Ozone Alteration of Membrane Permeability in Chlorella

I. PERMEABILITY OF POTASSIUM ION AS MEASURED BY ⁸⁶RUBIDIUM TRACER

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ROBERT L. HEATH AND PAULA E. FREDERICK
Department of Botany and Plant Sciences, University of California, Riverside, California 92521

ABSTRACT

The addition of ozone to a suspension of Chlorella sorokiniana causes a rapid loss of K⁺, as measured by efflux of ⁸⁶Rb from prelabeled cells. The efflux of the tracer is stimulated some 15 to 20 times over that of the control. For about 100 micromoles per liter ozone, about 25 minutes (6 × 10⁻⁴ moles O₃ delivered per minute) of exposure are required for a 50% depletion of the intracellular K⁺. The stimulation of K⁺ efflux is nearly linearly dependent upon the amount of ozone delivered into the solution. Following short pulses of ozone (lasting 1 to 5 minutes), efflux rates return to the control level but only after about 15 minutes.

While influx of K⁺ is ultimately inhibited by ozone, at low concentrations or for short exposure times the tracer influx is stimulated 100 to 200%. Ozone stimulation of an active pump mechanism is unlikely in view of a concomitant decrease in respiration. Thus, this influx may represent movement of K⁺ along its electrochemical gradient. Assuming that influx and efflux are in steady-state according to the Goldman equation, it was calculated that the membrane potential for K⁺ of −80 to −90 millivolts in control cells drops to −40 millivolts with ozone exposure and is accompanied by a calculated increased permeability to K⁺ of 2- to 3-fold.

It is nearly axiomatic to state that ozone affects membrane permeability (15); however, it is not yet understood how this may occur although it has been suggested that the membrane is made more permeable by ozone's oxidative action on either sulfhydryls of membrane proteins, or on the membrane lipid components (11, 15). Previously, ozone treatment of plant material has been shown to induce changes in the transport kinetics of many substances, including water (9), sugars (21, 22), and ions (6, 10). Accordingly, the loss of K⁺ has been shown to be one of the first indications of membrane permeability changes under a variety of stresses (5, 17, 18, 20).

The net loss of K⁺ from ozone-treated Chlorella cells suspended in a Tris buffer has been measured using a cation-specific electrode (6). The electrode method measured only the net loss of K⁺ at a low external K⁺ concentration. Thus, the effect of ozone upon the K⁺ influx and efflux could not be studied separately under physiological conditions. The use of Tris as a buffer induces K⁺ loss itself, thus making the interpretation of K⁺ loss difficult (7). This paper examines the effects of ozone on the influx and efflux of K⁺ in Chlorella sorokiniana by using ⁸⁶Rb as a tracer for K⁺ (2, 3). A comparison of changes in influx and efflux transport kinetics produced by various ozone dosages and a close examination of initial influx kinetics suggest that the mode of oxidant injury may be due not only to increased membrane permeability but also to a collapse of the membrane potential.

MATERIALS AND METHODS

Cultures of C. sorokiniana, strain 07-11-05, were grown as described previously (6). Cells were harvested, washed twice, and resuspended in a phosphate-buffered medium containing 4.2 mM KH₂PO₄, 0.82 mM K₂HPO₄, 1.0 mM CaCl₂, and 0.23 mM MgSO₄. 7H₂O at pH 6.0. This medium provided not only enough K⁺ for transport saturation (2, 3), but also the proper Ca²⁺/Mg²⁺ and proper pH buffer to maintain normal cell integrity. In addition, it contained no organic compounds or heavy metals which may react with ozone in solution (1). Cell concentrations were determined by Coulter Counter (model 2F, Coulter Electronics, Hialeah, Fla.). For influx measurements, 10⁷ cells were added to a total volume of 5 ml of medium in a stirred and temperature-regulated cuvette (38 C). Label (⁸⁶RbCl, New England Nuclear) was added prior to cell addition and subsequent gassing. Either oxygen or ozone in oxygen, produced and measured as previously described (6, 11), was introduced into the aspirated solution through a 50-μl micro-pipette. At specific times, a 200-μl aliquot of the cell suspension was withdrawn, filtered on a Millipore filter (0.45 μm), and washed with 5 ml of unlabeled medium. The cell sample on the filter was removed to a drying stand, bleached with 1 drop of Purex, and placed in a toluene-Triton cocktail for scintillation counting (14). uptake rates were calculated from the amount of ⁸⁶Rb present in the cells at various time intervals, based upon the external specific radioactivity of Rb⁺ and K⁺.

In experiments during which the initial uptake kinetics were examined, 1-ml aliquots from a batch of cells (10-25 ml) treated with O₂ or O₃ in the absence of label were taken at various times, and transferred to smaller, stirred and temperature-controlled vessels to which label was added. Smaller aliquots (200 μl) were then sampled from these vessels.

For efflux experiments, logarithmically growing cells were pre-labeled overnight (15-18 h) with ⁸⁶Rb in autotrophic growth medium (approximately 1 μCi/ml culture medium; 0.050 mCi/ mmol K⁺). Label was added to 70 ml medium initially containing 5 × 10⁴ to 10⁵ cells/ml. Cells absorbed less than 4% of the available K⁺ during the logarithmic growth phase and the t₅₀ for accumulation was about 2.5 h. For seven experiments, the average internal concentration of K⁺ was calculated to be 7.7 ± 1.2 (SD) × 10⁻¹⁵ eq/cell (390 μm using the cell's volume from ref. 14), by the internal label and external specific radioactivity. After preloading, cultures were harvested and washed as above. The procedure for sampling was the same as in the uptake experiments, except that both the supernatant and filtered cells were collected for counting. Efflux was measured as the rate of appearance of label in the external medium.

Cells were stored at room temperature in room light in phosphate buffer between experiments. The amounts of K⁺ moved into or out of the cells are reported as equivalents (or normality) since it is not yet known how or what other ions move with K⁺, or if electrical neutrality is always maintained.

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RESULTS

As we found previously using an electrode in Tris buffer (6), ozone greatly increases the rate of K+ loss from Chlorella cells (Fig. 1). The rate depends upon the time period between harvesting the cells and exposure to ozone: the longer this period, the faster the rate of K+ loss upon exposure to ozone. For each batch of cells this rate is constant and the rate for various batches varies from 1.05 x 10^{-16} to 1.67 x 10^{-15} eq/cell-min, or 10 to 15 times faster than control rates. The controls, treated with O_2 under the same conditions, also lose K+, but at a very slow rate. Less than 0.14 ± 0.01% of cell's total labeled K is lost per minute at an intracellular K+ concentration of 6.6 x 10^{-15} eq/cell. Although we have not specifically investigated the variability of efflux with time between harvesting and exposure, there is a greater variability in both respiration and in metabolic use of externally supplied glucose as this time increases. This is not surprising since the cells are held in a minimal nutrient medium at a suboptimal temperature with no energy source. In most experiments cells were exposed to the gasses as soon as possible after harvesting.

Under higher levels of ozone (200-250 μl/l, 5.6 x 10^{-7} mol ozone/min delivered into solution), the efflux rate is greatly increased, so that in autotrophic medium nearly all of the internal Rb is lost within 20 min (data not shown). In this case, the internal and external specific radioactivities become equal.

K+ influx also increases during ozone exposure (Fig. 2). At 100 μl/l, after a 3-min lag, the influx rate nearly doubles compared to that of the control. At the higher ozone level (150 μl/l), the linear rate likewise doubles but the lag time is not evident; later the influx rate becomes zero (and ultimately negative) after about 10 min. Although the amount of stimulation of K+ influx by ozone exposure varies from one cell culture to another, the average rate increases nearly 2-fold. For experiments with 12 separate cultures, the ratio of influx for ozone-treated cells to influx for O_2-treated cells was 1.9 ± 0.6 (so) at an ozone concentration of 100 μl/l (5.6 x 10^{-7} mol O_2/min delivered to the solution). The stimulated influx rate is about five to eight times less than the stimulated efflux rate (the maximum observed rate was 1.9 x 10^{-17} eq/cell-min). Thus, a net loss of K(Rb) always occurs. The increase in influx depends upon the ozone concentration and delivery rate (Fig. 2), as does the time at which the apparent influx rate reaches zero.

Several tens of minutes into influx experiments, the internal specific radioactivity becomes high enough to cause a loss of internal label; thus, we observe an apparent zero rate of influx, where the real influx is equal to the real efflux. To circumvent this problem, we treated cells with ozone in the absence of label, but then added label to aliquots of cells removed from the treatment vessel after specific times (see under "Materials and Methods"). The initial uptake rate was measured from 1 to 4 min. By keeping the internal concentration of ^86Rb low, we kept exchange processes minimal. Control uptake rates showed little change over 60 min (Fig. 3), while ozone treatment induced a stimulation of the K+ influx rate for up to 20 to 30 min of treatment. Thereafter, the influx rate was progressively inhibited, to a degree dependent upon the amount of ozone delivered into the solution.

Results (not shown here) using 27 μl/l ozone at a flow rate of 7.5 x 10^{-8} mol ozone delivered per minute closely duplicate those.
above at 100 μl/l and an equivalent delivery rate. After a 4- to 5-min lag, cells lose K⁺ at a high rate (2.4 ± 0.2 × 10⁻¹⁶ compared to the control of 2.8 ± 10⁻¹⁷ eq/cell-min), while the uptake is stimulated only about 2- to 3-fold. These data indicate that the rate of ozone delivered into the solution is the important parameter for K⁺ loss; its concentration in the gas stream is not.

Some researchers have noted an ozone-induced stimulation in respiration which could be linked to increased ATP production (19). This increased energy level might stimulate K⁺ pump activity leading to an elevated influx. After measuring the rate of [³⁵S]glucose utilization by Chlorella (via ¹⁴CO₂ release) under our conditions of ozone exposure, we found a decline in glucose metabolism. After 15 min at 100 μl/l (2 × 10⁻⁷ mol O₃/min), the rate is 59 ± 2% of the control, and at 30 min, 21 ± 2%. Further, the rate of O₂ consumption from endogenous reserves declines upon ozone exposure (Fig. 4). The delivery rates of ozone in both these cases compare with the higher ozone level in Fig. 2 and the lower level in Fig. 3.) We have never observed an increase in respiration of more than 10%, and only in both control and treated cultures within the first 2 to 3 min following exposure to the gases. After 20 min of exposure, the respiration rate is less than 10% of that of the control.

Efflux of K⁺ from Chlorella after exposure to various short pulses of ozone is seen in Figure 5A. As noted by Chimiklis and Heath (6), the rate of K⁺ loss returns to the control level after removal of ozone. By extrapolating the linear rate of loss (10–15 min following the pulse) back to zero, we can compare the amount of K⁺ loss with the amount of ozone delivered to the cell suspension (Fig. 5B). About 55% of the K⁺ is lost from 10⁶ cells (3.9 × 10⁻¹⁷ eq/cell) for about 7.5 × 10⁻⁷ mol of ozone delivered to the solution. This compares favorably with the amount of K⁺ lost from cells subjected to continuous gassing where 15.4 min of continuous gas is required to deliver 10⁻⁶ mol of ozone (Fig. 1).

The kinetics of the process by which the high rate of K⁺ loss returns to the control level, after removing the ozone (Fig. 5A), appears to be first order, with a t½ of about 3 to 3.5 min. This is similar to previous findings (6).

Ozone stimulates K⁺ influx 2-fold but the kinetic curves are difficult to interpret. The data are clarified if plotted as the amount of K⁺ uptake with ozone minus the amount of K⁺ uptake with O₂ alone (Fig. 6). The excess amount of K⁺ uptake depends somewhat on length of ozone exposure; however, the rate and extent of these curves reach a maximum for ozone pulses of 2 min and drop off for longer exposure times. This dropoff may be due to the cells' increasing inability to retain high levels of label. In all cases, a constant low value of K⁺ influx is reached about 15 to 20 min after the cessation of ozone exposure.

The rate of K⁺ influx is nearly doubled (2.2 ± 0.4) during the initial 5 to 10 min after 2 to 5-min pulses, and is significantly increased (1.3 ± 0.1) for shorter pulse times (0.5–1 min). This is similar to what is observed with continuous exposure to ozone (Fig. 2); in addition, it correlates with the lag time necessary to reach the maximum stimulation of the K⁺ influx.

**DISCUSSION**

Changes in K⁺ fluxes provide one of the first indications of membrane injury (5, 15). The loss of K⁺ due to changes in membrane permeability properties has been observed under oxidative stresses such as ozone exposure (7, 12), UV damage (5, 20), exposure to heavy metals (24), and peroxidation (18). We have shown in this paper that ozone dramatically affects the outward movement of K⁺ in Chlorella.

Potassium influx is also stimulated by ozone treatment, as it is
after γ-irradiation (2, 3). There are at least five possible reasons for the apparent increased influx: (a) ozone alters general membrane permeability to all species thus linking increased Rb transport to either an anion or cation exchange mechanism; (b) most of the influx is due to a pump (2, 3), and ATP levels elevated by increased metabolic activity (19) are used to move K+ inside more rapidly; (c) a lowered turgor pressure due to K+ loss increases the pump via a control mechanism (8); (d) external ozone reactions change the environment at the membrane level and these changes, in turn, stimulate a pump; (e) no active pump is changed by ozone, but the K+(Rb) movement follows a natural electrochemical gradient. We have no data regarding the first possibility. Both coand countercurrent exchange of Rb could be increased by a general increase in permeability to all ionic species. The only species in the resuspension medium likely to be involved in an exchange process are K+ and Cl−. It is possible that given an increase in permeability, Cl− is co-transported with Rb+.

Results shown in Figure 4 negate the second possibility. Respiration is rapidly inhibited by ozone treatment. After only 20 min of exposure, the respiratory rate is only 10% of the control; thus, about 10−18 mol of O2/cell-min are being used. If only glucose is respired, about (38 ATP/6 O2) × 10−18 mol ATP/cell-min or 6.3 × 10−18 mol ATP/cell-min would be produced. Since the postulated pump moves 1.6 × 10−17 eq K+/cell-min (Fig. 2), there is clearly not enough ATP provided by this respiration level to move K+ inward at the observed rate, providing that all K+ movement is due to a simple ATPase/pump.

Likewise, the third possibility can be eliminated. The influx increases by 2-fold after 2 to 3 min of ozone (Figs. 2 and 3), which would require an external increase in osmotic potential of −5 to

**Fig. 5.** A: efflux of 86Rb from Chlorella following pulses of ozone. Experiments were performed as described under "Materials and Methods" at an ozone level of 100 μl/l (delivery rate = 2.5 × 10−7 mol/min). The level of internal K+ in these cells at 0 min was 7.0 ± 0.3 × 10−15 eq/cell. Symbols, other than circles are ozone treatments (open and filled symbols represent duplicate experiments). Bars are standard deviations of control experiments. Numbers near curves represent minutes of ozone pulse. B: amount of K+ lost due to an ozone pulse is the extrapolated intercept (at 0 min) from Figure 5A (see text for more details).
ozonation, the of min (plus the active in Valonia, which induced an increased accumulation of K⁺ only after 30 min (13). Thus, the increased influx seems to occur too rapidly to be a response to only an internal turgor pressure change.

The fourth possibility is more difficult to test. Ozone decomposition in an aqueous environment often gives rise to an alkalinization (23). For example, ozone decomposed at or near the cell wall and locally increased the pH, influx would be stimulated (we measured a 2- to 3-fold increase in influx at pH 8.0 as compared to pH 6.0, in the absence of ozone). This localized pH shift would be difficult to monitor and would be extremely dependent upon the wall's buffering capacity.

The fifth possibility, that increased influx involves no pump, requires more detailed explanation. It is based on the assumption that, with no light under non-growth conditions, the pump does not operate at a high rate and is, therefore, a small component of the total influx. In addition, the influx-efflux rates are governed by both the accumulated K⁺ and the membrane potential, according to the Goldman equation (12).

To test this possibility, we need to know the membrane potential (ΔΨ) of this Chlorella. Unfortunately, two divergent membrane potential values have been measured in other Chlorella. Using a microelectrode, Barber (4) measured it at -40 mv (4). Using the rate of accumulation of tetrathylenepseudophosphorium ion, Komor and Tanner (16) recorded a value of -130 mv. Different algal strains might yield different measured potentials, although possible cell injury resulting from the insertion of a relatively large microelectrode might account for the low membrane potential measured by Barber (4).

Using our measured control efflux rate (2.62 x 10⁻¹³ mol/cm²-s, Fig. 1) with an internal K⁺ concentration of 390 mm (see above), the calculated membrane permeability (Pₐ) is either 3.4 x 10⁻⁷ cm²-s (for ΔΨ = -130 mv) or 3.0 x 10⁻⁸/cm-s (for ΔΨ = -40 mv) on the basis of the Goldman equation (12), assuming that the efflux is completely passive. Using the Pₐ to calculate the passive influx rate, we obtain either 2.1 x 10⁻¹⁷ mol/cell-min (for -130 mv) or 7.4 x 10⁻¹⁹ mol/cell-min (for -40 mv). The first value exceeds the measured influx and can be eliminated. Therefore, the ΔΨ must be less than -130 mv (in fact, calculated to be about -95 mv).

These data can be explained at least in two ways. If the membrane potential were low (ΔΨ = -35 mv) and stable during ozonation, increased influx is due to a 16-fold increase in Pₐ, accounting for our measured value. Then subsequently, the rate of the passive influx can be calculated to be 1.2 x 10⁻¹⁷ mol/cell-min (plus the active component), near our observed rate.

On the other hand, assuming that influx and efflux are in steady-state according to the Goldman equation, there is no pump in operation and the ΔΨ is nearly -95 mv. (A ΔΨ in this range is also near the equilibrium potential calculated according to the Nernst equation.) If ozone increases the Pₐ by 2- to 3-fold and dropped the ΔΨ to -40 mv, the influx would increase only 2-fold and the efflux 15-fold, likewise near our observed changes. This second explanation seems more likely in view of known membrane behavior. Further, the observed decrease in respiration suggests lowered ATP levels and consequently, membrane depolarization (25).

Under our conditions, the processes which maintain membrane permeability and membrane potential are interdependent, with changes in one predating changes in the other. In this case, the high rate of efflux is not only due to an increase in permeability to K⁺ but also reflects a declining membrane potential. Although measurement of total flux can be helpful in assessing changes in membrane properties, both influx and efflux measurements are necessary to formulate an accurate picture of membrane phenomena.

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