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CAFFEINE METABOLISM IN LIVER SLICES DURING POSTNATAL DEVELOPMENT IN THE RAT

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Abstract—The metabolism of caffeine was investigated in liver slices of young and adult rats. Liver slices from adult male rats metabolized caffeine at an initial rate of 48.31 ± 3.71 nmoles · (g liver)$^{-1}$ · hr^{-1} to four main metabolite fractions. By a combination of thin-layer radiochromatography and high performance liquid chromatography, theophylline, paraxanthine and 1,3,7-trimethyldihydrouric acid were identified as caffeine metabolites. Apparent V_{max} of the overall reaction was 83.30 nmoles caffeine metabolites formed · (g liver)$^{-1}$ · hr^{-1}. Theophylline competitively inhibited caffeine metabolism [the apparent K_m was 19.20 μM in the absence of theophylline, the apparent K_i was 36.50 μM in the presence of theophylline (100 μM)]. SKF 525-A inhibited caffeine metabolism; the formation of all of the metabolite fractions was inhibited to a similar extent. Allopurinol (100 μM) had no effect. The specific activity of the enzyme system was extremely low when liver slices of 2-day-old-rats were used [1.46 ± 0.08 nmoles caffeine metabolites formed · (g liver)$^{-1}$ · hr^{-1}]; the reaction velocity increased gradually with increasing age and reached a peak [52.26 ± 1.41 nmoles caffeine metabolites formed · (g liver)$^{-1}$ · hr^{-1}] at 30 days of age. Changes in the formation of the four metabolite fractions with age followed the pattern of the overall caffeine metabolism. These results demonstrate that the liver of the newborn rat has an extremely limited capacity to metabolize caffeine *in vitro* and are consistent with the proposed involvement of the liver microsomal cytochromes P-450 monooxygenase system in the metabolism of caffeine. *N*-Demethylation is the main pathway of *in vitro* caffeine metabolism in the rat liver at all ages.

The recent introduction of caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine) in the treatment of apnea of prematurity [1] has stimulated the study of the pharmacokinetics and the metabolism of methylxanthines in the neonate.

The plasma elimination half-life of caffeine is about twenty times longer in the newborn than in the adult human [2, 3]. Similar findings have been reported in an animal model [4]. As compared to other drugs, including theophylline, the magnitude of this developmental difference is exceptionally large.

Analyses of the urinary excretion of caffeine in the adult human, dog, rat and mouse have shown that this drug undergoes extensive biotransformation to methylated xanthines and uric acids [5–11]. As opposed to the situation in adults, *in vivo* studies in newborn humans and dogs show that a large fraction of caffeine is eliminated unchanged in the urine. The ratio of urinary caffeine metabolites to urinary caffeine increases gradually with age [12, 13†].

The present study was carried out to characterize further the enzyme system responsible for the metabolism of caffeine and its changes during postnatal maturation in the rat.

MATERIALS AND METHODS

Animals. Charles River rats were housed under standard laboratory conditions and were fed Purina chow and water *ad lib.* For adult experiments only male rats weighing 250–350 g were used.

Chemicals. [1-^{14}C-Methyl]caffeine, with a specific activity of 48.96 mCi/nmole, was obtained from the New England Nuclear Corp. (Boston, MA). Thin-layer radiochromatography of this material revealed that it was more than 99.83 per cent pure. Caffeine, theophylline and Krebs-bicarbonate buffer ingredients were obtained from commercial sources. Paraxanthine and 1,3,7-trimethyluric acid (purum) were obtained from Fluka, Switzerland. 1,3,7,-Trimethyldihydrouric acid‡ was supplied by Dr. M. J. Arnaud, La Tour de Peiltz, Switzerland. SKF 525-A and allopurinol were gifts from Smith, Kline & French, Philadelphia, PA, and Ikapharm Ltd., Israel, respectively. Solvents used for extraction and t.l.c. elution were analytical 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

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‡ 1,3,7-Trimethyldihydrouric acid was chemically identified by Dr. J. M. Arnaud as 4-amino-5-(*N*-methyl formylamine)-1,3-dimethyluracil (personal communication).

2-, 4- and 5-day-old rats, livers of individual litters were pooled. Following preliminary experiments (Fig. 1), conditions for the *in vitro* metabolism were determined. Unless otherwise specified, 150 mg of liver slices were incubated for 1.5 hr in 20-ml Erlenmeyer flasks containing 3 ml of Krebs-bicarbonate buffer (pH 7.4) with [^{14}C]caffeine to give a final concentration of 10 μM (0.029 μCi/μg), under an atmosphere of 95% O_2–5% CO_2, in a metabolic shaker (140 rpm) at 37°. For determination of kinetic values three to eight animals were used for each caffeine concentration (0.5–40 μM). In the indicated experiments, theophylline, SKF 525-A and allopurinol were preincubated with liver slices for 15 min before the addition of [^{14}C]caffeine. The enzymatic reaction was terminated by placing the incubation flasks on ice.

Analysis of in vitro *caffeine metabolites.* Tissue material was removed immediately at the end of the enzymatic reaction by centrifugation (4000 rpm for 10 min). The recovery of [^{14}C]caffeine following incubation was 85–90 per cent. The supernatant liquid was evaporated to dryness *in vacuo* at room temperature and the residue was extracted with 20 ml chloroform–methanol (9:1). The suspension was filtered and concentrated under a nitrogen stream at room temperature and streaked on silica gel plates. Precoated silica gel 60 F 254 TLC aluminum sheets (No. 5554, Merck, Darmstadt, FRG) were used. The solvent system consisted of chloroform–ethanol (9:1). Plates were developed three times in order to separate metabolites having close R_f values [7]. The distribution of radioactivity on the plate was determined by counting 0.25–0.5 cm strips of the chromatogram. Radioactivity of the strips was measured after addition of 1 ml H_2O and 10 ml of Triton–toluene scintillation mixture in a Packard Tri-Carb liquid scintillation spectrometer model 3330. Counting efficiency, as determined by external standard, was 70 per cent. The recovery of [^{14}C]caffeine from t.l.c. plates was 93 per cent.

High performance liquid chromatography (h.p.l.c.) analysis of caffeine and caffeine metabolites. Caffeine and caffeine metabolites were also analyzed by h.p.l.c. The instrument used was a Du Pont model 848 equipped with a 2.1 mm i.d. × 25 cm column of Zorbax ODS and a Pye Unicam LC u.v. detector. Solvent (10% acetonitrile in 0.01 M acetate buffer, pH 4) was pumped through the column at a flow rate of 5.4 ml/hr at room temperature and a pressure of 2000 psi. The incubation mixture was extracted as described above, part of the residue was dissolved in 100 μl elution solvent, and aliquots (20 μl) were injected into the high performance liquid chromatogram and read at a wavelength of 275 nm. The rest of the residue was chromatographed on silica gel plates as described. The individual radioactive fractions were analyzed by h.p.l.c. Caffeine metabolites were identified by comparing their R_f values (on t.l.c. plates) and retention times (h.p.l.c.) with values of reference compounds.

RESULTS

Figure 1 presents results obtained in preliminary experiments carried out to determine the optimal conditions of the reaction. Under the conditions established, liver slices from adult male rats metabolized caffeine (final concentration of 10 μM) at an intitial rate of 48.31 ± 3.71 nmoles of caffeine metabolites formed · (g liver)$^{-1}$ · hr^{-1}. Figure 2 shows the

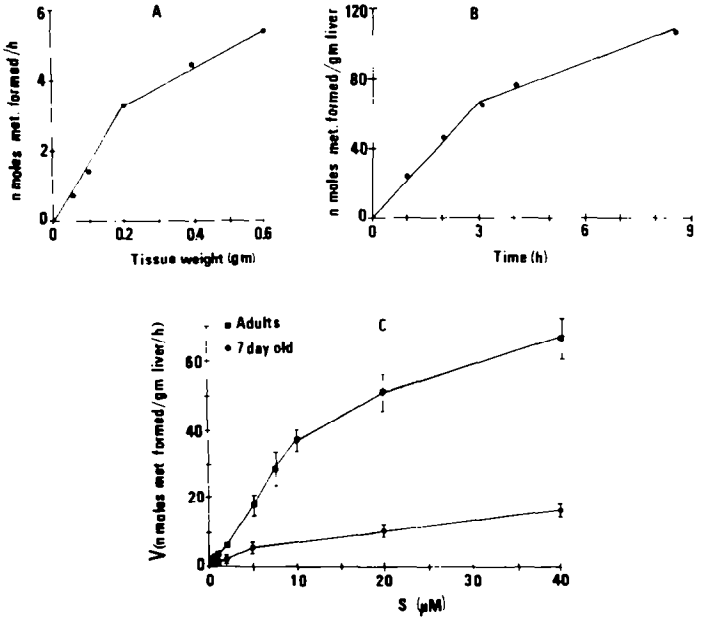

Fig. 1. Metabolism of caffeine using rat liver slices. (A) 5 hr incubation time, 10 μM caffeine, and various amounts of adult liver slices; (B) 150 mg adult liver slices, 10 μM caffeine, and various incubation times; (C) 150 mg liver slices of adult and 7-day-old rats, 1.5 hr incubation time, and various substrate concentrations. All caffeine concentrations had the same quantity of [^{14}C]caffeine, i.e. 200,000 cpm/incubation.

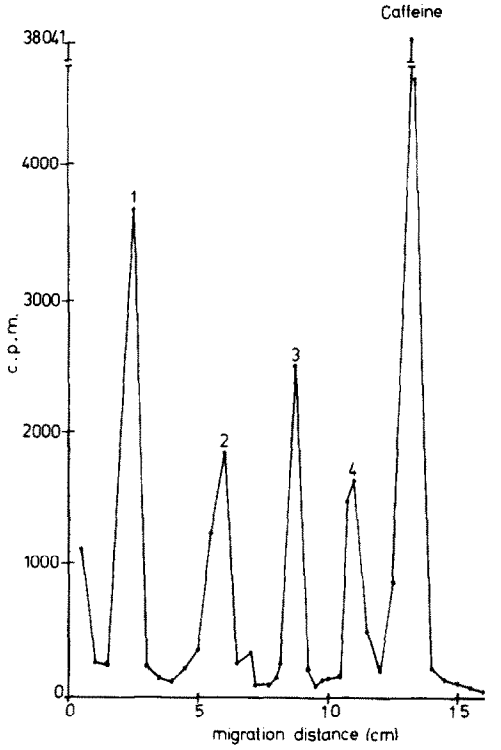

Fig. 2. Chromatographic distribution of radioactivity of incubation mixture extracts. Conditions were described in Materials and Methods.

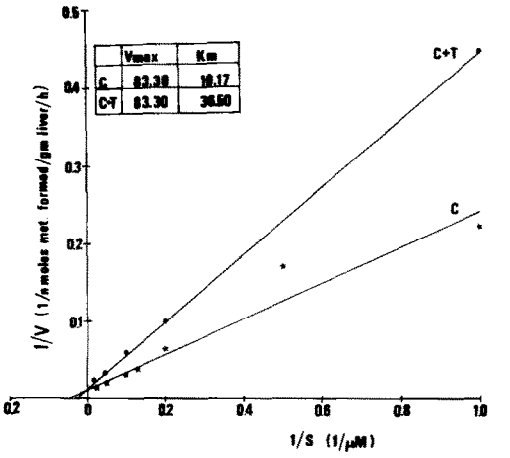

Fig. 3. Lineweaver–Burk plots of theophylline (T) inhibition of caffeine (C) metabolism *in vitro*. Lines were fitted by least squares regression analysis. Conditions were as described in Materials and Methods.

thin-layer radiochromatographic separation of compounds from the incubation mixture at the end of the reaction. Caffeine and four main metabolite peaks were recognized with R_f values of 0.156 (peak 1), 0.372 (peak 2), 0.543 (peak 3), 0.604 (peak 4) and 0.825 (caffeine). (R_f values were calculated following three developments of t.l.c. plates). The rate of formation of these four metabolite fractions was linear. By a combination of thin-layer chromatography and h.p.l.c. techniques peak 1 was identified as 1,3,7,-trimethyldihydrouric acid, peak 3 as paraxanthine and peak 4 as theophylline. Peak 2 was not finally identified; according to its R_f, it could correspond to 1-methylxanthine, 1,3- or 1,7-dimethyluric acid [7] (Table 1).

Preincubation with theophylline competitively inhibited caffeine metabolism. Figure 3 shows Lineweaver–Burk plots of the reactions in the presence and in the absence of theophylline.

The microsomal mixed-function oxidase inhibitor SKF 525-A (700 μM) caused a 63 per cent inhibition of the overall caffeine metabolism. The formation of all of the metabolite fractions was inhibited (Table 2). In contrast, the xanthine oxidase inhibitor allopurinol (100 μM) had no effect on the overall metabolism of caffeine; but the formation of one metabolite (metabolite fraction 2) was increased by 20 per cent (Table 2).

The specific activity of the enzyme system was extremely low when liver slices of 2-day-old rats were used [1.46 ± 0.08 nmoles metabolites formed · (g liver)$^{-1}$ · hr^{-1}. The reaction velocity increased gradually with increasing age of the rat and reached a peak [52.26 ± 1.41 nmoles metabolites formed · (g liver)$^{-1}$ · hr^{-1}] at 30 days of age (Fig. 4). A similar developmental pattern was obtained using a caffeine concentration of 40 μM. Caffeine and the same main four metabolite fractions were identified when livers of either newborn and young rats (2–30 days of age) or adult rats were analyzed. The

Table 1. High performance liquid chromatography retention times of caffeine and its metabolites

Compound	Retention time of authentic sample (min)	Retention time of compound purified by t.l.c. (min)
1,3,7-Trimethyldihydrouric acid* (peak 1)	15.0	15.1
Paraxanthine (peak 3)	29.8	30.0
Theophylline (peak 4)	30.0	30.0
Caffeine	69.0	69.0

* See footnote to the section on "Chemicals" in Materials and Methods.

Table 2. Effect of SKF 525-A and allopurinol on *in vitro* caffeine metabolism by adult rat liver slices

Metabolite fraction	Caffeine (10 μM) (control) Specific activity*	Caffeine (10 μM) + SKF 525-A (700 μM)		Caffeine (10 μM) + allopurinol (100 μM)	
		Specific activity	% of control	Specific activity	% of control
1	12.25 ± 0.98	4.08 ± 0.62†	33.7 ± 3.6	12.94 ± 1.52	105.2 ± 7.5
2	7.17 ± 0.77	2.06 ± 0.36†	28.3 ± 2.4	8.57 ± 1.02‡	119.4 ± 7.2
3	14.62 ± 0.87	6.11 ± 0.76†	41.2 + 3.5	15.59 ± 1.24	108.3 ± 6.2
4	8.97 ± 0.58	3.28 ± 0.41†	36.0 ± 3.4	9.61 ± 0.88	106.2 ± 7.2
Total metabolites	48.31 ± 3.71	17.40 ± 2.21†	34.03 ± 2.76	51.83 ± 4.86	104.2 ± 5.6

* Expressed in nmoles caffeine metabolites formed · (g liver)$^{-1}$ · hr^{-1}. Each result is the mean + S.E.M. of five experiments.

† $2P < 0.001$ of caffeine + SKF 525-A group vs control.

‡ $2P < 0.05$ of caffeine + allopurinol group vs control.

developmental curves of all four metabolite fractions followed the pattern of the overall caffeine metabolism (Fig. 5).

DISCUSSION

This study demonstrates that the liver of the new born rat has an extremely limited capacity to metabolize caffeine. This capacity increases gradually with increasing age and reaches a peak (greater than adult activity) following weaning, at 30 days of age.

The pattern of postnatal development of *in vitro* overall caffeine metabolism by rat liver slices closely resembles the increase in total body clearance of this drug after birth as described in the human and the dog [2–4]. The developmental pattern of caffeine metabolites formed and the close apparent K_m values in young and adult rats found in this study (Table 3) indicate that the slow rate of caffeine metabolism in young rats is due to a quantitative (rather than a qualitative) deficit of the metabolic pathways.

Although renal mechanisms play a role in caffeine elimination [5, 13, *], the activity of the liver mixed-function oxidase system seems to be the predominant factor that determines the rate of elimination of this drug. Caffeine is excreted in the urine of adult humans, rodents and dogs in the form of oxidated metabolites [5–11]. Caffeine plasma elimination is increased in rats following pretreatment with inducers of the cytochromes P-450 monooxygenases such as polycyclic aromatic hydrocarbons, Arochlor 1254 and phenobarbital [14, 15]. Aldridge and Neims [11] have also shown that phenobarbital and naphthoflavone pretreatment decrease considerably the serum half-life of caffeine in beagle dogs and stimulate the formation of selective demethylated urinary metabolites. Those results suggest that both cytochromes P-450 and P-448 are involved in the metabolism of caffeine. As shown, SKF 525-A (an inhibitor of the microsomal mixed-function oxidases) causes a marked inhibition of *in vitro* caffeine metabolism.

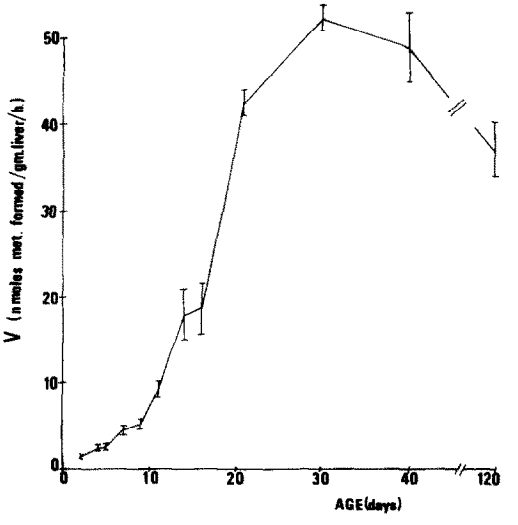

Fig. 4. Postnatal pattern of caffeine metabolism activity by rat liver slices. Caffeine concentration : 10 μM. Conditions were as specified in Materials and Methods. Each point represents the mean ± S.E. of three to ten separate experiments.

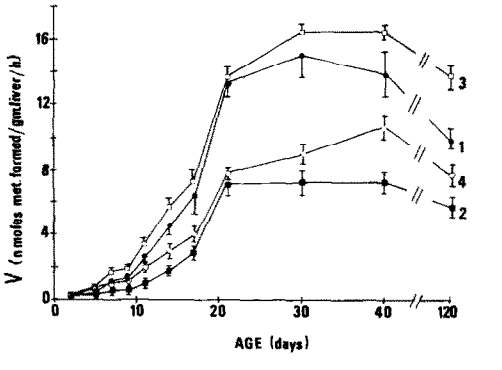

Fig. 5. Postnatal development pattern of formation of caffeine metabolite fractions. Numbers on the curves represent the respective peaks (Fig. 2). Each point indicates the mean ± S.E. of three to ten separate experiments.

* D. Warszawski, Z. Ben-Zvi and R. Gorodischer, in collaboration with M. J. Arnaud and I. Bracco, unpublished data.

Table 3. Apparent K_m and V_{max} values using liver slices from adult and 7-day-old rats*

Rats	Apparent K_m (μM)	Apparent V_{max} [nmoles caffeine metabolized · (g liver)$^{-1}$ · hr^{-1}]
Adult	19.20	83.30
7-Day-old	7.70	12.70

* Apparent K_m and V_{max} were calculated from Lineweaver–Burk plots from lines fitted by least squares regression analysis.

The *in vivo* apparent K_m of serum caffeine elimination in adult male rats (40 μM [14]) compares well with the finding in this study of an *in vitro* apparent K_m of 19.20 μM. Although dose-dependent kinetics in caffeine elimination have been described in the adult rat [14], but not in humans or in dogs [2–4], it is of significance that a large difference exists between neonates and adults both in the elimination half-life (in humans and in dogs) and in the *in vitro* metabolism of caffeine (in rats).

Quantitation and identification of the metabolites formed *in vitro* indicate that *N*-demethylation is the predominant pathway in caffeine metabolism. *N*-Demethylations on positions 3 and 7 (formation of paraxanthine and theophylline respectively) were demonstrated; the possibility of demethylation on position 1 could not be shown with the labeled substrate used ([1-^{14}C-methyl]caffeine) (Fig. 6). In the present study, allopurinol (an inhibitor of xanthine oxidase) had no effect on the rate of caffeine metabolism. This does not exclude, however, a role of xanthine oxidase in the production of minor caffeine metabolites not identified by the analytical system employed. Xanthine oxidase catalyzes the *in vitro* conversion of 1-methylxanthine to 1-methyluric acid [16, 17]; both of these compounds have been described as caffeine metabolites in the urine of rats [7, 10]. On the other hand, xanthine oxidase has not been found to play a role in the *in vitro* oxidation of di- and trimethylxanthines [16].

Caffeine and theophylline seem to be metabolized by a closely related enzyme system. In this study, theophylline (1,3-dimethylxanthine) competitively inhibited the *in vitro* metabolism of caffeine (1,3,7-trimethylxanthine). Similar *in vivo* and *in vitro* metabolites have been described following administration of either methylxanthine [5–11, 17]. Lohmann and Miech [17] found that rat liver slices metabolize theophylline at an initial rate of 6 nmoles · (g liver)$^{-1}$ · hr^{-1}; considering the somewhat different methodology, this value is close to the initial rate of caffeine metabolism reported here. Caffeine inhibits the plasma elimination and the metabolism of theophylline [17, 18] and, as shown in the present study, theophylline competitively inhibits *in vitro* caffeine metabolism. The metabolism and/or plasma clearance of both methylxanthines are stimulated by *in vivo* administration of similar inducers of mixed-function oxidases and are inhibited *in vitro* by a typical liver microsomal inhibitor (SKF 525-A) [11, 14, 15, 17 and present study]. Further work is necessary to fully characterize the enzyme systems involved in the metabolism of both methylxanthines.

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REFERENCES

1. J. V. Aranda and T. Turmen, *Clinics Perinatol.* **6**, 87 (1979).
2. J. V. Aranda, C. E. Cook, W. Gorman, J. M. Collinge, P. M. Loughman, E. W. Outerbridge, A. Aldrige and A. H. Neims, *J. Pediat.* **94**, 663 (1979).
3. R. Gorodischer, M. Karplus, D. Warszawski, H. Bark, S. W. Moses and C. Gordon, *Pediat. Res.* **11**, 1013 (1977).
4. D. Warszawski, R. Gorodischer, S. W. Moses and H. Bark, *Biol. Neonate* **32**, 138 (1977).
5. H. H. Cornish and A. A. Christman, *J. biol. Chem.* **228**, 315 (1957).
6. A. W. Burg and E. Werner, *Biochem. Pharmac.* **21**, 909 (1972).

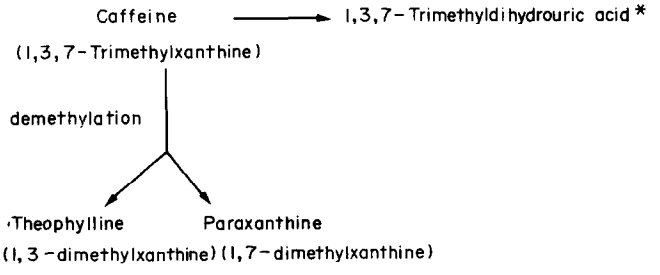

Fig. 6. Primary metabolic pathways of caffeine in rat liver slices. (*See footnote to the section on "Chemicals" in Materials and Methods.)

7. K. L. Khanna, G. S. Rao and H. H. Cornish, *Tox. appl. Pharmac.* **23**, 720 (1972).
8. G. S. Rao, K. L. Khanna and H. H. Cornish, *Experientia* **29**, 953 (1973).
9. M. J. Arnaud, *Experientia* **32**, 1238 (1976).
10. M. J. Arnaud, *Biochem. Med.* **16**, 67 (1976).
11. A. Aldridge and A. H. Neims, *Drug Metab. Dispos.* **7**, 378 (1979).
12. M. G. Horning, C. Stratton, J. Nowlin, A. Wilson, E. C. Horning and R. M. Hill, in *Fetal Pharmacology* (Ed. L. O. Boréus), pp. 355–73, Raven Press, New York (1973).
13. A. A. Aldridge, J. V. Aranda and A. H. Neims, *Clin. Pharmac. Ther.* **25**, 447 (1979).
14. A. Aldridge, W. D. Parsons and A. H. Neims, *Life Sci.* **21**, 967 (1977).
15. R. M. Welch, S. Y. Hsu and R. L. De Angelis, *Clin. Pharmac. Ther.* **22**, 791 (1977).
16. F. Bergmann and S. Dikstein, *J. biol. Chem.* **223**, 765 (1956).
17. S. M. Lohmann and R. P. Miech, *J. Pharmac. exp. Ther.* **196**, 213 (1976).
18. T. J. Monks, J. Caldwell and R. L. Smith, *Clin. Pharmac. Ther.* **26**, 513 (1979).