Effects of O₂ and CO₂ Concentration on the Steady-State Fluorescence Yield of Single Guard Cell Pairs in Intact Leaf Discs of *Tradescantia albiflora*¹

Evidence for Rubisco-Mediated CO₂ Fixation and Photorespiration in Guard Cells

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**ABSTRACT**

A procedure for following changes in the steady-state yield of chlorophyll a fluorescence (F₀) from single guard cell pairs in variegated leaves of *Tradescantia albiflora* is described. As an indicator of photosynthetic electron transport, F₀ is a very sensitive indirect measure of the balance of adenosine 5'-triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), producing reactions with the sink reactions that utilize those light-generated products. We found that F₀, under constant light is sensitive to manipulation of ambient CO₂ concentrations, as would be expected if either phosphoenolpyruvate carboxylase or ribulose-1, 5 bisphosphate carboxylase/oxygenase (Rubisco)-dependent CO₂ fixation is the sink for photosynthetic ATP and NADPH in guard cells. However, we also found that changing O₂ concentration had a strong effect on fluorescence yield, and that O₂ sensitivity was only evident when the concentration of CO₂ was low. This finding provides evidence that both O₂ and CO₂ can serve as sinks for ATP and NADPH produced by photosynthetic electron transport in guard cell chloroplasts. Identical responses were observed with mesophyll cell chloroplasts in intact leaves. This finding is difficult to reconcile with the view that guard cell chloroplasts have fundamentally different pathways of photosynthetic metabolism from other chloroplasts in *C₃* plants. Indeed, Rubisco has been detected at low levels in guard cell chloroplasts, and our studies indicate that it is active in the pathways for photosynthetic carbon reduction and photorespiration in guard cells.

Turgor-dependent movements of the guard cells of stomata control the diffusive conductance of leaf surfaces to gas exchange and play a key role in regulating the water and carbon balance of plants. Both environmental and physiological factors influence stomatal aperture, and although some sensory and response systems are known to reside in the guard cells themselves (e.g. the blue light response), others are not well understood. One of the most perplexing aspects of the overall control system is that the stomatal conductance of intact leaves appears to respond in parallel with changes in the photosynthetic capacity of the cells in adjacent mesophyll (29). This observation has led some workers to speculate that there may be a mesophyll message directing guard cell movements, and others to suggest that chloroplasts in the guard cells themselves might be directing the stomatal response to certain environmental and physiological cues (2).

Chloroplasts found in guard cells are smaller and have fewer grana than their mesophyll counterparts (16). Most of the information available concerning the properties of guard cell chloroplasts has been inferred from studies of Chl a fluorescence from intact systems, epidermal peels, or protoplasts, and from studies of enzyme activities in isolated guard cells, guard cell protoplasts, or epidermal peels (for a review, see ref. 13).

Many guard cell fluorescence studies have analyzed differences and similarities between the shapes of Chl a fluorescence induction curves in mesophyll and guard cells and their functional implications (9–11, 21, 30). These studies have established the presence of active PSI and PSII linked to linear electron transport from water to NADPH (6, 14, 21, 30), along with photophosphorylation (cyclic and noncyclic) in guard cell chloroplasts (23) as in mesophyll chloroplasts. Induction of Chl a fluorescence from intact leaves has been interpreted as reflecting the activation of reactions that serve as sinks for NADPH and ATP (7, 17), and, using this interpretation, it has been proposed that differences noted between the shapes of guard and mesophyll cell fluorescence induction curves indicate a difference either in the identity of the sink reactions or simply in the conditions under which similar sink reactions are operating.

Recent studies suggest that the latter interpretation may be incorrect. Immunocytochemical and immunolabeling evidence for the presence of Rubisco in guard cells (30), medium alkalization and oxygen evolution by guard cell protoplasts under white (4, 20, 24) and red light (23), and incorporation of ¹⁴CO₂ into PGA², RuBP, fructose, and sedoheptulose in guard

¹ This is Carnegie Institution of Washington, Department of Plant Biology publication number 1119.

² Abbreviations: PGA, 3-phosphoglycerate; F₀, fluorescence yield of chlorophyll a steady-state illumination; qₑ, quenching coefficient for "energy-dependent" quenching of chlorophyll a fluorescence; qₑ₀,
cells under red light (5) have indicated that photosynthetic CO₂ fixation may well occur in guard cell chloroplasts, although Outlaw (13) voices concern over the contributions of contaminating mesophyll cells and/or fragments to the results of these experiments. Other reactions, for instance synthesis of malic acid via PEPCase in the cytoplasm, could serve as the major sink for the products of electron transport from guard cell chloroplasts, whereas Rubisco, if present, may represent only a minor sink. For example, based on studies detailing very different concentration ratios of certain Calvin cycle enzymes in guard cells, Shimazaki et al. (22) suggest that guard cell chloroplasts could instead function to export ATP and reducing equivalents to the cytosol via a PGA/dihydroxyacetone phosphate shuttle. Cytoplasmic synthesis of malic acid from stored carbohydrates via PEPCase could then serve as a sink for the products of guard cell electron transport.

If pathways of carbon metabolism other than the Calvin cycle are dominant in guard cells, guard cell Chl fluorescence might be expected to reflect very different responses of electron transport to environmental conditions than that of mesophyll cell chloroplasts. Studies of the yield of Chl fluorescence with pulse-modulated fluorometers (17) have clearly established that restriction of the activity of ATP- and NADPH-consuming reactions in intact green leaves, for example by low CO₂ and O₂ concentrations, can result in down regulation of the rate of electron transport and changes in the quenching of Chl a fluorescence (3, 19, 28). We reasoned that it should be possible to obtain some additional insight into the nature of the sink for products of photosynthetic electron transport in guard cell chloroplasts by examining the response of fluorescence quenching to changes in gas concentration.

In this paper, we report a technique for following changes in the quenching of Chl a fluorescence from guard cell pairs in leaf discs under steady illumination. Presumably, if PEPCase and associated reactions are the main sink for ATP and NADPH generated in guard cell chloroplasts, decreasing CO₂ should restrict PEPCase activity, reducing ATP and NADPH utilization and energizing the thylakoids. Increased nonphotochemical quenching of steady-state fluorescence would then be expected. If, however, Rubisco and the Calvin cycle were the main sink for products of photosynthetic electron transport, changes in either CO₂ or O₂ concentration should affect the regulation of electron transport because both CO₂ and O₂ are substrates for Rubisco. This O₂ sensitivity of steady-state fluorescence should be most visible at low CO₂ concentrations and would be a clear signature for Rubisco’s activity as a major sink for ATP and NADPH in guard cell chloroplasts.

By examining stomata above white mesophyll in variegated leaves of Tradescantia alibiflora, we avoid both light contamination from green mesophyll chloroplasts and any artifacts caused by injury during peeling or protoplast isolation. It has been found that guard cell fluorescence patterns from epidermal peels or protoplasts are sensitive to various ions and ABA in their surrounding media (10, 12); by using intact leaf discs, we avoid subjecting stomata to ionic concentrations unlike those in the intact leaf. Most important, we can probe the nature of the sink for ATP and NADPH by observing changes in steady-state Chl a fluorescence in response to altered ambient CO₂ and O₂ concentrations in the leaf chamber.

**MATERIALS AND METHODS**

Tradescantia alibiflora plants were grown in greenhouses at the Carnegie Institution of Washington (on the Stanford campus) under shade netting that removed approximately two-thirds of the incident light. Plants were watered as necessary and fertilized weekly with Plantex 20–20–20 all purpose fertilizer (Plantex Inc., Brampton, Ontario, Canada). The youngest fully expanded leaves from growing shoots were used in experiments; for fluorescence determinations from guard cells, only leaves with white sectors devoid of both obvious pink anthocyanin accumulation and a yellow/green tinge were used. Before each guard cell experiment, 1-cm leaf discs were cut under water and then monitored for contaminating fluorescence from green mesophyll cells using blue epi-illumination from a mercury vapor lamp on the microscope.

Chl fluorescence measurements were obtained by two methods in green leaf areas; one method utilized a PAM fluorometer (PAM-101, H. Walz, D-8521 Effeltrich, Germany) and the other relied on the fluorescence signal captured through a microscope by a Hamamatsu R-928 photomultiplier (Hamamatsu Corporation, Middlesex, NJ) via a lock-in amplifier. Guard cell Chl fluorescence was measurable only under the microscope. Both methods selectively sense relative changes in the yield of Chl a fluorescence excited by a low-intensity modulated light directed to the sample and are insensitive to fluorescence excited by other light present in the environment.

Leaf chambers in each of the two experimental setups were flushed continuously with gas mixed from CO₂-free air, N₂, and 5% CO₂ in air with the use of mass-flow controllers (Tylan, Torrence, CA). N₂ and CO₂-free air were first humidified and passed through a Peltier-controlled condenser, then 5% CO₂ was added to produce the desired final CO₂ concentration. The oxygen concentration of the air was rapidly changed by simultaneously changing the CO₂-free air and N₂ mass flow controller set points, so that total flow to the humidifier remained constant while O₂ changed from 21 to 2%. CO₂ concentrations in the chambers were measured with a Li-Cor CO₂/H₂O analyzer (model LI-6262, Li-Cor, Lincoln, NE).

For measuring fluorescence with the PAM fluorometer, intact, green, attached leaves were placed in a clear-topped photosynthesis cuvette. The polyfurfurated fiber optic from the PAM fluorometer was positioned over the clear chamber top and used to monitor fluorescence levels from mesophyll chloroplasts in the intact leaf, as described by Schreiber et al. (17). The maximum fluorescence yield (with most PSII traps reduced; see ref. 8) was determined from dark-adapted leaves or from leaves under steady-state illumination by application.
of a pulse of 3600 μmol m⁻² s⁻¹ blue light from a xenon-arc lamp. The minimum fluorescence yield was measured by momentarily turning off actinic light and pulsing the leaf with far red light to oxidize most PSII. Leaves were illuminated with 50 μmol m⁻² s⁻¹ blue actinic light until steady-state fluorescence levels were reached and stomata were open. As gas concentrations were changed, F₅ was monitored, and the quenching coefficients qₑ and qₑP were determined as described by Bilger and Schreiber (1).

For microscopic studies, leaf discs were floated on distilled water in an enclosed, clear-topped chamber under a microscope (Olympus BH2-RFL) with a long-distance inverted microscope objective (Leitz NPL Fluor 30x). Modulated (300 Hz) broad-band blue (360–590 nm, Corning 9782 filter) light of 50 μmol m⁻² s⁻¹ (at the microscope stage) was provided from a highly regulated power supply and a quartz-halogen bulb (EKZ, GE, Inc., Cleveland, OH) via a fiber optic that fitted in place of the microscope’s illuminator lamp. The light was directed to the stage through the normal illuminator optics. Light incident on the sample was measured using a Ga-As photodiode (G1118, Hamamatsu Corp.) calibrated against a Li-Cor quantum probe (LI 185A, Li-Cor, Lincoln, NE). Fluorescence emitted from chloroplasts passed through the objective lens and through the tube of a mask (masking portion of Nanospec/10s UV-VIS-NIR microspectrophotometer, Nanometrics, Inc., Sunnyvale, CA).

The terminal set of mirrors in the mask consisted of four mirror pieces that could be adjusted to select light from a portion of the image to be directed to the photomultiplier. The remainder of the light was reflected via two sets of mirrors to a CCD camera (4810 series, COHU, Inc., San Diego, CA), which permitted viewing of the field and adjustment of the mirrors to select the area for Chl fluorescence measurement. Glass filters (Schott R-8) blocked the excitation light and passed Chl fluorescence to the photomultiplier and CCD element. Output from the CCD camera was displayed on a television monitor (Sony model PVM-1342Q), and images were captured and stored using a Data Translation DT2853 frame grabber (Data Translation, Inc., Marlboro, MA) in a NEC PowerMate Portable computer (NEC Information Systems, Boxborough, MA). The output from the photomultiplier was converted to voltage, fed through a lock-in amplifier (Ithaco Dynatrac Lock-In Analyzer, Model 393, Ithaco, Inc., Ithaca, NY), and the modulated signal was recorded on chart paper. The microfluorometer was suitable for measurement of F₅, but we were unable to obtain reliable measurement of qₑP and qₑ by the saturating pulse method of Schreiber et al. (17); guard cell chloroplasts were easily damaged by the pulses. Nevertheless, when the gas composition was changed, we observed significant time-dependent changes in the steady-state level of fluorescence (Fₛ), which reached different stable Fₛ levels at different gas concentrations.

RESULTS

We used parallel experiments with intact leaves in the Walz system to characterize the bases of these changes in Fₛ in response to changing ambient CO₂ or O₂. An attached leaf was clamped in a normal gas exchange chamber and monitored by the Walz fluorometer, and the microfluorometer was focused on green mesophyll cells of a leaf disc from the same plant. Gas of known concentration was fed simultaneously into the two chambers. The leaf in each chamber was illuminated with broad-band blue light (50 μmol m⁻² s⁻¹) in low CO₂ for approximately 0.5 h to open stomata. This step was necessary to ensure that gas concentrations inside the leaves changed quickly when the chambers were flushed with new gas mixes. A steady-state variable fluorescence level was reached during this 0.5 h, and was recorded for each system. For these comparisons, the amplification of the two fluorometers were adjusted to give identical output levels of Fₛ at the start of an experiment, and the Fₛ signals were displayed on a two-pen recorder (see Fig. 1).

Figure 1 shows two representative traces of Fₛ when gas concentrations were changed. The top trace was obtained from the Walz fluorometer and shows not only changes in variable fluorescence, but also spikes and troughs associated with measurement of maximum and minimum fluorescence yields with pulses of blue and far red light. Calculated values of qₑ and qₑP are shown along each spike. The lower trace was obtained by the photomultiplier from the green leaf disk under the microscope. Gas concentration changes are indicated at the arrows. It is clear that the changes in Fₛ associated with changes in CO₂ and O₂ concentration are virtually identical in the two traces. Sivak et al. (25) observed similar changes in Fₛ of green spinach leaves with changes in CO₂ concentration, and showed that these were directly related to alterations in scattering of 535-nm weak green light, an indicator of the energization state of the thylakoids. This energization is reflected in the energy-dependent component of qₑ (28).

As discussed by Schreiber et al. (17), Fₛ is primarily determined by the levels of photochemical and nonphotochemical quenching. Recently, several workers have established that changes in photochemical and nonphotochemical quenching regulate the quantum efficiency of PSII during steady-state photosynthesis (3, 19, 28). In the present work, the absorbed quantum flux is held constant, and we are interested in using Fₛ as monitored by the microfluorometer, to infer changes in fluorescence quenching processes (and, therefore, in the rate of electron transport) caused by changes in O₂ and CO₂ concentration.

In Figure 1, each time O₂ was decreased from 21 to 2% (or CO₂ was decreased), the Fₛ increased at first. This was probably associated with a decrease in qₑ. Over several seconds, the Fₛ level decreased. In the Walz trace taken from mesophyll, this reflected an increase in qₑP as qₑ returned to its previous level (about 0.9). These complementary changes in qₑP and qₑ have been observed in previous studies of transient responses to changes in CO₂ and O₂ concentration as the rate of oxygenation or carboxylation was restricted (19), and these are attributed to regulatory responses that follow a decrease in the capacity of a sink reaction to accept the products of electron transport. When the CO₂ was increased or O₂ concentration was returned to 21%, there was a rapid decrease in Fₛ, followed by a slow adjustment that returned Fₛ to near its original level with a complementary decrease in qₑP. This pattern of fluorescence change is indicative of an increase in the capacity of sinks to utilize products of electron transport.
Walz

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Figure 1. Traces taken simultaneously from green leaves of T. albiflora in the Walz fluorometer (upper) and the microfluorometer (lower). The gas stream was split and sent to each chamber; gas concentrations were changed at the arrows. The $F_5$ traces are displaced for comparison. The time courses of the responses of $F_5$ to changes in gas composition are identical; spikes and troughs on the upper trace reflect pulses of blue light (3600 μmol m$^{-2}$ s$^{-1}$) or far red light used in calculation of $q_{NP}$ and $q_P$.

Figure 2 shows the comparison of $F_5$ measured by the microfluorometer to $q_P$ and $q_{NP}$ (Walz trace, Fig. 1), which was taken when $F_5$ had reached a steady value following each change in gas concentration. It shows that changes in $F_5$ mainly reflect changes in $q_{NP}$; the transient changes in $F_5$ following changes in $CO_2$ or $O_2$ (Fig. 1) may reflect changes in $q_P$, but $q_P$ at steady state is relatively constant and drops only slightly under conditions of simultaneous limitation by low $O_2$ and $CO_2$ concentration. These results indicate that the observed changes in $F_5$ primarily reflect changes in the level of nonphotochemical quenching.

Figure 2. Graph of $q_P$ and $q_{NP}$ versus $F_5$ calculated from the data shown in Figure 1. Nonphotochemical quenching was highly correlated with changes in $F_5$ caused by altered $CO_2$ or $O_2$ concentration, whereas photochemical quenching changed only slightly with changes in $F_5$.

Figure 3. Epidermis over the white part of a T. albiflora leaf as viewed by the CCD camera in bright field (left) or Chl fluorescence illumination (right). During an experiment, light from the guard cells’ chloroplasts would be directed to the photomultiplier by moving the stage to bring the stomate into the central black square seen in bright field.
To follow fluorescence changes in guard cells, albino portions of leaves were floated in the microscope chamber and then treated with blue light and low CO₂ to open the stomata. An open stomate was positioned so that fluorescence from its guard cell chloroplasts (Fig. 3, right) would pass to the photomultiplier through the hole in the terminal reflecting mirrors of the mask. This positioning was accomplished by moving the stomate into the black area in the middle of the image viewed by the CCD camera (Fig. 3, left). The relative fluorescence yield was then monitored as gas concentrations were changed.

Figure 4 shows a fluorescence trace obtained from a guard cell pair and one obtained from an area of leaf just outside the stomatal complex. Gas concentrations were manipulated as marked. Fluorescent light levels from guard cells were extremely low, but although noise in these traces is much greater than that from green mesophyll traces, it is clear that stomatal variable fluorescence at 35 ppm CO₂ decreased reversibly when the concentration of O₂ was decreased from 21 to 2%. F₀, also increased when CO₂ was increased from 35 to 350 ppm. By analogy to the whole leaf experiments (Fig. 1), these results indicate that decreasing CO₂ or O₂ results in an increase in qNp. As a control, Figure 4 also shows that no fluorescence changes were observed when the microfluorimeter was focused on an albino section of the leaf area adjacent to the stomatal complex, and that gas concentrations were changed. This eliminates the possibility that the above fluorescence responses were due to stray light scattered from adjacent green areas of the leaf.

DISCUSSION

Our studies indicate that carboxylation and oxygenation of RuBP can act as strong sinks for products of photosynthetic reactions, and that the changes in fluorescence might reflect alterations in use of ATP and reducing equivalents generated in the chloroplast by electron transport. However, it should be possible to distinguish PEPCase from Rubisco because PEPCase cannot use O₂ as an alternative substrate (as Rubisco can). If PEPCase and associated reactions are the major sink for products of electron transport, O₂ should not be able to substitute for CO₂ in altering qNp. If, however, Rubisco and associated reactions are a major sink, changes in both O₂ and CO₂ should have an effect on the strong nonphotochemical quenching developed at low CO₂ concentrations (see Fig. 4).

Also, it should be possible to titrate out the effect of decreased O₂ with increasing CO₂ concentrations. An example of such a titration in both green mesophyll and guard cells is shown in Figure 5. At 35 ppm CO₂, a change from 21 to 2% O₂ induces a large change in guard cell and mesophyll F₀. However, when the CO₂ concentration is increased to 130 ppm, the same drop in O₂ from 21 to 2% has a much smaller effect on F₀. At the intermediate 85 ppm CO₂, an intermediate response to changing O₂ is evident. In experiments of this kind, a return to low CO₂ concentration was always accompanied by restoration of O₂ sensitivity (data not shown).

Under the conditions of our experiments, the total extent of the changes in F₀ and the time dependence of these changes in response to changes in O₂ and CO₂ are similar in the two systems.
electron transport in guard cells, as they do in mesophyll cells. Identical patterns of change in the F$_i$ level in response to changes in CO$_2$ or O$_2$ were seen with intact leaves using the Walz fluorometer, with mesophyll cells and guard cells using the microfluorometer (Figs. 1 and 5). In green mesophyll, q$_{NP}$ increased and q$_{P}$ changed only slightly (Fig. 2) when CO$_2$ or O$_2$ concentrations were decreased. Based on previous studies of fluorescence quenching and gas exchange (19, 28), this indicates a decrease in the rate of electron transport as the capacity for the carboxylation or oxygenation of RuBP is restricted. Although we were not able to measure q$_{NP}$ and q$_{P}$ with the microfluorometer, we are confident, based on the similar nature of the changes in F$_i$ observed with the Walz fluorometer and the microfluorometer (Fig. 1), that the fluorescence changes observed with guard cell chloroplasts (Figs. 4 and 5) indicate that CO$_2$ and O$_2$ can also act as sinks for the products of electron transport in guard cells.

Under the conditions used in our experiments, guard cell and mesophyll fluorescence quenching became relatively much less sensitive to changes in O$_2$ at CO$_2$ concentrations greater than 130 ppm (Fig. 5). This interaction of ambient O$_2$ and CO$_2$ concentrations on Chl fluorescence would be expected if Rubisco and associated reactions are major sinks for the products of electron transport. If O$_2$ were influencing fluorescence primarily through the Mehler reaction, it would be expected that a decrease in oxygen would cause a decrease in electron transport, and, thus, a decrease in thylakoid energization and q$_{NP}$, an increase in the Mehler reaction with increasing O$_2$ would enhance the buildup of ATP and the pH gradient, driving an increase in q$_{P}$. This pattern of sensitivity to changes in O$_2$ concentration is the opposite of that observed in our experiments. We conclude that the responses of guard cell fluorescence quenching to O$_2$ and CO$_2$ are consistent with the hypothesis that the pathways for photosynthetic carbon reduction and photorespiration are active in guard cells.

The significance of photosynthetic CO$_2$ fixation by guard cell chloroplasts has been questioned (14) because the rate of photosynthesis measured previously is far too low to be the primary contributor of hexoses for production of malic acid during stomatal opening (15). The present results do not alter this conclusion. Nevertheless, a possible regulatory role for guard cell chloroplasts in stomatal movement should be considered. In fact, the red-light opening response of stomata seems to be dependent on a Chl-mediated transduction chain that can be inhibited by DCMU but cannot be subsequently restored by the addition of ATP (18). Serrano et al. (18) suggest that a photosynthetic product other than ATP may be necessary for mediation of the red-light response. Our experiments indicate that Rubisco is active in guard cells and that a metabolite of the photosynthetic carbon reduction pathway or photorespiration in guard cells may well be able to play a role in regulation of guard cell turgor and stomatal aperture. We have no information on how this pathway is integrated into the complex carbon metabolism of guard cells.

In considering these results, it is important to note that we followed guard cell fluorescence in white sectors of leaf discs of T. albiflora. Several previous studies that examine the possible role of guard cell photosynthesis in affecting stomatal movements (see ref. 27) have made use of the obser-
vation that the stomata in white sectors of variegated leaves contain chloroplasts. The chimeral structure of the T. albitiflora leaf is of the green-white-green type; the epidermal tissues over green and over white areas are derived from a single "green" tunica layer in the leaf primordium (26). When viewed through the microscope, guard cell pairs over white areas are seen to be similar in size, chloroplast content, and spacing to those over green areas of the leaf. All of the stomata appear to respond to light, CO₂, and humidity. We believe that the fluorescence responses observed in guard cells over white mesophyll are representative of those of guard cells throughout the leaf. It was not possible to obtain measurements of guard cell fluorescence from green areas of these leaves because of the overwhelming interference of mesophyll fluorescence.

Although Mawson and Zeiger (10) found differences in fluorescence induction curves under blue and green light, we found no wavelength specificity in our steady-state experiments; F, responded similarly to alterations in CO₂ and O₂ concentrations under both green and blue illumination (data not shown). These experiments could only be conducted in leaves with open stomata; hence, we cannot eliminate the possibility that fluorescence from chloroplasts in closed or opening guard cells might behave differently.

ACKNOWLEDGMENTS

We thank Dr. Steven Boxer for suggesting the experimental approach and for the loan of the Nanopect microspectrophotometer mask; Dr. Sharon Long for loan of the Leitz inverted microscope objective; and Dr. Winslow Briggs for help in editing the manuscript. Support for this project was provided by a (National Defense Science and Engineering Graduate) fellowship to Z.G.C. and by the Carnegie Institution of Washington.

LITERATURE CITED