Ozone-induced Loss of Intracellular Potassium Ion from *Chlorella sorokiniana*¹,²

**PHROSENE E. CHIMIKLIS³ AND ROBERT L. HEATH**

Departments of Biology and of Biochemistry, University of California, Riverside, California 92502

**ABSTRACT**

The unicellular algae *Chlorella sorokiniana* was used as a model system to investigate the interaction of ozone with plant cell membranes. Ozone induces K⁺ leakage from *Chlorella sorokiniana* similar to the electrolytic loss observed from many higher plants under stressful conditions. The kinetics of this leakage indicate that ozone initially interacts reversibly (within sec) with sites on membranes allowing a passive efflux of K⁺. This efflux ceases within minutes after the ozone stress is removed. This return to normal efflux is temperature dependent. High intracellular osmolarity seems to be an important criterion of susceptibility to ozone injury in this model system, since rates of ozone-induced K⁺ leakage are less when the external osmotic potential is decreased by suspension of the cells in mannitol. Cell interaction with ozone is further complicated by a saturating-type dependence of the K⁺ efflux upon ozone concentration within the medium.

The leakage of electrolytes can be induced in plants and microorganisms by pathogens (29), phytotoxins (19), and environmental stresses such as low temperatures (11, 18, 28), heavy metals (17, 21), atmospheric oxidants (9), and UV light (7). Lee (20) has shown that mitochondria leak K⁺ ion after ozone exposure. This leakage symptom has generally been attributed to an increase in membrane permeability (20, 22, 27) and has been used as one criterion of injury. While these investigations have examined specific alterations to membranes (20, 21, 23, 25), many have not investigated the effect of these stresses on other permeability properties or general integrity of the membrane, or the significance of membrane alterations to repair and recovery mechanisms.

The unicellular green alga *Chlorella sorokiniana* is used here as a model system to study the kinetics of ozone-induced K⁺ efflux in order to understand the effects of air pollutants on green plants. Used as such an investigative system, this eukaryotic cell not only eliminates the variables introduced by stomates, cuticle, cellular differentiation, and translocation, but also provides a statistically uniform population which can aid in investigations of physiological activities. K⁺ leakage was monitored with a cation electrode coupled to an antilog device which provides continuous recording of K⁺ fluxes and allows rapid evaluation of the data during experimentation (12).

Preliminary reports have previously appeared (2, 3) in which the significance of K⁺ leakage as a primary deleterious response has been related to factors of susceptibility to ozone injury in *Phaseolus vulgaris* (13). This paper shows that the ozone-induced K⁺ efflux is very dependent upon the external environmental conditions, including temperature, osmotic pressure, and ozone concentration.

**MATERIALS AND METHODS**

*Chlorella sorokiniana* was cultured at 38°C as previously described (4), and harvested during exponential growth by centrifuging and washing cells in 10 mM tris, 1 mM CaCl₂ at pH 9. The washed cells were kept on ice until used, but not for periods longer than 6 hr.

All measurements of K⁺ concentration and efflux were performed, unless stated otherwise, in a 10-ml aliquot of the wash solution of 10 mM tris, 1 mM CaCl₂ (pH 9) at 38°C with a cation electrode which was coupled to an antilog converter (12). These suspension conditions do not greatly impair the physiological state of cells since the washed cells, after being kept on ice for 4 hr, achieve maximal growth rates within 4 hr after resuspension in normal autotrophic growth media.

Ozone was produced by passing O₂ over UV lamps, according to the method of Coulson and Heath (6). The concentration of ozone was determined by bubbling 10-ml aliquots of KI solution for various time intervals. The concentration of ozone was then calculated from the absorbance at 350 nm using an extinction coefficient of 24 mmoles/cm² (1), with the assumption that one I₂ molecule is produced by one ozone molecule (1). For most experiments reported here with an (O₂/O₃) gas flow of 13.5 ml/min, the ozone concentration was 26 μmoles of ozone/l of air flow.

While the concentration of ozone in the gas was high (630 μl/l), the amount of ozone in solution, calculated by Henry's Law and the Bensen Coefficient of solubility (6, 15), was relatively low (15.2 μM). For lower concentrations of ozone, the gas produced above was diluted with O₂ to reach the reported values.

The cell volume was measured with a Coulter Counter² as previously described (14). The cells were suspended in the media described above with the addition of mannitol (at required concentration) and 40 mM choline chloride to act as an electrolyte for the Coulter Counter. The size was determined immediately upon the addition of the cells to the medium.

**RESULTS**

As reported previously (13), cells of *C. sorokiniana* suspended in an alkaline tris solution exhibit an initial rapid exponential

¹ This project has been financed in part with federal funds from the Environmental Protection Agency under Grant R-801311.
² The contents of this paper do not necessarily reflect the views and policies of the Environmental Protection Agency.
³ Present address: National Ecological Research Laboratory, United States Environmental Protection Agency, Corvallis, Ore.
⁴ Mention of trade names or commercial products does not constitute endorsement or recommendation for use.
As previously shown (13), the steady state, tris-induced leakage of K\(^+\) from 38 C-grown cells, which is not altered by gassing with O\(_2\) or N\(_2\) (4, 13), persists for about 3 hr and ceases after the loss of most of the K\(^+\) from the cell. These same tris-induced, steady state kinetics are observed for concentrations from 0.5 to 3 \times 10^4 cells/ml. Since the rate of K\(^+\) efflux during ozone exposure is somewhat dependent upon cell concentration (Fig. 2), 10^7 cells/ml are routinely used. Also the time course of K\(^+\) loss is modified by varying the cell concentration (Fig. 2).

Cells which had been gassed for 10 min with 26 \(\mu\)moles (1 air flow)\(^{-1}\), washed, and resuspended resumed exponential growth within 8 hr. If the cells were gassed with ozone for 15 min, no growth was observed for 24 hr. Furthermore, the cells

leakage of K\(^+\) (over the first 20 min), followed by a constant steady state rate of K\(^+\) efflux (at \(8 \times 10^{-17}\) eq. cell\(^{-1}\) min\(^{-1}\) at 38 C) for over 3 hr (Fig. 1). The addition of ozone to the later, steady state rate of K\(^+\) efflux greatly increased the rate (Fig. 1; ref. 14). Ten-fold lower concentrations of ozone also induced a leakage of K\(^+\) but at a lower rate.

If the cells are preloaded with \(^{86}\)Rb as a tracer for K\(^+\), a similar efflux is observed (13). In addition, this ozone-induced efflux of \(^{86}\)Rb can be observed with cells suspended in normal autotrophic media and in Tricine buffer (neutralized with K\(^+\)) (Heath and Frederick, unpublished data). Low pH (pH 6) or high K\(^+\) in the media makes it difficult to observe K\(^+\) loss with the cation electrode (12).

When ozone is added to a suspension of cells at the onset of the initial exponential leakage (within 15 sec), the rate of K\(^+\) leakage is much greater than that of the exponential phase (control) of tris-induced leakage, and also is greater than that efflux observed when ozone is added later (during the steady state phase; Fig. 1). The lowered ozone-induced K\(^+\) efflux when cultures are gassed in the steady state phase of efflux (compared with the exponential phase) may be a consequence of the lower control rates of steady state leakage, and of the lower concentrations of K\(^+\) within cells, existing after the exponential control phase has released much of the K\(^+\) within the cell (nearly 50% in Fig. 1). The initial exponential phase of tris-induced K\(^+\) leakage exhibits sizable variation depending on the amount of time cells are stored on ice between experiments. More quantitative data can be obtained by examining the effects of ozone on the tris-induced, steady state leakage which is not subject to these variations. The kinetics and rates of steady state, ozone-induced leakage are repeatable for cells (in logarithmic phase) harvested on successive days.

**Fig. 1.** Ozone-induced K\(^+\) efflux from algal cells. The external K\(^+\) represented here has been corrected for the loss of K\(^+\) from the reference electrode (12). Ozone (at a concentration noted by the numbers by the side of the curves in moles/l air flow) was added to the suspension either initially or at the indicated point. The two different phases of the control curve (0 ozone) are shown on the graph. The noise of the trace (especially at higher K\(^+\) concentrations) has been eliminated from the drawing (12).

**Fig. 2.** Dependence of the rate of K\(^+\) efflux upon cell concentration. The cells at the indicated final concentration were suspended in the normal media. Rate per cell was calculated for two time periods of gassing (0-5 and 5-10 min) during the steady state phases (Fig. 1) and was corrected for the efflux with no ozone present.

**Fig. 3.** Dependence of K\(^+\) efflux upon temperature after a short exposure to ozone. The algal cells were suspended in the medium at the indicated temperature. The K\(^+\) efflux has been corrected for the loss of K\(^+\) from the reference electrode. Ozone exposure was at a concentration of 26 \(\mu\)moles/l for 5 min. The cells were gassed when the steady state phase of K\(^+\) efflux began (0 time on the graph represents 20 min after the cells were added to the media).
Fig. 4. Effect of mannitol on ozone-induced K+ efflux from algal cells. The rate of K+ efflux was calculated for varied time intervals from curves similar to those in Figure 1. A: rate of K+ efflux with varied times of ozone exposure. Mannitol (the concentration given as the number next to the curves in m) was added to the solution before the addition of the cells. The ozone flow was started 20 min after the cells were suspended in the media. B: dependences of ozone-induced K+ efflux and cell volume on mannitol concentration. The time required for the K+ efflux to reach half its initial value at 11.25 min (T50), and the reciprocal of the initial rate of increase in the K+ efflux rate (1/(Δ rate/Δ time)) is plotted as a function of mannitol concentration. These values are taken from Figure 4A. Also shown is the normalized volume of the cells (dark symbols), measured with the Coulter counter.

exhibit a light-induced bleaching of the Chl, indicative of cellular death. To better assess primary responses of this alga to ozone and to avoid secondary interactions, experiments were performed with a 5 min dosage of ozone.

When cells grown at 38 °C are gassed at different temperatures for 5 min during the steady state phase of leakage, two phenomena are observed (Fig. 3). With decreasing temperatures, the rate of K+ leakage from cells during both pre-ozone exposure and during ozone exposure decreases. At all temperatures examined, the ozone-induced efflux is eight times greater than the steady state control efflux. The Arrhenius plot of data similar to Figure 3 (13) is linear with an energy of activation of 17 Kcal/mole either with or without ozone. After the introduction of ozone into the solution ceases, the rates of leakage decrease and return to the steady state efflux (Fig. 3), except when experiments are performed at 15 °C. The high ozone-induced level of K+ leakage persists at 15 °C for very long periods.

Neither the rate of steady state control leakage from cells or of that induced by ozone is altered by pH (between 7.9 and 10.1), or by CaCl2 concentrations (to 11 mM). Mannitol, added to the measuring solutions before the cells, does not alter the steady state leakage to any great extent, but does reduce the initial rate of the ozone-induced leakage from the cells. With increasing mannitol concentrations (Fig. 4A), the initial rate of increase in rate of K+ leakage is progressively depressed, and a longer period of time of ozone exposure is required to reach maximum rates. The time required to reach 50% of the maximal rate and the initial rate of increase is related nearly linearly to the mannitol concentration (Fig. 4B), with the half-time increasing nearly 40-fold for the highest mannitol concentration (zero compared to 0.6 m). Ozone concentration in solution is probably not reduced greatly by the presence of mannitol since ozone solubility is lowered only 50% by an increase of concentration to 0.5 m NaCl (16). Moreover, the mannitol decreases the cell volume (also shown in Fig. 4B) and thus must increase the apparent K+ concentration within the cell.

As indicated in Figure 1, the level of ozone affects the kinetics of the K+ efflux. This phenomenon is more dramatically shown in Figure 5 as the rate of K+ efflux as a function of time of ozone exposure. At a high level of ozone (26 μmoles/l), the rate of K+ efflux increases immediately and remains at the high level for some time. At 10-fold lower ozone concentration, the rate is slow at first, rises to a higher value, and then declines again. Initially the rate of K+ efflux at the lower ozone concentration is only 25% of that at the higher ozone concentration; this rate rises to 82% of the control compared to the maximum rate of the ozone induced K+ efflux (measured at 6.25 min).

The initial rate of ozone-induced K+ efflux (Fig. 6) varies nearly linearly for low ozone concentrations, but then begins to tend towards saturation at higher levels. Treated as saturation-type kinetics, the data indicate a maximum rate of K+ efflux of \( 1 \times 10^{-11} \) eq. cell⁻¹ min⁻¹ with a half-saturation point of 22 μmoles/l ozone concentration.

**DISCUSSION**

The ozone-induced leakage of K+ from *Chlorella sorokiniana* occurs between 5 and 15 seconds after exposure (Fig. 1) and, for that reason, is most likely a symptom of primary ozone interaction with the plasmalemma rather than a secondary expression of cellular injury.

Although no other data are presented in this paper, preliminary
results indicate that ozone does not increase chloride movement to any great extent (as measured by a Beckman chloride electrode upon samples of gassed algae) but increases H+ influx (as measured by a pH electrode) (2). These data would argue that ozone stimulates a K+/H+ exchange. pH changes in an ozone-gassed aqueous solution are large without cells present (24), and therefore any pH changes are subject to possible artifacts. The changes in other ions and organic molecules within the algae upon exposure to ozone will be discussed in a future paper.

Initially ozone probably alters the membrane reversibly rather than randomly disorganizing it, since cells gassed for 10 min (at 20 μmol of O3/l) show exponential growth within 6 hr (compared with 4 hr for O2-gassed cells) and the high rates of K+ efflux cease with the removal of O3 (within minutes, Fig. 3). Furthermore, while cells lose their viability (ability to grow on an agar plate) upon ozone exposure, this viability loss occurs only after a short lag period (5 to 10 min) (10). In addition, the K+ efflux of cells exposed to O3 at 15°C does not return to its pre-exposure rate. These facts argue strongly for a temporary change of state or of fluidity of the membrane (22), induced by ozone.

Comparisons between various experiments are difficult since the rate of K+ loss depends upon ozone and cell concentration. Again this dependence is similar to viability changes (compare Figures 1 and 2 in ref. 10) and to visible injury in green plants (15).

The apparent tendency toward saturation of the initial rate (Figs. 2, 4, 5) argues for a finite number of sites per cell with which ozone can interact. The slow build-up of K+ efflux rate (Fig. 5) at low concentrations of ozone is puzzling; possibly the penetration of ozone through the wall and free space is a cause.

The addition of mannitol external to the cell to reduce the cellular turgor pressure (5) also decreases the rate of K+ efflux induced by ozone (Fig. 4). This indicates, as in other plant studies, the importance of water potential for cellular injury. At low water potentials (lowered turgor pressure), Ting and Dugger (26, 27) have shown that visible injury induced by ozone declines even though the stomates remain open. The cellular condition of high turgidity may function by decreasing the free space between membrane and wall, and physically stretching the plasmalemma such that more sites are made accessible to ozone on the membrane. High water potentials may also explain the age-dependent sensitivity to ozone injury of both Gossypium and Phaseolus (8). Both plants exhibit the greatest amounts of injury just before full leaf expansion, when rates of expansion are maximal (5, 9, 26, 27). During this period water potential is the driving force of expansion and is thought to be high (5). The concentration of K+ in Phaseolus is highest during this same period (13). The high level of K+ may cause the high water potential and thus form the hydrostatic pressure needed for expansion. From this, the high turgor pressure may cause the high sensitivity of the plant at this age to ozone injury. Stomatal closure, often observed after ozone fumigation (8), may be a consequence of ozone interaction with the highly turgid (and K+-laden) guard cells in that K+ leakage from these cells results in loss of turgor and stomatal closure.

K+ leakage may, as for other stresses, be a rapid indicator of sensitivity of cells to ozone stress; whether or not K+ leakage, and subsequent ionic imbalances and dehydration of the cells, is the primary event in ozone phytotoxicity awaits further experimental confirmation.

Acknowledgment—The authors would like to thank Dr. W. M. Dugger for his enthusiastic interest and continued support.

LITERATURE CITED

OZONE-INDUCED K+ EFFLUX