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ARYL ACYLAMIDASE OF MONKEY BRAIN AND LIVER: RESPONSE TO INHIBITORS AND RELATIONSHIP TO ACETYLCHOLINESTERASE

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Abstract—The serotonin sensitive aryl acylamidase (aryl acylamide amidohydrolase EC 3.5.1.13) of monkey brain was compared with the liver enzyme. Although the two enzymes showed some similarities in their properties such as pH optima, the effect of metal ions and thiol agents, they significantly differed in their mol. wt and response to inhibitors. The liver enzyme had a higher mol. wt as observed by gel filtration on Sepharose 6B and a greater heat stability. The brain enzyme was inhibited specifically by the amines serotonin and tryptamine as well as by acetylcholine and its analogues and homologues in a non-competitive manner. The liver enzyme was unaffected by the above mentioned amines or acetylcholine but it was non-competitively inhibited by indole-3-acetic acid and indole-3-propionic acid both compounds having no effect on the brain enzyme. Eserine, a strong competitive inhibitor of acetylcholinesterase, at 10^{-7} M inhibited the brain aryl acylamidase to 75 per cent leaving the liver enzyme unaffected. Eserine inhibition of the brain enzyme was non-competitive. From Dixon plots serotonin, acetylcholine and eserine were shown to act at the same site on brain aryl acylamidase. The inhibition of the brain enzyme by eserine and acetylcholine, the elution of both aryl acylamidase and acetylcholinesterase activities in the same fractions during gel filtration and the regional distribution of aryl acylamidase in the brain suggested the association of aryl acylamidase with acetylcholinesterase in the brain though not in the liver.

The sensitivity of the enzyme aryl acylamidase (aryl-acylamide amidohydrolase EC 3.5.1.13) from rat brain to serotonin and the insensitivity of this enzyme from the rat liver towards this amine have been demonstrated in earlier studies [1, 2]. The biological role of this enzyme in the mammalian system remains uncertain although Fujimoto [1] has suggested that the enzyme may serve to hydrolyze some antipyretic and analgesic drugs which are aryl acylamides. Fujimoto has also hypothesized that the brain aryl acylamidase could serve as a model for the study of the mechanism of action of serotonin in the mammalian central nervous tissues [1]. We have observed that the aryl acylamidase of sheep brain but not liver was significantly inhibited by not only serotonin but also by acetylcholine, butyrylcholine and a number of other analogues and homologues of acetylcholine [3]. Fujimoto has recently studied the aryl acylamidase activity of purified *Electrophorus electricus* acetylcholinesterase and suggested that the serotonin sensitive aryl acylamidase is associated with acetylcholinesterase [4].

The remarkable difference in the sensitivity to serotonin and acetylcholine between the brain and liver enzymes prompted us to study the characteristics of aryl acylamidase from the monkey brain and liver. Furthermore we have examined the possibility of the association of brain aryl acylamidase with acetylcholinesterase. The data presented in this paper indicate that the liver aryl acylamidase differs from the brain enzyme not only in its response to inhibitors but also in its mol. wt, heat stability and in its association with acetylcholinesterase.

MATERIALS AND METHODS

Monkey (Macaca radiata) brain and liver obtained immediately after sacrifice of the animal under nembutal anesthesia were stored frozen at −18° until required. Albino rats of either sex of this laboratory strain were decapitated and the brains and livers stored at −18° until required. The various amines, indole-3-acetic acid, indole-3-propionic acid, phenylacetic acid, *p*-hydroxyphenylpyruvic acid, acetylcholine and related choline derivatives, and eserine sulphate were obtained from Sigma Chemical Company, U.S.A. All other chemicals were obtained as described earlier [3].

O-Nitroacetanilide. *O*-Nitroacetanilide was prepared from *O*-nitroaniline as described earlier [3]. The final product was recrystallized thrice from hot water and had a melting point of 93–94°.

Preparation of monkey brain and liver aryl acylamidase. All operations were carried out at 0–4° unless otherwise mentioned.

Monkey brain or liver homogenate and the subsequent ammonium sulphate fractions obtained between 30–65 per cent saturation of ammonium sulphate were prepared as described earlier for sheep brain [3]. The ammonium sulphate fractions were dialyzed against 0.05 M potassium phosphate buffer pH 7.0 for 8 hr with four changes of buffer. Unless otherwise mentioned this ammonium sulphate fraction was used as the source of monkey brain and liver aryl acylamidase.

While studying the developing rat brain and liver,

the homogenate from these tissues was used as the source of aryl acylamidase.

The homogenate was also used as the enzyme source while studying the regional distribution of aryl acylamidase in the fresh monkey brain.

Sepharose 6B gel filtration. The brain or liver ammonium sulphate fractions (1.5 ml) were applied to a Sepharose 6B (2.1 cm × 41.5 cm) column equilibrated with 0.05 M potassium phosphate buffer pH 7.0 containing 0.1 M NaCl at a flow rate of 16 ml/hr. Fractions of 2 ml were collected.

DEAE-Cellulose chromatography. The brain (1.0 ml) or liver (0.7 ml) ammonium sulphate fraction was applied on a DEAE-cellulose (0.9 cm × 5.5 cm) column equilibrated with 0.05 M potassium phosphate buffer pH 7.0. After washing with the same buffer containing 0.5 per cent Triton X-100 the enzyme was eluted with a linear gradient of 0–0.5 M NaCl in the same buffer containing 0.5 per cent Triton X-100.

Assay of aryl acylamidase. The assay of aryl acylamidase was carried out as described earlier with 0.02–0.05 ml enzyme (0.05–0.1 ml enzyme in the case of rat liver and brain homogenate and monkey brain homogenate and 0.25 ml in the case of the Sepharose 6B effluent [3]). The reaction rate was linear with respect to time and protein concentrations in all the assays.

Assay of acetylcholinesterase. Acetylcholinesterase was assayed according to the method of Hestrin [5]. The assay mixture contained in a total volume of 0.5 ml, 50 μmoles potassium phosphate buffer pH 7.5, 200 μmoles NaCl, 50 μmoles $MgCl_2$, 4 μmoles acetylcholine iodide and 0.02 ml enzyme (or 0.16 ml enzyme of the fractions eluted from the Sepharose 6B column). The incubation period was 30 min at 37°.

Protein estimation. Protein was estimated according to the method of Lowry *et al.* [6] using crystalline bovine serum albumin as standard.

RESULTS

General characteristics and similarities of the monkey brain and liver aryl acylamidases. A number of similarities were observed between the monkey brain and liver aryl acylamidases. The enzyme from both sources was unaffected by a number of common divalent cations such as Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and EDTA up to 2 mM concentrations although Cu^{2+} and Hg^{2+} at this concentration were found to inhibit both the brain and liver enzyme 50 and 100 per cent respectively. The thiol reagents *p*-chloromercuribenzoate, iodoacetamide, and *N*-ethylmaleimide as well as dithiothreitol at concentrations up to 10 mM had no effect on the enzyme from either organ. The brain enzyme exhibited a pH optimum in the range of pH 7.0–8.0 and the liver enzyme in the range pH 7.5–8.0. Similar elution profiles for the brain and the liver enzymes were obtained on DEAE–cellulose chromatography.

Inhibition by amines and choline derivatives. The amines histamine, tyramine, dopamine, noradrenaline and benzylamine up to concentrations of 10 mM were found to be non-inhibitory to liver and brain aryl acylamidase. A 50 per cent loss in activity of the brain enzyme was noted with 0.2 mM serotonin and 8 mM tryptamine. Both these amines were found to have no effect on the activity of the liver enzyme. The amino acid tryptophan did not alter the aryl acylamidase activity from either brain or liver.

The brain but not the liver aryl acylamidase was found to be inhibited by choline and a number of its derivatives. The inhibition at 2 mM was found to be in the order butyrylcholine > succinylcholine > benzoylcholine > choline > acetylcholine > acetylthiocholine > acetyl-β-methylcholine > propionylcholine. Acetylcholine at a concentration of 4 mM produced approximately 50 per cent inhibition of the brain enzyme. The inhibition characteristics were similar to those of the sheep brain and liver enzyme reported earlier [3]. Table 1 shows the effect of serotonin, tryptamine and acetylcholine at various concentrations on the monkey brain and liver aryl acylamidase.

Inhibition of liver aryl acylamidase. The monkey liver aryl acylamidase was inhibited by indole-3-acetic acid and indole-3-propionic acid at 1 mM to 60 and 40 per cent respectively. Both these compounds had

Table 1. Inhibition of monkey brain and liver aryl acylamidase

Inhibitor (non-competitive)	Conc. M	% Inhibition Brain	Liver
Serotonin	1×10^{-4}	22	0
	1×10^{-3}	72	0
	2×10^{-3}	82	0
Tryptamine	1×10^{-3}	18	0
	2×10^{-3}	28	0
	1×10^{-2}	62	0
Acetylcholine	2×10^{-3}	26	0
	4×10^{-3}	53	0
	1×10^{-2}	72	0
Indole-3-acetic acid	5×10^{-4}	0	33
	1×10^{-3}	0	58
Indole-3-propionic acid	5×10^{-4}	0	23
	1×10^{-3}	0	40
	5×10^{-3}	0	65
Eserine	1×10^{-7}	74	4
	1×10^{-6}	92	14
	1×10^{-5}	95	38

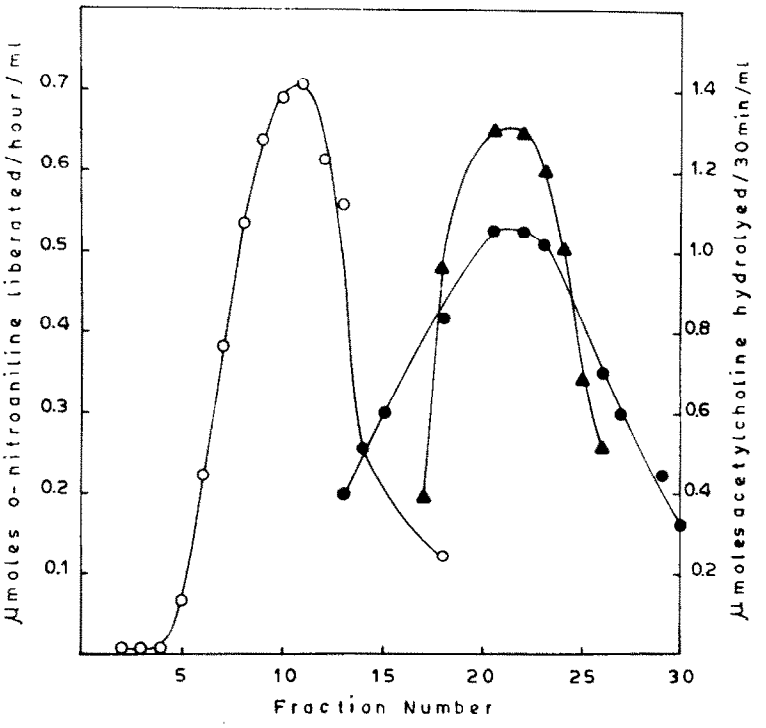

Fig. 1. Sepharose 6B chromatography of monkey brain and liver aryl acylamidase and monkey brain acetylcholinesterase. (See Methods). O——O Monkey liver aryl acylamidase; ●——● Monkey brain aryl acylamidase; ▲——▲ Monkey brain acetylcholinesterase.

no inhibitory effect on the brain enzyme (Table 1). From double reciprocal plots the inhibitions were found to be non-competitive in nature. At 1 mM phenylacetic acid and *p*-hydroxyphenylpyruvic acid, which have some structural similarity to the above compounds but which are devoid of the indole ring, were observed to have no effect on both the liver and brain enzymes.

Gel filtration on Sepharose 6B. Gel filtration on Sepharose 6B indicated that the liver enzyme was of a higher mol. wt than the brain enzyme (Fig. 1). The gel filtration experiments also showed that monkey brain aryl acylamidase activity and acetylcholinesterase activity eluted together in the same fractions (Fig. 1).

Thermostability of the enzyme. The brain and liver aryl acylamidases (at equal protein concentrations) were heated for intervals of 1, 2, 4, 5, 8, 10, 15 and 30 min at 56° and it was noted that at the various time intervals the percentage loss in enzyme activity was always greater for the brain enzyme than for the liver enzyme. A similar pattern of enzyme inactivation was observed at a temperature of 60° for the time intervals specified above up to 10 min. Heating the brain and liver enzymes at 56° for 20 min led to a loss of 75 and 60 per cent activity respectively while heating at 60° for 10 min resulted in activity losses of 95 and 80 per cent respectively.

Nature of inhibition by serotonin, acetylcholine and eserine. Eserine, a strong inhibitor of acetylcholinesterase was also found to be inhibitory to the brain aryl acylamidase, but much less effective on the liver enzyme (Table 1) [7]. At a concentration of 10^{-7} M it caused a 75 per cent inhibition of monkey brain aryl acylamidase, while the liver enzyme was practically unaffected at this concentration.

Lineweaver–Burk plots of the initial rates of reaction in the presence of the inhibitors serotonin, acetylcholine and eserine showed all three compounds to be non-competitive inhibitors of the brain aryl acylamidase.

Semenza and Balthazar [8] have shown that the Dixon plot of one inhibitor is changed by the presence of an additional inhibitor at constant concentration and that when two inhibitors compete with each other, the Dixon plots obtained in the presence and in the absence of the additional inhibitor are parallel. In Fig. 2 is shown the Dixon plots in the presence of the inhibitor serotonin as well as the additional inhibitors acetylcholine and eserine. From the parallel lines in Fig. 2 it was concluded that the non-competitive inhibitors serotonin, acetylcholine and eserine act at a common site on the brain aryl acylamidase.

Regional distribution of aryl acylamidase in the monkey brain. The specific activities of aryl acylamidase in various regions of the monkey brain are given in Table 2. The region of highest specific activity was found to be the basal ganglia.

A profile of aryl acylamidase in the developing rat brain and liver. A study of The developmental profile of the enzyme in the rat brain and liver showed that the specific activity remained approximately the same from birth until adulthood. The brain enzyme at all ages was found to be sensitive to serotonin and acetylcholine while the liver enzyme maintained its insensitivity to these two inhibitors throughout life.

Table 2. Regional distribution of aryl acylamidase in the monkey brain

Region	Aryl acylamidase activity*
Frontal lobe	0.039
Occipital lobe	0.032
Parietal lobe	0.032
Basal ganglia	0.348
Mid brain	0.142
Cerebellum	0.103
Pons	0.146
Brain stem	0.112

* μMoles *O*-nitroaniline liberated/hr/mg protein. Values are the mean of two samples.

DISCUSSION

Although some similarities were observed between the monkey brain and liver aryl acylamidases as described in the beginning of "Results" a number of differences characterised the enzymes from these two organs. Liver aryl acylamidase was found to be more heat stable and Sepharose gel filtration showed that it had a higher mol. wt than its counterpart from the brain. The most remarkable difference was in the response to inhibitors. While the monkey brain enzyme was significantly inhibited by serotonin, tryptamine and acetylcholine and its analogues in a non-competitive manner the monkey liver enzyme was unaffected by any of these compounds in accordance with the earlier observations made with sheep brain and liver [3]. The liver but not the brain enzyme was however non-competitively inhibited by indole-3-acetic acid and indole-3-propionic acid. suggested that a 2-(-3-indolyl) ethylamine moiety is the structural requirement for the inhibition of the brain enzyme and an indole ring with a with a $-CH_2COOH$ or $-CH_2CH_2COOH$ substituent for the liver enzyme.

Four lines of evidence supported the suggestion that the brain aryl acylamidase may be associated with acetylcholinesterase. (1) The potent inhibition of the monkey brain aryl acylamidase and the relative insensitivity of the liver aryl acylamidase to eserine. (2) the inhibition of the brain but not liver aryl acylamidase by acetylcholine (3) acetylcholinesterase activity was eluted in the same fractions as brain aryl acylamidase activivity from the Sepharose 6B column and that no acetylcholinesterase activity was detectable in the fractions in which liver aryl acylamidase activity was eluted (4) the regional distribution of monkey brain aryl acylamidase has a close similarity to the distribution of acetylcholinesterase [9].

The Dixon plots according to Semenza and Balthazar [8] suggested that the three non-competitive inhibitors of brain aryl acylamidase namely serotonin, acetylcholine and eserine have a common site of action on the enzyme. If aryl acylamidase and acetylcholinesterase are associated with the same protein with separate active centres for each activity, then the common site at which eserine, serotonin and acetylcholine act to inhibit aryl acylamidase activity noncompetitively could well be the active centre for acetylcholinesterase activity. The fact that eserine as well as serotonin have been shown to be competitive inhibitors of acetylcholinesterase and that acetylcholine is a substrate for acetylcholinesterase support such a hypothesis [7, 10].

Studies on the aryl acylamidases during the various

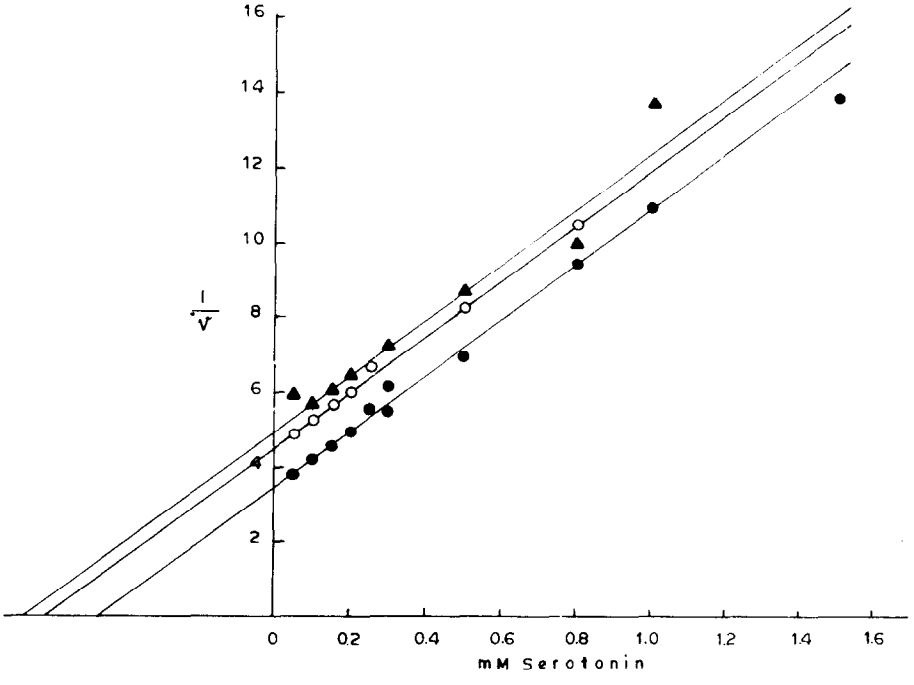

Fig. 2. Dixon plots of monkey brain aryl acylamidase for inhibitors mutually competing. Dixon plot of serotonin (●), serotonin with 2 mM acetylcholine iodide (○), serotonin with 4×10^{-5} M eserine sulphate (▲).

stages of development of rat brain and liver showed that the sensitivity and insensitivity of the brain and liver enzyme respectively to serotonin and acetylcholine was maintained throughout the period of growth from birth. It appears therefore that such differential inhibitions are inherent organ specificities.

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REFERENCES

1. D. Fujimoto, *Biochem. biophys. Res. Commun.* **61**, 72 (1974).
2. S. M. Paul, A. E. Halaris, D. X. Freedman and L. L. Hsu, *J. Neurochem.* **27**, 625 (1976).
3. A. Oommen and A. S. Balasubramanian, *Biochem. Pharmac.* **26**, 2163 (1977).
4. D. Fujimoto, *FEBS Lett.* **71**, 121 (1976).
5. S. Hestrin, *J. biol. Chem.* **180**, 249 (1949).
6. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
7. W. N. Aldridge and E. Reiner, in *Enzyme Inhibitors as Substrates*, p. 123. North-Holland Publishing Co., Amsterdam (1972).
8. G. Semenza and A. K. Balthazar, *Eur. J. Biochem.* **41**, 149 (1974).
9. A. M. Goldberg and I. Hanin, in *Biology of Cholinergic Function*, p. 14. Raven Press, New York (1976).
10. Y. S. Mohammed, M. Y. Osman and M. M. Mahfouz, *Biochem. Pharmac.* **25**, 1665 (1976).