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INFLUENCE OF SYMPATHETIC DENERVATION AND NERVE IMPULSE ACTIVITY OF TYROSINE HYDROXYLASE IN THE RAT SUBMAXILLARY GLAND

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Abstract—Tyrosine hydroxylase has been found in the rat submaxillary gland and superior cervical ganglion. Postganglionic sympathetic denervation resulted in a total disappearance of the enzyme activity in the submaxillary gland. Acute changes in sympathetic impulse activity produced by decentralization, bretylium, pempidine, or nerve stimulation did not markedly change tyrosine hydroxylase levels. Although alterations in impulse activity are known to produce changes in rate of norepinephrine synthesis and the rate-limiting step in norepinephrine synthesis is probably dopa formation from tyrosine, the rate of this reaction does not appear to be controlled by changes in tyrosine hydroxylase content of the tissue. A heat-labile, nondialyzable factor in the submaxillary gland was found to preserve tyrosine hydroxylase activity in extracts of rat adrenal *in vitro*. This factor is present in chronically sympathetically denervated submaxillary glands and may be a peroxidase. The factor is probably not present in sympathetic nerves, and it is unlikely that it plays any role in control of norepinephrine synthesis *in vivo*.

FORMATION of dopa from tyrosine is thought to be the rate-limiting step in norepinephrine synthesis.[1] The enzyme that catalyzes this reaction, *l*-tyrosine hydroxylase, is present in the brain, in the adrenal medulla, and in various sympathetically innervated tissues.[2] It has not been demonstrated whether this enzyme is present uniquely in adrenergic neurons of sympathetically innervated tissues.

The turnover rate and synthesis of norepinephrine appear to be related to impulse activity in adrenergic neurons.[3–7] Since dopa formation controls the rate of norepinephrine synthesis, one way in which this rate can be altered is by changes in levels of tyrosine hydroxylase.

The rat submaxillary gland can easily be sympathetically denervated, decentralized, or stimulated. This organ was therefore selected for a study of the influence of (a) sympathetic nerve degeneration and (b) sympathetic nerve impulse activity of tissue levels of tyrosine hydroxylase.

METHODS

Animals. Male or female Sprague–Dawley rats weighing 200–250 g were used in all experiments.

Surgical procedures. Unilateral sympathetic denervation or decentralization of salivary glands was performed on rats under ether anesthesia; a dissecting microscope

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was used. Unilateral postganglionic denervation was accomplished by removal of the superior cervical ganglion. These animals were killed 1 week later, and the tyrosine hydroxylase content of the submaxillary glands was determined.

Decentralization (preganglionic denervation) was accomplished by removal of a segment of the cervical sympathetic chain from 2 mm to 12 mm below the superior cervical ganglion. One day or eleven days after operation, the animals were killed by a blow on the head. The submaxillary glands and superior cervical ganglia were removed and assayed for tyrosine hydroxylase as described below.

Stimulation of the cervical sympathetic chain. The rats were anesthetized with pentobarbital, and a tracheal cannula was inserted. Rectal temperature was maintained at 37° by means of a heating lamp. The right cervical sympathetic chain and vagus nerve were dissected free from the common carotid artery and cut at the level of the clavicle. The vagus nerve was freed from the superior cervical ganglion and cut in order to interrupt afferent vagal impulses. Both nerve trunks were placed on a bipolar silver electrode immersed in paraffin oil and stimulated with 5 V, 5 msec duration, at a rate of 20 pulses/sec (pps), for 1 hr. After stimulation, the salivary glands were removed and assayed for tyrosine hydroxylase.

Drug treatment. Rats were given bretylium (50 mg/kg, i.p.) or pempidine (10 mg/kg, i.p.), and 6 hr later tyrosine hydroxylase was determined in the submaxillary glands.

Assay of tyrosine hydroxylase activity. Rats were killed by a blow on the head; one submaxillary gland, one adrenal gland, or two superior cervical ganglia were rapidly excised, cooled on ice, weighed, and homogenized in 1 ml of ice-cold 0·3 M sucrose in glass homogenizers. After centrifugation at 9000 *g* for 20 min at 0°, aliquots of the supernatant solutions (0·25 ml of adrenal homogenates, 0·5 ml of the other homogenates) were incubated with 200 μmoles sodium acetate buffer (pH 6·0), 6 μmoles aminodimethyltetrahydropteridine, 100 μmoles mercaptoethanol, and about 120 mμc of *l*-tyrosine-^{14}C (sp. act. 2·07 mc/mg) in a total volume of 1·2 ml. Incubation was carried out in an atmosphere of air with shaking in a water bath at 37° for 8 min. The reaction was stopped by the addition of 5 ml of 0·4 N perchloric acid.

After centrifugation to remove the protein precipitate, dopa-^{14}C formed during incubation was absorbed onto alumina according to the procedure used for catecholamines, described by Anton and Sayre.[8] The alumina and the solutions were poured onto a short alumina column (0·4 g alumina in 0·8 cm-diameter column). After washing the columns with 10 ml of 0·2 N sodium acetate buffer (pH 8·4) and three 10-ml portions of distilled water, the dopa-^{14}C was eluted with 10 ml of 0·2 N HCl. The eluate was evaporated in a stream of air at 80°, in vials suitable for liquid scintillation spectrometry. The dry residue was taken up in 0·2 ml of 1 N HCl, 4 ml ethanol, and 10 ml toluene containing 0·4% diphenyloxazole and 0·01% *p*-bis-12(5-phenoxazolyl)-1-benzene; and the ^{14}C was assayed in a liquid scintillation spectrometer.

RESULTS

Effect of chronic sympathetic denervation and boiling on dopa-^{14}C formation from d- *and* l-*tyrosine-^{14}C in rat salivary gland extracts*

l-Tyrosine-^{14}C incubated with salivary gland extracts resulted in significantly greater formation of dopa-^{14}C than in the absence of tissue (Table 1). When boiled tissue extracts were used, dopa-^{14}C formation was similar to that in the absence of

tissue. Incubation with extracts of chronically denervated salivary glands, however, resulted in significantly less dopa-^{14}C formation than with boiled tissue extracts and did not increase apparent dopa-^{14}C above that found in the tyrosine-^{14}C without incubation.

TABLE 1. DOPA-^{14}C FORMATION FROM TYROSINE-^{14}C IN RAT SALIVARY GLAND EXTRACTS

	Counts/min		
	Not boiled	Boiled	$P<$
1. No tissue, not incubated	157 ± 11		
2. No tissue, incubated	267 ± 15		
3. Denervated salivary gland	167 ± 15	261 ± 10	0·01
4. Innervated salivary gland	492 ± 21	270 ± 9	0·001

Results are the means ± S.E.M. for six experiments.
2 > 3, $P < 0{\cdot}01$
4 > 3, $P < 0{\cdot}001$
4 > 2, $P < 0{\cdot}001$

When *d*-tyrosine-^{14}C was used as a substrate, the same low *d*-dopa-^{14}C formation was obtained with extracts from normal and from denervated salivary glands. Again *d*-dopa formation was greater with boiled extracts or in the absence of tissue extracts.

These findings are similar to those of Nagatsu *et al.*[2] and suggest that factors in fresh extracts of denervated and intact salivary glands protect tyrosine from non-enzymatic oxidation to dopa-like material. Heated tissue extracts are not, therefore, suitable controls. In the subsequent experiments, dopa-^{14}C formation in incubation mixtures containing extracts from chronically denervated salivary glands were used as blanks.

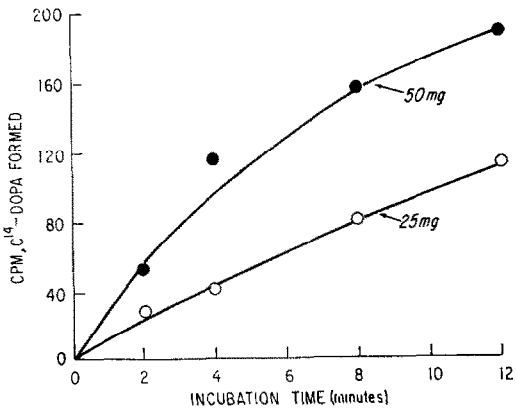

FIG. 1. Tyrosine hydroxylase activity in rat salivary gland.

Assay of tyrosine hydroxylase in the salivary gland and superior cervical ganglion

Figure 1 shows the formation of dopa-^{14}C during incubation of tissue extracts equivalent to 25 mg and 50 mg of intact salivary gland. The reaction proceeded almost linearly with time during the first 8 min, and the rate of dopa-^{14}C formation

appeared to be proportional to the amount of tissue (enzyme activity) present. Much higher activity was present in the superior cervical ganglion (Table 2), but the reaction was not linear with time and stopped within 4 min.

TABLE 2. EFFECT OF SYMPATHETIC NERVE STIMULATION ON TYROSINE HYDROXYLASE

	Dopa-^{14}C formed *in vitro* (counts/min/g)	
	Salivary gland	Superior cervical ganglia
Decentralized (1 hr)	3190 ± 650 (6)	21,200 ± 3200 (3 pairs)
Stimulated (20 pps, 1 hr)	3170 ± 530 (6)	19,000 ± 3600 (3 pairs)

Results are the means (±S.E.M.) for the number of experiments indicated in parentheses.

Effect of decentralization on the tyrosine hydroxylase content of the salivary gland and superior cervical ganglion

There was a significant decrease in tyrosine hydroxylase activity in the salivary gland of the rat one day after decentralization (Table 3). No further decrease in

TABLE 3. EFFECT OF DECENTRALIZATION OF TYROSINE HYDROXYLASE ACTIVITY IN THE RAT SALIVARY GLAND AND SUPERIOR CERVICAL GANGLION

	Dopa-^{14}C Formation (per cent control side)	
	Salivary gland	Superior cervical ganglia
Decentralized 1 day	64 ± 4* (6)	92 ± 8 (3 pairs)
Decentralized 11 days	74 ± 5* (6)	100 ± 11 (3 pairs)
		$P < 0{\cdot}02$

Results are the means (±S.E.M.) for the number of experiments indicated in parentheses.
* Differs from control.

enzyme activity was found 11 days after operation. Decentralization did not change the tyrosine hydroxylase activity in the superior cervical ganglia.

Effect of sympathetic nerve stimulation on tyrosine hydroxylase activity

Stimulation of the cervical sympathetic chain for 1 hr had no influence on the tyrosine hydroxylase activity of either the salivary gland or the superior cervical ganglia (Table 2).

Effect of bretylium and pempidine on tyrosine hydroxylase activity of the salivary gland

Six hours after administration of the ganglionic blocking agent, pempidine, there

was no change in tyrosine hydroxylase activity in the salivary gland (Table 4). Similarly, bretylium, which blocks release of norepinephrine in response to nerve impulses,[9] had no effect on the enzyme levels in this tissue.

TABLE 4. INFLUENCE OF BRETYLIUM AND PEMPIDINE ON TYROSINE HYDROXYLASE ACTIVITY IN THE RAT SALIVARY GLAND *IN VITRO*

	^{14}C-Dopa formed (counts/min/g)	$P<$
Controls	2670 ± 550	
Bretylium	2300 ± 320	0·5
Pempidine	2430 ± 160	0·5

Groups of six animals were killed 6 hr after i.p. injection of bretylium (50 mg/kg) or pempidine (10 mg/kg). Results are the means (±S.E.M.) for the number of experiments indicated in parentheses.

Effect of salivary gland extracts on tyrosine hydroxylase activity in adrenal gland or superior cervical ganglion homogenates

To assess possible inhibitory factors present in the salivary gland extracts, portions were added to homogenates of adrenal glands. The effect of increasing amounts

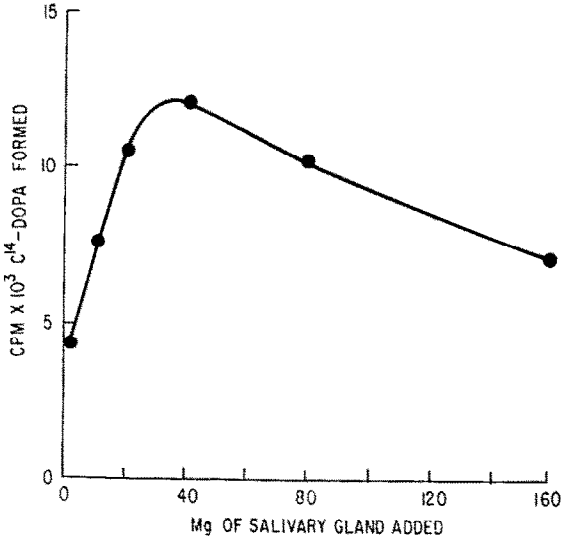

FIG. 2. Effect of salivary gland extracts on adrenal tyrosine hydroxylase activity *in vivo*. Adrenal and salivary glands were homogenized in ice-cold sucrose. A 0·25-ml aliquot of the adrenal gland homogenate was incubated with *l*-tyrosine-^{14}C and varying amounts of the salivary gland extract, as described in Methods.

of salivary gland extracts on adrenal tyrosine hydroxylase activity is shown in Fig. 2. Incubation with salivary gland extracts always increased the amount of dopa-^{14}C formed. The increase in activity produced by addition of a homogenate of 25 mg of

salivary gland (5000 counts/min) could not be accounted for by the tyrosine hydroxylase present in this amount of salivary gland (Fig. 1). Increased stimulation was apparent up to 40 mg of salivary gland, but larger amounts apparently inhibited the reaction (Fig. 2). Similar effects were found when the superior cervical ganglion was used as a source of tyrosine hydroxylase.

The effect of salivary gland extract on the time course of dopa-^{14}C formation during incubation of the adrenal gland extracts with tyrosine-^{14}C is shown in Fig. 3. There was

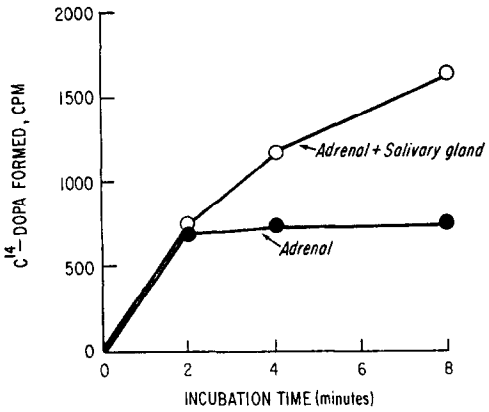

FIG. 3. Effect of salivary gland extract on adrenal tyrosine hydroxylase activity *in vitro*. Aliquots of adrenal gland homogenate were incubated for varying lengths of time with *l*-tyrosine-^{14}C with or without addition of extract of 40 mg salivary gland, as described in Methods.

no difference in the initial rate of tyrosine hydroxylation with or without the gland extract. When salivary gland extract was present, the reaction continued, but at a steadily dimishing rate. In the absence of gland extract, dopa-^{14}C formation was markedly decreased after 2 min (Fig. 3). The addition of salivary gland extract after 4 min of incubation failed to restore tyrosine hydroxylase activity.

Investigations on the nature of the salivary gland factor

Chronic sympathetic denervation decreased the amount of tyrosine hydroxylase activity in the salivary gland (Table 1) but had no effect on the ability of gland extracts to preserve the tyrosine hydroxylase activity of adrenal gland homogenates.

Additional pteridine cofactor, NADPH, a NADPH-generating system, or a pteridine–cofactor–regenerating system (sheep liver enzyme) all failed to preserve tyrosine hydroxylase activity.

The factor was found to be heat labile (2 min at 100° destroyed all activity) and nondialyzable. It was present in heart and liver as well as salivary gland. Dr. Menek Goldstein[10] suggested that the factor might be a peroxidase. Both peroxidase and catalase were found to result in apparent stimulation of tyrosine hydroxylase activity similar to that found when salivary gland extracts were used. When peroxidase or catalase was added together with denervated salivary gland extract, no further increase in dopa-^{14}C formation was seen.

DISCUSSION

Local norepinephrine synthesis is the major source of the catecholamines in various tissues.[11] Tyrosine hydroxylase catalyzes the formation of dopa from tyrosine, and this reaction is believed to be rate-limiting in norepinephrine synthesis.[2]

In the present study, tyrosine hydroxylase was found to be present in the rat salivary gland and superior cervical ganglia (Tables 1 and 2). The high enzyme activity in the ganglia, which contain predominantly adrenergic ganglion cells,[12] suggests that tyrosine hydroxylase is present in large amounts in the adrenergic neuron. Postganglionic sympathetic denervation resulted in a total disappearance of apparent enzyme activity in the salivary gland (Table 1). This decrease in tyrosine hydroxylase content parallels the decrease in norepinephrine content (unpublished observations). No decrease, however, in norepinephrine content[4] and only a slight decrease in tyrosine hydroxylase activity occurred after preganglionic denervation (Table 3). The disappearance of tyrosine hydroxylase after postganglionic denervation, therefore, appears to be a consequence of degeneration of the sympathetic neuron, rather than an interruption of impulse activity. The salivary gland tyrosine hydroxylase thus appears to be present uniquely in postganglionic sympathetic nerves. Tyrosine hydroxylase, which has previously been shown to be present in large amounts in the adrenal medulla, is therefore likely to be present only in catecholamine-storing cells.

In the present study, assays for tyrosine hydroxylase were performed *in vitro* with optimal concentrations of cofactor. During such conditions, changes in enzyme activity reflect enzyme content rather than enzyme activity, *in vivo*.

Pharmacological blockade of sympathetic ganglionic transmission, blockade of norepinephrine release from the nerve ending with bretylium, and a relatively rapid and long-lasting stimulation of the sympathetic nerves failed to alter levels of tyrosine hydroxylase in the salivary gland. Only chronic surgical decentralization resulted in a small, but significant, decrease in tyrosine hydroxylase activity. The slight decrease in tyrosine hydroxylase activity following sympathetic decentralization might be due to a slow adaptive decrease in enzyme content of the nerve endings after long-term functional inactivity. Neither decentralization nor stimulation altered the enzyme level in the superior cervical ganglion.

The content of tyrosine hydroxylase, therefore, bears no direct relationship to rapid changes in impulse activity. After decentralization, the turnover rate of norepinephrine in the superior cervical ganglia and the salivary gland is reduced, without any significant changes in the norepinephrine content.[4, 5] Stimulation of the cervical sympathetic chain increases norepinephrine synthesis from tyrosine-^{14}C in the rat salivary gland, *in vivo*.[7] These results indicate that the rate of norepinephrine synthesis in the adrenergic neuron is closely related to impulse activity.[3–6]

Since impulse activity is not related to tyrosine hydroxylase content, the control of norepinephrine synthesis cannot be mediated by changes in the content of this enzyme in the adrenergic neuron. As dopa formation appears to be rate-limiting, control of norepinephrine synthesis may be exerted through product inhibition[2] or alterations in levels of cofactors or inhibitors of tyrosine hydroxylase.

A factor that preserved the activity of adrenal and sympathetic ganglion tyrosine hydroxylase was found in the salivary gland and in other tissues. This factor was nondialyzable and heat labile. Activity of adrenal tyrosine hydroxylase was not preserved by other known cofactors, but was prolonged by peroxidase as well as

catalase. These enzymes might have protected tyrosine hydroxylase from destruction by hydrogen peroxide formed during incubation. The salivary gland contains high levels of peroxidase,[10] and extracts of salivary gland alone or in combination with peroxidase are not more effective in preserving tyrosine hydroxylase activity than is peroxidase alone. The presence of the factor in denervated salivary glands indicates that it is not confined to the sympathetic nerves; and it appears unlikely that this factor, possibly a peroxidase, plays any role in the control of dopa formation *in vivo*.

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