Fate of ^{14}C -Labeled Amino Acids Administered to the Seedlings of Red Radish with Special Regard to Anthocyanin Biosynthesis Studies on Anthocyanins, LII.*

by Nariyuki ISHIKURA and Kôzô HAYASHI**

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Using the experimental plants belonging to the genera, *Raphanus* (radish) and *Brassica* (turnip), attempt has been made by the present writers¹⁾ to disclose the metabolic background of the biosynthesis of aroylraphanusins. In the first place, a comparative survey was made on the change in quantities of component sugars, amino acids and aromatic acids during growth and development of plants both in red and white varieties in parallel. Consequently, a conspicuous difference was observed between the red and white varieties especially in the content of free amino acids, indicating that such a difference is pronounced by extensive synthesis of anthocyanins in the root. In other words, anthocyanin production is promoted by the administration of some amino acids, such as leucine, phenylalanine and valine.

According to the prevailing concept on biosynthetic scheme of anthocyanins²⁻⁵⁾, the $\text{C}_6\text{-C}_3\text{-C}_6$ unit seems to be formed by the condensation of cinnamic acid homologues which have to be derived through the shikimic-prephenic acid route⁶⁾ on the one hand, and the three acetic acid units on the other, whereby sucrose and glucose have been known as primary source for pigment synthesis.

Following our recent findings mentioned above, an interest points to a problem on the role of amino acids in anthocyanin biosynthesis. Of course, approach to this problem has once been done by the administration of ^{14}C -labeled amino acids to the seedlings of a red radish. The detail will be described in the following.

Material and Methods

General remark. Seedlings of a sort of radish (*Raphanus sativus* f. *comet*) were used as plant material, and the cultivation was made in the same way as described previously¹⁾. The seedlings used for tracer experiments were those of the 9 to 10 day growth after germination, namely at a stage of initiating synthesis of anthocyanins. Radioactivity measurement was made by means of the gas flow counter, Aloka Radiation Counter Model DC-4. The amino acids used were DL-phenylalanine-2- ^{14}C , DL-tyrosine-2- ^{14}C , and the uniformly labeled L-isomers of leucine, valine, threonine and aspartic acid. All of these compounds were the preparations from the Radiochemical Center of England. According to the preliminary examinations, these amino acids showed a promotive effect on anthocyanin synthesis, with an exception that tyrosine was more or less inhibitory. The relative effectiveness of these compounds for pigment synthesis was assessed in comparison with the radioactivity of the anthocyanin, which was synthesized after administration of a definite amount of phenylalanine-2- ^{14}C to the seedlings, since phenylalanine has been shown by many

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** Botanical Department, Faculty of Science, Tokyo Kyoiku University.

workers to be the most excellent precursor for the formation of the B-ring in anthocyanin molecule.

Administration of ^{14}C -labeled amino acids to the seedlings. Every four seedlings grown on a modified Hoagland's solution were used in each experiment. Each seedling was cut with a razor blade to remove a colorless part of the root. The cuttings were trimmed to a definite magnitude and placed in a small glass tube containing 0.4 ml (total activity being $2\mu\text{c}$) of the solution of ^{14}C -labeled amino acid (5×10^{-3} M), in which non-labeled acid was also added as carrier. The whole set was covered with a bell jar having two bent-holes; the lower one being used for the input of fresh air and the upper one for the output of $^{14}\text{CO}_2$ produced by test plant. The illumination was effected by standard cool white fluorescent lamps, and the total light intensity was adjusted to 8,000 lux. Under these conditions the seedlings were grown at $16 \pm 4^\circ$ for 9 to 24 hours under gentle ventilation. As a rule, test plant absorbs the whole solution (0.4 ml) within the first 2.5 hours, whereupon further three 0.4 ml portions of water were successively added through an inlet tube in order to effect complete absorption of the labeled compound. Finally, the seedlings were irrigated with about 4 ml of the medium.

Prior to extraction of anthocyanin from the plant, the radioactivity in excised seedlings was measured in the following manner: the seedlings were cut into two separate portions, cotyledon and hypocotyl, and each was rinsed in water, blotted, and put into a small glass cup for activity measurement. Then, the material was squashed with a glass rod together with a minimum amount of 1% methanolic hydrochloric acid, whereupon the whole material was dried in a vacuum and used for the measurement of radioactivity.

Extraction and purification of the anthocyanin moiety. Since anthocyanin occurs for the most part in hypocotyl, still colorless cotyledons were carefully removed before extraction.

The red pigmented portion of the seedlings was thoroughly extracted with 1% methanolic hydrochloric acid until it became colorless. The filtered extract was added with some anthocyanin carrier, which had been prepared from a red radish⁷⁾. From this solution the pigment was precipitated completely by the addition of a methanolic solution of basic lead acetate. The purple lead salt of anthocyanin was collected by suction, and washed with some ethanol. The product was converted into the chloride by dissolving in 5% methanolic hydrochloric acid, and precipitated with ether as an amorphous red powder. This was dissolved again in 1% methanolic hydrochloric acid and precipitated with ether. The radioactivity of this product was regarded as the total activity of the anthocyanin moiety, since no impurity was detected by chromatographic tests. Further, the pigment was used for the following tests.

Degradation of the anthocyanin. As already reported⁸⁾, *Raphanus*-anthocyanin is composed of five kinds of acylated raphanusin, a triglucoside of pelargonidin. In order to know about the distribution of ^{14}C in various components of the molecule, the pigment was subjected to hydrolysis by means of acid and alkali, and the individual fractions were examined for radioactivity in detail.

First, the anthocyanin fraction obtained above was saponified by 10% aqueous sodium hydroxide. After acidification, the organic acids liberated were transferred to an ethereal layer, from which they were separated into three components, *p*-coumaric, caffeic and ferulic acids, by the ascending mass paper chromatography using benzene/acetic acid/water (125:72:3, v/v). Individual acids separated on the

chromatogram were eluted with methanol from the respective bands. Each of the eluates was evaporated to dryness and applied for the measurement of radioactivity.

Next, an aqueous pigment solution obtained above was added with conc. HCl to about 20%, and boiled for 2.5 minutes for hydrolysis of the pigment (raphanusin). On cooling, the aglycone was transferred into *iso*-amyl alcohol, while sugar remained in the mother liquor. Both fractions were separated from each other and evaporated to dryness *in vacuo* over solid sodium hydroxide, and tested for radioactivity.

Finally, the aglycone (pelargonidin) was degraded according to Karrer⁹⁾ in the following way: The aglycone was weighed, and dissolved in 3 ml of 15% aqueous sodium hydroxide. After refluxing for 1.5 hours, the solution was acidified with hydrochloric acid, and shaken with ether. The ethereal extract was evaporated, and the residue was dissolved in a few drops of methanol, and streaked on a sheet of Tōyō No. 51 filter paper (15×60 cm). After irrigation with *n*-butanol/acetic acid/water (4:1:5, v/v) by the ascending method, the chromatogram was carefully compared with the standard which was prepared from a mixture of phloroglucinol and *p*-hydroxybenzoic acid (spots being developed with diazotized sulfanilic acid). The bands of phloroglucinol and *p*-hydroxybenzoic acid were cut from the chromatogram, eluted with ethanol, and subjected to radioactivity measurement.

Colorimetric determination of phloroglucinol and p-hydroxybenzoic acid in degradation products. Phloroglucinol eluted from the chromatogram was dissolved in a small amount of ethanol, and added with 1 ml of D-1 reagent* and 0.5 ml of NH₄OH solution (28%). The purplish red solution obtained was transferred to a 25 ml volumetric flask, and filled with ethanol up to the mark. With this solution the absorption at 514 mμ was measured with Beckman spectrophotometer (*cf.* Fig. 1),

Fig. 1. Absorption spectra of
— phloroglucinol (color being developed by D-1 reagent),
----- *p*-hydroxybenzoic acid (color being developed by D-2 reagent).

and the reading was compared with that of the standard solution of phloroglucinol prepared in the same way (*cf.* Fig. 2). Likewise, *p*-hydroxybenzoic acid which was prepared from the chromatogram was added with 1 ml of D-2 reagent** and 0.5 ml of NH₄OH solution (28%) to develop a purplish red color. The optical density was read at 514 mμ (*cf.* Fig. 1), and the amount of *p*-hydroxybenzoic acid was computed

* D-1 reagent: 4-Benzamido-2, 5-diethoxybenzenediazonium zinc (100 mg) was dissolved in a mixture of dioxane (5 ml) and water (10 ml) at 0° and filtered.

** D-2 reagent: *p*-Nitrobenzenediazonium fluoroborate (60 mg) was dissolved in a mixture of dioxane (5 ml) and water (10 ml) at 0° and filtered.

Fig. 2. Standard curve for the estimation of *p*-hydroxybenzoic acid (I) and phloroglucinol (II).

-○-○- (I), color being developed by D-2 reagent.

-△-△- (II), color being developed by D-1 reagent.

from a standard curve (*cf.* Fig. 2). The rate of recovery of the degradation products from pelargonidin was about 35% on an average.

*Distribution of ^{14}C in *p*-coumaric, caffeic and ferulic acids.* For this experiment, excised seedlings were fed with labeled phenylalanine and tyrosine, respectively, for a definite length of time. Cotyledons were cut off from the seedlings, and the remaining parts were extracted thoroughly with hot methanol. Then, the extract was evaporated to dryness, and the residue was saponified with 10% aqueous sodium hydroxide. The organic acids liberated therefrom were separated by chromatography as usual. The individual acids were eluted with methanol in a separate manner, and applied for the activity measurement with Radiation Counter.

Results

The radioactivities in a whole seedling as well as in cotyledons and hypocotyl, which was fed with each of the ^{14}C -labeled amino acids for 24 hours, are shown in Table 1, from which the major activity is to be seen in the hypocotyl. Among six amino acids tested, phenylalanine and aspartic acid seem to be most rapidly utilized for anthocyanin synthesis in the hypocotyl.

The radioactivities of the anthocyanin and its degradation products are shown in Tables 2 and 3. As already demonstrated in a variety of plants, phenylalanine has proved to be the best precursor of anthocyanin in radish as well. In spite of its close structural relationship, tyrosine was effective only about one-half to one-third as much as phenylalanine for the synthesis of pelargonidin. Activity measurements on alkaline degradation products of pelargonidin, *i.e.*, phloroglucinol and *p*-hydroxybenzoic acid, have indicated that the amino acids having $\text{C}_6\text{-C}_3$ skeleton are incorporated to form the B-ring and its adjacent three-carbon chain in anthocyanidin molecule. On the other hand, other amino acids such as leucine, threonine and aspartic acid are also shown to be effective for the biosynthesis of pelargonidin to a remarkable degree. Besides, threonine and aspartic acid have much to do with the same moiety

Table 1. Distribution of radioactivity between hypocotyl and cotyledon in radish seedlings fed with ^{14}C -labeled amino acid for 24 hours.

Amino acids administered to the seedlings	Radioactivity (c.p.m.) in		
	hypocotyl	cotyledon	whole plant
Phenylala.- $2\text{-}^{14}\text{C}$	239292	42268	281560
Tyr.- $2\text{-}^{14}\text{C}$	324112	182682	506800
Leu.- $\text{U-}^{14}\text{C}$	337284	228468	565752
Val.- $\text{U-}^{14}\text{C}$	359984	91836	451820
Threo.- $\text{U-}^{14}\text{C}$	377832	71956	449788
Asp. acid- $\text{U-}^{14}\text{C}$	230080	84876	314956

Table 2. Radioactivity (c.p.m.) of anthocyanins and degradation products thereof. (Pigment was isolated from excised radish seedlings fed with ^{14}C -labeled amino acids.)

Time length of feeding in hrs.	Amino acids administered to the seedlings	Total activity (c.p.m.) of				
		<i>Raphanus</i> -anthocyanin	raphanusin	pelargonidin (aglycone)	glucose	organic acids
9	Phenylala.- $2\text{-}^{14}\text{C}$	2301.6	960.4	822.4	74.8	1240.5
	Tyr.- $2\text{-}^{14}\text{C}$	657.0	338.6	269.0	53.0	238.0
	Leu.- $\text{U-}^{14}\text{C}$	754.9	536.0	516.0	34.8	129.4
	Val.- $\text{U-}^{14}\text{C}$	288.2	239.9	139.6	84.8	19.2
	Threo.- $\text{U-}^{14}\text{C}$	859.2	592.8	291.8	298.6	26.8
	Asp. acid- $\text{U-}^{14}\text{C}$	1141.4	877.6	268.6	476.3	53.8
24	Phenylala.- $2\text{-}^{14}\text{C}$	3511.3	1126.5	985.4	86.7	1812.4
	Tyr.- $2\text{-}^{14}\text{C}$	1196.6	616.0	554.0	60.5	238.0
	Leu.- $\text{U-}^{14}\text{C}$	1207.8	703.8	660.4	47.6	212.0
	Val.- $\text{U-}^{14}\text{C}$	566.9	375.2	299.4	43.1	101.1
	Threo.- $\text{U-}^{14}\text{C}$	1083.1	901.7	449.9	445.2	167.4
	Asp. acid- $\text{U-}^{14}\text{C}$	1186.6	793.6	490.9	238.3	149.3

Table 3. Distribution of radioactivity (^{14}C) in sugar-free pigment (pelargonidin).

Amino acids administered to the seedlings	Specific activity (c.p.m.) per mg of carbon of		
	aglycone	phloroglucinol (A-ring)	<i>p</i> -hydroxybenzoic acid (B-ring)
Phenylala.- $2\text{-}^{14}\text{C}$	167.9	3.9	5.3
Tyr.- $2\text{-}^{14}\text{C}$	94.4	5.4	4.9
Leu.- $\text{U-}^{14}\text{C}$	112.5	55.5	155.9
Val.- $\text{U-}^{14}\text{C}$	51.0	23.9	65.9
Threo.- $\text{U-}^{14}\text{C}$	76.6	23.7	91.1
Asp. acid- $\text{U-}^{14}\text{C}$	83.6	32.1	108.8

Time length of amino acid feeding being 24 hrs.

Table 4. Distribution of radioactivity (^{14}C) in *p*-coumaric, caffeic and ferulic acids, which were obtained from the excised radish seedlings fed with ^{14}C -labeled amino acids.

Amino acids administered to the seedlings	Total activity (c.p.m.) of the compounds					
	from the anthocyanin moiety			from the hypocotyl		
	<i>p</i> -coumaric	caffeic	ferulic	<i>p</i> -coumaric	caffeic	ferulic
Phenylala.- $2\text{-}^{14}\text{C}$	697.0	163.9	316.5	1686.0	332.2	756.6
Tyr.- $2\text{-}^{14}\text{C}$	39.8	13.6	32.3	363.8	1115.4	89.0
Leu.- $\text{U-}^{14}\text{C}$	16.2	17.8	24.2	76.9	83.2	75.2
Val.- $\text{U-}^{14}\text{C}$	21.6	37.2	34.6			
Threo.- $\text{U-}^{14}\text{C}$	26.6	38.6	50.8			
Asp. acid- $\text{U-}^{14}\text{C}$	21.8	32.4	46.4			

Time length of amino acid feeding being 24 hrs.

in raphanusin. On the other hand, leucine and valine are incorporated exclusively into the $\text{C}_6\text{-C}_3\text{-C}_6$ skeleton, especially into the B-ring of pelargonidin.

Table 4 shows the distribution of ^{14}C in *p*-coumaric, caffeic and ferulic acids, when the seedlings are fed with labeled phenylalanine and tyrosine, respectively. In the case of phenylalanine, *p*-coumaric acid exhibits the highest radioactivity, whereas the highest activity was measured in caffeic acid on administration of tyrosine. Therefore, it is likely that phenylalanine is rapidly transferred to a *p*-coumaric acid pool and tyrosine to a caffeic acid pool. However, when the labeled tyrosine was administered to the seedlings, the radioactivity of caffeic acid, which constitutes the acyl groups of anthocyanin, was found to be unexpectedly low.

Discussion and Conclusion

So far as the tracer experiments are concerned, six amino acids tested here seem to contribute to the biosynthesis of anthocyanins in radish seedling. But a question remains still open, whether all of these amino acids participate directly in the production of anthocyanin or not, since the comparative incorporation rate of individual acids into anthocyanin moiety varies from one to another.

In contrast to a remarkable effect of phenylalanine, tyrosine affects rather inhibitorily on anthocyanin synthesis. Although tyrosine can be absorbed by the plant as much as leucine, valine and threonine as shown in Table 1, it is almost ineffective for anthocyanin synthesis in spite of its closer structural relationship to the B-ring of pelargonidin, which is produced in this plant. Therefore, it appears that the introduction of a hydroxyl group into the 4'-position of the B-ring of anthocyanidin does not occur at a stage of phenylpropanoid in biosynthetic pathway. This may also be reflected by the results on the distribution of ^{14}C in the free and bound phenolic acids, as shown in Table 4. In fact, tyrosine is rapidly converted into caffeic acid, and phenylalanine into *p*-coumaric acid in radish seedlings. Therefore, it seems likely that *p*-coumaric, ferulic and caffeic acids, which are usually present in larger amount, are nothing but by-products formed during the process of anthocyanin synthesis.

In this connection, it must be added that the radioactivity of caffeic acid, which occurs in combination with anthocyanins in tyrosine-fed seedlings, was found to be

unexpectedly low. Perhaps it may be that the *endogenous* caffeic acid is utilized rather preferentially for the esterification of anthocyanin, since this acid is synthesized in larger amount in this plant.

In spite of their structural discordance, leucine, valine, threonine and aspartic acid are incorporated into anthocyanidin (pelargonidin) to an appreciable extent. Similar events have been frequently described in the recent literatures. G. Nétien and J. Lacharme^{10,11)} have shown that the anthocyanin content in radish germ may increase when the seeds are pretreated with antibiotics such as terramycin, streptomycin, etc. Similar result was also described by T. Yamada and K. Hori¹²⁾ in the seedlings of *Brassica chinensis*. On the other hand, T. Erdös and A. Ullmann¹³⁾ have shown that in cell-free extract of *Mycobacterium* streptomycin inhibits the transfer of amino acids from ribonucleic acid to protein. Moreover, according to W. Brucker and U. Drehmann¹⁴⁾, certain amino acids or protein metabolites play in *Phycomyces* an essential role in the synthesis of phenol-carboxylic acids.

From these findings, it is surmised that the biosynthesis of anthocyanins may proceed not only along the sugar-shikimic acid route^{4,5)} as already established, but also *via* another route, in which amino acids are involved as starting material.

Since aspartic acid and threonine are incorporated not only into pelargonidin itself but also into sugar moiety of the glycoside, these amino acids may be utilized for anthocyanin synthesis through somewhat round-about pathway; that is, aspartic acid may be once converted into citric acid, and threonine into pyruvate. On the other hand, leucine and valine are predominantly incorporated into aglycone (pelargonidin) and only a little into glucose moiety of the glycoside. In general, leucine and valine may be converted into acetoacetate and acetyl Co A. If these products contribute to the anthocyanin synthesis, the incorporation of these amino acids into pelargonidin may occur either exclusively in the A-ring of anthocyanin (according to H. Grisebach⁸⁾) or uniformly in the whole molecule. However, the incorporation of leucine and valine into pelargonidin in radish seedlings occurred predominantly in the B-ring rather than in the A-ring.

Recently, W. O. James *et al.*¹⁵⁾, E. H. Hess¹⁶⁾ and others have presumed the following scheme: oxidized polyphenols are capable of combining amino acids, peptides or simple protein to form certain addition compounds (adducts), which behave as catalysts for the oxidative deamination of amino acids.

According to this view, leucine and valine may be converted into the corresponding keto acids, and somewhat participate in anthocyanin synthesis through the route for the biosynthesis of steroid from mevalonyl Co A¹⁷⁾. A possibility of this reaction route involving oxygen consumption in the utilization of amino acids may be strengthened by the fact that anthocyanin production usually takes place in connection with higher intensity of respiration (F. Ebenhardt^{18,19)} and O. Björkman²⁰⁾).

Summary

The present work was carried out in order to settle the question: why several amino acids promote the anthocyanin synthesis in radish plant. Tracer experiments have disclosed the following facts:

1. DL-Phenylalanine-2-¹⁴C, DL-tyrosine-2-¹⁴C and uniformly labeled L-isomers of

leucine, valine, threonine and aspartic acid were fed separately to the excised seedlings of a red radish (*comet*). After feeding with individual labeled acids, anthocyanin was isolated from the plants, and its radioactivity was measured. Then, the anthocyanin was saponified into organic acids and raphanusin, and the latter was hydrolyzed into sugar and pelargonidin. Finally, the aglycone was degraded into two parts, *p*-hydroxybenzoic acid and phloroglucinol. The individual products obtained were subjected to the measurement of radioactivity.

2. Among six amino acids tested, phenylalanine was shown to be the most excellent precursor of anthocyanin, and the C₆-C₃ skeleton of this amino acid, and also of tyrosine, seems to be transferred to the B-ring and adjacent three-carbon chain of pelargonidin.

3. Four amino acids having no C₆-C₃ skeleton are also effective for anthocyanin synthesis. The incorporation of these amino acids into anthocyanin was discussed.

4. Threonine and aspartic acid also contribute to the biosynthesis of anthocyanin probably along the usual pathways of metabolic degradation. Leucine and valine are well incorporated into the C₆-C₃-C₆ unit, especially into the B-ring of anthocyanin. The precise mechanism has much to be investigated in future.

5. In spite of the close structural relationship, the effectiveness of tyrosine was only one-half to one-third as much as compared with that of phenylalanine. This seems to indicate that the introduction of *p*-hydroxyl group into the B-ring of pelargonidin takes place before the formation of phenylpropanoid chain is completed.

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摘 要

石倉成行・林 孝三： ダイコン芽生えのアントシアニン生成にあずかる標識アミノ酸の行方 (アントシアニン研究・第52報)

著者らは前報(本誌 **78**:481) で若干のアミノ酸がアントシアニン形成を促進することを報告したが、今回これを確認するために6種類の¹⁴C-アミノ酸(フェニルアラニン、チロシン、ロイシン、ヴァリン、スレオニン、アスパラギン酸)をそれぞれ赤ダイコンの芽えに供与して¹⁴Cの行方を追跡した。その結果、これらのアミノ酸は、いずれもアントシアニン(ラファヌシン)の生成に利用されることがわかった。したがって、アミノ酸による色素生産の促進効果は、他の共役代謝系を刺激するというような間接的なものではなく、むしろ直接アントシアニン合成系へ入るためとみられる。この事実は、既知のシキミ酸経路のほかに、アミノ酸を含む別個の生成経路の存在を暗示するものであり、これをめぐり若干の論議を試みた。因みに、色素部分への¹⁴Cの取込みは、予期のごとく、フェニルアラニンが最大であるが、ロイシン、アスパラギン酸、ス

レオニン，チロシンでもその約 1/3 程度の取込みがみられる (第 2 表). 特に注目すべきことは芳香環をもたないロイシン- ^{14}C の取込みである. このアミノ酸の ^{14}C はもっぱら色素配糖体のアグリコン部分 (ペラルゴニン) に取込まれ，糖部分や有機酸部分へはほとんど移行しない. 今後，この特異性を手がかりに ^{14}C の行方を詳細に研究する必要がある. (東京教育大学理学部植物学教室)