AMINO ACIDS IN THE BIOSYNTHESIS OF LEAF PIGMENTS IN HIGHER PLANTS I.*

by

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The role of carotenoids in the maintenance of the photostability of the photosynthetic apparatus in a well established fact *(1)*. Therefore, it is not surprising that a part of chlorophyll mutants with increased light sensitivity exhibits abnormal carotene metabolism: the concentration of carotenoids is surprisingly low in these mutants *(2)* and/or the double bonds in the carotenoid molecules contained by them are partly saturated *(3, 4)*. The block of carotenoid biosynthesis may affect the functioning and life time of clorophyll molecules in a number of ways. The best known consequence of decreased carotenoid content in green tissues in the irreversible photooxidation of chlorophylls *(5, 6)*. In some mutants this might take place even at light intensities lower than the compensation point of photosynthesis/respiration *(7)* and result in a poor differentiation *(8)* or structural destruction of chloroplasts *(9)*. Finally the plants perish.

Relatively little is known about the conditions of carotenoid biosynthesis taking place in the chloroplasts of higher plants. Studies on the biosynthesis of carotenoids were carried out mainly on *Phycomyces Blakesleanus* and fruits of *Lycopersicum (10)*. Mevalonic acid which proved to be an immediate precursor of isoprenoids in these studies hardly penetrates the chloroplasts *(11)*. Therefore, we might come to the conclusion that mevalonic acid is synthesized under natural conditions within the chloroplasts from intermediates of the photosynthesis or some other metabolic process.

In the course of the differentiation of chloroplasts – preceding the start of photosynthesis – as a stable precursor of carotene biosynthesis leucine might come into consideration. According to earlier investigations *(12, 13)* the part of the leucine molecule containing the C(4) C(5) and C(5') atoms appears almost eight times in the β-carotene of *Phycomyces B.* and to a smaller extent the C(3) and C(2) carbons are also incorporated into the isoprene units. The role of leucine in the pigment synthesis of higher plants is suggested by the abnormality of leucine content in chloroplast mutants *(14)*. The importance of leucine is also indicated by the observation that the altered submicroscopic structure of chloroplasts in xantha-23 barley mutants can be markedly „normalized" by feeding leucine to the seedlings. This treatment raises the chlorophyll content almost to the normal level *(15)*.

In the present study the incorporation of leucine into the leaf pigments was investigated in the course of chloroplast differentiation.

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Materials and methods

The experiments were carried out with the barley variety "*Bonus*". The seeds were germinated on filter paper in the dark at room temperature. The first leaves of 6,5-day old seedlings were cut into 5 mm long pieces and floated on a L-leucine-^{14}C(U) solution containing 1 per cent sucrose. The manipulations were done in week green light (absorption maximum 520 – 580 m μ). The concentration of leucine solution was 2,5 mM with a specific activity of 1 mC/mM. The chemical was supplied by the Radiochemical Centre, Amersham. The purity of the compound was tested by the chromatographic method of L e v y *(16)*. 0,5 μC gave a single spot on Kodak no-screen roentgen film upon expouse for 2 weeks. The leaf samples were kept in darkness for 1 hour, then one part was illuminated (2000 lux, tungsten filament lamp) and the other was kept further in darkness.

0,1 – 0,2 g samples were taken at given intervals, washed, dried with filter paper and measured. The pigments were extracted by a mixture of acetone and peroxide-free ether and chromatographed twice on Whatman 1 filter paper according to S a p o z h n i k o v *(17)*. The pigments were then eluted from the paper by ether and their ^{14}C-content was measured after plating. The residue was repeatedly extracted with 70 per cent alcohol, the extracts were pooled and used for the determination of free leucine content in the leaf pieces *(18)*. The leucine content incorporated into proteins was determined in the residue by plating with formic acid. Selfabsorption was measured by the use of casein models. The maximal time of exposure was 7 hours as longer incubation resulted in necrosis of the edges of leaf pieces.

Radioactivity was measured by a *FH 407* proportional counter of 50 per cent efficiency. The statistical error of the measurement was 1 – 10 per cent.

Representative examples from 6 repeated experiments are shown in the tables. The maximal difference between the various experiments was about 25 per cent.

Results and discussion

The distribution in various fractions of leucine absorbed by the leaves in a 7-hour experiment is shown in Table I.

Table I.

The distribution of radioactivity in barley leaves floated on L-leucine^{14} C(U) solutions (μM leucine/g fresh weight)

Treatment	Fractions						
	Alcoholic extract	%	Protein	%	Pigment	%	Total
Dark	1,13	91	0,13	8,4	0,007	0,6	1,237
Illuminated	1,32	83	0,26	15,5	0,024	1,5	1,604
Illuminated / Dark	1,18	0,91	2,00	1,88	2,86	2,50	1,30

It may be seen form the data that leucine is absorbed by both illuminated leaves and those kept in darkness. The amount of leucine taken up is considerable as compared to the endogenous level. The floating of leaves resulted in a

doubled tissue level of leucine *(19)*. The distribution of radioactivity deriving from leucine is different in dark and illuminated leaves. The illumination slightly stimulates the incorporation of radioactivity into the compounds contained by the alcoholic fraction. The stimulation is stronger with the protein fraction and even more marked in the leaf pigments.

The difference between the activity of alcoholic extracts is small. This suggests that a part of the leucine recovered in this fraction is taken up by the tissues aspecifically in the biological sense, i.e. by diffusion. To have a deeper insight into this problem one half of the labelled leucine was diluted with known amounts of inactive L−leucine and the other with the same amounts of D,L-leucine. In this second solution the specific activity of L-leucine was thus the double of the first one.

The distribution of radioactivity in the various fractions after floating on these diluted solutions in shown in Table II.

Table II.

Incorporation into the various fractions of barley leaves of L-leucine^{14} C(U) diluted with L- and D,L-leucine respectively

^{14}C content in various fractions of leaves floated on L-leucine-^{14}C(U) diluted with D,L-leucine

^{14}C content in various fractions of leaves floated on L-leucine-^{14}C(U) diluted with L-leucine

Treatment	Alcoholic extract	Protein	Pigment	Leucine solution
Dark	1,62	1,89	1,83	
Illuminated	1,60	1,86	1,94	1,97

It may be seen from Table II. that the ratio of radioactivity recovered from the alcoholic extract is lower than that of the other fractions. This indicates that the isomer discrimination is indeed not perfect. The ratio of leucine incorporated into the proteins and pigments is near to the ratio of specific activity of the solutions applied (calculated on the basis of L-leucine content). The small difference might probably be explained by the inhibition of the uptake of L-form by the D-configuration *(20)*.

From these data the amount of aspecifically absorbed leucine can be roughly calculated. The average of the ratios found in the proteins and pigments (1,88) may be regarded as the possible maximum figure in the presence of the D-form. „x" part of the alcohol soluble fraction penetrated the cells by isomere discrimination, i.e. by selective absorption. Therefore the ratio of the specific activity of leucine is 1,88 in this fraction. 1-x part penetrated the leaves without isomere discrimination. Thus the ratio of specific activity of leucine is 1. From the observed average ratio (1,61) the ratio of leucine taken up by an active process can be calculated on the basis of the following equation:

$$1{,}61 - 1{,}88x = 1\text{-}x \qquad x = 0{,}69$$

It might be concluded, therefore, that the amount of leucine taken up by passive processes is the range of 30−40%.

The incorporation into various pigments of ^{14}C supplied to the leaves as L-leucine-^{14}C(U) is shown in Table III.

Table III.

Incorporation of ^{14}C into the pigments of barley leaves floated on L-leucine-^{14}C (U) solution (10^3 cpm/g fresh weight)

Time hours	Dark			Illuminated			Light induced car./chlor.
	Carotenoids	Protochloro-phyllid	car./ protochlor.	Carotenoids	Chlorophylls	car./chlor.	
1	1,59	0,34	4,7	–	–	–	–
2	3,65	0,45	8,1	4,52	0,82	5,6	(2,7)
3	4,96	0,44	11,0	9,22	1,80	5,1	3,2
5	6,64	0,47	14,8	14,20	3,08	4,6	2,5
7	8,04	0,47	17,1	21,72	6,38	3,4	2,3

It might be concluded from the results presented in Table III. that leucine is incorporated into the carotenoids of both etiolated and illuminated leaves. The activity in the protochlorophyll + protochlorophyllid spots of leaves kept in darkness is small. By contrast, marked incorporation into the chlorophylls takes place upon illumination. The ratio of incorporation of ^{14}C in illuminated and dark leaves indicates that by the start of intensive chlorophyll synthesis (end of the experiment) about 2/3 of the leucine molecule is incorporated into the carotenoids and 1/3 into the chlorophyll. According to preliminary investigations a considerable part of the latter is located in the phytol moiety, although some activity can be also recovered from the porphyrine ring.

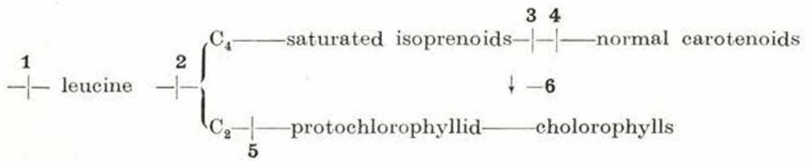
A comparison of the amount of ^{14}C incorporated into the carotenoids with the overall carotene content (given arbitrary as β-carotene) indicates that the results (in view of the short incubation periods) are in a good accordance with the data obtained with *Phycomyces (19)*. Calculated on the basis of the specific activity of leucine taken up in a specific manner, after 7 hours treatment the following percentual values of the theoretically possible maximal incorporaton were obtained: 4% in leaves kept in darkness, 6% in illuminated leaves and 10% in the carotenoids synthesized upon illumination. As to the labelling of the porphyrine ring it might be assumed that the glycine residue of the leucine molecule, which does not take part in the synthesis of isoprenoids, is used for the biosynthesis of δ-amino levulinic acid and thereby for porphyrine synthesis *(21)*. This idea is supported by the observation that the radiochromatography of alcoholic extracts from leaves kept in darkness indicate the presence of glycine in addition to that of leucine. Under identical conditions glycine could not be detected on the radiogramms of illuminated leaves.

By comparing the labelling extent of leucine in proteins and pigments we might conclude that the figures are of the same order of magnitude. The protein content of young barley leaves was found to be 16 per cent *(22)*. The leucine content accounts for 7 per cent of the protein nitrogen *(23)*. This corresponds to 11 μM leucine/g fresh weight. On the basis of the activity found in the proteins their labelling extent is about $1-2\%$ after feeding with leucine for 7 hours.

The favourable effect of leucine on the differentiation of chloroplasts in the barley mutant xantha-23 is explained by E r i k s s o n *(15)* by a stimulated protein synthesis. As may be seen from the results presented in this paper that in addition to the above effect the role of leucine as a powerful

precursor in carotenoid biosynthesis must also be taken into account. Thereby the photostability of chloroplasts is also affected.

On the basis of the above results and the data published so far the following working hypothesis can be advanced concerning the genetical block in the pathway of pigment synthesis in chloroplast mutants of higher plants with abnormal leucine and/or carotenoid content:



From the favourable effect of leucine on the pigment content of the barley mutant xantha-23 *(15)* it might be concluded that in this particular case the leucine synthesis is not sufficient by the time and/or at the place of chloroplast development „**1**". By contrast, the results obtained by F u j i i a n d O n o *(14)* indicate that the leucine content in albino wheat mutants might be increased. This finding suggests a block in the transformation of leucine „**2**". A n d e r s o n *(3)* reported that in rapidly bleaching corn mutants the biosynthesis of carotenoids is blocked after the formation of phytoen and phytofluen „**3**". In the corn strain used by F a l u d i *(4)* the genetical block is certainly located at a later stage of carotene biosynthesis, i.e. after ζ-carotene „**4**". The residue of leucine not utilized in carotene synthesis is channeled into the pathway of porphyrine biosynthesis which is blocked in some mutants, according to W e t t s t e i n *(24)* after the formation of protoporphyrine IX „**5**" Finally, the results by S m i t h *(25)* indicate that in some mutants the esterification of chlorophyll is not complete „**6**".

The sketch is of course, still a fairly rough one and further studies are necessary for its improvement.

Summary

The incorporation of L-leucine- $^{14}C(U)$ into the leaf pigments of etiolated barley leaves was investigated during the formation of the chloroplasts.

It has been found that L-leucine is incorporated into the carotenoids of both dark and illuminated leaves. Some incorporation into the chlorophyll molecule was also observed.

Leucine was found to be a potent precursor of carotenoids in higher plants during the formation of chloroplasts.

The biochemical nature of the genetic block of chloroplast mutants with abnormal leucine and carotenoid content is discussed.

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РЕЗЮМЕ

Мы изучали внедрение в равномерно отмеченного L-лейцина ^{14}C в пигменты ячменных листьев, в этиолированном состоянии и в течение дифференциации хлоропластов.

Нами было установлено, что L-лейцин внедрается в каротиноиды листьев, находящихся как и в темноте, так и освещенных.

По мере внедрения можно сделать вывод, что лейцин является одним из эффективных ранних прекурсоров каротиноидов, и так может являться ограничительным фактором пигментового синтеза в известных мутантах хлоропласта с аномальным лейциновым метаболизмом.

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