Developmental Delay of Lingual Lipase Expression after Guanethidine-Induced Sympathectomy (43346)

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Abstract. **Rat lingual lipase increases during postnatal development. To evaluate the role of the sympathetic nervous system in the control of lingual lipase during development, suckling rats were chemically sympathectomized by chronic treatment with guanethidine. This treatment was found to be effective in suppressing the developmental increase of lingual lipase. The effect was age dependent and also related to the dose of guanethidine given (i.e., the higher the dose, the more effective the suppression is, up to 40 μg/g body wt). The effect of guanethidine on lingual lipase suppression was not a result of induced stress, since simultaneous treatment with RU-38486, a known glucocorticoid receptor antagonist, did not prevent the decrease in lingual lipase activity. Ephedrine, a known sympathomimetic agent, restored the lingual lipase to a near normal level in guanethidine-treated animals, confirming that guanethidine acts through the sympathetic nerves. Furthermore, histochemical studies showed that guanethidine-treatment resulted in the reduction or elimination of catecholaminergic fibers in the von Ebner's glands. The effect of guanethidine was found to be transient, in that the lingual lipase activity showed complete recovery upon withdrawal of the treatment for 1 week. Together, the results indicated that sympathetic nerves have an important regulatory role in lingual lipase in rat pups during development.** [P.S.E.B.M. 1992, Vol 199]

Dietary lipids are acted upon by a series of lipolytic enzymes and cofactors in the gastrointestinal tract before assimilation. In neonatal rats and humans, the extremely low levels of pancreatic lipase (1) are compensated for by alternative sources of lipases. These include bile salt-stimulated milk lipase (2), lingual lipase (secreted by the serous glands of the tongue [3, 4], and gastric lipase (5, 6). In rats, lingual lipase undergoes significant developmental changes in activity during postnatal life (7). The mechanisms that regulate the development of lingual lipase are not known.

We have shown recently that glucocorticoids play a dual role in the regulation of lingual lipase in suckling rats. The elimination of glucocorticoid production (by adrenalectomy) prevents the maturation of lingual lipase, while exogenous administration of dexamethasone at high doses (=25 μg/100 g body wt) attenuates the maturation of the same enzyme (8). We further noted that even in the absence of adrenal glucocorticoids, lingual lipase still developed to about 60% of the control level. This suggests that in the rat, other factors besides adrenal glucocorticoids are involved in the control of lingual lipase development. In adult rats, bilateral sympathectomy decreases lingual lipase (9), suggesting a role of sympathetic innervation in the maintenance of this enzyme. It is not known whether sympathetic nerves also take part in the maturation process of lingual lipase in rat pups. The aim of the present study was to define the role of sympathetic nerves in the modulation of lingual lipase development in the neonatal rat.

Materials and Methods

Animals. Pregnant Sprague-Dawley rats were housed in individual cages and maintained on a 12:12-hr light:dark cycle. On the expected date of delivery,

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cages were checked every 2 hr for birth. The day of birth was regarded as Day 0. Pups were allowed to suckle freely until the time of sacrifice. Rats were sacrificed by decapitation at the age stated. After sacrifice, the posterior half of the tongue was dissected, and cartilage and muscle were carefully removed. Isolated glandular tissues were either used immediately or stored frozen at −70°C. Preliminary studies showed that storage at −70°C for several months did not result in appreciable loss of enzyme activity. The National Institutes of Health guidelines for the care and use of laboratory animals were followed for all protocols used to ensure that animals were not subjected to pain and discomfort.

Chemical Sympathectomy. Sympathectomy was carried out according to a schedule used previously (10, 11), with modifications. Guanethidine sulfate (Sigma Chemical Co., St Louis, MO) dissolved in phosphate-buffered saline (PBS) (pH 7.4) at various doses, as specified, was administered intraperitoneally to rat pups at the age indicated every 48 hr until the day before sacrifice. Age-matched littermate controls were given injections of equal volumes of PBS at the same times. The following schedules of injections were performed:

1. Critical period. In experiments designed to define whether there was a developmental period that was more sensitive to chemical sympathectomy, pups at 2, 4, 10, and 15 days of age were given 20 μg/g body wt of guanethidine sulfate every 48 hr. All animals were sacrificed at the age of 20 days.

2. Dose response. Pups at 4 days of age were given guanethidine sulfate in the following concentrations, 10, 20, 30, 40, and 80 μg/g body wt every 48 hr, and were sacrificed at 20 days of age.

3. Replacement Studies. To determine whether guanethidine acted through the sympathetic nerves, treated pups were supplemented with ephedrine, a sympathomimetic agent, to counteract the action of guanethidine. Littermates were divided into four different groups: (i) control PBS (Group 1), (ii) guanethidine (40 μg/g body wt) (Group 2), (iii) ephedrine (60 μg/g body wt, ip, dissolved in PBS) (Group 3), and (iv) guanethidine and ephedrine (Group 4); both were given at the same dosage as that for Groups 2 and 3, with ephedrine given immediately following guanethidine injection.

4. Recovery. To determine whether guanethidine causes permanent damage to the maturation of lingual lipase, two litters of 12 pups each were pooled at birth and redistributed at random to one of two dams, such that each dam had 12 pumps. They were then divided into two groups: (i) control PBS and (ii) guanethidine (40 μg/g body wt). Injections were started at the second day of age. At 18 days of age, three pumps from each group were sacrificed to give a baseline of guanethidine effect on lingual lipase development. The remaining control pups were continued on PBS treatment, while the guanethidine group was divided into two subgroups; Subgroup A was continued with the guanethidine treatment, but Subgroup B was given PBS instead. Representative pups from each of these groups were sacrificed at 25 and 30 days of age.

5. Assessment of sympathectomy. Pups were treated with PBS (control) or guanethidine (40 μg/g body wt) starting at two days of age. At 18 days of age, both control and guanethidine-treated pups were anesthetized with halothane (Halocarbon Laboratories, Inc., Hackensack, NJ). The vascular system was perfused with 60 cm^3 of a chilled washout solution (25 m*M* $NaHCO_3$, 118 m*M* NaCl, 6.6 m*M* KCl, 5.4 m*M* $MgCl_2$, 2.4 m*M* MgSO4, 0.18% glucose, 10% sucrose, and 2% glyoxylic acid [pH 7.3]), according to the method of Hwang and Williams (12), to prepare the tongue for histochemical staining for catecholamines. The tongues were removed immediately after perfusion. They were placed in the same solution in the refrigerator for 15 min and then frozen at 20°C until sectioning.

Cryostat sections were cut at 20 μm from the posterior portion of the tongue in the region of the circumvallate papilla and associated von Ebner's glands. Sections were placed on acid-cleaned, gelatin-subbed glass slides. The slides were subsequently dipped in a solution containing sucrose, KH_2 PO_4, glyoxylic acid, and double-distilled water (SPG), and processed following the method of De La Torre (13). SPG-treated sections of the tongue were then viewed and photographed on a Nikon Optiphot fluorescence microscope. The slides were observed by two investigators, neither of whom were aware of the code for control and sympathectomized groups.

Biochemical Determinations

The preparation of homogenates for enzyme and protein determinations was performed as described previously (8). Briefly, frozen tissue was partially thawed and minced in 10 vol of ice cold water. Tissue fragments were homogenized with a Polytron for 30 sec with the container immersed in crushed ice. Triton X-100 was added to the homogenate to a final concentration of 0.08%. The homogenate was treated with the Polytron for another 30 sec. The resulting homogenate was left on ice for 60 min, with occasional mixing. Following centrifugation at 1500*g* for 6 min, the supernatant fraction was recovered and used for lingual lipase and protein determinations. Lingual lipase was determined, as reported previously (8), by potentiometric titration (at a constant pH of 5.0) of ionized fatty acids liberated from tributyrin (20 m*M*) in a citrate-phosphate buffer (1 m*M*) with 0.01% Triton X-100. Units were expressed as micromoles of NaOH required to neutralize free fatty acids liberated per minute per mg of protein. Protein was determined by the method of Lowry *et al.*

(14) using bovine serum albumin fraction V as the standard.

Reagents

All reagents used for biochemical determinations were from Sigma Chemical Co., St. Louis, MO. RU 38486 (U486) was a gift from Dr. D. Martini of Roussel Uclaf, Romanville, France.

Statistics

Results are reported as the mean ± SD. The differences between the means of two groups were evaluated by Student's *t* test. For multigroup comparisons, analysis of variance was used. A *P*-value of ≤ 0.05 was considered significant.

Results

Duration of Treatment. The effect of guanethidine on lingual lipase development was dependent upon the age at which the treatment was initiated. In normal pups, lingual lipase is low at birth. A gradual increase in activity occurs before weaning at around 15 days of age, and is followed by a second increase after 15 days of age that eventually reaches the activity found in adults (8).

As summarized in Table I, treatment with guanethidine was ineffective when started at the age of 15 days. When guanethidine was started at 10 days of age, there was a 30% decrease in lingual lipase activities, compared with age-matched control littermates. The earlier the treatments were started, the more pronounced the effects were on lingual lipase. The greatest suppressive effect was seen when the treatment began at 2 days of age.

Two sets of experiments were performed to delineate whether the decrease in lingual lipase was due to the duration of treatment or the age when the treatment was initiated. In the first set, guanethidine injections (20 μg/g body wt, every 48 hr) began on the eighth day and the rats were sacrificed at 24 days of age. This duration of treatment was equivalent to that of the fourth to the 20th day. A second experiment was carried out to duplicate the dose received by initiating the guanethidine injection (20 μg/g body wt every 24 hr for a total of eight injections, i.e., the same number of injections as the 4–20-day group received); injections began on the eighth day and sacrificed on 17th day of age. As shown in Table I, Part B, both regimens resulted in a suppression of lingual lipase development.

Table I. Effect of Treatment Schedule on Lingual Lipase Development[a]

	Duration[b] (days)	Enzyme activity (μmol/min/mg protein)		Percentage of control
		Control	Guanethidine	
A	15–20	1.66 ± 0.20	1.6 ± 0.19	97.5
	10–20	1.63 ± 0.18	1.20 ± 0.06[c]	73.6
	4–20	1.58 ± 0.26	1.02 ± 0.11[c]	64.6
	2–20	1.63 ± 0.25	1.03 ± 0.09[c]	61.3
B	8–25	1.86 ± 0.33	1.28 ± 0.10[c]	68.8
	8–17[d]	1.66 ± 0.27	0.96 ± 0.16[c]	58.0
C	15–24[d]	1.98 ± 0.11	1.81 ± 0.06	91.4
	20–29[d]	2.23 ± 0.03	2.09 ± 0.06	93.7

[a] Each value represents the mean ± SD of three to six animals.
[b] First number denotes age of pups when first injected; the second number denotes the age they were sacrificed. All except the group in footnote *d* were given guanethidine (20 μg/g body wt every 48 hr) until 2 days before sacrifice.
[c] Values significantly different from control ($P \leq 0.05$).
[d] Pups received guanethidine (same dose, but every 24 hr) to duplicate the total dose received by the 4–20 day group.

In the second set of experiments, guanethidine injections (20 μg/g body wt, every 24 hr) were started on the 15th to 20th days of age and the rats were sacrificed on the 24th and 29th days of age, respectively. In these regimens, the rats received a total of eight injections, duplicating the number of injections received by the previous sets. Table I, Part C shows that such treatments had minimal, if any, effect on the lingual lipase development. These results suggest that although the duration of treatment and the total dose of guanethidine used were important factors, the age at which the treatment began was critical.

Effect of Guanethidine Doses. To examine the dose dependency of the guanethidine effect, pups at four days of age were given increasing doses of guanethidine (10, 20, 30, 40, and 80 μg/g body wt) and sacrificed at the age of 20 days. As shown in Table II, the decrease in lingual lipase was dose dependent. No effect was evident at 10 μg/g body wt. An effect was detected at 20 μg/g body wt. At 40 μg/g body wt, only about half of the lipase activity remained. At 80 μg/g body wt, guanethidine was extremely toxic and all pups died after three to four injections. Body weight did not show significant differences between the control and guanethidine-treated groups, except at 40 μg/g body wt.

Effect of Ephedrine Replacement. To determine whether the effect of guanethidine was indeed due to its action on the sympathetic nerves, pups receiving guanethidine were also given ephedrine, a sympathomimetic agent, as a replacement. In two separate experiments (Table III), ephedrine at the dose given counteracted the effect of guanethidine in terms of lingual lipase activity. Ephedrine at the same dose by itself had no effect on lingual lipase activity.

Effect of Glucocorticoid Receptor Antagonist. In view of our recent finding that high doses of dexamethasone and other glucocorticoids have an attenuating effect on lingual lipase development in rat pups (8), it is possible that guanethidine administration might have resulted in stress, which then secondarily affected the lingual lipase development by increasing the amount of circulating glucocorticoids. We first attempted to use

Table II. Effect of Guanethidine Dose on Lingual Lipase Development[a]

Guanethidine concentration (μg/g body wt)	Body wt (g)	Enzyme activity (μmol/min/mg protein)	Percentage of control
0	41.0 ± 0.6	1.63 ± 0.18	100
10	41.3 ± 1.0	1.68 ± 0.09	103
20	40.0 ± 0.7	1.26 ± 0.06[b]	77
30	39.7 ± 0.9	0.98 ± 0.05[b]	60
40	38.4 ± 1.3[b]	0.84 ± 0.09[b]	52

[a] Duration of treatment was from 4 to 20 days. Each value represents mean ± SD of three to four animals.
[b] Significantly different from control ($P \leq 0.05$).

Table III. Effect of Ephedrine Replacement[a]

Treatments	Enzyme activities (μmol/min/mg protein)			
Control	1.40 ± 0.08	(33.1 ± 1.8)[b]	1.78 ± 0.27	(39.8 ± 2.4)
Ephedrine only (60 μg/g wt)	1.35 ± 0.20	(31.0 ± 1.3)	1.75 ± 0.04	(36.5 ± 0.7)
Guanethidine only (20 μg/g wt)	1.08 ± 0.15[c]	(32.3 ± 1.0)	0.97 ± 0.18[c]	(36.7 ± 2.8)
Guanethedine and ephedrine (20 μg/g wt and 60 μg/g wt)	1.37 ± 0.06	(30.6 ± 1.9)[d]	1.44 ± 0.03[d]	(35.2 ± 2.6)

[a] Duration of treatment was 2–20 days. Each value represents the mean ± SD of three to four animals.
[b] Values in parentheses are body weights of pups in grams.
[c] Significantly lower than control groups ($P \leq 0.05$).
[d] Significantly higher than guanethidine only group ($P \leq 0.05$).

adrenalectomized animals to eliminate the involvement of the adrenal gland. Unfortunately, the adrenalectomized pups were extremely sensitive to guanethidine and all pups died within 3 days after the first guanethidine injection. Alternative experiments were then performed by giving U486, a known Type II glucocorticoid receptor antagonist (15, 16), to the guanethidine-treated pups. The results in Table IV show that U486 given simultaneously with guanethidine did not block the effect of guanethidine on lingual lipase development. By itself, U486 had no detectable effect on the development of lingual lipase.

Recovery from Guanethidine Treatment. To see if the adverse effect of guanethidine on lingual lipase was permanent or transient, pups were treated with guanethidine from 2 to 18 days of age, at which time some treated pups were allowed to recover until 25 and 30 days of age. As depicted in Table V, at 18 days of age (i.e., at the end of the first guanethidine treatment), lingual lipase showed the typical decrease in activity, compared with control pups. A recovery of lingual lipase activity was evident at 25 days of age, i.e., 7 days after withdrawal of guanethidine treatment. Pups continuing on guanethidine showed continual depression of lingual lipase compared with age-matched controls. However, these guanethidine-treated pups still exhibited an age-dependent increase in activity levels.

Histochemical Studies. The injection scheme used in this study has been reliably used in previous studies to produce an 80–92% reduction in the number of perikarya in the superior cervical ganglia (10, 11, 17). Histochemical fluorescence was assessed within the tongue in order to determine the effectiveness of the sympathectomy at the target organ level. As shown in Figure 1, catecholaminergic histofluorescent fibers were seen in both glandular (Fig. 1A) and vascular (Fig. 1C) regions from control rats. The vascular catecholaminergic staining appeared more punctate compared with the extensive arborizations found in the glandular regions. In the sympathectomized rats, glandular catecholaminergic fibers were absent (Fig. 1B) in all sections from all the rats observed. In the case of the vascular regions, catecholaminergic innervation was absent in many cases, but in a few sections, punctate staining could still be observed, albeit at greatly reduced levels.

Discussion

A previous report has documented that after bilateral sympathectomy in adult rats, lingual lipase activity decreases 40–50% in 1 week (9). In the present study in neonatal rats, guanethidine treatment suppressed lingual lipase development. The importance of sympathetic nerves in the maturation of the gastrointestinal tract (10, 18) and associated glands (11, 19) has been well documented. Chemical sympathectomy using

Table IV. Effect of U486[a]

Treatments	Enzyme activities (μmol/min/mg protein)	
	Experiment 1	Experiment 2
Control	1.44 ± 0.21 (28.8 ± 2.2)[b]	1.55 ± 0.23 (39.1 ± 0.9)
Guanethidine only (40 μg/g body wt)	0.95 ± 0.08[c] (27.7 ± 2.1)	1.13 ± 0.19[c] (37.3 ± 1.3)
Guanethidine and U486 (40 μg/g body wt and 30 μg/g body wt)	0.82 ± 0.15[c] (26.4 ± 0.8)	— —
U486 alone (30 μg/g body wt)	— —	1.53 ± 0.18 (39.4 ± 0.3)

[a] Each value represents the mean ± SD of three to four animals. Duration of treatment was 2–18 days.
[b] Values in parentheses are body weight of pups in grams.
[c] Significantly different from control ($P \leq 0.05$).

Table V. Recovery of Lingual Lipase after Guanethidine Treatment[a]

Treatments	Enzyme activities (μmol/min/mg protein)		
	18 days	25 days	30 days
Control	1.97 ± 0.11 (34.8 ± 2.7)	2.34 ± 0.32 (64.1 ± 3.6)	2.13 ± 0.14 (89.8 ± 6.9)
Guanethidine[b] (to day of sacrifice)	1.04 ± 0.05[c] (33.7 ± 3.4)	1.79 ± 0.02[c] (58.8 ± 6.8)	1.76 ± 0.10[c] (84.7 ± 8.3)
Guanethidine[d] (to Day 18 only)		2.68 ± 0.12 (59.0 ± 4.9)	2.14 ± 0.10 (91.6 ± 7.1)

[a] Each value represents mean ± SD of three to four animals. Values in parentheses are body weights of pups in grams.
[b] Guanethidine was given at 40 μg/g body wt every 48 hr starting the second day of age and continuing until 2 days before sacrifice.
[c] Significantly different from control ($P \leq 0.05$).
[d] Guanethidine was given at 40 μg/g body wt every 48 h starting the second day of age, but was stopped at the 18th day of age to allow for recovery.

chronic guanethidine administration decreases cell proliferation and alters cell morphology both in the parotid gland acini (11) and in the small intestine (10). Few studies have examined the biochemistry of specific components in the affected tissues. Denervated rat parotid gland contains less total RNA compared with the contralateral control gland (20). Chronic treatment with the sympathomimetic agent isoproterenol prematurely increases amylase in the rat parotid gland (21), and β-adrenergic receptors and various secretory proteins in the rat submandibular glands (22).

In most developmental systems, certain stages of the development cycle are particularly critical in that these periods are especially vulnerable to insults that lead to permanent damage, which may result in failure of the system to reach its genetic potential (23). The present studies show that chemical sympathectomy of rat pups at an early postnatal age significantly affects the maturation of lingual lipase. The net effect appears to be a delay, rather than a prevention, of lingual lipase development, since prolonged treatment beyond 20 days of age did not block the increase in the level of the enzyme entirely (see Table V). Termination of treatment also led to the prompt return of lingual lipase levels to those of the age-matched control pups, suggesting a transient disruption of maturation when innervation by the autonomic nervous system was interrupted experimentally.

In an attempt to evaluate whether there exists a period during the postnatal development that might be more sensitive to guanethidine treatment, injections were given at various ages. An initial protocol starting at 2–15 days of age and terminating at 20 days of age showed an apparent decrease in sensitivity with age, in that when treatments were started after 10 days of age, less suppression in lingual lipase development was seen (Table I, Part A). These differences were later shown to be more apparent than real, since the number of guanethidine injections received and the duration of treatment in these older groups were correspondingly decreased compared with those groups that received the treatment at an earlier age. Further experiments confirmed this interpretation, as pups started at a later age (8 days instead of 2 or 4 days of age) but given the same dose (in a shorter duration), or at an equivalent duration but sacrificed at a later age, showed depression of their lingual lipase activities compared with age-matched control littermates (Table I, Part B). Additional experiments that initiated the guanethidine treatment (with an identical dose) at an even older age (i.e.,

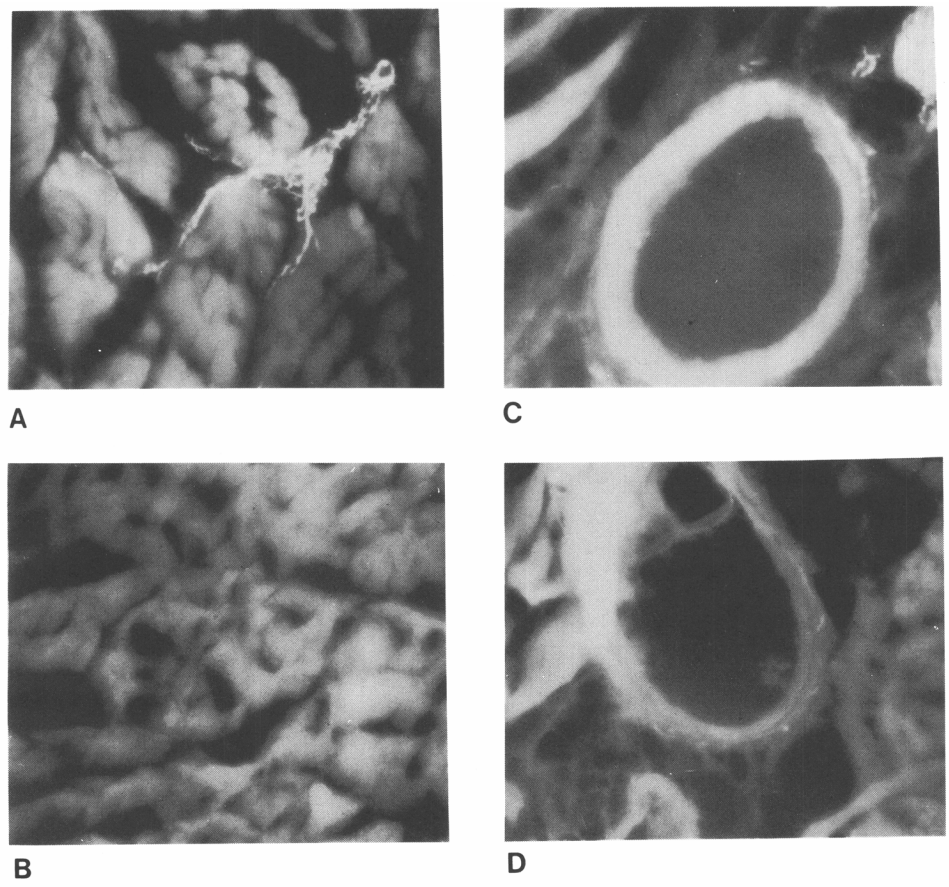

Figure 1. Catecholaminergic histofluorescence in von Ebner's glands of the tongue. Guanethidine (40 μg/g body wt) was administered every 48 hr starting at 2 days of age. Controls were littermates receiving only vehicle injection at the same time as the treated group was injected. Both guanethidine-treated and control pups were sacrificed at 18 days of age. (A) Von Ebner's glands from control rats demonstrate extensive catecholaminergic profiles. (B) In the sympathectomized rats, fibers were absent from the glandular areas. In most of the sections observed, catecholaminergic fibers were absent from the vascular region; catecholaminergic histofluorescence was only reduced in the (D) guanethidine-treated pups compared with (C) control (original magnification ×1870).

15 and 20 days) showed no effect on lingual lipase development (Table I, Part C). These later results indicate that there is a critical phase in which an intact sympathetic system is required for lingual lipase development. This period appears to be before 15 days of postnatal age.

Guanethidine could act through several possible mechanisms. It might have a general toxic effect that inhibits normal growth of the animal, which in turn would be reflected in a depression of lingual lipase. However, in our hands, the treatment schedule used did not result in any appreciable growth retardation, except at the highest dose reported (40 μg/g) (see Table II). Similar observations were noted in previous studies using lower doses of guanethidine (20 μg/g) (11, 17). The repeated injection of guanethidine might lead to undue stress followed by an increase in circulating glucocorticoid, which has recently been shown to attenuate lingual lipase development when given at moderate to high doses (8). If that were the case, the observed effect would be counteracted by a specific glucocorticoid receptor antagonist. Our experiment using U486 concomitantly with guanethidine administration, however, did not block the effect of guanethidine. We have shown previously in the same model that U486 was very effective in blocking the attenuating effect of dexamethasone treatment (8). Our present results would, therefore, indicate that the action was not via the adrenal through the release of glucocorticoids.

Guanethidine presumably could act on the serous glands by causing a loss of available sympathetic neurons and/or decreased sympathomimetic substances. Both of these actions have previously been shown to occur in other tissues. Guanethidine treatment in the neonatal period was found to result in a sharp reduction in ganglion cells in the superior cervical ganglion (11, 17, 24, 25). A similar treatment was also reported to lead to a 92% depression in norepinephrine content of the peripheral vasculature (26) and disappearance of norepinephrine from the submandibular glands (27) in rats. The present study revealed an almost complete absence of nerve endings following guanethidine treatment, supporting the concept of guanethidine acting through a loss of ganglion cells. The catecholaminergic

histofluorescence retained in the vascular regions may represent the areas of optimum regrowth following guanethidine-induced destruction of noradrenergic innervation. Alternatively, the vascular histofluorescence may represent differential susceptibility of noradrenergic fibers in different regions. Additionally, depression of sympathetic neurotransmitters in serous glands might also play a role, since replacement with ephedrine could alleviate the suppressive effect of guanethidine on the lingual lipase levels. In other glands, such as the major salivary glands, a trophic role for the sympathetic nervous system has been suggested. In those studies, normal cell proliferation, differentiation, and gland maturation were dependent on an intact sympathetic nervous system (28, 29). The effects of the sympathetic nervous system were only observed during postnatal development; sympathectomy of the adult did not significantly alter gland structure and function. Our results suggest that a similar sympathetic neural trophism may be required for the normal maturation of lingual lipase.

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