Plant Physiol. (1984) 74, 846–851
0032-0889/84/74/0846/06/$01.00/0

Peroxidase Release Induced by Ozone in *Sedum album* Leaves

INVOLVEMENT OF Ca^{2+}

Received for publication July 7, 1983 and in revised form November 18, 1983

FEDERICO J. CASTILLO*, CLAUDE PENEL, AND HUBERT GREPPIN
Laboratoire de Physiologie Végétale, Université de Genève, 3 Place de l'Université, 1211 Genève 4, Switzerland

ABSTRACT

The effect of ozone was studied on the peroxidase activity from various compartments of *Sedum album* leaves (epidermis, intercellular fluid, residual cell material, and total cell material). The greatest increase following a 2-hour ozone exposure (0.4 microliters O_3 per liter) was observed in extracellular peroxidases. Most of the main bands of peroxidase activity separated by isoelectric focusing exhibited an increase upon exposure to ozone. Incubation experiments with isolated peeled or unpeeled leaves showed that leaves from ozone-treated plants release much more peroxidases in the medium than untreated leaves. The withdrawal of Ca^{2+} ions reduced the level of extracellular peroxidase activity either in whole plants or in incubation experiments. This reduction and the activation obtained after addition of Ca^{2+} resulted from a direct requirement of Ca^{2+} by the enzyme and from an effect of Ca^{2+} on peroxidase secretion. The ionophore A23187 promoted an increase of extracellular peroxidase activity only in untreated plants. The release of peroxidases by untreated and ozone-treated leaves is considerably lowered by metabolic inhibitors (3-(3,4-dichlorophenyl)-1,1-dimethylurea and sodium azide) and by puromycin.

Peroxidase activity increases in plants in response to a great variety of stresses, including viral, microbial, or fungal infections, salt stress, wounding, or air pollution (9). Several pollutants such as ozone (5, 6, 22), SO_2 (14, 16), or NO_2 (13) are known to induce an enhancement of the total peroxidase activity of plants. The peroxidase increase following an exposure to ozone is different in different species and is a function of the resistance of the plant to ozone. Ozone-tolerant and ozone-sensitive cultivars have been described, the peroxidase activity of the former being less affected by ozone (5).

In a previous work (4), it was shown that there is a parallelism between the level of air pollution and the peroxidase activity measured in *Sedum album* leaves. This was demonstrated in plants grown in diversely polluted areas. It appeared that *S. album* is a suitable plant material for the study of the mechanism leading plants exposed to a pollutant to increase their peroxidase activity.

The present work was performed in the laboratory, under controlled environmental conditions and using standardized amounts of ozone as air pollutant. The dose applied to *S. album* (0.4 $\mu l\ l^{-1}$ for 2 h) is known to increase the total peroxidase activity in many plant species (21, 22). Peroxidase activity was measured in several leaf compartments. The data obtained showed that after an exposure to ozone the most significant increase of peroxidase activity occurred in the extracellular compartment. As the release of peroxidase was reported to be a calcium-dependent process in spinach cell suspensions (18), the possible involvement of this ion in the control of the extracellular peroxidases of *S. album* leaves was also investigated in relation with the response to ozone.

MATERIALS AND METHODS

Plant Material and Growing Conditions. *Sedum album* plants were generated by vegetative propagation from a single plant and grown in a glass-house in 10-cm pots containing ordinary garden soil. Plants were watered three times per week and fertilized weekly with a Hoagland nutrient solution. Four weeks after propagation, they were transferred to a controlled environment chamber at 20 ± 1°C and 75 ± 5% RH. A 8-h day/16-h night cycle was provided with irradiances of 20 w/m^2 supplied by Sylvania day-light fluorescent tubes. The leaves used for the extractions were always taken from stems containing about 20 leaves. The five younger and the five older leaves were discarded.

Ozone Exposure. Plants were exposed to ozone in a 50 × 50 × 50-cm vinyl chamber placed in a controlled environment chamber in order to maintain light, temperature, and RH at the same levels as in the growth chamber. Air was drawn through a charcoal filter, supplemented with the desired ozone concentration before entering the exposure chamber, at a rate of 8 l/min. Ozone was produced by passing a stream of charcoal-filtered air through a high voltage electric discharge ozonizer. The concentration of ozone was determined at the plant level by bubbling 10-ml aliquots of KI solution for various time intervals. The concentration of ozone was then calculated from the *A* at 350 nm using an extinction coefficient of 24 $mM^{-1}\ cm^{-1}$, with the assumption that one I_2 molecule is produced by one ozone molecule (3). Five-week-old plants were exposed for 2 h to 0.4 $\mu l\ O_3\ l^{-1}$, 2 h after the beginning of the light period. Following this treatment, they were returned to the growth chamber until harvested.

Preparation of Leaf Fractions. Peroxidase and G6PDH[1] activities were assayed in different leaf compartments obtained as follows. Samples containing 3 g tissue (20–30 leaves/g) were harvested 24 h after ozone exposure always at the middle of the light period to prevent variability in data arising from the possible photoperiodic rhythms. After washing with distilled H_2O, leaves (1 g) were peeled and epidermes were washed with 100 mM KCl and homogenized in a glass homogenizer with 0.5 ml ice-cold 40 mM MOPS-KOH, 100 mM KCl (pH 6.5) buffer. Enzymes from the intercellular spaces of leaves were extracted by the technique of Rathmell and Sequeira (17). One g peeled leaves were vacuum infiltrated (three periods of 30 s each) with distilled H_2O. The fluid recovered after centrifuging the leaves at 1,500*g*

[1] Abbreviations: G6PDH, glucose-6-phosphate dehydrogenase; MOPS, *N*-morpholino-3-propane sulfonic acid.

for 5 min at 4°C constituted the free extracellular peroxidases fraction. The remaining leaves were infiltrated again with 100 mM KCl and the fluid collected after centrifugation corresponded to the ionically bound extracellular peroxidases. Alternatively, extracellular peroxidases (free plus ionically bound) were obtained in one step by infiltration and centrifugation of another sample of leaves (1 g) with 100 mM KCl. The remaining leaf tissue was ground in liquid N_2 in a micro-dismembrator (Braun) with insoluble PVP (1 g/g tissue) in 40 mM MOPS-KOH, 100 mM KCl (pH 6.5) buffer. The extract was filtered and centrifuged at 10,000*g* for 10 min. The resulting supernatant constituted the soluble fraction of residual cell material. Total cell material (whole leaves) extract was obtained by grinding 1 g of washed intact leaves as indicated above. Intercellular fluid for the assay of G6PDH was obtained by vacuum infiltration of leaves with 60 mM Tris-HCl (pH 7.5) buffer, 2 mM EDTA, 20 mM 2-mercaptoethanol, and 100 mM KCl. Leaf extract was made by grinding 0.5 g of washed leaves with the same buffer added with insoluble PVP (1 g/g tissue).

For incubation experiments, peeled or unpeeled leaves were vacuum infiltrated with 100 mM KCl and centrifuged as indicated above. The remaining leaf tissue was distributed in 25-ml glass flasks (0.5 g leaves/flask) containing 10 ml of 100 mM sorbitol and various chemicals specified in each case and infiltrated again for one period of 30 s. Flasks were placed in a heavy duty Rotator (Cole-Parmer) and gently and continuously shaken under illumination with fluorescent day-light at 20°C. Peroxidase release was assayed by taking 100 μl samples at different times. Calcium chloride, lanthanum chloride, EGTA, NaN_3, DCMU, puromycin (Sigma), and calcium ionophore A23187 (Calbiochem-Behring Corp.) were added to the incubation medium at concentrations specified in each case using small volumes of stock solutions.

Enzyme Assays. Peroxidase assay was performed at 25°C in 3 ml of 60 mM phosphate buffer (pH 6.1) containing 16 mM guaiacol and 2 mM H_2O_2. Increase in absorbance was recorded at 470 nm with a Unicam SP 1700 Spectrophotometer. The reaction was linear for 30 min. G6PDH activity was assayed in 1 ml 60 mM Tris-HCl (pH 8.1) buffer with 150 mM $MgCl_2$, 6 mM NADP, 20 mM glucose-6-P, and 50 μl enzyme preparation. Increase in NADPH absorbance was monitored at 340 nm. Protein was determined using the Coomassie G-250 dye-binding assay (2) with crystalline BSA as a standard.

Isoelectric Focusing. Thin-layer isoelectric focusing was performed in agarose (LKB) gel using pH 3.5 to 9.5 (3.6%) and pH 9 to 11 (1.8%) ampholytes on 12 × 12 cm plastic sheets. Enzyme extracts (20–40 μl) were applied to the gel layer on paper pieces. Focusing was carried out at constant power (6 w) with a maximum voltage of 400 v for 2 h. Isozymes bands were stained with 2 mM benzidine in acetate buffer (pH 4.5). The reaction was initiated by adding 3 mM H_2O_2.

RESULTS

The distribution of peroxidase activity and soluble proteins among various fractions isolated from *S. album* leaves is shown in Table I. The peroxidase activity associated with isolated epidermis and that extracted from intercellular spaces with distilled H_2O represent only a minor part of the total activity. Most of the activity cannot be recovered by vacuum infiltration and corresponds to intracellular peroxidases and peroxidases trapped in the wall and liberated by cell disruption. Leaves collected 24 h after a 2-h ozone exposure exhibit an enhanced peroxidase activity in all the fractions tested. Free extracellular peroxidases show the greatest increase and, more generally, the extracellular peroxidases are more sensitive to ozone treatment than those from residual cell material. It was also determined that the transfer of plants to the exposure chamber without ozone did not affect peroxidase activity (data not shown). Soluble protein values do not show significant differences between control and treated plants, except in the free extracellular fraction which contains more proteins after an exposure to ozone. Thus, with the exception of this fraction, the comparison of enzyme activities is similar whether peroxidase activity is expressed on the basis of soluble protein or fresh tissue weight. Measurement of G6PDH, a typical cytosolic enzyme, in extracellular fraction and whole leaves, revealed that there is little leakage of cytoplasmic proteins upon vacuum infiltration and centrifugation of the leaves. This experimental procedure thus does not allow an artifactual leakage of peroxidases by damaging the cell membrane. It can be noticed that ozone treatment increases the total activity of G6PDH. No visible injury was observed within 72 h after ozone treatment (data not shown).

Peroxidase is known to occur as an isozyme. Figure 1 shows the isoperoxidase pattern of the various fractions described in Table I, separated by agarose gel isoelectric focusing. The main bands are found in the acidic part of the pH gradient (A_6, A_7, A_8) and near pH 11 (C_1). There are also several very minor bands (A_1, A_2, A_3, A_4, A_5, A_9) which are not always present and could be considered as artifacts. There is no great qualitative difference between the diverse fractions as far as the main bands are concerned. The cationic peroxidase C_1 is missing in epidermis. Exposure to ozone does not induce new isozyme bands but increases the intensity of the main acidic bands, whereas C_1 seems to be slightly decreased.

Figure 2 shows the increase of peroxidase activity in the intercellular fluid and in the total cell material, following a 2-h exposure to ozone. Whereas peroxidase activity remains almost constant in untreated plants, it progressively increases in the treated ones over a period of 3 d. The effect of ozone is much more pronounced on extracellular activity which is finally increased by 3.6 times than on the total activity which is only enhanced by 1.3 times.

As the control of the extracellular peroxidase level in cell suspension culture has been shown to be under the control of Ca^{2+} (18), the influence of this ion on the ozone-induced increase of extracellular peroxidase in *S. album* leaves was determined. For that purpose, whole plants were carefully depotted and their roots were dipped in distilled H_2O supplemented or not with different concentrations of EGTA or $CaCl_2$. Four h later, these plants were subjected to ozone for 2 h. Extracellular peroxidase activity was determined 24 h later. Table II shows that ozone exposure induces an increase of this fraction in leaves of plants dipped in distilled H_2O. EGTA, at a concentration of 1 mM, decreases the extracellular peroxidase activity of untreated plants and prevents the ozone-induced increase of extracellular peroxidase activity. Calcium chloride at three different concentrations has a slight promotive effect on the extracellular peroxidase activity. This effect is almost maximum at 5 mM $CaCl_2$ and is more pronounced in control plants. The inhibitory effect of EGTA on ozone-treated plants is suppressed by the presence of Ca^{2+}. It therefore appears that Ca^{2+} is required for the peroxidase response to ozone to occur.

Incubation experiments were also performed in which leaves, previously vacuum infiltrated and centrifuged in order to remove their extracellular peroxidases, were immersed in 100 mM sorbitol. Peroxidase activity was assayed in the incubation medium. It appears that leaves from plants exposed to ozone 24 h before harvesting release much more peroxidase in the incubation medium than unexposed leaves, although the extracellular spaces of leaves were freed from peroxidases at the beginning of incubation (Fig. 3). EGTA reduces the level of peroxidase activity found in the incubation medium. The addition of Ca^{2+} rapidly increases this activity and overcomes the inhibitory effect of EGTA.

A similar experiment was conducted with isolated leaf epider-

Table I. *Peroxidase and G6PDH Activities and Protein Content in Different Leaf Compartments Prepared from Control and Ozone-Treated Plants*

FEP, free extracellular peroxidases; IEP, ionically bound extracellular peroxidases; EP, extracellular peroxidases. For further explanations, see "Materials and Methods." The values are the average ± SD of at least 12 independent extractions (three measurements per extraction). Plants were harvested 24 h after ozone exposure.

Fraction		Epidermis	Intercellular Fluid			Residual Cell Material	Total Cell Material
			FEP (1)	IEP (2)	EP (1) + (2)		
Peroxidase activity (ΔA_{470} min^{-1} g^{-1} fresh wt leaves)	$-O_3$	0.49 ± 0.03	0.52 ± 0.03	2.07 ± 0.16	2.78 ± 0.22	27 ± 2	31 ± 2
	$+O_3$	0.69 ± 0.05	3.03 ± 0.39	4.36 ± 0.51	7.61 ± 0.54	31 ± 3	41 ± 3
G6PDH activity (nmol NADPH min^{-1} g^{-1} fresh wt leaves)	$-O_3$	Not tested	Not tested	Not tested	0.164 ± 0.013	Not tested	55 ± 5
	$+O_3$				0.173 ± 0.014		84 ± 7
Protein (µg g^{-1} fresh wt leaves)	$-O_3$	181 ± 16	3.11 ± 0.21	7.98 ± 0.41	12 ± 1	2114 ± 153	2157 ± 151
	$+O_3$	196 ± 17	4.84 ± 0.23	8.34 ± 0.32	13 ± 1	2144 ± 162	2219 ± 132

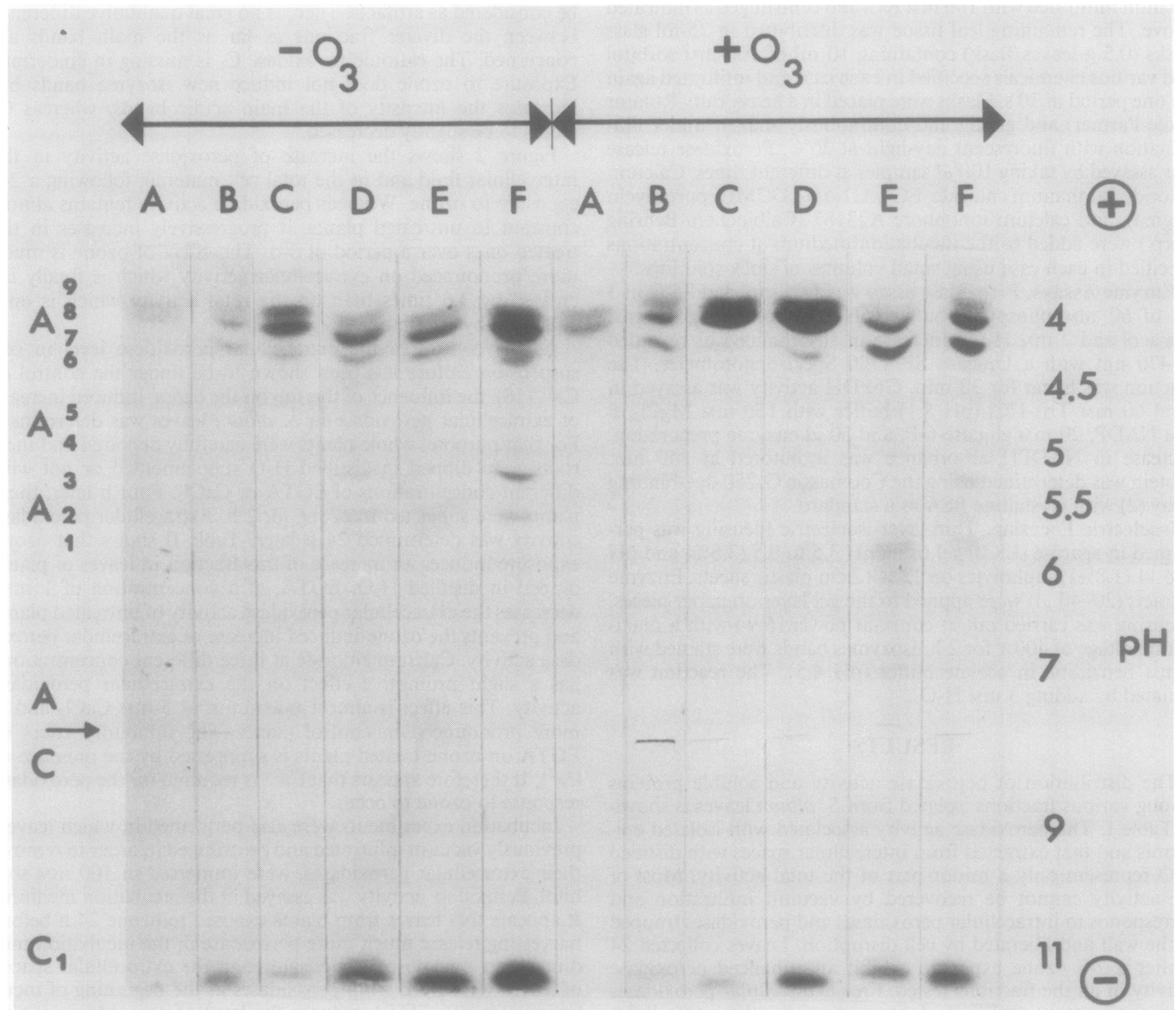

FIG. 1. Zymogram patterns of *S. album* peroxidase isozymes in several leaf compartments of control and ozone-treated plants, 24 h after a 2-h ozone exposure. Anode at the top. A, epidermis (20); B, free extracellular peroxidase (4); C, ionically bound extracellular peroxidases (4); D, extracellular peroxidases (4); E, residual cell material (40); F, total cell material (40). Values in parenthesis indicate the amount of protein in µg contained in the respective samples.

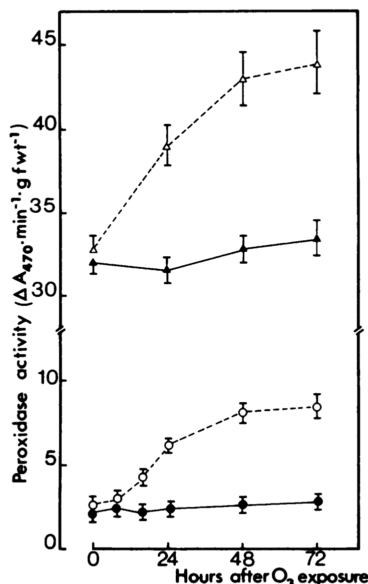

FIG. 2. Extracellular (●, ○) and total cell material (▲, △) peroxidase activities from control (●, ▲) and ozone-treated (○, △) plant leaves at different times after a 2-h ozone exposure. Values are the average of six different experiments (three replicates each) with SD indicated by bars.

Table II. *Influence of a Pretreatment with EGTA or Ca^{2+} on the Extracellular Peroxidases of Control and Ozone-Treated Plants*

Plants were depotted and their roots dipped in distilled H_2O supplemented or not with different concentrations of EGTA or/and Ca^{2+}, 4 h before the ozone treatment, and maintained in these conditions until harvesting 24 h after ozone treatment. Values are the average ± SD of four different experiments with three replicates in each experiment.

Treatments	$-O_3$	$+O_3$
	ΔA_{470} min^{-1} g^{-1} fresh wt leaves	
Control	4.13 ± 0.33	9.89 ± 0.73
0.1 mM EGTA	3.99 ± 0.27	8.51 ± 0.81
1 mM EGTA	3.17 ± 0.18	4.97 ± 0.43
1 mM Ca^{2+}	4.41 ± 0.39	10.12 ± 0.94
5 mM Ca^{2+}	5.03 ± 0.41	10.86 ± 0.85
10 mM Ca^{2+}	5.16 ± 0.53	11.01 ± 1.07
1 mM EGTA + 5 mM Ca^{2+}	4.28 ± 0.36	9.94 ± 0.86

mis and with leaves without their epidermis (Fig. 4). Peeled leaves release peroxidase activity at twice the rate exhibited by unpeeled leaves. The addition of 1 mM EGTA 95 min after the beginning of incubation rapidly inhibits the increase of peroxidase activity in the incubation medium. The addition of 5 mM $CaCl_2$ promotes this increase as in Figure 3. The cationophore A23187 enhances the effect of Ca^{2+} in the case of unexposed leaves but not in the case of ozone-exposed leaves. Isolated epidermis only releases a small amount of peroxidase activity.

The effect of Ca^{2+} on the peroxidases themselves was also tested. For that purpose, leaves were removed from their incubation medium after a 90-min incubation (Fig. 4) and aliquots of the medium were tested for peroxidase activity 25 min after the addition of Ca^{2+}, ionophore A23187, or EGTA. Table III shows the effects of these substances on the peroxidase activity compared to their effects obtained for the same period when leaves were present. It appears that Ca^{2+} activates peroxidases in the absence of leaves by 40%. But the enhancement of activity was greater in the presence of leaves (88%). A23187 activates the release of peroxidase activity in the medium containing unexposed leaves. It has a weak effect on ozone-exposed leaves and is without effect in the medium alone. The Ca^{2+}-chelating agent EGTA decreases the activity both in the presence and in the absence of leaves, but the decrease is less important in the presence of leaves. These data suggest a dual effect of Ca^{2+} on both the enzyme molecule, which is known to require Ca^{2+} for being active (11), and the leaf cells which release more peroxidase in presence of Ca^{2+}.

Table IV shows the effect of Ca^{2+}, EGTA, and La^{3+} on isolated extracellular and residual cell material peroxidases. Extracellular peroxidases are not responsive to Ca^{2+} addition and are inactivated by EGTA or La^{3+}. The activity of residual cell material peroxidases is enhanced by Ca^{2+} and inhibited by EGTA and La^{3+}. The inactivation of these peroxidases by EGTA is reversed

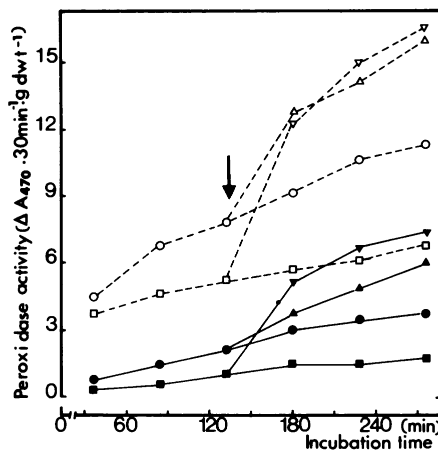

FIG. 3. Peroxidase activity released by whole leaves (with their epidermis) from control (●, ■, ▲, ▼) and ozone-treated (○, □, △, ▽) plants. Leaves were placed in the incubation medium without (●, ○) and with (■, □) 1 mM EGTA, 24 h after a 2-h ozone exposure. Ca^{2+} (5 mM) was added 135 min (arrow) after the beginning of the incubation period on control and ozone-treated leaves without (▲, △) and with (▼, ▽) EGTA. Values are the averages of four diffeent experiments with three replicates in each department. Standard deviations were less than 10% of the mean for control and ozone-treated plants.

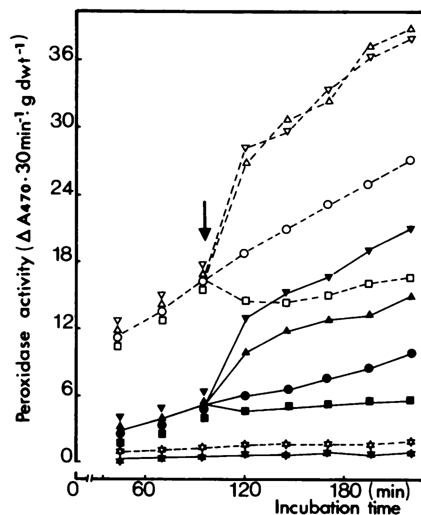

FIG. 4. Peroxidase activity released by epidermis (★, ☆) and peeled leaves (●, ○) from control (★, ●, ▲, ▼, ■) and ozone-treated (☆, ○, △, ▽, □) plants. Twenty-four h after a 2-h ozone exposure, leaves were harvested, peeled, infiltrated, centrifuged, and placed in the incubation medium. 5 mM Ca^{2+} (▲, △), 5 mM Ca^{2+} (▼, ▽) plus 1 µM A23187, and 1 mM EGTA (■, □) were added 95 min (arrow) after the beginning of the incubation to the flasks containing the peeled leaves. Values are the average of four different experiments with three replicates in each experiment. Standard deviations were less than 10% of the mean for control and ozone-treated plants.

Table III. *Effect of the Addition of Ca^{2+}, A23187, and EGTA on the Peroxidase Activity from the Incubation Medium in the Absence or in the Presence of Leaves*

Leaves were removed from or maintained in their incubation medium after a 90-min incubation and aliquots of the medium were tested 25 min after the addition of chemicals (see Fig. 4). One-hundred % peroxidase activity (ΔA_{470} 30 min^{-1} g^{-1} dry wt) of control and treated leaves were 5.23 ± 0.51 and 15.6 ± 1.31, respectively, measured in the incubation medium just before the addition of chemicals. Values are the average of four different experiments with three replicates in each experiment.

Treatments	Peroxidase Activity (% of the Controls)			
	$-O_3$		$+O_3$	
	Without leaves	With leaves	Without leaves	With leaves
Control	100	114	100	118
5 mM Ca^{2+}	140	188	141	173
1 μM A23187	100	135	100	123
5 mM Ca^{2+} + 1 μM A23187	137	245	142	182
1 mM EGTA	65	87	59	90
1 mM EGTA + 5 mM Ca^{2+}	131	177	135	168

Table IV. *In Vitro Effect of Ca^{2+}, La^{3+}, and EGTA on the Activity of Extracellular and Residual Cell Material Peroxidases of Control Plants*

Chemicals were added into the test tubes at the indicated concentrations. Peroxidase assays were carried out at 25°C, by taking aliquots from the test tubes. Values are the average ± SD of 10 measurements done at different intervals during a 4-h period. Similar results were obtained in three other experiments.

Treatments	Extracellular peroxidases	Residual cell material peroxidases
	ΔA_{470} min^{-1} g^{-1} fresh wt leaves	
Control	2.51 ± 0.12	28.01 ± 1.68
5 mM Ca^{2+}	2.57 ± 0.17	41.87 ± 3.35
5 mM La^{3+}	1.85 ± 0.09	19.61 ± 1.58
1 mM EGTA	1.92 ± 0.21	11.21 ± 0.91
1 mM EGTA + 5 mM Ca^{2+}	2.29 ± 0.22[a]	42.65 ± 4.23[a]
1 mM EGTA + 5 mM La^{3+}	0.23 ± 0.01[a]	4.96 ± 0.31[a]

[a] Ca^{2+} or La^{3+} were added 90 min after the addition of EGTA.

by the further addition of Ca^{2+}. Such a reversibility is only partial in the case of extracellular peroxidases. When La^{3+} and EGTA are added together, they strongly inhibit peroxidase activity in both cases. Thus, the sensitivity of peroxidases towards Ca^{2+} and EGTA is different, depending on their cellular localization within the leaves.

The above results do not unequivocally show whether the increasing extracellular peroxidase activity after ozone exposure depends on an activation of the enzyme or an activated release of preexisting wall peroxidases or on a release or secretion of enzymes from the cells. To answer this question, inhibitors were used in an incubation experiment with untreated and ozone-treated peeled leaves. It appears from the data presented in Figure 5 that NaN_3 and DCMU given together strongly inhibit the release of peroxidase by untreated or ozone-treated leaves. NaN_3 was used at a concentration which is not directly inhibitory for *S. album* peroxidases. Puromycin, at a concentration of 0.5 mM, increasingly inhibits peroxidase release. In the presence of puromycin, peroxidase activity in the incubation medium is only 50% of the activity measured in medium without puromycin. The kinetics of the inhibition in control and ozone-exposed leaves is similar. This result suggests that, in these conditions, the synthesis and secretion of peroxidases by peeled leaves proceed continuously and that the rate of one of these steps or both is accelerated by ozone.

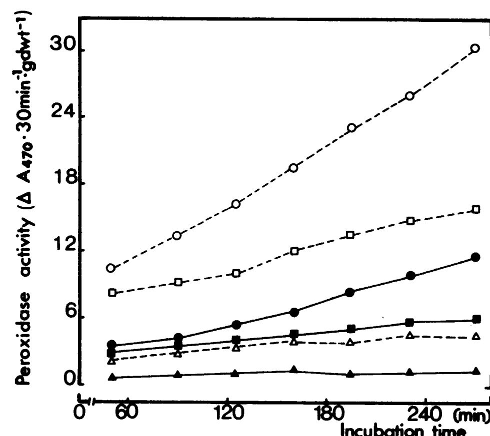

FIG. 5. Peroxidase activity released by peeled leaves from control (●, ■, ▲) and ozone-treated (○, □, △) plants, 24 h after a 2-h ozone exposure. Leaves were placed in the incubation medium alone (●, ○) or containing 0.5 mM puromycin (■, □) or 0.5 mM Na azide plus 0.05 mM DCMU (▲, △). Values are the average of four different experiments with three replicates in each experiment. Standard deviations were less than 10% of the mean for control and ozone-treated plants.

DISCUSSION

The data unequivocally show that extracellular peroxidase activity is strongly increased after an exposure of *S. album* to ozone. The extracellular peroxidases, which include enzymes present in the intercellular spaces and enzymes ionically bound to cell walls, are known to be involved in the polymerization of lignin precursors (10), suberization (1), and the cross-linking of proteins or other molecules with wall material (23). Peroxidases may also be useful by hardening cell membranes by the formation of bridges between tyrosine residues of membrane proteins (8). Such a reaction could allow polluted plants to reduce their cellular permeability. An increased peroxidase activity may also be linked to a decreased rate of growth (9).

Calcium seems to play a crucial role in the response of peroxidase to ozone. Plants fed with a solution of EGTA do not respond to the pollutant, whereas the presence of Ca^{2+} reverses this effect. The addition of A23187 with Ca^{2+} was without effect in ozone-treated plants, although the ionophore increases the effect of Ca^{2+} in plants which were not exposed to ozone.

In addition to these effects, there was also a direct effect of Ca^{2+} on the peroxidases themselves (Tables III and IV). Such an effect was already reported by Haschke and Friedhoff (11) who showed that the removal of Ca^{2+} from a horseradish root isoperoxidase reversibly inactivated its catalytic properties. The effect of EGTA and La^{3+} on *S. album* peroxidases confirms the specific requirement of these enzymes for Ca^{2+}. Extracellular peroxidases are weakly sensitive to changes in Ca^{2+} concentrations, while residual cell material peroxidases are strongly activated by Ca^{2+} addition. The peroxidases which diffuse outside the leaves to incubation experiments are also activated by Ca^{2+}. To explain these differences it may be hypothesized that an increase in the cytoplasmic Ca^{2+} concentration is required for peroxidase secretion (19). This involvement of Ca^{2+} in peroxidase secretion has already been described in plant cell suspensions (18). Newly secreted peroxidases would be activated by the incorporation of Ca^{2+} once they are in contact with extracellular spaces, rich in Ca^{2+}. In incubation experiments, leaves previously drained of their extracellular peroxidases and extracellular free Ca^{2+} by vacuum infiltration released intracellular peroxidases prone to

activation which can no more be activated in extracellular spaces. If this hypothesis is correct, ozone would trigger the secretion of peroxidases by raising the intracellular level of Ca^{2+}. In plants which are not exposed to ozone, a comparable increase of extracellular peroxidases can be obtained by applying the ionophore A23187 alone or with Ca^{2+} (Fig. 4; Table III). Such a treatment would result in an increase in the cytoplasmic Ca^{2+} concentration. An alternative explanation could be that Ca^{2+} activates a peroxidase pool located in periplasmic space and wall. Upon activation by Ca^{2+}, for example released by cells, these peroxidases become extractable by vacuum infiltration or diffuse towards intercellular spaces and the exterior of leaves. This second hypothesis, however, cannot explain the positive effect of A23187 on untreated plants.

The effect of metabolic inhibitors and puromycin (Fig. 5) indicates that the extracellular peroxidase increase observed after exposure to ozone is not a simple release or activation of enzymes in the walls. It may be concluded from the inhibition obtained by the addition of NaN_3 and DCMU that the secretion of peroxidases is an active, energy-requiring process. The important and rather rapid inhibition produced by puromycin suggests that the accelerated rate of peroxidase release by ozone-treated leaves is directly linked to a *de novo* synthesis of proteins. This inhibition was obtained in incubation experiments which include as a first step the withdrawal of preexisting extracellular peroxidases by vacuum infiltration. Thus, during the incubation time, the peroxidase activity released by leaves and measured in the medium could have as an origin either a reserve pool located in walls and periplasmic space or the cells themselves. The inhibition by puromycin allows the conclusion that these peroxidases come from the interior of cells and that cells of ozone-treated leaves synthetize and secrete more peroxidase than cells from untreated leaves.

Ozone does not damage cell membranes such that macromolecules can be lost from the cytoplasm. This is confirmed by the very low level of the cytosolic enzyme G6PDH found in extracellular spaces. However, it has often been reported that ozone induces a leakage of solutes (7, 12, 15, 20). Measurements of the electrical conductivity of extracellular fluids of *S. album* leaves have revealed a slight increase of 13% just after a 2-h exposure to ozone or 24 h later (data not shown). Also, extracellular potassium increased by 15% during this same period, as compared to untreated plants (data not shown). It is not possible at this time to correlate these small ionic redistributions with Ca^{2+} movements or with the changes observed in extracellular peroxidase level. The possible importance of injury-mediated Ca^{2+} influx into plant cells was recently suggested (24) but needs to be substantiated.

Acknowledgment—The technical assistance of Lydie Chané is gratefully acknowledged.

LITERATURE CITED

1. Borchert R 1978 Time course and spatial distribution of phenylalanine ammonia-lyase and peroxidase activity in wounded potato tuber tissue. Plant Physiol 62: 789–793
2. Bradford MM 1976 A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
3. Byers DH, BE Saltzman 1956 Determination of ozone in air by neutral and alkaline iodide procedures. Adv Chem 21: 93–102
4. Castillo FJ, H Greppin 1980 Comparaison entre différents "marqueurs" de l'environnement genevois: utilisation de la peroxydase. CR Soc Phys Nat Genève 15: 57–60
5. Curtis CR, RK Howell, DF Kremer 1976 Soybean peroxidases from ozone injury. Environ Pollut 11: 189–194
6. Endress AG, SJ Suarez, OC Taylor 1980 Peroxidase activity in plant leaves exposed to gaseous HCl or ozone. Environ Pollut Ser A 22: 47–58
7. Evans LS, IP Ting 1973 Ozone-induced membrane permeability changes. Am J Bot 60: 155–162
8. Foerder CA, BM Shapiro 1977 Release of ovoperoxidase from sea urchin eggs hardens the fertilization membrane with tyrosine crosslinks. Proc Natl Acad Sci USA 74: 4214–4218
9. Gaspar T, C Penel, T Thorpe, H Greppin 1982 Peroxidases 1970–1980. A survey of their biochemical and physiological roles in higher plants. University of Genève, Switzerland
10. Harkin JM, YR Obst 1973 Lignification in trees: indication of exclusive peroxidase participation. Science 180: 296–298
11. Haschke RH, JM Friedhoff 1978 Calcium-related properties of horseradish peroxidase. Biochem Biophys Res Commun 80: 1039–1042
12. Heath RL 1980 Initial events in injury to plants by air pollutants. Annu Rev Plant Physiol 31: 395–431
13. Horsman DC, AR Wellburn 1975 Synergistic effect of SO_2 and NO_2 polluted air upon enzyme activity in pea seedlings. Environ Pollut 8: 123–133
14. Horsman DC, AR Wellburn 1977 Effect of SO_2 polluted air upon enzyme activity in plants originating from areas with different annual mean atmospheric SO_2 concentrations. Environ Pollut 13: 33–39
15. McKersie BD, P Hucl, WD Beversdorf 1982 Solute leakage from susceptible and tolerant cultivars of *Phaseolus vulgaris* following ozone exposure. Can J Bot 60: 73–78
16. Pierre M, O Queiroz 1981 Enzymic and metabolic changes in bean leaves during continuous pollution by subnecrotic levels of SO_2. Environ Pollut 25: 41–51
17. Rathmell WG, L Sequeira 1974 Soluble peroxidase in fluid from the intercellular spaces of tobacco leaves. Plant Physiol 53: 317–318
18. Sticher L, C Penel, H Greppin 1981 Calcium requirement for the secretion of peroxidases by plant cell suspensions. J Cell Sci 48: 345–353
19. Tartakoff A, P Vassalli 1978 Comparative studies of intracellular transport of secretory proteins. J Cell Biol 79: 694–707
20. Ting IP, J Perchorowicz, L Evans 1974 Effect of ozone on plant cell membrane permeability. *In* M Dugger, ed, Air Pollution Effects on Plant Growth. American Chemical Society, Washington DC, pp 8–21
21. Tingey DT 1974 Ozone induced alterations in the metabolite pools and enzyme activities of plants. *In* M Dugger, ed, Air Pollution Effects on Plant Growth. American Chemical Society, Washington DC, pp 40–57
22. Tingey DT, RC Fites, C Wickliff 1975 Activity changes in selected enzymes from soybean leaves following ozone exposure. Physiol Plant 33: 316–320
23. Whitmore FW 1978 Lignin-carbohydrate complex formed in isolated cell walls of callus. Phytochemistry 17: 421–425
24. Zocchi G, JB Hanson 1982 Calcium influx into corn roots as a result of cold shock. Plant Physiol 70: 318–319