ELSEVIER

Biochimica et Biophysica Acta 1238 (1995) 29–33

BBA Biochimica et Biophysica Acta

Uptake and metabolism of Δ^1-piperidine-2-carboxylic acid by synaptosomes from rat cerebral cortex

Yung-Feng Chang *, Ambrose K. Charles [1]

Department of Biochemistry, University of Maryland Dental School, 666 West Baltimore Street, Baltimore, MD 21201, USA

Received 6 March 1995; accepted 24 March 1995

Abstract

Δ^1-Piperidine-2-carboxylic acid (P2C), an intermediate of the L-lysine metabolic pathway in the brain, was studied for its uptake and metabolism in the synaptosome of the rat cerebral cortex. The results of this study showed that the uptake of P2C into the synaptosome was NA^+-and temperature-dependent with a two-tier transport kinetic (K_m = 2.6 and 0.7 μM; V_{max} = 1.6 and 0.73 pmol/min/mg). P2C uptake was only moderately inhibited ($\approx$ 20%) by L-lysine and its metabolites, L-pipecolic acid and L-α-aminoadipic acid at up to 100 μM, and the putative amino acid neurotransmitters, γ-aminobutyric acid, L-glutamic acid and L-aspartic acid (25–31%) at 5–500 μM. The synaptosomal preparation only has a very low activity for metabolizing P2C to its product L-pipecolic acid. The metabolic activity for P2C was mainly contained in the 27000 $\times$ g supernatant S_2 fraction. Since P2C is the precursor of the putative neuromodulator L-pipecolic acid, the understanding of its uptake and metabolic characteristics in the brain should be of significance.

Keywords: Δ^1-Piperidine-2-carboxylate; Synaptosome; Transport; Metabolism; Neuromodulator

1. Introduction

Δ^1-Piperidine-2-carboxylic acid (P2C; the cyclic form of α-keto-ϵ-aminocaproic acid) is an intermediate of L-lysine metabolism through the pipecolic acid pathway which is different from the more familiar pathway involving saccharopine (ϵ-*N*-(glutaryl-2)-L-lysine) as a first product [1]. An L-amino acid oxidase L-amino acid: O_2 oxidoreductase (deaminating) from the microsome of chicken liver [2] and an L-lysine dehydrogenase L-lysine: nicotinamide-adenine dinucleotide (NAD)-oxidoreductase (deaminating) from the soluble fraction of human liver homogenate [3] have both been reported to metabolize L-lysine to P2C. In bacteria, however, P2C appears to be a product of D-lysine metabolism [4]. A nucleotide-linked enzyme, P2C reductase (L-Pipecolic acid: NAD $(NADP)_2$-oxidoreductase) responsible for the conversion of P2C to L-pipecolic acid has been described in both the mammals [5] and the bacteria [6]. In our preliminary study we have characterized and purified this enzyme to near homogeneity and it will be submitted for publication in the near future. Measurement of Δ^1-pyrroline-2-carboxylate reductase activity taken as pipecolic acid formation exhibits considerable regional differences in the central nervous system of the mouse, dog and monkey [7]. The regional distribution of the enzyme P2C reductase which is an actual precursor of pipecolic acid has not so far been studied.

L-Pipecolic acid has been shown to be a major product of L-lysine metabolism in the animal and human brains [8–10] with many neurological studies been reported [11–16]. The uptake and release of γ-aminobutyric acid (GABA) have been shown to be influenced by pipecolic acid [17–19]. In addition, pipecolic acid has been shown to modulate GABA binding of GABA to its brain membranes in the presence of barbiturates [20], and it also has a specific binding to the brain membranes [21]. However, despite P2C is a precursor of L-pipecolic acid, perhaps because P2C is not commercially available, no transport or neurochemical study has so far been reported. We report here a study which concerns the metabolism and uptake of P2C by the synaptosome-enriched fraction (P_3) prepared from the rat cerebral cortex. The effects of other lysine metabolites and putative amino acid neurotransmitters, GABA, L-glutamate and L-aspartate, on the uptake have also been investigated.

* Corresponding author. Fax: +1 (410) 7060193.

[1] Present address: Institute of Experimental Pathology and Toxicology, Albany Medical College, Albany, NY 12208, USA.

SSDI 0005-2736(95)00092-5

2. Experimental procedures

2.1. Materials

L-[1-^{14}C]Lysine was obtained from New England Nuclear Corporation; L-lysine, L-pipecolic acid, DL-pipecolic acid, DL-α-aminoadipic acid, L-aspartic acid and L-glutamic acid from Sigma; γ-aminobutyric acid from Mann Research Laboratories; D-amino acid oxidase (EC 1.4.3.3) from Sigma; Dowex 50 (H^+) from Bio-Rad Laboratories; Hydromix from Yorktown Research.

2.2. Preparation of synaptosome-enriched P3 fraction

Synaptosome-enriched P_3 fraction was prepared from the cerebral cortex of male Sprague-Dawley rats (150–200 g) according to a method combining the procedures of Wheeler [22] and Tamir et al. [23]. In general, the cerebral cortex (excluding mesencephalon, pons, and medulla) from rats was homogenized in 10 volumes of ice-cold 0.32 M sucrose by a glass tissue grinder equipped with a Teflon pestle driven by a motor at 650 rpm for 10–15 strokes. The homogenates were centrifuged at $1000 \times g$ for 10 min to remove the nuclei and other cell debris (P_1). The supernatant (S_1) was centrifuged again at $27\,000 \times g$ for 15 min to obtain the synaptosome-mitochondria-myelin pellet (P_2). The supernatant fraction (S_2) was saved for metabolism study. The P_2 fraction after suspension in 0.32 M sucrose was layered onto a discontinuous sucrose gradient prepared from 8 ml each of 0.8 and 1.2 M sucrose in half-strength Krebs-Ringer solution free of Ca^{2+}. After centrifugation at $55\,000 \times g$ for 60 min, the synaptosome-enriched P_3 fraction was recovered in the 0.8 M sucrose layer.

2.3. Synthesis of [1-^{14}C]P2C

[1-^{14}C]P2C (300 Ci/mol) was synthesized from DL-[1-^{14}C]pipecolic acid by exhaustive oxidation of the latter compound with D-amino acid oxidase as described previously [25]. DL-[1-^{14}C]Pipecolic acid was prepared from L-[1-^{14}C]lysine according to the method of Rodwell [24] as described previously [25]. Unlabeled P2C was prepared from DL-pipecolic acid similar to that of the labeled P2C [25]. Both labeled and unlabeled P2C were dissolved in 0.01 M HCl and kept frozen. The compound was neutralized immediately before use.

2.4. Uptake of [1-^{14}C]P2C by synaptosomes

A standard assay of P2C uptake was conducted by incubating 0.1 ml [1-^{14}C]P2C (0.5 M, 1.00 Ci/ml) with 0.2 ml synaptosome preparation (P_3) (0.2–0.5 mg/ml) in 3.7 ml of Krebs-Henseleit buffer, pH 7.4 (concentration of the ingredients in mM: NaCl, 127.2; KCl, 5.0; $MgSO_4$, 1.3; $CaCl_2$, 2.7; Tris, 25.0) for 5 min at 37°C. At the end of the incubation, P2C uptake was stopped by filtration through a Reeve Angel (Whatman) 934 AH glass filter (2.4 cm diameter) on a Hoefer vacuum filtration manifold. The filter was washed twice, each with 5 ml cold buffer. The dried filter with the retained synaptosomes was mixed with Hydromix and assayed for radioactivity in a Packard 2425 liquid scintillation spectrometer. The counting efficiency of the samples was estimated by the use of automatic external standardization. For the assay of P2C uptake at various concentrations, unlabeled P2C was added to obtain the desired concentrations. To test dependency of P2C uptake on Na^+, Na^+ ions were replaced by Tris in the Krebs-Henseleit buffer to maintain a constant osmolarity.

2.5. Metabolism of [1-^{14}C]P2C

[1-^{14}C]P2C (0.2 Ci) was incubated with the cerebral cortex crude homogenate, the S_2 or the P_3 fraction (each containing approx. 1 mg protein) at 37°C for 60 min in a final volume of 1 ml. After the incubation the mixtures were extracted with 4 volumes of 0.4 M perchloric acid. After neutralization with 0.4 M KOH and removal of precipitated perchlorate by centrifugation, the supernatant solution was flash evaporated to dryness under reduced pressure at 50°C. The residues dissolved in 0.01 M HCl were analyzed by a Technicon amino acid analyzer equipped with a Durrum DC-1A Column as described previously [8,9]. The column was eluted with buffers B and C of Pico-Buffer II (Pierce) programmed for 35 and 100 min, respectively. The column effluent collected by a fraction collector was counted for radioactivity as described previously [8,9]. Characterization of pipecolic acid and α-aminoadipic acid, the reaction products of P2C, was by means of amino acid analysis and paper chromatography as previously described [8,9,25,26].

3. Results

3.1. Uptake of [1-^{14}C]P2C by synaptosomes

The uptake of P2C by the synaptosomes of rat cerebral cortex was proportional to the incubation time (Fig. 1). The rate of this uptake remained linear in relation to the incubation time for up to 5 min under the experimental conditions described. The maximal tissue-medium ratio of approx. 3.6 was attained after 10 min of incubation.

The rate of synaptosomal uptake of P2C was temperature-dependent with a Q_{10} value of 1.2 (Fig. 2). The transport of P2C was only partially Na^+-dependent (Table 1). Even in the absence of Na^+, the synaptosomal P2C uptake rate was still 63% of that when Na^+ concentration was 127.2 mM.

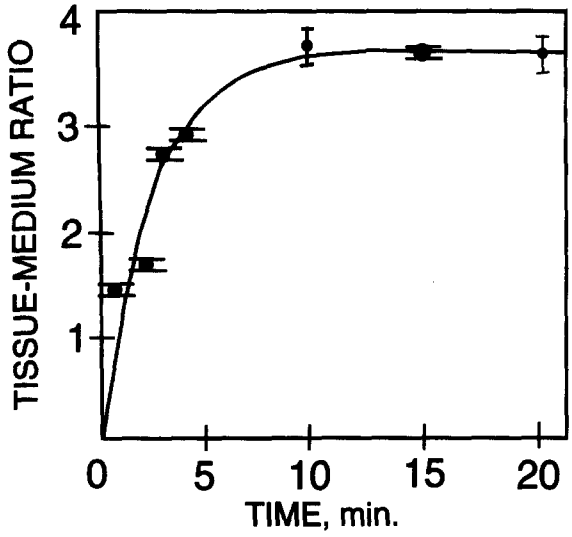

Fig. 1. Time-course of accumulation labeled P2C into rat cortical synaptosome. Experiment was conducted as in the standard assay except that samples were taken at time intervals as shown. Results are means ± S.E. from quadruplicate measurements.

Table 1
Dependency of P2C uptake into synaptosome on sodium ion

NaCl (mM)	P2C uptake (pmol/mg synaptosome)	Uptake rate (%)
127.2	1.49 ± 0.03	100 ± 1.9
50	1.06 ± 0.04	71.1 ± 2.4
20	1.06 ± 0.05	71.1 ± 3.6
0	0.94 ± 0.04	62.9 ± 2.7

P2C uptake was measured as described in the standard assay except that NaCl was replaced with equivalent amounts of Tris in the Krebs Henselit buffer when NaCl concentration was reduced from 127.2 mM. Values are means ± S.E. from quadraplicate experiments.

3.2. Kinetics of [1-^{14}C]P2C uptake into synaptosomes

Kinetic analysis of the uptake of P2C into synaptosomes was done by the double reciprocal plot of Lineweaver and Burk [27] as presented in Fig. 3. The K_m values calculated by the least square fit for the uptake of P2C were found to be 2.6 M and 0.74 μM. The V_{max}

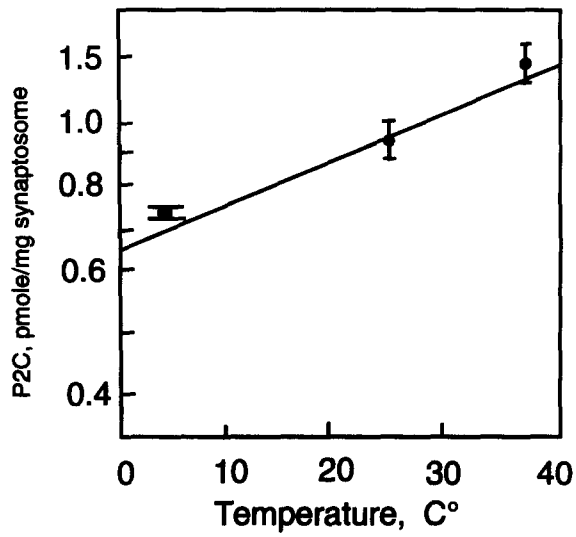

Fig. 2. Temperature dependency of labeled P2C uptake into rat cortical synaptosome. Experiment conditions were as those in the standard assay except that incubation was conducted at temperatures shown. Values are means ± S.E. from quadruplicate measurements.

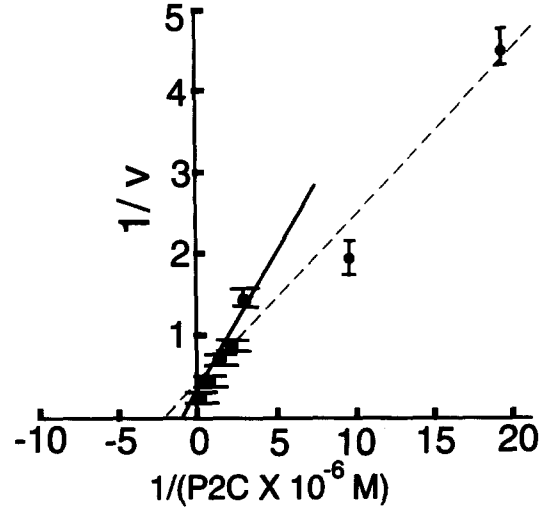

Fig. 3. Kinetic analysis of the uptake of labeled P2C into rat cortical synaptosome. Experimental conditions were same as those for the standard assay. Values are means ± S.E. of four measurements. The lines represent double reciprocal plots of Lineweaver and Burk of the computed least square fit. V: pmol/5 min/mg.

Table 2
Effect of L-lysine, L-pipecolate and L-α-aminoadipate on the uptake of P2C into synaptosome

Effector concentration (μM)	L-Lysine		L-Pipecolic acid		L-α-Aminoadipic acid	
	pmol/mg	inhibition %	pmol/mg	inhibition %	pmol/mg	inhibition %
0	1.44 ± 0.15	–	1.44 ± 0.15	–	1.44 ± 0.15	–
5	1.11 ± 0.05	23.1 ± 3.7	1.15 ± 0.02	20.2 ± 1.5	1.15 ± 0.05	20.4 ± 3.3
50	1.14 ± 0.08	21.3 ± 5.8	1.15 ± 0.06	20.5 ± 3.9	NA	NA
100	1.06 ± 0.06	26.9 ± 4.4	1.26 ± 0.08	12.6 ± 5.2	NA	NA
100	0.99 ± 0.06	31.5 ± 4.1	1.22 ± 0.07	15.7 ± 4.8	1.19 ± 0.09	17.3 ± 6.5

NA denotes 'not assayed'.
P2C uptake was measured as described in the standard assay except that NaCl was replaced with equivalent amounts of Tris in the Krebs Henselit buffer when NaCl concentration was reduced from 127.2 mM. Values are means ± S.E. from quadraplicate experiments.

Table 3
Effect of GABA, L-glutamate and L-aspartate on uptake of P2C into synaptosome

Effector concentration (μM)	GABA		L-Glutamate		L-Aspartate	
	pmol/mg	inhibition %	pmol/mg	inhibition %	pmol/mg	inhibition
0	1.49 ± 0.03	–	1.49 ± 0.03	–	1.49 ± 0.03	–
5	1.11 ± 0.04	25.4 ± 2.5	1.03 ± 0.05	30.5 ± 3.2	1.09 ± 0.04	26.6 ± 5.4
50	1.32 ± 0.02	11.5 ± 1.5	NA	NA	NA	NA
100	1.26 ± 0.13	15.1 ± 8.5	1.29 ± 0.17	13.5 ± 11.6	1.19 ± 0.07	19.7 ± 4.8
500	1.20 ± 0.04	19.1 ± 2.9	1.16 ± 0.05	21.9 ± 3.2	1.11 ± 0.06	25.5 ± 4.2

NA denotes 'not assayed'.
P2C uptake was measured as described in the standard assay except that NaCl was replaced with equivalent amounts of Tris in the Krebs Henselit buffer when NaCl concentration was reduced from 127.2 mM. Values are means ± S.E. from quadraplicate experiments.

values obtained from this analysis were 1.6 pmol/min/mg and 0.73 pmol/min/mg.

3.3. Effect of lysine metabolites, GABA, L-glutamate and L-aspartate on the uptake of P2C

P2C uptake into synaptosomes was inhibited 20 to 23% by L-lysine and two of its metabolic intermediates, L-pipecolic acid and L-α-aminoadipic acid at 5 μM (Table 2). This inhibition was increased to about 32% by L-lysine at 0.5 mM. The increase of L-pipecolate and L-α-aminoadipate concentrations not only did not increase but slightly reduced their inhibition on P2C uptake.

At 5 μM GABA inhibited P2C uptake by 25% while L-glutamic acid and L-aspartic acid by 31 and 27%, respectively (Table 3). The increase of the concentration of GABA and L-glutamate to 0.5 mM, however, slightly reduced their inhibition on P2C uptake.

3.4. Metabolism of P2C

Very active metabolism of P2C was observed in the crude homogenate of rat cerebral cortex and the 27 000 × g supernatant (S_2) (Table 4). After 60 min of incubation 51.5% of the starting material was converted to pipecolic acid by the crude homogenate, and 94.3% by the S_2 fraction. Other metabolic products of P2C were also detected in these two incubation mixtures. These products include α-aminoadipic acid and other uncharacterized metabolic intermediates which amount to about 19 and 4% of the total recovered radioactivity in the crude homogenate and S_2 incubations, respectively (Table 4). The synaptosomal fraction (P_3) has only very low activity of P2C metabolism. After 60 min of incubation, more than 93% of P2C was recovered with less than 3% of the starting material being converted to pipecolic acid, and about 4% to other metabolic products.

Table 4
Metabolism of [1-^{14}C]P2C by crude homogenate (27000× g) supernatant (S_2) and synaptosome (P_3) fractions of the rat cerebral cortex [a]

Preparation	% Radioactivity recovered		
	P2C	piecolate	other metabolites [b]
Crude homogenate	29.3	51.5	19.2
S_2	1.6	94.3	4.1
P_3	93.5	2.6	3.9

[a] 0.2 Ci of [1-$_{14}$]P2C was incubated with the crude cerebral cortex homogenate (27000× g) supernatant (S_2) or the synaptosome (P_3) fraction for 60 min at 37°C as described in the Experimental procedures. The incubation mixtures were extracted and analyzed by an amino acid analyzer as described. The overall recovery of radioactivity was approx. 70% of the starting material for every incubation.
[b] 'Other metabolites' refer to metabolic products of P2C other than pipecolic acid. These metabolites include α-aminoadipic acid and other compounds which are not yet clearly characterized.

4. Discussion

P2C displayed a temperature-and Na^+-dependent uptake by the synaptosome-enriched fraction (P_3) of the rat cerebral cortex, suggesting that this uptake is most likely a specific rather than a diffusional mechanism. The dependency on Na^+ for P2C uptake appears to be much less than that for the putative amino acid neurotransmitters L-glutamate and L-aspartate [28], or that for L-α-aminoadipate [29], another intermediate of the lysine metabolic pathway in the brain. It should be noted that the uptake of pipecolic acid, another lysine metabolite of neurological interest [13], is only partly inhibited when Na^+ concentration is lowered.

P2C uptake into the rat cortical synaptosomes appears to display an unusual two-tier transport kinetic (Fig. 3). Since both K_m values are very low (0.74 and 2.6 μM), they represent the high-affinity, low-capacity uptake. The reason that the low-affinity transport is not apparent for P2C might be due to masking by the high affinity uptake as reported by Davies and Johnson for D-aspartic uptake [30]. On the other hand, it is interesting to note that other lysine metabolites pipecolic acid and α-aminoadipic acid display a clear high- and low-affinity uptake [15,29].

The uptake of P2C is only partially inhibited by other

lysine intermediates and other amino acids tested (Table 2 and Table 3). Since the level of inhibition by either the alicyclic compound (i.e., L-pipecolic acid) or the aliphatic compounds (i.e., L-lysine and L-α-aminoadipic acid) are not much different, it suggests that there is no preference of P2C been transported in the cyclic form (i.e., Δ^1-piperideine-2-carboxylic acid) or in the aliphatic form (α-keto-ζ-aminocaproic acid). However, since the transport of an amino acid usually can be inhibited by several compounds with different degrees of structural similarity [31], the phenomenon observed above may be another example of low specificity inhibition common in amino acid transport. On this basis, it is interesting to note that proline, a close analog of pipecolic acid, at the concentration of 10^{-4} M inhibits pipecolic acid uptake by synaptosomes from mouse brain by 50% [15].

Active metabolism of P2C to pipecolic acid was observed in the crude homogenate and supernatant fraction (S_2) of rat cerebral cortex (Table 4). The slight activity of P2C metabolism to pipecolic acid and other metabolites observed in the P_3 fraction might be due to either a contamination of the S_2 fraction or the presence of P2C reductase inside the synaptosomes. The latter possibility is currently under investigation. As expected this conversion is most effective in the supernatant fraction since P2C reductase has been shown earlier to be a soluble enzyme both in the animals in our preliminary study mentioned above and in the bacteria [6]. Pipecolic acid is known to be metabolized to α-aminoadipic acid in the rat [8,9] and in the human and monkey [10]. This may explain the presence of α-aminoadipic acid and other metabolites in the incubation mixtures of the crude homogenate and the S_2 fraction when P2C was the substrate.

As noted by us previously [25], P2C is quite unstable. It polymerizes readily under storage at room temperature at neutral pH [25]. No P2C has yet been detected in the living system, indicating its transitory status as an intermediate in the lysine metabolic pathway, and its unstable nature. However, in view of the fact that the metabolic system for P2C is present as P2C reductase has been identified, purified and characterized in our preliminary study. P2C is actively taken up by the synaptosomes of rat brain, and that this uptake is influenced by other putative amino acid neurotransmitters as described in the present article, undoubtedly the uptake and metabolism of P2C play a significant role in neuronal function which has yet to be fully elucidated. More importantly P2C is the precursor of the putative neuromodulator L-pipecolic acid. The catabolism of pipecolate is defective due to the dysfunction of the peroxisomes. Several neurological diseases such as the hyperpipecolatemia, adrenoleukodystrophy and the cerebrohepatorenal syndrome of the Zellweger's (for review see Ref. [32]) are known to exist. Further study and understanding of P2C transport and its neurochemical characteristics are warranted.

Acknowledgements

This work is supported by the U.S. Public Health Grant NS11822 from the National Institute of Neurological Disorders and Stroke. The authors thank Ms. M. Dombrowski, Mrs. Sheila McNair and Ms. Joann Sonya Walker for their skillful secretarial assistance.

References

[1] Scriver, C.R. and Rosenberg, L.E. (1973) Amino Acid Metabolism and Its Disorders, pp. 250–255, Saunders, Philadelphia.
[2] Struck, J. and Sizer, I.W. (1960) Arch. Biochem. Biophys. 90, 22–30.
[3] Burgi, W., Richterich, R. and Colombo, J.P. (1966) Nature 211, 854–855.
[4] Chang, Y.F. and Adams, E. (1974) J. Bacteriol. 117, 753–764.
[5] Meister, A., Radhakrishnan, A.N. and Buckley, S.D. (1957) J. Biol. Chem. 229, 789–800.
[6] Payton, C.W. and Chang, Y.F. (1982) J. Bacteriol. 149, 864–871.
[7] Garweg, G., Von Rehren, D. and Hintze, U. (1980) J. Neurochem. 35, 616–621.
[8] Chang, Y.F. (1976) Biochem. Biophys. Res. Commun. 69, 174–180.
[9] Chang, Y.F. (1978) J. Neurochem. 30, 347–354.
[10] Chang, Y.F. (1982) Neurochem. Res. 7, 577–588.
[11] Charles, A.K. and Chang, Y.F. (1982) Life Sci. 29, 947–954.
[12] Charles, A.K. Chang, Y.F. and Myslinski, N.R. (1983) Neurochem. Res. 8, 1087–1096.
[13] Kase, Y., Takahama, K., Hashimoto, T., Kaisaku, J., Okano, Y. and Miyata, T. (1980) Brain Res. 193, 608–613.
[14] Nomura, Y., Okuma, Y., Segawa, T., Schmidt-Glenewinkel, T. and Giacobini, E. (1979) J. Neurochem. 33, 803–805.
[15] Nomura, Y., Schmidt-Glenewinkel, T. and Giacobini, E. (1980) Neurochem. Res. 5, 1163–1173.
[16] Miyata, T., Kamata, K., Noguchi, M., Okano, Y. and Kase, Y. (1973) Jpn. J. Pharmacol. 23 (Suppl.), 81.
[17] Nomura, Y., Okuma, Y. and Segawa, T. (1978) J. Pharm. Dyn. 1, 251–255.
[18] Nomura, Y., Okuma, Y., Segawa, T., Schmidt-Glenewinkel, T. and Giacobini, E. (1981) Neurochem. Res. 6, 391–400.
[19] Okuma, Y., Nomura, Y. and Segawa. T. (1979) J. Pharm. Dyn. 2, 261–265.
[20] Feigenbaum, P. and Chang, Y.F. (1986) Brain Res. 327, 176–179.
[21] Charles, A.K. (1986) Neurochem. Res. 11, 521–525.
[22] Wheeler, D.C. (1979) J. Neurochem. 32, 1197–1213.
[23] Tamir, H., Rapport, M.M. and Roizin, L. (1974) J. Neurochem. 23, 943–949.
[24] Rodwell, V.W. (1971) Methods Enzymol. 17B, 174–188.
[25] Chang, Y.F., Charles, A.K. and Tilkin, R.B. (1982) Anal. Biochem. 125, 376–395.
[26] Hernandez, M.F. and Chang, Y.F. (1980) Biochem. Biophys. Res. Commun. 93, 762–769.
[27] Lineweaver, H and Burk, D. (1934) J. Am. Chem. Soc. 56, 658–666.
[28] Bennett, J.P., Mulder, A.H. and Snyder, S.H (1974) Life Sci. 15, 1045–1056.
[29] Charles, A.K. and Chang, Y.F. (1981) J. Neurochem. 36, 1127–1136.
[30] Davies, L.P and Johnson, G.A.R. (1976) J. Neurochem. 26, 1007–1014.
[31] Bennett, J.P., Logan, W.J. and Snyder, S.H. (1973) J. Neurochem. 21, 1533–1550.
[32] Lazarow, P.B. and H.W. Moser (1989) in The Metabolic Basis of Inherited Disease (Schriver, C.R., Beudet, A.L., Sly, W.S. and Valle, D., eds.), 6th Edn., pp. 1479–1509, McGraw Hill, New York.