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BIOTRANSFORMATION OF CAFFEINE, PARAXANTHINE, THEOPHYLLINE, AND THEOBROMINE BY POLYCYCLIC AROMATIC HYDROCARBON-INDUCIBLE CYTOCHROME(S) P-450 IN HUMAN LIVER MICROSOMES

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ABSTRACT:

The microsomal metabolism of caffeine and its primary dimethylxanthine metabolites, paraxanthine, theophylline, and theobromine, was investigated in 15 different human livers, including those from two known nonsmokers and one known smoker. At least two distinct enzymes with differing substrate affinities have the potential to catalyze most methylxanthine *N*-demethylations and C_8-hydroxylations *in vitro*; however, at the low methylxanthine concentrations routinely encountered *in vivo*, participation by the high affinity site is expected to predominate. It appears that the high affinity enzyme is a polycyclic aromatic hydrocarbon-inducible isozyme of cytochrome P-450, based on competitive inhibition by 7-ethoxyresorufin and benzo[*a*]pyrene, and based on a significant ($p < 0.001$) correlation between 7-ethoxyresorufin-*O*-deethylation and methylxanthine demethylation rates. α-Naphthoflavone inhibited all methylxanthine demethylations in excess of 80% in two high activity livers, whereas 8-hydroxylations were generally inhibited less. Kinetic analysis of paraxanthine 7-demethylation in four different liver preparations resulted in similar K_m values of 1.2 ± 0.5 mM (mean ± SD), whereas V_{max} values varied 8-fold, compatible with participation by the same high affinity isozyme. Notable was the high degree of inter-liver variation in metabolic rates, with the known smoker showing the second highest activity among a 20-fold range in paraxanthine demethylation rates, consistent with polycyclic aromatic hydrocarbon-related enzyme induction. Maximal inhibition of paraxanthine 8-hydroxylation by α-naphthoflavone left similar residual activities in the 15 liver preparations, indicating the presence of an enzyme activity that was not inducible. Furthermore, in low activity livers, more than 80% of paraxanthine 8-hydroxylation was mediated by an isozyme of cytochrome P-450 insensitive to inhibition by α-naphthoflavone. Our *in vitro* data show that the proportion of demethylation relative to hydroxylation products of paraxanthine correlate with 7-ethoxyresorufin *O*-deethylation rates. Taken together, the data provide a rationale for a potential *in vivo* marker of polycyclic aromatic hydrocarbon-inducible cytochrome P-450 activity based on a urinary metabolite ratio of paraxanthine 7-demethylation to 8-hydroxylation products after caffeine intake.

In recent years, the widely ingested methylxanthines, caffeine, theophylline, and theobromine, have received considerable attention regarding their biodisposition in humans (1–8). Caffeine, a central nervous system stimulant prevalent in many beverages, undergoes oxidative *N*-demethylation in humans primarily to paraxanthine, and less so to theophylline and theobromine. Theobromine, a major constituent in chocolate, is also widely ingested by the general population. Theophylline, a commonly used bronchodilator in the treatment of asthma, is maintained during therapy at serum levels of 10–20 μg/ml (9), about 2–5 times higher than serum caffeine levels routinely maintained by the coffee-drinking public.

The investigation of caffeine, paraxanthine, theophylline, and theobromine metabolism is of importance in its own right because of the widespread and frequently copious intake of these substances. However, of particular interest to us is the potential use of one or more of these four methylxanthines as an *in vivo* probe for PAH[1]-inducible cytochrome(s) P-450 activity in humans. Considerable circumstantial evidence exists concerning the involvement of PAH-inducible cytochrome P-450 in the biotransformation of caffeine (10–15), theophylline (16–19), and theobromine (20) in PAH-exposed animals and humans. This isozyme is of toxicological importance because of its role in converting several common environmental pollutants to reactive intermediates, including ultimate carcinogens (21, 22), and because its activity can be increased upon exposure to a multitude of environmental contaminants.

Previously, we developed and reported (23) a method for investigating caffeine biotransformation by human liver microsomes. Because *in vitro* metabolism of caffeine yields only the primary metabolites paraxanthine, theophylline, theobromine, and 1,3,7-trimethyluric acid (23), elucidation of subsequent biotransformation pathways requires separate incubations with each of these primary metabolites as substrates. In this paper, we describe a method for investigating each of paraxanthine, theophylline, and theobromine metabolism *in vitro*, as well as report on enzyme kinetics and the effect of model inhibitors and substrates of PAH-inducible cytochrome P-450 such as ANF (24–26) and 7ER (27, 28).

Other investigators (10–12) have proposed the caffeine breath test as a probe for PAH-inducible P-450 activity. The caffeine

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[1] Abbreviations used are: PAH, polycyclic aromatic hydrocarbon; 137X, 1,3,7-trimethylxanthine (caffeine); 17X, 1,7-dimethylxanthine (paraxanthine); 37X, 3,7-dimethylxanthine (theobromine); 13X, 1,3-dimethylxanthine (theophylline); 137U, 1,3,7-trimethyluric acid; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; 1X, 1-methylxanthine; 7X, 7-methylxanthine; 3X, 3-methylxanthine; 13U, 1,3-dimethyluric acid; 37U, 3,7- dimethyluric acid; 17U, 1,7-dimethyluric acid; 1U, 1-methyluric acid; MC, 3-methylcholanthrene; ANF, α-naphthoflavone; 7ER, 7-ethoxyresorufin; EROD, 7-ethoxyresorufin-*O*-deethylase; mAb, monoclonal antibody.

breath test is based on a 2-h cumulative exhalation of $^{13}CO_2$ after administration of suitably labeled caffeine. This paper provides a rationale for the use of a urinary methylxanthine-derived metabolite ratio to assess PAH-inducible P-450 activity in humans because of its potential as a simple, inexpensive, and noninvasive method applicable to studies in biochemical epidemiology.

Materials and Methods

Chemicals. The compounds 137X, 17X, 13X, 37X, 1X, ANF, metyrapone, acetanilide, antipyrine, allopurinol, Tris-HCl, NADP, NADPH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma. In addition, 137U, 17U, 13U, 37U, and 1U were obtained from Adams Chemical; 7U from Tridon/Fluka AG; 7X from Pfaltz and Bauer; 7ER and resorufin from Molecular Probes; benzo[*a*]pyrene from Aldrich Chemical Co.; phenacetin from British Drug House; and chloroform (Accusolv) from Anachemia. All other chemicals were reagent grade products from Fisher Chemicals.

Monoclonal antibodies (gift of Dr. H. V. Gelboin, NIH, Bethesda, MD) were raised to purified rat liver MC-induced cytochrome P-450 isozymes using techniques described by Park *et al.* (29, 30). Control ascites fluid obtained from mice injected with hybridomas producing an anti-lysozyme mAb (HyH2-L-9) was used to control for nonspecific effects by undefined components of the ascites fluid.

Tissue Preparation. Human liver tissue, obtained at the time of kidney donation (K and KD series) or partial hepatectomy (DK series) was quickly immersed in 1.15% KCl at 0–4°C until it could be frozen in liquid nitrogen and stored at −70°C. Table 1 summarizes donor history and liver characteristics. Use of oral contraceptives among female donors was unknown.

Washed liver microsomes were prepared by the method of Meier *et al.* (31). Protein concentration of microsomal suspensions was determined by the Lowry method (32) using bovine serum albumin as the standard. Total cytochrome P-450 content was determined by the method of Estabrook *et al.* (33), with P-450 concentrations calculated from the absorbance difference between the 450 nm peak and 490 nm peak, using a molar extinction coefficient of 100 $mM^{-1}cm^{-1}$. The preparation of rat and mouse liver microsomes was the same as that for human liver. The MC induction of the animals followed standard procedures (34), whereby MC dissolved in corn oil (80 mg/kg) was injected ip, 3 days prior to death through cervical dislocation. Male Wistar rats and male C57BL/6N mice were purchased from Charles River suppliers.

Methylxanthine Purification. To purify paraxanthine, it was suspended in chloroform (100 mg/10 ml), heated to boiling, and dissolved by the dropwise addition of methanol. The solution was introduced to the top of a glass column slurry packed with silica (70–230 mesh, Merck/BDH) and eluted with chloroform/methanol (12:1, v/v). Those fractions free of impurities (as determined by HPLC analysis) were dried on a rotary evaporator at 40–50°C.

Theophylline was purified by injecting 5 ml of concentrated aqueous theophylline solution (8 g/liter) onto a Regis Prep-10 HPLC column (10-μm ODS) and eluting with water/methanol (80:20, v/v). Those fractions devoid of impurities were dried on a rotary evaporator at 40–50°C.

Theobromine, because of its poor solubility in water, was purified by three consecutive recrystallizations.

Enzyme Assays. Microsomal EROD activity was determined by a method modified after Burke and Mayer (24). For each incubation, 7ER dissolved in methanol was added to a cuvette, dried down under air, and resuspended in 50 mM Tris/150 mM KCl/1 mM EDTA buffer (pH 7.8). Appropriately diluted microsomal suspension was added, and the resultant mixture was preincubated for 5 min at 37°C. The reaction was initiated with NADPH (final concentration, 0.25 mM) dissolved in buffer, vortexed briefly, and inserted into the heated compartment (37°C) of a Zeiss Spectrofluorimeter (Model PM QII) to continuously monitor increasing resorufin fluorescence. The excitation wavelength was 550 nm and emission wavelength 584 nm. Depending on the protein concentration used, resorufin production rates were linear for the first 4–15 min of the incubation. EROD in washed microsomes were linear with final protein concentrations up to 250 μg/ml.

TABLE 1

Donor history and liver characteristics

Liver	Donor Gender	Donor Age	Cause of Death	Known Drug Intake	Total[a] P-450	EROD ± SD[b]
K5[c]	F	61	Cerebral hemorrhage		0.46	10.9 ± 2.0
K6[c]	M				0.62	86.2 ± 6.0
K7[c]	M	20	Broken neck	Phenothiazines	0.43	72.8 ± 6.0
K9[c]					0.48	25.9 ± 4.0
K10[c]	M	21	Seizures	Barbiturates, cimetidine, amitriptyline	0.56	28.8 ± 1.9
K12[c]	F	23	Cocaine overdose	Mannitol, dopamine	0.27	16.4 ± 1.6
K14[c]	F	35	Subarachnoid hemorrhage	Dopa, Dilantin, netilmicin, phenobarbital	0.57	48.0 ± 4.2
K15[c]	M	39	Motor vehicle accident	Dopamine, levorphanol, penicillin G, furosemide	0.55	137.4 ± 5.0
K16[c]	M	52	Subarachnoid hemorrhage	Mannitol, dopamine	0.37	13.4 ± 1.3
K18[c]	M	20	Drowning	Insulin, furosemide	0.39	237 ± 27.7
K19[d]	F	37	Cerebral bleeding	Dopamine, furosemide	0.69	16.8 ± 2.0
K20[d]	F	50	Postoperative cerebral edema	Diazepam, phenobarbital, dexamethasone	0.66	45.1 ± 5.1
K21[e]	F	50	Cardiac arrest	Propranolol, methyldopa, amitryptyline	0.50	137 ± 15
KD[c]					0.60	10.6 ± 1.6
DK2[c]	M		Donor alive (partial hepatectomy)		0.56	47.5 ± 7.0

[a] Total cytochrome P-450 expressed as nmol/mg washed microsomal protein.
[b] 7ER substrate concentration was 1 μM, units of velocity are pmol/min/mg.
[c] Unknown smoking status.
[d] Known nonsmoker.
[e] Known smoker.

The paraxanthine, theophylline, and theobromine assays were a modification of the *in vitro* caffeine assay described previously (23). Briefly, each 0.5-ml incubation volume contained microsomal suspension (300–900 μg of protein, 60 mM sucrose, 3 mM Tris-HCl), purified substrate, 150 mM KCl, 200 mM phosphate buffer (pH 7.4), and NADPH-generating system (2 mM MgCl, 4 mM glucose 6-P, 0.4 mM NADP, and 0.4 unit of glucose-6-P dehydrogenase). After 15 min of incubation at 37°C in a metabolic shaker, the reaction was stopped with 10 ml of the extraction solvent (chloroform/isopropyl alcohol, 85:15, v/v), to which was added 50 μl of internal standard and about 500 mg of ammonium sulfate. The mixture was vortexed for 30 sec, centrifuged for 5 min at 2500 rpm, and the organic phase was evaporated to dryness (45°C) under nitrogen. Metabolites were eluted isocratically from a Beckman Ultrasphere analytical column (ODS, 5-μm, reversed phase) with 0.05% acetic acid/methanol. Different HPLC conditions (table 2) were used for each assay to ensure adequate separation of metabolite peaks from each other and from a prominent unidentified peak that could otherwise have interfered with 1X, 7X, 3X, or 37U detection. Typical chromatographs of extracts from microsomal incubations for the four methylxanthine substrates are shown in fig. 1.

Monoclonal antibody inhibition studies were undertaken by preincubating mAb (1 mg/200 nmol of cytochrome P-450) or control ascites fluid with microsomal suspension for 15 min at 37°C. The addition of buffer, substrate, and NADPH-generating system initiated the reaction, and the per cent inhibition was expressed relative to control rates in the presence of ascites fluid. mAb inhibition of EROD activity in human and rat liver preparations is reported here for the first time, whereas the corresponding inhibition data for caffeine metabolism is from elsewhere (35).

Data Analysis. In cases where the enzyme kinetics appeared monophasic, kinetic constants were derived by least squares linear regression of data plotted on a Hanes plot (S *vs.* S/V) (36). In a Hanes plot, V_{max} is the reciprocal of the slope, and K_m is the negative value of the x intercept. In those instances, where Eadie-Hofstee plots (V/S *vs.* V) (37) display biphasic kinetics, a Gaushaus computer program was applied to the nonlinear data to provide weighted ($1/y^2$) least squares estimates of the parameters for the high affinity site. In determining kinetic parameters, caffeine, paraxanthine, and theophylline concentrations ranged from 0.05–15 mM and were chosen to optimize characterization of the high affinity site. Due to solubility limitations of theobromine, kinetic parameters were based on a more limited concentration range of 0.04–3 mM. The coefficient of variation between replicate incubations was 4–7%. The kinetic analysis of caffeine metabolism by K15 liver microsomes (fig. 4 and table 3) was reported previously (23) and is included in this paper only for rough qualitative comparison. The generation of caffeine metabolic data for K15 microsomes differed in that unwashed microsomes were used, and kinetic parameters were not subjected to Gaushaus computer analysis.

For the inhibition experiments, Dixon plots (38) (I *vs.* $1/V$) were used to determine the type of inhibition and the inhibition constant K_i. The K_i was determined graphically as the mean point of intersection of the three lines generated for the three substrate levels. In cases of ambiguity with respect to whether the inhibition was competitive or mixed-typed, the data were replotted on a Cornish-Bowden plot (39) (I *vs.* S/V) to distinguish between these two types of inhibition.

For the correlation experiments, correlation coefficients were determined based on log transformations of methylxanthine and EROD reaction rates. This was done to correct the skewness in the distribution of the data, and thereby avoid artificially inflating the correlation coefficient.

TABLE 2

HPLC conditions for methylxanthine assays

Substrate	Metabolite Detected	Internal Standard	Mobile[a] Phase	Flow	Column Temperature
				ml/min	*°C*
Caffeine (137X)	17X 37X 13X 137U	7X	88:12	1.3	40
Paraxanthine (17X)	1X 1U 7X 17U	37X	92:8	0.8	32
Theobromine (37X)	3X 7X 37U	13U	95:5	1.0	32
Theophylline (13X)	1X 3X 13U	37X	95:5	1.0	40

[a] The mobile phase was 0.05% acetic acid/methanol at the proportions (v/v) listed above.

Results

Time-activity and enzyme-activity studies performed for each of caffeine, paraxanthine, theophylline, and theobromine substrates established that *N*-demethylation and C_8-hydroxylation rates were linear at least for 20 min, and at protein concentrations of at least 2 mg of washed microsomal protein per ml incubation volume. All methylxanthine demethylations and 8-hydroxylations observable *in vitro* in human liver preparations were catalyzed almost exclusively (greater than 90%) by microsomal enzymes and were NADPH-dependent. In the absence of NADPH, biotransformations were less than 3% of those in the presence of NADPH.

Fig. 2 shows the effect of the prototype cytochrome P-450 inhibitor, metyrapone, on methylxanthine demethylations at low (1 mM) substrate concentrations. In contrast to the strictly inhibitory nature of metyrapone on demethylations, metyrapone inhibited all four methylxanthine 8-hydroxylations at inhibitor concentrations of less than 3 mM, but apparently stimulated these pathways at higher concentrations. Carbon monoxide (80:20 CO/O_2) inhibited all methylxanthine biotransformations by more than 70%. Furthermore, the effect of metyrapone and carbon monoxide on a methylxanthine at a high substrate concentration (20 mM) was examined using paraxanthine. Carbon monoxide and metyrapone (50 mM) resulted in more than 70% inhibition of paraxanthine demethylations and 8-hydroxylation also at the high substrate concentration, indicating that both high and low affinity sites are isozymes of cytochrome P-450.

Three human livers were characterized with respect to EROD activity. K_m values were very similar in K14, K15, and K18 (0.30 ± 0.09, 0.38 ± 0.05, and 0.37 ± 0.03 μM SD, respectively), whereas enzyme capacity, as represented by V_{max}, varied 4-fold (76 ± 20, 149 ± 16, and 244 ± 14 pmol/min/mg). ANF (0.3 μM) inhibited EROD activity at 1 μM 7ER substrate by more than 95% in all three livers.

The Eadie-Hofstee plots depicted in figs. 3 and 4 illustrate the biphasic nature of many methylxanthine demethylations. Fig. 3 depicts a representative Eadie-Hofstee plot indicative of biphasic kinetics, on which the computer-dissected slopes representing Michaelis-Menten constants (K_{m_1} and K_{m_2}) for the high and low affinity sites are drawn. At low substrate concentrations (1 mM), catalytic activity by the high affinity site contributed a high proportion of the total reaction velocity, whereas at high substrate concentrations (20 mM), the low affinity site mediated most of the total metabolic activity.

Table 3 summarizes kinetic parameters for the formation of metabolites from caffeine, paraxanthine, theophylline, and theobromine in human liver microsomes. The K_m values for the high

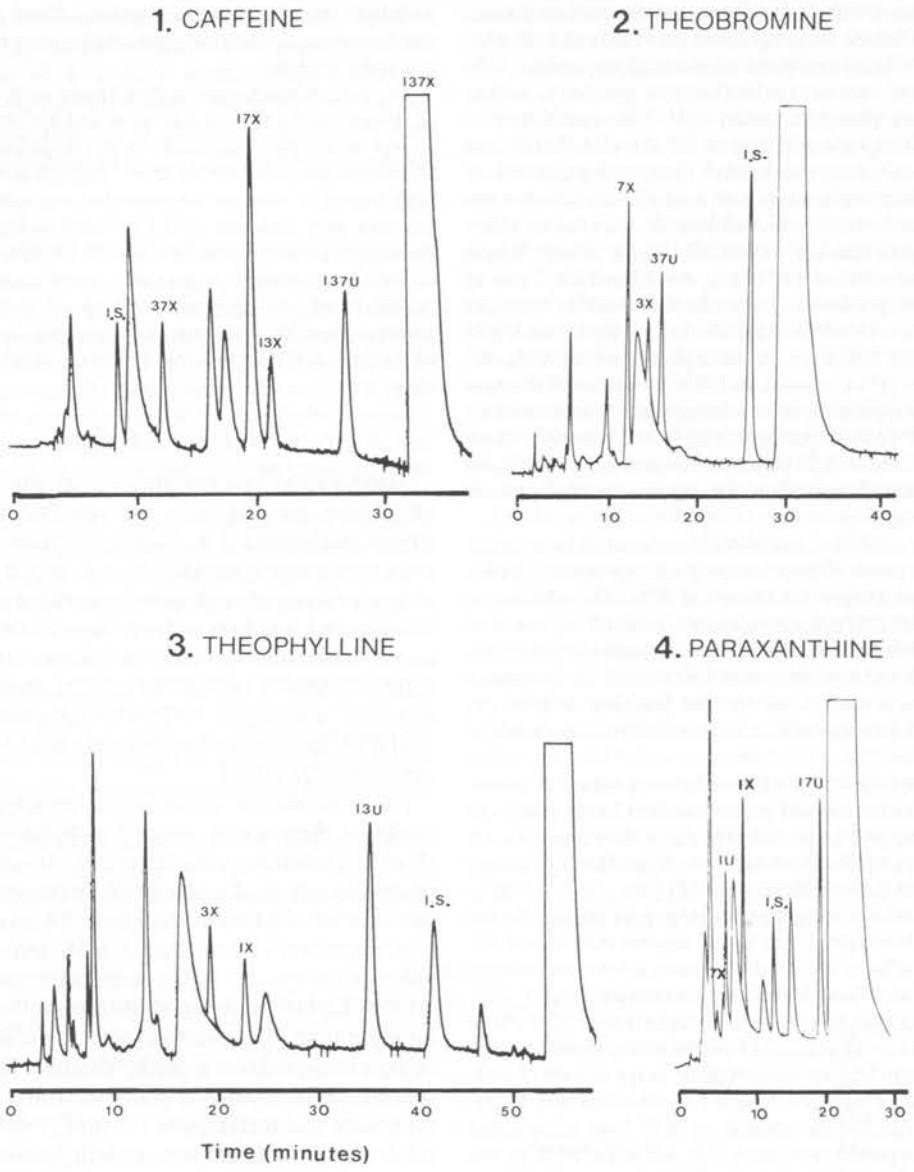

FIG. 1. *Typical HPLC chromatographs of extracts of microsomal incubations for caffeine, theobromine, theophylline, and paraxanthine substrates (1 mM).*

K18 human liver microsomes were used.

affinity sites involved in demethylations ranged from 0.3–1.9 mM (table 3), whereas K_m values for the low affinity site were approximately 10-fold higher, ranging from 4.0–35 mM. Listed in table 3 are kinetic parameters for metabolite formation from paraxanthine in microsomal preparations from four liver donors, including one known smoker (K21) and one known nonsmoker (K20). The K_m values for the 7-demethylations ($K_m = 1.2 \pm 0.5$, mean ± SD) were similar in all four livers, whereas the corresponding V_{max} values varied 8-fold, the V_{max} from the known nonsmoker being lowest.

The profile of demethylated metabolites produced from 1 mM caffeine, paraxanthine, theophylline, and theobromine was similar to that observed *in vivo* (3, 5, 7, 8), with caffeine 3-demethylation, paraxanthine 7-demethylation, theophylline 1- and 3-demethylation, and theobromine 3-demethylation, each being the dominant demethylation pathway both *in vitro* and *in vivo*. In contrast, 8-hydroxylation rates for each of the four methylxanthine substrates to their respective uric acid metabolites (137U, 17U, 13U, and 37U) was higher than expected based on *in vivo* data.

A series of inhibition experiments was undertaken involving methylxanthines and substrates known to be at least partially metabolized by PAH-inducible cytochrome P-450 such as 7ER (24), phenacetin (40), antipyrine (41), and benzo[*a*]pyrene (28). Phenacetin, antipyrine, and benzo[*a*]pyrene competitively inhibited EROD (table 4) and caffeine biotransformation (table 5) by human liver microsomes. Fig. 5 shows the complete inhibition of caffeine demethylation by benzo[*a*]pyrene and phenacetin in K18 microsomes, whereas 8-hydroxylation was inhibited about 80%.

Caffeine, paraxanthine, theophylline, and theobromine each competitively inhibited EROD (table 4), and, conversely, 7ER competitively inhibited all demethylation and 8-hydroxylations of caffeine, paraxanthine, theophylline, and theobromine (table

TABLE 3

Kinetic parameters of caffeine, paraxanthine, theophylline, and theobromine metabolism by human liver microsomes

Substrate	Metabolite Formed	Liver	$Km^a \pm$ SD	$V_{max}{}^a \pm$ SD
			mM	*pmol/min/mg*
Caffeine[b]	17X	K18	0.46 ± 0.17	570 ± 65
(137X)	13X	K18	0.31 ± 0.06	27 ± 5
	37X	K18	0.28 ± 0.05	53 ± 13
	137U	K18	0.41 ± 0.10	41 ± 11
Paraxanthine	1X + 1U	K15	1.05 ± 0.24	330 ± 88
(17X)	1X + 1U	K18	0.65 ± 0.09	1048 ± 134
	1X + 1U	K20	1.57 ± 0.26	125 ± 34
	1X + 1U	K21	1.75 ± 0.33	534 ± 111
	7X	K15	1.08 ± 0.22	19 ± 3
	7X	K18	0.70 ± 0.10	55 ± 4
	17U	K15	0.33 ± 0.28	94 ± 98
	17U	K18	0.71 ± 0.04	1207 ± 11
	17U	K20	0.41 ± 0.09	90 ± 18
	17U	K21	0.64 ± 0.10	322 ± 40
Theophylline	3X	K15	1.18 ± 0.21	105 ± 9
(13X)	3X	K18	0.35 ± 0.03	166 ± 14
	1X	K15	1.01 ± 0.14	75 ± 5
	1X	K18	0.36 ± 0.03	114 ± 10
	13U	K15	3.88 ± 0.28	1427 ± 79
	13U	K18	1.54 ± 0.22	1147 ± 103
Theobromine	7X	K15	1.17 ± 0.11	447 ± 27
	7X	K18	1.91 ± 0.06	1460 ± 30
	3X	K15	1.22 ± 0.14	84 ± 4
	3X	K18	1.21 ± 0.10	179 ± 11
	37U	K15	5.03 ± 0.42	632 ± 44
	37U	K18	2.58 ± 0.21	1720 ± 112

[a] Given for the high affinity site.

[b] Kinetic parameters for K15 are approximately as follows: K_m for 17X, 13X and 37X production, 0.5–1.0 mM; V_{max} for 17X, 13X, and 37X are about 140, 20, and 25 pmol/min/mg unwashed microsomal protein. Caffeine kinetic parameters for K15 are not directly comparable to data listed above (see *Materials and Methods* for explanation).

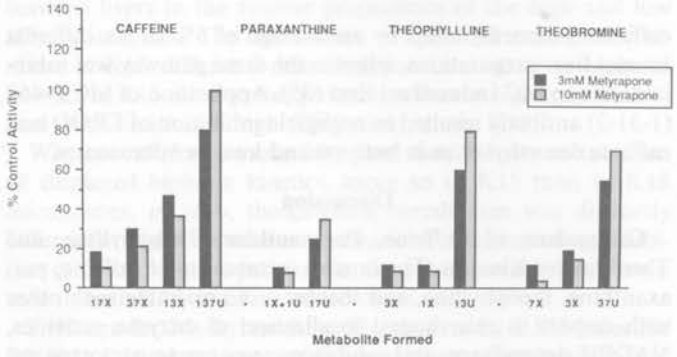

FIG. 2. *Effect of metyrapone on methylxanthine metabolism by high affinity site.*

Note the dose-dependent inhibition and stimulation of all four 8-hydroxylation pathways. The abscissa denotes metabolic pathway by product formed.

5). Furthermore, the inhibition constant (K_i) for each metabolite was close in value to its respective K_m.

Fig. 6 shows the effect of acetanilide, a model substrate of PAH-inducible cytochrome P_3-450 (42) on EROD activity in microsomes prepared from K18 human liver, MC-induced C57BL/6N mouse, and Wistar rat liver. Acetanilide almost completely inhibited EROD activity in all three livers. Furthermore, acetanilide competitively inhibited EROD activity in human, mouse, and rat liver microsomes (table 4), and competitively inhibited caffeine biotransformation (table 5).

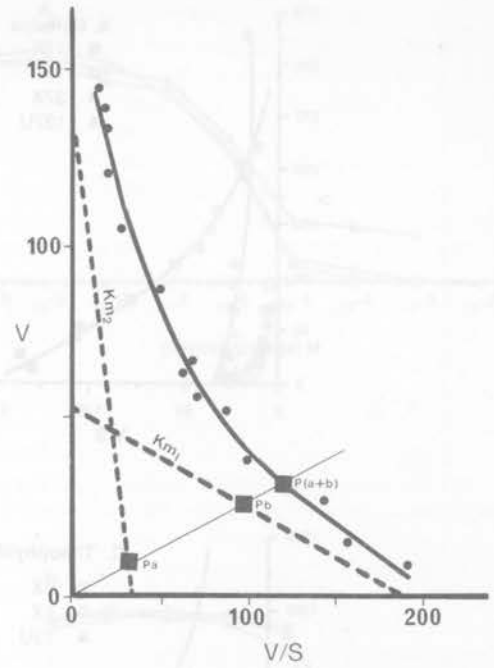

FIG. 3. *A representative Eadie-Hofstee plot showing biphasic kinetics of caffeine 1-demethylation by K18 microsomes.*

The slopes of the *dotted lines* represent the computer-derived estimates of K_{m_1} and K_{m_2} for the high and low affinity sites, respectively. The *heavy solid line*, based on observed reaction velocities, represents a composite (P_{a+b}) of the computer-dissected coordinates for each of the high (P_b) and low affinity (P_a) isozymes. Velocity (V) is expressed as pmols/min/mg and substrate concentration (S) as mM.

There was a 20-fold difference in EROD (table 1) and methylxanthine demethylation rates over 15 human livers. Mean EROD rates for males (89.0 ± 77.0, mean ± SD) were not significantly higher ($p = 0.1$) than for females (45.7 ± 47.2). Fig. 7 depicts the highly significant correlation ($r = 0.95$, $p < 0.001$) between paraxanthine 7-demethylation and EROD rates for 15 different human livers. The results of correlation studies (table 6) revealed strong correlations ($r = 0.84$–0.96, $p < 0.001$) between EROD and all methylxanthine demethylation rates, and generally poorer correlations with methylxanthine 8-hydroxylation rates ($r = 0.48$–0.76, $p > 0.001$). Similarly, demethylation rates among methylxanthines tended to correlate much better than 8-hydroxylation with demethylation rates.

Table 7 demonstrates that caffeine and its dimethylxanthine products mutually inhibited each other's metabolism in a competitive manner.

Fig. 8 shows that ANF generally inhibited methylxanthine 8-hydroxylations less than demethylations, particularly for theophylline and theobromine in K15 microsomes.

The relationship between C_8-hydroxylation and N-demethylation was investigated further using paraxanthine. Fig. 9 depicts 8-hydroxylation activity remaining in human liver microsomes after maximal inhibition by ANF. In low EROD activity livers, a large portion of 8-hydroxylation activity was not inhibited by ANF, whereas in high EROD activity livers, most 8-hydroxylation was inhibited by ANF. In fig. 10, it is seen that low EROD rates corresponded to a high 8-hydroxylation to 7-demethylation

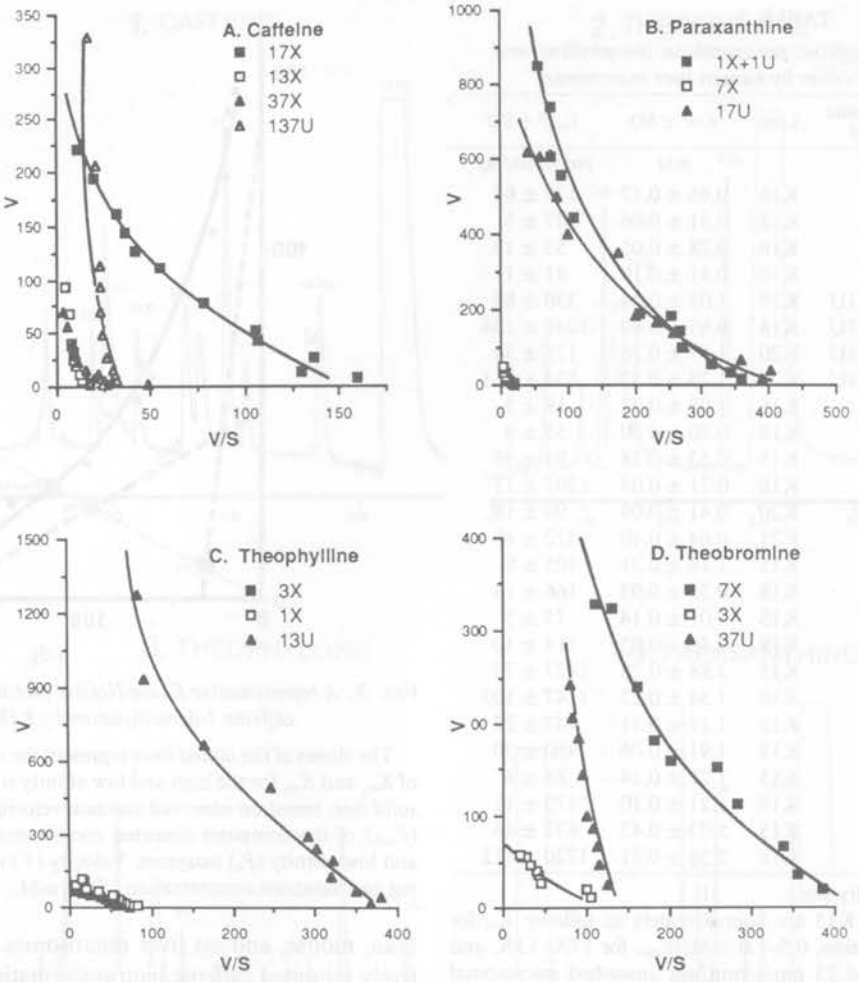

FIG. 4. *Comparison of Eadie-Hofstee plots for caffeine, paraxanthine, theophylline, and theobromine metabolism by K15 human liver microsomes.* Methylxanthine substrate concentrations (S) are expressed as mM, velocities (V) as pmol/min/mg.

TABLE 4

K_i values for competitive inhibitors of ethoxyresorufin O-deethylation by K18 microsomes

Inhibitor	K_i	K_m of Inhibitor When Acting as Substrate Alone
Phenacetin	30 μM	6.3 μM (high affinity site)[a] 248 μM (low affinity site)[a]
Antipyrine	5 mM	
Caffeine	0.75 mM	0.3–0.5 mM (*N*-demethylations) 0.4 mM (8-hydroxylation)
Paraxanthine	0.60 mM	0.6–0.7 mM (*N*-demethylations) 0.7 mM (8-hydroxylation)
Theophylline	0.80 mM	0.3–0.4 mM (*N*-demethylations) 1.5 mM (8-hydroxylation)
Theobromine	1.2 mM	1.2–1.9 mM (*N*-demethylations) 2.6 mM (8-hydroxylation)
Acetanilide	0.75 mM	0.55 mM (4-hydroxylation in mouse)[b]

[a] Source of published K_m values (42a).
[b] Source of published K_m values (42).

ratio (17U/1X + 1U), whereas at high EROD rates the inverse was true.

Application of monoclonal antibody MC P-450 (1-7-1) to K14, K16, and K18 human liver microsomes resulted in an average inhibition of EROD of 5%, whereas EROD was inhibited almost 80% in MC-induced rat liver microsomes. mAb 1-7-1 inhibited caffeine 3-demethylation by an average of 6% in six different human liver preparations, whereas the same pathway was inhibited 70% in MC-induced rat liver (35). Application of MC P-450 (1-31-2) antibody resulted in negligible inhibition of EROD and caffeine demethylation in both rat and human microsomes.

Discussion

Comparison of Caffeine, Paraxanthine, Theophylline, and Theobromine Kinetics. The *in vitro* metabolism of caffeine, paraxanthine, theophylline, and theobromine resemble each other with respect to microsomal localization of enzyme activities, NADPH dependence, and inhibitory response to prototype cytochrome P-450 inhibitors such as carbon monoxide and metyrapone. Lohmann and Miech (43) have previously demonstrated that theophylline metabolism by rat liver is cytochrome P-450-mediated.

Eadie-Hofstee analysis indicates the potential participation of at least two isozymes with differing substrate affinities in the production of individual metabolites from a given methylxanthine. However, because K_m values for the high affinity site generally exceed methylxanthine concentrations (around 0.05 mM) encountered *in vivo* during routine dietary intake, metabolite production at physiologically relevant methylxanthine concentrations likely varies as a linear function of substrate concentration, with negligible contribution by the low affinity site, and

TABLE 5

K_i values for competitive inhibitors of methylxanthine metabolism

Inhibitor	Substrate	Metabolite Formed	K_i (K15)	K_i (K18)
7ER	Caffeine (137X)	17X	2.6 μM	ND[a]
		13X	4.2 μM	ND
		37X	4.7 μM	ND
		137U	6.5 μM	ND
7ER	Paraxanthine (17X)	1X + 1U	2.4 μM	1.7 μM
		7X	2.0 μM	1.9 μM
		17U	3.3 μM	2.8 μM
7ER	Theophylline (13X)	3X	3.0 μM	3.0 μM
		1X	2.5 μM	3.0 μM
		13U	4.0 μM	4.5 μM
7ER	Theobromine (37X)	7X	1.4 μM	1.5 μM
		3X	1.6 μM	2.0 μM
		37U	3.8 μM	2.5 μM
Benzo[a]pyrene	Caffeine (137X)	17X	ND	3.5 μM
		13X	ND	—[b]
		37X	ND	—
		137U	ND	7.0 μM
Antipyrine	Caffeine (137X)	17X	ND	2.5 mM
		13X	ND	4.0 mM
		37X	ND	—
		137U	ND	—
Phenacetin	Caffeine (137X)	17X	ND	50 μM
		13X	ND	75 μM
		37X	ND	—
		137U	ND	80 μM
Acetanilde	Caffeine (137X)	17X	2.5 mM	ND
		13X	2.0 mM	ND
		37X	2.5 mM	ND
		137U	2.0 mM	ND

[a] ND, not determined.

[b] —, analytical interference prevented reliable detection of these metabolites.

compatible with *in vivo* observations (44). There are differences between livers in the relative proportions of the high and low affinity isozymes. The observation of apparent monophasic kinetics in K18 caffeine 3-demethylation in contrast to biphasic kinetics in K15 microsomes probably reflects a higher, nonsaturated concentration of high affinity isozyme in K18.

Whereas caffeine, paraxanthine, and theobromine metabolism all displayed biphasic kinetics, more so in K15 than in K18 microsomes, *in vitro*, theophylline metabolism was distinctly different at high substrate concentrations, demonstrating inhibition of demethylation pathways at concentrations greater than 6 mM in both K18 and K15 microsomes.

Evidence for Methylxanthine Metabolism by PAH-inducible P450. In this paper, we provide several lines of evidence indicating that all demethylation pathways for each of caffeine, paraxanthine, theophylline, and theobromine are mediated in large part by the same high affinity enzyme(s), and that this enzyme likely is PAH-inducible cytochrome(s) P-450. That the same high affinity isozyme mediates all methylxanthine demethylations is suggested by: 1) the results of the methylxanthine mutual inhibition study in which pairs of methylxanthines competitively inhibited the demethylation of each other, and 2) the strong correlations in the biotransformation rates for multiple methylxanthine pathways in different livers.

Evidence for the identity of the high affinity isozyme as PAH-inducible cytochrome P-450 is based on the following experimental observations: 1) 7ER competitively inhibited all caffeine, paraxanthine, theophylline, and theobromine demethylations; 2) conversely, each of caffeine, paraxanthine, theophylline, and theobromine competitively inhibited EROD activity; 3) EROD rates correlated highly ($r = 0.84$–0.96, $p < 0.001$) with all caffeine, paraxanthine, theophylline, and theobromine demethylation rates; 4) ANF inhibited caffeine, paraxanthine, theophylline, and theobromine demethylations in excess of 80% in two high activity livers; and 5) benzo[*a*]pyrene, antipyrine, and phenacetin each competitively inhibited detectable caffeine demethylations by human liver microsomes.

Mutually competitive inhibition between 7ER and a given

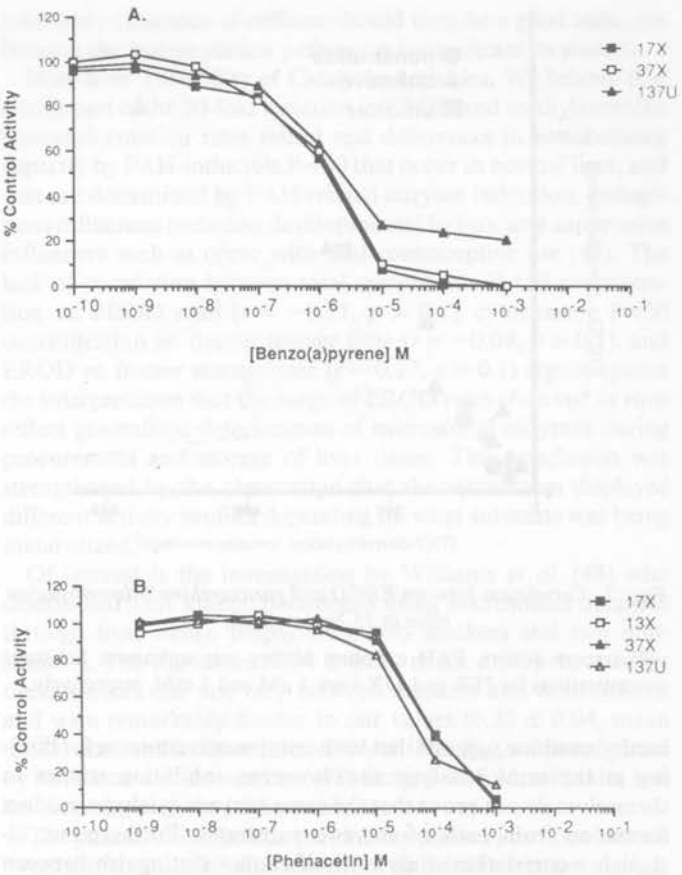

FIG. 5. *Inhibition of caffeine metabolism by benzo[a]pyrene and phenacetin.*

K18 microsomes were used. Caffeine concentration was 1 mM.

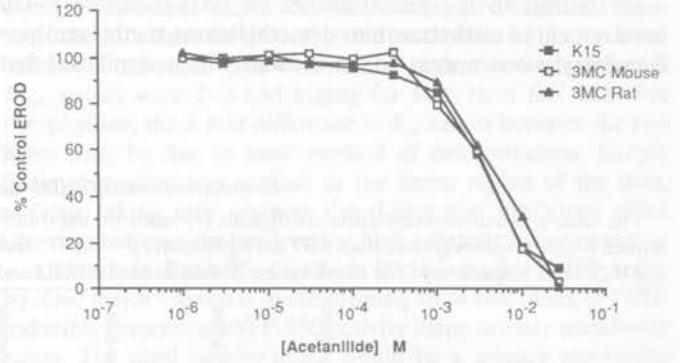

FIG. 6. *Acetanilide inhibition of EROD.*

Microsomes were prepared from K18 human liver, MC-induced rat, and mouse liver. 7ER substrate concentration was 1 μM.

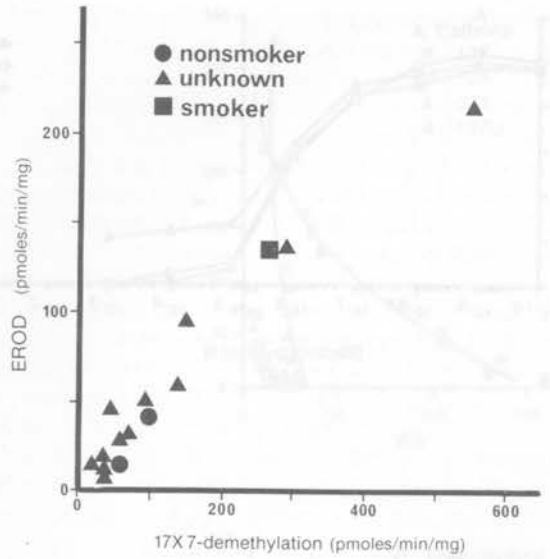

FIG. 7. *Correlation between EROD and paraxanthine 7-demethylation rates in 15 human livers.*

For most donors, PAH exposure history was unknown. Substrate concentrations for 7ER and 17X were 1 μM and 1 mM, respectively.

methylxanthine suggests that both compounds compete for binding at the same catalytic site; however, inhibition studies in themselves do not prove that the same enzyme catalyzes product formation from each of the two substrates. Furthermore, although a correlation study in itself cannot distinguish between single enzyme involvement or the involvement of multiple enzymes under the same regulatory control, the combination of inhibitor/correlation studies provides a powerful tool in implicating PAH-inducible P-450 in methylxanthine demethylations.

In contrast to the predominance of PAH-inducible P-450 involvement in methylxanthine demethylations, methylxanthine 8-hydroxylations appear to be mediated in a significant but variable part by another cytochrome P-450 isozyme in addition to PAH-inducible P-450. This conclusion is supported by the poor ability of ANF to inhibit this pathway in many different livers (fig. 9), by the generally poor correlation between EROD and methylxanthine 8-hydroxylation rates in different livers, and by the different response to metyrapone between demethylation and 8-hydroxylations.

This paper bases its conclusions for the involvement of PAH-inducible P-450 in methylxanthine demethylations on a classical approach involving kinetic analysis, competitive inhibition, and correlation studies. In our hands, application of MC-P-450 (1-7-1) monoclonal antibody to 7ER and caffeine incubation mixtures containing human liver microsomes resulted in little or no inhibition of EROD or caffeine demethylation, whereas the same pathways were greatly inhibited in MC-induced rat liver. One interpretation is that structural differences between analogous rat and human isozymes are sufficient to preclude equivalent mAb binding to antigenic sites required to inhibit enzyme activity. More useful for this type of study would be application of mAbs raised against human isozymes themselves.

In the mouse and rat, MC-type induction results in increased synthesis of (at least) two isozymes of cytochrome P-450. In the mouse, P_1-450 and P_3-450 (45, 46) mediate EROD (28) and acetanilide 4-hydroxylation (42), respectively, whereas in the rat, the analogous cytochomes are P-450c and P-450d (45). The ability of acetanilide to competitively inhibit EROD in both MC-induced mouse and rat liver microsomes (tables 4 and 5) suggests an overlapping substrate specificity between both PAH-inducible isozymes. Consequently, the use of classical inhibition and correlation studies cannot distinguish which, if not both, of the PAH-inducible isozymes are involved in methylxanthine metabolism by human liver microsomes. However, given that cytochromes P_1-450 and P_3-450 are under the same regulatory control, a urinary ratio of metabolite recovery after caffeine (discussed later) should nonetheless provide a reasonable index of PAH-induced cytochrome(s) P-450 activity.

Comparison with Published Data. Based on our *in vitro* find-

TABLE 6

Correlations among EROD and methylxanthine metabolic rates

The table summarizes correlation coefficients (r) based on log transformations of metabolic rates using microsomes prepared from 10 different human livers. R values greater than 0.77 are significant ($p < 0.001$). Substrate concentrations for the EROD and methylxanthine assays were 1 μM and 0.25 mM, respectively. The livers varied 20-fold in both EROD and methyxanthine demethylation rates.

	EROD	37X 7-Demethylation	37X 3-Demethylation	13X 8-Hydroxylation	13X 1-Demethylation	17X 8-Hydroxylation	17X 1-Demethylation	17X 7-Demethylation	137X 8-Hydroxylation	37X 7-Demethylation	137X 3-Demethylation
137X 1-demethylation	0.94	0.85	0.77	0.69	0.79	0.64	0.95	0.90	0.87	0.97	0.96
137X 3-demethylation	0.96	0.86	0.87	0.49	0.89	0.58	0.97	0.83	0.81	0.92	
137X 7-demethylation	0.92	0.90	0.73	0.72	0.75	0.71	0.90	0.91	0.79		
137X 8-hydroxylation	0.76	0.74	0.53	0.45	0.52	0.23	0.71	0.59			
17X 7-demethylation	0.94	0.89	0.84	0.68	0.62	0.87	0.83				
17X 1-demethylation	0.88	0.74	0.71	0.56	0.54	0.68					
17X 8-hydroxylation	0.61	0.70	0.72	0.67	0.40						
13X 1-demethylation	0.91	0.81	0.83	0.41							
13X 8-hydroxylation	0.48	0.51	0.65								
37X 3-demethylation	0.88	0.83									
37X 7-demethylation	0.84										

TABLE 7

Mutual methylxanthine inhibition using K18 human liver microsomes

Inhibitor	Substrate	Metabolite Formed from Substrate[a]	K_i
			mM
Caffeine (137X)	Theobromine (37X)	7X	0.5
		3X	0.6
		37U	0.6
Theobromine (37X)	Caffeine (137X)	17X	1.2
		13X	1.0
		137U	1.2
Caffeine (137X)	Paraxanthine (17X)	7X	0.8
		1X + 1U	0.9
		17U	0.8
Paraxanthine (17X)	Caffeine (137X)	37X	1.2
		137U	2.5
Caffeine (137X)	Theophylline (13X)	1X	0.6
		3X	0.6
		13U	1.3
Theophylline (13X)	Caffeine (137X)	17X	1.0
		37X	0.9
		137U	1.0

[a] Listed here are only those metabolites formed whose detection was not obscured by the high methylxanthine inhibitor concentrations used.

ings, it is predictable that corresponding *in vivo* biotransformation pathways in humans will be enhanced upon PAH-related induction, such as occurs during smoking. Human disposition studies have shown that total body clearance of caffeine was increased in smokers (13). In rats, PAH induction resulted in caffeine metabolism by a high affinity P-450 isozyme (14) in which all three demethylation pathways were greatly enhanced (15).

Studies of theophylline disposition in mice (18) and rats (19) demonstrated a 2–3-fold increase in theophylline clearance in PAH-induced, compared to noninduced, animals. Like caffeine, theophylline clearance in humans was increased in smokers compared to nonsmokers (16, 17). Smokers showed a 2-fold increase in both the theophylline 1- and 3-demethylations, whereas theophylline 8-hydroxylation was less enhanced than demethylations (16). This is consistent with our hypothesis for the predominance of PAH-inducible P-450 involvement in the two demethylation pathways and multiple enzyme involvement in 8-hydroxylation.

In a recent study of theobromine disposition in humans, theobromine total body clearance was enhanced in smokers compared to nonsmokers; however, the increased clearance by the 3-demethylation pathway (38%) was less than the 7-demethylation pathway (52%) (20). In light of our *in vitro* data demonstrating biphasic kinetics, one interpretation for the difference in the apparent degree of induction of the two demethylation pathways is that at the high theobromine concentrations (600 mg/day) used by Miners *et al.* (20), the low affinity isozyme contributed significantly to one of the demethylation pathways, thereby obscuring the actual degree of cytochrome P-450 induction.

Our *in vitro* study would suggest that a nonspecific parameter such as total body clearance of theophylline would be a poor indicator of PAH-inducible P-450 activity *in vivo* because about half of the ingested theophylline undergoes 8-hydroxylation to 13U (4), and because this pathway can be mediated significantly by an isozyme other than PAH-inducible P-450. In contrast, total body clearance of caffeine should then be a good indicator because the hydroxylation pathway is insignificant *in vivo* (3).

Inter-liver Variability of Catalytic Activities. We believe that a large part of the 20-fold variation in EROD and methylxanthine biotransformation rates reflect real differences in metabolizing capacity by PAH-inducible P-450 that occur in normal liver, and that are determined by PAH-related enzyme induction, endogenous influences including developmental factors, and suppressive influences such as occur with oral contraceptive use (47). The lack of correlation between total cytochrome P-450 concentration *vs.* EROD rates ($r = -0.11$, $p > 0.1$), cytochrome P-450 concentration *vs.* freezer storage time ($r = -0.09$, $p > 0.1$), and EROD *vs.* freezer storage time ($r = 0.27$, $p > 0.1$) argues against the interpretation that the range of EROD rates observed *in vitro* reflect generalized deterioration of microsomal enzymes during procurement and storage of liver tissue. This conclusion was strengthened by the observation that the same livers displayed different activity profiles depending on what substrate was being metabolized.[2]

Of interest is the investigation by Williams *et al.* (48) who determined 7ER kinetic parameters using microsomes obtained through liver wedge biopsy from two smokers and two nonsmokers. The K_m values (0.34 ± 0.06, mean ± SD) reported by these authors did not vary between smokers and nonsmokers, and were remarkably similar to our values (0.35 ± 0.04, mean ± SD) observed in three human livers. The V_{max} values reported by Williams and co-workers (48) ranged 30-fold, with the two smokers showing the highest values.

The paraxanthine kinetic parameters (table 3) for four human livers showing similar K_m values, but an 8-fold variation in V_{max}, are compatible with participation by a common high affinity isozyme, the amount of which varies among donors. It is noteworthy that V_{max} for paraxanthine 7-demethylation in the known smoker is considerably higher than that for the known nonsmoker, consistent with PAH-related enzyme induction. Similarly, the K_m values for demethylated metabolite formation from caffeine and theobromine were similar in the two livers, whereas V_{max} values were 2–3-fold higher for K18 than for K15. For theophylline, the 3-fold difference in K_m values between the two livers may be due to their method of determination. Simple linear regression was applied to the linear region of the data, without taking into account the differential inhibitory effect observed between the two livers at high substrate concentrations.

A Metabolic Ratio That Reflects PAH-inducible P-450 Activity. Our major interest is in establishing an *in vivo* index of PAH-inducible cytochrome(s) P-450 activity using urinary metabolite ratios. The ideal *in vivo* index would be a urinary metabolite ratio in which metabolite production from a PAH-inducible pathway is compared to production from a noninducible pathway. Based on our *in vitro* investigations, we did not observe any pathway in the biotransformation of caffeine, paraxanthine, theophylline, and theobromine that was not mediated at least in part by PAH-inducible P-450.

A large portion of paraxanthine 8-hydroxylation in low EROD activity livers appears to be mediated by a noninducible P-450, whereas 8-hydroxylation in the high EROD activity livers appear to be mediated predominantly by PAH-inducible P-450. We have previously reported that the *in vivo* administration of the xanthine oxidase inhibitor allopurinol to two subjects resulted in a slight decline in urinary excretion of 17U after caffeine (49),

[2] W. Kalow, unpublished observations.

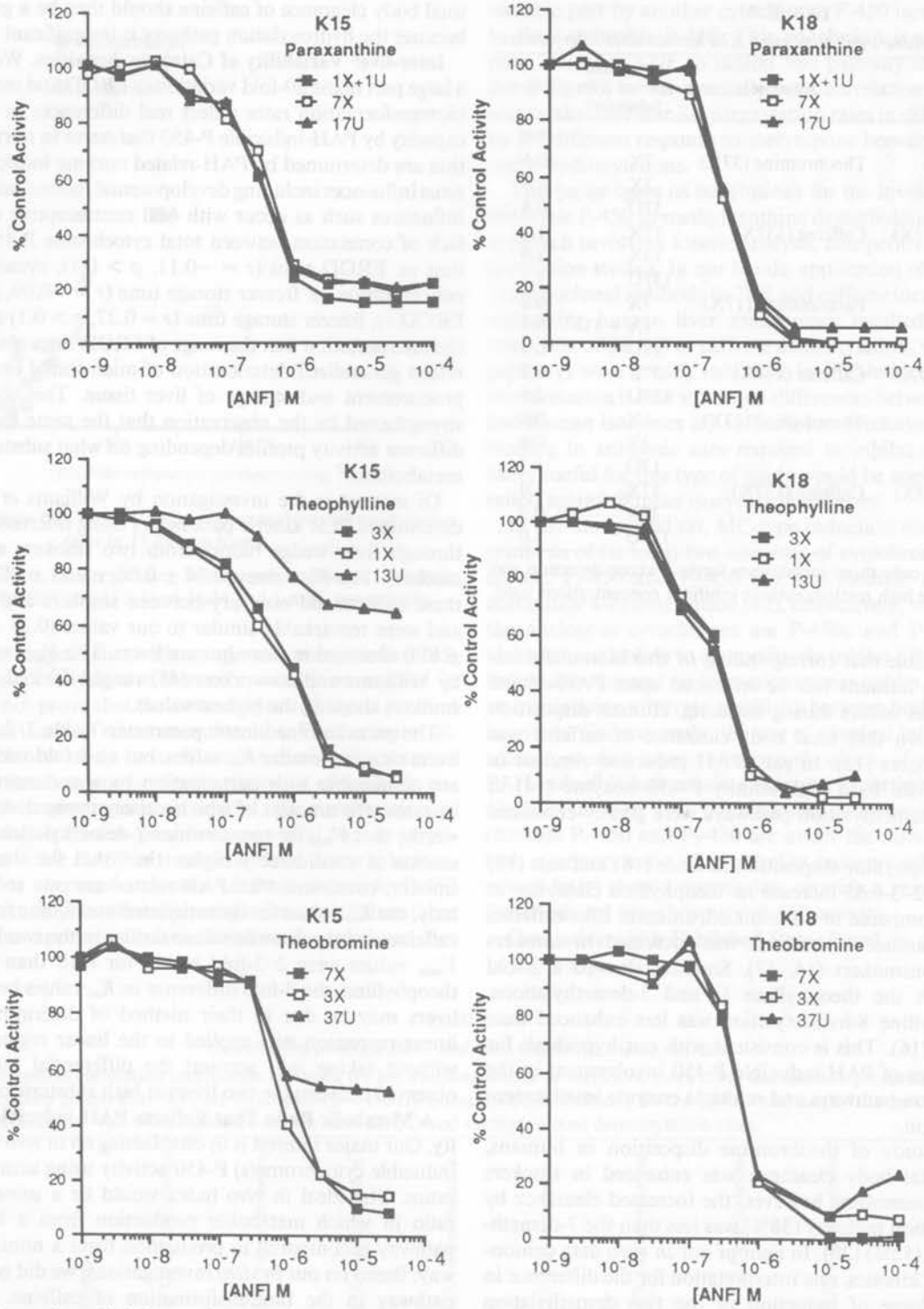

FIG. 8. *Comparison of inhibition of paraxanthine, theophylline, and theobromine metabolism by ANF for K15 and K18 human livers.*

All methylxanthine substrate concentrations were 1 mM.

raising as one possibility the involvement of xanthine oxidase in the 8-hydroxylation of paraxanthine. However, we were unable to inhibit microsomal 17U production in human liver at an allopurinol concentration 15 times higher than that which completely inhibited xanthine oxidase-mediated conversion of 1X to 1U, indicating that the low affinity enzyme participating in paraxanthine 8-hydroxylation is not xanthine oxidase.

The production of AFMU from paraxanthine was not observed *in vitro*, nor would its presence be expected during microsomal metabolism given the cytosolic location of *N*-acetyltransferase and its requirement for acetyl coenzyme A as an acetyl group donor (50). Consequently, the microsomal formation of 1X + 1U from paraxanthine is taken as a measure of paraxanthine 7-demethylation activity *in vitro*. *In vivo*, paraxanthine 7-demethylation is represented by the urinary recovery of 1X + 1U + AFMU (3).

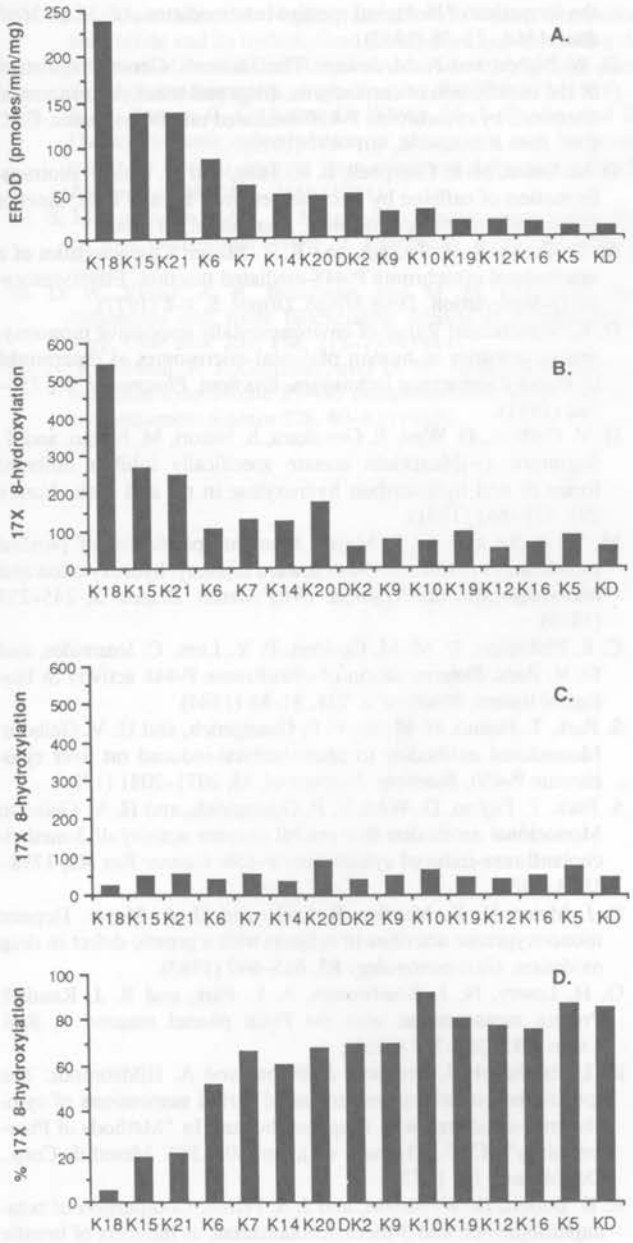

FIG. 9. *Comparison of EROD rates and paraxanthine 8-hydroxylation rates in the presence and absence of ANF in multiple human livers.*

A, EROD rates at 1 μM substrate; *B*, paraxanthine concentration was 1 mM, velocity expressed as pmol/mg/min; *C*, residual 8-hydroxylation activity remaining following maximal inhibition by ANF, velocity in pmol/min/mg; and *D*, the same residual activity expressed as per cent of control activity.

A plot of the ratio of *in vitro* paraxanthine 8-hydroxylation to 7-demethylation (17U/1X + 1U formation) rates *vs.* EROD rates for 12 different livers shows a distinct relationship (fig. 10). It is notable that high activity livers give a low 17U/1X + 1U ratio, whereas the low activity livers give a high ratio. One possible interpretation for this relationship is depicted in the hypothetical model of fig. 10. In the model, as PAH-inducible P-450 levels increase, whether by endogenous or PAH-related induction, the relative proportions of the two isozymes contributing to 17U production change. At low states of PAH induction, most of the 8-hydroxylation is mediated by a noninducible isozyme, whereas at states of high induction, most of the 8-hydroxylation pathway is predicted to be mediated by PAH-inducible P-450.

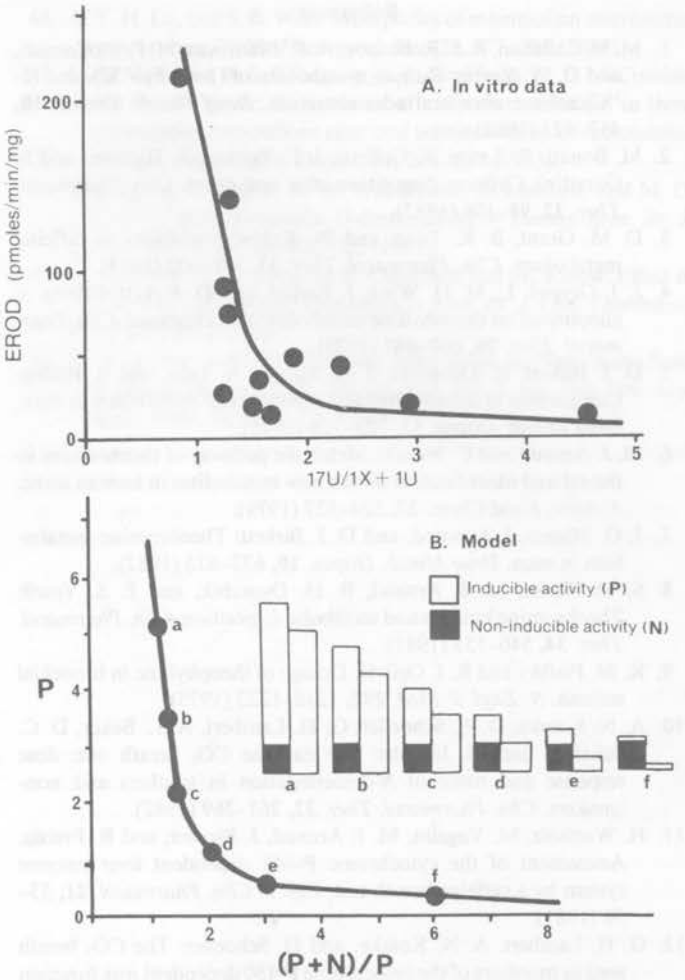

FIG. 10. *Comparison of paraxanthine metabolic ratios with EROD activities.*

A, a plot of paraxanthine 8-hydroxylation/7-demethylation (17U/1X + 1U) *vs.* EROD rates for 12 livers. *B*, this plot illustrates a hypothetical model comparable to the actual data generated *in vitro* in the *upper plot*. In the model, the *abscissa* of the line graph, and the *left side* of each of the pairs of *bars* in the histogram *inset* reflect 17U/1X + 1U formation from paraxanthine. The *ordinate* of the line graph and the *right side* of each of the pairs of bars reflect EROD (PAH-inducible P-450).

In conclusion, this paper provides an *in vitro* rationale for a potential *in vivo* index of PAH-inducible cytochrome P-450 activity in human populations. To establish whether a 8-hydroxylation to 7-demethylation ratio is sufficiently sensitive as an *in vivo* marker, we are currently comparing the corresponding urinary metabolite ratios after caffeine ingestion with caffeine clearance in subjects with known and varying PAH-exposure history.

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