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Reduction of Aromatic L-Amino Acid Decarboxylase Protein in Rats after Chronic Administration of *Alpha*-Methyldopa

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SUMMARY

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The effect of chronic administration of α-methyldopa (400 mg/kg per day for seven days) to rats on the activity and amount of aromatic L-amino acid decarboxylase (EC 4.1.1.28) was studied. Aromatic L-amino acid decarboxylase activity measured in dialyzed supernatants was substantially reduced relative to control levels in all tissues analyzed. This effect was not due to the presence of substances inhibiting enzyme activity or to an alteration in the affinity of aromatic L-amino acid decarboxylase for its substrate, L-dopa. The amount of aromatic L-amino acid decarboxylase in dialyzed supernatants was estimated by immunotitration with a specific antibody raised to the enzyme purified from hog kidney cortex. Chronic administration of α-methyldopa caused a substantial decrease in the amount of aromatic L-amino acid decarboxylase protein in all tissues. The extent of the reduction in enzyme activity and enzyme protein was very similar within each tissue. The turnover of total soluble protein, measured by double isotope incorporation, was not altered in heart, brain or liver after α-methyldopa administration, although small reductions in adrenal and kidney soluble protein turnover were detected. Potential mechanisms for the reduction of aromatic L-amino acid decarboxylase levels are discussed.

INTRODUCTION

Aromatic L-amino acid decarboxylase (AADC[1]) catalyzes the decarboxylation of many naturally occurring and synthetic aromatic amino acids, including L-dopa and L-5-hydroxytryptophan (1), precursors of the catecholamine neurotransmitters and serotonin, respectively. The enzyme requires pyridoxal phosphate for activity, a small amount of the cofactor being tightly bound to the enzyme (2, 3). AADC is widely distributed in mammalian tissues (4) and appears to be located in monoaminergic neurons and non-neuronal cells within the same tissue (5, 6).

Chronic administration of L-dopa (7-10) and L-5-hydroxytryptophan (8) to rats, mice, and cats has been reported to decrease AADC activity in homogenates of liver. Immunochemical titration with a monospecific antiserum to AADC has shown a reduction in the amount of AADC protein in liver homogenates from animals chronically treated with L-dopa (7). The mechanism by which this reduction occurs is not known, although dopamine and ser-

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[1] Abbreviations used: AADC, aromatic L-amino acid decarboxylase; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; L-dopa, 3-hydroxy-L-tyrosine.

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otonin and/or their nondeaminated metabolites have been implicated as possible causative agents (8).

L-α-methyldopa, a structural analogue of L-dopa, is widely used in the treatment of hypertension. The drug is an inhibitor of AADC, a rapid, dose-dependent reduction of enzyme activity being measured both in the intact animal (11) and in homogenates of tissues taken from these animals (12). Although the mechanism of α-methyldopa inhibition of AADC *in vivo* is not known, *in vitro* studies indicate that the inhibition may be either competitive or noncompetitive with substrate, depending on the order of addition of inhibitor, amino acid and pyridoxal phosphate to the enzyme (13–15). The *in vitro* inhibition may be reversed by extensive dialysis (13) or by addition of excess pyridoxal phosphate (16).

It appears to have been generally accepted, although not stated explicitly, that the inhibition of AADC activity that is observed after chronic administration of α-methyldopa to animals and which would be anticipated in hypertensive patients receiving the drug, occurs by a gradually reversible inhibition of existing AADC protein. However, reports that chronic administration of L-dopa reduces the amount of AADC protein in liver, suggest that chronic α-methyldopa treatment reduces AADC activity by lowering the level of enzyme protein. The present study is an investigation of the effect of chronic administration of α-methyldopa on the enzymatic activity and the amount of AADC in several tissues of the rat. The technique of immunotitration with a specific antibody raised to AADC was employed to determine levels of AADC protein in rat tissues.

MATERIALS AND METHODS

Animals. Groups of male Sprague-Dawley rats (150–200 g) were injected subcutaneously with a fine suspension of α-methyldopa (400 mg/kg) in 0.9% sodium chloride, given every 24 hours for seven consecutive days. Control rats received saline. Animals were killed by stunning and decapitation five hours after the final injection.

Preparation of tissues for AADC assay. Adrenal glands, heart, brain, liver, and kidneys were removed rapidly after sacrifice and the hearts perfused through the aorta with 5 ml of ice-cold saline in order to remove blood. Tissues were processed immediately for the assay of AADC enzyme activity or frozen at −20° for several days before use, a procedure that was found to retain 90–95% of the activity of fresh tissues.

The following operations were carried out at 0–4°. All tissues except adrenal glands were homogenized in 5 volumes of 0.005 M sodium phosphate, pH 7.2, using a Polytron PT-10 (Kinematica). Adrenal pairs were homogenized in 600 μl of the same buffer, using 10 strokes of a Kontes Duall all-glass homogenizer (no. 20). The homogenates were centrifuged at 27,000 × *g* for 30 min and the supernatants retained. Preliminary experiments showed that AADC activity in all tissues was completely solubilized by the disruption techniques described, since less than 5% of the total enzyme activity in homogenates was detected in washed pellets obtained after centrifugation at 27,000 × *g*. Portions of the supernatants were dialyzed for 16 hours against homogenization buffer (100 volumes, three buffer changes).

Assay of AADC activity. AADC activity was measured in dialyzed supernatants by the method of Lamprecht and Coyle (17), using a substrate concentration of 300 μM L-[1-^{14}C]dopa in a total volume of 500 μl. Blanks contained either tissue in the presence of 100 μM 4-bromo-3-hydroxybenzyloxyamine, an inhibitor of AADC (4), or homogenization medium; both blanks yielded similar values. Assay conditions were chosen so that product formation was linear with respect to protein concentration and incubation time. The volumes of dialyzed supernatants used for the assay were adrenals, 50 μl; heart and brain, 100 μl; liver and kidneys, 10 μl. The results are expressed as nmole $^{14}CO_2$ formed/hour/organ.

Purification of AADC from hog kidney cortex. AADC was purified from hog kidney cortex by a modification of the method of Christenson *et al.* (2). All procedures were carried out at 0–4°. Ammonium sulphate precipitation, heat treatment in the pres-

ence of substrates, and adsorption with alumina Cγ gel were performed exactly as described previously (2). The fraction obtained after elution from alumina Cγ gel was dialyzed for 16 hr against two changes of two liters of 0.05 M sodium phosphate, 0.01 M 2-mercaptoethanol, pH 7.2. The dialyzed eluate was then loaded onto a column of DEAE-Sepharose (2.5 × 31.5 cm) previously equilibrated with the same buffer. After washing with 10 ml of buffer, the column was developed at a flow rate of 18 ml/hour with a linear gradient of 0 to 0.5 M sodium chloride in a total volume of 1000 ml of buffer. Fractions (9 ml) were collected and monitored for AADC activity and protein, which was assayed by the method of Lowry *et al.* (18). Fractions containing maximal enzyme activity were pooled and concentrated by ultrafiltration in an Amicon Diaflo cell, using an XM-50 membrane. The concentrate was dialyzed for 16 hr against 200 volumes of 0.002 M potassium phosphate, 0.01 M 2-mercaptoethanol, pH 7.2 and loaded onto a freshly packed column of hydroxyapatite (1.5 × 30 cm) previously equilibrated in the same buffer. After washing the column with 12 ml of buffer, AADC was eluted with a concave gradient formed by a two-chamber device, extending from 0.002 to 0.3 M potassium phosphate, pH 7.2, containing 0.01 M 2-mercaptoethanol, in a total volume of 500 ml. Fractions containing maximal enzyme activity were stored at 4°, since a previous experiment had shown that freezing and thawing of purified AADC resulted in precipitation and denaturation of protein, with substantial loss of enzyme activity.

Polyacrylamide gel electrophoresis. Portions of the most active fractions obtained from hydroxyapatite chromatography, equivalent to 100 μg protein, were diluted to 200 μl with 0.005 M sodium phosphate, pH 7.2 and dialyzed against the same buffer to remove 2-mercaptoethanol. The dialyzed samples were subjected to polyacrylamide gel electrophoresis in Tris-glycine, pH 8.9 (19). Protein bands were visualized by staining with Coomassie Brilliant Blue G-250 in 2.5% perchloric acid (20).

When polyacrylamide gel electrophoresis was used to prepare an antigen for production of specific antibodies, aliquots of the dialyzed hydroxyapatite eluate (125 μl containing 125 μg protein) were electrophoresed as before and the major protein bands containing AADC visualized by fluorescence, after immersion of gels in 0.003% magnesium anilinonaphthalene sulphonate in 0.05 M sodium phosphate, pH 6.4 (21). These bands were excised from the gels, minced finely and homogenized by hand in 2 ml of 0.9% sodium chloride, using a Kontes Duall all-glass homogenizer. The resulting slurry was allowed to stand at 4° for 16 hours in order to elute protein from the gel fragments. Approximately 20% of the total protein obtained by electrophoresis was eluted into saline. Portions of the saline eluate were subjected to polyacrylamide gel electrophoresis as described above.

Immunization of rabbits. New Zealand male rabbits were used for the preparation of antisera. A volume of saline slurry obtained from the electrophoresis of 2 mg of the hydroxyapatite fraction was emulsified with an equal volume of complete Freund's adjuvant. Half of this mixture was injected into each of two rabbits, using one intramuscular injection in each limb and one subcutaneous injection in the neck region. Immunization was repeated at 4 and 10 weeks after the initial injection. The rabbits were exsanguinated by cardiac puncture seven days after the final injection and serum collected and stored at −20° in aliquots.

Immunodiffusion. Double immunodiffusion was performed by standard methods (22).

Immunotitration of AADC. The experimental design of the immunotitrations was based on the method of Christenson *et al.* (23). In this method, fixed amounts of tissue extract from control and drug-treated animals are incubated with increasing amounts of antibody. As more antibody is added, decreasing amounts of enzyme remain in the supernatant after removal of immunoprecipitates by centrifugation. Immunotitration curves are constructed by plotting supernatant enzyme activity on the ordinate against volume of antiserum on the abscissa. Extrapolation of the linear

portion of the curve to the abscissa gives an estimate of the volume of antiserum required to reach the point of complete removal of enzyme activity from solution. This has been labeled the "equivalence point" of the immunotitration (24).

Dialyzed supernatants were prepared from the tissues of six to eight control or drug-treated animals as described for the assay of AADC. Equal volumes of the supernatants within each treatment group were pooled and assayed for AADC activity. Each pooled supernatant from the control group was diluted with 0.005 M sodium phosphate, pH 7.2, containing an appropriate concentration of bovine serum albumin, in order to obtain a solution of equivalent enzyme activity and total protein per unit volume with respect to the pooled supernatant from the drug-treated group.

For the immunotitration of AADC, a fresh aliquot of antiserum to the hog kidney enzyme was thawed immediately before each incubation and diluted with four volumes of ice-cold phosphate-buffered saline (0.15 M sodium chloride, 0.01 M sodium phosphate, pH 7.4; PBS). Portions of pooled tissue supernatants (adrenals, heart, brain: 100 μl; kidneys: 20 μl) were then transferred to 5 × 50 mm polycarbonate tubes and increasing volumes of diluted antiserum added. Control serum diluted with PBS was added to give a constant serum volume, since maintenance of equivalent protein concentration in all tubes was critical for obtaining reproducible results. 2-Mercaptoethanol (final concentration 0.01 M) was also included to stabilize AADC activity and PBS was added to the incubates to give a final volume of 760 μl. Tubes were incubated for 30 min at 25° and 16 hr at 4°. Immunoprecipitates were removed by centrifugation at 27,000 × *g* for 30 min at 4°. AADC activity remaining in the supernatants was measured in duplicate 300 μl aliquots. The slopes and equivalence points of the immunotitration curves were determined by linear regression analysis.

Turnover of trichloroacetic acid-precipitable soluble protein. Groups of five rats were injected with α-methyldopa or saline for seven days as described above. Twenty-four hours before sacrifice they received intraperitoneal injections of 10 μCi L-[^{14}C] leucine, followed by 100 μCi of L-[^{3}H]leucine given intraperitoneally four hours before sacrifice and one hour after the last of the chronic drug injections. Incorporation of radioactivity into trichloroacetic acid-precipitable protein of supernatants obtained after centrifugation of tissue homogenates at 27,000 × *g* for 30 min was estimated by the method of Ciaranello and Axelrod (25).

Statistics. Student's *t*-test was used to determine the significance of the difference between means. The measure of variation in this study is the standard error of the mean.

The standard errors of the slopes and equivalence points of the immunotitration curves were calculated by standard statistical methods (26).

Materials. L-[1-^{14}C]dopa, spec. act. 23.5 mCi/mmol, L-[4,5-^{3}H]leucine, spec. act. 55 Ci/mmol and L-(U-^{14}C)leucine, spec. act. 348 mCi/mmol, were purchased from the Radiochemical Centre (Amersham, England). L-Dopa and pyridoxal 5′-phosphate were from Sigma Chemical Co. (St. Louis, Mo.) and Coomassie Brilliant Blue G-250 and magnesium anilino-naphthalene sulphonate from Serva (Heidelberg, Germany). DEAE-Sepharose was purchased from Pharmacia South Seas (North Ryde, Australia) and α-methyldopa was obtained from Merck, Sharp, and Dohme (Sydney, Australia). The hydroxyapatite gel was prepared by the method of Bernardi (27). All other reagents were of analytical grade.

RESULTS

Purity of AADC used as antigen. The fraction of highest specific activity obtained from hydroxyapatite chromatography catalyzed the formation of 12.32 μmole $^{14}CO_2$ per minute per mg protein when L-dopa was used as a substrate, representing a 246-fold purification over the initial crude supernatant, with a recovery of 22% (Table 1).

Polyacrylamide gel electrophoresis of this hydroxyapatite fraction revealed a major protein band containing AADC, with a

TABLE 1

Summary of the purification of AADC from hog kidney cortex

These values represent the results of a single experiment based on approximately 140 g wet weight of frozen hog kidney cortex. The enzyme and protein assays were carried out as described in METHODS.

	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	ml	*(mg)*	*μmole $^{14}CO_2$/min*	*μmole $^{14}CO_2$/min/mg protein*	*fold*	*%*
Crude supernatant	365	5658	308.59	0.05	—	—
32–49% ammonium sulphate fraction	50	1750	282.00	0.16	3.2	91
Heat supernatant	108	1685	282.86	0.17	3.4	92
Alumina Cγ gel eluate	39	243	135.21	0.56	11.2	44
Chromatography on DEAE-Sepharose (concentrate)	11.5	36	127.05	3.53	70.6	41
Chromatography on hydroxyapatite						
fraction 15	5.5	2.9	25.22	8.70	175.0	8
16	5.5	5.5	67.75	12.32	246.4	22
17	5.5	4.8	26.21	5.46	109.2	8

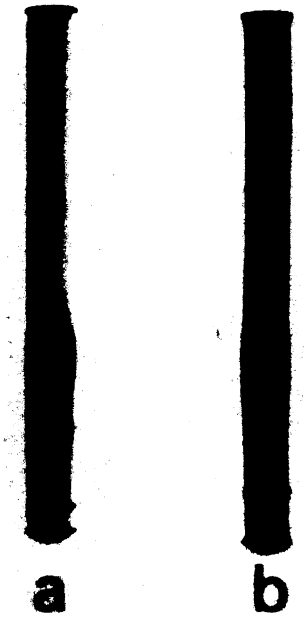

FIG. 1. *Polyacrylamide gel electrophoresis of AADC*

The direction of migration is from top to bottom. (a) Hog kidney AADC in hydroxyapatite fraction number 16 (100 μg protein) was electrophoresed in a 5% polyacrylamide gel column (7.5 cm) in Tris-glycine buffer, pH 8.9 (19). Gels were stained with Coomassie Brilliant Blue G-250 in 2.5% perchloric acid. The thin band near the bottom of each gel is the dye marker, bromphenol blue. (b) Hog kidney AADC in hydroxyapatite fraction number 16 (125 μg protein) was electrophoresed as described in (a). The major protein band was visualized, excised and a saline eluate subjected to a second electrophoresis as described in METHODS.

faint band which moved more rapidly than AADC and was located at the bottom of the gel (Fig. 1a).

Therefore further purification of the hydroxyapatite eluate was necessary before it could be used as an antigen for the production of specific antibodies to AADC. Separation of AADC from protein contaminants was achieved by polyacrylamide gel electrophoresis in the same buffer system as used previously. After elution of AADC from the gel segment and electrophoresis of the eluate, only a single major band of protein was observed (Fig. 1b), thus demonstrating the validity of the method for preparing a purified protein antigen.

Specificity of the antiserum directed against AADC. Ouchterlony double immunodiffusion was used to demonstrate the specificity of the antiserum raised against hog kidney AADC. Single precipitin lines were observed when the antiserum was diffused against purified AADC (Fig. 2a) or crude fractions obtained during the course of purification (Fig. 2b). A single, more diffuse line was also observed after diffusion of the antiserum against an ammonium sulphate fraction (32–49%) prepared from rat

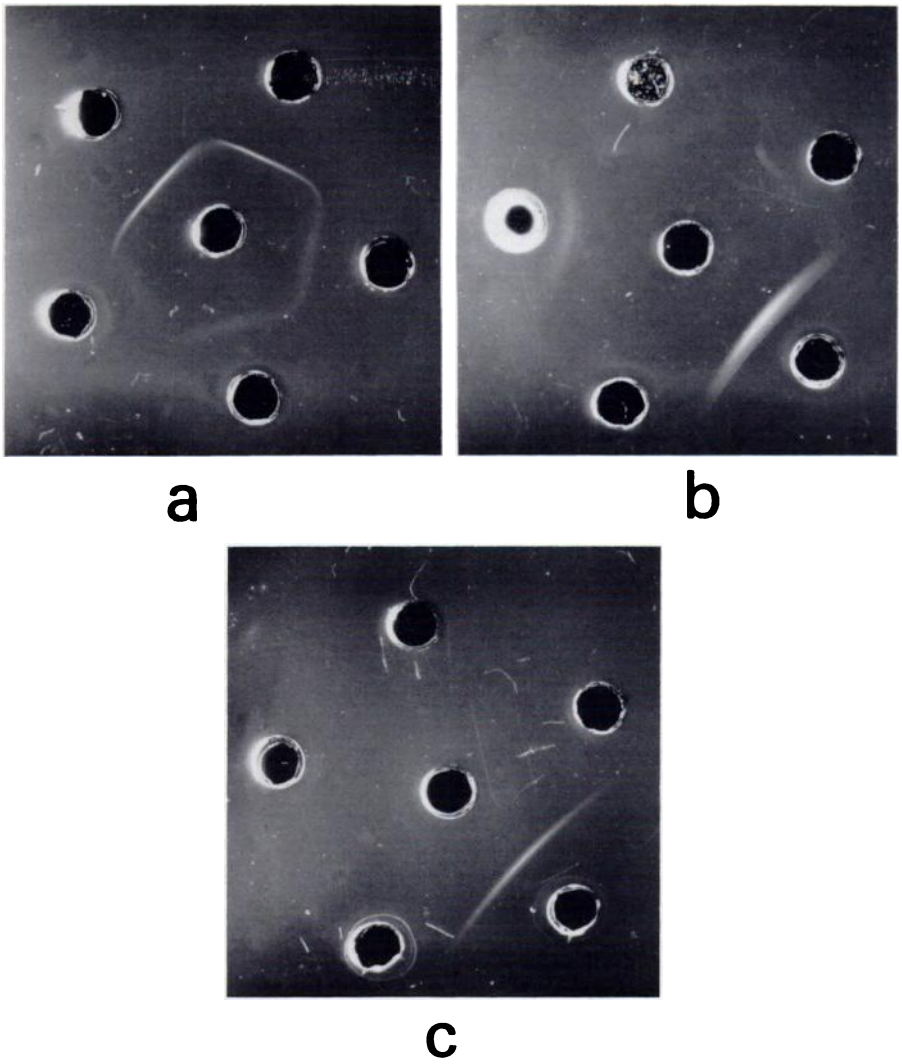

FIG. 2. *Ouchterlony double immunodiffusion*

Plates were developed for approximately 48 hours at 25°. (a) The center well contained 10 μl of rabbit antiserum to hog kidney AADC. The outer wells contained: 11 o'clock, 10 μg of purified hog kidney enzyme (hydroxyapatite fraction 16); from 11 o'clock in a clockwise direction, the remaining outer wells contained serial two-fold dilutions in saline of the same preparation. (b) The center well contained 10 μl of rabbit antiserum to hog kidney AADC. The outer wells contained: 10 o'clock, 10 μl of dialyzed 32–49% ammonium sulphate pellet; 11 o'clock, 10 μl of crude supernatant; 2 o'clock, 10 μl of alumina C_γ gel eluate; 5 o'clock, 10 μl of dialyzed, concentrated DEAE-Sepharose eluate; 7 o'clock, 15 μg of purified bovine adrenal medullary dopamine β-hydroxylase. (c) The center well contained 10 μg of purified hog kidney AADC (hydroxyapatite fraction 16). The outer wells contained: 5 o'clock, 10 μl of rabbit antiserum to hog kidney AADC; 7 o'clock, 10 μl of rabbit 1 antiserum to bovine adrenal medullary dopamine β-hydroxylase; 9 o'clock, 10 μl of rabbit 2 antiserum to dopamine β-hydroxylase; 11 o'clock, 10 μl of nonimmune rabbit serum.

kidney extract (not shown). Immunotitration of the antiserum to hog kidney AADC against supernatants from rat adrenals, heart, brain, liver, and kidneys showed that the antibody was able to precipitate over 90% of the original AADC activity.

No immunoprecipitin lines were observed when nonimmune rabbit serum or antiserum to dopamine β-hydroxylase was diffused against purified hog kidney AADC

(Fig. 2c). The antiserum to AADC did not inhibit the activities of the other enzymes of noradrenaline biosynthesis, tyrosine hydroxylase, or dopamine β-hydroxylase, obtained from rat adrenal glands and heart. There were no precipitin lines observed after diffusion of the antiserum to AADC against purified bovine adrenal medullary dopamine β-hydroxylase (Fig. 2b).

Effect of chronic administration of α-methyldopa on AADC activity. Subcutaneous administration of 400 mg/kg α-methyldopa to rats for seven consecutive days substantially reduced AADC activity in dialyzed supernatants of all tissues studied, measured five hours after the final injection (Table 2). The effect was most marked in adrenal and heart supernatants, where AADC activity decreased to about 20% of control values. Brain, liver, and kidney AADC activities were depressed to 40, 63, and 30%, respectively, of their control values by the same α-methyldopa treatment regime.

Since α-methyldopa is an inhibitor of AADC *in vitro* (1, 13), the reduction in AADC activity after chronic administration of α-methyldopa could have been due to inhibition of the enzyme by free α-methyldopa remaining in the tissues at the time of sacrifice. However this was unlikely since the dialysis procedure to which supernatants were subjected before assay was more than sufficient to completely reverse inhibition of AADC by free α-methyldopa. This was demonstrated in preliminary experiments by the addition of 1 mM α-methyldopa to control supernatants and subsequent dialysis for different time intervals before assay of AADC activity. Dialysis for as little as 6 hours against 100 volumes of buffer completely reversed the 95% inhibition of enzyme activity. The dialysis procedure used in all experiments was three buffer changes of 100 volumes during 16 hours.

TABLE 2

Effect of chronic administration of α-methyldopa on AADC activity

Rats were injected subcutaneously once daily for 7 consecutive days with saline or 400 mg/kg α-methyldopa and sacrificed 5 hours after the final injection. AADC activity of dialyzed supernatants of tissues was determined as described in Methods. Each value shown is the mean ± standard error of 6 or 7 animals.

Tissue	AADC activity		% of control
	Control	α-Methyldopa	
	μmole CO_2 formed/hour/organ		
Adrenals	0.283 ± 0.019	0.055 ± 0.021**	20
Heart	0.551 ± 0.026	0.115 ± 0.008**	21
Brain	2.34 ± 0.10	0.94 ± 0.06*	40
Liver	197.1 ± 27.7	124.7 ± 13.0*	63
Kidneys	23.82 ± 0.93	7.12 ± 0.49**	30

* $p < 0.01$.
** $p < 0.001$.

The results of mixing experiments indicated that the decreased AADC activity in the dialyzed supernatants of animals pretreated with α-methyldopa was not due to the presence of inhibitory substances. When equal volumes of supernatants from control and drug-treated rats were mixed and assayed for AADC activity, enzyme activities were additive. Further evidence that an inhibition of AADC by free α-methyldopa or other inhibitory substances was unlikely was obtained by measuring the activity of purified hog kidney AADC added to tissue supernatants. The recovery of purified enzyme activity from the supernatants of tissues taken from both control and α-methyldopa-pretreated rats was 95–100%.

The reduction in AADC activity after chronic administration of α-methyldopa was not due to a decreased affinity of the enzyme for its substrate, L-dopa, since the K_m for L-dopa was not altered by the drug treatment (Table 3). However the maximal velocity, V_{max}, of the reaction was significantly reduced relative to controls (Table 3), suggesting that the amount of AADC present had been lowered. A similar percentage reduction in AADC activity after α-methyldopa administration was observed when dialyzed supernatants were assayed in the presence of a wide range of pyridoxal phosphate concentrations (0.1 μM to 1 mM).

Immunotitration of AADC. In order to determine whether the reduction of AADC activity after chronic administration of α-methyldopa was due to an alteration in the amount of AADC protein or an inhibition of existing enzyme molecules, immunotitration studies using the antiserum to hog

kidney AADC were performed with dialyzed supernatants from both control and drug-treated rats.

TABLE 3

Effect of chronic administration of α-methyldopa on the kinetic parameters of kidney AADC

Dialyzed supernatants of kidneys were prepared from rats injected for 7 days with saline (controls) or 400 mg/kg α-methyldopa as described in METHODS. Aliquots (50 μl) of the supernatants were assayed for AADC activity in the presence of approximately 70 to 340 μM L-dopa and 10 μM pyridoxal phosphate. Kinetic parameters were determined using linear regression analysis of a double reciprocal plot. The method of Armitage (26) was used to calculate the standard error of these parameters; the number of degrees of freedom for each value was 9.

	Control	α-Methyldopa
K_m (L-dopa; μM)	118 ± 13	147 ± 10
V_{max} (nmole CO_2 formed/hour/ 50 μl dialyzed supernatant)	133.3 ± 5.8	61.5 ± 4.2*

* $p < 0.005$.

A typical immunotitration curve for rat adrenal AADC is shown in Figure 3. Chronic administration of α-methyldopa (400 mg/kg) to rats for seven days resulted in approximately a three-fold reduction in the equivalence point of adrenal AADC, since 20.3 μl of AADC antibody was required to remove enzyme activity from control supernatants, whereas only 6.5 μl of the same antibody completely precipitated AADC activity from the supernatants of α-methyldopa-treated rats.

This indicated that the amount of AADC protein present in adrenal glands was decreased approximately three-fold by α-methyldopa administration. The ratio of equivalence points (α-methyldopa:control, 0.32) was almost identical to the ratio of AADC activities in the adrenal supernatants used for immunotitration (α-methyldopa:control, 0.33; see Table 4).

Immunotitration of AADC from heart, brain, and kidneys also showed reductions of approximately four-, three-, and two-fold,

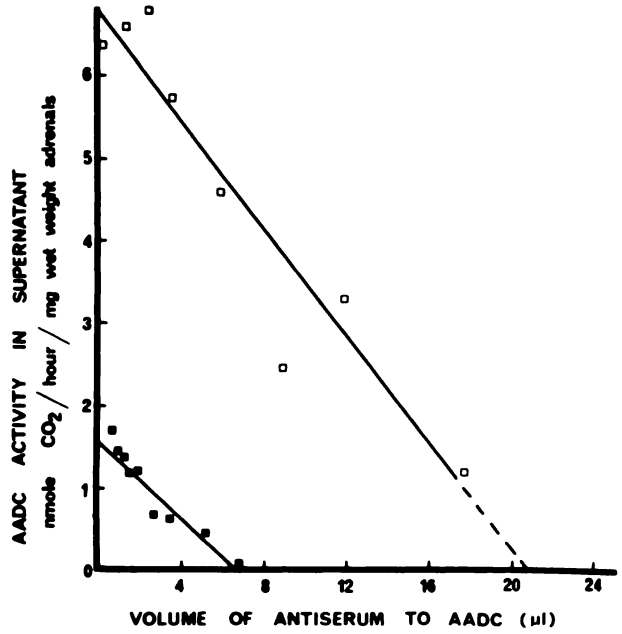

FIG. 3. *Immunotitration of adrenal AADC*

Rats were injected subcutaneously once daily for 7 consecutive days with saline or α-methyldopa, 400 mg/kg and sacrificed 5 hours after the final injection. Dialyzed adrenal supernatants from 5 rats per group were pooled for each treatment group and the control supernatants were diluted to contain the same enzyme activity per unit volume as the pooled supernatant from the treated group. Immunotitration of 100 μl of these supernatants was carried out as described in METHODS. Linear regression analysis was used to obtain the lines of best fit and equivalence points are indicated by the intersection of the extrapolated lines with the abscissa. Each point is the mean of duplicate incubations. □ Control; ■ α-Methyldopa.

respectively, in the equivalence point of the enzyme after α-methyldopa administration. For all tissues studied, the ratio of equivalence points (α-methyldopa:control) was very similar to the ratio of AADC activities in the initial supernatants (Table 4). This indicates that the reduction of AADC activity measured in tissue extracts after chronic administration of α-methyldopa is entirely attributable to a decrease in the amount of enzyme protein and not to an inhibition of existing enzyme molecules.

TABLE 4

Effect of chronic administration of α-methyldopa on AADC activity and enzyme protein

Rats were injected subcutaneously once daily for 7 consecutive days with saline or α-methyldopa, 400 mg/kg and sacrificed 5 hours after the final injection. AADC activity and equivalence point (a measure of AADC protein) were determined using the same dialyzed supernatants of tissues, as described in METHODS. Each equivalence point ratio shown is computed from individual values of the equivalence point (see the legend of Figure 3 for further details) and each enzyme activity ratio is estimated from the mean of 6 or 7 animals.

Tissue	Enzyme activity ratio (α-methyldopa:control)	Equivalence point ratio (α-methyldopa:control)
Adrenals	0.33	0.32
Heart	0.27	0.25
Brain	0.36	0.35
Kidneys	0.34	0.40

Turnover of TCA-precipitable soluble protein. The dual label incorporation technique of Arias *et al.* (28), as described by Ciaranello and Axelrod (25), was used to measure turnover of total soluble protein. The short-term incorporation of L-[^{3}H]leucine provides an estimate of the relative rate of protein synthesis, while the amount of L-[^{14}C]leucine incorporated into protein in the longer time interval before sacrifice gives an estimate of the relative rate of protein degradation. The ratio of tritium to carbon-14 incorporated into protein is therefore a measure of the relative rate of protein turnover.

Chronic administration of α-methyldopa (400 mg/kg) to rats for seven days did not

TABLE 5

Incorporation of radioactivity into TCA-precipitable soluble protein from control and α-methyldopa-treated rats

Rats were injected subcutaneously once daily for 7 consecutive days with saline or 400 mg/kg α-methyldopa. Intraperitoneal injections of 10 μCi L-(^{14}C-leucine) and 100 μCi L-(^{3}H)-leucine were given 24 and 4 hours, respectively, before sacrifice. Incorporation of radioactivity into TCA-precipitable soluble protein was measured as described in Methods. Each value shown is the mean ± standard error of 4 or 5 animals.

	Radioactivity		^{3}H:^{14}C
	^{3}H	^{14}C	
	dpm × 10^4/organ		
Adrenals			
Control	2.67 ± 0.14	0.26 ± 0.01	10.27 ± 0.32
α-methyldopa	3.10 ± 0.62	0.34 ± 0.06	9.12 ± 0.40
Heart			
Control	18.54 ± 0.77	1.91 ± 0.12	9.71 ± 0.22
α-methyldopa	11.75 ± 0.14**	1.28 ± 0.14*	9.18 ± 0.11
Brain			
Control	26.81 ± 1.02	3.10 ± 0.09	8.65 ± 0.23
α-methyldopa	24.64 ± 3.72	2.87 ± 0.33	8.59 ± 0.32
Liver			
Control	763.9 ± 66.2	72.95 ± 4.29	10.47 ± 0.42
α-methyldopa	694.5 ± 67.2	65.70 ± 5.69	10.57 ± 0.53
Kidneys			
Control	131.61 ± 5.59	11.61 ± 0.33	11.34 ± 0.40
α-methyldopa	99.12 ± 6.43***	10.45 ± 0.39	9.49 ± 0.32**

* $p < 0.05$.
** $p < 0.02$.
*** $p < 0.005$.

alter the turnover of soluble protein in heart, brain, or liver (Table 5). However, this drug administration schedule produced small, but statistically significant reductions of 16 and 19%, respectively, in the turnover of adrenal and kidney soluble protein (Table 5). The relative rates of synthesis and degradation of soluble protein in heart were also significantly decreased after chronic administration of α-methyldopa, but with no overall effect on the relative turnover (Table 5). A small reduction in the relative rate of synthesis of kidney soluble protein was also observed in animals which had received α-methyldopa.

DISCUSSION

Although the ability of α-methyldopa to inhibit AADC activity has been documented for many years, the mechanism by which *in vivo* inhibition of the enzyme occurs after chronic administration of the drug to animals is not known. It appears that the slowly reversible inhibition of AADC by α-methyldopa *in vitro* (13) has also been assumed to account for the *in vivo* inhibition of AADC activity after acute or chronic administration of the drug. However, the results of the present study indicate that chronic administration of α-methyldopa to rats reduces tissue AADC activity by decreasing the amount of AADC protein, measured by immunotitration with a specific antibody to the enzyme. Since the reductions in enzyme activity and enzyme protein following α-methyldopa treatment are very similar within each tissue, the alterations in AADC levels in the dialyzed tissue extracts appear to account entirely for the decreased enzymatic activities relative to controls. The reduction in AADC levels after chronic administration of α-methyldopa has been demonstrated in all tissues studied so far, including adrenals, heart, brain, and kidneys, although the extent of the reduction varies among tissues. Therefore it may be a general phenomenon expressed in many other tissues that also contain the enzyme. The present study does not differentiate between AADC apparently located in both neuronal and nonneuronal cells within some tissues (5, 6). Hence it is possible that AADC protein in many different cellular types may be similarly affected by α-methyldopa administration, although further experiments are required to test this hypothesis.

It is not known whether α-methyldopa itself or a metabolite is responsible for lowering AADC levels, or whether the causative agent acts directly within the cell or by some extracellular stimulus to lower the amount of enzyme protein. In this context it may be useful to compare the effects of chronic administration of α-methyldopa and the structurally analogous compound, L-dopa, on AADC levels. In contrast to the α-methyldopa-induced depression of AADC in all tissues studied so far, chronic administration of large doses of L-dopa (100 to 1,000 mg/kg per day) for several days to rats substantially reduces the amount of AADC protein in liver only, with very little or no effect on the enzyme in adrenals, brain, heart, and kidneys (7).[2] In mice, a still larger dose of L-dopa (1,500 mg/kg per day) given for several days substantially reduces the activity of AADC in extracts of liver and kidneys, with no significant effect on the brain enzyme (9). However, the ability of L-dopa to lower AADC levels appears to be confined mainly to the liver. Moreover, larger doses of L-dopa relative to those of α-methyldopa are required to produce a similar reduction in liver AADC levels.

The comparison between the effectiveness of L-dopa and α-methyldopa in lowering AADC levels is particularly interesting in view of the suggestion that increased levels of dopamine and/or a nondeaminated metabolite of dopamine are responsible for the effect of L-dopa on liver AADC (8). This proposal is based on the findings that decarboxylation of the administered amino acid is necessary for the effect to occur and also that a low dose of L-dopa that is ineffective by itself, in combination with a monoamine oxidase inhibitor to prevent oxidative deamination, produces a reduction in the amount of liver AADC protein similar to that seen after large doses of L-dopa (8). Since the decarboxylation product of α-methyldopa, α-methyldopamine, is

[2] A. J. Culvenor and B. Jarrott, manuscript in preparation.

not catabolized by monoamine oxidase (4), tissue levels of α-methyldopamine and its nondeaminated metabolites following α-methyldopa administration to animals may be higher than those of dopamine and its corresponding metabolites after giving L-dopa. Moreover, the tissue distribution of the decarboxylation products of L-dopa and α-methyldopa may be quite different, since a large proportion of L-dopa is metabolized in the liver, whereas α-methyldopa is less rapidly and less extensively decarboxylated by liver AADC (15). Therefore a greater fraction of administered α-methyldopa, relative to L-dopa, may be available to extrahepatic tissues. If such a relative tissue distribution of the amino acids does occur, and if L-dopa and α-methyldopa lower AADC levels by a similar mechanism, it may explain the greater and more widespread effect on AADC of α-methyldopa, relative to L-dopa. However comparative studies on the metabolism of the two amino acids in a variety of tissues will be required in order to evaluate this suggestion.

A decrease in AADC levels after chronic administration of α-methyldopa may reflect either a specific effect on the enzyme or a nonspecific change in total protein turnover. The finding that the drug treatment did not affect soluble protein turnover in heart, brain, or liver, with only small reductions in adrenal and kidney protein turnover, indicates that administration of α-methyldopa does not produce extensive changes in protein turnover within tissues. Therefore the reduction in AADC levels may be specific for that protein.

A decline in the amount of AADC protein may be due to either a decreased rate of synthesis or an increased rate of degradation. For example, binding of AADC by α-methyldopa may enhance the rate of degradation of the protein. In view of the strong affinity of AADC for α-methyldopa (15) and the low dissociation rate of the drug from the enzyme *in vitro* (13), a fairly stable enzyme-substrate or enzyme-product complex could be formed after chronic administration of α-methyldopa. If the continued binding of α-methyldopa to AADC induced a conformational change in the protein, this might render it more susceptible to intracellular degradative processes, since proteins with abnormal configurations tend to be more rapidly degraded (29). Alternatively, the enzyme-bound cofactor may be important in maintaining the structural integrity of AADC. Pyridoxal phosphate stabilizes other pyridoxal phosphate-dependent enzymes against denaturing conditions *in vitro* (30) and has been suggested to be involved in preserving the correct conformation of this class of enzymes (31). It is possible that the proposed binding of enzyme-bound cofactor by α-methyldopa may interfere with normal stabilization of AADC by the cofactor and hence lead to increased degradation of the enzyme. It is pertinent to note that an adequate supply of pyridoxal phosphate is essential for maintaining levels of AADC in some tissues, since pyridoxine-deficient rats have reduced amounts of AADC enzyme in liver and kidney (32).

α-Methyldopa has a greater tendency than L-dopa to favor the decarboxylation-dependent transamination reaction of AADC (33). In this reaction, loss of carbon dioxide from the enzyme-bound Schiff base may be followed by the protonation of a benzylic carbon atom of the amino acid substrate, rather than of the usual α-carbon atom. This results in the formation of an aldehyde or ketone and enzyme-bound pyridoxamine phosphate, which dissociates from AADC and releases the apoenzyme. The latter species is rapidly inactivated (33) and is probably more susceptible to degradation. However, although the probability of the transamination occurring in the presence of α-methyldopa is 100 times greater relative to L-dopa, this alternative reaction only occurs in about 2% of enzymatic decarboxylations of α-methyldopa (33) and is therefore unlikely to be an important means of lowering AADC levels.

Alternatively, chronic administration of α-methyldopa may lower AADC levels by reducing the rate of synthesis of the protein. One potential method of reducing the rate of transcription of messenger RNA for AADC is by repression of the operon responsible for its synthesis. In some systems, a protein coded for by a structural gene within an operon acts as a repressor protein

for that operon (34). It is possible that, if AADC acts as a repressor for the transcription of its own messenger RNA, binding of α-methyldopa to the enzyme may enhance the repression of the relevant operator gene.

In summary, it has been demonstrated conclusively that chronic administration of α-methyldopa to rats reduces the amount of AADC protein in a variety of tissues. The implications of this finding for hypertensive patients receiving chronic therapy with α-methyldopa need to be considered. Although doses of the drug administered to rats in the present study were higher on a weight per body weight basis than those used clinically, caution is required in extrapolating from animals to man. It may be possible to test the effects of α-methyldopa therapy on liver AADC by comparing the activities and levels of the enzyme in biopsy specimens taken from patients receiving α-methyldopa with those obtained from patients not receiving the medication.

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