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# Purification of Liver Microsomal Cytochrome P-450 Isozymes 3a and 6 from Imidazole-Treated Rabbits

## Evidence for the Identity of Isozyme 3a with the Form Obtained by Ethanol Treatment

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SUMMARY

Two forms of cytochrome P-450 have been purified to electrophoretic homogeneity from hepatic microsomes of rabbits treated with imidazole. Several criteria indicate that the cytochrome of higher electrophoretic mobility is identical with ethanol-inducible isozyme 3a. "Imidazole-3a" and "ethanol-3a" exhibit the same chromatographic characteristics and have identical electrophoretic mobilities upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Furthermore, the two protein preparations have the same absorbance maxima and absorption coefficients in the oxidized, reduced, and reduced-CO states. A single immunoprecipitin band exhibiting complete identity was observed upon reaction of imidazole-3a and ethanol-3a with the immunoglobulin G fraction from sheep immunized with the latter protein. The amino acid composition and first 10 residues of the amino terminus of the two protein preparations are indistinguishable, as are the high-performance liquid chromatographic maps of the peptides obtained upon cleavage with trypsin, *Staphylococcus aureus* $V_8$ protease, or Lys C endoproteinase. Furthermore, these preparations have very similar activities in the oxidation of ethanol to acetaldehyde and the *p*-hydroxylation of aniline. Evidence was obtained that the cytochrome of lower electrophoretic mobility isolated from imidazole-treated rabbits is probably identical with isozyme 6; the spectral characteristics, amino acid composition, and carboxyl-terminal sequence are described. As an inducer, imidazole has the advantage over ethanol of being less variable in its effects and requiring a shorter period of treatment. From the resulting liver microsomes, one can readily isolate, in addition to P-450 isozymes 3a and 6, isozymes 3c and 4 as well as epoxide hydrolase.

## INTRODUCTION

Chronic ethanol consumption by animals and man is believed to be associated with a variety of diseases and toxicities, many of which may be related to the induction of one or more forms of cytochrome P-450 (1, 2). While many substrates could in theory be used to monitor the ethanol-dependent induction of P-450 in hepatic microsomes, the oxidation of ethanol (2), the *p*-hydroxylation of aniline (3), and the demethylation of dimethylnitrosamine (2) have proven to be consistently increased following chronic ethanol treatment of experimental animals. We have recently purified a unique form of P-$450_{LM}$,[1] isozyme 3a, from livers of rabbits chronically exposed to ethanol (4), and have demonstrated that the purified cytochrome exhibits the greatest activity of the known isozymes for the oxidation of alcohols and the hydroxylation of aniline (3). Although isozyme 3a appears to represent only a small fraction of the total P-

[1] The abbreviations used are: P-$450_{LM}$, rabbit liver microsomal cytochrome P-450; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; IgG, immunoglobulin G. The isozymes of rabbit liver microsomal P-450 are numbered according to their relative electrophoretic mobilities. The ethanol-inducible isozyme is referred to as P-$450_{LM3a}$, or simply as form or isozyme 3a, and the preparations from ethanol- and imidazole-treated animals are referred to as ethanol-3a and imidazole-3a. Similarly, preparations of P-450 isozyme 6 are referred to as ethanol-6 and imidazole 6.

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450 as estimated by SDS-PAGE of microsomes from ethanol-treated rabbits, the high activity of the purified cytochrome with aniline and alcohols is consistent with the increase seen in these microsomal activities after alcohol administration (3). Robertson *et al.* (5) have obtained analogous results with P-450 form 5, which represents only about 12% of the total P-450 in microsomes from phenobarbital-treated rabbits but is responsible for greater than 80% of the activity associated with the conversion of 2-aminoanthracene to a mutagen.

It has been well established that the P-450-mediated metabolism of many compounds can be altered by the exposure of animals to structurally diverse xenobiotics. Increases in the demethylation of dimethylnitrosamine or the hydroxylation of aniline have been reported in liver microsomes following the administration of pyrazole (6), imidazole (7), phenylimidazole (7), acetone (8), isopropanol (8), or benzene (9). This laboratory has recently found that the aniline hydroxylase activity of liver microsomes is increased by treatment of rabbits with trichloroethylene, pyrazole, benzene, *m*-xylene, aniline, or imidazole (10). Ingelman-Sundberg *et al.* (9) have reported the isolation of an isozyme from benzene-treated rabbits which exhibits characteristics similar to those of isozyme 3a, but a detailed comparison of the two preparations was not made. Although the increase in the aniline hydroxylase activity of liver microsomes from animals exposed to these diverse compounds is suggestive that isozyme 3a is induced in all cases, it cannot be assumed *a priori* that this is the case. In the present paper we report the isolation of isozymes 3a and 6 from imidazole-treated rabbits and present a detailed comparison of ethanol- and imidazole-3a. Studies on the chromatographic, spectral, catalytic, and immunochemical properties as well as on some features of the primary structure indicate that the two proteins are identical. In addition, spectral and structural characteristics of isozyme 6 are presented.

## METHODS

*Purification of P-450 isozymes 3a and 6* - Adult New Zealand White male rabbits (2.0–2.5 kg in weight) were given drinking water containing 10% ethanol (v/v) for 1 week followed by 5% ethanol for an additional 2 weeks with free access to Purina rabbit chow. In other experiments, rabbits were treated with imidazole as described by Hajek and Novak (7); the animals received an i.p. injection of imidazole (200 mg/kg body weight) in aqueous solution adjusted to pH 7.0 on each of 4 days and were killed 24 hr after the final injection. Pyrophosphate-washed liver microsomes were prepared and solubilized with cholate (5 mg/mg of protein) and Tergitol NP-10 at 0.1% final concentration and treated with polyethylene glycol 6000 (11). The fraction precipitating from 7–14% polyethylene glycol was suspended in 10 mM Tris-acetate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, and 0.5% Tergitol NP-10.

Isozyme 3a was purified from the 7–14% polyethylene glycol 6000 precipitate as previously described (4), except that the 10 mM $K_2SO_4$ and 100 mM $K_2SO_4$ eluates obtained from the initial DEAE-cellulose column were concentrated by direct application onto a hydroxylapatite column (40 nmoles of P-450 per milliliter of resin) equilibrated with 10 mM phosphate buffer (pH 7.4) containing 20% glycerol and immediate elution with 600 mM phosphate buffer (pH 6.0) cotnaining 20% glycerol, 1 mM EDTA, and 0.5% Tergitol NP-10. The concentrated fraction was then dialyzed overnight against 200 volumes of 10 mM potassium phosphate buffer (pH 6.0) containing 20% glycerol, 1 mM EDTA, and 0.5% Tergitol NP-10, with one change of the buffer solution. The substitution of hydroxylapatite chromatography for the batchwise calcium phosphate procedure described previously (4) did not affect the subsequent steps of the purification procedure and resulted in about a 20-fold concentration of the protein with greater than 95% recovery while requiring less manual operation. The preparations from ethanol- and imidazole- treated rabbits behaved identically throughout the purification procedure.

Isozyme 6 was isolated from the 100 mM $K_2SO_4$ DEAE-cellulose eluate (4). The concentrated solution from the hydroxylapatite column, prepared as described above, appeared slightly turbid after dialysis, but fractionated well on CM-Sepharose at pH 6.0, as described for isozymes 3a and 3b (4, 11). The cytochrome was enriched in the fraction eluted with 150 mM phosphate buffer (pH 6.0) containing 20% glycerol, 1 mM EDTA, and 0.5% Tergitol NP-10. The enzyme solution was concentrated by use of calcium phosphate and dialyzed against 10 mM phosphate buffer (pH 7.7) containing 20% glycerol, 1.0 mM EDTA and 0.5% Tergitol NP-10. The slight turbidity was removed by the addition of cholate (0.2 mg/nmole of P-450) and Tergitol NP-10 at a final concentration of 0.5%, and the preparation was applied to a DEAE-Sepharose column previously equilibrated with the same buffer solution as used for dialysis. Isozyme 6 was bound tightly to the top of the column, and contaminating isozymes 3a and 3b were removed by washing the column with 10 column volumes each of 10 mM and then 20 mM phosphate buffer (pH 7.7) containing 20% glycerol, 1 mM EDTA, and 0.5% Tergitol NP-10. The column was then washed with 20 column volumes of a similar buffer solution containing 30 mM phosphate, and isozyme 6 was eluted from the column as a sharp peak when the phosphate was increased to 75 mM. The enzyme preparation was diluted 1:1 with 20% glycerol and applied to a hydroxylapatite-cellulose column (50 nmoles of P-450 per milliliter of resin) previously equilibrated with 10 mM phosphate (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, and 0.3% Tergitol NP-10. The column was washed with the equilibration buffer and 10 column volumes of a similar solution containing 45 mM phosphate, and then with the 45 mM phosphate buffer solution without Tergitol NP-10 to remove the bound nonionic detergent. As soon as the absorbance of the effluent at 276 nm was essentially zero, the detergent-free protein was eluted with 750 mM phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. The final preparation was dialyzed and concentrated in a Micro-ProDiCon apparatus containing 100 mM phosphate buffer (pH 7.4), 20% glycerol, and 0.1 mM EDTA.

*Amino acid analysis and carboxyl-terminal sequence analysis.* The amino acid composition of isozyme 6 was determined following hydrolysis of the samples of protein with 6 N HCl for 1, 2, and 3 hr at 150° (12). Triplicate samples were run at each time point. Cysteine was determined as cysteic acid after performic acid oxidation (12), tryptophan was determined fluorimetrically as described by Pajot (13), and nearest integer values were determined as described previously (4).

The COOH-terminal analysis of isozyme 6 was determined by digestion with carboxypeptidase Y, as described previously for other isozymes (4), in 100 mM sodium phosphate buffer (pH 7.0) containing 0.1% SDS. The carboxypeptidase Y was added in a 1:100 (w/w) ratio to P-450. At each time point, the analysis was in duplicate, and the recovery of amino acids released was corrected for the amounts in control mixtures in which the cytochrome or carboxypeptidase Y was omitted and for the recovery of amino acids from the analysis procedure using $\alpha$-aminobutyric acid as an internal standard.

*Peptide maps.* Proteolytic digests of imidazole- and ethanol-3a were prepared as described previously (4). Each isozyme was incubated with trypsin (1 $\mu$g/50 $\mu$g of P-450), *Staphylococcus aureus* $V_8$ protease (1 $\mu$g/12.5 $\mu$g of P-450), or endoproteinase Lys C (1 $\mu$g/25 $\mu$g of P-450) for 16 hr at 37° in 100 mM phosphate buffer (pH 8.0) with 4 M urea present in all cases.

*Preparation of antibodies.* Antibodies to ethanol-3a were elicited in yearling female sheep. Four animals were initially immunized by multiple i.d. injections in the flanks with 500 $\mu$g of the purified isozyme

emulsified in 2.0 ml of Freund's complete adjuvant containing 25 μg of killed tuberculosis mycobacterium. Each sheep received three additional immunization at monthly intervals with 500 μg of the protein in Freund's incomplete adjuvant and were bled by jugular venipuncture 2 and 3 weeks after the final injections. The γ-globulin was isolated from the immune serum by ammonium sulfate precipitation and anion exchange chromatography (14). Ouchterlony double-diffusion plates were run as described by Thomas *et al.* (15) except that the phosphate buffer concentration was increased to 100 mM to improve the solubility of isozyme 3a, and detergent was omitted from the buffer (15). The plates (7 × 50 mm with wells 4 mm in diameter) contained 5 ml of 0.9% low-electroendosmosis agarose. Microsomes were solubilized by the addition of 1 mg of sodium cholate per milligram of protein. Diffusion was allowed to proceed at 4° for 48–96 hr after which time the plates were stained with 0.05% aqueous Coomassie blue (14).

*Other methods.* The concentration of P-450 in crude fractions, the absolute spectrum of form 6, and the heme concentration were determined as previously described (11). Electrophoretically homogeneous NADPH-cytochrome P-450 reductase, which catalzyed the reduction of 50 μmoles of cytochrome *c*/min/mg of protein, was obtained from liver microsomes of phenobarbital-treated rabbits (16). Protein was determined by the method of Bensadoun and Weinstein (17). The procedure for SDS-PAGE and the preparation of the DEAE-cellulose, calcium phosphate, and hydroxylapatite (Type B), have been described (4, 11).

The catalytic activity of imidazole- and ethanol-3a and imidazole 6 was determined in a reconstituted system containing 50 mM phosphate buffer (pH 7.6), sonicated dilauroylglyceryl-3-phosphorylcholine (30 μg/ml), substrates at saturating concentrations, 0.3 μM NADPH-cytochrome P-450 reductase, 0.1 μM P-450, and 1 mM NADPH as the final addition. All incubations were carried out at 30° for times that represented the initial rate of product formation. Blank mixtures contained all of the components, but the reactions were quenched prior to the addition of NADPH. The procedure used for the measurement of the hydroxylation of aniline has been described elsewhere (3). The deethylation of 7-ethoxyresorufin was determined by direct measurement of the fluorescent product, resorufin, as described by Burke *et al.* (18). The oxidation of ethanol was measured by the semicarbazide-trapping method in center-well flasks as described previously (3).

*Materials.* 7-Ethoxyresorufin was obtained from Molecular Probes, Inc. (Junction City, Ore.), endoproteinase Lys C from Boehringer Mannheim (Indianapolis, Ind.), low-electroendosmosis agarose from Bio-Rad Laboratories (Richmond, Calif.), and the 80/100 Carbopack B/5% Carbowax 20 M column from Supelco (Bellefonte, Pa.). The sources of the materials used in the enzyme purification procedure and catalytic assays have been described (3, 4, 11). Phosphate buffers were the potassium salts unless otherwise noted.

## RESULTS

The treatment of rabbits with imidazole has been reported to increase the liver microsomal content of cytochrome P-450 and to increase the microsomal oxidation of dimethyaniline, *p*-nitroanisole, dimethylnitrosamine, ethanol, butanol, and aniline (7). Preliminary experiments in our laboratory have confirmed these findings; imidazole treatment resulted in a 1.7-fold increase in total P-450 and about a 2-fold increase in the aniline hydroxylase activity (10). In experiments not shown, a comparison by SDS-PAGE revealed that microsomes from ethanol- and imidazole-treated rabbits have an increase in a protein band with the same electrophoretic mobility, whereas this band is absent or present at very low levels in microsomes from untreated animals. Similar electrophoretic results have been reported by Hajek and Novak (7). We found that this band has the same electrophoretic mobility as isozyme 3a purified from ethanol-treated rabbits.

Attempts to purify isozyme 3a from liver microsomes of untreated animals or animals treated with isosafrole, phenobarbital, or 5,6-benzoflavone have been unsuccessful, probably as a result of the very low levels of this cytochrome. In contrast, when microsomes from imidazole-treated rabbits were subjected to the procedure developed for the isolation of isozyme 3a from ethanol-treated rabbits, a P-450 preparation was obtained with the same chromatographic and solubility properties as isozyme 3a from ethanol-treated animals (4). A summary of the purification results is given in Table 1; the final detergent-free isozyme 3a represents about 1% of the total P-450 in the microsomal fraction. The preparation exhibited a single major band upon gel electrophoresis and had the same mobility as ethanol-3a (Fig. 1, lanes *a* and *b*).

Electrophoretic examination of the initial DEAE-cellulose column fractions eluted with 100 mM $K_2SO_4$ re-

TABLE 1

*Purification of P-450$_{LM_{3a}}$ and P-450$_{LM_6}$ from liver microsomes of imidazole-treated rabbits*

| Preparation | Protein | Cytochrome P-450 content | | | Yield |
|---|---|---|---|---|---|
| | | Total P-450$_{LM}$ | P-450$_{LM_{3a}}$ | P-450$_{LM_6}$ | |
| | *mg* | *nmoles/mg protein* | | | *% total P-450* |
| Pyrophosphate-treated microsomes | 6660 | 4.40 | | | 100 |
| Polyethylene glycol precipitate (7–14%) | 3443 | 6.15 | | | 72 |
| DEAE-cellulose column eluate Fraction IV, 10 mM $K_2SO_4$ | 621 | | 4.1 | | 8.6 |
| CM-Sepharose column eluate, 150 mM phosphate | 63 | | 12.2 | | 2.6 |
| DEAE-Sepharose column eluate, 10 mM phosphate | 23.5 | | 13.3 | | 1.1 |
| Hydroxylapatite-cellulose column eluate | 12.7 | | 20.7 | | 0.9 |
| Fraction V, 100 mM $K_2SO_4$ | 1051 | | | 7.4 | 26.4 |
| CM-Sepharose column eluate, 150 mM phosphate | 139 | | | 13.0 | 6.1 |
| DEAE-Sepharose column eluate, 75 mM phosphate | 81.7 | | | 15.1 | 4.2 |
| Hydroxylapatite-cellulose column eluate | 75.4 | | | 16.2 | 4.1 |

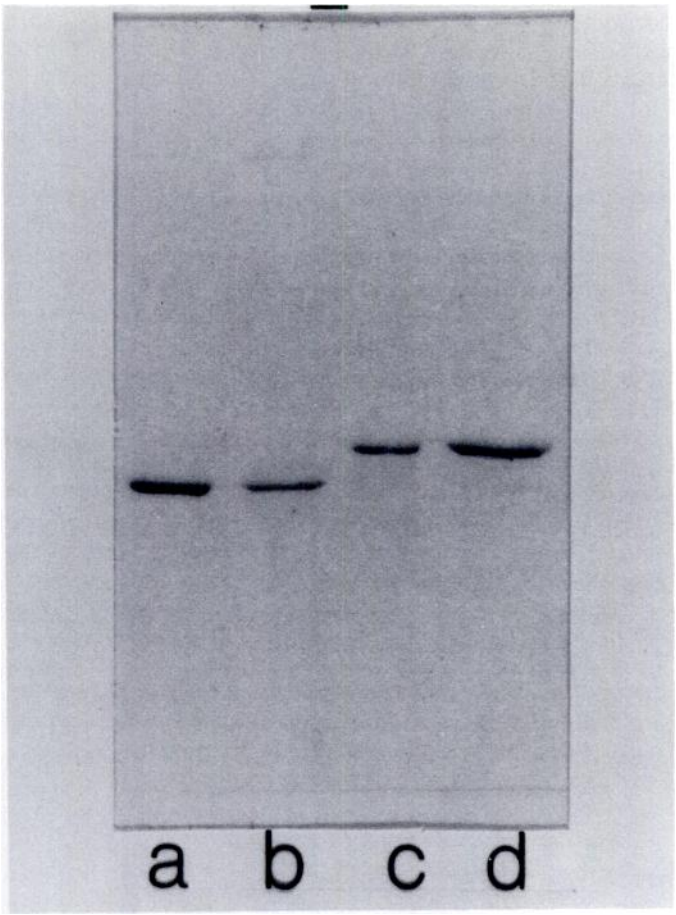


FIG. 1. *Slab SDS-PAGE of rabbit liver microsomes and P-450$_{LM}$ preparations*

The samples were analyzed at the protein levels indicated on gels 0.75 mm thick with 7.5% acrylamide separating gels. The gel shows a comparison of isozyme 3a purified from (*a*) imidazole-treated rabbits (1.0 μg of protein) or (*b*) ethanol-treated rabbits (0.5 μg of protein), and isozyme 6 isolated from imidazole-treated rabbits at loads of 0.5 and 1.0 μg of protein, respectively, in lanes *c* and *d*.

vealed a very high concentration of a protein with an electrophoretic mobility corresponding to that of authentic isozyme 6 (19, 20), kindly provided by Dr. Eric F. Johnson (La Jolla, Calif.). This cytochrome was purified by the procedure summarized in Table 1. The preparation, which exhibited a single major band upon gel electrophoresis as shown in Fig. 1, lanes *c* and *d*, had a specific content of 16.2 nmoles of heme/mg of protein.

In addition to isozymes 3a and 6, the method described permits the isolation of isozymes 3c and 4 and epoxide hydrolase[2] from the same preparation. Application of the previously published methods for the isolation of 3c and 4 (11) gave yields of 2% and 19% for forms 3c and 4, respectively.

The increase in the aniline hydroxylase activity of microsomes from imidazole-treated rabbits and the identical chromatographic and electrophoretic properties of imidazole- and ethanol-3a are very suggestive that the proteins are the same, but small differences may exist which are not revealed by these techniques. A comparison of the spectral properties of the two preparations, as presented in Table 2, shows they have identical absorption maxima and very similar absorption coefficients in the oxidized, reduced, and reduced carbonyl states. Imidazole-3a is isolated in the pentacoordinate, high-spin state in the presence of nonionic detergent, as has been reported for ethanol-3a (4). Thus, the structure of the protein in the vicinity of the heme, as reflected by the spectral properties, appears to be the same in both preparations.

Antibodies were produced to purified ethanol-3a in sheep as described under Methods. IgG purified from the antisera exhibited no cross-reactivity with isozymes 2, 3b, 3c, 4, or 6 in Ouchterlony double diffusion experiments or by radioimmunoassay.[3] Figure 2 shows the reactivity of this antibody with ethanol-3a and imidazole-3a and the hepatic microsomes from ethanol- and imidazole-treated rabbits. A single, well-defined immunoprecipitin band was formed between the two purified enzyme preparations, thus indicating that the antigenic determinants are the same. In addition, only a single precipitin band showing complete identity with both of the purified preparations was observed with microsomes from ethanol- and imidazole-treated rabbits. The lines of identity between the purified enzymes and microsomes and the single precipitin line with microsomes indicated that the antibody reacted with a single protein. The increased intensity of the precipitin band between the antibody and the microsomes from imidazole-treated rabbits when compared with the equivalent amount of microsomes from ethanol-treated rabbits is suggestive that more isozyme 3a is present in the former microsomes. Detergent was omitted from the Ouchterlony double-diffusion plate shown in Fig. 2 in order to enhance any lines of partial identity or weak cross-reactivity that might be inhibited by detergent (15). Similar results were obtained when detergent was included.

TABLE 2

*Spectral characteristics of isozyme 6 and imidazole- and ethanol-3a*

The absorption coefficients are based on the cytochrome concentration determined by analysis for heme.

| Isozyme | Oxidized | | Reduced | | Reduced-CO | |
|---|---|---|---|---|---|---|
| | $\lambda_{max}$ | $\epsilon$ | $\lambda_{max}$ | $\epsilon$ | $\lambda_{max}$ | $\epsilon$ |
| | *nm* | $mM^{-1}cm^{-1}$ | *nm* | $mM^{-1}cm^{-1}$ | *nm* | $mM^{-1}cm^{-1}$ |
| Imidazole-3a | 393 | 85 | 413 | 71.3 | 452 | 105 |
| | 648 | 4.5 | 547 | 14.6 | 554 | 12.9 |
| Ethanol-3a | 393 | 84.7 | 413 | 72.1 | 452 | 110 |
| | 648 | 3.9 | 547 | 14.5 | 554 | 12.4 |
| 6 | 416 | 91.5 | 410 | 69.3 | 448 | 102 |
| | 533 | 10.2 | 544 | 12.7 | 552 | 12.1 |
| | 568 | 9.6 | | | | |
| | 636 | 2.2 | | | | |

[2] The identification of band *1* (4) as epoxide hydrolase has been confirmed by purification to electrophoretic homogeneity. The final preparation catalyzed the hydration of 242 nmoles of benzo[*a*]pyrene-4,5-epoxide/min/mg of protein. The $NH_2$-terminal sequence found for the rabbit enzyme, Met-Leu-Leu-Glu-Leu-Leu-Leu-Ala-X-Val-Leu-Gly-Phe, is identical with that of the rat protein except for Trp in place of Leu, and Val in place of Leu at positions 2 and 6, respectively.

[3] D. R. Koop, G. D. Nordblom, and M. J. Coon, unpublished data.

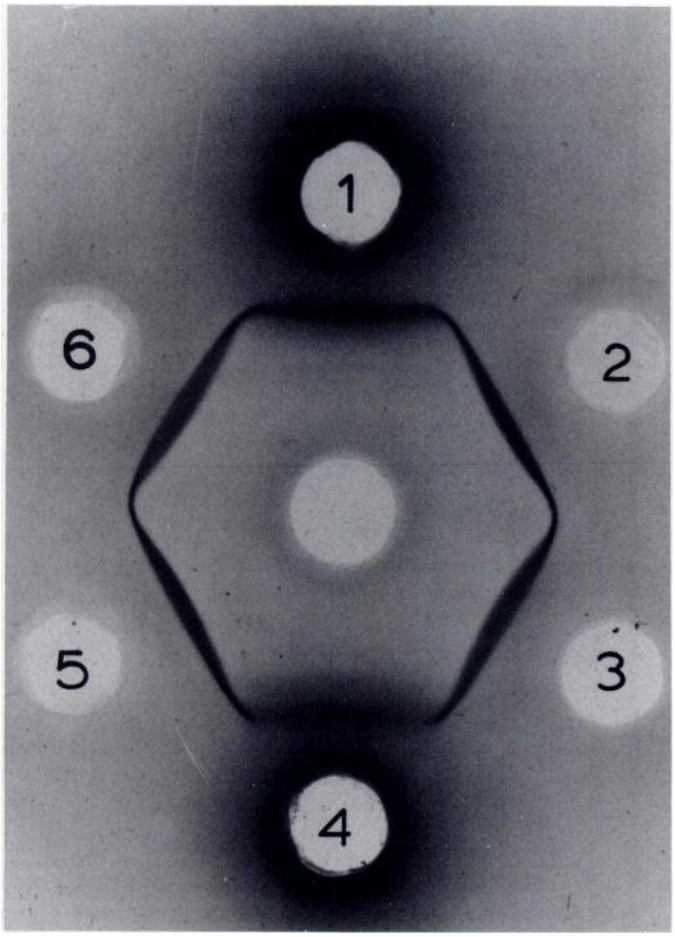


FIG. 2. *Immunochemical comparison of isozyme 3a purified from ethanol- and imidazole-treated rabbits*

Ouchterlony double-diffusion analysis was carried out in 0.9% agarose gels containing 100 mM phosphate buffer (pH 7.4) containing 1.0 M glycine, 86 mM NaCl, 15 mM sodium azide, and 0.2 mM EDTA. Microsomes were solubilized with 1 mg of cholate per milligram of protein in the same buffer system. The center well was loaded with 250 μg of anti-ethanol-3a IgG. Wells *1* and *4* were loaded with 200 μg of microsomal protein from imidazole- and ethanol-treated rabbits, respectively; wells *2* and *5* were loaded with 6 μg of imidazole-3a; and wells 3 and *6* were loaded with 6 μg of ethanol-3a. Diffusion was allowed to proceed at 4° for 48 hr in a moist chamber, and staining was then carried out with aqueous Coomassie blue.

The first 10 residues of the $NH_2$-terminal sequence of imidazole-3a, determined by manual Edman degradation (11), are Ala-Val-Leu-Gly-Ile-Thr-Val-Ala-Leu-Leu-, which are identical with the first 10 residues reported for ethanol-3a (4). Yuan *et al.* (21) have recently demonstrated that, although P-450$_b$ and P-450$_e$ purified from phenobarbital-treated rats have an identical $NH_2$-terminal sequence for the first 35 amino acids, 13 amino acid differences occur elsewhere between the two proteins. Since the complete sequence of imidazole- and ethanol-3a is not known, a comparison of their structures was made by peptide mapping with HPLC after proteolysis with trypsin, *Staphylococcus aureus* $V_8$ protease, and endoproteinase Lys C. This technique has proven to be extremely useful in revealing structural differences among the P-450 isozymes from rabbit liver (4, 11) and has been used to demonstrate very minor differences between strain variants of rabbit isozyme 3b (22) and between P-450$_b$ and P-450$_e$ from phenobarbital-treated rats (21). In results not shown, both trypsin and $V_8$ protease gave a mixture of small peptides which were not retained on 12.5% acrylamide gels, whereas with endoproteinase Lys C, large peptides (15,000–30,000 $M_r$) were still present after digestion of 3a as judged by PAGE under the same conditions. Different HPLC columns and solvent systems were examined to obtain maximal resolution of the peptides generated by each protease. The results obtained with *S. aureus* $V_8$ protease are shown in Fig. 3. The complex mixture of small peptides was well resolved, and no significant differences were observed between the two preparations of isozyme 3a. The profiles obtained after digestion with trypsin or endoproteinase Lys C were also indistinguishable (results not shown). The identity of the peptide maps with three different proteases further suggests that the amino acid composition of ethanol- and imidazole-3a is identical. In results not shown, this was confirmed when the amino acid composition of each isozyme was determined. These results indicate that the primary structure of the two preparations of isozyme 3a are highly similar if not identical.

When imidazole-3a was reconstituted with synthetic dilauroylglyceryl-3-phosphorylcholine and NADPH-cytochrome P-450 reductase, it oxidized ethanol and aniline at rates of 10 and 11 nmoles/min/nmole of P-450, respectively. These values are about the same as the rates obtained with ethanol-3a (3, 4); the small differences are within the variation we have observed with different preparations of the same isozyme from ethanol-treated animals.

As previously indicated, Johnson and co-workers (19, 20) have reported the isolation of isozyme 6 from TCDD-treated rabbits and have demonstrated the unique nature of this cytochrome. We have demonstrated the activity of this form in the activation of acetaminophen (23), but further characterization of the enzyme has not been reported. The wavelength maxima and absorption coefficients of purified isozyme 6 in the oxidized, reduced, and reduced-CO states are summarized in Table 2. Although the oxidized protein is predominantly low-spin, as indicated by the Soret maximum at 416 nm, the presence of a small charge transfer band at 636 nm indicates some high-spin character. The maximum of the Soret peak of the reduced protein is slightly blue-shifted as compared with the other isozymes isolated in the low-spin state, and the reduced-CO maximum occurs at 448 nm.

The amino acid content of isozyme 6 is presented in Table 3. The composition is similar to that of isozymes 2, 3a, 3b, 3c, and 4 (4), with a relatively high content of hydrophobic residues. The minimal molecular weight of isozyme 6 calculated from these data is 56,800, in good agreement with the value of 57,000 determined by calibrated PAGE. The time course of amino acid release during the limited carboxypeptidase Y digestion of isozyme 6 is shown in Figure 4. Alanine was released most rapidly, followed by Phe, Val, and Leu. The increase in Leu above Ala at 120 min apparently indicates the pres-

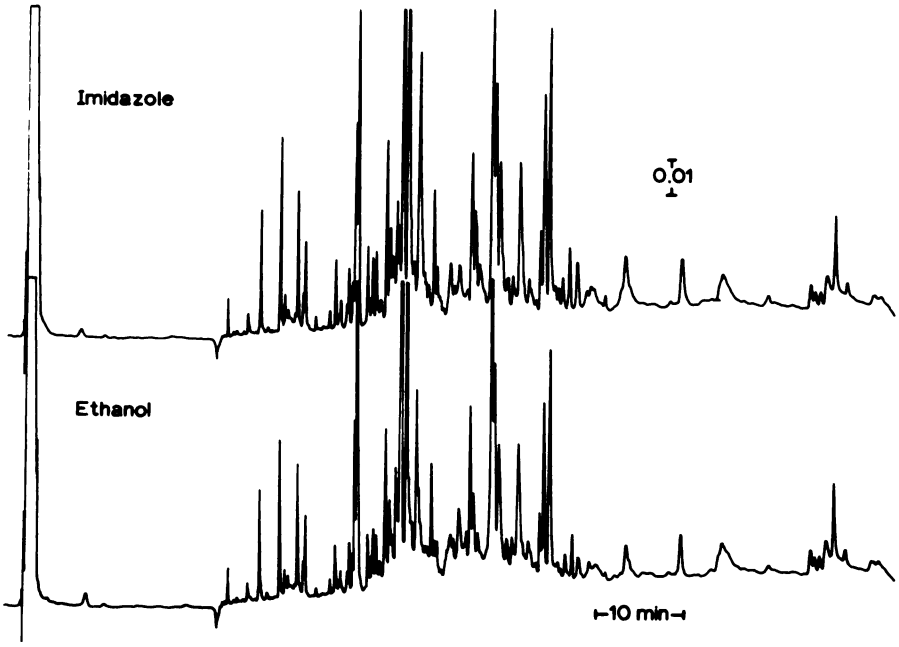


FIG. 3. *HPLC profiles of fragments resulting from proteolysis of isozyme 3a purified from imidazole- or ethanol-treated rabbits*

The purified isozymes were treated with *Staphylococcus aureus* $V_8$ protease, as described under Methods. After acidification with phosphoric acid (30 μl/ml of reaction), the peptides (100 μg of protein) were separated on an Altex Ultrasphere ODS column (5 μm, 4.6 × 250 mm) by use of gradient elution with 1% phosphoric acid adjusted to pH 2.2 with KOH (solvent A) and acetonitrile (solvent B). A linear gradient from 0 to 80% solvent B was generated over 90 min beginning 5 min after injection of the sample. The flow rate was 1.0 ml/min, and peptides were monitored in the column effluent at 214 nm.

TABLE 3

*Amino acid composition of isozyme 6*

| Amino acid | Isozyme 6 |
|---|---|
| | *residues/molecule P-450$_{LM_6}$* |
| Cys | 8 |
| Asx | 48 |
| Thr | 28 |
| Ser | 39 |
| Glx | 45 |
| Pro | 24 |
| Gly | 36 |
| Ala | 32 |
| Val | 40 |
| Met | 8 |
| Ile | 20 |
| Leu | 54 |
| Tyr | 14 |
| Phe | 31 |
| His | 16 |
| Lys | 22 |
| Arg | 36 |
| Trp | 6 |
| Total | 507 |

ence of two Leu residues near the COOH terminus. Electrophoretic examination of the cytochrome during the time course of digestion showed no evidence of any endoprotease activity.

We have previously reported the $NH_2$-terminal sequence of isozyme 6 isolated from ethanol-treated rabbits (24), with serine as the first residue. The results of proteolytic peptide mapping experiments similar to those presented for isozyme 3a indicated that imidazole- and ethanol-6 are identical proteins. However, the manual Edman analysis of imidazole-6 has not yielded a single $NH_2$-terminal residue. Preliminary results from the first 10 cycles of Edman degradation suggest that imidazole-6 is a mixture of an N- and (N-1)-form of the protein (where N-1 represents a portion of the protein that has lost the $NH_2$-terminal residue), whereas the ethanol-6 was predominantly an N-2 form. Further experiments

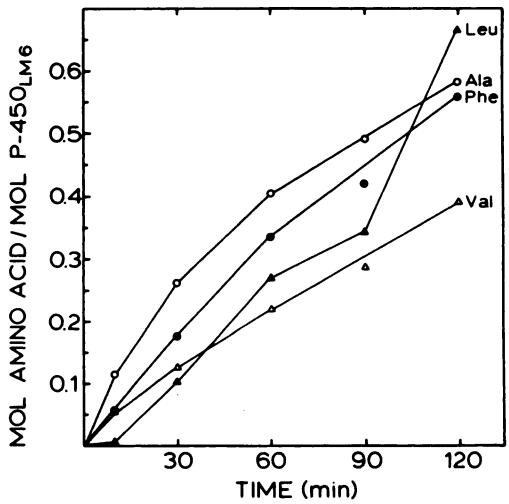


FIG. 4. *COOH-terminal amino acids released from purified isozyme 6*

The purified cytochrome was treated with carboxypeptidase Y at 37°, and the amino acids released were determined as described under Methods.

are needed to confirm this interpretation, but it should be noted that this laboratory has obtained similar results with isozymes 3b (11) and 4 (24), and Ozols *et al.* (25) have also reported that a preparation of form 3b was missing the $NH_2$-terminal Met residue.

Isozyme 6 has been reported to have the highest activity of the cytochromes purified from rabbit liver in the deethylation of 7-ethoxyresorufin (19, 20). Our preparation of isozyme 6 is also active toward this substrate, exhibiting a turnover number of 2.0 $min^{-1}$.

## DISCUSSION

Many of the isozymes of P-450 which have been purified from rat and rabbit liver microsomes are induced by treatment of the animals with structurally diverse compounds. Thomas *et al.* (15) have demonstrated immunochemically that in rats both P-450$_c$ and P-450$_d$ are induced by isosafrole, 3-methylcholanthrene, $\beta$-naphthoflavone, TCDD, phenothiazine, and the polychlorinated biphenyl mixture, Aroclor 1254. Similarly, isozyme 4, which is present in high levels in control and phenobarbital-treated rabbits, is identical with the isozyme induced by TCDD, 3-methylcholanthrene, $\beta$-naphthoflavone, and isosafrole as judged by spectral, catalytic, immunochemical, and structural studies on the purified enzyme (26). Applying similar criteria, Bonfils *et al.* (27) have demonstrated that isozyme 3b purified from triacetyloleandomycin-treated rabbits is identical with the isozyme purified from untreated rabbits (11). As indicated in the present paper, isozyme 6 isolated from isosafrole, ethanol-, or imidazole-treated rabbits is apparently identical with the protein isolated by Johnson and coworkers after TCDD treatment (19, 20) as determined by an exchange of proteins between our laboratories. There are numerous other examples of identity or possible identity of isozymes of P-450 which have been purified in different laboratories after different treatments but not compared directly (26). In many cases, the different nomenclature used by various laboratories for the same protein has led to confusion about the multiplicity and inducibility of each isozyme (26).

While immunochemical cross-reactvity is very strong evidence for the identity of two isozymes, in some cases a single such criterion is inadequate, since minor differences may exist between the two proteins. Vlasuk *et al.* (28) have reported the existence of four different forms of phenobarbital-inducible rat liver microsomal P-450 which exhibit immunochemical identity and greater than 95% fingerprint homology, and recent sequence work has demonstrated only 13 amino acid differences between P-450$_b$ and P-450$_e$ (21). Dieter and Johnson (22) have described the occurrence of a variant of rabbit form 3b which exhibits greatly diminished 6$\beta$-hydroxylase activity toward progesterone; structural differences were detected only by comparison of tryptic peptide fingerprints. As yet another example, treatment of rabbits with cholestyramine induces an isozyme of P-450 specific for the 7$\alpha$-hydroxylation of cholesterol (29, 30). This enzyme is electrophoretically identical with the 5,6-benzoflavone-inducible isozyme 4, but when partially resolved from the endogenous form 4, the 7$\alpha$-hydroxylase exhibits differences in peptide maps (29, 30) and amino acid composition (30).

The results presented in this paper describe the isolation of isozymes 3a and 6 from liver microsomes of imidazole-treated rabbits. Imidazole-3a was compared with ethanol-3a by use of a variety of criteria, including the chromatographic, electrophoretic, immunochemical, spectral, catalytic, and structural characteristics of the two proteins. While each criterion on its own is indicative that the two proteins are similar, the evidence taken together is compelling that imidazole- and ethanol-3a are identical isozymes of P-450.

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