農化學會誌 第九號 (1968)

Action Spectra for Light-Induced De-Epoxidation and Epoxidation of Xanthophylls in Spinach Leaf

KANG HO LEE

PUSAN FISHERES COLLEGE

(Received for publication Dec. 29, 1967)

李 康 浩

釜山水產大學

SUMMARY

The action Spectra for violaxanthin de-epoxidation and zeaxanthin expoxidation in New Zealand spinach leaf segments *Tetragonia expansa*, were determined at equal incident quanta of 2.0×10^{15} quanta cm^{-2} $second^{-1}$. The action spectrum for de-epoxidation had major peaks at approximately 480 and 648 nm. Blue light was slightly more effective than red light and little activity was observed beyond 700 nm. The action spectrum for epoxidation showed major peaks at around 440 and 670 nm. Blue light was more effective than red light and light beyond 700 nm showed definite activity.

The net result of de-epoxidation and epoxidation is a cyclic scheme, the violaxanthin cycle, which consumes O_2 and photoproducts. The action spectra indicate that the violaxanthin cycle is more active in blue than in red light and therefore could accout for O_2 uptake stimulated by blue light. The differences between the action spectra for de-epoxidation suggest that possibly two photosynthetic systems are involved. It was suggested that the violaxanthin cycle may functional a pathway for the consumption of excess photoproducts generated in blue light or the conversion of these photoproducts to other forms of energy.

INTRODUCTION

Violaxanthin appear to be involved in the photosynthetic oxygen metabolism of higher plants and green algae (1). Sapozhnikov *et al* (2) that violaxanthin and lutein, the two major xanthophylls in leaf, were interconvertible through light and dark reactions as shown in Scheme 1.

$$\text{violaxanthin} \underset{\text{dark, } O_2}{\overset{\textit{light, anaerobic}}{\rightleftharpoons}} \text{lutein}$$

Scheme 1

Yamamoto *et al* (3) confirmed this light-induced activity but found that the product of violaxanthin de-epoxidation was zeaxanthin rather than lutein and that antheraxanthin was an intermediate as summarized in Scheme 2.

$$\text{Violaxanthin} \underset{\text{dark, } O_2}{\overset{\text{light, } N_2}{\rightleftharpoons}} \text{antheraxanthin} \underset{\text{dark, } O_2}{\overset{\text{light, } N_2}{\rightleftharpoons}} \text{zeaxanthin}$$

Scheme 2

Yamamoto and Chichester(4) also showed that

molecular oxygen was incorporated directly into the epoxy group of antheraxanthin.

Part of the above scheme, the interconversion of antheraxanthin and zeaxanthin, was found in *Euglena gracilis*(5). However, later work established that the effect of light was indirect and that de-epoxidation was a dark enzymatic reductive reaction. Also, in contrast with leaf, epoxidation of zeaxanthin in *Englena* was reported to be a nonenzymatic light reaction(7).

Recently, Yamamoto *et al*(1) re-investigated the effect of light on xanthophyll interconversions in leaf and found that both de-epoxidation and epoxidation were dark enzymatic reactions mediated by photoproducts as shown in Scheme 3.

$$\text{violaxanthin} \underset{\text{photoproduct}+O_2}{\overset{\text{photoproduct}}{\rightleftharpoons}} \text{antheraxanthin} \underset{\text{photoproduct}+O_2}{\overset{\text{photoproduct}}{\rightleftharpoons}} \text{zeaxanthin}$$

Scheme 3

The net result of Scheme 3 is consumption of photoproducts and O_2. Thus it was suggested that this could be a pathway for light-induced uptake. In the present study action spectra for light-induced xanthophyll de-epoxidation and epoxidation in New Zealand spinach leaf segments were determined.

MATERIALS AND METHODS

Preparation of leaf segments. Mature New Zealand spinach leaves(**Tetragonia expansa**) grown in the field were picked at about 8 : 00 a.m., washed with distilled water, placed in polyethylene bags, chilled to 2°C and used for epoxidation experiments. In de-epoxidation experiments fresh leaves were "dark-adapted" overnight in total darkness at 2°C. Fresh leaves or dark-adapted leaves were cut into approximately 1 cm^2 segments, mixed, and distributed into beakers in amounts adequate for a single illumination. The beakers were capped with aluminum foil and kept in the dark until used. All procedures were carried out at 2°C under about 1×10^2 lux illumination.

Illumination procedure. Monochromatic light was obtained by filtering light from a quartz-iodine lamp (Sylvania DWY) through three heat absorbing glass filters (Ansco) and a series of ten interference filters (Baird Atomic Type B-1, 2×2 inches, half bandwidth 6-10 nm) from 400 to 720 nm. The illumination chamber was a water jacketed beaker, which held approximately 1 gram of leaf segments and was illuminated with monochromatic light through the bottom of the beaker. The atmosphere was controlled by continuous flushing with gases from compressed tanks and temperature was controlled by circulating water at 20°C. The beaker was protected from background light by a light-tight cover to which a 15-watt round fluorescent lamp was attached for epoxidation experiments. Light intensities were regulated with a powerstat. Monochromatic light intensity was measured with a YSI Model 65 Radiometer and white light intensity with a Spectra Candela foot-candle meter.

Action spectrum for de-epoxidation. About 1 gram of leaf segments was spread in the illumination beaker in a single layer with the upper surface of the leaf facing the monochromatic light. The beaker was flushed with humidified high-purity (minimum 96. 96%) N_2 at 600 cm^3 min^{-1} for 10 minutes in the dark. The leaf segments were exposed to monochromatic light for 30 minutes under N_2, inactivated in hot anhydrous methanol, heated to boiling, stored at -18°C, and analyzed for epoxide content the following day. The effect of monochromatic illumination was determined as the difference in percent epoxide content from an unilluminated control sample.

Action spectrum for epoxidation. The leaf segments were distributed in the same way as for de-epoxidation. To increase the nonepoxide content of the leaf segments, the leaf segments, the leaf segments were illuminated with white light from a round fluorescent lamp under N_2 before monochromatic illumination under air. This initial de-epoxidation was carried out by flushing with N_2 for 10 minutes in the dark, white light illumination for 20 minutes, and a final 10 minutes dark "rest" period. Following de-epoxidation, leaves were exposed to monochromatic light for minutes under 30 humidified air at 600 cm^3 min^{-1}, inactivated, stored and analyzed as described for de-epoxidation experiments. The control sample was a similarly treated sample except for substitution of darkness

for monochromatic light. The effect of monochromatic illumination on epoxidation was determined as the difference in percent epoxide content from the control.

Analysis of xanthophylls. The methanol extract was decanted, the nearly colorless segments were rinsed with a small amount of methanol, 6 g KOH was added to the extract and the mixture was stirred with a magnetic stirrer for about 15minutes in the dark. After saponification, the carotenoids were washed into peroxide free ethyl-ether, the extract was washed several times with distilled water and dried ether with powdered anhydrous sodium sulfate, or by freezing at -18°C for 3~4 hours. The extract was concentrated in a rotary vacuum evaporator, transferred to a small test tube, and adjusted to 0.5 ml. About 50 μl of pigment solution was applied as a narrow band on a glass slide coated with Micro-Cel C. The pigments were resolved in 20 minutes with 13% acetone-petroleum ether (BP 30-60°C) in an unsaturated chamber which was protected from light during chromatography. Clear separations of xanthophylls, comparable to column methods(8), were obtained. The carotenes were not resolved and migrated with the solvent front.

The bands of violaxanthin, antheraxanthin and lutein-zeaxanthin were scraped into 15 ml centrifuge tubes and eluted with 3.5 ml redistilled acetone. The mixture was agitated to insure thorough extraction of pigments and was centrifuged for 5 minutes in a clinical centrifuge. The concentration of xanthophylls in the clear supernatent solution was determined spectrophotometrically with a Beckman DU spectrophotometer at 440, 447, and 450 nm for violaxanthin, antheraxanthin, and lutein-zeaxanthin, respectively, with corresponding extinction coefficients ($E_1{}^{1\%}{}_{cm}$) of 2550, 2500, and 2500. The percent epoxide was calculated as percent by weight of violaxanthin and antheraxanthin to the total weight of violaxanthin fractions. Neoxanthin, one of the major epoxy xanthophylls in leaf was not included in the calculations because previous studies (1,2) have shown that the concentration of neoxanthin was not affected appreciably by light.

Preparation of Micro-Cel C thin layer slides. Ten gram of Micro-Cel C (Johns-Manville Co.) in 75 ml water was ground in a mortar, approximately 3 ml of the suspension was spread uniformly on a 2×20 cm glass slide, and excess water was evaporated at room temperature. The slides were activated in an oven at 90~100°C overnight before use. Adhesion of Micro-Cel C to glass is adequate without binder. Calcium sulfate appears to inactivate the resolving capacity of Micro-Cel C. Each slide can resolve approximately 10 g of leaf xanthophylls.

RESULTS

Action spectrum for de-epoxidation. Since only a single wavelength could be tested at a time, an average of 5 filters could be run in a day and a minimum of days were required to cover the entire range from 400 to 720 nm. Initial results using fresh leaves were disappointing because de-epoxidation activity under monochromatic illumination varied considerably from day to day, especially in red light. Modifications of the illumination procedure such as removing traces of oxygen from nitrogen gas, and varying the time and intensity of monochromatic illumination were ineffective in reducing this variation. However, improved consistency and de-epoxidation activity were obtained by dark adaptation of the leaves overnight in total darkness at 2°C.

The action spectrum for xanthophyll de-epoxidation in dark adapted spinach leaf segments at equal incident quanta of 2.0×10^{15} quanta cm^{-2} $second^{-1}$ from 400 to 720 nm is shown in Fig. 1.

The range of de-epoxidation activity at any given wavelength was fairly large but there were sufficient differences in activity at different wavelengths that an action spectrum was discernable. The action spectrum for de-epoxidation shows activity in the blue and red regions but with slightly more activity in the blue region. The major peaks were located at approximately 480 and 648 nm, and very little activity was observed beyond 700 nm.

Action spectrum for epoxidation. Preliminary experiments determined that fresh leaves had better epoxidation activity than dark adapted leaves and that the effect of monochromatic light was different depending on the intensity of white light used for the initial de-epoxidation. When 2.2×10^{3} lux was

used for the initial de-epoxidation, the effect of subsequent monochromatic illumination was to stimulate epoxidation at all wavelengths investigated. When a higher white light intensity of 6.5×10^3 lux was used, subsequent illumination with monochromatic light in the red region resulted in net de-epoxidation. These results were analogous to those reported earlier (1) on light induced epoxidation with white light, where it was shown that stimulation of epoxidation by white light was most evident when the intensity of white light used for the initial de-epoxidation treatment was low. Thus we presume that under relatively high white light intensity an excess of photoproducts for de-epoxidation is produced which masks epoxidation activity in the subsequent treatment.

The action spectrum for epoxidation from 400 to 720 nm at equal incident quanta of 2.0×10^{15} quanta cm^{-2} $second^{-1}$ is shown in Fig. 2. The intensity of white light used for the initial de-epoxidation was 2.2×10^3 lux.

Epoxidation was stimulated by blue and red light but blue light was considerably more effective than red. The peaks are located at approximately 440 and 670 and some activity is evident beyond 700 nm.

DISCUSSION

In earlier papers, de-epoxidation of violaxanthin and epoxidation of zeaxanthin were termed interconversion reactions. Since epoxidation does not appear to be a simple reversal of de-epoxidation (1, 4), we now prefer the more descriptive term "violaxanthin cycle" which is analogous to the term "antheraxanthin cycle" introduced by Krinsky (7) for reactions in *Euglena*.

The action spectra for de-epoxidation confirm, under conditions of monochromatic illumination, that both de-epoxidation of violaxanthin and epoxidation of zeaxanthin are light-induced reactions. The results also show that light in the regions of chlorophyll and carotenoid absorption are utilized for these reactions.

While both blue and red light are utilized for the violaxanthin cycle, it appears that generally blue light is more effective than red especially for epoxidation. Thus the overall cycle would be expected to be more active under blue than under red light.

French (9) recently summarized the numerous effects of blue light on photosynthetic and respiratory processes which have not been explained and suggested that they may be due to unidentified blue absorbing chloroplast pigments. Stimulation of respiration by blue light has been observed by Emerson and Louis(10) and Reid (11) in *Chlorella* and French and French and Fork (12) in a Chlamydomonas mutant. Reid (11) observed very large increases in respiration after 1 second exposure to blue light which lasted for as long as 8 minutes and consumed 500 times more oxygen than the amount evolved during light exposure. The results were interpreted as an activiation of an enzyme system of the cellular respiratory mechanism rather than an inherent part of the photosynthetic system. The fact that these effects thus far have been observed in photosynthetic cells which have epoxy xantophylls but not in *Porhyridium cruentum* (13) may be significant.

The possible role of epoxy xanthophyll interconversion in photosynthetic O_2 uptake has been discussed(1). The present results suggest that the violaxanthin cycle could account for photosynthetic O_2 uptake in blue light. However, it is clear that the violaxanthin cycle is not the path for photosynthetic system 1. Photosynthetic system 1 is more active in red than in blue light(14) and has been observed in Porhyridium cruentum (15) which is devoid of epoxy xanthophylls (16).

The function of epoxy xanthophylls in plants has not been determined although several proposals have been made(1, 6, 17-20). Krinsky (7) has proposed that the antheraxanthin cycle in *Euglena gracilis* could protect it from lethal photo-oxidation. Earlier Nakayama(21) and Calvin(22) discussed the possiblity that carotenoids might protect photosynthetic organisms against lethal oxidations by an undetermined cyclic regenerative system. Although epoxide cycles may serve in this role, it is clear that carotenoids can protect an organism against photo-oxidation by other means since photosynthetic bacteria (23) are protected apparently without the aid of epoxy carotenoids and even O_2 evolving

algae such as the Cyanophyceae (24) apparently do not contain epoxy carotenoids.

Calvin (22) has pointed out that protection may be an indirect effect of a reaction which removes the excitation energy in some useful way. The fact that reactions of the violaxanthin cycle are dark reactions tends to support the concept that the primary function of the cycle is something other than protection against photo-oxidation.

Since the results of the violaxanthin cycle are the consumption of photoproducts and O_2, this could be a pathway for the consumption of excess photoproducts generated in blue light or possibly a pathway for the conversion of these photoproducts to other forms of energy.

The differences in the action spectrum for de-epoxdiation and epoxidation suggest that the photoproducts for epoxidation and de-epoxidation are generated by different photosynthetic systems. Although the action spectrum for de-epoxidation and epoxidation differ from photosynthetic systems 1 and 2 in their response to blue light, the locations of the major peaks in the red region for de-epoxidation and epoxidation are similar to the locations of the major peaks for system 2 and system respective (17). These results therefore suggest a possible assoication of the components of the violaxanthin cycle with these photosynthetic systems.

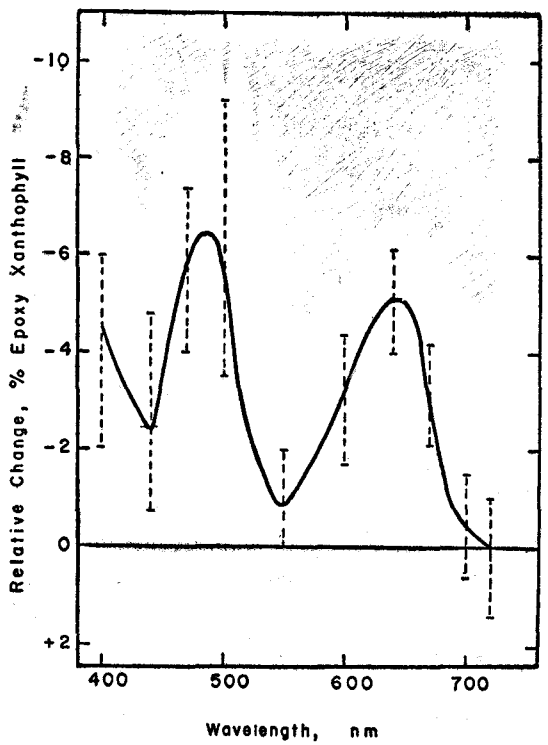

Fig. 1 The action spectrum for light-induced de-epoxidation at equal incident quanta of 2.0×10^{15} quanta cm^{-2} sec^{-1} in New Zealand spinach leaf segments. The curve is drawn through the average values from 5~6 experiments. The brokem lines show the range.

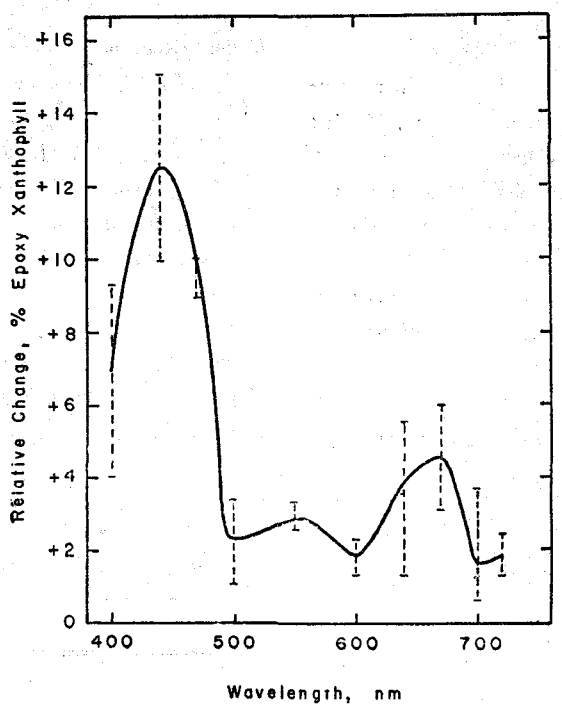

Fig. 2. The action spectrum for light-induced xanthophyll epoxidation at equal incident quanta of 2.0×10^{15} quanta cm^{-2} sec^{-1} in New Zealand spinach leaf segments. Initial illumination was 2.2×10^{3} lux. The curve is drawn through the average values from 2~3 experiments. The broken lines show the range.

要 約

뉴우질랜드 시금치(**Tetragonia expansa**)잎의 切片을 2.0×10^{15} quanta cm^{-2} sec. $^{-1}$의 일정 레벨의 에너지를 가지는 各波長의 光線을 쪼였을때 일어나는 violaxanthin의 de-epoxidation과 zeaxanthin의 epoxidation 反應의 action spectrum을 測定하였다. De-epoxidation의 action spectrum에서는 480 nm와 648 nm에 두개의 주 피이크를 나타 내었고 靑色光線은 赤色光線보다 더 有効하였으며, 700 nm 이상의 波長에서는 効果가 없었다. 한편 expoxidation의 spectrum은 440과 670 nm 近處에 피이크를 나타내었고 이것 또한 靑色光이 赤色光보다 有効하였으나 700 nm 이상의 波長에 있어서 確實히 効果를 나타 내었다.

Epoxidation과 de-epoxidation의 相反되는 두 反應의 正의 結果는 하나의 cyclic scheme 즉 酸素와 光反應生性物을 消費하는 "violaxanthin cycle"로 보아진다. Action spectra가 지시하는바와 같이 靑色光이 赤色光線보다 더 有効하다는 것은 靑色光에 의한 刺戟으로 進行되는 O_2-uptake로 看做된다. 두 action spectrum 間의 差異點들은 다분히 두개의 光合成機構가 同時에 關聯되고 있다는 것을 시사한다. 그러므로 violaxanthin cycle은 靑色光에 위해서 發生하는 過剩한 光合成生成物의 消費經路로서, 또는 그들 生成物을 어떤 다른 形態의 에너지로 轉換하는 役割을 하는것이라 보아진다.

REFERENCES

1. H.Y. Yamamoto, J.L. Chang, and M.S. Aihara, Biochim. Biophys. Acta(in press).
2. D.I. Sapozhnikov, T.A. Krasfvakaya, and A. N. Maevskaya, Dokl. Akad. Nauk, U.S.S.R. **113**. 465 (1956).
3. H.Y. Yamamoto, T.O.M. Nakayama, and C.O. Chichester, Arch. Biochem. Biophys. **97**, 168 (1962).
4. H.Y. Yamamoto and C.O. Chichester, Biochim,

Biophys. Acta **109**, 303 (1965).

5. N.I. Krinsky. Biochim. Biophy. Acta **88**, 487 (1964).
6. M.S. Bamji and N.I. Krinskys, J. Biol. Chem. **240**, 467 (1965).
7. N.I. Krinsky, in Biochemistry of Chloroplasts (T.W. Goodwin, Ed.), Vol. 1, p. 429, Academic Press, London (1966).
8. H.Y. Yamamoto, C.O. Chichester, and T.O.M. Nakayama, Photochem. Photobiol. **1** 53 (1962).
9. C.S. French, in Biochemistry of Chlorpolasts (T.W. Goodwin, Ed.), Vol. 1, p. 337, Academic Press, London (1966).
10. R. Emerson and C.M. Lewis, Am. J. Bot. **30**, 165 (1943).
11. A. Reid, Carnegie Institution of Washington Year Book **64**, 399 (1965).
12. C.S. French and D·C. Fork, Carnegie Institution of Washington Year Book **60**, **357** (1961)
13. C.S. French and D.C. Fork, Carnegie Institution of Washington Year Book **60**, 351(1961)
14. W. Vidaver and C.S. French, Plant Physiol. **40**, 7 (1965).
15. C.S. French, in "Photosynthesis Mechanisms in Green Plants" Pub. 1145, p. 355. National Academy of sciences, National Research Council (1963).
16. F.T. Haxo and C. Oh Cocha. in Proceedings of the Second International Seaweed Symposium (T. Braarud and N. A. Sorensen, Eds.)p. 23, Pergamon Press, London(1956).
17. L. Cholnoky,C. Gyorgyfy, E. Nagy, and M. Panczel, Nature **178**, 410 (1956).
18. G.D. Dorough and M. Calvin, J. Am. Chem. Soc. **73**, 2362 (1951).
19. D.I. Sapozhnikov, Dokl. Akad. Nauk S.S.S.R. **154**, 974 (1964).
20. C.O. Chichester, H.Y. Yamamoto, and K.L. Simpson. Bacteriol. Proc. 12 (1966).
21. T.O.M. Nakayama, in Physiology and Biochemistry of Algae(R.A. Lewin, Ed), p. 409, Academic Press, New York (1962).
22. N. Calvin, Nature **176**, 1215 (1955).
23. R.Y. Stanier, Harvey Lectures, Ser. **54**, 216 (1958~59).
24. T.W. Goodwin, in Chemistry and Biochemistry of Plant Pigments (T.W. Goodwin, Ed.,), p. 127, Academic Press, London (1965).