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RELATIONSHIP BETWEEN THE CLEARANCE OF CAFFEINE AND ITS 7-*N*-DEMETHYLATION IN DEVELOPING BEAGLE PUPPIES*

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Abstract—Caffeine and eleven of its potential metabolites have been assayed, by high performance liquid chromatography, in the blood and urine of developing beagle puppies. The clearance of caffeine from blood increased from 31.4 ± 6.0 ml·kg^{-1}·hr^{-1} at 2 days to 279.1 ± 45.4 ml·kg^{-1}·hr^{-1} at 29 days of age. This change reflected an increased first-order elimination rate constant since the apparent volume of distribution did not change significantly. Coincident with this increase in clearance, the fraction of a dose of caffeine excreted unchanged in urine decreased 18-fold. The remainder of the dose was metabolized to a series of di- and mono-methylxanthines and urates. In 2-day-old puppies, the caffeine metabolites potentially derived from the paraxanthine, theophylline or theobromine pathway accounted for 42, 33 and 14 per cent, respectively, of the identified products in urine. Between 2 and 22 days of age, the metabolite pattern of caffeine changed substantially, with those metabolites potentially derived from the theophylline pathway (theophylline 1-methylurate, 1-3-dimethylurate and 3-methylxanthine) increasing from 33 to 82 per cent. We suggest, therefore, that the change in caffeine clearance seen during development of the beagle puppy is determined primarily by the rate of maturation of caffeine-7-*N*-demethylase.

Caffeine (1,3,7-trimethylxanthine) is eliminated more slowly by premature and full-term newborn infants than by adult human beings [1, 2]. This finding in itself is not unexpected since it is well known that many drugs that require metabolic oxidation prior to efficient excretion are eliminated more slowly in the neonatal period than in adulthood [3]. However, the magnitude of the deficit with caffeine is more pronounced than that seen with other drugs. The half-life of caffeine in premature infants given the drug for treatment of apnea is approximately 100 hr [1], compared to about 6 hr in non-smoking adults [4]. This deficit is not due to prematurity *per se* or illness, since healthy full-term infants who had received caffeine transplacentally exhibited a mean half-life of caffeine of approximately 80 hr [2]. The urinary metabolite pattern of caffeine in newborn infants reveals a distinct lack of demethylated products, both after a theurapeutic dose of caffeine [5] and after transplacental acquisition [6]. Indeed, during the first month after birth, caffeine itself accounts for 85 per cent of the methylxanthines and methylurates excreted [5]. The fraction of caffeine excreted unchanged in the urine slowly decreases with age such that by age 7–9 months caffeine accounts for only 1–2 per cent of the total methylxanthines and methylurates recovered. The series of partially demethylated xanthines and urates which appear by this age are similar to the metabolites seen in adult urine [5, 7, 8].

Studies of the disposition of caffeine in animals have suggested that the metabolism of caffeine is catalyzed, at least in part, by the cytochrome(s) P-450 mono-oxygenase system [9–12]. Moreover, in rats, caffeine is a particularly good substrate for the form(s) of the enzyme complex induced by polycyclic aromatic hydrocarbons [10–12]. It is tempting, therefore, to postulate that the maturational deficit of caffeine elimination in man reflects the slow postnatal development of this form of the mono-oxygenase complex, but extrapolation between species is hazardous and *in vivo* experiments with the neonatal rat are difficult.

Warszawski *et al.* [13] have found that the elimination half-life of caffeine in newborn mongrel puppies is about 48 hr compared to an adult value of about 6 hr [13, 14]. These results prompted us to initiate detailed studies of the metabolism of caffeine in the dog. Beagle dogs metabolize caffeine primarily by an initial 7-*N*-demethylation to yield theophylline, with the subsequent excretion of 3-methylxanthine, 1,3-dimethylurate and 1-methylurate [15]. The metabolism of caffeine in these animals is stimulated not only by pretreatment with a polycyclic aromatic hydrocarbon, but also by pretreatment with phenobarbital [15]. Phenobarbital did not modify the urinary metabolite pattern, but β-naphthoflavone

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seemed to preferentially stimulate the 3-*N*-demethylation of caffeine. We have now extended our investigation to the beagle puppy in order to explore the relationship between the specific *N*-demethylations of caffeine and the maturation of caffeine clearance.

METHODS

The disposition of single intravenous doses of caffeine was studied in groups of four female beagle puppies at ages 2, 8, 15, 22 and 29 days. The same four puppies were used at 2, 8 and 15 days of age. By day 22 of age, two of the puppies were replaced by more vigorous animals matched for age, sex and weight; one additional substitution was required for study at age 29 days. Each subject was housed with mother and littermates in a separate pen and allowed to suckle feeely. Bitches were allowed free access to food and water.

Solutions of caffeine in 150 mM NaCl were sterilized by passage through a 0.45 μm Millipore filter. At each designated age, caffeine at a dose of 20 mg/kg of body weight was administered via the jugular vein over a 1-min interval. Blood samples (0.1–0.3 ml) were collected in heparinized syringes from the contralateral jugular vein at various times before and after the administration of caffeine and stored at 4° until assay. Urine samples (0.2–2.0 ml) were collected by gentle suprapubic pressure at the same time points as blood collections. Urine was stored at −15° until assay.

Blood (0.1–0.3 ml) was mixed with 0.3 g of ammonium sulfate and sufficient water to achieve a final volume of 0.5 ml before extraction with 15 ml of chloroform–isopropanol (85 : 15, v/v). The mixture was shaken for 30 min and then vortexed for 5 sec to separate the two layers. The upper aqueous layer was aspirated and discarded. Twelve millilitres of the organic layer were transferred to another test tube and evaporated to dryness under nitrogen at 50°. The residue was dissolved in water to give a volume twice that of the initial sample of blood. An aliquot of 50 μl was injected directly into a high performance liquid chromatograph (Waters Associates Inc., Milford, MA). Urine was extracted for caffeine and its metabolites as described previously [5]. Caffeine and eleven of its methylxanthine and methylurate metabolites were assayed as described previously [5] with one modification: a reversed phase silica column (RP-10; Brownlee Labs, Santa Clara, CA) was used instead of μBondapak c_{18} (Waters Associates Inc.) to improve the separation of theophylline and paraxanthine.

Standard curves in blood or water (for urine) were generated for all compounds involved. Caffeine and its metabolites were quantitated by peak height. Under the conditions described, the minimal concentration of each compound in blood or urine that could be detected with confidence was 0.1 μg/ml.

The elimination rate constant was computed using a log–linear least squares regression analysis. C_b^0, the blood concentration at time 0 hr, was estimated from the intercept of the extrapolated blood concentration–time curve. The apparent volume of distribution was calculated by dividing the dose by C_b^0. The clearance of caffeine from blood was calculated as the product of the apparent volume of distribution and elimination rate constant. The areas under the concentration (μg/ml)–time curves (AUC) for blood and urine were calculated by the trapezoidal rule, using the concentration at time 120 hr as 0. In order to assess the relative proportion of each compound excreted in urine, urinary AUC's were converted from $\mu g \cdot ml^{-1} \cdot hr$ to $moles \cdot l^{-1} \cdot hr$ and expressed as percentages of the total identified methylxanthines and methylurates. All values are reported as means ± S.E. No attempt at material balance was made because complete recovery of urine from these puppies was impractical.

Caffeine (as the free base) was obtained from the Sigma Chemical Co., St. Louis, MO. All other reference compounds and chemicals were obtained as described previously [5].

RESULTS

Regardless of age, the beagle puppies exhibited first-order elimination of caffeine when the compound was administered intravenously at a dose of 20 mg/kg (Fig. 1). The data were analyzed according to a one-compartment, open model. During the first few days after birth, caffeine disappeared from blood with an elimination rate constant of 0.035 ± 0.008 hr^{-1} (mean $T_{1/2}$, 23.3 hr; Table 1). The elimination

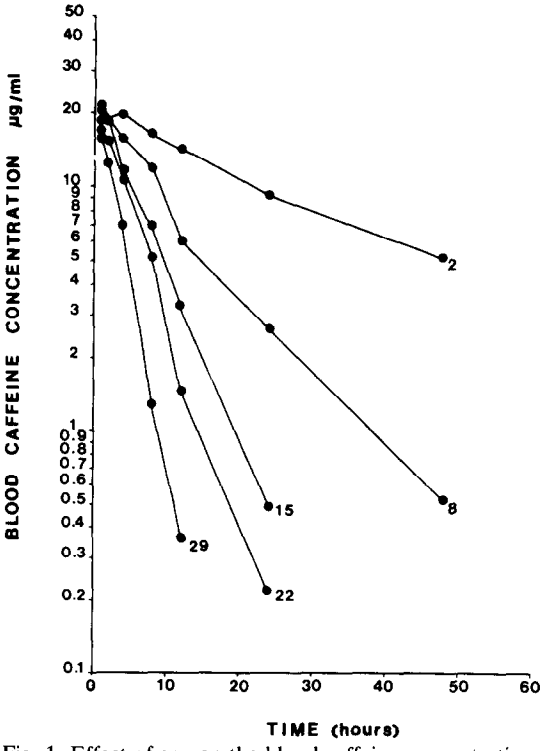

Fig. 1. Effect of age on the blood caffeine concentration–time curves in beagle puppies. Caffeine (20 mg/kg) was administered i.v. and assayed in whole blood by high performance liquid chromatography. Each point represents the mean caffeine concentration from four puppies. The number associated with each is the age, in days, of the puppies at the time of dosing.

Table 1. Pharmacokinetics of caffeine in developing beagle puppies*

Age at dosing (days)	Pharmacokinetic parameters†			
	k_{el}	$T_{1/2}$	AV_d	Cl
2	0.035 ± 0.008	23.3	0.928 ± 0.087	31.4 ± 6.0
8	0.114 ± 0.027	7.4	0.865 ± 0.113	98.8 ± 26.9
15	0.173 ± 0.019	4.2	0.776 ± 0.066	132.1 ± 12.4
22	0.234 ± 0.024	3.1	0.823 ± 0.157	187.1 ± 30.5
29	0.369 ± 0.022	1.7	0.781 ± 0.172	279.1 ± 45.4

* All values are derived from four individual puppies and expressed as means ± S.E.

† The pharmacokinetic parameters studied were elimination rate constant, k_{el} (hr^{-1}), elimination half-life, $T_{1/2}$ (hr), apparent volume of distribution, AV_d ($l \cdot kg^{-1}$) and clearance, Cl ($\mu g \cdot ml^{-1} \cdot hr^{-1}$). The method of calculation of each parameter is described in the text.

rate constant for caffeine increased with increasing age such that by 29 days of age the mean value was 0.369 ± 0.022 hr^{-1} (mean $T_{1/2}$, 1.7 hr). During the first month of life, the apparent volume of distribution of caffeine decreased from 0.928 ± 0.087 $l \cdot kg^{-1}$ (2 days) to 0.781 ± 0.172 $l \cdot kg^{-1}$ (29 days). Clearance of caffeine from blood increased from 31.4 ± 6.0 to 279.1 ± 45.4 $ml \cdot kg^{-1} \cdot hr^{-1}$ over the same time period. The increase in clearance reflected the increase in elimination rate constant since the apparent volume of distribution did not increase, but actually decreased slightly. These values for clearance (Table 1) are essentially the same as those obtained by dividing the dose by the area under the blood concentration–time curves, a finding that supports applicability of the one-compartment, open model.

The disappearance of caffeine from blood depends primarily upon metabolism because the majority of a dose of caffeine was excreted as metabolites in urine, with caffeine accounting for only a small percentage of the identified products. The three initial dimethylxanthine metabolites, paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine), were detected in blood in all age groups (Fig. 2). The peak concentration of theophylline in blood increased 6-fold with increasing age, whereas the increase for paraxanthine and theobromine was much less pronounced. The time to peak concentration of all three dimethylxanthine metabolites decreased with increasing age. The AUC's for caffeine and its metabolites in blood are shown in Table 2. The AUC for theophylline increased from 52.3

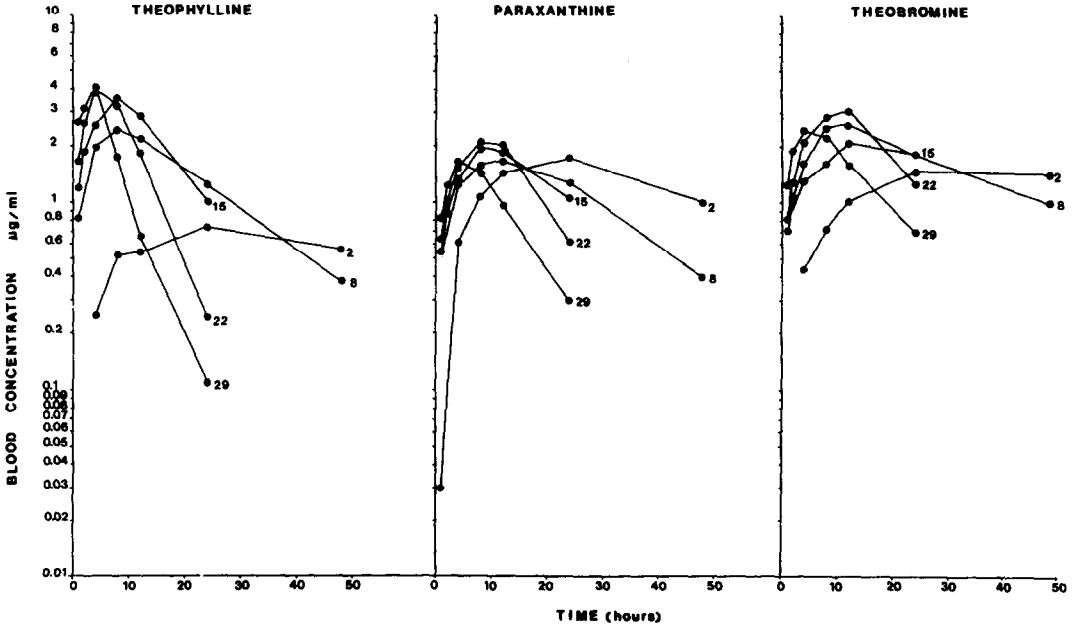

Fig. 2. Effect of age on the blood concentration–time curves of the three dimethylxanthine metabolites of caffeine in beagle puppies. Caffeine (20 mg/kg) was administered i.v. to each puppy, and theophylline, paraxanthine and theobromine were assayed in whole blood by high performance liquid chromatography. Each point represents the mean concentration of each metabolite from four puppies. The number associated with each curve is the age, in days, of the puppies at the time of dosing.

Table 2. Area under the blood concentration–time curves for caffeine and its metabolites in developing beagle puppies*

Compound	Age (days) at dosing				
	2	8	15	22	29
Caffeine	666.3 ± 115.3	280.6 ± 80.2	180.1 ± 23.0	132.7 ± 23.2	81.6 ± 9.9
Theophylline	52.3 ± 6.2	76.6 ± 12.2	103.2 ± 22.8	58.1 ± 7.2	38.3 ± 5.2
Paraxanthine	96.4 ± 18.7	67.8 ± 15.2	86.0 ± 10.1	65.3 ± 7.2	37.3 ± 5.3
Theobromine	130.1 ± 12.8	111.5 ± 10.5	138.8 ± 6.8	114.8 ± 9.9	71.3 ± 8.9
3-Methylxanthine		22.0 ± 9.5	59.6 ± 10.0	80.4 ± 19.0	94.3 ± 21.4

* Area under the blood concentration–time curves estimated by trapezoidal rule, assuming a concentration of zero at time 120 hr. All values are expressed as means ± S.E. ($\mu g \cdot ml^{-1} \cdot hr$) (N = 4).

to 103.2 $\mu g \cdot ml^{-1} \cdot hr$ during the first 15 days of life, and then decreased to 38.3 $\mu g \cdot ml^{-1} \cdot hr$ by 290 days of age. The AUC's for paraxanthine and theobromine remained relatively constant for the first 22 days after birth and then decreased. The only other metabolite of caffeine that was found and measured in blood was 3-methylxanthine. The AUC for this metabolite increased steadily during the first month of age (Table 2). These changes in AUC with development do not necessarily indicate a change in the amount of metabolite produced since AUC also depends on the rate of elimination of each compound.

Caffeine and eleven of its metabolites were found in urine (Table 3 and Fig. 3). In 2-day-old puppies, the major compounds excreted were 1-methylurate (18 per cent), caffeine (18 per cent) and 1,7-dimethylurate (16 per cent). With increasing age the metabolite ratio changed substantially. The amount of caffeine excreted decreased such that by 22 days of age caffeine represented only 1 per cent of the identified products. The only urinary metabolites which increased with age were 1-methylurate, 1,3-dimethylurate and 3-methylxanthine. The molar percentage of all other metabolites decreased with development. The fraction of caffeine excreted as 1,3-dimethylurate increased 5-fold (from 6 per cent at 2 days of age to 29 per cent at 22 days of age), and that as 3-methylxanthine increased 30-fold (from 1 to 30 per cent over the same time period). The change in fraction of 1-methylurate with development was not statistically significant. The fraction of metabolites retaining the 7-methyl grouping decreased during the first 3 weeks after birth from 59 to 16 per cent. Conversely, the fraction of metabolites containing the 1- or 3-methyl groupings excluding caffeine and 1,3,7-trimethylurate increased during the same time period (Table 4).

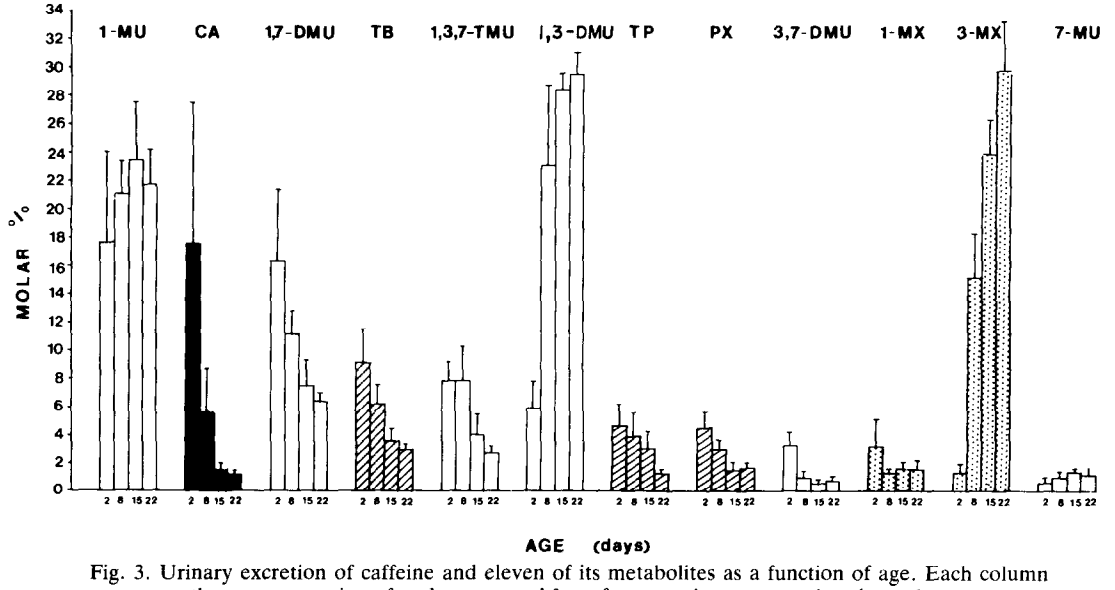

Fig. 3. Urinary excretion of caffeine and eleven of its metabolites as a function of age. Each column represents the mean excretion of each compound from four puppies, expressed as the molar percentage of all identified metabolites ± S.E. Caffeine (20 mg/kg) was administered i.v. to each puppy, and the methylxanthines and methylurates were assayed in urine by high performance liquid chromatography. The abbreviations used are: 1-MU, 1-methylurate; CA, caffeine; 1,7-DMU, 1,7-dimethylurate; TB, theobromine; 1,3,7-TMU, 1,3,7-trimethylurate; 1,3-DMU, 1,3-dimethylurate; TP, theophylline; PX, paraxanthine; 3,7-DMU, 3,7-dimethylurate; 1-MX, 1-methylxanthine; 3-MX, 3-methylxanthine; and 7-MU, 7-methylurate.

Table 3. Caffeine and its metabolites in urine of beagle puppies as a function of age*

Compound	Age (days) at dosing				P†
	2	8	15	22	
7-Methylurate	0.53 ± 0.31	0.95 ± 0.36	1.28 ± 0.15	1.14 ± 0.48	NS
1-Methylurate	17.72 ± 6.39	21.06 ± 2.38	23.52 ± 3.90	21.73 ± 2.41	NS
3,7-Dimethylurate	3.25 ± 0.90	0.93 ± 0.38	0.46 ± 0.19	0.66 ± 0.36	< 0.05
3-Methylxanthine	1.27 ± 0.58	15.04 ± 3.22	23.90 ± 2.34	29.61 ± 3.68	< 0.001
1-Methylxanthine	3.14 ± 1.81	1.32 ± 0.13	1.51 ± 0.41	1.51 ± 0.64	NS
1,3-Dimethylurate	5.99 ± 1.80	23.13 ± 5.45	28.36 ± 1.23	29.50 ± 1.57	< 0.001
Theobromine	9.12 ± 2.39	6.11 ± 1.41	3.64 ± 0.92	2.93 ± 0.44	< 0.05
1,7-Dimethylurate	16.32 ± 5.00	11.15 ± 1.56	7.51 ± 1.75	6.42 ± 0.60	< 0.05
Paraxanthine	4.57 ± 1.18	2.86 ± 0.83	1.49 ± 0.52	1.57 ± 0.20	< 0.05
Theophylline	4.65 ± 1.55	3.83 ± 1.84	2.90 ± 1.34	1.18 ± 0.20	< 0.05
1,3,7-Trimethylurate	7.85 ± 1.36	7.89 ± 2.48	4.11 ± 1.42	2.68 ± 0.52	< 0.01
Caffeine	17.51 ± 9.91	5.60 ± 3.11	1.37 ± 0.53	1.07 ± 0.27	NS

* All values are expressed as the molar percentage (mean ± S.E., N = 4) of the total identified compounds.

† Statistical comparisons are between groups of puppies at 2 and 22 days of age (unpaired Student's *t*-test). NS = not significant.

DISCUSSION

Caffeine is eliminated by a first-order process from the blood of 2- to 29-day-old beagle puppies in accord with a one-compartment, open model. During this time, the capacity to eliminate caffeine matures appreciably. The elimination rate constant increases 10-fold, from 0.035 hr^{-1} at 2 days of age to 0.369 hr^{-1} at 29 days of age, a value that even exceeds that of the adult beagle dog (0.174 hr^{-1}, [15]). Since the apparent volume of distribution changes insignificantly during the first month, the 9-fold increase in clearance reflects the change in elimination rate constant. Coincident with this increase in elimination rate, the fraction of a dose of caffeine excreted unchanged in the urine decreased 18-fold. This change is not due to a change in the efficiency with which the kidney excretes caffeine because the urine/blood caffeine concentration ratio in all experiments was found to be about 1.5, regardless of age. The sum of these findings strongly suggests that maturation of the process of caffeine elimination is due to an increase in the rate of metabolism of the trimethylxanthine.

Table 4. Urinary excretion of methylxanthines and methylurates in beagle puppies as a function of age and position of the methyl groups*

Position of methyl grouping	Age (days) at dosing			
	2	8	15	22
7-Methyl	59.15	35.49	19.86	16.47
3-Methyl	49.64	62.53	64.74	67.63
1-Methyl	77.75	76.84	70.77	65.66

* All values are expressed as the sum of the molar percentages of the excreted methylxanthines and methylurates containing either a 1-, 3- or 7-methyl grouping. Some metabolites contain more than a single specific methyl grouping; thus, the totals for each age group may exceed 100 per cent. The values do not include caffeine or 1,3,7-trimethylurate.

In the adult beagle dog the major route of metabolism of caffeine involves an initial *N*-demethylation at the 7-position to yield theophylline, which is further metabolized to 3-methylxanthine, 1,3-dimethylurate, 1-methylxanthine and 1-methylurate [15]. These compounds account for approximately 77 per cent of the total identified metabolites of caffeine in the urine of adult beagle dogs. These same compounds account for 82 per cent of the identified metabolites in the urine of 22-day-old beagle puppies. However, in the 2-day-old puppies a different metabolite pattern is observed. Aside from the increased fraction of caffeine noted above, one does not observe selectivity toward the initial 7-*N*-demethylation. Indeed, the potential metabolites of the paraxanthine, theophylline and theobromine pathways account for 42, 33 and 14 per cent of identified products respectively. Between ages 2 and 22 days, there is an overall 7-fold increase in the clearance of caffeine. The maturation of caffeine clearance seems to correlate closely with an increase in the rate of the 7-*N*-demethylation of caffeine to yield theophylline. Between 2 and 22 days of age, the proportion of 1,3-dimethyl derivatives increases 3-fold, whereas those of the 3,7- and 1,7-dimethyl metabolites decrease 3-fold. If one includes the monomethyl derivatives in the analysis, the same trend is indicated, but interpretation is complicated since each monomethyl metabolite could have been derived from either of two dimethylxanthines.

N-Demethylations of xenobiotics are characteristically catalyzed by the cytochrome(s) P-450 monooxygenase system [16, 17], and circumstantial evidence suggests the primary involvement of these enzyme(s) in the metabolism of caffeine [4, 10–12, 15]. In adult beagle dogs, pretreatment with either phenobarbital or β-naphthoflavone increases the clearance of caffeine. Phenobarbital stimulates the 7-*N*-demethylation of caffeine to theophylline, and β-naphthoflavone stimulates the 3-*N*-demethylation of caffeine to paraxanthine [15]. It is the development of a form(s) of cytochrome P-450 with substrate specificity similar to, or identical to, the phenobar-

bital-inducible caffeine-7-*N*-demethylase of adult dogs which seems to correlate with the maturation of caffeine clearance observed in the beagle puppy. The details of this conclusion may not apply to human infants since human beings first metabolize caffeine to paraxanthine via a 3-*N*-demethylation reaction [5, 7, 8].

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