Immunochemical Demonstration of Increased Accumulation of Tyrosine Hydroxylase Protein in Sympathetic Ganglia and Adrenal Medulla Elicited by Reserpine

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ABSTRACT Chronic administration of reserpine to rats increases, in sympathetic ganglia and adrenal medulla, the activity of tyrosine hydroxylase (EC 1.14.3.4), the enzyme catalyzing the rate-limiting step in the biosynthesis of catecholamines. Immunochemical titration of the enzyme in both adrenal gland and innervated superior cervical ganglia demonstrates that enhanced enzyme activity is entirely attributable to accumulation of more specific enzyme protein and not activation of pre-existent enzyme molecules.

In adrenal gland and sympathetic neurons the activity of tyrosine hydroxylase (EC 1.14.3.4), the enzyme catalyzing the rate-limiting step in the biosynthesis of catecholamines (1), is regulated, in large measure, by the background of nerve impulse activity. When discharge of sympathetic neurons is directly or reflexly augmented over several days, activity of the enzyme may be increased many fold (2-5). Because such increase depends upon the integrity of preganglionic innervation the phenomenon is often referred to as transynaptic induction (10). A commonly used mode of producing transynaptic induction of TH is chronic administration of reserpine (3, 5, 6).

It has been assumed on the basis of indirect evidence that the elevation in TH activity associated with prolonged nerve stimulation is due to an increase in the number of specific enzyme molecules (3, 10, 11). In support of this contention are the observations that the rise of enzyme activity in sympathetic ganglia and adrenal gland elicited by reserpine (3, 5, 6) can be blocked with treatment by inhibitors of protein biosynthesis (10) and is associated with a rise in Vmax of the enzyme associated with a change of Km (3). However, similar findings could be explained by mechanisms not involving accumulation of specific enzyme protein, for example, by synthesis of a protein deactivating a noncompetitive inhibitor of the enzyme. To show that new TH enzyme protein has accumulated would require demonstration by immunological methods of the presence of increased quantities of specific enzyme protein. However, until the present time a specific antibody to TH has not been available.

We have recently purified TH in sufficient quantities to produce a specific antibody to the enzyme. By immunochemical titration we have attempted to determine if the increase of TH activity in the adrenal medulla and superior cervical ganglion of rats elicited by reserpine results, in its entirety, from accumulation of specific enzyme protein.

MATERIALS AND METHODS

Materials and Assay of Enzymes. Reserpine (Serpasil, 5 mg/2-ml ampule) was obtained from CIBA Pharmaceutical Co., L-3,4-dihydroxyphenylalanine (L-DOPA) from Amersham/Searle Corp., 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine from Aldrich Chemical Co., catalase (EC 1.11.1.6) from Sigma Chemical Co., and alumina from M. Woelm Co., Germany.

TH activity was assayed by the method of Shiman et al. (12) as modified by Coyle (13). The assay mixture contained 50 μl of enzyme, 10 μl of 1 M phosphate buffer (pH 6.2), 10 μl of sheep liver dihydropteridine reductase purified by the method of Kaufman et al. (14), 1000 units of catalase in 10 μl, 5 μl of 10 mM TPNH, 5 μl of 6 mM dimethyltetrahydropteridine, and 10 μl of 2 mM L-[3H]tyrosine. The mixture was incubated for 20 min at 37°C; the reaction was stopped by addition of 6 ml of 0.4 N perchloric acid containing 6 μg of carrier L-dihydroxyphenylalanine (L-DOPA). After centrifugation at 1000 × g for 10 min, the supernatant was added to a mixture of 30 mg of sodium bisulfate, 5 ml of 2% EDTA, 1.5 ml of 0.35 M KH2PO4, and 200 mg of alumina and titrated to pH 8.6 with 1 N NaOH. The suspension was poured over columns of 200 mg of activated alumina and washed with 20 ml of H2O; [3H]DOPA was eluted with 2.5 ml of 0.2 N acetic acid. The total eluate was counted in 15 ml of Bray's solution. Activity was expressed as cpm or nmol of [3H]DOPA formed per 20 min. Protein was assayed by the method of Lowry et al. (15).

Preparation of Specific Antibody to TH. Bovine adrenal glands were obtained fresh from a slaughterhouse; the adrenal cortex was removed by dissection. The medulla was homogenized in 20 mM potassium phosphate buffer (pH 6.5) and cellular debris was removed. The homogenate was centrifuged at 40,000 × g for 60 min, yielding active enzyme in both the supernatant ("high-molecular-weight" form (16)) and the sediment ("low-molecular-weight" form (16, 17)). The enzyme was partially purified from the supernatant in the following manner:

Ammonium sulfate was added to the supernatant to 80% saturation. The precipitate was dissolved and dialyzed against the same buffer containing 5% ammonium sulfate. At this
stage the enzyme aggregates easily (16). Aggregation, however, was prevented by addition of 5% ammonium sulfate to the buffer, possibly as a consequence of increased ionic strength. The enzyme was further purified by passage over a Sephadex G-200 column. Active fractions were precipitated with 0.2 M sodium acetate buffer (pH 5.5) and then centrifuged at 8000 × g. The supernatant was collected, dialyzed against 20 mM potassium phosphate buffer (pH 6.5), and passed through another Sephadex G-200 column. The active fractions were pooled, and the protein was concentrated for disc-gel electrophoresis.

We purified the sediment enzyme by treating the sediment with trypsin as described by Petrack et al. (17) and then centrifuging at 100,000 × g for 60 min. The supernatant was collected, treated with ammonium sulfate, and sequentially chromatographed through a Sephadex G-200 and then through a Sephadex G-100 column.

For disc-gel electrophoresis, 100 μg of protein was added to each gel by the method of Davis (18) with a sodium acetate buffer and run at 3 mA per tube for 90 min. The small gel segments containing the active enzyme were pooled and homogenized in a glass tube with 2 ml of 0.9% NaCl. An equal volume of complete Freund's adjuvant was added; the mixture was thoroughly emulsified and then injected into the foot pad of white New Zealand rabbits. After 2–3 months, when the antibody titer had reached a high level, the antibody was harvested. Immunoglobulin was precipitated from the serum at 50% saturation with ammonium sulfate.

Specificity of the Antibody. An antibody to TH found in either the supernatant or sediment fractions could be produced. The immunogenicity of both forms was similar and both forms yielded antibodies with indistinguishable properties. In this study, for convenience, we used antibody to enzyme in the supernatant fraction. The antibody to TH appeared to be highly specific for the enzyme by the following criteria: (a) Immunoelectrophoresis of antibody run against either purified bovine-adrenal TH or a crude extract of the gland yielded a single precipitin arc (Fig. 1). On the other hand, no precipitin arcs formed when the antibody was run against other catecholamine-synthesizing enzymes also purified from bovine-adrenal medulla, including dopamine-β-hydroxylase, aromatic L-aminoacid decarboxylase, and phenylethanolamine-N-methyltransferase. (b) The antibody inhibited TH activity in purified or crude bovine-adrenal extract nearly 100% (Table 1). In addition, the antibody to TH from bovine-adrenal gland was highly crossreactive with TH in homogenates of adrenal gland, superior cervical ganglia, or brain from rats (Table 1). (c) The antibody did not inhibit the activities of dopamine-β-hydroxylase, DOPA-decarboxylase, or phenylethanolamine-N-methyltransferase from bovine or rat adrenal gland.

Treatment of Rats. Reserpine was administered (2 mg/kg intraperitoneally) for 4 successive days to male Sprague–Dawley rats weighing about 250 g. In some animals, anesthetized with halothane (3% in 100% O₂), the sympathetic and vagus nerves, alone or in combination, were transected. On the fifth day the animals were killed, and the superior cervical ganglia and adrenal glands were rapidly removed and weighed. Ganglia and adrenals were assayed individually to establish variation within the different groups. For immunoprecipitation experiments, paired ganglia from six or paired adrenal glands from two rats were pooled, weighed, homogenized with a glass homogenizer in 50 volumes (w/v) of 5 mM Tris·HCl buffer (pH 7.5) containing 0.1% Triton X-100, and centrifuged at 16,000 × g for 10 min. TH was assayed in the supernatant.

The immunoprecipitation procedure was adapted from that described by Feigelson and Greengard (19). In this method increasing amounts of the enzyme (tissue homogenate) are added to a constant amount of antibody, i.e., that amount which will precipitate out or inactivate all of the enzyme protein in the smallest amount of tissue that is used. Ideally this procedure results in disappearance of enzyme activity in the supernatant. As more tissue enzyme is added, it saturates the

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**TABLE 1. Inhibition of tyrosine hydroxylase activity in various tissues by antibodies to tyrosine hydroxylase**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TH activity (cpm)*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>With control rabbit IgG</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Purified bovine-adrenal TH</td>
<td>3200</td>
</tr>
<tr>
<td>Bovine-adrenal medulla homogenate</td>
<td>3200</td>
</tr>
<tr>
<td>Rat-adrenal gland (whole)</td>
<td>3150</td>
</tr>
<tr>
<td>Rat-superior cervical ganglia</td>
<td>3200</td>
</tr>
<tr>
<td>Rat brain: caudate nucleus</td>
<td>3150</td>
</tr>
<tr>
<td>locus coeruleus</td>
<td>3150</td>
</tr>
</tbody>
</table>

*Volumes of enzyme preparations were adjusted such that 50 μl of homogenate contained about 4000 cpm of enzyme activity. 10 μl of antibody IgG or control rabbit serum IgG was added to 50 μl of enzyme, and the mixture was allowed to stand at room temperature (25°) for 60 min with occasional shaking. The resultant antigen–antibody precipitate was removed by centrifugation, and 50 μl of the supernatant was assayed for TH activity. Enzyme allowed to stand in buffer at room temperature for 60 min lost no more than 10% of activity. Control rabbit IgG did not change enzyme activity.
available antibody and increasing amounts of active enzyme remain in the supernatant. The concentration of tissue enzyme that precipitates all the antibody is termed the equivalence point, and is graphically identified as the point on the abscissa at which enzyme activity in the supernatant appears.

In the usual experiment, 10 µl of TH antibody was added to 10–60 µl of the tissue homogenate and then sufficient buffer [5 mM Tris·HCl (pH 7.5)] was added to bring the final volume to 70 µl. The mixture was allowed to stand for 60 min at room temperature with occasional shaking and then centrifuged at 16,000 \( \times g \) for 10 min to remove the precipitate. TH activity was assayed in a 60-µl aliquot of the supernatant.

**RESULTS AND DISCUSSION**

Reserpine administered daily for 4 days (2 mg/kg intraperitoneally) produced the well-known (3–6, 10) rise of enzyme activity in the superior cervical ganglion and the adrenal gland. Enzyme activities increased to 2.7 times that of control (Table 2). In the ganglion, the reserpine-induced rise of activity was blocked by transection of the preganglionic axons, confirming observations by others (5) that the increase of the enzyme activity in the ganglion is transynaptic. Hence, the increase in enzyme activity in ganglion cells cannot be attributed to any direct action of reserpine, at least in the absence of preganglionic innervation.*

To determine if the increased TH activity in ganglia and adrenal medulla elicited by reserpine was due to accumulation of more specific enzyme protein, we analyzed, by immunochromatographic titration (19), the amount of enzyme required to bind the specific antibody to TH in treated and control animals. Two types of experiments were performed. In the first (Figs. 2 and 3A), increasing amounts of tissue homogenates from control and treated animals were added to a fixed amount of antibody and the equivalence point was determined. In the second type of experiment (Fig. 3B), the tissue homogenate from the reserpine-treated animals was diluted so that enzyme activity in an aliquot of homogenate from a treated animal was identical to that in an equal volume of homogenate from a control animal.

In the first experiment (Figs. 2 and 3A) reserpine treatment shifted the equivalence points of both innervated ganglia and adrenal to the left. Only 12 µl of tissue homogenate from treated animals, in contrast to 32 µl from control animals, was required to bind all of the antibody. These experiments indicate that each volume of tissue from reserpine-treated animals contains 2.7 times more specific enzyme protein than control animals. The 2.7-fold increase in enzyme protein in the innervated ganglia and also in the adrenal medulla corresponds exactly to the incremental rise of enzyme activity (2.7 times) induced by reserpine in the same tissues (Table 2).

The second experiment (Fig. 3B) was entirely confirmatory. Immunoprecipitation of adrenal homogenates from treated and control animals adjusted for equal activity per unit volume resulted in overlapping equivalence points.

The present study, therefore, demonstrates that the increase in the activity of TH induced transsynaptically by prolonged neural stimulation in sympathetic neurons and chromaffin cells of the adrenal medulla is entirely attributable to accumulation of more specific enzyme protein. Activation of preexisting enzyme molecules does not seem to occur. Moreover, the precise relationship between the incremental rise of enzyme activity and the amounts of specific antibody required for binding of the enzyme protein indicates that accumulated enzyme protein is antigenically identical to that of the parent enzyme.

It remains to be determined whether the increased accumulation of TH results from increased synthesis of the enzyme, reduced degradation, or a combination of both processes. Further studies in which the rates of synthesis or degradation (turnover) of the enzyme are measured by immunoprecipitation of specific enzyme protein tagged with radiolabeled precursors (22) will be required to answer this question. However, evidence, accrued by others, that the reserpine-induced increase of TH activity can be blocked by cycloheximide and actinomycin-D (10, 11) and that reserpine will increase the content of RNA in sympathetic ganglia within 4

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* Of interest was the finding that unilateral preganglionic sympathectomy resulted in a significant 20% increase of TH activity in the contralateral (unoperated) ganglion (Table 2). This rise of enzyme activity could not be attributed to operative trauma since contralateral vagotomy preserving the sympathetic nerve failed to produce the effect. Possibly it was the result of a reflexly increased sympathetic activity resulting from interruption of baroreceptor afferents in the aortic nerve, which, in rats, run along with preganglionic sympathetic fibers in the cervical sympathetic chain (20). Increased sympathetic nerve activity elicited by baroreceptor denervation in rabbits increases the activity of adrenal TH (21), and conceivably could also increase ganglion enzyme activity in rats.

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### Table 2. Effects of reserpine on tyrosine hydroxylase activity in innervated and denervated superior cervical ganglion and adrenal gland in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Activity*</th>
<th>Reserpine/ control Left/right</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reserpine</td>
<td>461 ± 11</td>
<td>468 ± 12</td>
</tr>
<tr>
<td>Right preganglionic denervation</td>
<td>1244 ± 25</td>
<td>1244 ± 29</td>
</tr>
<tr>
<td>Right preganglionic denervation plus reserpine</td>
<td>474 ± 21</td>
<td>561 ± 20</td>
</tr>
<tr>
<td>Right vagotomy</td>
<td>459 ± 11</td>
<td>1258 ± 19</td>
</tr>
<tr>
<td>Adrenal TH activity</td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Activity*</th>
<th>Reserpine/ control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2081 ± 31</td>
<td></td>
</tr>
<tr>
<td>Reserpine</td>
<td>5602 ± 65</td>
<td>2.69</td>
</tr>
</tbody>
</table>

* Enzyme activity expressed as nmol/mg of protein per 20 min ± SEM. * Enzyme activity expressed as nmol/pair of adrenal glands per 20 min ± SEM. * Differ significantly from control (P < 0.001). * Differ significantly from right ganglia and control (P < 0.01). * Differ significantly from right ganglia and control (P < 0.001). * No significant difference from control (P > 0.05).
ultimately, however, studies of sympathetic ganglia in tissue culture suggest that events initiated by membrane depolarization (24) and possibly mediated by cyclic AMP may be of importance (25).

Activation of the sympathetic nervous system with augmented release of norepinephrine from sympathetic neurons coupled to enhanced release of adrenomedullary catecholamines is a common denominator in behaviors in which animals are prepared for fight or flight. This view, proposed by Cannon (26), emphasizes the utility of catecholamine release for an immediate physiological adaptation of viscer metabolism to emotional arousal. Recent studies have demonstrated that when arousal is produced over days by brain stimulation (9) or by emotional stress (8), the activity of TH may be increased transynaptically in adrenal glands and even in brain. On the assumption that such increased TH activity is due to accumulation of more enzyme protein, Cannon’s concept can be expanded. The new findings suggest that, when sustained, behavior characterized by adrenomedullary hyperactivity can lead to accumulation of the rate-limiting enzyme required for synthesis of the transmitters themselves. The biological advantage of this accumulation would be to match synthesis to release, thereby increasing the availability of catecholamines at receptors. Such changes, in all probability, also occur at noradrenergic neurons in the brain under conditions of enhanced activity. The consequences of the changes in enzyme quantity, however, remain to be elucidated.

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