Vitamins C and E: An Antioxidative System against Herbicide-Induced Lipid Peroxidation in Higher Plants

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To study protection from in vivo lipid peroxidation in plants, the concentration of vitamin C (ascorbic acid) and vitamin E (α-tocopherol) in plants was determined, and the extent of peroxidative cell damage was measured as ethane production or water loss of cells, calculated as an increase of the dry weight to fresh weight ratio. To induce peroxidation, plant seedlings were treated with 1 kg/ha of the diphenyl ether herbicide oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene]. Peroxidation of membranes was directly related to both the concentration and the ratio of the vitamins C and E present in plants. Seedlings that had a substantial amount of both vitamins and a ratio of the vitamin C to the vitamin E concentration between 10 and 15:1 (wt/wt) were highly protected against the phytotoxic action of the herbicide. Cell damage dramatically increased in plants that have a much lower or a much higher ratio of the vitamin C to vitamin E concentration or a lower amount of both antioxidants. Therefore, oxyfluorfen-induced peroxidative cell damage seems to be highly controlled by antioxidative systems, such as the vitamins C and E.

INTRODUCTION

There is considerable evidence to show that several herbicides, such as the bipyridylium salts or *p*-nitrodiphenyl ethers, induce peroxidation of biomembranes in both plants and algae (Youngman et al., 1979; Kunert and Böger, 1981; Kunert, 1984a). Protection against the harmful peroxidation of lipids is provided by several antioxidative systems including a large array of enzymes and a variety of small molecules, such as glutathione, β-carotene, and the vitamins C and E (Foyer and Hall, 1976; Elstner, 1982; Kunert and Tappel, 1983; Kunert and Böger, 1984; Burton and Ingold, 1984).

From animal studies, several researchers have proposed that the water-soluble vitamin C is an antioxidant–synergist with the lipid-soluble vitamin E and that both vitamins can act together as a powerful antioxidative system in cells (Tappel, 1962; Leung et al., 1981). The antioxidative role can be explained by the interaction of both vitamins. Vitamin E acts as a primary antioxidant, while vitamin C reductively regenerates oxidized vitamin E. Recently, the free-radical interaction between these vitamins has been observed by pulse radiolysis techniques (Packer et al., 1979). Furthermore, a combination of both antioxidants has been found to be highly protective against methyl linoleate oxidation in an in vitro test system (Niki et al., 1983).

In plants, vitamin E is located mainly in the chloroplast (Bucke, 1976), and a high concentration of the lipid-soluble vitamin is found in the chloroplast envelope (Lichtenthaler et al., 1981). Vitamin C, a reducing agent, is present in a substantial amount in higher plants (Jones and Hughes, 1983), and the water-soluble vitamin is generally regarded as a normal cellular constituent. However, reports about the importance of both compounds in plants under peroxidative conditions are rare. Research to date has shown that both antioxidants seem to be protectors against peroxidation induced by air pollutants (Barnes, 1972; Lizada and Yang, 1981) or certain herbicides. Recently, we reported a highly significant correlation between in vivo lipid peroxidation, measured as ethane production, and the vitamin C content of mustard seedlings treated with diphenyl ethers or the bipyridylium salt paraquat (Kunert, 1984b). Further, Orr and Hess (1982) and Lambert et al. (1983) have shown that vitamin E prevents membrane damage when added to diphenyl ether treated cucumber cotyledons or herbicide-treated algae.

In the present study, we have demonstrated some new aspects of protection from in vivo lipid peroxidation by the vitamins C and E as an example for the action of a water-soluble and a lipid-soluble antioxidant present in higher plants. The sensitive measurements of both ethane, a decomposition product of ω3-unsaturated fatty acid hydroperoxides, and the increase of the dry weight to fresh weight ratio, were used to determine the antioxidative capacity of both vitamins under peroxidative conditions in plants. The peroxidation process was initiated by the *p*-nitrophenyl phenyl ether herbicide oxyfluorfen. Metabolism and translocation of oxyfluorfen are very low in plants, and the effects of the herbicide develop in a relatively short time.

MATERIALS AND METHODS

Herbicide. Oxyfluorfen was obtained from Rohm and Haas, Spring House, PA.

Cultivation of Plants. Plant seedlings of alfalfa (*Medicago sativa*), buckwheat (*Fagopyrum esculentum*), jimson weed (*Datura stramonium*), lamb's-quarter (*Chenopodium album*), morning glory (*Ipomoea purpurea*), mustard (*Sinapis alba*), pigweed (*Amaranthus retroflexus*), sicklepod (*Cassia obtusifolia*), and velvetleaf (*Abutilon theophrasti*) were grown in plastic pots containing vermiculite. Cultivation was done in a growth chamber, regulated to a 12 h photoperiod (170 W/m^2 supplemental light) with day and night temperatures of 22 °C and 18 °C, respectively. Seedlings that had developed the cotyledons, which normally occurred between 10 days and 15 days of age, were sprayed with an emulsifiable concentrate formulation of oxyfluorfen mixed with water. After herbicide application, seedlings were cultivated for another 6 days under the growth conditions described above.

Analysis. For determination of the dry weight of plants, cotyledons (0.2 g) were dried for 2 h at 105 °C. Cell damage was calculated as dry weight/fresh weight × 100 (= dry weight as percentage of the fresh weight). Percent values for untreated control plants were in the range of 10–16% in the different species. Increasing percentage values, due to membrane damage and water loss of cells,

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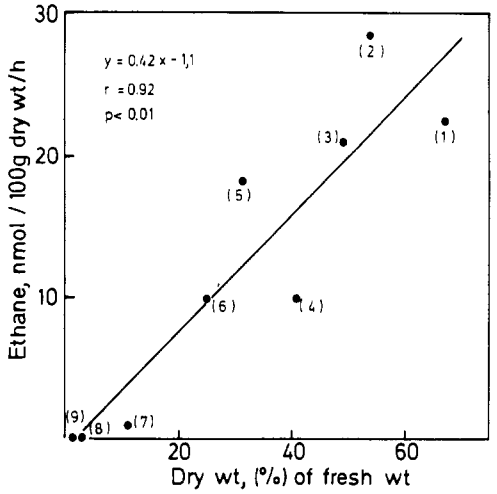

Figure 1. Ethane production and cell damage (dry wt as percentage of fresh weight, for calculation see Materials and Methods) of different plant species 6 days after treatment with 1 kg/ha of oxyfluorfen. Identification numbers for plant species used represent morning glory (1), jimson weed (2), pigweed (3), buckwheat (4), velvetleaf (5), lamb's-quarter (6), alfalfa (7), sicklepod (8), and mustard (9). Data shown for both ethane evolution and cell damage in plants represent the difference of the means of 6 untreated control plants and 6 herbicide-treated plants in each group.

indicate a higher level of cell destruction in plants after herbicide treatment.

The vitamin C content of the cotyledons was measured by dye titration according to the method of Kelly and Latzko (1980). The vitamin was titrated against 2,6-dichlorophenol–indophenol, as outlined by the Association of Official Analytical Chemists (1980).

The vitamin E content of the cotyledons was determined by HPLC with a fluorescence detector (Shimadzu Corp., Kyoto, Japan) at an excitation wavelength of 294 nm and emission wavelength of 325 nm (Cort et al., 1983). The vitamin was separated on a Nucleosil 50, 5-μm particle size, column (Machery-Nagel, Düren, Germany). Dioxane at 0.6% in hexane (v/v) was used as the mobile phase according to the method of Grumbach (1983). For sample preparation, cotyledons of seedlings (0.2 g) were homogenized in 80% ethanol, and the vitamin was extracted with 0.5 mL of hexane.

For determination of ethane evolution, cotyledons (0.2 g) were incubated 6 days after herbicide treatment in 10-mL vials sealed with rubber septa for 3 h at 20 °C under continuous light of 40 W/m^2. The hydrocarbon gas produced was analyzed according to the method of Kunert and Böger (1984).

Statistical Analysis. All estimates of sample variability are given in terms of the standard error (SE) of the mean. Comparisons of two means were calculated by using student's two-tailed t test. p values ≤ 0.05 were considered significant.

RESULTS

To determine if the herbicide-induced cell damage could be related to lipid peroxidation, ethane evolution was measured after oxyfluorfen treatment in different plant species (Figure 1). Ethane production correlated well with the increase in cell damage of plants. The relationship was linear and the correlation was highly significant ($r = 0.97$, $p < 0.01$).

Table I shows the amount of the vitamins C and E present in different plant species. The lowest concentration of both antioxidants was found in morning glory. This plant species had also the highest extent of cell damage

Table I. Antioxidant Content of Different Plant Species and Cell Damage after Treatment with Oxyfluorfen[a]

plant species	antioxidants, mg/100 g dry wt: vitamin C	antioxidants, mg/100 g dry wt: vitamin E	cell damage, % increase
morning glory (1)	2 ± 1	10 ± 3	67
lamb's-quarter (6)	58 ± 18	12 ± 3	25
alfalfa (7)	143 ± 12	10 ± 1	12
pigweed (3)	504 ± 24	10 ± 1	49
buckwheat (4)	537 ± 27	28 ± 2	41
mustard (9)	469 ± 24	50 ± 9	2
sicklepod (8)	861 ± 73	60 ± 6	3
velvetleaf (5)	92 ± 7	50 ± 4	31
jimson weed (2)	114 ± 29	83 ± 16	59

[a] Increase of cell damage (for calculation see Materials and Methods) of seedlings 6 days after treatment with 1 kg/ha oxyfluorfen and content of the vitamins C and E of untreated control seedlings at the same age as the herbicide-treated plants. The identification number of each plant species is identical with the number given for each species in Figures 1 and 2. Values given for the antioxidants represent the mean ±SE of 6 plants of each species. Values given for cell damage represent the difference of the means of 6 untreated control plants and 6 herbicide-treated plants in each group.

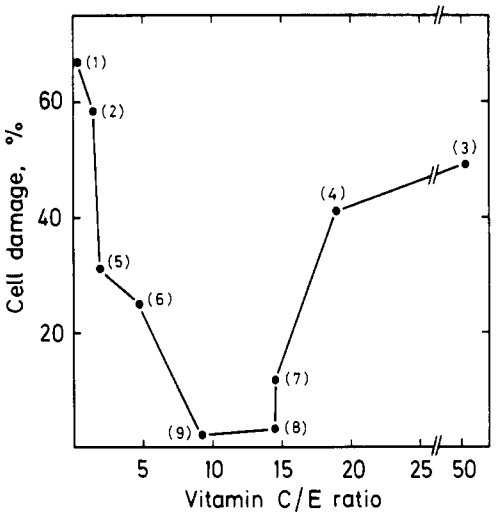

Figure 2. Ratio of the vitamin C concentration to the vitamin E concentration (wt/wt) and percent increase of cell damage in different plant species. Ratios for the vitamins were calculated by using the values for the concentrations of vitamin C and E in plants given in Table I. The identification number of each plant species is identical with the number given for each species in Figure 1 and Table I. Cell damage was determined as described under Materials and Methods. Data shown represent the ratio of the means of the vitamin C and E concentration of 6 untreated control plants in each group. Values given for cell damage represent the difference of the means of 6 untreated control plants and 6 plants treated with 1 kg/ha of oxyfluorfen in each group.

(67%) after oxyfluorfen treatment. The vitamin E content of lamb's-quarter and alfalfa was similar to the amount of vitamin E found in morning glory. Herbicide-induced cell damage was substantially less in both plant species than in morning glory. The vitamin C content, however, was significantly higher ($p < 0.01$) in lamb's-quarter and alfalfa than in morning glory. In pigweed was found both a higher amount of vitamin C and a higher degree of cell damage than in lamb's-quarter and alfalfa. A high content of vitamin C comparable to the amount in pigweed was also found in buckwheat, mustard, and sicklepod. However, only mustard and sicklepod had a substantial content of both vitamins and the lowest extent of cell damage of all plant species tested after oxyfluorfen treatment. Velvetleaf and jimson weed had almost an equal amount

of vitamin E but a significantly lower ($p < 0.01$) concentration of vitamin C than mustard and sicklepod. Cell damage, however, was higher in both plant species than in mustard or sicklepod.

Figure 2 shows peroxidative cell damage related to the ratio of the concentrations of vitamin C and vitamin E (wt/wt) present in different plant species. Herbicide-induced peroxidation was practically nonexistent in plants, such as mustard and sicklepod that had a ratio of the vitamin C content to the vitamin E content between 10 and 15:1. Oxyfluorfen-induced peroxidation dramatically increased when plants had a ratio of the concentrations of vitamin C to vitamin E lower or higher than between 10 and 15:1. Both plant species, morning glory and pigweed, that had a ratio of the vitamin C content to the vitamin E content of 0.2:1 and 50:1, respectively, were found to be most sensitive against the herbicide.

DISCUSSION

Peroxidation of polyunsaturated fatty acids induced by toxic compounds, such as the *p*-nitrophenyl phenyl ether herbicide oxyfluorfen, is usually investigated by the sensitive measurement of ethane, a decomposition product of ω3-unsaturated fatty acid hydroperoxides (Tappel, 1980; Kunert, 1984a). In our experiments, the increase of the hydrocarbon gas in plants was significantly correlated with an increase in cell damage. When membranes are damaged by peroxidation, plant cells lose a substantial amount of water as one toxic consequence of peroxidation.

The peroxidation process is ended either by a damaging reaction or by the action of antioxidants. Animal studies have shown that in vivo lipid peroxidation is inversely proportional to the amount of the antioxidant vitamin E (Downey et al., 1978; Kornbrust and Mavis, 1980). This result is not consistent with our observation in plants. In our experiments, the extent of peroxidative cell damage after herbicide treatment was determined by the amount of both vitamins present in plants and not by the amount of either vitamin E or vitamin C alone.

Generally, the vitamin C concentration of plants was higher than the concentration of vitamin E. However, the content of the vitamins was highly variable in different plant species, and peroxidation seems to be determined by both the amount and the ratio of the antioxidants present in plants. A low vitamin C concentration in plant cells potentiates the effect of an initiator on the peroxidation process. Sensitivity against the herbicide was lower in lamb's-quarter and alfalfa than in morning glory. Both species had a higher amount of vitamin C, but almost the same amount of vitamin E than morning glory. This is similar to the result we recently found in animals (Kunert and Tappel, 1983).

There is evidence that vitamin C represents a reservoir of antioxidant potential to regenerate directly, under peroxidizing conditions, the lipid-soluble primary antioxidant vitamin E (Packer et al., 1979; Leung et al., 1981). Under our culture conditions, a ratio of the vitamin C to vitamin E concentration between 10 and 15:1 was highly protective for plants against herbicide-induced peroxidation. Sensitivity against the herbicide dramatically increased in plant species that had either an extremely low vitamin C to vitamin E ratio, such as morning glory and jimson weed, or an extremely high ratio of the vitamins, such as buckwheat and pigweed. Although jimson weed had the highest vitamin E content of all plant species tested but a relatively low vitamin C concentration, this plant species was not protected against peroxidation. We assume that the antioxidative potential of vitamin C was too low in jimson weed to regenerate efficiently vitamin E.

Stimulation of lipid peroxidation by a high concentration of vitamin C is well documented (Haase and Dunkley, 1969; Dumelin and Tappel, 1977). Further increase of peroxidation in erythrocytes by high vitamin C supplementation of animals that could be counterbalanced by more vitamin E was recently reported by Chen (1981). We also have evidence that the prooxidative action of a high vitamin C concentration in plants is balanced by vitamin E. The substantial amount of vitamin C found in mustard and sicklepod was very likely balanced by more vitamin E in these plant species. Consequently, both the ratio of the vitamin C to vitamin E concentration and the extent of cell damage was significantly lower in mustard and sicklepod than in pigweed and buckwheat. Both plant species had a high vitamin C content but a lower vitamin E content than mustard and sicklepod.

Overall, phytotoxicity of the diphenyl ether oxyfluorfen seems to be controlled in plants by antioxidants, such as the vitamins C and E, and we assume that both the amount and the ratio of the antioxidants in plants are directly related to herbicide-induced peroxidation. But, the possibility cannot be excluded that other antioxidative systems than these antioxidative vitamins may also play an important role as protectors against the herbicide.

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Registry No. Vitamin C, 50-81-7; vitamin E, 59-02-9; oxyfluorfen, 42874-03-3.

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Isocyanate Formation in the Decomposition of Phenmedipham in Aqueous Media

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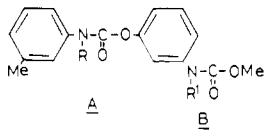
The alkaline hydrolysis of phenmedipham and one of its N-methylated derivatives, 3-[[(methoxycarbonyl)methyl]amino]phenyl *N*-(3-methylphenyl)carbamate, into *m*-hydroxyphenols and *m*-toluidine via *m*-tolylcarbamic acid was studied for hydroxide ion concentrations ranging from 10^{-6} to 6 N. The pH–rate profiles correspond to the rate laws of the ElcB or $B_{Ac}2$ reaction mechanisms that can be involved in the hydrolysis of carbamates. The positive activation entropy, the Brønsted β value of −1.21 for the hydrolysis of a series of aryl and alkyl *N*-(3-methylphenyl)carbamates, and the Hammett ρ value of 0.74 for the hydrolysis of a series of 3-[(methoxycarbonyl)amino]phenyl *N*-(substituted phenyl)carbamates are in favor of the involvement of a ElcB reaction scheme for phenmedipham hydrolysis. The importance of the formation of *m*-tolyl isocyanate during phenmedipham decomposition is underlined as this intermediate, although only transient, may lead to carbamylation reactions of enzymatic systems during the metabolism of the herbicide in plants or animals.

Phenmedipham, or 3-[(methoxycarbonyl)amino]phenyl *N*-(3-methylphenyl)carbamate **1**, is a herbicide of the bis(carbamate) family (Trebst et al., 1968) used for postemergence weed control in beet crops and strawberry plants (Arndt and Kötter, 1968).



R=R¹=H Phenmedipham
R=Me R¹=H Biscarbamate 2
R=H R¹=Me Biscarbamate 3

In acid soils, the herbicide decomposes into *m*-toluidine and methyl *N*-(3-hydroxyphenyl)carbamate with a half-life of ca. 28–55 days (Kossmann, 1970). In basic soils, Sonawane and Knowles (1971a) noted that methyl *N*-(3-hydroxyphenyl)carbamate subsequently decomposes into *m*-aminophenol.

In plants (Von Kassebeer, 1971) and the rat (Sonawane and Knowles, 1971b) the hydrolysis of the carbamate A function is one of the main pathways of in vivo bis(carbamate) metabolism.

The only data in the literature concerning phenmedipham stability in aqueous media are the values of the half-life measured at pH 7 ($t_{1/2} \simeq 5$ h) and pH 9 ($t_{1/2} \simeq 10$ min) at 30 °C (Martin and Worthing, 1974).

Previous studies on the stability of carbamates in alkaline media demonstrated a difference in the reactivity between N-monosubstituted and N,N-disubstituted compounds (Dittert and Higuchi, 1963). The latter compounds do not have a mobile proton in the carbonyl α position and are hydrolyzed via a $B_{Ac}2$ mechanism. The hydrolysis of the N-monosubstituted derivatives, however, may proceed via two reaction schemes: a bimolecular $B_{Ac}2$ pathway and a monomolecular ElcB pathway involving the formation of isocyanate (Hegarty and Frost, 1973; Williams, 1972) (Figure 1). Hence the hydrolysis of carbaryl (Vontor et al., 1972) and the O-(methylcarbamoyl) oximes (Mrlina and Calmon, 1980) involves the formation of methyl isocyanate whereas that of propham, chlorpropham, and swep follows a $B_{Ac}2$ reaction scheme (Bergon and Calmon, 1983).

During the hydrolysis of phenmedipham, any isocyanates that may be formed cannot be directly demonstrated because of their high reactivity in aqueous media (Williams and Ibrahim, 1981). We therefore carried out a kinetic study of this reaction in order to determine its mechanism (Ben Hamida et al., 1981, 1982).

As phenmedipham is characterized by the presence of two carbamate functions, two monoanions **4** and **5** may form in alkaline media (Figure 2). In order therefore to determine the ionization site of the phenmedipham molecule we examined, on the one hand, the effect of the substitution of the aromatic nucleus on the acidity of a series of methyl carbanilates including bis(carbamate) **2**, and on the other hand, the hydrolysis reaction kinetics of 3-[[(methoxycarbonyl)methyl]amino]phenyl *N*-3(methylphenyl)carbamate **3**, the N-methylated derivative on the phenmedipham B function.

Finally, in order to look for Hammett and Brønsted relationships of the type $\log k_{OH} = f(\sigma \text{ or } pK_a)$ with ρ and β values that characterize the hydrolysis mechanism of a carbamate function (Sartoré et al., 1977), we synthesized two families of carbanilates with a chemical structure analogous to that of phenmedipham: the 3-[(methoxycarbonyl)amino]phenyl carbanilates and 3-methylcarbanilic acid esters.

EXPERIMENTAL SECTION

Apparatus. A Unicam SP 1800 recording spectrophotometer fitted with a SP 1805 program controller and a thermostated multiple cell compartment or, for the more

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