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

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It was previously shown 1-2 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 3. It has been suggested that carotenoids may play an important role 4 in the protection of the chloroplasts from photodestruction. This would be in agreement with isorenoid abnormalities in chloroplast mutants with increased photosensitivity 5. Such abnormalities could consist of qualitative deviations in the carotenoid composition - as saturation of some double bonds 6,7 - or a low concentration of the normal isorenoid components 8-10. In the latter case it can be suggested that the carotenoid (or in general the isorenoid) synthesis is limited by a decreased production of some precursor. The early carotenoid precursors, among which leucine is considered important 11,12, were investigated mainly in fungi. In some chloroplast mutants, deviations from the normal amino acid and protein metabolism have been reported 13-15. 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 16. It is therefore of interest to study whether leucine can serve as a precursor in carotenoid (isorenoid) 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-14C (1 μC/μM).

- The L-leucine-14C 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 μμ. 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. The bulk carotenoid content was determined by measuring the light absorption at 480 μμ and nominally given as β-carotene 21. An aliquot of the extract was chromatographed twice in the faster direction on Whatman No. 1 paper with the solvent system of Sapozhnikov 22. 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 23.

The data reported represent the results of 4 independent incorporation experiments; differences 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 total 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 leuc-
**SHORT COMMUNICATIONS**

**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 × 10^4 cpm/μM).

<table>
<thead>
<tr>
<th>Time</th>
<th>Dark</th>
<th>Light</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM*/g</td>
<td>μM/g</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>—</td>
<td>0.67</td>
</tr>
<tr>
<td>10 s</td>
<td>0.15</td>
<td>...</td>
</tr>
<tr>
<td>20 s</td>
<td>0.22</td>
<td>...</td>
</tr>
<tr>
<td>40 s</td>
<td>0.35</td>
<td>...</td>
</tr>
<tr>
<td>1.0 h</td>
<td>0.51</td>
<td>0.92</td>
</tr>
<tr>
<td>1.5 h</td>
<td>0.61</td>
<td>1.00</td>
</tr>
<tr>
<td>2.0 h</td>
<td>0.74</td>
<td>1.12</td>
</tr>
<tr>
<td>3.0 h</td>
<td>0.84</td>
<td>1.35</td>
</tr>
<tr>
<td>5.0 h</td>
<td>1.01</td>
<td>2.05</td>
</tr>
<tr>
<td>7.0 h</td>
<td>1.13</td>
<td>2.29</td>
</tr>
</tbody>
</table>

In the experiments of Chichester et al., 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.

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 *Phycococcus* 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 (60μC/50μM L-leucine in the solution, illumination started after 1.0 h in the dark).

<table>
<thead>
<tr>
<th>Time</th>
<th>Dark 10^3 cpm in carotenoids per g fr.w.</th>
<th>Dark μM leuc. / μM carot.</th>
<th>Light 10^3 cpm in carotenoids per g fr.w.</th>
<th>Light μM leuc. / μM carot.</th>
<th>Light μM leuc. / μM carot.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0 h</td>
<td>1.59</td>
<td>0.111</td>
<td>0.04</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1.5 h</td>
<td>2.39</td>
<td>0.111</td>
<td>0.05</td>
<td>2.49</td>
</tr>
<tr>
<td></td>
<td>2.0 h</td>
<td>3.65</td>
<td>0.109</td>
<td>0.07</td>
<td>4.52</td>
</tr>
<tr>
<td></td>
<td>3.0 h</td>
<td>4.96</td>
<td>0.109</td>
<td>0.10</td>
<td>9.22</td>
</tr>
<tr>
<td></td>
<td>5.0 h</td>
<td>6.64</td>
<td>0.110</td>
<td>0.16</td>
<td>14.22</td>
</tr>
<tr>
<td></td>
<td>7.0 h</td>
<td>8.04</td>
<td>0.110</td>
<td>0.20</td>
<td>21.72</td>
</tr>
<tr>
<td></td>
<td>7.0 h — Difference (light — dark):</td>
<td>13.68</td>
<td>0.054</td>
<td>0.60 ± 0.24</td>
<td></td>
</tr>
</tbody>
</table>

(1) $10^3$ cpm/μM carotenoid · 6/4

$10^3$ cpm/μM leucine in the leaf tissue

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many cell generations. In such experimental conditions the mean incorporation value for the C₄ 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 metabolite 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, 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 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 [¹⁴C]labeled acetates into the lycopene was about 10⁻⁵ μM/μM carotenoid, thus at an order of magnitude less than the incorporation of the C₄ 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-[¹⁴C] into the carotenoids of barley leaves was investigated. It was found that the [¹⁴C] originating from uniformly labeled 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 assistance by Mrs Ingrid Lodén. The work was performed as an IAEA fellowship contract with A. F. Déniel and was also supported financially by the Knut and Alice Wallenberg Foundation and the Swedish Agricultural Research Council.


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30. Yokoyama, H., Chichester, C. O., Nakayama, T., Lukten, A., MacKinney, G.

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

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Recently we have described the preparation and some properties of sulfitolysed fibrinogen and fibrin. 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 Blomback and Blombäck, were used. Two types of reagents were employed for reduction: sodium borohydride and the mercaptans thioglycollic acid and mercapto-ethanol. Sodium borohydride was used mainly as described by Moore et al. 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. and by Anfinsen and Haber and Crestfield, respectively. The reaction mixture was 8 M in urea, 0.4 M in tris and 5 × 10⁻⁴ M in versene at a pH of 8.5 and room temperature. Protein concentration was 0.3—1.0%. Mercaptoan 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.

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, 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 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. 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 successive 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 alkylatation 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 alkylatation of fibrinogen and fibrin were almost quantitatively recovered. For these reasons, the mercaptolysed, alkylated products were

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