

- Kaguni, L. S., Rossignol, J.-M., Conaway, R. C., & Lehman, I. R. (1983a) *Proc. Natl. Acad. Sci. U.S.A.* 80, 2221-2225.
- Kaguni, L. S., Rossignol, J.-M., Conaway, R. C., Banks, G. R., & Lehman, I. R. (1983b) *Proc. Natl. Acad. Sci. U.S.A.* 80, 9037-9039.
- Kaguni, L. S., DiFrancesco, R. A., & Lehman, I. R. (1984) *J. Biol. Chem.* 259, 9314-9319.
- Karawya, E., Swack, J., Albert, W., Fedorko, J., Minna, J. D., & Wilson, S. H. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81, 7777-7781.
- Kronberg, H., Zimmer, H.-G., & Neuhoff, V. (1984) *Clin. Chem. (Winston-Salem, N.C.)* 30, 2059-2062.
- Laemmli, U. K. (1970) *Nature (London)* 227, 680-685.
- Magnussen, G., Pigiet, V., Winnacker, E. L., Abrams, R., & Reichard, P. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 412-415.
- Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Masaki, S., Koiwai, O., & Yoshida, S. (1982) *J. Biol. Chem.* 257, 7172-7177.
- Masaki, S., Tanabe, K., & Yoshida, S. (1984) *Nucleic Acids Res.* 12, 4455-4467.
- McHenry, C., & Kornberg, A. (1977) *J. Biol. Chem.* 252, 6478-6484.
- Pedrali-Noy, G., & Spadari, S. (1979) *Biochem. Biophys. Res. Commun.* 88, 1194-1202.
- Plevani, P., Foiani, M., Valsasnini, P., Badaracco, G., Cheerithundam, E., & Chang, L. M. S. (1985) *J. Biol. Chem.* 260, 7102-7107.
- Reckmann, B., Grosse, F., Urbanke, C., Frank, R., Blöcker, H., & Krauss, G. (1985) *Eur. J. Biochem.* 152, 633-643.
- Richter, A., Scheurich, R., & Otto, B. (1980) *Eur. J. Biochem.* 109, 67-73.
- Sauer, B., & Lehman, I. R. (1982) *J. Biol. Chem.* 257, 12394-12398.
- Siegel, L. M., & Monty, G. (1966) *Biochim. Biophys. Acta* 112, 346-362.
- Tanaka, S., Hu, S., Wang, T. S.-F., & Korn, D. (1982) *J. Biol. Chem.* 257, 8386-8390.
- Villani, G., Fay, P. J., Bambara, R. A., & Lehman, I. R. (1981) *J. Biol. Chem.* 256, 8202-8207.
- Wahl, A. F., Kowalski, S. P., Harwell, L. W., Lord, E. M., & Bambara, R. A. (1984) *Biochemistry* 23, 1895-1899.
- Wang, T. S.-F., Hu, S.-Z., & Korn, D. (1984) *J. Biol. Chem.* 259, 1854-1865.
- Wong, S. W., Parborsky, L. R., Fisher, P. A., Wang, T. S.-F., & Korn, D. (1986) *J. Biol. Chem.* 261, 7958-7968.
- Yurov, Y. B. (1980) *J. Mol. Biol.* 136, 339-342.
- Zahradka, P. (1987) *FEBS Lett.* 212, 259-262.

Genetic Polymorphism of Human Cytochrome P-450 (S)-Mephenytoin 4-Hydroxylase. Studies with Human Autoantibodies Suggest a Functionally Altered Cytochrome P-450 Isozyme as Cause of the Genetic Deficiency[†]

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ABSTRACT: The metabolism of the anticonvulsant mephenytoin is subject to a genetic polymorphism. In 2-5% of Caucasians and 18-23% of Japanese subjects a specific cytochrome P-450 isozyme, P-450 meph, is functionally deficient or missing. We have accumulated evidence that autoimmune antibodies observed in sera of patients with tienilic acid induced hepatitis (anti-liver kidney microsome 2 or anti-LKM2 antibodies) specifically recognize the cytochrome P-450 involved in the mephenytoin hydroxylation polymorphism. This is demonstrated by immunoinhibition and immunoprecipitation of microsomal (S)-mephenytoin 4-hydroxylation activity and by the recognition by anti-LKM2 antibodies of a single protein band on immunoblots of human liver microsomes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis or isoelectric focusing. The cytochrome P-450 recognized by anti-LKM2 antibodies was immunopurified from microsomes derived from livers of extensive (EM) or poor metabolizers (PM) of (S)-mephenytoin. Comparison of the EM-type cytochrome P-450 to that isolated from PM livers revealed no difference in regard to immuno-cross-reactivity, molecular weight, isoelectric point, relative content in microsomes, two-dimensional tryptic peptide maps, one-dimensional peptide maps with three proteases, amino acid composition, and amino-terminal protein sequence. Finally, the same protein was precipitated from microsomes prepared from the liver biopsy of a subject phenotyped in vivo as a poor metabolizer of mephenytoin. These data strongly suggest that the mephenytoin hydroxylation deficiency is caused by a minor structural change leading to a functionally altered cytochrome P-450 isozyme.

Large interindividual variations in drug response in the population are frequently caused by differences in drug oxidation capacity by liver cytochrome P-450 isozymes. Several

genetic polymorphisms in drug oxidation have been described (Mahgoub et al., 1977; Eichelbaum et al., 1979; Küpfer et al., 1979; Scott & Poffenbarger et al., 1979; Kleinbloesem et al., 1984). The hydroxylation polymorphism of the anticonvulsant drug mephenytoin is one of the best studied examples (Küpfer & Preisig, 1984; Inaba et al., 1984; Wedlund et al., 1984). It occurs in 2-5% of the white European and North American population and in 18-23% of Japanese subjects (Jurima et al.,

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1985; Nakamura et al., 1985). This deficiency is inherited as an autosomal recessive trait (Inaba et al., 1986) and affects one of the two major metabolic pathways of mephenytoin, namely, 4-hydroxylation of its phenyl ring. The other main reaction, N-demethylation, remains unaffected (Küpfert et al., 1984a,b).

Mephenytoin is a chiral compound and displays strongly stereoselective metabolism in vivo (Küpfert et al., 1981, 1982) and in vitro (Meier et al., 1985b). Thus, in normal or extensive metabolizers (EM)¹ (S)-mephenytoin is mainly hydroxylated and rapidly excreted as (S)-4-hydroxymephenytoin glucuronide, while the R enantiomer is preferentially N-demethylated and excreted as (R)-5-phenyl-5-ethylhydantoin (nirvanol). In genetically deficient subjects or poor metabolizers (PM) this stereoselectivity is virtually absent and both enantiomers are slowly excreted as the pharmacologically active nirvanol (Küpfert et al., 1984a,b; Wedlund et al., 1985). The same changes in stereoselective metabolism were found in microsomes isolated from EM and PM subjects (Meier et al., 1985a). The P-450 involved in mephenytoin hydroxylation has been functionally isolated and characterized from human livers by this (P-450 meph;² Gut et al., 1986) and another laboratory (P-450 MP-1 and MP-2; Shimada et al., 1986). Polyclonal antibodies raised in rabbits against P-450 meph recognized no difference in the extent of the immunochemical reaction on Western blots of microsomes from EM and PM individuals (Gut et al., 1986). This provided initial evidence that the mephenytoin hydroxylation polymorphism may be due to a functionally altered P-450 isozyme rather than to the total absence of such an isozyme.

In sera of patients suffering from tienilic acid (ticrynafen, a diuretic) induced hepatitis, high titers of novel "anti-liver kidney microsome" (anti-LKM2) antibodies have been detected (Homberg et al., 1984, 1985). These human autoimmune antibodies strongly inhibit the microsomal oxidation of tienilic acid and recognize specifically P-450₈ on Western blots of human liver microsomes (Beaune et al., 1987). It was previously shown that P-450₈ corresponds to P-450 meph and P-450 MP (Gut et al., 1986; Shimada et al., 1986). Because of the apparent relatedness of P-450 meph and P-450₈ preliminary studies were done in collaboration with P. Dansette (Université René Descartes, Paris), which revealed inhibition of microsomal mephenytoin hydroxylation by anti-LKM2 sera (Beaune et al., 1987).

In this paper we present evidence that anti-LKM2 antibodies indeed recognize specifically the P-450 responsible for microsomal (S)-mephenytoin 4-hydroxylation. Furthermore, this P-450 was purified by immunoaffinity to anti-LKM2 IgG from EM and PM liver microsomes.³ Biochemical comparison of the two isozymes from EM and PM livers revealed no sig-

nificant difference, indicating that a minor alteration in the PM isozyme causes the mephenytoin hydroxylation polymorphism.

EXPERIMENTAL PROCEDURES

Sera and IgG. Human sera containing anti-LKM2 autoantibodies were obtained from patients suffering from tienilic acid induced hepatitis. They were kindly provided by Dr. J.-C. Homberg (Laboratoire Central d'Immunologie et d'Hématologie, Hôpital Saint-Antoine, Paris). Human control serum was from a normal healthy individual (U.T.M., designated as TOM). The three-letter code in parenthesis behind the sera identifies the corresponding patient or control. IgG fractions from these sera were purified by protein A-Sepharose chromatography as described (Hjelm et al., 1972) and dialyzed against NaP_i (0.1 M).

Rabbit anti-P-450 meph sera and IgG were those previously described (Gut et al., 1986). Rabbit sera against P-450 meph-ip were raised in female New Zealand White rabbits as described (Gut et al., 1986) with minor modifications. One hundred micrograms of antigen cut out from SDS-PAGE gels after visualization with potassium chloride (Hager & Burgess, 1980) was used for immunization and boosts of the rabbits.

Preparation of IgG Covalently Coupled to Protein A-Sepharose (Immunobeads). IgG fractions from the different sera were adsorbed to protein A-Sepharose CL-4B beads by incubation for 2 h [0.3 mL of serum/mL of stock suspension of protein A-Sepharose (30% v/v)]. The whole procedure was carried out at room temperature. The beads were then sequentially washed twice with each of the following: (1) NaP_i (0.1 M) containing 0.2% (w/v) bovine serum albumin; (2) NaP_i (0.1 M) containing 0.5 N sodium chloride; and (3) NaP_i (0.1 M) alone. The IgG were coupled covalently to protein A by incubation with a 5% (v/v) glutaraldehyde (for electron microscopy, Merck) solution in NaP_i (10 mM) for 18 h. Residual glutaraldehyde was removed by extensive washing with NaP_i (0.1 M). Free aldehyde groups were then blocked by incubation with 0.5 M ethanolamine in NaP_i (0.5 M) for 18 h. After the beads were washed 5 times with NaP_i (0.1 M) containing 1 N sodium chloride, the IgG covalently coupled to protein A-Sepharose beads was kept as stock suspension (30% v/v) in the same buffer at 4 °C. The binding capacity, as tested by immunoprecipitation of the covalently coupled IgG, is the same as that of IgG noncovalently adsorbed to protein A-Sepharose beads, and the beads have been used up to 8 times in immunoprecipitation or immunoaffinity chromatography experiments without loss of binding capacity. These protein A-Sepharose beads carrying covalently coupled IgG are called "immunobeads" in the text.

Immunoprecipitation. Human liver microsomes were solubilized with either 2% (w/v) sodium cholate (protein:detergent = 1:6)/2% (v/v) Triton X-100/20% (v/v) glycerol or 1% (w/v) CHAPS (protein:detergent = 1:3). After centrifugation at 105000g the supernatant, corresponding routinely to 170 µg of microsomal protein or 100 pmol of P-450, was diluted by a factor of 3 with NaP_i (0.1 M) containing the above detergents. Twice the amount of supernatant was used without dilution in experiments where activity was reconstituted after detergent removal. Solubilized microsomes were then incubated with the indicated amounts of anti-LKM2 or human control immunobeads for 90 min at 4 °C. After immunoprecipitation and centrifugation, the supernatant was harvested for activity measurement and SDS-PAGE. The pellet was washed twice with the appropriate solubilization buffer, 5 times with NaP_i (0.1 M), 1 N sodium chloride, and 0.1% CHAPS, and twice with NaP_i containing no sodium chloride. The

¹ Abbreviations: EM, extensive metabolizer of mephenytoin; PM, poor metabolizer of mephenytoin; P-450, cytochrome P-450; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing; NaP_i, sodium phosphate buffer, pH 7.4; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IgG, immunoglobulin G; immunobeads, IgG covalently coupled to protein A-Sepharose beads; HPLC, high-performance liquid chromatography.

² Awaiting a generally accepted nomenclature of cytochrome P-450 isozymes, we have used the designations given by the respective authors: P-450 meph, Gut et al. (1986); P-450 MP-1 and MP-2, Shimada et al. (1986); P-450₈ and P-450₉, Wang et al. (1983); P450₈, Beaune et al. (1985); P450 meph-ip, referring to the P-450 immunopurified with the autoantibodies described in this paper.

³ If no further description is given, the expressions "EM microsomes" and "PM microsomes" denote that the microsomes were in vitro characterized regarding their (S)-mephenytoin 4-hydroxylation phenotype by the method of Meier et al. (1985a).

bound protein was eluted with 0.5% (w/v) SDS and submitted to SDS-PAGE. No elution was observed with 1 mM racemic mephenytoin, 2 N sodium chloride, 5 M magnesium chloride, pH 2.2 or 11 solutions, 5 M guanidine hydrochloride, and electrophoretic elution.

Immunoaffinity Column Chromatography. A column (0.9 cm in diameter) was packed with 6 mL of a 30% stock suspension of anti-LKM2 immunobeads (PLA) and equilibrated with solubilization buffer [NaP_i (0.1 M)/2% (w/v) sodium cholate/2% (v/v) Triton X-100/20% (v/v) glycerol]. Chromatography (flow rate 20 mL/h) was performed at room temperature, and the column eluate was monitored at 280 nm. Microsomes (30 mg of protein) were solubilized and centrifuged as described for immunoprecipitation and applied to the column at a concentration of 1 mg/mL. The column was sequentially washed with 40 mL each of solubilization buffer, NaP_i (0.1 M), NaP_i containing 2 N sodium chloride, and distilled water. Approximately 0.3 mg of protein could then be eluted by using 0.5% SDS in water. The eluted protein was extensively dialyzed against NaP_i (10 mM) and concentrated by ultrafiltration (Centricon 30, Amicon). This protein was designated P-450 meph-ip.

Isoelectric Focusing (IEF). IEF was performed in agarose gels as described by the manufacturer (Pharmacia Fine Chemicals, 1982) with the following modifications. Gels contained 5% (v/v) glycerol, 1% (w/v) CHAPS, 2% (v/v) Triton X-100, and 2.7% (w/v) ampholine (LKB, pH 3.5–10:pH 6–8 = 3:1). Microsomes were solubilized under the same conditions and centrifuged at 105000g before focusing. After IEF, gels were first stained for heme by benzidine (saturated solution in ethanol:50% acetic acid = 1:1) and 3% perhydrol solution in water. Thereafter, proteins were immediately fixed and stained by Coomassie blue as described (Pharmacia Fine Chemicals, 1982).

Immunoblotting on Nitrocellulose. Western blots were performed after SDS-PAGE of microsomal protein (100 $\mu\text{g}/\text{cm}$) and purified P-450s (2 $\mu\text{g}/\text{cm}$) as described by Towbin et al. (1979). After isoelectric focusing of microsomal proteins (200 $\mu\text{g}/\text{cm}$) they were transferred passively under non-denaturing conditions to nitrocellulose sheets ["native blot", Reinhart and Malamud (1982)]. The proteins on nitrocellulose were then stained with amido black and destained with acetic acid/methanol (7.5%/20%) before immunochemical reaction with rabbit antibodies. Before reaction with human antibodies nitrocellulose was treated directly with 10% fetal calf serum in 10% phosphate-buffered saline. Immunochemical reaction of the human or rabbit antibodies on blots treated differently yielded a high background stain. Antigens on nitrocellulose blots were incubated with sera diluted 1:100 and visualized either by peroxidase-labeled goat anti-human IgG (Nordic GAHu/Ig/PO) followed by peroxidase stain with 4-chloro-1-naphthol or by ^{125}I -protein A iodinated by the chloramine T procedure (Greenwood et al., 1963) and fluorography. For blocking, either 10% (v/v) fetal calf serum (peroxidase method) or 10% (w/v) defatted powdered milk (^{125}I -protein A method; Sanolait, Coop, Switzerland) in 10% phosphate-buffered saline was used as described elsewhere (Hauri & Bucher, 1987).

Mephenytoin 4-Hydroxylation and N-Demethylation Activity. Microsomal mephenytoin metabolism was measured by a HPLC assay as previously described (Meier et al., 1985b). P-450 meph was reconstituted by addition of NADPH-cytochrome P-450 reductase and α -dilauroylphosphatidylcholine as reported previously (Gut et al., 1986); the method for immunoinhibition studies is also described therein. IC_{50} values

were determined from concentration-dependent microsomal inhibition studies with the different anti-LKM2 sera under the conditions described in the legend for Figure 1. Activity on anti-LKM2 immunobeads was detected after extensive washing of the immunobeads in the presence of 1 μM NADPH-cytochrome P-450 reductase and incubation in a final volume of 250 μL at 400 μM (*S*)-mephenytoin in 0.25% (v/v) propylene glycol. Activity in solubilized microsomes with or without detergent removal and in proteins eluted from IEF gels was also measured in the presence of 1 μM NADPH-cytochrome P-450 reductase but at 800 μM mephenytoin in 0.5% (v/v) propylene glycol.

Electrophoretic Detergent Removal and Electroelution. An ISCO sample concentrator (Model 1750, ISCO, Lincoln, NE) was used to remove detergents from solubilized microsomes and elute proteins from IEF gels. The procedure was essentially the same as that published by Allington et al. (1978) with additional information found in Hunkapiller et al. (1983) and Bhowm and Bennett (1983). Briefly, gel slices from IEF or 100 μL of supernatants after immunoprecipitation, corresponding to 200 pmol of P-450 or 340 μg of microsomal protein of cholate-solubilized microsomes, were applied to the sample cup in the cathode compartment. The running buffer was Tris-acetate, pH 8.6, 40 mM/10 mM in the electrode/inner compartment of the electrophoresis tank. Spectrapor 2 dialysis membranes prevented the proteins from diffusion into the electrophoresis tank. Electroelution or electrophoretic transfer of proteins from nonionic detergents and concomitant electrodialysis of anionic detergents were carried out at constant power (3 W) for 4 h at 4 °C simultaneously in four sample cups per electrophoresis tank. The protein was found concentrated in 200 μL after this procedure. After elution from IEF gels 100 μL of this concentrated sample was used for activity reconstitution experiments, and after detergent removal 25 μL of this concentrated sample was used.

Other Methods. Human liver microsomes were prepared from kidney donor livers according to Meier et al. (1983) with slight modifications (Meier et al., 1985b). Their phenotype in regard to (*S*)-mephenytoin hydroxylation was determined by our published method (Meier et al., 1985a). Microsomes from liver wedge biopsies were isolated as described (Meier et al., 1985a). Dr. T. Cresteil (Hôpital Necker, Paris) kindly provided us with microsomes from human fetal liver (Cresteil et al., 1985). The purification and characterization of P-450 meph has recently been published (Gut et al., 1986). NADPH-cytochrome P-450 reductase was purified from liver microsomes of untreated rats (Yasukochi & Masters, 1976) and assayed at room temperature as described by Strobel and Dignam (1978). Two-dimensional peptide mapping was carried out on thin-layer chromatography sheets (Polygram, CEL 300, Macherey-Nagel, Düren, FRG) after tryptic digestion according to Elder et al. (1977). One-dimensional peptide maps were performed after partial digestion in the presence of 0.25% (w/v) SDS on SDS-PAGE gels (12.5% acrylamide) by the indicated amounts of α -chymotrypsin (Worthington), papain (Sigma), and *Staphylococcus aureus* V8 protease (Pierce) according to Waxman and Walsh (1983). Amino acid composition was determined from 0.2 and 0.4 μg of each P-450 meph-ip by H. W. Mewes (Max Planck Institute, Martinsried, FRG) according to the method of Tsugita and Scheffler (1982) and Mewes and Tsugita (unpublished results). N-Terminal protein sequences were determined by automated Edman degradation in a gas-phase sequencer (Applied Biosystems) and the phenylthiohydantoin amino acids analyzed by HPLC (Lottspeich, 1985). This was performed

Table I: Immunoinhibition by Anti-LKM2 Antibodies of (R)- and (S)-Mephenytoin Metabolism in EM and PM Microsomes and Reconstituted P-450 meph^a

IgG	4-hydroxylation			N-demethylation		
	none ^b	human control (%)	anti-LKM2 (%)	none ^b	human control (%)	anti-LKM2 (%)
(S)-mephenytoin						
EM microsomes	7.09	109	9	7.28	102	113
PM microsomes	1.07	93	32	18.34	94	92
reconstituted P-450 meph ^c	15.00	108	20	4.27	104	29
(R)-mephenytoin						
EM microsomes	0.54	106	69	5.26	80	75
PM microsomes	0.74	115	73	14.17	92	93
reconstituted P-450 meph ^d	2.00	129	35	2.76	119	50

^aOne milligram of IgG/nmol of P-450 [anti-LKM2 (PLA) and human control (TOM)], 35 pmol of microsomal P-450, and 80 pmol of purified P-450 meph were used. (R)- and (S)-mephenytoin concentrations were 800 μ M (0.5% propylene glycol). ^bActivity given in nmol of metabolite/(nmol of P-450)·h. ^cSubstrate concentration was 800 μ M (S)-mephenytoin in 1% propylene glycol. ^dSubstrate concentration was 1600 μ M (R)-mephenytoin in 1% propylene glycol.

on 200 pmol of each P-450 meph-ip by F. Lottspeich (Max Planck Institute, Martinsried, FRG). SDS-PAGE was carried out by using the Laemmli system (Laemmli, 1970) in 10% acrylamide gels of 1.5-mm thickness. Gels were routinely stained with silver (Merril et al., 1984). Bufuralol 1'-hydroxylation activity (Dayer et al., 1984) was determined in intact and solubilized microsomes by using cumene hydroperoxide as oxygen donor. Cytochrome P-450 and protein concentrations were measured as described by Omura and Sato (1964) and Lowry et al. (1951), respectively. Chemicals and reagents for the assay of microsomal mephenytoin metabolism by HPLC have been described in detail elsewhere (Meier et al., 1985b). (+)-Bufuralol and 1'-hydroxybufuralol were a gift from Hoffmann-La Roche, Basel, Switzerland, and Welwyn Garden City, U.K. Protein A-Sepharose CL-4B was purchased from Pharmacia. Other chemicals and reagents were obtained from Sigma, Merck, or Serva.

RESULTS

Immunoinhibition. EM liver microsomal (S)-mephenytoin 4-hydroxylation was almost completely inhibited by anti-LKM2 IgG in a concentration-dependent manner. Anti-P-450 meph IgG was 20 times less potent, and control IgG had no effect (Figure 1A). Moreover, all three IgG preparations affected neither (S)-mephenytoin N-demethylation (Figure 1B) nor bufuralol 1'-hydroxylation activities (data not shown). Anti-LKM2 IgG also inhibited (S)- and (R)-mephenytoin metabolism of purified reconstituted P-450 meph and mephenytoin 4-hydroxylation of microsomes from PM liver (Table I). These results are strikingly similar to those obtained by using anti-P-450 meph IgG at a concentration of 16 mg of IgG/nmol of P-450 (Gut et al., 1986). Polyclonal sera raised in rabbits against the denatured P-450 meph-ip exhibited no inhibition of microsomal mephenytoin metabolism.

Immunoprecipitation from EM and PM Microsomes. Anti-LKM2 IgG and human control IgG were covalently coupled to protein A-Sepharose and used for immunoprecipitation from solubilized microsomes as described under Experimental Procedures. This efficient procedure to our knowledge has not been described previously. It allows the identification of immunoprecipitated protein on SDS-PAGE without radiolabeling, and the antibodies can be reused several times. These anti-LKM2 immunobeads precipitated a single electrophoretically detectable protein from liver microsomes prepared from wedge biopsies of in vivo and in vitro phenotyped EM and PM individuals (Figure 2, lanes 2 and 3) and from microsomes prepared from in vitro phenotyped kidney donor livers (Figure 2, lanes 4 and 7). No detectable protein band could be precipitated by human control immunobeads

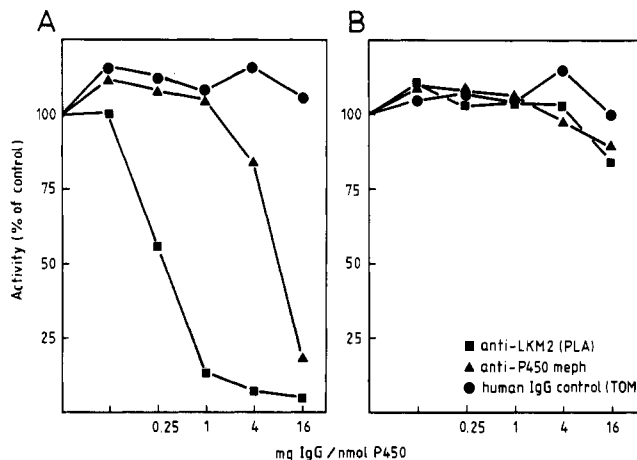


FIGURE 1: Immunoinhibition by anti-LKM2 antibodies of microsomal (S)-mephenytoin metabolism. Microsomal (S)-mephenytoin 4-hydroxylation (A) and N-demethylation (B) were measured in the presence of varying concentrations of IgG fractions prepared from anti-LKM2 (PLA), human control (TOM), and rabbit anti-P-450 meph serum. Twenty picomoles of microsomal P-450 was incubated with 200 μ M (S)-mephenytoin [0.125% (v/v) propylene glycol]. One hundred percent activity was (A) 6.95 nmol of 4-hydroxymephenytoin/(nmol of P-450)·h and (B) 12.35 nmol of nirvanol/(nmol of P-450)·h.

(Figure 2, lane 6). This immunoprecipitated protein was tentatively called P-450 meph-ip to denote an immunopurified band possibly corresponding to P-450 meph. No missing protein could be detected in supernatants of solubilized microsomes when compared before and after immunoprecipitation as tested by silver-stained SDS-PAGE (not shown).

Immunoprecipitation from Fetal Microsomes. Microsomes from human fetal liver have been shown to lack immunochemically detectable P-450₈ (Creteil et al., 1985), which appears to correspond to P-450 meph (Gut et al., 1986) and to P-450 MP-1 (Shimada et al., 1986). Anti-LKM2 immunobeads were not able to precipitate any protein from fetal liver microsomes (Figure 2, lane 8). These microsomes also exhibit very low (S)-mephenytoin 4-hydroxylase activity (Shimada et al., 1986) and a high R/S ratio of mephenytoin 4-hydroxylation near unity, characteristics common to microsomes of adult PM subjects (Meier et al., 1985a). In contrast, the R/S ratio of the demethylation reaction (mean \pm SD = 0.46 ± 0.16 , $n = 5$) was found to correspond to that in microsomes of adult EM subjects (0.45 ± 0.08 , $n = 13$; Meier et al., 1985a) although the absolute activity was also decreased [(R)- and (S)-mephenytoin N-demethylation = 1.60 ± 0.66 and 3.50 ± 0.64 nmol of nirvanol/(nmol of P-450)·h, respectively, $n = 5$]. These data suggest that the absent P-450 protein in fetal liver microsomes may be that affected by the

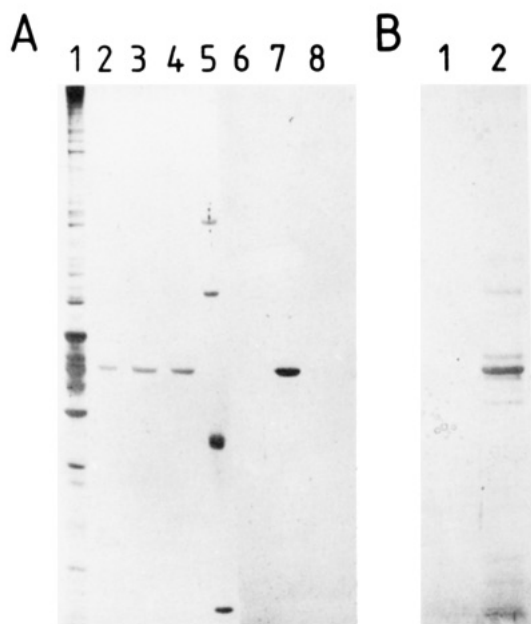


FIGURE 2: SDS-PAGE of immunoprecipitated P-450 meph-ip by anti-LKM2 immunobeads from solubilized microsomes. Human liver microsomes were solubilized by either 2% (w/v) cholate/2% (v/v) Triton X-100 (A) or 1% CHAPS (B) and incubated with anti-LKM2 immunobeads (panel A: lanes 2–4, 25 μ L; lanes 7 and 8, 50 μ L; panel B: lane 2, 30 μ L) or human control immunobeads (panel A: lane 6, 50 μ L; panel B: lane 1, 30 μ L). The immunoprecipitated protein was eluted and subjected to SDS-PAGE. (A) Lane 1, solubilized microsomes (7.5 μ g); lanes 2 and 3, precipitates from microsomes of in vivo and in vitro phenotyped EM and PM subjects, respectively; lanes 4, 6, and 7, precipitates from EM microsomes; lane 5, molecular weight markers (top to bottom, 92.5K, 66.2K, 45K, and 32K); lane 8, precipitate from fetal liver microsomes. (B) Lanes 1 and 2, precipitates from CHAPS-solubilized microsomes.

genetic defect in PM liver microsomes of adults.

Immunoprecipitation of Activity. Because the immunoprecipitated protein could only be eluted under denaturing conditions from anti-LKM2 immunobeads, we attempted to reconstitute activity of the protein while still attached to the immunobeads by addition of NADPH-cytochrome P-450 reductase. Despite the inhibitory potency of the anti-LKM2 antibodies, very low but concentration-dependent (*S*)-mephenytoin 4-hydroxylation activity could be measured (Figure 3A), whereas no activity was detected on human control immunobeads. No (*S*)-mephenytoin N-demethylation or (+)-bufuralol 1'-hydroxylation activity was found by using this procedure (not shown). Anti-LKM2 IgG from different patients exhibited varying capacities to precipitate (*S*)-mephenytoin 4-hydroxylase activity which correlated with the amount of protein precipitated and with their inhibitory potency and reactivity on native blots (Figure 6A). Moreover, the reconstituted (*S*)-mephenytoin 4-hydroxylation activity was only exhibited by the protein precipitated from EM but not by that from PM microsomes. This result suggests that the protein precipitated from PM microsomes has either a very low or no activity. After partial removal of detergent and addition of NADPH-cytochrome P-450 reductase, activity was also monitored in the supernatant from solubilized microsomes after immunoprecipitation. With increasing amounts of anti-LKM2 immunobeads, (*S*)-mephenytoin 4-hydroxylation activity progressively disappeared from supernatants, while (*S*)-mephenytoin N-demethylase and (+)-bufuralol 1'-hydroxylase (not shown) were not precipitated (Figure 3B). It was not possible either to measure a reduced cytochrome P-450 CO spectrum of P-450 still attached to the immunobeads or to determine spectrophotometrically the amount of

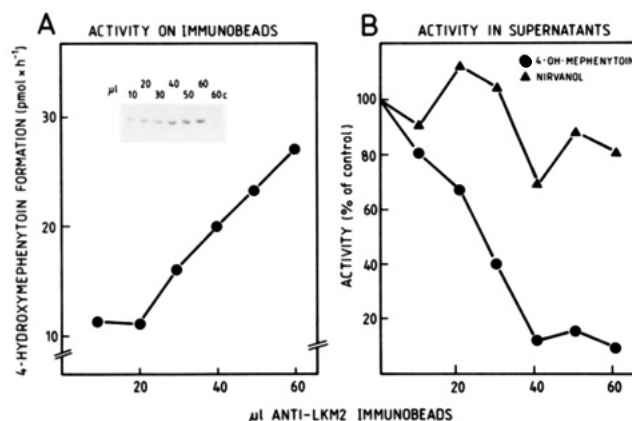


FIGURE 3: Immunoprecipitation by anti-LKM2 immunobeads of (*S*)-mephenytoin 4-hydroxylation activity. Cholate-solubilized EM microsomes were incubated with varying amounts of a 30% (gel v/v) suspension of anti-LKM2 immunobeads (PLA) and activity reconstituted after precipitation as described under Experimental Procedures. Inset: Silver-stained SDS-PAGE gel of the eluted P-450 meph-ip in relation to the amount of anti-LKM2 immunobeads used ("60c" refers to the eluate from 60 μ L of human control beads). (B) (*S*)-Mephenytoin 4-hydroxylation was measured in the supernatants after electrophoretic removal of detergents, as described under Experimental Procedures. One hundred percent activity corresponds to that measured after precipitation by 60 μ L of human control beads [4.12 nmol of 4-hydroxymephenytoin/(nmol of P-450)-h and 2.00 nmol of nirvanol/(nmol of P-450)-h].

P-450 disappearing from the supernatant after immunoprecipitation.

Immunoprecipitation from microsomes solubilized by 1% (w/v) CHAPS instead of 2% (w/v) sodium cholate/2% (v/v) Triton X-100/20% (v/v) glycerol yielded a fraction somewhat less homogeneous (Figure 2B). However, a much higher activity could be measured upon addition of NADPH-cytochrome P-450 reductase to CHAPS-solubilized microsomes than under the more stringent cholate/Triton X-100 solubilization conditions. Therefore, more extensive studies of the metabolism of (*R*)- and (*S*)-mephenytoin could be performed in supernatants after precipitation by anti-LKM2 immunobeads, as summarized in Table II. Under these conditions EM microsomes were characterized by a low *R/S* ratio (0.15) of mephenytoin 4-hydroxylation (due to preferential metabolism of the *S* enantiomer) while PM microsomes lacked most of the stereoselectivity for this reaction (*R/S* ratio = 0.92), in agreement with our previous findings (Meier et al., 1985a). After precipitation by anti-LKM2 immunobeads, the *R/S* ratio in EM microsomes was converted to a value near unity, mimicking a PM microsomal phenotype. This is in agreement with the concept that the precipitated protein indeed is the one affected by the mephenytoin hydroxylation polymorphism. Moreover, the *R/S* ratio of mephenytoin 4-hydroxylation was only slightly affected by precipitation from PM microsomes while the *R/S* ratio of the demethylation reaction remained almost unchanged in both phenotypes (Table II).

Immunoaffinity Column Chromatography. Anti-LKM2 immunobeads were further used to purify the antigen in larger amounts from EM and PM liver microsomes on an immunoaffinity column. The proteins purified by this procedure also exhibited one single band on SDS-PAGE (Figure 4). These P-450 meph-ip's exhibited a mean (\pm SD) M_r of 52 200 \pm 2100, which was determined on three different SDS-PAGE gels relative to the molecular weight standards. Although P-450 meph was initially reported to have a M_r of 55 000 (Gut et al., 1986), it had exactly the same mobility on SDS-PAGE as P-450 meph-ip (data not shown). The amount (mean \pm SD) of P-450 meph-ip isolated from EM microsomes was

Table II: Precipitation by Anti-LKM2 Immunobeads of (*R*)- and (*S*)-Mephenytoin Oxidation Activity from CHAPS-Solubilized EM and PM Liver Microsomes^a

immunoprecipitation	<i>(R)</i> -mephenytoin		<i>(S)</i> -mephenytoin		<i>R/S</i> ratio	
	4-hydroxy-mephenytoin [nmol/(nmol of P-450)·h]	nirvanol [nmol/(nmol of P-450)·h]	4-hydroxy-mephenytoin [nmol/(nmol of P-450)·h]	nirvanol [nmol/(nmol of P-450)·h]	4-hydroxy-mephenytoin	nirvanol
EM microsomes						
none	1.28	8.32	8.77	27.49	0.15	0.30
human control beads	1.05	8.16	6.40	22.73	0.16	0.36
anti-LKM2 beads	0.78	6.64	0.82	19.05	0.95	0.35
PM microsomes						
none	1.31	17.21	1.43	35.12	0.92	0.49
anti-LKM2 beads	0.98	14.41	0.74	35.69	1.32	0.40

^a Microsomal protein (170 μ g) was incubated with 100 μ L of immunobeads (30% v/v), and after precipitation activity was measured in supernatants in the presence of 1 μ M NADPH-cytochrome P-450 reductase and 1 mM (*R*)- or (*S*)-mephenytoin in 1% (v/v) propylene glycol.

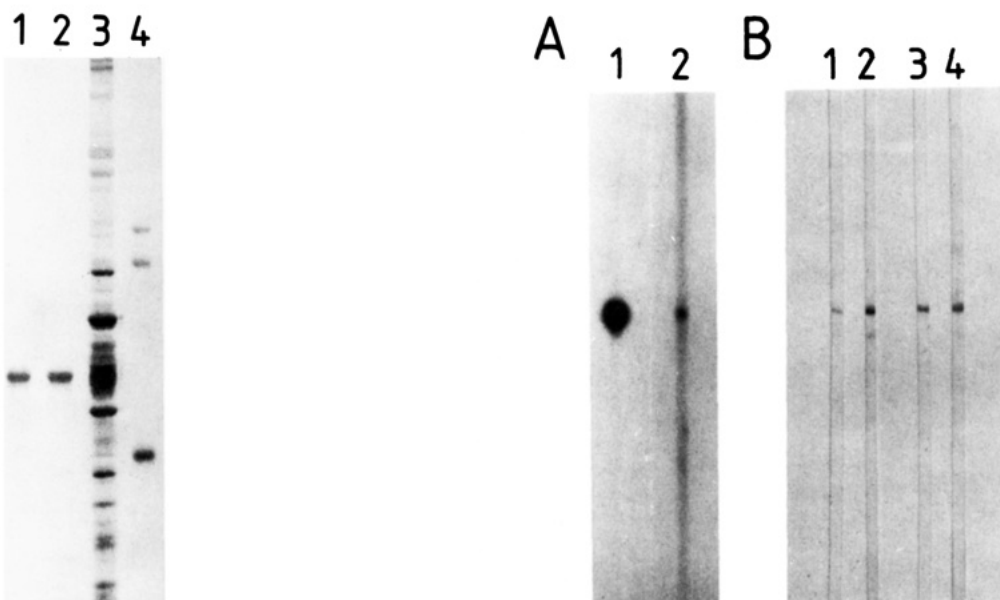


FIGURE 4: SDS-PAGE of P-450 meph-ip immunoaffinity purified from EM and PM microsomes: 1 μ g of P-450 meph-ip immunopurified from EM (lane 1) and PM (lane 2) microsomes. Lane 3: Microsomal protein (5 μ g). Lane 4: Molecular weight markers (77K, 66.2K, 45K, and 31K).

determined from four different preparations to be $1.1 \pm 0.1\%$ of total microsomal protein while the amount isolated from PM microsomes corresponded to 0.8%. The recovery was quantitative as it was not possible to precipitate any further P-450 meph-ip from the flow-through fractions of the affinity column. It was not possible to reconstitute activity in these proteins or to measure a characteristic P-450 difference spectrum since the proteins could only be eluted from the affinity column under denaturing conditions.

Immunochemical Reactivity on Nitrocellulose Blots. On Western blots of human liver microsomes anti-LKM2 sera faintly recognized a single band (Figure 5, lane 2) of the same relative molecular weight as was detected by anti-P-450 meph antibodies (Figure 5, lane 1). Furthermore, both the classically purified P-450 meph and the immunoaffinity-purified P-450 meph-ip were recognized on Western blots by polyclonal rabbit sera raised against either antigen (Figure 5B). Anti-LKM2 sera reacted only very weakly on Western blots of microsomes (Figure 5) and of the two purified proteins (not shown), but they exhibited a very high inhibitory potency (Figure 1 and Table I) and effectively precipitated a single protein. This may

FIGURE 5: Immunochemical relatedness of P-450 meph and P-450 meph-ip on Western blots. (A) After SDS-PAGE microsomes were blotted to nitrocellulose. Fluorographs of immunoreactions of rabbit anti-P-450 meph (lane 1) and human anti-LKM2 (KUT, lane 2) sera are shown. (B) Western blots of purified P-450 meph-ip (lanes 1 and 3) and P-450 meph (lanes 2 and 4) were incubated with either anti-P-450 meph (lanes 1 and 2) or anti-P-450 meph-ip (lanes 3 and 4) rabbit sera. Immunoreaction was visualized by peroxidase stain. The amounts of proteins and dilutions of sera were as indicated under Experimental Procedures.

indicate that these sera are mainly directed against a native protein. We therefore tested their reactivity on *native blots*. To do this, we separated microsomes by IEF in the absence of urea and blotted these proteins to nitrocellulose sheets under nondenaturing conditions (Reinhart & Malamund, 1982). Anti-LKM2 sera reacted strongly with a band focusing at pH 8.7 (Figure 6A). The intensity of the immunoreaction of anti-LKM2 sera from different patients seemed to correlate with their potency of inhibiting (*S*)-mephenytoin 4-hydroxylation as represented by their IC_{50} values (Figure 6A). No visible difference in the extent of immunochemical reaction on native blots was detected by anti-LKM2 serum between EM and PM liver microsomes (Figure 6B, lanes 1 and 2). The same results were obtained with anti-P-450 meph antibodies on native blots after the microsomal proteins had been denatured by amido black staining and destaining by acetic acid and methanol (Figure 6B, lanes 3 and 4). These data corroborate our previous findings with anti-P-450 meph antibodies on Western blots of microsomes from *in vivo* and *in vitro* phenotyped EM and PM subjects (Gut et al., 1986).

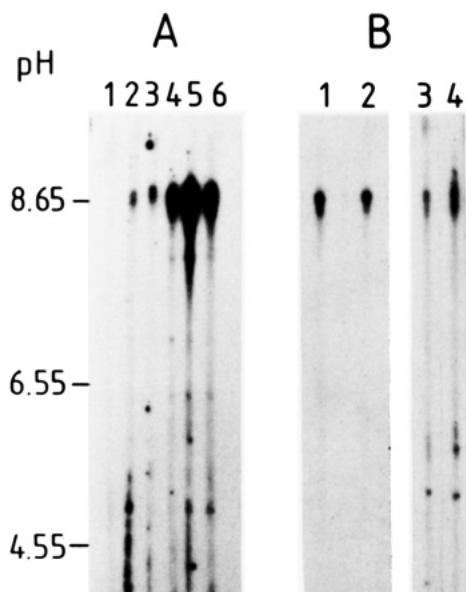


FIGURE 6: Immunoreactivity of anti-LKM2 and anti-P-450 meph sera on native blots of EM and PM microsomes. Human liver microsomes were isoelectrically focused in agarose gels and transferred to nitrocellulose sheets under nondenaturing conditions. Immunoreactivity of antibodies was visualized by ^{125}I -protein A and fluorography. pH indicates focusing positions of marker proteins with corresponding isoelectric points (see Figure 7A). (A) Native blots of EM microsomes incubated with human control serum TOM (lane 1) and anti-LKM2 sera FAR (lane 2, 76 μL), REY (lane 3, 60 μL), JOR (lane 4, 25 μL), CAI (lane 5, 16 μL), and PLA (lane 6, 14 μL). The second number in parentheses denotes the amount of serum needed per nanomole of microsomal P-450 for a 50% inhibition of (*S*)-mephenytoin 4-hydroxylation (IC_{50}). (B) Native blots of EM (lanes 2 and 4) and PM (lanes 1 and 3) microsomes incubated with anti-LKM2 serum PLA (lanes 1 and 2) or anti-P-450 meph serum (lanes 3 and 4). The amounts of proteins and dilutions of sera were as indicated under Experimental Procedures.

Isoelectric Focusing. Microsomal proteins were solubilized in CHAPS and Triton X-100 containing buffer and separated according to their isoelectric points in an agarose gel system containing the same detergents. Heme staining of the focused proteins revealed a major band (Figure 7, arrow, lane 3) at the same pH where anti-LKM2 and anti-P-450 meph antibodies had recognized a protein (Figure 6). A distinct protein band was also seen in that position after Coomassie blue staining of the same lane (Figure 7, lane 2). This band was cut out from the IEF gel, boiled in SDS-containing sample buffer, and subjected to SDS-PAGE. Besides a minor contamination at a molecular weight around 40 000, a single protein band was detected with the same molecular weight as P-450 meph-ip (Figure 7B, arrow). When solubilized EM and PM microsomes were focused, no difference in the stain intensity of the band at pH 8.7 could be observed either after heme stain or after Coomassie blue stain (not shown). Classically purified P-450 meph was only weakly detectable at pH 8.7 with heme staining after IEF and not at all with Coomassie blue staining (not shown). A reason for this could be that part of the purified P-450 meph seems to be denatured apoprotein as already discussed by Gut et al. (1986). This hypothesis is also supported by the fact that denatured immunopurified P-450 meph-ip could also not be detected after IEF by either staining method. No activity could be reconstituted with the protein band at pH 8.7 nor with proteins at any other pH after electroelution from IEF gels. The electroelution process was excluded as a cause for the activity loss mainly by two experiments: first, by testing for the eluted proteins on silver-stained SDS-PAGE gels and second by

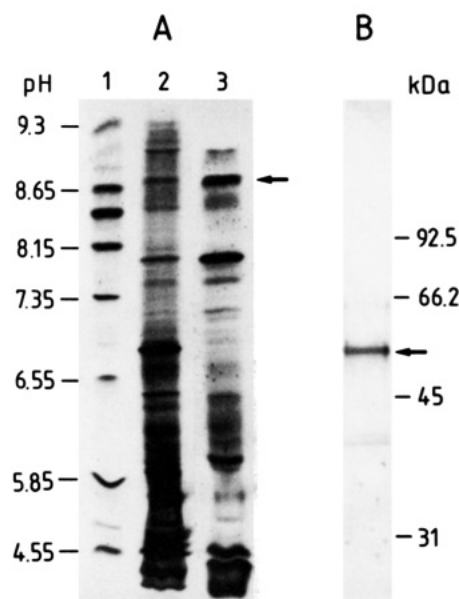


FIGURE 7: Isoelectric focusing (IEF) of microsomes and SDS-PAGE of the protein exhibiting an isoelectric point of 8.7. (A) IEF of EM microsomes (200 μg , lanes 2 and 3) and of calibration markers (Pharmacia, lane 1). Lanes 1 and 2 were stained by Coomassie blue, and lane 3 corresponds to lane 2 stained first for heme by benzidine (Experimental Procedures). The pH refers to the isoelectric points of the corresponding marker proteins (from top to bottom: trypsinogen, 9.30; lentil lectin—basic band, 8.65; lentil lectin—acidic band, 8.15; myoglobin—basic band, 7.35; human carbonic anhydrase B, 6.55; bovine carbonic anhydrase B, 5.85; soybean trypsin inhibitor, 4.55). Focusing conditions: Distance between electrodes was 9 cm, and gel thickness was 1 mm. Samples and marker proteins were applied after prefocusing on paper sample applicators 1.3 and 2.5 cm from anode, respectively. IEF was performed at constant power (8 W) for 2800 V·h at 10 $^{\circ}\text{C}$. (B) SDS-PAGE of the heme and protein band occurring at pH 8.7 excised from the IEF gel (arrow in panel A). Molecular masses of marker proteins are indicated. Arrow denotes position of P-450 meph and P-450 meph-ip.

measuring reconstituted activity in solubilized microsomes after their electroelution from control IEF gels that had not been focused before.

Peptide Maps. The two immunopurified P-450 meph-ip's from EM and PM microsomes were compared by ^{125}I -labeled tryptic two-dimensional peptide maps (Elder et al., 1977). Essentially the same tryptic fingerprints were found for both proteins, and repeatedly no difference could be observed between minor spots after varying exposure times of the X-ray films (data not shown). This method was reported to distinguish rat P-450s which are probably more than 99% homologous (Rampersaud & Walz, 1986; Vlasuk et al., 1982), suggesting that very minor differences could exist between P-450 meph-ip from EM and PM microsomes. It must, however, be realized that only peptides containing tyrosine, histidine, and phenylalanine residues can be detected by using this method. The two proteins were further analyzed by partial digestion in the presence of SDS, each by two different amounts of α -chymotrypsin (1 and 4 μg), *S. aureus* V8 protease (0.45 and 1.8 μg), and papain (0.03 and 0.12 μg). Comparison of the proteolytic peptides of the two proteins on SDS-PAGE gels stained with silver also revealed no visible difference (data not shown).

Amino Acid Composition. For further characterization of the two P-450 meph-ip preparations from EM and PM microsomes their amino acid compositions were determined (Table III). This was kindly performed by H. W. Mewes at the Max Planck Institute (Martinsried, FRG) and revealed no differences between the two proteins within the error limits

Table III: Amino Acid Compositions and N-Terminal Protein Sequences of P-450 meph-ip Immunopurified from EM and PM Microsomes

		1	2	3	4	5	6	7	8
EM		Met-Asp-Ser-Leu-Val-Val							
PM		Met-Asp-Ser-Leu-Val-Val-Leu-Val							
		N-terminus							
		no. of residues of P-450 meph-ip ^a				no. of residues of P-450 meph-ip ^a			
amino acid			amino acid						
	EM	PM		EM	PM				
Ala	31.0	31.0	Met	14.6	11.3				
Arg	30.4	28.1	Phe	30.7	29.1				
Asx	41.9	42.8	Ser	30.4	33.8				
Glx	43.1	48.7	Thr	25.4	25.1				
Gly	32.6	42.8	Tyr	17.7	15.5				
His	12.1	12.9	Val	37.5	33.6				
Ile	28.5	26.8	total ^b	461.5	464.9				
Leu	62.3	57.5							
Lys	23.3	25.9							

^aValues are means of duplicate determinations and are calculated relative to alanine assuming a M_r of 52 200. ^bNot including cysteine, proline, and tryptophan.

of the determination method (H. W. Mewes, and A. Tsugita, unpublished results).

Amino-Terminal Protein Sequences. The comparison of the N-terminal amino acid sequences of P-450 meph-ip from EM and PM microsomes revealed identity (Table III). They were also identical with the published N-terminal region of P-450 MP (Shimada et al., 1986) but differed from those of 4 other human P-450s (Jaiswal et al., 1985; Wrighton et al., 1986a,b; Beaune et al., 1986) and from 11 rat and 7 rabbit P-450 N-termini recently compared by Shimada et al. (1986).

DISCUSSION

The combined evidence derived from the experiments described in this study suggests that the genetic polymorphism of mephenytoin hydroxylation is due to the presence of a functionally altered cytochrome P-450 meph and not to a decreased amount of intact enzyme protein. This conclusion is based on two considerations. (1) High specific human antibodies against P-450 (S)-mephenytoin 4-hydroxylase were discovered, and these antibodies recognized the same amount of a protein in microsomes of livers from EM and PM subjects with very similar or almost identical structure. (2) Circumstantial evidence suggests that P-450 (S)-mephenytoin 4-hydroxylase is indeed the target of the genetic polymorphism.

Anti-LKM2 Antibodies Are Specifically Directed against P-450 (S)-Mephenytoin 4-Hydroxylase. Anti-LKM2 antibodies strongly inhibit (S)-mephenytoin 4-hydroxylation activity of reconstituted P-450 meph and in both EM and PM liver microsomes. The fact that the low activity in PM microsomes is inhibited indicates that the altered enzyme still remains some catalytic activity (Figure 1, Table I). Anti-LKM2 antibodies have no effect on the microsomal N-demethylation of mephenytoin. A quantitatively similar inhibition of (S)-mephenytoin 4-hydroxylation in microsomes and in reconstituted P-450 meph was previously observed with rabbit antibodies raised against P-450 meph and P-450₈ (Gut et al., 1986). Thus P-450 meph, P-450₈, and the anti-LKM2 antigen are immunologically related.

Shimada et al. (1986) have reported the purification and characterization of two very similar P-450s (MP-1, M_r 48 000, and MP-2, M_r 50 000) involved in (S)-mephenytoin 4-hydroxylation. P-450 MP-1 and MP-2 apparently exhibit a somewhat lower molecular weight than P-450 meph (Gut et

al., 1986), and their amino acid compositions revealed possible differences as compared to that of P-450 meph-ip, the protein purified by immunoaffinity to anti-LKM2 antibodies. Nevertheless, P-450 MP-1 and P-450 MP-2 were also shown to be immunochemically related to P-450₈, and their amino-terminal sequence is identical with that of P-450 meph-ip. Therefore, the anti-LKM2 antigen (P-450 meph-ip) is part of the same P-450 family as P-450 meph, P-450 MP-1, P-450 MP-2, and P-450₈ or is in fact identical with them.

Anti-LKM2 antibodies recognize a single protein on immunoblots of human liver microsomes after separation according to molecular weight (Western blots, Figure 5) and according to charge (native blots, Figure 6). The hemoprotein recognized on native blots after IEF appeared homogeneous on SDS-PAGE and had the same apparent molecular weight as P-450 meph and P-450 meph-ip (Figure 7). P-450 meph-ip, exclusively precipitated by anti-LKM2 antibodies from solubilized microsomes, was electrophoretically pure (Figure 2). Moreover, Beaune et al. (1987) recently have shown that anti-LKM2 antibodies specifically recognize human P-450₈ but not P-450₅ or P-450₉. P-450₈ is absent or present in extremely small amounts in human fetal liver microsomes (Creteil et al., 1985). Fetal microsomes indeed exhibit very low (S)-mephenytoin 4-hydroxylation activity (Shimada et al., 1986), and no P-450 meph-ip could be precipitated from them. All these data demonstrate the specificity of anti-LKM2 antibodies for the P-450 isozyme catalyzing (S)-mephenytoin 4-hydroxylation in human liver microsomes.

The specificity of the anti-LKM2 antibodies is presumably due to the mechanism of autoimmunity. They have been exclusively detected in sera of patients suffering from tienilic acid (ticrynafen) induced hepatitis. The formation of a highly reactive metabolite of tienilic acid that covalently binds to the P-450 by which it is metabolized and extracellular appearance of this altered P-450 by cellular necrosis are suspected mechanisms (Beaune et al., 1987). The inhibition of microsomal tienilic acid metabolism by anti-P-450₈ and by anti-LKM2 sera (Beaune et al., 1987) also supports this mechanism.

Mechanism of Mephenytoin Polymorphism. As already discussed, anti-LKM2 antibodies almost completely inhibit and precipitate (S)-mephenytoin 4-hydroxylation activity of human liver microsomes. They have a much smaller effect on the hydroxylation of (R)-mephenytoin and do not inhibit or precipitate N-demethylation of mephenytoin. The effect of anti-LKM2 antibodies on EM microsomes strikingly simulates the situation in liver microsomes of PM subjects where we observed a selective deficiency of (S)-mephenytoin 4-hydroxylation (Meier et al., 1985a). Because of this unique specificity for the P-450 isozyme affected by the mephenytoin polymorphism, we used anti-LKM2 autoantibodies to test for the presence or absence of immuno-cross-reactive protein in liver microsomes of PM subjects. An essentially identical quantity of an immuno-cross-reactive protein was found in EM and PM microsomes (Figures 2 and 6). Moreover, the protein from EM and PM microsomes could not be distinguished by extensive chemical analysis as outlined under Results. We therefore propose that minor structural differences in the P-450 isozyme isolated from EM and PM microsomes are responsible for the drastic loss of activity in PM livers.

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its publication. We gratefully acknowledge the gift of human fetal liver microsomes by T. Cresteil, the determination of amino acid compositions by H. W. Mewes, the determination of the amino-terminal sequences by F. Lottspeich, and the preparation of P-450 meph by J. Gut and T. Catin. We are also thankful to D. M. Grant for critically reading the manuscript and to M. Liechti for excellent secretarial work.

Registry No. Cytochrome P-450, 9035-51-2; (S)-mephenytoin 4-hydroxylase, 96779-46-3; (S)-mephenytoin, 70989-04-7.

REFERENCES

- Allington, W. B., Cordry, A., McCullough, G. A., Mitchell, D. E., & Nelson, J. W. (1978) *Anal. Biochem.* **85**, 188-196.
- Beaune, P., Flinois, J.-P., Kiffel, L., Kremers, P., & Leroux, J. P. (1985) *Biochim. Biophys. Acta* **840**, 364-370.
- Beaune, P. H., Umbenhauer, D. R., Bork, R. W., Lloyd, R. S., & Guengerich, F. P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8064-8068.
- Beaune, P., Dansette, P. M., Mansuy, D., Kiffel, L., Finck, M., Amar, C., Leroux, J. P., & Homberg, J. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 551-555.
- Bhown, A. S., & Bennett, J. C. (1983) *Methods Enzymol.* **91**, 450-455.
- Cresteil, T., Beaune, P., Kremers, P., Celier, C., Guengerich, F. P., & Leroux, J. P. (1985) *Eur. J. Biochem.* **151**, 345-350.
- Dayer, P., Gasser, R., Gut, J., Kronbach, T., Robertz, G.-M., Eichelbaum, M., & Meyer, U. A. (1984) *Biochem. Biophys. Res. Commun.* **125**, 374-380.
- Eichelbaum, M., Spannbrucker, N., Steincke, B., & Douglas, H. J. (1979) *Eur. J. Clin. Pharmacol.* **16**, 183-187.
- Elder, J. H., Pikett, R. A., II, Hampton, J., & Lerner, R. A. (1977).
- Greenwood, F. C., Hunter, W. M., & Glever, J. S. (1963) *Biochem. J.* **89**, 114-123.
- Gut, J., Meier, U. T., Catin, C., & Meyer, U. A. (1986) *Biochim. Biophys. Acta* **884**, 435-447.
- Hager, D. A., & Burgess, R. R. (1980) *Anal. Biochem.* **109**, 76-87.
- Hauri, H.-P., & Bucher, K. (1986) *Anal. Biochem.* **159**, 386-389.
- Hjelm, H., Hjelm, K., & Sjöquist, J. (1972) *FEBS Lett.* **28**, 73-76.
- Homberg, J.-C., André, C., & Abuaf, N. (1984) *Clin. Exp. Immunol.* **55**, 561-570.
- Homberg, J.-C., Abuaf, N., Helmy-Khalil, S., Biour, M., Poupon, R., Islam, S., Darnis, F., Levy, V. G., Opolon, P., Beaugrand, M., Toulet, J., Danan, G., & Benhamou, J.-P. (1985) *Hepatology (Baltimore)* **5**, 722-727.
- Hunkapiller, M. W., Lujan, E., Ostrander, F., & Hood, L. E. (1983) *Methods Enzymol.* **91**, 227-236.
- Inaba, T., Jurima, M., Nakono, M., & Kalow, W. (1984) *Clin. Pharmacol. Ther. (St. Louis)* **36**, 670-676.
- Inaba, T., Jurima, M., & Kalow, W. (1986) *Am. J. Hum. Genet.* **38**, 768-772.
- Jaiswal, A. K., Gonzalez, F. J., & Nebert, D. W. (1985) *Science (Washington, D.C.)* **288**, 80-83.
- Jurima, M., Inaba, T., Kadar, D., & Kalow, W. (1985a) *Br. J. Clin. Pharmacol.* **19**, 483-487.
- Kleinbloesem, C. H., van Brummelen, P., Faber, H., Danhof, M., Vermeulen, N. P. E., & Breimer, D. P. (1984) *Biochem. Pharmacol.* **33**, 3721-3724.
- Küpfer, A., & Preisig, R. (1984) *Eur. J. Clin. Pharmacol.* **26**, 753-759.
- Küpfer, A., Desmond, P., Schenker, S., & Branch, R. (1979) *Pharmacologist* **21**, 173.
- Küpfer, A., Desmond, P. V., Schenker, S., & Branch, R. A. (1982) *J. Pharmacol. Exp. Ther.* **221**, 590-597.
- Küpfer, A., Desmond, P., Patwardhan, Z., Schenker, S., & Branch, R. A. (1984a) *Clin. Pharmacol. Ther. (St. Louis)* **35**, 33-39.
- Küpfer, A., Patwardhan, Z., Ward, S., Schenker, S., Preisig, R., & Branch, R. A. (1984b) *J. Pharmacol. Exp. Ther.* **230**, 28-33.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685.
- Lottspeich, F. (1985) *J. Chromatogr.* **326**, 321-327.
- Lowry, O. H., Rosebrough, N. J., Farr, A. C., & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
- Mahgoub, A., Idle, J. R., Dring, L. G., Lancaster, R., & Smith, R. L. (1977) *Lancet* **2**, 584-586.
- Meier, P. J., Müller, H. K., Dick, B., & Meyer, U. A. (1983) *Gastroenterology* **85**, 682-692.
- Meier, U. T., Dayer, P., Male, P.-J., Kronbach, T., & Meyer, U. A. (1985a) *Clin. Pharmacol. Ther. (St. Louis)* **38**, 488-494.
- Meier, U. T., Kronbach, T., & Meyer, U. A. (1985b) *Anal. Biochem.* **151**, 286-291.
- Merril, C. R., Goldman, D., & Van Keuren, M. L. (1984) *Methods Enzymol.* **104**, 441-447.
- Nakamura, K., Goto, F., Ray, W. A., McAllister, C. B., Jacqz, E., Wilkinson, G. R., & Branch, R. A. (1985) *Clin. Pharmacol. Ther. (St. Louis)* **38**, 402-408.
- Omura, T., & Sato, P. (1964) *J. Biol. Chem.* **239**, 2370-2378.
- Pharmacia Fine Chemicals (1982) in *Isoelectric Focusing, Principles and Methods*, Vol. 1, pp 69-74, Ljungforetagen AG, Orebro, Sweden.
- Rampersaud, A., & Walz, F. G., Jr. (1986) *Biochim. Biophys. Acta* **869**, 293-303.
- Reinhart, M. P., & Malamud, D. (1982) *Anal. Biochem.* **123**, 229-235.
- Scott, J., & Poffenbarger, P. C. (1979) *Diabetes* **28**, 41-51.
- Shimada, T., Misono, K. S., & Guengerich, F. P. (1986) *J. Biol. Chem.* **261**, 909-921.
- Strobel, H. W., & Dignam, J. D. (1978) *Methods Enzymol.* **52**, 89-96.
- Towbin, H. T., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350-4354.
- Wang, P. P., Beaune, P., Kaminsky, L. S., Dannan, G. A., Kadlubar, F. F., Larrey, D., & Guengerich, F. P. (1983) *Biochemistry* **22**, 5375-5383.
- Waxman, D. J., & Walsh, C. (1983) *Biochemistry* **22**, 4846-4855.
- Wedlund, P. J., Aslanian, W. S., McAllister, C. B., Wilkinson, G. R., & Branch, R. A. (1984) *Clin. Pharmacol. Ther. (St. Louis)* **36**, 773-780.
- Wedlund, P. J., Aslanian, W. S., Jacqz, E., McAllister, C. G., Branch, R. A., & Wilkinson, G. R. (1985) *J. Pharmacol. Exp. Ther.* **234**, 662-669.
- Wrighton, S. A., Campanile, C., Thomas, P. E., Maines, S. L., Watkins, P. B., Parker, G., Mendez-Picon, G., Haniu, M., Shively, J. E., Levin, W., & Guzelian, P. S. (1986a) *Mol. Pharmacol.* **29**, 405-410.
- Wrighton, S. A., Thomas, P. E., Molowa, D. T., Haniu, M., Shively, J. E., Maines, S. L., Watkins, P. B., Parker, G., Mendez-Picon, G., Levin, W., & Guzelian, P. S. (1986b) *Biochemistry* **25**, 6731-6735.
- Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem.* **251**, 5337-5344.