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BIOTRANSFORMATION OF CAFFEINE BY MICROSOMES FROM HUMAN LIVER

KINETICS AND INHIBITION STUDIES*

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Abstract—The nature of the cytochrome P-450-dependent enzyme reactions giving rise to four primary metabolites of caffeine was investigated using microsomes isolated from livers of human kidney donors. Metabolite formation proceeded at a lower rate than that predicted from *in vivo* caffeine elimination half-lives, as has been observed in other species using this compound as a substrate in microsomal incubations. Kinetic experiments indicated that the formation of each of the N-demethylated metabolites paraxanthine, theobromine and theophyline was mediated by both a high- and a low-affinity catalytic site over a substrate concentration range from 0.05 mM to 80.0 mM, although only the high-affinity component is likely to be of any importance at normally encountered *in vivo* caffeine concentrations. 7-Ethoxyresorufin and acetanilide, selective substrates for two polycyclic aromatic hydrocarbon (PAH)-inducible isozymes of cytochrome P-450 in the mouse (P_1-450 and P_3-450, respectively) were each able to inhibit competitively the formation of caffeine metabolites by human liver microsomes, while caffeine could in turn similarly inhibit the biotransformations of these two compounds. The isozyme-selective P-450 inhibitor α-naphthoflavone (ANF) potently inhibited the high-affinity component of caffeine *N*-demethylations, while 1-phenylimidazole (PI) was a more potent inhibitor of the low-affinity component. The inhibition studies also indicated that the formation of 1,3,7-trimethyluric acid was mediated by both ANF-sensitive and PI-sensitive sites. Taken together, the data support suggestions from *in vivo* studies that a PAH-inducible isozyme of cytochrome P-450 plays a significant role in the biotransformation of caffeine in man.

A number of groups have recently been investigating the use of methylxanthines as potential *in vivo* probes for the assessment of liver drug-metabolizing enzyme functions in man. One approach which has been used in this regard is to monitor the exhalation of isotopically labelled CO_2 after administration of caffeine (1,3,7-trimethylxanthine, 137X,‡ Fig. 1) labelled in various methyl positions, as a measure of cytochrome P-450-dependent *N*-demethylation activity [1–3]. Others have concentrated on quantifying the urinary excretion of metabolites of caffeine [4, 5], theophylline [6, 7] or theobromine [8, 9] as potential indicators of a number of specific liver enzyme activities.

One reason behind the choice of caffeine as a suitable probe was considerable circumstantial evidence from *in vivo* studies in both man and animals to suggest the involvement of polycyclic aromatic hydrogen (PAH)-inducible isozyme(s) of cytochromes P-450 in its biotransformation [2, 3, 10–13]. Knowledge of the activity of such isozymes may be of some toxicological significance, since these have been shown in a number of animal systems to be responsible for the metabolic activation of certain chemicals, structurally related to the inducing agents, to reactive carcinogenic intermediates [14, 15]. In the mouse, for example, pretreatment with 3-methylcholanthrene, β-naphthoflavone or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) leads to activation of gene transcription for two major cytochrome P-450 isozymes, P_1-450 and P_3-450 [16, 17, 19], and ultimately results in increased susceptibility to certain chemically-induced tumors [14].

Since the induction of caffeine *N*-demethylation rates by PAHs has been shown to correlate exceedingly well with the inducibility of the aforementioned P-450 isozymes in genetically responsive and non-responsive strains of mice [13], it may be presumed that caffeine is biotransformed in man by an analogue or analogues of one of these murine enzymes. This then implies that certain parameters of caffeine biotransformation in man could be useful as predictors of PAH-inducible cytochrome P-450 activity. That this is indeed the case has been suggested by the results of the aforementioned breath tests [2, 3], which showed an increased rate of caffeine *N*-demethylations in human smoking subjects (exposed to PAHs in cigarette smoke) as compared to non-smokers.

In the mouse, PAH-inducible cytochromes P_1-450 and P_3-450 exhibit marked selectivity for the biotransformation of certain substrates, and thus

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‡ Abbreviations used: 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; 17U, 1,7-dimethyluric acid; 7X, 7-methylxanthine; PAH, polycyclic aromatic hydrocarbon; 7ER, 7-ethoxyresorufin; ANF, α-naphthoflavone (7,8-benzoflavone); PI, 1-phenylimidazole.

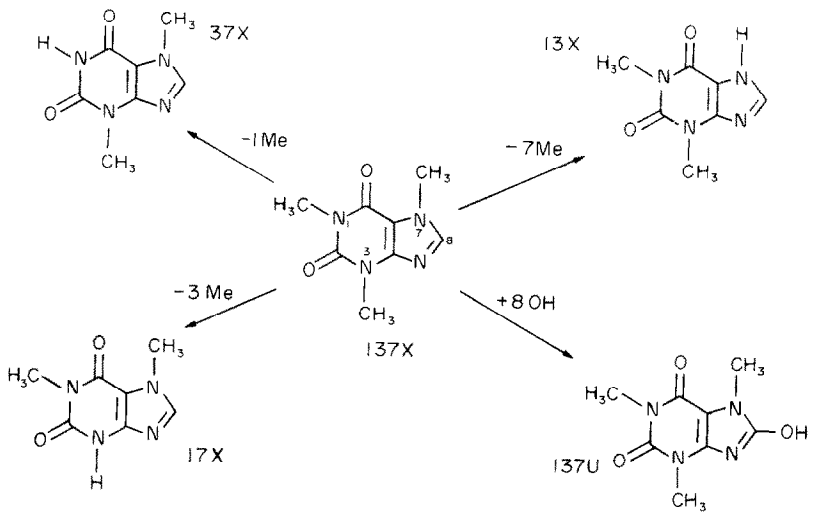

Fig. 1. Primary pathways of caffeine (137X) biotransformation in man.

prototype compounds have been identified which may be used to detect these enzymes in crude tissue preparations. The *O*-deethylation of 7-ethoxyresorufin (7ER), for example, may be used as an index of *in vitro* cytochrome P_1-450 activity, while acetanilide 4-hydroxylation appears to be relatively selectively mediated by cytochrome P_3-450 [16, 20]. The human counterpart(s) of these mouse enzymes could then be expected to mediate both of the above reactions, as well as presumably the *N*-demethylations of caffeine. In this case, each of the three substrates should competitively inhibit the metabolism of the others in incubations of human liver microsomes.

It is also known that certain other chemicals may act in a relatively isozyme-selective fashion to alter the activity of cytochromes P-450. One of these, 7,8-benzoflavone (α-naphthoflavone, ANF), potently inhibits PAH-inducible forms of P-450, while either stimulating or having no effect on constitutive or phenobarbital-inducible isozymes [21]. On the other hand, the imidazole derivative 1-phenylimidazole (PI) has recently been shown to display the opposite effect to that of ANF, namely more potent inhibition of drug metabolism in control or phenobarbital-induced rat liver microsomes than in PAH-induced microsomes [22].

In this report we present the results of studies demonstrating for the first time the kinetics of *in vitro* caffeine metabolite production by microsomes isolated from human livers. We also provide biochemical evidence, from experiments using the abovementioned selective cytochrome P-450 substrates and inhibitors, for the identity of the enzyme(s) taking part in the primary biotransformations of caffeine in man.

MATERIALS AND METHODS

Chemicals. The methylxanthines 137X, 17X, 37X and 13X, as well as ANF, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADP were obtained from Sigma Chemical Co., St. Louis, MO; 137U was from Tridom/Fluka AG, Buchs, Switzerland; 17U was from Adams Chemical Co., Round Lake, IL; 7X was from Pfaltz & Bauer, Flushing, NY; acetanilide and 4-hydroxyacetanilide were from Aldrich Chemical Co., Milwaukee, WI; 7ER and resorufin were from Molecular Probes Inc., Junction City, OR; PI was from Transworld Chemicals Inc., Chevy Chase, MD; chloroform and methanol (HPLC grade) were from Anachemia; and all other chemicals were reagent grade products from BDH or Fisher Chemical Co., Toronto.

Tissues. Healthy liver tissues from human kidney donors designated as brain dead (K series, Table 1) or from a surgical patient undergoing partial hepatectomy for hepatic carcinoma (DK series) were received through Dr W. A. Mahon or through the Department of Surgical Pathology, Toronto General Hospital, respectively. In the latter case, the tissue used for these studies was determined to be histologically normal. Pre-death dietary habits and exposure to environmental chemicals, including alcohol and tobacco, were not known. At death, liver tissue was removed and immersed in ice-cold 1.15% KCl for transportation to the laboratory. Livers were then cut into 1 cm slices, quickly frozen in either liquid N_2 or dry ice, and stored at −70° in sealed plastic containers.

Liver microsomes were prepared by the method of Meier *et al.* [23], and either used immediately or frozen in liquid N_2 and stored at −70°.

Caffeine biotransformation assay. Incubation mixtures (total volume 0.5 ml in 20 ml test tubes) contained 0.1 ml potassium phosphate buffer (200 mM, pH 7.4); 0.1 ml of 1.15% KCl; 0.1 ml of the substrate, 137X, dissolved in phosphate buffer; 0.1 ml of NADPH-generating system (containing 20 mM glucose-6-phosphate, 2 mM NADP, 2 U/ml glucose-6-phosphate dehydrogenase and 10 mM $MgCl_2$) dissolved in phosphate buffer; and 0.1 ml of liver microsomal suspension (at a protein concentration of 5–10 mg/ml in 0.3 M sucrose–0.015 M Tris–HCl, pH 7.6). Reactions were started, after a 5 min preincubation at 37°, with the addition of either substrate or tissue fraction to the remaining components

Table 1. Caffeine metabolite formation by human liver microsomes

Human liver source	Age/sex	Product formation rate (pmoles/min-mg protein) 17X	37X	13X	137U	P-450*
K6	—/M	86	19	14	39	0.42
K10	21/M	58	27	28	89	0.56
K12	23/F	22	8	7	28	0.12
K15	39/M	119	24	16	31	0.71
K18	20/M	269	45	27	48	0.39
DK2	—/M	46	16	9	23	0.34

Washed liver microsomes (1.5–2.0 mg protein per ml incubation volume) were incubated with 1 mM caffeine for 15 min at 37° and processed as described in Methods.
* nmoles/mg microsomal protein.

of the mixture. After incubation in a shaking water bath at 37° for 15 min under an air atmosphere, reactions were stopped by the addition of 10 ml of chloroform/isopropanol (85:15, v/v) and 0.05 ml of internal standard solution (7X and 17U, each at 5 mg/l in H_2O), followed immediately by vigorous mixing for 15 sec using a vortex mixer. A saturating amount (about 0.5 g) of ammonium sulfate was added to each tube, followed by further mixing for 30 sec and centrifugation to separate aqueous and organic phases. The latter was removed, taken to dryness at 40–50° under N_2, and the residue was resuspended in an appropriate volume of HPLC mobile phase solvent.

Metabolites were analysed by injection of an aliquot (50–100 μl) of the incubation extract into an HPLC system (Hewlett-Packard Model 1084B) equipped with a reverse-phase column (Beckman Ultrasphere ODS, 25 cm × 4.6 mm i.d.) which was eluted isocratically at a flow rate of 1.3 ml/min and a column temperature of 40° using a mobile phase of 0.05% acetic acid/methanol (88:12, v/v). Compounds were detected by u.v. absorbance at 273 nm, and quantified by comparison of peak height ratios, relative to internal standard, with those of incubation mixtures spiked with known amounts of 17X, 37X, 13X and 137U and processed as above but without any incubation. Typical chromatograms of microsomal extracts with and without incubation at 37° are shown in Fig. 2. Detection limits for production of caffeine metabolites were 1–2 pmoles/min per mg microsomal protein, and coefficients of variation for the assay procedure ranged from 3 to 6%.

Other assays. The 4-hydroxylation of acetanilide was measured by an HPLC method using identical incubation, extraction and analysis conditions as for the caffeine assay described above. Since the retention times of the substrate (21.0 min) and its product (6.5 min) under the conditions of the analysis gave complete separation from all caffeine-derived products, mutual inhibition studies were performed simultaneously using appropriate concentrations of caffeine and acetanilide as substrate/inhibitor in the same incubation tubes. The detection limit for the assay using K15 liver microsomes was about 10 pmoles/min per mg protein, with coefficients of variation from 4–8% over the concentration range studied.

7ER *O*-deethylase was assayed using a spectrofluorimetric method essentially as described by Burke and Mayer [24].

Incubations for all inhibition studies were initiated

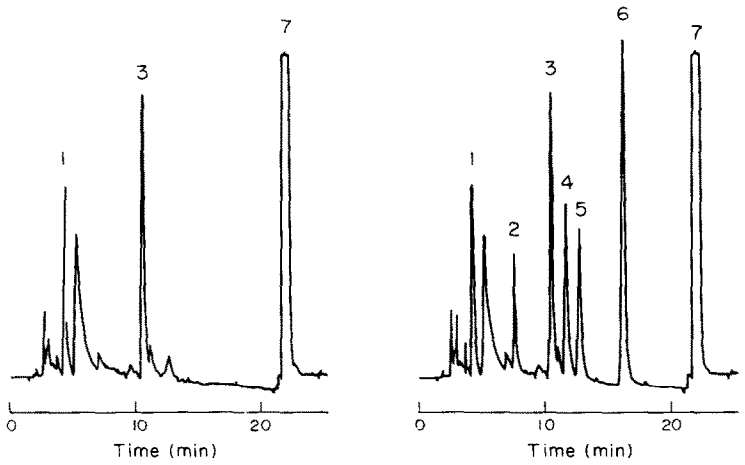

Fig. 2. HPLC chromatograms of extracts from *in vitro* tissue incubation mixtures using 10 mM 137X as substrate for human liver microsomes, before (left) and after (right) incubation for 15 min at 37°. Chromatographic conditions are as described in Methods. Identity of peaks is as follows: 1: 7X (internal standard); 2: 37X; 3: 17U (internal standard); 4: 17X; 5: 13X; 6: 137U; 7: 137X (substrate).

by the addition of tissue source to tubes containing both substrate and inhibitor at appropriate concentrations. Caffeine, acetanilide and PI were dissolved in phosphate buffer for addition to tubes. 7ER was dissolved in ethanol, an appropriate volume was added to tubes, taken to dryness, and the residue resuspended in phosphate buffer. ANF was dissolved in acetone, serially diluted and added to incubation tubes in a fixed volume of 5 μl to give the proper final concentrations.

Microsomal protein was measured by the method of Lowry *et al.* [25] using bovine serum albumin to construct standard curves. Cytochrome P-450 concentrations were determined by the reverse method of Estabrook *et al.* [26], using a Beckman DU-7 single-beam spectrophotometer, and a molar extinction coefficient of $100\,mM^{-1}\,cm^{-1}$ for cytochrome P-450.

RESULTS

All preliminary experiments suggested the involvement of cytochrome P-450 in the *in vitro* production of each of the four detectable primary metabolites of caffeine (17X, 37X, 13X and 137U) by human liver. This evidence included localization of activities in the microsomal subcellular fraction, heat lability (loss of activity after heating for 2 min at 80°), absolute dependence upon the presence of an NADPH-generating system for catalytic activity and inhibition by the prototype P-450 inhibitors carbon monoxide, SKF-525A and metyrapone. Reactions were linear with time to at least 20 min incubation and with microsomal protein concentration to at least 2 mg per ml final incubation volume. Total percent conversion of caffeine to its metabolites never exceeded 1% at a substrate concentration of 1 mM for any of the livers tested.

The relative rates of demethylated metabolite formation from 1 mM caffeine (Table 1) were compatible with those which can be deduced from *in vivo* plasma [27, 28] and urinary [29, 30] patterns of caffeine metabolites, with predominant N-3-demethylation to 17X. The generally higher rate of formation of 137U than predicted by *in vivo* data was due to the high substrate concentration necessary for these *in vitro* studies, in combination with the kinetics of formation of this metabolite (see below). The formation rate of total demethylated products (17X + 37X + 13X) correlated poorly with 137U production over the six livers tested ($r = 0.20$), whereas correlations between each of the three demethylation rates were considerably higher (17X vs 37X: $r = 0.92$, $P < 0.01$; 37X vs 13X: $r = 0.86$, $P < 0.05$; 17X vs 13X: $r = 0.62$, n.s.), suggesting common control over the latter reactions. Rates of formation of caffeine metabolites by human liver microsomes were generally higher (per nmole spectrophotometrically determined P-450) than those by microsomes from NZW rabbit or Wistar rat induced with either phenobarbital or β-naphthoflavone (data not shown).

Figure 3 shows the apparent hyperbolic kinetics of caffeine metabolite formation by microsomes from human liver K15. Production of the major *in vivo* metabolite 17X predominated, especially at the lower (closer to physiological) substrate concentrations (0.05–0.1 mM), and 137U formulation was virtually linear as a function of caffeine concentration in this range, implying a higher K_m for this reaction. When the kinetic data from five of the human livers were expressed in the form of Eadie–Hofstee plots, however (Fig. 4), the biphasic nature of the *N*-demethylation reactions became apparent. Production of each of the dimethylxanthines appeared to be mediated by separate high- and low-affinity catalytic sites, each present in varying proportions

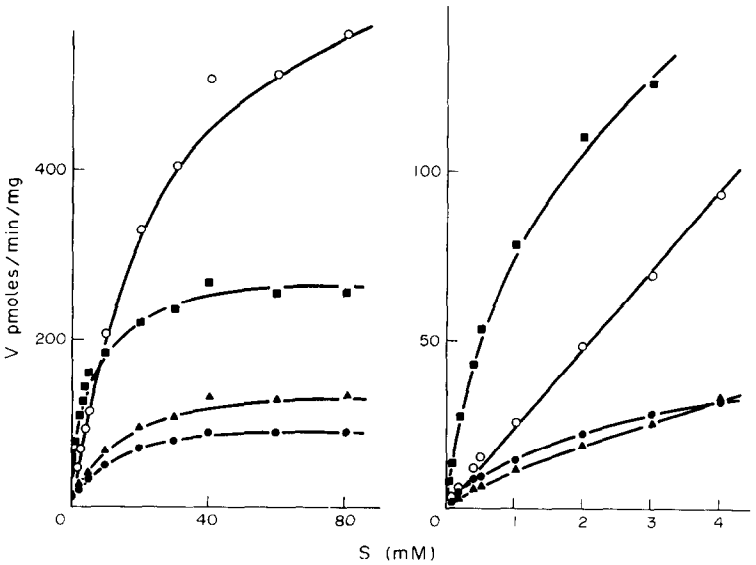

Fig. 3. Initial velocity of formation of the caffeine metabolites 17X (■), 37X (●), 13X (▲), and 137U (○) as a function of substrate concentration by microsomes isolated from human liver K15.

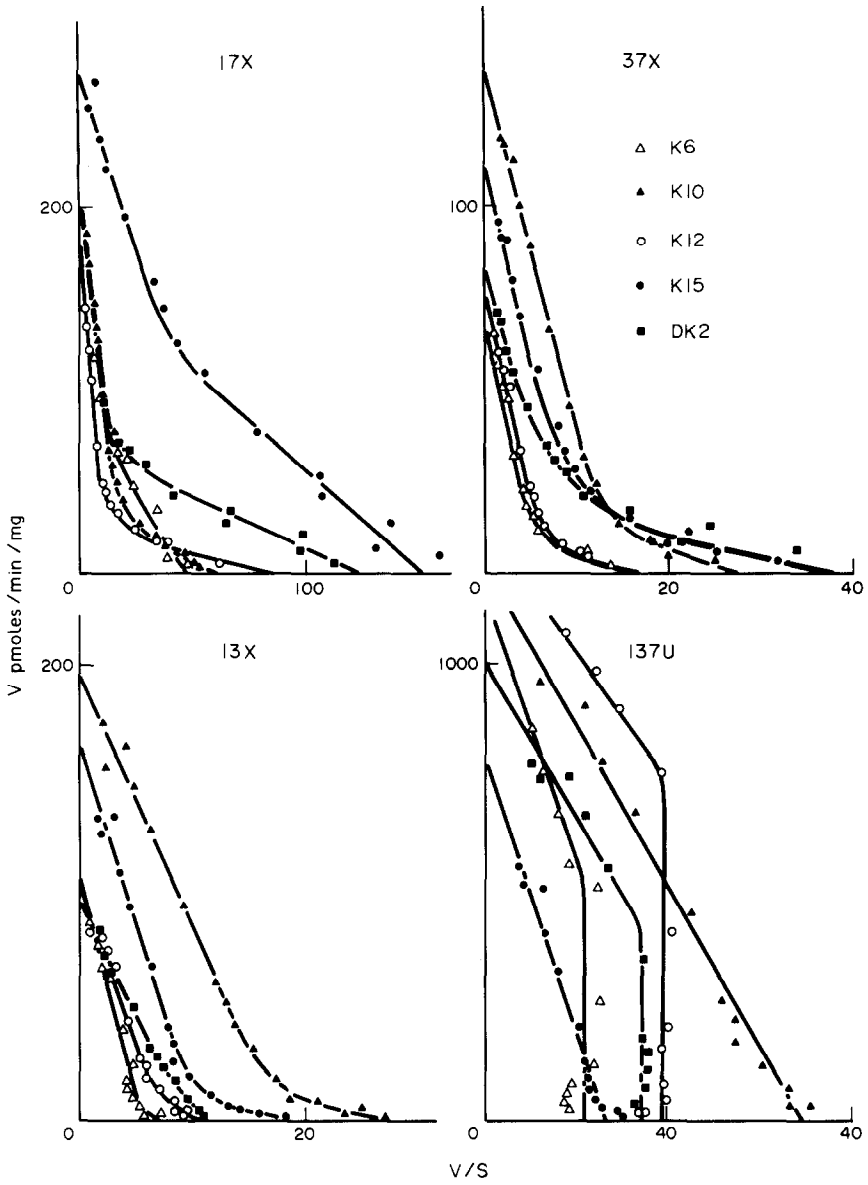

Fig. 4. Eadie–Hofstee plots of the kinetics of caffeine metabolite formation by microsomes from five different human livers.

amongst the different livers. In general, production of 17X displayed the largest proportion of high-affinity component, followed by 37X and then 13X, whose production appeared to be mediated mostly by an enzyme of lower affinity. Production of 137U, on the other hand, was apparently mediated in most livers by a single site of low affinity and high capacity, although for K15 (Fig. 4) and K18 (not shown) a higher affinity component was detected at lower substrate concentrations. From linear regression of the components of each curve, rough estimates of apparent K_m could be calculated to be about 0.5–1.0 mM for each of the high-affinity demethylations, and 15–30 mM for 8-hydroxylation. Of the low-affinity sites for *N*-demethylation, the K_m for *N*-3-demethylation to 17X was approximately 10 mM, while those for production of the other two dimethylxanthine were roughly 15 mM.

Figure 5 illustrates the mutual inhibition of caffeine, 7ER and acetanilide biotransformations in microsomes from human liver K15 (chosen for study on the basis of adequate supply and relatively high caffeine-metabolizing activity). Acetanilide and 7ER inhibited production of all metabolites of caffeine, with a suggestion that the inhibition of 137U formation might be maximal at less than 100% inhibition. Unfortunately, this could not be verified due to the limits of aqueous solubility of both of these compounds. 7ER was at least a 100-fold more potent inhibitor than acetanilide on a molar basis. Acetanilide and caffeine had a similar effect on 7ER *O*-deethylase activity, with caffeine displaying a

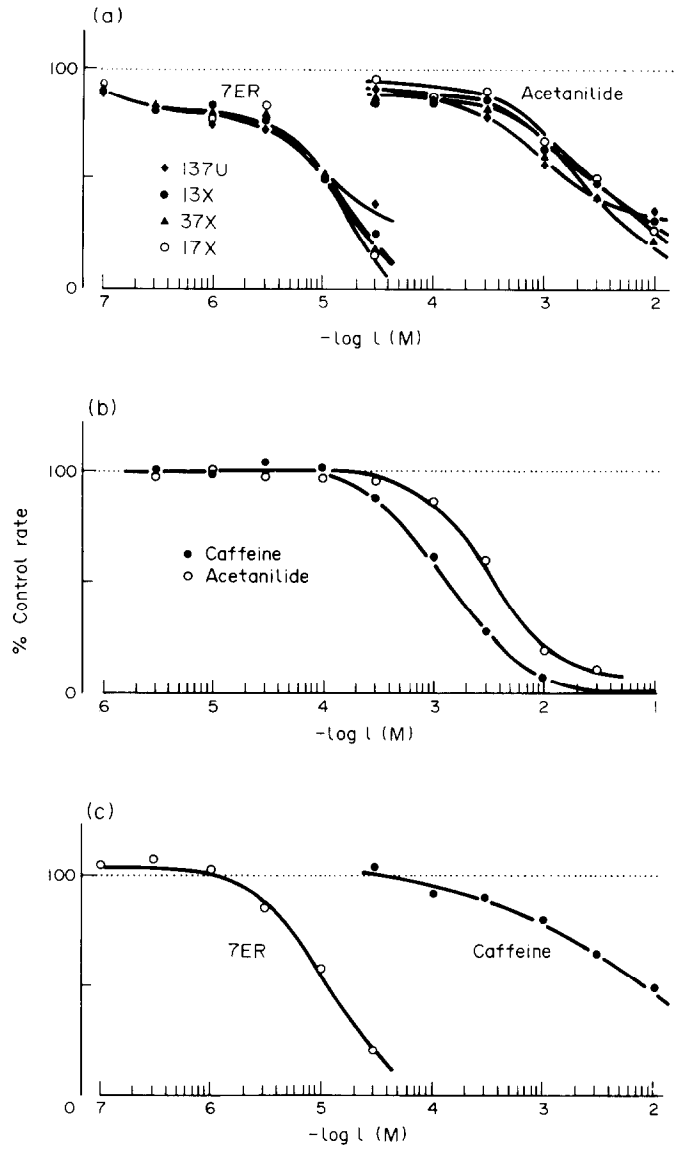

Fig. 5. Inhibition studies of caffeine, 7ER and acetanilide biotransformations by microsomes from human liver K15. (a) inhibition of caffeine metabolite production by 7ER and acetanilide; (b) inhibition of 7ER *O*-deethylase activity by caffeine and acetanilide; (c) inhibition of acetanilide 4-hydroxylase activity by 7ER and caffeine. Results are expressed as a percent of control rates in the absence of inhibiting agents.

slightly (3-fold) greater potency. Both 7ER and caffeine could also inhibit acetanilide 4-hydroxylase activity, again with 7ER showing considerably more potency than caffeine in this respect. The relative order of inhibitory potencies displayed by each of these compounds generally paralleled their apparent affinities as substrates for their biotransforming enzymes in human liver. Inhibition of caffeine metabolite production by 7ER was competitive according to Dixon plots (Fig. 6) and Cornish–Bowden plots (not shown) [31], with apparent K_i values ranging from 2.6 to 6.5 μM. Similar plots for acetanilide inhibition of caffeine metabolite production, and for the respective inhibitions of 7ER and acetanilide biotransformations, also gave evidence for their competitive nature (not shown). Apparent substrate affinities and inhibition constants for each of these three compounds in mutual inhibition studies using K15 liver microsomes are summarized in Table 2.

Figure 7 shows the effect of ANF, a selective inhibitor of PAH-inducible cytochrome P-450 isozymes, on caffeine metabolite formation at substrate concentrations of 1 mM and 5 mM. At low ANF concentrations production of all metabolites was potently inhibited (with IC_{50} values of about 3×10^{-7} M) at both substrate concentrations. At higher inhibitor concentrations, however, the production of 137U was selectively stimulated to well above control production rates. Maximum attainable

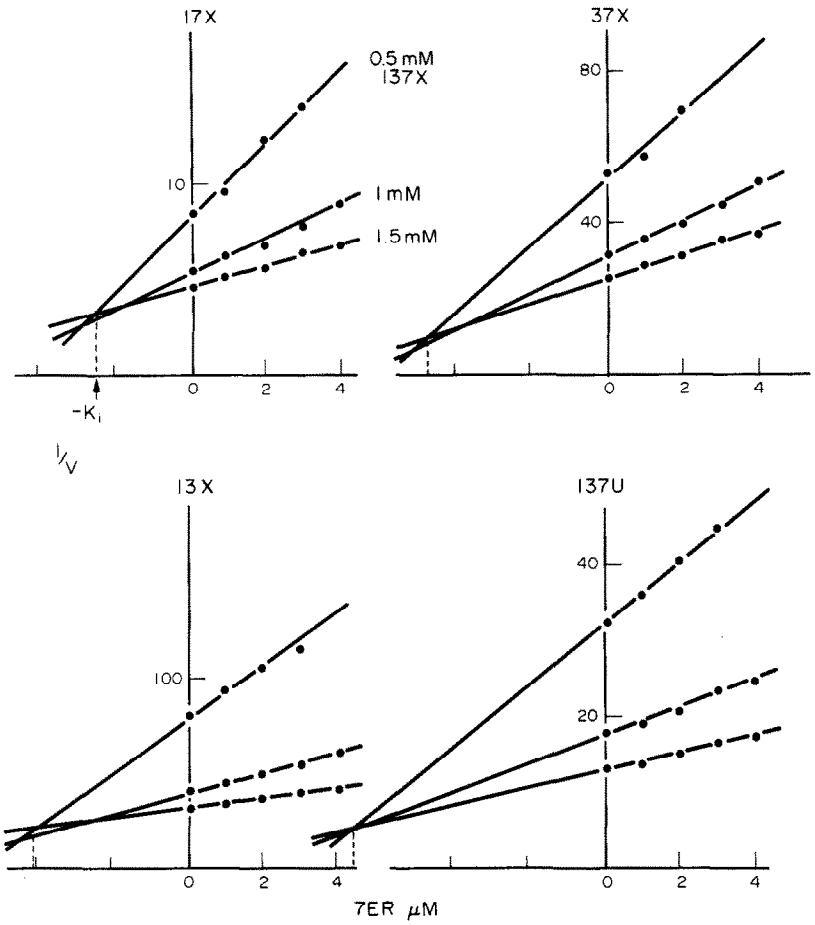

Fig. 6. Dixon plots: inhibition of formation of the caffeine metabolites 17X, 37X, 13X and 137U by 7ER in microsomes from human liver K15.

inhibition differed among the metabolites and between the two substrate concentrations. At 1 mM caffeine, maximal inhibition of 13X, 37X and 137U production was 80%, 95% and 70%, respectively, while corresponding values at 5 mM caffeine were 50%, 90% and 35%. Production of 17X was almost completely inhibited (98%) at both substrate concentrations.

Table 2. Mutual inhibition studies in K15 human liver microsomes

Substrate	K_m (mM)	Inhibitor	K_i (mM)
Caffeine	0.5–1.0	7ER	0.0026–0.0065
		Acetanilide	2.0–2.5
7ER	0.0004	Caffeine	0.75
		Acetanilide	0.75
Acetanilide	1.5	Caffeine	2.5
		7ER	ND

K_m values were determined by Eadie–Hofstee analysis of metabolite formation rates in the absence of inhibitors. K_i values were determined using Dixon plots as in Fig. 6. For caffeine data, substrate concentrations were chosen so that the high affinity components were predominantly responsible for metabolite formation.
ND = not determined.

Figure 8 shows the results obtained using PI as a putative selective inhibitor of constitutive or phenobarbital-inducible P-450 isozymes. PI could completely inhibit production of all metabolites at high concentrations (0.3 mM), whereas at lower concentrations the effects were more complex. Inhibition of 17X production was characterized by a steeply sloped log-dose inhibition curve in which product inhibition became complete over a 100-fold concentration range. Inhibition of 37X, 13X and 137U formation, however, displayed increasingly shallow curves to the point that inhibition of the latter compound's formation proceeded over almost four log cycles, or a 10,000-fold inhibitor concentration range.

DISCUSSION

The substrate concentrations required in these *in vitro* studies (0.5–5.0 mM) to produce accurately detectable levels of metabolic products are considerably higher than those which would be observed *in vivo* (<0.05 mM) with normal caffeine intake in man. This requirement is a consequence of the markedly low *in vitro* turnover rate of the enzyme(s) for production of caffeine metabolites, which can be

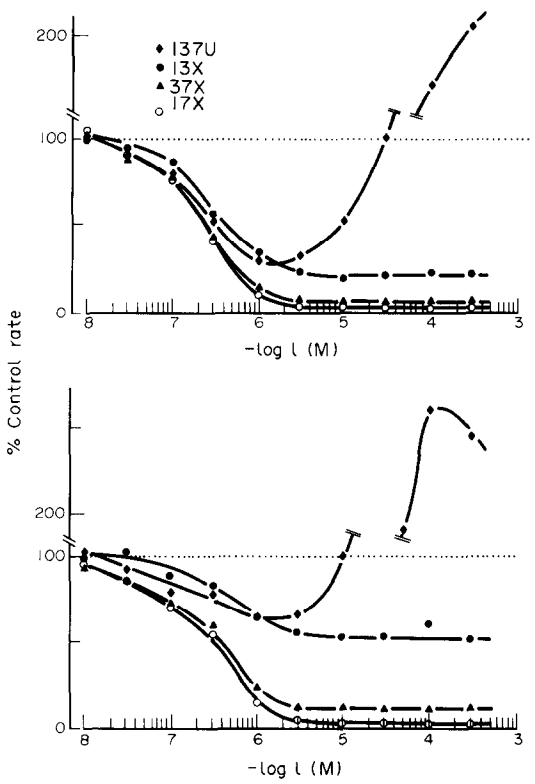

Fig. 7. Effect of ANF on production of caffeine metabolites by microsomes from human liver K15. Top, 1 mM substrate concentration; bottom, 5 mM substrate concentration. Control rate is that in the presence of acetone vehicle alone.

estimated from our data to be no more than about 6% of the rate expected from extrapolation of the *in vivo* elimination half-life of caffeine in an average adult human. This abnormally low rate of microsomal caffeine biotransformation has also been observed in other species [32–34], and is as yet unexplained.

It is therefore necessary to assume that the results of our experiments may be extrapolated to the lower concentrations that would be found in man. In light of the biphasic kinetics which we observed, and which have also been seen in the rat [31], this must be approached with caution. Nonetheless, the metabolites we observed using caffeine as an *in vitro* substrate for human liver microsomes correspond

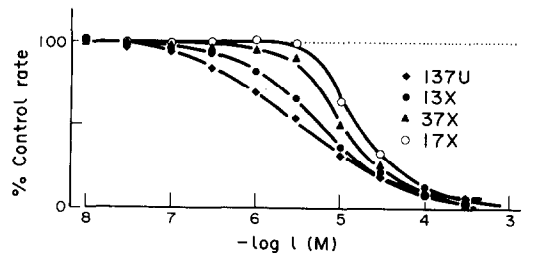

Fig. 8. Effect of PI on production of caffeine metabolites by microsomes from human liver K15. Substrate concentration was 1 mM.

well to those detected in the body fluids of man after caffeine intake. In particular, the *in vivo* selectivity for caffeine N-3-demethylation to paraxanthine-derived products [29, 30] is completely retained *in vitro* in isolated microsomes.

Part of the between-liver variability in caffeine metabolite production (Table 1) may be due to differences in general enzymatic activity of the tested livers as a result of variations in storage times, handling methods and efficiency of organ removal at the time of death. In addition, variation was also observed in the catalytic activity of samples taken from different regions of the same human liver, accompanied by differences in spectrally determined P-450 levels. Another part of the variability should, however, be due to true intersubject differences in catalytic activities before death, possibly reflecting differences in genetic constitution and environmental exposures (and their interaction). Both of these factors will contribute to the observed range of cytochrome P-450 contents in isolated microsomes. We saw no correlation between overall P-450 content and production of any of the individual caffeine metabolites, implying that a specific isozyme or isozymes of P-450, contributing varying but relatively minor amounts to the photometrically determined cytochrome, are responsible for the caffeine *N*-demethylations and 8-hydroxylation.

The lack of correlation between *N*-demethylation and 8-hydroxylation pathways suggests that different forms of cytochrome P-450 mediate these two reaction types. On the other hand, the much higher correlations between each of the demethylation pathways is compatible with a common isozyme or closely related isozymes contributing to all of the demethylation activities at the substrate concentration (1 mM) used here.

The kinetic studies indicate that at least two different microsomal enzymes with differing substrate affinities mediate production of the dimethylxanthines over the substrate concentration range we used. Obviously only the high-affinity site will be of any physiological relevance, and the *in vivo* results from other investigators [2, 3, 10] suggest that it is most likely a PAH-inducible isozyme of cytochrome P-450. Environmental exposures of the liver donors prior to death are unknown, but it would be tempting to speculate that a part of the variation observed in Fig. 4 may be due to factors such as differential smoking habits or exposures to other drugs and chemicals. It is also interesting to note in this regard that Bonati *et al.* [33] also detected biphasic kinetics of caffeine metabolite formation by rat liver microsomes, and demonstrated that 3-methylcholanthrene pretreatment of animals preferentially induced the high-affinity component of enzyme activity.

The results of the caffeine-acetanilide-7ER mutual inhibition studies provide compelling evidence that a common form or forms of cytochrome P-450 take part in the biotransformation of each of these substrates in man. Apart from the apparent competitive nature of the inhibition, the order of inhibitory potencies correlated relatively well with the apparent K_m values for the compounds as substrates in human liver microsomes. The observed discrepancies between apparent K_m values and K_i values as inhibi-

tors (e.g. for 7ER) could be explained by the likelihood of variable and overlapping substrate specificity for additional isozymes of cytochrome P-450.

The results of the inhibition studies using ANF and PI also deserve comment. ANF has been shown to selectively inhibit PAH-inducible enzyme activities and to have no effect on or to stimulate constitutive or phenobarbital-inducible enzymes [21]. Our result (Fig. 7) may be explained in the context of the biphasic kinetics for caffeine *N*-demethylations (Fig. 4) by assuming that ANF inhibits the high-affinity component of metabolite production but not the low-affinity component. At 5 mM caffeine, the latter contributes more to the production of 13X (and to a lesser degree 37X) than at 1 mM, so that the maximum inhibition is less at the higher substrate concentration. The production of 17X, on the other hand, is mediated almost exclusively by the high-affinity site at both 1 mM and 5 mM caffeine, so that ANF inhibition is essentially complete in both cases. By extrapolation to the caffeine concentrations that would be encountered *in vivo*, it can then be predicted that high-affinity, ANF-sensitive, and therefore presumably PAH-inducible enzyme(s) are by far predominating in each of the *N*-demethylations.

The result for 137U production is also compatible with the potential participation of at least two sites in 8-hydroxylation in microsomes from liver K15: one which is ANF-inhibited and one which is ANF-stimulated (and thus presumably a constitutive rather than a PAH-inducible form of P-450). Although it has not been tested, livers apparently lacking the high-affinity component of 8-hydroxylation activity (K6, K10, K12, DK2) may not show the inhibitory phase of the ANF effect on 137U formation observed with K15 (Fig. 7) and also with K18 (not shown). We also do not know which of these sites for 8-hydroxylation is important in the *in vivo* production of 137U, but this pathway is in any case of minor quantitative importance for man.

Further evidence for these concepts may be gathered from the results of the PI inhibition studies. PI inhibited 137U formation over a wide range of inhibitor concentrations, suggestive of a potent effect on one enzyme site and a weaker effect on another site. Inhibition of 17X production, on the other hand, was consistent with relatively weaker PI interaction with a single enzyme site. In the light of previous findings [22] that PI more potently inhibited constitutive or phenobarbital-inducible P-450 isozymes than PAH-inducible forms, this result is therefore consistent with the implications from the ANF studies. At 1 mM caffeine, 137U formation appears to be mediated by two sites, one a PAH-inducible site (ANF-inhibited and PI-insensitive), and the other a constitutive form (ANF-stimulated and PI-sensitive). Production of the demethylated metabolites, on the other hand, appears to proceed increasingly (13X < 37X < 17X) via the action of a PAH-inducible (ANF-inhibited and PI-insensitive) isozyme of P-450 at this particular substrate concentration. This interpretation would also explain why the PAH-inducible P-450 substrates 7ER and acetanilide were able to inhibit 137U production to a significant extent in microsomes from K15 liver.

Taken together, our results provide biochemical evidence to support *in vivo* implications that PAH-inducible isozyme(s) of cytochrome P-450 play an important part in the N-demethylations of caffeine at biologically meaningful concentrations. These isozymes may then be related to one recently characterized on the molecular level and designated as human cytochrome P_1-450 [18]. It follows that some *in vivo* measure of any of the primary caffeine demethylations should indeed give a potentially valuable index of PAH-inducible cytochrome P_1-450 activity and/or inducibility. Due to the complicated overlapping pathways of primary and secondary caffeine *N*-demethylations [4], however, a specific measure of a single *N*-demethylation reaction may be difficult to obtain in a practical sense. On the other hand, if any or all of the *N*-demethylations of the dimethylxanthines themselves were also shown to be mediated by the same P-450 isozyme, then use of one of these compounds as an *in vivo* probe would circumvent the problem of ambiguous reaction pathways. Further *in vitro* studies are currently being conducted to test this premise.

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