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The Rapid Activation of Adrenal Tyrosine Hydroxylase by Decapitation and its Relationship to a Cyclic AMP-dependent Phosphorylating Mechanism[1]

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SUMMARY

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Rat adrenal tyrosine hydroxylase appears to exist in two forms that differ primarily in their affinities for pterin cofactor. In the nonstressed rat that has been anesthetized either by halothane or pentobarbital, approximately 25% of adrenal tyrosine hydroxylase is in the activated (low K_m) form. Following decapitation, approximately 60% of the enzyme is found in the active (low K_m) form, suggesting that a considerable fraction of the less active form of the enzyme is rapidly transformed to the active form during stress. A similar, although more complete, transformation of the less active form of the enzyme to the active form can be produced if the soluble enzyme from either stressed or nonstressed rats is incubated in the presence of cyclic AMP-dependent protein phosphorylating system. In the presence of the cyclic AMP-dependent protein phosphorylating system, the enzyme from both stressed and nonstressed animals achieves a comparable degree of activation. These results suggest that stress related activation of tyrosine hydroxylase may be mediated by a cyclic AMP-dependent protein kinase reaction.

INTRODUCTION

Tyrosine hydroxylase (TH)[2] is considered to be the rate limiting enzyme in the synthesis of catecholamines in both the peripheral and central nervous systems (1). Changes in the activity of TH in catecholaminergic tissues have been attributed to two basic mechanisms: induction (2) and activation (3-6).

Induction of TH has been shown to occur following prolonged stress of the animal, including cold exposure (7, 8) and immobilization (9, 10); as well as following treatment with various drugs, such as carbachol (11), reserpine (12) and oxytremorine (13). The induction of TH requires many hours and appears to be associated with an increase in enzyme protein, as demonstrated by immunochemical titration techniques. The mechanism by which this induction takes place has been examined by Costa

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[1] A preliminary report of this work was presented at the annual meeting of the American Society of Neurochemistry, Denver, Colorado. (*Trans. Am. Soc. Neurochem.* **8:** 144, 1977).

[2] The abbreviations used are: TH, tyrosine hydroxylase; 6-MePtH$_4$, 6-methyltetrahydropterin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; TES, 2-{[tris-(hydroxymethyl)methyl]-amino} ethanesulfonic acid buffer.

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and coworkers (14) who claim that it is mediated by a cyclic AMP-dependent protein kinase reaction involving the translocation of the protein kinase into the nucleus. However, the validity of this hypothesis has been challenged (15).

Acute activation of TH differs from induction of the enzyme in that it can occur following brief periods of nerve stimulation (3). This type of activation has been shown to take place in various preparations, including following stimulation of the isolated hypogastric nerve-vas deferens preparation (3, 4), in the hippocampus following stimulation of the locus coeruleus (16), and in the striatum following administration of antipsychotic agents (6, 7).

Activation of TH also can be produced by a cyclic AMP-dependent protein phosphorylating system (3, 5, 18, 19). Soluble, crude enzyme may be activated by exposure to $MgCl_2$, ATP and cyclic AMP. In addition, treatment of various tissue preparations, including rat striatal slices (20, 21), striatal and mesolimbic synaptosomes (22), rat striatal homogenates (23) and intact vas deferens (3) with dibutyryl cyclic AMP or other cyclic AMP analogs is associated with an increase in TH activity in these more intact systems. Evidence for the phosphorylation of TH by a cyclic AMP-dependent phosphorylating mechanism has been provided by experiments utilizing purified enzyme from various tissue preparations, including bovine striatum (24), rat striatum (25), rat pheochromocytoma (26), bovine chromaffin cells (27) and bovine adrenal medulla (28). Earlier efforts to demonstrate direct phosphorylation of TH from either rat striatum (5) or bovine caudate (29) yielded negative results.

Weiner and co-workers (3, 30) have attempted to relate the *in vitro* activation of TH by the cyclic AMP-dependent protein kinase system to the enzyme activation associated with nerve stimulation. They have found that stimulation of the hypogastric nerve-vas deferens preparation produces an increase in TH activity within a fraction of a minute. The addition of cyclic AMP, ATP and Mg^{++} to soluble enzyme prepared from stimulated and control tissues produces an activation of both the less active enzyme from unstimulated preparations and a less dramatic increase in activity of the more active enzyme from stimulated preparations. The activity of TH from control and stimulated tissues in the presence of the cyclic AMP-dependent protein phosphorylating system is similar, suggesting that the effects of nerve stimulation may be mediated through a cyclic AMP-dependent mechanism.

The purpose of these experiments was to determine whether a similar activation can occur in adrenal medulla tissue as a consequence of the application of acute stress to the intact animal.

METHODS

Material

Animals. Male-Sprague-Dawley rats weighing 250 to 400 g were obtained from Charles River Laboratories (Wilmington, Massachusetts). Animals were housed in groups of four to six with free access to food and water.

Drugs and assay chemicals. Drugs were obtained from the following companies: sodium pentobarbital (Diamond Laboratories, Des Moines, Iowa); halothane (Ayerst Laboratories, New York, New York); ether (J. T. Baker Chemical Company, Phillipsburgh, New York); chlorisondamine hydrochloride (Ciba Pharmaceutical Company, Summit, New Jersey); and hexamethonium bromide (Sigma Chemical Company, St. Louis, Missouri).

Chemicals were purchased from the following companies: 6-methyltetrahydropterin HCl (6-MePtH_4), Calbiochem (San Diego, California); catalase, Boehringer-Ingelheim, Ltd. (Elmsford, New York); 1-^{14}C-L-tyrosine and gamma-[^{32}P]ATP, New England Nuclear (Boston, Massachusetts); dithiothreitol, histone, pyridoxal 5′-phosphate, NADPH, 3-iodotyrosine, cyclic AMP and ATP, Sigma Chemical Company (St. Louis, Missouri). All other reagents were of the highest purity available commercially.

Procedures for removal of adrenal glands from stressed and nonstressed animals

Anesthesia. Adrenal enzyme from non-

decapitated (nonstressed) animals was obtained by anesthetizing the animals with either pentobarbital, halothane or ether and removing the adrenal glands surgically. An adequate anesthetic state was defined as that level of anesthesia at which the animals exhibit no blink reflex or response to a small abdominal skin incision. Once adequate anesthesia was obtained, the abdomen was opened and the adrenal glands were removed, placed upon filter paper, and maintained at 4° on ice. The adrenal medulla was then dissected out and immediately frozen on dry ice.

Pentobarbital anesthesia was induced by the administration of 60 mg/kg of this compound intraperitoneally. Anesthesia with the volatile anesthetics was produced by placing the animals into a one gallon jar and injecting either halothane (0.066 volume %) or ether (0.2 volume %) onto gauze attached to the underside of the jar lid. The volatile anesthetic doses were calculated to achieve a partial pressure which would produce surgical anesthesia within a five minute period.

Decapitation stress procedures. In the studies in which the effects of pentobarbital were assessed, control animals were injected with 1 ml/kg of saline and five minutes later the rats were decapitated, using a small animal guillotine. In studies in which the effects of either ether or halothane were assessed, control animals were placed in a one gallon jar for a period of five minutes, removed and decapitated. The adrenals were removed and processed as described above.

Administration of ganglionic blocking agents. Chlorisondamine, 50 mg/kg, was administered subcutaneously 4 hours prior to either pentobarbital administration or decapitation. Control rats received saline, 1 ml/kg, subcutaneously, four hours prior to the removal of the adrenal gland. In some cases chlorisondamine (15 mg/kg) or hexamethonium (15 mg/kg) was administered one hour before killing the animals. Adrenal glands were removed as described above.

Tissue preparation and assay of TH. The adrenal medullae were weighed and homogenized in 9 volumes of 0.05 M Tris-acetate buffer, pH 6, containing 0.2% Triton X-100. The homogenate was centrifuged at 40,000 × *g* for 30 min at 4°. One-half milliliter of the supernatant was passed over a Sephadex-G25 column (28 cm × 0.9 cm) to remove catecholamines and other small molecules and the protein fraction was eluted with homogenization buffer. The one milliliter fraction following the void volume (4.5 ml) was collected and 20 μl aliquots of the eluate fraction containing TH, as determined in preliminary experiments, were employed in each assay.

The TH activity of the adrenal tissue was determined by means of the coupled decarboxylase assay (31) as modified by Zivković *et al.* (6). The standard assay mixture (100 μl) contained: 90 mM potassium phosphate buffer (pH 6.2), 20 mM Tris-acetate buffer (pH 6), 10 mM sodium phospate buffer (pH 7), 1 mM 6-MePtH$_4$, 0.5 mM NADPH, 4 mM pyridoxal 5′-phosphate, 2 μl hog kidney L-aromatic amino acid decarboxylase (prepared according to the method of Waymire *et al.* [31]), 1,666 units of catalase, 10 μl sheep liver pteridine reductase (prepared according to the method of Kaufman [32]). The Tris acetate buffers and sodium phosphate buffers are employed to dissolve the NADPH and 6-MePtH$_4$, respectively (6). The final pH in the assay was 6.2. When kinetic studies were performed, either the 6-MePtH$_4$ concentration was varied from 0.033 mM to 1 mM, or the tyrosine concentration was varied from 0.66 μM to 100 μM.

In cases where the "cyclic AMP-dependent protein phosphorylating system" was employed, the following compounds were added to the assay mixture in a final total volume of 100 μl: 0.1 mM cyclic AMP, 0.5 mM ATP, 0.8 mM theophylline, 20 mM NaF, 20 mM magnesium acetate, and 0.12 mM EGTA (17).

Each sample was assayed in duplicate. An additional tube, serving as a blank, contained ingredients identical to those in the assay tubes, plus 2 mM 3-iodotyrosine in 0.01 N HCl. To the assay mixture was added, at 4°, 20 μl of the adrenal enzyme (after Sephadex chromatography) and, finally, 1-[^{14}C]L-tyrosine. Except in those experiments where tyrosine concentration was varied, the final concentration of

[^{14}C]tyrosine employed was 0.1 mM (specific activity, 10 mCi/mmole).

Following the addition of substrate, the tubes were capped with a rubber septum from which a plastic well was suspended, and the reaction was initiated by placing the stoppered tubes into a 37° bath. Each sample was incubated for 20 min, and then transferred to an ice bath. This reaction was linear with enzyme concentration and with time for at least 30 min. At the end of 20 min, 50 μl of 20 mM 3-iodotyrosine in 1 M sodium phosphate buffer, pH 8.0, was added. The assay tube was then incubated for an additional 30 min at 37° to allow for the quantitative conversion of [^{14}C]dopa to $^{14}CO_2$ and dopamine. The reaction was stopped by injecting 0.1 ml 1.6 N perchloric acid into the assay tube. Two-tenths milliliter of NCS solubilizer (Amersham Searle Corporation, Arlington Heights, Illinois) was injected into the plastic well suspended from the rubber septum covering the top of the assay tube and the tube was incubated for an additional hour at 37° to allow for quantitative collection of the liberated CO_2. The plastic well was then removed and placed into a scintillation vial to which was added 10 ml scintillation fluid containing 0.5 g 1,4-bis(2-(4 methyl-5-phenyloxazolyl)) benzene (di-methyl POPOP), 4.9 g 2,5-diphenyloxazole (PPO) and 5 ml ethanol per liter of toluene. Radioactivity was counted by liquid scintillation spectrometry. Counting efficiency was 85%. Protein was determined by the method of Lowry *et al.* (33). Results are expressed as nmols product formed per mg protein per hour.

Assay of cyclic AMP: Two adrenal medullae were homogenized in one milliliter of 5% trichloroacetic acid to extract cellular cyclic AMP. The homogenate was centrifuged at 4,000 × *g* for 20 min at 4°. The supernatant was passed over a Dowex column according to the procedure of Su *et al.* (34) to separate cyclic AMP from other nucleotides. Cyclic AMP was assayed by the radioimmunoassay procedure of Harper and Brooker (35).

Assay of protein kinase. Protein kinase was assayed based on the method of Langan (36). Four adrenal medullae were homogenized using a teflon pestle in 15 volumes of buffer (pH 6.0) containing 10 mM potassium phosphate, 10 mM EGTA and 5 mM theophylline. The homogenate was centrifuged at 40,000 × *g* for 15 min at 4°. The protein kinase activity was assayed within 20 min in the presence and absence of 20 μM cyclic AMP. The assay mixture (240 μl) contained: 50 mM TES (pH 6.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 200 μg histone and 25 μl gamma-[^{32}P]ATP (specific activity, 45 μCi/mole). The reaction was initiated by placing 10 μl of supernatant into the assay mixture and placing the assay tubes into a 37° bath. The tubes were incubated for 5 min and the reaction was terminated by the addition of 2.25 ml of 28% TCA to each tube. Each sample was passed over a Millipore filter (0.45 μm) and washed with 16 ml of 25% TCA. Following the wash the filters were allowed to dry and then placed into 10 ml Omnifluor-toluene (0.4%) fluid and counted by liquid scintillation spectrometry.

Calculations and statistics. All differences between treatment groups were analyzed by the Student's *t*-test for paired samples with a program from the Olivetti Programma 101 computer. For the kinetic studies, the results were analyzed according to the method of Lineweaver and Burk and the kinetic constants were determined by weighted linear regression analysis according to the procedure developed by Wilkinson (37), employing the Olivetti computer program. All calculations from the kinetic data presented in this paper were performed to obtain the apparent K_m and the apparent V_{max} values.

RESULTS

The apparent K_m for tyrosine for the adrenal enzyme from untreated, decapitated rats is 58 μM when the assay is performed in the presence of 1 mM 6-MePtH$_4$. The Lineweaver-Burk plot of enzyme activity vs. tyrosine concentration yields a straight line, suggesting that the enzyme obeys Michaelis-Menten kinetics (Fig. 1). The apparent maximal velocity of the enzyme reaction is 68 ± 9 nmols product formed per hour per mg adrenal medulla supernatant protein.

Kinetic analysis of the adrenal medulla

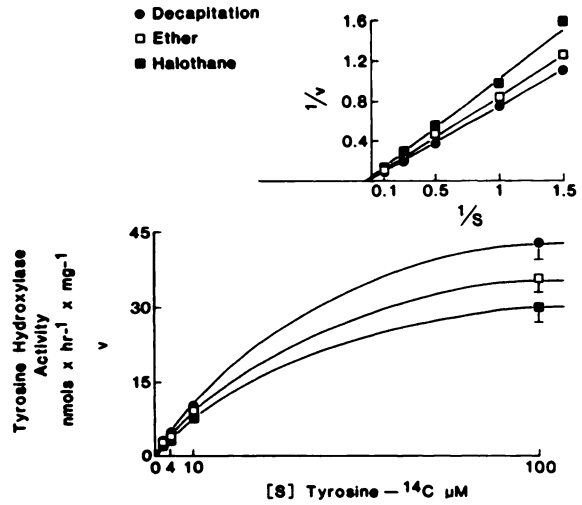

FIG. 1. *Kinetic analysis of the effects of decapitation, ether and halothane on adrenal tyrosine hydroxylase activity at various substrate concentrations*

The results are means from five experiments ± S.E.M. All values were determined at 1 mM 6-MePtH$_4$. Six tyrosine concentrations were employed. For clarity of the figures the 0.66 µM and 1 µM points were deleted from the S, V plot and the 100 µM point was deleted from the 1/S, 1/V plot.

enzyme from unanesthetized, decapitated rats, employing different concentrations of pterin cofactor, also indicates that the enzyme obeys Michaelis-Menten kinetics over a range of cofactor concentrations from 0.033 to 0.25 mM (Figs. 2 and 3). However, at 1 mM 6-MePtH$_4$, the reciprocal substrate-velocity relationship appears to deviate from linearity in a downward (higher enzyme activity) direction (Fig. 3). The apparent K_m for 6-MePtH$_4$ is calculated to be 89 µM and the apparent V_{max} in the presence of 0.1 mM tyrosine is 33 nmols product formed per hour per mg adrenal medulla supernatant protein.

Comparison of tyrosine hydroxylase activity in the adrenal gland following decapitation vs. anesthesia and surgical removal

Variations in tyrosine concentration. Table 1 summarizes the changes in the activity of adrenal TH prepared from decapitated and anesthetized animals as a function of different concentrations of tyrosine. No marked differences in the affinity of the enzyme for tyrosine were observed following any of the anesthetic procedures or decapitation. In contrast, in all

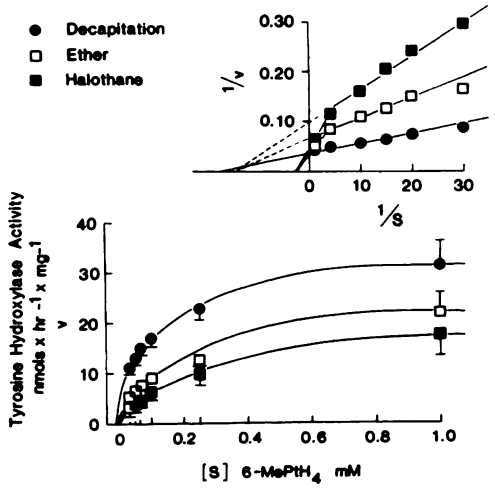

FIG. 2. *Kinetic analysis of the effects of decapitation, ether and halothane on adrenal tyrosine hydroxylase activity at various cofactor concentrations*

The results are means from four experiments ± S.E.M. All values were determined at 0.1 mM [1-^{14}C]L-tyrosine.

cases where the adrenal was removed surgically from the rat following anesthesia, the apparent V_{max} of the enzyme was significantly less than that seen following decapitation ($p < 0.05$). The apparent V_{max} of

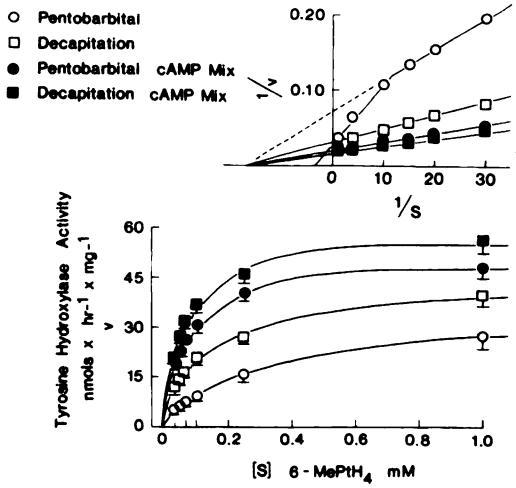

FIG. 3. *Kinetic analysis of the effects of decapitation and pentobarbital ± "cyclic AMP mix" on adrenal tyrosine hydroxylase activity at various cofactor concentrations*

The results are means from four experiments ± S.E.M. All values were determined at 0.1 mM [1-^{14}C]L-tyrosine.

TABLE 1

The effects of stress and a cyclic AMP-dependent protein kinase system on tyrosine hydroxylase of rat adrenal

Tyrosine as variable substrate. The values are the means from five experiments ± S.E.M. All kinetic values for tyrosine were determined at 1 mM 6-MePtH$_4$. V_{max} values are presented as nmoles CO_2 formed per hour per mg protein.

Treatment	K_m	V_{max}
	(mM)	
Series A		
Halothane	0.048 ± 0.009	41.3 ± 6.0[b, e]
Ether	0.051 ± 0.008	53.9 ± 8.3[a]
Decapitation	0.058 ± 0.008	67.8 ± 8.9
Series B		
Pentobarbital	0.048 ± 0.006	50.5 ± 2.1[a, d]
Decapitation	0.046 ± 0.007	70.7 ± 3.2[c]
Pentobarbital + cAMP mix	0.061 ± 0.010	98.8 ± 9.2
Decapitation + cAMP mix	0.062 ± 0.014	104.7 ± 6.3

[a, b] Significantly different from decapitation in the corresponding series of experiments $p < 0.05$ and $p < 0.01$, respectively.

[c, d] Significantly different from cAMP treatment $p < 0.05$, $p < 0.01$, respectively.

[e] Significantly different from ether, $p < 0.05$.

the enzyme prepared from animals anesthetized with halothane is also significantly lower ($p < 0.05$) than the corresponding value for the enzyme recovered from animals anesthetized with ether. These results suggest that partial activation of adrenal TH may be associated with ether anesthesia, whereas minimal or no activation of the enzyme occurs when the adrenals are removed following anesthesia with halothane.

The activity of adrenal TH prepared from animals anesthetized with pentobarbital was similar to that seen following anesthesia with halothane. There was no significant difference in the affinity of the enzyme for tyrosine between the pentobarbital anesthetized group and the group killed by decapitation. However, TH prepared from pentobarbital anesthetized animals exhibited a significantly lower reaction velocity, at 1 mM 6-MePtH$_4$, than was seen with comparable enzyme preparations prepared from decapitated rats.

Effect of different cofactor concentrations. When relatively low concentrations of pterin cofactor were employed in the assay, TH prepared from adrenals of decapitated rats exhibited significantly higher activity than the enzyme from anesthetized rats. However, at the highest concentration of pterin cofactor employed (1 mM), the differences in tyrosine hydroxylase activity between preparations obtained from anesthetized and decapitated rats were less pronounced (Figs. 2 and 3). When the activity of adrenal TH from rats anesthetized with either halothane, ether or pentobarbital was determined in the presence of different cofactor concentrations and plotted according to the method of Lineweaver and Burk, a nonlinear relationship between the reciprocal of enzyme velocity and the reciprocal of pterin cofactor concentration was obtained. The curves deviated downward considerably at the highest cofactor concentrations. This deviation was more marked for enzyme prepared from animals anesthetized with halothane or pentobarbital. According to Reiner (38), this type of relationship suggests that more than one enzyme type may be present and that these exhibit different affinities for the variable sub-

strate. Similar results have been obtained for other enzymes, including the high and low K_m forms of cyclic AMP phosphodiesterase (39), aldehyde dehydrogenase (40) and N-hydroxyphentermine reductase (41). Another possible interpretation of the downward deviation of the Lineweaver-Burk plot is that the enzyme exhibits negative cooperativity. In the present study, the interpretation that there are two forms of the enzyme present seems more likely, since the cyclic AMP activated form of TH enzyme shows a normal Lineweaver-Burk plot with no apparent cooperativity (Hill coefficient = 1.06) (42). This supports the notion that the enzyme does exist in two forms, although the possibility that the less active form may exhibit negative cooperativity cannot be excluded.

The kinetics of the enzyme were analyzed according to the method of Wilkinson (37). For enzyme from the adrenals of anesthetized animals, where the curvilinear Lineweaver-Burk relationships were apparent, only the lower cofactor concentrations were employed in these calculations. As is apparent from Figures 2 and 3, at low cofactor concentrations the straight line relationship obtained in all instances.

Using the equations suggested by Reiner (38) for the determination of the proportions of two enzymes with different affinities for substrate but similar maximal velocities, the apparent K_m value for the less active form of the enzyme was calculated. As was anticipated, the contribution of the less active form of the enzyme to the total enzyme activity becomes more prominent as saturating levels of cofactor are approached. At the higher levels of cofactor, a line is obtained with a slope equal to:

$$\frac{K_1V_1 + K_2V_2}{V_2} \quad (1)$$

where V = maximal velocity, $K = K_m$ and the subscripts refer to enzyme 1 (high affinity enzyme) and enzyme 2 (low affinity enzyme) and $V = V_1 + V_2$. the intercept of this line on the vertical axis, $\frac{1}{v}$ is $\frac{1}{V}$ and the tangent of the line segment intersecting the ordinate, when extended to the abscissa (−1/S) is equal to:

$$-1/S = -\frac{(V_1 + V_2)}{(K_1V_1 + K_2V_2)} \quad (2)$$

If we assume that K_1 is much less than K_2, then the equation may be simplified to:

$$S = \frac{K_2V_2}{V_1 + V_2} \quad (3)$$

(30). Using the above equations, the theoretical apparent K_m values for the less active form of the enzyme were obtained (Table 2, column 3).

Based upon the analysis of the kinetic properties of the adrenal enzyme obtained from animals either decapitated or exposed to halothane or ether, as indicated in Figures 2 and 3 and Table 2, it seems apparent that different proportions of the less and the more active forms of tyrosine hydroxylase may coexist among the several groups. The mean apparent K_m value for the high affinity enzyme is approximately 0.07–0.1 mM 6-MePtH$_4$, whereas that for the low affinity enzyme is approximately 0.7–0.9 mM. Thus, the high affinity enzyme exhibits a 7–10-fold greater affinity for pterin cofactor than does the low affinity form.

The effect of the cyclic AMP-dependent protein phosphorylation system on the activity of adrenal tyrosine hydroxylase prepared from decapitated and anesthetized rats

When soluble TH prepared from adrenals surgically removed from either decapitated or pentobarbital anesthetized animals was incubated with the cyclic AMP-dependent protein phosphorylating system in the presence of different concentrations of tyrosine, further activation of the enzyme was noted (Fig. 4). No change in the apparent affinity of the enzyme for tyrosine was seen with either preparation. However, for the enzyme prepared from both decapitated ($p < 0.05$) and pentobarbital anesthetized ($p < 0.01$) animals, the apparent maximal velocity of the reaction in the presence of the cyclic AMP-dependent protein kinase system was significantly greater than that seen in the absence of this system. Similar levels of enzyme activity were seen when TH from these two groups of animals was assayed in the presence of the cyclic AMP-

TABLE 2

The effects of stress and nonstress procedures ± cyclic AMP mix on the cofactor kinetic parameters of adrenal tyrosine hydroxylase

The values are the means from four experiments ± S.E.M. All kinetic values for cofactor were determined at 0.1 mM [1-^{14}C]L-tyrosine. K_m values are presented as mM 6-MePtH$_4$. V_{max} values are presented as nmoles CO_2 formed per hour per mg protein. High affinity K_m (column 1) and V_{max} (column 2) values were obtained by using the Wilkinson test (27). In cases where the treatments produced nonlinear reciprocal kinetics at high cofactor concentrations, only the values obtained with the low cofactor concentrations, which are on the linear portion of the plot, were used to determine the high affinity K_m and V_{max} values. The low affinity K_m values were determined by two procedures described in the text. The K_m values in column 3 were determined by assuming that the high affinity K_{m_1} was much less than the low affinity K_{m_2}. As a consequence, when a sufficient proportion of the enzyme is in the low affinity state, so that $K_{m_1} V_1 \ll K_{m_2} V_2$, $K_{m_1} V_1$ may be ignored in these calculations (equation 3). Values of S, V_1 and V_2 (equation 3) were determined by extrapolation of the linear portion of the lines obtained experimentally. The K_m values in column 4 were determined with the use of the high affinity K_m and V_{max} values presented in columns 1 and 2 which were obtained in the presence of the cyclic AMP-dependent phosphorylating system (cAMP mix). This minimizes the uncertainty of the extrapolation of the Lineweaver-Burk relationships, since deviation from linearity was insignificant, thus allowing one to obtain a more precise value for K_{m_1} and total V_{max} to use in the calculations. V_{max} values (column 5) were obtained by inserting the high affinity V_{max} values (column 2, + cAMP mix) into the following equation: V_{max} (total) = V_{max} (high affinity) + V_{max} (low affinity). For the pentobarbital and decapitation groups the corresponding V_{max} values obtained in the presence of the cyclic AMP-dependent protein phosphorylating system were used as estimates of total V_{max}. For the halothane and ether groups, total V_{max} was assumed to be that obtained in the presence of cAMP mix following decapitation of the animals.

Treatment	High Affinity		Low Affinity		
	K_m	V_{max}	K_m	K_m	V_{max}
Halothane	0.113 ± 0.006	8 ± 3[a]	0.97	0.67	48
Ether	0.083 ± 0.010	15 ± 8[a]	0.76	0.52	41
Decapitation	0.089 ± 0.009	33 ± 10			23
Decapitation + cAMP Mix	0.055 ± 0.010	56 ± 3			
Pentobarbital	0.071 ± 0.010	16 ± 1[a, c]	0.51	0.38	35
Decapitation	0.066 ± 0.005	35 ± 1[b]	0.64	0.55	21
Pentobarbital + cAMP Mix	0.059 ± 0.003	51 ± 6			
Decapitation + cAMP Mix	0.055 ± 0.010	56 ± 3			

[a] Significantly different from decapitation, $p < 0.05$.

[b] Significantly different from cAMP treatment, $p < 0.05$.

[c] Significantly different from cAMP treatment, $p < 0.01$.

dependent protein phosphorylating system.

The activity of TH obtained from decapitated animals and animals anesthetized with pentobarbital, measured in the presence and absence of the cyclic AMP-dependent protein kinase system, also was evaluated using variable cofactor concentrations. As indicated in Table 2, the K_m values of the enzyme in the presence of cyclic AMP, ATP and Mg^{++}, for the two groups, was not significantly different. Although the apparent V_{max} for the high affinity enzyme obtained from adrenals of rats following decapitation is significantly greater than that of anesthetized animals, no significant difference in the apparent V_{max} for this form of the enzyme was observed when the enzyme from the two groups was assayed in the presence of the cyclic AMP-dependent protein kinase system. The apparent V_{max} values for the high affinity enzyme from both groups of animals, when measured in the presence of cyclic AMP, ATP and Mg^{++}, was significantly greater than the apparent V_{max} of enzyme from pentobarbital treated animals ($p < 0.05$) measured in the absence of these constituents. The average apparent K_m values for the low affinity enzyme were 0.45 mM for the pentobarbital anesthetized group and 0.60 mM for the enzyme from decapitated rats. The low affinity enzyme exhibits an apparent K_m which is approximately 7–10-fold higher than the K_m value

for the high affinity form of the enzyme.

Assuming the cyclic AMP-dependent protein phosphorylating system is able to convert the entire population of TH molecules to the active form, the apparent K_m values for the less active form of the enzyme may be calculated by an alternative procedure, employing the reciprocal of equation 2.

$$S = \frac{K_1V_1 + K_2V_2}{V_1 + V_2} \quad (4)$$

For these calculations the values of K_m obtained by the Wilkinson weighted linear regression analysis were employed. In all experimental groups, when the enzyme was assayed in the presence of the cyclic AMP-dependent protein phosphorylating system, the reciprocal plots of enzyme velocity vs. cofactor concentration yield straight lines which resembled the high affinity enzyme form (Fig. 3). It was therefore assumed that the K_m and V_{max} values obtained for the enzyme assayed in the presence of the cyclic AMP-dependent protein phosphorylating system reflected the K_m and V_{max} values for the totally activated (high affinity) form of the enzyme and that the entire complement of enzyme was now in this form; i.e., $V_2 = 0$ and $V_1 = V_1 + V_2$. When this procedure is employed to analyze the activated enzyme, V and K_1 can be readily determined from the Lineweaver-Burk plot. The experimental data obtained in the absence of a cyclic AMP-dependent phosphorylating system are then inserted into equation (4) for different concentrations of cofactor, and the apparent K_m value for the less active form of the enzyme can be obtained. These results are presented in column 4 of Table 2 for the halothane, ether and pentobarbital anesthetized groups.

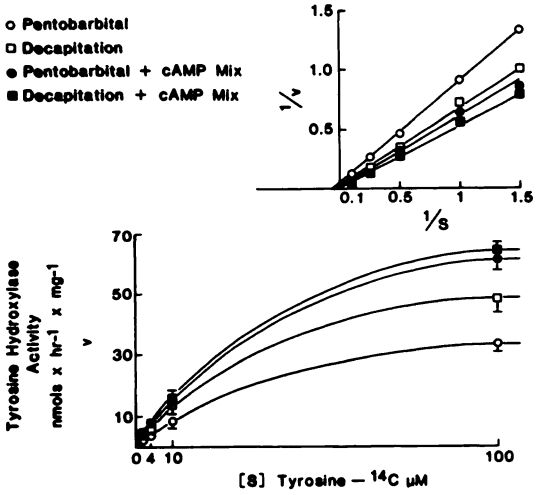

FIG. 4. *Kinetic analysis of the effects of decapitation and pentobarbital ± "cyclic AMP mix" on adrenal TH activity at various substrate concentrations*

The results are means from five experiments ± S.E.M. All values were determined at 1 mM 6-MePtH$_4$. Six tyrosine concentrations were employed. For clarity of the figures the 0.66 µM and 1 µM points were deleted from the S, V plot and the 100 µM point was deleted from the 1/S, 1/V plot.

Cyclic AMP levels and protein kinase activities in adrenal medulla following decapitation and pentobarbital anesthesia

Table 3 illustrates cyclic AMP levels and protein kinase activity in the adrenal medulla of rats subjected to decapitation or pentobarbital anesthesia before the adrenals were removed. There is no significant difference between the cyclic AMP levels in the glands from the two groups. However, there is a significant increase ($p <$

TABLE 3

The effects of stress on cyclic AMP levels and protein kinase activity in the adrenal medulla

The values are the means ± S.E.M. from 12 experiments for the cyclic AMP measurements and 5 experiments for the protein kinase data.

Treatment	Cyclic AMP	Protein kinase activity		
		−cAMP	+cAMP	−cAMP/+cAMP
	(*pmols/mg*)	(*nmols/hr/mg*)		
Pentobarbital	27 ± 10	42 ± 6[a]	141 ± 7	0.30 ± 0.03
Decapitation	39 ± 12	67 ± 9	136 ± 6	0.49 ± 0.04

[a] Significantly different from decapitation, $p < 0.05$.

0.05) in the cyclic AMP-independent protein kinase activity following decapitation as compared with adrenal protein kinase activity from animals anesthetized with pentobarbital.

Effects of chlorisondamine upon stress activated TH

The effect of chlorisondamine treatment on the stress-induced activation of TH in the adrenal medulla was determined. Chlorisondamine, 50 mg/kg, was administered subcutaneously 4 hours before killing the rats. This treatment did not appear to affect adrenal TH activity in the pentobarbital treated group of animals (Table 4). In contrast, the ganglionic blocking agent inhibited the activation of TH associated with decapitation by approximately 60%. This effect was apparent at both high and low cofactor concentrations. When chlorisondamine (15 mg/kg) or hexamethonium (15 mg/kg) was administered one hour before decapitation or anesthetization of the animals, similar results were obtained (data not shown).

TABLE 4

Stress-activation of adrenal medulla tyrosine hydroxylase

Effect of chlorisodamine. The values are the means from five experiments ± S.E.M. All values were determined at 0.1 mM [1-^{14}C]L-tyrosine. Data are presented as nmoles CO_2 formed per hour per mg protein. Chlorisondamine was given intraperitoneally (50 mg/kg), 4 hours prior to removal of the adrenal glands. In the lowest line of the table, the percentages represent the inhibition of adrenal tyrosine hydroxylase activation in the decapitation stressed animals which was seen following chlorisondamine treatment. Percentages were determined by using the average of the pentobarbital and pentobarbital + chlorisondamine groups as the 0% value and the decapitation group as the 100% value.

Treatment	[6-MePtH$_4$]	
	(*0.066 mM*)	(*1 mM*)
Pentobarbital	5.3 ± 1.0	26.8 ± 3.6
Pentobarbital + chlorisondamine	6.9 ± 1.0	29.8 ± 3.2
Decapitation	22.0 ± 3.5	69.7 ± 9.3
Decapitation + chlorisondamine	13.7 ± 2.4 (↓ 58%)	42.1 ± 8.2 (↓ 67%)

DISCUSSION

Rat adrenal TH can be activated rapidly as a consequence of decapitation of the animal. The activation of the enzyme appears to be related to a shift of a fraction of the less active form of the enzyme to a more active state. The notion that the enzyme ordinarily exists in two different states is suggested from the curvilinear Lineweaver-Burk kinetics seen in adrenal medulla supernatants prepared from animals that were anesthetized prior to killing (Figs. 2 and 3). Quantitative transformation of the enzyme to the active form can be produced by incubation of enzyme prepared from anesthetized rats in the presence of the cyclic AMP-dependent protein phosphorylating system. The addition of the cyclic AMP-dependent protein kinase system to the enzyme obtained from adrenals of both the stressed and nonstressed rats produces activation to approximately the same level. The elimination of the deviation from linearity of the Lineweaver-Burk plot of enzyme activity versus cofactor concentration suggests that the enzyme is now in the totally active state.

A greater proportion of the less activated form of TH is apparent in adrenals obtained from animals anesthetized with ether, pentobarbital or halothane, as compared with animals whose adrenals are removed following decapitation. That this effect is seen with all three anesthetics indicates that the lower activity of TH obtained following anesthesia is due to a "non-stressful" removal of the adrenal gland and not to some nonspecific effect of the anesthetics. We have added pentobarbital (1 μM to 1 mM) to soluble adrenal TH and observed no effect on TH activity. In addition, when adrenal TH from animals that were subjected to decapitation under pentobarbital anesthesia was examined, the activity of the enzyme was similar to that seen in preparations obtained from unanesthetized, decapitated animals. It thus appears that the differences in TH activation observed in the several groups examined in this study are due to the effects of the stress and are not related to the anesthetic itself.

Since protein kinase is activated by decapitation (Table 3), it is possible that TH

activity is enhanced artifactually either during homogenization of the tissue or during the assay. However, the preparation of the tissue for assay was conducted at 4°. We have demonstrated that the addition of cyclic AMP, ATP and Mg^{++} to the adrenal enzyme at 4° does not result in enzyme activation. In addition, the supernatant enzyme was routinely separated from small molecules by Sephadex chromatography prior to the assay. Thus, ATP and Mg^{++} would be separated from the enzyme and any cyclic AMP-independent protein kinase present would be unable to activate TH by phosphorylation subsequent to this step. Furthermore, when protein kinase is separated from TH by sucrose density centrifugation, the activation of the latter enzyme following decapitation can still be demonstrated. These results suggest that the activation of TH following decapitation is indeed occurring in the intact adrenal gland.

It has been reported that the effects of handling rats and injecting them intraperitoneally can itself be stressful (44; R. Kvetňanský, personal communication). This might explain the presence of a considerable fraction of the enzyme in the activated state in the anesthetized groups. However, it is possible that there is some proportion of activated enzyme present in the adrenal gland even in the absence of adrenal impulses and stress, because we observed no difference in TH activity between animals treated with pentobarbital and those treated with chlorisondamine plus pentobarbital. The proportion of the enzyme in the active state that is seen in the anesthetized groups may therefore represent the "basal level" of rat adrenal TH activity. Conversely, animals treated with chlorisondamine prior to decapitation exhibit a 60% decrease in adrenal TH activity as compared to the decapitated animals not treated with the ganglionic blocking agent. This suggests that the TH activation seen in the adrenal following stress is transsynaptically mediated.

Ether anesthesia was associated with a significant increase in TH activity over that seen with halothane anesthesia. This increase may be explained by the fact that ether produces a generalized sympathetic nervous system response and catecholamine release from the adrenal medulla (45, 46), whereas halothane does not exert this effect.

A considerable number of putative allosteric modulators of TH has been proposed and one or more of these substances may be responsible for the enhanced activity of this enzyme that is associated with neural activity or stress. Mandell *et al.* (47) noted that administration of either amphetamine, reserpine or alpha-methyl-p-tyrosine to rats is associated with a shift of caudate TH from the soluble to the particulate state, presumably as a consequence of membrane binding. This was associated with a modest activation of the enzyme. However, stresses that presumably enhance central neural activity did not reproduce the effects of the drugs on this enzyme.

Kuczenski and Mandell (48) observed that sulfate ions and heparin activated rat caudate soluble TH *in vitro*. Both the maximal velocity and the affinity of the enzyme for cofactor were enhanced by heparin. In addition, the K_i for dopamine was increased by this sulfated polysaccharide. The changes produced by heparin resulted in an enzyme whose kinetic properties resembled closely those of particulate enzyme. Kuczenski and Mandell (49) proposed that physiological stimuli may modify the soluble enzyme or its environment in a manner that allows the molecule to become membrane bound. As a consequence, the enzyme is activated. However, the relevance of this effect of heparin to neurally induced enzyme activation is questionable (50).

Other anions, such as, phosphatidyl-L-serine (51, 52), lysolecithin (52), and polyglutamic acid (53), are able to activate rat caudate TH. In most instances, activation involves an enhanced affinity of the enzyme for cofactor, a shift in the optimal pH of the enzyme toward the more physiological pH region, and a reduced affinity of the enzyme for dopamine. However, neither heparin nor phosphatidyl-L-serine affect the activity of TH from peripheral adrenergic neurons (54). In contrast, melanin is able to activate both rat brain and bovine adrenal TH (55).

The similar activation of the enzyme associated with stress and with the cyclic AMP-dependent phosphorylating system suggests that direct phosphorylation or the phosphorylation of some intermediate activator may be responsible for enzyme activation *in vivo*. This interpretation is based upon the assumption that, if the stress effect were through some independent mechanism, one might expect to see an additivity of the stress activation and the cyclic AMP-dependent activation of the enzyme. Various other treatments do appear to produce TH activation that is additive with cyclic AMP-dependent enzyme activation. These include the effects of calcium on TH in the hippocampus (16), stimulation of TH activity in striatal tissue by lysolecithin (43) and activation of TH in striatal synaptosomes by ouabain and veratridine (22). We have observed an additive effect of *in vitro* cyclic AMP-dependent activation of the enzyme and TH activation elicited in adrenal slices in the presence of elevated potassium concentrations (unpublished observations).

In the present studies, we observed no change in the apparent K_m value for tyrosine. However, a small increase in the apparent V_{max} was observed with variable substrate concentrations, following stress and during incubation of the soluble enzyme with cyclic AMP, ATP and Mg^{++}. This is probably because fully saturating cofactor concentrations, at least for the high K_m form of the enzyme (1 mM), were not employed. Zivković *et al.* (6) observed similar kinetic changes in rat striatal TH following neuroleptic administration, which may be explained in a similar manner.

The present study suggests that, in the rat adrenal medulla, two forms of TH coexist and the proportions of these two enzyme forms depends upon the manner in which the animals are manipulated prior to and during killing.

Table 2 illustrates the K_m values obtained for the activated enzyme form in each group. There are no significant differences among these K_m values, as would be expected if we assume we are dealing with the same activated enzyme form in each group. If we assume that the sum of the apparent V_{max} values presented in Table 2 for the high affinity and low affinity enzyme are indeed equal to the V_{max} for the cyclic AMP-dependent stimulated form (V_{max} high affinity + V_{max} low affinity = V_{max} cyclic AMP), then we can make the following statements: In the preparation from the nonstressed rat, the adrenal TH is approximately 25% activated; 75% is in the less active form. Subsequent to decapitation, the proportion of activated form in the adrenal increases to 60%. These values are calculated on the assumption that the apparent V_{max} is proportional to the quantity of the enzyme. Proof of this awaits further study directed toward the separation of the two forms of TH and quantitation of the amounts present by either immunotitration or other means. Based on preliminary immunotitration studies, the assumption appears to be valid.

Table 2 presents the calculated apparent K_m values for the less active form of adrenal TH. The average values are within the range of 0.45 to 0.8 mM. In previous studies using tissue obtained from striatum (17, 49, 56), nucleus accumbens, hypothalamus, brain stem (6, 56) and vas deferens (3), K_m values for the pterin cofactor (6-MePtH$_4$ or dimethyltetrahydropterin) for control tissues were within the range of 0.47 to 0.80 mM. The absence of reports from other laboratories that suggest the presence of two forms of the enzyme may be due to the fact that, in most tissues, the enzyme is mainly in the less active state. Various treatments, including nerve stimulation (3), heparin sulfate (48), antipsychotic drugs (17, 56) and melanin (55) appear to be associated with shifts in the apparent K_m for cofactor to values which are within the range of 0.11 to 0.29 mM. The infrequency of curvilinear reciprocal enzyme kinetics reported for the above mentioned activated forms of the enzyme may be due to the fact that these treatments yield an enzyme that is almost fully activated. However, demonstration of two forms of the enzyme may be difficult unless the proportion of the less common form of the enzyme is in excess of 20–25% of the total enzyme and unless a sufficiently broad range of cofactor concentrations is examined.

Lovenberg and Bruckwick (17) reported

that haloperidol pretreatment of rats produces a reduction in the K_m of striatal TH for the cofactor from 0.53 mM to 0.21 mM. The addition of the "cyclic AMP-dependent protein phosphorylation system" to the soluble striatal enzyme prepared from control rats produces a similar K_m change from 0.50 mM to 0.16 mM. It is apparent from these studies that enzyme activation *in vivo*, e.g., by haloperidol treatment, can produce TH activation in the central nervous system comparable to that produced by cyclic AMP-dependent protein kinase. However, based on the present study, the degree of activation of adrenal TH following decapitation is not as great as that attainable *in vivo* in the presence of a cyclic AMP-dependent protein phosphorylating system (Fig. 3).

Weiner *et al.* (3) have reported that curvilinear reciprocal kinetics are obtained with TH prepared from homogenates of unstimulated hypogastric nerve-vas deferens preparations, which is consistent with data from the adrenal gland. The curvilinear kinetics are less apparent for the enzyme prepared from stimulated vas deferens tissue. The apparent K_m value for cofactor is reduced from approximately 0.5 mM in the unstimulated preparation to 0.07 mM in the stimulated preparation.

Similar results have been reported by Nagatsu *et al.* (55) for rat brain TH and for purified bovine adrenal TH. Nonlinear Lineweaver-Burk plots were obtained in both these preparations and were converted to linear plots when the enzyme was assayed in the presence of melanin.

Table 3 illustrates that decapitation of rats produces an increase in protein kinase activity with no demonstrable change in cyclic AMP levels in the adrenal medulla. Concomitant with the protein kinase increase is an increase in TH activity (Fig. 3). Recently, we have observed that 5 min following the transorbital application of electroconvulsive shock to rats there is an increase in protein kinase and TH activities in the adrenal medulla with no concomitant change in cyclic AMP levels (57). However, 10 min following the administration of electroconvulsive shock to rats, a significant increase in cyclic AMP levels in the adrenal medulla does occur and is correlated with a significant increase in both protein kinase activity and TH activity in that tissue. Thus, following stress in the rat, the activation of adrenal medulla protein kinase appears to precede any measurable increase in tissue cyclic AMP level. A similar result has been reported by Corbin *et al.* (58) who found that low doses of epinephrine (0.11 μM) will produce an increase in protein kinase activity and glycerol release in fat pads without a demonstrable change in cyclic AMP levels. Nevertheless, the failure to demonstrate a significant increase in cyclic AMP at a time when TH is already activated raises the possibility that the enzyme activation is not mediated by cyclic AMP. It is possible that protein kinase is activated by a cyclic AMP-independent mechanism, e.g., by calcium-dependent regulator protein (59, 60).

Our results do not exclude the possibility that other mechanisms, such as anions (48, 49, 53) or phospholipid activation (51, 52) may be responsible for the activation of TH following decapitation.

Joh *et al.* (25) reported that phosphorylation of purified rat brain TH is associated with an increase in V_{max} of the enzyme, with no change in K_m for either tyrosine or pterin cofactor. However, in this laboratory (P. R. Vulliet, T. A. Langan and N. Weiner, unpublished observations), phosphorylation of purified enzyme from rat pheochromocytoma is associated with an increase in affinity for pterin cofactor. The basis for this discrepancy is not known, but it may be related to intrinsic differences in the enzyme from these two tissues.

When a severe stress, such as decapitation, is imposed on the animal, adrenal TH changes rapidly to a more activated form, suggesting that the adrenal enzyme possesses considerable capacity to respond rapidly to stress. The adrenal gland is responsive to a number of physiological stimuli, including cold, heat, alterations in plasma pH, asphyxia, hypotension, hypoglycemia, glucagon and physical and emotional stresses (61). It seems reasonable to suppose that adrenal TH is maintained in a state of partial activation and that it possesses the capacity for even greater activa-

tion following severe stress. Preliminary experiments in our laboratory have shown that adrenal TH can be activated by other stresses, including pain stress from subcutaneous formaldehyde injections and seizures produced by electroconvulsive shock.

In the present study we have provided further evidence to support the concept that a cyclic AMP-dependent protein phosphorylating system mediates TH activation, and that this activation can occur in the intact adrenal gland following decapitation. Although this effect is not associated with a demonstrable rise in cyclic AMP, there is a significant increase in cyclic AMP-independent protein kinase within the adrenal medulla. It remains to be determined whether the activation of the enzyme following stress or decapitation involves phosphorylation of the enzyme.

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