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Ethanol-, Fasting-, and Acetone-Inducible Cytochromes P-450 in Rat Liver: Regulation and Characteristics of Enzymes Belonging to the IIB and IIE Gene Subfamilies†

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ABSTRACT: Two major forms of hepatic microsomal cytochrome P-450 were purified from starved and acetone-treated rats. On the basis of amino acid sequence analysis, they were identified as P-450j and P-450b. Ethanol or acetone treatment of rats caused a 9-fold increase in the amount of P-450j in liver microsomes accompanied by similar increases in the rate of NADPH-dependent metabolism of carbon tetrachloride, acetone, and benzene. Immunological experiments indicated that P-450j constitutes the major catalyst of the microsomal metabolism of the latter agents and contributes by about 50% to microsomal P-450-dependent ethanol oxidation under the conditions used. The P-450j-dependent catalytic activities had a high rate of turnover. In contrast, this was not the case for the immunodetectable P-450j, indicating the occurrence of inactive forms of this protein in microsomes. Starvation or ethanol or acetone treatment caused 10–30-fold increases in the amount of both mRNA and apoprotein of P-450b,e compared to control. Run-on experiments and the concomitant increases of the P-450b,e gene products at the mRNA and protein levels indicated the appearance of mainly a transcriptional activation by acetone, ethanol, or starvation. Fasting exerted, in addition, a pronounced synergistic effect on acetone-dependent induction of P-450b,e mRNA (3-fold), apo-P-450b,e (4.3-fold), P-450j mRNA (2-fold), and apo-P-450j (2-fold). No increase of mRNA coding for P-450j, compared to control, was seen after acetone or ethanol treatment alone. The results indicate that effects of ethanol, acetone, and/or starvation on drug and xenobiotic metabolism are caused by the induction of P-450 forms belonging to at least two gene subfamilies.

Ethanol is known to affect drug metabolism in two principally different ways. The acute effect of the alcohol is mainly exhibited by inhibition of drug oxidation, probably caused by competitive interactions with cytochromes P-450 (Rubin & Lieber, 1968; Rubin et al., 1970, 1971). The chronic effect of ethanol on drug metabolism is the opposite, i.e., an increase of the metabolism rate [cf. Linnoila et al. (1979) and Khanna et al. (1976)], and includes a proliferation of the smooth endoplasmic reticulum (Iseri et al., 1966), as well as an increase in the amount of hepatic cytochrome P-450 (Rubin et al., 1968; Villeneuve et al., 1976; Ekström et al., 1986). The rate of drug clearance from the blood is enhanced after ethanol consumption [cf. Kater et al. (1969)], as examplified by the elimination of, e.g., meprobamate and pentobarbital from the blood of man or rats (Misra et al., 1971). The alcohol effect on clearance appears mainly due to an enhanced rate of hepatic metabolism of the compounds, as evidenced by studies with meprobamate (Misra et al., 1971), aminopyrine (Vesell et al., 1971), propanolol (Prichard & Schneck, 1977), and rifamycin (Grassi & Grassi, 1975).

Ethanol treatment of rats results in an enhanced rate of microsomal metabolism of halogenated hydrocarbons, e.g., carbon tetrachloride (CCl_4)[1] (Maling et al., 1975), chloroform

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[1] Abbreviations: P-450, cytochrome(s) P-450; PEG, poly(ethylene glycol); HFBA, heptafluorobutyric acid; TBA, thiobarbituric acid; A, rats treated with acetone (5 mL/kg) for 1 day; SA, rats starved for 24 h and subsequently treated with acetone (5 mL/kg) for 1 day; SA^2, rats starved for 24 h and subsequently treated with acetone (5 mL/kg) for 2 days; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; CCl_4, carbon tetrachloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid. The gene nomenclature is given in Nebert et al. (1987).

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(Sato et al., 1980; Sato & Nakajima, 1985), trichloroethylene (Sato & Nakajima, 1985), and 1,2-dichloroethane (Sato et al., 1980; Sato & Nakajima, 1985), and of hydrocarbons like, e.g., toluene (Wallen et al., 1984; Sato & Nakajima, 1985), benzene (Sato & Nakajima, 1985), and pentane (Terelius & Ingelman-Sundberg, 1986). The ethanol-dependent induction of the microsomal metabolism apparently causes a synergistic effect of ethanol on the toxicity of halogenated and aromatic compounds like CCl_4 (Hasumura et al., 1974), chlorpromazine (Teschke et al., 1980), halothane (Ishii et al., 1983), and benzene (Baarson et al., 1982). Induction of cytochrome P-450 as a cause of increased toxicity of ethanol is also evident with nitrosoamines (Maling et al., 1975; Olson et al., 1984; Yang et al., 1985) and acetaminophen (Sato et al., 1981; Walker et al., 1983).

The molecular basis for these synergistic effects of ethanol was clarified upon purification and characterization of an ethanol-inducible form of cytochrome P-450 from rabbit (P-450 3a) (Koop et al., 1982; Ingelman-Sundberg & Hagbjörk, 1982) and rat liver (P-450j) (Ryan et al., 1985; Patten et al., 1986). Compared to other forms of P-450, this type of high-spin cytochrome P-450 is very active in the metabolism of aniline (Morgan et al., 1982), ethanol (Morgan et al., 1982; Ingelman-Sundberg & Johansson, 1984), *N*-nitrosodimethylamine (Patten et al., 1986; Levin et al., 1986), acetone (Johansson & Ingelman-Sundberg, 1985b; Johansson et al., 1986; Koop & Casazza, 1985), acetaminophen (Coon et al., 1984), pentane (Terelius & Ingelman-Sundberg, 1986), and CCl_4 (Johansson & Ingelman-Sundberg, 1985a). The ethanol-dependent induction of this P-450 form thus provides an explanation for the synergistic effect of ethanol on the metabolism and toxicities of these compounds. Besides ethanol, structurally diverse compounds like pyrazole (Ingelman-Sundberg & Jörnvall, 1984), imidazole (Ingelman-Sundberg & Jörnvall, 1984; Koop et al., 1984), isoniazid (Ryan et al., 1985), benzene (Ingelman-Sundberg & Hagbjörk, 1982),[2] and acetone (Johansson & Ingelman-Sundberg, 1985b; Johansson et al., 1986; Koop & Casazza, 1985) are also inducers of P-450j or the homologous rabbit form P-450 3a.

We here present results indicating that ethanol and, in particular, acetone are inducers of cytochrome P-450b, the main phenobarbital-inducible form of P-450 in rat liver. Indeed, this P-450 type appears to be the quantitatively most important P-450 isozyme in liver microsomes from acetone-treated rats. In addition, the mechanisms of induction of P-450j and P-450b by acetone were investigated and compared to the effects seen upon starvation of the animals, since fasting or a low-carbohydrate diet has been described to exert ethanol-like inductive properties (Gadeholt et al., 1983; Rao et al., 1986; Miller & Yang, 1984; Teschke et al., 1981; Strubelt et al., 1981; Nakajima & Sato, 1979).

Experimental Procedures

Materials

Chemicals Used. Acetone and benzene were obtained from Merck, EDTA was from Fluka, NADPH was from Sigma, and TPCK-treated trypsin (212 units/mg) was from Cooper Biomedical. [^{14}C]Acetone (sp act. 25.5–57 mCi/mmol) and [^{14}C]benzene (sp act. 20–60 mCi/mmol) were purchased from New England Nuclear (NEN). Acetonitrile and heptafluorobutyric acid (HFBA) were purchased from Rathburn Chemicals (Walkerburn, Scotland).

[2] I. Johansson and M. Ingelman-Sundberg, submitted for publication.

Animals Used. Most studies were performed with male Sprague-Dawley rats (150–170 g). In total, nine different groups of animals were used. One group of rats was starved for 24 h and subsequently injected with acetone (5 mL/kg) intragastrically for 2 days (SA^2). These rats received no food but water ad libitum and were killed 18–24 h after the last injection. A second group of rats (SA) was treated with acetone only for 1 day but was otherwise identical with SA^2. Control rats to these animals (C) received food and water ad libitum until 24 h before sacrifice, from which time they were starved. In some studies, fed and watered rats treated with acetone (5 mL/kg) for 1 day were used (A). A fifth group of rats (E) was fed the liquid alcohol diet (Bioserv, Frenchtown, NJ), described by DeCarli and Lieber (1967), ad libitum for 20 days. Control rats to these animals (CE) were pair fed the control liquid diet, which was supplied once a day. Other rats (PB) were treated with phenobarbital (80 mg/kg) by intraperitoneal injections for 3 consecutive days and starved 24 h before sacrifice. The run-on experiments were performed with male Wistar rats (200 g) treated overnight with phenobarbital (100 mg/kg) or starved for 1 day and subsequently injected with an intragastric dose of acetone (5 mL/mg) (SA).

Methods

Isolation of Liver Microsomes. The livers were homogenized in 2 volumes of 10 mM sodium/potassium phosphate buffer, pH 7.4, containing 1.14% (w/v) KCl. The microsomes were isolated by centrifugation of the homogenate at 10000*g* for 10 min and by ultracentrifugation of the resulting supernatant at 100000*g* for 60 min. The microsomal pellet was washed once in the salt solution and subsequently suspended in 50 mM potassium phosphate buffer, pH 7.4. The microsomes were stored under nitrogen at −70 °C at a concentration of about 30 mg of protein/mL.

Purification of Cytochromes P-450. Cytochromes P-450 were purified from male rats that had been pretreated with acetone (SA^2) or phenobarbital. The livers were homogenized in 2 volumes of 1.14% KCl (w/v), containing 10 mM EDTA, and the microsomes were prepared by centrifugation (see above). The microsomal pellets were washed once in the KCl solution and subsequently suspended in 10 mM Tris-HCl, pH 7.4, containing 20% glycerol and 1 mM EDTA (buffer A) to a concentration of about 50 mg of protein/mL. The microsomes (usually corresponding to 2.5–3 g of microsomal protein) were solubilized with sodium cholate and fractionated with poly(ethylene glycol) (PEG) as described by Koop et al. (1982) for the purification of isozyme 3a from the rabbit. The PEG pellet was dialyzed against buffer A and subsequently solubilized with Renex 690 to a final concentration of 0.5%. The material was subjected to DEAE-Sepharose (4 × 25 cm, 2000 nmol of P-450 per column) equilibrated in buffer A containing 0.5% Renex 690. The columns were eluted with similar buffers containing 0, 10, 15, and 20 mM K_2SO_4.

Preparation of j-Type Cytochrome P-450. The 0 mM K_2SO_4 fraction (I in Figure 1) from the DEAE-Sepharose columns was dialyzed twice against 10 volumes of 10 mM potassium phosphate buffer, pH 6.5, containing 20% glycerol and 1 mM EDTA and submitted to CM-Sepharose chromatography, phosphocellulose chromatography, and calcium phosphate gel treatment, essentially according to the procedures described by Ryan et al. (1986).

Preparation of b,e-Type Cytochromes P-450. The cytochrome P-450 containing fractions from the 15 and 20 mM K_2SO_4 eluates of the DEAE-Sepharose columns (two pools with 60–200 nmol of P-450 each) were dialyzed twice against 4–5 volumes of 10 mM potassium phosphate buffer, pH 6.0,

containing 20% glycerol and 1 mM EDTA. The pH of the protein fractions was adjusted with HCl to 6.0. The fractions were then applied on separate CM-Sepharose columns (2 × 10 cm) equilibrated with the phosphate buffer containing 0.5% Renex 690. The columns were eluted with similar buffers containing 10, 25, 50, 100, 150, and 200 mM phosphate. Catalase was usually eluted in the 100 mM phosphate fraction. The 150 and 200 mM fractions were dialyzed against 15 volumes of 20% glycerol containing 1 mM EDTA. The protein fractions were applied to separate columns (1 × 6 cm) of hydroxylapatite–cellulose (1:1) equilibrated with 10 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol. The columns were washed free of detergent with the equilibration buffer, and cytochrome P-450 was eluted with 300 mM potassium phosphate, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, and 0.25% (w/v) sodium cholate. The preparations were finally dialyzed twice against 50 volumes of 100 mM potassium phosphate, pH 7.4, containing 20% glycerol and 0.2 mM EDTA.

Preparation of Anti-P-450 IgG. Male rabbits (2.5 kg) were immunized with 75 μg of either the b,e-type P-450 or the j-type P-450 isolated from liver microsomes of acetone-treated rats. The first injection was in 50% (v/v) Freund's complete adjuvant, whereas booster injections (at 4-week intervals) were in 50% Freund's incomplete adjuvant. The IgG fraction was prepared from the antisera by ammonium sulfate precipitation (Elshourbagy & Guzelian, 1980). Preimmune IgG was prepared in an identical manner.

Amino Acid Sequence Analysis. Proteins were subjected to liquid-phase sequencer degradations after application into glycine-precycled Polybrene (Jörnvall & Philipson, 1980), with a Beckman 890 D instrument. Peptides were similarly analyzed with an Applied Biosystems 790 A gas-phase sequencer. Phenylthiohydantoin derivatives were detected by HPLC on a Nucleosil C_{18} (5 μm) column utilizing an acetonitrile gradient elution system modified (Kaiser et al., 1988) from Zimmerman et al. (1977).

Oligonucleotide Probe. An oligodeoxyribonucleotide complementary to bp 771–820 of the P-450j sequence published by Song et al. (1986) was synthesized on an Applied Biosystems 380 B DNA synthesizer. The probe was 5′-end labeled with [γ-^{32}P]ATP (sp act. 3000 Ci/mmol, NEN) and T_4 polynucleotide kinase (NEN), essentially as described by Richardson (1965). The specific activities of the oligomers used for hybridization were (2–6) × 10^6 dpm/pmol.

RNA Blots. Total RNA was isolated from polysomes obtained after sucrose gradient centrifugation of homogenized livers in the presence of heparin (25 mg/mL) [cf. Blobel and Potter (1967) and Taylor and Schimke (1973)]. The polysomes were extracted with phenol–*m*-cresol–chloroform (1:0.14:1 by volume), and RNA was precipitated with ethanol. Blot hybridization to [α-^{32}P]UTP-labeled RNA probes complementary to P-450b,e mRNA (PB-1) was performed as described elsewhere (Scholte et al., 1985). Blot hybridization to ^{32}P-end-labeled oligonucleotide probe for P-450j was performed at 60 °C with 2 × 10^6 dpm/mL oligomer as described in Davis et al. (1986). The nitrocellulose filter was washed 3 times at 60 °C with 1× SCC containing 0.1% SDS.

Run-On Experiments. The run-on experiments were carried out according to Pike et al. (1985). Hepatic nuclei were isolated by differential centrifugation and stored at −70 °C in a sterile solution of 20 mM Tris-HCl, pH 7.8, containing 75 mM NaCl, 0.5 mM EDTA, 50% (w/v) glycerol, 0.15 mM PMSF, and 1 mM DTT. Transcription by nuclei in vitro was carried out in the presence of [α-^{32}P]UTP (Amersham).

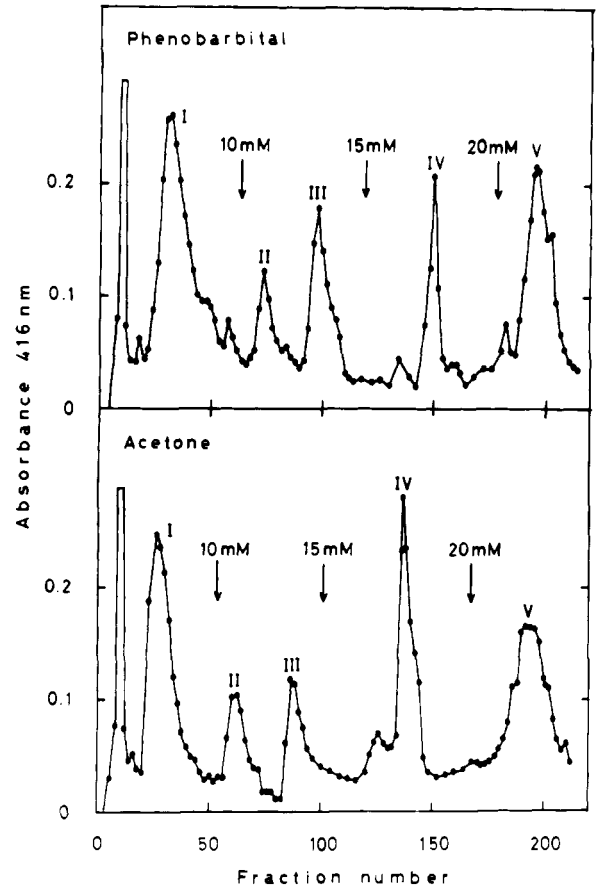

FIGURE 1: DEAE-Sepharose chromatography of cholate-solubilized and poly(ethylene glycol)-precipitated [7–14% (v/v)] liver microsomes from acetone-treated (bottom) or phenobarbital-treated rats (top). Elution was achieved by a stepwise increase of the K_2SO_4 concentration as indicated by the arrows. P-450j was isolated from fraction I (acetone-treated rats). Fraction V was further chromatographed on CM-Sepharose for isolation of the b type of P-450. Conditions are as described under Experimental Procedures.

Hybridization was performed to a linearized GEM 4 vector containing a full-length P-450b cDNA insert, immobilized on nitrocellulose filters [cf. Scholte et al. (1985)]. The linearized GEM 4 vector itself was used to estimate nonspecific hybridization.

Assay Methods. Published methods were followed in the assays given below: radial immunodiffusion (Thomas et al., 1979; Ingelman-Sundberg & Jörnvall, 1984), protein content (Lowry et al., 1951), acetone oxidation (Johansson et al., 1986), benzene oxidation (Johansson & Ingelman-Sundberg, 1983), ethanol oxidation (Lieber & DeCarli, 1970; Ingelman-Sundberg & Johansson, 1981), CCl_4-dependent lipid peroxidation (Johansson & Ingelman-Sundberg, 1985a), N-demethylation of benzphetamine (Nash, 1953), and cytochrome P-450 (Omura & Sato, 1964). Benzene was added from a stock solution in water. Incubations with CCl_4 and benzphetamine contained 10 μM EDTA in order to inhibit superoxide anion dependent lipid peroxidation [cf. Ekström and Ingelman-Sundberg (1986)]. Microsomal incubations with ethanol were performed in the presence of 0.5 mM sodium azide.

RESULTS

Structural Analysis of Purified Cytochromes P-450. Microsomes from acetone-treated and from phenobarbital-treated rats were solubilized with cholate, fractionated with poly(ethylene glycol), and chromatographed on DEAE-Sepharose. Figure 1 shows that the chromatograms obtained

Table I: N-Terminal Amino Acid Sequence Analysis of the Two Types of Cytochrome P-450 Isolated from Liver Microsomes of Acetone-Treated Rats and of Peptide Fractions Obtained after Digestion of Cytochrome P-450 (V-150) (Isolated from Acetone-Treated Rats, cf. Methods) with either CNBr or Trypsin[a]

	amino acid sequence
protein fraction	
j fraction	AVLGITIALLVWVAILLVI-
V-150	MEPSILLLLALLVGFLLLLVRG--KS-
peptide fraction	
CNBr-1	EKEKSNHHTEFHHEN-
CNBr-4	LKYPHVAEKVQKEIDQVIGSHRLPTLDDRSK-
	PYTDAVIHEIQR---L-P-GVPHRVTK-
CNBr-5	FRGYLLPKNTEVYPILSSALHDPQYF-
	PYTDAVIHEIQRFSDLVPIGVPHRVTKD-
trypsin-5	ATLDPSAP
trypsin-52	SNHHTEFHHENLMISLL-LFFAGTETS-

[a] The initial yields in the protein sequence analysis were 65% (j fraction) and 85% (V-150). The designation of the peptide fractions refers to Figures 3 and 4. In two cases, the peptide sequences were determined in a two-component mixture. Assignments for both sequences were then made by comparison of the relative intensities of the two amino acids in each cycle.

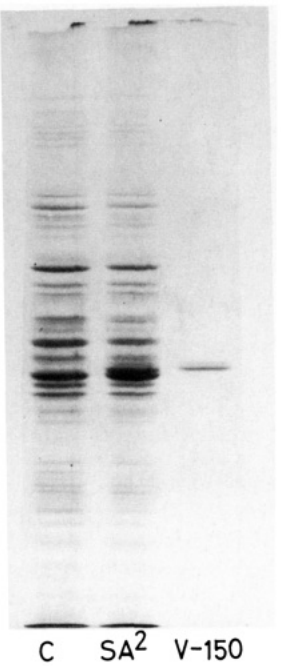

FIGURE 2: SDS–polyacrylamide gel electrophoretic analysis of liver microsomes isolated from control rats (C), acetone-treated rats (SA^2), and the major P-450 fraction purified from the latter type of microsomes (V-150). The electrophoresis was carried out in 8.5% polyacrylamide gels according to Laemmli (1970).

with both types of microsomal preparations were almost identical. However, fraction I had, in samples from acetone-treated rats, a high-spin component in the Soret band, which was absent from similar samles of phenobarbital-treated rats. The high-spin component was further purified to electrophoretic homogeneity (see Experimental Procedures). The final preparation was entirely in the high-spin form (not shown). N-Terminal sequence analysis revealed the amino acid sequence given in Table I. This sequence is identical with that reported for cytochrome P-450j (Ryan et al., 1986). The isolated protein fraction was therefore identified as P-450j.

Fraction V from both types of microsomes, and as obtained from DEAE-Sepharose columns, was chromatographed on CM-Sepharose at pH 6.0. Elution with 150 and 200 mM phosphate yielded fractions essentially homogeneous on SDS–polyacrylamide gel electrophoresis (cf. Figure 2).

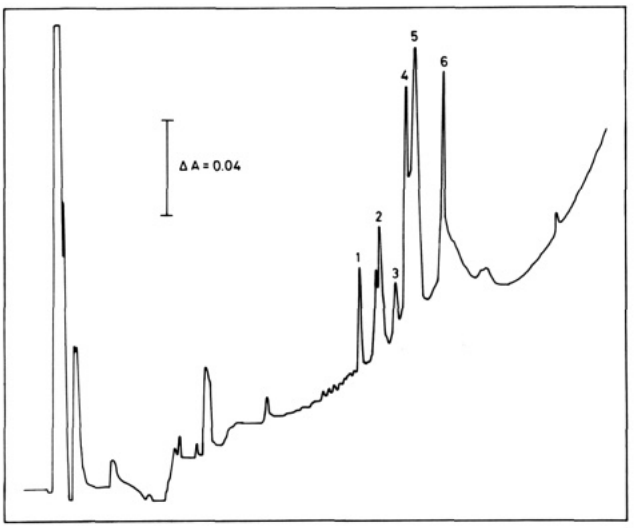

FIGURE 3: HPLC analysis of soluble peptides of the major cytochrome P-450 fraction (V-150) from liver microsomes of acetone-treated rats, after digestion with CNBr. The cytochrome P-450 fraction (5 nmol) was dialyzed against 100 volumes of 30% acetic acid overnight. The sample was taken to dryness under nitrogen, and the residue was dissolved in 1 mL of 70% (v/v) formic acid. CNBr (0.2 g) was added, and the digestion was performed overnight at room temperature. The solution was taken to dryness, and the residue was washed twice with 50% (v/v) formic acid. The residue was subsequently suspended in water containing 0.1% (v/v) HFBA. After centrifugation, aliquots of 200 μL of the supernatant were injected onto the HPLC column [Hibar, LiChrosorb RP-8 (7 μm), 0.4 × 25 cm (Merck)]. The peptides were eluted with a linear gradient (100 min, 1 mL/min) of water to 100% (v/v) 2-propanol–acetonitrile (1:3) (solvent B). The solvents contained 0.1% (v/v) HFBA. The absorbance at 254 nm was registered.

N-Terminal amino acid sequence analysis of the 150 mM fraction from acetone-treated rats (Table I) revealed an amino acid sequence identical with that described for cytochromes P-450b and P-450e (Yuan et al., 1983). SDS–polyacrylamide gel electrophoretic analysis of microsomes from control (C) and acetone-treated (SA^2) animals and of the 150 mM fraction indicates that the latter fraction corresponds to the major protein induced in liver microsomes upon treatment of the rats with acetone (Figure 2).

Since the cytochrome P-450b,e gene family has been shown to be composed of several members (Atchison & Adesnic, 1983; Mizukami et al., 1983), it was considered important to evaluate which form of cytochrome P-450 was isolated from acetone-treated animals. The 150 mM fraction was therefore subjected to digestion with CNBr and trypsin. HPLC analysis of the CNBr peptides revealed the occurrence of six major fractions (Figure 3). N-Terminal amino acid sequence analysis of three of the fractions were carried out, and the results are shown in Table II. The peptide sequences determined correspond to the amino acid sequence of cytochrome P-450b starting at methionine residues in positions 272 (CNBr-1), 314 (CNBr-4), 346 (CNBr-4 and CNBr-5), and 376 (CNBr-5) [cf. Yuan et al. (1983)].

The 150 mM fractions from both phenobarbital-treated and acetone-treated rats were treated with trypsin. HPLC analysis of the peptides showed an almost identical chromatographic pattern (Figure 3). N-Terminal amino acid sequence analysis of peptides 5 and 52 isolated from acetone-treated rats (Table I) revealed that these originated from positions 254–261 and 277–307, respectively, in the cytochrome P-450b sequence. Fraction 44 actually consisted of the heme group. Interestingly, the heme group reproducibly precipitated together with peptides 51 and 52 in preparations from acetone-treated rats (cf. Figure 4). Since peptide 52 includes the sequence of two

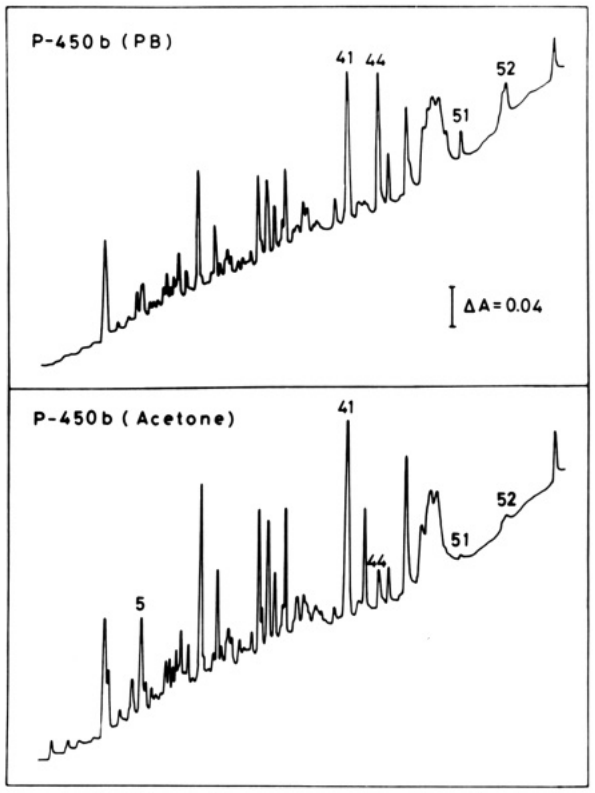

FIGURE 4: HPLC analysis of tryptic peptides from the cytochrome P-450b fraction obtained from liver microsomes of phenobarbital-treated rats (top) and from the same fraction isolated from acetone-treated rats (bottom). To each protein fraction (0.5 mg of protein) in 0.5 mL of 100 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.2 mM EDTA, was added 100 μL of trypsin (1 mg/mL). The tubes were incubated at 37 °C for 2 h and subsequently at room temperature for 16 h. Before HPLC analysis, the fraction from acetone-treated rats was centrifuged at 5000*g* for 15 min, yielding a clear colorless supernatant. Aliquots of 200 μL of the samples (supernatant in the case of acetone-inducible P-450) were injected onto the HPLC column [Hibar, LiChrosorb RP-8 (7 μm), 0.4 × 25 cm (Merck)]. Elution was performed as described in the legend to Figure 3, except that the linear gradient contained at maximum 70% of solvent B. The absorbance at 254 nm was registered. The fractions were numbered in order of elution.

histidine doublets at positions 279–280 and 284–285 and since the heme group most probably is bound to Cys-436 [cf. Black and Coon (1986)], is appears plausible that heme binds to these histidine residues. The selective precipitation of these peptides in preparations from acetone-treated rats remains unexplained.

In total, sequence analysis of 147 amino acids of the protein in the 150 mM fraction from acetone-treated rats were recovered. The material was found to be identical with corresponding published sequences of cytochrome P-450b. The area sequenced covers 9 out of the 12 amino acid substitutions between P-450b and P-450e. It is therefore concluded that the protein in the 150 mM fraction is identical with cytochrome P-450b.

Effect of Pretreatment of Rats on P-450 at Protein and mRNA Levels. Antibodies against cytochromes P-450b and P-450j isolated from acetone-treated rats were raised in rabbits and utilized for the quantification (by radial immunodiffusion) of these apoproteins in liver microsomes (Table II). The results showed an apparent 9-fold induction of apo-P-450j in microsomes from acetone- (SA^2) or ethanol-treated rats, compared to controls. By contrast, this protein was almost absent from microsomes of phenobarbital-treated animals. The level of cytochromes P-450b and P-450e, recognized by the P-450b antibodies, was on the other hand highest in microsomes from phenobarbital-treated microsomes (>200-fold higher than the control level), intermediate in microsomes from acetone-treated (SA^2) rats (>100-fold induction), and smaller in microsomes from ethanol-treated rats (3-fold higher than pair-fed controls).

Table II: Apparent Levels of mRNA and Apoprotein of Cytochromes P-450b,e and P-450j in Rat Liver Microsomes, As Determined by Dot Blot Analysis and Radial Immunodiffusion, Respectively[a]

animal treatment	mRNA level (%)[b]		amount of cytochrome P-450		
	P-450b,e	P-450j	P-450b,e (%)[c]	P-450j (%)[c]	total P-450 (nmol/mg of protein)[d]
control (C) (5)	<5	58	<0.6	11 ± 11	0.58 ± 0.07
starvation (S) (5)	13	nd[e]	8 ± 2	31 ± 14	0.9 ± 0.03
acetone (A) (4)	28	45	23 ± 11	56 ± 22	0.99 ± 0.22
acetone + starvation (SA^2) (5)	100	100	100 ± 16	100 ± 9	1.40 ± 0.13
phenobarbital (PB) (4)	310	48	225 ± 22	3 ± 2	2.01 ± 0.15
ethanol diet (E) (5)	10	67	8 ± 2	100 ± 8	1.15 ± 0.14
control diet (CE) (5)	3.5	20	<0.6	12 ± 8	0.46 ± 0.04

[a] The animal groups are specified under Materials. The number of animals is indicated within parentheses. [b] The mRNA level is determined from the dot blot experiments (cf. Figure 5) and related to the amount in starved and acetone-treated rats; two to three different animals in each group were used here; [c] The apoprotein levels are related to the amount present in starved and acetone-treated rats; the 100% value corresponds to about 0.4 nmol/mg for P-450j and 0.7 nmol/mg for P-450b; [d] Determined spectrophotometrically. [e] nd, not determined.

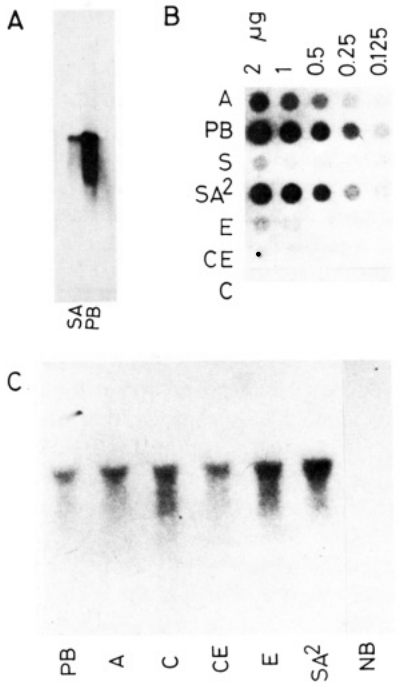

FIGURE 5: Northern blot (A and C) and dot blot (B) analysis of hepatic mRNA coding for P-450b,e (A and B) and P-450j (C) from control (C), acetone-treated (A), starved (S), phenobarbital-treated (PB), newborn (NB), fasted and acetone-treated (SA^2), ethanol-fed (E), or control liquid-fed (CE) rats. Northern blot analysis was carried out with 1 μg of hepatic poly(A)-rich RNA obtained by purification on poly(U)–Sepharose (A) or with 10 μg of total RNA (C). The agarose gel electrophoresis was performed under denaturing conditions.

Table III: Effect of Treatment of Ethanol, Acetone (SA^2), and Phenobarbital on the Rat Liver Microsomal Metabolism of Cytochrome P-450 Substrates[a]

substrate		rate of metabolism			
		control	ethanol	acetone (SA^2)	phenobarbital
acetone, 1.36 mM	Pr	0.20 ± 0.03	1.11 ± 0.23	1.53 ± 0.17	0.26 ± 0.07
	P-450	0.35 ± 0.07	0.81 ± 0.19	1.23 ± 0.20	0.15 ± 0.03
aminopyrine, 3.4 mM	Pr	3.77 ± 0.30	6.63 ± 0.55	7.13 ± 0.53	11.8 ± 0.87
	P-450	5.97 ± 0.63	4.83 ± 0.60	4.81 ± 0.59	6.65 ± 0.53
benzene, 25 μM[b]	Pr	0.009 ± 0.001	0.17 ± 0.03	0.19 ± 0.05	0.02 ± 0.003
	P-450	0.023 ± 0.007	0.12 ± 0.02	0.15 ± 0.04	0.01 ± 0.001
benzphetamine, 0.5 mM	Pr	3.10 ± 0.12	5.67 ± 0.70	14.2 ± 1.4	21.8 ± 1.3
	P-450	5.69 ± 0.40	4.05 ± 0.38	9.45 ± 1.0	12.2 ± 1.2
CCl_4, 2.15 mM[c]	Pr	0.19 ± 0.05	1.98 ± 0.30	3.46 ± 0.53	0.80 ± 0.07
	P-450	0.33 ± 0.09	1.71 ± 0.17	1.97 ± 0.20	0.46 ± 0.05
ethanol, 50 mM	Pr	4.72 ± 0.68	10.6 ± 1.33	15.5 ± 1.3	5.85 ± 0.74
	P-450	8.03 ± 0.95	7.59 ± 0.43	10.9 ± 1.2	3.28 ± 0.40

[a] Incubations were carried out with liver microsomes corresponding to 1 mg of protein (0.5 mg with CCl_4) in a total volume of 1 mL of 50 mM potassium phosphate buffer, pH 7.4 (2 mL with ethanol as substrate), containing 0.3–0.5 mM NADPH. The reactions were started by the addition of NADPH and performed at 37 °C for 2 (benzphetamine and CCl_4), 3 (aminopyrine), 8 (ethanol), 15 (benzene), or 20 min (acetone). Four to six animals in each group were used, and the rate of metabolism is expressed as nanomoles per milligram of protein (Pr) and per nanomole of cytochrome P-450 (P-450) per minute. [b] The amount of water-soluble products was quantified. [c] The values represent nanomoles of TBA-reactive substances formed in the presence of 10 μM EDTA.

The amount of mRNA coding for P-450b,e and P-450j in livers from control and treated rats was determined by dot blot and Northern blot analysis (Figure 5). The ^{32}P-labeled P-450b probe used [PB-1 (Scholte et al., 1985)] recognized mRNA with an approximate length of 2000 nucleotides in samples from both acetone- and phenobarbital-treated rats. The ^{32}P-labeled oligonucleotide coding for bp 771–820 in the P-450j mRNA sequence (Song et al., 1986) recognized mRNA with an approximate length of 2000 nucleotides in preparations from all types of animals examined (Figure 5). The signal was absent when RNA preparations from newborn rats were used [cf. Song et al. (1986)]. Treatment of the rats with phenobarbital caused a >80-fold induction of P-450b,e mRNA, whereas a >20-fold increase was registered after acetone treatment in combination with starvation. Starvation and acetone treatment separately induced P-450b,e mRNA 2.5- and 5.5-fold, respectively (Table II). Chronic ethanol treatment of the rats caused a 3-fold increase in the P-450b,e mRNA level compared to pair-fed controls. Accordingly, similar differences were seen between the different groups of animals both at the protein level and at the mRNA level (cf. Figure 5 and Table II). In contrast, this relationship was not true for P-450j. mRNA coding for P-450j was increased (2-fold) only in samples from starved and acetone-treated rats (Figure 5 and Table II). Ethanol, acetone, or phenobarbital treatment did not significantly influence the level of P-450j mRNA, which is interesting in view of the totally different effects of these agents on P450j at the protein level (Table II).

Contributions of Cytochromes P-450b,e and P-450j to Microsomal Oxidations. The different types of microsomes were incubated with various substrates in the presence of NADPH. As shown in Table III, ethanol or acetone treatment (SA^2) caused increases to similar extents of most enzyme activities, except for benzphetamine N-demethylation where acetone was a more potent inducer. Expression of the enzyme activities in relation to the amount of cytochrome P-450j revealed good correlation between the rate of benzene, acetone, or CCl_4 metabolism in the various liver microsomal fractions and the relative level of P-450j in microsomes from acetone-(SA^2) and ethanol-treated rats (cf. Tables II and III). A difference between microsomes from ethanol- and acetone-treated rats was seen in the rate of benzphetamine N-demethylation, probably caused by the different relative contents of P-450b in the two types of microsomes.

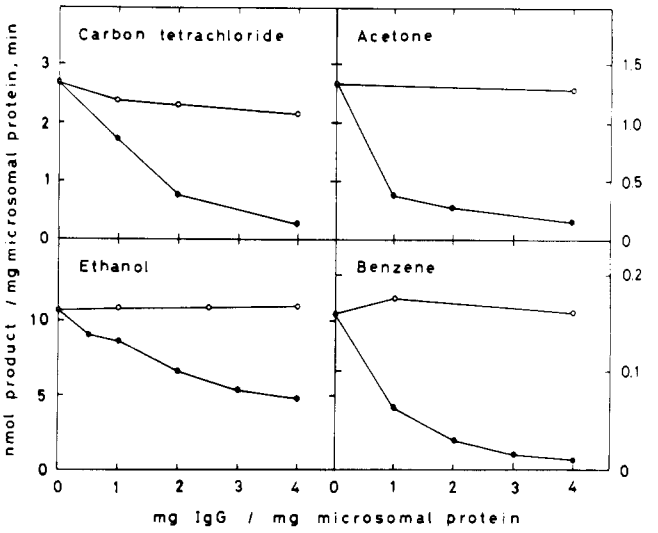

FIGURE 6: Effect of anti- (P-450j) IgG (●) on the NADPH-dependent rate of metabolism of acetone, benzene, ethanol, and CCl_4 in liver microsomes from starved and acetone-treated rats (SA^2). Control incubations were performed with preimmune IgG (○).

The relative contribution of cytochrome P-450j to the microsomal NADPH-dependent metabolism of CCl_4, acetone, ethanol, and benzene was further evaluated by incubations of microsomes from starved and acetone-treated rats (SA^2), in the presence of progressive amounts of anti-P-450j IgG (Figure 6). At 4 mg of anti-P-450j IgG per milligram of microsomal protein an almost complete inhibition of the NADPH-dependent metabolism of CCl_4, acetone, and benzene was achieved, whereas the rate of ethanol oxidation at maximum was decreased by 50%. This corresponds to an almost complete inhibition of the inducible activity (cf. Table III). Preimmune IgG had no effects. These results thus indicate the contribution of other types of cytochromes P-450 than P-450j to the microsomal ethanol oxidation. However, P-450j appears almost exclusively responsible for the activity of the three other microsomal activities examined.

Effect of Time on Induction of P-450 Enzymes and Activities. An attempt to investigate mechanisms behind the induction of P-450b,e and P-450j as well as of their enzyme

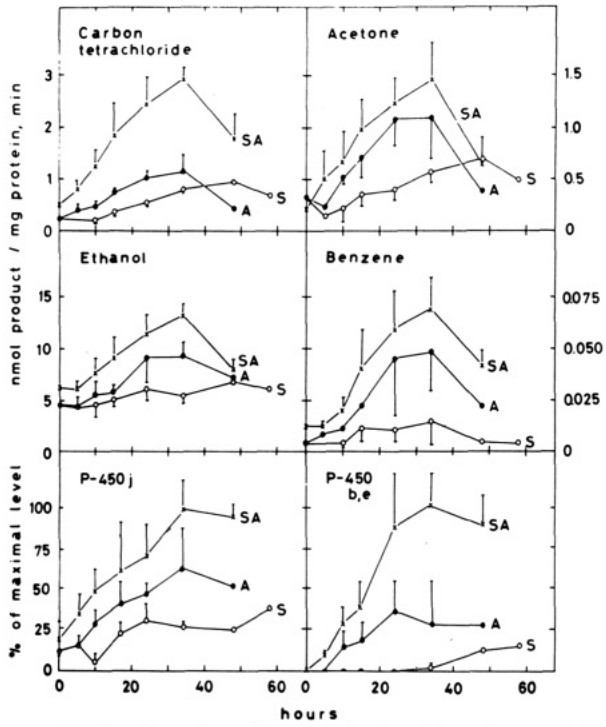

FIGURE 7: Time dependency for the induction of cytochromes P-450j and P-450b,e as well as cytochrome P-450 dependent metabolism of acetone, benzene, ethanol, and CCl_4 in liver microsomes after treatment of rats with acetone (A), by starvation (S), or with acetone and starvation (SA). Acetone-treated rats (A) were injected intragastrically at 8 a.m. with 5 mL of acetone/kg and received food without restriction. Another group of rats (SA) was initially starved for 24 h and subsequently treated with 5 mL/kg acetone at 8 a.m., whereafter they continued their starvation. The third group of rats (S) was placed on starvation at 8 a.m. (time zero in the figure). The content of b,e-type P-450 and j-type P-450 in the microsomes was determined by radial immunodiffusion [cf. Ingelman-Sundberg and Jörnvall (1984)] with antiserum raised in rabbits. Three to five rats were used in each group. When four or more rats were used, the values are shown as mean ± SD.

activities was carried out by determination of these variables at different time intervals after treatment of rats by starvation and/or acetone (Figure 7). Starvation alone caused an induction of both types of cytochromes P-450 and their enzyme activities examined, with a maximal effect after about 50 h. Acetone treatment of fed rats resulted in a 5–10-fold increase in the rate of CCl_4, acetone, and benzene metabolism as well as of the level of P-450j and P-450b. A 2-fold increase was observed in the rate of ethanol oxidation. A lag phase of about 10 h was seen, and the maximal effect was registered after about 30 h. Starvation of the rats for 24 h before acetone treatment (SA) had a pronounced effect on the extent of induction of enzyme activities and protein levels. In general, the induction caused by acetone treatment was amplified by 50–250%. The synergistic effect of starvation was especially significant for induction of P-450b and P-450e. After 30 h, the enzyme activities examined soon returned to basal or near-basal levels, whereas this was not true for the apoenzyme levels (Figure 7).

Run-on Transcriptional Analysis. To study the cause of the increased P-450b,e mRNA levels in the livers of acetone-treated rats, run-on experiments were performed with isolated nuclei. As shown in Figure 8, an enhanced production of P-450b-type sequences was seen in nuclei isolated from livers of starved and acetone-treated rats (SA) as well as in nuclei from livers of phenobarbital-treated rats, compared to control (C). The apparent relative transcription rates inferred from the run-on experiments correlated with the relative mRNA

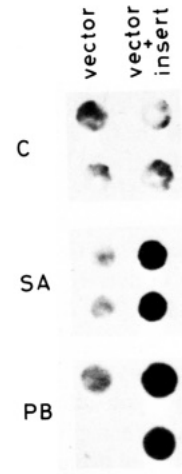

FIGURE 8: Transcriptional run on assay of the P-450b gene. The GEM 4 vector, containing a full-length cDNA insert of cytochrome P-450b, and the GEM 4 vector itself were linearized, bound to nitrocellulose filters, and hybridized to in vitro labeled transcripts from nuclei isolated from control (C), phenobarbital-treated (PB), and acetone-treated (SA) rats. The amount of label bound to the filters was, after washing, visualized by autoradiography.

levels in the dot blot experiments.

Discussion

The results indicate that acetone and ethanol in rat liver induce proteins belonging to at least two different cytochrome P-450 gene subfamilies. Acetone preferentially causes induction of P-450b,e and to a lesser extent P-450j, whereas the situation is reversed in rats on an ethanol diet. The number of members in the gene subfamily corresponding to the P-450j-type of protein seems restricted to 1 or 2 (Song et al., 1986; Wrighton et al., 1986; Khani et al., 1987), whereas 9–11 members of the cytochrome P-450b,e gene subfamily have been described (Fujii-Kuriyama et al., 1982; Adesnic & Atchinson, 1986). Accordingly, it was considered important to identify which member of this latter gene subfamily was induced following treatment with acetone. The major form of cytochrome P-450 isolated from acetone-treated rats was therefore digested with trypsin and CNBr. Sequence analysis of 147, out of a total of 491, amino acid residues revealed an amino acid sequence identical with that published for P-450b (Yuan et al., 1983; Fujii-Kuriyama et al., 1982). On the basis of the facts that (i) the chromatographic profile on DEAE-Sepharose of microsomes from acetone-treated rats was identical with that obtained with microsomes from phenobarbital-treated rats (Figure 1), (ii) the major isolated protein fraction of microsomes from acetone-treated rats corresponds to the major protein band induced in the microsomes according to SDS–polyacrylamide gel electrophoresis, and (iii) a high content of this type of protein in microsomes from acetone-treated rats has been immunologically determined (Figure 7 and Table II), we suggest that P-450b is the quantitatively dominating type of P-450 in these microsomes. Results indicative of induction of P-450b by acetone have also recently been reported by Song et al. (1986).

Cytochromes P-450b and P-450e have previously been shown to be induced by exogenous compounds other than phenobarbital, e.g., by polychlorinated biphenyls (Parkinson et al., 1983; Scholte et al., 1985), *trans*-stilbene oxide (Thomas et al., 1981), and 1,1-bis(chlorophenyl)-2,2-dichloroethylene (Yoshioka et al., 1984). This induction has hitherto been coupled to an increase in the level of the corresponding mRNA (Hardwick et al., 1983; Morohashi et al., 1984; Scholte et al., 1985), indicating the involvement of a transcriptional acti-

vation. In the present study, acetone, ethanol, phenobarbital, and starvation all induced the mRNA corresponding to P-450b (Figure 5 and Table II). The relative level between the amount of mRNA present and the content of P-450b in the microsomes was strikingly similar in the different groups (Table II). The run-on experiments (Figure 8) indicated a transcriptional P-450b gene activation after acetone and phenobarbital treatment in parity to the relation of the P-450b,e mRNA level in the two groups. These results therefore indicate that acetone induces P-450b almost exclusively at the transcriptional level and that the enhanced P-450b,e mRNA level after starvation and ethanol treatment is compatible with a transcriptional activation also in these cases.

Measurements of catalytic activities (Table III) indicated that N-demethylation of benzphetamine to a great extent appears to be related to the presence of cytochrome P-450b in microsomes from acetone- (SA^2) and phenobarbital-treated rats. An immunological support for this suggestion was not obtained, since the antisera produced did not inhibit P-450b-dependent activities (not shown). By contrast, immunochemical evidence was obtained indicating that P-450j catalyzes all microsomal metabolism of CCl_4, benzene, and acetone (Figure 6). The extent of induction and the temporal behavior of these activities after acetone treatment (SA) correlated well with the apparent level of P-450j in the microsomes (Figure 7) up to 30 h after acetone injection. In addition, all inducible microsomal NADPH-dependent ethanol oxidation was, on the same grounds, dependent on the presence of P-450j in the membranes. However, 50% of the rate of ethanol oxidation in microsomes from acetone-treated rats (SA^2) and apparently 100% of the similar activity in microsomes from control rats (C) were dependent on other, presently unknown, forms of cytochrome P-450 or due to activity of oxygen radicals generated by the presence of chelated non-heme iron in the microsomal preparations [cf. Ingelman-Sundberg and Johansson (1984)].

Several differences in the regulation of P-450b and P-450j and their corresponding enzyme activities are apparent from the present study. P-450b induction by acetone exhibits a lag phase, whereas P-450j is more promptly induced (Figure 7). The P-450j-dependent catalytic activities rapidly return to basal levels, indicating a high rate of turnover of the protein. This is in accordance with the rapidly declining rate of ethanol oxidizing activity of liver microsomes following removal of ethanol from the rat diet (Morgan et al., 1981). However, immunologically detectable P-450j in the microsomal fraction remained constant even 50 h after the treatment (Figure 7). This indicates that the antibodies recognize inactive forms of the protein and suggests the action of regulatory components, such as specific proteases, in the endoplasmic reticulum or in other intracellular compartments.

A difference in the regulation of P-450b and P-450j was also observed in relation to starvation, which had a much more pronounced synergistic effect on acetone-dependent induction of P-450b than of P-450j (Figure 7). Recent results by Song et al. (1986) have indicated that induction of P-450j by pyrazole and acetone involves activation of posttranscriptional levels, since induction of the protein determined immunologically did not correlate to a comparably increased amount of the corresponding mRNA. Furthermore, results by Hong et al. (1987) indicate that fasting causes an enhancement of mRNA corresponding to P-450j in rat liver. On the basis of the observed increase of P-450j mRNA seen after acetone treatment only in combination with starvation (Figure 5 and Table II), it appears likely that the synergistic effect of starvation in this case is caused by an enhanced rate of P-450j gene transcription or P-450j mRNA stabilization. Stress hormones, such as thyroxine, appear likely candidates as participants in the regulation of P-450b and P-450j at the mRNA level, although much work is necessary in order to examine this possibility. However, the primary sites of action of ethanol, acetone, and other inducers of P-450j are, according to results reached in hepatocyte cultures, at the protein level, where they upon binding to P-450j apparently induce a stabilization of this protein (Eliasson et al., 1988).

Phenobarbital-treatment reduced the level of P-450j to 30% of the control value but did not have any effect on the amount of P-450j mRNA (Table II). It remains to be established whether the turnover time of P-450j is decreased under these conditions.

In conclusion, our results suggest that P-450b is induced mainly, if not exclusively, at the transcriptional level by the endogenous compound acetone, by ethanol, and by hormonal or metabolic conditions that are present under conditions of fasting. This indicates a role of this type of P-450 in the metabolism of endogenous compounds as well as of drugs under alcoholic conditions. In contrast, P-450j seems to be regulated at several cellular levles, most importantly by posttranscriptional mechanisms. This protein appears of considerable importance for the synergistic action of ethanol on the toxicity of aromatic and halogenated hydrocarbons.

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Defective Catabolism and Abnormal Composition of Low-Density Lipoproteins from Mutant Pigs with Hypercholesterolemia†

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ABSTRACT: Metabolic and chemical properties of low-density lipoproteins (LDLs) were studied in a strain of pigs carrying a specific apo-B allele associated with hypercholesterolemia and premature atherosclerosis. LDL mass was significantly greater in mutant than in control pigs (400 ± 55 mg/dL vs 103 ± 26 mg/dL), as was LDL cholesterol. When normal and mutant LDLs were injected into the bloodstream of normal pigs, the fractional catabolic rate (FCR) of mutant LDL was about 30% lower than that of control LDL. In mutant pigs, the mean FCRs of mutant and control LDL were similar, although they were much lower than the corresponding FCRs observed in normal pigs. The density profile of LDL particles differed in control and mutant pigs; the peak LDL flotation rate was shifted from $S_f^0 = 5.3 \pm 1.9$ in controls to a more buoyant 7.4 ± 0.5 in mutants. The elevation of LDL in the mutants was restricted to the most buoyant LDL subspecies. This subpopulation of mutant LDL was enriched with cholesteryl ester (47% vs 37%) and depleted of triglyceride, relative to LDL of similar density and size in controls. The lipid compositions of the denser LDL subpopulations ($\rho > 1.043$ g/mL) were similar in mutants and controls. We conclude that the hypercholesterolemia of these mutant pigs is accounted for by defective catabolism of LDL. The buoyant cholesterol ester enriched LDL subspecies that accumulate in plasma may contribute to the accelerated atherogenesis that occurs in these animals.

Hypercholesterolemia is a major risk factor for coronary heart disease. Population genetic studies have estimated a heritability coefficient for hypercholesterolemia of 0.5–0.6 (Berg, 1983; Goldbourt & Neufeld, 1986). Despite the apparently high genetic contribution in determining cholesterol levels, the molecular basis underlying the genetic contribution is unknown for the vast majority of individuals.

Hypercholesterolemia in humans usually involves an elevation in the plasma concentration of low-density lipoprotein (LDL). Elevated LDL concentrations result from increased production and/or defective clearance of LDL (Grundy et al., 1985). In some instances, hypercholesterolemia is clearly related to a decrease in receptor-mediated LDL clearance. The best understood genetic cause of impaired receptor-mediated catabolism of LDL is familial hypercholesterolemia, which results from mutations in the gene encoding the LDL receptor (Brown & Goldstein, 1986). Diminished activity of the LDL receptor leads to a marked elevation in LDL levels in two ways: (1) LDL is inefficiently removed from the bloodstream, and (2) hepatic receptor-mediated clearance of intermediate density lipoprotein (IDL), the metabolic precursor of LDL, is decreased, leading to increased conversion of IDL to LDL (Bilheimer et al., 1982). Although mutations in the gene for LDL receptors are common relative to other known human mutations, the frequency of these mutations, 0.002, is considerably less than the frequency of hypercholesterolemia

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