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CHANGES IN THEOPHYLLINE METABOLISM DURING POSTNATAL DEVELOPMENT IN RAT LIVER SLICES

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Abstract—The metabolism of theophylline was studied in liver slices of young and adult rats. Theophylline and six metabolite fractions were recognized in adult liver by thin-layer radiochromatography and high performance liquid chromatography: 1-methyluric acid; 1-methylxanthine; 1,3-dimethyluric acid and/or 3-methylxanthine; caffeine; a uracil derivative and two unknown polar compounds. Preincubation with caffeine or theobromine inhibited theophylline metabolism. Allopurinol decreased the formation of three metabolite fractions but markedly increased the production of 1-methylxanthine. SKF 525-A inhibited the overall metabolism of theophylline. The specific activity of the enzyme system was 3.2 ± 0.4 nmoles · (g liver)$^{-1}$ · hr^{-1} in the 4- to 5-day-old rat and increased to a peak of 25.7 ± 1.7 in the 28-day-old; values for K_m and V_{max} in the 7- and 28-day-olds were 132.1 and 67.5 μM, and 23.9 and 52.1 nmoles · (g liver)$^{-1}$ · hr^{-1} respectively. Theophylline and the same six metabolites were identified in young and adult rats, but the development pattern was not uniform. Peak age-related activity and involvement of mixed-function oxidase system are features which are common to theophylline and caffeine metabolism. Xanthine oxidase played a role in theophylline metabolism. Formation of caffeine from theophylline was not dependent on a lack of activity of other pathways.

Theophylline (1,3-dimethylxanthine) is used therapeutically for bronchodilation in humans of all ages as well as for the prevention of apnea of premature and older infants. Incomplete data exist regarding its biotransformation in early postnatal life. It has been found that caffeine (1,3,7-trimethylxanthine) is a major metabolic product of theophylline in the fetus [1] and the newborn infant [2–6], while in the adult human theophylline undergoes extensive biotransformation to mono- and dimethyluric acids and monomethylxanthines [7].

A previous study from this laboratory describes the *in vitro* metabolism of caffeine in the adult and in the young rat [8]. The purpose of this investigation was to characterize further the enzyme system responsible for the metabolism of theophylline in the adult and its changes during postnatal development in the rat.

MATERIALS AND METHODS

Animals. Charles River rats were housed under standard laboratory conditions and fed Purina chow and water *ad lib.* Rats were weaned at 21 days. For experiments with rats younger than 21 days, both sexes were used, whereas for experiments with older rats, only males were used.

Chemicals. [8-^{14}C]Theophylline with a specific activity of 25 mCi/mmole was obtained from Amersham (England). Thin-layer chromatography of this material revealed that it was more than 98% pure. Theobromine and caffeine were obtained from BDH Chemicals Ltd. (Poole, England); theophylline was from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); and 1-methylxanthine, 3-methylxanthine, 1-methyluric acid and 1,3-dimethyluric acid (purum) were from Fluka (Switzerland). 4-Amino-5-(*N*-methylformylamine)-1,3-dimethyluracil (or 1,3,7-DAU) was supplied by Dr. M. J. Arnaud (NESTEC, Vevey, Switzerland). SKF 525-A and allopurinol were gifts from Smith, Kline & French Laboratories (Philadelphia, PA, U.S.A.) and Ikapharm Ltd. (Israel) respectively. Solvents for extraction and TLC were analytical grade; solvents for high performance liquid chromatography (HPLC) were HPLC grade.

In vitro *metabolism.* Following decapitation of the rats, the livers were rapidly removed and placed in ice-cold Krebs-bicarbonate buffer (pH 7.4). Liver slices, approximately 0.5 mm thick, were prepared at 4° and weighed immediately. For experiments using 4-, 5- and 7-day-old rats, livers of individual litters were pooled. Following preliminary experiments, conditions for the *in vitro* metabolism were determined. Unless otherwise specified, liver slices (300 mg) were incubated for 5 hr in 25-ml Erlenmeyer flasks containing 3 ml of Krebs-bicarbonate buffer (pH 7.4) with [8-^{14}C]theophylline to give a final concentration of 40 μM (0.1 μCi) under an atmosphere of 95% O_2–5% CO_2 in a metabolic shaker (100 rpm) at 37°. For determination of kinetic values, seven to thirteen animals (older than 7 days) or pooled litters (up to 7 days old) were used for each theophylline concentration. In the indicated experiments, liver slices were preincubated with SKF 525-A, allopurinol, caffeine and theobromine for

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30 min before the addition of [8-^{14}C]theophylline. The enzymatic reaction was terminated by placing the incubation flasks on ice and centrifugation.

Analysis of in vitro *theophylline metabolites.* Tissue material was removed immediately at the end of the enzymatic reaction (3000 rpm for 10 min). Following centrifugation, 0.3 g ammonium sulfate was added to the supernatant liquid which was extracted thereafter with 15 ml chloroform–isopropanol (85:15, v/v). The organic phase was removed and evaporated under a nitrogen stream at 37°. The residue was redissolved with 200 μl chloroform–ethanol (9:1, v/v) and streaked on precoated silica gel plates (60F-254 TLC aluminum sheets 5554, Merck, Darmstadt, F.R.G.). The solvent system consisted of chloroform–ethanol (9:1, v/v). Plates were developed twice in order to separate metabolites which have close R_f values. The distribution of radioactivity on the plates was determined by counting 0.5-cm strips of the chromatogram. Radioactivity was measured after addition of 1 ml H_2O and 10 ml of Triton–Toluene scintillation cocktail in a Packard Tri-Carb liquid scintillation spectrometer model 3330. Counting efficiency, as determined by external standard, was 60%. Peak number 3 (Fig. 1) was scraped off the plate, extracted and restreaked on a TLC plate. The plate was eluted two-dimensionally according to Arnaud [9] and cut into 0.5 cm^2 squares whose radioactivity was estimated as described above.

HPLC analysis of theophylline and theophylline metabolites. Theophylline and theophylline metabolites were also analyzed by HPLC. The instrument used was a Knauer HPLC pump 64 equipped with a 4.1 mm i.d. × 30 cm column RP-C 18 μBondapak 10 micron (Altech Assoc., IL) and a Pye Unicam LC u.v. detector. Mobile phase (5% acetonitrile in 0.01 M acetate buffer, pH 4) was pumped through the column at a flow rate of 1 ml/min at room temperature and a pressure of 6 mPa. Radioactive fractions scraped off from the TLC plates were dissolved in 100 μl mobile phase, and aliquots (20 μl) were injected into the HPLC and detected at a wavelength of 275 nm.

Theophylline metabolites were identified by comparing their R_f values (on TLC plates) and retention times (HPLC) with values of reference compounds.

RESULTS

The TLC separation of theophylline and its metabolites is shown in Fig. 1. Theophylline and six main metabolite fractions were recognized. These six metabolites constituted 30% of the radiolabeled substrate incubated.

Peak 3 (Fig. 1) was resolved into two materials by two-dimensional TLC; the amount of radioactivity in the two spots was similar. One of those spots had the chromatographic properties of 1,3,7-DAU. The R_f values of DAU in the two-dimensional TLC were 0.42 (first elution) and 0.39 (second elution). For further identification, peak 3 was rechromatographed on a TLC plate, scraped off, extracted and analyzed by HPLC. The double peak obtained (the two rotamers of 1,3,7-DAU) had the same R_f as the authentic sample (Fig. 2). Other diaminouracils were not studied as potential metabolic products. Peak 4

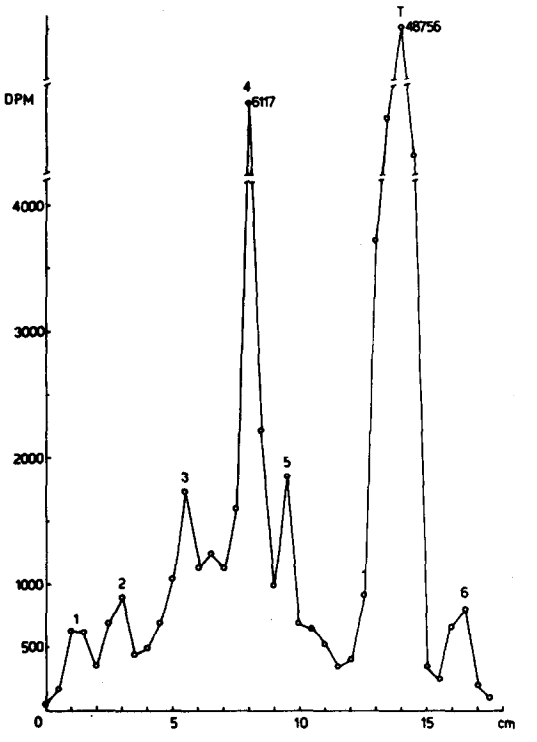

Fig. 1. Thin-layer chromatography of radioactivity of incubation mixture extracts (conditions were as described in Materials and Methods). Peak 1: 1-methyluric acid; peak 2: unknown metabolite; peak 3: 1,3,7-DAU and unknown metabolite; peak 4: 3-methylxanthine and/or 1,3-dimethyluric acid; peak 5: 1-methylxanthine; T: theophylline; and peak 6: caffeine.

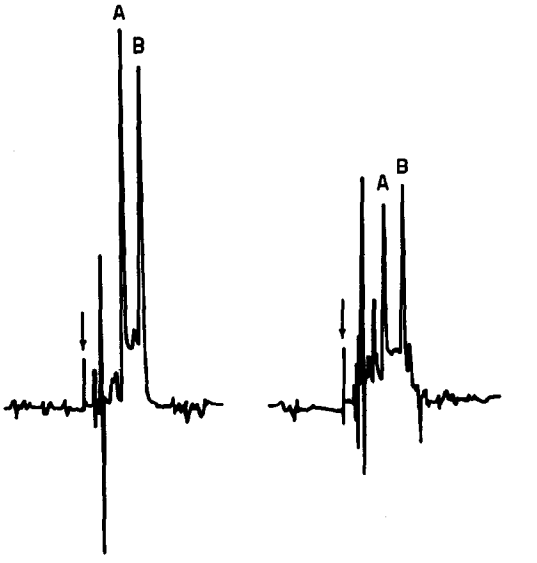

Fig. 2. High performance liquid chromatography of TLC peak 3 (Fig. 1) following rechromatography by TLC (right) and of 1,3,7-DAU (authentic sample, left) showing the two typical rotamers (A and B). Arrows indicate injections to chromatograph. (Conditions were as described in Materials and Methods.)

Table 1. Effect of inhibitors on *in vitro* theophylline metabolism by adult rat liver slices

Metabolite fraction (peak)	[^{14}C]Theophylline (% control)			
	Theophyl. (40 μM) + Caffeine (400 μM)	Theophyl. (40 μM) + Theobr. (400 μM)	Theophyl. (40 μM) + SKF 525-A (700 μM)	Theophyl. (40 μM) + Allopurinol (100 μM)
1	71.1 ± 6.0*	35.0 ± 5.0*	23.7 ± 5.8*	53.4 ± 11.9
2	33.3 ± 9.6*	48.6 ± 1.5*	22.1 ± 5.4*	51.1 ± 17.5*
3	28.0 ± 5.4*	75.2 ± 4.4*	35.0 ± 5.4*	156.5 ± 25.0*
4	11.3 ± 2.6*	19.8 ± 6.3*	41.9 ± 7.4*	77.4 ± 11.4*
5	13.8 ± 3.0*	36.9 ± 3.9*	29.5 ± 6.6*	1044.0 ± 9.0*
6	81.4 ± 13.7	51.2 ± 4.9*	52.9 ± 5.6*	42.9 ± 24.1*
Total metabolites	27.8 ± 1.7	42.7 ± 2.1	35.4 ± 2.6	118.7 ± 11.8

Results are given as mean ± S.E. Metabolite assignments are the same as in Fig. 1.
* $P < 0.05$.

was not finally identified; according to its R_f it could correspond to 3-methylxanthine and/or 1,3-dimethyluric acid.

Preincubation of liver slices with caffeine (1,3,7-trimethylxanthine) or with theobromine (3,7-dimethylxanthine) inhibited theophylline metabolism (Table 1). Figure 3 shows Lineweaver–Burk plots of the metabolic reaction in the presence and in the absence of caffeine.

The microsomal mixed-function oxidase inhibitor SKF 525-A inhibited the overall theophylline metabolism and the formation of each one of its metabolites (Table 1). On the other hand, allopurinol (a xanthine oxidase inhibitor) inhibited the formation of peaks 2, 4 and 6, while the formation of peak 5 (1-methylxanthine) was increased considerably (Table 1).

The specific activity of the enzyme system was extremely low when livers of 4- to 5-day-old rats were used [(3.2 ± 0.4 (nmoles metabolites formed) · (g liver)$^{-1}$ · hr^{-1}]. The reaction velocity increased with increasing age and reached a peak [25.7 ± 1.7 (nmoles metabolites formed) · (g liver)$^{-1}$ · hr^{-1}] at 28 days of age (Fig. 4). The K_m of the enzyme system was 67.5 μM in the 28-day-old and 132.1 μM in the 7-day-old rat. The V_{max} changed 2-fold [52.1 vs 23.9 nmoles · (g liver)$^{-1}$ · hr^{-1}] between these two ages.

Theophylline and the same six main metabolite fractions were identified when livers of young or adult rats were analyzed. The developmental pattern of the formation of theophylline metabolites is shown in Fig. 5.

DISCUSSION

Six metabolite fractions were identified with the use of [8-^{14}C]theophylline in the present study. They included demethylated xanthines and uric acids, the

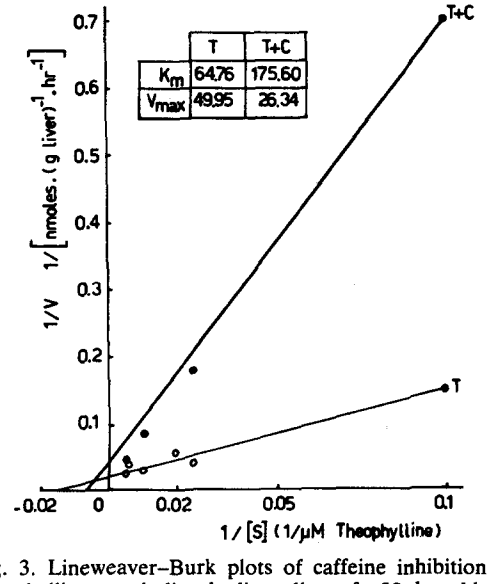

Fig. 3. Lineweaver–Burk plots of caffeine inhibition of theophylline metabolism by liver slices of a 28-day-old rat. Lines were fitted by least squares analysis. (Conditions were as described in Materials and Methods.) T: theophylline; and C: caffeine, 400 μM.

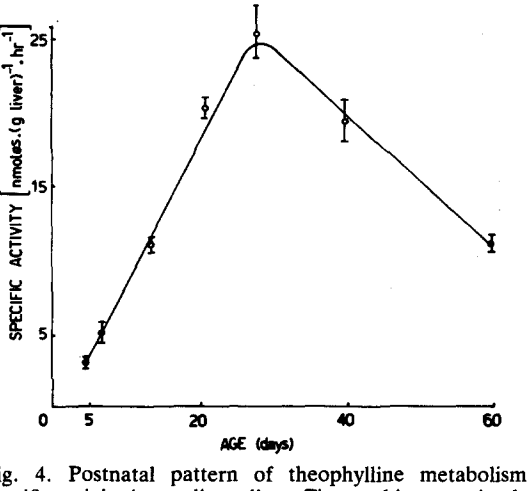

Fig. 4. Postnatal pattern of theophylline metabolism specific activity by rat liver slices. Five to thirteen animals (older than 7 days) or pooled litters (4- to 7-days-old) were used for each age point.

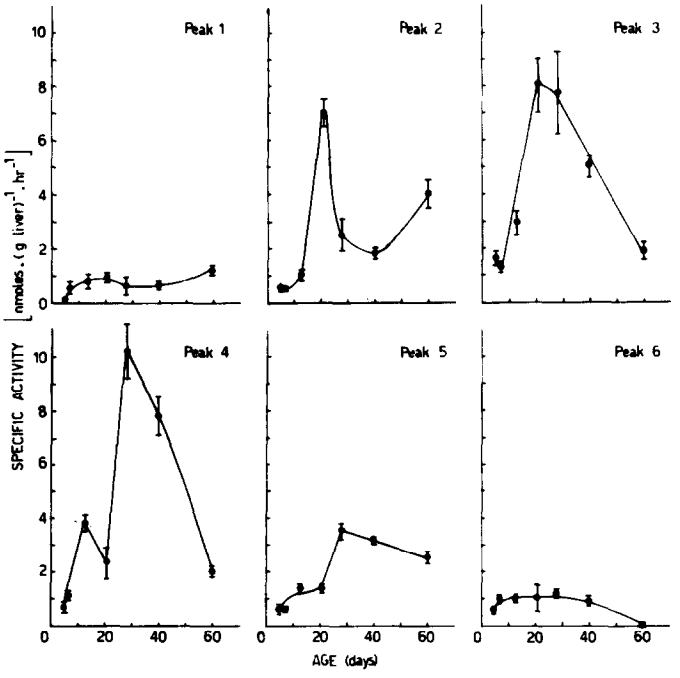

Fig. 5. Postnatal development pattern of formation of theophylline metabolite fractions. Five to thirteen animals (older than 7 days) or pooled litters (4- to 7-days-old) were used for each age point. Number identifying metabolite peaks, as in Fig. 1.

uracil derivative 1,3,7-DAU, caffeine and unknown polar compounds. The unidentified products (peak 2 and part of peak 3 of Fig. 1) may represent secondary metabolites of theophylline or of caffeine.

The methylation of theophylline to caffeine is known to occur in the human [1, 7], particularly in the premature neonate [2–6]. Therefore, the formation of 1,3,7-DAU as a metabolic product from theophylline demonstrated in this study is not surprising since it is a known caffeine metabolite [7, 8, 10]. However, this uracil derivative was not identified in previous studies on theophylline metabolism. Other caffeine metabolites, such as theobromine, have been found in infants treated with theophylline [3, 5].

Although the major metabolites formed from theophylline (this study) and from caffeine [8] are different, the developmental pattern of the overall metabolism of theophylline by rat liver slices is very similar to that of caffeine [8]. As with caffeine, activity of theophylline metabolism peaked after weaning, at about 28 days of age; this coincided with the peak formation of peak 5 (1-methylxanthine), peak 4 (3-methylxanthine and/or 1,3-dimethyluric acid) and peak 3 (1,3,7-DAU and an unknown compound). Since the formation of caffeine increased gradually during the first 2 weeks of life (Fig. 5), it was not dependent on the lack of activity of other pathways, as suggested in other studies [4] but on an intrinsic metabolic capability of the 7-methylation pathway which increased progressively in early postnatal life.

The rate of theophylline metabolism in liver slices from adult rats was greater than twice the rate obtained in 7-day-old rats. The phenomenon of very low metabolism in the young organism relative to the adult one is common to many drugs metabolized via the mixed-function oxidase cytochrome P-450 system and it is particularly pronounced in the case of caffeine [8]. These rates of methylxanthine biotransformation are consistent with the differences in plasma elimination half-lives of theophylline and of caffeine in young infants as compared with the adult human [11, 12].

The metabolism of theophylline in the rat is enhanced by pretreatment with 3-methylcholanthrene [13, 14], which indicates involvement of the cytochrome P-450 system. In addition, Lohmann and Miech [14] showed that preincubation of liver slices with the cytochrome P-450 inhibitor SKF 525-A causes a 50% inhibition in the total metabolism of theophylline. This latter finding was confirmed in the present study by demonstrating inhibition of the formation of all of the theophylline metabolites by 50–70% (Table 1). It is of interest that, although the formation of caffeine is not an oxidative reaction, it was inhibited by SKF 525-A, by 50% as well. SKF 525-A is known to inhibit caffeine metabolism [8].

The xanthine oxidase inhibitor allopurinol considerably affected theophylline metabolism (Table 1). The formation of peaks 2 (unknown metabolite), 4 (3-methylxanthine and/or 1,3-dimethyluric acid) and 6 (caffeine) was inhibited while the formation of peak 5 (1-methylxanthine) was enhanced over 10-fold. The inhibition of theophylline metabolism by allopurinol has already been demonstrated in the human [15] and in the rat [14]. As opposed to its marked effects on theophylline metabolism, allopurinol has only a marginal effect on the metabolism of caffeine [8]. The major caffeine metabolites reported [8, 17] are different from those identified in this study. The four main caffeine metabolites

formed by rat liver slices were theophylline, paraxanthine (1,7-dimethylxanthine), 1,3,7-DAU [8] and 1,3,8-trimethylallantoin [17]; none of these compounds is a good substrate for xanthine oxidase [16].

As shown here, preincubation of liver slices with theobromine (3,7-dimethylxanthine, 400 μM) and with caffeine (1,3,7-trimethylxanthine, 400 μM) inhibited theophylline metabolism by 60 and 70% respectively (Table 1). Caffeine inhibition of theophylline metabolism has also been shown by Lohmann and Miech [14]. The mechanism of inhibition of theophylline biotransformation by these two methylxanthines is probably competitive because of the similarity in their chemical structure and the nature of the enzyme system involved. We have shown previously that theophylline competitively inhibits caffeine metabolism [8].

In summary, this study shows similarities as well as differences between theophylline and caffeine biotransformation [8] by rat liver. In particular, it indicates that both the liver microsomal mixed-function oxidase system and xanthine oxidase are involved in theophylline metabolism, and that the formation of caffeine from theophylline is not dependent on the activity of other pathways in early postnatal life. The maximal rate of overall theophylline metabolism is reached at approximately 28 days of postnatal life, as shown previously for caffeine. Further work is required in order to characterize fully the various metabolic pathways of theophylline and related methylxanthines.

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