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STEREOCHEMICAL ASPECTS OF *para*-CHLOROAMPHETAMINE METABOLISM

RABBIT LIVER MICROSOMAL METABOLISM OF *RS*-, *R*(−)-, AND *S*(+)-*para*-CHLOROAMPHETAMINE*

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Abstract—With the aid of a chiral derivatizing reagent and a sensitive and specific nitrogen–phosphorus gas chromatographic assay, metabolism of *para*-chloroamphetamine (PCA) enantiomers has been studied following incubation of racemic (*RS*)-, *R*(−)- and *S*(+)-PCA with rabbit liver microsomal preparations. Significant metabolism of racemic PCA and the individual enantiomers was observed following incubation in rabbit liver microsomal preparations. Metabolism required viable microsomes, NADPH and molecular oxygen, and the rate of metabolism increased following pretreatment with phenobarbital. Incubation of racemic PCA resulted in more rapid metabolism of the *S*(+) enantiomer than of the *R*(−) enantiomer. When the enantiomers were incubated individually, each enantiomer was metabolized more rapidly than when incubated as part of a racemic mixture, and the *R*(−) enantiomer was metabolized more rapidly than the *S*(+) enantiomer.

Acute administration of *para*-chloroamphetamine (PCA)§ to rats and mice causes reversible short-term reduction in brain concentrations of serotonin and 5-hydroxyindoleacetic acid [1, 2], and long-term neurotoxicity in serotonergic neurons of the central nervous system [3–5]. After partial recovery of serotonin and 5-hydroxyindoleacetic acid concentrations following a single i.p. injection (10 mg/kg), long-term depletion (up to 4 months) is observed, and there is significant reduction of tryptophan hydroxylase activity characterized by reduced amounts of available enzyme rather than reduced activity of existing enzyme [3]. Long-term neurotoxicity is associated with irreversible cytopathological cell damage to serotonergic B-9 cell groups [3, 6–8]. Long-term neurotoxic effects differ markedly for *R*(−)- and *S*(+)-PCA. The *S*(+) enantiomer depletes brain serotonin [9] and 5-hydroxyindoleacetic acid [5] for longer periods of time, and to a greater extent than does the *R*(−) enantiomer. Similarly, the decrease in tryptophan hydroxylase activity following administration of *S*(+)-PCA is greater and of longer duration than that observed following administration of *R*(−)-PCA [9].

The possibility that long-term neurotoxicity of PCA is mediated by formation of a chemically reactive metabolite rather than parent drug has been considered. PCA does not inhibit brain tryptophan hydroxylase *in vitro* [10]. The half-life of parent drug in the brain is 8.5 hr [5], yet significant radioactivity is detected in the brain 800 hr after administration of radiolabeled PCA [11]. Chemically stable PCA metabolites (such as the oxime and beta-hydroxyl derivatives) are not neurotoxic [9, 12], with the exception of the hydroxylamine which is reduced *in vivo* to parent PCA [9]. It has been suggested [11, 13] that neurotoxicity may be mediated by the reactive 3,4-epoxide of PCA, and the expected "NIH-shift" metabolite 3-chloro-4-hydroxyamphetamine has been identified as a urinary metabolite in the rat [13]. We have shown that rat liver microsomes will convert PCA to a reactive metabolite (in the presence of NADPH and O_2) which covalently binds to microsomal proteins [14]. The irreversible damage observed in adrenergic neurons after administration of 6-hydroxydopamine has been described as identical to that observed in serotonergic neurons following administration of PCA [7]. The neurotoxicity of 6-hydroxydopamine is widely believed to involve oxidation of parent molecule to reactive oxygen species [15] and to reactive 6-hydroxydopamine metabolites which covalently bind to proteins [16], similar to our findings with PCA [14].

There are no detailed studies on PCA metabolism in the literature, and none with regard to stereochemical aspects of PCA metabolism. Stereoselective metabolism of many drugs, including amphetamines, is well documented [17] and may effect pharmacological activities of such agents. Because of the probable role of metabolism in PCA neurotoxicity, and the stereochemical nature of that neurotoxicity, we are studying stereochemical aspects of PCA metabolism. The studies reported have been designed to detect differences in metab-

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§ Abbreviations: PCA, *para*-chloroamphetamine; MTPA-Cl, (−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride; g.c., gas chromatography; and i. p., intraperitoneal.

olism of PCA enantiomers in liver microsomal preparations under oxidative conditions. Rabbit liver microsomes were selected for these studies because they metabolize PCA more avidly than similar preparations from rats (see below), the species employed for most neurotoxicity studies. Both species metabolize amphetamines under oxidative conditions [18], and both species have been reported to metabolize enantiomers of drugs differently [17]. We here report studies characterizing metabolism of PCA enantiomers following incubation of racemic, *R*(−)-, and *S*(+)-PCA in rabbit liver microsomal preparations.

MATERIALS AND METHODS

Materials. *RS*-PCA hydrochloride was purchased from the Regis Chemical Co. (Chicago, IL), phenobarbital from the Sigma Chemical Co. (St. Louis, MO), thionyl chloride and (−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid from the Aldrich Chemical Co. (Milwaukee, WI), and NADPH from Sigma Chemical Co. All column packings were purchased from Applied Sciences (State College, PA). Racemic and *S*(+)-amphetamine (free base and sulfate) were provided by Smith, Kline & French Laboratories (Philadelphia, PA) and the National Institute on Drug Abuse. All solvents were glass distilled and obtained from Burdick & Jackson Laboratories (Muskegon, MI).

Resolution of PCA enantiomers. PCA was resolved with *N*-acetyl-L-leucine and *N*-acetyl-D-leucine as previously described [19]. *N*-Acetyl-L-leucine was purchased commercially, *N*-acetyl-D-leucine was prepared from D-leucine and acetic anhydride [20]. In a typical resolution experiment, PCA hydrochloride (3.0 g, 0.015 M) was dissolved in 30 ml water. *N*-Acetyl-L- or *N*-acetyl-D-leucine (1.5 g, 0.0087 M) was dissolved in 8.7 ml of 1 N NaOH (0.0087 M) and 10 ml water to yield the sodium salt (pH 7). The *N*-acetyl-leucine was slowly added dropwise to the stirring PCA solution. Solids appeared after addition of approximately two-thirds of the *N*-acetyl-leucine solution. Stirring was continued for several minutes after complete addition. Solids were filtered under vacuum, washed with a minimum volume of ice-cold water, and dried on the filter. A small portion of the diastereomeric salt was hydrolyzed (NaOH) and extracted (benzene) for gas chromatographic enantiomeric analysis following derivatization with MTPA-Cl (see Results). The remainder of the salt was recrystallized from 95% ethanol. When *N*-acetyl-L-leucine is used in the above procedure, the diastereomeric salt of *R*(−)-PCA and *N*-acetyl-L-leucine preferentially precipitates from the aqueous solution, while *N*-acetyl-D-leucine yields the diastereomeric salt containing *S*(+)-PCA. The average yield of diastereomeric salts in these experiments was 47 per cent. Optical purity of resolved *R*(−)-PCA was greater than 97 per cent; optical purity of resolved *S*(+)-PCA was greater than 90 per cent. Optical purity of *S*(+)-PCA was limited possibly because of racemization during preparation of *N*-acetyl-D-leucine.

Animals and preparation of hepatic microsomes. Male Sprague–Dawley rats (150–250 g) and male New Zealand white rabbits (1.5–2.0 kg) were used for all metabolism studies. Microsomal enzymes were induced by pretreatment of animals with phenobarbital (80 mg/kg, i.p.) daily for 3 days. Animals were killed 24 hr later. Microsomes were prepared by differential centrifugation as described by Ernster *et al.* [21]. Protein concentrations were determined by the method of Lowry *et al.* [22].

Incubations. Microsomal incubations consisted of the following components present in final concentrations as stated: microsomal protein (2–3 mg/ml), $NADP^+$ (2.0 mM), glucose-6-phosphate (118 mM), glucose-6-phosphate dehydrogenase (2 units/ml), and substrate (25–50 μM) in Tris-buffer (pH 7.4, 250 μM) in a final volume of 6 ml. Incubations were carried out at 37° in a shaking water bath. After a 2-min preincubation of mixture and substrate, the reaction was started by addition of microsomes. Portions (0.5–1.0 ml) were removed at appropriate times and added to 6 ml benzene.

Sample workup procedure. To incubation samples (containing incubation aliquot and 6 ml benzene) were added 0.5 ml of 20% NaOH and 10 μl of a 1 μg/μl benzene solution of *S*(+)-amphetamine as internal standard. Samples were shaken on a mechanical shaker for 20 min and centrifuged for 15 min (1000 g). The organic layer was transferred to a 12-ml conical centrifuge tube. After addition of 100 μl butanol, samples were concentrated under a gentle stream of nitrogen to approximately 100 μl. Samples were then directly analyzed by g.c. for total PCA or derivatized for enantiomeric analysis by g.c. as described below.

Derivatization procedure. The acid chloride of (−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA-Cl) was prepared using freshly distilled thionyl chloride and the acid as described previously [23]. The resulting oil was stored in the cold as a solution in methylene choloride. To 12-ml conical centrifuge tubes containing the concentrated organic extract was added 50 μl of MTPA-Cl dichloromethane solution and pyridine (20 μl). Tubes were capped and heated at 70° for 30 min. Tubes were cooled in an ice-water bath, 1 N hydrochloric acid was added (1 ml), and the tubes were shaken for 5 min. Following centrifugation (1000 *g*) the aqueous phase was discarded and 15% ammonium carbonate (0.5 ml) was added with additional dichloromethane (100 μl). The tubes were again shaken for 5 min. Following centrifugation (1000 *g*), an aliquot (1–2 μl) of the organic layer was analyzed by gas chromatographic analysis (see below).

Gas chromatographic analysis. Underivatized PCA was analyzed using microprocessor-controlled Hewlett Packard 5840A gas chromatograph equipped with a nitrogen–phosphorus detector. Glass columns (2 ft by 2 mm i.d.) were silanized and packed with 10% Carbowax 20 M/2% KOH on Supelcoport 80/100 mesh. Injector and detector temperatures were 235° and 300° respectively. Hydrogen gas flow-rate was 3 ml/min, air 90 ml/min and helium 30 ml/min. An oven temperature program was employed (140–180°, 10°/min). The same instrument was employed for analysis of derivatized enantiomers of PCA. Glass columns (6 ft × 2 mm i.d.) were silanized and packed with SP-2250 on Supelcoport

80/100 mesh. Instrument conditions (temperatures and flow rates) were as described above, except that oven temperature was maintained at 235° during the analysis.

RESULTS

Analytical methodology. To carry out the metabolism studies, it was necessary to develop an analytical technique which would allow measurement of total PCA and, in addition, the *R*(−) and *S*(+) enantiomers of PCA present in the same sample. Derivatization of PCA with the chiral derivatizing agent MTPA-Cl afforded the diastereomeric product amides which were readily resolved by gas chromatographic analysis. MTPA-Cl has been used previously to resolve enantiomers of amphetamines for gas chromatographic analysis [23]. Use of a nitrogen–phosphorus detection provided sensitive and specific analysis of total PCA and the derivatized enantiomers. *S*(+)-Amphetamine served as internal standard both for assays of total PCA and for assay of PCA enantiomers following derivatization. Chromatograms of samples taken from microsomal incubations containing racemic PCA and *S*(+)-amphetamine following analysis for total PCA (analyzed without derivatization) and for the enantiomers of PCA (analyzed after derivatization with MTPA-Cl) are illustrated in Fig. 1A and 1B respectively. Base line resolution of the derivatized enantiomers was obtained (Fig. 1B). There was no racemization, as evidenced by appearance of the second diastereomeric product observed in chromatograms from any experiments in which only one enantiomer was added to control incubation mixtures. A standard curve obtained after addition of different amounts of the *R*(−) and *S*(+) enantiomers of PCA to a control (no $NADP^+$) microsomal incubation using amphetamine as an internal standard is shown in Fig. 2. Identical curves were obtained when racemic PCA was added rather than a mixture of the enantiomers. Similar standard curves for total PCA, analyzed without derivatization, were obtained with the nitrogen–phosphorus g.c. assay.

Characterization of PCA microsomal metabolism. We studied first the metabolism of PCA (without regard to enantiomeric composition) by rat and rabbit liver microsomal preparations. Rat liver microsomes even from animals pretreated with phenobarbital only minimally metabolized PCA (Table 1). Rabbit liver microsomal preparations readily metabolized PCA (50 μM). Metabolism was dependent on molecular oxygen, NADPH, and viable microsomes (Table 1). Metabolism was linear for approximately 60 min (Fig. 3) and dependent upon the concentration of microsomal protein (data not shown). The rate of metabolism was increased by pretreatment with phenobarbital (Table 1).

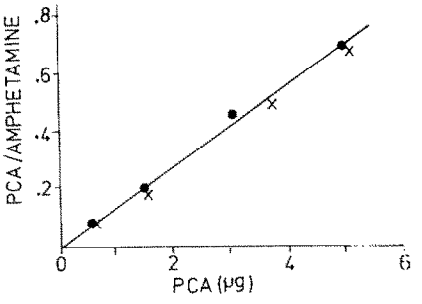

Fig. 2. Standard curve prepared by adding 10 μg *S*(+)-amphetamine and 0–5 μg *S*(+)- (×——×) and *R*(−)-(●——●) PCA to control microsomes. Samples were analyzed by extraction, derivatization with MTPA-Cl, and nitrogen–phosphorus gas chromatography.

Metabolism of R(−)- *and* S(+)-*PCA.* Using the MTPA-Cl derivatization assay, we studied rabbit liver microsomal metabolism of *R*(−)- and *S*(+)-PCA following incubation of racemic (*RS*)-PCA and following incubation of the individual PCA enantiomers. When racemic PCA (50 μM total, 25 μM each enantiomer) was incubated, the time course of disappearance of the enantiomers differed markedly. *S*(+)-PCA was metabolized at a more rapid rate than *R*(−)-PCA under these conditions (Fig. 4). When *R*(−)- and *S*(+)-PCA were incubated separately with microsomes (25 μM each), a different pattern of metabolism was observed (Fig. 5). The *R*(−) enantiomer was metabolized more rapidly than the *S*(+) enantiomer when they were incubated sep-

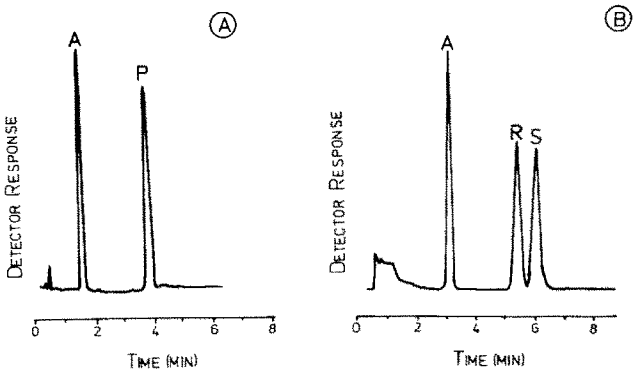

Fig. 1. Nitrogen–phosphorus gas chromatograms of a microsomal extract containing 10 μg racemic PCA and 10 μg *S*(+)-amphetamine following (A) analysis of total PCA (P) and *S*(+)-amphetamine (A) on a 2 ft 10% Carbowax 20 M/2% KOH column or (B) derivatization with MTPA-Cl and analysis of *S*(+)-PCA (S), *R*(−) PCA (R) and *S*(+)-amphetamine (A) on a 6 ft 3% SP-2250 column.

Table 1. Liver microsomal metabolism of PCA*

	Incubation conditions	Concn (μM)	Disappearance (nmoles·mg^{-1}·min^{-1})
Rat	Complete	25	0.004
	Induced microsomes	25	0.046
Rabbit	Complete	50	0.152 ± 0.026
	− NADPH	50	0.001 ± 0.000
	− O_2	50	0.000 ± 0.000
	Boiled microsomes	50	0.000 ± 0.000
	Induced microsomes	50	0.253 ± 0.046

* Incubation mixtures contained 3 mg of microsomal protein. Results of all experiments are expressed as rate of disappearance of *total* PCA during 30-min incubations. Results for each rabbit study are expressed as the mean of three experiments ± S.E.M. Results of the rat study are for one experiment each using microsomes prepared from three rats.

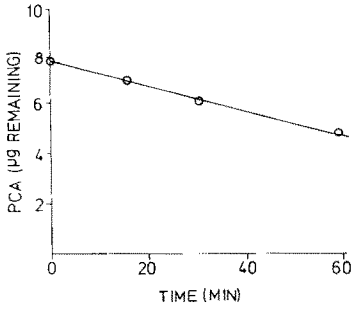

Fig. 3. Total PCA metabolism by rabbit liver microsomal preparation. Substrate concentration was 50 μM.

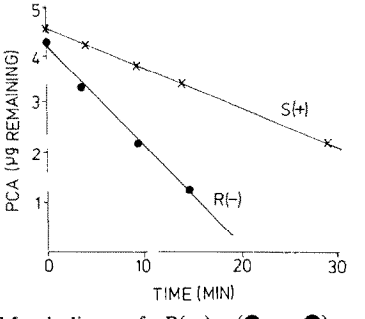

Fig. 5. Metabolism of $R(-)$- (●——●) and $S(+)$- (×——×) PCA by rabbit liver microsomal preparation following addition of $R(-)$-PCA (25 μM) and $S(+)$-PCA (25 μM).

arately, in contrast to findings when racemic PCA was added to incubation mixtures. In addition, the rate of metabolism of each enantiomer was greater when incubated separately than when the enantiomers were incubated together as the racemic mixture. Data in Figs. 4 and 5 were obtained from experiments carried out with the same microsomal preparation to allow visual comparison. Results from all experiments on metabolism of PCA enantiomers are summarized in Table 2.

DISCUSSION

For these studies on stereochemical aspects of PCA metabolism, we developed an analytical method which allows sensitive (< 1 μg) detection of either PCA enantiomer in the presence of a large excess of its antipode. This allowed us to determine the metabolic fate of PCA enantiomers when incubated together as the racemate, as well as when added individually to incubation mixtures. When racemic PCA was incubated with fortified rabbit liver microsomal preparations, PCA enantiomers were metabolized at different rates, with the $S(+)$ enantiomers being metabolized more rapidly. In contrast, incubation of the individual enantiomers resulted in a more rapid rate of metabolism for $R(-)$-PCA. Our studies were carried out at only one substrate concentration, and we cannot define the mechanism by which differences in enantiomer metabolism occur

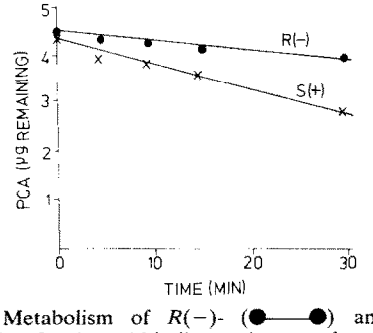

Fig. 4. Metabolism of $R(-)$- (●——●) and $S(+)$- (×——×) PCA by rabbit liver microsomal preparation following addition of racemic (RS)-PCA (50 μM) to incubation mixture.

Table 2. Metabolism of PCA enantiomers by rabbit liver microsomes following incubation of RS-, $R(-)$-, and $S(+)$-PCA*

Substrate	Concn (μM)	Disappearance (nmoles·mg^{-1}·min^{-1}) $R(-)$	$S(+)$
RS (racemic) PCA	50	0.037 ± 0.008	0.145 ± 0.020
$R(-)$	25	0.329 ± 0.024	
$S(+)$	25		0.258 ± 0.029

* Incubation mixtures contained 3 mg of microsomal protein. Results are expressed as rate of disappearance of enantiomers during 15-min incubations. Each value is the mean of at least three experiments ± S.E.M.

in microsomal preparations. However, the accelerated rate of enantiomer metabolism observed when they were incubated separately, particularly for the *R*(−) enantiomer, suggests that the presence of one enantiomer or its metabolites inhibits metabolism of its antipode. This has been proposed for similar observations with amphetamine [24] and the hallucinogenic amphetamine 1-(2,5-dimethyoxy-4-methyphenyl)-2-aminopropane [25].

We have characterized microsomal oxidative metabolism of PCA, demonstrated differences in the metabolism of the enantiomers, and demonstrated an interaction between the two enantiomers with regard to their metabolism. Because we employed rabbit liver microsomal preparations, we cannot directly relate our metabolism findings to differences in the neurotoxicity of PCA enantiomers in the rat. However, it should be noted that, while quantitative differences in stereochemical aspects and oxidative pathways of amphetamine metabolism are found between rats and rabbits (and humans), these species metabolize these compounds in the same qualitative manner [17]. When combined with previously discussed studies on the nature of PCA neurotoxicity, we feel our studies provide an important initial observation suggesting further studies on the involvement of stereochemical aspects of PCA metabolism in PCA neurotoxicity. If the reactive metabolite is very unstable, the liver may serve as a model for PCA metabolism studies, while metabolic activation of PCA may have to take place in the brain for neurotoxicity to occur. We previously characterized cytochrome P-450 in rat brain microsomal preparations [26] and, while concentrations are much lower than in liver, brain preparations do metabolize amphetamines [27] and indole alkylamines [28]. Continuing work on PCA metabolism includes studies on stereochemical aspects of *in vitro* PCA metabolism in the rat and rabbit, and the relationships to metabolic activation of PCA in the rat.

REFERENCES

1. A. Pletscher, M. DaPrada, W. P. Burkard and J. P. Tranzer, *J. Pharmac. exp. Ther.* **154**, 64 (1966).
2. R. W. Fuller, C. W. Hines and J. Mills, *Biochem. Pharmac.* **14**, 483 (1965).
3. E. M. Gal, P. A. Christiansen and L. M. Yunger, *Neuropharmacology* **14**, 31 (1975).
4. E. Sanders-Bush, D. A. Gallager and F. Sulser, *Adv. Biochem. Psychopharmac.* **10**, 185 (1974).
5. H. J. Sekerke, H. E. Smith, J. A. Bushing and E. Sanders-Bush, *J. Pharmac. exp. Ther.* **193**, 835 (1975).
6. R. W. Fuller, H. P. Snoddy, K. W. Perry, F. P. Bymaster and D. T. Wong, *Biochem. Pharmac.* **27**, 193 (1977).
7. J. A. Harvey and S. E. McMaster, *Psychopharmac. Bull.* **12**, 62 (1976).
8. J. A. Harvey, S. E. McMaster and L. M. Yunger, *Science* **187**, 841 (1975).
9. M. E. Smith, E. P. Burrows, D. D. Miano, C. D. Mount, E. Sanders-Bush and F. Sulser, *J. med. Chem.* **17**, 416 (1974).
10. E. Sanders-Bush, J. A. Bushing and F. Sulser, *Biochem. Pharmac.* **21**, 1501 (1972).
11. C. J. Parli, *Psychopharmac. Bull.* **12**, 54 (1976).
12. R. W. Fuller, K. W. Perry, J. C. Baker, C. J. Parli, N. Lee, W. A. Day and B. B. Molloy, *Biochem. Pharmac.* **23**, 3267 (1974).
13. J. J. Parli and B. Schmidt, *Res. Commun. Chem. Path. Pharmac.* **10**, 601 (1975).
14. M. M. Ames, S. D. Nelson, W. Lovenberg and H. A. Sasame, *Commun. Psychopharmac.* **1**, 455 (1977).
15. R. E. Heikkila and G. Cohen, in *Chemical Tools in Catecholamine Research* (Eds. G. Jonsson, T. Malmfors and C. Sachs), Vol. 1, p.7. North-Holland, Amsterdam (1975).
16. C. R. Creveling, A. Rotman and J. W. Daly, in *Chemical Tools in Catecholamine Research* (Eds. G. Jonsson, T. Malmfors and C. Sachs), Vol. 1, p. 23. North-Holland, Amsterdam (1975).
17. P. Jenner and B. Testa, *Drug Metab. Rev.* **2**, 117 (1973).
18. J. Caldwell, *Drug Metab. Rev.* **5**, 214 (1976).
19. P. W. Feit and H. Brunn, *Archs int. Pharmacodyn. Thér.* **170**, 443 (1967).
20. R. M. Herbst and D. Shemin, *Org. Synth.*, Collective Vol. 2, p. 11 (1943).
21. L. Ernster, P. Siekevits and G. Palade, *J. Cell Biol.* **15**, 541 (1962).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
23. J. Gal, *J. pharm. Sci.* **66**, 169 (1977).
24. J. Gal, J. Wright and A. K. Cho, *Res. Commun. Chem. Path. Pharmac.* **15**, 525 (1976).
25. N. P. McGraw, P. S. Callery and N. Castagnoli, *J. med. Chem.* **20**, 185 (1977).
26. H. A. Sasame, M. M. Ames and S. D. Nelson, *Biochem. biophys. Res. Commun.* **78**, 919 (1977).
27. L. Demisch and N. Seiler, *Biochem. Pharmac.* **24**, 575 (1975).
28. S. A. Barker, J. A. Monti and S. T. Christian, *Biochem. Pharmac.* **29**, 1049 (1980).