Polymorphic *N*-acetylation of a caffeine metabolite

In the course of investigations into variability in the metabolism of caffeine in human populations, urinary levels of 5-acetylamino-6-formylamino-3-methyluracil (AFMU), a newly discovered ring-opened metabolite of caffeine, were found to be both bimodally distributed and interethnically variable in samples (Caucasian: n = 42; Oriental: n = 26) from the Toronto population. To test the premise that the polymorphic N*-acetyltransferase enzyme (E.C.2.3.1.5) could be responsible for the production of AFMU, 20 of the subjects were phenotyped for acetylator status using sulfamethazine (SMZ). Concordance for all subjects between AFMU production and SMZ acetylation strongly suggests that the acetylation polymorphism is involved in the formation of AFMU in man.*

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Caffeine (1,3,7-trimethylxanthine) enjoys world-wide popularity as a central stimulant in beverages and in over-the-counter stay-awake aids. In addition, in recent years its use has been increasing in the treatment of neonatal apnea.[2, 3] Nevertheless, the full elucidation of caffeine's biotransformation pathways in man has yet to be realized. Since the initial studies of Cornish and Christman in 1957,[12] a number of reports have verified that caffeine undergoes oxidative demethylation and hydroxylation reactions, mediated by microsomal mixed-function monooxygenases containing cytochromes P-450, to produce a variety of methylated xanthine and uric acid derivatives.[1, 6, 8, 10, 11] More recently it was discovered that caffeine and some of the dimethylxanthines may also undergo ring cleavage, yielding substituted uracilic metabolites.[4, 5, 6, 11, 22]

Our investigations were concerned with interindividual variability in the metabolism of caffeine in man and its assessment by analysis of urinary caffeine metabolites.[20] In the course of these ongoing studies, a major metabolite was discovered, isolated, and later identified as 5-acetylamino-6-formylamino-3-methyluracil (AFMU).[27] The production of this acetylated metabolite was highly variable between subjects.

We report here on the nature of the variability in urinary AFMU levels after oral caffeine in a sample (n = 68) from the Toronto population. The results show, first, that AFMU excretion follows a distinctly bimodal pattern both in an overall sample and in Caucasian and Oriental groups taken separately. Furthermore, a difference in the frequencies of "high excretors" and "low excretors" between Caucasian and Oriental samples, coupled with our knowledge of the

Supported by the Medical Research Council of Canada, Ottawa, grant MA-4763.

Received for publication Sept. 10, 1982.

Accepted for publication Oct. 7, 1982.

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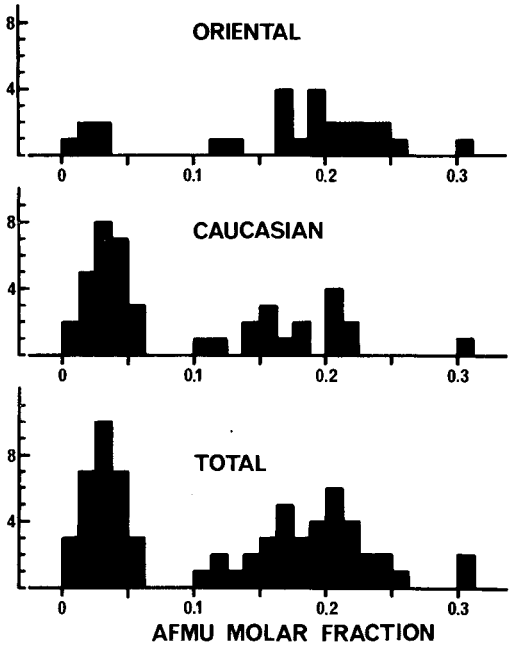

Fig. 1. Frequency histograms of urinary AFMU excretion in Oriental (n = 26) and Caucasian (n = 42) samples, and for both groups combined. The *horizontal axis* represents the molar fraction AFMU/(1U + 1X + 17U + 17X + AFMU), that is, the proportion of AFMU in the sum total of the major metabolites of caffeine excreted in 24 hr after 300 mg caffeine.

acetylated nature of AFMU, led us to postulate the involvement of the polymorphic *N*-acetyltransferase enzyme (E.C.2.3.1.5)[17, 28] in AFMU formation. To test this, we chose subjects from the original study group and determined their acetylator phenotypes using sulfamethazine (SMZ) as a test drug.[15, 18] The complete concordance found between AFMU production and SMZ acetylation appears to verify the above postulate.

Methods

The subjects who enrolled in the original investigations[20] were from the Toronto area and included university students, staff members, and others from a variety of occupational and age groups. Data were available from 68 unrelated subjects who were all healthy nonsmokers. Forty-two were Caucasians and 26 were of Oriental (mostly Chinese) descent. There were 35 men and 33 women in the sample, and the overall mean age was 26.6 yr, with a range from 16 to 65 yr.

Details of the experimental protocol will be presented elsewhere.[20] Briefly, after a methylxanthine-free period, all subjects ingested a 300-mg dose of caffeine and a pooled 24-hr urine sample was collected. After volume measurement, aliquots were stored frozen for subsequent analysis.

Analytical procedures. HPLC analyses for demethylated xanthine and uric acid metabolites and for AFMU will be outlined in another publication.[20] In short, xanthines and uric acids were quantified, after chloroform:isopropanol (85:15,v/v) extraction of urine, using a 5μ reversed-phase column eluted isocratically with 0.05% acetic acid:methanol (88:12,v/v) at a flow rate of 1.2 ml/min. A similar method was used for AFMU determination, except that the extracting solvent was 100% chloroform and the concentration of methanol in the HPLC mobile phase was increased to 15%(v/v). Total amounts of each metabolite excreted in 0- to 24-hr urine samples were expressed either as a molar fraction of the ingested dose or as a fraction of the five major metabolites excreted (1-methylxanthine [1X], 1-methyluric acid [1U], 1,7-dimethyluric acid [17U], 1,7-dimethylxanthine [17X], and AFMU).

Acetylator phenotyping. Subjects were chosen for further study from the above sample on the basis of their level of excretion of AFMU in the urine, determined as above. A total of 20 subjects were selected, 10 from each of the high and low modes of AFMU excretion.

The method for determination of acetylation status using SMZ as a test drug was modified from Evans,[18] as suggested by du Souich et al.,[15] with the following protocol. Subjects were requested to refrain from eating for 2 hr before and after taking drug. Subjects voided and ingested 10 mg/kg of SMZ in gelatin capsules. Pooled urine was collected for the next 6 hr, at which time a single 10-ml blood sample was collected into oxalate-containing tubes and plasma was separated by centrifugation. Plasma and urine aliquots were stored frozen for later analysis.

Concentrations of SMZ and *N*-acetylsulfamethazine (NSMZ) in plasma and urine were

determined by the classical Bratton-Marshall procedure[9] as modified by du Souich et al.,[14] except that acid incubations for determination of NSMZ were carried out for 30 min instead of for 5 min. Plasma and urine acetylation indices (PI_6 and UI_6, respectively), denoting the percentage of SMZ in the acetylated form, were calculated as described.[15, 18]

Results

From Fig. 1 it is evident that the excretion of AFMU was clearly divisible into high and low modes when expressed as a molar fraction of the total of the five major metabolites of caffeine, both in the total sample (n = 68) and also in each of the ethnic groups. The same sharp bimodality could be seen when AFMU excretion was expressed as a ratio to the excretion of 1X (not shown). An arbitrary antimode was defined at 0.08 on the histograms. That is, subjects excreting less than 8% of the major recovered metabolites as AFMU were classified as low excretors, and those producing more than 8% were assigned to the extensive excretor mode.

The histograms also illustrate a significant difference in proportions in the two modes between Caucasian and Oriental groups ($x^2 = 9.22$, $P < 0.005$). Low excretors made up 19.2% of the Oriental sample and 59.5% of the Caucasian sample. It was noted that these modal frequencies were similar to those derived from the reported allele frequencies for the human acetylator polymorphism for various Caucasian and Oriental populations.[21] However, mean excretion of AFMU within each mode did not differ ($P > 0.05$) between the two ethnic groups, with recoveries of 3.2 ± 1.4% and 2.2 ± 0.9% in the low mode and 17.9 ± 4.6% and 20.2 ± 4.2% in the high mode for Caucasians and Orientals.

Using the described acetylator phenotyping procedure, all 20 of the retested subjects could be definitively classified as either fast or slow acetylators on the basis of either SMZ plasma or urine acetylation indices. Better separation of phenotypes was achieved by the plasma index; mean PI_6 values for high and low modes were 74.1% and 22.4% SMZ acetylated, while corresponding values for the urine index were 87.7% and 55.7%. These results are in close agreement with those of other authors.[15, 18, 21]

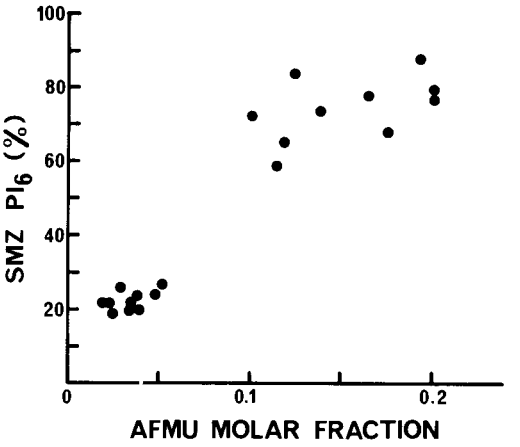

Fig. 2. Relationship between SMZ acetylation and excretion of AFMU in man. *Abscissa* is as in Fig. 1; the *ordinate* represents the percentage of SMZ in plasma in the acetylated form.

Fig. 2 shows that the segregation of fast and slow SMZ acetylation was in complete accordance with AFMU excretion mode for all 20 of the subjects ($x^2 = 16.20$, $P << 0.001$). The correlation between the plasma indices for SMZ acetylation and AFMU excretion is clearly visible, with a nonparametric (Spearman) rank correlation coefficient of 0.881 ($P < 0.001$; n = 20).

Discussion

Our findings came to light during studies into variability in the metabolic fate of caffeine in man. The purpose of the latter was to investigate caffeine's potential as a probe drug for the assessment of hepatic-metabolizing capacity in human populations and were deemed desirable in light of the widespread use of caffeine, its relative safety, and the variety of microsomal enzyme reactions that might be monitored by following the excretion of its urinary metabolites. In fact, other authors have recently taken a different approach in proposing a similar use for caffeine in $^{14}CO_2$ or $^{13}CO_2$ breath tests, which appear to monitor 3-methylcholanthrene–inducible cytochrome P-448–dependent enzyme activity.[7, 29, 30]

The unusual finding that caffeine also undergoes an *N*-acetylation reaction mediated by the

polymorphic *N*-acetyltransferase system carries a number of clinical implications. Many drugs, such as isoniazid, some sulfonamides, hydralazine, and nitrazepam, as well as certain endogenous compounds including serotonin, are known to be substrates for this enzyme.[13, 16, 28] In addition, it is becoming increasingly apparent that the acetylation of certain foreign chemicals, most notably arylamines, may be of toxicologic significance in the production and in the elimination of potential carcinogens.[19, 24] It is therefore important to determine the association between the acetylator phenotype and therapeutic and toxic responses to drugs and other foreign chemicals. As such, knowledge of acetylator status for individuals may be of value in risk assessment as well as in therapeutic drug regimen formulation. The ubiquitous use of caffeine opens up the possibility of developing a very widely applicable acetylation phenotyping test for such purposes.

On the other hand, much debate surrounds studies on the mutagenic and teratogenic effects of caffeine itself.[23, 25] The biologic activity and toxicity of AFMU are at present unknown, although it has been reported that 5-acetylamino-6-amino-3-methyluracil (AAMU), the nonbiologically deformylated compound arising from AFMU,[27] was not as potent as caffeine in enhancing ultraviolet light-induced cytotoxicity in hamster cells.[26] It has been noted, however, that certain of the uracilic metabolites of caffeine bear a structural resemblance to antimetabolites such as 5-fluorouracil, which are probably teratogenic.[22] There may, then, be an association between acetylator status and some of the toxic effects of caffeine. It is also likely that there are interactions between caffeine and therapeutic and toxic responses to other drugs and chemicals that are polymorphically acetylated.

In conclusion, we have shown that the urinary excretion of AFMU, an *N*-acetylated metabolite of caffeine in man, follows a bimodal and interethnically variable pattern in Caucasian and Oriental samples from the Toronto population. The complete concordance found between AFMU excretion and SMZ acetylation also provides strong evidence for the participation of the polymorphic *N*-acetyltransferase enzyme in the formation of AFMU in man. These findings widen the scope of caffeine's potential as a probe for hepatic enzyme activities and also raise a number of questions of therapeutic, toxicologic, and theoretical interest. Further studies may be aimed at (1) determining the influence of caffeine's acetylation on its therapeutic and toxic effects, (2) further developing caffeine as a probe for the assessment of acetylator status as well as of cytochrome P-450–dependent enzyme activities, and (3) elucidating the biochemical pathways giving rise to this new metabolite.

We are grateful to Dr. T. Inaba for his assistance in blood collections. The continuing cooperation of the subjects who participated in these studies is much appreciated.

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