Acta Biologica Szeged 18 (1—4), pp. 81—91 (1972)

LIGHT-INDUCED TRANSFORMATIONS OF PIGMENTS II. THE ROLE OF WATER IN THE TRANSFORMATION OF CAROTENOIDS

I. MARÓTI and IRÉN SZAJKÓ

Department of Botany, Attila József University, Szeged

(Received January 15, 1972)

Introduction

Carotenes and xanthophylls are generally to be found in the choroplasts of photosynthetically active plants, where they undergo continual transformation. The carotenoids have a very important role in the photosynthetic system, the following transformation reactions being of outstanding importance for the performance of their active functions:

a) cyclisation of the acyclis carotene precursors,
b) hydroxylation and dehydroxylation,
c) epoxidation and desepoxidation.

The effects of light, O_2 and N_2 on the synthesis and transformation of carotenoids have been dealt with by STRAIN (1959), SAPOZHNIKOV et al. (1965, 1969), KRINSKY (1966), HAGER (1966, 1967), DONOHUE et al. (1967), CLAES (1967), GOODWIN (1969), and many others. Nevertheless, no investigations are known dealing with the joint effect of light and the water contents of the plast and leaf. It is to be expected on the basis of several observations that the transformation of carotenoids is influenced by water.

a) It has been verified experimentally (NAGY et al. 1967, DEROCHE—COSTES, 1966, 1969) that carotenoids, like chlorophylls, are linked to the multi-enzyme complexes of thylakoids in various ratios and by various types of bond. The different apolar and polar binding forces (hydrogen binding, apolar binding, ionic interactions, van der Waals' forces) depend to a large extent upon the water content of the multi-enzyme surfaces.

b) The carotenoid distributions of the photosynthetic pigment system I (PS I) and pigment system II (PS II) are very characteristic.

	ch—a	ch—b	car.	lut.	viol.	neo
Thylakoids	100	38	13	14	5	2
PS I	100	22	15	7	7	0,5
PS II	100	54	11	21	4	3,5

The relative pigment contents of thylakoids of the chloroplast of *Spinacia oleracea*, and of the two pigment systems (PS I and PS II); the numbers of other pigment molecules are those corresponding to 100 chlorophyll a molecules (according to LICHTENTHALER 1969).

According to the hypothesis of SAPOZHNIKOV (1969), the light-induced desepoxidation reactions of violaxanthin can take place in two ways:

a) The photosensitized oxidation of epoxygroups in the state of aerobiosis, in the course of which molecular oxygen is formed, is connected with the second pigment system (PS II).

b) Desepoxidation under anaerobic conditions is related with the first pigment system (PS I) and photoreduction takes place; water is therefore formed the oxygen of the epoxygroup.

It is presumed that in plasts which become dry during strong illumination, the desepoxidation of a part of the violaxanthin in PS II can also take place through photoreduction, as a result of the loss of water.

c) Several reports and assumptions can be found in the literature (KESZTHELYI 1970, CHUA 1970), according to which water occurs in the living organism in a socalled „anomalous" structure. It is known that anomalous water is formed in the capillaries, from the vapour phase. It is presumed that the transformation of the xanthophylls will also be influenced by whether the isolated leaves float on the surface of liquid water, or are in a highly humid, or dry environment during illumination. In a humid environment, the water molecules may diffuse to approximately the same extents from any direction of space into the leaf-disks, or to the surface of the thylakoid membrane. Starting from this fact, it is assumed that during illumination the symmetrical molecules (e. g. violaxanthin) change less or accumulate in the leaves in the vapour phase. As the leaf-disks float on the water, the current of water molecules is nearly unidirectional, and this favours the formation of asymmetrical pigment molecules (lutein, antheraxanthin).

The joint effect of light and water on photosynthesis is, of course, quite involved; at present, we have only begun to study its influence on the transformation of carotenoids. In the course of our investigations it was desired answer the following questions:

1. Do the heterogeneity of the carotenoids and the strength of binding forces change as a result of strong illumination and a decrease in the water content?

2. How does strong illumination (40—60,000 Lux) exert its effect during short experiments on the change in the carotenoid content of the leaves floating on the surface of water or in a very humid or dry environment?

Materials and Methods

Isolated leaves were used from the field-grown plants *Nonea lutea* (DESR.) DC and *Rumex acetosella* L. The completely developed, but not old leaves were collected at 6 a. m. Disks of 1 cm diameter were prepared from the leaves with a cork-borer, by the „leaf-half" method, with the following considerations:

1. The sample was taken from the middle part of the leaf-half, as the pigment-distribution changes from the leaf base towards the apex.

2. The leaf-veins do not contain any pigments, and therefore symmetrical leaves were generally used so that the indiviual samples would contain the same amount of leaf-veins.

3. In general four, and more rarely six disks were cut from each leaf.

4. Sixteen leaf-disks were used in each experiment. The sixteen disks originated from sixteen leaves. The fresh weight of the sixteen leaf-disks was 0,340 g, the dry weight was 0,045 g and the surface was 12,56 cm^2. Attention was also paid to the homogeneous distribution of the leaf-disks.

The leaf-disks were illuminated in a glass vessel of 6,8 cm diameter of our own design. During the illumination, the leaf-disks lay upper surface upwards in the vessel on a net made of plastic in humid, dry of wet environments. The vessel was covered with a colourless polyethylene foil. For illumination two 1000 W iodine-vapour lamps (Tungsram Halogen) were used, that together gave a light of 60,000 Lux even after water filtration. The illumination generally lasted for the following times: 5, 10, 15, 20 or 40 minutes. After illumination the temperature in the vessel was 20 ± 3 °C.

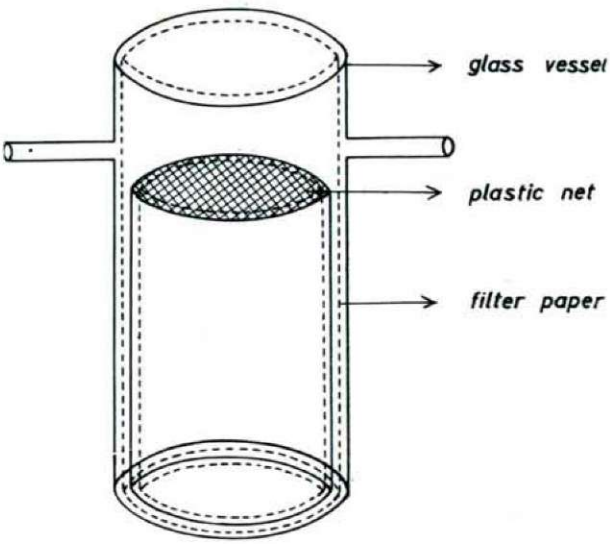

Fig. 1. Glass vessel for the experiments.

The controll was kept in the dark at room temperature (20 °C) under conditions appropriate for the sample, and the pigments were extracted immediately from the leaf-disks either of two ways:

1. The extraction was performed by the method of MARÓTI and GABNAI (1971).

2. The heterogeneity of the carotenoids and the enhancement of this on the action of light were investigated by another method of pigment extraction. The pigments were obtained from samples and controls treated in the same way with various solvents:

a) with absolute petroleum ether,

b) with a 99 : 1 mixture of petroleum ether : ethanol,

c) by method (2) (2 ml absolute acetone and about 8 ml petroleum ether).

In the case of methods *(a)* and *(b)*, only a fraction of the pigments was contained in the extract. The residual pigments were extracted by repeated rubbing as in method (2) until the whole of the pigment content was in the extracts. The extract volume was adjusted in all cases to 5 ml, and can be used immediately for separation by thin-layer chromatography. The chlorophylls, carotenes, and xanthophylls were separated in this way (MARÓTI and GABNAI, 1971).

The individual pigments were eluted and measured in the previously described manner (MARÓTI nad SZEMENKEI, 1972).

Results and Discussion

Role of light and water in the heterogeneity of carotenoids

The heterogeneity of chlorophylls has been definitely proved (SHLYK—FRADKIN, 1962; SHLYK—NIKOLAEVA, 1962; SHLYK et al., 1969; NAGY et al., 1967). Chlorophyll a in an in vivo state in the chloroplast divergent, while chlorophyll b is bound more strongly than chlorophyll a in the multi-enzyme complexes.

There are only few data available as to the binding of carotenoids, but the results so far (DEROCHE—COSTES 1966; 1969; NAGY et al., 1967) have demontstrated convincingly that the carotenoids can be bound in the thylakoid membrane in several ways.

On the basis of theoretical considerations and their experimental results, PAULING (1961) and KLOTZ (1962) assume that water plays a role in the carotenoid-protein binding the water forming well-conducting hydrotactoid layer on the multi-enzyme surfaces.

The dependence of the stability of the carotenoid-protein complex on the temperature was investigated by NAGY et al. (1967). They demonstrated that the carotenoids were bound more weakly in the proteo-lipid complexes than in chlorophylls.

DEROCHE—COSTES (1969) extracted pigments from fresh leaves and freeze-dried chloroplasts with solvents of increasing polarity at different temperatures. They demonstrated that, on the basis of the binding, there are two kinds of β-carotene molecules in the plast. One of them is weakly bound in the complex (ca. 99%), the other (ca. 1%) more strongly.

The heterogeneity of violaxanthin and lutein in green leaves has also been observed. No data could be found in the literature to show what part is played by light, or by light and the water-content together, in the heterogeneity of the carotenoids. It is assumed that light weakens the pigment-protein binding by exciting the pigment molecules, and in this way the different nature of the bindings is revealed even more clearly. It was obvious that the complete separation of the effects of light and heat was impossible. Nevertheless, an attempt was made to reduce to a minimum the effect of the change in temperature, in the following way:

1. The glass vessel in which the experiment was carried out was cooled, together with the leaf-disks, to + 4°C before illumination.

2. The illumination lasted for 20 minutes (60,000 Lux) and the temperature of the humid air in the vessel became 24°C. It is known (NAGY et al., 1967) that the „melting point" of the carotenoid-protein complex is about 40—50°C.

3. After illumination, the disks were again cooled to 4°C (ten minutes) and the pigments were then eluted with cooled solvents.

The amount of carotenoid extracted depends upon the quantity of solvent (NAGY et al., 1967). In our experiments 2 ml acetone + 8 ml petroleum ether was used for complete extraction of the pigments from the sixteen leaf-disks of 0,340 g fresh weight; and 10 ml 99 : 1 petroleum ether : ethanol or 10 ml

petroleum ether for the differential extraction of the free and bound pigments. The pigment extract volume was always adjusted to 10 ml. The results are given in Table I. The amounts of carotenes, lutein + zeaxanthin, neoxanthin and viloaxanthin, kept in the dark and obtained with a complete extraction, were taken as 100%.

Table I. Effect of illumination on the amount of carotenoids extracted from *Nonea lutea* leaves by solvents of different polarities.

solvent	treat-ment	carotenes %	antheraxanthin lutein zeaxanthin %	neoxanthin %	violaxanthin %
petroleum ether 100%	dark	38,1	9,2	—	19,3
	illuminated	76,9	10,4	—	—
99 : 1 petroleum ether : ethanol	dark	49,2	5,6	—	8,5
	illuminated	76,0	9,6		—
2 : 8 acetone : petroleum ether	dark	100	100	100	100
	illuminated	120,3	148,0	91,6	34,1

As a result of illumination, the following changes may be observed in the heterogeneity of the carotenoids in a leaf of *Nonea lutea:*

1. 40—50% of the carotenes is in a readily-soluble fraction, that is, it is bound only weakly to the macromolecular complexes of the chloroplast. This result differs from that of DEROCHE and COSTES (1969), who found 99%. The cause of the difference may be that they worked with a 13 day wheat seedling, and we with a completely developed leaf of *Nonea lutea.* Further, they obtained this high weakly-bound carotene fraction from a freeze-dried chloroplast, and in that state of the carotenoid-protein complex water can have played only a small part in the strength of the binding.

2. As a result of illumination, the total carotenoid content increases, or the individual components are transformed, and therefore for the illuminated samples we have calculated the amounts of carotene, lutein + zeaxanthin, neoxanthin and violaxanthin in the extracts obtained with petroleum ether and petroleum ether-ethanol, as compared with the illuminated control. On illumination, the readily-soluble carotene fraction increases considerably.

3. In the samples kept in the dark, the weakly-bound violaxanthin occurs as 8—20%; in the illuminated leaf-disks, however, no violaxanthin or neoxanthin was obtained with the eluting materials applied. The weakly-bound violaxanthin molecules are presumably the first to take part in the light-induced desepoxidation violaxanthin ⟶ antheraxanthin ⟶ zeaxanthin.

4. No essential change occurred in the binding of the zeaxanthin and lutein in the complexes as a consequence of the illumination.

Transformation of carotenoids

The transformations of carotenoids are primarily light-induced reactions, but they also depend on the composition of the gases in contact with the plant, the temperature and the water content. The transformations of carotenoids

were listed and classified by DONOHUE et al. (1967) on the basis of the literature data and their own experimental results. We too have observed, the order of the reactions of transformations suggested by them, and have supple-

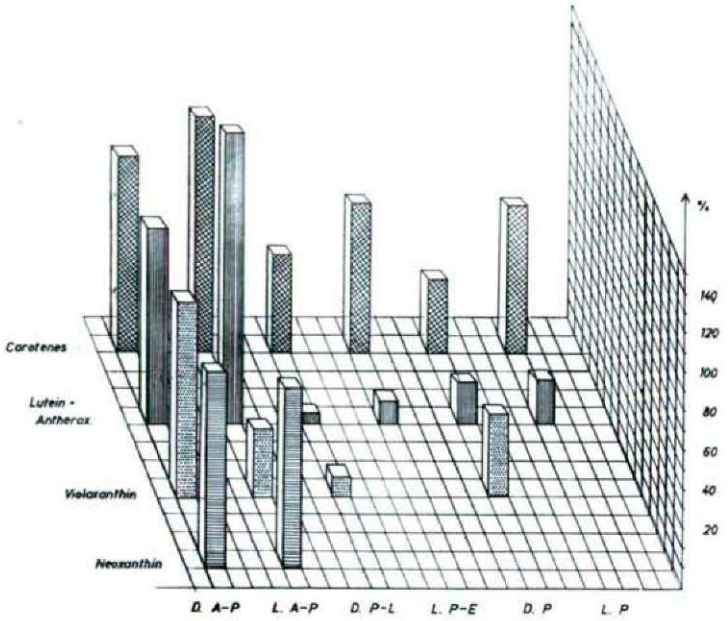

Fig. 2. Effects of light and dark upon the heterogeneity of carotenoids. The change in the carotenoid-protein binding is indicated by the pigment amounts soluble in solvents of different polarities.
D = dark; L = lighted; A—P = acetone-petroleum ether (2 : 8); P—E = petroleum ether-ethanol (99 : 1); P = petroleum ether.

mented the Table with our results (MARÓTI and SZEMENKEI 1972). In contrast with the literature data, we assume that zeaxanthin and lutein are dehydroxylated on the action of light, and consequently carotenes develop. The opposite occurs in the dark. The increase of carotenes can of course also be the result of new synthesis. For example, in the etiolated leaves of yellow corn weak light (25 Lux) promotes the synthesis of α- and β-carotenes, and strong light (60,000 Lux) increases the amount of antheraxanthin, too (HORVÁTH et al., 1972). On the other hand it is assumed that in the case of fully-developed leaves newly-synthesized carotene forms only an insignificant part of the carotene increase.

Effect of water on the light-induced reactions

In the carotenoid transformations the roles of light and water have been stressed. A study was made of the change in the water content of the leaf, during the illumination, but, in accordance with the theoretical assumptions

mentioned in the introduction with primary regard to the physical state of the water. The experiments were made under aerobic conditions, and the vapour content of the air was varied. Four experiments were carried out simultaneously, with 4×16 leaf-disks. The pigments were extracted immediately after collection from one of the samples (dark control). The other three samples were illuminated under the following conditions (10 minutes):

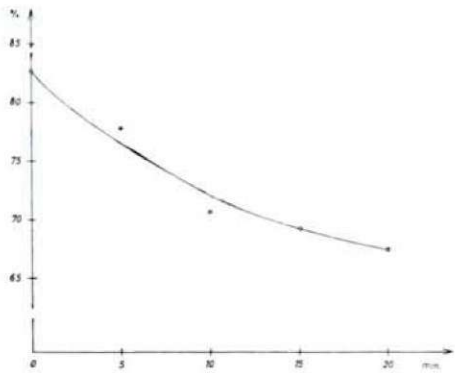

Fig. 3. Change of water content of a leaf of *Nonea lutea* during illumination (60,000 Lux).

a) In dry air, with anhydrous $CaCl_2$ in the vessel.

b) In a vessel saturated with water vapour.

c) The leaf-disks were floating on the surface of water.

The weight of leaf-disks floating on the water surface did not shange after being illuminated for ten minutes. The evaporation of the leaf-disks of course, was different in the dry vessels and in those saturated with water vapour.

In addition to *Nonea lutea,* similar experiments were made with other plant species, too. The transformation of carotenoids in *Rumex acetosella* L. is shown in the following Table.

Table II. Change in the carotenoid content of *Nonea lutea* leaves on the action of light (10 minutes, 60,000 Lux).

Illumination condition	carotenoids	zeaxanthin lutein antheraxanthin	neoxanthin	violaxanthin	total carotenoids
	%				
dark control	100	100	100	100	100
dry	101,3	117,6	97,8	73,6	100,6
humid	98,8	108,2	103,6	87,6	100,5
wet	103,3	125,6	95,6	59,4	100,6

Table III. Change in the carotenoid content of *Rumex Acetosella* leaves on the action of light (10 minutes, 60,000 Lux).

Illumination condition	carotenoids	zeaxanthin lutein antheraxanthin	neoxanthin	violaxanthin	total carotenoids
			%		
dark control	100	100	100	100	100
dry	108,1	117,5	80,2	73,4	99,5
humid	106,8	115,8	75,9	77,9	98,8
wet	113,1	127,5	79,2	63,6	102,1

Under indentical conditions, the transformations of the two species on illumination are similar. The extent of desepoxidation is decreased by a humid environment, as compared to both the liquid water and the dry environment. In the leaf-disks floating on the water surface, the percentages of carotenes and zeaxanthin + lutein are the highest. The effect of the humid environment in decreasing the rate of the violaxanthin ⟶ antheraxanthin ⟶ zeaxanthin reaction cannot be explained by the different degree of evaporation of the water content of the leaves, because an increased desepoxidation can be observed even in the isolated leaves illuminated under dry conditions. This change is also demontsrated by the percentage distribution of the carotenoids (Table IV).

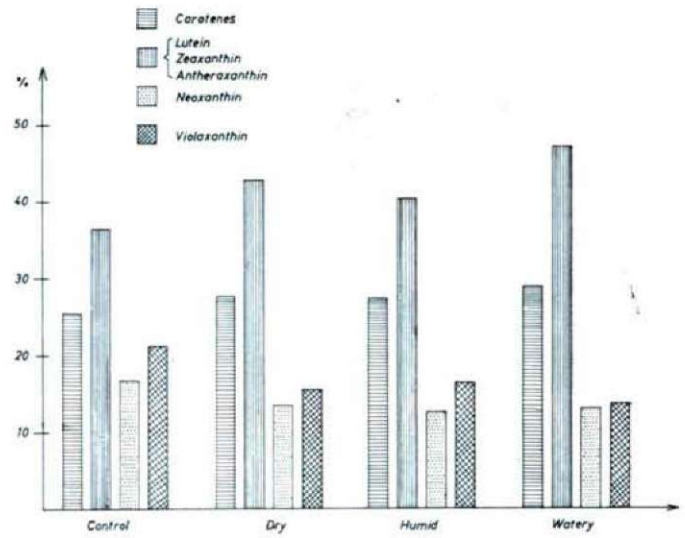

Fig. 4. Percentage distribution and change in the ratio of the carotenoid content of *Nonea lutea* leaves as a result of illumination under various conditions (10 minutes, 60,000 Lux).

Table IV. Percentage distribution and change in the ratio of the carotenoid content of *Nonea lutea* leaves as a result of illumination under various conditions (10 minutes, 60,000 Lux).

Illumination condition	carotenoids	zeaxanthin lutein antheraxanthin	neoxanthin	violaxanthin
		%		
dark control	22,4	38,4	13,8	23,3
dry	24,7	45,1	13,5	17,2
humid	24,2	41,6	14,3	20,4
wet	25,3	48,2	13,2	13,9

It is assumed that the structure of the water reaching the surface of the multi-enzymes from the vapour-saturated air differs from that from the liquid water. In the humid environment the water molecules (or associates) can move symmetrically from any direction of the space to approximately the same extent into the leaf-disks, or to the surface of the thylakoid membranes of the plast. As a result of such a symmetrical effect the symmetrical pigment molecules, e. g. violaxanthin, are bound more strongly in the proteo-lipid complex and change less on illumination.

In the case of *Nonea lutea*, the quantity of neoxanthin increases compared to the control on illumination in a humid environment, and here it is the highest. This was not observed in our repeated experiments with *Rumex Acetosella*. The cause may be the characteristics of the species, or the histological

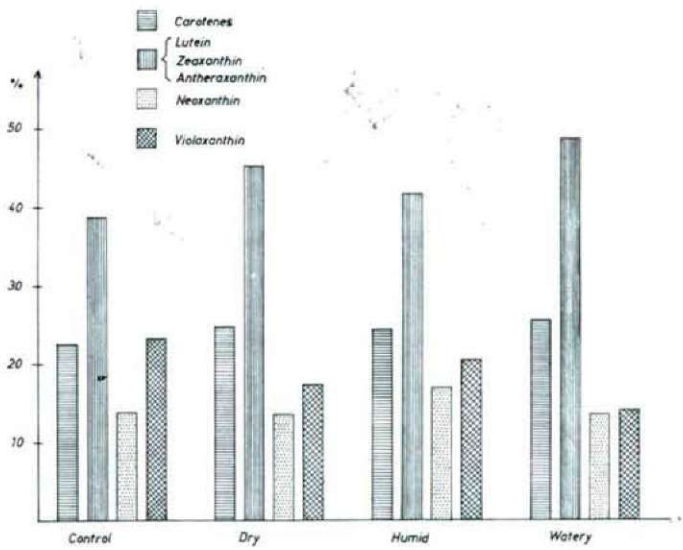

Fig. 5. Percentage distribution and change in the ratio of the carotenoid content of *Rumex Acetosella* leaves as a result of illumination under various conditions (10 minutes, 60,000 Lux).

and cytological structure of the leaves. On both the upper and lower surfaces of *Nonea lutea* leaves much down can be found: there are no trichomes however, on the epidermis of *Rumex* leaves.

In the isolated leaves in contact with liquid water (in the case of both species) the quantities of lutein + zeaxanthin and antheraxanthin increase significantly as a result of illumination.

A study was also made of the effects of longer illuminations (20—40 minutes) upon the transformation of the carotenoids.

As a result of an illumination for 20—40 minutes, the transformations discussed above are revealed even more strongly. At the end of 40 minutes, the total carotenoid content may have increased by as much as 8—12%. As a result of illumination under dry conditions, the quantity of carotenes increases to a greater extent than in humid or wet environments.

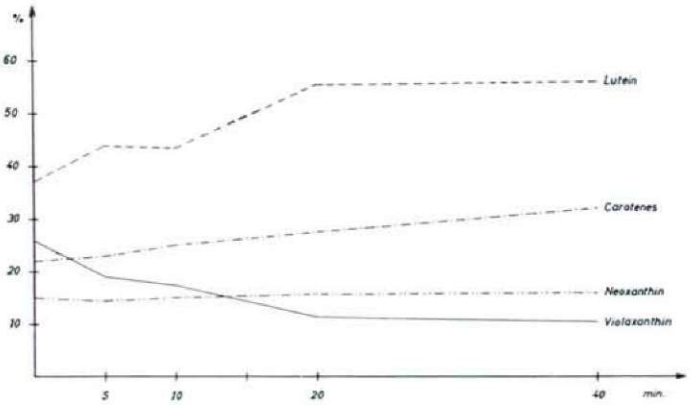

Fig. 6. Changes in the carotenoids of *Nonea lutea* leaves in a humid environment on the action of light.

Summary

1. Illumination and a decrease in water content considerably change the heterogeneous binding of carotenoids in the thylakoid membrane of the plast.

a) As a result of illumination the readily-soluble carotene fraction increases to the largest extent.

b) It is assumed that primarily the weakly-bound violaxanthin molecules take part in the desepoxidation reaction.

2. On the action of strong illumination for 20—40 minutes the total carotenoid content in isolated leaves increased by 8—12%.

3. Compared to liquid water and dry air, a humid environment diminishes the extent of the light-induced desepoxidation.

4. In isolated leaves in contact with liquid water the amounts of lutein, zeaxanthin, and antheraxanthin increase, considerably as a result of illumination.

References

CHUA, K. .S (1970): Structure of anomalous water and its mechanism. — Nature *227*, 834—836.

CLAES, HEDWIG (1967): Action spectrum of light-dependent carotenoid synthesis in *Chlorella vulgaris*. — Biochemistry of chloroplasts. Ed.: T. W. Goodwin, Acad. Press. London—New York 341—344.

DEROCHE, M. E.—COSTES, C. (1966): Hétérogénéité du β-carotene dans les choloroplastes. — Ann. Physiol. Vég. *8*, 259—269.

DEROCHE, M. E.—COSTES, C. (1969): Heterogeneity of carotenoids in chloroplasts. Progress in Photosynthesis Research. — Ed.: Metzner, Tübingen II, 681—693.

DONOHUE, H. V.—NAKAYAMA, T. O. M.—CHICHESTER, C. O. (1967): Oxygen reactions of xanthophylls. — Biochemistry of chloroplasts. Ed.: T. W. Goodwin, Acad. Press. London—New York 341—440.

GOODWIN, T. W. (1969): Carotenoid biosynthesis in chloroplasts. — Progress in Photosynthesis Research. Ed.: H. Metzner. Tübingen II, 669—674.

HAGER, A. (1966): Die Zusammenhänge zwischen lichtinduzierten Xanthophyll-Umwandlungen und Hill-Reaktion. — Ber. dtsch. bot. Ges. *79*, 94—107.

HAGER, A. (1967): Untersuchungen über die Rückreaktionen im Xanthophyll-Cyclus bei *Chlorella*, *Spinacia* und *Taxus*. — Planta (Berl.) *76*, 138—148.

HORVÁTH, G.—KISSIMON, J.—FALUDI-DÁNIEL, Á. (1972): Effect of light intensity on the formation of carotenoids in normal and mutant maize leaves. — Phytochemistry, *11*, 183—187.

KESZTHELYI, L. (1970): Az anomális víz (Anomalous water). — Fizikai Szemle 7, 193—196.

KLOTZ, I. M. (1962): Water horizons in biochemistry, 523—550.

LICHTENTHALER, H. K. (1969): Localization and functional concentrations of lipoquinones in chloroplasts. — Progress in Photosynthesis Research. Ed.: H. Metzner, Tübingen I, 304—314.

MARÓTI, I.—GABNAI, É. (1971): Separation of chlorophylls and carotenoids by thin-layer chromatography. — Acta Biol. Szeged (In the press).

NAGY, A. H.—FALUDI-DÁNIEL, Á. (1967): On the nature of the binding forces stabilizing carotenoid-protein and chlorophyll-protein complexes in vivo. — Photosynthetica *1*, 69—75.

PAULING, (1961): A molecular theory of general anaesthesia. — Science *134*, 15—21.

SAPOZHNIKOV, D. J.—CHERNYAEVA, J. J. (1965): Influence of compounds with various oxidation-reduction potentials in the dark reaction of the interconversions of xanthoyhylls. — Dokl. Akad. Nauk. Tadch. SSSR, *8*, 34—37.

SAPOZHNIKOV, D. I. (1969): Transformations of xanthophylls in the chloroplasts. — Progress in Photosynthesis Research. Ed.: H. Metzner, Tübingen II, 694—700.

SHLYK, A. A.—FRADKIN, L. I. (1962): O skorosti metabolisma chlorofilla w selenom rastenii. — Biofizika, 7, 281—291.

SHLYK, A. A.—NIKOLAEVA, G. N. (1962): Metabolicheskaya heterogennost chlorofilla w rasteniy. — Dokl. Akad. Nauk. SSSR. *143*, 460—463.

SHLYK, A. A.—PRUDNIKOVA, J. V.—FRADKIN, L. I.—NIKOLAEVA, G. N. and SAVCHENKO, G. E. (1969): Relationship between chlorophyll metabolism and heterogeneity of pigment apparatus. — Progress in Photosynthesis Research. Ed.: H. Metzner, Tübingen II, 572—591.

STRAIN, H. H. (1959): Chloroplast pigments and chromatographic analysis, p. l. Penn. State Univ. Press. Univ. Park, Penn.

Addresses of the authors:
Dr. I. MARÓTI,
IRÉN SZAJKÓ
Department of Botany, A. J. University,
6722 Szeged, Hungary