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# CAFFEINE AND THEOPHYLLINE METABOLISM IN NEWBORN AND ADULT HUMAN HEPATOCYTES; COMPARISON WITH ADULT RAT HEPATOCYTES

FRANÇOIS BERTHOU,*† DAMRONG RATANASAVANH,‡ DOMINIQUE ALIX,§ DOMINIQUE CARLHANT,‖ CHRISTIAN RICHE‖ and ANDRÉ GUILLOUZO‡

*Laboratoire de Biochimie-Faculté de Médecine, BP-815-29285, Brest Cédex, France; ‡Unité U-49 INSERM, CHR de Pontchaillou, Rennes, France; §Service de Pédiatrie-CHR de Brest, France; and ‖Laboratoire de pharmacologie, Faculté de Médecine de Brest, France

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**Abstract**—Cultured hepatocytes from newborn human (three samples), adult human (eight samples) and adult rat livers were used to study the metabolism of theophylline and caffeine, two drugs of which the metabolic pathways are known to be cytochrome P-450-dependent.

Known metabolic pathways of caffeine *in vivo* were qualitatively maintained. However, only the primary metabolites were formed through oxidative *N*-demethylation giving theophylline, paraxanthine and theobromine and, through C-8 hydroxylation, giving 1,3,7-trimethyluric acid and a ring-opened compound the 6-amino-5[*N*-formylmethylamino]1,3-dimethyl uracil. The ratio of the three dimethylxanthine metabolites was dependent upon the species (human, rat), development stage (newborn, adult) and environmental factors.

Similarly, theophylline was metabolized as *in vivo* by the demethylation pathway giving, preferentially, 3-methylxanthine and not 1-methylxanthine, and by a C-8 oxidation giving 1,3-dimethyluric acid. In newborn hepatocytes, all pathways were absent except the well-known methylation to caffeine. Moreover, such a methylation also occurred in adult human hepatocytes. This result was explained by the very low metabolic capacity of cultured cells, allowing the detection of only direct metabolites. Indeed, the overall biotransformation of both the methylxanthines by primary cultures of hepatocytes was remarkably weak, confirming previous studies with liver microsomal incubations. Thus the metabolism rate did not exceed about 30 nmoles/$10^6$ cells/24 hr in human adults, except for two subjects which were characterized by an extensive metabolism and a different metabolic profile. These two subjects were probably induced *in vivo* by environmental compounds.

Both quantitative and qualitative data obtained from this study were roughly correlated with other *in vivo* and *in vitro* studies. Overall the experimental model of cultured human hepatocytes was shown to be capable of assessing the metabolic profile of two methylxanthines which is in agreement with the situation encountered *in vivo*. This example suggests that a breakthrough may be brought in new drugs development by the predictability from human hepatocyte culture model to the *in vivo* human situation.

Caffeine (1,3,7-TMX¶) and theophylline (1,3-DMX) are pharmacologically active alkaloids that occur naturally in a variety of plants. These two methylxanthines have been widely used for many decades in the treatment of asthma and more recently in the therapy of apnea in the human newborn [1]. Both compounds are extensively metabolized *in vivo* into a wide number of metabolites [2]. Many studies have demonstrated that the biotransformation of theophylline and caffeine occurred principally in the liver via microsomal cytochrome P-450 monooxygenases [2] and via the soluble enzyme xanthine oxidase for the formation of 1-methyluric acid from 1-methylxanthine [3] which is a metabolite of theophylline as well as of caffeine [4]. A large degree of interindividual variability in the metabolic profiles exists in the adult [5, 6] and neonate [7] human. This variability appears to involve genetic factors [8, 9] in addition to various environmental factors [8]. This has led to propose that methylxanthines, especially caffeine, are model compounds for measuring liver function [10].

Although caffeine and theophylline are reputedly the world's most widely consumed drugs, detailed studies of their biotransformation pathways in man have been scarce until recently. If these two methylxanthines are known to be extensively metabolized *in vivo* [2], many *in vitro* studies carried out, either with explants of fetal human liver [11] or with slices or microsomes from rat [12–17], mouse [18] or human [19, 20] liver, showed a markedly limited metabolism. Only the perfused isolated liver extensively metabolized caffeine [14] as *in vivo*. As noted by the authors themselves [14, 19], such discrep-

† Author to whom correspondence should be addressed.

¶ Abbreviations: 1,3,7-TMX, 1,3,7-trimethylxanthine (CA or caffeine); 1,7-DMX, 1,7-dimethylxanthine (PX or paraxanthine); 1,3-DMX, 1,3-dimethylxanthine (TP or theophylline); 3,7-DMX, 3,7-dimethylxanthine (TB or theobromine); 1,3,7-TMU, 1,3,7-trimethyluric acid (TMU); 1,3,7-DAU, 6-amino-5-[*N*-formylmethylamino]1,3-dimethyluracil; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; 1,3-DMU, 1,3-dimethyluric acid; 3-MX, 3-methyl xanthine; 1-MX, 1-methylxanthine. The other methylxanthines are abbreviated according to the position of the methyl substituent on the xanthine nucleus (MX) or the uric acid nucleus (MU).

ancies remain, as yet, unexplained. Since the *in-vitro* systems including liver slices or microsomes exhibit rapid functional alterations, it may be postulated that intact hepatocytes could represent a more appropriate model to study hepatic metabolism of methylxanthines. Parenchymal cells from various species including man at different ages can be easily prepared and cultured for periods of days or weeks depending on the culture conditions used.

The present study was carried out in order: (i) to evaluate the ability of isolated human hepatocytes to metabolize caffeine and theophylline and (ii) to compare the metabolic pathways of these two methylxanthines in primary cultures of newborn and adult human hepatocytes in comparison with those found in cultured rat hepatocytes.

## MATERIALS AND METHODS

*Chemicals.* Methylxanthines were purchased from commercial sources, Sigma (St. Louis, MO, U.S.A.) or Fluka (Buchs, Switzerland); AFMU and 1,3,7-DAU were kindly supplied by Dr. M. J. Arnaud (Nestec, La-Tour-du-Peilz, Switzerland). The purity of theophylline and caffeine purchased from Sigma was checked by high performance liquid chromatography (HPLC) as described below and exceeded 99.9% with respect to the other methylxanthines.

[$8^{14}$C]Theophylline (Amersham, U.K.) was pure at a level higher than 99.95% by thin-layer-chromatography (TLC). [$8^{14}$C]caffeine was prepared by N-7 methylation of theophylline with methyl iodide according to a method previously described [21]. When necessary these two radioactive methylxanthines were purified by TLC on silicagel F-254 (Merck, Darmstadt, F.R.G.) with three migrations by chloroform/ethanol (9/1; v/v) mixture. No radioactivity was found at retention times similar to those of other methylxanthines.

*Liver samples.* Human livers were obtained from three post-mortem neonates (less than 3 weeks old), eight kidney transplantation donor adults (18–41 years old) who died from traffic accidents. Sampling was made following the recommendations of the local ethical committee. Dietary habits and exposure to environmental chemicals before death were not known. After brain death, liver was removed and immediately processed for hepatocyte isolation. Rat livers were obtained from 200–300 g male Sprague–Dawley animals.

*Cell isolation and culture.* Adult and newborn human hepatocytes were prepared by the two-step collagenase perfusion method as previously described [22]. The cells were seeded at the density of $2.5 \times 10^6$ cells per 28 $cm^2$ Petri dish in 4 ml of nutrient medium composed of a mixture of 75% minimum essential medium and 25% medium 199 containing 10 $\mu$g/ml bovine insulin, 0.2% bovine serum albumin and 10% foetal calf serum. The medium was changed 16 hr later and was supplemented with $3 \times 10^{-6}$ M hydrocortisone hemisuccinate (Roussel-UCLAF, Paris, France).

Rat hepatocytes were isolated by the collagenase perfusion of the whole liver [23]. The cells were seeded and cultured according to the conditions described for human cells. The medium supplemented as described above was renewed 4 hr after cell seeding.

*Incubation of hepatocytes with theophylline or caffeine.* Four and 16 hr after cell seeding, respectively, rat and human hepatocytes were incubated with methylxanthines dissolved in the culture medium at concentrations ranging between $10^{-4}$ and $10^{-3}$ M. These concentrations were found to be non-toxic over a 24-hr incubation in preliminary studies.

Some experiments were performed using [$8^{14}$C]theophylline (specific activity 3.72 mCi/mmol) or [$8^{14}$C]caffeine (specific activity 1.8 mCi/mmole). Other experiments were carried out with identical concentrations of cold substrate.

*Determination of uptake.* In order to determine cell uptake of methylxanthines, hepatocytes were incubated with radioactive caffeine or theophylline at the concentrations of $10^{-4}$, $7.5 \times 10^{-4}$ and $10^{-3}$ M. After a 24-hr incubation, cells and media were separated and their radioactive content was determined. Metabolic profiles of cellular extracts were compared to those of media so that the uptake could be calculated from the radioactivity measured in cells relative to the total radioactivity.

*Evaluation of overall metabolism.* Overall biotransformation was expressed by the percentage of substrate transformed into known metabolites relative to the untransformed substrate, and both the parent drug and metabolites were determined only in culture media. The rate of caffeine or theophylline metabolism was expressed as nanomole/$10^6$ cells/24 hr of transformed substrate.

In order to evaluate possible effects of reaction products on the biotransformation of substrate, the following experiment was carried out: adult rat hepatocytes were incubated for 24 hr with a mixture of substrate and metabolites which consisted of [$8^{14}$C]caffeine at $10^{-4}$ M and the three cold DMX (TP–PX–TB) each at $1.5 \times 10^{-6}$ M. The overall metabolism and metabolic profiles were evaluated by means of the radioactivity contained in all the HPLC peaks.

Control plates contained the nutrient medium supplemented with methylxanthine substrates in the absence of hepatocytes. Similarly, blank plates contained culture medium and hepatocytes without methyxanthines. All the plates were incubated according to the same procedure.

*Metabolic profiles of caffeine and theophylline.* After 24 hr incubation, an aliquot of 4 ml of culture medium and cells were collected and stored at −80° until analysis. The cells were harvested by scraping with a rubber policeman after two washes with HEPES buffer pH 7.4. After thawing they were suspended in phosphate buffer 0.1 M pH 7.4 and sonicated for 30 sec. Culture media and sonicated cells were saturated by ammonium sulfate and then extracted by two volumes of chloroform–isopropanol mixture (85:15; v/v) using a mechanical shaker for 2 hr. After centrifugation at 2500 rpm for 5 min the organic phase was removed, taken to dryness at 40° under nitrogen stream and redissolved in HPLC mobile phase.

*Quantification of methylxanthine metabolites by HPLC.* A model SP-8700 ternary solvent delivery system equipped with a SP-8780 autosampler (Spec-

Table 1. Theophylline and caffeine uptake by cultured hepatocytes

| Origin of cells | Concentration (M) | Theophylline (%) | Caffeine (%) |
|---|---|---|---|
| Rat | $10^{-4}$ | 1.8 ± 0.6 (5) | 1.1 ± 0.1 (4) |
| | 7.5 $10^{-4}$ | 1.5 ± 0.7 (5) | 1.3 ± 0.7 (6) |
| | $10^{-3}$ | 2.4 ± 0.7 (6) | 1.8 ± 0.6 (9) |
| Human newborn | $10^{-4}$ | 3.3 ± 0.8 (5) | 3.6 ± 0.2 (5) |
| | 7.5 $10^{-4}$ | 4.0 ± 0.9 (10) | 4.0 ± 1.0 (11) |
| Human adult | $10^{-4}$ | 2.2 ± 0.5 (6) | 2.2 ± 0.6 (8) |
| | 7.5 $10^{-4}$ | 1.6 ± 0.6 (3) | 1.7 ± 1.0 (3) |
| | $10^{-3}$ | 2.4 ± 0.8 (4) | 1.3 ± 0.1 (4) |

Results are expressed as the percentage of substrate detected in cells relative to culture medium after a 24-hr incubation. SD was calculated from the number (indicated by brackets) of plates incubated with cold or radioactive substrate.

tra-Physics, San Jose, CA, U.S.A.) was used. The column packed with Nucleosil C-18, 5 μm particle size, 25 cm × 4.6 mm I.D. (Macherey-Nagel, Duren, F.R.G.) was eluted at a flow-rate of 1 ml/min. Eluates were monitored by UV absorbance at 280 nm by means of a LDC-Spectromonitor III (LDC, Riviera Beach, FL, U.S.A.) detector. Metabolites were separated using a gradient elution system, the eluent for pump A being tetrahydrofuran, acetic acid and acetonitrile in water (5:4:15:976; v/v) and for pump B tetrahydrofuran, acetonitrile and acetic acid in water (7.5:150:4:838.5; v/v). Solvent A was pumped for 20 min before starting a linear gradient to 30% B in 30 min.

Amounts of each metabolite were expressed as molar percentage of all metabolites quantified in the sample with respect to their relative UV response factor. Metabolites were identified by their retention times compared with those of standards.

## RESULTS

### *Uptake*

Theophylline and caffeine uptake levels by cultured human and rat hepatocytes are shown in Table 1. For both methylxanthines it was very low, not exceeding 4% of the amount of incubated substrate. No obvious difference was observed whatever the origin of cells, age of donors, substrate concentration and nature of methylxanthine. In addition, intra-experiment variation did not exceed 30%. Longer incubation time period (48 hr) did not result in a significant increase of uptake (not shown).

### *Overall metabolism*

Table 2 shows the overall metabolism of caffeine and theophylline at three concentrations. Biotransformation was shown to be remarkably low in all the samples whatever the species, age of donor

Table 2. Overall metabolism of theophylline and caffeine by hepatocyte cultures

| | Concentration (M) | Patients | Sex | Age | Caffeine % | Caffeine Metabolism rate | Theophylline % | Theophylline Metabolism rate |
|---|---|---|---|---|---|---|---|---|
| Human newborn | $10^{-3}$ | N3 | * | | 1.0 | 16.0 | 0.16 | 2.5 |
| | 7.5 $10^{-4}$ | N2 | * | | 1.5 | 18.0 | 0.3 | 3.6 |
| | 7.5 $10^{-4}$ | N1 | M | 1 day | 1.7 | 20.4 | NM | — |
| | $10^{-4}$ | N2 | | | 10.6 | 17.0 | 1.2 | 2.0 |
| Human | $10^{-3}$ | A1 | F | 18 yr | 9.5 | 152.0 | 5.0 | 80.0 |
| | $10^{-3}$ | A2 | M | 41 yr | 15.5 | 248.0 | 9.8 | 156.0 |
| | $10^{-3}$ | A4 | F | 35 yr | 0.5 | 8.0 | 0.7 | 11.2 |
| | $10^{-3}$ | A5 | M | 18 yr | 1.7 | 27.2 | 1.6 | 26.4 |
| | $10^{-3}$ | A7 | M | 36 yr | 0.7 | 11.2 | 0.9 | 14.4 |
| | $10^{-3}$ | A8 | F | 32 yr | 0.3 | 4.8 | NM | — |
| Adult | 7.5 $10^{-4}$ | A4 | F | 35 yr | 0.7 | 8.4 | NM | — |
| | 7.5 $10^{-4}$ | A6 | M | 34 yr | 1.5 | 18.0 | 0.3 | 3.6 |
| | $10^{-4}$ | A3 | F | 32 yr | 2.5 | 4.0 | 1.1 | 1.8 |
| | $10^{-4}$ | A6 | M | 34 yr | 7.7 | 12.3 | 3.3 | 5.3 |
| | $10^{-3}$ | (N = 1) | | | 0.7 | 11.2 | ND | — |
| Rat | 7.5 $10^{-4}$ | (N = 3) | | | 1.1 ± 0.3 | 13.2 ± 3.6 | 0.20 ± 0.05 | 3.0 ± 0.6 |
| | $10^{-4}$ | (N = 4) | | | 5.5 ± 2.0 | 8.7 ± 3.2 | 0.70 ± 0.05 | 1.1 ± 0.2 |

Results are expressed as the percentage of transformed substrate (%) or as nmole of transformed substrate/$10^6$ cells/24 hr. Values represent the mean of three replicates for each experiment.

ND, not determined; NM, not measurable; *, details not available.

and concentration of substrate. However, important interindividual variations were observed in adult samples. Two human cell populations (subjects A1 and A2) were found to metabolize both methylxanthines at levels much greater than the others: up to 9.5 and 15.5% of $10^{-3}$ M caffeine were metabolized vs 0.8% ± 0.6 (four subjects) in the same incubation conditions. The same finding was observed with theophylline.

The percentages of transformed substrate were dependent on substrate concentration in the range tested. However, when expressed as the metabolism rate, very close values were obtained whatever the concentration (Table 2), suggesting that the enzyme(s) was (were) saturated. This was confirmed by an additional experiment with lower methylxanthine concentrations. Thus, when caffeine was incubated with adult rat hepatocytes at concentrations of 0.8 and 9 μM, 8 and 7.5% of substrate were metabolized, respectively and the rates were 0.1 and 1.1 nmole/$10^6$ cells/24 hr, respectively vs 12.2 ± 3.4 (N = 4 rat samples) for concentrations in the range of $10^{-4}$–$10^{-3}$ M (results not shown).

Human newborn hepatocytes transformed caffeine at a rate of about 18 nmoles/$10^6$ cells/24 hr whereas in human adult cells this rate varied between 4 and 27.2 nmoles/$10^6$ cells/24 hr, except in the two highly active adult cell populations mentioned above. Caffeine metabolism velocity was about 10 nmoles/$10^6$ cells/24 hr in adult rat hepatocytes.

The overall metabolism of theophylline was slightly lower than that of caffeine. Its metabolic rate was between 1.8 and 26.1 nmoles/$10^6$ cells/24 hr and about 3 nmoles/$10^6$ cells/24 hr in adult and newborn human hepatocytes, respectively. The overall metabolism was not increased by increasing the incubation time. Thus adult human hepatocytes incubated with theophylline at $10^{-3}$ M metabolized 1.5% of substrate for a 24-hr incubation vs 1.67% for a 48-hr incubation (results not shown).

When adult rat hepatocytes were incubated for 24 hr with a mixture of substrates that contained [$8^{14}$C]caffeine at $10^{-4}$ M and the three DMX at $1.5 \times 10^{-6}$ M, each at a concentration close to that obtained after a 24-hr incubation (see below), the overall metabolism did not decrease. Indeed 8.5% of added caffeine was transformed at a rate of 13.4 nmoles/$10^6$ cells/24 hr. The respective relative ratio of TB : PX : TP was slightly modified during the incubation period from the initial value of 33 : 33 : 33 to the final one of 36 : 43 : 21.

*Metabolic profiles of caffeine*

Fourteen known metabolites retaining their intact purine ring can be formed by *in vivo* biotransformation of caffeine and theophylline [2]. Moreover caffeine may undergo imidazole ring cleavage resulting in the formation of two uracilic metabolites, namely AFMU [24] and 1,3,7-DAU [25]. Because all these compounds are structurally related, a specific and sensitive assay is required for their separation and quantification. Figure 1 illustrates the separation of these compounds that was achieved with the outlined analytical procedure from both the standard mixture (Fig. 1a) and control extracts (Fig. 1b). No interferences with HPLC peaks of all the metabolites were found in control samples.

Metabolic profiles of caffeine were different according to species and development stage. The first step of metabolism consisted of demethylation reactions on one of the three nitrogen atoms bearing a methyl group. The predominant pathway in adult human hepatocytes consisted of N-3 demethylation to paraxanthine (Fig. 1e) except for the cells of higher metabolizers (cases A1 and A2) which exhibited mostly a N-7 demethylation to theophylline (Fig. 1d). Rat hepatocytes gave a nearly equal proportion of TB, PX and TP with a slight predominance of TB (Fig. 1f). Newborn human hepatocytes transformed caffeine only into theophylline (Fig. 1c).

The relative ratio of the first demethylated metabolites of caffeine is shown in Figure 2. In human adult hepatocytes from cases A3 to A8 the main metabolite was paraxanthine that represented more than 55% (range 41–72%) of the three DMX. The metabolic profile of the cells from the two active metabolizers was characterized by high levels of TP. It appears very close to that found in human newborn hepatocytes which formed only this metabolite. The percentages of TB : PX : TP were found, identical in media and cell extracts from rat hepatocytes: 37 : 40 : 23. They were 18 : 61 : 21 and 16 : 55 : 29 in media and cell extracts, respectively from human hepatocytes (cases A3 to A8).

Only five primary metabolites of the 16 caffeine metabolites found in human urine were detected in culture media. Although the three DMX represented quantitatively the most important metabolites, two other oxidated compounds were accurately measured at $10^{-3}$ M. These corresponded to TMU formed by C-8 oxidation and to 1,3,7-DAU derived from hydratation of the 8,9 double bond followed by the opening of the imidazole ring. The main chromatographic property of this latter compound was that it gave two HPLC peaks [25] due to the equilibrium between two rotamers (Fig. 1). In human hepatocytes from cases A3 to A8 these two metabolites represented 3.5%, relatively to the three DMX and the ratio between TMU and 1,3,7-DAU was 2. They represented 20% for rat hepatocytes and their ratio was 0.5.

Secondary metabolites of caffeine such as monomethylxanthines, except for 3-MX, dimethyluric and methyluric acids were not detected. Similarly AFMU was not identified although PX, which was the major caffeine metabolite found both *in vivo* and *in vitro*, has been suggested to be the precursor of this uracilic compound [8].

*Metabolic profiles of theophylline*

Two major metabolites of theophylline, 1,3-DMU and 3-MX, were clearly detected in rat and adult human hepatocytes. They were formed by 1-N demethylation giving 3-methylxanthine and by oxidation on carbon 8 giving 1,3-dimethyluric acid (Fig. 3). The 1,3-DMU : 3-MX ratio was 2.3 ± 0.7, range 1.5–3.3 (Fig. 4) in six adults (cases A3 to A8). In one of the human samples characterized by higher metabolic activity (case A1) this ratio was 13 while in the other 1,3-DMU was not found. Quantitative differences in the metabolic profile were observed in

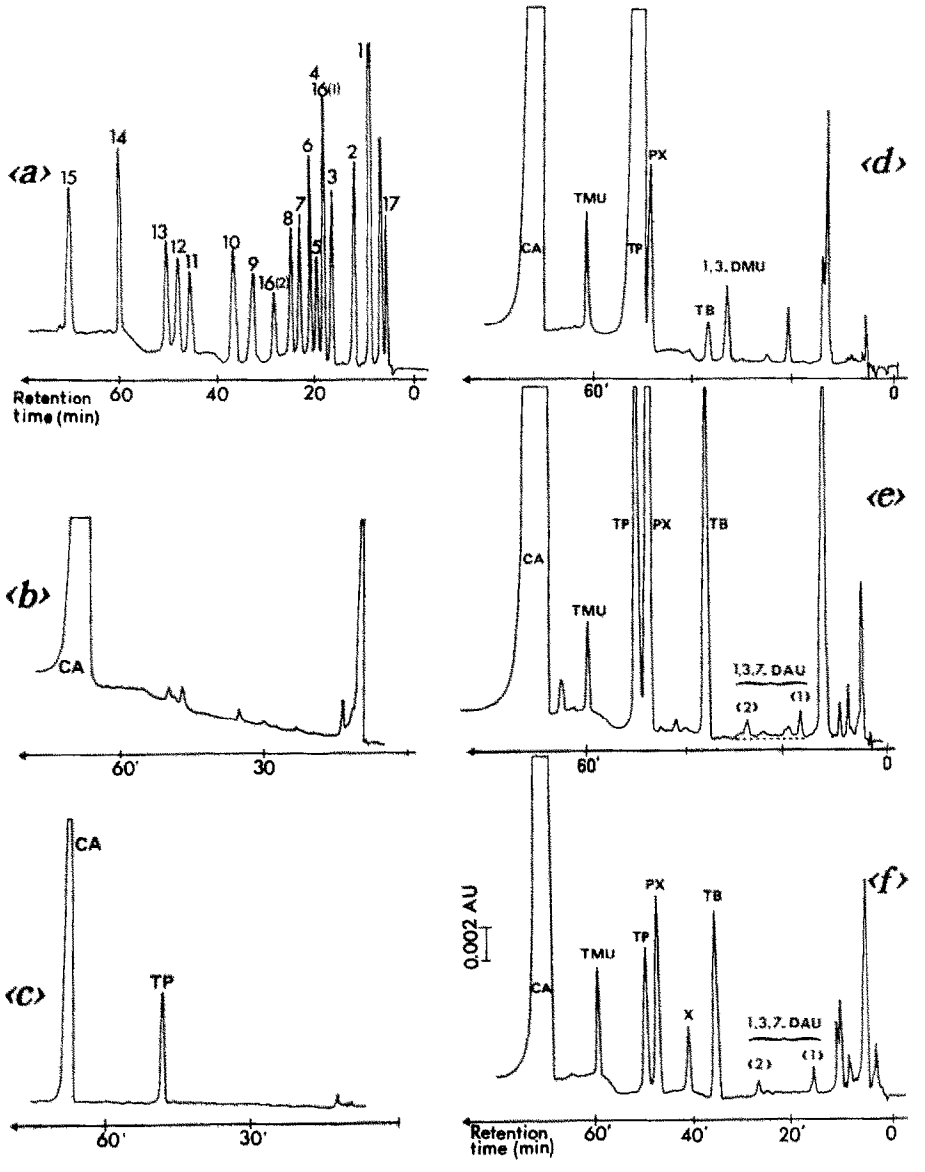


Fig. 1 Typical HPLC chromatograms of extracts of culture medium after a 24-hr incubation of 1 mM caffeine with cultured hepatocytes. (a) HPLC profile of a standard mixture containing 17 possible metabolites. Numbered peaks correspond to the following metabolites: 1, AFMU; 2, 3-MU; 3, 7-MU; 4, 7-MX; 5, 1-MU; 6, 3-MX; 7, 3,7-DMU; 8, 1-MX; 9, 1,3-DMU; 10, TB; 11, 1,7-DMU; 12, PX; 13, TP; 14, 1,3,7-TMU; 15, CA; 16(1) and (2), 1,3,7-DAU; 17, AAMU. (b) HPLC profile of a control plate incubated with caffeine without hepatocytes. (c) Newborn human subject N-2. (d) Adult human subject A-2, extensive metabolizer. (e) Human adult subject A-5. Note the difference in the ratio of the three dimethylxanthines (TB/PX/TP). (f) Adult rat. ×, Indicates a compound not identified yet. For identification of human samples, see Table 2.

adult rat hepatocytes: the 1,3-DMU : 3-MX ratio was 8.8 ± 1.4 (N = 3). 1-Methylxanthine was formed in such low amounts that it was not generally detected although it was not transformed into 1-methyluric acid by the xanthine oxidase (Fig. 3).

In newborn human hepatocytes only the methylation of theophylline to caffeine was demonstrated. This N-7 methylation accounted for about 0.3% of the incubated substrate at $7.5 \times 10^{-4}$ M, i.e. a transformation rate of 3.6 nmoles/$10^6$ cells/24 hr. However preliminary observations suggested that other metabolites could be detected when hepatocytes were cultured for several days (results not shown).

An interesting finding was the substantial metabolic conversion of theophylline to caffeine by adult hepatocytes. This observation was in agreement with the detection of 1,3,7-TMU at trace levels in some samples. Moreover this product could not be detected in control and blank plates (Fig. 3). This pathway was twofold and even tenfold higher than

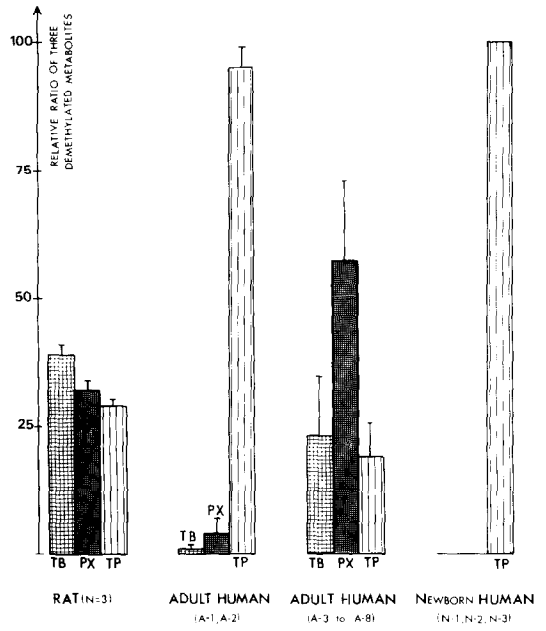


Fig. 2. Relative ratio of three dimethylxanthine (DMX) metabolites formed from caffeine incubated with hepatocyte cultures. TB, theobromine; PX, paraxanthine; TP, theophylline. The relative ratio was calculated as indicated for example: PX/(PX + TP + TB)%. For identification of human samples, see Table 2.

the demethylation reaction to 3-MX in the two categories of studied humans (cases A3 to A8 and cases A1 and A2, respectively) (Fig. 4). In adult rat cells the ratio between caffeine and 3-methylxanthine formed from theophylline was about 4.5.

## DISCUSSION

Theophylline and caffeine are weak bases with low $pK_a$; accordingly they will be unionized in the culture medium. Consequently, though having relatively poor lipophilicity (log P = −0.07 and −0.02 for caffeine and theophylline, respectively), they readily cross biological membranes and will be distributed in body fluids and tissues in approximate proportion to their water content by simple diffusion [26, 27]. As hepatocytes at $0.6 \times 10^6$ cells/ml represent a volume as low as about 1.5% compared to culture medium, their content in methylxanthines should be in the same order of magnitude. Indeed the uptake of caffeine and theophylline by adult human hepatocytes was never greater than about 3%. The similar ratio of demethylated metabolites of caffeine measured into hepatocytes and media are in agreement with the observation that no physiological barriers limit the passage of methylxanthines through tissues [28].

Our results give the first demonstration of the ability of cultured hepatocytes to metabolize caffeine and theophylline. However, they demonstrated that the level of transformation was very low. The substrate concentrations required in these *in-vitro* studies (0.1–1 mM) to produce accurately UV detectable levels of metabolites were considerably higher than those used *in vivo* (0.02–0.1 mM) during therapeutic treatment. This could be related to the low turnover rate of enzyme(s) involved in caffeine or theophylline biotransformation. This low turnover was not due to the accumulation of reaction products in culture medium as demonstrated by the simultaneous incubation of the substrate and metabolites. Similarly low biotransformation of both methylxanthines was obtained by incubating liver slices and microsomes from the same donor (unpublished results), thereby confirming previous observations. Indeed, calculated from the available data reported in the studies conducted with liver microsomes, the following values of overall metabolism were obtained. Caffeine was metabolized between 1.3% [13] and 3% [14] in rat, 1.4% [18] in mouse, about 2% in rabbit [29] and less than 1.5% in ten adult humans (our results) and less than 1% in 6 other adult human [19] livers. Similarly, theophylline was metabolized to a limited extent, below the limit of detectability in control rat [12], about 5% in rabbit [30] and no more than 0.8% in adult humans [20]. In addition, a derivative of theophylline, doxophylline, was transformed at about 5% [31]. *In-vitro* incubation of liver explants from human fetus with theophylline also resulted in the transformation of no more than 1% of substrate [11]. By contrast, caffeine had a higher metabolism in perfused rat liver and after 3 hr only 5% of substrate remained unchanged in the perfusion liquid [14]. Also, it is well known from Cornish and Christman's study [32] that no more than 2% of caffeine was excreted unchanged in human urine [2]. The low metabolic capacity of different *in vitro* models compared to the isolated perfused liver and *in vivo* still remained to be elucidated.

However, these discrepancies between *in vitro* models and *in vivo* could be only apparent. Indeed, a correlation between the different models can be established by considering three parameters, namely the total amount of drug to be transformed, time needed to metabolize a given amount of drug and the number of hepatocytes. This implies that the metabolism rate has to be expressed as nmole of substrate transformed by $10^6$ hepatocytes for 24 hr. Thus, the metabolism rate of caffeine can be calculated and compared in the different systems by postulating that 1 g liver approximately contains $10^8$ cells [33] or 10 mg of microsomal proteins. For example it can be calculated from table 1 of Ref. 19 that the mean biotransformation rate of caffeine by human liver microsomes was 180 pmoles/min·mg protein (in agreement with our unpublished results, 160 ± 95, N = 10 adult humans), i.e. about 260 nmoles/24 hr/mg microsomal protein or $10^7$ cells. The calculated results are summarized in Table 3. These data clearly demonstrate that all the *in-vitro* models such as liver microsomes and hepatocyte cultures metabolize caffeine at the same rate as *in-vivo* and perfused liver. Identical correlation can be obtained with theophylline. Accordingly, although these data are approximate, they allow a clear relationship to be established between *in-vivo* metabolism and metabolism in the hepatocyte system.

Regarding the metabolic profiles of caffeine, our results fit well, from a qualitative point of view, with those obtained in man. Metabolic profiles of caffeine

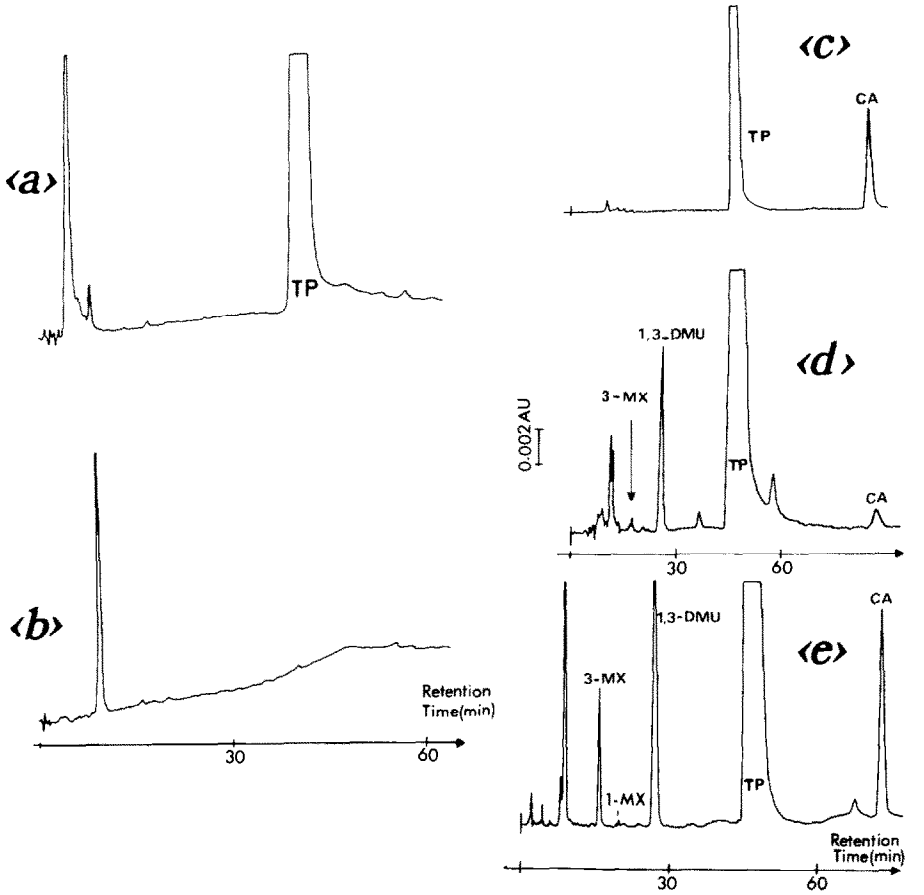


Fig. 3. Typical HPLC chromatograms of extracts of culture medium after a 24-hr incubation of $7.5.10^{-4}$ M theophylline with cultured hepatocytes. (a) Control plate (culture medium with theophylline without hepatocytes); (b) blank plate (culture medium with hepatocytes without theophylline); (c) newborn human subject N-2; (d) adult rat; (e) adult human subject A-5. For identification of human samples, see Table 2.

in human hepatocyte cultures corresponded to those detected in human body fluids after drug intake. In particular, the *in-vivo* selectivity for caffeine N-3 demethylation to paraxanthine was completely retained. This process represented 58% (±15%) of caffeine demethylations in human hepatocytes and for the 37–70% range in human plasma [25, 28, 34–36]. The other pathways of demethylations with the formation of TB and TP, respectively accounted for 23% (±12) and 19% (±6) in human hepatocytes vs

Table 3. Metabolism rates of caffeine calculated from *in vivo* and in three *in vitro* models

| | Liver microsomes Adult human† | Cultured hepatocytes Adult human‡ | Perfused liver Rat§ | *In vivo* Adult human |
|---|---|---|---|---|
| Total amount of drug (μmole) | 0.5 | 0.4 | 1 | 2800* |
| Percent of drug metabolized (time) | 0.5 (15 min) | 7.7 (24 hr) | 95 (3 hr) | 50 (6 hr) |
| Total hepatocytes | $10^{7}$** | $2.5\ 10^{6}$ | $10^{9}$*** | $150\ 10^{9}$**** |
| Metabolic rate nmole/$10^{6}$ cells/ 24 hr | 24 | 12.3 | 7.6 | 17 |

† Results from Ref. [19].
‡ Our results from case A6.
§ Results from Ref. [14].
* Total amount of drug = plasmatic concentration × distribution volume, i.e. 80 μM × 0.5 l/kg × 70 kg.
** 1 mg of microsomal protein, i.e. 0.1 g liver.
*** Whole rat liver = 10 g.
**** Whole adult human liver = 1500 g.

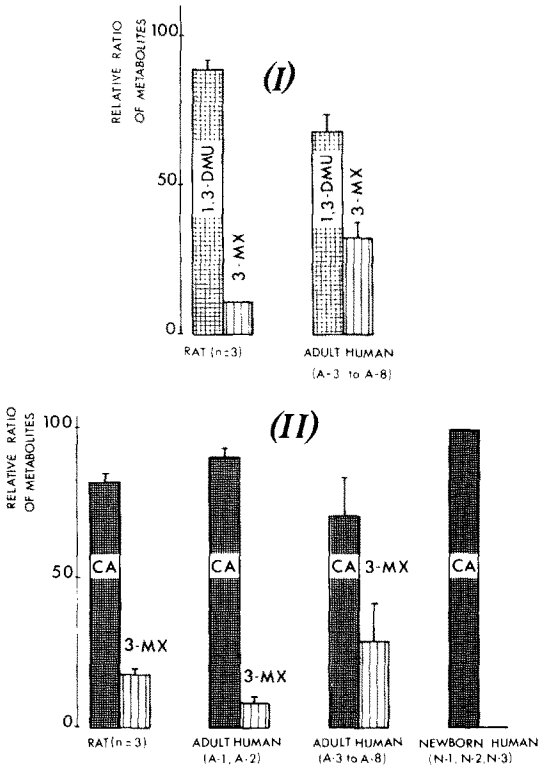


Fig. 4. Relative ratio of 1,3-DMU/3-MX formed from theophylline incubated with hepatocytes (I) and relative ratio of the methylation/demethylation pathways (CA/3-MX) from theophylline substrate (II). For identification of human samples, see Table 2.

about 20% (12–26% range) and 10% (8–11% range), respectively in human plasma.

Species differences between rat and man were also noted. While first demethylations of caffeine were of nearly similar quantitative importance in rat hepatocytes, paraxanthine was produced as a much larger fraction in human hepatocytes, as indicated above. In addition, rat hepatocytes produced a much larger fraction of metabolites formed by C-8 oxidation and hydratation of the 8,9 double bond of caffeine than human cells [36, 37]. These observations agree with those reported *in vivo* [28, 38, 39]. However, this was not true for the two human cell populations (cases A1 and A2) which formed 20 times more metabolites than the six others. The high metabolic activity could be related to *in-vivo* induction of drug metabolizing enzymes. Environmental compounds such as polyaromatic hydrocarbons (PAH) of tobacco smoke have been reported to induce enzymes involved in caffeine metabolism [38–41]. Previous results demonstrated [42] that the higher metabolizers showed a higher (3.5 fold) 7-ethoxyresorufin *O*-deethylation (EROD) activity than normal adults, thereby confirming the correlation between EROD activity and methylxanthine demethylations [43]. This high metabolic capacity was correlated with a dramatic change in the metabolic profile characterized by a predominant theophylline formation. That was not due to the presence of this methylxanthine in the hepatocytes at the time of culturing as demonstrated by the blank incubation. Such a finding suggests the involvement of a cytochrome P-450 isozyme different from that involved in the two other demethylation reactions. This isozyme could be the same as that which mediates caffeine demethylation in the newborn hepatocytes. Such an interpretation is supported by the finding that a substantial part of theophylline formation in human liver microsomes is mediated by a cytochrome P-450 isozyme which is not inhibited by alpha-naphthoflavone [19] contrary to paraxanthine and theobromine formation. Moreover, it was shown by using a caffeine breath test [36] that the 7-demethylation resulting in the formation of theophylline clearly discriminated between smokers and non-smokers.

The production of AFMU from caffeine via paraxanthine was observed with neither hepatocytes nor microsomes [43] probably because it was below the limit of detectability and required acetyl coenzyme A as acetyl donor group [43].

Newborn human hepatocytes were able only to convert caffeine to theophylline, thereby confirming *in-vivo* observations [44]. This suggests that after birth the enzymatic equipment involved in caffeine biotransformation is not fully operative [45]. Our preliminary observations showing that some additional metabolites may be detected after several days of culture fit well with this conclusion and give further support to studies which have reported a transient maturation of fetal newborn hepatocytes in primary culture.

When theophylline, a primary metabolite of caffeine, was used as substrate, the 1,3-DMU was found to be the major metabolite. The second important metabolite was 3-MX resulting from N-1 demethylation. The ratio between C-8 hydroxylation and N-1 demethylation, determined with adult human hepatocytes, was in agreement with those measured *in vivo* after drug intake: 2.3 (±0.7) in human hepatocytes vs 2.8 in human urine [25, 46, 47] and 2.2 in human plasma [48]. The lack of detection of 1-MX or its direct derivative 1-MU was somewhat surprising. Indeed it was suggested that the N-1 and N-3 demethylations of theophylline are carried out by the same cytochrome P-450 isozyme [20, 47, 49]. As our analytical procedure could easily detect 1-MX or 1-MU, we may conclude that either the relative percentages of these two demethylations are very different or 1-MX is a metabolite not produced in cultured hepatocytes.

The overall metabolism of theophylline in cultured hepatocytes was lower than that of caffeine. In addition, as *in vivo* [7], the rate of theophylline biotransformation was significantly lower in newborn hepatocytes than in adult hepatocytes. Indeed about 10% of theophylline is excreted unchanged in urine of adult human after drug intake [2, 46] (vs 2% for caffeine) and 98% in newborn urine [49]. Moreover *in-vitro* incubation of liver explants from 15 weeks old aborted fetuses demonstrated [11] that only 0.04% of theophylline was transformed at a very low rate of 2.5 nmoles/mg protein/24 hr. This low metabolic capacity in the newborn explains the high elimination half-life, 30 hr vs 7 hr in adults [7]. Theophylline

was metabolized by newborn human hepatocytes according to unusual pathways; it was only methylated into caffeine, whereas there was a total absence of *N*-demethylations and oxidative pathways. The methylation pathway has been demonstrated *in vivo* while the occurrence of oxidative metabolites of theophylline in neonate is still controversial [49, 50, 52]. This discrepancy between published results could be explained by the different stages of maturity of different cytochrome P-450 isozymes involved in the metabolism of theophylline and methylxanthines in general [49]. These isozymes are probably PAH-inducible [43] sharing high EROD activity. When this latter enzyme activity was measured in liver microsomes from newborn studied, it was found to be much lower (4.5-fold) than in normal adult (cases A3 to A8) counterpart (results not shown).

Although demonstrated by the results of Tang-Liu and Riegelman [53] the methylation of theophylline was believed to be unique to the newborn [7]. It is possible that such a reaction would be difficult to obtain evidence for adults owing to the fast and extensive metabolism of caffeine. As described above, in hepatocyte cultures the metabolism of methylxanthines was very weak. Thus, the products formed by the first step of biotransformation did not undergo a secondary transformation during a 24-hr incubation, allowing the detection of caffeine.

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