ETHANOL-INDUCED CYTOCHROME P-450: CATALYTIC ACTIVITY AFTER PARTIAL PURIFICATION.

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SUMMARY

Cytochrome P-450 was partially purified from liver microsomes obtained from control, ethanol, phenobarbital, and 3-methylcholanthrene-treated rats. Benzphetamine demethylation, benzpyrene hydroxylation and aniline hydroxylation activities were assayed in a reconstituted system using fixed amounts of reductase and lipids, and increasing amounts of cytochrome P-450 from each source. Cytochrome P-450 from ethanol-fed rats showed substrate specificity differing from cytochrome P-450 obtained from control, phenobarbital and 3-methylcholanthrene-treated rats.

INTRODUCTION

The liver microsomal mixed function oxidase system has been solubilized and resolved into three components, cytochrome P-450, NADPH-cytochrome c reductase and lipid (phosphatidylcholine), all of which are essential for the hydroxylation of a variety of substrates (1-5). Using this reconstituted system, the cytochrome P-450 induced by the administration of PB[1] and that induced by the administration of MC[1] have been shown to differ by their spectral (2), catalytic (6,7,8) and antigenic (8,9) properties, as well as by their apparent molecular weight (7).

Chronic ethanol administration increases liver microsomal proteins and phospholipids (10), cytochrome P-450 content (11) and the activity of various drug-metabolizing enzymes (12,13). It is still unknown whether the enhancement by ethanol of the activity of various microsomal drug metabolizing enzymes can be attributed to an increase in cytochrome P-450 content and/or to the induction of a catalytically more active form of cytochrome P-450. A cytochrome P-450

[1]Abbreviations used are: PB: phenobarbital; MC: 3-methylcholanthrene.

species showing high affinity for cyanide has been reported as preferentially induced by ethanol (14,15). Ullrich recently reported that tetrahydrofurane specifically inhibited 7-ethoxycoumarin O-dealkylation in ethanol-treated rats, and postulated the appearance of a particular species of cytochrome P-450 after ethanol treatment (16). However, there is so far no direct evidence that ethanol treatment results in the appearance of a form of cytochrome P-450 different from others by its catalytic activity.

In the reconstituted system, using fixed amounts of the same reductase and lipid, and varying the source of the hemoprotein, it is possible to compare the relative catalytic activity of cytochrome P-450 from various sources. If the hemoproteins are identical, the rate of metabolism of various substrates should be the same, when equal amounts of hemoproteins are used. Using this system, we provide evidence that cytochrome P-450 from ethanol-treated rats has a catalytic activity different from that of cytochrome P-450 obtained from control, PB and MC-treated rats.

MATERIAL AND METHODS

Female Sprague-Dawley rats weighing 125-150 g were fed a nutritionally adequate liquid diet (17) for 21 days and divided in four groups: a control group, an ethanol-fed group, in which ethanol (36% of total calories) was isocalorically substituted for dietary carbohydrates, a MC-treated group (25 mg/kg i.p. daily on days 18-19-20), and a PB-treated group (75 mg/kg i.p. daily on days 18-19-20). Rats of each group had similar caloric intake (46-48 cal./day). All animals were sacrificed on day 21, and liver microsomes were prepared as described by Lu et al. (1). For each group of rats, cytochrome P-450 was partially purified according to Levin et al. (2). NADPH-cytochrome c reductase (5) and lipid fraction (1) were partially purified from microsomes obtained from ethanol-fed rats.

Cytochrome P-450 was measured by the method of Omura and Sato (18) as modified by Van der Hoeven et al. (4). NADPH-cytochrome c reductase activity was determined according to Masters et al. (19), and phospholipids by the method of Rouser et al. (20). One unit of reductase is defined as the amount of reductase that reduces one nmole of cytochrome c per minute. Benzphetamine N-demethylation was assayed by following the rate of NADPH oxidation (3). Benzpyrene hydroxylation was determined by measuring the rate of formation of the radioactive products of benzpyrene (21) and aniline hydroxylation by the formation of p-aminophenol (22). Assay conditions are given in the legends of figures. Protein was determined by the method of Lowry et al. (23).

RESULTS AND DISCUSSION

Results of the purification procedure of each cytochrome P-450 are given in Table I. The final preparation (step III) resulted in a 3.2 to 4.7-

TABLE I.

PARTIAL PURIFICATION OF CYTOCHROME P-450 FROM RAT LIVER MICROSOMES

	cytochrome P-450			reductase	
	nmoles/mg protein		relative purification	units/nmole P-450	
	microsomes	step III		microsomes	step III
Control [a]	0.83	3.19	3.8	101	18
Ethanol-treated[a]	1.32	4.32	3.3	105	19
PB-treated[b]	1.49	4.77	3.2	97	18
MC-treated[b]	1.16	5.48	4.7	74	21

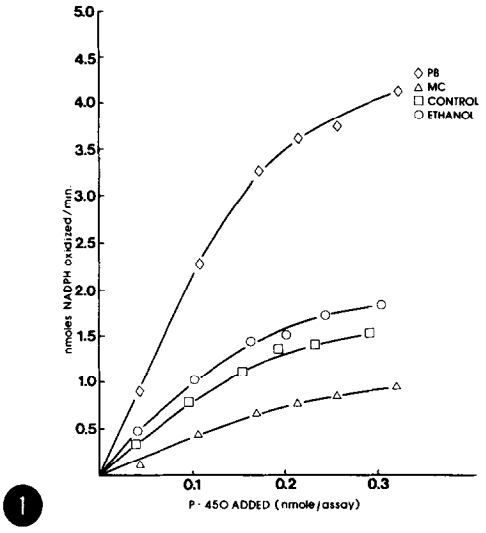
[a]mean of 6 purification experiments

[b]one purification experiment

fold increase in specific activity. This degree of purification is in perfect agreement with that reported by Levin et al. (2) using the same method. In the final preparations, contaminating NADPH-cytochrome c reductase, when expressed in units per nmole of cytochrome P-450, accounted for ±20% of the reductase activity in microsomes (Table I). The content of cytochrome b_5 and phospholipid, when expressed per nmole of cytochrome P-450, had been respectively reduced to 10% and 3% of the initial microsomal content.

The purification procedure for NADPH-cytochrome c reductase originating from microsomes of ethanol-treated rats resulted in a 10 fold increase in enzyme activity; the preparation had a specific activity of 900 to 1100 units per mg of protein, and contained no cytochrome P-450. The lipid fraction, also prepared from microsomes of ethanol-treated rats, was free of cytochrome P-450 and NADPH-cytochrome c reductase.

The activity of each cytochrome P-450 was tested in the presence of benzphetamine, benzpyrene and aniline in the reconstituted system. These



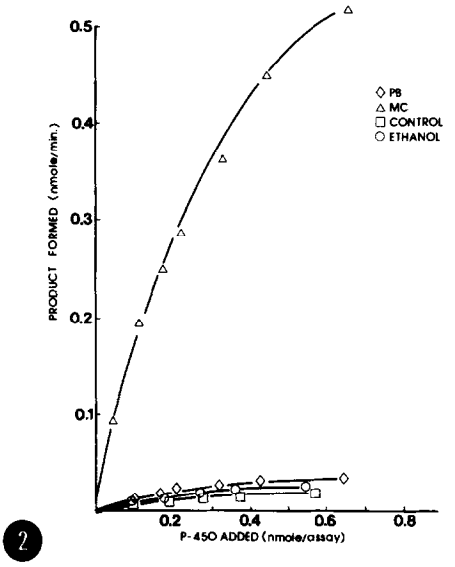


Figure 1. Benzphetamine N-demethylation.
The reaction mixture, in a final volume of 1.0 ml, contained potassium phosphate buffer 100 µmoles (pH 7.4, 30°C), $MgCl_2$ 3 µmoles, benzphetamine 1 µmole, NADPH 0.1 µmole, reductase 150 units, lipid fraction 29 µg phospholipids and the indicated amounts of hemoproteins. The reaction was initiated by the addition of NADPH.

Figure 2. Benzpyrene hydroxylation.
The reaction mixture, in a final volume of 1.0 ml, contained potassium phosphate buffer 100 µmoles (pH 7.0, 37°C), $MgCl_2$ 3 µmoles, [^{3}H]-3,4-benzpyrene 10 nmoles (0.5 µCi), NADPH 0.4 µmole, reductase 150 units, lipid fraction 29 µg phospholipids, and the indicated amounts of hemoproteins. The reaction was initiated by the addition of NADPH.

activities were assayed using fixed amounts of reductase and lipid, and increasing amounts of each cytochrome P-450.

PB-cytochrome P-450 was much more active for the N-demethylation of benzphetamine (fig. 1), MC-cytochrome P-450 was more active for the hydroxylation of 3,4-benzpyrene (fig. 2) and ethanol-cytochrome P-450 was more active for the hydroxylation of aniline (fig. 3). Control-cytochrome P-450 had low activity in the presence of all three substrates.

Since the amounts of reductase and lipid were kept constant in these experiments, we can therefore conclude that the various hemoproteins have different substrate specificity. Such differences have been repeatedly

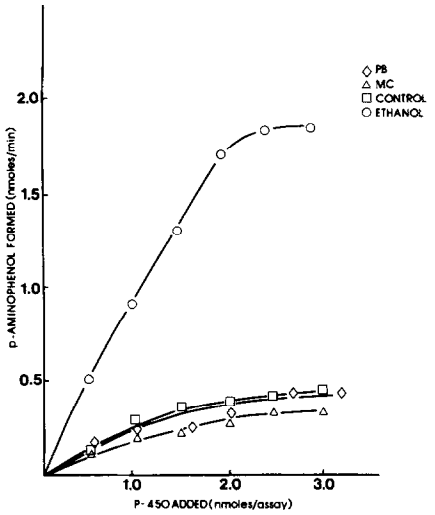


Figure 3. Aniline hydroxylation.
The reaction mixture, in a final volume of 1.0 ml, contained Tris-HCl buffer 50 µmoles (pH 7.4, 37°C), glycerol 10% v/v, $MgCl_2$ 10 µmoles, aniline 8 µmoles, NADP 1 µmole, glucose-6-phosphate 5 µmoles, glucose-6-phosphate dehydrogenase 2 units, reductase 400 units, lipid fraction 29 µg phospholipids, and the indicated amounts of hemoproteins. The reaction was initiated by the addition of the NADPH-generating system.

demonstrated for PB-cytochrome P-450, MC-cytochrome P-450 and control cytochrome P-450 (2,3,4,6,7,24). The present study provides direct evidence that chronic ethanol feeding results in the appearance of a form of cytochrome P-450 differing by its catalytic activity from cytochrome P-450 of control, PB or MC-treated rats.

Reported increases in microsomal benzphetamine demethylation (13) and benzpyrene hydroxylation (12) in ethanol-treated rats can be explained mainly by quantitative changes in microsomal cytochrome P-450 content. By contrast, we have recently reported that ethanol increases cytochrome P-450 content (52%) much less than it enhances aniline hydroxylation activity (169%) (25). The appearance, after ethanol, of a new form of cytochrome P-450, showing a high catalytic activity for aniline, explains the apparent discrepancy between the reported effects of chronic ethanol administration on cytochrome P-450 and aniline hydroxylation activity (25).

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