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Biotransformation of caffeine in human liver microsomes from foetuses, neonates, infants and adults

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1 Caffeine metabolism was studied in human liver microsomes from foetuses (n = 10), neonates (n = 10), infants (n = 9) and adults (n = 5). Caffeine and its metabolites, 1-3-7-trimethyluric acid, paraxanthine, theophylline and theobromine, were assayed by h.p.l.c. Methoxyresorufin-*O*-demethylase activity (MEROD) was determined and immunoquantifiable levels of CYP1A2 were measured.

2 The formation of the dimethylxanthines by *N*-3, *N*-7 or *N*-1-demethylation was significantly less in foetuses, neonates and infants than in adults, as shown previously *in vivo*. The formation of 1-3-7-trimethyluric acid (C-8-hydroxylation) was not significantly different between age groups. The production of total dimethylxanthines, paraxanthine and theophylline increased significantly with age within the neonate-infant group over at least the 0–300 day range (r_s = 0.739, 0.667, 0.682, respectively). These data differ from those reported *in vivo* which suggested that *N*-3 and *N*-7-demethylations matured at about 120 days. The difference in maturational profiles of each metabolic pathway suggests that the reactions depend on different isoenzymes. The delay in the maturation of *N*-1 compared with *N*-3 and *N*-7-demethylation is in agreement with previous *in vivo* data.

3 In the neonate-infant group, only *N*-3-demethylation correlated with both MEROD activity (r_s = 0.681; $P < 0.05$) and CYP1A2 microsomal concentration (r_s = 0.454; $P \approx 0.05$), suggesting that, as in adults, this reaction depends on CYP1A2.

4 In the foetal samples, the production of total dimethylxanthines, paraxanthine and theobromine decreased significantly (r_s = –0.879, –0.767, –0.708, respectively) with increasing gestational age. Only CYP3A has previously been detected in human foetal liver; neither CYP1A1 nor CYP1A2 were present, suggesting that the metabolic pathways of caffeine depend on CYP3A at this stage of development.

Keywords caffeine metabolism human microsomes maturation

Introduction

In vivo, numerous metabolites of caffeine have been detected in urine [1, 2], but *in vitro* only the primary metabolites are formed. These are paraxanthine (1-7X), theophylline (1-3X), theobromine (3-7X) and 1-3-7-trimethyluric acid (1-3-7U), obtained from *N*-3-, *N*-7-, *N*-1-demethylation and C-8-hydroxylation, respectively (Figure 1) [3–7]. Developmental changes in caffeine elimination and metabolism have been described in 0 to 19 month old children [8–10]. The profiles of the maturational changes observed *in*

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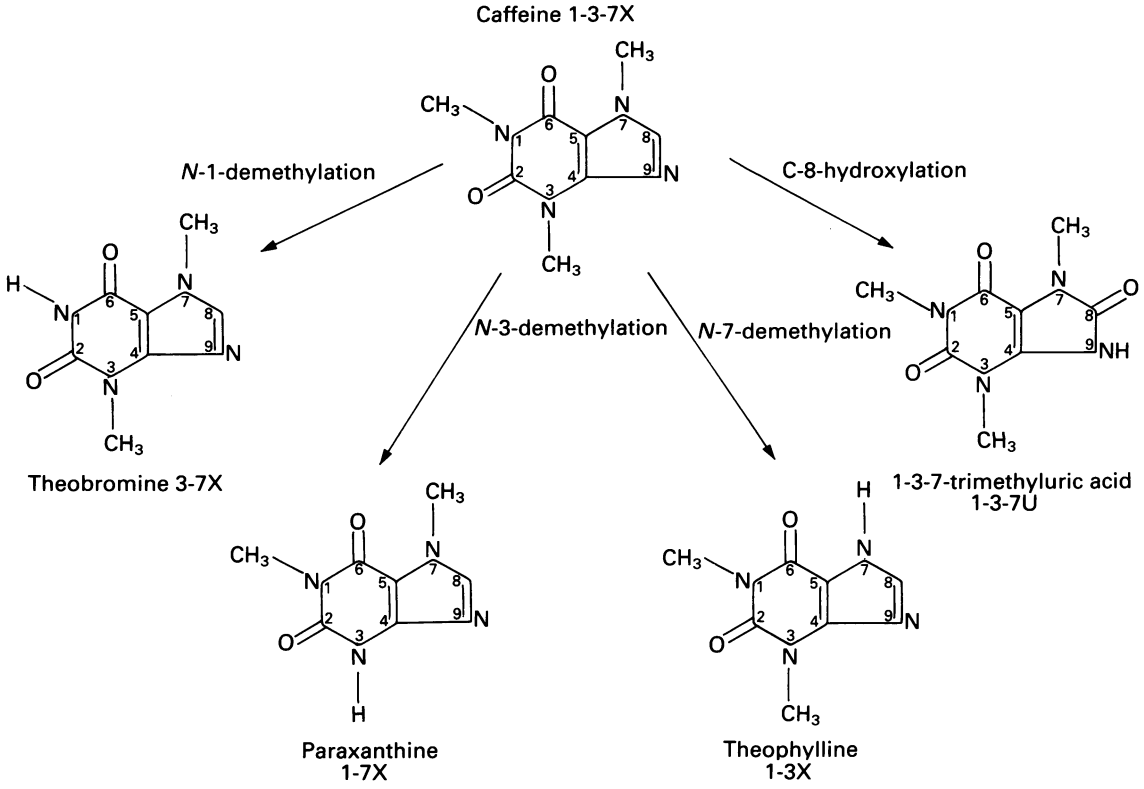

Figure 1 Primary metabolic pathways of caffeine.

vivo vary according to the metabolic pathway, suggesting that they may depend on different cytochromes P450. Total demethylation, *N*-3- and *N*-7-demethylation increase exponentially with post-natal age, a plateau being reached by 120 days. *N*-1-demethylation shows no variation with post-natal age suggesting that maturation of *N*-1-demethylation occurs later than 19 months of age. C-8-hydroxylation is mature as early as 1 month [10].

The aim of this study was to describe the maturational changes of the different metabolic pathways in foetuses, in neonates and in infants using hepatic microsomal preparations, to verify if they are consistent with those previously described *in vivo*, and to determine the relationship between these changes and those of CYP1A2 which is considered to be primarily responsible for caffeine *N*-3-demethylation in adults [11–13].

Methods

Chemicals

Caffeine (1-3-7X) and its metabolites (1-3-7U, 1-3X, 1-7X, 3-7X) as well as NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, acetic acid and paracetamol were obtained from Sigma Chemical Co. (St Louis, MO); $MgCl_2$, KCl, $SO_4(NH_4)_2$, acetonitrile, tetrahydrofuran, chloroform and isopropanol were from Merck (Nogent, France).

Microsomes

Human liver samples were obtained post-mortem from foetuses (n = 10), neonates (n = 10), infants (n = 9) and adults (n = 5) according to the recommendations of the Ethics Committee of INSERM. Sex, age and causes of abortion are summarized in Table 1. Liver donors and mothers of foetuses had no history of drugs known to modify hepatic drug metabolism. No information was available on the smoking habits of the mothers.

Human livers were obtained within the first hour after death and microsomes were prepared as described previously [14]. No difference in the time between death and tissue freezing was noted between age groups. Protein concentration was measured according to Lowry *et al.* [15], cytochrome P450 by the procedure of Omura & Sato [16], methoxyresorufin-*O*-demethylase activity according to Namkung *et al.* [17] and immunoquantified CYP1A2 according to Treluyer *et al.* [18] using a rabbit polyclonal antibody raised against rat CYP1A1. Anti-rat CYP1A1 antibodies reacted with both human CYP1A1 and 1A2 proteins giving two distinct bands on Western blots.

Aliquots of a few milligrams of the microsomal preparations were stored at –80° C until studied.

Biotransformation of caffeine

The metabolism of caffeine *in vitro* was determined by the method of Grant *et al.* [3], with slight

Table 1 Clinical data of liver donors

	Samples	Postnatal age	Gestational age (weeks)	Sex	Cause of death or abortion; remarks
Foetuses	1	–	19	F	Down syndrome
	2	–	22	M	Down syndrome
	3	–	22	F	Down syndrome
	4	–	22	M	Miscarriage, triplet
	5	–	22	F	Miscarriage, triplet
	6	–	24	F	Miscarriage, triplet
	7	–	26	F	Polymalformation
	8	–	33	–	Abnormality of chromosome 12
	9	–	35	M	Down syndrome
	10	–	35	M	Dwarfism
Neonates	11	0.5 h	35	F	Tumour of the respiratory tract
	12	12 h	28	M	Prematurity
	13	18 h	26	M	Twin, 850 g
	14	3.5 d	40	M	Neurologic abnormalities
	15	6 d	39	M	Down syndrome, jaundice
	16	7 d	29	M	Twin,.leukomalacia
	17	7 d	30	M	Infection *E. coli*, haemodynamic problems
	18	8 d	26	M	880 g, cranium bifidum, jaundice
	19	4 wk	29	M	Hydrocephalus, generalised infection
	20	4 wk	42	M	Trisomy 18, hypotrophy
Infants	21	6 wk	29	M	Respiratory distress syndrome
	22	2 mo	–	M	Sudden infant death syndrome
	23	2 mo	–	–	No data available
	24	3 mo	–	–	Sudden infant death syndrome
	25	4 mo	–	M	Sudden infant death syndrome
	26	5 mo	–	F	Myocarditis
	27	5 mo 10 d	–	M	Sudden infant death syndrome
	28	9 mo	–	F	Gastroenteritis, death due to dehydration
	29	10 mo	–	F	Sudden infant death syndrome
Adults	30	–	–	–	Traffic accident
	31	–	–	–	Traffic accident
	32	–	–	–	Traffic accident
	33	–	–	–	Traffic accident
	34	–	–	–	Traffic accident

modifications. Incubation mixtures (total volume 0.5 ml in 10 ml test tubes) contained 0.1 ml potassium phosphate buffer (200 mM, pH 7.4), 0.1 ml of 1.15% w/v KCl in distilled water, 0.1 ml 5 mM caffeine, 0.1 ml of a NADPH generating system (containing 20 mM glucose-6-phosphate, 2mM NADP, 2 u ml^{-1} glucose-6-phosphate dehydrogenase and 10 mM $MgCl_2$) dissolved in phosphate buffer, and 0.1 ml homogeneized liver microsome suspension (at a protein concentration of 3.75 mg ml^{-1} in phosphate buffer). Reactions were started after a 5 min preincubation at 37° C, with the addition of the microsomal suspension. After incubation in a shaking water bath at 37° C for 30 min under air, reactions were stopped by the addition of 5 ml of the extraction solvent (chloroform/isopropanol:85/15; v/v) followed by vigorous vortex mixing for 20 s. The internal standard solution (paracetamol 5 μg ml^{-1} in water) was added 50 μl in assays of microsomes from foetuses, neonates and newborns and 100 μl in assays of adult microsomes) and vortexed. The aqueous phase was saturated by adding about 0.5 g of ammonium sulphate and vortexed for 30 s. After mixing for 30 min and centrifugation, the organic phase was removed and taken to dryness at 50° C under nitrogen. The residue was dissolved in 100 μl of h.p.l.c. mobile phase (solvent B).

Metabolites were assayed by h.p.l.c. according to Berthou *et al.* [6], using a nucleosil C 18 column (SFCC, 250× 4.6 mm, type N 225), two pumps (114M Solvent Delivery System, Beckman), a 421 Controller (Beckman), a u.v. detector (used at 270 nm Spectra 100, Spectraphysics) and an integrator (SP 4000 or Chromjet Integrator, Spectraphysics). Gradient elution was performed at a flow rate of 1 ml min^{-1}, solvent A was tetrahydrofuran-acetic acid-acetonitrile-water (3.3–2.7–10–984) and solvent B was tetrahydrofuran-acetic acid-acetonitrile-water (7.5–2.75–75–914.75). The initial mobile phase consisted of 90% A for 5 min, then a linear gradient to 80% B was performed in 30 min with a step to 50% B in 20 min. Standards were prepared of a mixture of the metabolites (1-7X, 1-3X, 3-7X, 1-3-7U) in a 100 μl volume, each at a concentration of 2 μg ml^{-1}, the internal standard solution (100 μl i.e. 0.5 μg) and phosphate buffer (200 mM, pH 7.4:400 μl for the assays of adult microsomes and 350 μl for the other assays). The detection limit of the method was 0.01 μg ml^{-1} for each metabolite; coefficients of variation were 0.87%, 1.86%, 0.93%

and 1.69% for 3-7X, 1-7X, 1-3X and 1-3-7U, respectively, at a concentration of 2 μg ml^{-1}. Standard curves were linear from 0.05 to 2 μg ml^{-1} for each metabolite. Extraction recoveries were 97% for 3-7X and 1-3X and 96% for 1-7X and 1-3-7U. Retention times of paracetamol, 3-7X, 1-7X, 1-3X, 1-3-7U, 1-3-7X were 22, 25, 33, 34, 38 and 48 min, respectively. This method also allowed detection of most of the other metabolites of caffeine (1-methylxanthine, 3-methylxanthine, 7-methylxanthine, 1,3-dimethyluric acid, 1,7-dimethyluric acid, 3,7-dimethyluric acid, 1-methyluric acid, 3-methyluric acid, 7-methyluric acid, 6-amino-5-*N*-methylformylamino 1,3-dimethyluric acid), but they were not observed in incubation mixtures.

Extent of metabolism:

The extent of metabolism was calculated from:

$$\frac{\text{total amount of metabolites formed (pmol mg}^{-1})}{\text{amount of caffeine substrate in the incubation mixture (pmol)}} \times 100$$

Statistical analysis

Data for the different groups (foetus, neonate, infant, adult) were compared using the non-parametric test of Kruskal and Wallis. When a significant difference was found, pairwise comparisons were then performed using the Mann-Whitney Test.

Relationships between age and metabolite concentrations were assessed in the neonate-infant group and in the foetus group using Spearman's rank correlation (r_s).

Results

Biotransformation of caffeine

Only the primary metabolites of caffeine (1-7X, 1-3X, 3-7X, 1-3-7U) were detected in the different age groups (Table 2). The extent of metabolism was

Table 2 Caffeine metabolite formation by liver microsomes from foetuses, neonates, children and adults

	Sample number	*1-3X*	*1-7X*	*3-7X*	*1-3-7U*	*DMX*	*Total*
Foetuses	1	0.09	1.25	1.66	11.55	3	14.55
	2	0.29	1.98	0.74	24.63	3.01	27.64
	3	0.33	1.02	0.40	2.82	1.75	4.57
	4	0.33	1.54	1.27	18.12	3.14	21.26
	5	0.17	2.01	0.29	22.53	2.47	25.00
	6	0.17	0.27	1.21	24.00	1.65	25.65
	7	0.06	0.61	0	12.26	0.67	12.93
	8	0.19	0	0	23.83	0.19	24.02
	9	0.06	0	0.36	0.26	0.42	0.68
	10	0	0	0.16	7.87	0.16	8.03
Neonates	11	0.12	0	0	3.29	0.12	3.41
	12	0.05	0.45	0.27	14.48	0.77	15.25
	13	0	0.67	0.56	16.17	1.23	17.40
	14	0.32	0	0.59	13.14	0.91	14.05
	15	0	2.14	0.57	7.67	2.71	10.38
	16	0	0.86	0.08	72.84	0.94	73.78
	17	0.07	0	0.05	3.28	0.12	3.40
	18	0	0	0	30.19	0	30.19
	19	2.17	0.29	1.13	26.93	3.59	30.52
	20	1.68	1.35	0.57	12.07	3.60	15.67
Infants	21	1.13	0.46	0	6.36	1.59	7.94
	22	0.31	0	0	1.34	0.31	1.65
	23	1.65	1.49	1.22	10.33	4.36	14.69
	24	0.98	1.09	1.64	11.17	3.71	14.88
	25	2.06	3.59	1.85	6.93	7.50	14.44
	26	0.89	1.32	0	5.49	2.21	7.70
	27	1.35	4.10	0.90	5.78	6.35	12.13
	28	4.06	3.98	1.37	24.45	9.41	33.87
	29	1.17	3.61	0.80	6.65	5.58	12.23
Adults	30	3.84	11.03	3.24	8.48	18.11	26.59
	31	11.58	45.65	9.48	27.36	66.71	94.07
	32	3.92	34.50	5.12	7.28	43.54	50.82
	33	4.12	13.48	3.44	10.89	21.04	31.93
	34	3.05	15.92	2.97	8.80	21.94	30.74

Incubations were performed for 30 min, using a final concentration of 0.75 mg ml^{-1} of microsomal protein and a final concentration of 1 mM caffeine.

Activities are expressed in pmol min^{-1} mg^{-1}; DMX = 1-7X + 1-3X + 3-7X; total = DMX + 1-3-7U.

low and variable in each group: 0.098 ± 0.058% (0.004–0.166) in the foetus group, 0.128 ± 0.124% (0.020–0.433) in the neonate group, 0.080 ± 0.053% (0.010–0.203) in the infant group and 0.281 ± 0.168% (0.160–0.564) in the adult group.

Influence of maturation

Within the adult group, the main metabolite was 1-7X which represented 50.4% of the total; 1-3-7U represented 27.6% and 1-3X and 3-7X 11.4 and 10.6%, respectively (Table 3). In contrast, the main metabolite formed in foetal, neonatal and infant samples was 1-3-7U (respectively 82.9, 91.2, 66.6% of the total), followed by 1-7X (3.9% in neonates; 15.7% in infants) and 3-7X (9% in foetuses).

The amounts of 1-7X, 1-3X and 3-7X, considered separately, were significantly lower in the foetus, neonate and infant groups compared with adults, as was total cytochrome P450. The amounts of 1-7X and 1-3X were significantly higher in the infant group than in the foetus and neonate groups (Table 3). There was no significant difference in the appearance of 1-3-7U across the four age groups.

Within the neonate-infant group, the appearance of total DMX and of 1-7X and 1-3X increased with postnatal age (respectively, $r_s = 0.739$, $r_s = 0.667$, $r_s = 0.682$, $P < 0.01$) (Figure 2). The appearance of these metabolites was lower in the oldest infants than in the adult group (Table 2). No influence of age on the appearance of 3-7X ($r_s = 0.432$, NS) and 1-3-7U ($r_s = -0.228$, NS) was observed.

Within the foetus group, the production of total DMX, 1-7X and 3-7X decreased with gestational age ($r_s = -0.879$, $r_s = -0.767$, $P < 0.01$ and $r_s = -0.708$, $P < 0.05$, respectively) (Figure 3), whereas there was no significant influence of gestational age on the production of 1-3X and 1-3-7U ($r_s = -0.503$ and -0.261, respectively; NS).

In the neonate-infant group only *N*-1-demethylation correlated with the concentration of total cytochrome P450 ($r_s = 0.674$). In contrast, in this group *N*-3-demethylation was correlated with MEROD activity ($r_s = 0.681$, $n = 12$, $P < 0.05$) and, at the limit of significance ($r_s = 0.454$, $P \approx 0.05$), to the immunoquantified microsomal content of CYP1A2. *N*-7-demethylation correlated significantly with immunoquantified microsomal content of CYP1A2 ($r_s = 0.501$), but *N*-1-demethylation ($r_s = 0.035$) and C-8-hydroxylation ($r_s = -0.348$) did not (data not shown).

Discussion

Only the primary metabolites of caffeine were detected using liver microsomes, while fourteen metabolites were recovered *in vivo* in the urine of adult subjects [1, 2]. Consistent with previous findings, only 0.004 to 0.564% of caffeine was metabolized per mg of microsomal protein *in vitro* [3–7]. The metabolic pattern and its interindividual variability in microsomes from adult subjects were similar to those reported previously [3–7, 19].

Rates of reactions (using a substrate concentration of 1 mM) have been compared between age groups. However, in adult human liver microsomes, caffeine exhibits biphasic demethylation kinetics and at a substrate concentration of 1 mM both the high affinity and low affinity isoforms will contribute to caffeine demethylation [2, 4, 6]. Because the liver samples were small we could not characterize caffeine kinetics (K_m, V_{max}) in foetal, neonatal and infant livers. Therefore, we assumed that complexities in the kinetics did not influence the interpretation of the comparative rate data in the various age groups.

We have shown that caffeine metabolism by human liver microsomes undergoes maturational changes, similar to those reported *in vivo* [8–10]. However, the maturation of *N*-3 and *N*-7-demethylation occurs over at least a 0–300 day age range, while the *in vivo* data

Table 3 Comparison of the production of each metabolite of caffeine in the different age groups and total cytochrome P450 content of liver microsomes

	Foetuses n = *10*	*Neonates* n = *10*	*Infants* n = *9*	*Adults* n = *5*	P *(K.W.)*
1-7X	0.87 ± 0.58 (0.81)*† (5.90 ± 4.80 (6.71))	0.58 ± 0.51 (0.71)*† (3.90 ± 4.75 (6.64))	2.18 ± 1.25 (1.62)* (15.67 ± 8.97 (11.67))	24.12 ± 19.35 (15.59) (50.40 ± 13.51 (10.88))	< 0.05
1-3X	0.17 ± 0.09 (0.12)*† (2.30 ± 2.21 (3.09))	0.44 ± 0.57 (0.80)*† (2.60 ± 2.68 (3.75))	1.51 ± 0.82 (1.07)* (12.22 ± 2.54 (3.31))	5.30 ± 4.38 (3.53) (11.40 ± 2.99 (2.41))	< 0.05
3-7X	0.61 ± 0.41 (0.58)* (9.00 ± 11.37 (15.90))	0.38 ± 0.26 (0.37)* (2.30 ± 1.39 (1.95))	0.86 ± 0.55 (0.72)* (5.56 ± 3.75 (4.88)	4.85 ± 3.38 (2.72) (10.60 ± 1.10 (0.89))	< 0.05
1-3-7U	14.79 ± 6.55 (9.15) (82.90 ± 13.73 (19.20))	20.01 ± 14.71 (20.57) (91.20 ± 6.39 (8.93))	8.72 ± 5.03 (6.54) (66.56 ± 10.05 (13.08))	12.56 ± 10.39 (8.37) (27.60 ± 9.80 (7.89))	NS
P450	0.126 ± 0.016 (0.022)	0.106 ± 0.036 (0.051)	0.127 ± 0.063 (0.082)	0.348 ± 0.078 (0.063)	

Results are expressed as mean ± t.s.d./$\sqrt{n}$ (s.d.) where t stands for Student's coefficient corresponding to $(n - 1)$ degrees of freedom and $(1 - \alpha)$ levels of confidence ($\alpha = 5\%$).
Metabolites of caffeine are expressed as pmol min^{-1} mg^{-1} and as (percentage of all metabolites detected, (P450 as nmol mg^{-1} protein)).
K.W.: Kruskal-Wallis test.
Mann-Whitney Test: * = significant/adults, † = significant/infants.

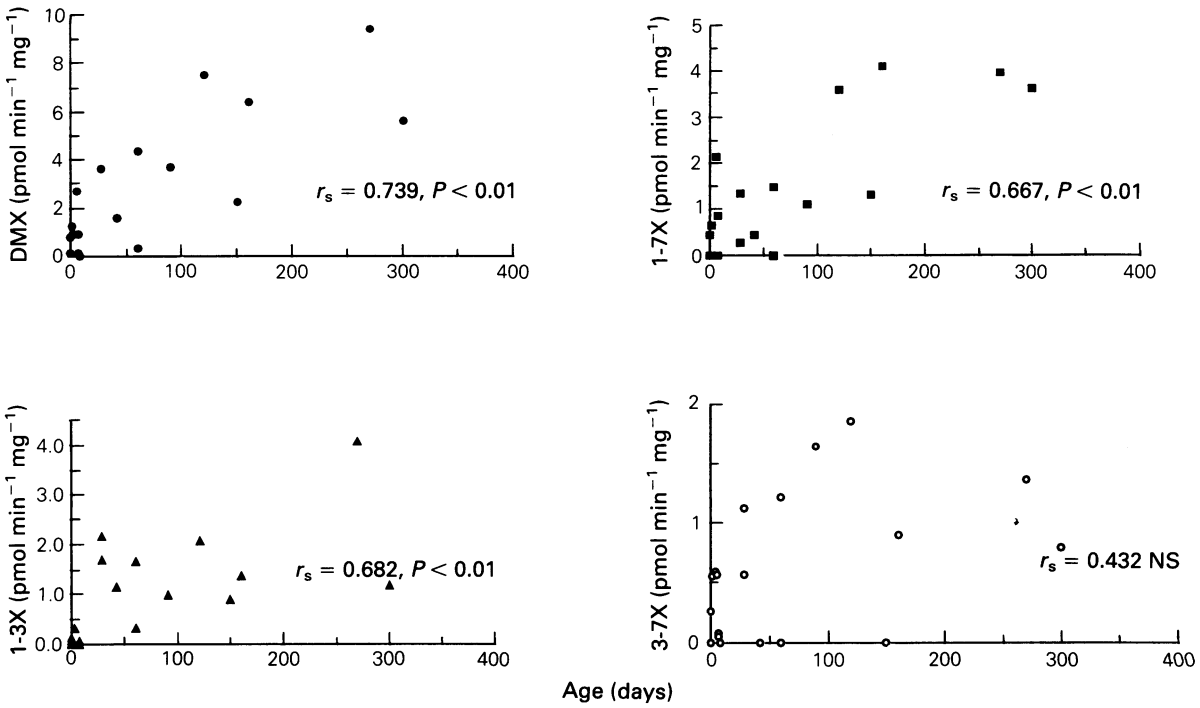

Figure 2 Postnatal changes in caffeine *N*-demethylations in liver microsomes from neonates and infants. Incubation were performed for 30 min, with a protein concentration of 0.75 mg ml^{-1} and a caffeine concentration of 1 mM.

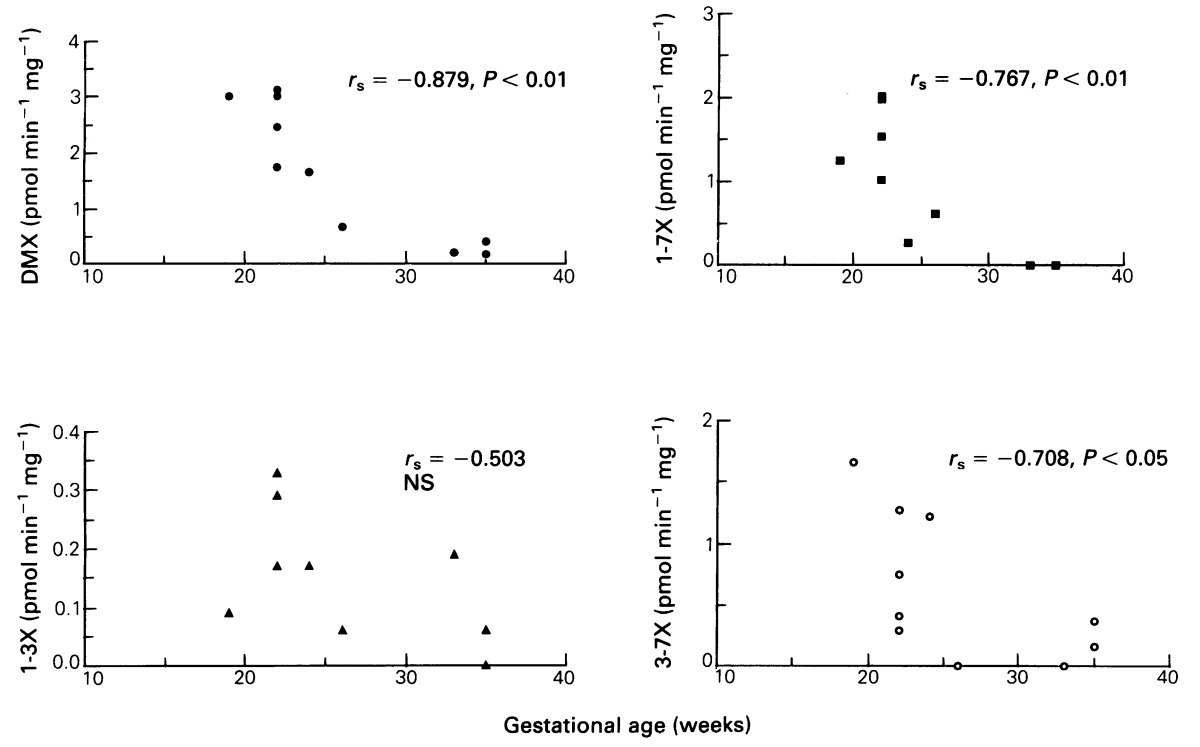

Figure 3 Maturational changes in caffeine *N*-demethylations in foetal liver microsomes. Incubation were performed for 30 min, with a protein concentration of 0.75 mg ml^{-1} and a caffeine concentration of 1 mM.

suggested that these demethylations reach maturity at around 120 days of age [8, 10]. This discrepancy may be attributable to the relatively higher weight of the liver in infants than in adults, the *in vitro* data being expressed with regard to the specific activity of enzyme-containing preparations while *in vivo* data also take the total amount of liver tissue into account. No influence of maturation on *N*-1-demethylation was detected in the 0–300 days age range, while the production of theobromine was higher in adults than in

children suggesting that, as shown *in vivo*, maturation of *N*-1-demethylation appears to be delayed compared with *N*-3 and *N*-7-demethylation [8, 10]. No influence of maturation was found *in vitro* on C-8-hydroxylation while *in vivo* data suggest that maturational changes occur within the first 19 months of life [10]. This apparent discrepancy may be related to the fact that production of 1-3-7U was measured *in vitro* while 1-7U was estimated *in vivo*. These two metabolites may be formed by different cytochromes P450.

The differences in the maturation of the different caffeine metabolic pathways (*N*-3 and *N*-7-demethylations *vs* *N*-1-demethylation and *vs* C-8-hydroxylation) suggest that the reactions depend on different cytochromes P450. In the neonate-infant group, only *N*-1-demethylation was correlated with total cytochrome P450 content, while *N*-3-demethylation was correlated with both MEROD activity (a specific marker of CYP1A2 [17], and the immunoquantified microsomal content of CYP1A2, suggesting that in children as well as in adults [11–13], *N*-3-demethylation is mediated by CYP1A2. *N*-7-demethylation was correlated with the immunoquantified microsomal content of CYP1A2, but *N*-1-demethylation and C-8-hydroxylation were not. Taken together these data are consistent with a dependence, although not exclusive, of *N*-3-demethylation on CYP1A2.

Surprisingly, within the foetus group caffeine *N*-demethylation activity was detectable yet no CYP1A1 nor CYP1A2 has been detected in foetal liver (Cresteil *et al.*, manuscript in preparation). This suggests that caffeine metabolism may depend in the foetus on the major foetal liver cytochrome, CYP3A [14, 20–23]. The C-8-hydroxylation of caffeine may also be mediated by CYP3A. *N*-demethylation activity decreased with increasing gestational age and this may be explained by a decrease in the transcription of the relevant cytochromes P450, possibly due to an hormonal influence during late pregnancy. Such a decrease in the liver content of a cytochrome P450 has already been described. Thus, Kitada *et al.* [22] showed that CYP3A7 content (formerly P450HFLa) varied from 0.08 to 0.04 nmol mg^{-1} liver homogenate protein in foetal livers and was less than 0.03 nmol mg^{-1} (mean: 0.01) in adult livers. Further investigations using foetal liver microsomes are indicated to study the relationship between changes in the content of specific cytochromes P450 and those involved in caffeine metabolism during maturation.

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