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ACTIVATION OF RAT BRAIN TRYPTOPHAN HYDROXYLASE BY POLYELECTROLYTES*

DONALD M. KUHN, MARY ANNE MEYER and WALTER LOVENBERG

Section of Biochemical Pharmacology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20014, U.S.A.

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Abstract— The *in vitro* activity of rat brain tryptophan hydroxylase is increased 2-fold by heparin and 4-fold by dextran sulfate. Other polysaccharides including hyaluronic acid, chondroitin sulfate and dermatan sulfate, as well as the unsulfated polymer dextran, do not alter hydroxylase activity. The effect of heparin or dextran sulfate on tryptophan hydroxylase is manifested kinetically as a decrease in the apparent K_m of the enzyme for both substrates 6-methyl-tetrahydropterin (6-MPH_4) and tryptophan, as well as increases in the V_{max} values. A variety of polyanions (DNA, glycogen, poly-*d*- and poly-*l*-glutamic acid) have no effect on tryptophan hydroxylase, whereas salts [NaCl, KCl, $(NH_4)_2SO_4$ and $MgSO_4$] inhibit the enzyme, indicating that the effects of heparin and dextran sulfate on tryptophan hydroxylase are not mediated by increases in ionic strength *per se*. Several lines of data suggest that tryptophan hydroxylase binds ionically to these polyelectrolytes: (1) the activation produced by heparin and dextran sulfate diminishes as the ionic strength of the assay medium increases, (2) tryptophan hydroxylase binds to heparin-substituted Sepharose 4B and is eluted by increasing the ionic strength of the eluant buffer, and (3) large molecular weight dextran sulfate (mol. wt = 500,000) dramatically shifts the elution profile of tryptophan hydroxylase from a K_{av} of 0.41 to a K_{av} of 0.10 on a Sepharose/CL-6B column. Taken together, these data suggest that the binding of certain polyelectrolytes to tryptophan hydroxylase may induce a conformational change in the enzyme which results in increased catalytic activity.

The *in vitro* activity of tryptophan hydroxylase can be increased by a variety of substances, including the membrane detergent sodium dodecyl sulfate (SDS) [1], phospholipids [2] and ATP-Mg^{2+} [3, 4]. Upon close examination, these seemingly different treatments may have a common effect on the enzyme. In fact, insight into the molecular mechanisms by which these treatments alter tryptophan hydroxylase can be gained from the results of experiments on a similar enzyme, tryosine hydroxylase. For example, heparin, phospholipids and certain biological membranes [5–8] stimulate the activity of tyrosine hydroxylase. These structurally unrelated substances are all polyanions which apparently activate tyrosine hydroxylase via salt reversible, "electrostatic" interactions with the enzyme [5]. In an effort to understand more completely how the activity of tryptophan hydroxylase is altered by polyanions, including SDS and phospholipids, the effects of various acidic mucopolysaccharides and the model polyelectrolyte, dextran sulfate, on tryptophan hydroxylase were investigated.

MATERIALS AND METHODS

Chemicals. Heparin, chondroitin sulfate, dermatan sulfate, hyaluronic acid, ethanolamine, dextrans, poly-*l*- and poly-*d*-glutamic acid, glycogen, DNA, SDS and phosphatidylserine were purchased from the Sigma Chemical Co. (St. Louis, MO). Sepharose 4B and Sepharose/CL-6B were obtained from Pharmacia (Piscataway, N.J.). Cyanogen bromide (CnBr) was purchased from the Eastman Kodak Co. (Rochester, NY). Heparin-substituted Sepharose 4B was prepared by the method of Shapiro *et al.* [9], and the amount of heparin (Sigma) covalently bound was estimated to be 1.4 mg/ml of gel. CnBr-activated Sepharose 4B treated with ethanolamine served as a control for the heparin Sepharose. Dithiothreitol (DTT), 6-methyl-tetrahydropterin (6-MPH_4) and catalase were purchased from CalBiochem (La Jolla, CA). 6-MPH_4 was prepared as described previously [10]. Phosphatidylserine was delivered in chloroform/methanol (95:5). The solvent was removed prior to use by a stream of nitrogen gas, and the phosphatidylserine was resuspended in 0.05 M Tris–HCl (pH 7.4). All other substances were freshly prepared in 0.05 M Tris–HCl (pH 7.4).

Assays. Male Sprague–Dawley rats (150–200 g) from Zivic Miller Laboratories, (Allison Park, PA) were decapitated, and the mesencephalic tegmentum was rapidly dissected from the rest of the brain, frozen on dry ice and stored in liquid N_2 until used. Frozen brain tissue was weighed and homogenized by hand in conical glass homogenizers in 4 vol. of 0.05 M Tris–HCl (pH 7.4) containing 0.002 M DTT. Homogenates were centrifuged at 40,000 *g* for 20 min at 4° and the supernatant fraction was used as the crude enzyme source. Tryptophan hydroxylase activity was measured by the method of Friedman *et al.* [11] as modified by Baumgarten *et al.* [12]. The standard reaction mixture contained the following in a total volume of 50 μl: 150–170 μg of tissue extract, 2.5 μmoles Tris–HCl (pH 7.4), 0.01 μmole dithiothreitol, 400 units catalase (bovine liver) and 0.02 μmole *l*-tryptophan. Reactions were initiated with the addition of 5 nmoles of the

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cofactor 6-MPH_4 (subsaturating), and incubations were carried out for 15 min at 37°. Hydroxylation of tryptophan is linear with respect to the time parameters and protein concentrations presently used. Reactions were terminated by the addition of 10 μl of 6 N perchloric acid. Precipitated protein was removed by centrifugation for 2 min in a Beckman 152 microfuge and a 40-μl aliquot of the supernatant fraction was added to 100 μl of 8 N HCl. The fluorescence of the solution was measured at excitation/emission wavelengths of 295/540 nm. The amount of 5-hydroxytryptophan (5-HTP) formed was calculated by least squares regression analysis of a standard curve of 5-HTP carried through the entire procedure. Reaction tubes incubated without 6-MPH_4 served as blanks. All test substances (below) were added directly to the reaction mixtures without preincubation. When the kinetic properties of tryptophan hydroxylase were studied, tryptophan concentrations were held constant at 0.4 mM while 6-MPH_4 varied from 20 μM to 0.5 mM; likewise, 6-MPH_4 was held constant at 0.5 mM while tryptophan was varied from 20 μM to 0.4 mM. Apparent Michaelis–Menten kinetic constants were calculated from least squares regression analyses of the appropriate Lineweaver–Burk plots. Proteins were estimated with the Biorad protein assay kit using bovine serum albumin as the standard.

Affinity chromatography and gel filtration. Columns (0.6 × 3 cm) of heparin- and ethanolamine-substituted Sepharose 4B were exhaustively washed with 0.05 M Tris–HCl (pH 7.4) containing 0.002 M DTT. Approximately 1.2 mg of crude enzyme was passed over either column, and the gel was washed with 5 bed volumes of the same buffer. Fractions of 0.5 ml were collected. The column was then eluted with 5 bed volumes of 0.05 M Tris–HCl (pH 7.4) containing 1.0 M NaCl and 0.002 M DTT, and 0.5-ml fractions were collected. Each fraction was subsequently assayed for trytophan hydroxylase as described above. Gel filtration was carried out on a column (0.9 × 29 cm) of Sepharose/CL-6B equilibrated with 0.05 M Tris–HCl (pH 7.4) containing 0.002 M DTT. The column was pumped in a descending direction at a linear flow rate of 15.5 ml/cm^2/hr and 6 drop (0.38 ml) fractions were collected. The crude enzyme (2.5 to 3.0 mg protein) was layered over the gel by adding either dextran or dextran sulfate (mol. wt = 500,000 for each) to the tissue extract to a concentration of 5.0 mg/ml. All fractions were assayed for tryptophan hydroxylase as described above. The void volume of the Sepharose/CL-6B column was marked with Blue Dextran. All chromatographic procedures were carried out in a cold room (4°).

RESULTS

Effects of polyanions and salts on tryptophan hydroxylase activity. The effects of a variety of mucopolysaccharides on the activity of tryptophan hydroxylase are presented in Fig. 1. Only heparin appeared to alter enzymic activity; at a concentration of 1.0 mg/ml, the velocity of the reaction was almost twice that of controls. Chrondroitin sulfate, dermatan sulfate and hyaluronic acid, in concentrations of up to 20 mg/ml, did not alter tryptophan hydroxylase activity. The effects of dextran sulfate (mol. wt = 500,000) on tryptophan hydroxylase are also shown in Fig. 1. It can be seen that dextran sulfate is more effective than heparin as an activator of the hydroxylase, producing a 4-fold stimulation of enzymic activity at a concentration of 0.5 mg/ml. The effect of a lower molecular weight dextran sulfate (mol. wt = 40,000) could be superimposed on the dextran sulfate plot in Fig. 1, while dextran itself had no effect.

In addition to the mucopolysaccharides, other polyanions including glycogen, poly-*l*- and poly-*d*-glutamic acid, and DNA were tested for stimulation of tryptophan hydroxylase. Although, in some cases, these same polyanions produce greater increases in tyrosine hydroxylase activity than does heparin [5], they are largely without effect on tryptophan hydroxylase under the conditions presently used. Only poly-*l*-glutamic acid produced an increase in enzyme activity at concentrations exceeding 5–10 mg/ml.

Since heparin and dextran sulfate are polyanions, increases in the concentrations of these substances would increase the ionic strength of the medium. However, it can be seen in Fig. 2 that increasing the salt

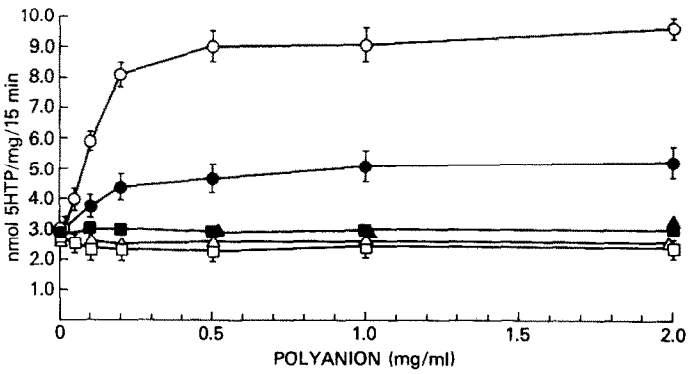

Fig. 1. Effects of polyanions on the activity of tryptophan hydroxylase. The enzyme was assayed with 0.4 mM tryptophan and 0.1 mM 6-MPH_4. The following substances were added in the final concentrations indicated on the abscissa: dextran sulfate (○), heparin (●), dermatan sulfate (■), hyaluronic acid (▲), chondroitin sulfate (△) and dextran (□). Enzyme activity is expressed as nmoles of 5-HTP/mg/15 min. Each point is the mean of at least two separate determinations in duplicate.

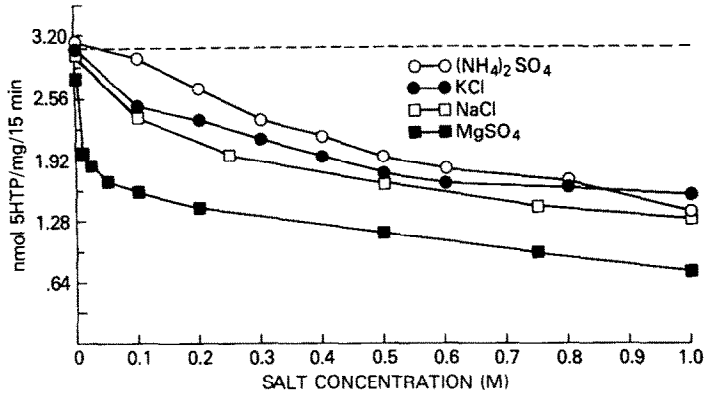

Fig. 2. Effects of salts on the activity of tryptophan hydroxylase. The ionic strength of the assay mixture was adjusted to the indicated molar (M) concentrations with the following salts: $(NH_4)_2SO_4$ (○), KCl (●), NaCl (□), or $MgSO_4$ (■). The enzyme was assayed with 0.4 mM tryptophan and 0.1 mM 6-MPH_4

concentration with NaCl, KCl, $MgSO_4$ or $(NH_4)_2SO_4$ resulted in as much as 50 per cent inhibition of hydroxylase activity at ionic strengths of 0.75–1.0 M. Thus, increases in ionic strength *per se* produced by heparin or dextran sulfate cannot explain the effects seen in Fig. 1.

Effects of heparin and dextran sulfate on the kinetics of tryptophan hydroxylase. The enzyme reaction displayed hyperbolic kinetics for both 6-MPH_4 and tryptophan in the presence or absence of the polyanions. It can be seen from the double reciprocal plots of the kinetic data in Fig. 3 that heparin (1.0 mg/ml) decreased the apparent K_m for 6-MPH_4 from 217 μM to 112 μM. The V_{max} was also increased from 0.525 to 0.778 nmole/mg/min. Heparin had a smaller effect on tryptophan kinetics, decreasing the apparent K_m from 132 μM to 91 μM and increasing the V_{max} from 0.50 to 0.77 nmole/mg/min. The effects of dextran sulfate (0.5 mg/ml) on the kinetic properties of tryptophan hydroxylase are qualitatively the same as those of heparin except that its effects on V_{max} are somewhat larger in magnitude (data not shown).

Affinity chromatography and gel filtration studies. The use of a crude tissue extract as an enzyme source necessitates the demonstration that heparin and dextran sulfate are interacting directly with tryptophan hydroxylase and not with some other possible modulator substance. The enzyme–polyanion interaction was investigated by affinity chromatography of the hydroxylase on heparin-substituted Sepharose 4B and gel filtration on Sepharose/CL-6B. The results of the experiments with affinity chromatography are presented in Table 1. It can be seen that tryptophan hydroxylase binds to some extent to the heparin-Sepharose. Approximately 44 per cent of the total recovered activity was bound to the gel. The addition of heparin (2 mg/ml) to either the equilibrating buffer or the enzyme solution prior to chromatography completely blocked the binding of trytophan hydroxylase to the gel (data not shown). The ionic nature of the binding is indicated by the NaCl (1.0 M)-induced elution of the enzyme. Furthermore, if the heparin-Sepharose was equilibrated with buffer containing 1.0 M NaCl, the enzyme did not bind to the column (data not shown). Finally, tryptophan hydroxylase did not bind to ethanolamine-treated CnBr-Sepharose 4B.

The results of gel filtration studies are presented in Fig. 4. It was assumed that, if heparin and dextran sulfate were binding to tryptophan hydroxylase, the polyanion–enzyme complex should chromatograph

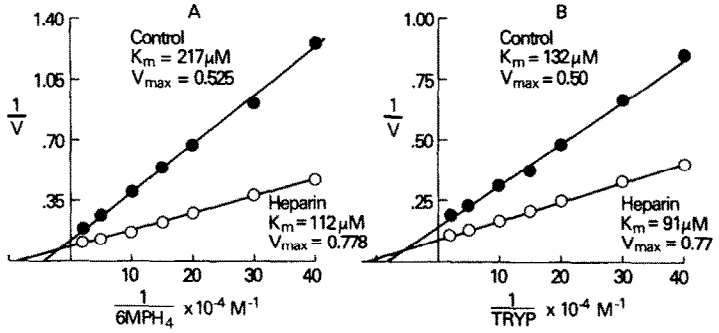

Fig. 3. Effects of heparin on the kinetic properties of tryptophan hydroxylase. Kinetic parameters for 6–MPH_4 (A) and tryptophan (B) were determined as described in Materials and Methods. Velocity (V) is expressed as nmoles of 5-HTP/mg/min. Heparin was added in a final concentration of 1.0 mg/ml. This experiment was repeated with similar results on three separate occasions.

Table 1. Affinity chromatography of tryptophan hydroxylase on heparin-Sepharose*

	Enzyme activity (nmoles 5-HTP/30 min)	Protein (mg)	%Activity bound†
Total enzyme applied	13.50	1.20	
Heparin-Sepharose			
0.05 M Tris, pH 7.4 (effluent)	5.31	0.410	
0.05 M Tris, pH 7.4, + 1.0 M NaCl (eluate)	4.11	0.37	44
Ethanolamine-Sepharose			
0.05 M Tris, pH 7.4 (effluent)	12.0	0.653	
0.05 M Tris, pH 7.4, + 1.0 M NaCl (eluate)	0	0.001	0

* Enzyme activity was measured in each fraction as described in Materials and Methods except that the reaction volume was increased to 100 μl and the incubation time was increased to 30 min. This experiment was repeated three times with essentially the same results.

† Per cent activity bound refers to that fraction of the total amount of recovered enzyme activity (approximately 70 per cent) which was eluted from the gels by NaCl.

quite differently from the native enzyme. The use of a large molecular weight dextran sulfate (mol. wt = 500,000) made this type of experiment technically feasible. It is evident in Fig. 4 that, when chromatographed with dextran (5mg/ml), tryptophan hydroxylase emerged in a retained fraction. The peak tube was at fraction 31 which corresponds to a K_{av} of 0.41. However, if dextran sulfate was added to the enzyme solution (5 mg/ml) prior to chromatography, the enzyme was eluted at fraction 22, corresponding to a K_{av} of 0.10. The shoulder on the low molecular weight side of the enzyme + dextran sulfate elution curve probably represents enzyme activity which was not bound to dextran sulfate. This dramatic shift in the elution profile of the hydroxylase indicates that the enzyme was bound to dextran sulfate.

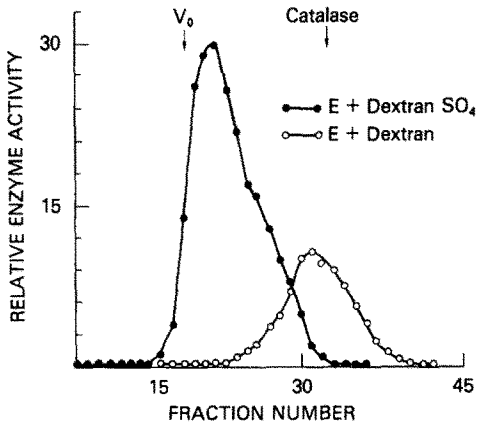

Fig. 4. Gel filtration of tryptophan hydroxylase. Dextran (○) or dextran sulfate(●) was added to the enzyme preparation (2.5 to 3.0 mg protein) in a final concentration of 5 mg/ml, and the solution was layered over a Sepharose/CL-6B column (0.9 × 29 cm) equilibrated with 0.05 M Tris–HCl (pH 7.4) containing 2 mM DTT. Fractions were assayed as described in Materials and Methods except that the incubations were carried out for 30 min. The column void volume (V_0) was determined with Blue Dextran. Enzyme activity is expressed in arbitrary units on the ordinate. This experiment was repeated with the same results on four separate occasions.

*Inhibition of polyanion stimulation of tryptophan hydroxylase by salt.*It is well known that the surface charge potential of high density polyelectrolytes can be compromised by increases in the concentration of a mobile counterion [13]. Based on this fact, as well as the observation that NaCl prevents binding of tryptophan hydroxylase to heparin-Sepharose (above), it was predicted that the increase in tryptophan hydroxylase activity produced by either heparin or dextran sulfate would diminish with increases in tonic strength. The results presented in Fig. 5 support this prediction. The enzyme activation produced by either heparin or dextran sulfate was completely blocked by NaCl ($I = 0.2$ M).

DISCUSSION

The results of the present series of experiments suggest that the polyelectrolytes, heparin and dextran sulfate, bind ionically to tryptophan hydroxylase, producing a 2- to 4-fold increase in enzymic activity. These results are in agreement with the studies of Katz *et al.* [5] on the polyanion-induced stimulation of tyrosine hydroxylase from bovine caudate nucleus. Of the polysaccharides currently tested, only heparin and dextran sulfate altered tryptophan hydroxylase activity. Chondroitin sulfate and dermatan sulfate are both sulfated polysaccharides [14], and at least chondroitin sulfate can activate tyrosine hydroxylase [5, 6]. However, neither these polysaccharides nor hyaluronic acid was capable of increasing tryptophan hydroxylase activity. The studies with dextran (Fig. 1) point out clearly that the common, salient factor responsible for the increases in tryptophan hydroxylase activity produced by heparin and dextran sulfate is the high negative charge density produced by the sulfate groups. Heparin contains an average of 1.6–3 sulfates per disaccharide [14], whereas dextran sulfate has an average of 4.6 sulfates per disaccharide. Although some polyanions such as poly-*l*-glutamic acid produce a larger activation of tyrosine hydroxylase than heparin [5], not one of the variety of polyanions currently tested, including poly-*l*- and poly-*d*-glutamic acid, glycogen and DNA, altered tryptophan hydroxylase. Apparently, the increase in tryptophan hydroxylase activity produced by poly-

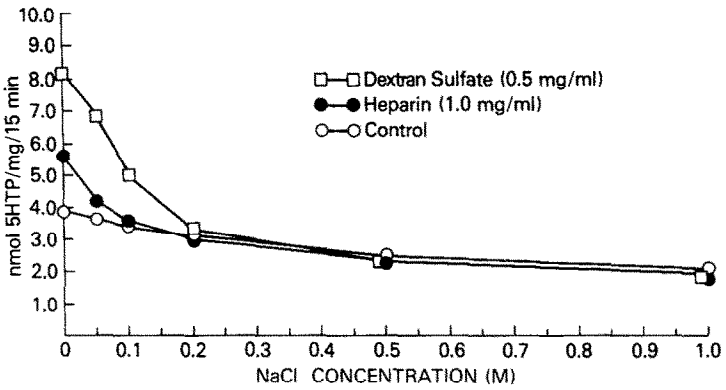

Fig. 5. Effects of increasing ionic strength on the stimulation of tryptophan hydroxylase by dextran sulfate and heparin. The ionic strength of the assay mixture was adjusted to the indicated molar (M) concentrations with NaCl. Dextran sulfate (□) was added in a final concentration of 0.5 mg/ml, and the heparin (●) concentration was 1.0 mg/ml. The enzyme was assayed with 0.4 mM tryptophan and 0.1 mM 6-MPH_4. Enzyme activity is expressed as nmoles of 5HTP/mg/15 min on the ordinate.

anions is dependent on higher charge densities and is also more specific than the polyanion-induced activation of tyrosine hydroxylase [5]. Perhaps tryptophan hydroxylase can now be added to the list of macromolecules which bind specifically to certain glycosaminoglycans [14].

The ionic nature of the interaction between polyelectrolytes and tryptophan hydroxylase can be inferred from a variety of supporting evidence. First, the increase in hydroxylase activity produced by either heparin or dextran sulfate diminishes as the salt concentration (and ionic strength) of the medium is increased (see Fig. 5). Second, the hydroxylase binds to heparin-Sepharose at low ionic strengths and is eluted at high ionic strengths. Heparin itself also prevents the binding of the enzyme to the gel (unfortunately the amount of enzyme bound to and recovered from the gel and the extent of purification by heparin–Sepharose chromatography was too low to make this a practical step in a purification scheme for tryptophan hydroxylase). Third, gel filtration of the hydroxylase with the high molecular weight dextran sulfate added (5.0 mg/ml) to the enzyme prior to chromatography drastically shifted the elution profile of the enzyme. The native enzyme eluted with a K_{av} of 0.41, whereas the enzyme–dextran sulfate complex eluted very close to the void colume with a K_{av} of 0.10 (Fig. 4). Taken together, these data suggest that tryptophan hydroxylase, like tyrosine hydroxylase [5], interacts with polyanions through electrostatic effects (dependent on both charge density and ionic strength). In contrast to results with tyrosine hydroxylase [5], some salts in very high concentrations have inhibitory effects on tryptophan hydroxylase. These effects are probably not related to the increases in tryptophan hydroxylase activity in brain slices produced by $MgCl_2$ or $BaCl_2$ [15].

Based on the similarities between the stimulation of tryptophan hydroxylase and tyrosine hydroxylase by polyanions, the inferences drawn about various structural features of tyrosine hydroxylase [5] can perhaps be extended to tryptophan hydroxylase: (1) the enzyme itself, like most proteins, is a polyelectrolyte which must have local areas of relatively high positive charge density facilitating its interaction with polyanions; and (2) tryptophan hydroxylase may exist in different activity states depending on its association with other polyelectrolytes and the extent of its protonation. It is implicit in these assumptions that the binding of tryptophan hydroxylase to polyanions induces a conformational change in the enzyme which leads to activation. The salt-induced inhibition of the hydroxylase may also involve a conformational change in the enzyme which instead deactivates the enzyme. Based on the results of studies of the partition of tryptophan hydroxylase between aqueous mixtures of polyethylene glycol and dextran [16], and of isoelectric focusing experiments [17], it is possible that the enzyme is negatively charged at neutral pH. Thus, tryptophan hydroxylase may, to some extent, behave like a linear polyelectrolyte such as dextran sulfate. At low ionic strengths the polyelectrolyte is extended due to intramolecular repulsion [13]. As the ionic strength increases, the surface electrostatic potential decreases and the polyelectrolyte "shrinks".

It is not likely that the stimulation of tryptophan hydroxylase specifically by heparin or dextran sulfate plays an important role *in vivo*. In addition, the salt concentrations which prevent the increases in tryptophan hydroxylase activity produced by heparin and dextran sulfate are within the physiological range (0.1 to 0.2 M). However, the interaction of tryptophan hydroxylase with anionic cellular and membrane components could serve as a mode of regulation. In fact, immunocytochemical studies have demonstrated what appears to be an association of tryptophan hydroxylase with cytoplasmic organelles in dorsal raphe neurons [18]. Furthermore, sulfated mucopolysaccharides are present in both soluble and particulate brain subfractions [19, 20], and phospholipids have received a great deal of attention recently as activators of tryptophan hydroxylase [2].

Finally, the role of protein phosphorylation in activating tryptophan hydroxylase has been investigated only recently [3, 4]. If brain tryptophan hydroxylase is analogous to hepatic phenylalanine hydroxylase, phosphorylation of the enzyme may alter its ionic nature [21], and presumably its structure [22], as a result of

the enzymatic attachment of the anionic phosphate group to the hydroxylase. Studies are underway with tryptophan hydroxylase to assess this possibility.

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