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Active Dimers of Dopamine β-Hydroxylase in Human Plasma

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SUMMARY

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Normal human plasma and serum have been shown to contain two active species of dopamine β-hydroxylase (EC 1.14.17.1) by gradient ultracentrifugation and gel filtration. About 75-80% of the dopamine β-hydroxylase activity in plasma sediments almost exactly like bovine dopamine β-hydroxylase, and has a molecular weight of 289,000. The remainder of the dopamine β-hydroxylase activity in plasma or serum (20-25%) has a molecular weight of 147,000, or one-half that of the principal species. No interconversion is observed between these two species at pH 7.4, ionic strength 0.15. Thus a significant amount of dopamine β-hydroxylase activity in plasma is in the form of an active dimer of the basic subunit of this enzyme.

INTRODUCTION

Dopamine β-hydroxylase (EC 1.14.17.1) catalyzes the conversion of dopamine to norepinephrine in the catecholamine biosynthetic pathway. It has been purified from bovine adrenal medulla (1-6) and from human pheochromocytoma tissue (7, 8). The bovine enzyme is a glycoprotein with a molecular weight of 290,000 and is composed of four subunits (1, 9, 10). Several species of dopamine β-hydroxylase have been isolated from human pheochromocytoma tissue (7, 8). The major species has a molecular weight similar to that found for the bovine enzyme. Additional species with molecular weights of approximately one-half and twice that of the principal species have also been found. It is not known, however, whether these species are in equilibrium with each other (7, 8) or whether the additional forms of the enzyme are artifacts of the purification procedure.

Enzymatically active dopamine β-hydroxylase is known to occur in human plasma (11). Considerable controversy exists regarding the relationship of the plasma concentration of this enzyme to various disease states, particularly hypertension, and the use of plasma dopamine β-hydroxylase as an index of sympathetic activity in humans (12, 13). During purification of the enzyme from normal human plasma we have observed that gel filtration resolves the enzymatic activity into two species (14). In order to characterize these two species further as well as to resolve the question of the biological relevance of the multiple forms of human dopamine β-hydroxylase, we have examined behavior of this enzyme in both whole human plasma and serum.

MATERIALS AND METHODS

All sedimentation coefficients were determined by ultracentrifugation at 40,000 rpm for 16-20 hr with a Beckman SW 41 rotor on linear (5-20%) sucrose gradients, by the procedure of Martin and Ames (15).

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The gradients also contained 0.15 M NaCl and ionic strength 0.01 potassium phosphate, pH 7.4. β-Galactosidase (Worthington), bovine liver catalase (Calbiochem), rabbit skeletal muscle aldolase (Sigma), and ovalbumin (Schwarz/Mann) were used as standards. Bovine dopamine β-hydroxylase (adrenal medulla) was purified by the procedure of Foldes *et al.* (2) as modified by Wallace *et al.* (10). $s_{20,w}$ values for the standard proteins were from published data (16). $s_{20,w}$ values for bovine and human plasma dopamine β-hydroxylases were calculated from a linear least-squares fit of the $s_{20,w}$ vs. distance migrated data for the standards. Gel filtration studies were performed using a column packed with Ultragel AcA-22 (0.9 × 88 cm) equilibrated with the 0.15 M NaCl–ionic strength 0.01 postassium phosphate buffer pH 7.4. Fifteen-drop fractions were collected, and the column was pumped at 3 ml/hr. This column was standardized with *Busycon canniculatum* hemocyanin (mol wt approximately 6,500,000) and phenol red to mark the excluded and included volumes, respectively. β-Galactosidase, catalase, aldolase, and ovalbumin (see above for sources), as well as apoferritin (Calbiochem), albumin (human serum), peroxidase (Worthington), and myoglobin (Miles), were used as the standards. Myoglobin (2 mg/ml) was used as an internal standard in all chromatographic runs and was detected by its absorbance at 410 nm. Molecular weights and $D_{20,w}$ values for the standard proteins were from published data (16). Stokes radii for the standard proteins were calculated from the expression $A = kT/6\pi\eta D_{20,w}$. Apparent molecular weights and Stokes radii for the dopamine β-hydroxylase species were calculated from linear least-squares fits to the appropriately treated data for the standard proteins (17). The protein contents of both the sample and standard gradients and column fractions were measured by a micro-Lowry procedure (18). Dopamine β-hydroxylase activity (human) was determined with a two-step assay using phenylethanolamine *N*-methyltransferase (19). Saturating amounts of catalase (bovine liver, Calbiochem) were added in order to eliminate any possible artifacts that might arise from the presence of catalase in some of the fractions (20). Each assay tube contained the following components: in step 1, 0.05 M sodium acetate (pH 5.5), 1 mM pargyline, 5 mM *N*-ethylmaleimide, 10 mM sodium fumarate, 6 mM ascorbic acid, 0.4 mM tyramine HCl, 1 μM $CuSO_4$, and 12,000 IU of catalase in a volume of 1 ml; in step 2, 200 mM potassium phosphate (pH 8.6), 67 mM cysteine, 13 mM sodium EDTA, 0.78 mM *S*-adenosyl-L-[*methyl*-^{14}C]methionine (20 mCi/mmole), and 0.005 unit of phenylethanolamine *N*-methyltransferase in a total volume of 1.5 ml.

Reagent-grade chemicals were used whenever possible. Sucrose was ultrapure special enzyme grade (Schwarz/Mann). Ascorbic acid, fumaric acid, and *N*-ethylmaleimide (Aldrich), L-cysteine HCl and *S*-adenosyl-L-methionine iodide (Calbiochem), and pargyline (Abbott Laboratories) were used without further purification. Tyramine HCl (Aldrich) was recrystallized from hot ethanol. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine was obtained from New England Nuclear Corporation. Phenylethanolamine *N*-methyltransferase (bovine adrenal medulla) was purified through the G-100 step according to a slight modification of the procedure of Connett and Kirshner (21), in which the sulfaethyl-Sephadex step was omitted. Bovine dopamine β-hydroxylase was assayed by the procedure of Wallace *et al.* (10).

In the initial sedimentation experiments, freshly obtained plasma was diluted 1:3 with the gradient buffer, and 100–200 μl of the diluted plasma were layered on top of each gradient. After centrifugation approximately 39 five-drop fractions were collected from the bottom of each gradient and stored frozen (−20°) until assayed. In the initial gel filtration experiments, freshly obtained serum was diluted 1:3 with the column buffer, and approximately 0.7–0.8 ml was loaded onto the column. After chromatography the fractions were assayed as soon as possible and then stored at −20° until further use. For the resedimentation and rechromatographic experiments, the appropriate fractions from the initial gradients or column runs

were pooled, dialyzed against the ionic strength 0.01 potassium phosphate–0.15 M NaCl buffer, pH 7.4, and concentrated approximately 10-fold by ultrafiltration, using an XM-50 membrane.

RESULTS

The dopamine β-hydroxylase activity in human plasma could be fractionated into two species by ultracentrifugation on a linear sucrose gradient. The distribution of activity over a typical gradient is illustrated in Fig. 1, which also shows the distribution of total protein. Approximately 75–80% of the activity sedimented in the major band (fraction I), which had an $s_{20,w}$ value of 10.3 ± 0.1 S (Fig. 1, inset, and Table 1). As the data in Table 1 indicate, the predominant species of dopamine β-hydroxylase in human plasma sedimented exactly like the bovine enzyme (adrenal medulla). The remainder of the activity appeared as a shoulder on the low $s_{20,w}$ side of the major band ($s_{20,w}$ 6.8 ± 0.2 S; designated fraction II). Although the data reported here were determined using plasma from one individual with a high plasma dopamine β-hydroxylase content, we have observed similar activity profiles on sucrose gradients with plasma from other individuals with medium to high plasma concentrations of this enzyme. The observed activity profile also appeared to be independent of the age of the plasma, at least up to 3–4 weeks. Thus the apparent existence of two species of active dopamine β-hydroxylase in normal human plasma would appear to be a general phenomenon.

In order to establish whether these two forms of the enzyme were in equilibrium, and also whether both forms were intrinsically active, each fraction was separately resedimented. Resedimentation of fraction I revealed no evidence for a species corre-

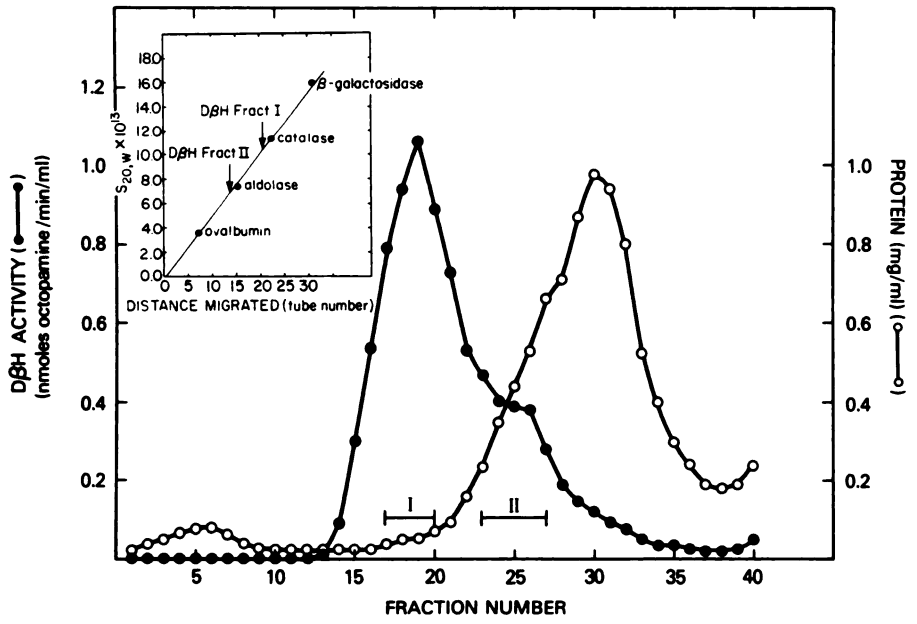

FIG. 1. *Sedimentation of whole human plasma*

Human plasma diluted 1:3 with potassium phosphate (ionic strength 0.01) and 0.15 M NaCl buffer, pH 7.4 (150 μl), was layered over a linear 5–20% sucrose gradient containing the above buffer and then centrifuged at 40,000 rpm and 4° for 20 hr. The dopamine β-hydroxylase (DβH) activity (●——●) and total protein concentration (○——○) of each fraction were determined as described in MATERIALS AND METHODS. Inset: $s_{20,w}$ determination of dopamine β-hydroxylase species in human plasma by sucrose gradient ultracentrifugation. Samples (150 μl) containing either ovalbumin (3.8 mg/ml), aldolase (3.7 mg/ml), catalase (5.1 mg/ml), and β-galactosidase (2.5 mg/ml) or a 1:3 dilution of human plasma were centrifuged and analyzed as described in MATERIALS AND METHODS.

TABLE 1

Sedimentation coefficients of dopamine β-hydroxylase

Dopamine β-hydroxylase	$s_{20,w}$ (S)
Fraction I (whole plasma)	10.3 ± 0.1 (11)[a]
Fraction I (resedimented fraction I)	10.3 ± 0.2 (3)
Fraction I (resedimented fraction II)	10.1 ± 0.2 (3)
Fraction II (resedimented fraction II)	6.8 ± 0.2 (3)
Bovine (adrenal medulla, purified)	10.1 ± 0.1 (6)

[a] Numbers in parentheses represent the number of determinations.

sponding to fraction II (Fig. 2B). All the activity was observed at an $s_{20,w}$ identical with that of the original fraction I. Resedimentation of fraction II (Fig. 2C) showed that most of the activity sedimented with an $s_{20,w}$ identical with that of the original fraction II. However, a distinct band of activity having a sedimentation coefficient identical with that of fraction I was also observed. The presence of some of this species ($s_{20,w}$ = 10.3 S) was to be expected, since originally fraction II had not been cleanly resolved from fraction I (Fig. 2A). In fact, by comparing the normalized activity profiles derived from the curves shown in Fig. 2A and B, the minimum amount of activity associated with the high $s_{20,w}$ dopamine β-hydroylase species contained in fraction II could be estimated. On this basis we can account for over 80% of the activity observed at the position of fraction I when fraction II was resedimented. The results of the two resedimentation experiments make it unlikely that the two species of the enzyme are in equilibrium with each other, and thus support the independent existence of an active species of dopamine β-hydroxylase having an $s_{20,w}$ of 6.8 S. However, the activity profile obtained upon the resedimentation of fraction II clearly indicates that there are only two active species of the enzyme in human plasma.

The dopamine β-hydroxylase activity in human serum could be clearly resolved into two species by gel filtration in our system (Fig. 3). On the average, 78% of the

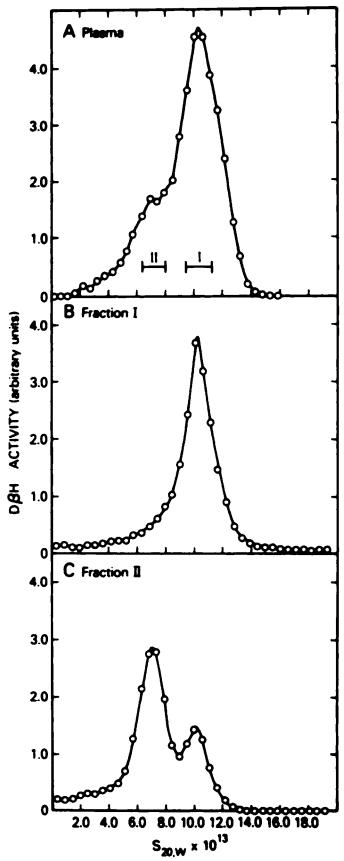

FIG. 2. *Resedimentation of active dopamine β-hydroxylase (DβH) fractions in human plasma on linear 5-20% sucrose gradients*

A. Sedimentation of whole plasma (1:3). B. Resedimentation of pooled and concentrated material (see MATERIALS AND METHODS) from fraction I. C. Resedimentation of pooled and concentrated material (see MATERIALS AND METHODS) from fraction II. Enzymatic activity was measured as described in MATERIALS AND METHODS.

activity was eluted in the major band (fraction I). The remainder of the activity (22%) was eluted as a smaller species (fraction II). No other species having dopamine β-hydroxylase activity was observed. The activity profile observed upon gel filtration was remarkably similar to that seen when whole plasma was sedimented on linear sucrose gradients (Fig. 1). Two peaks of dopamine β-hydroxylase activity were observed, and the major portion of the activity was associated with a larger molecular

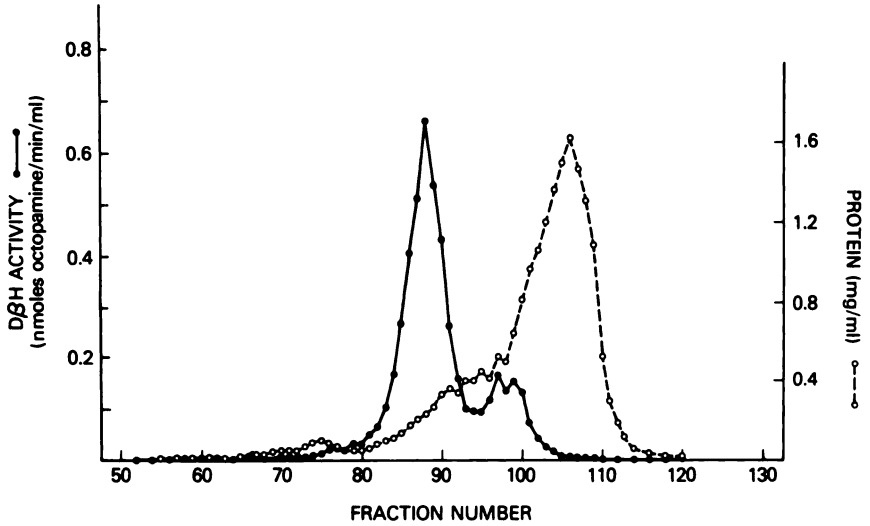

FIG. 3. *Ultragel AcA-22 chromatography of whole human serum*

Human serum diluted 1:3 with potassium phosphate (ionic strength 0.01) and 0.15 M NaCl buffer, pH 7.4 (0.7 ml), was layered on the top of the column and eluted with the same buffer. Dopamine β-hydroxylase (DβH) activity (●——●) and protein content (O- - -O) in each fraction were determined as described in MATERIALS AND METHODS.

species in each case. In addition, the distribution of activity between the larger and smaller species was also practically identical.

Since the initial resolution of these two fractions was good, rechromatographing each fraction separately could provide a conclusive test as to their interconvertibility. Rechromatography of fraction I revealed no evidence for an active species corresponding to fraction II (Fig. 4B). Similarly, rechromatography of fraction II (Fig. 4C) yielded activity only at the elution volume for the original fraction II. In order to check for a possible concentration-dependent association-dissociation phenomenon, the material from fraction I was not concentrated but merely pooled before the rechromatography experiment described above. On the other hand, the material from fraction II was concentrated before application to the column. The activity profiles in Fig. 4B and C indicate that no concentration-dependent effects were observed. Thus, under the conditions of our experiments, both species of the enzyme were independently active and there was no interconversion between them.

The apparent molecular weights of the human serum and bovine dopamine β-hydroxylases were obtained by comparing their elution behavior with those of standard proteins (Fig. 5A). Fractions I and II had apparent molecular weights of 560,000 and 189,000, respectively, while bovine dopamine β-hydroxylase had an apparent molecular weight of 425,000. As was found in the sedimentation studies, fraction I and bovine dopamine β-hydroxylase appeared to be quite similar. The value we found for the apparent molecular weight of the bovine enzyme is 50% larger than the accepted value (1). This discrepancy, however, could be due to an inherent asymmetry in the dopamine β-hydroxylase molecule (see below) or to its being a glycoprotein (22).

A more realistic estimation of the molecular weights of the various dopamine β-hydroxylase species could be obtained by combining both the sedimentation and gel filtration data. According to the theory of Laurent and Killander (17), the elution volume (or K_{av}) of a protein is related to its Stokes radius. Thus we could use the gel filtration data to determine the Stokes radii for the various dopamine β-hydroxylase species (Fig. 5B). The values for the

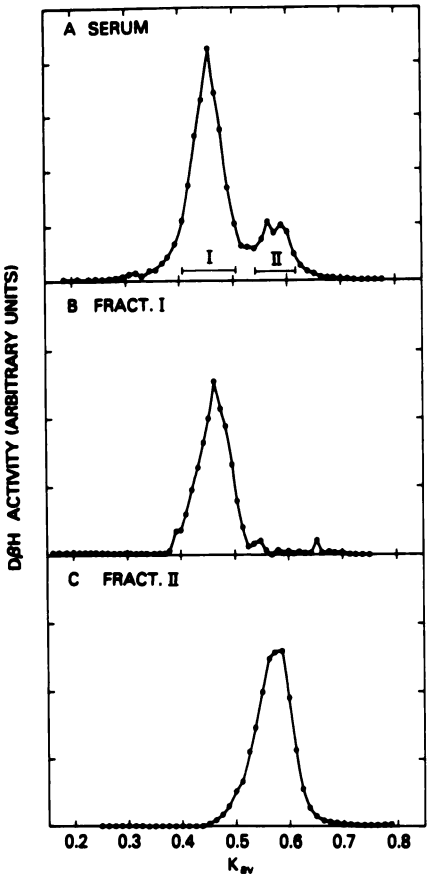

FIG. 4. *Rechromatography of active dopamine β-hydroxylase (DβH) fractions in human serum on Ultragel AcA-22*

A. Elution of whole serum (1:3). B. Rechromatography of pooled material from fraction I. C. Rechromatography of pooled and concentrated material (see MATERIALS AND METHODS) from fraction II. Enzymatic activity was measured as described in MATERIALS AND METHODS.

Stokes radii of fraction I, fraction II, and the bovine enzyme determined in this manner were 65.1 ± 1.4, 50.1 ± 1.2, and 61.3 ± 1.4 A, respectively. These data also indicate that fraction I and bovine dopamine β-hydroxylase are quite similar, although the bovine enzyme appeared to be slightly smaller. In our experiments the bovine enzyme was consistently eluted several fractions behind fraction I of the human serum enzyme.

Based on the independently determined value of the sedimentation coefficient and the Stokes radius, it is possible to calculate the molecular weights of the dopamine β-hydroxylase species (23). The molecular weights we calculated for fractions I and II as well as for the bovine enzyme are summarized in Table 2. The values obtained for fractions I and II, 289,000 and 147,000, respectively, clearly imply that fraction I is a dimer of fraction II. The value we obtained for the bovine enzyme, 267,000, is slightly smaller but in reasonable agreement with the accepted value of 290,000, considering the sensitivity of the calculation to parameters such as $\bar{\nu}$. In our estimation of $\bar{\nu}$ we neglected contributions due to the carbohydrate residues in dopamine β-hydroxylase [4% for the bovine enzyme (10)]. The frictional ratios f/f_0 of around 1.35 indicate that these enzymatic species are rather asymmetrical. Whether this apparent asymmetry reflects the inherent shape of the enzyme molecule or is related to the arrangement and/or solvation of the carbohydrate groups on the surface of the molecule (25) cannot be resolved on the basis of our results. However, either factor could explain why the enzyme was eluted well ahead of its true molecular weight in our experiments.

DISCUSSION

Our findings indicate that it is very likely that there are two independent species of dopamine β-hydroxylase in normal human plasma that are catalytically active. These results are mostly consistent with those of Park *et al.* (8) regarding the nature of dopamine β-hydroxylase isolated from human pheochromocytoma tumors. However, we found no evidence for any active species larger than fraction I in plasma or serum. Thus it is possible that the species ($s_{20,w}$ = 13 S) found by Park *et al.* (8) was an artifact of their purification procedure, as we have observed that treatment of either the bovine or the human plasma enzyme with ammonium sulfate can cause aggregation to species larger than fraction I.[1] Alternatively this discrepancy may reflect the different sources of

[1] R. C. Rosenberg, unpublished observations.

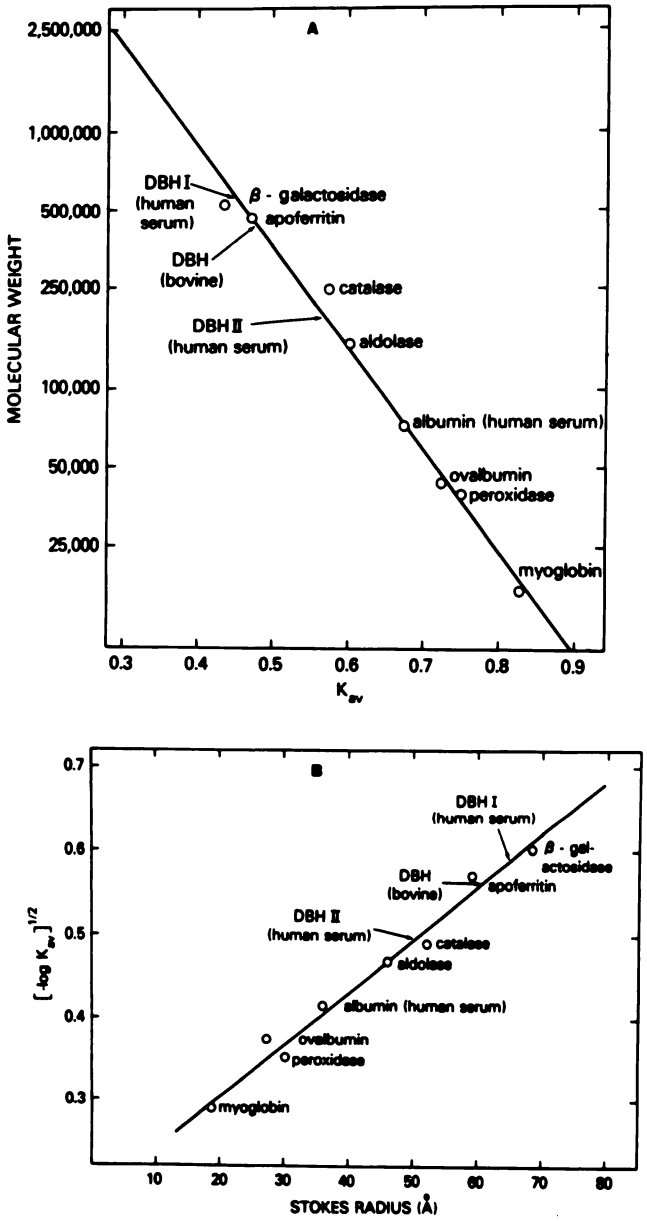

FIG. 5. *Determination of apparent molecular weight and Stokes radius of dopamine β-hydroxylase species by Ultragel AcA-22 chromatography.*

A. Determination of apparent molecular weight of dopamine β-hydroxylase (DBH) species. B. Determination of the Stokes radii of dopamine β-hydroxylase species, using the model of Laurent and Killander (17).

dopamine β-hydroxylase used in these studies.

Our finding that there was no apparent interconversion between the two active species of human plasma dopamine β-hydroxylase is also consistent with the results of Park *et al.* (8) for the human pheochromocytoma enzyme. However, these studies appear to differ from the findings of Stone *et al.* (7), who reported that dopa-

TABLE 2

Molecular parameters of dopamine β-hydroxylase

Species	Stokes radius (A)	$s_{20,w}$	Mol wt[a]	f/f_0[b]
	A	*S*		
Fraction I (human serum)	65.1	10.3	289,000	1.37
Fraction II (human serum)	50.1	6.8	147,000	1.32
Bovine enzyme (adrenal medulla)	61.3	10.1	267,000	1.33

[a] Molecular weights were calculated from the relationship (23)

$$\text{Mol wt} = \frac{6\pi\eta N A s_{20,w}}{(1 - \bar{\nu}\rho)}$$

where η = solvent viscosity, ρ = solvent density, and $\bar{\nu}$ = partial specific volume of the enzyme. $\bar{\nu} = 0.731$ was calculated from the amino acid composition of the bovine enzyme.

[b] Frictional ratio was calculated from the relationship (23)

$$\frac{f}{f_0} = A\left[\frac{4\pi N}{3 \times \text{mol wt}\,(\bar{\nu} + \delta/\rho)}\right]^{1/3}$$

where δ = solvation factor, with an assumed value of 0.2 (24).

mine β-hydroxylase from human pheochromocytoma reversibly dissociates or aggregates as a function of enzyme concentration. Since the conditions of pH and ionic strength of these three studies were not comparable, the apparent inconsistency in the behavior of the human enzymes could reflect the different conditions under which the experiments were done. Alternatively, these differences could reflect the fact that we could detect only enzymatically active dopamine β-hydroxylase. A systematic study of the effects of pH and ionic strength on human dopamine β-hydroxylase is clearly needed before these apparent inconsistencies can be completely resolved.

The values we found for the sedimentation coefficients of both the human plasma (10.3 S for fraction I) and bovine dopamine β-hydroxylases (10.1 S) differ significantly from the values reported by Park *et al.* (8) (8.9 S for both the bovine and human enzymes). As mentioned above, however, there are considerable differences in the experimental conditions employed in these two studies. Our experiments were done at pH 7.4 and an ionic strength of 0.15 in order to model the conditions in plasma, while theirs were done at pH 6.5 and an ionic strength of less than 0.01. In this regard, the value we found for the sedimentation coefficient of bovine dopamine β-hydroxylase is identical with the value (10.2 ± 0.1 S) found by Foldes *et al.* (26) at pH 7.2 and an ionic strength of 0.1, whereas Friedman and Kaufman (1), working at pH 6.8 and an ionic strength of 1.0, found the sedimentation coefficient of the bovine enzyme to be 8.9 S. These results clearly indicate that the sedimentation coefficient of bovine dopamine β-hydroxylase is dependent on pH and/or ionic strength. Yet all workers have consistently obtained a molecular weight of around 290,000 for the bovine enzyme. Thus it is possible that the shape of the enzyme in solution is sensitive to changes in the surrounding medium. Similar behavior was observed for the principal species of human dopamine β-hydroxylase. It is apparent that under specific conditions of pH and ionic strength the bovine enzyme and the principal species of human dopamine β-hydroxylase (our fraction I) are practically indistinguishable, at least as regards their sedimentation and elution properties.

Since the native form of bovine dopamine β-hydroxylase is known to be tetrameric (9, 10), and since the bovine and human enzymes appear to be so similar, it is reasonable to assume that the species in plasma we have called fraction I is tetra-

meric, as has been proposed for the pheochromocytoma enzyme (7, 8).[2] On this basis we tentatively conclude that the species we have called fraction II is an active dimer of dopamine β-hydroxylase. These species correspond to fraction II (tetramer) and fraction I (dimer) identified by Park *et al.* (8) in their preparation of dopamine β-hydroxylase from human pheochromocytoma tissue.

Craine *et al.* (9) found that native bovine adrenal dopamine β-hydroxylase consisted of two dimers linked by hydrogen bonds and that each dimer was made up of two monomers linked to each other by disulfide bonds. To our knowledge no one has found activity associated with the dimeric form of bovine dopamine β-hydroxylase. Our results indicate that the dimeric species of human dopamine β-hydroxylase occurs *in situ* in an active form (at least in plasma). The present data are insufficient to resolve the question whether the dimer and the tetramer of the human enzyme have identical catalytic properties. Our results and those of Park *et al.* (8) indicate only that the dimeric form of the enzyme is active. Park *et al.* (8) found a lower specific activity for the dimer, but unfortunately they did not analyze their preparations for copper. Our work, using whole plasma, precluded such an analysis because of the presence of other copper-containing proteins, notably ceruloplasmin. Resolution of this question will have to await a determination of both the specific activity and metal content of the purified dimeric and tetrameric human dopamine β-hydroxylase species.

The genesis of the two species of dopamine β-hydroxylase we have found in plasma is not clear. Our results and those of Park *et al.* (8) indicate that a dissociative mechanism is unlikely, since no interconversion of the two enzymatic species was evident over a relatively wide range of conditions. While it is possible that the tetrameric form of the enzyme was degraded between the time the blood was drawn and the plasma was fractionated on the sucrose gradients, we do not consider this to be very likely. If the dimeric form of the enzyme had been generated after the blood sample was taken, we would have expected to see some time dependence in the relative amounts of enzymatic activity in the two forms. However, as mentioned above, the relative proportions of the two forms of the enzyme in plasma remained constant. Thus we conclude that both the dimeric and tetrameric forms of the enzyme exist in the bloodstream. Currently available data do not permit us to eliminate the possibility that the dimeric form of dopamine β-hydroxylase results from some degradative or dissociative process occurring before the enzyme enters the bloodstream. In this regard Stone *et al.* (7) have reported that human pheochromocytoma dopamine β-hydroxylase reversibly dissociates to dimers as a function of enzyme concentration at pH 5.0. Chromaffin storage vesicles (bovine adrenal medulla) have been reported to have an internal pH of 5.5 (27). A similarly acidic environment at the site(s) of origin of human plasma dopamine β-hydroxylase (nerve terminal storage vesicles) could give rise to some of the dimeric form of the enzyme. This distribution of dimeric and tetrameric species could become fixed when the enzyme encounters the more neutral pH values of the extracellular body fluids, giving rise to the mixture of species we have observed in plasma.

[2] On several occasions we have purified human plasma dopamine β-hydroxylase almost to homogeneity. Analysis of these preparations by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol revealed a high proportion of a protein species with a molecular weight of around 75,000 (W. Lovenberg and E. A. Bruckwick, unpublished observations).

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