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PHOTO-OXIDATION OF UNSATURATED LIPIDS IN CUCUMIS LEAF DISCS DURING CHILLING

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SUMMARY

Unsaturated lipids in *Cucumis* leaf discs at 1 °C undergo a photo-oxidative degradation which can be measured with the thiobarbituric-acid test. Kinetics of thiobarbituric-acid-reactivity (TBAR) show a lag phase followed by a rapidly increasing phase and a final decreasing phase. DCMU inhibits the photo-oxidative increase of TBAR. Among all combined fatty acids of *Cucumis* leaf discs linolenic acid is the most abundant (72.6%).

The fatty acid composition of *Cucumis* leaf discs shows little alteration after 8 hours light at 1 °C but after 24 and 48 hours light there is a fast degradation of linolenic acid as compared to the rest of the fatty acids. A crude action spectrum shows a high maximum of TBAR in the blue and a lower maximum in the red spectral region and minimal TBAR in the green and in the far red region. It is suggested that chlorophyll and carotenoids are sensitizers of unsaturated fatty acid photo-oxidation. Possible connexions between chlorophyll and photo-oxidative fatty acid degradation are discussed.

1. INTRODUCTION

Exposing discs from leaves of *Cucumis sativus* to low temperature higher than 0 °C (chilling) causes irreparable damage. Light enhances the damage during chilling treatment. One of the effects is bleaching of the leaf pigments, carotenoids and chlorophylls. It was concluded from previous experiments (VAN HASSELT 1972) that the pigment degradation was caused by photo-oxidation. The loss of leaf pigments causes the death of the cells by starvation but cannot account for the rapidly occurring damage shown by the leaf discs after chilling in the light.

Damage to cell membranes has been considered to be the primary cause of photo-oxidative injury (DWORKIN 1958, DODGE 1971). The chloroplast lamellae contain the chlorophylls and most of the carotenoids (KIRK 1967) and in addition they have a remarkable high content of unsaturated fatty acids (ALLEN et al. 1966). Photo-oxidation of unsaturated fatty acids is catalysed by chlorophyll in vitro (KAHN et al. 1954, RAWLS & VAN SANTEN 1968).

HEATH & PACKER (1968) describe a photo-induced cyclic peroxidation of unsaturated fatty acids (measured as malonaldehyde production) and of chlorophyll in isolated spinach chloroplasts at 25 °C. A similar photo-oxidation of unsaturated lipids might cause irreparable damage to the chloroplast membranes of *Cucumis* leaf discs during chilling. Moreover, a prefential photo-oxidation of unsaturated fatty acids could explain the lag phase in the photo-oxidative de-

gradation of leaf pigments observed in previous experiments (VAN HASSELT 1972).

The effect of light on unsaturated fatty acids of *Cucumis* leaves during chilling was investigated. The results indicate a photo-oxidation of unsaturated fatty acids sensitized by chlorophyll and the carotenoids.

2. MATERIAL AND METHODS

Material

Discs 7 millimetres in diameter were punched between the largest veins of the first leaves of ± 14 days old plants (*Cucumis sativus* L. cv. Kleine Groene Scherpe). Plants were grown in a greenhouse with a minimum night temperature of 20 °C and a minimum day temperature of 25 °C.

Incubation

Discs were incubated at 1 °C as described before (VAN HASSELT 1972). During incubation a light intensity of 20,000 lux was obtained by using a 400 W high pressure mercury lamp type (HPLR; Philips).

Determination of chlorophyll

Samples of 10 discs were dried between filter paper and ground in 2 ml ice-cold acetone for 1 minute in a microchamber of a Sorvall Omnimixer equipped with a microattachment. The extract and acetone washings were added to a 15 ml calibrated centrifuge tube.

The solution was made up to 8 ml with acetone and 2 ml demineralized water was added to obtain 80% acetone. All operations were carried out in a cool room in dim light. The 80% acetone extract was centrifuged for 10 minutes at 900 × g to remove cell debris and the concentration of chlorophyll a + b in the supernatant was measured spectrophotometrically and calculated according to BRUINSMA (1963).

Measurement of unsaturated fatty acid degradation

Unsaturated fatty acid degradation was measured indirectly by the 2-thiobarbituric-acid method and directly by analysing the fatty acids with the aid of gas-liquid chromatography.

Determination of thiobarbituric-acid-reactivity (TBAR)

Thiobarbituric acid (TBA) reacts with malonaldehyde, a decomposition product of the oxidation of poly-unsaturated fatty acids (KWON et al. 1965).

TBAR was determined by adapting the method used by HEATH & PACKER (1968) who measured TBAR of isolated chloroplasts. Samples of 20 discs were ground in a mortar with 2 ml of reagent (0.25% TBA in 10% trichloroacetic acid) in a cool room. The suspension and two reagent washings were added to a 15 ml calibrated centrifuge tube. The tube was made up to 5 ml with reagent and incubated for 20 minutes in an oil bath at 100 °C.

During incubation the tubes were attached to water cooled condensors to prevent loss of vapour. The centrifuge tubes were cooled to room temperature with running tap water. The extract was adjusted to a volume of 5 ml with demineralized water, mixed, and centrifuged for 10 minutes at 900 × g. TBAR was determined in the supernatant by correcting the absorbance at 532 nm for non-specific turbidity by substracting the absorbance at 600 nm.

Fatty acid analysis

Lipids from samples of 200 discs were extracted with 20 ml ice-cold chloroform-methanol (1:2, by vol.) according to the procedure of ALLEN et al. (1966) except that the chloroform-methanol extract was washed with 0.1 M KCl instead of distilled water for quantitative yield of lipids.

Saponification of the lipids and methylation of the fatty acids with BCl_3 in methanol were as described by KUIPER (1970). Separation of the fatty acid methyl esters by gas-liquid chromatography and their identification were according to KUIPER & LIVNE (1972).

Action spectrum

A crude action spectrum of the increase of TBAR in *Cucumis* leaf discs at 1°C was determined by using broad band interference filters as described before (VAN HASSELT 1973).

3. RESULTS

Effect of temperature

The effect of temperature on TBAR during light and darkness is shown in *fig. 1.*

In the dark there is relatively little alteration of TBAR from 0°C to 25°C. In the light, however, there is a considerable increase of TBAR at 5°C and especially at 1°C. At 10°C there is no increase, while at 15, 20 and 25°C a slight increase of TBAR is observed.

Effect of oxygen concentration

The data of *table 1* show the effect of oxygen concentration in the light and in

Table 1. Effect of oxygen concentration on TBAR. Leaf discs were floated on 1% sucrose at 1°C under mixtures of nitrogen and oxygen for 28 hours in the light and in the dark. Light intensity was 20,000 lux. The figures are the mean of four samples with their standard error.

Gasphase % O_2 in N_2	% TBAR of control (100%)	
	light	dark
0	90 ± 10	84 ± 11
6	216 ± 6	71 ± 5
20	332 ± 15	85 ± 4
100	451 ± 24	93 ± 15

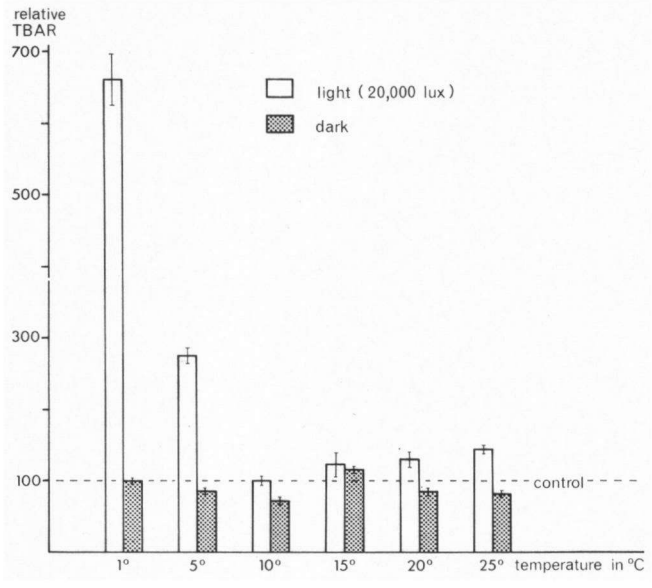

Fig. 1. The effect of temperature on TBAR. Discs were incubated for 48 hours in the light and in the dark. The mean of two determinations is given.

In this figure as well as in the following figures and tables relative TBAR means the TBAR relative to an untreated control, which is adjusted to 100% for reasons of comparison.

the dark on TBAR at 1°C. TBAR does not increase in the dark and in the absence of oxygen (under N_2) in the light.

However, when both oxygen and light are present, an increase in TBAR is observed. As oxygen and light are needed simultaneously it can be concluded that the light-induced increase of TBAR at 1°C is due to a photo-oxidative process.

Kinetics of the increase of TBAR and simultaneous chlorophyll degradation

In *fig. 2* simultaneous time courses of the increase of TBAR and chlorophyll degradation at 1°C in the light are shown. Kinetics of chlorophyll degradation show the biphasic shape known from previous experiments (VAN HASSELT 1972). The time course of TBAR increase also shows a lag phase preceding a fast phase. The lag phase of TBAR is, however, shorter than the lag phase of chlorophyll degradation: TBAR reaches a maximal value of six times the control after 48 hours and thereafter declines to about four times the control after 72 hours.

It can be concluded that the photo-oxidative increase of TBAR shows three phases: first a lag phase, then a second, fast phase which is followed by a third phase in which TBAR decreases.

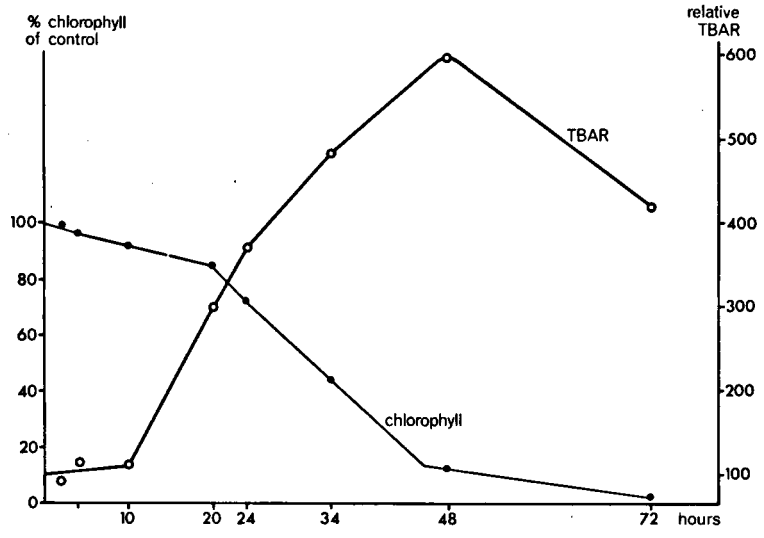

Fig. 2. Simultaneous time courses of the relative TBAR increase and chlorophyll degradation in the light at 1 °C.

Effect of DCMU (3(3,4-dichloro-phenyl)-1, 1-dimethylurea)

DCMU has an inhibiting effect on the light induced increase of TBAR at 1°C *(fig. 3)*.

The maximal inhibitive effect is reached at a concentration of 10^{-5} M.The inhibition cannot be attributed to an effect of the ethanol used to dissolve DCMU as the maximal ethanol concentration used (1 % at 10^{-4} M DCMU) has virtually no effect.

Fig. 4 shows that 10^{-5} M DCMU has no inhibitive effect in the dark and in light under a nitrogen atmosphere. DCMU inhibits only when light as well as oxygen are present. It can therefore be concluded that DCMU inhibits the photo-oxidative process which causes the increase of TBAR at 1°C.

Fatty acid composition

The fatty acid composition of Cucumis leaf discs after different periods of incubation at 1°C is represented in *table 2*.

The fatty acid composition of the untreated control shows that the major part (72.6 %) of the total fatty acids is linolenic acid (C18:3). Palmitic acid (C16:0) is the next most abundant fatty acid. The remaining fatty acids constitute less than 5 % each. Little alteration in fatty acid compostion is observed after 8 hours of light and after 48 hours of darkness. After 24 and 48 hours of light, however, the relative amount of linolenic acid is decreased while the relative amount of the other fatty acids is increased.

It can be concluded that light at 1°C causes a fast degradation of linolenic acid as compared with the rest of the fatty acids.

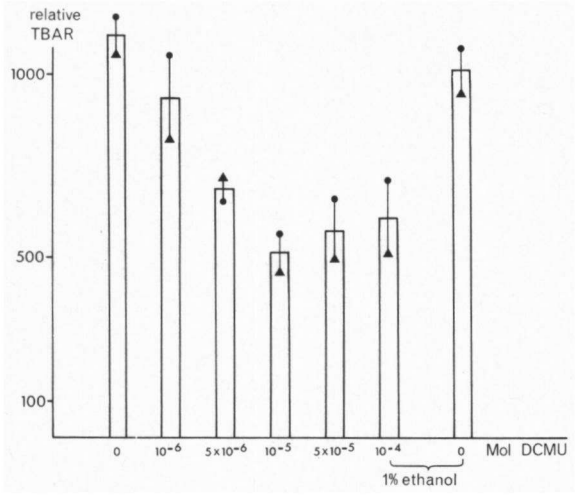

Fig. 3. Effect of DCMU concentration on TBAR after 40 hours at 1 °C in the light. The mean of two experiments (dots and triangles respectively) is given.

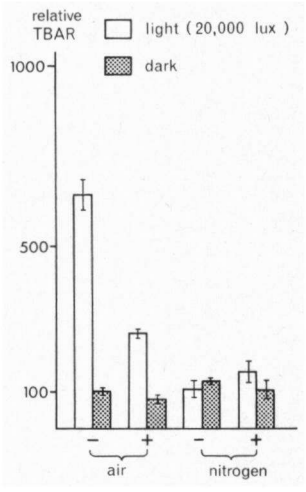

Fig. 4. Effect of DCMU on TBAR. The leaf discs floated on 10^{-5}M DCMU in water (+), on water alone (−), in air or in nitrogen atmosphere during 20 hours in the dark or in the light. The mean of three experiments is given with their standard error.

Kinetics of linolenic acid degradation

Fig. 5 shows that the kinetics of the photo-oxidative degradation of linolenic acid correspond with the kinetics of the TBAR increase. In both cases there is a lag phase of at least 8 hours followed by a fast phase.

This and the high percentage of linolenic acid suggest that the increase of TBAR is to a large extent due to the photo-oxidation of linolenic acid.

Table 2. The fatty acids in leaf discs of *Cucumis* after floating on demineralized water at 1 °C in the light for different times. Light intensity 20,000 lux. The figures give the percentage by weight of the total fatty acids.

Hours at 1°C	Fatty acids (carbons: double bonds).								
	12:1	14:0	14:2	16:0	16:1	18:0	18:1	18:2	18:3
untreated control	0.9	1.1	1.1	13.1	2.9	1.3	2.2	4.8	72.6
8 hours light	0.9	0.8	1.0	13.5	2.7	1.6	2.6	5.8	71.5
24 hours light	1.0	1.3	1.5	15.6	3.3	1.7	2.5	5.0	67.9
48 hours light	1.2	2.3	2.2	23.6	7.5	3.8	5.5	7.6	46.3
48 hours dark	1.0	1.2	1.3	13.1	2.6	1.5	1.3	5.2	71.8

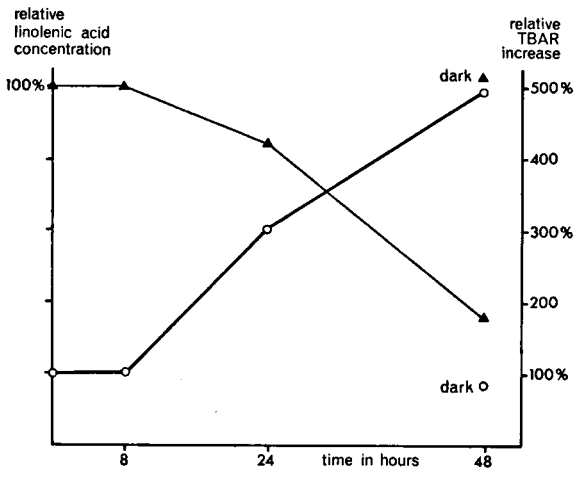

Fig. 5. Simultaneous time courses of relative TBAR increase (O——O) and relative linolenic acid degradation (▲——▲) in the light at 1 °C. Relative linolenic acid concentration is given as the ratio between linolenic acid and palmitic acid. For comparative reasons the value of untreated leaf discs has been adjusted to 100%.

Action spectrum

The action spectrum of the photo-oxidative increase of TBAR shows maxima in the blue (K1) and in the red (K5, K6) spectral region and minima in the green (K4) and in the far red (K7) region *(fig. 6)*. There is a remarkably high effect of blue light (K1, K2) as compared with red light (K5, K6).

4. DISCUSSION

The results clearly show that a substantial photo-oxidative degradation of unsaturated fatty acids takes place in *Cucumis* leaf discs during chilling.

FRIEND & ACTON (1967) have shown that light stimulates hydroperoxide formation and breakdown in sugar beet chloroplasts while HOLDEN (1965) observed that extracts from legume seeds bleached chlorophyll during the breakdown of hydroperoxides of unsaturated fatty acids formed by lipoxidase action. The slight increase of TBAR observed in the light at temperatures above 10 °C *(fig. 1)* is accompanied by chlorophyll degradation (unpublished result). The increase could be attributed therefore to light-stimulated hydroperoxide formation by lipoxidase action.

Linolenic acid is mainly found in the galactolipids of the chloroplast lamellae. The high percentage of linolenic acid in the total fatty acids of the *Cucumis* leaf *(table 2)* is in agreement with the high percentages of linolenic acid observed in chloroplasts of other species (CROMBIE 1958, WOLFF et al. 1962). The high percentage of linolenic acid in the total fatty acids, the resemblance of the kinetics of photo-oxidative linolenic acid degradation, and the increase of TBAR *(fig. 5)*, suggest that the increase of TBAR is due mainly to photo-oxidative degradation of linolenic acid. This agrees with the results of WILBUR et al. (1949) who found that among the saturated and unsaturated C16 and C18 fatty acids linolenic acid is most sensitive in the TBA test.

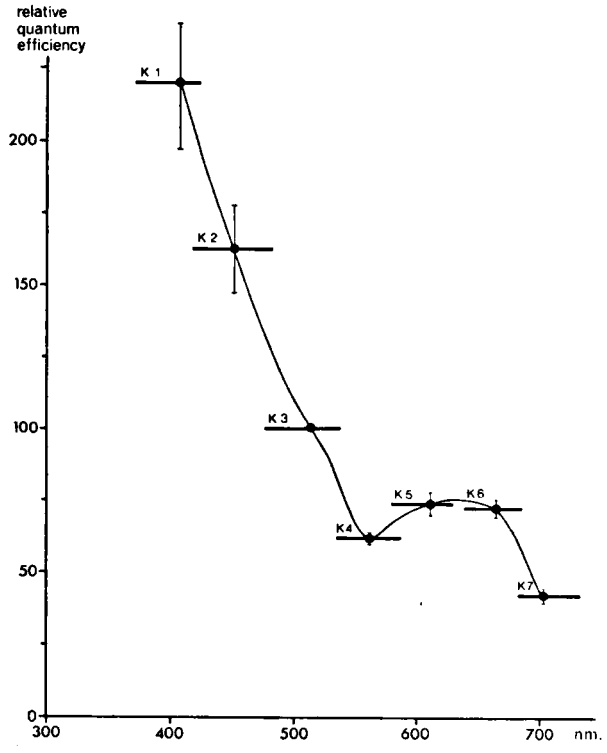

Fig. 6. Action spectrum. Relative quantum efficiency is expressed as the increase of TBAR after 30 hours at 1 °C in light of different spectral regions minus the TBAR of a dark control. The effect of the blue spectral regions (K1, K2 and K3) was compared at 57×10^{14} quanta cm^{-2} sec^{-1}. The effect of the green and red spectral regions (K3, K4, K5, K6 and K7) was compared at 115×10^{14} quanta cm^{-2} sec^{-1}. Twice as much quanta were needed to obtain an increase of TBAR in the green and the red regions during 30 hours of exposition.

The effect of the blue and the red spectral region is compared by relating all values to the value of K3. The value of K3 was about twice as much when twice as many quanta were given and is adjusted to 100 in all experiments. The mean of four experiments with their standard error is given.

A correlation between photo-oxidative injury to living cells and membrane damage has been observed previously (DWORKIN 1958, DODGE 1971). Photo-oxidation of membrane linolenic acid might be an explanation for this observation.

Kinetics of photo-oxidative increase of TBAR show a lag phase which precedes a fast phase *(fig. 2)*. This corresponds with previous data on photo-oxidation during chilling (VAN HASSELT 1972, 1973). A lag phase preceding a fast phase during photo-oxidation can be explained by preferential photo-oxidation of another substance. It has been suggested that carotene protects against photo-oxidation in this way (KRINSKY 1968).

However, carotene photo-oxidation in *Cucumis* leaf discs during chilling shows a short lag phase (VAN HASSELT 1972) and it seems probable that other

substances have a protective function during the initial period of the lag phase.

Phenolic antioxidants protect unsaturated lipids against photo-oxidation in vitro (SCOTT 1965). The possibility of a primary protective function for naturally occurring phenolic antioxidants such as the tocopherols, which are located in the grana lamellae, should be considered.

The lag phase of TBAR increase is shorter than the lag phase of chlorophyll degradation *(fig. 2)*. Preferential photo-oxidation of unsaturated fatty acids cannot be the origin of the lag phase of chlorophyll degradation during the period when both processes show a lag phase. Such a protective function cannot be excluded during the last period of the lag phase of chlorophyll degradation when TBAR increases. On the other hand, HOLDEN (1965) observed chlorophyll bleaching by legume seed extracts during the break-down of unsaturated fatty acid hydroperoxides. The possibility of a similar action by reactive intermediate products of unsaturated fatty acid photo-oxidation on chlorophyll degradation deserves further attention.

The decreasing rate of TBAR after 20 hours *(fig. 2)* can be related to a leakage of TBA reactive substances from the cells. Such a leakage is observed after 72 hours, when part of the TBAR which disappears between 48 and 72 hours is found in the medium. On the other hand, the fast photo-oxidative degradation of the main sensitizer, chlorophyll, after 20 hours may cause a decreasing rate of TBAR.

The inhibiting effect of DCMU, which acts specifically on the reducing side of photosystem II in the chloroplast (PS II), indicates that light energy absorbed by PS II induces photo-oxidation during chilling. The involvement of PS II and the resemblance of the action spectrum with the absorption spectrum of chlorophyll suggest that chlorophyll is the main sensitizer for the photo-oxidation of unsaturated fatty acids.

However, the relatively high quantum efficiency in the blue-green region of the spectrum where chlorophyll absorbs relatively little energy, suggest that carotenoids as well as chlorophylls are sensitizers.

A similar action spectrum was observed for the photo-oxidative decrease of TTC reducing capacity in *Cucumis* leaf discs during chilling treatment in the light (VAN HASSELT 1973). This suggests that in both cases photo-oxidative damage is due to light energy absorbed by the same sensitizers.

The high effect of blue light in photo-oxidation during chilling needs further explanation. Blue light of high intensity is more effective in photo-inhibition of photosynthesis than in photosynthesis of *Chlorella* (KOK 1956). However, no difference in sensitizing pigments is supposed. KANDLER & SCHÖTZ (1956) also observed a stronger action of blue light than of red light in the photobleaching of α and β carotene lacking *Chlorella* mutants.

They suggest that beside chlorophylls yellow pigments like xanthophylls and riboflavins sensitize light energy, which induces photo-oxidation.

The actual mechanism by which light absorbed by chlorophyll initiates unsaturated fatty acid oxidation is unknown. Two probable mechanisms can be distinguished:

1. Unsaturated fatty acid photo-oxidation is caused by free radicals. Free radicals are able to initiate hydrogen abstraction from unsaturated fatty acids, which resulting in a free radical chain peroxidation (SCOTT 1965). Free radicals might be generated by light in the photosystems when insufficient electrons are available due to an inhibitive effect of low temperature on the water splitting system. On the other hand, enzymatic drain of electrons into the Calvin cyclus will be inhibited by low temperature: an excess of electrons in the electron transport chain will be the result.

This could initiate oxidation of reduced intermediates by atmospheric oxygen with concomitant H_2O_2 production (HEBER & FRENCH 1968, RIDLEY & LEECH 1970). H_2O_2 can produce damaging hydroxyl and peroxy radicals.

2. Unsaturated fatty acid oxidation is initiated by excited (singlet) oxygen which is generated by quenching of triplet chlorophyll by ground state oxygen. There is circumstantial evidence for participation of the sensitizer triplet state in photo-chemical reactions (SPIKES 1968) and for generating singlet oxygen by quenching of the sensitizer triplet state by ground state oxygen (OGRYZLO 1970). The occurrence of the triplet chlorophyll state will be favoured by the inhibition of enzymatic conversion of electromagnetic into chemical energy at low temperature. This would explain the high rate of photo-oxidation at low temperatures. The participation of singlet oxygen in unsaturated fatty acid photo-oxidation is supported by the data of RAWLS & VAN SANTEN (1968), who found common products for both chlorophyllsensitized photo-oxidation of methyllinoleate and the direct oxidation of methyllinoleate by singlet oxygen. In addition they observed that photo-oxidation of methyllinoleate was inhibited by singlet oxygen quenchers.

Electron transport is needed in the first mechanism while it is not necessary in the singlet oxygen generating mechanism. Therefore only the first mechanism offers an explanation for the inhibitive effect of DCMU *(figs. 3,4)*. However, DCMU inhibits only partially. Thus a combination of both mechanisms cannot be excluded. The elucidation of the primary photo-oxidative processes during chilling needs further study.

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REFERENCES

ALLEN, C. F., P. GOOD, H. F. DAVIS, P. CHISUM & S. D. FOWLER (1966): Methodology for the separation of plant lipids and application to spinach leaf and chloroplast lamellae. *J. Am. Oil Chem. Soc.* **43**: 223–230.

BRUINSMA, J. (1963): The quantitative analysis of chlorophylls a and b in plant extracts. *Photochem. Photobiol.* **2**: 241–249.

CROMBIE, W. M. (1958): Fatty acids in chloroplasts and leaves. *J. Exp. Bot.* **9**: 254–261.

DODGE, A. D. (1971): The mode of action of the bipyridylium herbicides paraquat and diquat. *Endeavour* **30**: 130–135.

DWORKIN, M. (1958): Endogenous photosensitization in a carotenoidless mutant of Rhodopseudomonas spheroides. *J. Gen. Physiol.* **41**: 1099–1112.

FRIEND, J. & G. J. ACTON (1967): The oxidation of unsaturated lipids by isolated sugar beet chloroplasts. *Brookhaven Symposia in biology* **19**: 485–489.

HASSELT, PH. R. VAN (1972): Photo-oxidation of leaf pigments in Cucumis leaf discs during chilling. *Acta Bot. Neerl.* **21**: 539–543.

— (1973): Photo-oxidative damage to triphenyl-tetrazolium chloride reducing capacity of Cucumis leaf discs during chilling. *Acta Bot. Neerl.* **22**: 546–552.

HEATH, R. L. & L. PACKER (1968): Photoperoxidation in isolated chloroplasts. *Arch. Biochem. Biophys.* **125**: 189–198.

HEBER, U. & C. S. FRENCH (1968): Effects of oxygen on the electron transport chain of photosynthesis. *Planta* **79**: 99–112.

HOLDEN, M. (1965): Chlorophyll bleaching by legume seeds. *J. Sci. Fd Agric.* **16**: 312–325.

KAHN, N. A., W. E. TOLBERG, D. H. WHEELER & W. O. LUNDBERG (1954): Photochemical oxidation of fatty acid esters with and without chlorophyll. Ultraviolet and infrared studies of products. *J. Am. Oil Chem. Soc.* **31**: 460–466.

KANDLER, O. & F. SCHÖTZ (1956): Untersuchungen über die photooxidative Farbstoffzerstörung und Stoffwechselhemmung bei Chlorellamutanten und panaschierten Oenotheren. *Z. Naturschg.* **11b**: 708–718.

KIRK, J. T. O. (1967): In *The Plastids*: 22. W. H. Freeman and Co., London and San Fransicso.

KOK, B. (1956): On the inhibition of photosynthesis by intense light. *Biochim. Biophys. Acta* **21**: 234–244.

KRINSKY, N. I. (1968): Protective function of carotenoid pigments. In A. C. GIESE (ed.). *Photophysiology* III: 145. Academic Press, New York-London.

KUIPER, P. J. C. (1970): Lipids in alfalfa leaves in relation to cold hardiness. *Plant Physiol.* **45**: 684–687.

— & A. LIVNE (1972): Differences in fatty acid composition between normal human erythrocytes and hereditary spherocytosis. *Biochim. Biophys. Acta* **260**: 755–758.

KWON, T., D. B. MENZEL & H. S. OLCOTT (1965): Reactivity of malonaldehyde with food constituents. *J. Fd. Sci.* **30**: 808–813.

OGRYZLO, E. A. (1970): Physical properties of singlet oxygen. In A. C. GIESE (ed.). *Photophysiology* V: 35. Academic Press, New York-London.

RAWLS, H. R. & P. J. VAN SANTEN (1968): Singlet oxygen and the initiation of fatty acid autoxidation. *Tetrahedron letters* **14**: 1675–1678.

RIDLEY, S. M. & R. M. LEECH (1970): Light dependent consumption of oxygen by Vicia faba chloroplasts. *Arch. Biochem. Biophys.* **139**: 351–360.

SCOTT, G. (1965): In *Atmospheric oxidation and antioxidants:* 13–14. Elsevier Publ. Company, Amsterdam-London-New York.

SPIKES, J. D. (1968): Photodynamic action. In A. C. GIESE (ed.). *Photophysiology* III: 53–57. Academic Press, New York-London.

WILBUR, K. M., F. BERNHEIM & O. W. SHAPIRO (1949): The thiobarbituric acid reagent as a test for the oxidation of unsaturated fatty acids by various agents. *Arch. Biochem. Biophys.* **24**: 305–313.

WOLFF, F. T., J. G. CONIGLIO & J. T. DAVIS (1962): Fatty acids of spinach chloroplasts. *Plant Physiol.* **37**: 83–85.