On the Role of Leucine in the Carotenoid Synthesis of the Chloroplasts of Higher Plants

L. EHRENBERG and A. F. DÁNIEL

Institute of Organic Chemistry and Biochemistry, University, Stockholm, Sweden

It was previously shown [1-5] that a considerable group of the chloroplast mutants of higher plants possess the ability of chlorophyll synthesis, but that in ordinary light intensities their leaves bleach. Such chloroplast mutants also show an incomplete structural differentiation of the chloroplasts [6]. It has been suggested that carotenoids may play an important role [7-9] in the protection of the chloroplasts from photodestruction. This would be in agreement with isoprenoid abnormalities in chloroplast mutants with increased photosensitivity [10]. Such abnormalities could consist of qualitative deviations in the carotenoid composition — as saturation of some double bonds [11, 12] — or a low concentration of the normal isoprenoid components [13-15]. In the latter case it can be suggested that the carotenoid (or in general the isoprenoid) synthesis is limited by a decreased production of some precursor. The early carotenoid precursors, among which leucine is considered important [16,17], were investigated mainly in fungi. In some chloroplast mutants, deviations from the normal amino acid and protein metabolism have been reported [18-23]. Recently it was reported that the barley mutant xantha-23 can be brought to normal green, *i.e.* with more or less normal chloroplasts, if the seedlings are grown in a leucine containing solution [24]. It is therefore of interest to study whether leucine can serve as a precursor in carotenoid (isoprenoid) synthesis in the leaves.

Experimental. Normal barley seeds (Bonus variety) were sown in Petri dishes and germinated in the dark at room temperature (20°). The leaves of the 6.5 day old seedlings (height 6—7 cm) were cut in 5 mm long pieces, rinsed for 0.5 h in tap water and placed in an aq. dest. solution of 1 % sucrose containing uniformly labelled 2.5 mM L-leucine-^{14}C (1 μC/μM). — The L-leucine-^{14}C was purchased from the Radiochemical Centre, Amersham. The operations were made under weak green light produced by means of an interference filter (Balzer's Broad Band No. 4) with a main transmission between 520 and 580 mμ. One portion of the leaves was kept in the dark continuously, the other was exposed to the light (about 2000 lux) from three 150 W tungsten lamps.

Leaf samples between 0.1 and 0.2 g were taken out from the leucine solution at different times, transferred to a glass filter and washed thoroughly, blotted and weighed. The pigments were extracted with acetone and peroxide-free ether [25]. The bulk carotenoid content was determined by measuring the light absorption at 480 mμ and nominally given as β-carotene [26]. An aliquot of the extract was chromatographed twice in the faster direction on Whatman No. 1 paper with the solvent system of Sapozhnykov [27]. The carotenoid spots were eluted, plated, and their radioactivity measured in a proportional counter (FH 407) with a standard error varying from 1 to 10 %.

The remainder of the extract was repeatedly treated with 70 % ethanol. The combined extracts were used for the determination of the radioactive leucine inside the leaves.

Separate experiments were made with non-labelled leucine to determine the leucine and carotenoid content in the leaves. The leucine content was determined by separating the leucine by repeated one-dimensional paper chromatography and measuring optical density of the leucine-ninhydrin-Cu $^{2+}$ complex [28].

The data reported represent the results of 4 independent incorporation experiments; differencies as great as 25 % have been found between the different experimental series.

Results and discussion. From the radioactivity of the alcoholic extracts it was clear that leucine is taken up rapidly by the leaves (Table 1).

The data in Table 1 demonstrate that the leucine uptake seems to proceed nearly at the same rate in the dark and the light and the tota leucine content was not markedly influenced by the light conditions applied. Saturation of the tissue with leucine solution was reached in the first hour of floating. Later, the specific activity of the leucine was about the same, which indicates that a considerable amount was remaining in the intercellular space, and the incorporation of the leucine into non-soluble compounds was small and/or compensated by the intracellular non-radioactive leu-

* Present address: Institute of Phylogenetics and Genetics, Eötvös Loránd University, Budapest, Hungary.

Table 1. Leucine-^{14}C(*) uptake, leucine content per g fresh weight, and specific activity of leucine in barley leaves floated on leucine solution (1.1×10^6 cpm/μM).

Time	Dark			Light		
	μM*/g	μM/g	$\frac{10^3 \text{ cpm}}{\mu\text{M}}$	μM*/g	μM/g	$\frac{10^3 \text{ cpm}}{\mu\text{M}}$
0 min	—	0.67	—	—	≡ dark	—
10 »	0.15	...	...	0.16	...	...
20 »	0.22	...	...	0.24	...	...
40 »	0.35	...	...	0.40	...	...
1.0 h	0.51	0.92	609	0.53	0.92	634
1.5 »	0.61	1.00	668	0.63	1.00	688
2.0 »	0.74	1.12	727	0.79	1.20	726
3.0 »	0.84	1.35	685	0.88	1.30	743
5.0 »	1.01	2.05	543	1.04	2.09	547
7.0 »	1.13	2.29	542	1.32	2.29	635

cine-pool. In the experiments of Chichester *et al.* [16] it was shown that in *Phycomyces blakesleeanus* grown for 7 days in a medium containing ^{14}C-labelled leucine, only a fragment containing three or at the most four out of the six carbon atoms is utilized directly in the carotenoid synthesis. Consequently, estimating the labelling extent of the carotenoids in the leaves, this part of the molecule had to be considered.

It appears from Table 2 that the ^{14}C of the leucine is incorporated into the carotenoids both in the dark and in the light, with increasingly higher values in the light. Consequently, leucine is used in the light induced carotenoid synthesis. This offers a plausible explanation of the leucine-conditioned normalization of the mutant, xantha-23 [24].

Considering the fact that the carotenoids contain eight isoprenoid units, the highest possible value of the leucine incorporation may be 8. In the investigations using *Phycomyces* [16, 29, 30] labelled DL-leucine was applied, and the duration of the experiments included

Table 2. Incorporation of the ^{14}C into the carotenoids of barley leaves floated on leucine-^{14}C solution (50μC/50μM L-leucine in the solution, illumination started after 1.0 h in the dark).

Time	Dark			Light		
	10^3 cpm in carotenoids per g fr.w.	$\frac{\mu\text{M carot.}}{\text{g fr. w.}}$	$\frac{\mu\text{M leuc.*}(1)}{\mu\text{M carot.}}$	10^3 cpm in carotenoids per g fr. w.	$\frac{\mu\text{M carot.}}{\text{g fr.w.}}$	$\frac{\mu\text{M leuc.*}(1)}{\mu\text{M carot.}}$
1.0 h	1.59	0.111	0.04	—	—	—
1.5 »	2.39	0.111	0.05	2.49	0.108	0.05
2.0 »	3.65	0.109	0.07	4.52	0.105	0.09
3.0 »	4.96	0.109	0.10	9.22	0.112	0.17
5.0 »	6.64	0.110	0.16	14.22	0.134	0.29
7.0 »	8.04	0.110	0.20	21.72	0.164	0.31
7.0 »	Difference (light — dark):			13.68	0.054	0.60 ± 0.24

(1) $\frac{10^3 \text{ cpm/ } \mu\text{M carotenoid} \cdot 6/4}{10^3 \text{ cpm/ } \mu\text{M leucine in the leaf tissue}}$

many cell generations. In such experimental conditions the mean incorporation value for the C_4 fragment of the leucine was found to be 3.37 (3.96 for C(4), C(5), C(5′) and 1.59 for C(3), respectively). Considering that only the L-form of the leucine possesses biological activity, the extent of incorporation found in the *Phycomyces* thus approximates the ideal value. The extent of carotenoid labelling found in the barley leaves was 3 % in the dark, and 8 % in the light induced synthesis, respectively, of the possible maximum in the case of the incorporation of 4 leucine carbons into each of 8 isoprene units per carotenoid molecule. The relatively low incorporation found may be due to the short time of treatment (7 h *versus* the 7 days used in the *Phycomyces* experiments). Furthermore, on entering the cells, the leucine is partly taken up by other active metabolic pools before it can reach the site of the carotenogenesis. It is also possible that some carotenoids are formed from non-radioactive immediate precursors already present in the cells, [17] and that the duration of our experiment was too short to exhaust them. It is clear that in our experiments different carotenoids may utilize leucine to a very different extent; here, the average incorporation has been determined. The present experiment — working with etiolated and illuminated leaves — could be compared with the studies of Braithwaite and Goodwin [31] performed on the precursors of lycopene in tomato slices. Calculating their data by the method applied here it can be concluded that the incorporation of the $^{14}CO_2$ and of the ^{14}C-labelled acetates into the lycopene was about 10^{-2} $\mu M/\mu M$ carotenoid, thus at an order of magnitude less than the incorporation of the C_4 fragment of the leucine into the carotenoids of barley leaves, indicating that the latter is a rather effective early precursor in the carotenoid synthesis in the leaves of higher plants.

Conclusion and summary. The incorporation of the L-leucine-^{14}C into the carotenoids of barley leaves was investigated.

It was found that the ^{14}C originating from uniformly labelled L-leucine is incorporated into the carotenoids of barley leaves.

The extent of labelling was greater in illuminated leaves than in leaves kept in the dark.

It was demonstrated that leucine may serve as an early precursor of carotenoids in the chloroplasts of the leaves of higher plants.

Acknowledgement. Our thanks are due to Professor D. von Wettstein for fruitful discussions. We also appreciate valuable assistence by Mrs Ingrid Lodén. The work was performed as an IAEA fellowship contract with A. F. Dániel and was also supported financially by the *Knut and Alice Wallenberg Foundation* and the *Swedish Agricultural Research Council.*

1. Koski, V. M., Smith, J. H. C. *Arch. Biochem. Biophys.* **34** (1951) 189.
2. Holm, G. *Acta Agr. Scand.* **4** (1954) 457.
3. Röbbelen, G. *Z. Vererbungslehre* **90** (1959) 50.
4. Lyastchenko, I. I. *Biokhimiya* **22** (1957) 942.
5. Faludi, B., Dániel, F. A., Gyurján, I., Száraz, I. *Ann. Univ. Sci. Budapestiensis Sect. biol.* **4** (1961) *(In press).*
6. Wettstein, D. v. *Hereditas* **43** (1957) 303.
7. Lynch, V. H., French, C. S. *Arch. Biochem. Biophys.* **70** (1957) 382.
8. Milner, M., French, C. S., Milner, H. V. *Plant Physiol.* **33** (1958) 367.
9. Bishop, N. I. *Plant. Physiol.* **33** (1958) Suppl.
10. Euler, H. v., Burström, D., Hellström, H. *Hereditas* **18** (1933—34) 225.
11. Anderson, I. C., Robertson, D. S. *Plant Physiol.* **35** (1960) 531.
12. Faludi, B., Dániel, A. F., Kelemen, G. *Physiol. Plantarum* **13** (1960) 227.
13. Tulpule, Sh. H. *Am. J. Botany* **41** (1954) 294.
14. Smith, J. H. C., Durham, L. J., Wurster, C. F. *Plant Physiol.* **34** (1959) 340.
15. Crane, F. L. *Plant Physiol.* **34** (1959) 121.
16. Chichester, C. O., Nakayama, T., MacKinney, G., Goodwin, T. W. *J. Biol. Chem.* **21** (1955) 515.
17. Goodwin, T. *Biochem. J.* **70** (1960) 5.
18. Euler, H. v., Hellström, H., Löfgren, N. *Z. physiol. Chem. Hoppe-Seylers'* **234** (1935) 151.
19. Seltman, H. *Plant Physiol.* **30** (1955) 258.
20. DeKock, P. C., Morrison, R. I. *Biochem. J.* **70** (1958) 266.
21. Fujii, T., Ono, Y. *Proc. Japan. Acad.* **36** (1960) 610.
22. Mossberg, G. Lecture given at the Meeting of the Swedish Mutation Research Group, 26th January, 1962.
23. Holmgren, P. *Ann. Roy. Agr. Coll. Sweden* **22** 354.
24. Eriksson, G., Kahn., A., Walles, B., Wettstein, D. v. *Ber. deut. botan. Ges.* **74** (1961) 221.
25. Koski, V. M. *Arch. Biochem. Biophys.* **29** (1952) 339.

26. Withrow, R. B., Klein, W. H., Price, L., Elstad, W. *Plant Physiol.* **28** (1953) 1.
27. Sapozhnykov, D. I., Bronstein, I. A. and Krasnovskaya, I. A. *Biokhimiya* **20** (1955) 286.
28. Fischer, G. F., Dörfel, H. *Biochem. Z.* **324** (1953) 65.
29. Wuhrman, J. J., Yokoyama, H., Chichester, C. O. *J. Am. Chem. Soc.* **79** (1958) 4569.
30. Yokoyama, H., Chichester, C. O., Nakayama, T., Lukten, A., MacKinney, G. *J. Am. Chem. Soc.* **79** (1957) 2029.
31. Braithwaite, G. D., Goodwin, R. W. *Biochem. J.* **76** (1960) 1.

Received June 8, 1962.

On the Preparation and Properties of Disulfide-Reduced, Alkylated Fibrinogen and Fibrin

AGNES HENSCHEN

Chemistry Department II, Karolinska Institutet, Stockholm, Sweden

Recently we have described the preparation and some properties of sulfitolysed fibrinogen and fibrin [1]. In order to evaluate the completeness and specificity of the sulfite reaction of these proteins, it was desirable to obtain other disulfide-modified derivatives and to compare their properties. In the present investigation, we have chosen to cleave the disulfide bonds by reduction and to block the resulting sulfhydryl groups with an alkylating reagent. Different reducing agents were tried under different conditions.

Bovine fibrinogen and fibrin, purified according to the method of Blombäck and Blombäck [2], were used. Two types of reagents were employed for reduction: sodium borohydride or the mercaptans thioglycollic acid and mercapto-ethanol. Sodium borohydride was used mainly as described by Moore *et al.*[3] The reaction was performed in an 8 M urea solution at a pH between 9 and 11 at + 40°C. Protein and reagent concentrations were about 0.4 %. Sometimes versene or tris were added to the reaction mixture. Thioglycollic acid and mercapto-ethanol were used mainly as described by Sela *et al.*[4] and by Anfinsen and Haber [5] and Crestfield [6], respectively. The reaction mixture was 8 M in urea, 0.4 M in tris and 5×10^{-3} M in versene at a pH of 8.5 and room temperature. Protein concentration was 0.3—1.0 %. Mercaptan was added in approximately 150-fold molar excess regarding the half-cystine content; the solution was kept in a sealed vessel for 4 to 5 h. Alkylation was performed according to the methods of the same authors [3-6]. Only iodoacetic acid was tried. When borohydride was used for reduction, the excess was destroyed by acidification. The reagents and the protein-product were separated by gel-filtration on Sephadex G-50 [7], by dialysis or by precipitation with acetone. The number of unchanged disulfide bonds or freed sulfhydryl groups was determined by electrometric titration. The yields of protein in the procedures were calculated from spectrophotometric values [8] and from the weights of the lyophilized material.

The reduction procedure with sodium borohydride never acted both quantitatively and selectively on the disulfide bonds. Under the conditions described by Moore *et al.*[3] a complete cleavage of the disulfide bonds could be obtained. At the same time, however, some peptide bonds were split as revealed by the formation of new N-terminal amino acids. This side reaction occurred even in the presence of versene. In all these experiments with borohydride the final pH was 10 to 11. In order to obtain a more constant and slightly lower pH some reductions were performed in the presence of tris or with succesive additions of hydrochloric acid in a pH-stat. In these cases no complete cleavage of the disulfide bonds was obtained.

The reduction procedure with the mercaptans thioglycollic acid and mercapto-ethanol acted quantitatively on the disulfide bonds as determined by electrometric titration after alkylation and isolation. In spite of the absence of a nitrogen barrier there was no sign of (not reversed) reoxidation. N-Terminal amino acid analysis of the products gave the same results as with the unmodified proteins. The derivatives obtained by mercaptolysis and alkylation of fibrinogen and fibrin were almost quantitatively recovered. For these reasons, the mercaptolysed, alkylated products were