MOLECULAR PHARMACOLOGY, 7, 136–146

Different Molecular Forms of Bovine Adrenal Tyrosine Hydroxylase

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(Received October 15, 1970)

SUMMARY

The sedimentation coefficients of native and trypsin-treated bovine adrenal tyrosine hydroxylase were determined by sucrose density gradient centrifugation and compared with protein standards whose sedimentation coefficients were known. Native tyrosine hydroxylase was found to have a sedimentation coefficient of 9.20 $s^{0.725}_{20,w}$, and that of trypsin-treated tyrosine hydroxylase was 3.45 $s^{0.725}_{20,w}$. Using this information and known molecular weights of the protein standards, it was possible to make a rough estimation of the molecular weights of the two forms of the enzyme.

Sephadex gel filtration was also used to estimate the molecular weights of the two forms of tyrosine hydroxylase. A Stokes radius of 23.7 A and a frictional ratio of 1.12 were also determined for trypsin-treated tyrosine hydroxylase by gel filtration; these determinations were not possible for the native form of the enzyme because of the presence of urea, which was necessary to avoid the aggregation of tyrosine hydroxylase.

The sedimentation coefficients and the Stokes radius of trypsin-treated tyrosine hydroxylase were used to calculate the molecular weight of this enzyme, which was found to be 34,000. It was also determined that the molecular weight of native tyrosine hydroxylase is approximately 4 times that of the trypsin-treated enzyme. These results indicate that trypsin-treated tyrosine hydroxylase is only a fragment of the native form.

INTRODUCTION

Depending on the isolation procedure used to obtain bovine adrenal tyrosine hydroxylase, marked differences have been observed in some of the physicochemical characteristics of the enzyme. Under certain conditions, tyrosine hydroxylase was found to produce aggregates which obscure the subcellular distribution of the enzyme and are an obstacle to its purification (1, 2). It was also found that solubilization of the so-called "particle-bound tyrosine hydroxylase" by tryptic digestion, as described by Petrack *et al.* (3), produced an enzyme apparently much smaller than the native tyrosine hydroxylase (4).

The possibility of artificially producing different molecular forms of tyrosine hydroxylase during the purification procedure is an important problem and certainly deserves further investigation. The inadvertent use of artificial forms of the enzyme would be highly misleading, since a modified molecular form of tyrosine hydroxylase, even if enzymatically active, may conceivably show modified kinetic behavior. Obviously, in order to characterize tyrosine hydroxylase completely and to study *in vitro* the mechanisms which may control the enzyme activity *in vivo*, it will be necessary to isolate

This work was supported by Grant 5 R01 AM 13128 from the National Institutes of Health.

[1] Research Scientist Awardee of the United States Public Health Service (Grant 1-K2-MH-17,885).

the native form of the enzyme, or prove that there are no kinetic differences between the native and the trypsin-treated tyrosine hydroxylase.

The present study was undertaken in order (a) to extend the initial observations that the purification procedure may produce artificial forms of tyrosine hydroxylase and (b) to determine some of the molecular parameters of the different forms.

MATERIALS AND METHODS

Chemicals. The artificial cofactor for the assay of tyrosine hydroxylase, 6,7-dimethyl-5,6,7,8-tetrahydropterine, was purchased from Calbiochem. [3,5-^{3}H]-L-Tyrosine (1000 mCi/mmole) was purchased from Amersham/Searle; it was purified by passage through a Dowex 50W-X4(K^+) column, and the purified material was diluted to a specific activity of 10 mCi/mmole. Sephadex G-100 and G-200 and the chromatographic columns were obtained from Pharmacia Fine Chemicals, Inc. Tris(hydroxymethyl)aminomethane (Trizma base) was purchased from Sigma Chemical Company, and all other chemicals were obtained from standard commercial sources. Deionized, glass-distilled water was used for the preparation of all reagents and buffers.

Protein standards. Alcohol dehydrogenase and catalase were purchased from Worthington Biochemical Corporation. Cytochrome *c*, myoglobin, chymotrypsinogen A, ovalbumin, and albumin were obtained from Mann Research Laboratories. Hemoglobin was prepared from a 60–95% ammonium sulfate saturation of hemolyzed bovine red blood cells; the hemoglobin was resuspended in 5 mM Tris-HCl, pH 7.0, dialyzed overnight against the same buffer, and frozen until used. The molecular parameters of the protein standards are shown in Table 1.

TABLE 1

Molecular parameters of protein standards

Protein	Molecular weight	$s_{20,w}$	Stokes radius	References
			A	
Hemoglobin	64,500	4.3		5, 6
Alcohol dehydrogenase	150,000	7.4		7
Catalase	250,000	11.3		8
Cytochrome *c*	12,400		17.4	9
Chymotrypsinogen A	25,000		22.4	9
Ovalbumin	45,000		28.0	9
Albumin	67,000		36.0	9
Myoglobin	17,800			9

Radioactive assay of tyrosine hydroxylase. Tyrosine hydroxylase activity was determined by the method of Nagatsu *et al.* (10), with modifications described by Wurzburger and Musacchio (11).

Enzyme preparation. The native form of tyrosine hydroxylase was prepared by homogenization of fresh bovine adrenal glands in 2 volumes of isotonic KCl–5 mM Tris-HCl, pH 7.0. The homogenate was centrifuged at 100,000 × *g* for 1 hr, and the fat-free supernatant fraction, containing the enzyme, was used. This supernatant fraction usually contained 60% of the total tyrosine hydroxylase activity and had a specific activity of 19.0 mμmoles of ^{3}HOH formed in 10 min per milligram of protein. An ammonium sulfate fraction of tyrosine hydroxylase was prepared by a modification of the method of Nagatsu *et al.* (12), as described by Wurzburger and Musacchio (11). The specific activity of the ammonium sulfate fraction was 25.0 mμmoles of ^{3}HOH formed in 10 min per milligram of protein. Trypsin-treated tyrosine hydroxylase, prepared by the procedure of Petrack *et al.* (3), had a specific activity of 73.0 mμmoles of ^{3}HOH formed in 10 min per milligram of protein.

Sucrose density gradients. Continuous linear sucrose density gradients were used to determine the sedimentation coefficients and to estimate the molecular weights of the different forms of tyrosine hydroxylase. The gradients were prepared in cellulose nitrate tubes with a Buchler universal density gradient mixer, using equal amounts of 5 and 20% (w/v) sucrose solutions. The sucrose solutions consisted of 1 mM 2-mercaptoethanol, 5 mM KCl, and 0.05 M Tris-HCl; the pH was adjusted to 7.0. The reliability of the gradient mixer to prepare linear sucrose density gradients was verified by the addition of sodium 2,6-dichlorobenzenoneindophenol to a 20% sucrose

solution. The gradients were centrifuged for 15 or 18 hr in a Spinco model L2-65B centrifuge, using either a Spinco SW 27 or SW 41 rotor. The rotor was accelerated very slowly by setting the speed control knob at 3000 rpm, in order to avoid the initial jolt. After approximately 15 sec, the speed control knob was set at either 27,000 or 41,000 rpm. At the end of the run, the centrifuge was stopped without the use of the brake. The total volume of the gradient in the SW 27 rotor consisted of 37.0 ml of sucrose and 1.0 ml of sample; in the SW 41 rotor, 12.2 ml of sucrose and 0.3 ml of sample were used.

After centrifugation, a hole was pierced in the bottom of the cellulose nitrate tubes and fractions of equal volume were collected with a Buchler fraction collector. Fractions of 1.0 ml were collected from the gradients run in the SW 27 rotor, and fractions of 0.5 ml were collected from the gradients run in the SW 41 rotor. All procedures were carried out at 4°.

A protein layered on a sucrose gradient migrates from the top layer (or last gradient fraction collected) toward the lower layers (or earlier fractions collected); therefore, the distance migrated by the protein is proportional to the difference between the total gradient volume and the volume of elution of the protein. The distance traveled by the different forms of tyrosine hydroxylase and different protein standards was measured from the center of the layered sample at the top of the gradient to the mode of the peak fraction. This distance (D_m), measured in milliliters, was calculated by the following equation:

$$D_m = G_V - \tfrac{1}{2}S_V - P_{ev}$$

where G_V = the total gradient volume, S_V = the sample volume, and P_{ev} = the elution volume of the protein. The mode was calculated by the procedure described by Croxton (13).

Protein standards employed in the sucrose density gradients were hemoglobin, alcohol dehydrogenase, and catalase. The standards were dissolved in isotonic KCl–5 mM Tris-HCl, pH 7.0, and mixed with a tyrosine hydroxylase preparation. The protein concentration of the sample did not exceed 20 mg/ml. Hemoglobin content was determined at 414 mμ in a Beckman DU-2 spectrophotometer. Alcohol dehydrogenase activity was determined by the method of Vallee and Hoch (14), and catalase activity was determined as described by Beers and Sizer (15).

Sephadex gel filtration. Gel filtration was used to estimate the molecular weights of native and trypsin-treated tyrosine hydroxylase and to determine the Stokes radius and frictional ratio of trypsin-treated tyrosine hydroxylase. Sephadex G-100 and G-200 were used; the gel was allowed to swell in water for 3 days, and the fines were then decanted several times in the presence of the proper buffer. The chromatographic columns were packed and then run by upward flow elution with the proper buffer for 18–24 hr prior to use, in order to guarantee proper equilibration. The dimensions of the column packed with Sephadex G-200 were 2.5 × 25 cm, and those of the column packed with Sephadex G-100 were 2.5 × 90 cm. The flow rate of both columns was approximately 15.0 ml/hr. Buffers used were (a) isotonic KCl–1 mM 2-mercaptoethanol–5 mM Tris-HCl, pH 7.0, and (b) isotonic KCl–1 mM 2-mercaptoethanol–2.0 M urea–5 mM Tris-HCl, pH 7.0. The void volume of the columns was determined with a 0.2% solution of blue dextran, which was read at 620 mμ in a Beckman DU-2 spectrophotometer.

The tyrosine hydroxylase preparation was mixed with the protein standards, which had previously been dissolved in buffer, and then applied to the column; 1.0 ml of sample was applied to the G-200 column and 5.0 ml of sample were applied to the G-100 column. The protein concentration of the sample did not exceed 30 mg/ml. Immediately after the application of the sample, 2.0 ml of a 10% sucrose solution were applied to the column to prevent tailing. Fractions equal in size to the sample volume were collected with a Buchler fraction collector. When urea was present, the fractions were dialyzed overnight against 80–100 volumes of 5 mM Tris-HCl, pH 7.0, in order to remove the urea, which interferes with the determination of tyrosine hydroxylase. All procedures were carried out at 4°.

Besides alcohol dehydrogenase and catalase, additional protein standards of lower molecular weights were used during gel filtration. They included cytochrome *c*, myoglobin, chymotrypsinogen A, ovalbumin, and albumin; their elution peaks were determined in a Beckman DU-2 spectrophotometer. Cytochrome *c* was determined at 400 mμ; myoglobin determination was done at 414 mμ; chymotrypsinogen A, ovalbumin, and albumin were determined at 280 mμ. The mode of each protein peak was determined by the procedure described by Croxton (13).

Calculations. According to Martin and Ames (16), the distance migrated by a protein in a linear sucrose density gradient is directly proportional to its sedimentation coefficient. Therefore, by using the appropriate protein standards, the sedimentation rates of the different forms of tyrosine hydroxylase can be calculated according to the formula

$$\frac{D_u}{D_s} = \frac{s^{0.725}_{20,w} \text{ of unknown}}{s^{0.725}_{20,w} \text{ of standard}} \quad (1)$$

where D_u = the distance migrated by the unknown, D_s = the distance migrated by the protein standard, and $s^{0.725}_{20,w}$ is defined as the $s_{20,w}$ calculated on the assumption of a partial specific volume of 0.725 ml/g. This assumption results in an error of less than 3% in the estimation of $s_{20,w}$ for most proteins. The calculation of the sedimentation coefficient permits an estimation of the molecular weights (MW_u) of the different enzyme forms by the use of the following equation (16):

$$\frac{s^{0.725}_{20,w} \text{ of unknown}}{s^{0.725}_{20,w} \text{ of standard}} = \left(\frac{MW_u}{MW_s}\right)^{2/3} \quad (2)$$

where MW_s = the molecular weight of the protein standard. Since the molecular weight calculated by this method is only a crude estimation, gel filtration was employed to obtain additional information about the various molecular parameters of the different enzyme forms and allow more precise calculations of the molecular weights. The K_{av} values of the different forms of tyrosine hydroxylase and of the protein standards were calculated from gel filtration experiments as described by Laurent and Killander (17). The K_{av} values were plotted against their respective molecular weights on a logarithmic scale; the molecular weights of the different forms of the enzyme were then read from the graph.

The results from sucrose density gradients and gel filtration could also be combined to give an accurate measurement of the molecular weight of a protein. The molecular weight of trypsin-treated tyrosine hydroxylase, calculated with Eq. 3 (18, 19), was obtained by combining the sedimentation coefficient with the Stokes radius determined from gel filtration (20).

$$M = 6\pi\eta \frac{Nas}{1 - \bar{v}\rho} \quad (3)$$

where M = molecular weight, η = viscosity of water at 20°, N = Avogadro's number, a = Stokes radius, s = sedimentation coefficient, ρ = density of water at 20°, and $\bar{v}$ = partial specific volume. The partial specific volume of 0.725 ml/g, representative of most proteins and selected by Martin and Ames (16), was used.

The frictional ratio (f/f_0) of trypsin-treated tyrosine hydroxylase was calculated from Eq. 4 (18, 19).

$$\frac{f}{f_0} = \frac{a}{(3\bar{v}M/4\pi N)^{1/3}} \quad (4)$$

Protein standards of known Stokes radii (Table 1) were used to calibrate a Sephadex G-100 column and determine the effective gel pore radius. The Stokes radius of the trypsin-treated tyrosine hydroxylase was then calculated by the method of Ackers (20).

RESULTS

Sedimentation patterns of different forms of tyrosine hydroxylase. The native form of tyrosine hydroxylase is contained in the 100,000 × *g* supernatant fraction of the adrenal medulla. Upon prolonged high-speed centrifugation, the enzyme sediments faster than endogenous hemoglobin and forms a rather sharp peak (see Fig. 1). Although there is a small amount of aggregated enzyme, as indicated by some scattered activity in the lower regions of the gradient

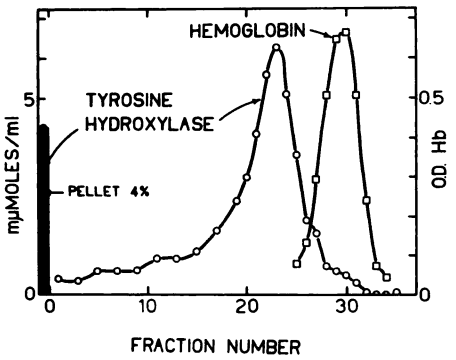

FIG. 1. *Comparison of sedimentation patterns of native tyrosine hydroxylase and hemoglobin*

The 100,000 × *g* fresh adrenal gland supernatant fraction was centrifuged in an SW 27 rotor for 18 hr at 27,000 rpm. The pellet was resuspended in 1.0 ml of 5 mM Tris-HCl buffer, pH 7.0, and contained 4% of the total tyrosine hydroxylase activity. Tyrosine hydroxylase activity is expressed as millimicromoles of ^{3}HOH produced in 10 min per milliliter. Hemoglobin distribution is expressed by the optical density produced at 414 mμ.

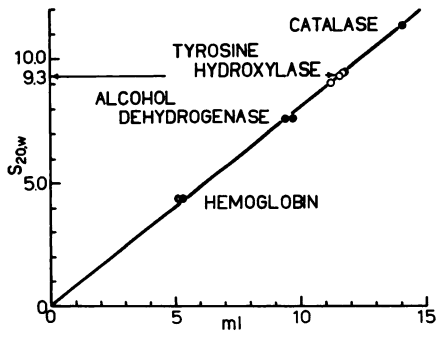

FIG. 2. *Determination of sedimentation coefficient* ($s^{0.725}_{20,w}$) *of native tyrosine hydroxylase*

The abscissa represents distance migrated (milliliters) from the top of the gradient. The 100,000 × *g* fresh adrenal gland supernatant fraction and the markers indicated were centrifuged in an SW 27 rotor for 18 hr at 27,000 rpm. Results from six different sucrose density gradients are plotted in this graph. Hemoglobin was determined in six gradients, alcohol dehydrogenase in two, and catalase in three. The distance migrated by tyrosine hydroxylase in four gradients was 11.56 ± 0.13 ml (mean ± standard error); this value was used to determine the sedimentation coefficient of the enzyme. The results were so reproducible that individual points are not always distinguishable.

and in the pellet collected at the bottom of the tube, this experiment demonstrates that tyrosine hydroxylase contained in the 100,000 × *g* supernatant fraction is almost entirely in a soluble form. In other experiments, catalase and alcohol dehydrogenase—proteins with well-known sedimentation coefficients—were used as standards as well as the hemoglobin contained in the 100,000 × *g* supernatant fraction. The distance migrated by each protein from the top to the bottom of the gradient was measured as described in MATERIALS AND METHODS and was plotted against the sedimentation coefficients of the standards (see Fig. 2). The average sedimentation coefficient of tyrosine hydroxylase, calculated from eight different sucrose density gradients, was found to be $s^{0.725}_{20,w} = 9.2$.

When a partially purified ammonium sulfate fraction of tyrosine hydroxylase was centrifuged in a sucrose density gradient, a completely different pattern of distribution from the one shown in Fig. 1 was obtained. As indicated in Fig. 3, 44% of the enzyme sedimented with the pellet and a considerable amount was found in the lower portions of the gradient. In a similar experiment, not illustrated here, 54% of the tyrosine hydrox-

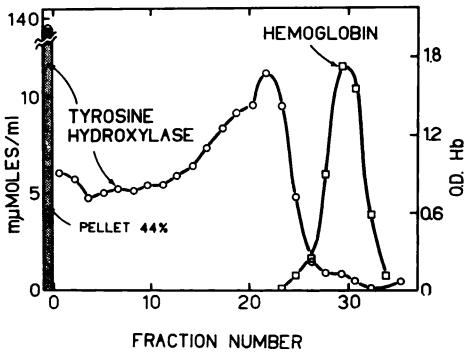

FIG. 3. *Comparison of sedimentation patterns of an ammonium sulfate fraction of tyrosine hydroxylase and hemoglobin*

The ammonium sulfate-precipitated enzyme was prepared as described in MATERIALS AND METHODS, diluted with an equal volume of isotonic KCl–5 mM Tris-HCl, pH 7.0, containing hemoglobin, and centrifuged in an SW 27 rotor for 18 hr at 27,000 rpm. The pellet was resuspended in 1.0 ml of 5 mM Tris-HCl, pH 7.0, and contained 44% of the total tyrosine hydroxylase activity. Tyrosine hydroxylase activity is expressed as millimicromoles of ^{3}HOH produced in 10 min per milliliter. Hemoglobin distribution is expressed by the optical density at 414 mμ.

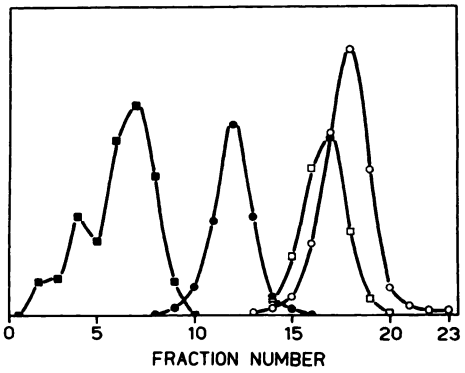

FIG. 4. *Sedimentation pattern of trypsin-treated tyrosine hydroxylase and protein standards*

Trypsin-treated tyrosine hydroxylase was mixed with the different protein standards and centrifuged in an SW 41 rotor for 18 hr at 41,000 rpm. The activity of the enzyme and standards was determined as indicated in MATERIALS AND METHODS. The peak activity for trypsin-treated tyrosine hydroxylase (○) was 69.95 mμmoles of ^{3}HOH formed per milliliter in 10 min; the hemoglobin (□) peak was 283 absorbance units at 414 mμ; alcohol dehydrogenase (●) peak activity was 4.88 units/ml; catalase (■) peak activity was 40.6 units/ml.

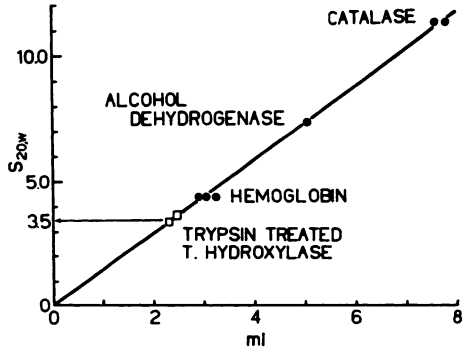

FIG. 5. *Determination of sedimentation coefficient* ($s^{0.725}_{20,w}$) *of trypsin-treated tyrosine hydroxylase*

The abscissa represents distance migrated (milliliters) from the top of the gradient. Trypsin-treated tyrosine hydroxylase, prepared as described by Petrack *et al.* (3), and the markers indicated were centrifuged in an SW 41 rotor for 15 hr at 41,000 rpm. Results from four different sucrose density gradients are plotted in this graph. Hemoglobin was determined in three gradients, alcohol dehydrogenase in one, and catalase in two. The distance migrated by trypsin-treated tyrosine hydroxylase in three gradients was 2.50 ± 0.11 ml (mean ± standard error); this value was used to determine the sedimentation coefficient of the enzyme.

ylase activity was found in the pellet when an older enzyme preparation was used. It is evident from Figs. 1 and 3 that the peak of the ammonium sulfate fraction of tyrosine hydroxylase is skewed and displaced to the denser regions of the gradient when compared with the peak produced by the native form of the enzyme. This displacement of the peak and the sedimentation of a considerable fraction of the total enzyme layered on the gradient suggest the existence of enzyme aggregates. The sedimentation pattern of trypsin-treated tyrosine hydroxylase is illustrated in Fig. 4, and, contrary to expectations, a homogeneous, symmetrical peak was obtained; the probable explanation for this finding will be considered under DISCUSSION.

In sharp contrast with the native form and with the ammonium sulfate tyrosine hydroxylase fraction, the trypsin-treated tyrosine hydroxylase has a much smaller sedimentation coefficient, as indicated in Fig. 5. The average sedimentation coefficient of trypsin-treated tyrosine hydroxylase, calculated from seven different sucrose density gradients, was found to be $s^{0.725}_{20,w}$ = 3.45. It is noteworthy that this light form of the enzyme was also obtained by tryptic digestion of an ammonium sulfate fraction of partially purified tyrosine hydroxylase.

In order to test the possibility that the enzyme fragments obtained after trypsin treatment may be able to reassociate, two different experiments were performed. In the first, a mixture of a 100,000 × *g* supernatant fraction and the trypsin-treated enzyme was incubated at 37° for 10 min; the sedimentation coefficients of the different enzyme forms were unchanged, as indicated in Fig. 6. In the second experiment, which is not illustrated here, trypsin-treated tyrosine hydroxylase was incubated with all and with different combinations of the components used to detect the enzyme activity; the different incubation mixtures and controls were then layered on top of a sucrose density gradient and centrifuged as before. The sedimentation characteristics of the enzyme fragment were not changed by the incubation with the different substances

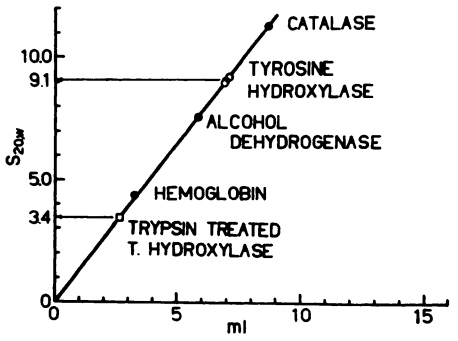

FIG. 6. *Determination of sedimentation coefficient* ($s^{0.725}_{20,w}$) *of native and trypsin-treated tyrosine hydroxylase*

In some gradients the two forms of tyrosine hydroxylase were mixed, incubated, and centrifuged together, as described in MATERIALS AND METHODS. The abscissa represents distance migrated (milliliters) from the top of the gradient. The 100,000 × *g* fresh adrenal gland supernatant fraction, trypsin-treated tyrosine hydroxylase, and the markers indicated were centrifuged in an SW 41 rotor for 18 hr at 41,000 rpm. Results from six different sucrose density gradients are plotted in this graph. Hemoglobin was determined in six gradients, alcohol dehydrogenase in three, and catalase in three. The distance migrated by trypsin-treated tyrosine hydroxylase in four gradients was 2.65 ± 0.02 ml (mean ± standard error); the distance migrated by native tyrosine hydroxylase in four gradients was 6.95 ± 0.06 ml. These values were used to determine the sedimentation coefficients of the different forms of the enzyme. The results were so reproducible that individual points are not always distinguishable.

used, and no indications of enzyme aggregation were found in the gradient profiles.

A crude estimation of the molecular weight of a protein can be obtained from the sedimentation constants alone by using Eq. 2. The molecular weights of the different forms of tyrosine hydroxylase calculated with this equation, using different standards, are shown in Table 2. The errors involved in these calculations will be considered under DISCUSSION.

Estimation of different molecular parameters by gel filtration. Sephadex gel filtration experiments were performed to estimate the molecular weight of different forms of tyrosine hydroxylase and to determine the Stokes radius and frictional ratios of the

TABLE 2

Molecular weights of native and trypsin-treated tyrosine hydroxylase determined by sucrose density gradient centrifugation

Protein standard	Molecular weight		A:B
	Native (A)	Trypsin-treated (B)	
Catalase	183,000	43,000	4.3
Alcohol dehydrogenase	208,000	48,000	4.4
Hemoglobin	202,000	46,000	4.4
Average	198,000	45,000	4.4
Trypsin-treated tyrosine hydroxylase[a]	147,000		4.4

[a] The molecular weight and sedimentation coefficient of trypsin-treated tyrosine hydroxylase (Table 4) were used as standard.

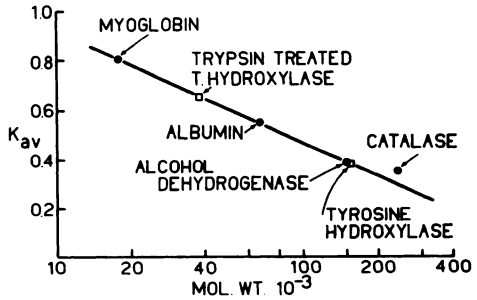

FIG. 7. *Estimation of molecular weight of native and trypsin-treated tyrosine hydroxylase on Sephadex G-200 in the presence of 2.0 M urea*

The graph is a summary of five different experiments run with different markers each time. Myoglobin was determined three times, albumin once, alcohol dehydrogenase five times, catalase once, trypsin-treated tyrosine hydroxylase three times, and native tyrosine hydroxylase twice. The K_{av} of trypsin-treated tyrosine hydroxylase was 0.66 ± 0.015 (mean ± standard error), and that of the native enzyme was 0.38 ± 0.015. These values were used to estimate the molecular weights of the different forms of the enzyme. The results represent the average of the number of determinations indicated.

trypsin-treated enzyme. The native form of tyrosine hydroxylase contained in the 100,000 × *g* supernatant fraction aggregates upon gel filtration (11). Therefore, the gel

TABLE 3

Molecular weights of native and trypsin-treated tyrosine hydroxylase estimated by gel filtration

Sephadex gel	Molecular weight of tyrosine hydroxylase		A:B
	Native (A)	Trypsin-treated (B)	
G-200 (2.0 M urea)	155,000	38,000	4.1
G-200		32,000	
G-100		33,000	

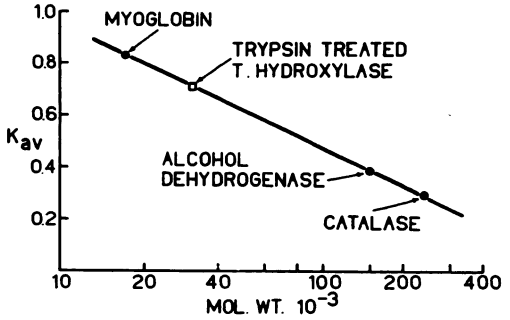

FIG. 8. *Estimation of molecular weight of trypsin-treated tyrosine hydroxylase in Sephadex G-200*

The graph is a summary of five different experiments run with different markers each time. Myoglobin was determined three times, alcohol dehydrogenase five times, catalase once, and trypsin-treated tyrosine hydroxylase four times. The K_{av} of trypsin-treated tyrosine hydroxylase was 0.71 ± 0.017 (mean ± standard error); this value was used to estimate the molecular weight of the enzyme. The results represent the average of the number of determinations indicated.

filtration experiments to estimate the molecular weight of the native form of the enzyme were carried out in the presence of 2.0 M urea, which is known to inhibit aggregation of tyrosine hydroxylase. The semilogarithmic plot of K_{av} with respect to the molecular weights of the protein standards and native and trypsin-treated tyrosine hydroxylase is illustrated in Fig. 7; the estimated molecular weights for the two enzyme forms are shown in Table 3. The presence of 2.0 M urea, although necessary to keep the native form of tyrosine hydroxylase in solution, may introduce errors into the molecular weight estimations. These errors will be considered under DISCUSSION.

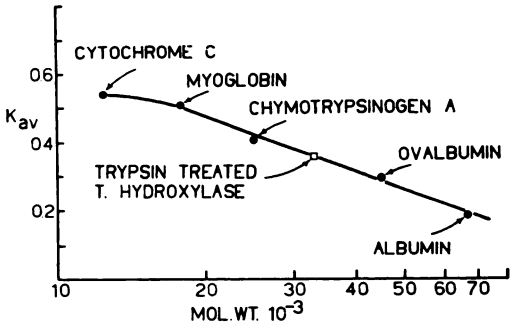

FIG. 9. *Estimation of molecular weight of trypsin-treated tyrosine hydroxylase in Sephadex G-100*

Each point represents one determination.

TABLE 4

Molecular parameters of trypsin-treated tyrosine hydroxylase calculated from sucrose density gradient centrifugation and gel filtration

Parameter	Value
Molecular weight	34,000
$s^{0.725}_{20,w}$	3.45
Stokes radius (A)	23.7
f/f_0	1.12

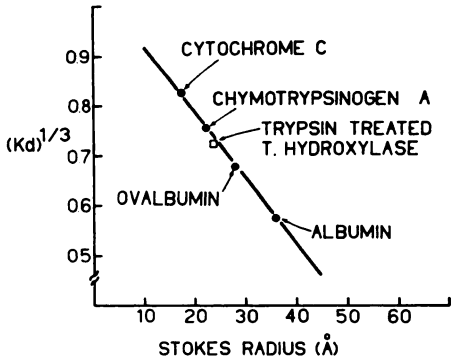

FIG. 10. *Stokes radius of trypsin-treated tyrosine hydroxylase*

The results are plotted according to the correlation described by Porath (21). The K_d values for the protein standards and trypsin-treated tyrosine hydroxylase were determined in the experiment described in Fig. 8. The K_d was calculated as described by Gelotte (22), and the Stokes radius of the trypsin-treated enzyme was measured according to the method of Ackers (20).

The molecular weight of trypsin-treated tyrosine hydroxylase was also estimated in Sephadex G-200 and G-100 without the addition of urea. The semilogarithmic plot of

the K_{av} values and molecular weights of the different protein standards and the enzyme are illustrated in Figs. 8 and 9. The results obtained with the two different gels are in close agreement, but they are slightly different from the values obtained in the presence of urea (see Table 3).

The Stokes radius for trypsin-treated tyrosine hydroxylase (Table 4) was obtained as described in MATERIALS AND METHODS and was found to follow the correlation described by Porath (21), as shown in Fig. 10. The molecular weight and frictional ratio of trypsin-treated tyrosine hydroxylase, calculated as described in MATERIALS AND METHODS, are given in Table 4.

DISCUSSION

The results obtained with sucrose density gradient centrifugation and with Sephadex gel filtration clearly indicate that trypsin-treated bovine adrenal tyrosine hydroxylase is a smaller molecule than the native form of the enzyme. With the sucrose density gradient method of Martin and Ames (16), it is possible to determine the sedimentation coefficients of enzymes in a crude extract. The determination of a sedimentation coefficient with this technique is accurate when the partial specific volume of the protein is known; if the partial specific volume of the protein is unknown, the average value of 0.725 ml/g can be used, since only a small error (up to 3% for most proteins) is introduced (16). A crude estimation of the molecular weight can then be obtained from the sedimentation constant alone, as shown in MATERIALS AND METHODS. The variations in the molecular weights obtained with this method (Table 2) are due to two factors (16): (a) the protein standards are not perfect spheres, and (b) there are some inaccuracies in the reported molecular weights of the protein standards. Therefore, the molecular weights obtained should only be considered approximations. At any rate, these results illustrate the marked molecular weight differences between the native and trypsin-treated forms of the enzyme.

For a series of proteins of similar frictional ratios and partial specific volumes, there is a linear relationship between the various elution parameters and the logarithm of the molecular weight (18). Urea may disrupt this relationship by the unfolding effect it has on some proteins, as described by Nozaki and Tanford (23); however, its use is necessary to prevent aggregation of the native form of tyrosine hydroxylase. The unfolding effect of urea changes the elution pattern (24) and, in consequence, produces an apparent increase in the molecular weight of some proteins. This phenomenon is shown in our experiments, in which there was an apparent increase in the molecular weight of trypsin-treated tyrosine hydroxylase, from 32,000 to 38,000. Urea may also have produced an apparent increase in the molecular weight of the native form of tyrosine hydroxylase; therefore, the molecular weight of 155,000 should be considered an approximation, since it might have been overestimated. Despite this limitation, it can be concluded that trypsin-treated tyrosine hydroxylase has a much smaller molecular weight than the native form of the enzyme (Table 3).

The limitations of sucrose density gradient centrifugation and gel filtration can be overcome by using only the reliable information that each method provides, i.e., the sedimentation coefficients and the Stokes radii. By combining both methods, an accurate determination of the molecular weight can be obtained (20). The sedimentation coefficient of trypsin-treated tyrosine hydroxylase and its Stokes radius were used to calculate the molecular weight of the trypsin-digested enzyme (18, 19); the calculated values are very close to the ones obtained with gel filtration (see Tables 3 and 4). This agreement is probably due to the fact that the frictional ratios of trypsin-treated tyrosine hydroxylase and the globular protein standards used to calibrate the column are similar.

Unfortunately, an accurate estimation of the molecular weight of the native form of tyrosine hydroxylase cannot be made with the information available, since the presence of urea during gel filtration will not permit an accurate determination of the Stokes radius. However, the results obtained with the sucrose density gradients and gel filtra-

tion, and all the calculations performed with this information, strongly indicate that the native form of tyrosine hydroxylase has a molecular weight between 4.0 and 4.4 times the molecular weight of the trypsin-treated enzyme. Since the value of 34,000 is a reliable determination of the molecular weight of trypsin-treated tyrosine hydroxylase, the molecular weight of the native form of the enzyme is probably between 135,000 and 155,000.

Despite the wide differences in the molecular weights of both enzymes, preliminary kinetic studies have shown only minor differences between both enzyme forms.[2]

The tendency of bovine adrenal tyrosine hydroxylase to form aggregates has been extensively investigated (2, 11). In the present study, the aggregation of tyrosine hydroxylase produces the sedimentation of a considerable fraction of enzyme and causes asymmetry in the pattern of distribution in the sucrose density gradient. The enzyme aggregates contain tyrosine hydroxylase of a specific activity higher than the fraction from which they were obtained. This increase in specific activity is probably due to the fact that only a fraction of all the protein molecules in solution will meet the physicochemical requirements to aggregate with the enzyme (11). The degree of aggregation in the fresh 100,000 × *g* adrenal gland supernatant fraction is negligible, since only minor peak asymmetry and sedimentation of only 4% of the total enzyme was observed. The trypsin-treated tyrosine hydroxylase sedimented in a homogeneous, symmetrical peak; this was rather surprising, considering that trypsin digestion should be expected to produce enzyme fragments of different sizes. The enzyme fragments possess approximately one-fourth the molecular weight of the native enzyme. The uniformity of the size of the fragments could be explained if there is only one peptide bond, per catalytic site, which could be hydrolyzed by trypsin. This problem cannot be solved definitely until a pure sample of native tyrosine hydroxylase is subjected to trypsin digestion.

In conclusion, trypsin-treated tyrosine hydroxylase is a product of the tryptic digestion of tyrosine hydroxylase. It is noteworthy that the same form of enzyme is obtained by digestion of either the "particle-bound" tyrosine hydroxylase, which is an artifact produced by adsorption to particles and aggregation (11), or the "soluble" enzyme. Trypsin-treated tyrosine hydroxylase is a smaller molecule than the native form of the enzyme, and it can not reassociate into larger aggregates; this gives strong support to the idea that it is only a fragment of the native enzyme and should not be considered the "solubilized particle-bound tyrosine hydroxylase." Obviously, the enzyme fragment contains at least one catalytic site; however, it is not clear at the present time what relationship the fragment has to the native enzyme subunits or the regulatory and catalytic sites.

[2] C. A. McQueen and J. M. Musacchio, unpublished observations.

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