Variability in caffeine metabolism

Urinary metabolites excreted after oral caffeine were quantified in a healthy sample (n = 68) from the Toronto population by HPLC analyses. The profile of metabolites, assessed by examining particular metabolite ratios, was found to differ widely among subjects. Ratios denoting cytochrome P-450–dependent activities were shown to be interethnically variable between Oriental and Caucasian groups, whereas those indicative of xanthine oxidase activity exhibited neither significant interindividual variation nor an ethnic difference. It was also shown that a ratio providing an index of polymorphic N-*acetyltransferase activity holds promise as a simple marker for acetylator status in man.*

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Clinically relevant variability in the response to drugs is common, often manifesting itself either as an unexpected lack of therapeutic response or as toxic side effects after drugs given in the generally accepted dose range. Such interindividual differences in drug response, whether mediated by genetic or environmental influences or by combinations thereof, may be due to variability in drug metabolizing capacity.[24,33,38] For a number of drugs, there are well-defined genetic polymorphisms in biotransformation, such as those regulating isoniazid acetylation and paraoxon hydrolysis.[25,35] More recently, such monogenic control of drug metabolism has also been demonstrated for selected cytochrome P-450–dependent enzyme activities,[17,18,23] implying that deficiencies in particular forms of P-450 may occur and can be detected.

Although caffeine (1,3,7-trimethylxanthine; 137X) is reputedly the world's most widely used drug, detailed studies of its biotransformation pathways in man have been scarce until recently. It is now established, however, that in various species, including man, caffeine undergoes oxidative demethylation and hydroxylation reactions to yield a number of urinary excretion products[1,3,5,9,12,15,16,30] (Fig. 1). Mixed-function monooxygenases containing cytochromes P-450, and in particular the polycyclic hydrocarbon–inducible P-448 (P_1-450), are involved in the metabolism of caffeine and other methylxanthines, as indicated in a number of animal and human studies.[2,4,31,34,40–42] It has also been shown, at least in the case of theophylline (13X), that the soluble enzyme xanthine oxidase is responsible for the conversion of 1X to 1U[21,31]; this reaction likely proceeds analogously for caffeine as well. It has recently also been noted that some of the xanthines may undergo imidazole ring cleavage to produce

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Abbreviations used

137X:	Caffeine (1,3,7-trimethylxanthine)
137U:	1,3,7-Trimethyluric acid
13X:	Theophylline (1,3-dimethylxanthine)
37X:	Theobromine (3,7-dimethylxanthine)
17X:	Paraxanthine (1,7-dimethylxanthine)
13U:	1,3-Dimethyluric acid
17U:	1,7-Dimethyluric acid
37U:	3,7-Dimethyluric acid
1X:	1-Methylxanthine
3X:	3-Methylxanthine
7X:	7-Methylxanthine
1U:	1-Methyluric acid
3U:	3-Methyluric acid
7U:	7-Methyluric acid
AFMU:	5-Acetylamino-6-formylamino-3-methyluracil

substituted uracilic metabolites in significant quantities.[5,6,8,10,15,30] The mechanism of this reaction is presently unknown.

In light of the ubiquitous use of caffeine, its relative safety, and its degradation by two or more enzymes (which one could hope to monitor), we chose to investigate caffeine as a probe for the assessment of variability in biotransformation capacity in human populations. Kinetically, caffeine displays many desirable features of a suitable probe drug[39]: rapid and complete gastrointestinal absorption, distribution throughout the total body water, low plasma protein binding, virtually complete biotransformation in the liver, negligible first-pass effect, and minimal renal elimination.[14]

Recent work has already indicated caffeine's potential as a test drug for liver function assessment in a breath test.[7,29,41] The exhalation of $^{14}CO_2$ or $^{13}CO_2$ after administration of suitably labeled caffeine has been monitored in rats and in man in basal and induced states, as well as under conditions of impaired liver function. The results indicate that the use of specific methyl labels may furnish an index of cytochrome P-448 activity, providing a complementary measure to the aminopyrine breath test,[22,36] which appears to monitor phenobarbital-inducible P-450 activity.

Our study of caffeine was undertaken with an approach differing from those mentioned earlier. Our aim was to investigate the utility of measurements of caffeine metabolites in urine as indicators of several hepatic enzyme functions in man and to determine the degree of variability in metabolite production in healthy subjects. Quantification of particular metabolites in comparative studies promises to illuminate discrete enzyme reactions, whereas measures of t½ or clearance of unchanged drug are generally composites of a number of reactions taking place concurrently or sequentially. Such an approach required the development of rapid, yet sensitive and precise, analytical methods for the quantification of urinary caffeine metabolites in large numbers of samples. The HPLC methods we have developed for this purpose will be presented here.

Our study included an interethnic comparison between Canadians of either Caucasian or Oriental extraction. This was stimulated by previous observations in this laboratory of interethnic differences in metabolizing capacities for amobarbital, debrisoquine, and paraoxon.[25–28]

In the course of this work, a new uracilic metabolite of caffeine was isolated and identified as 5-acetylamino-6-formylamino-3-methyluracil (AFMU).[37] It was subsequently demonstrated that the urinary excretion of this compound was both bimodally distributed and interethnically variable and that the polymorphic liver *N*-acetyltransferase enzyme was involved in its formation in man.[20] Our original analysis of population variability in caffeine metabolite excretion[19] has therefore been expanded to include AFMU and to take into account the acetylator phenotype of all subjects, as determined by their level of AFMU production.

Materials and methods

Our subjects in this investigation included university students, staff members, and others from a variety of occupational and age groups in the Toronto area. The sample comprised 68 unrelated, healthy, nonsmoking subjects, of whom 42 were Caucasians and 26 were of Oriental (mostly Chinese) descent. There were 35 men and 33 women, and the overall mean

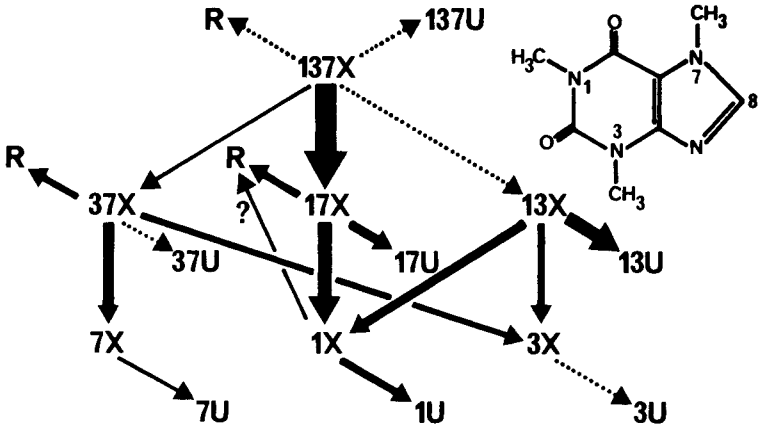

Fig. 1. Pathways of caffeine *(137X)* metabolism in man. Size of *arrows* indicates relative importance of pathways. *X* = xanthine; *U* = uric acid (8-OH); *R* = uracil (ring-opened xanthine); *1, 3, and 7* = positions of methyl groups.

Table I. *Caffeine metabolite recoveries*

No.	Compound	Retention time (min)	Recovery: (n = 10*)			
			Relative recovery (%)		Absolute recovery (%)	
			Mean ± SD	CV(%)	Mean ± SD	CV(%)
1	3U	4.0	58.5 ± 4.0	6.9	54.8 ± 3.0	5.5
2	7U	5.5	64.2 ± 8.5	13.2	60.0 ± 6.8	11.4
3	7X	6.1	97.3 ± 1.7	1.7	91.2 ± 2.7	3.0
4	1U	6.8	80.6 ± 4.9	6.0	75.5 ± 3.3	4.4
5	3X	7.3	98.8 ± 1.8	1.8	92.6 ± 2.6	2.8
6	37U	7.7	97.8 ± 2.1	2.1	91.7 ± 2.6	2.8
7	1X	9.1	100.2 ± 2.2	2.2	93.9 ± 2.9	3.1
8	I.S.	10.8	—	—	93.8 ± 2.3	2.5
9	13U	11.7	100.5 ± 1.8	1.8	94.2 ± 2.6	2.8
10	37X	12.4	101.3 ± 2.2	2.2	94.9 ± 3.2	3.4
11	17U	19.2	100.4 ± 1.4	1.4	94.2 ± 2.5	2.7
12	17X	20.8	100.8 ± 1.8	1.8	94.6 ± 2.4	2.5
13	13X	22.8	101.0 ± 1.2	1.2	94.7 ± 2.0	2.1
14	137U	31.0	100.9 ± 1.8	1.8	94.6 ± 2.5	2.6
P1†	AFMU	3.6	84.5 ± 3.6	4.3	83.3 ± 3.7	4.4

CV = coefficient of variation; I.S. = *N*-acetyl-*p*-aminophenol internal standard.

*Separate single determinations on different days over a period of 2 mo, at a concentration of 20 mg/*l* for a mixture of 13 metabolites in H_2O.

†AFMU analysis as outlined in methods section; n = 5 at 25mg/*l*.

age was 26.6 yr, with a range from 16 to 65 yr. Rough estimates of normal daily caffeine consumption were recorded for all subjects and were converted to arbitrary units (cups of coffee) based on relative strengths and caffeine content in beverages.

Subjects were required to refrain from all methylxanthine-containing foods and beverages for at least 48 hr before, and for the duration of, the study. Each subject collected a methylxanthine-free 24-hr pooled urine sample to serve as an analytical blank in the procedure to follow. At the end of this blank collection, subjects ingested 300 mg caffeine (Sigma Chemical) dissolved in a small volume of distilled water, and urine was collected for an additional 24 hr. Urine volumes were measured, and aliquots were stored at −20° for later analysis. A similar

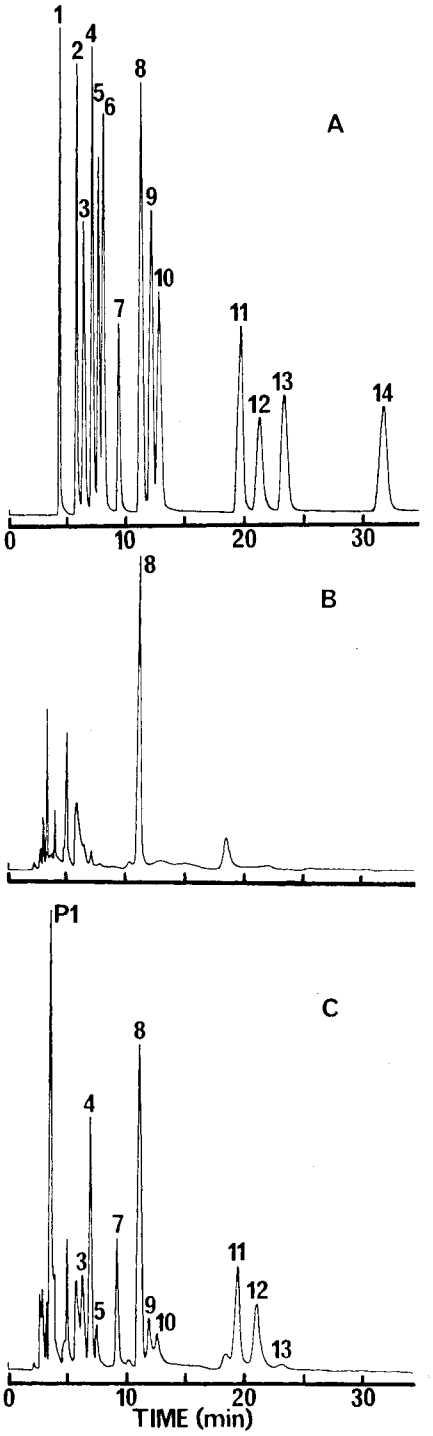

Fig. 2. HPLC chromatograms. *A*, Standard mixture of 13 caffeine metabolites plus internal standard. *B*, Extract of 24-hr blank urine from representative subject. *C*, Extract of 24-hr urine from same subject after ingestion of 300 mg caffeine. For identity of peaks, refer to Table I.

procedure was used for ingestion studies of caffeine metabolites in a single rapid-acetylator subject, except that urine collections were taken for particular intervals during the 24-hr period.

Analytical procedures. Two similar HPLC methods were developed for the analysis of the demethylated xanthines and urates and for AFMU, respectively. These methods were based on those of Aldridge et al.[1] and Muir et al.,[32] with modifications made not only to improve the ease and speed of analysis for large numbers of samples but also to maximize the recovery and resolution of the various metabolites.

A 0.2-ml volume of the urine to be analyzed was added to a 15-ml centrifuge tube, followed by a saturating amount (~120 mg) of ammonium sulfate, and the tube was vortexed briefly. For methylxanthine and urate analysis, 6 ml chloroform/isopropanol (85:15, v/v) and 0.2 ml *N*-acetyl-*p*-aminophenol internal standard solution (120 mg/*l* in chloroform) were added, and the tube was vortexed vigorously for 30 sec. AFMU was extracted in a similar manner, except that 2 × 6 ml of 100% chloroform was used as the extracting solvent. After centrifugation for 5 min at 2500 rpm, the organic phase was removed, taken to dryness at 40° to 45° under N_2, and resuspended in HPLC mobile-phase solvent; 20 μl was then injected immediately into the liquid chromatograph.

A Model M-45 Solvent Delivery System equipped with a Model 440 fixed-wavelength UV absorbance detector (Waters Scientific) was used to quantify the xanthine and uric acid metabolites, and a Model 1084B liquid chromatograph (Hewlett-Packard) was used in the AFMU analysis. In both analyses, Ultrasphere ODS, 5-μm particle size, 25 cm × 4.6 mm ID reversed-phase columns (Beckman), were eluted isocratically with 0.05% acetic acid/methanol (88:12, v/v, for xanthines and urates; 85:15, v/v, for AFMU) at a flow rate of 1.2 ml/min and monitored by UV absorbance at 280 nm.

Samples of blank urine spiked with a mixture of 13 demethylated xanthine and uric acid metabolites at concentrations up to 80 mg/*l*, or with AFMU to 50 mg/*l*, were taken through procedures identical to those described earlier, and standard curves relating peak heights of standards to that of the internal standard were

Table II. *Precision and accuracy of the assay*

	Concentration added (mg/l)							
	10		*20*		*40*		*80*	
Compound	C_{obs}*	*CV(%)*	C_{obs}	*CV(%)*	C_{obs}	*CV(%)*	C_{obs}	*CV(%)*
3U	9.6	8.9	20.0	3.3	40.6	0.5	79.7	3.2
7U	8.9	8.5	19.6	4.0	42.6	5.8	78.9	5.6
7X	10.1	3.5	20.0	1.3	39.9	2.7	78.9	5.6
1U	9.6	6.3	19.8	2.1	40.4	2.0	79.9	2.0
3X	9.7	1.6	20.0	0.4	40.5	1.1	79.8	0.2
37U	10.0	0.7	19.9	0.2	40.2	0.5	79.9	0.5
1X	9.9	0.5	20.1	0.1	39.9	1.4	80.0	0.6
13U	9.9	1.4	20.0	0.8	40.1	0.2	80.0	0.5
37X	10.1	1.4	19.9	0.9	39.9	1.3	80.1	0.5
17U	10.3	2.1	19.8	1.5	39.9	1.6	80.1	1.0
17X	10.7	2.6	19.9	0.9	39.1	1.7	80.4	1.2
13X	10.4	2.9	19.9	2.4	39.5	1.2	80.3	0.9
137U	ND	—	ND	—	ND	—	ND	—

C_{obs} = mean observed concentration; CV = coefficient of variation; ND = not determined.
*n = 3.

used for quantification. Corrections were made for any small interfering peaks in subjects' 24-hr blank urine collections. Total amounts of each metabolite excreted in 24-hr urine samples were expressed either as a molar percentage of the ingested dose or as a percentage of the five major recovered metabolites (1U, 1X, 17U, 17X, and AFMU).

Sources of metabolites and reagents. The compounds 137X, 13X, 17X, 37X, 3X, and *N*-acetyl-*p*-aminophenol were from Sigma Chemical; 13U, 17U, 37U, and 1U were from Adams Chemical; 137U, 3U, and 7U were from Tridom/Fluka AG; and 1X and 7X were from Pfaltz and Bauer. AFMU was isolated and purified to approximately 96% purity (by HPLC analysis).[37] Chloroform and methanol (Omnisolv) were from BDH Chemicals, and all other chemicals were reagent grade from Fisher Chemicals.

Data analysis. The data were checked for normality of distribution with the use of probit plots and tests for skewness and kurtosis. Statistical analyses were then based on the assumption that the data (or logarithmic transformations thereof) were normally distributed. Means, standard deviations, standard errors, and coefficients of variation were calculated for levels of all quantifiable metabolites and for various ratios of the five major metabolites (1U, 1X, 17U, 17X, and AFMU) in arithmetic or logarithmic forms.

Table III. *Molar percentage recoveries of caffeine metabolites in 24-hr urine (n = 68, except where indicated)*

Compound	*Mean ± SD*	*CV(%)*	*Range*
AFMU	4.3 ± 3.4	70.8	0.1-11.0
3U	ND	—	—
7U	ND	—	—
7X(n = 59)	2.5 ± 1.4	56.0	ND-8.2
1U	11.8 ± 5.0	42.4	1.5-27.0
3X(n = 61)	1.5 ± 0.7	46.7	ND-3.6
37U	ND	—	—
1X	10.1 ± 4.1	40.6	1.2-19.8
13U	1.2 ± 0.4	33.3	0.4-2.3
37X(n = 63)	1.1 ± 0.6	54.5	ND-3.5
17U	6.0 ± 1.9	31.7	1.3-10.0
17X	4.8 ± 2.4	50.0	1.2-11.3
13X	<1.0	—	—
137U	<1.0	—	—
Total	42.8 ± 11.9	27.8	10.4-71.1

CV = coefficient of variation; ND = not detectable.

The equality of means of these variables between sexes, ethnic groups, and acetylator phenotypes was evaluated using unpaired Student's t tests (two-tailed), with significance of the F statistic as the criterion for the choice of separate- or pooled-variance tests. Parametric and nonparametric correlation coefficients were cal-

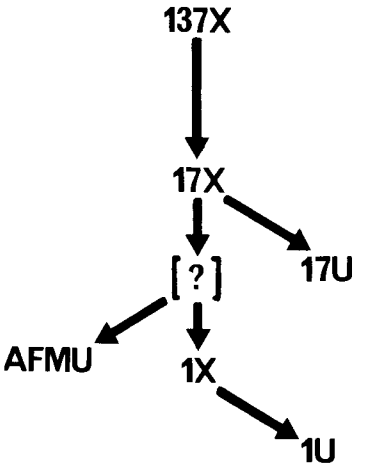

Fig. 3. Proposed pathways of formation of major metabolites of caffeine in man, including AFMU.

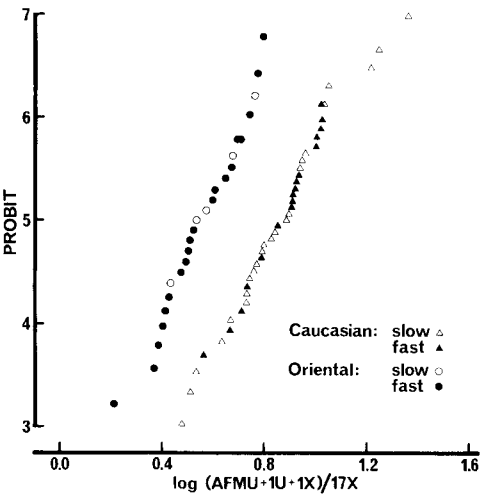

Fig. 4. Probit transformation of data relating to metabolism of caffeine in Caucasian and Oriental subjects. Abscissa denotes logarithm of ratio representing 7-demethylation activity as defined in text; ordinate is cumulative frequency distribution in probit units.

culated for a number of variable pairs to determine relationships between age, caffeine dose, normal daily caffeine consumption, urine flow, and any of the measured metabolite levels or ratios. In addition, two- and three-way analyses of variance were used, with race, sex, and acetylator phenotype as the independent, nonmetric variables. Age, caffeine dose, 24-hr urine volume, daily caffeine consumption, and total recovery of the ingested dose were introduced as metric covariates, either singly or together, to control for possible effects of these nonmetabolic factors on the dependent variables.

Results

Fig. 2 illustrates the separation that was achieved with the outlined procedure for the xanthine and uric acid metabolites, not only for the aqueous standard (Fig. 2, *A*), but also for urine extracts that were essentially free of major interferences for all metabolites of quantitative importance (Fig. 2, *B* and *C*). Standard curves plotting peak height ratios vs urinary metabolite concentration were linear to 80 mg/*l*, with correlation coefficients greater than 0.9999 for all compounds except 7U ($r = 0.998$) and 17X ($r = 0.9997$) and with the y intercepts not significantly different from zero. Relative and absolute recoveries from aqueous solution are shown in Table I. Table II documents the precision and accuracy of the assay for the analysis of spiked urine samples. Detection limits were between 0.3 and 0.5 mg/*l* for all metabolites in urine.

The finding that AFMU (peak Pl in Fig. 2, *C*) was well extracted in pure chloroform[37] led to the development of a chloroform extraction that eliminated essentially all of the endogenous interfering peaks in this polar region of the chromatograms.[37] The standard curve relating the peak height of AFMU to internal standard in spiked urine was linear to 50 mg/*l* ($r = 0.997$). Relative recovery of AFMU in this assay ranged from 80.6 ± 1.3 to 84.8 ± 2.1% at concentrations between 5 and 50 mg/*l* in aqueous solution.

Peaks corresponding to 1U, 1X, 17U, and 17X made up the major proportion of recovered xanthine and uric acid metabolites (Fig. 2, *C*), with lesser amounts of 7X, 3X, 13U, 37X, and 13X present. The remaining metabolites (3U, 7U, 37U, and 137U) were generally beneath the detection limits of the assay for all subjects. These results are qualitatively in agreement with previously published results,[1,9,12,15,30] indicating that an initial 3-demethylation of caffeine to paraxanthine is the predominant pathway in man.

Percentage recovery of metabolites in 24 hr after 300 mg caffeine is listed in Table III for

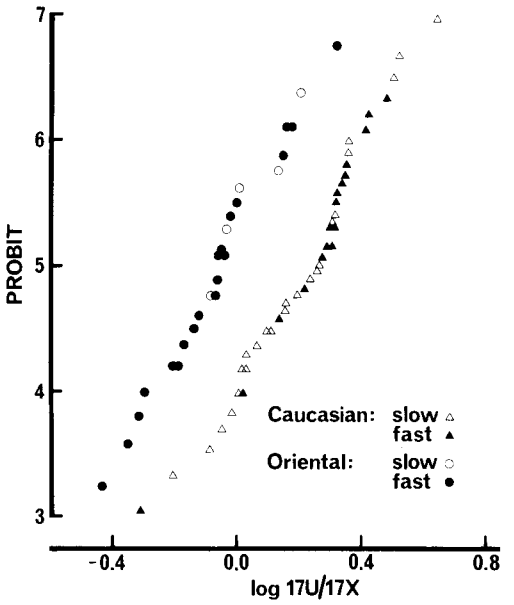

Fig. 5. As in Fig. 4, abscissa denotes logarithm of ratio representing 8-hydroxylation activity.

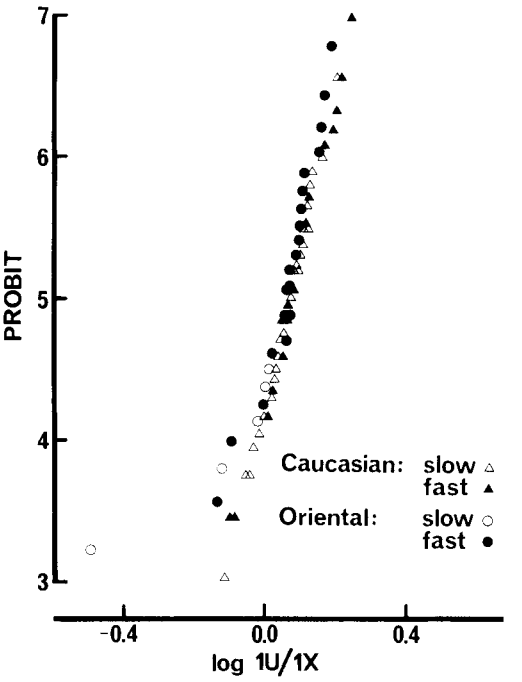

Fig. 6. As in Fig. 4, abscissa denotes logarithm of ratio representing xanthine oxidase activity.

the total sample. The levels are somewhat lower than those reported previously[12,15]; the reason for this discrepancy may be partly explained by the shorter urine collection time we used. Of particular note, however, are the degrees of variability in metabolite recoveries as evidenced by the large standard deviations, coefficients of variation, and ranges.

The peak corresponding to AFMU was present in widely variable amounts among subjects (Table III). As stated earlier, it has recently been shown that the production of AFMU is related to the polymorphic liver enzyme *N*-acetyltransferase.[20] In that study the molar fraction of the five major recovered metabolites present as AFMU (that is, the ratio of AFMU to 1U + 1X + 17U + 17X + AFMU) allowed the discrimination between "high" and "low" producers of AFMU as two distinct groups, and complete concordance was subsequently found with the polymorphic acetylation of sulfamethazine, a widely used probe of acetylator status. Therefore the ratio defined above was used by us to classify all subjects as either fast or slow acetylators.

To elucidate the pathways of AFMU formation, we performed metabolite ingestion studies with a single fast-acetylator subject. The results clearly indicated that AFMU was formed after caffeine or 17X but not after 37X, 13X, 1X, or 17U, although the latter result may be complicated by poor oral bioavailability of 17U. Since the structure of AFMU suggests that demethylation must take place from 17X, it is proposed that AFMU is formed from an unstable intermediate (possibly ring-opened) arising after 7-demethylation, which in fast acetylators is quickly acetylated and thereby stabilized in an open-ring structure (Fig. 3). In slow acetylators, on the other hand, the intermediate tends to reclose to form 1X. Support for this proposal comes from the finding that, in our sample, slow acetylators excreted more 1X + 1U (27.4 ± 7.6% of dose) than did fast acetylators (17.7 ± 7%, $P < 0.001$, Student's t test) but that the overall excretion of AFMU + 1X + 1U did not differ between the groups.

In light of the above, we proceeded to define ratios of the major metabolites (Fig. 3) as indices of particular enzyme activities. This approach was attempted only for the pathways that followed the initial 3-demethylation of caffeine

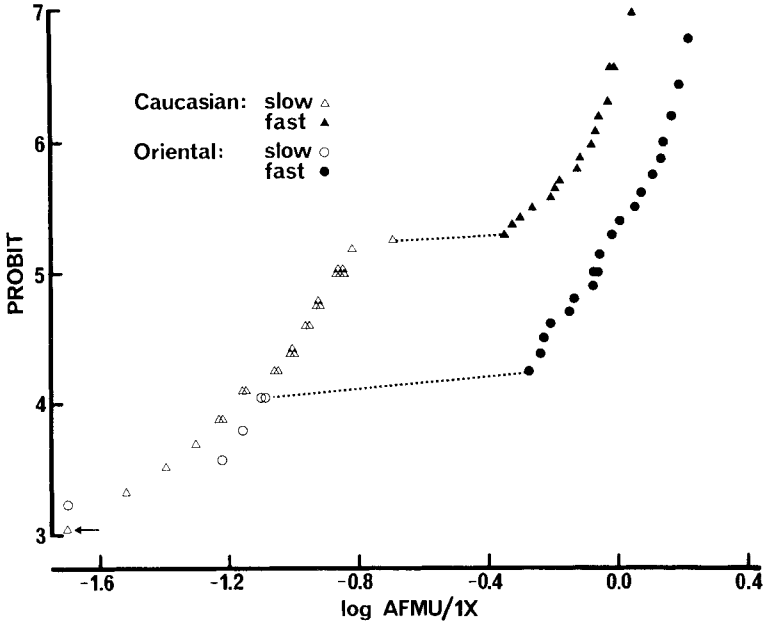

Fig. 7. As in Fig. 4, abscissa denotes logarithm of ratio representing *N*-acetyltransferase activity.

to 17X, since analytical error at low levels of detection might distort results from the minor 1-and 7-demethylation pathways. The ratio of AFMU + 1U + 1X to 17X was taken to indicate a P-450–dependent 7-demethylation capacity; the 17U/17X ratio defined an index of an 8-hydroxylation activity that may be microsomally mediated (since 17X is apparently a poor substrate for xanthine oxidase[11]); and the 1U/1X ratio was assumed to yield information on nonmicrosomal xanthine oxidase activity, since 1X is a good substrate for this enzyme.[11] In addition, the AFMU/1X ratio was investigated as a marker of acetylator status and therefore of *N*-acetyltransferase activity. Any contribution of 1X to these pathways from 13X was assumed to be negligible, because production of the latter from caffeine was very low in the sample studied (13X < 1%; 13U = 1.3%).

Probit analyses (transformations of cumulative frequency distributions) of the ratios defined earlier compared fast and slow acetylators for the two ethnic groups (Figs. 4 to 7). In Fig. 4 the logarithm of the 7-demethylation ratio yielded separate, linear, and parallel plots for the two ethnic groups, indicating that they were log-normally distributed and equally variable but had different mean values (values for Caucasians were higher; $P < 0.001$). There was no trend, however, according to acetylator phenotype for either race. The 8-hydroxylation ratio (Fig. 5) gave a similar result in terms of both variability (slope) and ethnic difference ($P < 0.001$). In this case a slight trend toward a higher ratio for slow acetylators in the Oriental group can be seen, but it was not statistically significant.

In contrast, the plot of the xanthine oxidase ratio (Fig. 6) illustrates not only a considerably smaller degree of variability (steeper slopes) but also a lack of any ethnic difference (superimposable lines). In addition, a difference between fast and slow acetylators for the Oriental group can be seen.

Both the high degree of variability associated with AFMU excretion and its bimodal, interethnically variable nature are illustrated in Fig. 7. Probit plots of the AFMU/1X ratio (in logarithmic form) were discontinuous for both ethnic groups, and segregation of fast and slow acetylators was achieved in complete accordance with the previously defined method.[20] Better separation between phenotypes was obtained in the Oriental group. The different modal frequencies between the ethnic groups are also evident.

No statistically significant differences were found between Oriental and Caucasian samples or between acetylator phenotypes with respect to age, weight, or volume of urine excreted per 24 hr, but the races differed ($P = 0.010$) in normal daily caffeine consumption, with Caucasian intake (3.0 ± 2.1 cups a day) almost twice that of Orientals (1.7 ± 1.5 cups a day), albeit with large interindividual differences. On the premise that differing rates of consumption could be the cause of the ethnic differences in metabolite profile, we tested for correlations or covariate effects between caffeine consumption and any of the metabolite recoveries or ratios. No significant correlations whatsoever were found, implying that there is no causal relationship between habitual caffeine consumption and its metabolite profile—or at least that 2 days of abstention from caffeine is sufficient to eliminate any existing effects.

The possibility of a sex difference in caffeine metabolism was also investigated, but none was found for any metabolite or metabolite ratio. No correlations were found between age or weight of subjects and metabolite recoveries or profiles. There was, however, a positive correlation between 24-hr urine volume and the excretion of 17X ($r = 0.50$, $P < 0.00001$, $n = 68$), suggesting a flow-dependent elimination of 17X that was not observed for any other metabolite.

Discussion

The analytical method used has a number of advantages over those that have been reported for the quantification of urinary caffeine metabolites.[1,12,15,30] First, resolution of all of the demethylated xanthines and uric acids was possible (Fig. 2) with the more highly resolving 5-μm column introduced by Muir et al.[32] for theophylline metabolite analysis, whereas methods using 10-μm columns do not separate certain pairs of metabolites (i.e., 1X-37U; 17X-13X; 7X-1U[1,15]). Second, the present method avoids the use of elution programming, a procedure that adds considerable time to the overall analysis because of the necessity for column reequilibration between runs.

In addition, modification of the extraction method of Aldridge et al.[1] improved the speed, recovery, and precision of the extraction (Table I), most notably for the major metabolite IU, which had been reported by these authors to have a low and highly variable recovery. An obvious drawback to our method is that we are not able to quantify unchanged caffeine along with its metabolites. This does not present a problem, however, since in all studies to date, urinary caffeine does not amount to more than approximately 1% of a dose in normal man[1,12,15,30] and is therefore of minor quantitative importance. Moreover, if it were deemed desirable under certain clinical circumstances,[1] urinary caffeine could be quickly and easily quantified by means of a separate isocratic HPLC system.

Some of the variability in 24-hr recovery of metabolites (Table III) may be attributed to differences in overall caffeine elimination t½, since urine collections were not exhaustive. Caffeine plasma t½ has been reported to vary between 3 and 10 hr in healthy, nonsmoking subjects,[34] and excretion t½ of its metabolites may be more than twice as long.[15] Therefore in some subjects the elimination of caffeine and metabolites would be essentially complete within 24 hr, whereas in others considerable amounts would remain in the body and would not be quantified under the present experimental protocol. This is a consequence of the simplified design of this study. It remains to be seen whether the rates of metabolite production or excretion or the plasma (or salivary) t½ or clearance of caffeine relate most usefully to the urinary metabolite profiles. Large-scale population assessment requires a compromise between simplification of protocol and maximization of information derived.[25]

An advantage of metabolite ratios as used in this study is that they relate the levels of metabolites to each other and are essentially independent of recovery. Therefore any variability in a ratio implies variation in an enzyme pathway, provided that the ratio is defined on a sound biologic basis. Empirical support for some merit to our approach may be seen in the fact that different ratios display different interindividual and interethnic variability.

Published data suggest that 7-demethylase activity can be induced by a polycyclic aromatic hydrocarbon and is therefore likely to be a cyto-

chrome P-448–dependent activity.[13,29,34,40,41] The AFMU + 1U + 1X/17X ratio, which we therefore propose as an index of P-448–dependent 7-demethylase activity, was shown to be highly variable in both Caucasian and Oriental subjects, with a range of 0.89 to 25.54—almost 3000%—when taken over the group as a whole (n = 68). The reason for this variation has yet to be studied but probably involves largely environmental influences such as diet and inducing agents. Validation of this ratio as an index of P-448 activity requires further tests; of interest in this regard would be the effects of smoking, a well-known inducer of P-448 and of caffeine elimination.[29,34] It should be noted that the ethnic difference in this ratio is independent of the ethnic variation in acetylator status.

The 17U/17X ratio, denoting an 8-hydroxylation activity, also demonstrated both a high degree of variability and an interethnic difference. It will be of interest to determine the nature of the enzyme responsible for the conversion of 17X to 17U and to see whether factors that alter the 7-demethylase activity have the same or a different effect on this ratio. It is possible that this reaction may be mediated by a form of P-450 which is distinct from that responsible for the demethylation of 17X.

An interesting result is the relative lack of variability or ethnic difference in the ratio assumed to denote nonmicrosomal xanthine oxidase activity. It is possible that this reflects a teleologic difference between constitutive enzymes and inducible systems such as P-450, which must maintain higher plasticity for response to environmental pressures. It is not presently clear, however, whether the 1U/1X ratio actually represents true xanthine oxidase activity. In the metabolism of theophylline, for example, it appears that once 1X is formed by 3-demethylation, it is quickly and virtually completely converted to 1U by xanthine oxidase; administration of the potent xanthine oxidase inhibitor allopurinol produces a ratio of 1U to 1X more closely resembling the ratio produced after ingestion of caffeine.[21] It may be, then, that the ratio in the latter instance is a reflection of an enzyme activity that has been partially inhibited, possibly by one or more of the other metabolites of caffeine. In this light it would be of interest to determine the profile of caffeine metabolites after allopurinol treatment.

It is also clear from this study that the ratio of AFMU to 1X gives a clear indication of acetylator phenotype. This finding is of some importance because it represents a considerable simplification of the method previously used to segregate fast and slow acetylators of caffeine.[20] It also opens up the possibility of developing a widely applicable and simple acetylation phenotyping test using caffeine as a test drug. We are presently optimizing rapid experimental and analytical methods for this purpose.

On a more theoretical note, it is interesting that acetylator status, and hence level of AFMU production, can influence the production of other metabolites of caffeine, namely 1X and 1U. As noted earlier, slow acetylators excrete more 1X + 1U than do fast acetylators. It appears, then, that the genetic characteristics of an individual can create a "channeling" toward pathways giving rise to particular metabolites. One could envision a toxicologic significance to such a phenomenon, whereby preferential formation of certain toxic metabolites in fast or slow acetylators may indicate a predisposition toward adverse drug reaction.

In conclusion, our results indicate that there is a large degree of variability, some of which is genetic in origin, in the profile of metabolites formed in man after the ingestion of caffeine. It appears that the use of caffeine metabolite ratios may provide simultaneous indices of a number of different enzyme activities. The subjects in this study were normal, healthy, nonsmoking persons from the Toronto population. Further studies will be aimed at validating metabolite ratios and investigating the effects of perturbations such as smoking, diet, and disease states on particular pathways of caffeine metabolism with a view toward elucidating determinants of the observed variability.

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