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PRECURSORS IN THE BIOSYNTHESIS OF PELARGONIDIN 3-GLUCOSIDE

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Abstract—Acetic acid-1-^{14}C, cinnamic acid-β-^{14}C, cinnamic acid-α-^{14}C, D-glucose-U-^{14}C, D-fructose-U-^{14}C, DL-phenylalanine-(ring labelled)-^{14}C, DL-phenylalanine-COOH-^{14}C, quinic acid-U-^{14}C, shikimic acid-U-^{14}C and L-tyrosine-U-^{14}C were administered to detached strawberries and the incorporation of the label into the anthocyanin was measured. Cinnamic and shikimic acids were the most efficient precursors followed by the sugars, phenylalanine, quinic acid, acetic acid and tyrosine. Degradation of the labelled pigment indicated that ring A can be formed from acetic acid, glucose or fructose, ring B from cinnamic acid, shikimic acid, quinic acid, phenylalanine, tyrosine, glucose or fructose, and the sugar moiety of the pigment from acetic acid, shikimic acid, glucose or fructose. Evidence was obtained that the C_6–C_3 skeleton of cinnamic acid and phenylalanine was incorporated intact.

INTRODUCTION

THE literature on the occurrence and biosynthesis of flavonoid compounds has been covered in several excellent reviews[1–4] and books.[5–8] Most of the information on the origin of the carbon in the C_6–C_3–C_6 skeleton has come from biochemical experiments using ^{14}C-labelled precursors.[9–11]

The work of Underhill *et al.*,[12] Watkin *et al.*,[13] Geissman and Swain[14] on the biosynthesis of quercetin in buckwheat showed that ring A of quercetin was formed from acetate and ring B from a C_6–C_3 compound, such as phenylalanine, cinnamic acid or *p*-coumaric acid, which was incorporated as a unit. Zaprometov[15] reported that acetate was incorporated mainly into ring A and shikimic acid was incorporated mainly into ring B of catechin in young shoots of tea plants. Comte *et al.*[16] also showed that β-^{14}C-cinnamic acid is incorporated into (+)-catechin by *Prunus avium*.

Hutchinson *et al.*[17] reported that phenylalanine was incorporated into ring B and acetate

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[1] L. BOGORAD, *Ann. Rev. Plant Physiol.* **9**, 417 (1958).

[2] A. GEISSMAN and E. HINREINER, *Botan. Rev.* **18**, 77 (1952).

[3] K. PAECH, *Ann. Rev. Plant Physiol.* **6**, 281 (1955).

[4] R. SESHADRI, *Ann. Rev. Biochem.* **20**, 487 (1951).

[5] A. GEISSMAN (Ed.), *The Chemistry of Flavonoids Compounds.* Pergamon Press, Oxford (1962).

[6] P. BERNFELD, *Biogenesis of Natural Compounds.* Pergamon Press, Oxford (1963).

[7] J. B. HARBORNE (Ed.), *Biochemistry of Phenolic Compounds.* Academic Press, New York (1964).

[8] T. W. GOODWIN, *Chemistry and Biochemistry of Plant Pigments.* Academic Press, New York (1965).

[9] H. GRISEBACH, *Z. Naturforsch.* **12b**, 227 (1957).

[10] J. E. WATKIN, E. W. UNDERHILL and A. C. NEISH, *Can. J. Biochem. Physiol.* **35**, 229 (1957).

[11] T. A. GEISSMAN and T. SWAIN, *Chem. & Ind. (Rev.)* 984 (1957).

[12] E. W. UNDERHILL, J. E. WATKIN and A. C. NEISH, *Can. J. Biochem. Physiol.* **35**, 219 (1957).

[13] J. E. WATKIN, E. W. UNDERHILL and A. C. NEISH, *Can. J. Biochem. Physiol.* **35**, 229 (1957).

[14] T. A. GEISSMAN and T. SWAIN, *Chem. & Ind. (Rev.)* 984 (1957).

[15] M. N. ZAPROMETOV, *Biokhimia* **27**, 366 (1962).

[16] P. COMTE, A. VILLE, G. ZWINGELSTEIN, J. FAVRE-BONING and C. MENTZER, *Bull. Soc. Chim. Biol.* **42**, 1079 (1960).

[17] A. HUTCHINSON, C. D. TAPER and G. H. N. TOWERS, *Can. J. Biochem. Physiol.* **37**, 901 (1959).

into ring A of phloridzin in leaf disks of Malus. Avadhani and Towers[18] showed that α-^{14}C-cinnamic acid was also readily incorporated into phloridzin. Grisebach[9] studied the biosynthesis of cyanidin in red cabbage seedlings. Acetate-1-^{14}C was found to enter mainly into ring A and the label was concentrated in the oxygen-bearing carbon. When acetate-2-^{14}C was fed, the ^{14}C was concentrated in the nonoxygen-bearing carbon of ring A. This shows that ring A is formed by head-to-tail condensation of acetate units. Grisebach[9] also showed that the label of phenylalanine-U-^{14}C is found in ring B and the attached carbon.

Grisebach and Bopp[19] reported that both quercetin and cyanidin were formed simultaneously from COOH-^{14}C-phenylalanine. Later, Grisebach and Patschke[20] showed that a chalcone may serve as a precursor of both cyanidin and quercetin.

Strawberries were selected as the experimental material in this work because they contain a typical anthocyanin, pelargonidin 3-glucoside, the biogenesis of which is practically unknown,[21] and because fruits have not previously been used in similar investigations. A number of ^{14}C-labelled compounds were administered into maturing fruits at their pink-white stage. The relative effectiveness of these compounds as precursors of the pigment was assessed by calculating the percentage ^{14}C converted. The pelargonidin-3-glucoside was degraded to locate the ^{14}C in the molecule.

RESULTS AND DISCUSSION

The efficiency of the various ^{14}C compounds which were tested as precursors of the strawberry anthocyanin is shown in Table 1.

TABLE 1. RELATIVE EFFICIENCY OF VARIOUS COMPOUNDS AS PRECURSORS OF PELARGONIDIN-3-GLUCOSIDE OF STRAWBERRIES

Compound fed	% ^{14}C converted*
Acetic acid	0·3
L-Tyrosine-U-^{14}C	0·2
DL-Phenylalanine-^{14}C (ring labelled)	0·9
D-Glucose-U-^{14}C	1·6
D-Fructose-U-^{14}C	1·7
Quinic acid ^{14}C (generally labelled)	0·4
Cinnamic acid-α-^{14}C	3·3
Shikimic acid-^{14}C (generally labelled)	2·2

$$* \ \% \ ^{14}\text{C converted} = \frac{\mu\text{c in pelargonidin-3-glucoside}}{\mu\text{c in compound fed}}.$$

Cinnamic acid and shikimic acid were the best precursors among the compounds tested as shown by their higher percentage ^{14}C conversion. Glucose and fructose came next, followed by phenylalanine. If only one of the two stereoisomers of phenylalanine was used, the conversion factor should be doubled. Tyrosine was the poorest of them all. Acetic acid and quinic acid were only slightly more efficient than tyrosine as precursors of the pigment.

The distribution of ^{14}C in the three components, glucose, phloroglucinol and *p*-hydroxybenzoic acid, into which the anthocyanin was chemically degraded is shown in Table 2.

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[18] P. N. AVADHANI and G. H. N. TOWERS, *Can. J. Biochem. Physiol.* **37**, 1605 (1961).
[19] H. GRISEBACH and M. BOPP, *Z. Naturforsch.* **14b**, 485 (1959).
[20] H. GRISEBACH and L. PATSCHKE, *Z. Naturforsch.* **16b**, 645 (1961).
[21] G. E. LIVINGSTON and P. MARKAKIS, *Science* **124**, 28 (1956).

These results support the hypothesis of Birch and Donovan[22] that flavonoids originate from the addition of three acetate units to cinnamic acid or related C_6–C_3 compounds. At least one such compound, shikimic acid, has been found in the strawberry fruit.[23, 24]

TABLE 2. DISTRIBUTION OF ^{14}C IN PELARGONIDIN 3-GLUCOSIDE OF STRAWBERRIES AFTER ADMINISTRATION OF VARIOUS ^{14}C-LABELLED COMPOUNDS

	Labelled degradation product (+)		
Compound fed	Phloroglucinol	*p*-Hydroxy-benzoic acid	Glucose
Acetic acid-1-^{14}C	+	trace	+
L-Tyrosine-U-^{14}C	−	+	−
DL-Phenylalanine-^{14}C (ring labelled)	−	+	−
DL-Phenylalanine-COOH-^{14}C	−	−	−
D-Glucose-U-^{14}C	+	+	+
D-Fructose-U-^{14}C	+	+	+
Quinic acid-U-^{14}C	−	+	−
Cinnamic acid-α-^{14}C	−	−	−
Cinnamic acid-β-^{14}C	−	+	−
Shikimic acid-U-^{14}C	trace	+	+

The fact that phloroglucinol and *p*-hydroxybenzoic acid were not radioactive when cinnamic acid-α-^{14}C or DL-phenylalanine-COOH-^{14}C were administered while the *p*-hydroxybenzoic acid was labelled when cinnamic acid-β-^{14}C or ring-labelled DL-phenylalanine-^{14}C were fed to the strawberries indicates that the entire C_6–C_3 skeleton of these precursors was used in the synthesis of the anthocyanidin. After alkaline fusion carbons 3 and 4 of the aglycon are not part of the phloroglucinol and *p*-hydroxybenzoic acid and the label of cinnamic-α-^{14}C and phenylalanine-1-^{14}C is lost, probably as acetate.

The labelling of both ring A and B in addition to the glucose moiety of the pigment when glucose and fructose were administered is indicative of the active and diverse metabolism of these sugars in the fruit. Tyrosine, in spite of its close chemical resemblance to ring B, is not utilized well by the strawberry fruit for the synthesis of the anthocyanin.

EXPERIMENTAL

Materials

Strawberries of the Everbearing 107 variety were grown in a greenhouse. The following ^{14}C-labelled compounds were used in the feeding experiments: Acetic acid-1-^{14}C, cinnamic acid-β-^{14}C, cinnamic acid-α-^{14}C, D-glucose-U-^{14}C, D-fructose-U-^{14}C, DL-phenylalanine-(ring-labelled)-^{14}C, DL-phenylalanine-COOH-^{14}C, quinic acid-U-^{14}C, shikimic acid-U-^{14}C and L-tyrosine-U-^{14}C.

All radiochemicals, except cinnamic acid, were purchased from New England Nuclear Corporation, Boston, Mass. Cinnamic acid was purchased from Merck, Sharp and Dohme of Canada, Montreal, Canada.* Their specific activity was in the range of 1 to 5 mc/mmole.

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* The authors gratefully acknowledge the donation by Dr. A. C. Neish, National Research Council of Canada, of a sample of radioactive cinnamic acid which was used in preliminary experiments.

[22] A. J. Birch and F. W. Donovan, *Australian J. Chem.* **6**, 360 (1953).

[23] A. C. Hulme and L. S. C. Wooltorton, *Chem. & Ind.* 659 (1958).

[24] P. Markakis and R. J. Embs, *J. Food Sci.* **29**, 629 (1964).

Administration of ^{14}C-Labelled Compounds to Strawberries

At least ten strawberries were used in each experiment. Maturing strawberries in the pink–white stage were removed from the plant by cutting the stems under water with a razor blade so that about 1 in. of stem remained attached to the berry. Individual berries were placed in a solution of the desired radioactive compound contained in a 5 mm i.d. glass tube 1 in. long. The ^{14}C-labelled compounds were administered as their aqueous solution. Cinnamic acid and acetic acid were administered as the sodium salt. The precursor solution, 50–100 μl, was sucked up by the strawberry within 3–4 hr. Water was added every time the tube was about to dry out, so that the last trace of the ^{14}C-labelled compounds was washed into the plant and a continuous supply of water was available to the fruit. The strawberries were allowed to metabolize for 2 days at room temperature; by that time they were very red. The pigments were then extracted and purified.

Isolation and Purification of the Pigments

The red strawberries were extracted thoroughly with several portions of hot *n*-butanol containing 0·1% HCl. The anthocyanins were precipitated from the combined butanolic extract by addition of about 10 vol. of light petrol (b.p. 30–60°). The pigments were then dissolved in 1 ml of methanol and the solution was streaked on Whatman No. 3 MM paper and developed with *n*-butanol–acetic acid–water (16:4:5) descendingly for 18 hr. The chromatograms were dried and the pigments, cyanidin 3-glucoside (R_f=0·29) and pelargonidin 3-glucoside (R_f=0·41), were each cut from the paper and extracted several times with methanol containing 0·01% HCl. The combined methanolic extract was concentrated under reduced pressure in a flash evaporator at 38°. The pelargonidin 3-glucoside was again purified by descending paper chromatography using 2% acetic acid. Further purification was carried out by paper electrophoresis.[25] The Durrum type paper electrophoresis cell by Spinco was used, with a voltage of 480 V in 0·5 N acetic acid on Whatman 3 MM paper. Another descending paper chromatography with *n*-butanol–acetic acid–water (16:4:5) was carried out as a final purification step. The purified pigment was taken up and diluted to the desired volume with 0·1% methanolic HCl.

The absorptivity of this solution was measured with a Beckman DU spectrophotometer at 506 mμ. The concentration was determined by means of a standard curve obtained with crystalline pelargonidin 3-glucoside.

Measurement of the Radioactivity

A measured volume of the pigment was transferred into a planchet, the solvent was evaporated under an i.r. lamp and the radioactivity of the pigment was determined with a Nuclear Chicago Corporation Decade Scaler (Model 181B) with automatic sample changer (Model C-110-B) and printing timer (Model C-111-B). A mixture of 98·7% helium and 1·3% butane was used as the carrier gas. The total pigment counted was controlled to be within 0·0015 to 0·01 mg/cm^2 of planchet. Within this range of solid material the counting efficiency of the counter is about 11 per cent.

Acid Hydrolysis of the Pigment

Enough 6 N HCl was added to the aqueous solution of the pigment to make a 10% HCl solution. The mixture was placed in a boiling water bath for 30 min and subsequently cooled

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[25] P. MARKAKIS, *Nature* **187**, 1092 (1960).

quickly. The pelargonidin was separated from the sugar by extraction with a few drops of isoamyl alcohol. The colorless aqueous layer was spotted on a Whatman No. 1 paper and developed in a descending direction for 48 hr in *n*-butanol–acetic acid–water (16:4:5) solvent. Pure glucose was run side-by-side with the sugar of the pigment.

After the paper was thoroughly dry, the portion of the paper containing the sugar was cut as a narrow strip and passed through an automatic chromatogram scanner (Vanguard, Model 880) to determine whether the sugar was labelled or not.

Alkaline Degradation of Pelargonidin

The pelargonidin was precipitated from the isoamyl alcohol extract by addition of about 10 vol. of benzene. The pelargonidin was then subjected to alkaline degradation by the method of Comte *et al.*[16]

The degradation products were extracted with ether, and the extract was chromatographed on Whatman No. 1 paper for 4 hr with the upper layer of *n*-butanol–benzene–acetic acid–water (2:10:2:1). A mixture of authentic phloroglucinol ($R_f = 0{\cdot}24$) and *p*-hydroxybenzoic acid ($R_f = 0{\cdot}75$) was run next to the degradation products. The portion of the chromatogram containing the degradation products was cut as a strip and passed through the automatic radiochromatogram scanner to locate the radioactivity.

The phloroglucinol and *p*-hydroxybenzoic acid were visualized by spraying with diazotized sulfanilic acid followed by 15% Na_2CO_3. Phloroglucinol gives a reddish brown spot and *p*-hydroxybenzoic acid gives an orange spot with these sprays.

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