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Inhibition of the Photosynthetic Capacity of Isolated Chloroplasts by Ozone[1]

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ABSTRACT

Isolated spinach chloroplasts have been used as a model system for studying the interaction of ozone, a component of photochemical smog, with plant membranes. Ozone bubbled into a suspension of isolated chloroplasts inhibits electron transport in both photosystems without uncoupling ATP production. Photosystem I (reduced 2,6-dichlorophenolindolphenol → $NADP^+$) is a little more sensitive than photosystem II (H_2O → 2,6-dichlophenolindolphenol). Ozone does not act as an energy transfer inhibitor, since the drop in ATP production and high energy intermediate (measured by amine-induced swelling) is nearly parallel to the decline in electron transport. A reasonable hypothesis is that ozone disrupts the normal pathway of energy flow from light-excited chlorophyll into the photoacts by a disruption of the components of the membrane but not a general disintegration of the membrane. In addition, ozone does not seem to penetrate into the grana region through the outer membrane of intact plastids, since ozone lowers the bicarbonate-supported O_2 evolution but does not affect the rate of ferricyanide reduction in the same plastids after osmotic disruption. This would indicate that the effect of ozone on green plants, at low concentrations, may be due to the interaction of ozone with the first membrane it contacts and not directly with internal metabolic processes.

Plant tissues are injured by ozone at concentrations which occur commonly in the smoggy atmosphere above many of our major cities (9). Fumigation of dicotyledonous plants with comparable levels of this photochemical oxidant produces visible injury to leaf tissue in the form of a gross necrosis on the palisade cells (8) many hours after fumigation. The morphological indication of ozone injury to pinto bean plants is a granulation within the chloroplast stroma region (28). In addition, ozone in pinto beans appears to affect only chloroplast ribosomes and not the cytoplasmic ribosomes, perhaps as a result of impaired photosynthesis (3). Even though these data indicate that photosynthetic sites are involved in ozone-induced injury, the plasmalemma is certainly implicated by other work as a possible site of ozone damage (8).

Photosynthetic physiology is extensively affected by ions: enhancement of photosynthesis by two different wavelengths of light is stimulated by Mg^{2+} (27), efficient photophosphorylation requires cations (7), and the extent of swelling and shrinking of chloroplasts is dependent upon the ionic environment (6, 7). Therefore, it is conceivable that an ozone-induced alteration in the water and ion permeability of the chloroplast outer membrane would affect the internal milieu of this organelle and, consequently, its function as a whole. In fact, the effect of ozone on the chloroplast morphological structure may well be due to these ionic and osmotic imbalances of the whole cell due to changes in the cell plasmalemma.

Even if it is not the primary site of ozone attack, the chloroplast is still valuable as a model system. The isolated grana stacks of chloroplasts represent a biological system containing lipids, proteins with sulfhydryls, and antioxidants within a highly organized and functionally defined membrane structure (1, 12). As such, the isolated chloroplast offers a perfect opportunity to study how biochemicals in their normal physiological surroundings may be altered by ozone in aqueous solution. Thus, biochemical and physiological studies on the isolated chloroplast may well elucidate, at least in part, the mechanism of oxidant injury to green plants.

This paper describes how ozone introduced into an aqueous solution will damage the functional capacity of the photosynthetic apparatus, and what site or sites of electron transport are involved. In addition, isolated, intact chloroplasts are used to ascertain how deep ozone can actually penetrate organelles in order to cause injury.

MATERIALS AND METHODS

Introduction of Oxidant to a Solution. To obtain reproducible results when gassing aqueous solutions, a "gas-dilution" system was constructed which conveniently produces and delivers ozone into a solution (shown schematically in Fig. 1). After passage through Drierite and activated charcoal (to remove impurities), oxygen flow into an ozone generator through either flowmeter 1, 2, or 3, is controlled by three-way valves A, B, C, D, and E. The oxygen flow rates could be from 3 to 8000 ml/min. The ozone generator was constructed from an aluminum cylinder coated inside with Teflon spray and contained three mercury vapor lamps (GE type G4S11) to produce UV radiation in order to convert some oxygen into ozone. The ozone-plus-oxygen mixture would then flow into the mixing chamber (a 3-liter glass sphere) where it would be diluted by air flowing from either flowmeter 1 or 2.

From the ozone generator to the solution, gas flowed through stainless steel tubing and valves (shown as dotted lines in Fig. 1). The junction between reducing valves 3 and 4 allowed the excess gas mixture to be bled off such that the gas flow into the sample (controlled at reducing valve 3)

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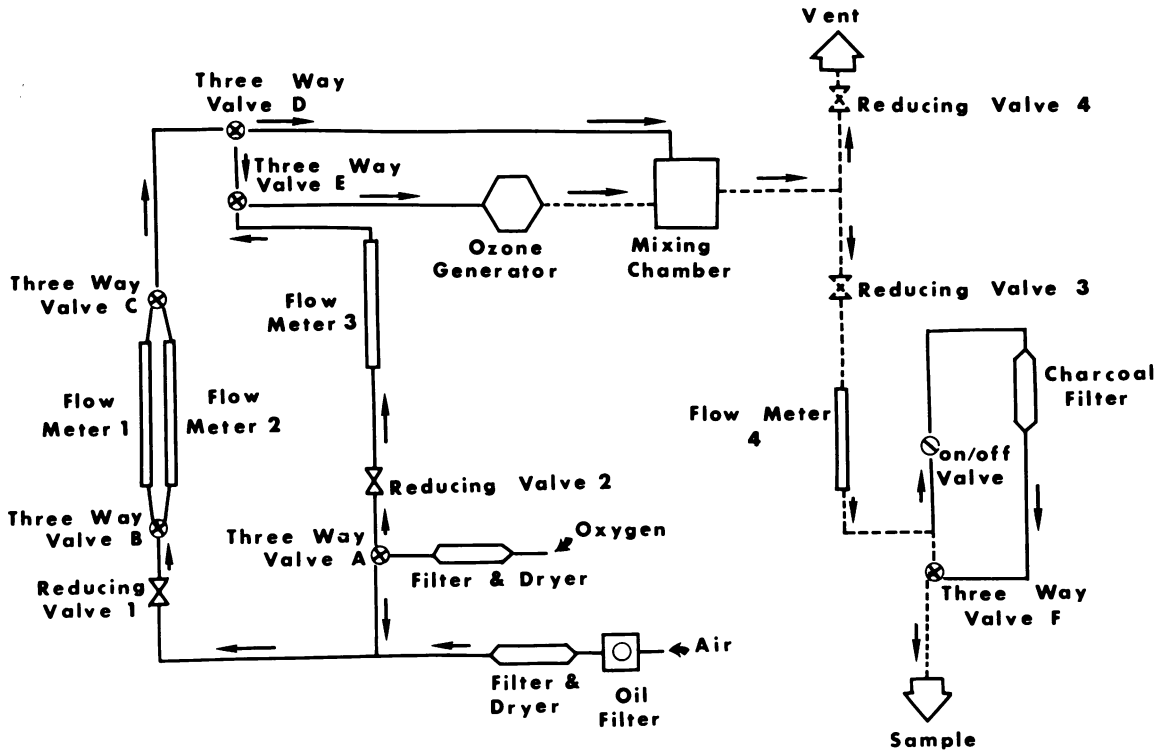

FIG. 1. Schematic diagram of gas-dilution system for delivery of ozone. Dotted lines represent one-quarter inch K.D. stainless steel tubing (San Diego Valve and Filtering Co., San Diego, type 316). Valves are: 1, 2: IRS4; 3: 2IRS2-316; 4: 4RD-316; A, B, C, D, E: 42X54; F: 42X54-316; ON/OFF: 42S4 (Whitey Co., Oakland, Calif.). Flow meters are: 1: 448-308; 2: 448-209; 3, 4: 448-001 (Fischer & Porter Co., Hatboro, Pa.) Mixing chamber: 3-liter glass sphere with three inlets. See text for description of ozonator.

could be less than the total flow out of the mixing chamber. The on-off valve and valve F allowed the gas to flow through an activated charcoal filter to remove ozone (8). This bypass circuit allowed the controls (without ozone) to be run conveniently. The gas mixture then was passed into the aqueous solution through a 25-μl pipette.

The 1-ml sample to be gassed (chloroplasts at 180 μg Chl in a solution of 0.2 M sucrose, 6 mM TES, 5 mM $MgCl_2$, and 20 mM NaCl, pH 7.4) was placed in a 10- $\times$ 100-mm test tube supported rigidly in an ice bath. The position of the 25-μl pipette was fixed to insure that its position in the sample was always the same (2 mm above bottom of tube). For the experiments described here, the flow rate was 10 ml/min for a total gassing time interval of 5 min at 0 C.

The dose of ozone (nanomoles) given to the solution during gassing (see "Results") represents the total amount of ozone passing into the solution as measured by the KI method (23). An increased dose is caused by increasing the partial pressure of ozone within the delivered air. Much of the ozone (90% or greater) passes through the chloroplast solution unabsorbed and unreacted.

Chloroplast Preparation and Functional Capacity Assays. Chloroplasts isolated from commercial spinach were prepared according to Hind *et al.* (14). Intact class I plastids were obtained by the method of Jensen and Bassham (17) with the modification that the leaves were chopped with an electric knife fitted with razor blades.

Reduction of potassium ferricyanide or DCIP[3] by isolated chloroplasts was measured on a Cary 15 spectrophotometer at 420 and 620 nm, respectively. The photomultiplier was shielded from the actinic light by both cut-off (Corning No. 4-94) and interference (Baird-Atomic, 420 or 620 nm) filters. Actinic illumination was provided at a 90° angle to the measuring beam by a 500-w tungsten-halogen projector with a red (Corning No. 2-92) filter (intensity, 60 kergs/$cm^{-2}\cdot sec^{-1}$). The isolated chloroplasts (20 μg Chl) were suspended in 1.8 ml of 0.2 M sucrose, 6 mM TES, 5 mM $MgCl_2$, and 20 mM NaCl (pH 7.4).

Bicarbonate-stimulated oxygen evolution by isolated class I chloroplasts was measured with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio, model 53). Chloroplasts (70 μg Chl) were added to 3.5 ml of deoxygenated reaction media of 0.33 M sorbitol, 2 mM $NaNO_3$, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM KH_2PO_4, 5 mM $Na_4P_2O_7$, and 50 mM Tricine (pH 8.1). The solution was illuminated with red actinic light as described above after the addition of 5.7 mM sodium bicarbonate (pH 8.1).

Light-induced increases in average chloroplast volume were measured by the use of a Coulter counter coupled to a pulse height analyzer (13). Sodium chloride (40 mM) was added to the electron transport assay media for electrical conductivity. Absorbance of chloroplast solutions was measured with the Cary 15 spectrophotometer at a wavelength of 540 nm.

The light-induced esterification of Pi was measured according to a modification of the method described by Huang (15). Chloroplasts (50 μg Chl) were suspended in 2 ml of 20 mM sucrose, 5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 6 mM Tricine buffer (pH 8.2), plus either 400 μM FeCN or 20 μM phenazine methosulfate, and (for certain experiments) ADP (2 mM) $H_3{}^{32}PO_4$ (0.5 μc). After illumination (1 min) the reaction was terminated by the addition of perchloric acid (final concentration, 6.3%). To assay the organic ^{32}P, 100 μl of the reaction solution were added to 2 ml of 2 mM KH_2PO_4 and 200 μl of 7.5 g of

[3] Abbreviations: DCIP and $DCIPH_2$: oxidized and reduced 2,6-dichlorophenolindolphenol; FeCN: potassium ferricyanide.

activated charcoal and 0.2 g of soluble starch in 75 ml of water. After shaking, this solution was then filtered through a Millipore filter (25 mm, 0.8 μm pore size, previously washed with 2 mM KH_2PO_4), and the filtrate was washed with 22 ml of cold KH_2PO_4 (2 mM). The radioactivity left on the filter was then counted by a Geiger-Mueller tube. For P/e_2 ratios, the esterification was carried out as above, with the exceptions that 20 μg of Chl were used, and the reaction was carried out in the Cary spectrophotometer for periods longer than 1 min.

RESULTS

Electron Transport. The bubbling of ozone into a suspension of isolated class II chloroplasts inhibits electron transport from water to ferricyanide (Fig. 2); as the dose of ozone increases, the rate of electron transport declines. This inhibition occurs at a lower ozone dose if the plastids are uncoupled by ammonia. Provided the bubbling is carried out in the same manner, the inhibition pattern is highly reproducible from preparation to preparation. The inhibition of the rate of ferricyanide reduction from basal electron flow (without added uncoupler) is observed only above an oxidant dose of about 500 nmoles. However, in other experiments with $NADP^+$ as an electron acceptor, a slight inhibition of coupled plastids does occur at a lower dose of ozone (with low rates of electron flow). No increase in the rate of FeCN reduction was observed in the absence of ammonium chloride when plastids were gassed with either oxygen or ozone. Thus, ozone does not function as a classical uncoupler (17) by increasing the electron flow, as observed with the addition of ammonium ion (see control rates in legend).

Ozone also functions as an inhibitor of uncoupled electron flow to $NADP^+$ when electrons come from either water (photosystem I + II) or reduced DCIP (photosystem I) as shown in Table I. The rate of $NADP^+$ production is inhibited quite strongly when the electrons come from water splitting (photosystem I + II activity) and less strongly (about half) when the electrons come from reduced DCIP (photosystem I activity). For example, in one experiment the percentages of $NADP^+$ reduction inhibition were 45 and 28%, respectively. Both photosystems are inhibited by ozone. In addition, the percentage inhibition for photosystem II + I electron flow is nearly that observed for FeCN reduction at the several doses of ozone shown (Fig. 2).

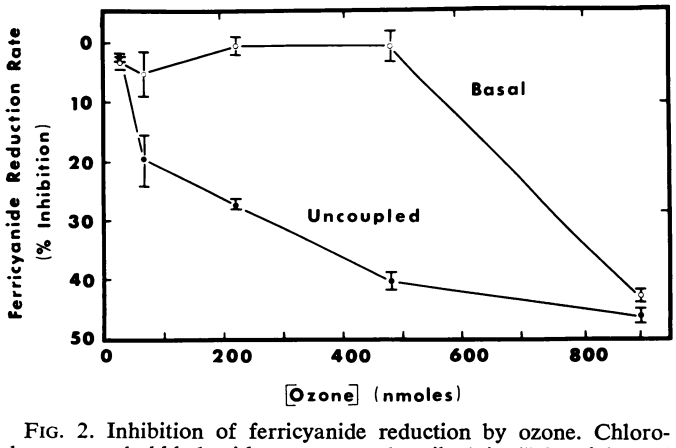

FIG. 2. Inhibition of ferricyanide reduction by ozone. Chloroplasts were bubbled with ozone, as described in "Materials and Methods", with a total dose given in Fig. 1. Control rates were measured under similar bubbling conditions with no ozone present and were: basal ($-NH_4^+$): 230 μeq/mg $Chl^{-1} \cdot hr^{-1}$; uncoupled (NH_4^+ at 10 mM): 560 μeq/mg $Chl^{-1} \cdot hr^{-1}$.

Table I. *Inhibition of Electron Flow in the Photosystems of Isolated Chloroplasts by Ozone*

The chloroplast suspension was gassed as described in "Materials and Methods," and the $NADP^+$ reduction rate was assayed for the chloroplasts (6 μg Chl/ml) in 0.2 M sucrose, 5 mM $MgCl_2$, 40 mM NaCl, 6 mM TES buffer (pH 7.4), 0.8 μM Na ascorbate, 300 μM $NADP^+$, 3 mM NH_4Cl for H_2O as electron donor. For $DCIPH_2$ as the donor, the same media was used with the addition of 3 mM Na ascorbate, 7 μM DCMU, and 8 μM DCIP. The reduction rate for FeCN or DCIP was assayed. The chloroplasts (10 μg Chl ml) were suspended in 0.2 M sucrose, 5 mM $MgCl_2$, 40 mM NaCl, 6 mM TES (pH 7.4), 5.6 mM NH_4Cl using either 25 μM DCIP or 400 μM FeCN as the electron acceptor. Spectrophotometry was carried out as described in "Materials and Methods," with the exception that DCIP reduction was measured by turning off the spectrophotometer during actinic illumination and not using blocking filters for the phototube. Illumination was provided for 20 sec and the amount of DCIP reduction was measured in the subsequent dark period. This was repeated several times to find an average rate.

Experiment	O_3	Reduction Rate	
		$H_2O \rightarrow NADP^+$	$DCIPH_2 \rightarrow NADP^+$
	nmoles	*μeq/mg $Chl^{-1} \cdot hr^{-1}$*	
1	0	220	58
	140	175 (20%)[1]	51 (12%)
	720	120 (45%)	42 (28%)
2	0	190	52
	140	144 (24%)	42 (19%)
	720	81 (57%)	38 (27%)
		$H_2O \rightarrow DCIP$	$H_2O \rightarrow FeCN$
3	0	87	630
	140	82 (6%)	440 (31%)
	720	72 (18%)	350 (45%)

[1] Numbers in parentheses: percentage inhibition of ozone-treated chloroplasts compared with the control (no O_3).

Photosystem II is also inhibited (Table I); the rate of electron flow from water to DCIP is a measure of photosystem II activity (12, 27). The percentage inhibition of photosystem II is less than that observed for ferricyanide reduction under the same ozone concentration. Apparently, the percentage inhibition for both photosystems adds and seems to be constant for different preparations of chloroplasts; for example, the 28% for photosystem I plus the 20% for photosystem II is nearly equal to the 50% for photosystems I + II, measured together.

Photophosphorylation. The ozone-induced inhibition of electron flow through photosystem I (using reduced DCIP to $NADP^+$) could be due to an alteration of the cytochrome system's redox poise (11). When phenazine methosulfate is used as a cofactor to induce cyclic photophosphorylation through photosystem I, the electron transport rates are much higher than those produced by reduced DCIP and $NADP^+$ (10), and changes in redox poise seem to be minimal. Since no stimulation of the basal electron flow was noted with ozone (Fig. 2) and ozone does not seem to be an uncoupler, cyclic photophosphorylation might be a better indication of photosystem I activity. Gassing plastids with 200 nmoles of ozone leads to a 26% reduction in cyclic photophosphorylation capacity (supported by phenazine methosulfate), slightly higher than that of the ozone-induced inhibition of electron flow

through photosystem I (15–20%), as in Table I. Ozone could be inhibiting electron flow more in this system or acting similarly to the so-called energy transfer inhibitors such as phlorizin or Dio-9 (10), which do not stimulate electron flow but do inhibit ATP production.

The effect of ozone on FeCN-supported photophosphorylation should indicate which of these two possibilities was correct. The P/e_2 ratios (10, 16) are shown in Table II for plastids gassed with ozone. Gassing by itself (with the oxygen control) reduces the rate of FeCN reduction; however, the rate of phosphate esterification is similarly reduced, and, thus, the P/e_2 ratio remains constant at about 0.67, similar to that found by other workers (17). P/e_2 does not remain the same when ozone is added to the gassing mixture. Ozone reduces the rate of FeCN reduction but, in addition, inhibits the rate of photophosphorylation more strongly. A low P/e_2 ratio defines an uncoupler; however, since ozone does not stimulate electron flow, it appears to act as an energy transfer inhibitor (17).

Izawa *et al.* (16) believe that the P/e_2 ratio should be calculated by subtracting the basal rate of FeCN reduction (that without added ADP) from the electron flow rate shown in Table II. If that is done (see Table II, column 5), ozone then causes no net decline in the ratio, which remains about 2.0. Unfortunately for the conclusion, the relatively high basal rate produces a high corresponding variability in the ratio. However, this result would indicate that ozone is only an inhibitor and does not uncouple or act as an energy transfer inhibitor.

The possibility exists that a lowered photophosphorylation rate is due to an increased ATP hydrolysis, which thereby lowers net ATP production. However, neither oxygen nor ozone at any concentration tested led to a breakdown of ATP in the dark.

Photoinduced, Amine-supported Swelling. The formation of a high energy intermediate prior to phosphorylation (either chemiosmotic H^+ gradient [22] or the chemical X-I intermediate, [26]) by electron transport is well established (10). If ozone inhibits ATP production in a manner similar to that of an energy transfer inhibitor, ozone should not affect this high energy intermediate (5). It is this high energy intermediate which seems also to be responsible directly for the gross swelling of chloroplasts which occurs in the light if amines are present (5). Problems in buffering the chloroplast suspension during ozone gassing prevent straightforward experiments on the H^+ movement itself using pH electrodes. Experiments along this line are now in progress.

Table II. *Inhibition of Ferricyanide-supported Photophosphorylation by Ozone*

Chloroplasts were treated and ferricyanide reduction and Pi esterification were measured as in "Materials and Methods." The variation of rates was about 5 to 10%.

Condition	Ferricyanide Reduction	Pi Esterification	P/e_2	
			Uncorrected	Correct for basal[1]
	$\mu eq/mg\ Chl^{-1} \cdot hr^{-1}$	$\mu moles/mg\ Chl^{-1} \cdot hr^{-1}$		
No gassing	490	164	0.67	1.7
O_3 gassed				
0	303	103	0.68	2.0
620 nmoles	254	70	0.54	2.5
900 nmoles	154	16	0.21	2.6

[1] The P/e_2-basal ratio was calculated according to Izawa *et al.* (16) by correcting the electron flow rate for uncoupled (−ADP) rate.

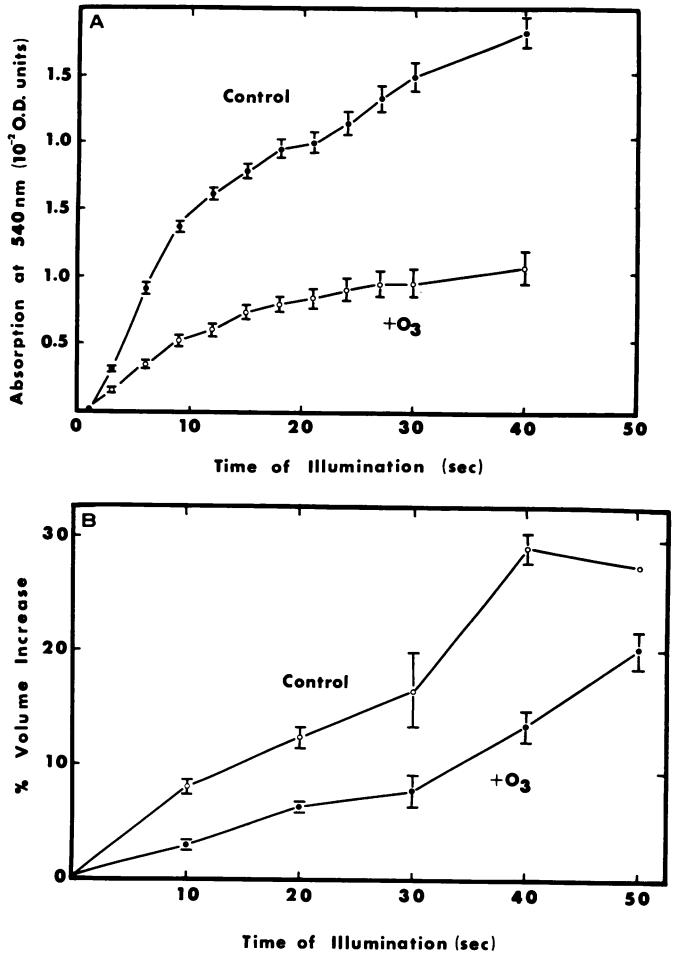

FIG. 3. Inhibition of the amine-supported, light-induced swelling of isolated Chl by ozone. Chloroplasts were bubbled with ozone (900 nmoles) as described in "Materials and Methods." A: Absorbance changes. The absorbance axis is for a decrease in absorption although plotted as increasing values. B: Coulter counter model volume change. Per cent volume increase in the per cent increase in the median of the size distribution of plastids (22).

Light scattering or absorbance studies (at a neutral wavelength) are identified with the formation of just such a high energy intermediate and swelling of the plastids (5, 6). Amine-induced swelling is accompanied by a decrease in absorbance at 540 nm (6). As seen in Figure 3A, ozone gassing into a suspension of plastids does decrease the extent of light-induced absorbance change by about 60% (at all times) without changing the apparent half-time of the rise. This should be compared with the percentage rate drop in FeCN reduction by ozone of 45% (Fig. 2) and in ATP production by ozone of 85% (Table II). The same percentage inhibition in swelling is observed if actual volume changes are measured by Coulter counter methods (13), as in Figure 3B. Ozone lowers the swelling extent by about 50 to 60% for each time interval, using a dose of 900 nmoles. In the dark, however, the total absorbance at 540 nm of plastids bubbled with ozone declines about 0.05 (A units, out of about 0.80) although no gross volume changes can be detected with the Coulter counter.

Attempted Reversal of Ozone Inhibition with Dithiothreitol. Sulfhydryls are easily oxidized by ozone in chemical systems,

as shown by Mudd (23); however, the oxidation often proceeds beyond the disulfide stage. Koukel *et al.* (19) showed that sulfhydryls are oxidized by peroxyactyl nitrate (another oxidant of photochemical smog), but this oxidation could be reversed by sulfhydryl reagents. The addition of dithiothreitol (1 mM) to the plastid suspension several minutes following gassing fails to stimulate electron transport. Thus, if sulfhydryls are being oxidized, they are oxidized beyond disulfides. Dithiothreitol is still present in suspension several minutes after its addition, as judged by its ability to reduce ferricyanide. Therefore, the lack of stimulation of electron transport by dithiothreitol was not due to its removal by any nonspecific reactions.

Bicarbonate-stimulated O_2 Evolution from Intact Plastids. One of the basic problems in the investigation of ozone injury to green plants is the depth of ozone penetration into the cell. In order to answer partially that question, intact chloroplasts with complete outer membranes were isolated from spinach. These plastids were subjected to ozone in the same manner as the grana stacks but, because of the fragility of the plastids, were gassed for only 1 min. These plastids were then tested to find (*a*) how ozone affected bicarbonate-stimulated O_2 evolution, and (*b*) how ozone affected the electron transport within the grana stacks after osmotically rupturing the intact plastids.

A trace of O_2 evolution from intact plastids (17) shows a time lag with little evolution, followed in several minutes by the development of the maximal rate of O_2 evolution. Ozone does not affect the lag time, but it does inhibit the maximal rate of O_2 evolution. It was noted that the time following plastid isolation had a great effect on the amount of apparent ozone inhibition of O_2 evolution. This is shown in Figure 4 for two separate preparations. The control rate of bicarbonate-stimulated O_2 evolution decreases quite rapidly after isolation (to about 20% of the original activity in about 3 hr). The apparent inhibition by ozone is much greater immediately after isolation compared to that observed at later times, as seen in the figure. However, the reduction of the maximal linear rate of bicarbonate-stimulated O_2 evolution is constant at 35%, regardless of the preparation over the time period shown in this figure.

The intact plastids were subjected to a dose of ozone and then osmotically ruptured so that the intact grana stacks could be used for studies of electron transport by the grana. While ozone reduced the amount of bicarbonate-stimulated O_2 evolution, the FeCN reduction rate after osmotic bursting was unaffected (Table III). As expected, the rate of bicarbonate-stimulated O_2 evolution falls as the dose of ozone increases. However, as shown in Table III, columns 2 and 3, for chloroplasts bubbled with either O_2 or O_3 the reduction rate after osmotic bursting is exactly the same. The percentage inhibition induced by ozone is statistically insignificant (Table III, column 4). Therefore, ozone at these concentrations does not penetrate the membrane and damage the grana stacks.

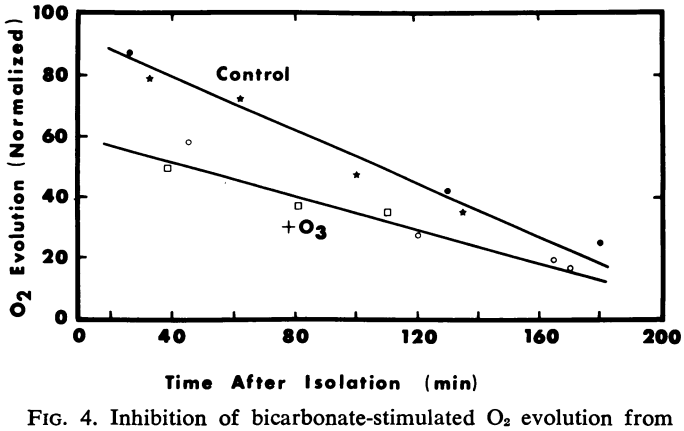

FIG. 4. Inhibition of bicarbonate-stimulated O_2 evolution from intact plastids. For experimental conditions see "Materials and Methods." Doses of O_3 were 400 nmoles for 1 min. The values for two preparations (● and ★: no O_3; ○ and □: O_3) are normalized by 105 and 92 μeq/mg Chl^{-1}·hr^{-1}.

Table III. *Effect of Ozone on Electron Transport Function in Intact Chloroplasts*

Ozone Added[1]	Ferricyanide Reduction[2]		Inhibition[4]
	Control[3]	+O_3	
moles	μeq/mg Chl^{-1}·hr^{-1}		%
0	—	443 ± 24	
40	426 ± 6	395 ± 15	7 ± 4
80	433 ± 9	416 ± 12	3 ± 3
240	354 ± 9	321 ± 9	9 ± 3
480	271 ± 3	262 ± 6	3 ± 2

[1] Ozone bubbled through solution as described in "Materials and Methods." Intact chloroplasts were used as described in "Materials and Methods" with approximately 85% intact, as judged by microscopic examination.

[2] Ferricyanide reduction was assayed as described in Figure 1. The rate of ferricyanide reduction without gassing and without rupturing was 212 ± 12 μeq/mg Chl^{-1}·hr^{-1}. The average is for four experiments.

[3] Control rate was obtained for chloroplasts gassed in the same manner as the chloroplasts (+O_3) but with the O_3 removed by activated charcoal.

[4] $\text{Inhibition} = \frac{\text{rate (control)} - \text{rate } (O_3)}{\text{rate (control)}} \times 100\%$.

DISCUSSION

This paper is addressed to two basic questions which have arisen in connection with the mechanisms of ozone injury to green plants. The first question revolves around membrane damage by ozone. Many studies (8, 20) have indicated that the primary site of ozone attack is the membrane; however, it is not yet clear whether ozone generally disrupts the membrane system such as a detergent or specifically affects certain sites, thereby making the membrane "leaky." The second question is concerned with depth of ozone penetration into the cell. If the primary site of ozone injury is the cell plasmalemma, are the other effects which are noted by electron microscopy (28) and metabolic studies (3, 8) due to an ionic imbalance caused by leakage from the primary site or are these other alterations due to a secondary attack site within the cell?

The results presented in this paper indicate that ozone-induced membrane disintegration is not general (at the concentrations employed by this study). Photophosphorylation is usually the most labile system within the chloroplast (12). High light intensities, high temperature, detergents, and uncouplers inhibit photophosphorylation before inhibiting the electron transport. In fact, often as photophosphorylation is inhibited, electron transport is concurrently stimulated, similar to the uncoupling action of ammonium ion. Even with energy transfer inhibitors (10) the formation of ATP production still does decrease.

The percentage inhibition of system I-linked electron flow (Table I) is very similar to percentage inhibition of photophosphorylation catalyzed by cyclic electron flow. Thus, it appears

that the inhibition of cyclic photophosphorylation is due to an inhibition of electron transport within photosystem I. In this regard, Table II is more informative; however, the interpretation of this table leads directly into a major controversy regarding the number of sites of ATP production in photosystems I and II (16). Most investigators (10) take the rate of phosphate esterification divided by the observed rate of electron flow to find the P/e_2 ratio. As shown in Table II which was calculated by this method, the P/e_2 ratio does, in fact, drop. However, if the P/e_2 ratio is calculated from a corrected electron flow rate (observed electron flow rate minus the basal electron flow rate) (16), then within limits of the experiment's variability, the P/e_2 ratio actually remains the same. Ozone is a very ineffective energy transfer inhibitor, if it is one at all; in fact, the rate of ATP production inhibition drops just a little faster than the rate of electron flow with added ozone.

In addition, if ozone were an energy transfer inhibitor such as phlorizin and Dio-9, then the high energy intermediate of phosphorylation would not be disrupted and only the formation of ATP (10) would be affected, not the pH gradient (22). In fact, the high energy intermediate should be increased. The plastids are gassed by ozone in a buffered media in order to prevent any nonspecific pH changes and, thus, the external measurement of pH changes is not possible.

A possible measurement is that of amine-supported, photoinduced, high amplitude swelling (5, 6), which is measured by both the Coulter counter and absorbance (Fig. 3) and is inhibited by ozone. Thus, the concentration of the high energy intermediate is decreased by ozone, probably because of reduced electron flow. The final conclusion must be that ozone inhibits only electron transport and that its effect upon photophosphorylation must be due to that inhibition.

Ozone does not seem to lead to a significant increase in proton permeability of the membrane; Figure 3, A and B, shows that even with 50% inhibition of electron transport, the build-up of a high energy intermediate, a proton gradient, or both (22) can still occur. Therefore, the inhibition must be due to an alteration in the transfer of photon energy from the collecting Chl to the photosynthetic trap. In other words, energy from the light is not getting in to the electron transfer pathway, a fact caused either by a disruption of the organization of the Chl (27), by a possible chemical alteration of the pigments by ozone (18), or by both. Furthermore, Table I shows that the percentage inhibition of electrons within the two photosystems by ozone seems to add, indicative of a degree of the inhibitions independence. Experiments relating to ozone-induced changes in the flow of light energy into the traps are now in progress and will be reported in a later publication.

Ozone does not seem to penetrate membranes readily. While it depresses the rate of bicarbonate-stimulated oxygen evolution (Fig. 4), the electron flow capabilities of the grana stacks within the chloroplasts are not altered (Table III). Thus, the ozone-induced damage to intact plastids is separate and distinct from the ozone-induced damage to isolated grana stacks. It is not the subject of this paper to inquire into how ozone inhibits bicarbonate-stimulated oxygen evolution from intact plastids, but rather to discover whether or not penetration of ozone occurs. Nobel *et al.* (24) have shown that a selective permeability increase for certain compounds is caused by ozone in intact plastids. The escape of intermediates into the suspending media seems a likely possibility.

The lack of penetration of ozone would indicate that, for moderate concentrations, the initial site of ozone injury is not beyond the plasmalemma. Thus, the alterations observed in metabolic studies (3, 8) and in electron microscopy (28) must be due to osmotic and ionic imbalances caused by a leaky plasmalemma. Furthermore, water-stressed plants (21) show the same inhibition of growth and cell wall elongation as that observed in ozone injury (4, 25). Obviously, work needs to be done to determine how ionic and water imbalances induce metabolic alterations.

Finally, the problem exists of comparing physiological doses of ozone in solution and in air. For atmospheric environments, injury to green plants is observed (in bean plants) after an exposure of plants to approximately 0.1 to 0.2 μl/liter ozone for 1 hr. However, in the experiments reported here, the maximal dose is given by a 5-min exposure of the aqueous solution to 400 μl/liter ozone. We believe that this dose is, in fact, physiological for aqueous solutions since most of the ozone (90%) passes through the solution unchanged. Indeed, the actual ozone concentration within the solution is governed by the partial pressure of the ozone above it (2). From the Bensen coefficient of ozone at O C (2) and the partial pressure of ozone above the aqueous solution, the ozone concentration within the solution can be calculated to be about 10 μM. This concentration is low and, expressed on the basis of number of ozone molecules per number of water molecules, comes out to be approximately 0.2 μl/liter for the highest dose rate. Therefore, the number of ozone molecules impinging upon the membrane surface per number of solute molecules is roughly the same for a chloroplast in an aqueous solution and a leaf cell in the physiological, aerial environment. In addition, the absence of any physiological response of chloroplasts to ozone for concentrations of ozone (bubbled through the solution) lower than a total dose of approximately 20 nmoles (Fig. 2) would argue that the region above this is physiological and that doses of ozone from 50 to 500 nmoles or exposure to a gaseous environment of 400 μl/liter or less ozone for 5 min is also physiological.

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