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Human Pheochromocytoma Dopamine-*Beta*-Hydroxylase: Purification and Molecular Parameters of the Tetramer

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SUMMARY

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Several investigators have reported multiple molecular forms of dopamine-β-hydroxylase purified from human pheochromocytoma and human plasma. We purified the soluble dopamine-β-hydroxylase from the chromaffin vesicles of two human pheochromocytomas, using carbohydrate affinity chromatography on Concanavalin A-Sepharose. The purified products were electrophoretically homogeneous. No evidence of multimeric forms of the enzyme were found by analytical polyacrylamide gel electrophoresis, preparative polyacrylamide gel electrophoresis with activity elution, analytical gel filtration chromatography, or zonal sedimentation on sucrose density gradients. By combining the hydrodynamic parameters of $s_{20,w}$ and Stokes radii, the tumor enzyme was characterized as having a molecular mass of 300,000 to 317,000 daltons, as compared to the bovine adrenomedullary enzyme at 282,000 daltons. Its frictional ratio was 1.68 to 1.69, leading to anomalously high molecular weights when estimated by gel filtration alone. The tetrameric enzyme was composed of monomeric subunits of 78,000 to 80,500 daltons each, joined by disulfide linkages to form dimeric subunits of 155,000 to 160,000 daltons, two of which join by noncovalent interaction to form each tetramer.

INTRODUCTION

The molecular forms of the enzyme dopamine-*beta*-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating) EC 1.14.17.1) in human tissues have been the subject of much recent interest. Several groups (1-3) including our own (4) have recently reported in human plasma 2 catalytically active species of the enzyme, corresponding in size to the intact tetramer (molecular weight 290,000 to 300,000) and the dimer (molecular weight 142,000 to 147,000).

In human pheochromocytoma, the published observations are, however, conflicting. One of us, in publishing the initial purification to homogeneity of the enzyme from human pheochromocytoma (5) observed that, while the enzyme elutes from gel filtration media as a single symmetric peak, suggesting one molecular form, its behavior during sedimentation equilibrium in the analytical ultracentrifuge suggests several molecular forms weighing 145,000 to 500,000. This contrasts with the bovine adrenomedullary enzyme, which is a tetramer of weight 290,000, over a wide concentration range, as determined by sedimentation equilibrium (5).

In contrast, Park *et al.* (6) claim to have isolated from human pheochromocytoma

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at least three distinct forms of the enzyme on sucrose density gradients. Their estimated sedimentation coefficients were 6.20, 8.96, and 13.47, with molecular weights judged very approximately (from sedimentation coefficient alone) to be 164,000, 286,000 and 524,000, respectively. Furthermore, on resedimentation, these multiple forms of active dopamine-β-hydroxylase appeared not to be readily interconvertible. The possibility that these forms were artifacts of their purification process has not, however, been excluded.

Because the human plasma enzyme does exist in two forms, dimer and tetramer (1-4) and because the adrenal gland is one possible source of the plasma enzyme, we elected to study the possibility of multiple forms of the enzyme from a rich source of human adrenal dopamine-β-hydroxylase, the tumor pheochromocytoma. Our approach to the problem included: (a) purifying to homogeneity the enzyme from two separate tumors by Concanavalin A affinity chromatography, (b) examining the purified product by gel electrophoresis to see if enzymatic activity is present in one or multiple bands and (c) characterizing the chromaffin vesicle lysate by zonal sucrose density gradient velocity sedimentation and by gel filtration, to estimate molecular weights of all dopamine-β-hydroxylase species present without introduction of purification procedure artifacts.

MATERIALS AND METHODS

Reagents. All chemicals were reagent grade unless otherwise specified. For standardization of molecular weight determinations, the following materials were used: phosphorylase A, horse spleen apoferritin, horse skeletal muscle myoglobin, ovalbumin, bovine liver catalase and bovine serum albumin were purchased from Sigma Chemical Company. Chymotrypsinogen, ribonuclease A, rabbit skeletal muscle aldolase, and blue dextran were purchased from Pharmacia Fine Chemicals.

Bovine adrenal medullary dopamine-β-hydroxylase was prepared by the method of Foldes *et al.* (7). Human hemoglobin was prepared by freeze-thaw lysis of human red blood cells, followed by centrifugal membrane removal.

Assays. Protein was determined by the method of Bradford (8). Dopamine-β-hydroxylase activity was determined by the spectrophotometric method of Nagatsu and Udenfriend (9) using tyramine as substrate, with enzyme activities expressed in units of micromoles of octopamine formed per minute (per milligram of protein, for *specific* activities).

Tumors. Two pheochromocytoma tumors, from two patients, were obtained at the time of surgical excision and placed on ice immediately. Tumor 1 weighed 17.0 g, and 2 weighed 17.5 g.

Preparative methods. Each tumor enzyme was purified and characterized separately. Chromaffin vesicles were prepared from the tumors by the method of Stone *et al.* (5). The purified vesicle pellets were lysed in ice-cold 0.005 M sodium phosphate, pH 6.5, whereupon the lysate was centrifuged at 100,000 $\times$ *g* for 1 hour in a Beckman model L-2-65B ultracentrifuge, to separate soluble from membrane-bound enzyme. Enzyme activity was measured in the lysate before and after centrifugation (to determine the relative amounts of membrane bound and soluble enzyme). Thereafter, only the soluble form of the enzyme was further purified and characterized.

Concanavalin A-Sepharose chromatography. The lysate from tumor 1 was dialyzed extensively against 0.5 M NaCl, 0.01 M sodium phosphate pH 6.5, then pumped at 10 ml/hour onto a 10 $\times$ 0.9 cm column of Concanavalin A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.) previously equilibrated with the same buffer. The column was washed with the same buffer until the absorbance of the eluate at 280 nm declined to zero. Then the column was eluted with 10% (w/v) α-methyl-D-mannopyranoside in 0.5 M NaCl, 0.01 M sodium phosphate, pH 6.5, at 3 ml/hour, collecting 2 ml fractions. To the lysate of tumor 2 (30 ml) was added 3 ml of 0.1 M sodium phosphate, pH 6.5, to yield a final low ionic strength solution buffer of 0.01 M sodium phosphate, pH 6.5. The lysate was then adsorbed onto a 10 $\times$ 0.9 cm column of Concanavalin A-Sepharose equilibrated

with the same buffer. The column was washed with this buffer until the absorbance of the eluate at 280 nm declined to zero; then the column was eluted with 10% (w/v) α-methyl-D-mannopyranoside in 0.01 M sodium phosphate, pH 6.5, at 6 ml/hour, collecting 2 ml fractions.

Gel filtration chromatography. The peak fractions (10.4 ml) from the Concanavalin A-Sepharose elution of the lysate from tumor 2 were pooled and concentrated to a volume of 2 ml on an Amicon ultrafiltration device using a YM-10 membrane (Amicon Corporation, Lexington, Mass.). This sample was then applied to a 90 × 1.5 cm column of Sephacryl S-200 gel filtration resin (Pharmacia Fine Chemicals, Piscataway, N.J.), equilibrated with 0.01 M sodium phosphate pH 6.5 and eluted with the same buffer at 8 ml/hour, collecting 2 ml fractions.

Analytical Methods. Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis, to assess purity of native enzyme preparations, was performed according to the method of Davis (10). After running, gels were stained with Coomassie blue G-250 in trichloroacetic acid, and destained in 5% acetic acid (11). When eluting gels for enzyme activity after electrophoresis, the gels were first pre-electrophoresed in 0.42 M Tris buffer, pH 8.9, at 4 mA/gel for 2 hours in order to remove excess persulfate (12) and then run at 4°. When enzymatic activity peaks were desired, the pre-electrophoresed gels were run, and then, rather than staining, they were sliced in 5 mm pieces, crushed and eluted overnight at 4° in 200 μl of 0.3 M Na phosphate, pH 6.5. 100 μl of supernatant over the crushed gel was assayed, as previously described (9).

For determinations of subunit molecular weights, the sodium dodecyl sulfate phosphate system of Weber and Osborne (13) was used and calibrated with protein standards. Multimeric aldolase molecular weight standards were prepared by the dimethylsuberimidate cross-linking method of Davies and Stark (14). For detection of intersubunit disulfide bonds, each purified protein was run both in the presence and absence of β-mercaptoethanol. Sodium dodecyl sulfate gels were stained with Coomassie blue R-250 in 10% acetic acid and 45% methanol, and destained in 7½ acetic acid.

Sucrose Gradient Ultracentrifugation. A modification of the method of Martin and Ames (15) was used. Five hundred microliter aliquots of each chromaffin vesicle lysate, with no further purification, were centrifuged on sucrose gradients in a Beckman model L-2-65B ultracentrifuge, using an SW-41 rotor. Isokinetic sucrose gradients of 10–30% were prepared in 0.1 M NaCl, 0.01 M sodium phosphate, pH 6.5. It should be noted that the usual gradients of 5–20% used in the SW-39 rotor specified by Martin and Ames are not isokinetic in the longer SW-41 rotor (16) because a steeper gradient is needed in the longer tube to prevent convective disturbances (17). After centrifugation at 40,000 rpm for 19 hours at 4°, the centrifuge was stopped without braking, and 460 μl fractions were collected. Dopamine-β-hydroxylase activity and protein were measured in each fraction. Standard proteins run simultaneously were bovine serum albumin, bovine dopamine-β-hydroxylase (7), rabbit skeletal muscle aldolase, and bovine liver catalase. Reference $s_{20,w}$ values were obtained from the literature (18–20). The $s_{20,w}$ values for the bovine and human dopamine-β-hydroxylases were obtained by interpolation from a linear least squares fit of the plot of $s_{20,w}$ versus distance migrated for the standards.

Analytical gel filtration chromatography. Analytical gel filtration, using the theory of Laurent and Killander (19), as applied by Siegel and Monty (20), was performed on an 89 × 1.6 cm column of Ultrogel ACA-22 resin equilibrated in 0.1 M NaCl, 0.01 M Na phosphate pH 6.5. This resin was chosen because dopamine-β-hydroxylase is well separated from the void volume (1), facilitating determination of molecular weight and Stokes radius by interpolation between appropriate standard proteins. Five hundred microliter aliquots of the chromaffin vesicle lysate of each pheochromocytoma, with no further purification, were chromatographed in the above buffer, at 8 ml/hour and 1.5 ml fractions were collected. Dopamine-β-hydroxylase activity was measured in the eluted fractions. Standard proteins run on this column were

bovine pancreatic ribonuclease A, equine skeletal muscle myoglobin, human hemoglobin, human serum albumin, rabbit skeletal muscle aldolase, bovine liver catalase, horse spleen apoferritin, bovine fibrinogen, and bovine adrenal dopamine beta hydroxylase (7). Blue dextran and KCl were used to determine the void volume and total internal volume of the column, respectively. K_{av} values for each protein were computed from the relation $K_{av} = Ve - Vo/Vt - Vo$, where Ve = the elution volume of the species in question, Vo = void volume of the column (66.0 ml by calibration with blue dextran) and Vt = the total volume of the column, 178.9 ml. Reference molecular weights and Stokes radii for the standards were obtained from the literature (18–20). Apparent molecular weights for each dopamine-β-hydroxylase species were determined by interpolation following linear least squares analysis of the plot of K_{av} versus molecular weight for the standards. Stokes radius for each dopamine-β-hydroxylase species was obtained by interpolation from a linear least squares fit of the plot of $(-\log_{10} K_{av})^{1/2}$ versus Stokes radius for the standards (19, 20).

Determination of molecular weight and frictional ratio. Determination of molecular weight (M) and frictional ratio (f/f_o) for each species of dopamine-β-hydroxylase was done by combining the hydrodynamic parameters of the enzyme derived from the separate methods of sedimentation velocity $s_{20,w}$ and gel filtration (Stokes radius) (20). $s_{20,w}$ and Stokes radius were obtained for each species by the interpolative methods noted above.

Molecular weights for each species of dopamine-β-hydroxylase (human pheochromocytoma tumors 1 and 2, and bovine adrenal) were calculated from the expression (20):

$$M = 6\pi\eta N A s_{20,w}/(1 - \bar{v}\rho)$$

where M = molecular weight in grams/mole, η = solvent viscosity in poise (g/cm·sec), N = Avogadro's number in molecules/mole, A = Stokes radius in cm, $s_{20,w}$ = sedimentation coefficient in Svedberg units (10^{-13} sec), $\bar{v}$ = partial specific volume in cm^3/g, and ρ = density of medium in g/cm^3. $\bar{v}$ for bovine dopamine-β-hydroxylase, calculated from the amino acid composition (21), is 0.731. $\bar{v}$ for human pheochromocytoma dopamine-β-hydroxylase, calculated from our previously reported amino acid composition (5), is 0.72. For each species of dopamine-β-hydroxylase so analyzed, the A value was that obtained from analytical gel filtration chromatography, while the $s_{20,w}$ value was that obtained from sucrose gradient ultracentrifugation.

The frictional ratio for each dopamine-β-hydroxylase species is calculated from the expression (20):

$$f/f_o = A \Big/ \left(\frac{3\bar{v}M}{4\pi N}\right)^{1/3}$$

where f/f_o = frictional ratio (dimensionless) and other symbols are as noted above. For each dopamine-β-hydroxylase analyzed, A was obtained from analytical gel filtration chromatography and M by the expression set forth above.

RESULTS

Enzyme purification. The enzyme was purified to homogeneity in each case. The initial step involved removal of the chromogranins by Concanavalin A-Sepharose affinity chromatography. During purification of the enzyme from tumor 1, 89% of the initial activity was lost during dialysis of the lysate against high salt buffer prior to Concanavalin A affinity chromatography. Subsequent studies indicated that elimination of the high salt dialysis step obviated this activity loss. Adsorption of the dialyzed lysate to Concanavalin A Sepharose resulted in complete retention of enzymatic activity, and elution with α-methyl-D-mannoside yielded 62% of the applied enzymatic units. The product migrated as one band on analytical polyacrylamide gel electrophoresis (Fig. 1), and catalyzed the formation of octopamine from tyramine at a rate of 0.40 μmoles/min/mg protein.

The lysate from tumor 2 was adjusted to pH 6.5 with buffer containing no salt and immediately adsorbed to Concanavalin A-Sepharose with complete retention of activity. Fifty percent of the applied activity could be eluted with α-methyl-D-manno-

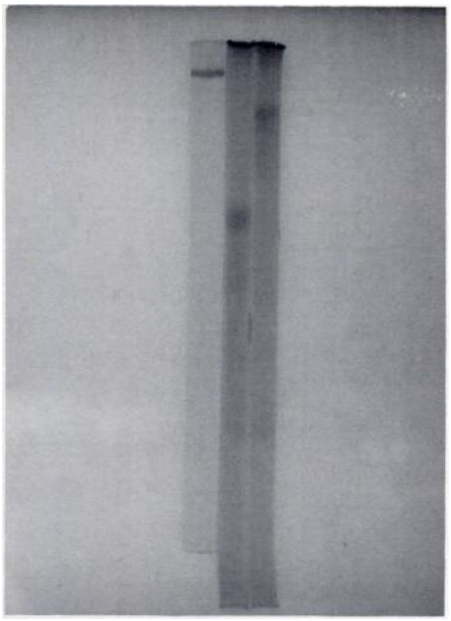

FIG. 1. *Polyacrylamide gel electrophoresis of purified pheochromocytoma dopamine-β-hydroxylase*

From left to right, gel 1 is a native gel (8) representing tumor 1 dopamine-β-hydroxylase after Concanavalin A-Sepharose. Gels 2 and 3 are SDS-phosphate gels (15) representing β-mercaptoethanol reduced tumor 1 dopamine-β-hydroxylase and unreduced tumor 1 dopamine-β-hydroxylase. Migration is from top to bottom.

side. The elution profile from Concanavalin A Sepharose was virtually identical to that of the Tumor 1 enzyme. The product, on analytical gel electrophoresis, showed 3 bands, only the slowest migrating of which possessed enzymatic activity (*vide infra*). The peak fractions were concentrated and applied to the Sephacryl S-200 column and the peak activity fractions from gel filtration had a specific activity of 2.34 units/mg protein, and migrated as one band on analytical disc gel electrophoresis.

Enzyme characterization. Polyacrylamide gel electrophoresis of the enzyme from tumor 1 after Concanavalin A-Sepharose chromatography showed a single band (Fig. 1). The enzyme from tumor 2 showed 3 bands after the Concanavalin A step, only one of which had enzymatic activity (Fig. 2). Further purification on S-200 yielded one protein band (not shown), corresponding to the gel electrophoretic activity peak (Fig. 2).

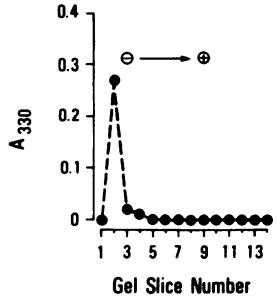

FIG. 2. *Preparative polyacrylamide gel electrophoresis of tumor 2 dopamine-β-hydroxylase*

Enzymatic activity (●) in each gel slice is indicated by absorbance at 330 nm after spectrophotometric assay.

SDS-polyacrylamide gels were performed with and without BME[1] (Fig. 1) and molecular weights were estimated by interpolation between migration values of standard proteins (Fig. 3). With BME, subunit molecular weights for the enzyme from tumors 1 and 2 were 78,000 and 80,500 daltons, respectively. Without β-mercaptoethanol, species of slower migrating apparent molecular weights of 155,000 and 160,000 daltons were noted, suggesting unreduced dimer in each case. For the bovine enzyme, reduced and unreduced molecular weights were 73,000 and 145,000 respectively (Figure 3).

Sucrose gradient sedimentation for the enzymes in the chromaffin vesicle lysates, unsubjected to any possible purification artifacts, as well as for purified bovine hydroxylase, are displayed in Figure 4, and showed a single activity peak in each case. $S_{20,w}$ values were obtained for the two human pheochromocytoma enzymes and for a purified bovine hydroxylase by interpolation between sedimentation distances of standard proteins (Fig. 5). $S_{20,w}$ values for tumors 1 and 2 were 10.0 and 10.3 respectively (Table 1), and that for the bovine enzyme was 10.0.

The technique of gel filtration chromatography was used to estimate apparent molecular weights and Stokes radii, according to the theory of Laurent and Killander (19) as applied by Siegel and Monty (20) for the purified bovine hydroxylase and for

[1] The abbreviation used is: BME, β-mercaptoethanol.

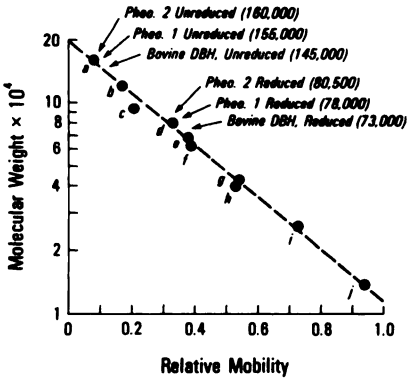

FIG. 3. *SDS-phosphate polyacrylamide gel electrophoresis of human pheochromocytoma and bovine dopamine-β-hydroxylase species*

The relative mobilities of the β-mercaptoethanol reduced and unreduced enzymes are shown on a scale calibrated with molecular weight standards as follows: (1) aldolase tetramer (160,000); (b) aldolase trimer (120,000); (c) phosphorylase A (94,000); (d) aldolase dimer (80,000); (e) bovine serum albumin (69,000); (f) catalase (60,000); (g) ovalbumin (43,000); (h) aldolase (40,000); (i) chymotrypsinogen (25,700); (j) ribonuclease A (13,700).

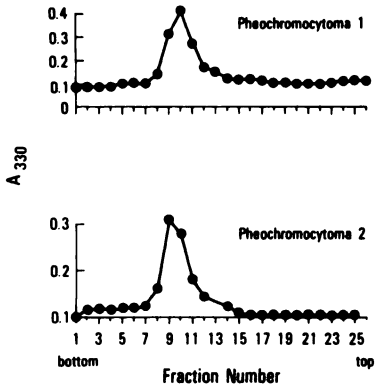

FIG. 4. *Sucrose gradient ultracentrifugation of chromaffin vesicle lysate dopamine-β-hydroxylase from the two pheochromocytomas*

Enzyme activity (●) is indicated by absorbance at 330 nm after spectrophotometric assay.

the undisturbed chromaffin vesicle lysate enzymes of the tumors. A plot of apparent molecular weights versus K_{av} for the standard proteins and the dopamine-β-hydroxylase samples is shown in Figure 6. The apparent molecular weights for tumor enzymes 1 and 2 were 461,000 and 510,000 daltons, respectively, whereas the apparent

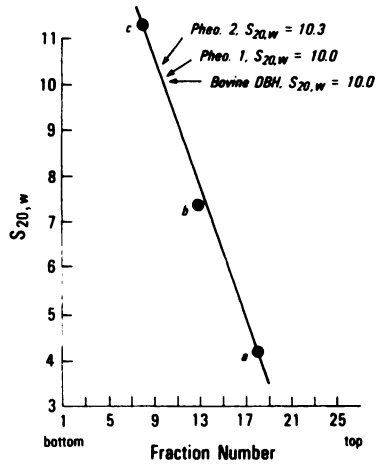

FIG. 5. *Estimation of the sedimentation coefficient of pheochromocytoma and bovine dopamine-β-hydroxylase*

$S_{20,w}$ is the sedimentation coefficient obtained from calibration with $S_{20,w}$ standards: (a) bovine serum albumin (4.20); (b) aldolase (7.35); (c) catalase (11.3).

molecular weight for the bovine hydroxylase was 324,000. The activity elution peak for each tumor enzyme was single and symmetric (Fig. 7).

Stokes radii were obtained by interpolation on a plot of $(-\log_{10}K_{av})^{1/2}$ versus Stokes radii for several standard proteins (Fig. 8). The values obtained for tumor enzymes 1 and 2 were 74.3 and 76.1 Å, respectively, while the bovine enzyme was 67.1 Å.

A molecular weight was determined for each enzymatic species by combining the independently derived hydrodynamic parameters $S_{20,w}$ and Stokes radius. Molecular weights for tumor enzymes 1 and 2 were 300,000 and 317,000, respectively, while bovine dopamine-β-hydroxylase yielded a value of 282,000 (Table 1).

Frictional ratios, derived from the molecular weight and Stokes radius, were 1.68 and 1.69 for tumor enzymes 1 and 2, while the bovine enzyme f/f_o was 1.55 (Table 1).

The molecular weight estimations for subunit, disulfide-linked subunit, and native species for both pheochromocytoma enzymes, with the bovine enzymes for comparison, are shown in Table 2.

DISCUSSION

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TABLE 1

Molecular parameters of dopamine-β-hydroxylase

Species	Strokes radius Å	$S_{20,w}$	Molecular weight	f/f_o
Human pheochromocytoma (tumor 1)	74.3	10.0	300,000	1.68
Human pheochromocytoma (tumor 2)	76.1	10.3	317,000	1.69
Bovine enzyme (adrenal medulla)	67.1	10.0	282,000	1.55

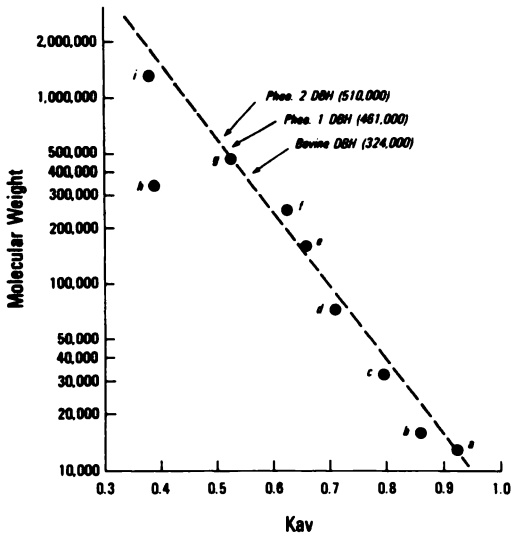

FIG. 6. *Estimation of apparent molecular weight of pheochromocytoma and bovine dopamine-β-hydroxylase on an Ultrogel ACA-22 gel filtration column*

Molecular weights are obtained from a column calibrated with: (a) ribonuclease A (13,700); (b) myoglobin (16,000); (c) hemoglobin half-mer (32,000); (d) human serum albumin (72,000); (e) aldolase (160,000); (f) catalase (250,000); (g) apoferritin (467,000); (h) fibrinogen (340,000); (i) Fe-ferritin complex (1,300,000). The regression line was calculated for globular proteins, excluding fibrinogen.

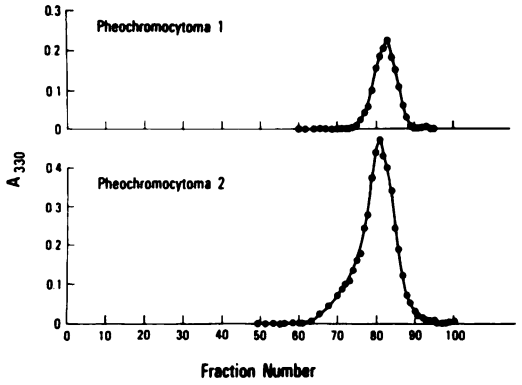

FIG. 7. *Gel filtration chromatography of pheochromocytoma chromaffin vesicle lysate and bovine dopamine-β-hydroxylase on Ultrogel ACA-22*

Enzyme activity (●) is indicated by absorbance at 330 nm after spectrophotometric assay.

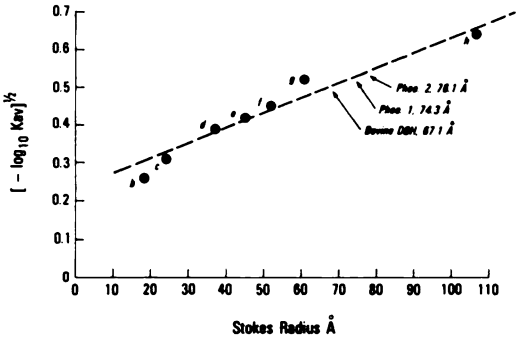

FIG. 8. *Determination of Stokes radius for pheochromocytoma and bovine dopamine-β-hydroxylase on the Ultrogel ACA-22 column calibrated with protein standards of known Stokes radius, in Å*

(b) Myoglobin (18.8); (c) hemoglobin half-mer (24); (d) human serum albumin (37); (e) aldolase (45); (f) catalase (51); (g) apoferritin (61); (h) fibrinogen (107).

other species seems to exist as a tetramer with molecular weight of approximately 290,000 (21, 22), there has been uncertainty as to possible multimeric states of human dopamine-β-hydroxylases. Several groups have reported enzymatically active dimer and tetramer in human plasma (1–4). Human pheochromocytoma dopamine-β-hydroxylase is described by Park *et al.* (6) as being composed of active, noninterchangeable dimer, tetramer and octamer. In contrast, Stone *et al.* (5) report reversible self-association and dissociation of the human pheochromocytoma enzyme in the analytical ultracentrifuge, with observed molecular weights ranging from 145,000 to 500,000.

We addressed this issue in several ways: Dopamine-β-hydroxylase was purified to homogeneity from two pheochromocytoma tumors. In each case, analytical polyacryl-

TABLE 2

Molecular weight estimations for bovine and pheochromocytoma dopamine-β-hydroxylase using hydrodynamic parameters and SDS gel electrophoresis, with and without β-mercaptoethanol

Enzyme Source	SDS with β-mercaptoethanol (monomer)	SDS without β-mercaptoethanol (dimer)	Hydrodynamic (gel filtration + sedimentation velocity) M (tetramer)
Pheochromocytoma 1	78,000	155,000	300,000
Pheochromocytoma 2	80,500	160,000	317,000
Bovine adrenal medulla	75,000	145,000	282,000

amide gel electrophoresis of the purified product revealed one homogeneous band; this finding argued against multimeric forms of the active enzyme. Furthermore, both sucrose gradient sedimentation and gel filtration of each chromaffin vesicle lysate showed one symmetric peak without auxiliary peaks or shoulders. Finally, elution of enzymatic activity from preparative polyacrylamide gel electrophoresis showed one symmetric peak of activity. These four consistent observations on each of two tumor enzymes weight heavily in favor of a single enzymatically active form of soluble pheochromocytoma dopamine-β-hydroxylase and preclude the probability of multimeric forms of enzymatically active protein in these tissues. These studies were performed on the soluble vesicle lysate protein in each case and need not necessarily pertain to the membrane bound enzyme.

Our results are compatible with those of Stone *et al.* (5) because they described a symmetric peak of enzymatic activity eluting from the gel filtration column used in their experiments. Their analytical ultracentrifuge data on purified pheochromocytoma dopamine-β-hydroxylase cannot immediately be reconciled with our studies on the pheochromocytoma enzyme, however. The introduction of artifacts during their purification scheme cannot be precluded.

Likewise, the results of Park *et al.* (6) appear to be inconsistent with ours. Several aspects of their characterization differ from ours: (a) their enzyme preparation utilized detergent solubilized tissue and hence released membrane bound hydroxylase, the properties of which may well be different from those of the soluble form of the enzyme (23); (b) their purification procedure involved precipitation of the enzyme with ammonium sulfate, a step that has been observed to cause the aggregation of human dopamine-β-hydroxylase to high molecular forms (1); (c) gradient centrifugation in the study by Park *et al.* was performed in 5–20% sucrose gradients in an SW-40 rotor. Recent studies (16, 17) indicate that a shallow 5–20% gradient, originally designed for the short radius SW-39 rotor, is not isokinetic in the longer SW-40 rotor and may give rise to convective disturbances. Doubt is thus cast on the reliability of their $s_{20,w}$ determinations.

We utilized the independently determined hydrodynamic parameters, $s_{20,w}$ and Stokes radius, to calculate native molecular weight (20). Molecular weights of 300,000 and 317,000 were obtained for the two pheochromocytoma enzymes, with a molecular weight of 282,000 for the bovine enzyme. These data are in reasonable agreement with our previous determinations (4), and those of Rosenberg and Lovenberg (1), on the human plasma and bovine hydroxylase, using these same techniques. We reported a human plasma enzyme and a bovine enzyme molecular weight of 302,000 and 268,000, respectively; the values obtained by Rosenberg and Lovenberg were 289,000 and 267,000 respectively. Differences may be due to measurement problems inherent with these techniques (1).

The consistent (1, 4) observation that human dopamine-β-hydroxylases are larger than bovine dopamine-β-hydroxylases is of interest. In the current study, as well as past studies of the human plasma enzyme and the bovine enzyme, $s_{20,w}$ values are similar, whereas the human enzymes appear to have larger Stokes radii (1, 4). In our study, the Stokes radii of the pheochromocytoma enzymes were 74.3 and 76.1 Å, both larger than the bovine value of 67.1 Å. The observed Stokes radius differences between the two tumor enzymes were small but reproducible upon repeated gel filtration experiments. The significance of these small

differences is not known but could represent differences in the primary structures of the proteins, as well as minor differences in the carbohydrate side chain composition of these tumor products.

The frictional ratios of the human pheochromocytoma enzyme, 1.68 and 1.69 were also larger then the 1.55 obtained for the bovine enzyme, also consistent with plasma enzyme observations (1, 4). The high frictional ratios may be related to a high degree of solvent hydration of the enzymes (24), to carbohydrate side chain induced asymmetry (1), or to asymmetry inherent in the quaternary structure of the protein. These possibilities cannot be distinguished on the basis of our data.

The molecular asymmetry and consequent large frictional ratios of the dopamine-β-hydroxylase species give rise to high Stokes radii and elution from gel filtration media earlier than would be expected on the basis of actual molecular weight. For example, bovine dopamine-β-hydroxylase—with a molecular weight of 290,000 by sedimentation equilibrium (21, 22) and 282,000 by combined hydrodynamic parameters—when interpolated in the plot of globular protein molecular weights versus K_{av} for the analytical gel filtration column shows an apparent molecular weight of 324,000. Likewise, the pheochromocytoma dopamine-β-hydroxylase, with a molecular weight of 300,000 to 317,000, had an apparent molecular weight by gel filtration alone of 461,000 to 510,000 (Fig. 7). Similar anomalously high apparent molecular weights by gel filtration column have been observed for the plasma enzyme (1, 4).

Our purification scheme demonstrated some difference between the proteins in the two pheochromocytoma chromaffin vesicle lysates. Dopamine-β-hydroxylase could be obtained from the lysate of tumor 1 in homogeneous form using Concanavalin A alone, whereas three proteins from the lysate of tumor 2 (two without enzymatic activity), bound to Concanavalin A, necessitating the additional step of gel filtration before an electrophoretically homogeneous preparation was obtained. The enzymatically inactive, Concanavalin A bound protein bands may represent dopamine-β-hydroxylase degradation products, or other glycoproteins in the chromaffin vesicle lysate.

The complete interaction with immobilization of each pheochromocytoma enzyme by Concanavalin A indicates that each tumor enzyme is a glycoprotein, and specifically a glycoprotein with terminal mannose and/or glucose residues (25). The observation that the pheochromocytoma enzyme interacts with Concanavalin A confirms a preliminary report of Miras-Portugal (26), although the technique has not heretofore found use in the purification of pheochromocytoma dopamine-β-hydroxylase.

Our observations of native and subunit molecular weights of human pheochromocytoma dopamine-β-hydroxylase lend credence to the supposition that, like the bovine enzyme (27), the native form of the soluble human pheochromocytoma enzyme is a tetramer composed of two dimeric subunits, each of which is held together by disulfide bonds, and which are in turn dissociated by sulfhydryl reagents into a total of four monomeric subunits per tetramer (Table 2).

In summary, the soluble dopamine-β-hydroxylase from two pheochromocytomas have been purified by using carbohydrate affinity chromatography on Concanavalin A-Sepharose. The purified products are homogeneous as judged by polyacrylamide gel electrophoresis. No evidence of multimeric forms of either the crude or the purified enzyme could be detected by analytical polyacrylamide gel electrophoresis, preparative polyacrylamide gel electrophoresis with activity elution, analytical gel filtration chromatography or zonal sedimentation on sucrose density gradients.

The tumor enzyme was characterized by using the combined hydrodynamic parameters of $s_{20,w}$ and Stokes radius as having a molecular weight of 300,000 to 317,000, as compared to the bovine adrenal medullary enzyme at 282,000. Its frictional ratio was 1.68 to 1.69, leading to anomalously high apparent molecular weights estimated by using filtration alone at 461,000 to 510,000.

Characterization of the subunit structure revealed the tetrameric enzymes to be composed of monomeric subunits of 78,000 to 80,500 daltons each, joined by disulfide linkages to form dimeric subunits of 155,000 to

160,000 daltons, two of which join by noncovalent interaction to form each tetramer.

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