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OXIDATION OF GLYCEROL TO FORMALDEHYDE BY RAT LIVER MICROSOMES

EFFECTS OF CYTOCHROME P-450 INDUCING AGENTS

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Abstract—Glycerol was shown recently to be metabolized to formaldehyde by microsomes from chow-fed control rats (Winters *et al., Biochem Biophys Res Commun* **153**: 612–617, 1988). In the present study, experiments were carried out to evaluate the oxidation of glycerol by microsomes isolated from rats treated with inducers of different isozymes of cytochrome P-450. The oxidation of glycerol to formaldehyde was increased in microsomes from rats treated with pyrazole, ethanol or acetone relative to their respective controls, but not after treatment with phenobarbital or 3-methylcholanthrene. This reaction was sensitive to inhibition by carbon monoxide and was inhibited by compounds known to be effective substrates for P-450j, e.g. aniline, ethanol, pyrazole and 4-methylpyrazole. Treatment with pyrazole caused an increase in V_{max} for glycerol oxidation but did not affect the K_m (about 15 mM) for glycerol, as compared to saline controls. Evidence that the product of glycerol metabolism is formaldehyde was provided by the observation that this product served as a substrate for the glutathione-dependent formaldehyde dehydrogenase, and the amount of formaldehyde detected was identical to that detected by the Nash reaction. By utilizing [^{14}C]glycerol, and coupling the formaldehyde dehydrogenase reaction to the formate dehydrogenase reaction, $^{14}CO_2$ could be detected, indicating that the formaldehyde produced was derived from the added glycerol. These results suggest that glycerol is not metabolically inert when added to microsomes but serves as an effective substrate for the cytochrome P-450j isozyme, extending the alcohol substrate specificity of this enzyme to poly-ols. The production of formaldehyde from glycerol may require caution since glycerol is often present in microsomal or reconstituted systems.

Pyrazole and 4-methylpyrazole affect the metabolism of certain drugs by the mixed-function oxidase system [1–4]. Microsomes isolated from pyrazole-treated rats display elevated activity with substrates such as ethanol, 2-butanol, dimethylnitrosamine, aniline, and *p*-nitroanisole but not with aminopyrine or ethoxycoumarin [5–7]. These results are similar to those obtained with microsomes from chronic ethanol-fed rats [2, 8–10], suggesting that ethanol and pyrazole induce the same isozyme of cytochrome P-450. The isozyme induced by pyrazole treatment has been purified to homogeneity and its properties and specificity were found to be similar to that of the ethanol-induced isozyme [11]. In addition to the substrates listed above, this isozyme of cytochrome P-450, P-450j or P-450IIE1, is active with carbon tetrachloride [12] *n*-pentane [13], diethyl ether [14], acetone and acetol [15], and nitrosamines [16, 17].

In vitro, glycerol is an important stabilizing compound protecting against denaturation and conformational changes that may lead to inactivation of proteins [18]. Glycerol, which is completely miscible in water, forms hydrogen bonds with the water, thus reducing the motion of the water molecules [19]. Glycerol, a product of the metabolism of triglycerides in adipose tissue, is converted to glycerol-3-phosphate by the enzyme glycerol kinase [20]. Through a series of reactions, glycerol-3-phosphate can be converted to glyceraldehyde-3-phosphate, which is an intermediate of both the glycolytic and gluconeogenic pathways. We recently described the metabolism of glycerol to formaldehyde by liver microsomes from chow-fed control rats [21]. In this report, we have further characterized this microsomal oxidation of the glycerol pathway. Rates of glycerol oxidation were evaluated in microsomes from pyrazole- and 4-methylpyrazole-treated rats, as well as in microsomes isolated from rats treated with a variety of other cytochrome P-450 inducing agents such as acetone and ethanol, which induce the same isozyme of cytochrome P-450 as pyrazole, and phenobarbital and 3-methylcholanthrene, which induce other isozymes of P-450 [22–25]. Experiments were also conducted to validate that formaldehyde is the product of the oxidation of glycerol by microsomes, and that the formaldehyde is indeed derived from the added glycerol.

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MATERIALS AND METHODS

Liver microsomes were isolated from male Sprague–Dawley rats weighing about 150 g that were given one of the following treatments intraperitoneally: pyrazole, 200 mg/kg body weight/day for 2 days; 4-methylpyrazole, 200 mg/kg body weight/day for 3 days; or 3-methylcholanthrene, 25 mg/kg body weight for 3 days. Comparable volumes of saline or corn oil were given as controls.

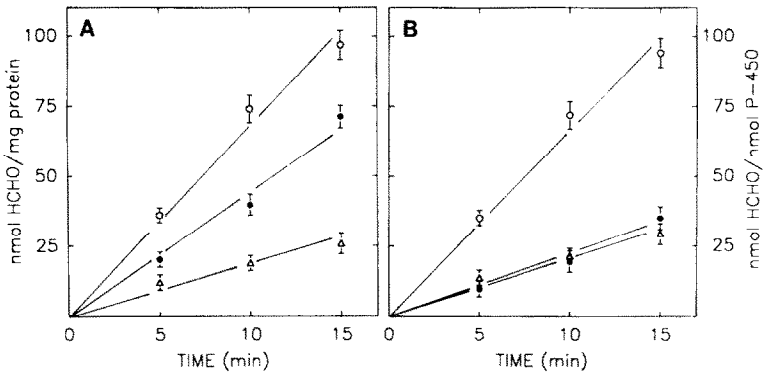

Fig. 1. Effect of incubation time on glycerol oxidation. The oxidation of 100 mM glycerol was assayed at 37°, as described under Materials and Methods, with increasing incubation time with microsomes from rats treated with saline (△), 4-methylpyrazole (●), and pyrazole (○). Results (mean ± SE) are from three experiments and are corrected for zero-time controls. Activity is expressed either per mg microsomal protein (A) or per nmol total cytochrome P-450 (B).

Other rats received either 0.1% (w/v) sodium phenobarbital or 1% (v/v) acetone in the drinking water for 7 and 10 days, respectively. Controls received an equivalent volume of water. These rats were starved overnight prior to being killed. For the chronic ethanol treatment, littermates were fed a liquid diet in which ethanol provided 36% of the total caloric intake, protein 18%, fat 35%, and carbohydrate 11% [26] for 3–4 weeks. Pair-fed controls were given the same diet except that carbohydrate isocalorically replaced the ethanol. The rats received their respective diets *ad lib.* on the day prior to being killed.

Microsomes were prepared by differential centrifugation, washed twice, resuspended in 0.125 M KCl, and stored at −70° at a protein concentration of about 10 mg/mL.

Oxidation of glycerol was assayed at 37° in a reaction mixture containing 100 mM potassium phosphate, pH 7.4, 10 mM $MgCl_2$, 0.4 mM $NADP^+$, 10 mM glucose-6-phosphate, 2.3 units of glucose-6-phosphate dehydrogenase, 100 mM glycerol and about 0.75 mg of microsomal protein in a total volume of 1 mL, unless stated otherwise. Reactions were initiated by the addition of glucose-6-phosphate plus glucose-6-phosphate dehydrogenase and terminated by the addition of trichloroacetic acid (final concentration of 6%, w/v). The reactions were routinely carried out for 10 min. Protein was removed by centrifugation in a table top centrifuge, and an aliquot of the supernatant fraction was used for determination of formaldehyde by the Nash reaction [27]. All values were corrected for zero-time controls in which the acid was added before initiation of the reaction with the NADPH-generating system. Experiments involving the effect of nitrogen or carbon monoxide were carried out as previously described [21].

For determination of formaldehyde involving formaldehyde dehydrogenase, the microsomes were removed by ultracentrifugation using an Airfuge (Beckman Instruments), and the supernatant fraction was incubated at 37° in the presence of 100 mM sodium phosphate, pH 8.0, 1.0 mM NAD^+, 2.0 mM glutathione and 1 unit of formaldehyde dehydrogenase until there was no further increase in absorbance at 340 nm [28]. The formate produced from this reaction was then converted to CO_2 by the addition of formate dehydrogenase plus NAD^+ [29]. When [^{14}C]glycerol was used, a 0.113 M solution of glycerol containing 20 dpm/nmol was added to the reaction mixture. The CO_2 formed as a result of the coupled formaldehyde plus formate dehydrogenase enzyme systems was trapped in 0.5 mL of 2 N NaOH and converted to $BaCO_3$ by the addition of a 5% $BaCl_2$ solution. The precipitate formed was collected on filter paper and counted in an LKB RackBeta 1217 scintillation counter. Parallel reactions were run with unlabeled glycerol for Nash determination.

Kinetic parameters were estimated from rate versus substrate curves by non-linear regression analysis using the HYPER program of Cleland [30] which was converted from FORTRAN to BASIC.

Protein was estimated by the method of Lowry *et al.* [31] using bovine serum albumin as standard. Cytochrome P-450 was determined by the method of Omura and Sato [32]. Values for product from the Nash reaction are based on a standard curve with known amounts of formaldehyde.

Glycerol, spectrophotometric grade, was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Formaldehyde dehydrogenase and formate dehydrogenase were from the Sigma Chemical Co. (St Louis, MO). All buffers were passed through a Chelex-100 column (Bio-Rad Laboratories, Richmond, CA) to remove contaminating iron. [^{14}C]Glycerol, uniformly labeled, was from the Amersham Corp. (Arlington Heights, IL).

RESULTS

The metabolism of glycerol was linear for at least 15 min in microsomes from rats treated with saline, pyrazole and 4-methylpyrazole (Fig. 1). The rate of glycerol oxidation to formaldehyde was highest in microsomes from rats treated with pyrazole and lowest in the saline control microsomes, when results were expressed per mg microsomal protein (Fig. 1A). Although pyrazole treatment does not change

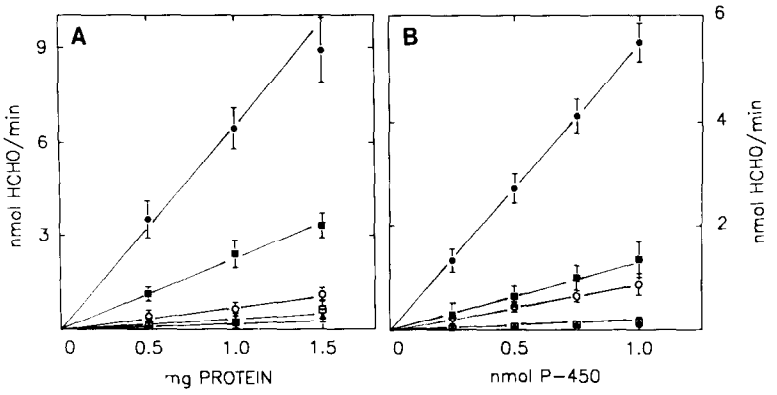

Fig. 2. Effect of microsomal protein concentration on glycerol oxidation. The oxidation of 100 mM glycerol was assayed at 37°, as described under Materials and Methods, with increasing concentrations of microsomal protein from rats treated with pyrazole (●), 4-methylpyrazole (■), saline (○), phenobarbital (□), and 3-methylcholanthrene (▲). Results are the mean (±SE) of three experiments with different preparations of microsomes and are corrected for controls which contained no microsomal protein. Activity is expressed either per mg microsomal protein (A) or per nmol total cytochrome P-450 (B).

significantly the total content of cytochrome P-450, 4-methylpyrazole treatment doubles the content of P-450 [6]. When rates of glycerol oxidation were expressed per nmol P-450, the turnover number for glycerol oxidation was increased about 4-fold after pyrazole treatment, whereas there was no significant change relative to the saline controls after 4-methylpyrazole treatment (Fig. 1B). No formaldehyde was produced in the absence of glycerol, microsomes or any of the components of the NADPH-generating system.

Rates of glycerol oxidation were linear with microsomal protein concentration up to at least 1.5 mg and were as much as three times higher in microsomes after pyrazole treatment than after 4-methylpyrazole treatment and eight times higher than that found in the saline controls (Fig. 2A). Similar to the time–course studies, rates with 4-methylpyrazole microsomes were also higher than saline controls over this range of protein when expressed per mg protein but not when expressed per nmol P-450 (Fig. 2, A and B).

Rates of glycerol oxidation to formaldehyde by microsomes isolated from rats treated with either phenobarbital or 3-methylcholanthrene were lower than in the controls when expressed in terms of nmol formaldehyde per min per mg protein (Fig. 2A). Since the total content of P-450 was elevated markedly by treatment with these agents, rates of glycerol oxidation by microsomes after treatment with phenobarbital or 3-methylcholanthrene were especially low when expressed per nmol cytochrome P-450 (Fig. 2B). In contrast to phenobarbital or 3-methylcholanthrene, treatment with acetone or chronic ethanol induces cytochrome P-450j [15, 25, 33], which appears to be identical to the pyrazole-induced P-450 isozyme [11]. In line with the above induction pattern for glycerol oxidation, rates of formaldehyde production from glycerol were increased about 3-fold by microsomes from rats treated chronically with either ethanol or acetone, as compared with their respective controls (Table 1). The elevated rates of glycerol oxidation were observed when results were expressed per mg microsomal protein or per nmol P-450 (Table 1).

Increasing the concentration of glycerol over the range of 2 to 100 mM resulted in an increase in formaldehyde production, and typical saturation kinetics were observed for the reaction (Fig. 3). The relative kinetic constants for glycerol metabolism were determined by non-linear regression of a Hanes–Wolf plot of the data (Fig. 3, inset, correlation coefficient of 0.99). With microsomes from pyrazole-treated rats, an apparent K_m value for glycerol of about 15 mM was calculated, whereas apparent V_{max} values were (nmol formaldehyde/min) 9.4 per mg microsomal protein or 6.8 per nmol cytochrome P-450 (Table 2). Typical saturation kinetics and linear Hanes–Wolf plots were also observed with microsomes from saline controls or rats treated with 4-methylpyrazole (data not shown). Microsomes from 4-methylpyrazole-treated and saline control rats had apparent K_m values for glycerol of about 11 and 18 mM respectively. V_{max} values per mg protein were about 3.6 and 2.6 nmol formaldehyde/min for the 4-methylpyrazole-treated and saline controls, respectively, while V_{max} values per nmol P-450 were about 2.1 and 3.5 respectively (Table 2). The increase in glycerol oxidation to formaldehyde by the pyrazole treatment appears to be associated with an increase in V_{max} rather than a change in the affinity for glycerol.

The effects of various modifiers of cytochrome P-450 function on glycerol metabolism by microsomes from pyrazole-treated rats are shown in Table 3. The effect of carbon monoxide on microsomal oxidation of glycerol was compared to an appropriate nitrogen control in order to rule out an anaerobic effect. Under an atmosphere of either 33 or 67% CO, the production of formaldehyde from glycerol was inhibited about 75% (Table 3). The cytochrome P-450j isozyme effectively catalyzes the oxidation of

Table 1. Effect of chronic treatment with ethanol or acetone on microsomal oxidation of glycerol

Treatment	Rate of glycerol oxidation		Effect of inducer	
	per mg protein	per nmol P-450	per mg protein	per nmol P-450
	nmol/min		%	
Pair-fed control*	1.15	2.27		
Chronic ethanol	3.58	5.71	+211	+151
Acetone control†	0.85 ± 0.18	1.15 ± 0.13		
Acetone	2.99 ± 0.40‡	4.28 ± 0.62‡	+251	+272

The oxidation of glycerol was assayed as described under Materials and Methods. The effect of inducer is based on the corresponding control taken as 100%.

* Results are the mean values of duplicates from two experiments; the difference between experiments did not exceed 10%.

† Results are the mean values from three experiments ± SD.

‡ $P < 0.01$ compared to acetone control.

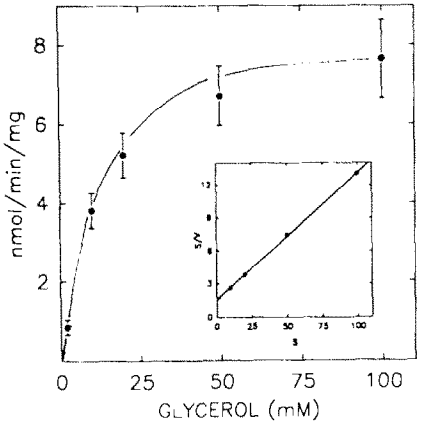

Fig. 3. Effect of the concentration of glycerol on the rate of metabolism of glycerol by microsomes from pyrazole-treated rats. Incubations were for 10 min at 37° with microsomes corresponding to 0.75 mg protein per sample. Results are the mean (±SE) of three experiments and are corrected for rates found in the absence of added glycerol. The inset shows a Hanes–Wolf plot of the substrate concentration curve ($r = 0.99$).

certain other drug substrates such as aniline, ethanol and pyrazole [9, 24, 25, 34]. The rate of glycerol oxidation to formaldehyde was decreased in the presence of aniline, ethanol, pyrazole, 4-methylpyrazole and imidazole (Table 3). Metyrapone, which is an effective inhibitor of drug oxidation catalyzed by the phenobarbital-induced P-450 isozyme, but not oxidations catalyzed by P-450j, had no effect on the oxidation of glycerol (Table 3).

4-Methylpyrazole was shown recently to be especially effective as an inhibitor of the oxidation of ethanol by microsomes isolated from rats treated with inducers of cytochrome P-450j [35, 36]. A concentration–response curve for 4-methylpyrazole inhibition of glycerol oxidation by microsomes from pyrazole-treated rats is shown in Fig. 4A. The kinetics of inhibition of glycerol oxidation by 4-methylpyrazole proved to be complex as 4-methylpyrazole increased the K_m for glycerol while lowering the V_{max} (Hanes–Wolf plot shown in Fig. 4B). The K_m for glycerol was elevated from a control value of 13.6 mM in the absence of 4-methylpyrazole to values of 24 and 31 mM in the presence of 0.5 and 3.0 mM 4-methylpyrazole respectively. The V_{max} (nmol formaldehyde per min per mg microsomal protein) was lowered from a control value of 7.2 in

Table 2. Kinetic parameters associated with the oxidation of glycerol by rat liver microsomes

Liver microsomes	K_m (mM)	V_{max} (nmol formaldehyde)	
		min/mg protein	min/nmol P-450
Control	17.60 ± 0.62	2.55 ± 0.63	3.53 ± 1.23
Pyrazole	15.25 ± 1.07	9.38 ± 1.88*	6.79 ± 0.42†
4-Methylpyrazole	10.87 ± 2.12	3.55 ± 0.82	2.10 ± 0.83

Rates of glycerol oxidation were determined using standard assay conditions over concentrations of glycerol ranging from 2 to 100 mM. The results are the means ± SD from duplicate experiments with three different preparations of microsomes from saline controls or rats treated with pyrazole or 4-methylpyrazole.

* $P < 0.005$ compared to control.

† $P < 0.01$ compared to control.

Table 3. Effects of various additions on the oxidation of glycerol by microsomes from pyrazole-treated rats

	Addition	Concentration (mM)	Rate of Glycerol oxidation (nmol/min/mg)	Inhibition (%)
(A)	None		4.99	
	Aniline	1.0	1.63	67
		3.0	1.03	79
	Ethanol	10	2.83	44
		50	2.68	46
	Pyrazole	0.5	2.60	47
		3.0	2.51	50
	4-Methylpyrazole	0.5	1.85	63
		3.0	1.38	72
	Imidazole	5	3.49	30
	Metyrapone	1	4.61	8
(B)	N_2 33%		6.95 ± 0.743	
	CO 33%		1.95 ± 0.495	72
	N_2 67%		5.93 ± 0.655	
	CO 67%		1.30 ± 0.127	78

Experiments in (A) were carried out using standard assay conditions with 25 mM glycerol. No inhibitor present was taken as 100%. All values were corrected for results obtained with the indicated additions but in the absence of glycerol. None of the additions in the absence of glycerol gave rise to significant Nash reactive material. Results are the mean values of two experiments where the difference between experiments did not exceed 10%. (B) Glycerol oxidation was assayed in the presence of 100 mM glycerol under standard conditions. N_2 controls were taken as 100%. Rates of glycerol oxidation under air were about 7 nmol per min per mg. Results are the mean values ± SD from three separate experiments.

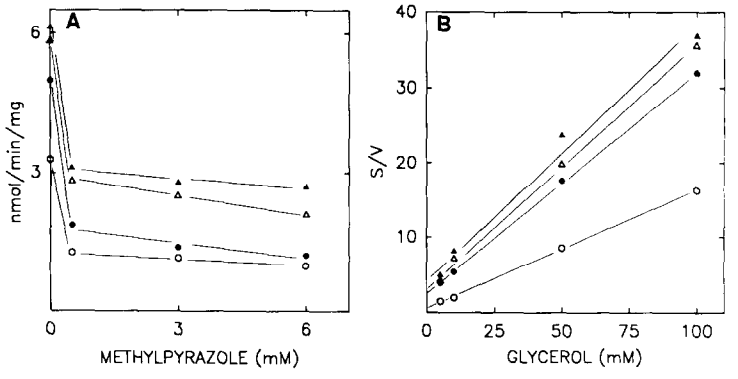

Fig. 4. Inhibition of microsomal oxidation of glycerol by the addition of 4-methylpyrazole. The effect of various concentrations of 4-methylpyrazole (0.5, 3 or 6 mM) on the rate of glycerol metabolism at glycerol concentrations of 5 mM (○), 10 mM (●), 50 mM (△), and 100 mM (▲) (panel A) is shown. A Hanes–Wolf plot of the data in panel A is shown in panel B.

the absence of 4-methylpyrazole to values of 6.4 and 5.9 in the presence of 0.5 and 3.0 mM 4-methylpyrazole respectively. An apparent K_i for 4-methylpyrazole as an inhibitor of glycerol oxidation of about 2.5 mM was calculated from plots of the slopes or the intercepts on the vertical axis of the Hanes–Wolf plots versus concentration of 4-methylpyrazole [37].

To verify that the Nash reactive material formed from the oxidation of glycerol by the microsomes was formaldehyde, a glutathione-dependent formaldehyde dehydrogenase was used to analyze the product. This enzyme is specific for formaldehyde and catalyzes the NAD^+ plus glutathione-dependent conversion of formaldehyde to formate. As is shown in Fig. 5, NAD^+ reduction coupled to the oxidation of the product of glycerol metabolism by microsomes can be demonstrated in the presence of formaldehyde dehydrogenase for a 20-min reaction sample, whereas no or very little reduction of NAD^+ was observed with a zero-time sample. The increase in absorbance at 340 nm was dependent on NAD^+, formaldehyde dehydrogenase and glutathione, strongly suggesting that the product of glycerol metabolism was indeed formaldehyde. The amount of formaldehyde produced from glycerol oxidation as detected by formaldehyde dehydrogenase was

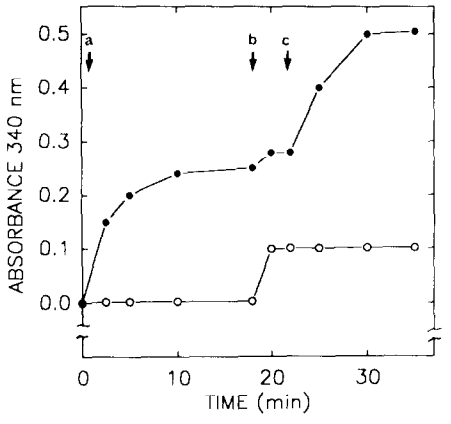

Fig. 5. Increase in absorbance at 340 nm during the formaldehyde and formate dehydrogenase reactions. Glycerol oxidation was assayed for 0 (○) and 20 (●) min, as described under Materials and Methods. The microsomes were removed by centrifugation, and the supernatant fraction was used for formaldehyde dehydrogenase and formate dehydrogenase reactions. The absorbance at 340 nm was initialized to zero, formaldehyde dehydrogenase was added (a) and the absorbance followed until there was no further increase. Upon addition of more NAD^+ (b), there was an increase in absorbance which levelled off quickly. This was followed by the addition of formate dehydrogenase (c) and the absorbance change followed until there was no further increase.

very similar to the amount of formaldehyde produced in duplicate samples as detected by the Nash procedure.

Further validation that the product of glycerol metabolism by the microsomes was formaldehyde was the observation that this product, after oxidation by formaldehyde dehydrogenase (presumably to formate), could be further oxidized by formate dehydrogenase in an NAD^+-dependent manner. As shown in Fig. 5, when the formaldehyde dehydrogenase reaction reached completion, additional NAD^+ (point b, Fig. 5) was added followed by formate dehydrogenase (point c, Fig. 5). There was an increase in absorbance at 340 nm for the 20-min experimental sample but not for the zero-time control. The net increase in absorbance by the formate dehydrogenase reaction (0.23 O.D. units) was identical to the net increase in absorbance by the formaldehyde dehydrogenase reaction (0.24 O.D. units).

A final set of experiments was conducted to demonstrate that the formaldehyde produced was actually derived from the added glycerol. [^{14}C]Glycerol was utilized as the substrate and the $^{14}CO_2$ produced from the combined formaldehyde and formate dehydrogenase reactions was trapped in sodium hydroxide, converted to $BaCO_3$, and counted for radioactivity (Table 4). Controls consisted of reactions kept on ice rather than at 37° or where no NADPH-generating system was added. In four separate experiments from reactions with incubation times of 20 min, approximately 30 nmol of CO_2 produced was calculated (Table 4). Formaldehyde was determined by the Nash reaction on parallel reactions with unlabeled glycerol. Approximately 80 nmol formaldehyde was detected using this method. Therefore, approximately 40% of the glycerol counts were recovered in the form of CO_2 which was somewhat lower than the 68% recovery found when standard [^{14}C]formaldehyde was used. This may reflect lack of completion of the coupled enzymatic reactions.

DISCUSSION

Cytochrome P-450IIE1 (P-450j) is thought to be induced under a variety of conditions that include fasting, acetone, chronic ethanol, isoniazid, pyrazole and 4-methylpyrazole treatment [9, 11, 15, 22–25, 33, 38–40]. Evidence for the various treatments inducing the same isozyme come from a variety of data including similarities in catalytic, spectral and immunological properties, amino-terminal sequence and, more recently, in complementary DNA sequence [23, 41]. The substrate specificity for this isozyme is broad and includes such diverse compounds as pyrazole, acetone, ethanol, 2-butanol, dimethylnitrosamine, aniline, *p*-nitroanisole, carbon tetrachloride, *n*-pentane, and diethyl ether. We have shown recently that glycerol can be oxidized to formaldehyde by control microsomes by a carbon monoxide-sensitive reaction [21]. The increase in rates of glycerol oxidation (per nmol P-450) in microsomes from rats treated with pyrazole, acetone and ethanol, over those of corresponding controls, suggests that cytochrome P-450j plays an important role in glycerol oxidation by microsomes. By contrast, the phenobarbital- or 3-methylcholanthrene-inducible P-450 isozymes do not appear to be effective in metabolizing glycerol to formaldehyde. Recent studies showed that an antibody raised against the purified pyrazole P-450 isozyme recognizes a 52,000 M_r antigen in control microsomes; the intensity of the immunoblot is increased with microsomes isolated from rats treated with pyrazole, 4-methylpyrazole, acetone or ethanol, but is decreased with microsomes from rats treated with phenobarbital or 3-methylcholanthrene [11]. These results suggest that the increased oxidation of glycerol induced by the former agents, but not the latter, reflects induction of the P-450j isozyme. It appears that glycerol may be added to the increasing number of low molecular weight substrates which are preferentially oxidized by the cytochrome P-450IIE family. In experiments to be described elsewhere (Clejan and Cederbaum, manuscript submitted for publication), cytochrome P-450IIE1 purified from pyrazole-treated rats oxidized glycerol to formaldehyde when reconstituted with NADPH–cytochrome P-450 reductase plus dilaurylphosphatidylcholine.

That cytochrome P-450 is involved in the oxidation of glycerol to formaldehyde by pyrazole-induced (Table 3) or control microsomes [21] can be demonstrated by the sensitivity to carbon monoxide. Moreover, a variety of compounds that are known to be effective substrates for the cytochrome P-450IIE1 isozyme were also inhibitors of the reaction with glycerol. In other experiments, we tested whether the reaction of glycerol with hydroxyl radicals could

Table 4. Oxidation of [^{14}C]glycerol to formaldehyde and subsequent conversion to $^{14}CO_2$

Reaction condition		Radioactivity dpm	Radioactivity Net dpm*	Formaldehyde (nmol)
[^{14}C]Glycerol in water†		688		
Reaction kept on ice		1532		
Minus NADPH-generating system		1865		
Minus microsomes		678		
20-min Reaction‡	(a)	2170	471	23.6
	(b)	2284	585	29.3
	(c)	2473	774	38.7
	(d)	2371	672	33.6

* Net dpm was obtained by subtracting the average of the controls with no generating system and the reaction kept on ice from the actual dpm (1699 dpm average).

† [^{14}C]Glycerol was placed in water, and the reaction with formaldehyde and formate dehydrogenase was carried out.

‡ Represents four different samples in which the initial reaction with microsomes was carried out for 20 min.

give rise to formaldehyde (Tsuei and Cederbaum, unpublished observations). Hydroxyl radicals were generated either via the ferric-EDTA catalyzed autooxidation of ascorbate, the coupled oxidation of hypoxanthine by xanthine oxidase, or by NADPH-dependent microsomal electron transfer in the presence of ferric-EDTA [42]. With all three reaction systems, there was little or no production of formaldehyde from glycerol, whereas formaldehyde was readily generated from dimethyl sulfoxide (DMSO) (a known product of the interaction of ·OH with DMSO [43]). It appears that glycerol oxidation to formaldehyde reflects a cytochrome P-450-mediated catalytic activity rather than interaction with ·OH generated by the microsomes.

Although 4-methylpyrazole treatment increased the oxidation of glycerol when results were expressed per mg protein, this increase appeared to be due to the increase in total P-450 content produced by the administration of 4-methylpyrazole; no significant increase in glycerol oxidation was observed when results were expressed per nmol P-450. These results with glycerol are similar to results observed with ethanol and 2-butanol as substrates [6, 7]. The 4-methylpyrazole treatment increased the intensity of two or three bands in the P-450 region as shown by sodium dodecyl sulfate (SDS)-gel electrophoresis, one of which corresponded to the P-450 induced by pyrazole [7]. Recent results suggest that in addition to inducing P-450j, 4-methylpyrazole may also induce isozymes similar to those induced by phenobarbital [23]. Since glycerol is an effective substrate for oxidation by P-450j, but not by the phenobarbital-inducible isozyme (Fig. 2), it is likely that any increased oxidation of glycerol by induction of P-450j is compensated by enrichment of non-glycerol-preferring isozymes of P-450 by the 4-methylpyrazole treatment. Hence, no increase in glycerol oxidation occurs when results are expressed as a turnover number, per nmol of total P-450.

When added *in vitro*, 4-methylpyrazole was an effective inhibitor of ethanol oxidation by microsomes from rats treated with inducers of P-450j, but not microsomes from rats treated with phenobarbital or 3-methylcholanthrene [35, 36]. The effective inhibition of glycerol oxidation by added 4-methylpyrazole (Fig. 4) most likely reflects this enhanced binding and inhibitory capability of 4-methylpyrazole in microsomes from rats treated with pyrazole and other inducers of P-450j. The mixed type of inhibition kinetics against glycerol oxidation was also observed for inhibition of ethanol oxidation by 4-methylpyrazole [35, 36].

The Nash reaction is relatively but not totally specific for formaldehyde [27]. Since glyceraldehyde could theoretically be a product (e.g. via hydroxylation at carbon one or three of glycerol, followed by loss of H_2O), we tested glyceraldehyde in the Nash reaction, and found that although it was slightly reactive, it was only reactive at very high concentrations and the color produced in the reaction with glyceraldehyde was not enough to account for the absorbance at 412 nm that we observed following glycerol oxidation. By use of a coupled enzyme system, further evidence that the product of glycerol oxidation was indeed formaldehyde was provided. The formaldehyde produced from the glycerol was converted to formate by the NAD^+-glutathione-dependent enzyme formaldehyde dehydrogenase. The formate thus produced was converted to CO_2 by the enzyme formate dehydrogenase. The amount of formaldehyde produced was equivalent to the amount of formate oxidized by formate dehydrogenase and equal to the amount of formaldehyde detected by the Nash reaction. When [^{14}C]glycerol was used as the substrate in the initial reaction, $^{14}CO_2$ was produced as a result of the coupled enzyme system, thus indicating that the formaldehyde produced as a consequence of the monooxygenase reaction was derived from the added glycerol.

In summary, these results suggest that glycerol is not metabolically inert when added to microsomes, but rather, glycerol can serve as an effective substrate for oxidation by cytochrome P-450j. Previous results indicated that primary [9, 22, 39] and secondary [10]

alcohols are good substrates for this P-450; this appears to be the first indication that a poly-ol is also an effective substrate. Indeed, the K_m for ethanol oxidation by microsomes from either saline controls or pyrazole-treated rats was about 14 mM, which is very similar to the K_m values for glycerol oxidation by these microsomal preparations. It is also possible that glycerol may compete with certain substrates for metabolism, especially if oxidation is occurring primarily via the P-450j isozyme. Preliminary experiments indicate that glycerol, albeit at high levels, inhibits the oxidation of ethanol and of pyrazole by microsomes from pyrazole-treated rats. Yoo *et al.* [44] have already demonstrated that glycerol is a competitive inhibitor of the metabolism of *N*-nitrosodimethylamine by microsomes isolated from rats treated with acetone.

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