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RAT BRAIN ARYL ACYLAMIDASE: MULTIPLE FORMS AND INHIBITION EFFECTS OF LSD, SEROTONIN AND RELATED COMPOUNDS

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At least two forms of aryl acylamidase (E.C.3.5.1.13, AAA) were separated from rat brain extracts by ammonium sulfate precipitation (33-60% saturation) and subsequent Bio-Gel column chromatography. Fraction AAA-1 showed pH optimum at 7.5 whereas AAA-2 showed a pH optimum at 5.5. AAA-1 activity was markedly inhibited at pH 7.5 by d-LSD and 2-Br-LSD, moderately inhibited by 5-HT and slightly inhibited by tryptamine but it was not affected by l-LSD, at 0.1 mM concentration. AAA-2 was only moderately inhibited at pH 5.5 by d-LSD and 2-Br-LSD but not affected by l-LSD, 5-HT or tryptamine at the same concentrations. Catecholamines and their structurally related drugs had no significant effects on either enzyme activity. Kinetic studies with AAA-1 indicated competitive inhibition by d-LSD with a K_i value of 4.90 ± 0.61 μM.

Enzymatic deacylation of aryl acylamides in various mammalian tissues such as liver and kidney has been known for many years. In 1909, Minkowski (1) first reported the hydrolysis of acetanilide and phenacetin by rabbit liver and kidney extracts. About forty years later, Krebs et al. (2) described the deacetylation of acetylsulfanilamides in animal tissues. During the next 20 years several authors studied the de-acetylation of 2-acetylamino fluorene by various mammalian liver preparations (3-5). Krisch (6) isolated a de-acetylase from pig liver microsomes that hydrolyzed acetanilide. Kiese and Renner (7) reported on the hydrolysis of acetanilide and some of its derivatives by enzyme preparations obtained from the microsomal and soluble fractions of the liver of various species. Mahadevan and Tappell (8) investigated the subcellular distribution arylamidases in rat liver and kidney. Nimmo-Smith (9) showed that homogenates of chick kidney were able to de-acetylate a variety of acylamidides. In 1971, Hoagland and Graf (10) detected the enzyme activity in brain tissues using acetanilide as substrate.

More specifically, Fujimoto (11) described a serotonin-sensitive aryl acylamidase (E.C.3.5.1.13, AAA) in rat brain. Our group has reported on the stereospecific inhibition of rat brain AAA by d-lysergic acid diethylamide (d-LSD), serotonin and tryptamine related compounds (12,13). In an attempt to further purify and characterize rat brain AAA, we succeeded in separating at least two forms of this enzyme by ammonium sulfate precipitation and Bio-Gel column chromatography. We also report on the inhibitory effects of biogenic amines as well as of several drugs including psychotomimetics on the enzyme activity of these two forms of rat brain AAA.

Materials and Methods

Adult male Sprague-Dawley rats (Madison, Wis.), 150-200 g in weight, were used.

Animals were kept in an air-conditioned room at 22°C and 55% relative humidity with a 7 a.m. to 7 p.m. light-dark cycle. The rats were housed six per cage and had ad libitum access to Purina rat chow and water. O-Nitroacetanilide (ONAC) was purchased from Eastman Kodac Chemical Company, serotonin creatinine sulfate (5-HT), tryptamine, dopamine (DA), norepinephrine (NE), histamine, 5-methoxy-tryptamine and 3', 5'-cyclic-adenosinemonophosphate (cAMP) were obtained from Sigma Chemical Company. Quipazine was obtained from Miles Laboratories. Mescaline was obtained from Regis Chemical Co., Chicago. d- and l-lysergic acid diethylamide (LSD), 2-Br-LSD (BOL), N,N-dimethyltryptamine (DMT), 5-hydroxy-N, N-dimethyltryptamine (bufotenine) and d-amphetamine had been supplied by the Center for Studies of Narcotic and Drug Abuse, NIMH. Bio-Gel A-5m (200 mesh) was purchased from Bio-Rad Company. Other chemicals were purchased from standard sources.

Rats were decapitated and their brains (excluding the pineal gland) were quickly removed. The brains were homogenized in 5 volumes of ice cold 0.05 M sodium phosphate (pH 5.5) containing 0.5% (V/V) Triton X-100. In order to avoid the possible effects of citrate or succinate ions that have stronger buffering power at pH 5.5 than phosphate, we chose to use Na-phosphate at pH 5.5 and we made up all the relevant solutions in Na-phosphate at pH 5.5. The homogenate was kept at 0°C for 1 hour and centrifuged at 8,500 x g for 20 minutes. The resulting supernatant was subjected to ammonium sulphate (saturated solution in Na-phosphate, pH 5.5) precipitation and the fraction (PP_2) precipitating between 33% and 60% saturation was redissolved in 2.5-3.0 mls of 0.05 M sodium phosphate (pH 5.5) and dialyzed over night against 200 volumes of the sodium phosphate (pH 5.5). The dialyzed precipitate (PP_2D) was used to determine time course, protein concentration, and pH effects. In all other experiments PP_2 obtained from 3 pooled rat brains was redissolved in 1.5 ml of 0.05 M sodium phosphate (pH 5.5) containing 25% of sucrose and the mixture was applied to a Bio-Gel A-5m column (2.5 x 24 cm) which had been equilibrated with 0.05 M sodium phosphate, pH 5.5. Protein elution was performed with the same solution and the eulate was collected in 2 ml fractions up to 90 ml followed by 1.0 ml fractions up to 130 ml. AAA enzyme activity was assayed in every other fraction at both pH 5.5 and 7.5. Fractions containing high enzyme activity from both protein peaks (1 & 2) were pooled separately for studies of pH and drug effects on the enzyme activity. The protein concentration in each fraction was determined by the method of Lowry et al. (14).

Enzymatic activity was assayed by the method of Hoagland and Graf (10) with a slight modification. The incubation mixture contained between 0.5 and 4 mM o-nitroacetanilide as substrate and 0.25 to 1.0 mg (100 λ) of enzyme protein in 0.05 M sodium phosphate buffer. The final incubation volume was 2 or 3 ml. Incubation was carried out at 37°C for 2 hours unless otherwise indicated. Drugs were dissolved in 0.05 M Na-phosphate at pH 5.5 or 7.5 and 0.2 ml aliquots were added to the mixture just prior to incubation. Blanks included buffer, enzyme and substrate at 0°C (13). Enzymatic activity was terminated by immersion of the reaction tubes in an ice bath. The extent of de-acetylation was measrued by determining the net absorbance between samples and blanks at 430 nm on a Coleman 124 spectrophotometer, corresponding to the formation of o-nitroaniline (ONA) (10). The molar absorptivity of ONA was previously determined as 4000 L cm^{-1} M^{-1} at 430 nm in 0.05 M phosphate buffer at pH 7.0.

Results

1. AAA activity as a function of protein concentration, incubation time and pH: The enzyme activity in PP_2D was linear with protein concentrations up to 2.0 mg, and with incubation time up to 2 hours (Fig. 1). The pH optimum was at 5.3 but there was a small peak activity at pH 7.5 (Fig. 2).

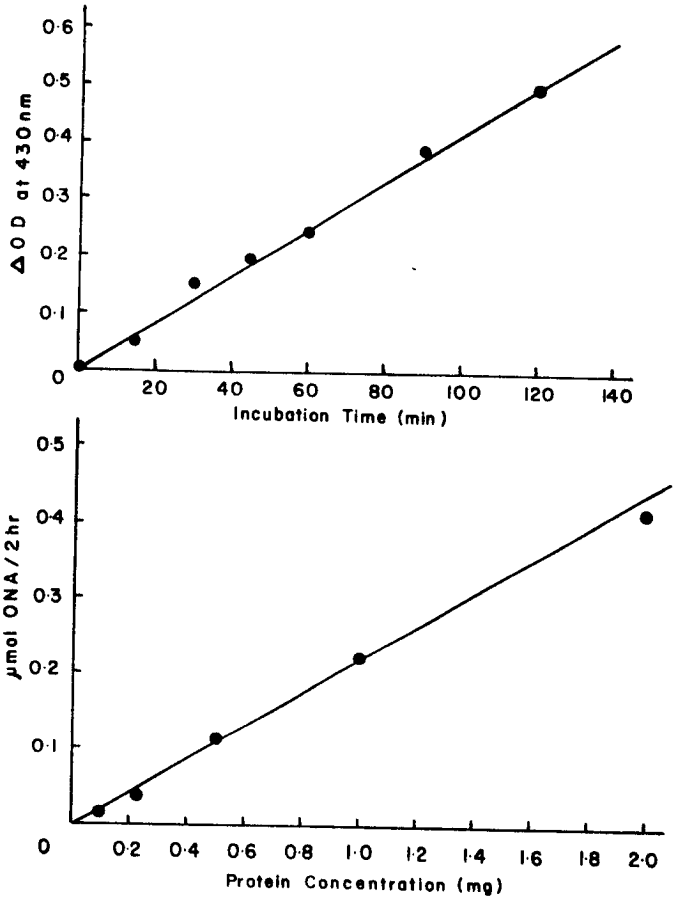

FIG. 1

Top: Time course of AAA Activity. The reaction mixture contained 4 mM ONAC, an aliquot of PP_2D (0.6 mg) and 0.05 M Na-phosphate (pH 7.5) in a final volume of 3.0 ml. Incubation temperature was 37°C. Same reaction mixtures kept at 0°C for the corresponding length of time served as blanks.

Bottom: Protein concentration curve of AAA activity in PP_2D. The reaction mixture contained 4 mM ONAC and various amounts of PP_2D in 3.0 ml of 0.05 M Na-phosphate buffer (pH 7.5). The reaction mixtures were incubated for 2 hours at 37°C. The respective reaction mixture kept at 0°C for 2 hours served as a blank. Each point represents the average of duplicate determinations.

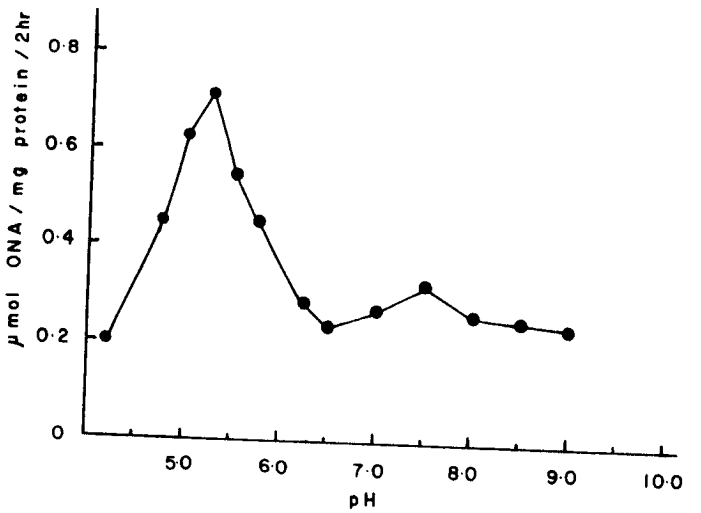

FIG. 2

AAA activity after ammonium sulfate precipitation as a function of pH. The reaction mixture contained 4.0 mM ONAC, an aliquot of PP_2D (0.6 mg) and 0.05 M Na-phosphate buffer at various pH values in a final volume of 3.0 ml. The reaction mixtures were incubated for 2 hours at 37°C. Reaction mixtures at respective pH values kept at °C for 2 hours served as blanks. Each point represents the average of duplicate determinations.

2. Separation of two AAA activity peaks by Bio-Gel column chromatography: To further purify and characterize brain AAA activity, whole brains were homogenized in 0.05 M sodium phosphate at pH 5.5, PP_2 was obtained (see Methods) and subjected to the Bio-Gel column fractionation. Two protein peaks were obtained with 0.05 M sodium phosphate elution at pH 5.5. A typical protein elution and enzyme activity pattern is shown in Fig. 3. There were at least two major AAA activity peaks corresponding to the two protein peaks when their activity was assayed at either pH 5.5 or pH 7.5. The first enzyme activity peak (AAA-1) occurred between fractions 20 and 35, whereas the second peak (AAA-2) occurred between fractions 55 and 80. At pH 5.5, the peak specific activity of AAA-1 was comparable to that of AAA-2. However, at pH 7.5 the peak specific activity of AAA-1 was twice that of AAA-2. Column fractionation did not markedly enrich AAA activity at either pH optimum (see legend to Fig. 3).

3. Effect of pH on the specific activity of AAA-1 and 2: AAA-1 activity increased with pH and reached a plateau at pH 7.5 (Fig. 4). AAA-2 activity increased with pH, but peaked at pH 5.5 and dropped thereafter. AAA-1 is more active under basic conditions whereas AAA-2 is more active under slightly acidic conditions.

4. Effects of various compounds on the specific activity of AAA-1 and 2: In initial attempts to characterize AAA-1 and AAA-2, the effects of various compounds were investigated (Table 1). AAA-1 activity was most potently inhibited by d-LSD and 2-Br-LSD, moderately inhibited by 5-HT, and slightly by tryptamine;

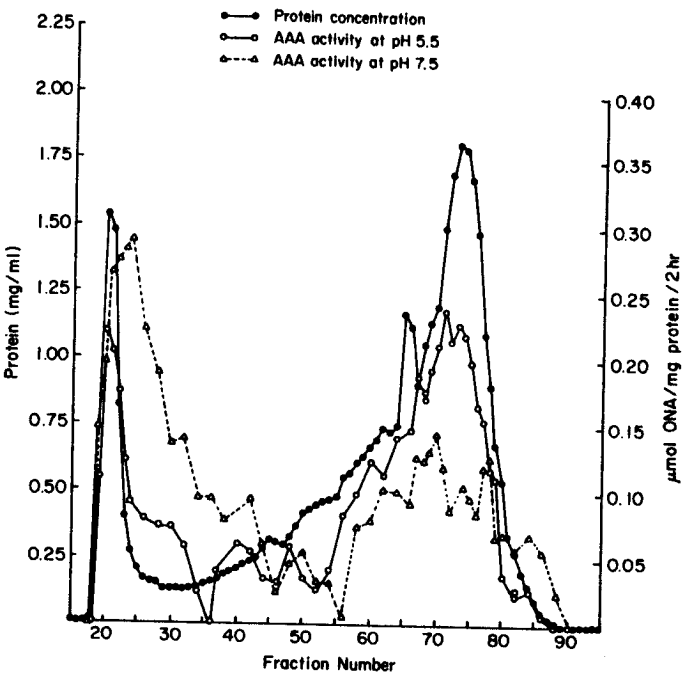

FIG. 3

A typical profile of protein elution pattern and enzymatic activity of AAA in the fractions obtained from Bio-Gel filtration: PP_2 from 3 pooled rat brains was applied to Bio-Gel column and the protein was eluted with 0.05 M Na-phosphate solution, pH 5.5. The elution volume was 2.0 ml per fraction from fractions 1 to 45 and 1.0 ml per fraction from fractions 46-90. Protein concentration was assayed in each fraction by the method of Lowry et al. (1951). AAA specific activity was assayed in every other fraction (see Methods). AAA activity in PP_2D was 0.167 and 0.128 µmol ONA per mg protein per 2 hours at pH 5.5 and 7.5, respectively. Each point represents the average of duplicate determinations.

it was not affected by 0.1 mM 1-LSD. On the other hand, AAA-2 was only moderately inhibited by the same concentration of d-LSD and 2-Br-LSD and was unaffected by 1-LSD, 5-HT or tryptamine.

5. Kinetic studies of the inhibitory effect of d-LSD on AAA-1: To further characterize the inhibitory effect of d-LSD on AAA-1, the kinetics of AAA-1 at various concentrations of both substrate (ONAC) and inhibitor (d-LSD) were

TABLE I EFFECTS OF VARIOUS COMPOUNDS (0.1 mM) ON THE SPECIFIC ACTIVITY OF AAA-1 AND AAA-2

Compounds	AAA-1 (at pH 7.5) (unit/2 hr)	Percent Inhibition	AAA-2 (at pH 5.5) (unit/2 hr)	Percent Inhibition
None	0.400 ± 0.028	---	0.142 ± 0.007	---
d-LSD	0.129 ± 0.011	*68	0.109 ± 0.008	*24
BOL	0.181 ± 0.011	*55	0.112 ± 0.010	*24
1-LSD	0.372 ± 0.023	7	0.130 ± 0.008	9
5-HT	0.297 ± 0.019	*26	0.136 ± 0.005	5
Bufotenine	0.380 ± 0.029	5	0.135 ± 0.008	5
5-Methoxy-tryptamine	0.390 ± 0.024	3	0.133 ± 0.010	7
DMT	0.380 ± 0.017	5	0.133 ± 0.004	7
Tryptamine	0.342 ± 0.019	*15	0.136 ± 0.004	5
DA	0.408 ± 0.028	---	0.134 ± 0.004	6
NE	0.400 ± 0.019	---	0.130 ± 0.005	9
d-Amphetamine	0.383 ± 0.025	5	0.133 ± 0.007	7
Mescaline	0.400 ± 0.019	---	0.134 ± 0.004	6
Histamine	0.385 ± 0.017	4	0.134 ± 0.004	6
cAMP	0.384 ± 0.017	4	0.133 ± 0.005	7
Quipazine	0.389 ± 0.028	3	0.135 ± 0.005	5

AAA-1 and AAA-2 were obtained by Bio-Gel column chromatography as described in the legend to Fig. 3. Fractions with relatively high specific enzyme activity of AAA-1 and AAA-2 were pooled separately for these studies. Each incubation mixture contained 4.0 mM ONAC, an aliquot of AAA-1 or AAA-2 (0.2-0.3 mg protein), with or without 0.1 mM of various compounds and 0.05 M Na-phosphate buffer in a final volume of 2 ml. The reaction mixtures were incubated at 37°C for 2 hr. The enzyme activity was expressed as unit/2 hr where 1 unit = 1 μmol ONA produced per mg protein. Each value represents the mean of 3 independent determinations ± S.E.

*$p < 0.02$ vs control (Mann-Whitney U-test).

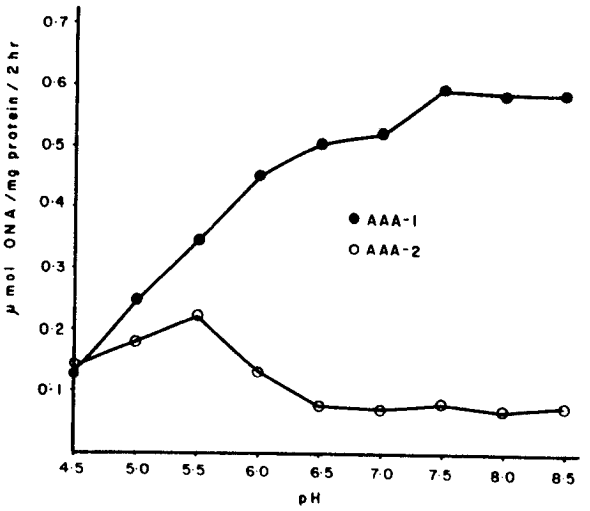

FIG. 4

AAA specific activity as a function of pH after Bio-Gel column chromatography. Bio-Gel fractions were obtained as described in Fig. 3. Fractions with relatively high specific enzyme activity for AAA-1 and AAA-2 were pooled separately for the pH studies. Each incubation mixture contained 4.0 mM ONAC, an aliquot of AAA-1 or AAA-2 (0.2-0.3 mg protein) and 0.05 M Na-phosphate buffer at various pH values in a final volume of 3.0 ml. Incubation time was 2 hours at 37°C. Each point represents the average of duplicate determinations.

studied. The Dixon plot (15) in Fig. 5 demonstrated competitive inhibition of AAA-1 activity by d-LSD. The inhibitor constant (K_i) was calculated by simultaneously solving a pair of linear equations from the Dixon plot: $y = ax + b$ and $y' = a'x + b'$, where a and a' were the slopes; b and b' were the $\frac{1}{v}$ values in the absence of inhibitor, at two different substrate concentrations. When $y = y'$, $x = -K_i = -\frac{b-b'}{a-a'}$

Five pairs of equations were obtained from Dixon plots by Linear Regression program using the Monroe 1860 calculator. The mean value of five K_i's thus calculated was 4.90 ± 0.61 μM. Similarly the mean K_i value obtained directly from Dixon plot was 4.83 ± 0.17 μM.

Discussion

The pH effects on AAA activity utilizing the partially purified enzyme preparation PP_2D clearly demonstrated an optimum at pH 5.3 and a small peak at pH 7.5. This observation suggested the possible existence of multiple forms of AAA in the rat brain. With further purification of brain AAA by Bio-Gel chromatography, two enzyme activity peaks at either pH 5.5 or 7.5 were consistently obtained (Fig. 3). AAA-1 reached a plateau at pH 7.5 whereas AAA-2 demonstrated a pH optimum at 5.5 (Fig. 4). The specific activity of AAA-1 is higher than that of AAA-2 at all pH values studied, indicating that the former may be purer than

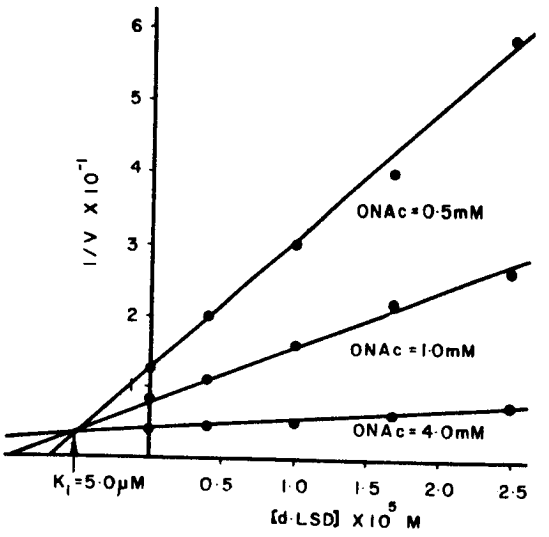

FIG. 5

A typical Dixon plot showing competitive inhibition of d-LSD on the AAA-1 enzyme activity with ONAC as the substrate. AAA-1 was obtained from Bio-Gel column chromatography as described in the legend to Fig. 3. Fractions with relatively high AAA-1 activity were pooled for the kinetic study. The AAA-1 specific activity was determined at pH 7.5 in the absence or presence of various concentrations of d-LSD and various concentrations of ONAC as indicated. Each point represents the average of duplicate determinations. The mean K_i value thus obtained from three independent determinations was 4.83 ± 0.17 μM. The K_i value calculated by slope ratio method from Dixon plot was 4.90 ± 0.61 μM (See Text).

the latter. There is a relative abundance of AAA-2 in the crude PP_2D which may account for the pH optimum observed for PP_2D (Fig. 2).

The effects of drugs on the two forms of AAA differ. AAA-1 was markedly inhibited by d- and 2-Br-LSD, moderately by 5-HT and slightly by tryptamine. AAA-2 activity was much less affected by all of these compounds. It is thus possible that these two forms of brain AAA are different and that AAA-1 may not be an aggregate of the lower molecular weight AAA-2. The lack of further enrichment of AAA specific activity after Bio-Gel fractionation may be due to loss of enzyme activity during the procedure. It remains to be determined whether the two forms differ in substrate specificity, especially with respect to naturally occurring N-acetylated biogenic amines (16,17). Franklin et al. (18) reported an Mn^{2+}-dependent and an Mn^{2+}-independent AAA activity in rat kidney using p-acetamidobenzoic acid as substrate. However, in our laboratory, the Mn^{2+} ion (0.1 mM) only slightly inhibited (10%) both AAA-1 and AAA-2 activity in brain

when ONAC was used as the substrate (unpublished data). Therefore, these multiple forms of AAA in rat brain may not be associated with those in rat kidney. The ratio of AAA-1 and AAA-2 distribution in the brain has not been investigated, although in our laboratory total AAA activity is found to be uniformly distributed in corpus striatum, hippocampus, midbrain, brain stem and cerebellum.

Furthermore, our preliminary results from subcellular fractionation studies indicated that the brain AAA activity was mainly associated with the cytosol and microsomal fractions. When assayed at pH 7.5, the specific activity of AAA in the microsomal fraction was about three times higher than that in the cytosol. When assayed at pH 5.5, the specific activity of AAA in the microsomal fraction was about four times less than that in the cytosol. These observation suggested that AAA-1 might be rich in microsomal fraction and AAA-2 might be mainly associated with the cytosol fraction.

The fact that LSD competitively inhibits (Fig. 5) AAA-1 activity may be due to d-LSD being a substrate of the enzyme, and competing with ONAC for the active site of the enzyme. Identification of biotransformation of d-LSD after incubation with the enzyme preparation would help clarify this point. In vitro inhibition of AAA-1 activity by d-LSD gave a K_i value of 4.90 μM. Whether this stereospecific inhibitory effect of d-LSD occurs in vivo remains to be determined.

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References

1. E. MINKOWSKI, Doctoral dissertation, Universitat Munchen (1909).
2. A.H. KREBS, W.O. SYKES, and W.C. BARTLEY, Biochem. J. 41 622-630 (1947).
3. J. BOOTH and E. BOYLAND, Biochem. J. 66 73-78 (1957).
4. H.T. NAGASAWA and H.R. GUTMAN, Biochim. Biophys. Acta 25 186-189 (1957).
5. C.C IRWING, Fed. Proc. 24 152 (1965).
6. K. KRISCH, Biochem. Zs. 337 531-545 (1963).
7. M. KIESE and G. RENNER, Naunyn Schmiedeberg Arch. Exp. Pathol. 252 480-500 (1966).
8. S. MAHADEVAN and A.L. TAPPELL, J. Biol. Chem. 242 2369-2374 (1967).
9. R.H. NIMMO-SMITH, Biochem. J. 75 284-293 (1960).
10. R.E. HOAGLAND and G. GRAF, Enzymologia 41 313-319 (1971).
11. D. FUJIMOTO, Biochem. Biophys. Res. Comm. 61, 72-74 (1974).
12. S.M. PAUL and A.E. HALARIS, Biochem. Biophys. Res. Comm. 70 207-211 (1976).
13. S.M. PAUL, A.E. HALARIS, D.X. FREEDMAN and L.L. HSU, J. Neurochem. 27 625-627 (1976).
14. O.H. LOWRY, N.J. ROSENBOROUGH, A.L. FARR and R.J. RANDALL, J. Biol. Chem. 193 265-275 (1951).
15. M. DIXON and E.C. WEBB, "Enzymes" 2nd ed. esp. chaps. 2,4,8 & 9, Academic, New York (1964).
16. G.A. BUBENIK, G.M. BROWN, I. UHLIR and L.J. GROTA, Brain Res. 81 233-242 (1974).
17. S.H. KOSLOW, In: Serotonin--New Vistas, adv. in Biochem. Pharmac. 11 95-100, Raven Press, New York (1974).
18. M.R. FRANKLIN, J.W. BRIDGES and R.T. WILLIAMS, Biochem. J. 114 6p (1969).