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Electrophoretic Properties of Dopamine β-Hydroxylase in Several Tissues from Three Species

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SUMMARY

Dopamine β-hydroxylase (EC 1.14.2.1) from tissue homogenates of three species was subjected to electrophoresis on polyacrylamide gel and starch blocks. In both systems dopamine β-hydroxylase in crude homogenates of solid tissues exhibited two electrophoretically distinguishable peaks of activity. The smaller, more slowly migrating peak was eliminated by high-speed centrifugation prior to electrophoresis, suggesting that this peak represented a form of the enzyme bound to the membrane or to a subcellular constituent. This interpretation is supported by partial conversion of dopamine β-hydroxylase activity from the slowly moving peak to the fast-migrating peak by Triton treatment. Also in harmony with this view are the observations that a nonparticulate tissue such as serum contained only the faster-migrating peak and that prior treatment with reserpine produced elevations of both peaks in solid tissues. The main peak of dopamine β-hydroxylase activity from human, cow, and rat adrenals differed in electrophoretic mobility, but within each species the major peak of activity from all tissues, including serum, had the same mobility. Human serum dopamine β-hydroxylase activity migrated with the mobility of a β-globulin. The major peak of enzyme activity from each species was broad and asymmetrical, and possibly included several distinct molecular forms of the enzyme.

INTRODUCTION

Dopamine β-hydroxylase (EC 1.14.2.1) catalyzes the final reaction in the biosynthetic pathway leading to norepinephrine (1). Recent development of a sensitive enzymatic assay for dopamine β-hydroxylase (2, 3) has facilitated determination of its activity in homogenates of sympathetically innervated tissues and study of the possible role of changes in the activity of this enzyme in the regulation of catecholamine biosynthesis. For example, reserpine administration leads to a transsynaptically mediated increase in dopamine β-hydroxylase activity in the adrenal medulla, sympathetic ganglia, and sympathetically innervated tissues (3, 4) as the result of an increase in the quantity of enzyme protein (5).

Two of the enzymes involved in catecholamine biosynthesis and metabolism, phenylethanolamine *N*-methyltransferase and catechol *O*-methyltransferase, exhibit marked species differences in electrophoretic

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mobility; multiple molecular forms of catechol *O*-methyltransferase have been identified within a single species (6). The present study was undertaken to investigate certain properties of dopamine β-hydroxylase and to determine what role, if any, multiple forms of the enzyme might play in the regulation of its activity. In addition, the electrophoretic mobilities of dopamine β-hydroxylase from different tissues of the same species as well as from different species were compared.

MATERIALS AND METHODS

Animals and tissues. Sprague-Dawley rats (150–200 g) of either sex were used as one source of tissue. Frozen bovine adrenal glands were obtained from Pel-Freez, Inc., Rogers, Ark., and were stored at −20°. Human adrenal glands were obtained within 8 hr of death and were immediately frozen. They were obtained from a 19-year-old girl who died of Ewing's sarcoma with metastases, a 61-year-old man with chronic obstructive pulmonary disease, and a 54-year-old woman with rheumatic heart disease, who died at the time of operation. All experiments were repeated at least three times with different tissue preparations.

Chemicals. Tyramine HCl and octopamine HCl were purchased from Calbiochem. Catalase was obtained from Boehringer/Mannheim. The monoamine oxidase inhibitor pargyline was provided by Abbott Laboratories, and *S*-adenosyl-L-[*methyl*-^{14}C]-methionine (specific activity, 42–48 mCi/mmole) was purchased from New England Nuclear Corporation. All reagents for polyacrylamide gel electrophoresis were obtained from Canalco, Rockville, Md. Potato starch was purchased from Fisher Scientific Company. Triton X-100 was a product of Packard Instrument Company, and tris-(hydroxymethyl)aminomethane (trizma base) was purchased from Sigma Chemical Company. Reserpine (Serpasil) was obtained from Ciba Pharmaceutical Company.

Polyacrylamide gel electrophoresis. Polyacrylamide gels were made and electrophoresis performed as described by Davis (7). The separating gel (7.5%) was made with riboflavin (1 mg/100 ml) instead of persulfate. The pH of the gel was 8.9, and that of the buffer solution was 8.3.

Individual stellate ganglia were homogenized in 0.1% Triton X-100 containing 0.005 M Tris-HCl buffer, pH 7.4. Samples were prepared for electrophoresis by mixing 100 μl of homogenate with 50 μl of 37% sucrose and 5 μl of tracking dye (0.001% bromophenol blue). A 100-μl aliquot of this mixture, containing less than 100 μg of protein, was layered on the spacer gel. Electrophoresis was performed at 1 mamp/tube at 3° until the tracking dye had moved 45–50 mm into the gel. This usually required 2–2.5 hr. The gels were removed from the tubes and cut with horizontal and vertical gel slicers (Canalco). The vertical slicer produced a stack of segments, each 1.6 mm thick, each of which was divided into four sections to facilitate diffusion of substrates, cofactors, and products of the dopamine β-hydroxylase reaction into and out of the gel. Gels treated in this way gave the same results as obtained when the gel slices were homogenized. The four pieces from each segment were transferred into tubes containing 200 μl of cold 0.1 M sodium acetate buffer, pH 4.5. This acidic buffer was necessary to reduce the pH of the gel to about pH 6, which is optimal for the enzyme assay.

Starch block electrophoresis. Starch blocks were prepared in 0.05 M sodium barbital buffer, pH 8.6, as previously described (8). One experiment was also performed in a starch block prepared in phosphate buffer, pH 7.5, 0.05 M. Tissues were homogenized either in ice-cold water or in 0.1% Triton X-100, and the homogenates were centrifuged at 10,000 *g* for 10 min in a Sorvall refrigerated centrifuge. The supernatant fluid (1–2 ml) either was applied directly to the starch block or was centrifuged again at 100,000 × *g* for 1 hr in a refrigerated Spinco ultracentrifuge; 1–2 ml of the final supernatant fluid were then applied to the block. Electrophoresis was carried out at 4° for 16 hr at 360 V and 80 mamp. The starch block was then cut into 0.5-inch segments, and the enzyme was eluted from each segment with 1 ml of ice-cold 0.1% Triton X-100. Between 0.6 and 1.0 ml of 0.1 M sodium acetate buffer, pH 4.5, was added to the fluid eluted from each segment of a given starch block

to bring the pH to approximately 6. The volume of buffer added to the eluate from each segment of a given starch block was constant.

Protein assay. Protein concentrations were determined with the method of Lowry *et al.* (9), using bovine serum albumin as a standard.

Dopamine β-hydroxylase assay. Dopamine β-hydroxylase activity was determined using a sensitive two-step enzymatic assay (3), in which tyramine is converted to octopamine. The octopamine is then *N*-methylated by the enzyme phenylethanolamine *N*-methyltransferase, with *S*-adenosyl-L-[*methyl*-^{14}C]-methionine as a methyl donor. The labeled *N*-methyloctopamine (synephrine) formed is separated from the *S*-adenosylmethionine by solvent extraction, and its radioactivity is determined.

The reaction mixture consisted of 200 μl of enzyme and 1.2 μmoles of sodium fumarate (pH 6), 1.2 μmoles of ascorbate (pH 6), 200 μg of catalase, 0.16 μmole of pargyline, 0.3 μmole of tyramine (pH 6), 10 μmoles of Tris-HCl buffer (pH 6), and sufficient $CuSO_4$ to give optimal activity, in a final volume of 310 μl. The amount of $CuSO_4$ required varied with the tissue and the amount of protein applied to the electrophoresis block or gel.

The dopamine β-hydroxylase portion of the reaction was allowed to proceed for 20 min when polyacrylamide gel segments were being assayed, and for 30 min when the activity from starch block segments was being determined. The reaction was then stopped, and the phenylethanolamine *N*-methyltransferase reaction was initiated by changing the pH of the mixture to 8.6 by the addition, in a total volume of 100 μl, of 80 μmoles of Tris-HCl buffer (pH 8.6), 1 mμmole of [^{14}C]*S*-adenosylmethionine (0.05 μCi), and 40–50 μg of the transferase, which had been purified according to Axelrod (10) and then passed over a Sephadex G-200 column. The *N*-methylation step was stopped after 30 min by the addition of 0.5 ml of 0.5 M borate buffer, pH 10. The radioactive synephrine formed was extracted into 6 ml of toluene–isoamyl alcohol (3:2, v/v), and the sample was evaporated to dryness at 80° in a chromatography oven. The sample was then dissolved in 1 ml of absolute ethanol; 10 ml of a phosphor containing 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)] benzene per liter of toluene were added, and the radioactivity of the sample was determined in a Beckman scintillation counter at a counting efficiency for ^{14}C of 65%. A blank value was determined with each assay by carrying an identical enzyme sample heated to 95° for 5 min through the entire assay procedure. A standard consisting of 40 ng of octopamine HCl added to a complete assay mixture, including 200 μl of sample heated to 95°, was also determined. Results were expressed either as counts per minute per gel slice per 20-min incubation (polyacrylamide gel electrophoresis) or as nanomoles of octopamine per milliliter of buffer eluted from the block per 30-min incubation (for starch block electrophoresis) (1 nmole/ml/30 min represents 1 unit of dopamine β-hydroxylase activity).

Statistics and kinetics. Standard methods of statistical analysis were used in these studies (11). K_m values were determined by Wilkinson's method (12), using a FORTRAN program written by Cleland (13) and an IBM 1620 digital computer.

RESULTS

Homogenates of rat stellate ganglia yielded two bands of dopamine β-hydroxylase activity on polyacrylamide electrophoresis (Fig. 1). The stellate ganglia from rats treated with reserpine showed enhanced activity which had the same electrophoretic mobilities as that seen for untreated animals (Fig. 1).

Dopamine β-hydroxylase activity has been shown to exist in a bound and a soluble form in both the adrenal medulla and sympathetic nerves (14, 15). To examine the possibility that the two electrophoretically distinguishable peaks shown in Fig. 1 might correspond to these two forms, respectively, stellate ganglia were homogenized in distilled water. When the homogenate was centrifuged at 100,000 × *g* for 1 hr, 54 ± 6% of the dopamine β-hydroxylase activity remained in the supernatant fraction. Poly-

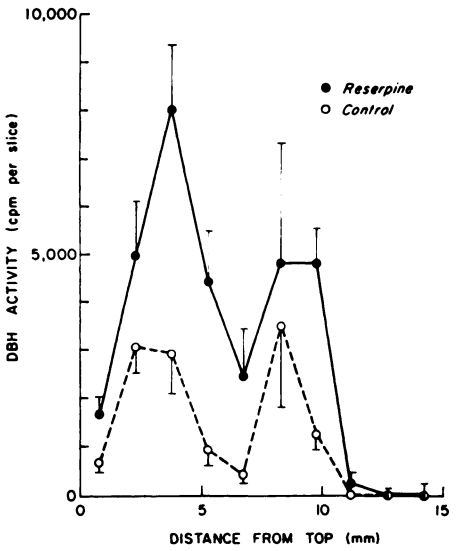

FIG. 1. *Polyacrylamide gel electrophoresis of homogenates of rat stellate ganglia before and after treatment with reserpine*

Rats that had been treated by injection with either 0.9% NaCl or reserpine (2.5 mg/kg daily for 3 days) were killed on the fourth day. Each pair of stellate ganglia was homogenized in 0.5 ml of 0.1% Triton X-100. No $CuSO_4$ was added to the reaction mixture for assay of stellate ganglion dopamine β-hydroxylase (DBH) activity after polyacrylamide gel electrophoresis. Each point represents the mean ± standard error of five pairs of ganglia (five gels). Electrophoresis was performed for 2.5 hr at 1 mamp/tube.

acrylamide gel electrophoresis of this fraction and the pellet solubilized by treatment with 0.1% Triton X-100 showed that the mobility of the supernatant enzyme was similar to that of the fast-moving band. The mobility of the sedimentable enzyme was similar to that of the slowly moving band (Fig. 2).

An attempt was made to determine whether the sedimentable, slowly moving activity could be converted into the faster-moving peak. A homogenate of eight ganglia was prepared in 2 ml of 0.1% Triton, and after centrifugation at 16,000 × *g* the supernatant fraction was applied to five duplicate gels (Fig. 3). The results showed the usual two peaks, with 40% of the activity located in the fast-moving peak and 60% in the slowly moving peak. A portion of the homogenate was stored in Triton X-100 at 4° for 3 hr and then at −10° for 16 hr. This treatment produced a reversal in the relative activity in the two bands; 64% of the activity was now located in the fast-moving peak (Fig. 3), with a concomitant fall in the slowly moving peak. The total activity recovered on the gels after this prolonged treatment with Triton differed by less than 5% from that recovered after 0.5 hr.

Dopamine β-hydroxylase activity occurs in the sera of various species (16, 17). When human serum dopamine β-hydroxylase was subjected to polyacrylamide gel electrophoresis, the peak migrated in a position intermediate between those of the two peaks observed in rat stellate ganglia. Because of the high molecular weight of this enzyme and its relatively poor penetration into the gel, another supporting medium, starch blocks, in which separation is independent of molecular weight, was used. Rat adrenals were first examined for dopamine β-hydroxylase activity on starch blocks in barbital

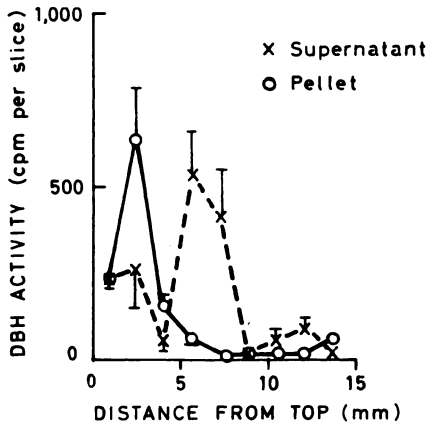

FIG. 2. *Polyacrylamide gel electrophoresis of rat stellate ganglion dopamine β-hydroxylase (DBH) after high-speed centrifugation*

One pair of ganglia was homogenized in 1.0 ml of distilled water, and the homogenate was centrifuged at 100,000 × *g* for 1 hr. The pellet was rehomogenized in 1.0 ml of 0.1% Triton X-100 and centrifuged at 16,000 × *g* for 10 min. The supernatant fraction from each centrifugation was subjected to electrophoresis for 2 hr at 1 mamp/tube. Each point represents the mean ± standard error of three gels.

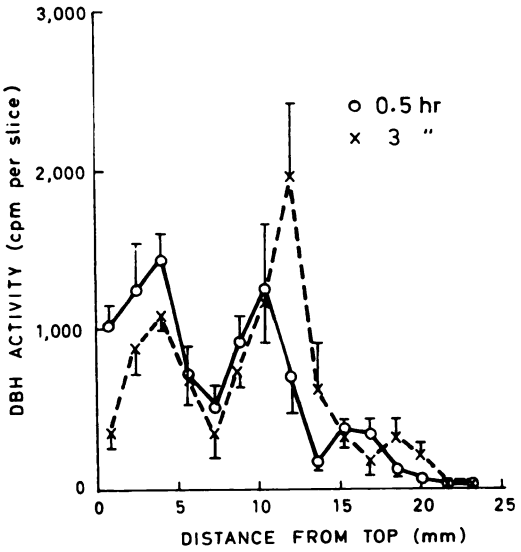

FIG. 3. *Effect of prolonged exposure of rat stellate ganglia to 0.1% Triton X-100*

Eight ganglia were homogenized in 2.0 ml of 0.1% Triton X-100 and then centrifuged at 16,000 × *g* for 10 min. Electrophoresis was carried out after 0.5 hr on five 100-μl aliquots of the supernatant sample (○). The remainder of this fraction was stored for 3 hr at 4° and then for 16 hr at −10°. Electrophoresis of four 100-μl aliquots (×) was carried out after thawing the sample. DBH, dopamine β-hydroxylase.

buffer, 0.05 M, pH 8.6. In the lower portion of Fig. 4 the rat adrenals, after homogenization in Triton X-100 and centrifugation at 10,000 × *g* for 10 min, exhibited two electrophoretically distinguishable peaks of activity (also see Fig. 5, bottom). In the upper portion of Fig. 4 the rat adrenals, after homogenization in water and centrifugation at 100,000 × *g* for 1 hr, revealed a single electrophoretic peak of activity in the supernatant fraction, without evidence of the more slowly migrating, presumably bound, peak. This result was similar to that shown in Fig. 1 for homogenates of stellate ganglia on polyacrylamide gel electrophoresis.

The electrophoretic mobilities of dopamine β-hydroxylase on starch blocks were compared in various tissues of the rat (Fig. 5). The mobility of the major peak of activity was identical in all three tissues examined: adrenal gland, stellate ganglia, and serum (Fig. 5). The absence of a slowly moving peak in the stellate ganglion 10,000 × *g* supernatant fractions was unexpected but was repeatedly observed on starch blocks, although, as shown in Fig. 1, stellate ganglia yield two distinct peaks on polyacrylamide gel electrophoresis. Since these two techniques differ in numerous ways, heterogeneity of proteins on acrylamide is not unusual even though homogeneity occurs on starch blocks, as illustrated by the examples of human haptoglobins, transferrins, and group-specific component. The absence of

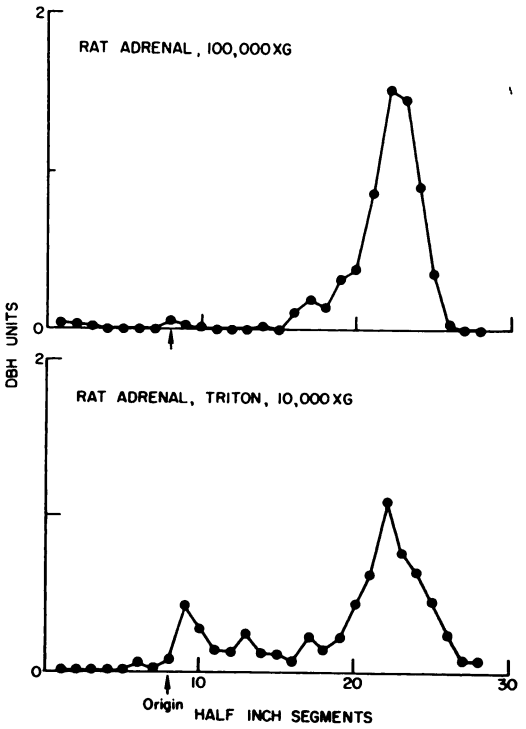

FIG. 4. *Starch block electrophoresis of rat adrenal β-hydroxylase after high-speed centrifugation.*

Twenty-four rat adrenal glands were homogenized in 4 ml of distilled water, and six rat adrenals were homogenized in 0.1% Triton X-100. Both were centrifuged at 10,000 × *g* for 10 min. The supernatant fluid of the Triton X-100 homogenate was applied directly to a starch block, while that of the water homogenate was centrifuged at 100,000 × *g* for 1 hr prior to application of the supernatant fraction to the same block. Both samples were assayed for dopamine β-hydroxylase (DBH) activity in the presence of a final $CuSO_4$ concentration of 12 μM.

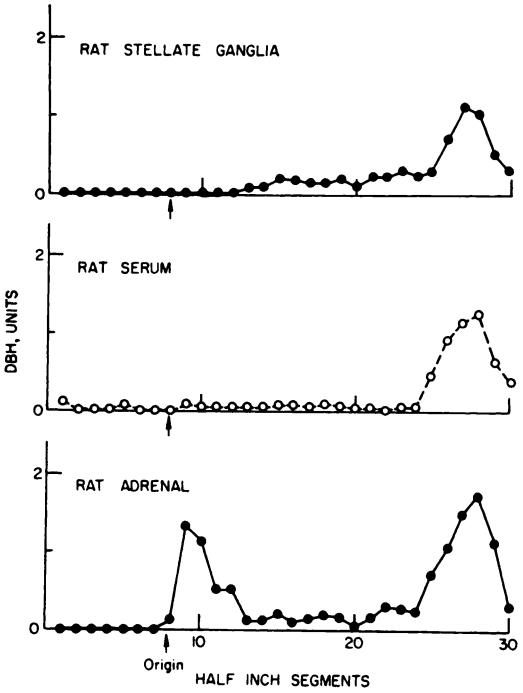

FIG. 5. *Starch block electrophoresis of rat tissue*

Six rat adrenal glands and 12 stellate ganglia were each homogenized in 2 ml of 0.1% Triton and centrifuged for 10 min at 10,000 × *g*. The supernatant fraction, as well as 2 ml of rat serum, was applied to a starch block. After 16 hr of electrophoresis, 200-μl aliquots of the fluid eluted from 0.5-inch segments of the block were assayed for dopamine β-hydroxylase (DBH) activity with a final $CuSO_4$ concentration of 12 μM for solid tissue and 25 μM for serum.

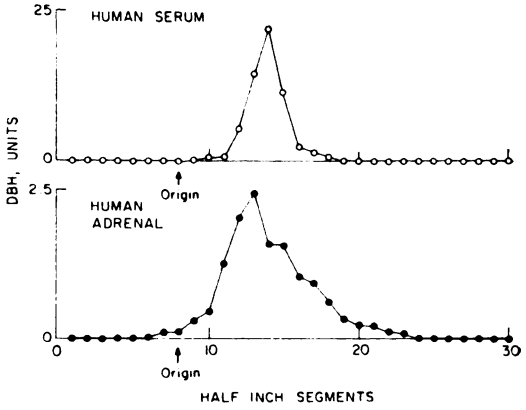

FIG. 6. *Starch block electrophoresis of human adrenal gland and human serum.*

After centrifugation at 100,000 × *g* for 1 hr of a homogenate of 100 mg of human adrenal glands in 20 ml of distilled water, the supernatant fluid was applied to a starch block with 0.3 ml of human serum. The final $CuSO_4$ concentration used for serum was 3 μM, and for the adrenal gland, 12 μM. Because of the high activity present in serum, the samples were diluted 1:4 with ice-cold water before assay. DBH, dopamine β-hydroxylase.

the slowly moving peak in stellate ganglia on starch block electrophoresis and its presence in adrenals suggest that the nature of the binding of dopamine β-hydroxylase may differ in these two tissues.

Dopamine β-hydroxylase from two human tissues, serum and adrenal, showed the same electrophoretic mobility, but the peak from

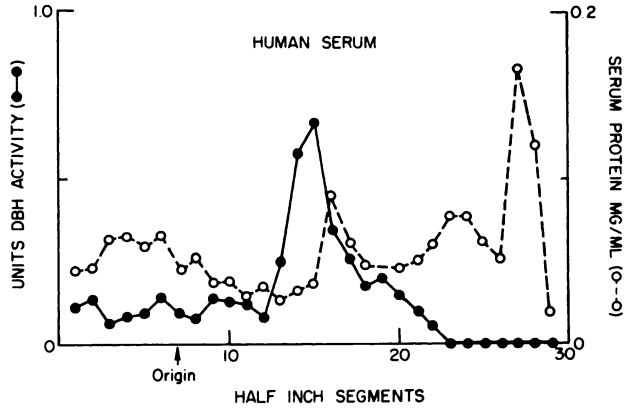

FIG. 7. *Protein concentrations and dopamine β-hydroxylase (DBH) activity after starch block electrophoresis of human serum.*

The peaks for human serum, beginning from the right, correspond to albumin, α-globulin, and γ-globulin.

the adrenal glands was broader and less symmetrical than that from serum (Fig. 6). Human serum enzyme activity migrated with the mobility of a β-globulin (Fig. 7).

Species differences in the electrophoretic mobility of dopamine β-hydroxylase were examined. The electrophoretic mobilities of the adrenal enzyme differed in the three species studied (Fig. 8), that from the rat being fastest and that from man the slowest. A broad, asymmetrical peak of activity was consistently observed. This characteristic suggests heterogeneity.

The supernatant fraction from crude homogenates of rat adrenal glands was also separated on a starch block prepared in phosphate buffer, pH 7.5, 0.05 M; the size and shape of the two peaks of dopamine β-hydroxylase activity remained the same in phosphate as in barbital buffer, 0.05 M, pH 8.6. Furthermore, when the main, fast-moving peak of activity from human adrenals was isolated after starch block electrophoresis in barbital buffer and rerun on another starch block under identical electrophoretic conditions, no change occurred in either its mobility or shape. As another control, to check for the role of protein interactions in producing the broad, asymmetrical, fast-moving peak, an experiment was performed in which supernatant fractions from homogenates of rat and bovine adrenals were mixed in equal amounts and the mixture was separated by starch block electrophoresis. The difference in the electrophoretic mobilities of dopamine β-hydroxylase from rat and bovine adrenals shown in Fig. 8 also occurred in the mixture, and the asymmetrical shape of the fast-moving peak of both rat and bovine adrenals was also preserved.

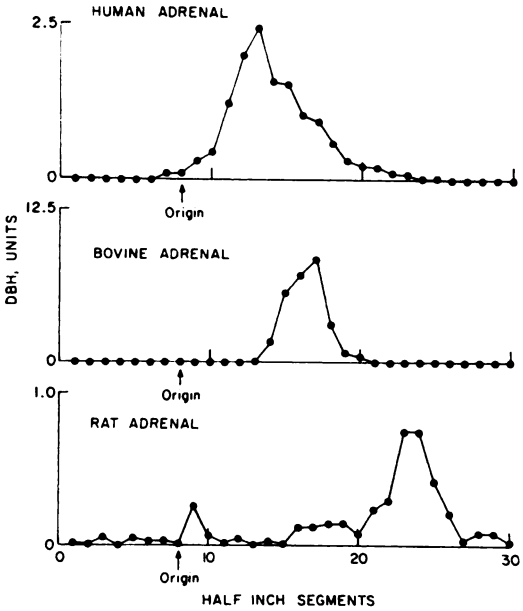

FIG. 8. *Starch block electrophoresis of adrenal homogenates from various species.*

Bovine and human adrenals (100 mg) were homogenized in 20 ml of distilled water, and 10 rat adrenal glands were homogenized in 3 ml of water. All homogenates were centrifuged for 1 hr at 100,000 × *g*, and 1 ml of the supernatant fluid from bovine and human and 2 ml of that from rat adrenals were applied to a starch block. The $CuSO_4$ concentraton was 12 μM. DBH, dopamine β-hydroxylase.

TABLE 1

After starch block electrophoresis, tissue samples containing the major peak of dopamine β-hydroxylase activity were pooled. The activity of the pooled samples was determined at five concentrations of $CuSO_4$, from 0 to 32 μM. K_m values for tyramine were determined at 37° in the presence of air, using the concentration of $CuSO_4$ which had given optimal activity. Four concentrations of substrate, from 0.12 to 0.96 mM, were used to determine the K_m values.

Tissue	K_m
	$M \times 10^4$
Rat adrenal	4.7 ± 1.7
Rat serum	6.3 ± 0.8
Human adrenal	8.1 ± 1.8
Human serum	6.5 ± 2.1
Bovine adrenal	5.6 ± 0.9

To characterize further the nature of the enzyme, K_m values for substrate were determined on the main peak of dopamine β-hydroxylase activity isolated by starch block electrophoresis from various tissues and species (Table 1). The K_m values were not statistically different. These kinetic values are similar to those reported elsewhere for the highly purified enzyme isolated from the bovine adrenal (1), for serum (17), and for stellate ganglia.[4]

[4] P. B. Molinoff, S. Brimijoin, and J. Axelrod, manuscript in preparation.

DISCUSSION

Two electrophoretically distinguishable forms of dopamine β-hydroxylase activity in crude homogenates of several solid tissues have been described here for the first time. Previously the highly purified bovine adrenal enzyme yielded a single electrophoretic peak (1). However, Gibb *et al.* (18) reported two electrophoretic peaks for highly purified bovine adrenal dopamine β-hydroxylase; the faster moving peak contained little protein or enzymatic activity and was attributed to altered native enzyme (18). In these respects it differed from our results on crude homogenates of solid tissues, where the two peaks of activity may possess physiological significance, the more slowly migrating peak corresponding to a bound form and the faster-moving peak to a soluble form. The first reason for this interpretation is that the presumably bound form can be eliminated by high-speed centrifugation of the supernatant fraction from homogenates of crude tissues and fails to appear altogether in a tissue such as serum, which contains little particulate material. The second reason is that treatment with a solubilizing agent such as Triton partly converts dopamine β-hydroxylase activity from the slower to the faster-moving peak. Furthermore, this interpretation is in harmony with previous descriptions (14, 15) of a free and a bound form of the enzyme in both adrenal medulla and sympathetic nerves. It also agrees with our observation that prior treatment with reserpine elevates both peaks of activity, since the existence of major structural differences between the molecular species constituting these two peaks might lead to major differences in their response to an inducing agent. The broad, asymmetrical shape of the fast-moving peak of dopamine β-hydroxylase activity may conceal several distinct molecular forms. Preservation of its mobility and asymmetry on a second electrophoretic run and in an experiment in which supernatant fractions from rat and bovine adrenal homogenates were mixed and separated electrophoretically suggests that the asymmetry of this fast-moving peak is not attributable to metastable polymers or to binding to different protein molecules.

Heterogeneity of another variety occurred in interspecies studies. The major peak of dopamine β-hydroxylase activity in the adrenal glands of each of the three species we examined (man, cow, and rat) differed in electrophoretic mobility. However, their Michaelis constants were similar. These kinetic similarities are consistent with earlier studies on the cofactor, oxygen, and copper requirements of human serum dopamine β-hydroxylase as compared to the purified bovine adrenal enzyme (17). Within a given species the activity from different tissues was similar in both electrophoretic mobility and substrate affinity.

Recent reports of dopamine β-hydroxylase activity in serum (16, 17) have raised the question of its relationship in blood to the enzyme in adrenal glands and sympathetic nerves. In the rat, serum dopamine β-hydroxylase activity rises during stress that increases the excretion of catecholamines (19); in these experiments the adrenal glands are not necessary either for the maintenance of normal serum enzyme activity or for the increase occurring with stress (19). Furthermore, in rats the administration of 6-hydroxydopamine, a drug which destroys sympathetic nerve terminals (20), leads to decreased serum dopamine β-hydroxylase activity (21). The observation that the activities of this enzyme in human and rat serum have electrophoretic mobilities similar to those of the enzyme from other tissues from the same species provides additional evidence that serum dopamine β-hydroxylase activity originates from the chromaffin granules of the adrenal gland, the catecholamine storage vesicles of sympathetic nerves, or both.

Reserpine administration causes a transsynaptic increase in dopamine β-hydroxylase activity in the adrenal medulla and in the sympathetic cell bodies and terminals (2, 4). This increase appears to result from elevated sympathetic nerve activity over a prolonged time. The present studies reveal that reserpine causes elevation of both the slowly moving, presumably bound, form of the enzyme and the fast-migrating, presumably soluble, peak.

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