The Appearance of Transient Species of Cytochrome c upon Rapid Oxidation or Reduction at Alkaline pH*

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SUMMARY

The rapid reduction and oxidation of horse heart cytochrome c has been studied in the pH range 7 to 12 by stopped flow spectrophotometry. Above pH 8 both the oxidation of ferrocytochrome c by ferricyanide, and the reduction of ferricytochrome c by dithionite, show biphasic kinetics in which electron transfer occurs rapidly in the first phase with a rate dependent on oxidant or reductant concentration. The rate of the second phase is independent of the reductant which electron transfer occurs rapidly in the first phase with a rate dependent on oxidant or reductant concentration. The rate of the second phase is independent of the reductant or oxidant concentration, and the amplitude of the absorbance changes is pH-dependent with an apparent pK near 9.3. Reduction of ferricytochrome c by dithionite at pH 10.7 produces a transient form of ferrocytochrome c characterized by a sharper and more intense Soret peak centered at 418 nm ($\epsilon_M \geq 173,000$), a more intense $\alpha$-band centered at 550 nm ($\epsilon_M \geq 31,000$) and a reduced $\beta$-band near 520 nm ($\epsilon_M \leq 14,500$). During the second phase, this transient form of ferrocytochrome c changes to the classical stable form with a rate constant of 2 to 8 s$^{-1}$, depending upon the pH.

Rapid oxidation of ferrocytochrome c by ferricyanide at pH 10.5 produces a transient species of ferricytochrome c with a 695-nm band absorption and an EPR spectrum characteristic of ferricytochrome c at neutral pH. With time, both the EPR spectrum and the 695-nm band absorption change to the forms normally observed at pH 10.5, and the rate constant of this change is 0.8 s$^{-1}$. A model is proposed which features a change in the sixth ligand of cytochrome c as being responsible for the changes observed during the second phase.

A complete understanding of the function and mechanism of action of an electron transfer protein will include both the detailed three-dimensional description of the static reduced and oxidized forms of the protein, and a dynamic description of the intermediate molecular events occurring as an electron is conducted to and from the active site during reduction and oxidation. The first condition has been realized for cytochrome c through the elegant x-ray crystallographic work of Dickerson et al. on the oxidized horse (1) and reduced tuna (2) heart proteins. The second description remains in the speculative stage, but a comparison of the amino acid sequences of cytochrome c from widely divergent species (3, 4) together with chemical modification studies (for examples, see Refs. 5 to 8) have identified several amino acid residues critical for the electron transfer function, although the specific role of many of the residues remains to be elucidated. Realization that the heme prosthetic group is buried within the protein has prompted proposals that an electron can reach the iron only through the amino acid residues, particularly the aromatic amino acids (2, 6).

Recently, the technique of pulse radiolysis has been used to study the reduction of ferricytochrome c by hydrated electrons (9-11) and there is agreement that the rate of reduction is nearly diffusion-controlled in the pH range 7 to 11. These observations are very interesting in that the rapidity of the electron transfer process places constraints on the conformation changes that could occur during reduction, and because a variety of evidence, to be reviewed later, suggests that Met-80 is not coordinated with the iron of ferricytochrome c at alkaline pH. Met 80 has been assigned a crucial role in a mechanism of reduction presented by Dickerson et al. (2). Subsequent to the extremely rapid phase of reduction occurring during pulse radiolysis experiments, slow spectral changes ($k_{obs} < 10$ s$^{-1}$) are also observed under certain conditions (9, 10). However, there are contradictory reports on the character of those secondary changes.

The observations made in pulse radiolysis experiments suggested to us that (a) the "nonreducible" form of ferricytochrome c appearing at alkaline pH (12, 13) can be readily reduced by the hydrated electron, and perhaps other powerful reductants and (b) the reduction may be followed by a ligand change, accounting for the slow spectral changes seen. The experiments we describe show that dithionite indeed reduces the alkaline form of ferricytochrome c and that, at alkaline pH, both rapid oxidation and reduction of cytochrome c lead to transient species with spectra significantly different from the static spectra expected. We have interpreted these results by assuming, with Gupta and Koenig (14), that Met-80 is still the sixth ligand of the stable form of ferrocytochrome c at alkaline pH, but a different ligand, perhaps the c-aminogroup of Lys-79, is the stable ligand for the alkaline form of ferricytochrome c.
MATERIALS AND METHODS

The sources of the materials used were: horse heart ferricytochrome, type VI, Sigma; sodium dithionite, Hardman and Holdman, Manchester; sodium tetraborate, Baker and Adamson; and potassium ferricyanide, J. T. Baker Chemical Co. All solutions were prepared in glass-distilled water.

Studies on Ferricytochrome c—Ferricytochrome c was dissolved in 30 mM phosphate buffer, pH 7.4, then equilibrated with 0.6 mM NaCl on a Sephadex G-25 column. Aliquots of the ferricytochrome c solutions were diluted with equal volumes of buffer of the desired pH to give 1.0 mM ferricytochrome c in 25 mM sodium tetraborate, plus 0.30 mM NaCl. The stock buffers at various pH values were prepared by mixing 50 ml of 0.1 mM sodium tetraborate with the appropriate quantity of 0.4 mM NaOH or 0.4 mM HCl and then diluting to 100 ml. After at least 10 min of equilibration, the pH of each sample of ferricytochrome c was determined using a Radiometer pH meter standardized against buffers of pH 7.0 and 10.0, the absorption spectrum in the 695-nm region was recorded using a Zeiss DMR-21 recording spectrophotometer, and an aliquot was frozen in a quartz EPR tube by immersing the tube into liquid nitrogen (10 s).

Aliquots of the ferricytochrome solutions used in the 695-nm and EPR absorption studies were diluted 125-fold for stopped flow experiments between 400 and 500 nm, or 25-fold for experiments between 500 and 600 nm, using buffers of approximately the same pH of 25 mM sodium tetraborate plus 0.30 mM NaCl. The pH of each solution of diluted cytochrome c was measured, and then each solution was placed in a glass tonometer and purged of oxygen by equilibrating the gas and liquid phases six times with purified nitrogen (<0.1 ppm of oxygen). Each solution of cytochrome c was reacted with 50 mM sodium dithionite of approximately the same pH prepared by dissolving solid sodium dithionite in oxygen-free 25 mM sodium tetraborate. Sodium chloride was not added to the dithionite solution so that mixing with cytochrome c in the stopped flow experiments would occur at approximately constant ionic strength. The exact pH of each dithionite solution was determined after the stopped flow experiment and was found in all cases to be within 0.1 unit of the corresponding cytochrome c solution. The stopped flow system used has been described previously (15).

Studies on Ferrocytochrome c—Ferrocytochrome c was prepared by reducing ferricytochrome c in 30 mM phosphate, pH 7.4, with the minimum amount of dithionite. Dithionite was removed and the ferrocytochrome c was equilibrated with 0.6 mM NaCl by chromatography on Sephadex G-25. Aliquots of the effluent were mixed with the desired buffer and stored under nitrogen until used. The ferrocytochrome c in the effluent was at least 95% reduced.

Changes in the EPR spectrum of ferrocytochrome c for a function of pH and time, and subsequent to rapid oxidation of ferrocytochrome c by ferriyanide, were observed on samples prepared by using the apparatus and techniques for rapid mixing and freezing described previously (16, 17).

Determination of Cytochrome c Concentration—All static spectra of cytochrome c solutions were recorded at room temperature in a Zeiss DMR-21 recording spectrophotometer. Concentrations of cytochrome c were calculated from the absorption at 550 nm of dithionite-reduced samples using ε = 27.6 mM cm⁻¹ (18).

RESULTS

Reduction of Ferricytochrome c by Dithionite at Alkaline pH—The absorbance changes resulting from the rapid reduction of ferricytochrome c by 50 mM sodium dithionite were followed from 390 to 600 nm in the pH range 7.2 to 11.7. At pH 7.2, the changes were complete in 10 ms, giving the classical spectrum described by Margolish and Frohwirt (18).

Above pH 8, the changes in absorbance were biphasic and similar to those described (9) for the reaction of ferricytochrome c with the hydrated electron at alkaline pH. A high concentration of sodium dithionite (50 mM) resulted in the first phase being complete within 10 ms and this was followed by a second phase which was complete within a few seconds and whose rate was independent of the dithionite concentration. Thus the two phases could be easily distinguished, and the absorbance changes of the second phase at selected wave lengths were obtained by extrapolating the plots of absorbance versus time back to the time flow stopped. These absorbance changes are shown in Fig. 1 for experiments near pH 9.0 and 10.7, and are presented as a difference spectrum between the transient and stable forms of ferrocytochrome c. As can be seen (Fig. 1), the amplitude of the second phase is pH-dependent, and the maximum changes are observed near pH 10.7.

Fig. 2A shows the spectrum of the transient form of reduced cytochrome present at the end of the first phase, obtained by adding the changes in absorbance occurring in the second phase (15, pH 10.7) to the spectrum of the stable form of reduced cytochrome c at pH 10.7 (Fig. 2B). By referring to Fig. 2, the changes in the absorbance due to rapid reduction of ferricytochrome c by dithionite at pH 10.7 can be deduced. This is done by proceeding from Curve C to A (first phase) and then to B (second phase). For example, at 550 or 418 nm, an increase in absorbance during the first phase is followed by decrease in the second. At 515 and 428 nm, increases in absorbance are observed in both phases. The direction of the absorbance changes observed during the second phase reverses at 413 and 424 nm. Light absorption by sodium dithionite prevented observations below 390 nm. A comparison of the main spectral features of the transient and stable forms of ferrocytochrome c at pH 10.7 are tabulated in Table I.

The kinetic data of the second phase are pH-dependent below pH 9 and near 11, the changes follow first order kinetics with rate constants of 8.5 and 2 s⁻¹, respectively. At intermediate...
tinction coefficients were derived assuming $E^\infty = 129.1 \text{ mW cm}^{-1}$ used to construct Curve C from and oxidized cytochrome c recorded in a Zeiss DMR-21
cytochrome c by 50 mM dithionite, and has been constructed by spectrum of the ferricytochrome c upon completion
photometer. Curve A is the spectrum of the transient species of excess of ferricyanide at pH 10.5 was nearly complete within the
dead-time of the stopped flow experiment, and Curve C is the spectrum of the stable form of ferrocytochrome c at pH 7.5 in 0.25
pH values, semilogarithmic plots of $\Delta$ absorbance versus time are curved showing that the reaction slows more rapidly than expected for a first order reaction. At a given pH the rate constants for the slow phase are independent of the dithionite
transfer event between ferrocytochrome c and ferricyanide is not pH-dependent. However, at 695 nm and pH 10.5 we observed a rapid increase in absorbance, which follows the same kinetics as the 550-nm band, followed by a decrease in absorbance with a first order rate constant of 0.8 s$^{-1}$. Curves A and B in Fig. 3 show the stable reduced and oxidized spectra of cytochrome c
is characterized by $g$ values of 3.07, 2.23, and 1.21 (the latter absorption is not shown), in good agreement with the
spectrum observed by Salmeen and Palmer (20). Above pH 10.98 (Curve E) , but a signal at $g = 3.6$ is seen as a shoulder of the
changes at 550 nm, Brandt et al. (13) concluded that the electron transfer event between ferrocytochrome c and ferricyanide is not pH-dependent.
Rapid Oxidation of Ferrocytochrome c by Ferricyanide—At pH 7.3, the oxidation of 0.2 mM ferrocytochrome c by a 2-fold excess of ferricyanide was nearly complete within the dead time of the stopped flow experiment. When both reactants were diluted 10-fold most of the reaction could be observed, and it was readily apparent that the disappearance of the 550-nm band and the appearance of the 695-nm band followed the same kinetics and were complete within 30 ms. Based on the rate of absorbance
TABLE I
Comparison of spectral properties of transient and stable forms of ferrocytochrome c at pH 10.7

<table>
<thead>
<tr>
<th></th>
<th>Transient</th>
<th>Stable$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$</td>
<td>418 nm</td>
<td>416 nm</td>
</tr>
<tr>
<td>$\varepsilon_M$</td>
<td>$\geq 173,000$</td>
<td>129,000</td>
</tr>
<tr>
<td>$\sigma_1, \lambda_{\text{max}}$</td>
<td>550</td>
<td>550</td>
</tr>
<tr>
<td>$\varepsilon_M$</td>
<td>$\geq 31,000$</td>
<td>27,600</td>
</tr>
<tr>
<td>$\beta_1, \lambda_{\text{max}}$</td>
<td>520</td>
<td>520</td>
</tr>
<tr>
<td>$\varepsilon_M$</td>
<td>$\leq 14,400$</td>
<td>15,900</td>
</tr>
</tbody>
</table>

Difference spectra

<table>
<thead>
<tr>
<th></th>
<th>413, 424.5 nm</th>
</tr>
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<tbody>
<tr>
<td>Maxima</td>
<td>418.5, 555 nm</td>
</tr>
<tr>
<td>Minima</td>
<td>514, 408, 429 nm</td>
</tr>
<tr>
<td>Spectra coincident</td>
<td>445-495, 525-535, 555-600 nm</td>
</tr>
</tbody>
</table>

$^a$ According to the method of Margoliash and Frohwirt (18).
The pH dependence of the 695-nm absorption of ferricytochrome c (c), \( g = \frac{3.07}{2} \) signal amplitude (O), and amplitude of the 418-nm absorbance change in the second phase (X) subsequent to the rapid reduction of ferricytochrome c by dithionite. The data are expressed on a per cent basis assuming that the changes are complete between pH 7.5 and 11.0—for example, the 695-nm absorption is maximal at pH 7.5 and decreases to a minimum at 11.0. The smooth curve is a theoretical curve plotted for a transition with a pK of 9.3.

The results we have obtained at alkaline pH, together with those reported earlier by others, can be logically interpreted in terms of Scheme 1 where S represents the sulfur of Met-80 and the amine is speculated to be that of Lys-79.

That the sixth ligand of both ferri- and ferrocytochrome c at neutral pH (Species A and F) is Met-80 has been unambiguously established by x-ray crystallography (1, 2). However, optical spectroscopic titrations of ferricytochrome c (see Ref. 5 for review), particularly in the 695-nm region, have shown a heme-linked protonic ionization with a pK of about 9.3, and kinetic studies have shown that neither ascorbate (12, 21) nor ferrocyanide (13) can reduce the alkaline form of ferricytochrome produced by this transition. Further manifestations of this transition include changes in the thermodynamic properties of ferricytochrome c (22, 23, see also summary of earlier work in Ref. 5) occurring with a pK of about 9.3.

There are several compelling lines of evidence that the pK 9.3 transition in ferricytochrome c is a result of a ligand change at the sixth coordination site. First, either titrating ferricytochrome c to alkaline pH, or adding liganding agents of iron such as cyanide and azide at neutral pH cause a reduction in the 695-nm absorption (data summarized in Ref. 5). These effects together with the z-polarization of the 695-nm absorption (24), and the appearance of the band when the heme peptide from cytochrome c is formed in a complex with N-acetyl methionine (25) are consistent with the view that the 695-nm absorption is ligand-specific and originates in the S-Fe coordination. Second, high resolution NMR experiments have shown that the contact-shifted NMR resonance of the methyl group of Met-80 disappears with a pK of about 9 (Ref. 14), or as the azide ferri-cytochrome c complex is formed (26). Finally, the EPR g values of ferricytochrome c change with increasing pH, again with a pK of about 9, and the EPR spectrum of the major species at alkaline pH is consistent with methionine having been replaced by an —NH3 function (27). By the optical and EPR criteria, ferricytochrome c at alkaline pH is still low spin. In agreement with others (14, 19), we believe the major species of ferricyto-
chrome c formed in the pK 0.8 transition is coordinated with the ε-amino group of Lys-79 (Species C of Scheme 1). Unambiguous proof of this assignment may best be obtained by x-ray crystallographic studies of ferricytochrome c at alkaline pH.

In contrast to ferricytochrome c, the stable species of ferrocyanochrome c apparently has Met-80 as the sixth ligand in the pH range of 4 to 12. Thus neither the optical visible absorption spectrum (5, 28) nor the contact-shifted NMR resonance of the methyl group of Met-80 (14) is sensitive to pH values less than 12. Further, liganding agents such as cyanide and azide do not form stable complexes with ferrocyanochrome c and the protein is not auto-oxidizable (5).

According to the scheme presented, oxidation and reduction changes in cytochrome c at neutral pH would involve interconversion between Species A and F, and neither a ligand change nor a heme-linked ionization would occur. As the pH of the ferrocyanochrome c solution is increased, two major stable forms of ferrocyanochrome c are in equilibrium (Species A and C) with the apparent pK of approximately 9.3 describing the equilibrium mixture. Reduction of Species C to give the stable form of ferrocyanochrome c (Species F) can obviously proceed in two ways. The first involves conversion of C to A by the appropriate ligand change in ferrocyanochrome c, followed by the electron transfer event (A + F). This pathway is applicable for the reduction of ferrocyanochrome c by ascorbate and ferrocyanide at alkaline pH, and the biphasic kinetics observed above pH 8 (12, 13) is composed of the first phase in which the proportion of ferrocyanochrome c in Form A is rapidly reduced, followed by a slower second phase consisting of the sequence C → B → A → F in which conversion of C to A is rate-limiting. The form of this interpretation is basically that presented previously (12, 13, 29) differing only in that we assign specific ligands to both the neutral and alkaline species of ferrocyanochrome c.

The second pathway for conversion of C to F proceeds through the sequence C → D → E → F with the electron transfer event preceding the ligand change. The evidence presented in Figs. 1 and 2 suggests that electron transfer can occur at a rate much faster than the ligand change, leading to a species of cytochrome c (Species D) with spectral properties different than those expected (summarized in Table I). In comparison with ferrocyanochrome c, the Lys-79 species of ferrocyanochrome c is less stable so that replacement by Met-80 occurs even at pH values where lysine should be completely deprotonated. Model studies have shown that the sulfur of methionine has a much stronger affinity for ferrous heme as compared to the ferric form (30).

The observations on rapid reduction of ferrocyanochrome c at alkaline pH are very similar to those obtained with cyanoferricytochrome c in that the cyanide complex is reduced by sodium dithionite at neutral pH to give the cyanoferricytochrome c complex (31, 32). The latter complex has a distinctly different spectra from ferrocyanochrome c with absorption maxima shifted from those seen for ferrocyanochrome c. However, the reduced complex is not stable and dissociates slowly in a pseudo-first order process, giving the classical absorption spectrum of ferrocyanochrome c. This process obviously must involve the replacement of cyanide as the sixth ligand by Met-80.

The data for the rapid reduction of the C species of ferrocyanochrome c by dithionite is in partial agreement with the results of pulse radiolysis (9, 10) where ferrocyanochrome c is reduced by hydrated electrons. The major difference concerns observations near neutrality where Pecht and Faraggi (10) reported slow secondary spectral changes while we, in agreement with Land and Swallow (9), saw none. However, Pecht and Faraggi may not have had proper control of pH as they were working with unbuffered solutions (10). In further agreement with Land and Swallow (9) we find that the magnitude of the secondary changes increased in the pH range above 8, with an approximate pK of 9.3 (Fig. 5). Our rate constants for the secondary phase changes at pH 8.5 and near 11 are in good agreement with the results of the pulse radiolysis experiments (9, 10). We also observe secondary changes in absorbance between 500 to 600 nm which follow the same kinetics as the changes in the Soret region and which were not reported in the pulse radiolysis work. However, these changes are much smaller in magnitude in comparison with...
the changes in the Soret region, and may not be easily observed in pulse radiolysis due to the intense light absorption by hydrated electrons above 500 nm, and the low concentration of cytochrome c used. We conclude from these observations that the concept that the alkaline form of ferrocytochrome c is not reducible needs to be qualified as it is clearly reducible by the hydrated electron, dithionite, and reduced lumiflavin 3-acetate.\(^1\) The failure of ascorbate or ferrocyanide to reduce this form may be due to either kinetic or thermodynamic reasons.

That the rapid oxidation of ferrocytochrome c by ferriyanide at alkaline pH would result in the transient appearance of the 695-nm band absorption is implicit in the work by Czerlinski and Bracokova at 633 nm (29). We have confirmed that the pathway proceeds from F to C through A by showing that rapid oxidation of ferrocytochrome c at pH 10.5 results in the immediate formation of a species with the 695-nm absorbance and the EPR spectrum expected for Species A of Scheme 1. This species then changes to the expected alkaline form of ferricytochrome c (Species C) with a rate constant of 0.8 s\(^{-1}\). These results further support the suggestion by Streanathan and Taylor (33) that the 695-nm band is ligand-specific, and we propose that changes in the 695 absorption reported to be due to perturbation of conformation (see Ref. b) may be better rationalized in terms of alteration of the equilibrium between Forms A and C. This alteration could result either from changes in the pK of the Lys-79 or the relative affinity of Lys-79 and Met-80 for the iron site, with both types of changes expected to show sensitivity to changes in conformation.

By Scheme 1, the experimentally determined heme-linked pK of approximately 9.3 in ferrocytochrome represents an over-all pK for the interconversion of Species A and C. Gupta and Redfield have found (20) that while the neutral form of ferrocytochrome c will form a complex with azide, the alkaline form will not, giving an indication that the affinity of the deprotonated amine Lys-79 is greater than that of Met-80 for the sixth site. Assuming a pK > 10 for the amine of Lys-79, and given these relative affinities, it is apparent that the concentration of Species B will be low at all pH values and that methionine coordination at the sixth site of ferrocytochrome c at neutral pH is largely due to the e-amino group of lysine being protonated. For ferro- and alkaline forms of ferricytochrome c, Met-80 is inferred to be a much stronger ligand than the deprotonated amine of Lys-79 since conversion of E to D does not occur even at pH 11 to 12. However, carboxymethylation of Met-80 should greatly reduce the affinity of the sulfur for the iron, and recent work by Brunori et al. (34) has shown that the ferro form of diacarmethylcytochrome c changes from high spin to low spin with a pK of 7.16. Brunori et al. interpreted this result as being due to a change from penta- to tetra-coordinated iron to coordination at the sixth site by the amine of Lys-79. Koller et al. have recently suggested (35) that the ferric form of diacarmethylcytochrome c is also coordinated with Lys-79 at neutral pH. The tendency of the Lys-79 amine to act as the sixth ligand at neutral pH in both the oxidized and reduced forms of diacarmethylcytochrome c can be attributed to a lowering of the pKa of Lys-79 caused by the charged group at the sulfur of Met-80, and the removal of the sulfur of Met-80 as a good coordinating group.

We know of no firm experimental evidence that species of cytochrome c other than A and E have a physiological role, although a role in oxidative phosphorylation for Met-50 (36, 37) or Met-80 and Lys-79 (38) has been proposed. Further, the kinetic data (12, 13, 29) of the transitions between the neutral and alkaline forms of ferricytochrome c (\(< 1 \text{ s}^{-1}\)) are much slower than the turnover rate of cytochrome c in respiring mitochondria. However, the findings that hydrated electrons, dithionite, and reduced lumiflavin can very rapidly reduce ferricytochrome c when Lys-79 is the probable sixth ligand does not support Dickerson’s proposal that methionine is a part of the electron-conducting pathway to the iron (2). Of course, the study of the mechanism of reduction of ferrocytochrome c by nonphysiological reductants may not be pertinent to the physiological mechanism, since the electrons may enter ferrocytochrome c at site (or sites) remote from the physiological site. However, a recent study by Yu et al. (39) of the reaction between ferrocytochrome c and ferrocyanide at pH 10.5 showed that the rate of electron transfer was still rapid (20% of the rate at neutrality), in spite of the drastic changes that must have occurred at the surfaces of the protein, and despite the fact that most of the ferricytochrome c should be in the alkaline form. In analogy with the ascorbate reduction of ferrocytochrome c at alkaline pH (12), it would be of interest to know whether the total reaction involves a fast phase in which the portion of cytochrome c possessing Met-80 as the sixth ligand reacts, followed by a slower phase of reduction occurring as Lys-79 is replaced by Met-80 as the sixth ligand.

Although we feel that the properties of cytochrome c in the pH range 7 to 11 are rationalized by Scheme 1, further complexities obviously exist. Thus Blumberg et al. (19) report further EPR spectral changes at high pH which they interpret as being due to a ligand replacement at the histidine coordination site, and the NMR spectrum of ferricytochrome c also shows a second pK near 11 (14). However, we have found that the reduction of ferrocytochrome c by dithionite is still rapid at pH 12, leading to slower secondary changes similar to the ones described in this paper.

REFERENCES

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\(^1\) D. O. Lambeth and G. Palmer, unpublished observations.

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