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Purification and Immunochemical Characterization of Dopamine β-Hydroxylase from Human Pheochromocytoma

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SUMMARY

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Dopamine β-hydroxylase was purified from a human pheochromocytoma. Following sucrose density gradient centrifugation the enzyme was isolated in three different molecular forms. The bulk of the enzymatic activity was associated with fraction II (approximate mol wt 286,000), which possessed the highest specific activity. The specific activity of dopamine β-hydroxylase in fraction I (approximate mol wt 164,000) and in fraction III (approximate mol wt 524,000) was 4 times lower than in fraction II. A specific antiserum to human dopamine β-hydroxylase isolated from fraction II was produced in rabbits (antiserum H). Electrophoretic and immunological evidence supports the contention that dopamine β-hydroxylase in fraction II was isolated in pure form. The lower homospecific activity of dopamine β-hydroxylase in fraction I as compared with fraction II and the immunological titration data indicated that fraction I contained more enzyme protein than was apparent from the enzyme activity. The immunological titration studies showed that antiserum H, as well as the antiserum to bovine adrenal dopamine β-hydroxylase (antiserum B), reduced the activity of the homologous enzyme more effectively than the activity of the heterologous enzyme. The poor interspecies cross-reactivity suggests that for measurements of human serum dopamine β-hydroxylase levels by radioimmunoassay a homologous system is required.

INTRODUCTION

The enzyme dopamine β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase, EC 1.14.17.1) has been purified by various procedures from bovine adrenal glands (1-3). The bovine adrenal enzyme has been characterized as a copper enzyme (4, 5), and it has been shown that the valence of the copper undergoes cyclic changes during the enzymatic β-hydroxylation reaction (4, 5). Specific antisera to bovine adrenal dopamine β-hydroxylase have been produced (6, 7) and used for measuring serum levels of the enzyme by a radioimmunoassay (8, 9) as well as for its neuronal localization by immunofluorescent techniques (10-12). However, the loss of cross-immunoreactivity between species could limit the usefulness of bovine adrenal dopamine β-hydroxylase for human studies. We have therefore purified dopamine β-hydroxylase from human pheochromocytoma and used this preparation to induce specific antibodies in rabbits. In this paper we describe the purification and characterization of various molecular forms of human pheochromocytoma

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dopamine β-hydroxylase. Partial characterization of the various molecular forms of the enzyme was accomplished by physicochemical and immunological analyses. The interaction of rabbit antiserum directed toward the human enzyme with human and bovine antigen was investigated. Preliminary reports on these studies have been presented (13, 14).

MATERIALS AND METHODS

Purification. About 100 g of a human pheochromocytoma tumor obtained immediately after surgery were used for each enzyme preparation. The tumor was suspended in 3 volumes of 0.25 M sucrose containing 20 mM potassium phosphate buffer (pH 6.5) and homogenized in a Waring Blendor for 1 min. The homogenate was centrifuged for 10 min at 700 × *g*, and the sediment was discarded. The supernatant was filtered through four layers of cheesecloth to remove the fat and then centrifuged at 100,000 × *g* for 1 hr. The sediment was suspended in 150 ml of 20 mM potassium phosphate buffer (pH 6.5) containing 0.1% Triton X-100. The suspension was homogenized gently with a ten Broeck tissue grinder, then centrifuged at 100,000 × *g* for 1 hr. The same process was repeated once more, and the supernatant fractions were combined. The enzyme was purified from the supernatant fraction by $(NH_4)_2SO_4$ fractionation as previously described (1, 2). Following $(NH_4)_2SO_4$ fractionation the enzyme was chromatographed on a DEAE-Sephadex A-25 column (1.6 × 60 cm) equilibrated with 5 mM potassium phosphate buffer (pH 6.5), and was eluted with a linear gradient containing equal volumes of 5 mM and 300 mM potassium phosphate buffer (pH 6.5). The most active fractions were combined and concentrated by Amicon Diaflo filtration (XM-100A membrane). The concentrated fractions were further purified by centrifugation on a linear surose density gradient. The gradients were prepared with a Büchler universal density gradient mixer, using equal amounts of 5% and 20% (w/v) sucrose solutions in 5 mM potassium phosphate buffer (pH 6.5). The gradients were centrifuged for 18 hr at 40,000 rpm in a Beckman model L-2-65B ultracentrifuge, using an SW 40 rotor. After centrifugation fractions of equal volume were collected from the top to the bottom of the gradient. The enzymatically active fractions were stored at 70° in plastic tubes.

Enzyme assay. Enzyme activity was determined spectrophotometrically (15), using a procedure as modified by Nagatsu and Udenfriend (16). Unless otherwise stated, each incubation mixture contained the following components (in micromoles): sodium acetate buffer (pH 5.5), 100; tyramine, 10; ascorbic acid, 10; fumaric acid, 10; *N*-ethylmaleimide, 10; and pargyline HCl 0.75. To each incubation mixture 700 units of catalase (bovine liver, Schwarz/Mann, 6600 units/mg of protein) were added. After addition of the enzyme solution the final volume was adjusted to 1 ml and the reaction mixture was incubated at 37° for 20 min. The specific activity is expressed as micromoles of octopamine formed per minute per milligram of protein at 37°. The immunoreactive dopamine β-hydroxylase was estimated by solid-phase radioimmunoassay (9, 10), utilizing an antibody covalently bound to glass beads (17). ^{125}I-Labeled human dopamine β-hydroxylase was prepared by the method of Greenwood *et al.* (18). To a solution of 40–50 μg of purified dopamine β-hydroxylase (purified on a DEAE-Sephadex A-25 column) in 0.5 M phosphate buffer (pH 7.5) were added 2 mCi of Na ^{125}I (specific activity, 17 Ci/mg) and 0.1 mg of Chloramine-T in 10 μl of phosphate-buffered NaCl solution. The reaction was stopped after 30 sec by the addition of 0.2 mg of sodium metabisulfite in 0.2 ml of phosphate-buffered NaCl, and then 2 mg of KI in 0.1 ml were added. The solution was passed through a column (1 × 22 cm) of Sephadex G-25 equilibrated with phosphate-buffered NaCl, and the eluted, iodine-labeled protein was then subjected to linear sucrose density gradient centrifugation as described above. The ^{125}I-labeled dopamine β-hydroxylase recovered from the middle portion of the gradient was used as a tracer in the radioimmunoassay. ^{125}I-Labeled bovine adrenal dopamine β-hydroxylase was prepared by the same procedure.

Protein was assayed by the fluoresca-

mine method (19), using bovine serum albumin as a standard, except in monitoring column effluents, when the protein was measured by determining the optical density at 280 nm.

Polyacrylamide gel electrophoresis was performed by the method of Davis (20) and Ornstein (21). Electrophoresis was carried out in 0.4 M glycine-Tris buffer (pH 8.3) at 3 mamp/tube at 2–5°, using 5% gel if not otherwise stated. Coomassie brilliant blue was used to stain the protein on the gel. To test enzyme activity, gels were sliced, and each slice was dispersed in 0.2 ml of 0.1 M sodium acetate buffer (pH 4.5). The activity was measured in these mixtures using a sensitive isotopic procedure (22).

Immunization of rabbits. About 0.2–0.3 mg of purified human dopamine β-hydroxylase purified by sucrose density gradient centrifugation (fraction II) was subjected to disc gel electrophoresis, loading 50–100 μg of protein on each gel. The protein band (2–3 mm wide) was cut out of the gel, extruded through on a 20-gauge needle, and allowed to stand in an equal volume of 0.9% NaCl for a few hours at 4°. An equal volume of complete Freund's adjuvant was added and thoroughly mixed. The entire mixture (1 ml) was divided into four equal portions, and each portion was injected into the footpads of the rabbits. The immunization procedure was repeated every 2–4 weeks for 3–6 months as described above, but the antigen was injected intramuscularly in four places on the backs of the rabbits. Control preimmune serum was obtained from each rabbit prior to the immunization. The antibodies in the serum were tested against the antigen by Ouchterlony immunodiffusion-analyses (23) and microimmunoelectrophoresis (24). The antisera to bovine adrenal dopamine β-hydroxylase were prepared in a similar manner. The antisera to human pheochromocytoma dopamine β-hydroxylase (antisera H) and the antisera to the bovine adrenal enzyme (antisera B) were stored at −70°.

Immunochemical titration. The immunochemical titrations were performed by adding increasing volumes of dopamine β-hydroxylase antisera to fixed aliquots of antigen. Aliquots of control sera were added to each sample to bring them all to the same volume. The antisera and antigen were incubated at 4° for 24 hr, and following centrifugation the supernatant fraction was assayed for dopamine β-hydroxylase activity.

Determination of sedimentation coefficients. Linear sucrose density gradient centrifugation was used for determination of the sedimentation coefficients and to estimate the molecular weights of the different forms of human pheochromocytoma dopamine β-hydroxylase. Protein standards employed in the sucrose gradients were dissolved in 5 mM potassium phosphate buffer (pH 6.5) and mixed with the enzyme preparation. The sedimentation rates of different forms of the enzyme were calculated as previously described, and the molecular weights were estimated (25). Fumarase (from pig heart, Sigma Chemical Company) activity was determined by measuring the enzymatic conversion of L-malate to fumarate (biochemical catalogue, Boehringer, 1970). γ-Globulin (human, Schwarz/Mann) and fibrinogen (bovine, Schwarz/Mann) were measured by determining the optical density at 280 nm, and bovine ^{125}I-labeled dopamine β-hydroxylase was measured in a γ-counter.

RESULTS

Purification of enzyme. A summary of the results of the purification of dopamine β-hydroxylase from human pheochromocytoma is shown in Table 1. Following $(NH_4)_2SO_4$ fractionation the enzyme was further purified on a DEAE-Sephadex A-25 column, which resulted in an over-all 34-fold purification with 11% recovery of enzyme activity. Further purification of the enzyme was achieved by centrifugation in a sucrose density gradient. Three fractions of enzymatic activity were obtained. The bulk of the enzymatic activity was recovered in the middle portion of the gradient (fraction II). A small percentage of the total activity was recovered in the upper portion of the gradient (fraction I) and in the lower portion of the gradient near the bottom (fraction III). The most active enzyme was recovered from fraction II and had a specific activity of approximately 30 units/mg of protein when assayed under standard conditions. How-

ever, when the protein in this fraction was assayed by the procedure of Lowry *et al.* (26), the specific activity was found to be only 15.7 units/mg. The specific activity of dopamine β-hydroxylase was approximately 4 times higher in fraction II than in fractions I and III. Using the same procedure, the enzyme was purified from two other pheochromocytoma tumors and similar results were obtained.

Electrophoretic and immunoelectrophoretic studies. Polyacrylamide disc gel electrophoresis of dopamine β-hydroxylase in fraction II obtained after sucrose density gradient centrifugation revealed a single stained protein band. No enzymatic activity was detected in the other sections of the gel. An immunoelectrophoretic analysis of antiserum H against purified human dopamine β-hydroxylase (fraction II) or against the partially purified human enzyme preparation purified by $(NH_4)_2SO_4$ fractionation showed a single precipitin arc (Fig. 1).

Estimation of different molecular parameters. To determine the average sedimentation coefficients of the different forms of dopamine β-hydroxylase the enzyme as well as various standard proteins were centrifuged in a sucrose density gradient. The sedimentation coefficient of the human pheochromocytoma enzyme with the highest specific activity (fraction II) was identical with the sedimentation coefficient of bovine adrenal dopamine β-hydroxylase. The molecular weights of the different forms of the enzyme were calculated from the sedimentation coefficients (Table 2). The enzyme activities in fractions I, II, and III correspond approximately to average molecular weights of 164,000, 286,000,

TABLE 1

Purification of dopamine β-hydroxylase from human pheochromocytoma tumor

Step	Volume	Total protein	Total activity	Specific activity	Yield	Purification
	ml	*mg*	*units*	*units/mg*	*%*	*-fold*
$(NH_4)_2SO_4$, 80% ppt	51.0	342.21	114.6	0.335	100	
$(NH_4)_2SO_4$, 25–45% ppt	10.3	105.58	60.3	0.570	52.6	1.7
Peak from DEAE-Sephadex A-25 (concentrated on XM-100A membrane)	2.1	1.126	13.0	11.5	11.3	34.4
Sucrose density gradient centrifugation						
Fraction I	1.47	0.036	0.249	6.92	0.22	20.7
Fraction II	3.67	0.194	5.83	30.1[a]	5.1	89.7
Fraction III	0.79	0.017	0.124	7.28	0.11	21.7

[a] The specific activity was found to be 48% lower when the protein was estimated by the procedure of Lowry *et al.* (26).

FIG. 1. *Immunoelectrophoretic analyses of purified and partially purified human pheochromocytoma dopamine β-hydroxylase against 50 μl of rabbit antiserum H*

The buffer was sodium barbital, pH 8.6; ionic strength 0.1. The upper well contained 20 μg of partially purified enzyme. The lower well contained 10 μg of purified enzyme. The anode is at left.

TABLE 2

Molecular parameters of human pheochromocytoma dopamine β-hydroxylase and protein standards calculated from sucrose density gradient centrifugation

Protein	$s_{20,w}$	Mol wt
	S	$\times 10^{-5}$
Pheochromocytoma enzyme		
Fraction I	6.20	1.64
Fraction II	8.96	2.86
Fraction III	13.47	5.24
Adrenal enzyme (bovine)	8.95	2.90 (2.90)[a]
Fibrinogen (bovine)	9.72	3.29 (3.30)
γ-Globulin (human)	5.46	1.40 (1.60)
Fumarase (pig)	7.17	2.09 (1.94)

[a] The values in parentheses were reported in the literature.

and 524,000, respectively. The enzyme activity recovered in fractions I and II was concentrated by ultrafiltration, and after mixing with standard proteins, these preparations were separately centrifuged in a sucrose density gradient. The analysis of the gradient containing fraction I revealed a single enzyme activity peak corresponding to the position of fraction I in the first gradient. The gradient containing fraction II showed a single major peak (approximately 96% of the total activity) corresponding to the position of fraction II in the first gradient. A small amount of enzyme activity was also recovered near the bottom of the gradient.

Homospecific activity of various molecular enzyme forms. To determine the enzyme homogeneity in the three fractions recovered after centrifugation in the sucrose density gradient, we measured the homospecific activity in each fraction (the ratio of enzyme activity to immunoreactive enzyme protein). The results in Table 3 show that in fraction II there was only a slight difference between the amount of total protein and the amount of immunoreactive enzyme. In fraction I immunoreactive dopamine β-hydroxylase could account for 60% of the total protein, while in fraction III it could account for only 17% of the total protein. The homospecific activity of dopamine β-hydroxylase in fraction II was twice as high as in fraction I and lower than in fraction III.

Immunological characterization. In double-diffusion reactions antiserum H was tested against partially purified human pheochromocytoma dopamine β-hydroxylase and bovine adrenal dopamine β-hydroxylase as well as purified human pheochromocytoma tyrosine hydroxylase. A single precipitin line was observed against human and bovine dopamine β-hydroxylase and none against human tyrosine hydroxylase.

The dose-dependent immunotitration curves of human pheochromocytoma and bovine adrenal dopamine β-hydroxylases by antisera H and B are shown in Figs. 2 and 3. The amounts of the human pheochromocytoma and bovine adrenal enzymes were adjusted so that their activities were approximately the same. The immunotitration curves of human pheochromocytoma dopamine β-hydroxylase with respect to antiserum H and of the bovine adrenal enzyme with respect to antiserum B are similar. The apparent biphasic decline of enzymatic activity in both heterologous titration curves may suggest that the antibodies have high and low binding affinities for the corresponding antigens. It can be seen from Fig. 2 that 1.5 μl of antiserum H are required to decrease the human pheochromocytoma dopamine β-hydroxylase activity by approximately 50% whereas about 7 μl of antiserum H are required for approximately 50% reduction of the bovine adrenal enzyme activity. Similar studies were done with antiserum B. It can be seen from the results in Fig. 3 that 0.25 μl of antiserum B is required to decrease the bovine adrenal enzyme activity by 50% while about 30 times more antiserum is needed to decrease the human pheochromocytoma enzyme activity by only 40%.

In separate experiments the dopamine β-hydroxylase in fractions I and II (enzyme preparations purified by sucrose density gradient centrifugation) were titrated with antiserum H. By extrapolating the linear portion of the immunotitration curve to the abscissa, the equivalence points were estimated. The immunotitration curve of the fraction II enzyme showed a much sharper decline than the immunoti-

TABLE 3

Homospecific activity of various molecular forms of dopamine β-hydroxylase isolated after sucrose density gradient centrifugation

Enzyme fraction	Total protein	Specific activity	Total immunoreactive enzyme protein[a]	Homospecific activity
	μg	*units/mg protein*	*μg*	*units/mg immunoreactive protein*
I	17.2	6.92	10.34	11.51
II[b]	92.4	30.10	110.60	25.11
III	8.2	7.28	1.35	43.70

[a] Determined by radioimmunoassay.

[b] In two subsequent experiments the differences between the amount of total protein and the amount of total immunoreactive protein were 11% and 14%.

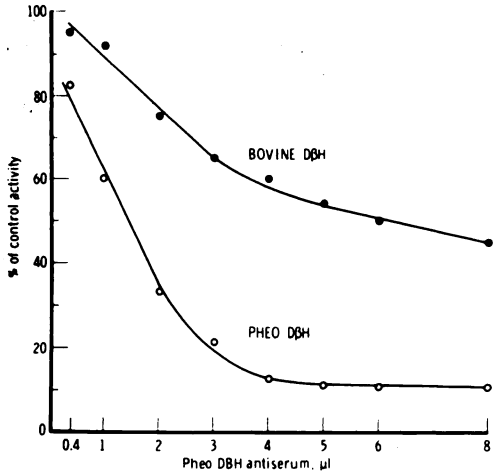

FIG. 2. *Titration of human pheochromocytoma (Pheo) and bovine adrenal dopamine β-hydroxylase (DβH) with antiserum H*

The results are the means from three experiments ± 3–5% (SE). The amounts of human pheochromocytoma and bovine enzymes were adjusted so that the activities of both were similar. The initial activities are expressed as micromoles of octopamine formed per assay in 10 min at 37°: human pheochromocytoma enzyme (100% of controls), 0.043; bovine adrenal enzyme (100% of controls), 0.046.

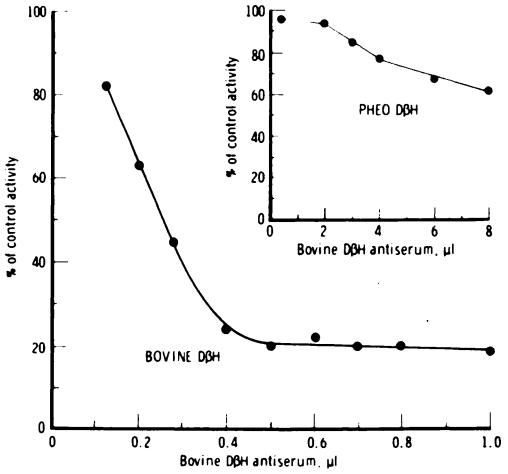

FIG. 3. *Titration of bovine adrenal and human pheochromocytoma (Pheo) dopamine β-hydroxylase (DβH) with antiserum B*

Results are the means from three experiments ± 3–6% (SE). The initial activities were the same as indicated in Fig. 2.

tration curve of the fraction I preparation (Fig. 4). The fraction I enzyme required 1.9 times more antiserum to reach the equivalence point than the enzyme in fraction II.

DISCUSSION

While this study was in progress (13, 14) another report described the purification of human dopamine β-hydroxylase from a pheochromocytoma tumor (27). The two procedures differ in some aspects. Stone *et al.* (27) purified the soluble form of the enzyme obtained from osmotic disruption of the chromaffin vesicles, while we purified a mixture which contains the soluble form and the enzyme bound to the granule membrane, liberated by detergent solubilization. The purification steps were also different in these two studies: we purified the enzyme by $(NH_4)_2SO_4$ fractionation, DEAE-Sephadex A-25 chromatography, and sucrose density gradient centrifugation, whereas Stone *et al.* (27) purified it by DEAE-cellulose and Sephadex G-200 chromatography. The specific activity of the human enzyme purified by the procedure described in this paper is much higher than the specific activity reported in the other study (27). It is noteworthy that the

specific activity of the human dopamine β-hydroxylase is comparable to the specific activity of bovine adrenal dopamine β-hydroxylase purified by affinity chromatography (28). The high specific activity obtained in this study is probably due in part to isolation of the enzyme by a gentle procedure (DEAE-Sephadex instead of DEAE-cellulose column chromatography[2]) and in part to separation of the enzymatically active forms from the inactive forms by sucrose gradient centrifugation.

Recent studies have shown that homospecific activity can be used as an index of homogeneity during enzyme purification (28). The lower homospecific activity of dopamine β-hydroxylase in fraction I as compared wth fraction II, as well as the immunological titration data, indicates that fraction I contains more enzyme protein than is apparent from the enzyme activity. This could imply that fraction I contains enzymatically inactive dopamine β-hydroxylase, or that the specific activity of the dimer (fraction I) is lower than the specific activity of the tetramer (fraction II), or that the dimer has more active sites exposed to interaction with antiserum H than the tetramer. Since antiserum H was obtained from immunization of rabbits against the tetramer, the latter possibility seems unlikely to us. The highest homospecific activity of the enzyme in fraction III does not necessarily indicate that this fraction contains the lowest amount of immunoreactive dopamine β-hydroxylase by radioimmunoassay per unit of enzyme activity, since it may be attributable to the lower immunological potency of antiserum H toward the aggregated antigen. Alternatively, the aggregated antigen may have fewer binding sites for interaction with antiserum H than the tetramer. The immunotitration experiments of antiserum H with dopamine β-hydroxylase in fracton III yielded inconsistent results, probably because of variations in the aggregation stage of the enzyme.

As we have reported (13, 14), the results obtained with sucrose density gradient centrifugation show that human dopamine β-hydroxylase occurs in different molecular forms. The findings that after recentrifugation on a sucrose density gradient fraction I was recovered in the same position as in the first gradient, and that following recentrifugation of fraction II no enzymatic activity was recovered in the gradient position of fraction I, suggest that fraction I is not an artifact formed during the centrifugation procedure. However, it is not yet clear whether fractions I and III are artifacts formed during the over-all purification procedure or whether they occur naturally. The lowest molecular weight form of the enzyme obtained in this study corresponds to the molecular weight of the dimer which was isolated in the presence of 6 M guanidine HCl from the bovine adrenal (29, 30) and human (27) enzymes. Furthermore, we have shown that the dimer (13, 14) and the aggregated form of the human enzyme have catalytic activity.

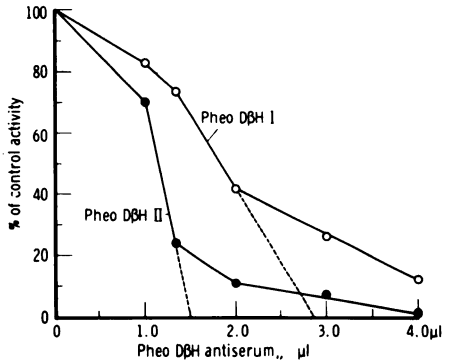

FIG. 4. *Titration of human pheochromocytoma dopamine β-hydroxylase (Pheo DβH) fractions I and II with antiserum H*

Results are the means from four experiments ± 2–5% (SE). The initial activities are expressed as micromoles of octopamine formed per assay in 10 min at 37°: fraction II, 0.063; fraction I, 0.054.

Immunization of rabbits with purified human dopamine β-hydroxylase yielded a highly potent antibody. This antiserum has been shown to be specific, since it interacts by enzymatic and immunological criteria with dopamine β-hydroxylases derived from tissues of various species but not with other enzymes involved in the biosynthesis of catecholamines. It is noteworthy that the sharpest decrease in activity was obtained when antiserum H was titrated against the fraction II preparation, indicating that antiserum H (antiserum to dopa-

[2] M. Goldstein and D. H. Park, unpublished observations.

mine β-hydroxylase in fraction II) has some specific determinants to this antigen. The immunological titration data show that the antibody to human dopamine β-hydroxylase, as well as the antibody to the bovine enzyme reduces the activity of the homologous enzyme more effectively than the activity of the heterologous enzyme. The poor interspecies cross-reactivity is manifest in the findings that the quantity of antibody to bovine dopamine β-hydroxylase which reduces the activity of the bovine adrenal enzyme by approximately 80% is not at all sufficient to reduce the activity of the human serum enzyme. This raises the question whether antibodies to bovine dopamine β-hydroxylase can be utilized for measuring the human serum enzyme by a radioimmunoassay. We have consistently shown a significant correlation between human serum dopamine β-hydroxylase activity and immunoreactive enzyme levels when the latter were measured by a radioimmunoassay system employing human ^{125}I-labeled enzyme and antibodies to the human enzyme (9, 31). Rush *et al.* (32) found no correlation between human serum dopamine β-hydroxylase activity and immunoreactive enzyme level when the latter was measured by a radioimmunoassay employing antibodies to bovine dopamine β-hydroxylase. More recently Rush *et al.* (33) measured human serum immunoreactive dopamine β-hydroxylase levels with a radioimmunoassay employing antibodies to the human enzyme and confirmed our original finding that there is a good correlation between human serum dopamine β-hydroxylase activity and immunoreactive enzyme level. It is obvious from the results of the present study that antibodies to bovine dopamine β-hydroxylase cross-react poorly with the human enzyme and therefore a radioimmunoassay which utilizes bovine antibodies and ^{125}I-labeled bovine enzyme may not be sensitive enough to measure specifically the content of dopamine β-hydroxylase in human tissues.

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REFERENCES

1. Levin, E. Y., Levenberg, B. & Kaufman, S. (1960) *J. Biol. Chem.*, **235**, 2080–2086.
2. Goldstein, M., Lauber, E. & McKereghan, M. (1965) *J. Biol. Chem.*, **240**, 2066–2072.
3. Foldes, A., Jeffrey, P. L., Preston, B. N. & Austin, L. (1972) *Biochem. J.*, **126**, 1209–1217.
4. Blumberg, W. E., Goldstein, M., Lauber, E. & Peisach, J. (1965) *Biochim. Biophys. Acta*, **99**, 188–190.
5. Friedman, S. & Kaufman, S. (1965) *J. Biol. Chem.*, **240**, 4763–4773.
6. Gibb, J. W., Spector, S. & Udenfriend, S. (1967) *Mol. Pharmacol.*, **3**, 473–478.
7. Livett, B. G., Geffen, L. B. & Austin, L. (1968) *J. Neurochem.*, **15**, 931–939.
8. Rush, R. A. & Geffen, L. B. (1972) *Circ. Res.*, **31**, 444–452.
9. Ebstein, R. P., Park, D. H., Freedman, L. S., Levitz, S. M., Ohuchi, T. & Goldstein, M. (1973) *Life Sci.*, **13**, 769–774.
10. Geffen, L. B., Livett, B. G. & Rush, R. A. (1969) *J. Physiol. (Lond.)*, **204**, 593–605.
11. Hartman, B. K. & Udenfriend, S. (1970) *Mol. Pharmacol.*, **6**, 85–94.
12. Fuxe, K., Goldstein, M., Hökfelt, T. & Joh, T. H. (1971) *Prog. Brain Res.* **34**, 127–138.
13. Goldstein, M., Anagnoste, B., Freedman, L. S., Roffman, M., Ebstein, R. P., Park, D. H., Fuxe, K. & Hökfelt, T. (1973) in *Frontiers in Catecholamine Research* (Usdin, E. & Snyder, S. H., eds.), pp. 69–78, Pergamon Press, London.
14. Park, D., Das, S., Ebstein, R. P. & Goldstein, M. (1974) *Fed. Proc.*, **33**, 1587.
15. Pisano, J. J., Creveling, C. R. & Udenfriend, S. (1960) *Biochim. Biophys. Acta*, **43**, 566–568.
16. Nagatsu, T. & Udenfriend, S. (1972) *Clin. Chem.*, **18**, 980–983.
17. Kashimoto, T., Park, D., Ebstein, R. P., Goldstein, M., Levitz, M. & Yaverbaum, S. (1974) *Experientia*, **30**, 1363–1364.
18. Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.*, **89**, 114–123.
19. Bohlen, P., Stein, S., Dairman, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 213–220.
20. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.*, **121**, 404–427.
21. Ornstein, L. (1964) *Ann. N. Y. Acad. Sci.*, **121**, 321–349.
22. Goldstein, M., Freedman, L. S. & Bonnay, M. (1971) *Experientia*, **27**, 632–633.
23. Ouchterlony, O. (1958) *Prog. Allergy*, **5**, 1–78.
24. Scheidegger, J. J. (1955) *Int. Arch. Allergy Appl. Immunol.*, **7**, 103–110.

25. Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.*, **236**, 1372–1379.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
27. Stone, R. A., Kirshner, N., Reynolds, J. & Vanaman, T. C. (1974) *Mol. Pharmacol.*, **10**, 1009–1015.
28. Rush, R. A., Kindler, S. H. & Udenfriend, S. (1974) *Biochem. Biophys. Res. Commun.*, **61**, 38–44.
29. Craine, J., Daniels, G. & Kaufman, S. (1973) *J. Biol. Chem.*, **248**, 7838–7844.
30. Wallace, E. F., Krantz, M. J. & Lovenberg, W. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, **70**, 2253–2255.
31. Ebstein, R. P., Freedman, L. S., Lieberman, A., Park, D. H., Pasternack, B., Goldstein, M. & Coleman, M. (1974) *Neurology*, **24**, 684–687.
32. Rush, R. A., Thomas, P. E., Nagatsu, T. & Udenfriend, S. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 872–874.
33. Rush, R. A., Thomas P. E. & Udenfriend, S. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, **72**, 750–752.