Incorporation of ^{14}C -Labeled Leucine into the Molecular Framework of Anthocyanins Appearing in Red Radish Studies on Anthocyanins, LIII*.

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According to the prevailing concept, biosynthesis of anthocyanins and flavones in higher plants resides in a concurrence of two different biochemical pathways¹⁾ leading to the formation of precursors for A- and B-rings in anthocyanins; the one being a poly- β -keto-acid intermediate produced by head-to-tail condensation of acetate units^{2,3)}, and the other being certain C_9 -intermediate formed through the shikimic-prephenic acid route as already pointed out by Davis^{4,5)}. Recently, some works on anthocyanin production have shown that in corn endosperm the pigmentation is promoted by some amino acids such as aspartic acid and cysteine, while it is suppressed by riboflavine and methionine to some extent⁶⁾, showing an apparent agreement with a previous view presented by Thimann *et al.*^{7,8,9)} using a small floating plant, *Spirodela oligorrhiza*.

Based on the preliminary analyses of various components in radish (*Raphanus sativus*) and turnip (*Brassica rapa*)¹⁰⁾, attempt has been made by the present authors to disclose the biosynthetic relationship between anthocyanin (acylated triglucoside of pelargonidin^{11,12)}) and some of the amino acids in these plants. As described in the preceding paper¹³⁾, the administration of ^{14}C -labeled amino acids to the seedlings has proved that not only phenylalanine but also leucine and valine are efficiently utilized in the biosynthesis of anthocyanin skeleton. While it has been established that phenylalanine serves as an excellent precursor for the formation of the B-ring of flavonoids in general, the role of other amino acids still remains to be investigated.

The present paper deals with this problem by the use of ^{14}C -labeled leucine in order to clarify the distribution of radioactivity within the molecule of anthocyanin itself as well as in a number of related compounds appearing in radish seedlings.

Material and Methods

Material. Seedlings of a red radish (var. *comet*) were grown in a manner as described previously¹²⁾. Radioactive leucine used in this experiment was a preparation labeled with ^{14}C either uniformly or only in carboxyl-C.

Administration of leucine- ^{14}C and preparation of the plant extract. The administration of leucine- ^{14}C to the excised seedlings was done as described previously. Every four seedlings were fed with leucine- ^{14}C under exposure to light (8,000 lux) for a definite length of time. The temperature was kept at $20^\circ \pm 2^\circ$, at which the pigment production was most pronounced. After each period of illumination, several seedlings were sampled out and washed twice with distilled water. The cotyledons were cut off, and the remaining hypocotyls were extracted with boiling 80% ethanol

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for three repeated times. The combined extract was evaporated to dryness *in vacuo*, and the residue was dissolved in water and the solution was passed through the resin columns of Amberlite IR-120 (H^+) and IR-4B (OH^-) one after another. By this treatment, all the free sugars remained in solution, and the organic acids are retained in resin columns. Therefore, free amino acids were liberated from the IR-4B resin column by eluting with 1 N Na_2CO_3 , and subsequently by passage through the IR-120 (H^+) column in order to remove sodium carbonate.

Paper chromatography and measurement of radioactivity. The fraction of free amino acids was chromatographed two-dimensionally on a large sheet of Tōyō No. 51 filter paper, using phenol saturated with water in one direction, and *n*-BuOH/AcOH/ H_2O (4:1:5, v/v) in the other. The air-dried chromatogram was printed on a X-ray film (Sakura, non-screen) for 3-4 weeks. Then, each of the radioactive spots on the chromatogram was eluted throughly with a small amount of hot water. Each fraction was evaporated to dryness, and radioactivities were measured with gas flow counter (Aloka Radiation Counter, Model DC-4).

The fractions of sugars and organic acids were subjected to one-way, ascending chromatography using the solvent mixtures: *n*-BuOH/pyridine/ H_2O (6:4:3, v/v) and *n*-BuOH/AcOH/ H_2O (4:1:5, v/v). Air-dried chromatograms were autoradiographed, and then each of the radioactive spots was eluted and the activity was measured as usual. Individual components of radioactive sugars, amino acids and organic acids were identified in the usual way by the use of the reagents specific for each group of compounds.

Isolation of anthocyanin and its hydrolysis. The isolation of anthocyanin and subsequent hydrolysis were made in the usual way. The sugar-free pigment (pelargonidin) was decomposed further by means of hydrogen peroxide and hot 15% aqueous sodium hydroxide, respectively. Here, the process may proceed according to the following scheme:

The degradation of pelargonidin with 15% aqueous sodium hydroxide and the radioactivity measurement of the resulting products, *i. e.*, phloroglucinol (I) and *p*-hydroxybenzoic acid (II), were made as described previously. The oxidation of pelargonidin with hydrogen peroxide was done according to the method of Chandler and Harper¹⁴), and the reaction products were chromatographed by irrigation with *n*-BuOH/AcOH/ H_2O (4:1:5, v/v). Individual products separated on the chromatogram were shown in Table 1. The radioactive spots were eluted with methanol in a separate manner, and the activity was measured as before.

Table 1. Paper-chromatographic behaviors of the degradation products of pelargonidin by means of hydrogen peroxide (by the one-way, ascending method, on Tōyō No. 51 filter paper, at ca. 20°).

Degradation products	Rf-value		Color reactions of the spots with		
	<i>n</i> -BuOH/ AcOH/H ₂ O (4:1:5, v/v)	C ₆ H ₆ /AcOH/ H ₂ O (123:73:3, v/v)	diazotised sulfanilic acid	Mo-reagent* (+NH ₃ gas)	Mn-reagent**
Compound III	0.18	0.05	diamine-brown	grayish blue (deep blue)	yellow
Compound II	0.89	0.73	zinc-orange	light blue (blue)	yellow
<i>p</i> -Hydroxy- benzoic acid (as control)	0.88	0.73	zinc-orange	light blue (blue)	yellow

* Mo-reagent: 2% aqueous solution of H₃PMo₁₂O₄₀·29H₂O.

** Mn-reagent: 1% aqueous solution of potassium permanganate.

Compound III: probably, 2, 4, 6-trihydroxyphenylacetic acid (or 2, 4, 6-trihydroxyphenyl- α -hydroxy-acetic acid) of the A-ring origin (according to Karrer and de Meuron¹⁶); freely soluble in water, ethanol, and ether, and insoluble in chloroform and benzene.

Compound II: *p*-hydroxybenzoic acid derived from the B-ring. The recovery of this compound was nearly equal to that of *p*-hydroxybenzoic acid obtained by degradation with 15% NaOH aq..

Results

The distribution of ¹⁴C in three kinds of molecular components of *Raphanus*-anthocyanin are summarized in Table 2. When leucine-1-¹⁴C was fed to the excised radish seedlings, about 70% of ¹⁴C were incorporated into the sugar-free moiety (pelargonidin). Similar result was also obtained in the case where leucine-U-¹⁴C was fed to the seedlings. The rate of ¹⁴C-incorporation into the bound organic acid moieties (*p*-coumaric, ferulic and caffeic acids) was exceedingly higher than that found in the sugar moiety (glucose). In Table 3, the distribution of ¹⁴C in the aglycone (pelargonidin) was examined in detail. When the seedlings were fed with leucine-U-¹⁴C, pelargonidin produced was labeled predominantly in the B-ring. A similar result was obtained in the case of leucine-1-¹⁴C. Accordingly, the carboxyl-C of leucine should participate in the formation of the B-ring *via* some other route that is still unexplored.

The alkaline degradation of pelargonidin leading to the formation of both phloroglucinol and *p*-hydroxybenzoic acid has proved that the incorporation of leucine-¹⁴C into the B-ring was three to four times as high as that into the A-ring of anthocyanidin. According to the result of H₂O₂-degradation, compound III, which may be composed of A-ring plus 3-C and 4-C of pelargonidin, has proved to be appreciably high in radioactivity. Since the activity of phloroglucinol itself, which has been derived from the A-ring of pelargonidin, is quite low as shown above, a higher radioactivity in compound III must be due to the activity of 3-C and/or 4-C in pelargonidin molecule. Besides, the experiment with leucine-U-¹⁴C showed that the activity of 3-C and/or 4-C of pelargonidin was more than three times as high as that obtained in the case of leucine-1-¹⁴C. This fact seems to indicate that the γ -pyran ring in pelargonidin is formed from unknown intermediate involving two or more carbon atoms, which must have originated from leucine.

Table 2. Distribution of radioactivity in the components of *Raphanus*-anthocyanin, which is produced in radish seedlings after administration of leucine- ^{14}C for 24 hrs..

Compounds administered to plants	Anthocyanin and its hydrolytic products	Total c. p. m.	Activity in % of total ^{14}C in anthocyanin	Specific activity in c. p. m. per mg of carbon
leucine-U- ^{14}C	anthocyanin	1359.7	100	
	{ organic acids	211.0	15.5	
	{ glucose	65.0	4.8	17.4 c. p. m./mg C
	{ pelargonidin	1066.8	78.5	181.7 c. p. m./mg C
leucine-1- ^{14}C	anthocyanin	1212.6	100	
	{ organic acids	196.0	16.2	
	{ glucose	86.0	7.1	23.0 c. p. m./mg C
	{ pelargonidin	842.0	69.4	143.4 c. p. m./mg C

Table 3. Distribution of radioactivity within the molecule of *Raphanus*-anthocyanin obtained after 24 hr culture. Activity of pelargonidin itself and degradation products thereof by means of 15% NaOH aq. or hydrogen peroxide.

Compounds administered to plants	Pelargonidin itself and the degradation products thereof	Total c. p. m.	Activity in % of total ^{14}C in pelargonidin	Specific activity in c. p. m. per mg of carbon
leucine-U- ^{14}C	with NaOH			
	pelargonidin	1066.8	100	181.7
	A-ring (I)	73.0	6.8	82.0
	B-ring (II)	274.0	25.7	322.4
leucine-1- ^{14}C	with NaOH			
	pelargonidin	842.0	100	143.4
	A-ring (I)	68.0	8.1	77.0
	B-ring (II)	197.2	24.4	232.0
leucine-U- ^{14}C	with H_2O_2			
	pelargonidin	1087.6	100	185.3
	A-ring+C-C (III)	213.5	19.6	—
	B-ring (II)	279.0	25.7	320.7
leucine-1- ^{14}C	with H_2O_2			
	pelargonidin	970.0	100	165.2
	A-ring+C-C (III)	113.0	11.6	—
	B-ring (II)	242.0	24.9	207.8

Tables 4 and 5 show the distribution of ^{14}C of labeled leucine in various components in radish hypocotyls. The activity of ^{14}C in free organic acid moieties was higher than that located in the neutral fraction containing free sugars. In the fraction containing free organic acids, radio-activity was found especially high in malic acid, and in succinic and malonic acids to a lesser extent. In the fraction of free amino acids, a higher activity was always found in leucine. In view of a predominant conversion of leucine into valine, the C-C fragment involving carboxyl-C

Table 4. Distribution of radioactivity in the 80% ethanol-soluble fractions obtained from radish hypocotyls, which were fed with leucine- ^{14}C .

Time of feeding	Fractions	Total c. p. m.	Activity in % of total ^{14}C in each fraction	Activity in % of total ^{14}C in 80% EtOH extract
9 hrs.	Aliphatic acid fraction	3708.0	100	1.7
	{malic acid	1021.8	27.6	0.5
	{succinic acid	237.0	6.4	—
	{malonic acid	101.0	2.7	—
	Amino acid fraction	184372.5	100	84.4
	{leucine	131900.1	71.5	60.4
	{valine	1036.2	0.6	0.5
	Neutral fraction	2158.0	100	1.0
	{glucose	84.0	3.9	—
	{unknown sbst.	126.4	5.8	—
	80% EtOH extract	218480.0	—	100
24 hrs.	Aliphatic acid fraction	4460.0	100	1.7
	{malic acid	1163.0	26.1	0.4
	{succinic acid	356.8	8.0	—
	{malonic acid	63.0	1.4	—
	Amino acid fraction	169400.0	100	62.9
	{leucine	126626.5	74.8	47.0
	{valine	2659.6	1.6	1.0
	Neutral fraction	4550.0	100	1.7
	{glucose	136.8	3.0	—
	{unknown sbst.	311.0	6.8	—
	80% EtOH extract	269506.0	—	100
	Anthocyanin fraction	1359.7	—	0.5

may be removed from leucine and the resulting product may be converted into pyruvic acid; such a reaction scheme seems to proceed gradually in radish plant. However, conversion of leucine into free sugars remains still open to further investigation. In this connection, it may be worthy of note that a high radioactivity was also observed in unknown substance in the neutral fraction, which reacted with neither silver nitrate nor with aniline hydrochloride. For the present, it is surmised that leucine participates in the biosynthesis of anthocyanin without preliminary conversion into simple sugars.

Discussion

The previous studies^{10,13)} have shown that in radish seedlings the biosynthesis of anthocyanin is conspicuously promoted by feeding with leucine as well as with phenylalanine.

The results obtained by the present experiment have shown that leucine- ^{14}C contributes preferentially to the make-up of 3- and 4-C in the central γ pyran ring and also of the B-ring of pelargonidin molecule. Of course, a high radioactivity located in the 3-C and/or 4-C of pelargonidin is due to the labeled carbon atoms of leucine. Moreover, it is evident that either of these two radioactive carbons is nothing but the carboxyl-C of leucine, so that leucine-1- ^{14}C contributes to the formation of the heterocyclic ring.

Table 5. Distribution of radioactivity in 80% ethanol-soluble fraction obtained from radish hypocotyls, which were fed with leucine-1-¹⁴C.

Time of feeding	Fractions	Total c. p. m.	Activity in % of total ¹⁴ C in each fraction	Activity in % of total ¹⁴ C in 80% EtOH extract
9 hrs.	Aliphatic acid fraction	2965.0	100	1.4
	{malic acid	856.0	28.9	0.4
	{succinic acid	77.0	2.6	—
	{malonic acid	25.5	0.9	—
	Amino acid fraction	129910.0	100	62.8
	{leucine	95185.1	73.3	47.0
	Neutral fraction	1144.0	100	0.6
	{glucose	97.5	8.5	—
24 hrs.	{unknown sbst.	322.5	28.2	—
	80% EtOH extract	206920.0	—	100
	Aliphatic acid fraction	3327.0	100	1.3
	{malic acid	1487.5	44.7	0.6
	{succinic acid	57.5	1.7	—
	{malonic acid	394.5	11.9	—
	Amino acid fraction	162115.0	100	62.8
	{leucine	106692.5	73.8	41.4
	Neutral fraction	3436.0	100	1.3
	{glucose	157.5	4.6	—
	{unknown sbst.	458.0	13.3	—
	80% EtOH extract	257994.0	—	100
	Anthocyanin fraction	1212.6	—	0.5

Now, it becomes plausible that pyruvic acid, which may also be derived from leucine, is transduced into the heterocyclic ring of pelargonidin. In this case, a probable conversion of leucine into pyruvate may proceed along the well known route of degradation: leucine→acetyl-CoA→pyruvic acid. Here, it must be added that the incorporation of ¹⁴C into the B-ring of pelargonidin is effected by leucine-1-¹⁴C at a rate much higher than that into the A-ring. This indicates that the carboxyl-C of leucine plays a significant role also in the building-up of the side benzene nucleus of pelargonidin. The real mechanism of this phenomenon is still open to further investigation.

Recently, Grisebach¹⁵⁾ has pointed out a possibility of cyclization of malonyl-CoA into the A-ring of flavonoid compounds in general. In this connection, it is interesting that the incorporation of leucine-¹⁴C into free organic acids used to occur at a higher rate in malic, succinic and malonic acids. Among other things, malic acid is particularly significant, since an inverse correlation seems to exist between the accumulation of this acid and the production of anthocyanins in radish plant. This may also provide an important key to a new problem of anthocyanin biogenesis.

Summary

1. Stimulatory effect of leucine on anthocyanin formation in radish seedlings was studied in detail by the use of leucine-U-¹⁴C and leucine-1-¹⁴C, in relation to the distribution of radioactivity in the molecular components of aroyl-raphanusin (acylated triglucoside of pelargonidin).

2. Leucine- $U-^{14}C$ is incorporated preferentially into 3- and/or 4-carbon atoms and also into the B-ring of pelargonidin. Quite similar is the case where leucine- $1-^{14}C$ was administered to the seedlings.

3. The carboxyl- C of leucine is transduced into the γ -pyran ring in pelargonidin. In this case, pyruvic acid, which is derived from leucine, seems to serve as an effective intermediate for the synthesis of pelargonidin.

4. Leucine may take part in the biosynthesis of anthocyanin without preliminary conversion into sugars.

5. The conversion of leucine into malic acid occurs more or less at a higher rate. Anthocyanin formation seems to occur at the expense of malic acid, which may have been derived from leucine.

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摘 要

石倉成行・林 孝三：アカダイコンにおけるアントシアニンの分子骨格へのロイシン ^{14}C 標識物のとり込み（アントシアニンの研究・第53報）

さきに著者らは、アカダイコンではアントシアニン形成に及ぼすロイシンの効果がフェニルアラニンのそれに匹敵することを報告したが、今回、その生合成的機作を調べるために、ロイシン- $U-^{14}C$ およびロイシン- $1-^{14}C$ のアントシアニンへの取り込みが色素分子のどの部分におこるか、また同時に、諸種の共在成分にその ^{14}C がどのように分布していくかを調べた。

ロイシン- $1-^{14}C$ およびロイシン- $U-^{14}C$ は、いずれもペラルゴニジンの複素環を構成している C_8 -部分とB環とに大量に取り込まれ、A環への取り込みは非常に少ない。 C_8 -部分には、ロイシン- $1-^{14}C$ によっても高い放射活性が示されることから、その構成にロイシンのカルボキシル炭素が効果的に使われるものとみられる。したがって、その取り込みはロイシンからビルビル酸を経て進行するものと考えられる。

ロイシンの遊離糖への転入は、有機酸への転入よりも少ないことからすると、アントシアニン形成に及ぼす促進的な効果は、ロイシン→糖→アントシアニンの過程を経るのではなく、むしろロイシン→有機酸→アントシアニンの過程をたどるものとみられる。なかんずく、有機酸区分中で最も高い放射活性がリンゴ酸にあることからすると、アントシアニン合成系とリンゴ酸代謝系との関係が今後の課題となるであろう。（東京教育大学理学部植物学教室）