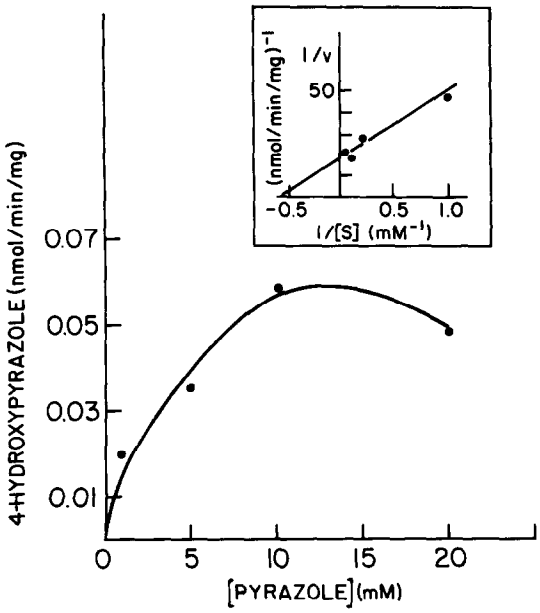

Fig. 2. Production of 4-hydroxypyrazole as a function of the concentration of pyrazole. Reactions were carried out for 10 min as described in Materials and Methods in the presence of either 1, 5, 10 or 20 mM pyrazole. The insert is a Lineweaver–Burk plot of these data.

dependent pathway in pyrazole oxidation by intact hepatocytes, the effects of classical inhibitors of mixed-function oxidase activity, and of competitive drug substrates were determined. The production of 4-hydroxypyrazole was decreased by 70% by aniline or aminopyrine (Table 1). Metyrapone, a potent inhibitor of certain cytochrome P-450 isozymes, also produced strong inhibition of pyrazole oxidation (Table 1). The ability of carbon monoxide to block pyrazole oxidation was compared to that of nitrogen, since pyrazole oxidation was very sensitive to anaerobiosis. In the presence of 33% carbon monoxide, the production of 4-hydroxypyrazole was depressed by 50% as compared to a mixture containing 33% N_2 (Table 1). Increasing the percentage of CO produced a further decrease in pyrazole oxidation; however, part of this decrease reflected anaerobiosis since increasing the percentage of nitrogen also caused inhibition of pyrazole oxidation. Nevertheless, CO was more inhibitory than N_2 at all concentrations evaluated (Table 1).

Pyrazole blocks the metabolism of ethanol *in vivo* and by a variety of *in vitro* systems including isolated hepatocytes and microsomes [3, 4, 17–21]. Since ethanol also serves as a substrate for cytochrome P-450 [22, 23], the ability of ethanol to inhibit pyrazole oxidation was evaluated. Ethanol proved to be an effective inhibitor of pyrazole oxidation, as 50% inhibition was observed at an ethanol concentration of 5 mM (Table 2). Ethanol may inhibit oxidation of drugs at the level of cytochrome P-450 or by interfering with the availability of the NADPH cofactor [24, 25]. However, the latter mechanism occurs only in the fasted nutritional state, not the fed state [25], in which these experiments were conducted. Further verification that availability of cofactor was not limiting for pyrazole oxidation under these conditions was the lack of effect of added metabolic substrates such as pyruvate or xylitol (Table 2) which are known to stimulate hepatic drug metabolism in the fasted but not the fed state [26, 27].

Induction of pyrazole oxidation. Results with isolated microsomes demonstrated that prior treatment with pyrazole causes an induction of pyrazole oxidation [8]. Similar experiments with hepatocytes isolated from pyrazole-treated rats could not be carried out successfully since even 24 hr after the last dose of pyrazole there were large amounts of 4-hydroxypyrazole present in the hepatocytes, and the subsequent addition of pyrazole produced only a variable, small change over these endogenous levels. The induction of pyrazole oxidation by prior treatment with pyrazole appears to be due to an increase in a particular isozyme of cytochrome P-450 [8]. This isozyme appears to be the same as the cytochrome P-450 isozyme induced by treatment with several diverse agents, including acetone [10, 14, 28, 29]. We therefore evaluated the ability of acetone treatment to induce the metabolism of pyrazole by isolated

Table 1. Effects of competitive drug substrates and inhibitors of cytochrome P-450 on the production of 4-hydroxypyrazole

Addition	Concn	Rate of production of 4-hydroxypyrazole (nmol/min/mg liver cell protein)	Inhibition by addition (%)
Control		0.062 ± 0.003	
Aniline	5 mM	0.019 ± 0.004	69
Aminopyrine	5 mM	0.018 ± 0.001	71
Metyrapone	1 mM	0.006 ± 0.001	90
Nitrogen	33%	0.058 ± 0.002	6
Carbon monoxide	33%	0.032 ± 0.003	48
Nitrogen	50%	0.026 ± 0.007	58
Carbon monoxide	50%	0.011 ± 0.003	82
Nitrogen	67%	0.020 ± 0.006	67
Carbon monoxide	67%	0.002 ± 0.001	97

The oxidation of 10 mM pyrazole by hepatocytes from fed rats was assayed for 10 min as described in Materials and Methods in the absence and presence of the indicated additions. Values are means ± SEM, N = 3.

Table 2. Effects of ethanol and metabolic substrates on the production of 4-hydroxypyrazole

Addition	Concn (mM)	Rate of production of 4-hydroxypyrazole (nmol/min/mg liver cell protein)	Inhibition by addition (%)
Control		0.063 ± 0.003	
Ethanol	5	0.032 ± 0.001	49
Ethanol	10	0.006 ± 0.001	90
Pyruvate	10	0.049 ± 0.010	22
Xylitol	10	0.046 ± 0.007	27

The oxidation of 10 mM pyrazole by hepatocytes from fed rats was assayed for 10 min as described in Materials and Methods in the absence and presence of the indicated additions. Values are means ± SEM, N = 3.

hepatocytes. Preliminary experiments with microsomes isolated from acetone-treated rats indicated an approximate 2-fold increase in the oxidation of pyrazole to 4-hydroxypyrazole as compared to controls drinking water, thus validating the ability of acetone to induce pyrazole oxidation. However, in contrast to the results with isolated microsomes, hepatocytes isolated from rats treated with acetone oxidized pyrazole at a lower rate than controls (Fig. 3). Essentially, similar results were obtained when 4-methylpyrazole (200 mg/kg/day for 3 days) was utilized as the inducing agent. Rates of 4-hydroxypyrazole production from pyrazole by hepatocytes from the 4-methylpyrazole-treated rats were about one-half that found with control hepatocytes (data not shown). To attempt to explain this unexpected finding, the kinetics of pyrazole oxidation were determined. As shown in Fig. 4, the rate of 4-hydroxypyrazole production by hepatocytes from the acetone-treated rats was lower than that by fed controls at all concentrations of pyrazole evaluated. Kinetic constants as calculated by regression analyses of a Lineweaver–Burk plot (Fig. 4, insert) indicated that the acetone treatment slightly lowered the V_{max} for pyrazole oxidation (control, 0.053 ± 0.009; acetone-treated, 0.038 ± 0.002 nmol 4-hydroxypyrazole per min per mg hepatocyte protein), whereas the apparent K_m for pyrazole was increased more than 3-fold (control, 1.55 ± 0.25; acetone-treated, 5.2 ± 1.8 mM). Correlation coefficients were 0.96 for controls and 0.93 for acetone-treated.

Recent experiments by Yang and co-workers [14, 30, 31] suggested the possibility that fasting induces a cytochrome P-450 isozyme which is similar to the isozyme induced by ethanol, acetone and pyrazole. The oxidation of pyrazole by hepatocytes isolated from rats fasted for 48 hr was therefore evaluated. Hepatocytes from fasted rats oxidized pyrazole at rates that were 2- to 3-fold higher than rates found with hepatocytes from fed rats (Fig. 3). In the fasted state, rates of pyrazole oxidation were linear for shorter reaction times than those found in the fed state (Fig. 3). This probably reflects the limiting amount of cofactor available under these conditions [24, 26].

At all concentrations of pyrazole tested, the pro-

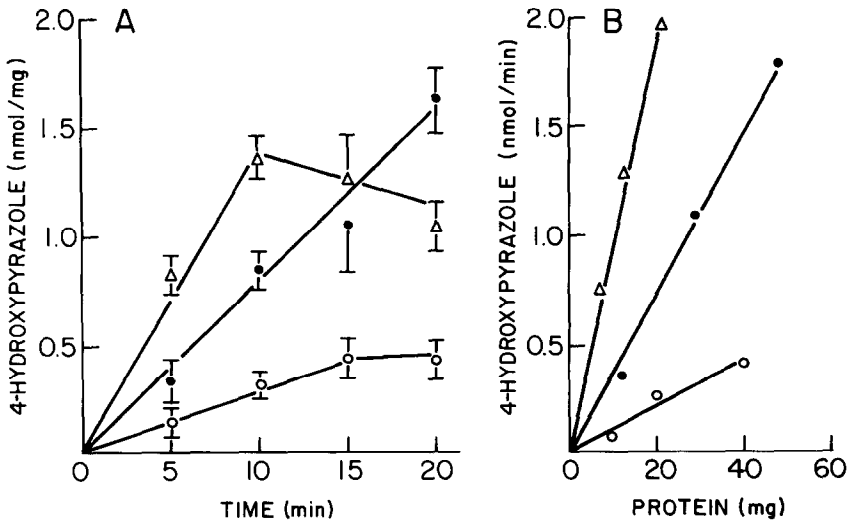

Fig. 3. Time course and protein concentration curve for the oxidation of pyrazole by hepatocytes from fasted rats (△—△) and from rats treated with acetone (○—○). For comparative purposes, results with hepatocytes from fed rats (●—●) are included. Results are from either three (means ± SEM) (A) or two (B) experiments.

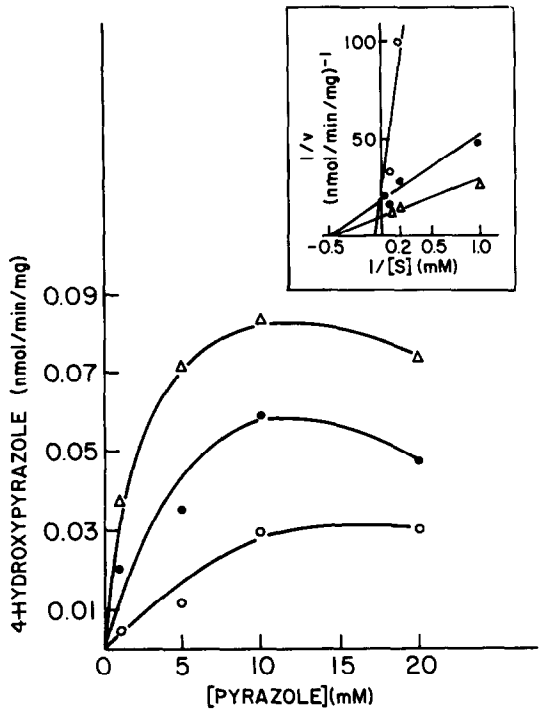

Fig. 4. Effect of pyrazole concentration on the production of 4-hydroxypyrazole by hepatocytes isolated from fasted rats (△—△). fed rats (●—●), or rats treated with acetone (○—○). The insert is a Lineweaver–Burk plot of these data.

duction of 4-hydroxypyrazole was greater with hepatocytes isolated from fasted rats as compared to those from fed rats (Fig. 4). A Lineweaver–Burk plot indicated that fasting caused a near 2-fold increase in the V_{max} for pyrazole oxidation (0.09 ± 0.01 nmol 4-hydroxypyrazole per min per mg hepatocyte protein), whereas the K_m for pyrazole (1.5 ± 0.6 mM) was not altered when compared to results with hepatocytes from fed rats (Fig. 4 insert, correlation coefficient of 0.99).

Table 3 compares the effects of several modifiers on pyrazole oxidation by the hepatocytes isolated from fasted rats and rats treated with acetone. Analogous to the results found with hepatocytes from the fed rats, pyrazole oxidation was sensitive to inhibition by metyrapone, ethanol and 4-methylpyrazole.

DISCUSSION

Intact hepatocytes were found to catalyze the oxidation of pyrazole to 4-hydroxypyrazole by a cytochrome P-450-dependent process. The inhibition of pyrazole oxidation by carbon monoxide and metyrapone, as well as by competitive drug substrates such as aniline and aminopyrine, implicates a role for cytochrome P-450 in the generation of 4-hydroxypyrazole from pyrazole. Pyrazole binds to cytochrome P-450 and produces a type 2 spectral change [21, 32] and, *in vitro*, pyrazole inhibits the oxidation of a variety of drugs, including microsomal oxidation of ethanol [20, 21]. *In vivo* treatment with pyrazole results in increased microsomal metabolism of drugs such as aniline [33], *N*-nitrosodimethylamine [34, 35], and alcohols such as ethanol and 2-butanol [15, 16]. The present results extend previous observations of pyrazole oxidation [8, 9] and the interactions of pyrazole with microsomes to the intact hepatocyte and identify the hepatic cytochrome P-450-dependent mixed-function oxidase system as the enzyme system responsible for the oxidation of pyrazole to 4-hydroxypyrazole.

The addition of ethanol to the hepatocytes produced a strong inhibition of pyrazole oxidation to 4-hydroxypyrazole. This inhibition was similar with hepatocytes isolated from either fed or fasted rats, which suggests that the inhibition probably reflects competition for metabolism at the level of cytochrome P-450 rather than interference with the availability of cofactor. The failure of pyruvate or xylitol to stimulate pyrazole oxidation with hepatocytes from fed rats also suggests that cofactor availability is not limiting in this state [25, 26]. The inhibition by ethanol of pyrazole oxidation by hepatocytes is likely to be responsible for the ability of ethanol *in vivo* to inhibit the production of 4-hydroxypyrazole from pyrazole and to increase the half-life of pyrazole [5, 7]. Thus, there are mutual inhibitory interactions between ethanol and pyrazole in blocking each other's metabolism, with the primary locus of pyrazole inhibition of ethanol oxidation being alcohol dehydrogenase, and perhaps to a lesser extent, the microsomal cytochrome P-450 system, while the lat-

Table 3. Effects of ethanol, metyrapone and 4-methylpyrazole on the production of 4-hydroxypyrazole by hepatocytes isolated from fasted rats and rats treated with acetone

	Rate of production of 4-hydroxypyrazole (nmol/min/mg liver cell protein)		Inhibition by addition (%)	
Addition	Fasted	Acetone-treated	Fasted	Acetone-treated
Control	0.083	0.031		
5 mM Ethanol	0.062	0.019	25	39
10 mM Ethanol	0.039	0.009	53	71
1 mM Metyrapone	0.021	0.004	75	87
5 mM 4-Methylpyrazole	0.043	0.009	48	71

The oxidation of 10 mM pyrazole by hepatocytes from fasted rats or rats treated with acetone was assayed as described in Materials and Methods in the absence and presence of the indicated additives.

ter reflects the locus of ethanol inhibition of pyrazole oxidation.

Attempts to demonstrate induction of pyrazole oxidation in hepatocytes by agents such as pyrazole, 4-methylpyrazole and acetone proved to be complex. These compounds, along with other agents such as isoniazid or chronic ethanol treatment, appear to induce the same cytochrome P-450 isozyme [10–16]. With pyrazole as the inducing agent, the residual presence of large amounts of 4-hydroxypyrazole in the hepatocytes complicated the accurate determination of newly generated 4-hydroxypyrazole and kinetic assays. With acetone and 4-methylpyrazole as inducing agents, the rates of pyrazole oxidation were lower than that of controls, which probably reflects the presence of residual inducer in the hepatocyte preparations. Kinetic studies with hepatocytes from acetone-treated rats indicated an increase in apparent K_m for pyrazole coupled to a small decrease in the apparent V_{max} when compared to values found with controls, consistent with the presence of an endogenous inhibitor of pyrazole oxidation. Whether this inhibitor is the inducing agent (acetone, 4-methylpyrazole) itself, or some other compound induced by the treatment is not known.

Fasting has been shown to induce a specific form of cytochrome P-450 which appears to be identical to the acetone-induced isozyme [30, 31]. Hepatocytes isolated from rats fasted for 48 hr proved to be more active than those isolated from fed rats in catalyzing the oxidation of pyrazole. The increase in pyrazole oxidation caused by fasting was associated with about a 2-fold increase in V_{max}, whereas the affinity for pyrazole was not altered. Hong *et al.* [30, 31] recently reported that fasting for 48 hr results in a 116% increase in rat liver microsomal *N*-nitrosodimethylamine demethylase activity, an increase similar to the increase for pyrazole oxidation by hepatocytes from fasted rats. This substrate appears to be specific for the isozyme induced by isoniazid, pyrazole, acetone, ethanol and this class of inducers [36]. Of considerable interest is the finding that the mechanism for induction of cytochrome P-450 by fasting (elevated mRNA levels) is different from the induction by acetone treatment (no change in mRNA levels) [30, 31]. These differing mechanisms may explain why fasting but not treatment with acetone (or 4-methylpyrazole) is effective in demonstrating induction of pyrazole oxidation to 4-hydroxypyrazole by intact hepatocytes. It should be pointed out that, with isolated, well-washed microsomes (in contrast to the hepatocyte studies), the oxidation of pyrazole to 4-hydroxypyrazole is induced about 3-fold after treatment with pyrazole, 4-methylpyrazole, acetone or chronic ethanol [8].

In summary, isolated rat hepatocytes can oxidize pyrazole to 4-hydroxypyrazole by a mechanism involving cytochrome P-450. Mutual inhibitory interactions between ethanol and pyrazole can be observed in hepatocytes, and it is likely that cytochrome P-450 is responsible for the *in vivo* oxidation of pyrazole to 4-hydroxypyrazole and is the enzyme sensitive to ethanol inhibition of pyrazole oxidation. Induction of pyrazole oxidation by hepatocytes can be observed when fasting is the inducer, which probably reflects the induction of the cytochrome P-450j isozyme. Other inducers of this isozyme, however, are not effective with hepatocytes, in contrast to microsomes, probably because of the presence of an endogenous inhibitor in the intact cells.

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