# AN IMMUNOCHEMICAL STUDY OF THE INDUCTION OF TYROSINE HYDROXYLASE IN RAT ADRENAL GLANDS

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Received March 4,1974

SUMMARY. Tyrosine hydroxylase-specific protein is increased in rat adrenal medulla by immobilization stress, cold exposure, and 6-hydroxydopamine administration. The kinetic properties of the induced enzyme are identical to those of the control enzyme.

INTRODUCTION. A wide variety of physiological stresses and drug treatments cause an increase in the synthesis and release of catecholamines in adrenal medulla (1,2,3). In some of these conditions an increase in the activity of the catecholamine biosynthetic enzymes, tyrosine hydroxylase (4) and dopamine-β-hydroxylase (5), has been observed. Repeated immobilization (4), cold exposure (6), hypoglycemia (7), reserpine (8), phenoxybenzamine, 6-hydroxydopamine (9), and aminophyllin (10) lead to an increase in tyrosine hydroxylase activity. A common mechanism may be involved since in all cases the effect can be inhibited by severing the preganglionic neurons that innervate the adrenal medulla (9). A previous immunochemical study has demonstrated an increase in tyrosine hydroxylase protein subsequent to reserpine administration (11). In this report we present evidence for an increase in this specific enzyme protein following other forms of trans-synaptic induction. Kinetic data suggest that the properties of the induced enzyme are identical to those of the enzyme obtained from the control animals.

MATERIALS AND METHODS. Male Sprague Dawley rats, 120-150 g., were allowed free access to food and water. Some animals were immobilized in an apparatus previously described (3) for a single 2 1/2 hour period on each of 5 or 6

consecutive days. Immediately following the last immobilization, the animals were sacrificed by decapitation. The cold stressed animals were placed in a room at 3-4° C for four days. Less than 1/4 inch of bedding was placed in the cages, and the animals' fur was ruffled gently with water twice daily. 6-hydroxydopamine hydrobromide (Regis Chemical), 120 mg. free base/kg., was administered intraperitoneally (12) on two consecutive days, and the animals sacrificed 48 hours after the beginning of the experiment. Control animals were injected with diluent (0.1% ascorbic acid in 0.9% saline). Immediately following sacrifice, the adrenals were removed and most of the cortex trimmed away from the medulla. Using corticosterone as a marker for cortical tissue (10), and epinephrine as a marker for the medulla (4), we estimate that 83 $\pm$ 2.2% of the cortex was removed and only 9.4 $\pm$ 2.0% of the medulla was lost. Corticosterone was assayed by the method of Silber *et al.* (13), and epinephrine was assayed, following alumina chromatography (14), by the method of Weil-Malherbe and Bigelow (15).

Following the dissection, the medullary tissue from each group of animals (6-8 rats) was pooled* and homogenized manually in a Duall homogenizer in 5 ml. of potassium phosphate buffer, 0.05 M, pH 7.6. The homogenate was centrifuged at 24,000 g. for 20 minutes and cold saturated ammonium sulfate, pH 6.8, was added to bring the supernatant fraction to 80% of saturation with respect to ammonium sulfate. The pellet obtained after centrifugation at 17,000 g. for 15 minutes was dissolved in 0.05 M potassium phosphate buffer, pH 7.6.

The immunochemical titrations were performed by addition of increasing volumes of tyrosine hydroxylase antisera to fixed aliquots of the rat adrenal extracts. To ensure that each sample contained the same amount of serum,

*The adrenal medullae from the rats in each group of animals were pooled so that sufficient quantities of enzyme could be obtained to perform the antibody titrations and kinetic studies. Although the pooling procedure precluded statistical analysis of the data, we reproduced the immobilization effect in eight similar experiments and the effect of cold exposure and 6-hydroxydopamine in two and three experiments respectively. It has previously been demonstrated that each of these treatments cause statistically significant increases in tyrosine hydroxylase activity (4,6,9).

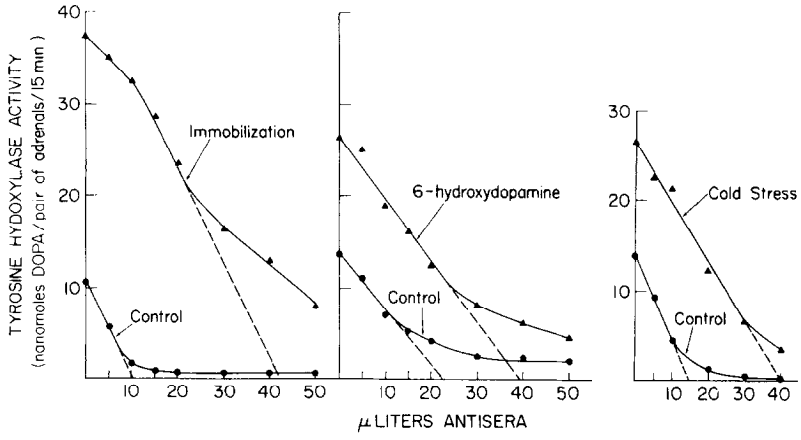


Figure 1. Titration of Tyrosine Hydroxylase with its Specific Antiserum.
The indicated volumes of antiserum were added to fixed aliquots of enzyme corresponding to one quarter of a single rat adrenal medulla. Control sera was added to each sample and the incubation performed as described in Materials and Methods. 2-amino-4-hydroxy-6-methyltetrahydropteridine was used as the cofactor in concentrations of 1.0 mM for the immobilization experiment, 0.95 mM for 6-hydroxydopamine, and 0.75 mM for cold stress. Final volume of the reaction mix was 1.0 ml. β-mercaptoethanol (14 mM) was included in the assay mixture to maintain the pteridine cofactor in reduced form. Initial tyrosine concentration was 0.05 mM.

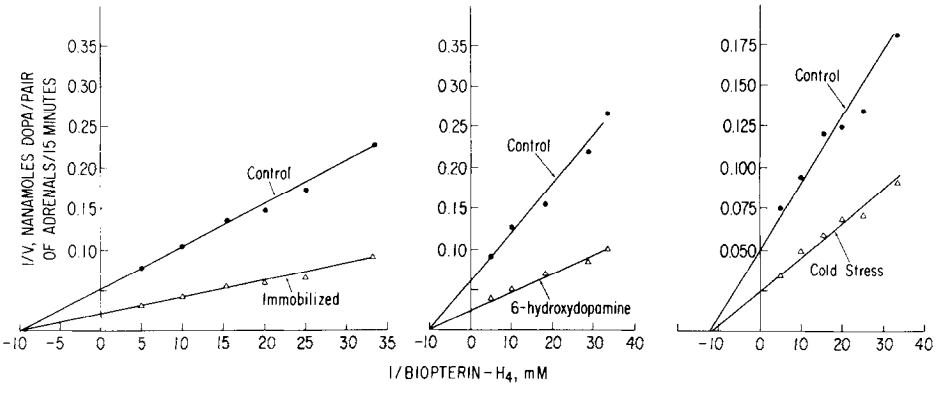


Figure 2. Determination of Km of Tyrosine Hydroxylase for Tetrahydrobiopterin.
Tyrosine hydroxylase was assayed at various concentrations of tetrahydrobiopterin which was maintained in its reduced form by dihydropteridine reductase (excess) and TPNH (0.5 mM). The reaction volume was 0.5 ml. Initial tyrosine concentration was 0.05 mM. The assay was performed with larger volumes of control enzyme (corresponding to one third of a single adrenal medulla) than induced enzyme (corresponding to one sixth of a single adrenal medulla), so that the amount of enzyme activity assayed was roughly equivalent for the various groups of animals. The velocity of the reaction is expressed in nanamoles of DOPA formed per pair of adrenal medulla during a 15 minute incubation.

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control serum was added to bring them all to the same volume. The antisera and the enzyme were incubated at 25° C for 15 minutes and at 0° C for 3 hours, and then tyrosine hydroxylase activity was assayed by the tritium release

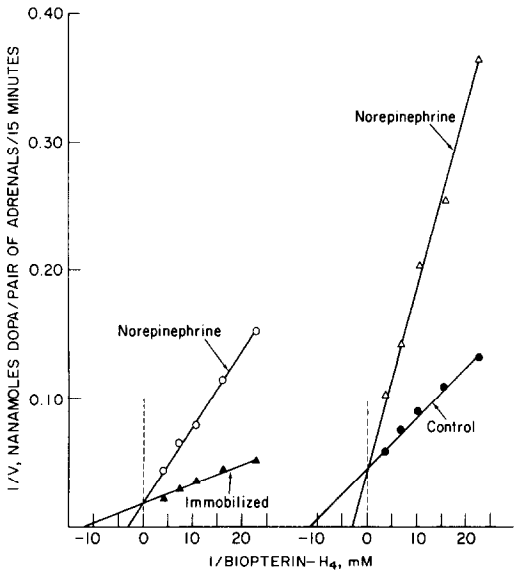


Figure 3. Competitive Inhibition of Tyrosine Hydroxylase by Norepinephrine. Tyrosine hydroxylase was assayed at various concentrations of tetrahydrobiopterin which was maintained in its reduced form by dihydropteridine reductase (excess) and TPNH (0.5 mM). Tyrosine concentration was 0.04 mM and norepinephrine was added to some tubes at 0.075 mM. The reaction mixture volume was 0.4 ml. The velocity of the reaction is expressed in nanamoles of DOPA formed per pair of adrenal medulla during a 15 minute incubation.

method (16) as employed in this laboratory (17). All enzyme assays were performed at 37° C for 15 minutes. (3,5-$^{3}$H) L-tyrosine (specific activity 48.2 Ci/mM) was purchased from New England Nuclear Corporation. The production of tyrosine hydroxylase antisera has been described previously (18). Protein was determined on aliquots of the dissolved ammonium sufate pellets by the method of Lowry et al. with the use of bovine serum albumin as a standard (19).

RESULTS AND DISCUSSION. Repeated immobilization led to a 2.5-3.5 fold increase in total tyrosine hydroxylase, whereas 6-hydroxydopamine and cold stress caused approximately a 2-fold increase in enzyme activity (Table I). The changes are less dramatic when expressed on a mg. protein basis, presumably because these procedures also lead to some medullary hypertrophy (4) with concomitant increases in medullary protein content. To determine if the tissue extracts from the treated animals contain increased numbers of tyrosine

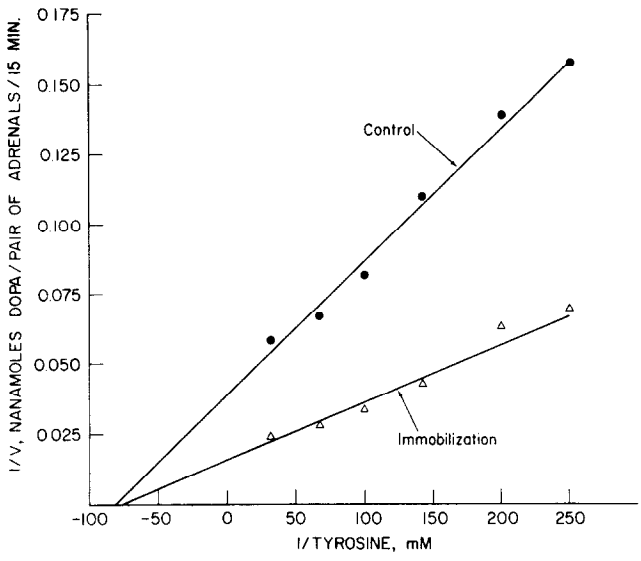


Figure 4. Determination of Km of Tyrosine Hydroxylase for Tyrosine.
Tyrosine hydroxylase was assayed at various concentrations of tyrosine, maintained at a constant specific activity of 140,000 cpm/nanamole. Tetrahydrobiopterin was present at a concentration of 0.2 mM. The reaction volume was 0.5 ml. The enzymatic regenerating system was employed as described in the legend to Figure 2. The assay was performed with larger volumes of control enzyme (corresponding to one third of a single adrenal medulla) than induced enzyme (corresponding to one sixth of a single adrenal medulla), so that the amount of enzyme activity assayed was roughly equivalent for the two groups of animals. The velocity of the reaction is expressed in nanamoles of DOPA formed per pair of adrenal medulla during a 15 minute incubation.

hydroxylase molecules, the extracts were titrated with a specific antiserum to the hydroxylase (Figure 1). By extrapolating the linear portions of the antibody titration curves to the abscissa it is possible to estimate the equivalence point, that amount of antiserum required to precipitate all of the tyrosine hydroxylase molecules present (20). The enzyme extracts from the immobilized animals required 3.5 times more antiserum to reach equivalence as did the enzyme extracts from the control animals. There was a two-fold increase in the amount of antiserum required to precipitate all of the tyrosine hydroxylase molecules from extracts obtained from cold stressed and 6-hydroxydopamine treated animals. Thus, for each of the three conditions, the increase in enzyme activity is associated with a parallel increase in the volume of antiserum needed for complete precipitation of the enzyme. These results

Table I. The Induction of Tyrosine Hydroxylase in Rat Adrenal by Immobilization, Cold Stress, and 6-Hydroxydopamine.

Tyrosine hydroxylase was extracted and assayed as described in Methods and Materials. 2-amino-4-hydroxy-6,7-dimethyl pteridine (0.8 mM) was used as a cofactor. β-mercaptoethanol (14 mM) was included in the assay mixture to maintain the pteridine cofactor in reduced form. Initial tyrosine concentration was 0.05 mM. Data are expressed as nanamoles of DOPA formed during a 15 minute incubation.

| | Tyrosine Hydroxylase Activity pair of adrenal medulla | Tyrosine Hydroxylase Activity mg. protein |
|---|---|---|
| Immobilization | 19.3 | 42.9 |
| Control | 6.5 | 23.2 |
| Cold Stress | 11.2 | 24.9 |
| Control | 5.8 | 16.0 |
| 6-hydroxydopamine | 12.6 | 26.8 |
| Control | 6.9 | 18.1 |

indicate that the increases in tyrosine hydroxylase activity seen under these experimental conditions are due to the presence of an increased quantity of tyrosine hydroxylase enzyme protein. An immunochemical study of the induction of tyrosine hydroxylase by reserpine led to a similar conclusion (11).

The experimentally elicited increases in tyrosine hydroxylase levels could result from enhanced synthesis and/or decreased degradation of the enzyme protein. There are indications that the turnover rate of tyrosine hydroxylase is slow, with a half-life estimated at 6-8 days (21). This result suggests that any alteration in protein degradation would affect enzyme levels significantly only after a time interval of this magnitude. Yet reserpine and 6-hydroxydopamine cause a two-fold increase in tyrosine hydroxylase after

18 (21) and 24 (22) hours respectively. It thus seems unlikely that the observed changes could result only from an alteration in the rate of enzyme degradation. Increased synthesis of the specific enzyme protein is further suggested by studies with inhibitors of nucleic acid and protein synthesis. Inhibition of RNA synthesis with actinomycin D prevents the increase in tyrosine hydroxylase activity elicited by insulin (7) and reserpine (21). Systemic administration of a protein synthesis inhibitor, cycloheximide, also blocks the reserpine effect (21).

Our immunological titration data, when taken together with the results of these inhibitor studies, strongly support the conclusion that the increased tyrosine hydroxylase activity observed in the adrenals of the treated animals is due, at least in part, to increased synthesis of hydroxylase molecules. It was of interest to determine whether the newly synthesized molecules have the same kinetic characteristics as the enzyme in control animals. It is possible, for example, that part of the increase in hydroxylase activity might be the result of the accumulation of enzyme molecules with more favorable catalytic or regulatory properties - such as a lower Km for tetrahydrobiopterin or a decreased sensitivity to feedback inhibition by norepinephrine. The induced enzyme, (actually, a mixture of "control" and "induced" enzyme molecules) however, has the same Km for tetrahydrobiopterin (0.09 mM) (Figure 2) and the same Ki for norepinephrine (0.022 mM) (Figure 3) as does the enzyme obtained from control animals. In agreement with previous data (23), the inhibition of the enzyme by norepinephrine is competitive with tetrahydrobiopterin. The induced enzyme also has an unaltered Km for tyrosine (0.013 mM) (Figure 4). Like the bovine adrenal enzyme (17), both the control and induced rat adrenal enzyme are inhibited by tyrosine concentrations above 0.05 mM when tetrahydrobiopterin is used as a cofactor. A kinetic study of rat adrenal tyrosine hydroxylase after reserpine treatment also failed to reveal any difference in the affinity of the new enzyme for tyrosine or 6,7-dimethyltetrahydropteridine (8). Tetrahydrobiopterin was employed in the present study since this is probably the natural

cofactor in adrenal medulla (24) and because several kinetic properties are exhibited by the enzyme only in the presence of this pteridine (17). In this regard, it was reassuring to find that the enhanced tyrosine hydroxylase activity elicited by these treatments could be demonstrated when the enzyme was assayed in the presence of tetrahydrobiopterin (Figure 3). Furthermore, even in the presence of the naturally occurring cofactor, the kinetic properties of the new enzyme molecules synthesized in these various experimental conditions are indistinguishable from those of the control enzyme.

Acknowledgement: Gratitude is expressed to Dr. Sheldon Milstien for providing the tetrahydrobiopterin.

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