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Turnover Rate of Tyrosine Hydroxylase during Trans-synaptic Induction

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SUMMARY

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The tyrosine hydroxylase of adrenal medulla was induced by exposing the rats to 4° for 4 hr. The synthesis rate of normal and induced tyrosine hydroxylase was measured by radiochemical and immunoprecipitation methods. Incorporation of ^3H into the enzyme was normal 6 hr after the beginning of the stress but was greater than normal at 10 hr and reached a maximum increase of 65% at 16 hr. Beginning 20 hr after stress application the rate of ^3H incorporation declined and approached normal values between 30 and 50 hr. The tyrosine hydroxylase activity was not yet increased 10 hr after the beginning of the stress; however, it was increased at 16 hr and a maximal increment was reached at 24 hr. This new steady state was maintained for the following 24 hr. The enzyme activity then declined, with a half-life of about 3 days. The radioactivity incorporated into tyrosine hydroxylase in glands of normal and stressed rats decayed exponentially, with a half-life of 68 hr. From this first-order rate constant ($k_d = 0.01$) and from the amount of enzyme present the synthesis rate (K_s) of tyrosine hydroxylase was calculated. The synthesis rate was increased in the adrenals of stressed rats. This increase failed to occur if adrenal denervation preceded the stress.

INTRODUCTION

The rate-limiting step for the synthesis of the neurotransmitters norepinephrine and dopamine is catalyzed by tyrosine hydroxylase (ED 1.14.16.2). In adrenal medulla and superior cervical sympathetic ganglia of rats, cold exposure (1, 2), immobilization stress (3), and the administration of certain drugs (4–6) can cause a delayed but long-lasting increase of tyrosine hydroxylase activity. In adrenals the increment in tyrosine hydroxylase activity elicited by cold is mediated by trans-synaptic activation of the cholinergic nicotinic receptors and can be prevented either by blocking these receptors or by severing the splanchnic nerve (6–8). This increase in tyrosine hydroxylase activity has been termed trans-synaptic induction because it requires RNA transcription and ribosomal translation (9). In fact, it has been shown by immunochemical titration that the trans-synaptic increase of enzymatic activity is due entirely to an accumulation of enzyme molecules (10, 11).

We have recently estimated the turnover rate of proteins endowed with tyrosine hydroxylase activity in adrenals of normal and cold-exposed rats, using a specific antibody and the technique of immunoprecipitation combined with labeling of the amino acid pool (12). Our data indicated that exposure for 4 hr at 4° increased the rate of amino acid incorporation into adrenal tyrosine hydroxylase after a time delay of 4–5 hr from the termination of the cold exposure. Moreover, although the enzyme activity was not increased at this

time, it was increased a few hours later. The first-order rate constant for the degradation of tyrosine hydroxylase molecules was unchanged by cold exposure, suggesting that an increase in the rate of synthesis was the cause for the trans-synaptic induction. The present report concerns experiments designed to obtain a better understanding of the mechanisms involved in the trans-synaptic induction of adrenal tyrosine hydroxylase.

MATERIALS AND METHODS

Chemicals. L-[4,5-^{3}H]Leucine (30–50 Ci/mmole) and L-[1-^{14}C]tyrosine (40–60 mCi/mmole) were purchased from New England Nuclear Corporation. Sepharose 4B, ovalbumin, aldolase, α-chymotrypsinogen, and ribonuclease were products of Pharmacia. Sodium dodecyl sulfate was obtained from Bio-Rad Laboratories. Cyanogen bromide, acrylamide, and *N,N'*-methylenebisacrylamide were purchased from Eastman Kodak.

Measurements of [^{3}H]leucine uptake into adrenal medulla and incorporation rate of [^{3}H]leucine into medullary proteins. Male rats (Zivic Miller, Allison Park, Pa.) (about 100 g) were exposed to 4° and 4 hr later were brought back to 25°. At 10, 16, and 28 hr after the beginning of cold exposure, these rats and control rats kept at 25° were treated with [^{3}H]leucine (2.5 mCi/kg intraperitoneally) and decapitated 30 min later. The adrenal cortex was trimmed away from the medulla as described (6), and each pair of the dissected medullae was homogenized in 0.5 ml of 20 mM potassium phosphate (pH 7.4), 140 mM KCl, 1 mM $MgCl_2$, and 0.2% Triton X-100 (medium A). An aliquot of 40 μl of the above homogenate was taken for the determination of protein (13). To an aliquot of 400 μl of the homogenate was added 1.0 ml of 7.5% TCA,[1] and the mixtures were heated at 80° for 30 min. The precipitated proteins were separated by centrifugation at 20,000 × *g* for 20 min. The supernatant fraction was counted for radioactivity in 15 ml of Aquasol solution (New England Nuclear). The protein pellets were collected on a Millipore filter (0.45 μm) and counted in a toluene-based scintillation fluid containing Triton X-100.

Measurement of rate of synthesis of adrenal tyrosine hydroxylase. Male rats (about 100 g), intact or unilaterally adrenal denervated, were obtained from Zivic-Miller Laboratories.

Rats with the left splanchnic nerve severed 5 days before the experiment were placed, one per cage, at 4° for 4 hr, and 16 hr later the rate of amino acid incorporation into adrenal tyrosine hydroxylase was measured. Another group of rats with monolateral denervation of adrenals was kept at 25° and served as controls. Both groups then received 7 mCi/kg of [^{3}H]leucine intraperitoneally and were killed 90 min thereafter. Both adrenal glands from each rat were removed, and the amount of ^{3}H incorporated into the tyrosine hydroxylase of intact and denervated adrenals was determined by immunoprecipitation. The detailed procedure for the immunoprecipitation assay was described previously (12). Rabbit antiserum specific to tyrosine hydroxylase, which was purified from bovine adrenal medulla, was prepared according to Kaufman and his co-workers (14, 15), with a slight modification (12).

The incorporation of ^{3}H was also measured in the soluble adrenal protein, which was precipitated with 5% TCA, collected on a Millipore filter, and counted. Tyrosine hydroxylase activity was assayed using the method of Waymire *et al.* (16).

Disc gel electrophoresis of immunoprecipitate. A group of 12 rats each received 500 μCi of [^{3}H]leucine intravenously 16 hr after the beginning of the cold exposure and were killed 90 min later. Their adrenals were pooled and homogenized in 6 ml of medium A. The homogenate was centrifuged at 10,000 × *g* at 4° for 20 min, and the supernatant was further centrifuged at 100,000 × *g* for 1 hr. One milliliter of rabbit antiserum was added to 5 ml of the high-speed supernatant and 250 μg of purified tyrosine hydroxylase protein from bovine adrenals. The adrenals from 12 rats kept at 25° were processed in parallel, using an identical procedure. The two mix-

[1] The abbreviations used are: TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate.

tures were incubated at 37° for 1 hr and at 4° for 16 hr. The precipitate was separated by centrifugation at 10,000 × *g* for 20 min, and the pellet was washed three times with 6 ml of medium A containing 0.2% bovine serum albumin. The final precipitate was dissolved in 200 μl of buffer containing 10 mM sodium phosphate (pH 7.0), 2% SDS, and 4% 2-mercaptoethanol. A 150-μl aliquot of each solubilized precipitate was used for disc gel electrophoresis.

SDS-gel electrophoresis was performed on 8-cm polyacrylamide gels (10%) in the presence of SDS (1%). The polymerization was catalyzed by ammonium persulfate. Electrophoresis was carried out at 10 mamp/gel for 8 hr in 100 mM sodium phosphate buffer containing 0.1% SDS at pH 7.0. Bromphenol blue was used as tracking dye. After electrophoresis, the gel was sliced into 2-mm sections. Each slice was dissolved by heating at 60° in 0.5 ml of 30% hydrogen peroxide and counted for radioactivity in 15 ml of Aquasol. The protein standards listed in Fig. 3 were used as markers for the molecular weight determination, which was carried out according to Weber and Osborn (17). After electrophoresis the protein bands were visualized by staining with Coomassie brilliant blue.

Affinity chromatography of adrenal soluble [^3H]proteins on Sepharose conjugated with antibody to tyrosine hydroxylase. Rabbit γ-globulin fraction containing antibody to tyrosine hydroxylase was isolated by ion-exchange chromatography as described (18). Four milliliters of packed Sepharose 4B were activated by cyanogen bromide according to the procedures of Cuatrecasas and Anfinsen (19). Twenty milligrams of the isolated immunoglobulin in 4 ml of 100 mM potassium phosphate, pH 7.4, were added to the CNBr-activated Sepharose. The mixture was rotated gently for 24 hr at 4°. The Sepharose was sedimented by centrifugation, and 4 ml of 1 M ethanolamine, pH 8.0, were added. The mixture was left overnight to allow complete reaction with the remaining CNBr-Sepharose. The suspension was washed twice with medium A and poured into a small column (1 × 5 cm). The column was washed extensively with 0.1 M sodium acetate, pH 4.5, containing 1.0 M NaCl, then with 0.1 M sodium bicarbonate, pH 7.6, containing 1.0 M NaCl. After three washing cycles, the column was equilibrated with medium A.

Soluble [^{3}H]proteins were prepared from adrenals and liver of rats treated with [^{3}H]leucine (6 mCi/kg intraperitoneally). These tissues were homogenized in medium A and then centrifuged at 100,000 × *g*. One milliliter of the supernatant, containing 5.2 mg of protein, was placed in each column and was washed with medium A. The [^{3}H]protein adsorbed by the Sepharose–anti-tyrosine hydroxylase was eluted with 6 M guanidine HCl, pH 3.0, and was immediately neutralized to pH 7.0 with Tris buffer.

Disc gel electrophoresis of adrenal [^3H]proteins adsorbed by Sepharose–anti-tyrosine hydroxylase. The [^{3}H]proteins eluted by 6 M guanidine HCl were pooled and extensively dialyzed against medium A. The dialysate was lyophilized, and the residues were redissolved in 200 μl of medium A. The sample was again dialyzed against medium A and then subjected to disc gel electrophoresis as described by Davis (20), using riboflavin as the catalyst. Each slice of the gel (2 mm) was counted for radioactivity as described above.

As a marker for tyrosine hydroxylase, 25 pairs of adrenal medullae of rats dissected from adrenal cortex were pooled and homogenized in 200 μl of medium A. The homogenate was centrifuged at 100,000 × *g*, and the supernatant was subjected to disc gel electrophoresis in parallel with the sample of [^{3}H]proteins adsorbed by Sepharose–anti-tyrosine hydroxylase. Each slice of gel was immersed overnight in 200 μl of 100 mM potassium phosphate, pH 6.2. Aliquots of 100 μl were used for the assay of tyrosine hydroxylase activity.

Measurement of rate of tyrosine hydroxylase degradation. Rats were exposed to 4° for 4 hr and then returned to 25°; these rats were kept at 25° for 44 hr and then treated with [^{3}H]leucine (10 mCi/kg intraperitoneally). Groups of six rats each were killed 9, 24, 48, 60, and 94 hr after the injection, and their adrenal glands were removed. The ^{3}H incorporated into adrenal tyrosine

hydroxylase was determined by immunoprecipitation (12).

RESULTS

Uptake and incorporation of [^3H]leucine into adrenal medullary proteins after 4 hr of cold exposure. The data reported in Table 1 show that 4 hr of cold exposure enhanced the incorporation of [^{3}H]leucine into the total medullary proteins 10, 16, and 28 hr after the beginning of the cold stress. At these times the uptake of [^{3}H]amino acids in adrenal medulla was not changed. The ratio of protein ^{3}H to free ^{3}H was significantly higher in the medullae of stressed rats than in those of normal rats. The increased incorporation of ^{3}H into protein was not due to an increased delivery of the labeled amino acid to the adrenal gland. Moreover, our previous study (12), using the technique of double labeling, had shown that cold stress does not change the amino acid precursor pools involved in the synthesis of adrenal tyrosine hydroxylase and of adrenal soluble proteins.

Effect of cold exposure on rate of ^3H incorporation into adrenal tyrosine hydroxylase. The results in Fig. 1 show that in rats exposed to 4° for 4 hr the amount of ^{3}H incorporated into soluble adrenal proteins precipitated by tyrosine hydroxylase antiserum was greater than that incorporated into soluble adrenal proteins of rats kept at 25°. This increase was detected from 10 to 30 hr but was no longer present at 48 hr. The duration of the increment in ^{3}H incorporation into proteins precipitated by tyrosine hydroxylase antiserum differed from the time course of the increase in this enzyme activity (Fig. 1). The activity of adrenal tyrosine hydroxylase was still unchanged about 10 hr after the beginning of the stress; at this time the incorporation of ^{3}H in the immunoreactive protein was already elevated. The enzyme activity began to increase at about 16 hr, when the increment in incorporation of ^{3}H into immunoreactive protein had already reached its peak. The elevation of the enzyme activity reached a maximum between 30 and 48 hr, but at 30 hr the incorporation of ^{3}H into immunoreactive protein had declined and was approaching basal values. The enzyme activity declined with a half-life of about 3 days and reached basal values about 10 days after the stimulus. Thus the increment in the rate of ^{3}H incorporation into tyrosine hydroxylase preceded the increase in enzymatic activity; the latter per-

TABLE 1

Effects of cold exposure on uptake and incorporation of [^3H]leucine into adrenal medulla proteins

Measurements of the uptake of [^{3}H]leucine into adrenal medulla and the incorporation of the label into medullary proteins are described in MATERIALS AND METHODS. Each value represents the mean ± standard error of six or seven experiments.

Time after beginning of cold exposure	Environmental temperature during stress	[^{3}H]Leucine in TCA-insoluble fractions (A)	[^{3}H]Leucine in TCA-soluble fractions (B)	A:B
hr		*cpm/mg protein*	*cpm/mg protein*	
10	25°	3836 ± 307	2040 ± 136	1.90 ± 0.12
	4°	5475 ± 297[a]	2190 ± 80	2.52 ± 0.16[b]
16	25°	3956 ± 339	2625 ± 247	1.52 ± 0.13
	4°	5033 ± 220[c]	2316 ± 202	2.19 ± 0.15[a]
28	25°	4183 ± 198	2090 ± 117	2.01 ± 0.08
	4°	5417 ± 367[c]	2321 ± 99	2.41 ± 0.06[a]

[a] $p < 0.01$ compared with controls.
[b] $p < 0.02$ compared with controls.
[c] $p < 0.05$ compared with controls.

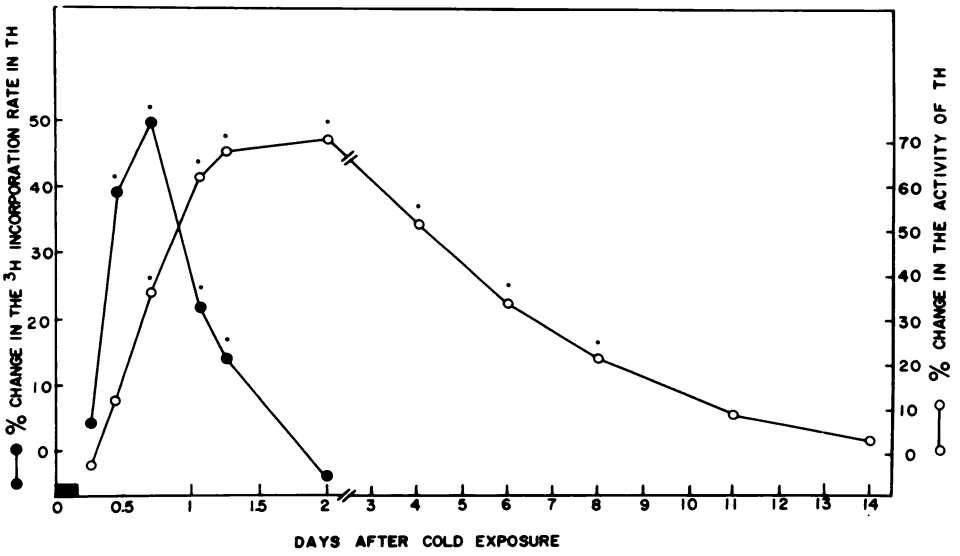

FIG. 1. *Time course of increase in synthesis rate of tyrosine hydroxylase (TH) and enhancement of enzymatic activity in adrenal medullae of rats exposed to cold*

Normal male rats (about 100 g) were kept at 4° for 4 hr, after which they were returned to 25°. At various times after stress, and also 90 min prior to death, each rat received 700 μCi of [^{3}H]leucine. The incorporation of ^{3}H into tyrosine hydroxylase and the activity of the enzyme were determined as described previously (12). Each point represents the percentage increase of the mean (five to seven experiments) obtained in stressed rats compared with that obtained in normal rats. ■, time at 4°.

* $p < 0.05$ when the values of stressed rats were compared with those of rats kept at room temperature.

sisted for several days after the incorporation of ^{3}H into immunoreactive protein had returned to normal.

Since the induction of adrenal tyrosine hydroxylase by cold exposure can be blocked by adrenal denervation (7), we examined the effect of cold stress on the incorporation of ^{3}H into adrenal tyrosine hydroxylase in rats with a monolateral adrenal denervation. These rats were exposed to cold for 4 hr on the fifth day after denervation. The rate of ^{3}H incorporation into tyrosine hydroxylase was measured at 16 hr, and enzyme activity was assayed 30 hr after the beginning of cold exposure. The data reported in Table 2 indicate that in the denervated adrenals of rats exposed to 4° the enzyme activity and rate of incorporation of [^{3}H]leucine into the immunoreactive protein were not significantly higher than those found in adrenals of rats kept at 25°. However, in the contralateral intact adrenal both values were significantly enhanced by cold exposure. The data of Table 2 also show that the incorporation of ^{3}H into total soluble proteins in either intact or denervated adrenals from rats exposed to 4° was greater than in adrenals from rats kept at 25°.

Characterization of immunoreactive proteins. The radioactivity that was precipitated by the rabbit antiserum to tyrosine hydroxylase added to the supernatant of adrenal gland homogenates was analyzed by electrophoresis on a polyacrylamide gel in the presence of SDS, which dissociates a protein into its subunits and permits determination of their molecular weight. Only two radioactive protein peaks were found in the gel, one (α) more abundant than the other (β). The electrophoretic profiles of the immunoprecipitates prepared from rats kept at 25° (Fig. 2A) and from the rats killed 16 hr after cold exposure (Fig. 2B) were quite similar. In adrenals of rats exposed to 4° the incorporation of ^{3}H into α and β proteins was greater than in adrenals of rats kept at 25°. The molecular weights of these two proteins were determined by comparing their mobility in the

TABLE 2

Effect of cold exposure on activity and [^{3}H]leucine incorporation into tyrosine hydroxylase protein in intact and denervated adrenal glands

The incorporation of [^{3}H]leucine into tyrosine hydroxylase and other adrenal proteins was determined 16 hr after the beginning of cold exposure. Ninety minutes prior to death the rats received [^{3}H]leucine (700 μCi/rat, intraperitoneally). Tyrosine hydroxylase activity was measured 30 h after the beginning of cold exposure. For details, see MATERIALS AND METHODS. Each value represents the mean ± standard error of six experiments.

Adrenals	Temperature	[^{3}H]Leucine in tyrosine hydroxylase	Tyrosine hydroxylase activity	[^{3}H]Leucine in adrenal soluble proteins
		cpm/adrenal pr	*nmoles dopa formed/ 30 min/adrenal pr*	*cpm/adrenal pr*
Intact	25°	965 ± 100	2.9 ± 0.2	21,040 ± 2,960
	4°	1,530 ± 105[a]	4.8 ± 0.4[a]	34,180 ± 3,100[b]
Denervated	25°	1,005 ± 160	2.7 ± 0.2	20,160 ± 2,130
	4°	1,290 ± 125	3.1 ± 0.5	28,280 ± 2,280[c]

[a] $p < 0.01$ compared with controls.
[b] $p < 0.02$ compared with controls.
[c] $p < 0.05$ compared with controls.

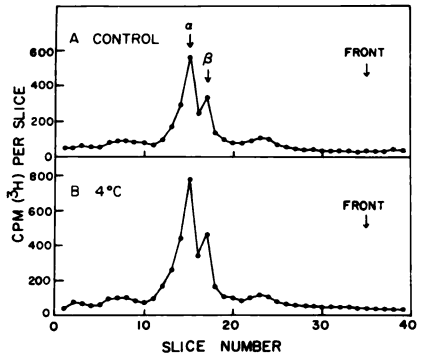

FIG. 2. *Gel electrophoresis of adrenal immunoprecipitate in the presence of SDS*

The samples were prepared either from normal rats (A) or from rats exposed to cold (B), and electrophoresis was carried out as described under MATERIALS AND METHODS.

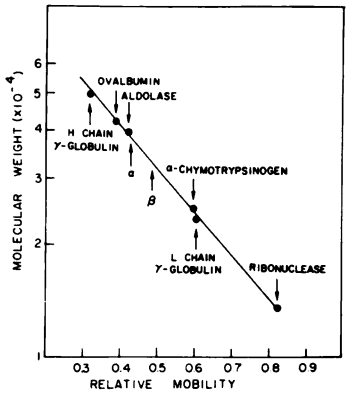

FIG. 3. *Determination by SDS–polyacrylamide gel electrophoresis of molecular weights of α and β proteins precipitated by antiserum*

●, marker proteins: heavy (H.) chain of γ-globulin (mol wt 50,000), ovalbumin (mol wt 45,000), aldolase (mol wt 39,500), α-chymotrysinogen (mol wt 25,000), light (L.) chain of γ-globulin (mol wt 23,500), and ribonuclease (mol wt 13,700). Molecular weights of proteins α and β were estimated from the standard straight line. For details, see MATERIALS AND METHODS.

gel with those of a number of proteins of known molecular weight (Fig. 3). The molecular weights of proteins α and β were thus estimated to be about 38,000 and 33,000, respectively.

In another experiment the immunoreactive proteins present in adrenals were characterized by affinity chromatography. The immunoglobulin purified from tyrosine hydroxylase antiserum was coupled to Sepharose with the cyanogen bromide linkage. The adrenal 100,000 × *g* soluble [^{3}H]proteins adsorbed to the Sepharose–anti-tyrosine hydroxylase were eluted with 6 M guanidine HCl. The results (Fig. 4A) show that the [^{3}H]proteins retained (fractions 24–28) represented about 4% of

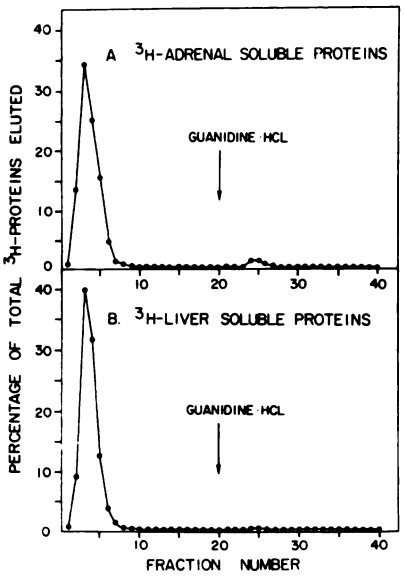

FIG. 4. *Chromatography of 100,000 × g soluble [³H]proteins from adrenals and livers of rats on Sepharose conjugated with anti-tyrosine hydroxylase immunoglobulins*

Samples in 1 ml of medium A, containing 5.2 mg of protein from adrenals (A) or liver (B), were loaded on the column. The radioactivity in adrenal and liver proteins which were chromatographed was 125,300 cpm and 84,800 cpm, respectively. The radioactivity present in each 1.0 ml of the eluate is plotted as a percentage of the total radioactivity loaded on the columns. Aliquots of 100 μl of each fraction were taken, and the proteins were precipitated with 5% TCA. The precipitates were collected on a Millipore filter and counted for radioactivity. For details, see MATERIALS AND METHODS.

the total soluble adrenal [³H]proteins chromatographed. More than 90% of the original enzyme activity was adsorbed to the Sepharose-antiserum (data not shown). When the soluble [³H]proteins prepared from liver were employed, practically no radioactivity could be eluted from the Sepharose column by guanidine HCl, supporting the specificity of the elution profile of the soluble adrenal proteins (Fig. 4B). Since exposure to 6 M guanidine HCl completely abolished the enzyme activity (data not shown), fractions 24–28 of the guanidine HCl eluate were extensively dialyzed and subjected to disc electrophoresis with rat medullary tyrosine hydroxylase as a marker. Figure 5B shows that the radioactivity migrated as a single, distinct peak on the gel. Moreover, the mobility of the radioactive peak was identical with that of the tyrosine hydroxylase contained in the homogenate (Fig. 5A).

Rate of degradation of adrenal tyrosine hydroxylase after cold exposure. Rats received 10 mCi/kg of [³H]leucine intraperitoneally 48 hr after the beginning of cold exposure, when the incorporation of radioactivity into the proteins precipitated by rabbit antiserum was equal to that in rats kept at 25°; the decay of radioactivity in the tyrosine hydroxylase proteins was determined. It is shown in Fig. 6 that the ³H content in these proteins declined exponentially and that the first-order rate constant for this radioactivity decay was essentially identical in rats stressed 48 hr before this measurement and in rats kept at 25°. From

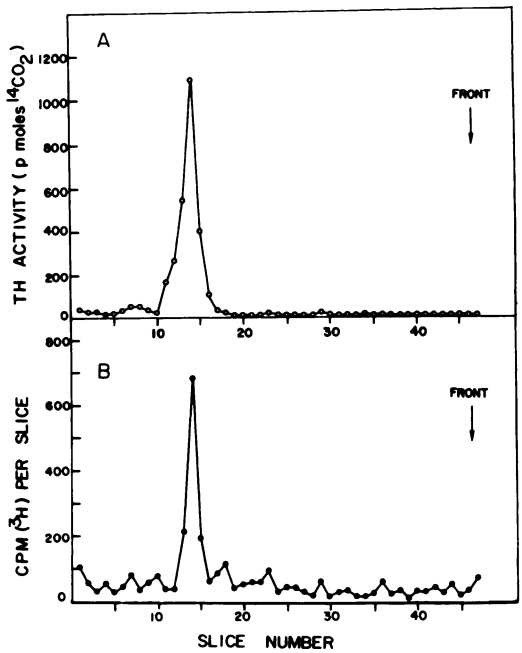

FIG. 5. *Disc gel electrophoresis of tyrosine hydroxylase (TH) in rat adrenal medullae and [³H]proteins adsorbed by Sepharose–anti-tyrosine hydroxylase*

Medullary 100,000 × g supernatant (A) and [³H]proteins eluted by guanidine HCl from Sepharose–anti-tyrosine hydroxylase (B) were subjected to disc gel electrophoresis according to Davis (20). O——O, tyrosine hydroxylase activity per 30 min per slice of gel; ●——●, counts per minute per slice of gel. For details, see MATERIALS AND METHODS.

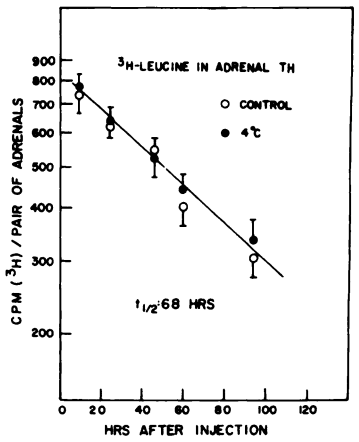

FIG. 6. *Decay of radioactivity in tyrosine hydroxylase (TH) from adrenal medullae of normal and cold-exposed rats*

Rats received [^{3}H]leucine 48 hr after the beginning of cold exposure. For details, see MATERIALS AND METHODS. Each value represents the mean ± standard error (brackets) of six pairs of adrenals.

the plots of Fig. 6, the half-life of adrenal tyrosine hydroxyase was estimated to be about 68 hr.

DISCUSSION

We have previously shown that in adrenals of rats exposed to 4° for 4 hr and then returned to 25°, the rate of tyrosine hydroxylase synthesis is increased 10 hr after the beginning of the stress whereas the enzyme activity begins to increase at about 16 hr (12). In the same rats the tyrosine hydroxylase activity of sympathetic ganglia fails to increase and the synthesis of the enzyme remains unchanged (12). Thus we had suggested that the increased tyrosine hydroxylase activity may be causally related to an increase in the synthesis rate of the enzyme. This view is further supported by the results with rats monolaterally denervated and exposed to 4° for 4 hr (Table 2). In the denervated adrenal glands neither the activity nor the incorporation of ^{3}H into tyrosine hydroxylase was enhanced. Moreover, the data of Table 2 show that in denervated adrenals the synthesis of adrenal soluble protein that is not precipitated by the antiserum is enhanced by cold stress, whereas the synthesis rate of the protein precipitated by the antiserum is not increased. This suggests that after stress the synthesis of soluble adrenal proteins may be accelerated by a number of mechanisms. The synthesis regulation of some of these proteins, including tyrosine hydroxylase, may be trans-synaptic while that of other proteins may be hormonal (21). Moreover, these data suggest that the enhancement of tyrosine hydroxylase activity elicited by cold is due to increased synthesis of the enzyme.

The increased incorporation of ^{3}H into adrenal tyrosine hydroxylase elicited by cold exposure is unrelated to enhanced uptake of labeled amino acid by the medullary cells. The data in Table 1 indicate that the uptake of [^{3}H]amino acid into adrenal medullae was unchanged between 10 and 28 hr after cold stress; at these times significant increments of ^{3}H incorporation into adrenal tyrosine hydroxylase and other medullary proteins were observed. Moreover, using the technique of double labeling (12), we had demonstrated that the increased incorporation of ^{3}H into adrenal tyrosine hydroxylase and adrenal soluble proteins is not due to an artifactual change in the compartmentation of amino acid pools. The duration of the increased synthesis in adrenal tyrosine hydroxylase differs from that of the total medullary proteins and adrenal soluble proteins. The increased synthesis of adrenal tyrosine hydroxylase reached a maximum around 16 hr after stress application, whereas the synthesis of total medullary proteins was increased to about the same extent at 10, 16, and 28 hr (Table 1). At 29½ hr after cold exposure the synthesis of adrenal soluble proteins is practically back to normal (12).

The specificity of the antiserum to tyrosine hydroxylase was investigated by experiments reported in Figs. 4 and 5. The labeled soluble proteins which were eluted from the Sepharose–anti-tyrosine hydroxylase column by the addition of guanidine HCl yielded a distinct peak on disc gel electrophoresis. The mobility of this radioactive peak was identical with that of the activity present in rat adrenal medulla. Furthermore, when soluble liver proteins of the same rats were chromatographed on

a Sepharose–anti-tyrosine hydroxylase column little or no [^{3}H]protein was eluted from the column by the addition of guanidine HCl. It should be stressed that only 4% of the ^{3}H associated with adrenal soluble proteins was retained by the Sepharose–anti-tyrosine hydroxylase column; this proportion is very close to that obtained by the method of immunoprecipitation used routinely in our experiments. Since the label in 100,000 × g supernatant proteins represented only about 40% of the label in the proteins of the total adrenal homogenate, it can be approximated that tyrosine hydroxylase constituted about 1.5% of the adrenal proteins synthesized in 90 min. When the labeled proteins precipitated by the antiserum to tyrosine hydroxylase were solubilized by SDS and resolved into their respective subunits, the SDS-gel electrophoresis analysis revealed a major (α) and a minor (β) protein. The molecular weight of the α protein was estimated to be 38,000, and that of β, to be 33,000. Musacchio *et al.* (22) reported that adrenal tyrosine hydroxylase has a molecular weight of about 150,000. Moreover, indirect evidence suggests that native tyrosine hydroxylase protein includes four subunits (22, 23). It seems appropriate to assume that the α and β proteins shown in Fig. 2 represent subunits of the enzyme. This view is also supported by the finding that cold exposure enhanced the incorporation of [^{3}H]leucine into proteins α and β by an equal extent.

The degradation rate of adrenal tyrosine hydroxylase does not seem to be reduced by cold stress. This was previously shown by the technique of double labeling (12) and has now been confirmed by the experiment reported in Fig. 6. Rats received [^{3}H]amino acid 48 hr after cold exposure. At this time the number of tyrosine hydroxylase molecules in the adrenals of stressed rats is increased and maintained at a new steady state. From the exponential decay of ^{3}H incorporated into tyrosine hydroxylase (Fig. 6) the half-life ($t_{1/2}$) of the enzyme was estimated to be about 68 hr in adrenals of normal and stressed rats. The first-order degradation constant (k_d) can be calculated to equal 0.01 hr^{-1}. From the steady-state relationship $K_s = k_d E$, where E is the tyrosine hydroxylase content, one can estimate K_s, which is the zero-order rate for tyrosine hydroxylase synthesis. In Table 3 we report the results of these calculations.

These results confirm our previous observation that the k_d of adrenal tyrosine hydroxylase remains unchanged after cold exposure (12). However, the absolute values of k_d estimated by the double-labeling procedure (12) were higher than those obtained in the present study. Since only two time points were sampled in our previous experiment (12), the absolute value of this estimation may have a high level of uncertainty. In normal rats the number of tyrosine hydroxylase molecules synthesized per hour by a pair of adrenals can catalyze the formation of 0.058 nmole of dopa per hour; the corresponding value in the adrenals of stressed rats is higher (0.096 nmole of dopa per hour). The data of Table 3 show that the turnover time (T_t) of tyrosine hy-

TABLE 3

First-order rate constant of degradation (k_d), zero-order rate constant of synthesis (K_s), and turnover time (T_t) of adrenal tyrosine hydroxylase in rats kept at 25° or exposed for 4 hr at 4°

k_d was determined from the exponential decay of ^{3}H incorporated into tyrosine hydroxylase while the enzyme was in the normal or the induced steady state. For details, see MATERIALS AND METHODS. K_s was calculated from the steady-state relationship $K_s = k_d E$, where E is the enzyme content (units per gland). The data in Table 1 show that a pair of normal adrenals produced 5.8 nmoles of dopa per hour, whereas in the steady state during enzyme induction a pair of adrenals produced 9.6 nmoles of dopa per hour. Thus a pair of normal adrenals synthesized in 1 hr enough enzyme units to form 0.058 nmole of dopa per hour. A pair of adrenals in stressed rats synthesized in 1 hr enough tyrosine hydroxylase to form 0.096 nmole of dopa per hour.

Rats	k_d	K_s	T_t[a]
	hr^{-1}	hr^{-1}	hr
Normal	0.01	0.058	100
Stressed	0.01	0.096	100

[a] T_t is the time required for destruction of 65% of tyrosine hydroxylase molecules and was obtained from the relationship $T_t = 1/k_d$.

droxylase is 100 hr in the adrenals of either normal or stressed rats. Since, by definition, T_t is the time required to destroy 65% of the enzyme molecules, and since cold exposure routinely elicited a 70% increase in the amount of enzyme present, it can be calculated that about 10 days may be required to re-establish a normal enzyme content after the increase in tyrosine hydroxylase synthesis induced trans-synaptically. The experimental results shown in Fig. 1 indeed confirm this calculation. From the data of Fig. 1, about 3 days are required to curtail by 50% the increase in tyrosine hydroxylase activity. These data are in good agreement with the half-life of the enzyme determined immunologically (68 hr). Moreover, the data of Fig. 1 are in agreement with the half-life of 3 days estimated by Kvetňanský and colleagues (3) for adrenal tyrosine hydroxylase.

The data of Fig. 1 show that the apparent increase in ^3H incorporation into tyrosine hydroxylase precedes the increment in enzymatic activity. The time delay between the two phenomena could be due either to the slow turnover time (100 hr) of the enzyme protein or to the possibility that tyrosine hydroxylase is synthesized in a precursor form which is either partially or totally devoid of enzymatic activity. At present we cannot decide which of these two possibilities reflects the situation *in vivo*. The occurrence of maximal ^3H incorporation into tyrosine hydroxylase 16 hr after the beginning of cold exposure is consistent with the report by Otten *et al.* (24) that the transcriptional phase of tyrosine hydroxylase induction is complete by 16 hr after stress application.

It was shown that immediately after cold exposure the medullary content of cyclic 3′,5′-AMP suddenly increases whereas the medullary content of cyclic 3′,5′-GMP decreases (7, 25–27). Furthermore, excellent correlations were shown to exist among induction of adrenal tyrosine hydroxylase, the duration of the increase in cyclic AMP to cyclic GMP ratio, and the subsequent activation of cyclic AMP-dependent protein kinase activity (27). These correlations strongly indicate that the activation of a protein kinase in the cytosol of adrenal medulla is an obligatory event that leads to the increase of tyrosine hydroxylase synthesis elicited trans-synaptically (27). Our current hypothesis is that, as in other models (28, 29), the activated protein kinase in adrenal medulla may be translocated from the cytosol to the nucleus, thereby phosphorylating either some nuclear proteins or RNA polymerase. The phosphorylation of specific nuclear proteins may promote the new synthesis of messenger RNA specific to tyrosine hydroxylase. We are currently investigating these possibilities.

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