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LIGHT-INDUCED $^{18}O_2$ UPTAKE BY EPOXY XANTHOPHYLLS IN NEW ZEALAND SPINACH LEAVES (*TETRAGONIA EXPANSA*)*

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SUMMARY

1. The effects of light on $^{18}O_2$ incorporation by epoxy xanthophylls in spinach leaves (*Tetragonia expansa*) were investigated. The major epoxy xanthophylls incorporated $^{18}O_2$ more rapidly in the light than in the dark. Incorporation was highest in antheraxanthin, less in violaxanthin and only slight in neoxanthin. This light-stimulated incorporation which occurred without large net changes in pigment concentrations shows that the major epoxy xanthophylls derived their epoxy oxygen from O_2 and confirms recent interpretations of kinetic data that the violaxanthin cycle takes up O_2 through light-induced reactions.

2. The rate of $^{18}O_2$ incorporation under 3770 lux light and 33 % O_2 atmosphere was determined. The rates of $^{18}O_2$ incorporation by antheraxanthin, violaxanthin and neoxanthin were 7, 100 and 5 mμmoles epoxy oxygen/μmole chlorophyll per h, respectively.

INTRODUCTION

Epoxy xanthophylls are present in most O_2 evolving photosynthetic organisms[1,2] but their role in photosynthesis has not been established. It has been suggested that they function in O_2 transport[3], as intermediates in O_2 evolution[4], in protecting against lethal photooxidations[5] and in removing excess photoproducts produced by blue light or converting these photoproducts to other forms of energy[6].

Evidence for the photobiochemical activity of violaxanthin was first reported by SAPOZHNIKOV, KRASOVSKAYA AND MAEVSKAYA[7]. They found that de-epoxidation of violaxanthin occurred under light and anaerobiosis, and that resynthesis occurred in the dark under O_2. Later YAMAMOTO, NAKAYAMA AND CHICHESTER[8] showed that the product of violaxanthin de-epoxidation was zeaxanthin with antheraxanthin as intermediate. Recently it was observed that light stimulated both the forward de-epoxidation and backward epoxidation reactions. Under some conditions the effects of light persisted for a time after the light was turned off in the case of de-epoxidation and was stimulated by low-intensity light in the case of epoxidation. Thus it was

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concluded that the action of light was indirect and that both de-epoxidation and epoxidation were dark reactions mediated by light-generated photoproducts as summarized in the following scheme[9]:

$$\text{Violaxanthin} \underset{\text{photoproduct} + O_2}{\overset{\text{photoproduct}}{\rightleftharpoons}} \text{Antheraxanthin} \underset{\text{photoproduct} + O_2}{\overset{\text{photoproduct}}{\rightleftharpoons}} \text{Zeaxanthin}$$

Scheme 1. Violaxanthin cycle.

The above scheme suggests that the incorporation of O_2 by epoxy xanthophylls is light induced, thus the participation of the violaxanthin cycle in photosynthetic O_2 uptake was proposed[9]. This hypothesis was tested in studies on incorporation of $^{18}O_2$ by xanthophylls of detached New Zealand spinach leaves (*Tetragonia expansa*).

MATERIALS AND METHODS

All chemicals used were of reagent grade. Methanol and acetone were distilled before use. Light petroleum (b.p. 30–60°) was passed through silica gel to remove ultraviolet absorbing impurities. Anhydrous sodium sulfate and Micro-Cel C (Johns-Manville Co.) were dried at 130 and 100°, respectively, before use. The $^{18}O_2$ was generated by electrolysis of $H_2{}^{18}O$ (5–20 atoms % ^{18}O) obtained from the Weizmann Institute, Israel. The illumination apparatus consisted of the light source, small volume illumination chambers and gas manifold. The light source was a row of 12, 4-ft fluorescent lamps and various light intensities were obtained by adjusting the distance of the lamps from the illumination chambers. Light intensity was measured by a Spectra Candela footcandle meter (Photo Research Corporation). The illumination chamber consisted of two, 12 × 24 × 0.5 inch glass plates separated by a 0.25 inch neoprene rubber gasket. The volume of the chamber was about 860 cm^3 and the chamber housed approx. 100 g of leaves.

A manifold was used for nitrogen flushing and admitting $^{18}O_2$. A mercury and water manometer attached to the manifold was used to check for leaks, measure vacuum, and the amount of O_2 admitted. Preliminary tests showed that dilution of added $^{18}O_2$ by non-isotopic photosynthetically evolved O_2 was negligible.

Fresh New Zealand spinach leaves (*Tetragonia expansa*) from a plot outside the Food Science and Technology building were picked in the morning, washed in distilled water, put in polyethylene bags and stored at 2° for 2 h before use. The leaves were spread in the illumination chambers and the chambers were sealed, using "C" clamps. After obtaining an air-tight seal, the chambers were flushed 6–10 times by alternate evacuation to 420 mm Hg and filling to atmospheric pressure with nitrogen. Following final evacuation, $^{18}O_2$ was added (30–50 % O_2) and the pressure of the chamber was raised to 100–200 mm water with nitrogen. These procedures were carried out under reduced light of about 54 lux. The leaves were exposed to various experimental conditions, inactivated in hot absolute methanol containing 0.5 % (w/v) potassium hydroxide to insure against epoxide isomerization by plant acids, and stored overnight at −18°.

All analytical work was done under reduced light. The partially extracted leaves were ground in methanol with a Waring blender, centrifuged, and the residue washed

twice with absolute methanol. Methanol washings were combined, KOH pellets added to make a 10 % solution, and the solution saponified for 30 min in the dark on a magnetic stirrer. The pigments were washed into anhydrous ethyl ether with an equal volume of water, and the ether layer washed 5 times with distilled water. The extract was then dried over anhydrous sodium sulfate, evaporated in a vacuum evaporator, redissolved in a small volume of 5 % acetone–light petroleum, and chromatographed on Micro-Cel C using 13 % acetone–light petroleum[10]. The isolated pigments were dried, dissolved in anhydrous ethyl ether and centrifuged to remove insoluble material, such as Micro-Cel C. The pigments were transferred to pyrolysis tubes, the solvent evaporated under a stream of nitrogen, and the pigments dried overnight *in vacuo*. Since approx. 10 μmoles CO_2 were required for mass spectrometric analysis, benzoic acid (primary standard, Baker Analyzed Reagent) was added as diluting agent. The mixture was pyrolyzed to CO_2 with $HgCl_2$ according to RITTENBURGH AND PONTECORVO[11] and the CO_2 purified as described elsewhere[10].

The ^{18}O content of the O_2 and the CO_2 was determined with a Consolidated–Nier mass spectrometer Model 21–201. The atom percent ^{18}O of the control sample containing lutein and zeaxanthin isolated from untreated leaves and benzoic acid was used as background values for computing atoms percent excess in ^{18}O in the experimental CO_2 samples.

RESULTS AND DISCUSSION

Previous studies were carried out by varying the gas phase and light intensity, causing either a forward or backward net change in the xanthophyll concentration. According to Scheme I, light should stimulate the incorporation of ^{18}O into antheraxanthin and violaxanthin without net changes in the pigment concentrations. Therefore New Zealand spinach leaves (*Tetragonia expansa*) were incubated with $^{18}O_2$ (50 % O_2 containing about 7.8 atoms % excess ^{18}O) under light (5380 lux) and dark conditions for 30 min.

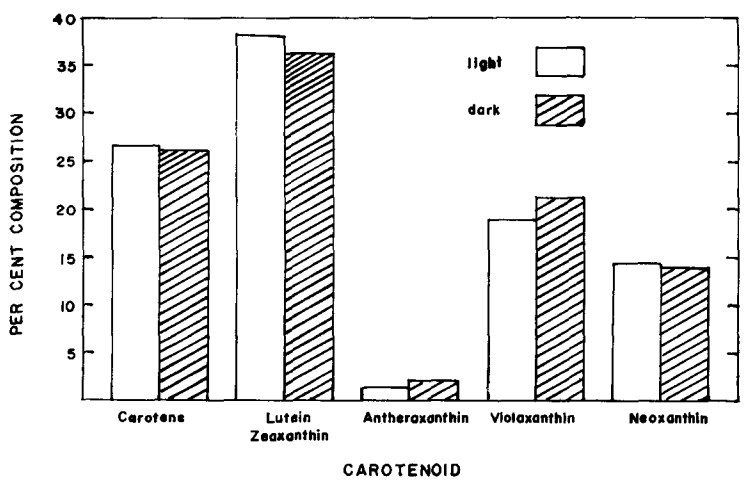

Fig. 1. The effect of light and dark on the carotenoid composition of detached New Zealand spinach leaves. The light sample was exposed to 5380 lux light. Incubation was for 30 min in 50 % O_2 atmosphere with 7.8861 atoms percent excess ^{18}O.

As seen in Fig. 1, the pigment compositions in the light and dark samples at the end of the incubation period were nearly identical. However, Fig. 2 shows that there was considerably more ^{18}O incorporated by leaves exposed to light than leaves kept in the dark. Incorporation was highest in antheraxanthin, less in violaxanthin, and only slight in neoxanthin. Thus, in accord with the proposed scheme, light stimulated the turnover of the violaxanthin cycle and consequently O_2 uptake *via* this pathway.

The rates of ^{18}O incorporation by the epoxy xanthophylls in detached spinach leaves were also investigated. Leaf segments were exposed to approximately 3770 lux light, in 33 % O_2 (about 4.6 atoms % excess ^{18}O) for up to 60 min. Fig. 3 shows

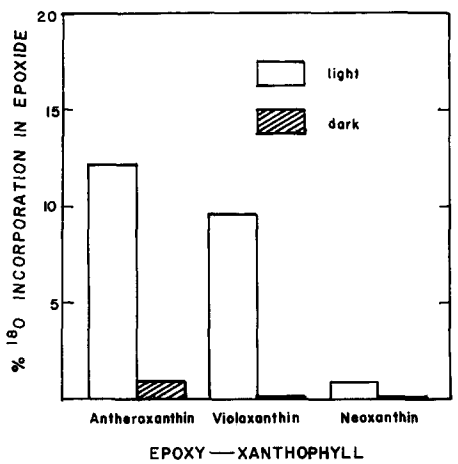

Fig. 2. The effect of light and dark on the incorporation of $^{18}O_2$ by the epoxy xanthophylls of detached New Zealand spinach leaves from experiment described in Fig. 1. The total incorporation of ^{18}O by lutein–zeaxanthin was 0.11 and 0.23 % in the light and dark samples, respectively.

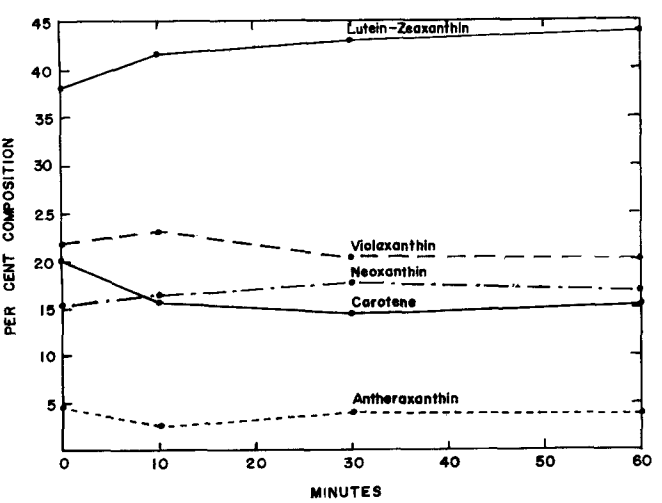

Fig. 3. Carotenoid changes in detached New Zealand spinach leaves under 33 % O_2 (containing 4.6505 atoms percent $^{18}O_2$) and 3770 lux light.

the pigment composition in the leaf segments over the 60-min incubation period. Again, only slight changes in the xanthophyll concentrations occurred during light exposure with possibly slight net de-epoxidation as indicated by the small increase in the lutein–zeaxanthin fraction. Fig. 4 shows the ^{18}O incorporation over the 60-min interval. As was the case with the light–dark experiment, there was considerable incorporation of ^{18}O into the epoxy xanthophylls, and the highest incorporation was in antheraxanthin followed by violaxanthin and neoxanthin. The ^{18}O incorporation appeared to approach a steady state level after about 30 min. We estimated the epoxidation activity of the violaxanthin cycle based on the incorporation for the first 10 min. The rates of epoxidation in mμmoles epoxide/μmole chlorophyll per h were

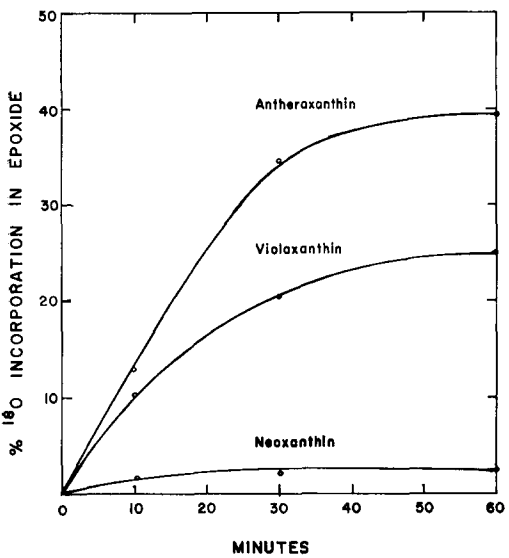

Fig. 4. The rate of ^{18}O incorporation by epoxy xanthophylls in New Zealand spinach leaves from experiment described in Fig. 3. Total incorporation into lutein–zeaxanthin was 1.38, 0.36 and 0.94% after 10, 30 and 60 min incubation, respectively.

7 for antheraxanthin, 100 for violaxanthin, and 5 for neoxanthin. The value for violaxanthin is in agreement with the maximum rate observed in kinetic experiments[9]. Although these rates of oxygen incorporation by the epoxide xanthophylls are very low as compared with most other photosynthetic processes, it does not necessarily follow that the rate of O_2 uptake by the violaxanthin cycle is low. In kinetic studies the rate of concentration change is observed and in ^{18}O studies the rate of ^{18}O incorporation into the pigment pool is followed. In either case it is assumed that all pigment molecules have equivalent activity and the process is in steady state. In a highly organized structure such as the chloroplast, these assumptions probably are not valid entirely. It is more likely that some pigments are more reactive than others depending on their proximity to reaction sites. Thus the rates reported herein show the rates of $^{18}O_2$ incorporation by the pigment pool and not the rate of $^{18}O_2$ uptake of the violaxanthin cycle.

Although we have shown that the violaxanthin cycle undergoes light-stimulated $^{18}O_2$ incorporation and therefore light-stimulated O_2 uptake, other evidence indicates

that the violaxanthin cycle probably is not the pathway for photosynthetic O_2 uptake (System I). A crude action spectrum obtained recently indicates that blue light is more effective than red light[6]. Also System I O_2 uptake has been observed in *Porphyridium cruentum*[12], a unicellular red alga, which does not contain epoxy carotenoids. It is also unlikely that the violaxanthin cycle is a system for protecting cells from lethal photooxidation since the reactions are all dark reactions.

SAPOZHNIKOV[4] suggested that violaxanthin could be a pathway for photosynthetic O_2 evolution based on evidence showing light-stimulated incorporation of ^{18}O from water into epoxy xanthophylls of photosynthesizing algae. However, YAMAMOTO, CHICHESTER AND NAKAYAMA[10] and SHNEOUR AND CALVIN[13] have shown that no ^{18}O from water was incorporated into violaxanthin by isolated chloroplasts in the Hill reaction. The present results not only show that the violaxanthin incorporated O_2 but also that this incorporation was light stimulated. Therefore a plausible explanation for SAPOZHNIKOV's results[4] is that light-stimulated incorporation of ^{18}O from water occurred through reincorporation of photosynthetically produced $^{18}O_2$.

Although the violaxanthin cycle is not the pathway for O_2 evolution, it appears to be associated with System II. It has been shown that System II particles produced from the action of detergents on chloroplasts had a higher violaxanthin content than System I particles[14,15]. Thus, at least one O_2 uptake system appears to be localized with the O_2 evolution system. From this standpoint it is interesting that WARBURG AND KRIPPAHL[16] consider a "backward" O_2 reaction essential for O_2 evolution. However, there is presently no evidence supporting this function for the violaxanthin cycle.

Some investigators have proposed that neoxanthin could be an intermediate between violaxanthin and antheraxanthin[17,18]. Although neoxanthin appears to have appreciable activity in some algae[17] and isolated chloroplasts[19], in leaves, kinetic studies have indicated that neoxanthin was inactive and not part of the violaxanthin cycle[8,9]. The present studies show that although neoxanthin undergoes epoxide reactions similar to violaxanthin, as indicated by the small light-stimulated ^{18}O incorporation, the rate of epoxidation is very low and only a small fraction of the pigment appears to be active. Also the recently established allenic structure for neoxanthin[20] gives further support to the view that neoxanthin is not an intermediate of the violaxanthin cycle.

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