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EXTRAPINEAL AMINE *N*-ACETYLATION IN RAT BRAIN

REGIONAL AND SUBCELLULAR DISTRIBUTION AND ENZYME KINETICS

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Abstract—Analysis by thin-layer chromatography showed that [^{14}C]*N*-acetyltryptamine and [^{14}C]*N*-acetyl-β-phenylethylamine are formed from the incubation of [^{14}C]acetyl-CoA with tryptamine or β-phenylethylamine, respectively, in the presence of rat brain extracts. The specific activity of the *N*-acetyltransferase in fifteen discrete regions of rat CNS ranged from 1·64 ± 0·05 nmoles of product formed/mg of protein/hr in cerebellum to 0·57 ± 0·05 nmole in occipital cortex with tryptamine as substrate, and from 2·80 ± 0·30 nmoles in cerebellum to 0·91 ± 0·13 nmole in cervical cord with β-phenylethylamine as substrate. Comparison of the regional specific activities in the presence of the respective substrates yielded a correlation coefficient of 0·83 ($P < 0·01$). In cerebellum *N*-acetyltransferase activity appears exclusively in cytosol. At two stages of purification (i.e. after Bio-Gel fractionation as well as after ammonium sulfate precipitation), the enzyme exhibited biphasic kinetics with respect to acetyl-CoA in the presence of tryptamine or β-phenylethylamine and with respect to either substrate in the presence of acetyl-CoA.

N-acetylation of endogenous or exogenous compounds by means of the hepatic enzyme acetyl-CoA arylamine *N*-acetyltransferase (EC 2.3.1.5) has been documented as a significant systemic detoxification process [1–6]. The finding of aromatic amine *N*-acetylating activity in brain outside the pineal gland [7–11] and occasional reports of *N*-acetylated metabolites of several endogenous and exogenous amines in peripheral and/or CNS tissue [12–17] have prompted us to continue our investigation of extrapineal *N*-acetylation of aromatic amines in rat brain.

We have reported that rat brain extract catalyzed the production of *N*-acetyltryptamine from acetyl-CoA and tryptamine at a pH optimum of 7·9, and that the reaction was linear with incubation time for as long as 60 min and with protein concentration up to 0·5 mg. We also determined the relative specific activities of progressively purified enzyme preparations in the presence of various amines [11]. Now we have examined the specific activity of *N*-acetyltransferase (NAT) in fifteen discrete regions of rat CNS as well as in six standard subcellular fractions from cerebellum, the region of highest NAT activity. In addition, we have studied the kinetics of the enzyme with regard to acetyl-CoA in the presence of either tryptamine or β-phenylethylamine ($\beta\Phi$EA) as well as with regard to each substrate.

MATERIALS AND METHODS

Animals and materials. Adult male Sprague–Dawley rats (150–200 g each) were obtained from Carworth Farms, Portage, Mich. [^{14}C]acetyl-CoA (3·7 or 58 mCi/m-mole) was obtained from Amersham Searle, Arlington Heights, Ill. Acetyl-CoA and $\beta\Phi$EA were obtained from Sigma Chemical Co., St. Louis, Mo.; tryptamine from Regis Chemical Co., Morton Grove, Ill. Acetic anhydride was purchased from Mallinckrodt Chemical Works, Los Angeles, Calif. Bio-Gel A-1·5 m was purchased from Bio Rad Co., Richmond, Calif., and Silica gel F-254 thin-layer chromatographic (t.l.c.) sheets were purchased from EM Laboratories, Elmsford, N.Y. Other chemicals, of optimal purity, were obtained from standard sources.

Standard *N*-acetyltryptamine and *N*-acetyl-$\beta\Phi$EA were synthesized in our laboratory by *N*-acetylation of tryptamine and $\beta\Phi$EA, respectively, with acetic anhydride in alkaline ethanol solution [18]. The products were extracted into dichloromethane, and in each case the solvent was subsequently evaporated under nitrogen atmosphere. On t.l.c., *N*-acetyltryptamine showed a single spot under shortwave u.v. light, with R_f values of 0·81 when developed in butanol–1 N acetic acid–water (4:1:1) and 0·56 when developed in chloroform–methanol–1 N acetic acid (97:3:1); standard tryptamine showed R_f values of 0·38 and 0·00 with the respective solvent systems. *N*-acetyl-$\beta\Phi$EA showed a single spot with R_f values of 0·78 when developed in butanol–1 N acetic acid–water (4:1:1) and 0·81 when developed in isopropanol–1 N ammonium hydroxide–water (8:1:1); standard $\beta\Phi$EA showed R_f values of 0·40 and 0·23 in the respective solvent systems.

Enzyme preparation for product analysis and kinetic studies. Rats were decapitated, and their brains were quickly removed, special care being taken to remove the pineal gland from the sample. Then the brains were homogenized in 5 vol of ice-cold double-distilled water and centrifuged at 100,000 *g* for 40 min. A portion of the supernatant was dialyzed against 100 vol of 0·05 M potassium phosphate buffer (pH 7·9) for 1 hr, and the dialyzed fraction was used

as the enzyme source for product analysis. Another portion of the supernatant was saturated with ammonium sulfate (50–75 per cent), and the precipitate (Pp_2) was picked up in appropriate amounts of 0·05 M potassium phosphate buffer (pH 7·9) and dialyzed against 200 vol. of the same buffer for 1 hr. Aliquots of the dialyzed precipitate (Pp_{2D}) were used for the kinetic studies described below. In other experiments Pp_2 was picked up in a minimal amount of 0·05 M potassium phosphate buffer (pH 7·9) and applied to a Bio Gel A-1·5 m column (2·5 × 20 cm) which was equilibrated with the same buffer. Protein was eluted with the same buffer and collected in fractions of 2·0 ml for 3 hr. Fractions with the highest specific enzyme activities were pooled for kinetic studies as described below.

Enzyme activity was assayed by the method of Deguchi and Axelrod [19] with slight modification. The incubation mixture contained 0·1 mM [^{14}C]acetyl-CoA (3·7 mCi m-mole), 2·5 mM substrate (if not otherwise indicated), and an aliquot of enzyme protein in 0·05 M potassium phosphate buffer (pH 7·9) with a total volume of 0·2 or 0·3 ml. We used boiled enzyme blanks as controls, having previously established [11] that they yielded higher activity than blanks consisting of either buffer plus enzyme without substrate or buffer plus substrate without enzyme. Mixtures were incubated at 37° for 0·5 or 1 hr. Reactions were stopped by the addition of 0·5 ml of 0·5 M sodium borate buffer (pH 10·0). The radioactive products were extracted into 6 ml toluene containing 3% isoamyl alcohol, with an efficiency of 100 per cent. Aliquots (4 ml) of the organic phase were transferred to scintillation vials containing 10 ml of counting solution (POPOP* and PPO in toluene and ethanol), and radioactivity was measured in a Beckman liquid scintillation spectrophotometer. In all experiments, protein concentrations were determined according to the method of Lowry *et al.* [20].

Product analysis. For each product, organic phases from two identical incubations were pooled and combined with 0·1 mg of the corresponding cold *N*-acetylated amine. The combination was evaporated to dryness in a vacuum oven at room temperature, and the residue was taken up in 0·2 ml ethanol. Aliquots of the ethanol solutions were applied in lines 2·5 cm long alongside 10 μg of the corresponding standards on Silica gel F-254 t.l.c. sheets (5 × 8 cm). Identical t.l.c. sheets were then developed in two different solvent systems until the fronts were about 1 cm from the top. After drying, each band visualized under u.v. light was scraped from the sheet and mashed in 2 ml ethanol for measurement of radioactivity. Strips of equal size from between the bands, including the solvent front and origin, were treated in the same way.

Subcellular fraction preparation. Three fresh rat cerebella were homogenized in 10 vol of 0·32 M sucrose in 0·001 M potassium phosphate buffer (pH 7·9) in a Thomas glass Teflon homogenizer with clearance of 0·025 cm. Homogenate was then centrifuged and fractionated according to the method of Gray and Whittaker [21] with slight modifications [22]. The nuclear pellet was taken after centrifugation at 900 *g* for 10 min, and the supernatant was spun at 14,000 *g* for 20 min to yield the crude mitochondrial pellet. The remaining supernatant was centrifuged at 100,000 *g* for 1 hr to yield the microsomal pellet and the soluble cytosol fraction. The crude mitochondrial pellet was washed once with 0·32 M sucrose, suspended in 5 ml sucrose, layered over the modified sucrose gradient (12·0 ml of 1·2 M; 8 ml of 0·8 M; 2 ml of 0·6 M; 2 ml of 0·4 M) and centrifuged at 100,000 *g* for 45 min. Myelin was taken from the interface between the layers of 0·4 and 0·6 M sucrose and centrifuged at 100,000 *g* for 40 min to yield a pellet. The synaptosomal fraction was obtained from the interface between the layers of 0·8 and 1·2 M sucrose, diluted with 2 vol. of cold 0·001 M potassium phosphate buffer (pH 7·9), and centrifuged at 100,000 *g* for 40 min to yield a pellet. The mitochondrial pellet at the bottom of the gradient was rinsed with 0·32 M sucrose. All the rinsed pellets were resuspended in minimal amounts of 0·32 M sucrose in 0·001 M potassium phosphate buffer, and NAT activity was assayed in all fractions as described above.

Regional fraction preparation. Brain regions were dissected freehand over ice immediately after the rats were sacrificed, according to *Craigie's Neuroanatomy of the Rat* [23], as follows. Representative wet weights are given in parentheses. Gentle pulling apart of the cerebral cortices stretched the corpus callosum (25 mg) which, after being teased away from its lateral insertions, was pinched away from the hippocampus, any gray matter being excluded. The corpus striatum (80 mg), including most of the caudate and the putamen and some globus pallidus, was excised from the cortices. Both the lateral and medial septal nuclei (20 mg) were cut free in one piece. After the cingulate region (100 mg) was taken, the frontal cortex (180 mg) was separated from the parietal cortex (78 mg). Similarly, the occipital cortex (60 mg) was taken after the entorhinal and posterior cingulate had been discarded. The dorsal hippocampi (100 mg) were separated at the midline and cut to include only dorsal hippocampus proper and dentate gyrus. The cerebellum (280 mg) was removed intact. The medulla (130 mg) extended from the clava up to the caudal border of the trapezoid body. The pons trapezoid (60 mg) was bordered rostrally by the caudal aspects of the interpeduncular fossa and the inferior colliculi. The midbrain piece (130 mg) extended rostrally up to the mamillary bodies and included the corpora quadrigemini. The hypothalamus (30 mg) was defined ventrally by the outline of the tuber cinereum and dorsally by the top of the third ventricle. For dissection of the cervical spinal cord and lumbosacral cord, a dorsal incision was made forward from the tail, exposing the vertebrae. The musculature was then dissected away from the point of decapitation back to the seventh vertebra and from the first lumbar vertebra to the fourth sacral vertebra. The cervical segment was cut from the vertebral column at the space between the seventh cervical vertebra and first thoracic vertebra as defined by its relationship to the tall spine of the third thoracic vertebra. The lumbosacral segment was severed anterior to the first lumbar vertebra and posterior to the fourth sacral vertebra. The vertebral segments were opened with scissors, and the cord was removed with forceps.

* POPOP = *p*-bis[2-(5-phenyloxazolyl)]-benzene; PPO = 2,5-diphenyloxazole.

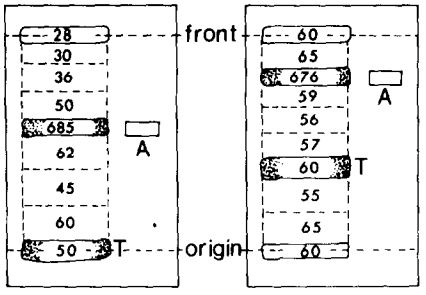

Fig. 1. T.l.c. identification of reaction product. A = standard *N*-acetyltryptamine. T = tryptamine. The numbers are the cpm in the bands. On the left the solvent system was chloroform–methanol–1 N acetic acid (97:3:1). On the right the solvent system was butanol–1 N acetic acid–water (4:1:1).

Like regions from four to eight rats were pooled, homogenized in 5–7·5 vol of double-distilled water at 4°, and centrifuged at 100,000 *g* for 40 min. The supernatants were dialyzed for 2 hr against 200 vol. of 0·05 M potassium phosphate buffer (pH 7·9), and the NAT activity was assayed as described above. Regardless of region, boiled enzyme blanks yielded approximately 1125 cpm; counts in each regional sample were at least twice those in the corresponding boiled enzyme blank.

RESULTS

Products. The radioactive product resulting from the reaction between tryptamine and [^{14}C]acetyl-CoA was isographic with standard *N*-acetyltryptamine on TLC chromatograms developed in two different solvent systems (Fig. 1). The radioactive product from the reaction between *βΦ*EA and [^{14}C]acetyl-CoA was isographic with *N*-acetyl-*βΦ*EA after development in the two solvent systems described in Materials and Methods. Over 80 per cent of the radioactivity applied to the t.l.c. plates was recovered from all the bands measured, and almost all of such radioactivity was localized in the *N*-acetylated product bands. No other bands with appreciable radioactivity were observed.

Table 1. Regional NAT activity in dialyzed soluble fractions from rat brain*

Sample	Specific activity (nmoles product/mg protein/hr)	
	Tryptamine	*βΦ*EA
Cerebellum	1·64 ± 0·05	2·80 ± 0·30
Whole brain	1·15 ± 0·14	1·98 ± 0·13
Corpus callosum	1·04 ± 0·08	1·96 ± 0·11
Frontal cortex	1·02 ± 0·08	1·40 ± 0·15
Hippocampus	0·97 ± 0·03	1·60 ± 0·16
Parietal cortex	0·91 ± 0·10	1·29 ± 0·13
Pons/trapezoid	0·89 ± 0·08	1·34 ± 0·13
Remainder of brain	0·88 ± 0·07	1·43 ± 0·12
Cingulate cortex	0·86 ± 0·05	1·91 ± 0·17
Medulla	0·82 ± 0·06	1·57 ± 0·16
Midbrain	0·76 ± 0·09	1·47 ± 0·11
Septal nuclei	0·74 ± 0·06	1·03 ± 0·09
Lumbosacral cord	0·72 ± 0·06	1·69 ± 0·08
Hypothalamus	0·69 ± 0·10	1·18 ± 0·11
Corpus striatum	0·63 ± 0·06	1·32 ± 0·08
Cervical cord	0·59 ± 0·06	0·91 ± 0·13
Occipital cortex	0·57 ± 0·05	1·46 ± 0·14

* Values are the means of four to six independent determinations ± S. E. M. Protein concentrations were between 0·15 and 0·25 mg/0·2 ml of reaction volume. Regional supernatants from 100,000 *g* centrifugation, dialyzed for 2 hr, were incubated for 0·5 hr with 0·1 mM [^{14}C]acetyl-CoA and 2·5 mM tryptamine or *βΦ*EA in 0·05 M potassium phosphate buffer (pH 7·9).

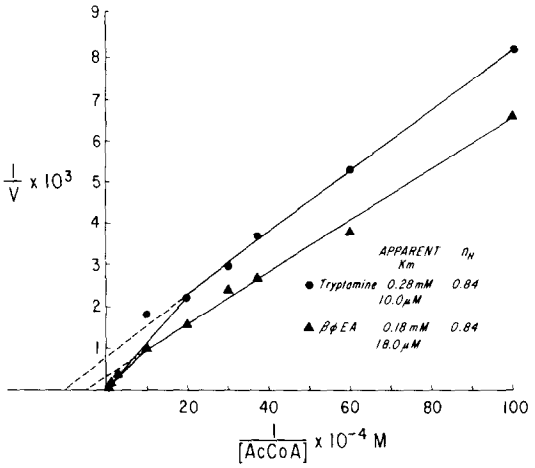

Fig. 2. NAT activity as a function of acetyl-CoA in the presence of tryptamine or *βΦ*EA. Combined Bio-Gel fractions were the enzyme source. The incubation mixtures contained 0·19 mg protein, 2·5 mM of either substrate, and various amounts of [^{14}C]acetyl-CoA in a total volume of 0·30 ml of 0·05 M potassium phosphate buffer (pH 7·9). Reaction velocity is expressed as 10^{-3} × pmoles of *N*-acetylated amine produced/mg of protein/hr. Each point represents the mean of duplicate determinations. n_H = Hill slope.

Subcellular localization. With tryptamine as substrate, we assayed the enzyme activity in subcellular fractions from cerebellum because preliminary regional studies had shown that NAT activity was relatively high in that region. In the cerebellum NAT activity appeared exclusively in the cytosol: the specific activity was 1·05 ± 0·06 nmoles of *N*-acetyltryptamine formed/mg of protein/hr. No activity was detected in the nuclear, microsomal, myelin, synaptosomal or mitochondrial fractions.

Regional distribution. As shown in Table 1, *N*-acetyltryptamine formation is not uniformly distributed in rat brain. Specific activities in fractions from fifteen discrete regions of the CNS plus the remainder of the brain ranged from 1·64 ± 0·05 nmoles of *N*-acetyltryptamine formed/mg of protein/hr in cerebellum to 0·57 ± 0·05 nmole in occipital cortex. Regional distribution of *N*-acetyl-*βΦ*EA formation was only slightly more uniformly distributed, with a range of 2·80 ± 0·30 nmoles of product formed/mg of protein/hr in cerebellum to 0·91 ± 0·13 nmole formed in cervical cord. When we performed a statistical comparison of the regional NAT activity in the presence of these two substrates, we obtained a correlation coefficient of 0·83 ($P < 0·01$).

Kinetic properties of partially purified NAT. Lineweaver–Burk double reciprocal plots of the reactions incorporating Bio-Gel fractions as the enzyme source

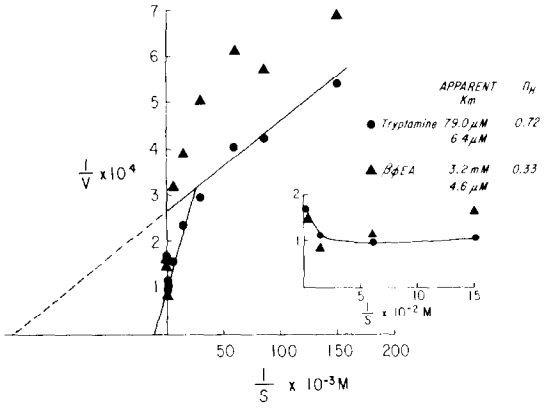

Fig. 3. NAT activity as a function of substrate amines. Combined Bio-Gel fractions were the enzyme source. The incubation mixtures contained 0·12 mg protein, 0·30 mM [^{14}C]acetyl-CoA, and various amounts of either tryptamine or $\beta\Phi$EA in a total volume of 0·30 ml of 0·05 M potassium phosphate buffer (pH 7·9). Reaction velocity is expressed as 10^{-4} × pmoles of *N*-acetylated amine produced/mg of protein/hr. Each point represents the mean of duplicate determinations. n_H = Hill slope.

show biphasic kinetics with regard to acetyl-CoA in the presence of either tryptamine or $\beta\Phi$EA (Fig. 2). The partially purified enzyme shows similar biphasic kinetics with regard to either substrate (Fig. 3). The enzyme activity was inhibited at concentrations of either tryptamine or $\beta\Phi$EA ranging from 6·6 to 33 mM. The interaction of the enzyme with acetyl-CoA in the presence of either tryptamine or $\beta\Phi$EA yielded Hill plots with slopes less than 1·0 (Fig. 4) [24]. The interaction of the enzyme with tryptamine or $\beta\Phi$EA also yielded Hill plots with slopes less than 1·0 (Fig. 4). Similar kinetic data were obtained when Pp_{2D} was used as the enzyme source (Table 2).

DISCUSSION

Although *N*-acetylation of various exogenous amines is a well-described systemic detoxification

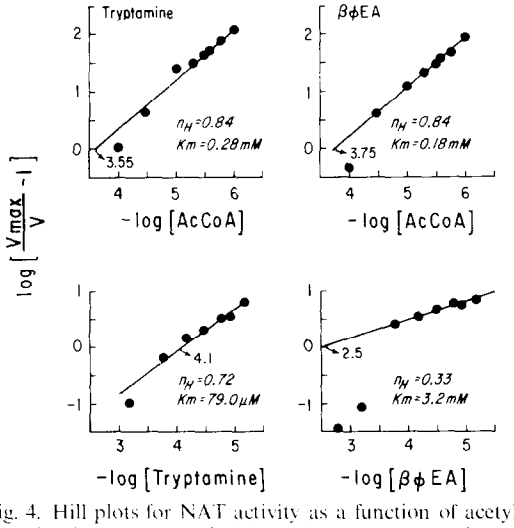

Fig. 4. Hill plots for NAT activity as a function of acetyl-CoA in the presence of either substrate (top) and as a function of either substrate (bottom). The data were taken from Figs. 2 and 3 respectively.

Table 2. Apparent K_m and n_H values for NAT with Pp_{2D} as the enzyme source*

	Acetyl-CoA		Amines	
Substrate	K_m	n_H	K_m	n_H
Tryptamine	0·14 mM	0·91	1·99 mM	0·33
	2·20 μM		0·25 mM	
βΦEA	0·22 mM	0·76	0·10 mM	0·67
	7·10 μM		7·10 μM	

* K_m and n_H values were obtained from Lineweaver Burk double reciprocal plots and Hill plots similar to those in Figs. 2–4. In determinations with respect to acetyl-CoA, the incubation mixture contained 0·28 mg protein, 2·5 mM of either substrate, and various amounts of acetyl-CoA in a total volume of 0·2 ml of 0·05 M potassium phosphate buffer (pH 7·9). In determinations with respect to the amines, the incubation mixtures contained 0·28 mg protein, 0·1 mM acetyl-CoA and various amounts of either tryptamine or $\beta\Phi$EA in a total volume of 0·2 ml of 0·05 M potassium phosphate buffer (pH 7·9).

mechanism [25, 26], relatively little is known about *N*-acetylation by any organ other than the liver [27] with regard to the inactivation of endogenous amines. McIsaac and Page [28] estimated from studying urinary indoleamine metabolites in patients with carcinoid tumors that between 5 and 25 per cent of serotonin is rendered inactive by *N*-acetylation, the majority of that presumably by hepatic enzymatic activity. Moreover, in the pineal gland, serotonin concentration is apparently regulated by *N*-acetylation mediated through β-receptor stimulation by norepinephrine [19, 29–34]. A similar mechanism could be speculated to exist for serotonin metabolism in the brain itself, specifically in those structures that receive both serotonergic and adrenergic input [35]. Our product analysis by t.l.c. clearly showed that *N*-acetyltryptamine and *N*-acetyl-$\beta\Phi$EA were formed from the incubation of acetyl-CoA with tryptamine and $\beta\Phi$EA, respectively, in the presence of brain extracts.

In our examination of amine *N*-acetylation in fifteen discrete regions of the rat CNS, we found a 3-fold difference in activity between the least and most active regions with either substrate. It seems that the high *N*-acetylating activity we found in rat cerebellum might be associated with the *N*-acetylalkylamines localized there by immunohistological methods after pinealectomy [36]. NAT activity being relatively high in cerebellum, cortical areas, and corpus callosum suggests that the enzyme is not distributed with axons and nerve endings of known aminergic systems in the brain. Unlike the activity of enzyme known to be related to putative neurotransmitter biosynthesis and degradation, this activity was relatively low in corpus striatum.

The activity we report, ranging from 1·64 ± 0·05 nmoles of *N*-acetyltryptamine formed/mg of protein/hr in cerebellum to 0·57 ± 0·05 nmole in occipital cortex, is higher than the activity reported by Yang and Neff [10], the range of which was 840 ± 80 to 370 ± 70 pmoles in seven unspecified brain regions. Ellison *et al.* [7] also reported lower *N*-acetylating activity (170, 150 and 140 pmoles of *N*-acetylserotonin formed/mg of tissue in rat olfactory bulb, cerebellum and cerebrum respectively). Allowing

for the 10–20 per cent protein content of brain tissue, Ellison *et al.* [7] found activity comparable to that reported by Yang and Neff [10]. Although Yang and Neff [10] do not specify the pH of their assay, Ellison *et al.* [7] used pH 6·8. We found that at pH 6·5, 40 per cent of the [^{14}C]acetyl-CoA decomposed in a 0·5-hr incubation. Such a loss could decrease the rate of the *N*-acetylation reaction, yielding lower enzymic specific activity.

The enzyme is apparently relatively unstable too. In our assays for regional enzyme activity, the soluble fractions were dialyzed for 2 hr, which was only half the dialysis time of our earlier assays, [11] and the shorter dialysis time nearly doubled the measurable enzymatic activity.

In the regional and subcellular experiments, our concentration of acetyl-CoA was 0·1 mM, which turned out to be between the two apparent K_m values in reactions with either substrate. In the presence of tryptamine, the K_m values were 0·28 mM (low affinity for acetyl-CoA) and 10·0 μM (high affinity for acetyl Co-A); with $\beta\Phi$EA they were 3·2 mM and 4·6 μM respectively (Fig. 2). With regard to substrate concentrations, it turned out that our concentration of tryptamine (2·5 mM) was adequate to elicit enzymatic activity with both high and low affinity for substrate, whereas our concentration of $\beta\Phi$EA (2·5 mM) fell between the apparent K_m values in reactions with that substrate (Fig. 3). Hence, NAT specific activity in brain regions might be different if higher or lower concentrations of acetyl-CoA or $\beta\Phi$EA were to be used. It seems that only the high-affinity enzyme activities were being measured under our assay conditions: the differential distributions of high- and low-affinity NAT activities in the brain remain to be determined. However, the biphasic kinetics at two stages of purification suggest that the enzyme may be polymorphic or that it may include non-identical peptide chains that have active sites with different binding constants as isozymes do [37].

The extrapineal presence of both 5-methoxytryptamine [38, 39] and 5-methoxy-*N*-acetyltryptamine (melatonin) [39] has been reported. Our finding [11], also reported by others [7, 10], that the brain is indeed capable of *N*-acetylating biogenic amines raises the possibility that extrapineal melatonin is in fact synthesized in the brain itself rather than transported from the pineal gland or from other structures in the region of the lamina intercalaris [39, 40]. Moreover, we have some preliminary data showing that after intraventricular infusion of labeled tryptamine or labeled $\beta\Phi$EA, the corresponding *N*-acetylated products are present in rat brain.

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