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Dopaminergic Neurons: Activation of Tyrosine Hydroxylase by a Calcium Chelator

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SUMMARY

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Addition of the calcium-chelating agent ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) to a high-speed supernatant preparation obtained from rat striatum produced a dramatic increase in the activity of tyrosine hydroxylase assayed in the presence of subsaturating concentrations of tyrosine and reduced pterin cofactor. This activation appeared to be mediated by changes in the kinetic properties of tyrosine hydroxylase. In the presence of EGTA (50 μM) the K_m of the enzyme for tyrosine decreased nearly 8-fold (from 54 μM to 9 μM), the K_m for 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine decreased 7-fold (from 0.89 mM to 0.13 mM), and the K_i for dopamine increased 700-fold (from 0.11 mM to 74 mM). No significant change in V_{max} was observed. All these kinetic alterations could be reversed by the addition of calcium to the assay medium, while magnesium, even in high concentrations (1 mM), was ineffective. A similar activation was observed with tyrosine hydroxylase isolated from other dopamine-containing regions of rat brain (median eminence, olfactory tubercle) and in the dopamine-rich pedal ganglion of *Mercenaria mercenaria* (Mollusca). EGTA produced no significant change in the activity of tyrosine hydroxylase prepared from central (medulla-pons) or peripheral noradrenergic neurons. These results suggest that tyrosine hydroxylase associated with dopaminergic neurons may differ from the enzyme found in noradrenergic neurons. This unique response of the tyrosine hydroxylase present in dopaminergic neurons may serve as a useful biochemical marker for identifying these neurons.

INTRODUCTION

The regulation of dopamine synthesis in dopaminergic neurons of the mammalian central nervous system displays unusual features under conditions in which the initiation or conduction of impulses in these neurons is blocked. For example, if firing of the dopaminergic fibers of the nigro-neostriatal pathway is blocked by systemic administration of a drug such as γ-hydroxybutyrate (1, 2) or by a direct injection into the nigro-neostriatal pathway of a local anesthetic such as xylocaine (2, 3), there is a dramatic increase in dopamine levels in the striatum, amounting to 200% of that of controls (4–9). Interruption of the nigro-neostriatal tract by transection or electrothermic lesion also produces a similar

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increase in dopamine formation in the region of the nerve terminals (10–12). In the presence of a decarboxylase inhibitor it has been possible to show an enhanced accumulation of dopa in the striatum following interruption of normal impulse flow (2, 13–15). This observation suggests that an increase in tyrosine hydroxylase activity may be responsible for the marked accumulation of dopamine which occurs in the striatum under the conditions described above (14, 15). Such a finding is quite unexpected, since noradrenergic neurons do not readily increase the level of transmitter in their terminals when nerve conduction is blocked (16–19).

Studies on dopamine formation in slices of rat striatum also indicate a paradoxical situation. Here the formation of dopamine from tyrosine in slices of striatum, incubated in Krebs-Ringer phosphate, is enhanced when calcium ions are removed from the medium (20, 21), whereas synthesis of norepinephrine in slices of rat cortex remains unchanged under these conditions (21, 22). Thus it appears that the absence of calcium ions from the incubation medium produces alterations in striatal slices which lead to an increase in dopamine formation. In view of the fact that calcium ions are known to mediate release of transmitter during nerve activity (23–26), the behavior of dopaminergic neurons *in vivo*, in the absence of nerve impulses, suggests that a lack of calcium influx may trigger this dramatic increase in formation of dopamine in the nerve terminal. Since tyrosine hydroxylase is the rate-limiting enzyme in the formation of catecholamines (27), the foregoing observations prompted us to investigate the effects of calcium and the calcium-chelating agent EGTA[2] on the activity of soluble tyrosine hydroxylase prepared from the rat striatum.

The findings reported here indicate that tyrosine hydroxylase in dopaminergic neurons differs from the enzyme found in noradrenergic neurons in its response to calcium ions and EGTA. Whereas calcium ions activate tyrosine hydroxylase from noradrenergic neurons, as reported earlier (28), they have little effect on striatal tyrosine hydroxylase. On the other hand, the calcium chelator EGTA activates tyrosine hydroxylase from striatum but is without effect on the enzyme isolated from norepinephrine-containing neurons.

[2] The abbreviations used are: EGTA, ethylene glycol bis(ρ-aminoethyl ether)-*N,N'*-tetraacetic acid; $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,-7,8-tetrahydropteridine; NSD-1055, 3-hydroxy-4-bromobenzyloxyamine dihydrogen phosphate.

METHODS

Materials. L-[3,5-^{3}H]Tyrosine, 30 Ci/mmole (New England Nuclear), was purified by passage through aluminum oxide (British Drug Houses) at pH 8.4 and was diluted to a specific activity of 0.1 Ci/mmole with L-tyrosine (Schwarz/Mann). Synthetic cofactor, $DMPH_4$, was obtained from Calbiochem. Purified bovine catalase (lyophilized powder form), NADPH, and Tris base (ultrapure) were products of Schwarz/Mann. EGTA and 3,4-dihydroxyphenylethylamine HCl were purchased from Sigma Chemical Company. The aromatic amino acid decarboxylase inhibitor 3-brocresine was a gift of Lederle Laboratories. Sheep liver dihydropteridine reductase was purified through the first ammonium sulfate fractionation according to Kaufman (29). All other agents were of the highest purity available commercially.

Preparation of tissue. Male Sprague-Dawley rats, 250–300 g (Charles River Breeding Laboratories), were killed by decapitation. The brains were removed and placed on ice. The corpora striata were dissected according to Bunney *et al.* (30), and the medulla-pons and cerebral cortex were also removed. The median eminence was exposed and dissected as described by Porter and Smith (31). Pedal ganglia were dissected from 200 specimens of *Mercenaria mercenaria* (32), freshly obtained from a fish market (Gambardella's, New Haven, Conn.). Immediately following dissection, all tissues were frozen on Dry Ice and stored at −70° for periods up to 2 weeks until assay.

At the time of assay, tissues from an appropriate number of animals were pooled and homogenized (5–10 strokes) in 10 volumes (w/v) of ice-cold 0.05 M Tris-acetate, pH 6.0, using a glass homogenizer (Kontes Duall grinder) with 0.010-cm clearance. The homogenate was centrifuged at

104,000 × *g* for 90 min, and the supernatant served as the source of tyrosine hydroxylase.

Tyrosine hydroxylase assay. Tyrosine hydroxylase was assayed by a modification of the methods of Shiman *et al.* (33) and Coyle (34) as described in detail by Morgenroth *et al.* (28). The supernatant (100 μl) was added to a 15 × 100 mm glass test tube containing a reaction mixture of 200 μmoles of acetate buffer (pH 6.0), 0.1 μmole of NSD-1055,[2] 3300 units of catalase, 100 μl of sheep liver dihydropteridine reductase in 0.025 M Tris-HCl buffer (pH 7.4), 1.0 μmole of NADPH in distilled water, and $DMPH_4$ in ice-cold 0.005 N HCl in the concentrations shown below. Catalase, $DMPH_4$, and NADPH were prepared fresh just prior to use. Test solutions or water were added in 100 μl. The total volume of the reaction mixture was 1.0 ml. Blanks consisted of either supernatant boiled for 20 min or complete reaction mixtures to which 50 μl of glacial acetic acid had been added. After a 5-min preliminary incubation at 37°, the reaction was initiated by the addition of substrate L-tyrosine in the concentrations indicated below. The reaction was stopped after 45 min by the addition of 50 μl of glacial acetic acid. The formation of ^3H_2O was followed as a measure of dopa formed.

Protein was assayed according to Lowry *et al.* (35), using bovine serum albumin as standard. All calculations were performed on a Hewlett Packard programmable calculator, model 9810A. Statistical estimates of K_m were performed according to Wilkinson (36). Kinetics was determined on linear portions of the time course and protein concentration curves.

The concentration of calcium in the high-speed supernatant prepared from the striatum was assayed using a model 330 Perkin-Elmer atomic absorption spectrometer. The calcium concentration in the high-speed supernatant obtained from a 10% striatal homogenate was 63 μM. Thus the starting concentration of calcium in our incubation mixture was about 6 μM.

RESULTS

The rate of L-dopa formation in all incubation media used in the following experiments was linear with time for at least 1 hr, and with protein concentration from 25 to 750 μg/ml.

The results in Table 1 indicate that the activity of tyrosine hydroxylase in high-speed supernatants prepared from brain regions rich in dopamine, i.e., the median eminence, olfactory tubercle, and corpus striatum, was markedly increased by the presence of 50 μM EGTA in the incubation media.

The effect of different concentrations of EGTA on the activity of tyrosine hydroxylase isolated from several regions of rat brain and from pedal ganglia of the clam *M. mercenaria* is shown in Fig. 1. Maximal activation of tyrosine hydroxylase was observed in the striatum at 5 μM EGTA, in the olfactory tubercle at 10 μM EGTA, in the median eminence at 1 μM EGTA, in the cerebral cortex at 10 μM EGTA, and in pedal ganglia of *M. mercenaria* at 1 μM EGTA. In contrast to the marked effects of EGTA on tyrosine hydroxylase activity from brain areas or ganglia rich in dopamine, the activity of tyrosine hydroxylase prepared from the medulla-pons, a brain region which contains noradrenergic but few, if any, dopaminergic neurons, was unaltered. When equal amounts of the high-speed supernatants obtained from the striatum were mixed with the high-speed supernatants obtained from the medulla-pons, the medulla-pons enzyme did not appear susceptible to activation by addition of EGTA. In a similar fashion, mixing of these high-speed supernatant preparations obtained from the medulla-pons and striatum did not cause the striatal tyrosine hydroxylase to develop sensitivity to the activating effects of added calcium ions.

In order to study the mechansim responsible for the increase in tyrosine hydroxylase activity observed in the presence of EGTA, the kinetics of tyrosine hydroxylation was determined using enzyme prepared from corpus striatum. The K_m for the substrate, L-tyrosine, was determined according to Lineweaver and Burk (37), using 1 mM $DMPH_4$. In the presence of EGTA (50 μM) the K_m for tyrosine was shifted 5-fold, from a control value of 54 μM to 9 μM (Fig. 2). The change in K_m for

TABLE 1

Effect of EGTA on tyrosine hydroxylase activity

Tyrosine hydroxylase activity was determined in the presence of 10 μM tyrosine and 100 μM $DMPH_4$. Each value is the mean ± standard error of triplicate assays carried out on four separate tissue samples.

Treatment	Tyrosine hydroxylase activity		
	Corpus striatum	Olfactory tubercle	Median eminence
	pmoles dopa/min/mg protein		
Control	25.9 ± 2.6	8.4 ± 1.3	63.2 ± 8.6
EGTA (50 μM)	85.6 ± 4.3	31.6 ± 3.5	204.7 ± 45.6
EGTA (50 μM) + Ca^{++} (100 μM)	24.2 ± 3.1	7.9 ± 0.3	65.9 ± 7.2
EGTA (50 μM) + Mg^{++} (1 mM)	83.9 ± 5.7	29.9 ± 2.7	234.1 ± 53.7

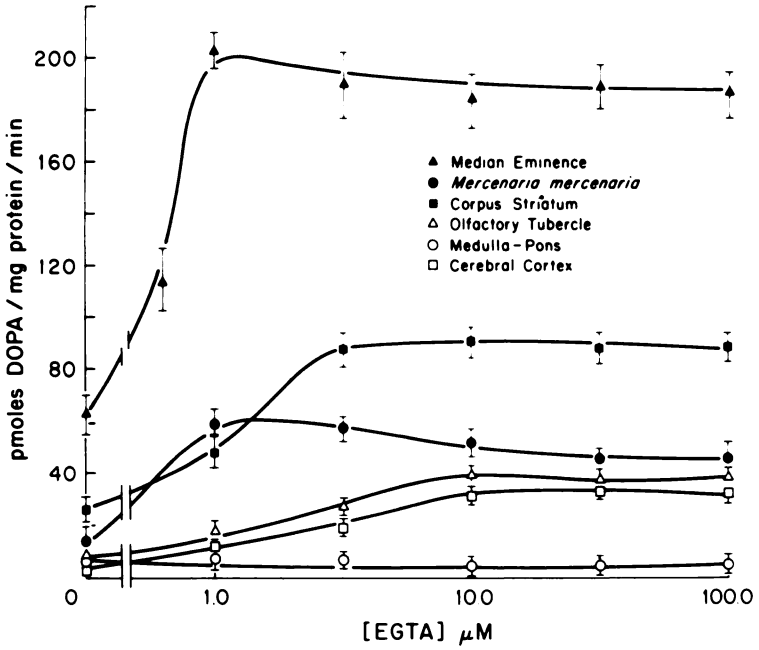

FIG. 1. *Effect of EGTA on tyrosine hydroxylase activity in selected regions of rat brain and pedal ganglion of Mercenaria mercenaria (Mollusca)*

Tyrosine hydroxylase was measured in the high-speed supernatants of tissue homogenates in the presence of subsaturating concentrations of tyrosine (10 μM) and $DMPH_4$ (100 μM). The results are expressed as the mean picomoles of dopa formed per milligram of protein per minute ± the standard error of six determinations.

tyrosine produced by EGTA could be completely reversed by addition of 100 μM calcium to the reaction medium. In no case was there a significant change in the maximum velocity.

The results shown in Fig. 3 demonstrate a similar effect of EGTA on the kinetics of tyrosine hydroxylase for the artificial cofactor $DMPH_4$. The K_m for $DMPH_4$ was shifted 8-fold, from 0.89 mM to 0.13 mM, in the presence of EGTA (10 μM). There was no significant change in the maximal velocity in the presence of EGTA.

The effect of EGTA on the K_i of striatal tyrosine hydroxylase for dopamine was determined according to Dixon (38) at three $DMPH_4$ concentrations (Fig. 4). In the presence of EGTA the K_i of tyrosine hydroxylase for dopamine was increased from a control value of 0.11 mM to 74 mM, indicating that the EGTA-treated enzyme had about a 700-fold decrease in its affinity for

dopamine. This kinetic alteration produced by EGTA was completely reversed by the addition of calcium (100 μM) (Fig. 4).

Tyrosine hydroxylase prepared from other brain regions rich in dopamine, namely, median eminence and olfactory tubercle, showed similar kinetic changes in the presence of EGTA (50 μM) (Table 2). In both tissues EGTA produced a 5-fold decrease in the K_m for tyrosine, an 8-fold decrease in the K_m for synthetic cofactor, $DMPH_4$, and an approximately 750-fold increase in the K_i for dopamine. There was no significant change in V_{max}.

DISCUSSION

The results presented in this paper suggest that the increase in tyrosine hydroxylase activity seen in preparations from median eminence, olfactory tubercle, and corpus striatum after adding EGTA *in vitro* may be attributed to the removal of calcium from tyrosine hydroxylase or some other protein present in the incubation medium. The effect of EGTA on tyrosine hy-

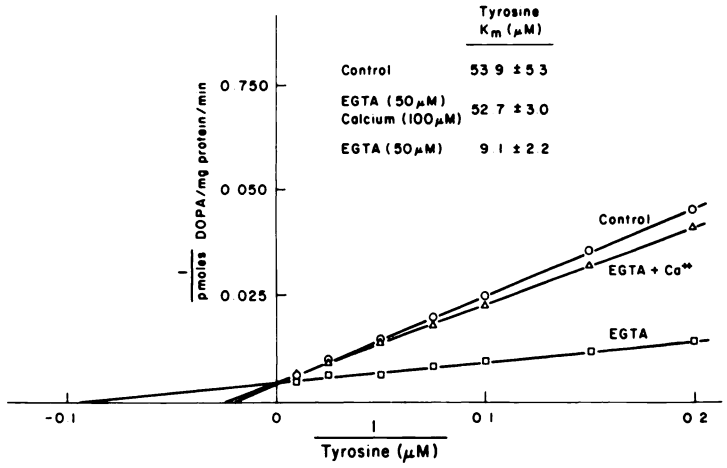

FIG. 2. *Effect of EGTA on K_m for tyrosine of tyrosine hydroxylase isolated from rat striatum*

The K_m for tyrosine was determined according to Lineweaver and Burk (37) at a $DMPH_4$ concentration of 1.0 mM and seven tyrosine concentrations, ranging from 5 to 100 μM. Each K_m value is the mean of intercepts generated from six separate lines.

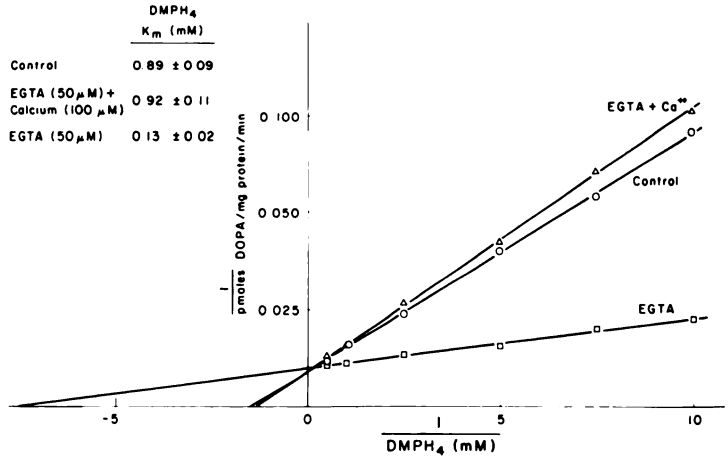

FIG. 3. *Effect of EGTA on K_m for $DMPH_4$ of tyrosine hydroxylase ioslated from rat striatum*

The K_m for $DMPH_4$ was determined according to Lineweaver and Burk (37) at a tyrosine concentration of 100 μM and at six $DMPH_4$ concentrations, ranging from 0.1 to 2 mM. Each K_m value is the mean of intercepts obtained from six separate lines.

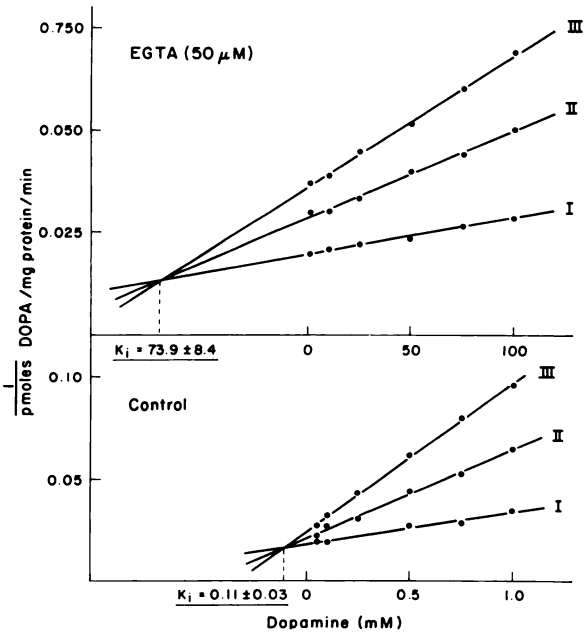

FIG. 4. *Effect of EGTA on K_i for dopamine of tyrosine hydroxylase isolated from rat striatum*

The K_i values for dopamine were determined by the method of Dixon (38) at three concentrations of $DMPH_4$ [100 μM (I), 10 μM (II), and 1 μM (III)] and six concentrations of dopamine, and are expressed as the means ± standard errors of six determinations.

TABLE 2

Effect of EGTA on kinetic properties of tyrosine hydroxylase from median eminence and olfactory tubercles

The K_m for tyrosine was determined by the method of Lineweaver and Burk (37) at a $DMPH_4$ concentration of 1.0 mM and at seven tyrosine concentrations, ranging from 0.5 to 100 μM. The K_m for $DMPH_4$ was determined by the method of Lineweaver and Burk (37) at a tyrosine concentration of 0.1 mM and at six $DMPH_4$ concentrations, ranging from 0.05 to 1.0 mM. Both K_m values are the means ± standard errors of six determinations. The K_i value was determined by the method of Dixon (38) at a tyrosine concentration of 0.1 mM, at $DMPH_4$ concentrations of 100, 10, and 1 μM, and at six dopamine concentrations, ranging from 0.01 to 1 mM for controls and 1 to 100 mM for EGTA-treated enzyme.

System	K_m tyrosine	K_m $DMPH_4$	K_i dopamine
	μM	*mM*	*mM*
Median eminence			
Control	50.6 ± 3.7	0.80 ± 0.11	0.13 ± 0.03
EGTA (50 μM)	13.0 ± 0.5	0.15 ± 0.06	77.5 ± 5.1
Olfactory tubercles			
Control	55.7 ± 5.1	0.96 ± 0.13	0.19 ± 0.02
EGTA (50 μM)	6.0 ± 0.2	0.09 ± 0.04	72.6 ± 3.8

droxylase is concentration-dependent and can be completely reversed by addition of excess calcium. Since the tyrosine hydroxylase reaction itself is linear with time and protein concentration in the presence and absence of EGTA, it follows that the increase in tyrosine hydroxylase activity seen with EGTA is not due to some type of stabilization of the enzyme in solution.

The reversal of the EGTA effect appears to be specific for calcium, since magnesium, in concentrations as high as 1 mM, does not reverse the activation produced by EGTA. Other calcium chelators, such as 7,8-dihydroxychlorpromazine (39), have been shown to increase the activity of tyrosine hydroxylase isolated from striatum to an extent similar to that produced by

EGTA. This activation is also reversed by addition of calcium. When the striatal enzyme is incubated with concentrations of EGTA producing maximal effects, addition of other calcium chelators has no further effect.

In preliminary investigations the increase in tyrosine hydroxylase activity produced by EGTA was found to be significantly greater if the assay was conducted at low concentrations of tyrosine and cofactor. These results suggested that EGTA may increase tyrosine hydroxylase activity by a kinetic activation of the enzyme (increased affinity for substrate and cofactor) rather than by some type of stimulation or increase in maximal velocity. The kinetic analysis of substrate and cofactor binding confirmed this hypothesis. The K_m for tyrosine was decreased nearly 6-fold, and the K_m for $DMPH_4$, about 7-fold. These changes in the affinity of the enzyme for substrate and cofactor occurred with no significant change in V_{max}. Similar results have recently been obtained using either 6-methyltetrahydropterin or tetrahydrobiopterin as cofactor. In fact, EGTA produces an even greater activation of tyrosine hydroxylase in the presence of either of these cofactors.[3]

The alterations of the K_m of tyrosine hydroxylase for both substrate and cofactor are probably sufficient to account for the increase in tyrosine hydroxylase activity observed *in vitro*. The results obtained with the soluble enzyme have shown that the activation by EGTA occurs only when subsaturating concentrations of substrate and/or cofactor are used. On the other hand, the increase in catecholamine biosynthesis observed in slices of striatum incubated in calcium-free media occurs in the presence of saturating concentrations of tyrosine in the medium (20, 21), suggesting that, in intact tissue, the alteration in the K_i for dopamine or the K_m for cofactor may be more important in explaining the observed increase in catecholamine synthesis.

The kinetic changes reported in this paper involve only the soluble form of tyrosine hydroxylase. Kuczenski and Mandell (40) have recently reported differences in the kinetic properties of the soluble and membrane-bound forms of tyrosine hydroxylase in the rat striatum. They have presented evidence that micromolar amounts of the mucopolysaccharide heparin alter the kinetics of the soluble enzyme, causing it to behave in a fashion similar to that of the bound enzyme. Taken together with the present findings, these results indicate that many factors may be involved in the regulation of tyrosine activity in dopaminergic neurons.

The kinetic alterations of tyrosine hydroxylase seen in the presence of EGTA were observed in all dopamine-rich brain regions studied. Similar changes have been noted in the pedal ganglion of the mollusc *M. mercenaria*, which is known to be rich in dopamine (32). However, EGTA has no effect on the activity of tyrosine hydroxylase isolated from peripheral noradrenergic neurons (41) or from brain regions containing noradrenergic but few, if any, dopaminergic terminals. These results suggest the possibility that the activation of tyrosine hydroxylase by EGTA may serve as a useful biochemical marker for the presence of dopaminergic neurons. In addition, the selective effect of EGTA on the tyrosine hydroxylase from dopamine-containing neurons indicates that this enzyme may be different from the tyrosine hydroxylase present in central and peripheral noradrenergic neurons.

[3] V. H. Morgenroth, III, and R. H. Roth, unpublished observations.

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