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METHAMPHETAMINE-INDUCED ALTERATION IN THE PHYSICAL STATE OF RAT CAUDATE TYROSINE HYDROXYLASE*

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Abstract—Methamphetamine was found to induce a shift of a portion of rat striate (and not midbrain) tyrosine hydroxylase activity from the 11,000 *g* supernatant to the "synaptosomal" and "mitochondrial" particulate fractions with no change in total measurable enzyme activity, although the particulate enzyme demonstrated a greater affinity for the synthetic cofactor, 6,7-dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$). The magnitude of this shift appeared dose-related up to 5 mg/kg. It demonstrated a shortest latency of 10 min and a longest duration of 8 hr. It could not be produced *in vitro* by homogenizing the striate area in varying concentrations of methamphetamine or catecholamines. The effect could not be abolished by pretreatment with cycloheximide or intraventricular colchicine. It could not be induced by the administration of imipramine, footshock, electroconvulsive shock, or behaviorally activating intraventricularly-infused norepinephrine (NE). However, this change could be produced by the administration of α-methyltyrosine and reserpine. The reserpine-induced change differed in that in addition to the shift of activity from the soluble to the particulate fraction, there was an increase in total measurable enzyme activity as well. It was suggested that a drug-induced reduction in intraneuronal catecholamines may be the common stimulus for the observed relative increase in rat caudate particulate tyrosine hydroxylase. The particulate state of caudate tyrosine hydroxylase generally resisted narrow clearance homogenization, hypotonic shock and sonication but varied in a complex way with varying ion concentrations (especially Ca^{2+} and Mg^{2+}) *in vitro*. Varying membrane binding of soluble tyrosine hydroxylase via the manipulation *in vitro* of divalent cations led to apparent decreases in the specific activity of tyrosine hydroxylase. However, other recent studies in our laboratory have demonstrated that membrane binding of tyrosine soluble hydroxylase activates the enzyme allosterically for $DMPH_4$ binding as well as for the competitive inhibition of the affinity of this cofactor site by dopamine (DA) and norepinephrine (NE). It is within this context that the amphetamine-induced alteration in physical state of tyrosine hydroxylase assumes potential regulatory significance.

WHEN tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of norepinephrine, was isolated from the adrenal medulla and brain by Nagatsu *et al.*,[1,2] the enzyme was characterized as particulate. This physical state and the inferred intravesicular location of the enzyme made the hypothesized control mechanism by product-feedback inhibition by norepinephrine (NE) plausible, in that adequate levels of NE to fit the kinetics of this inhibition might obtain in that subcellular location.[3] Subsequent work by Laduron and Belpaire[4] and Wurzburger and Musacchio[5] has suggested that, in the adrenal medulla, tyrosine hydroxylase is almost entirely cytoplasmic (soluble) with the adrenal enzyme's tendency to adsorb to subcellular organelles during

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homogenization and differential centrifugation under some conditions probably responsible for the initial reports of its particulate state. In studies of bovine caudate nucleus, however, there have been reports that tyrosine hydroxylase distributes for the most part as a particulate enzyme in the synaptosomal-enriched subcellular fractions with very little in the cytoplasmic fraction. Using marker enzymes, electron microscopy, and Ficoll gradients, Fahn *et al.*[6] were unable to discern whether the tyrosine hydroxylase, which they described as bound to the synaptic vesicles, was inside or outside these organelles. McGeer *et al.*[7] have demonstrated a similar pattern of subcellular distribution for striate tyrosine hydroxylase in man. In our previous studies of the effects of methamphetamine administration on the specific activity of whole brain tyrosine hydroxylase,[8,9] we observed an increase of 20 per cent in 2 hr after drug administration. These studies were carried out using the particulate fraction following centrifugation at 20,000 *g* for 20 min after homogenization in 0·32 M sucrose and an initial 1000 *g* spin to remove nuclei and cell debris. However, when we used a 25–50% ammonium sulfate precipitate of solubilized whole brain enzyme in our assays, the short-latency methamphetamine-induced increase in the specific activity of brain tyrosine hydroxylase disappeared. This suggested that the methamphetamine-induced apparent increase in activity was dependent upon its physical state which could be altered by solubilization and ammonium sulfate precipitation. This speculation was consistent with some of our recent studies which have suggested that alterations in the physical state of striate tyrosine hydroxylase could either inhibit or activate the enzyme.[10–12] The following report describes studies of the effects of the administration of methamphetamine and related drugs, and treatments on the subcellular distribution of rat caudate tyrosine hydroxylase. A necessary common mechanism to produce this effect will be adduced and the potential regulatory significance of this change will be discussed.

MATERIALS AND METHODS

A standardized region of the rat striate cortex (130–150 g, Sprague–Dawley rats) including a substantial amount of the caudate, the putamen and globus pallidus, together weighing approximately 0·03 g from each hemisphere, was routinely dissected free bilaterally immediately after the sacrifice of the experimental animals. Brain area weights varied about 20 per cent but not systematically with any treatment. The "caudate" parts were homogenized in 0·32 M sucrose, w/v of 1 to 25 using a Thomas glass-Teflon homogenizer with a 0·025 cm clearance ("wide clearance") using 10 strokes over 2 min at 2000 rpm. The homogenization conditions were standardized as much as possible for all experiments. Samples were then centrifuged in a Sorvall RC-2B at 1000 *g* for 10 min, and the low-speed pellet containing nuclear material and cellular debris was discarded. This fraction, which contained 10–20 per cent of the region's total tyrosine hydroxylase activity, was arbitrarily excluded from the following studies due to the structural heterogenity of this fraction. The supernatant was then centrifuged at 11,000 *g* for 20 min. The supernatant and washed pellet fractions from this separation were used as the enzyme sources in some experiments. In other experiments, the 11,000 *g* pellet was washed twice with 3 cm^3 of 0·32 M sucrose followed each time by a 11,000 *g* spin for 20 min. The pellet was then suspended in 5 cm^3 of 0·32 M sucrose and layered on discontinuous sucrose gradients consisting of 15 cm^3 of 0·8 M, and 7·5 cm^3 each of 1·2 and 1·4 M sucrose. These

gradients were spun at 50,000 *g* in a SW-25 swinging bucket rotor in a Beckman L2-65B ultracentrifuge for 2 hr. The bands, corresponding to the P_2A, P_2B and P_2C fractions of Gray and Whittaker[13] were collected with a Pasteur pipette. Each band was pelleted by centrifugation in a Ti-60 fixed-angle rotor at 94,000 *g* for 15 min. Each band was then suspended in 0·32 M sucrose and served as an enzyme source for the appropriate experiments. When enzyme free of subcellular organelles was desired, a 25–50% ammonium sulfate precipitate of the 11,000 *g* supernatant fraction of solubilized pooled caudate homogenate was used which was dialyzed for 12 hr against 100 vol. of 0·001 M potassium phosphate buffer, pH 7·0, before use.

Tyrosine hydroxylase assays were done using a modification of the method of Nagatsu *et al.*[1,2] L-Tyrosine-3,5-^{3}H, 35 c/m-mole was purchased from New England Nuclear and purified on a Dowex 50W-X4 (H^+) column. 6,7-Dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$) was purchased from Cal-Biochem. The incubation mixture in the assay contained 1 nmole of tyrosine-3,5-^{3}H (containing 10^6 cpm), 380 nmoles of $DMPH_4$, 20 μmoles of mercaptoethanol, 2,8 μmoles of $FeSO_4$, and 50 μmoles of sodium acetate buffer, pH 6·1. Fifty or 100 μl of the enzyme source was added. This mixture was brought to a final volume of 500 μl and incubated for 20 min at 37°. The reaction was stopped using 50 μl of glacial acetic acid. Three hundred μl of this mixture was placed on a 5·0 cm Dowex 50W-X4 protonated column. The column was washed twice with 400 μl of water. The column eluate and washings were mixed with scintillation fluid and 50 μl of saturated ascorbic acid, and counted in a Beckman LS-250 liquid scintillation fluid contained in 1 l. of 150 ml of Biosolv BBS-3 (Beckman) and 850 ml of toluene containing 3·4 g 2,5-diphenyloxazol (PPO) and 0·41 g 1,5-bis[2-(5-phenyloxazolyl)]benzene (POPOP). A rat brain homogenate pool served as the enzyme source of reference for each group of assays. Enzyme activity was linear for time and protein concentration within the parameters of the assay. Experiments were run with substrate concentration over a 50-fold range (up to 5×10^{-4} M tyrosine-3,5-^{3}H) without altering the findings. Dialysis against 100 vol. of 0·32 M sucrose or 0·002 M potassium phosphate buffer, pH 7·5, for 12 hr did not change manifested enzyme activity in any of the brain tissue fractions from control and drug-treated animals. This suggested that, unlike the case with adrenal tissue, catecholamines need not be routinely removed before assaying brain tissue. The failure of a wide range of substrate concentrations *in vitro* as well as dialysis to change measurable enzyme activity in drug-treated and control animals suggest that drug-induced alterations in endogenous substrate concentrations were not responsible for the observed effects. Protein concentrations were determined using the method of Lowry *et al.*[14] Statistical tests of significance were carried out using the Mann–Whitney–U.[15]

Methamphetamine hydrochloride was obtained from Burroughs & Welcome; α-methyltyrosine from Regis Chemical; imipramine hydrochloride from Geigy; reserpine from CIBA; *dl*-norepinephrine hydrochloride from CalBiochem.

RESULTS

Methamphetamine-induced shift. The administration of methamphetamine led to a shift in the subcellular distribution of tyrosine hydroxylase activity. A significant amount of enzyme activity appeared to shift from the 11,000 *g* supernatant to the P_2B and P_2C[13] particulate fractions with no change in total measurable activity. Table 1 summarizes the results of a typical experiment in which the caudate areas

TABLE 1. EFFECT OF AMPHETAMINE ON THE SUBCELLULAR DISTRIBUTION OF TYROSINE HYDROXYLASE*

	Total activity (%)	
	Saline	Amphetamine
11,000 *g* Supernatant	69	36
P_2A	0	0
P_2B	16	35
P_2C	14	27
	Total activity ($\mu\mu$moles dopa)	
Saline	102·3 ± 19·4	
Amphetamine	81·0 ± 16·7	

* Total activity and subcellular distribution of rat striatal tyrosine hydroxylase studied 2 hr after the administration of methamphetamine hydrochloride, 1 mg/kg, subcutaneously or an equivolume amount of saline. The values equal the means of three pools of three caudate areas each. The subcellular distribution is expressed as the mean per cent of total measurable activity, contained in each fraction. Amphetamine produced no effect on total activity which is expressed as micromicromoles of dopa synthesized per hr. Note the shift of enzyme activity from the soluble to the "synaptosomal" and "mitochondrial" fractions.

(as described in Materials and Methods) were studied 2 hr after the subcutaneous administration of methamphetamine hydrochloride, 1 mg/kg. Note that there is a non-significant change in total activity at the same time that there is a shift of the measurable activity into the P_2B and P_2C fractions. Because this induced change occurred to a similar extent in both these fractions and the P_2A fraction manifested no enzyme activity, further studies were carried out on the post low-speed spin, 11,000

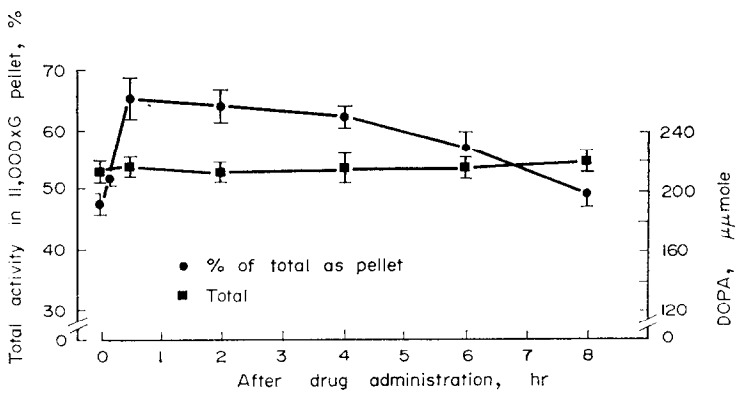

FIG. 1. Total measurable striatal tyrosine hydroxylase activity (micromicromoles of dopa synthesized per hr), and the per cent of that total in the post low-speed spin, 11,000 *g* pellet at various times after methamphetamine hydrochloride, 5 mg/kg. Each point represents the mean and the standard error of the mean of six caudate pairs (six animals). Whereas total measurable activity was not changed by the administration of methamphetamine, the per cent in the pellet rose rapidly reaching its peak in 30 min and remained significantly different from saline controls (which were not effected) until 6 hr.

g "crude mitochondrial" pellet. Figure 1 represents the results of a series of experiments using this cruder separation. Following a dose of 5 mg/kg of amphetamine, a shift of tyrosine hydroxylase from the soluble to the particulate fraction was observed which was maximal at 30 min and lasted 6 hr. As can be seen, there was no change in total measurable activity at any time. It was of interest that midbrain tyrosine hydroxylase (which using the same fractionation methods appeared to be over 80 per cent soluble and < 20 per cent particulate) did not manifest this shift with drug treatment, although it is possible that a comparable relative change in particulate could not emerge over the error term in the disproportionate distribution in the midbrain. Chronic treatment with amphetamine with increasing daily doses (5–50 mg, twice a day) resulted in the disappearance of the amphetamine-invoked shift on day 8.

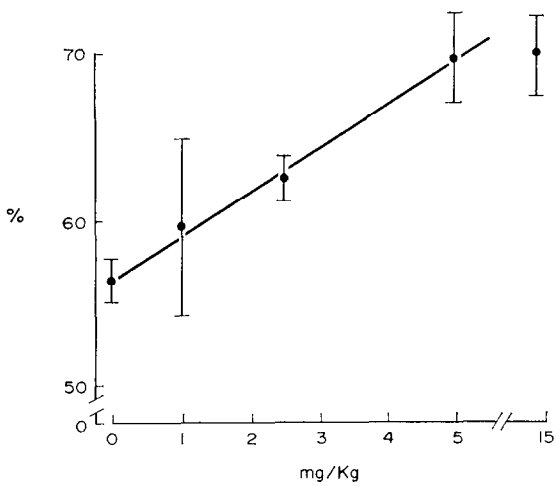

FIG. 2. Effect of the administration of various amounts of methamphetamine hydrochloride on the per cent of the total measurable striatal area tyrosine hydroxylase in the post low-speed spin, 11,000 *g* pellet studied 1 hr after drug administration. Each point represents the mean and standard error of the mean for eight caudate pairs (eight animals). Saline-injected controls did not change significantly over non-injected controls. The increase achieved significance at 2·5 mg/kg ($P < 0.05$). The 1 mg/kg group characteristically had a high variance as though this dose was at the threshold of a response with individual differences more easily seen. Fifteen mg/kg failed to produce a bigger effect than 5 mg/kg.

Dose-related relationship. The magnitude of the methamphetamine-induced shift appeared influenced by the dose. In a series of experiments, varying amounts of drug, from 1 to 15 mg/kg was administered to groups of eight animals each. Equivolume amounts of saline were administered to the control groups. Each group of animals was sacrificed 1 hr after drug administration, and the percentage of the total caudate tyrosine hydroxylase activity in the 11,000 *g* pellet (excluding the low-speed pellet) was determined. Figure 2 is a summary of the results. There appeared to be a dose-related increase in the shift of tyrosine hydroxylase activity into the crude mitochondrial pellet up to 5 mg/kg beyond which there was no greater effect.

Time course and latency of the shift. An experiment was carried out to explore the limits of the time dimensions of the methamphetamine-induced shift. Using the highest dose, 15 mg/kg, the latency and duration of the effect were determined. Figure 3

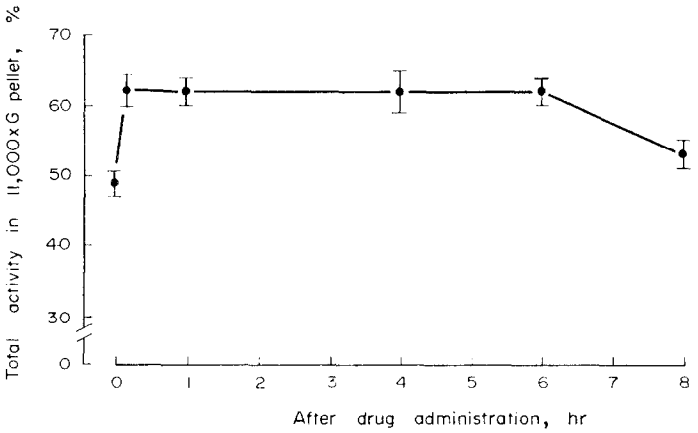

FIG. 3. Latency and duration of the methamphetamine-induced relative increase in particulate tyrosine hydroxylase at various times after the highest administered dose of the drug (15 mg/kg). Each point represents the mean and the standard error of the mean for six pairs of caudate (six animals) of the per cent of total striatal tyrosine hydroxylase in the previously defined 11,000 *g* pellet. Note that the effect appeared to reach its peak in 10 min and returned to control levels at 8 hr.

summarizes the effect of the administration of methamphetamine, 15 mg/kg, on the per cent of the total caudate tyrosine hydroxylase activity in the 11,000 *g* pellet at various times after the administration of the drug. Highest values were obtained at 10 min and they returned to control values at 8 hr. There is a slight suggestion that both latency and duration are dose-related in that the values following 5 mg/kg took longer to reach the peak and returned to control levels more quickly (see Fig. 1).

Methamphetamine in vitro. Experiments were conducted in an effort to explore whether this drug-induced shift could be produced *in vitro* as an effect of the drug on brain tissue during homogenization or subcellular fractionation rather than a drug-altered physiological mechanism. Methamphetamine hydrochloride, in concentrations from 0·01 to 0·25 mg/cm^3 was added to the caudate area at the time of homogenization. There were no observable changes in the subcellular distribution of tyrosine hydroxylase activity after this treatment.

Effect of cycloheximide and colchicine pretreatment. Although the very short latency of the methamphetamine-induced shift made either alterations in protein synthesis or fast axoplasmic flow unlikely mechanisms, drug studies were undertaken to explore these possibilities. Cycloheximide (2 mg/kg), sufficient to inhibit 75–80 per cent of the incorporation of ^{14}C-leucine into brain TCA precipitable protein, failed to alter the magnitude of the shift. Varying doses of colchicine given intraventricularly in an effort to bind microtubular protein and impede axoplasmic flow[16] failed to alter the amphetamine-induced shift in doses high enough to produce severe neurological symptoms. This drug would probably reach the striatum due to the close proximity of these structures to the ventricular system.

Effect of norepinephrine in vitro. Since much of the speculation concerning the action of amphetamine involves its influence on norepinephrine dynamics,[17] studies *in vitro* of the effects of altering norepinephrine concentration on the subcellular distribution of caudate tyrosine hydroxylase were carried out. The 11,000 *g* pellet and supernatant fractions were each dialyzed against 100 vol. of 0·32 M sucrose in an

effort to wash out tissue norepinephrine. After this dialysis, these fractions were recentrifuged without significant change in the distribution of the subcellular fractions. In other experiments, the post low-speed supernatant was suspended in 0, 20, 40 and 80 $\mu g/cm^3$ of *dl*-norepinephrine hydrochloride at 4° for 24 hr. This fraction was then centrifuged at 11,000 *g* for 30 min and the consequent supernatant and pellet fractions were dialyzed and assayed. The subcellular distribution of the enzyme was not altered by these varying amounts of norepinephrine *in vitro*.

Effect of imipramine. A number of studies have suggested that the major mechanisms of action of the amphetamine-like drugs on neural tissue is their ability to release and blockade reuptake of synaptic norepinephrine.[17] A drug which mimics some aspects of the effect of the amphetamines on brain norepinephrine was studied in relationship to its effect on the subcellular distribution of norepinephrine in the rat caudate. The tricyclic antidepressant imipramine (15 mg/kg) in a dose demonstrated to block the uptake binding of isotope NE to brain[18] failed to induce a shift as determined 2 hr after drug administration.

Effect of electroconvulsive shock and intermittent footshock. In an effort to mimic the non-specific neural activation effects of amphetamine, electroconvulsive shock was administered to groups of rats using bitemporal electrodes and a constant current source (30 mA at a pulse rate of 150 cps for 1 msec). Suitably handled groups were used as controls. Animals were sacrificed at varying times after this procedure, and the specific activity and subcellular distribution of caudate tyrosine hydroxylase noted in these experiments. A similar attempt to mimic non-specific activation was the use of intermittent footshock for 1 hr followed by studies of caudate tyrosine hydroxylase. A subcellular shift in enzyme was not noted.

Effect of reserpine. In a continuing effort to discern the necessary conditions to produce the methamphetamine shift, additional agents which share some of the actions of the amphetamines were tried. Reserpine, a depleter of intraneuronal NE as well as an agent which is thought to lead to the acute flooding of the biogenic amine receptors,[19] was administered at 5 mg/kg. As can be seen in Table 2, the

TABLE 2. EFFECT OF RESERPINE ADMINISTRATION ON THE SUBCELLULAR DISTRIBUTION OF RAT CAUDATE TYROSINE HYDROXYLASE*

Treatment	Time (hr)	Per cent 11,000 *g* particulate (mean ± S.E.)
Saline	2	59·5 ± 1·2
Reserpine	2	61·2 ± 1·4
Reserpine	16	64·6 ± 2·7
Reserpine	24	68·6 ± 0·7

* Effect of the acute administration of a large dose of reserpine, 5 mg/kg, on the per cent of total measurable caudate tyrosine hydroxylase in the 11,000 *g* particulate fraction. Each point is the mean and standard error of the mean of five caudate pairs (five animals). The relative increase in particulate tyrosine hydroxylase reaches significance at 16 hr ($P < 0{\cdot}02$). At 24 hr, the total measurable enzyme is up significantly by 25 per cent; all in the particulate.

percentage of total measurable tyrosine hydroxylase in the 11,000 *g* particulate increased over 24 hr, reaching significance at 16 hr. In this instance, however, in contrast to the amphetamine effect, the total measurable striatal enzyme activity increased, almost entirely in the particulate fraction. Since there was not a comparable decrease in total supernatant activity, it appeared that this effect was not due to a redistribution of enzyme from the soluble to the particulate fractions but rather an increase in particulate enzyme activity. This phenomenon may be explained by the previously described binding activation of tyrosine hydroxylase. We have shown in previous studies that membrane binding, the addition of a specific sulfated mucopolysaccharide (heparin), or incubation with trypsin leads to an allosteric activation of soluble tyrosine hydroxylase. This activation was manifested by an increased sensitivity to the activating effects of $DMPH_4$ and the inhibitory effects of either DA or NE.[11,12]* This sort of activation due to an apparent alteration of the conformation of the active site may be the mechanism of the reserpine-induced relative and absolute increase in particulate tyrosine hydroxylase activity.

Effect of intraventricular NE infusion. In previous studies[20] techniques were derived for the behavioral activation of freely moving rats by the intraventricular infusion of NE, slowly, at varying doses, and involving negligible volumes. The dose-dependent behavioral activation could be viewed as a model with which to study a part of the mechanism of the methamphetamine effect. Both amphetamine administration and intraventricular infusion of NE would produce activation of adrenergic receptors. In the instance of NE infusion, however, the intraneuronal NE concentrations would probably either not change or be increased via the presynaptic uptake mechanism, in contrast to the effects of the amphetamines which acutely deplete stores in high doses.[21] When saline and NE infusion groups were compared 1 hr after the infusion treatment as previously described at doses which maximally activated the animals behaviorally,[20] no statistically significant change was seen in the subcellular distribution of striatal tyrosine hydroxylase.

Effect of α-methyltyrosine. Another drug that significantly alters catecholamine levels in the brain is α-methyltyrosine. A number of reports have indicated that α-methyltyrosine administration acutely reduces NE levels in brain by inhibiting the enzyme, tyrosine hydroxylase. In addition, pretreatment with α-methyltyrosine prevents the behaviorally activating effects of amphetamine.[22] Four groups of ten animals each were studied in an effort to look at the particulate enzyme activity correlates of this double drug experiment. Each were given two injections, 1 hr apart and sacrificed 1 hr later. The usual separation procedures and assay followed dialysis in studying the effect of α-methyltyrosine (150 mg/kg) on the subcellular distribution of tyrosine hydroxylase in the striatal areas. The goals of these experiments were to determine the effect of α-methyltyrosine itself and the effect of α-methyltyrosine pretreatment on the amphetamine (5 mg/kg) effect. Table 3 shows that α-methyltyrosine produced a shift of enzyme activity into the 11,000 *g* particulate that was comparable to the amphetamine effect and was, in addition, additive to the amphetamine effect, in spite of the fact that this dose antagonized the amphetamine-induced behavioral activation. This suggested that the shift into the particulate fraction of striate tyrosine hydroxylase activity was not a non-specific neurobiological concomitant of the behaviorally activating effects of amphetamine.

* R. Kuczenski and A. J. Mandell, manuscript in preparation.

TABLE 3. RESULTS OF DOUBLE DRUG EXPERIMENT*

Injection No. 1	Injection No. 2	Shift over saline (%)
Saline	Saline	0 ± 3·5
Saline	Methamphetamine	11·8 ± 0·5
α-MT	Saline	11·8 ± 1·1
α-MT	Methamphetamine	15·4 ± 2·3

* Effect on the size of the methamphetamine-induced difference between the per cent of the total measurable caudate tyrosine hydroxylase in the 11,000 *g* pellet and the 11,000 *g* supernatant fractions of pretreatment with 150 mg/kg of α-methyltyrosine (α-MT). Since these fractions usually equal each other (about 50 per cent in the 11,000 *g* supernatant and particulate), no drug effect would make the difference equal approximately zero. Each number represents the mean and standard error of the mean of ten caudate pairs. The pretreatment ("Injection No. 1") preceded "Injection No. 2" by 1 hr; the animals were sacrificed 1 hr after the second injection. The difference between the per cent of total activity in the pellet and the supernatant after two saline injections was arbitrarily set at zero and subtracted from the following single or double drug experiments. Note that methamphetamine (as seen previously) increases the relative amount of enzyme activity in the 11,000 *g* pellet as does α-methyltyrosine. These effects were partially additive.

Effect of hypotonic shock, sonication and narrow clearance homogenization on the methamphetamine-increased particulate enzyme. The methamphetamine-augmented particulate tyrosine hydroxylase was shocked in 0·002 sodium phosphate buffer (pH 7·9) without significant increase in enzyme activity. Sonication with parameters that released over 80 per cent of synaptosomal soluble protein[23] did not alter the subcellular distribution or activity of striate tyrosine hydroxylase significantly. More rigorous homogenization of the caudate area using a narrow clearance (0·009 cm) glass–Teflon homogenizer, found destructive to brain synaptosomes, failed to alter the subcellular distribution or activity of the enzyme. The per cent of the total measurable tyrosine hydroxylase in the 11,000 *g* pellet was roughly the same whether the brain tissue was homogenized in 0·32 M sucrose or hypotonic 0·002 M sodium phosphate buffer (pH, 7·4). We have recently reported studies of the physical properties of rat striate tyrosine hydroxylase[11,12] demonstrating that systematic variation of buffer composition, ionic strength, pH and temperature have all failed to lower the "particle bound" tyrosine hydroxylase activity below 30–50 per cent depending upon the technique. Decrements in the particulate fraction enzyme activity after various manipulations *in vitro* were relatively the same for the control or methamphetamine-augmented levels. The observed particulate enzyme values for both the control and experimental animals could be augmented by increasing Ca^{2+} concentration during homogenization and/or centrifugation. These and our previous studies[11,12] have suggested that striate tyrosine hydroxylase is not particulate by entrapment in synaptosomes but rather due to binding to nerve ending membranes—the extent to which in the striate area can be altered by methamphetamine.

Binding and activity. As noted previously, alterations in the subcellular distribution of caudate tyrosine hydroxylase may be produced *in vitro* by some changes in ionic composition of the dialysis media and by divalent cations, especially Ca^{2+}. Although

the relationship between the specific activity of tyrosine hydroxylase and its subcellular disposition appears quite complex,[10–12] the data support the possibility of regulatory mechanisms involved in the association of the enzyme with membrane fractions. For example, the addition of divalent cations to the 3000 *g* supernatant consistently decreased the level of measurable tyrosine hydroxylase by 40–60 per cent. If a 17,000 *g* spin for 20 min was then performed and the Ca^{2+} removed by dialysis, the activity was recovered in the particulate fraction; this series of experiments suggested a binding phenomenon associated with inhibition. On the other hand, when the K_m values of the original 11,000 *g* supernatant and particulate fractions for $DMPH_4$ were determined, it appeared that the enzyme was relatively activated in the particulate state ($2{\cdot}71 \times 10^{-4}$ vs. $8{\cdot}78 \times 10^{-5}$ M).[11,12] Systematic exploration of ion, membrane, and enzyme systems *in vitro* continues in our effort to more systematically characterize the relationship between physical state and specific activity of striatal tyrosine hydroxylase. Recent work suggests that rat brain tyrosine hydroxylase is allosterically activated by sulfated mucopolysaccharides,[11] membrane binding,[12] and an apparent alteration in its tetrameric form to monomeric form.

DISCUSSION

A rather considerable amount of current interest has been focused on the subcellular location of tyrosine hydroxylase in various adrenergic systems. This issue is particularly important because this rate-limiting enzyme in the biosynthesis of catecholamines has been speculated to be under product-feedback control by the cytoplasmic catecholamine pool.[3,24–26] The relatively high K_i of tyrosine hydroxylase for catecholamines compared to the general cytoplasmic level of these substances, however, makes such issues as the subcellular location of the enzyme of particulate relevance, since the proximity of this enzyme to such modifiers as well as substrate and cofactors becomes critical, particularly in brain. A recent report by Musacchio *et al.*[27] suggesting that, in the adrenal, a pteridine reductase system coupled to the tyrosine hydroxylase increases the sensitivity to catecholamine product feedback, may not be relevant to the brain's nerve ending enzyme which we have shown to have a different set of kinetics as a function of its physical state.[11,12]

Much contradictory data have been collected concerning the subcellular distribution of tyrosine hydroxylase which appears to vary as a function of organ, species, and preparatory techniques. Nagatsu *et al.*[1,2] described the enzyme as particle bound in both adrenal and brain. Stjarne and Lishajko[28] using splenic nerve showed tyrosine hydroxylase to be over 90 per cent in the high-speed supernatant fraction. Petrack *et al.*[29] found that 90 per cent of the total tyrosine hydroxylase activity of bovine adrenal medulla was particulate. Musacchio[30,31] has shown that rat and bovine adrenal gland tyrosine hydroxylase was localized in the soluble fraction, findings which had been independently confirmed by Laduron and Belpaire.[4] Recently Petrack *et al.*[32] have reported a soluble form of tyrosine hydroxylase from guinea pig brain. Fahn *et al.*[6] on the other hand have reported that a significant fraction of tyrosine hydroxylase in beef striatal cortex was particulate. Nagatsu *et al.*[33] in their most recent studies have confirmed that a large percentage of bovine caudate nucleus tyrosine hydroxylase is particle bound. In a recent set of studies, Wurzburger and

Musacchio[5] and Musacchio *et al.*[34] have rather elegantly outlined the preparatory artifacts that might be operating to confuse the subcellular studies of adrenal tyrosine hydroxylase. These include the enzyme's tendency to aggregate as well as to bind to the coarse particles of the low-speed (700 *g*) sediment, particularly in non-ionic (sucrose) media. Their studies seem not to be relevant to the brain or to other than the "coarse particle fraction" in that they report that sucrose media did not influence the composition of the other subcellular fractions (specifically the ones examined in these studies).

These above reviewed studies have been directed toward an accurate physical characterization of brain tyrosine hydroxylase. We have been preoccupied by the potential regulatory significance of this physical state as could be approached through modification *in vitro*. This report focuses on a drug-induced phenomenon *in vivo* which may be an example of this sort of physical state alteration—enzyme regulatory change. This report documents a systematically obtainable shift of enzyme activity from the 11,000 *g* supernatant to the P_2B and P_2C particulate fractions from rat caudate area following immediately upon the administration of amphetamine and α-methyltyrosine *in vivo* but not *in vitro*. This kind of physical change in tyrosine hydroxylase was not observed in the midbrain. A relatively high density of cell bodies in the midbrain and of nerve endings in the caudate area of the catecholamine cell systems in the brain[35] might account for this regional difference. The high percentage of soluble tyrosine hydroxylase in an area relatively dense in catecholamine-containing cell bodies is consonant with the subcellular distribution of tyrosine hydroxylase reported by Wurzburger and Musacchio[5] for the adrenal medulla, an area also relatively dense in cell bodies. The relatively higher particulate fraction in the nerve-ending dense caudate area is consonant with the physical state of tyrosine hydroxylase in beef caudate reported by Fahn *et al.*[6] and Nagatsu *et al.*[33] In addition, McGeer *et al.*[7] in humans and Kuczenski and Mandell in rats[12] have reported similar findings. The amphetamine-induced shift may be a characteristic of the nerve-ending dominated caudate area. The small particulate fraction in the midbrain did not seem to vary with drug treatment but whether this was due to very small base line values (< 10 per cent particulate) and too much variance or the specificity of this event for nerve endings in the striate area is as yet indeterminant.

Although varying norepinephrine concentration *in vitro* did not alter the subcellular distribution of the enzyme, since amphetamine has been speculated to function in a myriad of ways including the release and blockade of the reuptake of NE by noradrenergic nerve endings, various drugs and treatments were used as analogues to see which if any of the features of amphetamine's actions were necessary to produce the shift. The general neurological activation effects of methamphetamine were initiated by the experiments using intermittent footshock and electroconvulsive shock. Neither of these is a perfect analogue, in that regional or neurophysiological system factors are not strictly comparable. It was nonetheless of interest that neither treatment led to the shift. This negative finding is also of interest in that both footshock and electroconvulsive shock have been shown to increase norepinephrine turnover in a way similar to the effect of amphetamine but do not significantly reduce intraneuronal NE.[36,37] Imipramine was used to mimic the potentiation of synaptic NE by re-uptake blockade; this would have the effect of increasing the amount of NE available to the post-synaptic receptor without decreasing intraneuronal NE. Apparently this facet of

the action of amphetamine was not responsible for the shift in that imipramine failed to alter the subcellular distribution of striate tyrosine hydroxylase. An experiment directed toward a similar portion of the amphetamine mechanism was that making use of intraventricularly infused NE. In this circumstance, the amount of NE available to receptors would increase without the concomitant decrease in intraneuronal NE. As a matter of fact, it is likely that this sort of infusion would if anything increase intraneuronal NE via the presynaptic uptake mechanism.[17] This sort of infusion has been shown to produce a dose-dependent behavioral activation not unlike that seen with amphetamine administration.[20] In addition, the failure to induce a significant shift with NE infusion suggested that the synaptic potentiation of NE by methamphetamine was probably not responsible for the shift. Only α-methyltyrosine, which significantly reduces intraneuronal NE, and reserpine which does the same thing demonstrated the relative increase in particulate tyrosine hydroxylase seen after amphetamine. In the case of reserpine, however, the total activity went up as well. A large and sustained decrease in intraneuronal NE produced by amphetamine (in doses used in this study), reserpine, and α-methyltyrosine, although accomplishing this by different mechanisms,[17,22] all appeared to share the resulting relative increase in tyrosine hydroxylase activity in the 11,000 *g* particulate fraction of rat striatal brain areas.

The precise physical state and/or subcellular location of the methadrine-augmented particulate tyrosine hydroxylase has not been ascertained. However, the general resistance to change of the particulate enzyme to narrow clearance homogenization, hypotonic shock and sonication probably rules out an intravesicular location or entrapment in intact synaptosomes. This suggested that the particulate state was probably due to binding to synaptic membranes or subcellular organelles in a way similar to that reported for tyrosine hydroxylase in beef caudate.[6] Recent preliminary experiments in our laboratory, demonstrating the binding of striatal soluble tyrosine hydroxylase *in vitro* to a specific synaptosomal membrane fraction isolated independently on Ficol gradients,[38] were consistent with this possibility.

It is not possible at the present time to other than speculate concerning the functional significance of the rather fast relative increase in rat striatal particulate tyrosine hydroxylase after the administration of drugs which by various means reduce intraneuronal NE. There have been some recent studies which may be relevant and suggest the possibility of either an activating or inhibitory function of such an adaptation under conditions *in vivo*. Besson *et al.*[39] have demonstrated non-competitive inhibition of dopamine biosynthesis in rat striatal brain slices after pretreatment with amphetamine, 5 mg/kg, with a very short latency. It is not likely that this is the result of the direct inhibition of the enzyme, in that our studies have shown that methamphetamine at concentrations up to 10^{-2} M did not alter measurable activity *in vitro*. Recently Fibiger and McGeer[40] reported that both a single dose or repeated doses of methamphetamine every 12 hr for 2 days resulted in a relatively fast decrease in measurable tyrosine hydroxylase activity in corpus striatum and no change in the hypothalamus. Their assays were conducted on whole tissue homogenized in 0·32 M sucrose, and a functional correlate of a physical state change was certainly not ruled out. Their failure to find these changes in the hypothalamus is consistent with our failure to find a physical state change in catecholamine cell body areas. The reserpine-induced increase in particulate enzyme activity was not quite the same as that seen with metham-

phetamine and α-methyltyrosine. The increase in apparent particulate activity without the associated loss in activity in the soluble was comparable to our findings after the acute administration of propranolol as reported by Sullivan *et al.*[41] It is tempting to speculate that an immediate alteration in the physical state of the rate-limiting biosynthetic enzyme in catecholamine biosynthesis leading to activation or inhibition would be a way of immediately adapting to primary drug effects on intraneuronal catecholamine concentration.

In conclusion, methamphetamine, in moderate to large doses, and other drugs which significantly deplete intraneuronal catecholamines (such as α-methyltyrosine and reserpine) lead to a short-latency relative increase in rat brain striatal (but not midbrain) particulate tyrosine hydroxylase activity which appears to be the result of drug-induced membrane binding of the enzyme. Drugs and treatments which imitate other amphetamine-like effects such as imipramine, intraventricular NE infusion, electroconvulsive shock and intermittent footshock fail to produce this effect. Previous studies *in vitro* of ion-promoted membrane binding of striatal tyrosine hydroxylase suggest that this binding (if applicable) could lead to either enzyme activation or inhibition. This drug-induced binding may be a regulatory mechanism in the control of the biosynthesis of catecholamines by nerve endings in central nervous system.

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