Evaluation of the Biochemical Effects Produced *in Vivo* by Inhibitors of the Three Enzymes Involved in Norepinephrine Biosynthesis

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SUMMARY

Inhibitors of tyrosine hydroxylase, dopa decarboxylase, and dopamine-β-oxidase have been compared with respect to the *in vivo* conversion of tyrosine-^{14}C and dopa-^{3}H to norepinephrine. Tyrosine hydroxylase inhibitors were found to be the most effective in blocking formation of norepinephrine from tyrosine-^{14}C. The lowering of norepinephrine levels in guinea pig tissues by α-methyltyrosine was found to be directly related to the degree of inhibition of tyrosine hydroxylase. Furthermore, with α-methyltyrosine, the measured inhibition of norepinephrine synthesis from tyrosine was found to be exactly the same as the calculated inhibition of tyrosine hydroxylase. This could be true only if tyrosine hydroxylase were the rate-limiting step in the overall biosynthesis of norepinephrine.

INTRODUCTION

After the isolation of the enzyme tyrosine hydroxylase (1) evidence was presented that it is the rate-limiting step in the conversion of tyrosine to norepinephrine (2). Several potent inhibitors of purified tyrosine hydroxylase have been obtained (3, 4), and some have been shown to lower tissue levels of norepinephrine in animals (5, 6). In the present study data are presented which demonstrate that on administration of α-methyltyrosine the lowering of norepinephrine levels in guinea pig tissues is directly related to the degree of inhibition of tyrosine hydroxylase. Several inhibitors of tyrosine hydroxylase, dopa decarboxylase, and dopamine-β-oxidase have been compared with respect to the *in vivo* conversion of tyrosine-^{14}C and dopa-^{3}H to norepinephrine. These studies show that tyrosine hydroxylase inhibitors are the most effective in blocking formation of norepinephrine from the dietary precursor, tyrosine. All the data support the hypothesis that tyrosine hydroxylase is the rate-limiting step in norepinephrine biosynthesis and that the reduction of tissue catecholamine stores by compounds such as α-methyltyrosine is a direct consequence of their inhibition of tyrosine hydroxylase.

MATERIALS AND METHODS

We wish to thank the following for making available some of the compounds used in this investigation: Merck, Sharp, and Dohme for α-methyl-L-tyrosine, α-methyl-DL-phenylalanine, and α-methyl-DL-dopahydrazine (3,4-dihydroxy-DL-hydrazinophenylalanine, α-MDH); Warner-Lambert Research Institute for benzyloxyamine; Smith Nephews Ltd. for 3-hydroxy-4-bromobenzyloxyamine (NSD-1055), and Dr. I. Kopin for antabuse.

L-Tyrosine-^{14}C (uniformly labeled, 334

μC/μmole) was obtained from New England Nuclear Corporation and DL-dopa-^{3}H (generally labeled, 192 μC/μmole) was purchased from Nuclear Chicago Corporation. α-Methyl-L-tyrosine-^{3}H (uniformly ring labeled, 236 μC/μmole) was a gift from Merck, Sharp and Dohme.

Hartley strain guinea pigs weighing 230–260 g were used in all the experiments. Food was removed from the cages the night before each experiment to exclude exogenous sources of tyrosine. All drugs were administered by the intraperitoneal route, except tyrosine-^{14}C, which was injected intravenously. Solutions for injection of α-methyltyrosine (100 mg/kg) were prepared as described previously (5); the L-isomer was used throughout. Disulfiram (Antabuse) for injection (400 mg/kg) was suspended in 1% carboxymethylcellulose in saline, except for the first experiment with tyrosine-^{14}C, where it was suspended in saline and administered orally. The other drugs, α-methylphenylalanine (200 mg/kg), α-MDH (300 mg/kg), NSD-1055 (200 mg/kg), and benzyloxyamine (200 mg/kg) were dissolved or suspended in saline. All inhibitors were administered 0.5 hr before injection of the labeled amino acid unless indicated otherwise.

Animals were killed 1.5 hr after the injection of tyrosine-^{14}C (45 μC in 0.5 ml saline) or ^{3}H-dopa (200 μC in 1 ml 0.002 N HCl), and the individual catecholamines in the tissues were isolated and assayed for radioactivity as described elsewhere (7). α-Methyltyrosine, which appeared in the effluents of the alumina columns, was adsorbed on Dowex 50 (H^+) and eluted with 4 N ammonia. After evaporation, the residues were dissolved in water and aliquots were assayed for total tyrosine (tyrosine plus α-methyltyrosine) by the nitrosonaphthol method (8). Tissue levels of α-methyltyrosine were obtained by difference, using as a base line the normal tyrosine content of each tissue. The latter was obtained by independent assays on tissues from ten untreated animals. Radioactivity of the isolated tyrosine was also measured.

α-Methyltyrosine-^{3}H (120 mg/kg, 1392 μC/kg) was administered to guinea pigs; animals were killed at various intervals and the tissues were homogenized in 5% trichloroacetic acid. To each sample was added 100 μg of α-methylnorepinephrine, as carrier, and the catecholamine was isolated by the procedure used for isolation of norepinephrine (7). This involves adsorption on and elution from alumina, passage over IRC-50 to remove any α-methyldopa and finally adsorption onto Dowex 50 (H^+) and elution with 2 N HCl. The radioactive α-methylnorepinephrine in the eluates was identified by chromatography on paper using butanol–acetic acid–water (4:1:1). In this system the R_f for α-methylnorepinephrine is 0.35, for α-methyldopa 0.30, for α-methyltyrosine 0.45 and for α-methyldopamine 0.59. The amount of α-methylnorepinephrine formed from the administered α-methyltyrosine-^{3}H was determined from the radioactivity isolated through the catecholamine isolation procedure, corrected for recovery of the carrier and for the loss of 25% of the tritium during conversion to the catecholamine.

Norepinephrine and α-methylnorepinephrine were assayed by a modification of the trihydroxyindole method (9). Dopamine was assayed by the method of Drujan *et al.* (10).

RESULTS

Effects of Various Enzyme Inhibitors on Catecholamine Formation in the Intact Guinea Pig

When tyrosine-^{14}C is administered to an animal it rapidly mixes with the endogenous tyrosine pool in the tissues (11) which is the initial precursor in the conversion to catecholamines. It has been shown that a single dose of tyrosine-^{14}C yields maximal labeling within 2 hr in the catecholamines in guinea pig tissues (7). The extent of labeling from tyrosine-^{14}C, under standard conditions, can be taken as an indication of the rate of endogenous catecholamine biosynthesis. Dopa-^{3}H is also incorporated into tissue catecholamines. The extent of its incorporation is a meas-

TABLE 1

Effects of enzyme inhibitors on tissue catecholamine levels

All compounds were administered by the intraperitoneal route and animals were killed 2 hr later. The values represent the mean of the number of animals given in parentheses. The control values are given with the standard deviation. NE = norepinephrine, DA = dopamine, Epi = epinephrine.

Compound administered	Enzyme inhibited	Catecholamine levels (μg/g)				
		Brain NE	Brain DA	Heart NE	Adrenal Epi	Spleen NE
None (9) (control)	None	0.22 ±0.03	0.51 ±0.07	1.74 ±0.16	250 ±35	1.10 ±0.14
α-Methyltyrosine (7), 100 mg/kg	Tyrosine hydroxylase	0.13	0.26	1.73	269	1.20
α-Methylphenylalanine (2), 200 mg/kg	Tyrosine hydroxylase	0.15	0.37	1.80	—	—
α-MDH (3), 300 mg/kg	Decarboxylase	0.24	0.82	3.40	270	1.69
NSD-1055 (4), 200 mg/kg	Decarboxylase and dopamine-β-oxidase	0.22	0.62	1.55	225	1.02
BOA (2), 200 mg/kg	Dopamine-β-oxidase	0.19	0.52	1.68	225	1.12
Antabuse (6), 400 mg/kg	Dopamine-β-oxidase	0.20	0.54	1.58	243	1.10

ure of the last two steps in catecholamine biosynthesis, dopa decarboxylase, and dopamine-β-oxidase.

Inhibitors of the three enzymes involved in norepinephrine synthesis were administered to animals 0.5 hr prior to tyrosine-^{14}C and the animals were killed 1.5 hr after the amino acid. This short interval of time was chosen to permit maximal enzyme inhibition and yet minimize the resulting decline of the catecholamines as is known to occur, particularly with tyrosine hydroxylase inhibitors. It can be seen in Table 1 that only the two inhibitors of tyrosine hydroxylase lowered tissue levels to a significant extent, and this was limited to brain norepinephrine and dopamine. The rapid lowering of the catecholamines in the central nervous system by tyrosine hydroxylase inhibitors has been noted before (5) and is consistent with the rapid turnover of the amines in the brain (7). The apparent elevation in some of the tissue catecholamine (nonradioactive) levels produced by the decarboxylase inhibitor α-MDH reflects contamination with catecholamine products derived from the inhibitor itself, as it is a catechol. However, the norepinephrine content of most tissues was not altered during the short interval following the administration of these inhibitors. The subsequent radioactive values were not corrected for the decreases which were achieved in some tissues with tyrosine hydroxylase inhibitors.

Studies with the inhibitors were carried out so that in each series two untreated controls were also given the labeled precursor. The radioactivity in the catecholamines in the various tissues of the two controls was measured, and the average of the two duplicate values for each tissue was taken as the uninhibited control (100%). Reduction in the incorporation of radioactivity produced by an inhibitor was based on such uninhibited controls which were run simultaneously with each experiment. Although there were significant differences from experiment to experiment in the extent of radioactivity incorporated into the tissue catecholamines of control animals, the duplicate controls run on a given day were generally in good agreement. Data for typical duplicate controls are shown in Table 2. In most tissues agreement between duplicate controls was as good or better than shown in Table 2. The largest variations between duplicate controls were generally observed in the adrenal epinephrine values.

TABLE 2

Incorporation of tyrosine-^{14}C and dopa-^{3}H into catecholamines in various tissues of the intact guinea pig

With each labeled compound the two figures for a tissue (A and B for tyrosine and C and D for dopa) represent values obtained on duplicate experiments. These values are typical of those which were averaged to obtain the controls used to calculate the inhibition obtained with the various inhibitors shown in Tables 3 and 4.

		Radioactivity incorporated (cpm/organ)			
		From Tyrosine-^{14}C		From Dopa-^{3}H	
Tissue	Catecholamine	A	B	C	D
Brain	Norepinephrine	1175	1162	3854	4105
Brain	Dopamine	3040	2995	3938	5076
Heart	Norepinephrine	2212	2833	5248	7259
Adrenal	Epinephrine	4776	7830	2669	4561
Spleen	Norepinephrine	546	464	2871	3602

TABLE 3

Effect of enzyme inhibitors on the conversion of tyrosine-^{14}C to catecholamines in various tissues of the intact guinea pig

The inhibitors were administered 0.5 hr prior to tyrosine-^{14}C at the same dosage as shown in Table 1. Animals were sacrificed 1.5 hr after the intravenous administration of 45 μC of tyrosine-^{14}C. The total radioactivity in catecholamines in each organ was measured as described under Materials and Methods and the per cent reduction in labeling was calculated from the following equation:

$$\text{per cent reduction in labeling} = \frac{C - E}{C} \times 100$$

where C equals the average value for radioactivity in duplicate controls (no inhibitor) run simultaneously and E equals the radioactivity obtained following the administration of the inhibitor. Values in parentheses represent the number of animals used with each inhibitor.

		Per cent reduction in labeling				
Compound	Enzyme inhibited	Brain NE	Brain DA	Heart NE	Adrenal Epi	Spleen NE
α-Methyltyrosine (4)	Tyrosine hydroxylase	91	96	91	97	91
α-Methylphenylalanine (2)	Tyrosine hydroxylase	55	69	60	—	—
α-MDH (2)	Dopa decarboxylase	0	0	19	29	0
NSD-1055 (2)	Dopa decarboxylase and dopamine-β-oxidase	0	81	82	83	73
BOA (1)	Dopamine-β-oxidase	2	0	0	0	0
Antabuse (2), oral	Dopamine-β-oxidase	3	0	0	5	5
Antabuse (2), I.P.	Dopamine-β-oxidase	40	0	25	7	55

Experiments with Tyrosine-^{14}C as Precursor

The effects of the different inhibitors on the conversion of tyrosine to catecholamines is shown in Table 3. It can be seen that the tyrosine hydroxylase inhibitor α-methyltyrosine produced almost complete inhibition in all tissues. It should also be noted that α-methyltyrosine was administered at a lower dosage than any of the other inhibitors. Appreciable inhibition was also produced by α-methylphenylalanine, another tyrosine hydroxylase inhibitor. The compound NSD-1055, which is both a dopamine-β-oxidase inhibitor and a decarboxylase inhibitor, also produced marked

TABLE 4

Effect of enzyme inhibitors on the conversion of dopa-^3H to catecholamines in various tissues of the intact guinea pig

Experimental details are the same as in Table 3 except that 200 μC of dopa-^{3}H were administered as the catecholamine precursor. Negative values represent an increase over controls.

Compound	Enzyme inhibited	Per cent reduction in labeling				
		Brain NE	Brain DA	Heart NE	Adrenal Epi	Spleen NE
α-Methyltyrosine (3)	Tyrosine hydroxylase	−8	−56	−31	−12	16
α-MDH (1)	Dopa decarboxylase	−257	−363	88	−142	86
NSD-1055 (2)	Dopa decarboxylase dopamine-β-oxidase	82	89	88	92	87
BOA (2)	Dopamine-β-oxidase	62	37	52	4	63
Antabuse (2)	Dopamine-β-oxidase	41	9	38	66	47

inhibition in most tissues. The failure of NSD-1055 to inhibit brain norepinephrine formation was surprising but was observed in two separate experiments. By contrast α-MDH, a potent decarboxylase inhibitor, and BOA, one of the most potent dopamine-β-oxidase inhibitors, had little effect. Antabuse, which apparently inhibits dopamine-β-oxidase by complexing copper (12) had little effect when given orally. When given intraperitoneally it had some effect on synthesis of norepinephrine in brain and spleen and less effect on synthesis in the heart and adrenal gland. As would be expected, it did not decrease brain dopamine formation.

Experiments with Dopa-^3H as Precursor

These were carried out to determine, first that the *in vivo* effects of tyrosine hydroxylase inhibitors were limited to the initial step. As shown in Table 4, α-methyltyrosine had no inhibitory action when catecholamine formation bypassed tyrosine hydroxylase and began with dopa. There was actually observed a significant increase in dopa-^{3}H incorporation into catecholamines following administration of the tyrosine hydroxylase inhibitor. This is to be expected and has already been noted and commented upon (5).

A second purpose of the experiments with dopa-^{3}H was to determine whether inhibitors of the other enzymes, at the dosages employed, were effective once the initial step had been bypassed. It can be seen that the inhibitory actions of NSD-1055 were even greater with dopa-^{3}H as a precursor, proving that its site of action was beyond the initial step. BOA, which had no effect on synthesis from tyrosine-^{14}C, had an appreciable effect on synthesis from dopa, consistent with its site of action. Although antabuse had an appreciable effect on synthesis from dopa it was not as marked as NSD-1055 and was not much more than occurred with tyrosine as precursor. The decarboxylase inhibitor α-MDH produced anomalous results, inhibiting markedly in heart and spleen but greatly increasing the labeling in brain and adrenal gland. The latter effect is explained in the subsequent experiment.

Effect of Inhibitors on the Uptake of Tyrosine-^{14}C and Dopa-^3H by Tissues

The anomalous effects observed with some of the drugs prompted us to investigate their action on the uptake of the radioactive precursors into the tissues. Levels of tyrosine-^{14}C and dopa-^{3}H were measured 2 hr after administration of each drug and 1½ hr after each labeled amino acid. Values for total tissue tyrosine were also determined. With tyrosine-^{14}C the specific activities were the same as those of controls and ranged from 1.4 to 1.8 $\times 10^5$ cpm/μmole in the hearts and from 2.0 to 2.4 $\times 10^5$ cpm/μmole in the brains. With α-methyltyrosine it was not possible to measure specific activity because of inter-

ference with the fluorometric assay. However, in this case, the amounts of tyrosine-^{14}C which were found in heart and brain were the same as in controls. The specific activities of tyrosine in hearts and brains

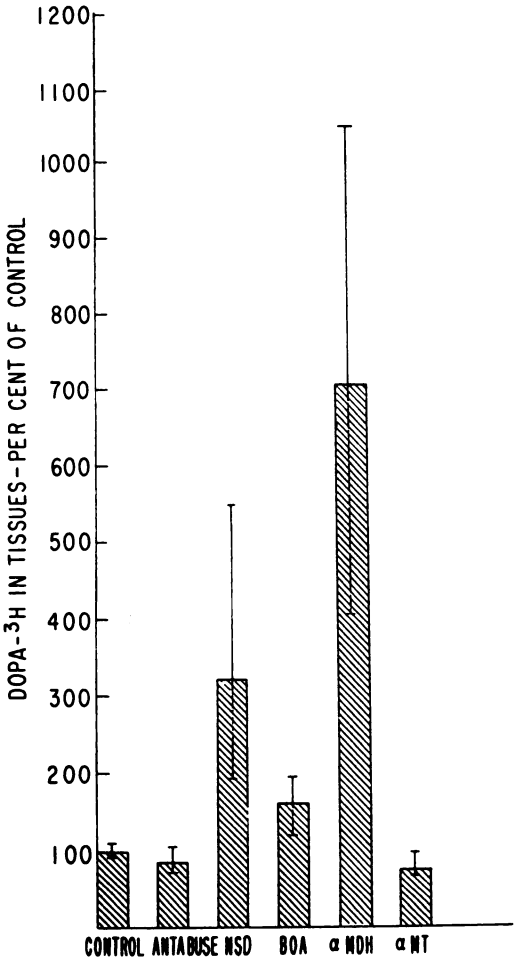

FIG. 1. *Effect of inhibitors on the uptake of dopa-^{3}H by tissues*

The concentration of dopa-^{3}H in heart, brain, and spleen following administration of 200 μC of dopa-^{3}H was 9811 cpm/g for heart, 3946 cpm/g for brain, and 18,356 cpm/g for spleen. The values for each of the tissues were arbitrarily set at 100%. This is defined as the control. For each inhibitor the bar represents the mean value for dopa-^{3}H content of the three tissues in all the experiments given in Table 4. The bracket above and below the mean represents the range of observed values. α-MT = α-methyltyrosine.

of α-MDH treated animals were slightly higher than the other values. With dopa-^{3}H marked increases in uptake over controls were produced by α-MDH and NSD-1055 (Fig. 1). A small increase was produced by BOA. There is no explanation for these effects. However, the increased amounts of dopa-^{3}H would, of course, tend to increase synthesis of norepinephrine and therefore reverse the effects of the inhibitor.[1] One must assume therefore, that the calculated inhibitions reported for α-MDH, NSD-1055 and BOA in Table 4 are far too low and that these compounds are even more effective inhibitors of the *in vivo* conversion of dopa to norepinephrine.

Correlation of in Vivo Actions of α-Methyltyrosine on Norepinephrine Synthesis with Tyrosine Hydroxylase Inhibition

It has been shown that α-methyltyrosine can deplete tissues of catecholamines, and some evidence was presented to indicate that this effect was related to inhibition of norepinephrine synthesis at the tyrosine hydroxylase step (5). However, it was also shown that α-methyltyrosine was, to some extent, oxidized to α-methyldopa. The latter depletes tissues of catecholamines by a totally different mechanism which involves conversion to the "false-transmitter" α-methylnorepinephrine (13–15). The following experiments were designed to ascertain which of the two mechanisms explained the *in vivo* actions of α-methyltyrosine.

Guinea pigs were given α-methyltyrosine (100 mg/kg) and each of the animals was given tyrosine-^{14}C (45 μC) 1.5 hr before it was to be killed. Animals were killed at

[1] The K_m values for dopa decarboxylase and dopamine-β-oxidase are 5×10^{-4} M and 5×10^{-3} M, respectively. Approximately 1 μmole of dopa-^{3}H was administered per 250 g, giving a calculated tissue concentration of 4×10^{-6} M (based on uniform distribution). Observed values of dopa-^{3}H were much lower even where they were elevated above control levels. Since endogenous tissue levels of dopa and dopamine are negligible (except in brain) decarboxylase and dopamine-β-oxidase would be expected to vary directly with tissue levels of dopa and dopamine.

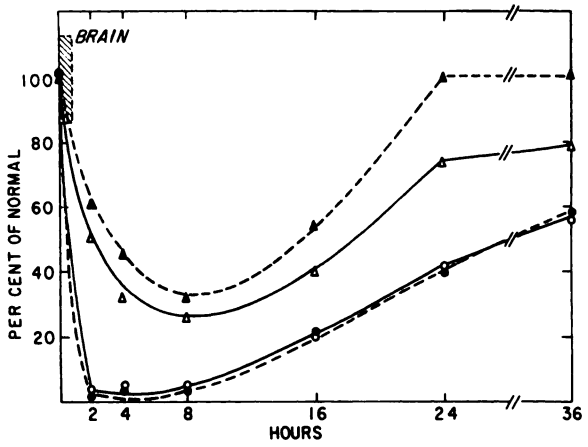

FIG. 2. *The effect of α-methyltyrosine on the conversion of tyrosine-^{14}C to catecholamines and on endogenous catecholamine levels in brain*

The cross-hatched area on the ordinate represents the range of endogenous catecholamine levels in control guinea pig brain (see Table 1). Each point represents a single animal. ▲----▲, endogenous dopamine; △——△, endogenous norepinephrine; ●----●, dopamine-^{14}C; ○----○, norepinephrine-^{14}C.

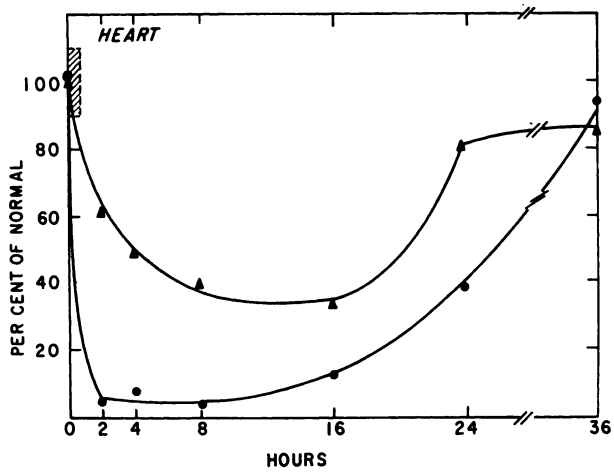

FIG. 3. *The effect of α-methyltyrosine on the conversion of tyrosine-^{14}C to norepinephrine and on endogenous norepinephrine levels in the heart*

The range of endogenous norepinephrine levels in control guinea pig hearts (cross-hatched area) is based on values shown in Table 1. Each point represents a single animal. ▲——▲, endogenous norepinephrine; ●——●, norepinephrine-^{14}C.

various time intervals after receiving the inhibitor. Tissues were removed and catecholamines were isolated and assayed fluorometrically, and their radioactivity was measured. α-Methyltyrosine levels were also determined in each of the tissue samples. As shown in Figs. 2 and 3 the ability to convert tyrosine-^{14}C to norepinephrine-^{14}C and dopamine-^{14}C immediately fell to negligible values in both tissues, remained at these low levels for several hours, then rose and returned to control values by 36 hr in the heart and later in the brain. Catecholamine levels in these tissues fell

more gradually,[2] reached minimal values in 8–16 hr, then returned almost to control levels as the synthetic ability returned. It is of interest that in the brain norepinephrine and dopamine levels were back to normal at a time when synthetic activity was still significantly reduced. What is important however, is that in these tissues as well as in spleen, catecholamine levels paralleled the extent of conversion of tyrosine-^{14}C to norepinephrine-^{14}C and dopamine-^{14}C.

Another interesting correlation was made. Tissue levels of α-methyltyrosine were determined and these values, the normal tissue levels of tyrosine, and the previously determined K_m and K_i values for tyrosine and the inhibitor (3) were used to calculate the degree of tyrosine hydroxylase inhibition in the tissues at each time interval (see legend to Fig. 4). As shown in Figs. 4 and 5 the calculated inhibitions agreed closely with the measured inhibitions at all but one point where measurements were possible. Calculated inhibitions were not meaningful at 36 hr because the assay was not sufficiently sensitive to measure the residual α-methyltyrosine.

Significance of α-Methyldopa

The following experiment was designed to see whether the catecholamine decrease observed with α-methyltyrosine was caused by α-methylnorepinephrine produced from α-methyldopa formed by the action of tyrosine hydroxylase on α-methyltyrosine. Guinea pigs were given α-methyltyrosine-^{3}H and individual animals were killed at stated intervals; the tissues were homogenized and nonradioactive α-methylnorepinephrine was added as carrier, as described under Materials and Methods. The radioactivity migrated with the carrier when chromatographed on paper as described under Materials and Methods. The material was eluted from the paper, and its specific activity was determined. As shown in Table 5, not only were the amounts of α-methylnorepinephrine extremely small compared to the norepinephrine deficit produced by α-methyltyrosine, but the appearance of α-methylnorepinephrine did not coincide with the changes in tissue norepinephrine levels. Little α-methylnorepinephrine was present when the decline in norepinephrine was most rapid. Furthermore, α-methylnorepinephrine levels rose and reached their peak after the norepinephrine levels had returned to normal.

[2] In this experiment heart norepinephrine levels were significantly lowered 2 hr after the α-methyltyrosine. The 2-hr value represents a single determination. In dozens of other experiments this dose had no significant effect.

TABLE 5

Comparison of tissue levels of norepinephrine and α-methylnorepinephrine following administration of α-methyltyrosine

Guinea pigs were given 120 mg/kg of α-methyltyrosine and killed at the specified time intervals. This is a higher dose of inhibitor than was used in the previous experiments.

	Heart (mμmoles/g)		Brain (mμmoles/g)	
Time (hr)	Norepinephrine	α-Methylnorepinephrine	Norepinephrine	α-Methylnorepinephrine
0	9.8	0	1.50	0
2	6.0	0.1	0.74	0.2
4	4.8	0.2	0.50	0.3
8	3.8	0.2	0.39	0.4
16	3.3	0.4	0.60	0.4
24	8.0	0.6	1.10	0.7
36	8.3	—	1.17	—
48	—	0.9	—	0.4

Thus, although, α-methyltyrosine does give rise to α-methyldopa and thereby to α-methylnorepinephrine, quantitatively and qualitatively the latter does not coincide with the changes in norepinephrine produced by administered α-methyltyrosine.

DISCUSSION

In previous studies when labeled tyrosine, dopa, and dopamine were perfused through the isolated guinea pig heart in increasing concentrations and the labeling in norepinephrine was assayed, maximal rates were achieved only with tyrosine (2). Furthermore, the apparent V_{max} obtained at saturation coincided with the *in vivo* rate of formation of heart norepinephrine, and the apparent K_m (one-half maximal rate)

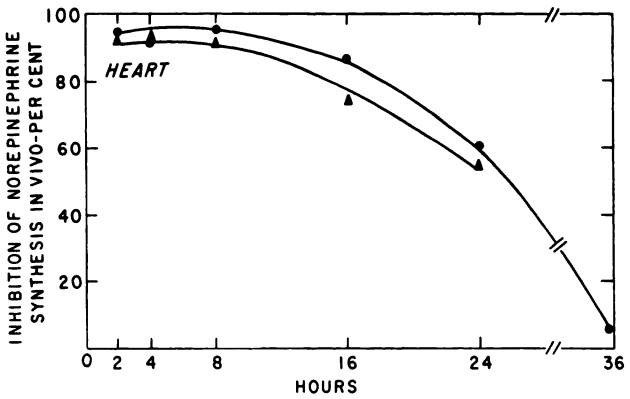

FIG. 4. *Correlation between the observed inhibition of the conversion of tyrosine-^{14}C to norepinephrine* (●——●) *and the calculated* (▲——▲) *inhibition of tyrosine hydroxylase in heart*

Each point ▲ equals

$$100\left(1 - \frac{V_1}{V_0}\right)$$

where V_0 is the normal rate of formation and V_1 the rate in the presence of inhibitor;

$$\frac{V_1}{V_0} = \frac{K_m + S}{K_p + S}$$

where $K_m = 6.25 \times 10^{-5}$ M (for tyrosine) and $S = 5 \times 10^{-5}$ M measured concentration of tyrosine in heart.

$$K_i = 1.7 \times 10^{-5} \text{ M (for } \alpha\text{-methyltyrosine)}$$

where $i =$ measured concentration of α-methyltyrosine at

$$K_p = \frac{i + K_i}{K_i} \times K_m$$

The K_m for tyrosine and the K_i for α-methyltyrosine were reported in a previous publication (3). The calculations are based on equations for competitive inhibition in Dixon and Webb (16).

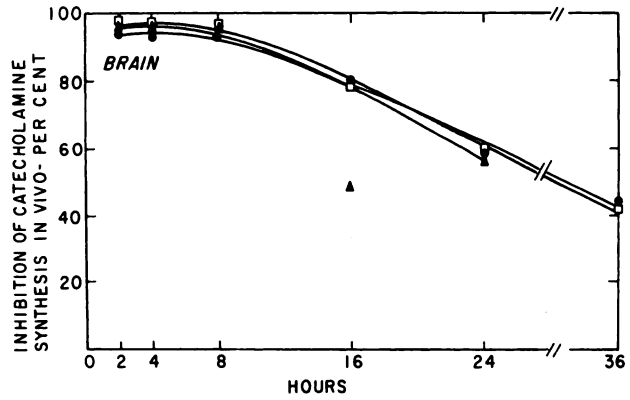

FIG. 5. *Correlation between the observed inhibition of conversion of tyrosine-^{14}C to norepinephrine* (●——●) *and dopamine* (□——□) *and the calculated* (▲——▲) *inhibition of tyrosine hydroxylase in brain*

Calculated points were obtained as described in the legend of Fig. 4.

for tyrosine in the perfused heart agreed with that obtained for purified tyrosine hydroxylase (*ca.* $\times 10^{-5}$ M).[3] These findings suggested that tyrosine hydroxylase was the rate-limiting step in the biosynthesis of norepinephrine. If this were true, inhibitors of tyrosine hydroxylase should block endogenous norepinephrine synthesis (from tyrosine-^{14}C) more effectively than inhibitors of any of the other enzymes. The data in Table 3 show that α-methyltyrosine, a potent competitive inhibitor of tyrosine hydroxylase, was by far the most effective agent for blocking endogenous norepinephrine production. α-Methylphenylalanine, a less potent *in vitro* inhibitor, was also fairly active in this *in vivo* study. Of the other inhibitors only NSD-1055, a potent inhibitor of both dopa decarboxylase and dopamine-β-oxidase was effective in the overall conversion from tyrosine. However, it was less active than α-methyltyrosine. Furthermore, inhibition with NSD-1055 was even greater when dopa-^{3}H was used as precursor for catecholamine synthesis (Table 4), indicating that NSD-1055 acts subsequent to the tyrosine hydroxylase step. Nevertheless, by effectively inhibiting both the second and third enzymes NSD-1055 does block endogenous synthesis of norepinephrine. It should be pointed out that NSD-1055 has been shown to lower catecholamine levels *in vivo* (17) more effectively than any of the compounds listed except the tyrosine hydroxylase inhibitors. Except for NSD-1055, decarboxylase and dopamine-β-oxidase inhibitors, at high dosage, were not effective in blocking endogenous formation of norepinephrine. However, when tyrosine hydroxylase was bypassed by giving dopa-^{3}H, dopamine-β-oxidase inhibitors and particularly benzyloxyamine became effective. Antabuse, although reported to be a potent inhibitor of dopamine-β-oxidase (12), was not a very active inhibitor *in vivo* with either precursor. α-Methyltyrosine did not inhibit conversion of dopa-^{3}H to catecholamine, proving that its site of action *in vivo* was at the tyrosine hydroxylase step. This was already reported in an earlier communication (5). It is difficult to compare α-MDH with the other drugs because its interference with catecholamine assays and influence on amino acid uptake by tissues are different. However, it is clear that although it is a potent inhibitor of decarboxylation *in vivo* (18), it does not significantly inhibit the conversion of tyrosine to norepinephrine.

The subsequent studies showed that following α-methyltyrosine administration the marked inhibition of tyrosine hydroxylase *in vivo* (as shown by conversion of tyrosine-^{14}C to norepinephrine-^{14}C and dopamine-^{14}C) was accompanied by a parallel drop in tissue catecholamine levels. The observed inhibitions of norepinephrine synthesis and the calculated inhibitions of tyrosine hydroxylase (Figs. 4 and 5) were also in excellent agreement, showing that α-methyltyrosine functioned in the same manner *in vivo* as *in vitro*. It should be emphasized that when labeled tyrosine is administered the overall conversion to norepinephrine requires three steps. However, the measured inhibition of overall norepinephrine synthesis from tyrosine was exactly the same as the inhibition calculated for tyrosine hydroxylase itself. This can be true only if tyrosine hydroxylase is the rate-limiting step.

The small amount of α-methylnorepinephrine produced through hydroxylation of α-methyltyrosine to α-methyldopa is of interest but has no bearing on the observed depletion of catecholamines in these short-term studies. It may prove to be of significance in long-term experiments when α-methyltyrosine is introduced for human use.

It should be noted (Table 1) that only α-methyltyrosine and α-methylphenylalanine lowered tissue catecholamine levels significantly during the relatively short period of 2 hr. However, these effects were seen mainly in brain. These observations corroborate previous findings (5) and show that once tyrosine hydroxylase is inhibited tissue norepinephrine levels fall at a rate

[3] The K_m of tyrosine for tyrosine hydroxylase has been found to vary from 1.0 to 6.9 $\times 10^{-5}$ M depending on the concentration of added tetrahydropteridine cofactor (Ikeda, Fahien, and Udenfriend, to be published).

determined by the normal turnover of the catecholamine in a given tissue. Since brain norepinephrine and dopamine have been shown to turn over much more rapidly than the catecholamines of the other tissues examined (7), it is not surprising that the effects of tyrosine hydroxylase inhibition are seen most rapidly in the brain. The earliest and most prominent effects of α-methyltyrosine are on the central nervous system (5).

It should be pointed out that the conversion of administered tyrosine-^{14}C to norepinephrine can be taken as a measure of the endogenous rate of norepinephrine biosynthesis. This assumption is valid not only because tyrosine hydroxylase is the rate-limiting step, but also because the trace amounts of radioactive tyrosine which are administered do not significantly increase the pool size of tyrosine. Administration of labeled dopa, on the other hand, would increase the pool size of dopa and thereby increase the rate of norepinephrine synthesis. Administered labeled dopamine would also be expected to increase the pool of endogenous dopamine. For this reason labeled dopa and dopamine cannot be used to measure normal rates of norepinephrine biosynthesis *in vivo*.

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