Carcinogenesis vol.13 no.9 pp.1561–1568, 1992

Foreign compound metabolism capacity in man measured from metabolites of dietary caffeine

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Caffeine is sequentially metabolized by cytochrome P4501A2 (CYP1A2), *N*-acetyltransferase (NAT) and/or xanthine oxidase (XO). In the present study the activity of these three enzymes was estimated from ratios of the metabolites formed from dietary caffeine and excreted into the urine collected as spot samples. In the urine samples from 10 out of 377 subjects concentrations of caffeine metabolites were too low to allow reliable measurements of the ratios. In 335 healthy subjects the NAT activity showed a typically bimodal distribution with 47% fast acetylators and 53% slow acetylators, consistent with a Danish population. The ratios reflecting CYP1A2 and XO activities were log normal and normal distributed, respectively. In 103 non-smoking men and 90 non-smoking women the ratio of caffeine metabolites expressing CYP1A2 activity was 4.7 ± 1.6 and 4.3 ± 1.9 as compared to 7.8 ± 2.5 and 7.3 ± 3.0 in 31 male and 25 female subjects smoking 10 cigarettes/day or more respectively, verifying induction of CYP1A2 by tobacco ($P < 0.05$), but minimal sex-related differences. In 12 non-smoking pregnant women and in 28 women using oral contraceptives the CYP1A2 ratio was 29 and 20% reduced respectively ($P < 0.05$). In a multivariate analysis the only significant predictor of the XO ratio was the consumption of caffeine with an increase of 2% per cup of coffee or equivalent ($P < 0.05$). In 23 healthy male subjects 30 days of vigorous exercise increased the CYP1A2 ratio by 70% and the XO ratio by 42% ($P < 0.05$), but left the NAT ratio unchanged. In nine healthy volunteers daily ingestion of 500 g of broccoli for 10 days increased the CYP1A2 ratio by an average of 12% ($P < 0.05$), compared to a control period with ingestion of an equivalent weight of non-cruciferous green vegetables. The ratios of metabolites from dietary caffeine in spot urine samples offer ethical, non-invasive and reliable estimates of CYP1A2, NAT and XO. These enzymes are highly relevant for the bioactivation of potentially toxic compounds and the formation of oxygen radicals. The method is applicable in large-scale epidemiological studies, allowing, for example, prospective testing of the relationship between these enzyme activities and the development of disease. Exercise may increase CYP1A2 activity to a magnitude corresponding to heavy smoking, as well as XO by mechanisms that remain to be clarified.

Introduction

Most carcinogens relevant for man, including those encountered in the diet, require metabolic activation to exert their effect (2–7). The enzymes involved in activation and detoxification reactions often show up to a 100-fold or more interindividual variability, which may relate to the apparent large variability in cancer susceptibility. The activity of the various enzymes is genetically controlled but may be modified by environmental factors (8). The latter include lifestyle factors—diet, exercise, occupational exposure to chemicals, disease, age, etc. (9). Such factors are the major determinants of the activity of some enzymes.

A number of epidemiologic studies relate foreign compound metabolism capacity to cancer susceptibility (4,7,9,10). So far, however, all studies have been cross-sectional, comparing patients with established cancer with healthy controls or patients with non-malignant disease. Usually, the metabolic capacity related to activation and/or detoxification has been assessed by measurement of the pharmacokinetics of a test compound, more or less specifically metabolized by an (or group of) enzyme(s) (9). The major problem related to this design is that the cancer disease *per se* is likely to affect the test result. Thus, the epidemiologic evidence must come from prospective cohort studies. Such large-scale studies require simple methods for the assessment of enzyme activities relevant for metabolic activation of carcinogens. For ethical and practical reasons the method should not be based on administration of a drug as test compounds.

Caffeine is metabolized along several pathways and a number of metabolites are excreted into the urine. The major part of ingested caffeine is demethylated to 1,7-dimethylxanthine (17X) by cytochrome P4501A2 (CYP1A2). Subsequently, 17X may be hydroxylated to 1,7-dimethyluric acid or demethylated once more by CYP1A2, leading to an *N*-acetylated ring split product or to 1-methylxanthine (1X), which may be hydroxylated to 1-methyluric acid by xanthine oxidase (XO). Thus, the urinary ratios of some of the caffeine metabolites express the activity of CYP1A2, *N*-acetyltransferase (NAT) and XO in the liver (Figure 1; refs 11–19). The two former enzymes are important for bioactivation and/or detoxification of many important carcinogens, such as aromatic amines (6,16,20–22), whereas the latter enzyme has been implicated in oxygen radical generation with resulting tissue damage as well as formation of reactive quinones (23–25). Thus, urinary metabolic ratios of dietary caffeine may be a candidate for epidemiological studies of activating enzyme activities and the development of disease.

A number of host factors have been shown to influence the clearance of caffeine and/or the metabolic ratio, both expressing the CYP1A2 activity. Thus, smoking increases and oral contraceptives decreases both indices (14,17,19,26–30), whereas pregnancy has been shown to decrease the elimination rate of caffeine (31). A diet rich in cruciferous vegetables and physical activity are other factors known to influence the activity of cytochrome P450 enzymes, probably including CYP1A2 (32,33).

In the present study we investigated factors influencing the metabolic ratios of dietary caffeine. Demographic variables and

*Abbreviations: 17X, 1,7-dimethylxanthine; CYP1A2, cytochrome P4501A2; 1X, 1-methylxanthine; XO, xanthine oxidase; NAT, *N*-acetyltransferase; 17U, 1,7-dimethyluric acid; AFMU, 5-acetyl-6-formylamino-3-uracil; 1U, 1-methyluric acid.

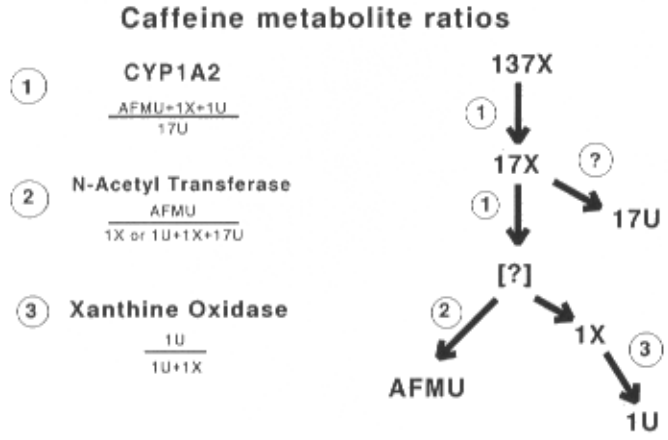

Fig. 1. Relevant pathways of the metabolism of caffeine (1,3,7-trimethylxanthine: 137X) and the urinary metabolic ratios expressing the involved enzymes, CYP1A2, NAT and XO.

host factors, including lifestyle factors, were investigated in a cross-sectional population sample, whereas the effect of exercise and a diet rich in cruciferous vegetables was studied longitudinally with subjects serving as their own control.

Materials and methods

Protocols

The protocols were approved by the local ethics committees. All subjects participated after giving informed consent. In all experiments sampling of 5 ml of urine was preceded by ingestion of 1–4 cups of coffee, or equivalent, within 2–6 h (11,19). Caffeine ingestion should not exceed 4 cups of coffee, because the metabolism is saturable (19,34). Sampling of urine was accomplished before noon and most subjects had taken sufficient coffee or tea at breakfast (35,36). Those who had not were offered a cup of coffee and returned for sampling 2 h later. The urine samples were immediately acidified with HCl to pH 3.5 and stored at −20°C for subsequent HPLC analysis.

In a cross-sectional experiment urine samples were collected from 377 healthy volunteers, who filled in a questionnaire regarding age, height, weight, use of medicine, the consumption of tobacco, alcohol beverages, coffee, tea, cola beverages, protein and cruciferous vegetables, possible occupational exposure to chemicals and exercise during the preceding 2 weeks. Most of subjects were recruited among medical students and faculty academics and technicians. Twenty-seven subjects were excluded due to ingestion of drugs, except oral contraceptives, during the week preceding urine sampling, whereas 10 subjects, i.e. 2.7% (1.3–4.9%; 95% confidence interval) were excluded because the concentrations of caffeine metabolites in their urine sample were too low to allow reliable measurements of the ratios. Five subjects failed to fill in the questionnaires. Of the remaining 335 subjects 171 were women, 12 of whom were pregnant in the second or third trimester and 28 used oral contraceptives. The consumption of cola beverages and the occupational exposure to chemicals were extremely low, whereas the recording of protein consumption was inadequate in this population sample and these data were thus not included in the statistical analysis.

In a longitudinal second experiment urine samples were collected twice from 23 healthy men (age 22 ± 2 years; body weight 75 ± 6 kg). One sampling was preceded by a common sedentary life, whereas the other sampling was preceded by a Danish army program for physical training consisting of 30 days with 8–11 h vigorous exercise per day. At both sampling occasions the subjects filled in questionnaires as described above.

In a longitudinal third experiment, spot urine samples were collected three times from nine healthy subjects (age 33 ± 5 years; body weight 69 ± 14 kg), five of whom were women. Each urine collection was preceded by three 10 day periods with (i) the habitual diet, (ii) a diet supplemented with 500 g of broccoli per day or (iii) a diet supplemented with 500 g of non-cruciferous vegetables. The order of the dietary regimens was random and separated by at least 4 weeks. In the women sampling was accomplished on the same day of three menstrual cycles. The vegetables were served lightly steamed and were distributed evenly to lunch and dinner. The non-cruciferous vegetables included 300 g of green beans, 100 g of peas and 100 g of cucumber and/or lettuce and no cruciferous vegetables were allowed. Except for the supplementation, the subjects were instructed to keep their diet, exercise and consumption of stimulants as constant as possible in the three periods and record it carefully. None of the subjects smoked and they consumed only minimal amounts of alcohol.

The vegetables were commercial products claimed free of pesticides and bought on one occasion at the Copenhagen vegetable market. They were cleaned and quickly blanched in boiling water before freezing for later consumption during the diet interventions. At the time of ingestion a sample of the broccoli contained after freeze drying glucobrassicin (9.0 μmol/g), neoglucobrassicin (6.4 μmol/g), glucoiberin (5.5 μmol/g) and glucoraphanin (5.5 μmol/g) as measured by HPLC (37,38).

Analysis of caffeine metabolites

The five relevant metabolites of caffeine—5-acetyl-6-formylamino-3-uracil (AFMU), 1X, 1-methyluric acid (1U), 17X and 1,7-dimethyluric acid (17U) were assayed in duplicate in the urine samples by HPLC as described by Campbell *et al.* (14). Standards were prepared in urine collected from a subject after 3 days of xanthine-free diet. Genuine compounds were used as reference: 1X, 1U and 17X were purchased from Sigma (St Louis, MO) and 17U was from Fluka (Büchs, Switzerland). AFMU was kindly provided by Dr B.K.Tang, Toronto, Canada.

After addition of 120 mg ammonium sulphate 200 μl of urine was extracted with 6 ml chloroform/isopropanol (90:10 v/v). The organic phase was dried at 40°C under nitrogen. The residue was reconstituted in the eluent (0.05% acetic acid/methanol, 90:10 v/v). The analytical column, a Beckman Ultrasphere ODS (5 μm, 25 cm) was eluted at 1 ml/min. The effluent was monitored at 280 nm. During preparation and extraction and after reconstitution all samples were kept at 0°C in order to conserve AFMU. When using the present caffeine metabolite ratio for assessing acetylator phenotype, the instability of AFMU should be taken into account. The problem may be solved by immediate acidification of urine samples and storage at lowest possible temperature or by deliberately converting AFMU to 5-acetyl-6-amino-3-methyluracil by alkalinization and assaying this compound (11,12,39). Since the metabolic ratios derive from the same chromatographic run, no internal standard was used. The analytical inter-day coefficient of variation of the metabolic ratios was 5%.

The metabolic ratios of caffeine were calculated according to Figure 1. The XO activity was previously assessed from the ratio of 1X to 1U (11,13). However, the addition of the substrate, 1U, in the denominator, as used in the present study and another recent paper (19) will give a more accurate reflection of the enzyme activity.

Statistics

The distribution of the three metabolic ratios of caffeine was investigated by means of probit analysis. The fit to the normal distribution before and after log transformation of the data was tested by Kolmogorov–Smirnoff and chi-squared tests.

The relationship between the recorded host factors and the log of the ratios was investigated by multivariate regression and covariate analysis. Spearman rank correlation coefficients between the recorded variables were also determined.

The subjects were stratified in three levels according to smoking habits: non-smokers, smokers of 1–9 cigarettes/day and smokers of ≥10 cigarettes/day. For the CYP1A2 ratio the women were divided in three groups according to the presence of pregnancy, the use of oral contraceptives or none of these factors. The (log of) the metabolic ratios were compared between the groups by means of one-way analysis of variance followed, if significant, by a comparison of the means by the method of least-significant differences.

The effect of exercise on the metabolic ratios was investigated by a paired *t*-test. The effect of the three diets was not normally distributed and thus statistically tested by means of Friedman two-way analysis of variance followed, if significant, by the Wilcoxon test. Probability values <0.05 were considered to indicate statistical significance.

Results

Distribution of metabolic ratios

The ratio between AFMU/1X assessing the acetylator status showed a typically bimodal distribution with 54% slow acetylators (rr) and 46% intermediate (Rr) and fast (RR) acetylators separated by an antimode of 0.55 (Figure 2). A second antimode discriminating Rr and RR could not be identified. After division of the data according to the antimode, the data from the slow acetylators fitted a normal distribution, whereas the Rr and RR data fitted a log-normal distribution. From the tail area probability of the density functions and the antimode of 0.55 it was estimated that 1% of rr would be phenotyped as intermediate acetylators, whereas 3% of Rr would be classified as slow acetylators.

The ratio reflecting CYP1A2 activity fitted a log-normal distribution (Figure 2). The XO ratio was normally distributed without signs of polymodality or single outliers (Figure 2).

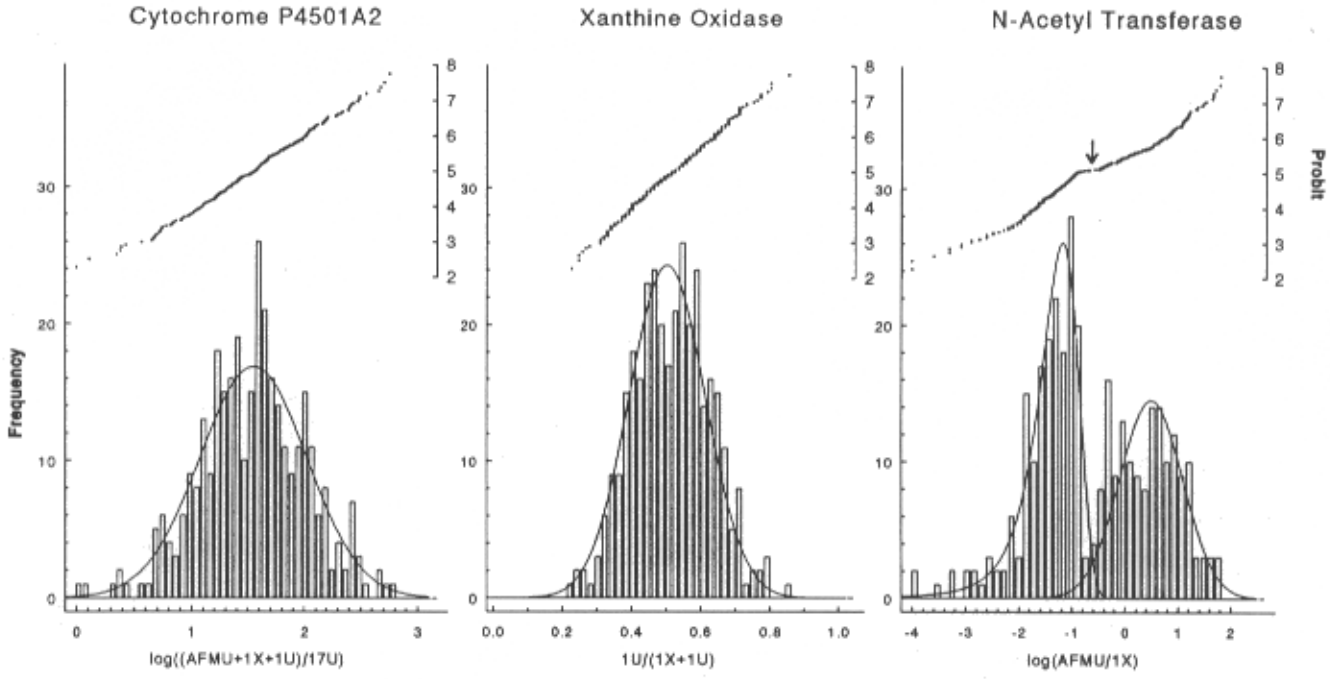

Fig. 2. Frequency distribution with overlaying density functions and probit plots of CYP1A2, XO and NAT as assessed by the urinary metabolic ratios of dietary caffeine in 335 healthy subjects. The arrow indicates the antimode separating slow acetylators from fast acetylators. The density function of each of these two populations is shown.

Table I. Spearman rank correlation matrix of urinary metabolite ratios from dietary caffeine expressing the activity of NAT, CYP1A2 and XO in relation to host factors in 335 health subjects

	No. or median (range)	NAT 0.42 (0–6.3)	CYP1A2 4.8 (0.5–16.1)	XO 0.50 (0.22–0.83)
Sex (M/F)	n = 164/171	0.18*	0.23*	−0.07
Pregnancy	n = 12	−0.01	−0.22*	−0.09
Oral contraceptive use	n = 28	−0.06	−0.17*	0.08
Age (year)	25 (18–70)	−0.08	0.007	0.15*
Height (cm)	175 (155–196)	0.18*	0.17*	−0.12*
Weight (kg)	67 (48–100)	0.23*	0.17*	−0.06
Smokers (M/F)	n = 63/41	−0.06	0.46*	0.22*
Coffee + tea consumption[a]	4 (0–20)	−0.04	0.12*	0.27*
Alcohol (drinks/day)	1 (0–6)	0.12*	0.13*	0.11*
Exercise (h/week)	4 (0–40)	−0.03	−0.08	−0.11
Cabbage (portions/week)	1 (0–12)	0.02	−0.07	−0.03

[a]Cups of coffee + 0.6 × cups of tea per day (35).
*$P < 0.05$.

Factors affecting metabolic ratios

A number of host factors recorded on the questionnaires were significantly correlated with the caffeine metabolite ratios (Table I). The Spearman rank-correlation coefficients and Pearson product-moment correlation coefficients (not shown) were of similar magnitude and statistical significance. The multivariate regression analysis showed that some of these correlations probably were a result of interactions between the variables, e.g. smoking and alcohol consumption were correlated.

The significant independent predictors of the CYP1A2 ratio were sex, pregnancy, oral contraceptive use and cigarette smoking (Table II). Identical regression coefficients were obtained for sex and smoking with or without inclusion of pregnant and oral contraceptive using women in the analysis. In Figure 3 the subjects are stratified according to sex and smoking habits, and the women also according to the presence of pregnancy or use of oral contraceptives. A dose-dependent inducing effect of smoking on the CYP1A2 ratio is shown as well as additive inhibitory effects of oral contraceptives and pregnancy. According to the regression coefficients men have a 16% (5–21%; 95% confidence interval) higher ratio than women; smoking increases the ratio by 58% (46–70%), whereas pregnancy or use of oral contraceptives reduces the ratio by 32% (3–62%) or 29% (9–50%) respectively. Assuming a linear model smoking increased the CYP1A2 ratio by 4% (3–5%) per cigarette per day.

In the multivariate analysis the only significant independent predictor of the XO ratio was the consumption of caffeine (Table II). Assuming a linear model, the XO ratio increased by 2% per cup of coffee or equivalent. Inclusion of the sum of the concentrations of the four relevant caffeine metabolites in the regression model did not affect the relationship between caffeine consump-

Table II. Coefficients of multiple regression models of the independent significant predictors of the urinary metabolite ratios from dietary caffeine expressing the activity of NAT, CYP1A2 and XO in 335 healthy subjects

	No. or mean ± SD	CYP1A2 5.2 ± 2.5	XO 0.50 ± 0.10	NAT 1.1 ± 1.2
Sex (M/F)	164/171	0.6 (0.1 to 1.1)*	–	–
Pregnant (no.)	n = 12	−1.3 (−2.6 to −0.1)*	–	–
Oral contraceptive user	n = 28	−1.3 (−2.2 to −0.4)*	–	–
Weight (kg)	68 ± 11	–	–	0.03 (0.01 to 0.04)*
Smokers (M/F)	n = 63/41	−2.5 (2.0 to 3.0)*	–	–
Coffee + tea consumption[a]	5 ± 3	–	0.010 (0.006 to 0.014)*	–
Constant		−4.3 (3.9 to 4.7)*	0.460 (0.44 to 0.48)*	−0.7 (−1.5 to 0.1)

[a]Cups of coffee + 0.6 × cups of tea per day (35).
*$P < 0.05$; 95% confidence intervals are given in parenthesis.

tion and the XO ratio. Thus, the effect of caffeine consumption is not likely to be related to increased concentrations of caffeine metabolites in the urine *per se*.

The body weight was the only significant predictor of the acetylator ratio and of modest statistical significance.

Effect of exercise

In the intervention period the average daily exercise amounted to 66 ±17 h week compared to 11 ±11 h week during the control period. After 30 days of this vigorous exercise the CYP1A2 was increased by 70% (47−94%) on average from the base level ($P < 0.0001$). As shown in Figure 4 the effect of exercise was additive to the inducing effect of smoking, which was kept constant during the study period. During the exercise period the XO ratio increased by 42% (124−159%; $P < 0.0001$) on average. The NAT ratio did not change significantly (87−114%; $P > 0.05$) and all subjects maintained their phenotype assignment during the exercise period. After the exercise period the sum of the urinary concentrations of the four caffeine metabolites used in the metabolic ratios was 89% (61−117%) of the value before the period ($P > 0.05$).

Effect of diet

The effect of the vegetable diets on the caffeine metabolite ratios is shown in Figure 5. After a diet supplemented with 500 g of broccoli per day for 10 days the CYP1A2 ratio was increased by median 12% (range 2−210%) compared to a corresponding period with a diet supplemented with 500 g of green beans, peas and lettuce ($P < 0.001$). The CYP1A2 ratio was not significantly different during any of the vegetable diets compared to the home diet period. The XO and NAT ratios were not significantly changed. One subject transgressed the antimode from rr to Rr acetylator status after the broccoli diet. The residual standard deviation of the CYP1A2 and XO ratios after two-way analysis of variance was 18 and 13% of the grand mean respectively. These indices of intra-individual variation are comparable to the 17% intra-individual variation reported by Kalow *et al.* (19).

Discussion

The present study investigated factors influencing the ratios of metabolites from dietary caffeine in spot urine. These estimates of CYP1A2, NAT and XO activity are easily obtained non-invasively and thus well suited for large-scale epidemiological investigations. In the present study the reliability of the indices was demonstrated by the expected distribution of the NAT ratio and effects of smoking, pregnancy, oral contraceptive use, exercise and a diet rich in cruciferous vegetables on the CYP1A2 ratio.

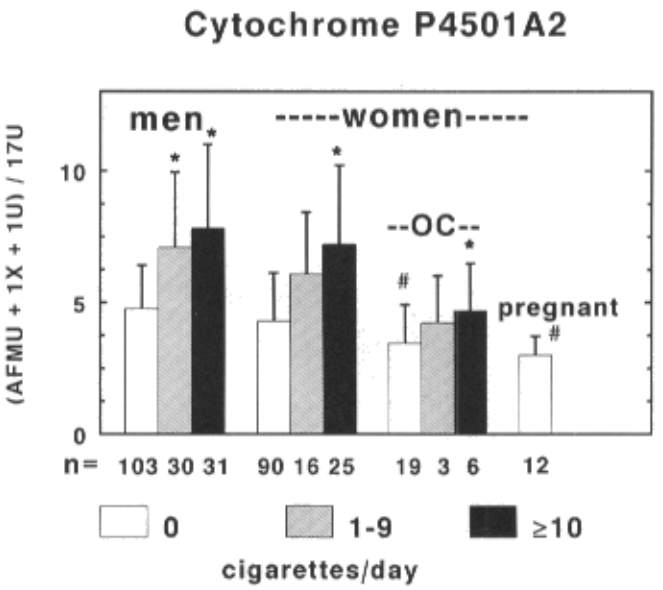

Fig. 3. The urinary metabolic ratio of dietary caffeine expressing CYP1A2 activity in 335 healthy subjects, stratified after gender, tobacco smoking, use of oral contraceptives and pregnancy. Values are group means with SD.

CYP1A2

In agreement with a study by Kalow *et al.* (17,19) the present population distribution of the CYP1A2 ratio was log-normal, i.e. there was no sign of a polymorphism. As shown in previous studies of caffeine elimination and the use of the metabolic ratio, the CYP1A2 ratio was increased by smoking (14,17,19,26,27,30), which increases the hepatic content of CYP1A2 (40). However, it should be noted that the effect of smoking was variable and some intensive smokers had ratios well within the range of the non-smokers. Thus, information on smoking habits is not sufficient for the assessment of the capacity of CYP1A2 for carcinogen activation, the actual capacity induced by smoking must be considered more relevant.

The reducing effect of oral contraceptives on the CYP1A2 ratio was also reproduced in the present data (14,28,29). The presence of pregnancy conveyed a reduced ratio as expected from studies showing decreased caffeine clearance in pregnant women (31). The difference in the ratio between men and women unrelated to pregnancy and oral contraceptives has not been shown significantly in the earlier smaller studies (14,41). However, the interindividual variation was much larger than the gender related difference, which may not reach statistical significance in smaller population samples. Moreover, as shown for theofylline, another

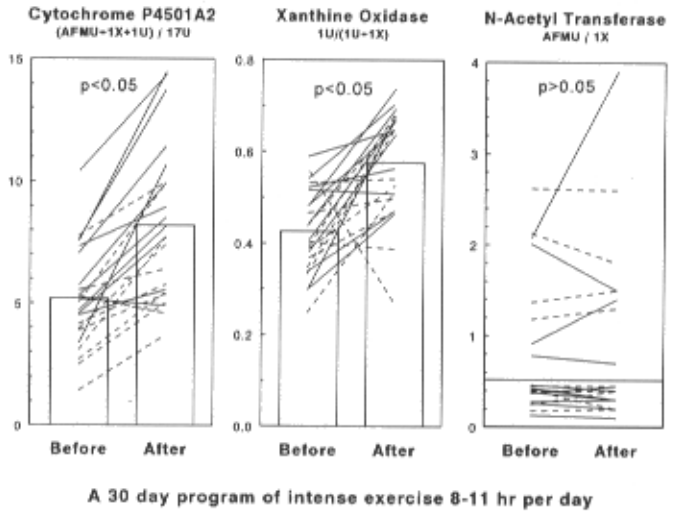

Fig. 4. Effects of a 30 day program with 8–11 h intense exercise per day on the urinary metabolic ratios of dietary caffeine expressing the activity of CYP1A2, XO and NAT in 23 healthy subjects. Smokers are shown as fully drawn lines, non-smokers as broken lines.

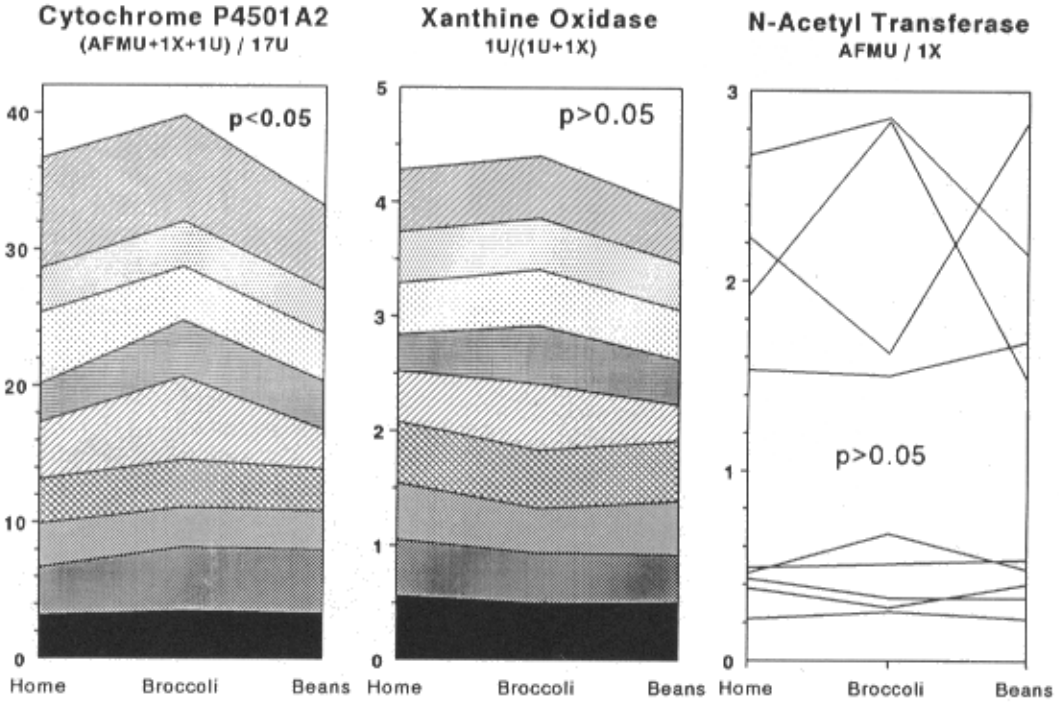

Fig. 5. The urinary metabolic ratios of dietary caffeine expressing the activity of CYP1A2 (summed), XO (summed) and NAT after 10 periods with the home diet, a diet supplemented with 500 g of broccoli and a diet supplemented with 500 g of beans and other non-cruciferous vegetables in nine healthy subjects.

substrate for CYP1A2, the clearance decreases during the middle of the menstrual cycle concurrent with the estrogen–progestogen surge (42), which is comparable to the result of oral contraceptive use. So far any relation between caffeine metabolism and the menstrual cycle has not been published.

Vigorous exercise increased the CYP1A2 to the same extent as smoking ≥ 10 cigarettes/day, by an additive effect. In agreement the elimination of antipyrine and aminopyrine, also cytochrome P450 substrates, was increased by exercise in a manner correlating to the physical fitness (33). In the population sample exercise was not a significant predictor of the CYP1A2 ratio in the multivariate analysis. However, the average extent of exercise was much less than in the control situation of the exercise investigation and thus probably not relevant. The mechanism behind an exercise-related induction is unknown but could be related to increased levels of circulating catecholamines (33).

A diet rich in the cruciferous vegetable broccoli increased the CYP1A2 ratio. This was expected since a diet containing comparable amounts of Brussels sprouts and cabbage induced the elimination of antipyrine and phenacetin (32). The effect in the present study was rather small possibly due to the fact that the broccoli was processed very freshly. Thus, the supposed active constituents, indolyl glucosinolates, may have been only partly degraded which is paramount to their inducing effect, as demonstrated in a rat study (unpublished observations). After storage cruciferous vegetables may have a greater enzyme inducing potency, yet amounts unrealistic in a daily diet are

required to be comparable with the effects of smoking and exercise.

In vitro CYP1A2 catalyzed hydroxylation leads to bioactivation of carcinogens, particularly aromatic amines, including azo dyes, cigarette smoke and cooked food mutagens (16,20–22), and to a lesser extent polycyclic aromatic hydrocarbons and aflatoxin (41,43,44). Many of these aromatic carcinogens are also inducers of the enzyme activity *per se* (40,45–48). CYP1A2 has also been shown to be involved in the bioactivation of paracetamol to its hepatotoxic metabolite (49).

The risk of contracting lung cancer is increased in smokers with high inducibility of the Ah-receptor controlled CYP1A subfamily as measured in lymphocytes (50). This inducibility and cancer risk also correlate with the elimination rate of antipyrine, an unspecific probe and substrate of several hepatic cytochrome P450 forms, probably including CYP1A2 (51). Similarly, the CYP1A2 ratio of caffeine metabolites would be expected to reflect AHH inducibility, although this has yet to be proven and the ratio shows no polymorphism in the population. Considering the importance of CYP1A2 in carcinogen activation and the epidemiological data available so far, the CYP1A2 ratio must have considerable implications for health and the risk of development of diseases, that however remain to be demonstrated in man.

Xanthine oxidase

Little is known of the regulation of XO. During tissue ischaemia xanthine dehydrogenase transforms to XO, which is then available for oxidation of accumulated hypoxanthine, a process that generates hydrogen peroxide and free oxygen radicals, once oxygen is resupplied (23). This mechanism is thought to be important in reperfusion injury (23) and may act similarly in infected tissue and other inflammatory damage (24). Allopurinol inhibits XO and decreases the XO caffeine metabolite ratio as verified by Grant *et al.* (13).

In the present 335 subjects the distribution of the XO ratio was normal and unimodal. By contrast, Kalow *et al.* found very low ratios in 4/178 subjects (19). In that study as well as in the present data there were no gender-related effects on the XO ratio. However, in a population study of XO in human liver tissue preparations the activity was 21% higher in samples from men than from women with a suggestion of bimodality of the distribution in the latter group (52).

In the present population study the only independent predictor of the XO ratio was the caffeine consumption, the effect being rather small, i.e. a 2% increase per cup of coffee equivalent per day; however, the 30 day training program increased the ratio by 42%. This effect could be related to tissue hypoxia during the vigorous exercise. In the population sample, exercise of modest average extent was not a significant predictor of the XO ratio in the multivariate analysis, suggesting that only intense exercise is relevant. In agreement, uric acid concentration in plasma, presumably a product of XO, is increased by only near-maximal exercise workloads (53,54). Moreover, only exhaustive exercise eliciting maximal oxygen consumption has been shown to increase markers of free oxygen radical damage (55).

The implications of increased XO activity for health and the risk of disease has yet to be determined. Considering the available data, XO could be relevant for the pathogenesis of some of the many diseases, including ageing and cancer, in which free oxygen radicals have been implicated and antioxidant therapy may prove important (56,57). Moreover, xanthine oxidase is also involved in the formation of reactive metabolites, such as semi- and hydroquinones from estrogen quinones (25). The involvement of XO in disease has yet to be proven in man. However, the caffeine metabolite ratio provides a powerful tool for large scale investigations in this respect.

N-Acetyl transferase

The activity of NAT in the liver is controlled by two alleles at a single autosomal gene locus, with slow acetylators being homozygous (rr) for the recessive allele (6). The 53% frequency of slow acetylators in the present study is in complete agreement with previous phenotyping of Danish population samples by means of sulfamethazine (58). The antimode was in complete agreement with the published values (11,12). The NAT ratio discriminated slow acetylators with only 1% error according to the density function whereas the heterozygous were phenotyped as slow acetylators in 3/100 cases. The longitudinal parts of the present study also confirmed the reproducibility of the phenotyping shown previously by others (11,12,59,60). The present distribution did not show the originally described second antimode (11,12,18,61), that presumably discriminates homozygous (RR) fast acetylators from heterozygous (Rr) intermediate acetylators. However, several other studies have also failed to find this second antimode (59,60,62).

Acetylation of carcinogenic arylamines and hydroxylamines from cooked food or industrial exposure to compounds such as azo dyes may be a toxification or detoxification process dependent on the organ system involved, e.g. colon and bladder respectively (6). Thus, the slow acetylator phenotype occurs at increased frequency among patients with bladder cancer, particularly in those occupationally exposed to carcinogenic arylamines (6). By contrast, the fast acetylator phenotype is overrepresented among colon cancer patients (63,64).

Potential limitations of the caffeine metabolite ratios

Less than 3% of the subjects participating in the present study presented with insufficient caffeine metabolites in their urine samples for estimation of the ratios. These subjects may have ingested less than assumed caffeine, e.g. by inadvertent use of low strength or decaffeinated coffee or herbal tea. Alternatively, the metabolic pathways related to the metabolite ratios were only minor in these subjects. Thus, caffeine is also hydroxylated to 1,3,7-trimethyluric acid as well as 3- and 7-demethylated (11). Little is known of the regulation of the enzymes involved in these pathways. Moreover, the availability of the substrate, 17X, for the metabolite ratios relies on CYP1A2 catalyzed demethylation of caffeine (Figure 1), representing a potential complication.

Possible applications of the caffeine metabolite ratios

The enzyme activities measurable from caffeine metabolism are especially relevant for the bioactivation of potentially toxic compounds and have retrospectively been linked to a variety of disease states, particularly cancer (4,7,9,10). The assessment of caffeine metabolism by means of spot urine ratios of metabolites from dietarily ingested caffeine is ideal for studies of the regulation and subsequent health aspects of the enzymes metabolizing foreign compounds. Moreover, this simple and non-invasive method may be expanded with similar supplementary indices, e.g. the 6β-hydroxycortisol to cortisol ratio in urine, reflecting cytochrome P4503A activity (65). Thus, investigation of relations between the enzyme activities and the development of disease in large-scale prospective studies or nested case control designs, using urine samples from biological banks, is ethically, technically and practically feasible. Other important applications of the caffeine metabolite ratios and similar non-invasive measurements of the enzyme activities relevant for (de)toxifica-

tion of xenobiotics include studies of the effects of environmental factors, such as occupational exposure to chemicals, diet intervention and physical activity.

In conclusion, the metabolite ratios of dietarily ingested caffeine proved reliable estimates of enzyme activities important for bio(in)activation of foreign compounds. In addition to the expected effects on CYP1A2 of smoking, a diet rich in cruciferous vegetables, use of oral contraceptives and pregnancy, the activity of this enzyme as well as XO was substantially increased by intense exercise.

Acknowledgements

We are grateful to Jeanette Otte, Chemistry Department, Royal Veterinary and Agricultural University, Copenhagen, Denmark, for the analysis of glucosinolates in broccoli. Parts of the material were presented at IV International Symposium on Biological Reactive Intermediates IV (1) and XII European Workshop on Drug Metabolism. Supported by the Danish Medical Research Council (grant no 12-9374) and The Foundation of Max and Anne Friedman. We would like to thank Captain Thorjin Eriksen of the Danish Army for his help with the exercise study.

References

1. Vistisen,K., Loft,S. and Poulsen,H.E. (1990) Cytochrome P450 IA2 activity in man measured by caffeine metabolism: effect of smoking, broccoli and exercise. *Adv. Exp. Med. Biol.*, **283**, 407–411.
2. Guengerich,F.P. and Liebler,D.C. (1985) Enzymatic activation of chemicals to toxic metabolites. *Crit. Rev. Toxicol.*, **14**, 259–307.
3. Parke,D.V. (1987) Activation mechanisms to chemical toxicity. *Arch. Toxicol.*, **60**, 5–15.
4. Guengerich,F.P. (1988) Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res.*, **48**, 2946–2954.
5. Shimada,T., Iwasaki,M., Martin,M.V. and Guengerich,F.P. (1989) Human liver microsomal cytochrome P-450 enzymes involved in the bioactivations of procarcinogens detected by umu gene response in *Salmonella typhimurium* TA 1535/pSK1002. *Cancer Res.*, **49**, 3218–3228.
6. Hein,D.W. (1988) Acetylator genotype and arylamine-induced carcinogenesis. *Biochim. Biophys. Acta*, **948**, 37–66.
7. Forrester,L.M. and Wolf,C.R. (1990) Genetic susceptibility to environmental insults. In Cohen,R.D., Lewis,B., Alberti,K.G.M.M. and Denman,A.M. (eds), *The Metabolic and Molecular Basis of Acquired Disease*. Ballière Tindall, London, pp. 3–18
8. Gonzales,F.J. (1988) The molecular biology of cytochrome P450s. *Pharmacol. Rev.*, **40**, 243–288.
9. Loft,S. (1990) Metronidazole and antipyrine as probes for the study of foreign compound metabolism. *Pharmacol. Toxicol.*, **66**, 1–31.
10. Gonzales,F.J., Jaiswal,A.K. and Nebert,D.W. (1987) P-450 genes: evolution, regulation and relationship to human cancer. In *Cold Spring Harbor Symposia on Quantitative Biology: Molecular Biology of Homo Sapiens*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 879–890.
11. Grant,D.M., Tank,B.K. and Kalow,W. (1983) Variability in caffeine metabolism. *Clin. Pharmacol. Ther.*, **33**, 591–602.
12. Grant,D.M., Tank,B.K. and Kalow,W. (1984) A simple test for acetylator phenotype using caffeine. *Br. J. Clin. Pharmacol.*, **17**, 459–464.
13. Grant,D.M., Tang,B.K., Campbell,M.E. and Kalow,W. (1986) Effect of allopurinol on caffeine disposition in man. *Br. J. Clin. Pharmacol.*, **21**, 454–458.
14. Campbell,M.E., Spielberg,S.P. and Kalow,W. (1987) A urinary metabolite ratio that reflects systemic caffeine clearance. *Clin. Pharmacol. Ther.*, **42**, 157–165.
15. Campbell,M.E., Grant,D.M., Inaba,T. and Kalow,W. (1987) Biotransformation of caffeine paraxanthine theophylline and theobromine by polycyclic aromatic hydrocarbon-inducible cytochrome(s) P-450 in human liver microsomes. *Drug Metab. Dispos.*, **15**, 237–249.
16. Butler,M.A., Iwasaki,M. and Guengerich,F.P. (1989) Human cytochrome $P\text{-}450_{PA}$ (P-450IA2), the phenacetin *O*-deethylase, is primarly responsible for the hepatic 3-demethylation and *N*-oxidation of carcinogenic arylamines. *Proc. Natl. Acad. Sci. USA*, **86**, 7696–7700.
17. Kalow,W. and Tang,B.K. (1991) Caffeine as a metabolic probe: exploration of the enzyme-inducing effect of cigarette smoking. *Clin. Pharmacol. Ther.*, *49*, 44–48.
18. Tang,B.K., Kadar,D., Iriah,J., Yip,J. and Kalow,W. (1991) Caffeine as a metabolic probe: validation of its use for acetylator phenotyping. *Clin. Pharmacol. Ther.*, **49**, 648–657.
19. Kalow,W. and Tang,B.K. (1991) Use of caffeine metabolite ratios to explore CYP1A1 and xanthine oxidase activities. *Clin. Pharmacol. Ther.*, **50**, 508–519.
20. McManus,M.E., Felton,J.S., Knize,M.G., Burgess,W.M., Roberts-Thomsen,S., Pond,S.M., Stupans,I. and Veronese,M.E. (1989) Activation of the food-derived mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by rabbit and human liver microsomes and purified forms of cytochrome P-450. *Carcinogenesis*, **10**, 357–363.
21. Butler,M.A., Guengerich,F.P. and Kadlubar,F.F. (1989) Metabolic oxidation of the carcinogens 4-aminobiphenyl and 4,4'-methylene-bis(2-chloroaniline) by human hepatic microsomes and by purified hepatic cytochrome P-450 monooxygenases. *Cancer Res.*, **49**, 25–31.
22. Wallin,H., Mikalsen,A., Guengerich,F.P., Ingelman-Sundberg,M., Solberg,K.E., Rossland,J. and Alexander,J. (1990) Differential rates of metabolic activation and detoxification of the food mutagen 2-amino-1-methyl-6-phenenylimidazo[4,5-*b*]pyridine by different cytochrome P450 enzymes. *Carcinogenesis*, **11**, 489–492.
23. Simpson,P.J. and Lucchesi,B.R. (1987) Free radicals and myochardial ischaemia and reperfusion injury, *J. Lab. Clin. Med.*, **110**, 13–30.
24. Oda,T., Akaike,T., Hamamoto,T., Suzuki,F. and Maeda,H. (1989) Oxygen radicals in influenza-induced pathogenesis and treatment with pyran polymer-conjugated SOD. *Science*, **244**, 97–976.
25. Roy,D., Kalyanaraman,B. and Liehr,J.G. (1991) Xanthine oxidase-catalyzed reduction of estrogen quinones to semiquinones and hydroquinones. *Biochem. Pharmacol.*, **42**, 1627–1631.
26. Parsons,W.D. and Neims,A.H. (1978) Effect of smoking on caffeine clearance. *Clin. Pharmacol. Ther.*, **24**, 40–45.
27. Callahan,M.M., Robertson,R.S., Branfman,A.R., McComish,M.F. and Yessair,D.W. (1983) Comparison of caffeine metabolism in three nonsmoking populations after oral administration of radiolabelled caffeine. *Drug. Metab. Dispos.*, **11**, 211–217.
28. Rietveld,E.C., Brockman,M.M., Houben,J.J., Eskes,A.B. and van Rossum,J.M. (1984) Rapid onset of an increase in caffeine residence time in young women due to oral contraceptives. *Eur. J. Clin. Pharmacol.*, **26**, 371–373.
29. Abernethy,D.R. and Todd,E.L. (1985) Impairment of caffeine clearance by chronic use of low-dose oestrogen-containing oral contraceptives. *Eur. J. Clin. Pharmacol.*, **28**, 425–428.
30. Joeres,R., Klinker,H., Heusler,H., Epping,J., Hofstetter,G., Drost,D., Reuss,H., Zilly,W. and Richter,E. (1987) Factors influencing the caffeine test for cytochrome P 448-dependent liver function. *Arch. Toxicol.*, **60**, 93–94.
31. Knutti,R., Rothweiler,H. and Schlatter,C.H. (1981) Effect of pregnancy on the pharmacokinetics of caffeine. *Eur. J. Clin. Pharmacol.*, **21**, 121–126.
32. Pantuck,E.J., Pantuck,C.B., Garland,W.A., Min,B.H., Wattenberg,L.W., Anderson,K.E., Kappas,A. and Conney,A.H. (1979) Stimulatory effect of Brussels sprouts and cabbage on human drug metabolism. *Clin. Pharmacol. Ther.*, **25**, 88–95.
33. Boel,J., Andersen,L.B., Rasmussen,B., Hansen,S.H. and Døssing,M. (1984) Hepatic drug metabolism and physical fitness. *Clin. Pharmacol. Ther.*, **36**, 121–126.
34. Cheng,W.S.C., Murphy,T.L., Smith,M.T., Cooksley,W.G.E. Halliday,J.W. and Powell,L.W. (1990) Dose-dependent pharmacokinetics of caffeine in humans: relevance as a test of quantitative liver function. *Clin. Pharmacol. Ther.*, **47**, 516–524.
35. Nagy,M. (1974) Caffeine content of beverages and chocolate. *J. Am. Med. Assoc.*, **229**, 337.
36. Lelo,A., Miners,J.O., Robson,R. and Birkett,D.J. (1986) Assessment of caffeine exposure: caffeine content of beverages, caffeine intake, and plasma concentrations of methylxanthines. *Clin. Pharmacol. Ther.*, **39**, 54–59.
37. Sørensen,H. (1990) Glucosinolates: structure-properties-function. In Shahidi,F. (ed.), *Rapeseed/Canola: Production, Chemistry, Nutrition and Processing Technology*. Van Nostrand Reinhold, Boston, MA, pp. 149–172.
38. Bjerg,B. and Sørensen,H. (1987) Quantitative analysis of glucosinolates in oilseed rape based on HPLC of desulfoglucosinolates and HPLC of intact glucosinolates. In Wathelet,J.-P. (ed.), *World Crops: Production, Utilization, Description, Glucosinolates in Rapeseeds: Analytical Aspects*. Martinus Nijhoff/Kluwer, Dordrecht, pp. 125–150.
39. Tang,B.K., Kadar,D. and Kalow,W. (1987) An alternative test for acetylator phenotyping with caffeine. *Clin. Pharmacol. Ther.*, **42**, 509–513.
40. Sesardic,D., Boobis,A.R., Edwards,R.J. and Davies,D.S. (1988) A form of cytochrome P450 in man ortologous to form d in the rat catalyse the *O*-deethylation of phenacetin and is inducible by cigarette smoking. *Br. J. Clin. Pharmacol.*, **26**, 363–372.
41. Shimada,T., Martin,M.V., Pruess-Schwartz,D., Marnett,L.J. and Guengerich,F.P. (1989) Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo(*a*)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(*a*)-

pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Res.*, **49**, 6304–6312.
42. Bruguerolle,B., Toumi,M., Faraj,F., Vervloet,D. and Razzouk,H. (1990) Influence of the menstrual cycle on the theophylline pharmacokinetics in asthmatics. *Eur. J. Clin. Pharmacol.*, **39**, 59–61.
43. Faletto,M.B., Koser,P.L., Battula,N., Townsend,G.K., Maccubbin,A.E., Gelboin,H.V. and Gurtoo,H.L. (1988) Cytochrome P_3-450 cDNA encodes aflatoxin B_1-4-hydroxylase. *J. Biol. Chem.*, **263**, 12187–12189.
44. Forrester,L.M., Neal,G.E., Judah,D.J., Glancey,M.J. and Wolf,C.R. (1990) Evidence for involvement of multiple forms of cytochrome P-450 in aflatoxin B1 metabolism in human liver. *Proc. Natl. Acad. Sci. USA*, **87**, 8306–8310.
45. Pelkonen,O. and Nebert,D.W. (1982) Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis. *Pharmacol. Rev.*, **34**, 189–222.
46. Degawa,M., Tanimura,S., Agatsuma,T. and Hashimoto,Y. (1989) Hepatocarcinogenic heterocyclic aromatic amines that induce cytochrome P-448 isozymes, mainly cytochrome P-448H (P-450IA2), responsible for mutagenic activation of the carcinogens in rat liver. *Carcinogenesis*, **10**, 1119–1122.
47. Ayrton,A.D., McFarlane,M., Walker,R., Neville,S. and Ioannides,C. (1990) The induction of P450 I proteins by aromatic amines may be related to their carcinogenic potential. *Carcinogenesis*, 11, 803–809.
48. Ayrton,A.D., McFarlane,M., Walker,R., Neville,S., Coombs,M.M. and Ioannides,C. (1990) Induction of the P-450 I family of proteins by poycyclic aromatic hydrocarbons: possible relationship to their carcinogenicity. *Toxicology*, **60**, 173–186.
49. Raucy,J.L., Lasker,J.M., Lieber,C.S. and Black,M. (1989) Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2. *Arch. Biochem. Biophys.*, **271**, 270–283.
50. Kouri,R.E., McKinney,C.E., Slomiany,D.J., Snodgrass,D.R., Wray,N.P. and McLemore,T.L. (1986) Positive correlation between high aryl hydrocarbon hydroxylase activity and primary lung cancer as analyzed in cryopreserved lymphocytes. *Cancer Res.*, **42**, 5030–5037.
51. Kellermann,G., Jett,J.R., Luyten-Kellermann,M., Moses,H.L. and Fontana,R.S. (1980) Variation of microsomal mixed function oxidase(s) and human lung cancer. *Cancer*, **45**, 1438–1442.
52. Guerciolini,R., Szumlanski,C. and Weinshilboum,R.M. (1991) Human liver xanthine oxidase: nature and extent of individual variation. *Clin. Pharmacol. Ther.*, **50**, 663–672.
53. Sutton,J.R., Toewa,C.J., Ward,G.R. and Fox,I.H. (1980) Purine metabolism during strenuous muscular exercise in man. *Metabolism*, **29**, 254–260.
54. Hellsten,Y., Ahlborg,G., Jensen-Urstad,M. and Sjödin,B. (1988) Indication of in vivo xanthine oxidase activity in human skeletal muscle during exercise. *Acta Physiol. Scand.*, **134**, 159–160.
55. Lovlin,R., Cottle,W., Pyke,I., Kavanagh,M. and Belcastro,A.N. (1987) Are indices of free radical damage related to exercise intensity. *Eur. J. Appl. Physiol.*, **56**, 313–316.
56. Ames,B.N. (1989) Endogenous oxidative DNA damage, aging, and cancer. *Free Rad. Res. Commun.*, **7**, 121–128.
57. Halliwell,B. (1991) Drug antioxidant effects—a basis for drug selection? *Drugs*, **42**, 569–605.
58. Mommsen,S., Barfod,N.M. and Aagaard,J. (1985) *N*-Acetyltransferase phenotypes in the urinary bladder carcinogenesis of a low-risk population. *Carcinogenesis*, **6**, 199–201.
59. Hardy,B.G., Lemiux,C., Walker,S.E. and Bartle,W.R. (1988) Interindividual and intraindividual variability in acetylation: characterization with caffeine. *Clin. Pharmacol. Ther.*, **44**, 152–157.
60. El-Yazigi,A., Chaleby,K. and Martin,C.R. (1989) Acetylator phenotypes of Saudi Arabians by a simplified caffeine metabolites test. *J. Clin. Pharmacol.*, **29**, 246–250.
61. Kilbane,A.J., Silbart,L.K., Manis,M., Beitins,I.Z. and Weber,W.W. (1990) Human *N*-acetylation genotype determination with urinary caffeine metabolites. *Clin. Pharmacol. Ther.*, **47**, 470–477.
62. Hildebrand,M. and Seifert,W. (1989) Determination of acetylator phenotype in Caucasians with caffeine. *Eur. J. Clin. Pharmacol.*, **37**, 525–526.
63. Lang,N.P., Chu,D.Z.J., Hunter,C.F., Kendall,D.C., Flamming,T.J. and Kadlubar,F.F. (1986) Role of aromatic amine acetyltransferase in human colorectal cancer. *Arch. Surg.*, **121**, 1259–1986.
64. Ilett,K.F., David,B.M., Detchon,P., Castleden,W.M. and Kwa,R. (1987) Acetylation phenotype in colorectal carcinoma. *Cancer Res.*, **47**, 1466–1469.
65. Ged,G., Rouillon,J.M., Pichard,L., Combalbert,J., Bressot,N., Bories,P., Michel,H., Beaune,P. and Maurel,P. (1989) The increase in urinary excretion of 6β-hydroxycortisol as a marker of human hepatic cytochrome P450IIIA induction. *Br. J. Clin. Pharmacol.*, **28**, 373–387.

Received on March 2, 1992; revised on June 15, 1992; accepted on June 17, 1992