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EVIDENCE FOR THE INVOLVEMENT OF SEVERAL CYTOCHROMES P-450 IN THE FIRST STEPS OF CAFFEINE METABOLISM BY HUMAN LIVER MICROSOMES

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ABSTRACT:

Caffeine biotransformation and four monooxygenase activities involving cytochrome P-450IA2, namely ethoxy- and methoxyresorufin O-dealkylases, phenacetin O-deethylase, and acetanilide 4-hydroxylation were studied in 25 human liver microsomes. All these activities were highly significantly intercorrelated ($r > 0.72, p < 0.001$) and correlated with the level of immunoreactive P-450IA2 content ($r > 0.65; p < 0.001$). P-450IA content was measured by immunoblotting with anti-rat P-450 β -naphthoflavone-B, an antibody that detects only a single band corresponding to P-450IA2. The formation rate of two

caffeine metabolites, namely paraxanthine and theobromine, was correlated with the four monooxygenase activities measured and P-450IA2-specific content ($r > 0.75$). However, inhibition studies of caffeine metabolism by phenacetin, a specific substrate of P-450IA2, clearly indicated that only the N-3 demethylation of caffeine was supported by this enzyme. These *in vitro* data demonstrate that P-450IA2 is predominantly responsible for the major metabolic pathway of caffeine and that the formation of other demethylated metabolites is mediated, at least partly, by other P-450 enzymes.

Caffeine, which is probably the most widely ingested drug in beverages and foods, is mainly metabolized by PAH¹-inducible P-450 enzymes (1–4). This P-450 family contains only two enzymes, named P-450IA1 and P-450IA2 according to the recommended nomenclature (5), that exhibit marked selectivity for the biotransformation of specific substrates such as PAH, ethoxresorufin, acetanilide, and phenacetin. These two enzymes are present in variable proportions in mammalian species (6, 7), while only P-450IA2 is expressed in human liver (7, 8).

In vitro studies have shown that caffeine biotransformation yields three primary demethylation products, namely paraxanthine, theophylline, and theobromine, as well as the C-8 hydroxylation product TMU (2–4). Caffeine 3-demethylation leading to the formation of PX was found to be the major pathway as found *in vivo*. It has been suggested from correlation and inhibition analyses that the same isozyme supports all the primary demethylations of this trimethylxanthine (9, 10). However, by using 3-MC-treated human hepatocyte cultures, we recently came to the conclusion that at least two P-450 enzymes are involved in these initial demethylation steps (4). In order to understand such a discrepancy, we have further examined the nature of P-450 enzymes involved in caffeine metabolism by human liver microsomes, using different experimental ap-

proaches including measurement of monooxygenase activities, inhibition studies, and enzyme immunoblotting.

In this report, we show that four monooxygenase activities known to be supported by P-450IA2 are strongly correlated with caffeine N-3 demethylation, *i.e.* paraxanthine formation. Phenacetin, which is known to be specifically deethylated by the P-450IA2 enzyme (8, 11, 12), is able to inhibit competitively caffeine biotransformation with an apparent K_i of 60 μM . However, only the N-3 demethylation pathway is significantly inhibited by phenacetin. In addition, we show that PX formation from caffeine is correlated with the relative immunoreactive P-450IA2. All these *in vitro* data demonstrate that the N-3 demethylation of caffeine is mediated by P-450IA2, as recently shown (11), while the other metabolic pathways involve other P-450 enzymes.

Materials and Methods

Ethoxyresorufin and resorufin were from Boehringer (Mannheim, FRG). Methoxyresorufin was synthesized by methylation of resorufin with iodomethane according to the procedure described by Prough *et al.* (13). Acetanilide, 4-hydroxyl-acetanilide, and 3-hydroxylacetanilide were from Aldrich (Steinheim, FRG). [¹⁴C]phenacetin was prepared by ethylation of [¹⁴C]paracetamol (60 mCi·mmol⁻¹, from CEA, Gif-sur-Yvette, France). In outline, 0.45 μmol paracetamol was dissolved in 1 ml of *N,N'*-dimethylacetamide and 0.1 ml of tetramethylammonium hydroxide (24% v/v in methanol); after formation of ammonium salt, paracetamol was alkylated by addition of 0.2 ml of iodoethane. The reaction was stopped after 30 min by adding 0.25 N KOH, and phenacetin was extracted by 2 \times 10 ml of diethyl ether; its purity, checked by HPLC, was greater than 99.8%. Electrophoresis products were purchased from Bio-Rad (Richmond, CA) and nitrocellulose Hybond-C from Amersham (Amersham, UK). Anti-rabbit IgG immunoglobulins and horseradish peroxidase anti-peroxidase complex were from ICN Biomedicals (Bucks, England). Nucleosil C-18 HPLC columns (25 cm \times 4.6 mm) were from Machery-Nagel (Düren, FRG). All other reagents were from Merck (Darmstadt, FRG) or Prolabo (Paris, France). Rat P-450 β -NF-B and the

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¹ Abbreviations used are: PAH, polycyclic aromatic hydrocarbon; PX, 1,7-dimethylxanthine (paraxanthine); TP, 1,3-dimethylxanthine (theophylline); TB, 3,7-dimethylxanthine (theobromine); TMU, 1,3,7-trimethyluric acid; P-450, cytochrome P-450; 3-MC, 3-methylcholanthrene; EROD, 7-ethoxyresorufin O-deethylase; MROD, 7-methoxyresorufin O-demethylase; POD, phenacetin O-deethylase; β -NF, β -naphthoflavone; PBS, phosphate buffer saline.

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corresponding antibody were prepared as described by Guengerich *et al.* (14).

Liver Donors. Human liver samples were obtained from 23 adult organ donors immediately after death (19 males, 4 females; mean age 38 ± 12 years) and two children: 5 months old (Br023) and 2 years old (Br025). Sampling was made in accordance with French legal considerations. Ethical committee approval was obtained prior to the study. Pre-death dietary habits and exposure to environmental chemicals were not known. Liver fragments were frozen immediately and stored in liquid nitrogen. Microsomal fractions were prepared as previously described (3) and stored at -80°C until use.

Determination of Monooxygenase Activities. Protein content of microsomes was determined by the method of Lowry *et al.* (15). Total cytochrome P-450 levels were measured according to Omura and Sato (16) with molecular extinction coefficient of 91 mM⁻¹·cm⁻¹. MROD and EROD activities were determined spectrofluorometrically according to the method of Prough *et al.* (13). The substrates were added in 10 µl of dimethylsulfoxide solution to 2 ml of 0.1 M potassium phosphate buffer, pH 7.4, in order to obtain a final concentration of 5 µM. Protein concentration was 0.075 mg·ml⁻¹. The reaction was initiated by addition of 0.5 mM NADPH. Fluorescence was measured directly in cuvettes under stirring for 2 min. Calibration was made by addition of 53 pmol resorufin to the reaction medium. Phenacetin O-deethylation was measured with 4 µM [¹⁴C]phenacetin (25 mCi·mmol⁻¹) in 75 mM Tris buffer, pH 7.4, containing 3 mM MgCl₂ and 0.2 mg·ml⁻¹ microsomal protein. The reaction was initiated by addition of 1.2 mM NADPH. After 10 min, reaction was stopped by addition of CHCl₃/isopropanol mixture, 85:15 (v/v). The organic phase taken to dryness under nitrogen stream at 40°C was analyzed by HPLC on a Nucleosil C-18 5 µm (25 cm × 4.6 mm) after dissolution in HPLC solvent containing 0.5 µg of 4-hydroxyacetanilide and phenacetin. The C-18 column was eluted with an elution gradient of 85% A to 95% B in 30 min. The eluent A was acetic acid/tetrahydrofuran/acetonitrile/water, 2.75:7.5:95:894.75 (v/v/v/v); the eluent B was the same mixture, except with 30% acetonitrile. Phenacetin and 4-hydroxyacetanilide detected by UV spectrophotometry at 250 nm were collected and measured by liquid scintillation spectrometry. Acetanilide 4-hydroxylation was measured at substrate concentration to 1.5 mM in 0.1 M phosphate buffer, pH 7.4, containing 1.15% KCl and 1 mM MgCl₂. The protein concentration was 1 mg·ml⁻¹. Reaction was started by addition of 2.4 mM NADPH. After 20 min at 37°C, the reaction was stopped by ammonium sulfate. Metabolites were extracted by chloroform/isopropanol, 85:15 (v/v). The major metabolite, 4-hydroxyacetanilide, was measured by HPLC-UV detection at 250 nm. HPLC was carried out using a column, 25 cm × 4.6 mm, packed with Nucleosil C-18 5 µm. Mobile phase consisted of acetic acid/tetrahydrofuran/acetonitrile/water, 2.75:7.5:95:894.75 (v/v/v/v). Quantification was made by means of an internal standard, namely 3-hydroxylacetanilide, added to the extraction solvent.

Caffeine metabolism was studied as previously described (3). In outline, [¹⁴C]caffeine (specific activity: 0.16 mCi·mmol⁻¹) was added at 1 mM concentration of 0.05 M phosphate buffer, pH 7.4, containing 1 mg of microsomal protein per ml. Reaction was started by addition of 1.2 mM NADPH. After 30 min, metabolites were extracted by chloroform/isopropanol, 85:15 (v/v) after saturation of medium by ammonium sulfate. HPLC separation of caffeine metabolites was conducted as detailed earlier (3). TB, PX, TP, TMU, and caffeine peaks were collected for radioactivity determination. Phenacetin, when used as a chemical inhibitor, was dissolved into phosphate buffer before starting the reaction. In all the monooxygenase assays, data were evaluated from the initial linear portion of the kinetic plots and expressed as pmol of product formed per mg of microsomal protein per min.

Immunoblot Analysis. Protein samples (40 µg) were separated by electrophoresis on 9% SDS-polyacrylamide gel according to Laemmli (17) and transferred electroforetically to a nitrocellulose sheet (18). After incubation at 37°C for 30 min by 3% bovine serum albumin and 10% newborn calf serum in PBS, the nitrocellulose sheet was sequentially treated with anti-P450-β-NF-B antibody overnight at 4°C, washed with PBS, then with PBS containing anti-rabbit IgG, and with PBS containing

horseradish peroxidase antiperoxidase complex raised against rabbit immunoglobulins. Finally, the sheets were washed six times with PBS buffer and the peroxidase activity was detected with 4-chloro-1-naphthol and H₂O₂. The relative concentration of P-450IA2 was determined by densitometry. The integrated peak area relative to the various microsomal preparations was converted to arbitrary units by reference to 1 pmol of total P-450 in liver microsomes from β-NF treated rats. It was expressed relatively to the amount of proteins.

Statistical Analysis. The values are means ± SD from 24 cases; the 5-month-old sample (Br023) was not included. Correlation coefficients were calculated using an analysis of variance table by the least-squares regression analysis from the raw data. Such a statistical analysis assumes a normal gaussian distribution in the population. Therefore, because the population is skewed due to the polymorphism of the P-450 family studied (see discussion below), correlations were considered significant only at the *p* < 0.001 level.

Results

Microsomal samples were prepared from liver tissue of 23 adult human organ donors and, in addition, from the liver of two young children. Because of the quite different metabolic profile of caffeine obtained with the youngest subject (5-month-old), this was excluded from correlation studies.

Human Hepatic Microsomal Oxidation of Caffeine. Twenty-five human hepatic microsomal samples were used for *in vitro* reactions to determine caffeine metabolism and four monooxygenase activities, *i.e.* EROD, MROD, POD, and acetanilide 4-hydroxylation (table 1). The rate of caffeine oxidation ranged from 77 to 420 pmol of substrate transformed per min per mg of protein. An excellent correlation between caffeine biotransformation and phenacetin O-deethylase was observed (fig. 1) (*r* = 0.88; *n* = 24; *p* < 0.001), suggesting that the same P-450 enzyme catalyzes both reactions. Microsomal caffeine transformation was also significantly correlated with the O-dealkylations of methoxy- and ethoxyresorufin and acetanilide 4-hydroxylation (table 2). All these activities are known to be associated with P-450IA; they were not significantly correlated with the total P-450 (table 2).

Correlations Between Caffeine Biotransformation, Monooxygenase Activities, and Immunochemically Determined Levels of P-450IA2. The rate of caffeine biotransformation in individual human hepatic microsomal preparations was compared to the immunochemically determined level of P-450IA family by immunoblotting with rabbit anti-rat P-450 β-NF IgG. The antibody raised to rat cytochrome P-450 β-NF-B recognized only a single form of cytochrome P-450 in all microsomal fractions of 24 adult human livers, *M_r* 54,000, very close to the molecular weight of rat P-450d run in the same electrophoresis conditions (fig. 2). The correlation of microsomal caffeine metabolism with the relative levels of immunoreactive P-450 β-NF-B was highly significant (*r* = 0.87, *p* < 0.001) (fig. 3). Similarly, dealkylations of methoxy- and ethoxyresorufin, 4-hydroxylation of acetanilide, and O-deethylation of phenacetin were highly significantly correlated with the amount of P-450IA2 protein present (table 3).

Metabolic Pathways of Caffeine and Immunoreactive P-450IA2 Content. Caffeine undergoes three oxidative *N*-demethylations leading to the formation of PX, TP, and TB and C-8 oxidation, giving TMU in human liver microsomes. Only the N-3 and N-1 demethylations were intercorrelated (*r* = 0.86), while the other metabolic pathways of caffeine were not significantly intercorrelated (*r* = 0.60 between PX and TP; *r* = 0.53 between PX and TMU; and *r* = 0.59 between TP or TB and TMU). Strong correlations (*r* = 0.80–0.86; *p* < 0.001) between four

TABLE 1
Cytochrome P-450 and monooxygenase activities in 25 human liver microsomes

Activities are expressed as pmol·min⁻¹·mg⁻¹ protein. EROD: ethoxresorufin O-deethylation; MROD: methoxyresorufin O-demethylation; POD: phenacetin O-deethylation; Acetanilide 4-hydroxylation; Caffeine: overall caffeine biotransformation; PX, TB, TP, TMU: paraxanthine, theobromine, theophylline, and trimethyluric acid formation from caffeine.

Subjects	Sex, Age	Proteins mg/ml	Total P-450 pmol/mg	EROD	MROD	POD	Acetanilide	Caffeine	PX	TB	TP	TMU
FH1	M, 18 y	9.83	269	314	266	41	1030	204	153	20	18	12
FH2A	M, 47 y	16.84	527	675	589	79	2220	420	248	38	46	84
FH3	M, 41 y	16.16	470	120	208	35	610	160	66	14	14	21
Br015	M, 43 y	11.5	414	172	203	24	1260	114	71	13	11	18
Br016	M, 44 y	11.66	369	213	296	48	1520	156	105	19	14	19
Br017	M, 26 y	14.78	593	477	557	137	2450	365	245	58	29	37
Br018	F, 45 y	15.46	334	37	137	18	1000	77	34	15	11	16
Br019A	M, 49 y	9.05	241	312	243	ND ^a	1980	138	88	14	18	18
Br021	M, 49 y	23.15	257	54	134	24	1420	78	42	8	12	12
Br022	M, 23 y	9.48	286	71	78	11	750	105	26	17	16	46
Br024	F, 15 y	14.23	505	112	96	10	830	108	35	15	24	35
Br025	M, 2 y	13.53	110	163	159	17	900	101	56	8	21	15
Br027	M, 27 y	10.4	100	206	149	15	910	91	42	13	23	12
Br028	M, 27 y	10.44	165	409	400	46	1510	172	108	22	22	17
Br029	M, 36 y	15.12	77	35	76.5	10	480	72.5	29	13	19	12
Br031	M, 23 y	8.17	154	183	166	26	990	89	27	14	21	26
Br032	M, 36 y	7.54	286	189	223	18	1196	104.5	34	20	18	31
Br033	M, 44 y	11.22	363	614	610	89	1846	280	168	39	31	39
Br034	M, 32 y	8.22	286	509	549	38	1453	210	113	32	27	36
Br035	M, 35 y	13.8	252	210	224	9	1424	117	43	18	18	39
Br036	M, 57 y	13.5	153	204	245	11	812	108	53	11	32	12
Br037	M, 56 y	7	230	82	226	4	1263	96	37	13	24	21
Br038	F, 51 y	5.62	120	84	171	5	781	115	61	14	25	15
Br039	F, 46 y	14.94	538	425	546	23	1097	150	81	18	17	35
Mean		12.14	296	244	273	31	1238	149	81	19	21	26
± SD		3.94	150	184	171	31	501	89	64	11	8	16
Br023	F, 5 m	7.69	228	5	8.5	45	670	75	9	11	26	29

^a ND, not determined.

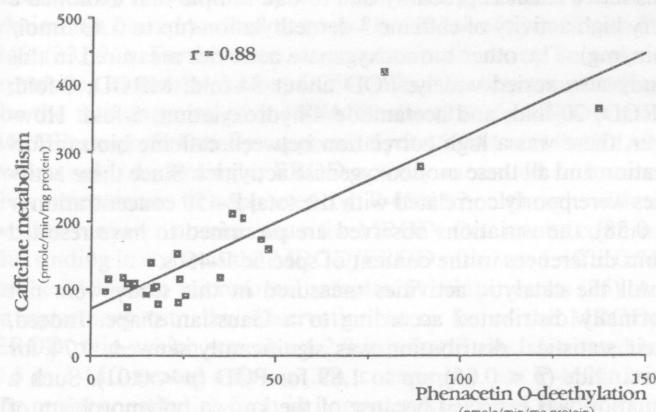


FIG. 1. Correlation between overall caffeine metabolism and phenacetin O-deethylation in 24 human hepatic microsomal preparations.

The correlation coefficient (*r*) was determined by the least-squares linear regression analysis (*N* = 24). Caffeine and phenacetin were used as substrates at 1 mM and 4 μ M, respectively.

monooxygenase activities and N-3 demethylation of caffeine (table 4) were observed while these activities were less significantly correlated with N-1 demethylation. By contrast, both N-demethylation and 8-hydroxylation of caffeine were not correlated with the four monooxygenase activities.

Among the four metabolic pathways of caffeine, only the N-3

TABLE 2

Correlation coefficient of four monooxygenase activities and caffeine biotransformation in 24 human hepatic microsomal samples

Correlation coefficients (*r*) were calculated by the least-squares linear regression method for 24 samples. *r* values greater than 0.72 were highly significant (*p* < 0.001). Monooxygenase activities are expressed as pmol·min⁻¹·mg⁻¹ protein and total P-450 content as pmol/mg microsomal protein.

	Caffeine	EROD	Acetanilide	MROD	Phenacetin
EROD	0.88				
Acetanilide	0.78	0.73			
MROD	0.84	0.94	0.72		
Phenacetin	0.88	0.73	0.75	0.75	
Total P-450	0.57	0.43	0.46	0.52	0.56

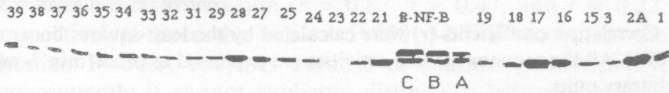


FIG. 2. Western blots with anti-rat P-450 β -NF-B of microsomal proteins from human livers.

Microsomal proteins (40 μ g) were separated by SDS-polyacrylamide gel electrophoresis and probed with anti-rat P-450 β -NF-B. Patient numbers are given in table 1. Lanes A, B, C: 1, 2, 4 pmol of total P-450 from liver microsomes of β -NF-treated rats.

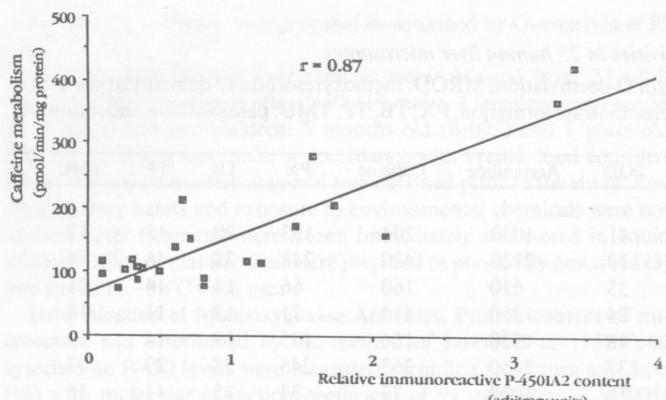


FIG. 3. Correlation between overall caffeine biotransformation and immunochemically determined content of human P-450IA2.

Microsomal preparations from 24 human liver samples were analyzed for caffeine oxidations and P-450IA2 content by immunoblot analysis of 40 μ g of microsomal protein using anti-rat P-450 β -NF-B. Intensity units are arbitrary values determined by densitometry.

TABLE 3

Correlation coefficients between monooxygenase activities measured with different substrates and immunoreactive P-450IA2 content in 24 human liver microsomal samples

Correlation coefficients (r) were calculated by the least-squares linear regression method. r values greater than 0.62 were highly significant ($p < 0.001$). All the monooxygenase activities are expressed as pmol \cdot min $^{-1}$ \cdot mg $^{-1}$ protein; the immunoreactive P-450IA2 content in 24 microsomal samples is expressed in relative arbitrary units.

Substrates for Monooxygenase Activities	Immunoreactive P-450IA2 Content
Caffeine	0.87
EROD	0.67
MROD	0.65
Phenacetin	0.85
Acetanilide	0.72

and N-1 oxidative demethylations were significantly correlated with P-450IA2 content ($r = 0.91$ and 0.75, respectively).

Inhibition Studies. To further assess whether or not the caffeine oxidation pathways are catalyzed by the P-450 enzyme involved in phenacetin *O*-deethylation, i.e. P-450IA2, the effect of phenacetin on these reactions was determined. Fig. 4 illustrates the competitive inhibition of phenacetin on overall caffeine biotransformation in microsomes from human liver FH2A, chosen on the basis of its high caffeine-metabolizing activity. Inhibition of caffeine metabolism by phenacetin was of competitive type according to Dixon plots (fig. 4) with an apparent K_i of about 60 μ M. In humans, the predominant caffeine metabolic pathway is

N-3 demethylation, leading to PX. In this study, it represented about $50.8 \pm 13.3\%$ of total metabolites produced in the liver microsomal samples. More than 50% inhibition of this pathway was obtained when 0.5 or 1 mM caffeine was incubated in the presence of 128 μ M phenacetin (fig. 5A and B) while it did not exceed 35% with 2 mM caffeine (fig. 5C). The two other demethylations on N-1 (TB formation) and N-7 (TP formation) positions of xanthine ring represented about $13.3 \pm 3.2\%$ and $16.3 \pm 6.4\%$ of total metabolites, respectively, in 24 human liver microsomal samples. These two pathways were not markedly affected by 2–200 μ M phenacetin (fig. 5). The rate of formation of TMU was generally higher than TB and TP formation: it represented $18.9 \pm 9\%$ of total metabolites. This 8-hydroxylation pathway was not inhibited by phenacetin in the 2–200 μ M range. Furthermore, at the highest inhibitor concentration used, the rate of TMU production was selectively stimulated at a level largely above those found in controls, particularly at 2 mM caffeine concentration (fig. 5C).

Discussion

Many studies (19–21) have shown that there are important interindividual variations in both P-450-dependent catalytic activities and amounts of P-450 enzymes in human liver microsomes. The results reported here emphasize these previous observations. They show a wide variability of the ability of the 24 human liver microsomal samples studied to catalyze biotransformation of caffeine, *O*-dealkylations of methoxyresorufin, ethoxresorufin, and phenacetin, and hydroxylation of acetanilide. Overall caffeine biotransformation varied 6-fold while its 3-demethylation varied 10-fold and its N-1 and N-7 demethylations no more than 6-fold. These values are in agreement with those published by Grant *et al.* (2), who found a 6-fold interindividual variation in the 3-demethylation of caffeine, while Butler *et al.* (11) observed up to a 57-fold variation of this pathway; this latter result is probably due to one sample that exhibited a very high activity of caffeine 3-demethylation (up to 0.43 nmol/min/mg). The other monooxygenase activities measured in this study also varied widely: POD about 34-fold; MROD, 8-fold; EROD, 20-fold, and acetanilide 4-hydroxylation, 5-fold. However, there was a high correlation between caffeine biotransformation and all these monooxygenase activities. Since these activities were poorly correlated with the total P-450 concentration ($r < 0.58$), the variations observed are presumed to have resulted from differences in the content of specific P-450s.

All the catalytic activities measured in this study were not normally distributed according to a Gaussian shape. Indeed, their statistical distribution was significantly skewed: 0.74 for acetanilide ($p < 0.05$), up to 1.89 for POD ($p < 0.01$). Such a situation was expected because of the known polymorphism of

TABLE 4

Correlation coefficients between the four caffeine metabolites (PX, TB, TP, and TMU) and monooxygenase activities and immunoreactive P-450IA2 content in 24 human hepatic microsomal samples

Correlation coefficients (r) were calculated by the least-squares linear regression analysis. r values greater than 0.62 were highly significant ($p < 0.001$). All the monooxygenase activities are expressed as pmol \cdot min $^{-1}$ \cdot mg $^{-1}$ protein; the immunoreactive P-450IA2 content is expressed in relative arbitrary units.

Metabolite Formed	EROD	MROD	POD	Acetanilide 4-hydroxylation	Immunoreactive P-450IA2 Content
PX	0.85	0.82	0.90	0.78	0.91
TB	0.77	0.80	0.92	0.75	0.75
TP	0.66	0.57	0.46	0.43	0.42
TMU	0.61	0.55	0.43	0.49	0.47

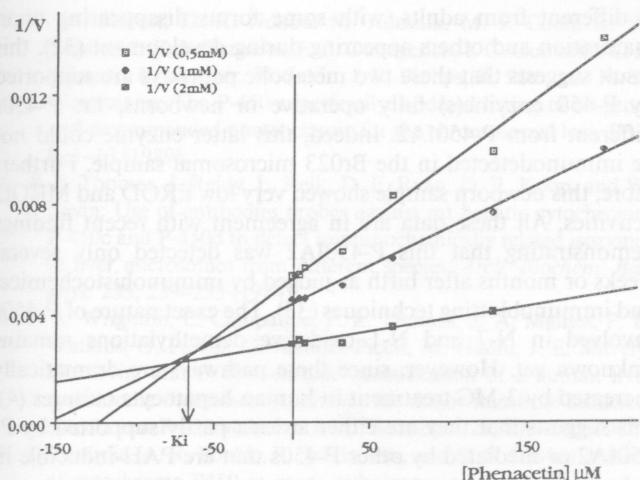


FIG. 4. Dixon plot: inhibition of caffeine biotransformation by phenacetin in microsomes from human liver FH2A.

V , expressed as $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, represents the sum of four caffeine metabolites, i.e. PX, TP, TB, and TMU. V_{\max} value was, respectively, 576, 465, and 517 pmol per min per mg protein in these conditions.

phenacetin *O*-deethylation (21, 22). Despite those limitations, the approach of using linear regression analysis does suggest a link between monooxygenase activities and individual P-450 enzymes.

As both enzymes of the P-450IA family share overlapping specificities for substrates used in this study, it is difficult to provide a definitive distinction between the contributions of each only by measuring the biotransformation of these compounds. However, given that only one enzyme, very likely P-450IA2 (7, 8, 11, this study), is expressed in human liver, it is obvious that the strongly intercorrelated four monooxygenase activities are mediated by P-450IA2 enzyme. Thus, ethoxresorufin *O*-deethylation has been described as an *in vitro* index of cytochrome P-448 (23, 24). Furthermore, it has been shown by means of expression in monkey kidney COS-1 cells of human genes coding for the two enzymes of P-450IA family (25) that EROD and MROD are preferentially supported by P-450IA1 and P-450IA2, respectively. Accordingly, EROD activity measured in human liver microsomes is supported by P-450IA2, although with a weaker turnover number than for MROD. Our results confirm this finding in so far as the MROD/EROD ratio was 1.40 ± 0.74 in the 24 human microsomal samples. Previous results (26) are in agreement with this observation in so far as the MROD/EROD ratio, which was about 2.8 in control rats, decreased to 0.5 in 3-MC-treated animals (26), confirming that P-450IA2 is the major constitutive form in normal rats (12) and P-450IA1 is the most induced in 3-MC-treated rats (27). Similarly, acetanilide 4-hydroxylation appears to be selectively mediated by cytochrome P₄₅₀ (25, 28, 29), i.e. P-450IA2 (5). Likewise, it has been previously established that the high-affinity component of phenacetin-*O*-deethylase activity in humans is catalyzed by a P-450 enzyme orthologous to P-450d in the rat (8) or P-450_{PA}, identified as P-450IA2 enzyme (11). The results reported here agree with the assertion that POD activity is strongly correlated with EROD, MROD, and acetanilide 4-hydroxylation.

Among the three forms of P-450 (P-450IA, P-450IIIA, and P-450IIE1) measured in the 24 microsomal samples studied by Western blots using corresponding specific antibodies (results not shown), only P-450IA2 content was significantly correlated with

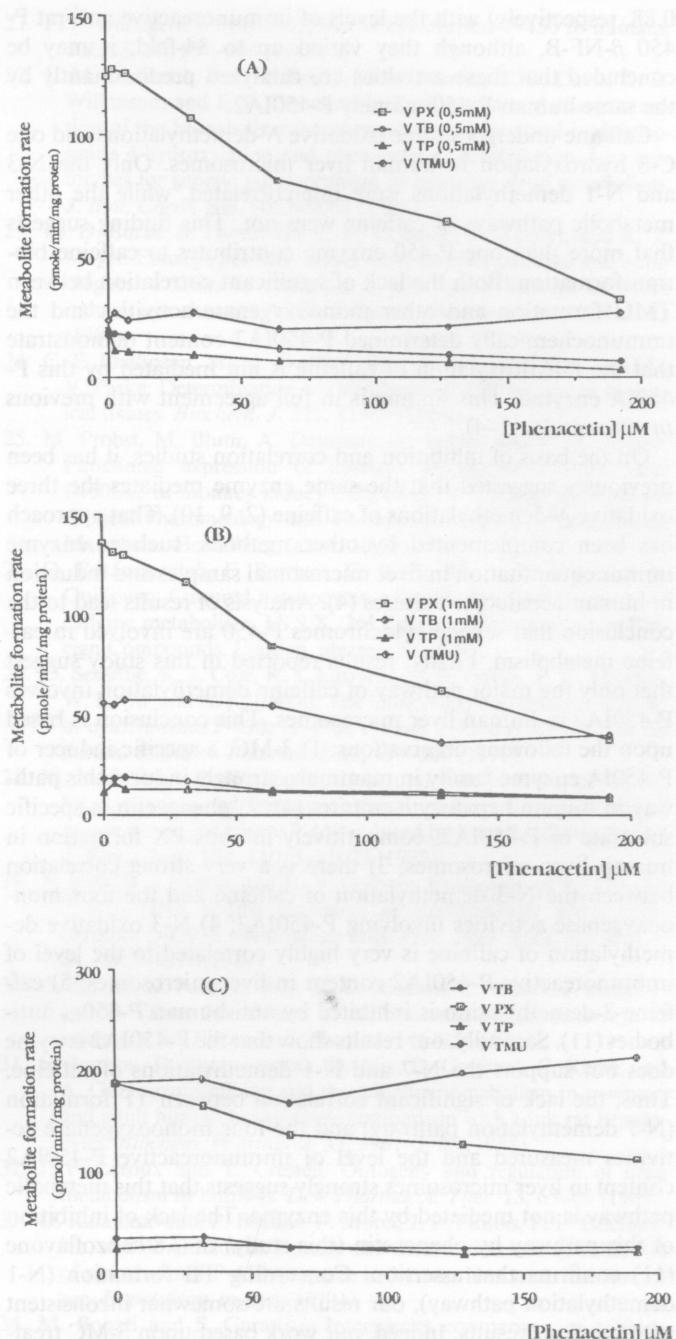


FIG. 5. Effects of phenacetin on caffeine oxidations by the FH2A liver microsomal preparation.

0.5 mM (A), 1 mM (B), and 2 mM (C) caffeine concentrations were tested.

caffeine biotransformation ($r = 0.87$, $r = 0.47$, and $r = 0.22$, respectively). Since the anti-rat P-450 β -NF-B recognizes the human P-450IA enzymes because of immunocrossreactivity, consequently it cannot explicitly distinguish between the two enzymes of the IA subfamily. However, this antibody recognizes a single polypeptide of electrophoretic mobility very close to the rat P-450d, thereby confirming that only a form of the IA subfamily is expressed in human liver (7, 8) and that this form is very likely P-450IA2. Furthermore, as both caffeine biotransformation and POD activity were highly correlated ($r = 0.87$ and

0.88, respectively) with the levels of immunoreactive anti-rat P-450 β -NF-B, although they varied up to 34-fold, it may be concluded that these activities are catalyzed predominantly by the same human P-450, namely P-450IA2.

Caffeine undergoes three oxidative N-demethylations and one C-8 hydroxylation in human liver microsomes. Only the N-3 and N-1 demethylations were intercorrelated, while the other metabolic pathways of caffeine were not. This finding suggests that more than one P-450 enzyme contributes to caffeine biotransformation. Both the lack of significant correlation between TMU formation and other monooxygenase activities and the immunochemically determined P-450IA2 content demonstrate that the 8-hydroxylation of caffeine is not mediated by this P-450IA enzyme. This finding is in full agreement with previous *in vitro* results (2-4).

On the basis of inhibition and correlation studies, it has been previously suggested that the same enzyme mediates the three oxidative N-demethylations of caffeine (2, 9, 10). That approach has been complemented by other methods, such as enzyme immunoquantitation in liver microsomal samples and induction in human hepatocyte cultures (4). Analysis of results lead to the conclusion that several cytochromes P-450 are involved in caffeine metabolism. Firstly, results reported in this study suggest that only the major pathway of caffeine demethylation involves P-450IA2 in human liver microsomes. This conclusion is based upon the following observations: 1) 3-MC, a specific inducer of P-450IA enzyme family in mammals, strongly induces this pathway in human hepatocyte cultures (4); 2) phenacetin, a specific substrate of P-450IA2, competitively inhibits PX formation in human liver microsomes; 3) there is a very strong correlation between the N-3 demethylation of caffeine and the four monooxygenase activities involving P-450IA2; 4) N-3 oxidative demethylation of caffeine is very highly correlated to the level of immunoreactive P-450IA2 content in liver microsomes; 5) caffeine 3-demethylation is inhibited by anti-human P-450_{PA} antibodies (11). Secondly, our results show that the P-450IA2 enzyme does not support the N-7 and N-1 demethylations of caffeine. Thus, the lack of significant correlation between TP formation (N-7 demethylation pathway) and the four monooxygenase activities measured and the level of immunoreactive P-450IA2 content in liver microsomes strongly suggests that this metabolic pathway is not mediated by this enzyme. The lack of inhibition of this pathway by phenacetin (this study) or 7,8-benzoflavone (11) confirms that assertion. Concerning TB formation (N-1 demethylation pathway), our results are somewhat inconsistent with previous results. Indeed, our work based upon 3-MC treatment of human hepatocyte cultures (4) suggested that TB formation is mediated by a P-450 enzyme different than that involved in PX formation. Correlation studies of the present study may suggest the opposite, showing the limits of correlation studies (see table 4). However, the lack of inhibition of TB formation by phenacetin confirms our previous assertion. Furthermore, the distinctive maturation of the three caffeine demethylation steps in newborns, especially N-1 demethylation, implies that they are dependent on different P-450 enzymes (30). Such a finding agrees with the fact that liver microsomes of the Br023 newborn showed a metabolic profile of caffeine very different from that observed in adults. Thus, TMU and TP, respectively, represented 39% and 35% of total metabolites. Previous results based upon newborn hepatocyte cultures (31) have already shown that TP was the major demethylated product of caffeine in newborns. Although the P-450 content of newborns

is different from adults, with some forms disappearing upon maturation and others appearing during development (32), this result suggests that these two metabolic pathways are supported by P-450 enzyme(s) fully operative in newborns, *i.e.* P-450s different from P-450IA2. Indeed, this latter enzyme could not be immunodetected in the Br023 microsomal sample. Furthermore, this newborn sample showed very low EROD and MROD activities. All these data are in agreement with recent findings demonstrating that this P-450IA2 was detected only several weeks or months after birth as judged by immunohistochemical and immunoblotting techniques (33). The exact nature of P-450s involved in N-7 and N-1 oxidative demethylations remains unknown yet. However, since these pathways are dramatically increased by 3-MC treatment in human hepatocyte cultures (4), this suggests that they are either at least partly supported by P-450IA2 or mediated by other P-450s that are PAH-inducible in culture conditions.

In adult humans, the predominant metabolic pathway of caffeine is N-3 demethylation. In the bloodstream, PX represents about 70-83% (34-36) of caffeine demethylations. As this metabolite formation involves PAH-inducible P-450 enzymes, especially P-450IA2 (11, this study), determination of urinary caffeine metabolite ratios seems to be a good criterion for measuring P-450IA activity in humans (9, 37). However, this urinary (AFMU + 1-MU + 1-MX)²/1,7-DMU ratio would be reliable if all these metabolites of caffeine formed via PX involve the same enzyme or at least enzymes that are not variable in humans (7-demethylation of PX, xanthine oxidase, N-acetyltransferase and C-8 oxidation). Until now, this has not been clearly demonstrated. Our work links the human hepatic P-450IA2 level and activity with the 3-demethylation of caffeine while the other primary metabolites of caffeine are probably mediated by other enzymes. Thus, as the individual's ability to 3-demethylate caffeine appears to be a very reliable means of measuring hepatic P-450IA2 activity, the plasmatic PX/caffeine or PX/(PX + TP + TB) ratios need to be further studied. Indeed, this inducible enzyme is of toxicological and pharmacological importance because it is known to be involved in activation of carcinogens (38, 39). Thus, caffeine may be proposed as a safe probe for measuring the relative P-450IA2 activity in human populations.

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²AFMU = 5-acetylamino-6-formylamino-3-methyluracil; 1-MU = 1-methyluric acid; 1-MX = 1-methylxanthine.

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