of changes in the activities of these enzymes may be known only after detailed analysis for free and protein amino acids, in vivo concentration of different metabolites, particularly in the vicinity of various enzymes, and chasing the fate of labeled NH_4^+, glutamine, and asparagine. On the basis of the results presented, it may, however, be suggested that in developing wheat grain, both the glutamate synthase cycle and the GDH pathway may be operative in ammonia assimilation, the former predominating during the early stages and the later playing a more active role during the later stages.

Registry No. GDH, 9001-46-1; GOGAT, 65589-88-0; GS, 9023-70-5; AS, 9023-69-2; GPT, 9000-86-6; GOT, 9000-97-9; APT, 9067-72-5.

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Peroxidative Activity of Oxyfluorfen with Regard to Carotenoids in *Scenedesmus*

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Autotrophic *Scenedesmus* cells were treated with difunon, a carotenogenesis inhibitor, thereby lowering the content of colored carotenoids by 50%. Photosynthetic electron transport remained effective to activate the *p*-nitrodiphenyl ether oxyfluorfen [2-chloro-4-(trifluoromethyl)phenyl 3-ethoxy-4-nitrophenyl ether) with subsequent peroxidation. Both carotenoid-deficient and normal cells exhibited the same peroxidative activity with oxyfluorofen. In both samples, peroxidative hydrocarbon formation was completely suppressed by an inhibitor of photosynthetic electron transport. We take this as evidence that in *Scenedesmus* and other green tissues light activation of *p*-nitrodiphenyl ethers essentially takes place through electron donation by the intact photosynthetic redox chain and not by direct interaction of diphenyl ethers with sensitized carotenoids.

Visible light is required for the phytotoxic activity of oxyfluorfen [2-chloro-4-(trifluoromethyl)phenyl 3-ethoxy-4-nitrophenyl ether; Kunert and Böger, 1981; Orr and Hess, 1981] as was previously described for other nitrodiphenyl ethers (Matsunaka, 1969a,b; Fadayomi and Warren, 1976). Peroxidations directed against membrane constituents apparently cause the essential phytotxic effects (Lambert et al., 1981; Orr and Hess, 1982). Peroxidative activity is decisively determined by substituents neighboring the *p*-nitro group (Lambert et al., 1983).

Obviously, a radical has to be originated by interaction of the *p*-nitrodiphenyl ether with a light-absorbing system. Right now there is no accordance as to how this may happen. Matsunaka originally proposed the essential role of (sensitized) carotenoids, since seedlings of white, carotenoid-free rice mutants exhibited tolerance, while yellow mutants were susceptible to *p*-nitrodipenyl ethers. Orr and Hess (1982), using etiolated, carotenoid-containing cucumber cotyledons, reported light-induced membrane leakage in the presence of peroxidizing diphenyl ethers. A decrease of colored carotenoids by a carotenogenesis inhibitor led to protection. Kunert and Böger (1981), however, treating the green microalga *Scenedesmus* with oxyfluorofen in the light, substantially prevented peroxidation and phytotoxicity by concurrent addition of diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. The latter herbicide blocks the photosynthetic electron flow, which was consequently postulated as being necessary to effectively initiate radical formation mediated by the *p*-nitrodiphenyl ether, giving rise to subsequent peroxidation of, e.g., polyunsaturated fatty acids (Sandmann and Böger, 1982a,b). Furthermore, peroxidation proceeded with red light (>610 nm), which did not activate carotenoids (Lambert et al., 1983a).

This paper resumes our studies with the alga *Scenedesmus*. Levels of colored carotenoids were decreased artifically by pregrowing cells in the presence of difunon [5-[(dimethylamino)methylene]-2-oxo-4-phenyl-2,5-di-

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Table I. Carotenoid Fractions [in μg (mL of Packed Cell Volume)$^{-1}$] from *Scenedesmus* Treated with Difunon and Norflurazon over 5 h under Culture Conditions

additions	xanthophylls	α- and β-carotene	phytoene	phytofluene
control	96	35	0	0
+difunon, 10 μM	70	6	50	10
+norflurazon, 10 μM	75	5	84	n.d.[a]

[a] n.d. = nondetectable.

hydro-3-furancarbonitrile], thus corroborating our hypothesis that in photosynthetically active organelles the electron-transport system, not merely carotenoids, is essential for the activation of peroxidative diphenyl ethers.

MATERIALS AND METHODS

Cultivation. The green microalga, *Scenedesmus acutus* (strain no. 276-3a, Algae Culture Collection, University of Göttingen, West Germany), was cultivated autotrophically in a sterile mineral medium by using a growth apparatus of Kniese-Edwards (Marburg, West Germany) with a light intensity of about 30 W/m^2 (Sandmann et al., 1979). The cell density was measured by packed cell volume (pcv) (Sandmann et al., 1981). The stock culture was pregrown for 48 h, reaching 5–7 μL of pcv/mL, to maintain the algae in the logarithmic growth phase. At the start of the experiment, the stock culture was diluted to a density of 1 μL of pcv/mL. Pure oxyfluorfen was dissolved in ethanol and difunon in a mixt. of acetone–methanol (1:9 v/v), while the final concentraton of the solvent was kept below 0.1% after addition to the culture medium.

Carotenoids and Chlorophyll. These pigments were extracted from intact cells and their contents determined as described by Sandmann and Böger (1983). The total colored carotenoids were determined in petroleum ether (bp 60–80 °C) by using an average extinction coefficient of $E^{1\%}_{1\,cm}$ = 2500 at 445 nm. Carotenoids were further separated by thin-layer chromatography on silica gel plates according to Lambert and Böger (1983) using extinction coefficients as given by Davies (1976). A certain loss of total carotenoids occurred, but the quantitative relationship between the fractions was not altered.

Physiological Parameters. Photosynthetic oxygen evolution was measured at 22 °C with a Clark-type oxygen electrode under saturating-light conditions (800 W/m^2, measured by a YSI radiometer, Model 65A) by using a red cutoff filter (RG-610) and a heat filter (KG-1), both from Schott, Mainz (Böger and Schlue, 1976). When indicated, light intensity was decreased down to 80 W/m^2 by neutral density filters (NG-4 and NG-11).

For determination of C_2 hydrocarbons as makers of peroxidation, 2-mL aliquots of the *Scenedesmus* culture with a density of 1.5 μL/mL were illuminated (approximately 100 W/m^2) in its original nutrient medium for 15 h in gas-tight vials without any additional bicarbonate by using a Warburg apparatus [for details and the determination, see Lambert et al. (1983b)]. *Scenedesmus* cells with lowered content of colored carotenoids were obtained by growing them in the presence of 10 μM difunon for 5 h. Unless indicated otherwise, the cells were washed 2 times and eventually resuspended in nutrient medium of the herbicide-free control.

Each experiment was repeated 6 times with separate culture batches. Data of each parameter given are means, with a tolerance of ±6% from the average (±10% for the data in Table I). Chemicals, analytical grade, were purchased from Merck, Darmstadt, West Germany.

RESULTS AND DISCUSSION

Figure 1A demonstrates the increase in the amount of colored carotenoids in the control culture during a 9-h

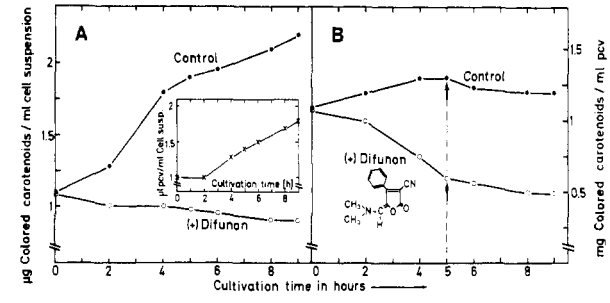

Figure 1. Influence of 10 μM difunon on colored-carotenoid content expressed as μg (mL of cell suspension)$^{-1}$ (A) and mg (mL of packed cell volume, pcv)$^{-1}$ (B), during a cultivation period of 9 h. The inset illustrates the identical growth as observed during the experimental time for the control and cells moderately treated with difunon.

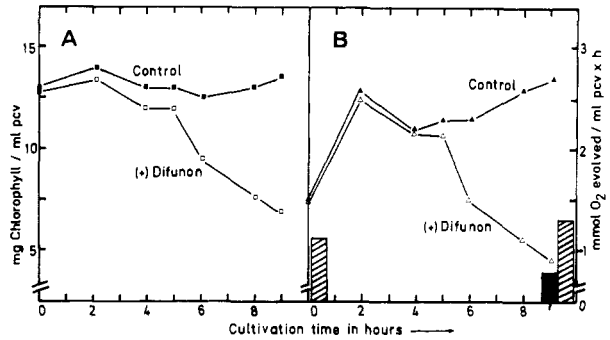

Figure 2. Influence of 10 μM difunon on chlorophyll content (A) and oxygen evolution at saturating light (curves) (B) over a cultivation period of 9 h. The hatched columns indicate the oxygen evolution of the control; the solid column indicates the difunon-treated culture measured at nonsaturating light intensity (of 80 W/m^2).

growth period while the presence of 10 μM difunon prevented formation of colored carotenoids. Instead, a small decrease was observed. After 5 h of growth, the control-culture volume contained about twice as much colored carotenoids as the difunon-treated culture. Growth, expressed as packed cell volume per milliliter of culture volume, was identical for both cultures during this short experimental time (Figure 1, inset). The content of carotenoids, when referenced to pcv, was halved in difunon-treated cells after 5 h (part B of Figure 1).

It was shown previously (Urbach et al., 1976; Clarke et al., 1982) that difunon is an effective inhibitor of desaturation during carotenogenesis. Table I shows the carotenoid pattern under the influence of this experimental herbicide. Under the conditions applied here, the content of the α- and β-carotene was lowered to about 17% of the control, while saturated precursors, both the colorless phytoene and some phytofluene, were accumulated. Xanthophylls were much less affected. Difunon exhibits an activity similar to norflurazon [4-chloro-5-(methylamino)-2-[3-(trifluoromethyl)phenyl]pyridazin-3(2*H*)one] (Sandmann and Böger, 1983). These data are included for comparison.

Figure 2A shows the decrease of chlorophyll that apparently was not directly affected by difunon, since it

Table II. Formation of C_2 Hydrocarbons by Normal and Difunon-Treated *Scenedesmus* under the Influence of the *p*-Nitrodiphenyl Ether Oxyfluorfen during a 15-h Light Incubation Period, in the Warburg Apparatus[a]

additions	pregrown minus difunon		pregrown plus difunon	
	not washed	washed	not washed	washed
(1) control	0.7	0.7	0.7	0.8
(2) + oxyfluorfen, 10 μM	22	24	6.2	26
(3) + oxyfluorfen, 10 μM, + diuron, 1 μM	n.d.	1	n.d.	n.d.

[a] Data are expressed in nmol (mL of packed cell volume)$^{-1}$ (15 h)$^{-1}$; n.d. = nondetectable. In the last column, 10 μM difunon was present for 5 h only and then removed by washing, and the cells were subsequently exposed to oxyfluorfen for 15 h; in samples not washed (next to the last column), the difunon content was maintained in addition to oxyfluorfen (and diuron as in line 3).

began to drop only after 5 h of treatment. Its decrease is explained by secondary photooxidation due to the absence of protecting carotenoid, especially α- and β-carotenes (Sandmann and Böger, 1982a). Chlorophyll degradation was accompanied by a decrease of photosynthetic oxygen evolution even exceeding the degree of chlorophyll loss, while the control showed little increase of photosynthesis after the 2-h lag phase common to both samples (part B). (This lag phase is due to inoculation, i.e., dilution of the cell density.)

When photosynthesis was measured with *non*saturating light intensities (e.g., 80 W/m^2), which came close to the light conditions during growth in Figure 2A, the decrease of photosynthesis between the fifth and ninth hour of difunon treatment was much less. At the end, activity was still 50% of the control (see solid column in part B). These data show that during the difunon treatment as performed here, substantial electron-transport activity is preserved. At 100 W/m^2 (this intensity is used for the peroxidation assay) the same photosynthesis rate is observed. Pretreatment with difunon has to be done carefully. Loss of colored carotenoids of more than 50% leads to almost complete decay of photosynthetic activity (data not shown).

Table II presents data on light-induced peroxidative formation of ethane and ethylene due to the presence of oxyfluorfen. Checking for short-chain hydrocarbons over a 15-h period, we found that their amount was decreased to 6.2 nmol (mL of pcv)$^{-1}$ (15 h)$^{-1}$ in the sample with difunon present vs. 22–24 nmol (mL of pcv)$^{-1}$ (15 h)$^{-1}$ in the difunon-free control. This low peroxidation is explained by decreased electron transport that—as we have demonstrated above—shows up after difunon treatment of more than 5 h [the rate was only 0.2 μmol of O_2 (mL of cell suspension)$^{-1}$]. Therefore, the cells were freed of difunon by thorough washing after a 5-h incubation time and then incubated with oxyfluorfen. Now, C_2-hydrocarbon evolution by cells having an altered carotenoid level was the same as with the untreated control with its normal carotenoid inventory. After difunon had been removed and oxyfluorfen (10 μM) added (lines 2 and 3), no carotenoid biosynthesis was resumed (data not shown). As shown previously (Kunert and Böger, 1981), oxyfluorfen, even at 1 μM, effectively degrades carotenoids. A 15-h incubation was necessary to reproducibly determine volatile hydrocarbon formation. For the experiment just mentioned, we have proven that, after this 15-h incubation, photosynthesis was 1.2-1.4 μmol of O_2 (mL of cell suspension)$^{-1}$ h^{-1} for both the washed difunon-pretreated and nontreated samples (when both samples did not contain oxyfluorfen). It should, however, be mentioned that photosynthesis was completely inhibited by a 15-h incubation period with oxyfluorfen present, regardless of whether the cells had been pretreated with difunon or not. Diuron effectively inhibited peroxidative activity induced by oxyfluorfen when using either difunon-pretreated or untreated cells (Table II, line 3).

Recently, Devlin et al. (1983) reported protection of corn seedlings against oxyfluorfen after a more than 95% bleaching of pigments by norflurazon treatment. According to our experiments, such a strong bleaching abolishes electron transport and, thereby, activation of oxyfluorfen. In our view, this "protection" has no bearing on carotenoids as possible sensitizers. The reported activating role of carotenoids as mentioned in the introduction may be most relevant in etiolated tissues. Using green cucumber cotyledons treated with a peroxidizing diphenyl ether, Orr and Hess (1982) did not observe interference with herbicidal activity of the diphenyl ether, when low concentrations of diuron and dibromothymoquinone were present together. Similar experiments were performed by Matsunaka (1969a) using a weakly peroxidizing *p*-nitrodiphenyl ether and a symmetric triazine as photosynthesis inhibitor.

In summary, photosynthetic electron transport is essentially responsible for activating oxyfluorfen in autotrophic *Scenedesmus* and chloroplasts (Lambert and Böger, 1981), thereby initiating peroxidation. If oxyfluorfen were directly activated by (illuminated) carotenoids, (1) an altered inventory of these pigments should change peroxidative activity and (2) direct inhibition of photosynthesis by diuron should not protect against oxyfluorfen. apparently, multiple activation mechanisms have to be assumed within the plant kingdom.

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Registry No. Oxyfluorfen, 42874-03-3.

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Direct Gas Chromatographic Analysis of Aqueous Citrus and Other Fruit Essences

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An analytical method has been developed in which the characteristic flavor and aroma constituents of natural fruit essences can, for the first time, be quantitatively and qualitatively determined from a direct injection of aqueous essence into a gas chromatograph. This objective method can be used to evaluate essence strength and quality, determine effects of fruit varieties, temperature, storage, and unusual weather, control seasonal blending and production efficiency, and detect adulteration. Commercial aqueous orange essence samples were analyzed to determine strength, quality, processing differences, and effects of mild freeze damage to the fruit. Although this method was developed for evaluating citrus essences, we have also demonstrated its applicability to essences from apples, grapes, pineapples, strawberries, and bananas.

Citrus essence (aroma) is an aqueous distillate collected from the first stage of an evaporator during the production of juice concentrate from the corresponding fresh juice. The aqueous essence is separated from an oily layer (essence oil) prior to storage. Citrus juice volatiles concentrated in the aqueous fraction reflect both quantitatively and qualitatively the flavor and aroma of the parent juice. The aqueous fraction is thus a desirable flavoring material. Although aqueous essences are produced from all major citrus fruit, orange essence is the most commercially important and widely used. It is added to orange concentrate to restore "fresh" flavor and aroma and to synthetic drinks and other products to impart a natural flavor and aroma.

Ten million pounds of aqueous orange essence are currently being used annually in the United States. The potential annual production, based on the quantity of orange concentrate produced, is approximately 20 million lb. Realization of the full commercial potential of orange and other citrus essences has not materialized primarily because of the difficulty in evaluating their strength and quality and thus in producing a consistent, standard product (Shaw, 1977). Strength and quality characteristics of aqueous essences vary from lot to lot because of variations in processing methods, cultivar, season of harvest, and maturity. Many of the analytical methods previously reported for evaluating aqueous essences have included concentration of essence constituents before analyses. These include solvent extractions (Wolford et al., 1962; Shultz et al., 1964; Moshonas and Shaw, 1973). Other means of concentration included liquid–liquid extraction (Attaway et al., 1962) and adsorption on organic polymer powders (Moshonas and Lund, 1971; Shultz et al., 1971; Dravnieks and O'Donnell, 1971). Colorimetric techniques were reported by Attaway et al. (1967), Braddock and Petrus (1971), Peleg and Mannheim (1970), and Ismail and Wolford (1970). Attempts to obtain accurate quantitative and qualitative analysis by injecting orange essence on packed columns was reported by Lund and Bryan (1977). However, these and other methods have had inherent limitations and have not been satisfactory to the citrus industry for calculating essence strength and quality. Consequently, subjective organoleptic evaluations are still necessary to adequately evaluate these essences for use in flavoring citrus products.

The present study reports a simple, objective method for evaluating strength and quality of aqueous fruit essences. This gas chromatographic (GC) method makes it possible, for the first time, to obatin detailed quantitative and qualitative analyses of these essences from a direct injection of a small quantity of the whole essence. The production of fused silica capillary columns coated with a cross-linked, nonpolar liquid phase helped in the development of this method. These columns resist bleeding and degradation associated with aqueous sample analyses attempted on earlier GC columns.

EXPERIMENTAL SECTION

Gas Chromatography. GC data were obtained with a Hewlett-Packard Model 5880A instrument equipped with a flame ionization detector, a 50-m, wide-bore (0.031–0.032-mm i.d.) capillary fused silica cross-linked SE-54 column (Hewlett-Packard, Avondale, PA), and a capillary inlet system fitted with a splitless liner that allows helium to flow down through the liner to the head of the column. There the flow divides, with 1.5 mL/min going through the column while the rest is vented. The normal three-stage operation of the splitless mode was not used.

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