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PIGMENTS OF CENTROSPERMAE—IV

ON THE BIOGENESIS OF INDICAXANTHIN AND BETANIN IN *OPUNTIA FICUS-INDICA* MILL.

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Abstract—Indicaxanthin and betanin were isolated from *Opuntia ficus-indica* fruits after incubation with labelled proline and β-(3,4-dihydroxyphenyl)alanine (DOPA). From measurements of the distribution of radioactivity in the pigments and degradation experiments it can be deduced that DOPA acts as precursor of the hydrogenated pyridine moiety of the pigments. Proline has been shown to be specifically incorporated in the proline moiety of indicaxanthin.

INTRODUCTION

IT IS well known that plants belonging to the order Centrospermae contain red-violet and yellow nitrogenous pigments which have been called, respectively, betacyanins and betaxanthins.[1] Chemical investigation of these compounds has proceeded very slowly, and it is only recently that one of them, betanin, has been isolated in a crystalline state from red beetroot.[2–4] Its aglycone, betanidin, has been shown to have structure I.[5,6] All the betacyanins studied until now have been shown to derive from betanidin or its diastereoisomer isobetanidin.[7,8] More recently a betaxanthin (indicaxanthin) has been isolated from *Opuntia ficus-indica* Mill. and has been shown to have structure II.[9] Two other betaxanthins isolated from *Beta vulgaris* L., were suggested to have structures III and IV.[10]

Dreiding[6,11] has suggested that betanidin itself might be formed from two molecules of β-(3,4-dihydroxyphenyl)alanine (DOPA), according to Scheme I. Hörhammer *et al.*[12] have in fact shown that DOPA is incorporated in the betanin occurring in the root and hypocotyl of *Beta vulgaris*, but since no degradation experiments were made, these observations are not sufficient to confirm Dreiding's hypothesis, since the radioactivity might be located solely in the 2,3-dihydro-5,6-dihydroxyindole moiety.

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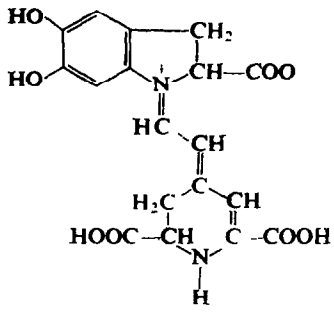
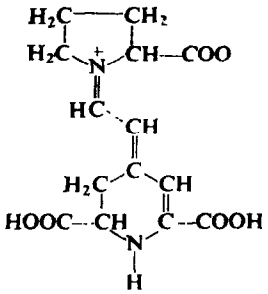


I



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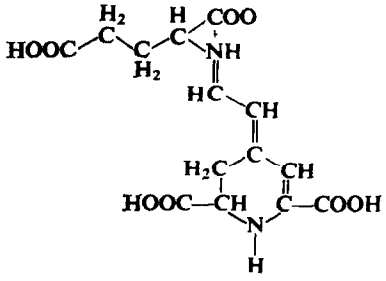

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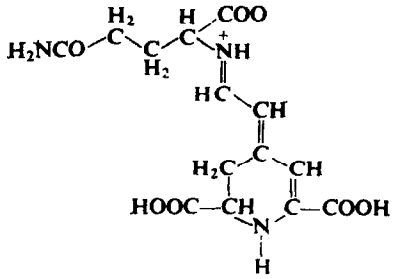
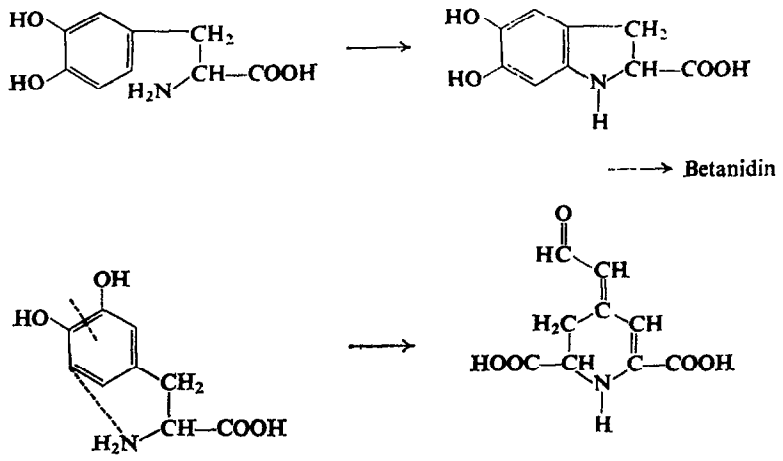


IV

This paper describes experiments on the incorporation of labelled precursors into the indicaxanthin and betanin of *Opuntia ficus-indica* fruits. Results obtained from the partial degradation of indicaxanthin formed from both proline and DOPA are also described.



SCHEME 1.

RESULTS AND DISCUSSION

Opuntia ficus-indica fruits were previously shown to contain two nitrogenous pigments (indicaxanthin and betanin),[8,9] the relative amounts of which determine the colour of the fruit, which may vary from yellow to violet.

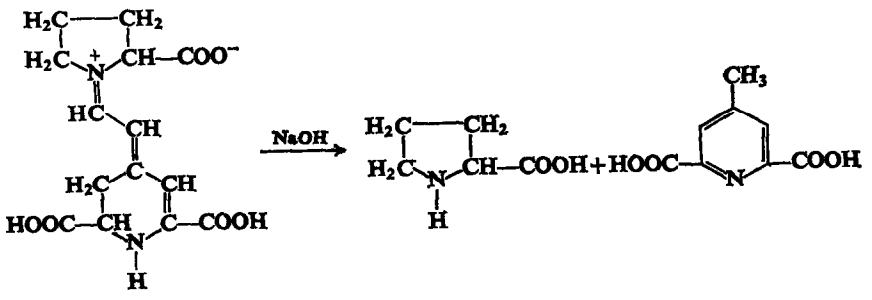
Indicaxanthin and betanin were isolated from *Opuntia ficus-indica* following administration of generally labelled L-proline. The distribution of radioactivity in these compounds, isolated at various times after administration of the amino acid, is shown in Table 1. The

TABLE 1. INCORPORATION OF L-PROLINE-^{14}C(U) INTO INDICAXANTHIN AND BETANIN BY *Opuntia ficus-indica* FRUIT

Amount administered (μC)	Incubation period (hr)	Compound isolated*	Amount of compound isolated (mg)	Specific activity (cpm/μM)	Incorporation (%)
2	8	I	0·56	2360	0·24
2	4	I	1·02	1860	0·30
		B	0·43	327	0·028
2	13	I	0·65	4440	0·43
		B	1·01	385	0·041
2	24	I	0·88	5400	0·75
		B	0·26	460	0·013
2	8	I	0·72	1400	0·18
		B	0·64	0	0
5	8	I	0·77	4420	0·25
		B	0·68	390	0·013
5	8	I	0·71	4200	0·22

* I = indicaxanthin; B = betanin.

formation of radioactive indicaxanthin after administration of labelled proline and the relatively high incorporation indicate that this amino acid is a good precursor of the betaxanthin. The specific activity (2140 cpm/μM) of the proline* obtained by degradation of the indicaxanthin according to Scheme 2, was 95% of the specific activity (2250 cpm/μM)



SCHEME 2.

* 4-Methylpyridine-2,6-dicarboxylic acid could not be obtained in pure condition, because of the small quantities obtained.

of the pigment, and it is clear therefore that proline is specifically incorporated into the proline moiety of indicaxanthin. The little activity which appeared in betanin is evidently due to randomization.

The data concerning the experiments of administration of DL-β-(3,4-dihydroxyphenyl)-alanine-2-^{14}C to *Opuntia ficus-indica* fruits are summarized in Table 2. The incorporation of radioactivity into both indicaxanthin and betanin suggests that the hydrogenated pyridine ring of the pigments may derive from DOPA according to Dreiding's hypothesis. The specificity of DOPA incorporation into the hydrogenated pyridine moiety of indicaxanthin was confirmed by the result obtained by alkaline fusion of a radioactive sample of the pigment formed from labelled DOPA. In this case the isolated proline contained only 2% of the activity present in indicaxanthin.

TABLE 2. INCORPORATION OF DL-β-(3,4-DIHYDROXYPHENYL)ALANINE-2-^{14}C INTO INDICAXANTHIN AND BETANIN BY *Opuntia ficus-indica* FRUIT

Amount administered (μC)	Incubation period (hr)	Compound isolated*	Amount of compound isolated (mg)	Specific activity (cpm/μM)	Incorporation (%)
5	8	I	0·88	9300	0·59
		B	0·55	21000	0·53
5	14	I	0·62	9300	0·41
		B	0·53	21500	0·53
5	24	I	0·50	15000	0·58
		B	0·36	57000	0·94
2	8	I	0·56	1560	0·16
		B	0·44	5600	0·26
5	8	I	0·73	2520	0·15
		B	0·73	15600	0·37
5	8	I	0·45	5920	0·22
		B	0·58	17700	0·44

* I = indicaxanthin; B = betanin.

EXPERIMENTAL

Materials

Opuntia ficus-indica fruits were purchased from the local markets. L-proline-^{14}C(U) (hydrochloride; specific activity, 49 mC/mM) and DL-β-3,4-dihydroxyphenyl)alanine-2-^{14}C (specific activity, 5·7 mC/mM) were obtained from Philips-Duphar, Amsterdam.

Administration of Labelled Precursors

Pulp of *Opuntia ficus-indica* fruits (ranging from yellow to red-orange) (10 g) was disintegrated in a Potter homogenizer. To the homogenate a solution of the radioactive compound in phosphate buffer (pH 6·8) was added and the resulting mixture was gently stirred at 35°. All the experiments were carried out under ordinary room illumination and aseptic conditions. The amount of radioactive material used in each experiment is indicated in Tables 1 and 2.

Measurement of Radioactivity

The compounds were counted as solid samples with a Tracerlab windowless flow counter (Model S.C. 16). The radioactive solutions were plated on aluminium planchets over a 2 cm^2 area. Planchets were dried on a warm plate at 50°. The plated materials were corrected for self-absorption by using an appropriate correction curve. Each solution was assayed in duplicate and at least 2000 counts above background recorded.

Isolation of Indicaxanthin and Betanin

When the incubation period was over (see Tables 1 and 2), methanol (30 ml) was added to the homogenate. The mixture was then filtered and the solid residue washed with 50% aq. methanol (10 ml). The combined filtrate and washing were adjusted to pH 3 with N HCl and the pigments were absorbed onto a column of Dowex 50W-X2 (H^+ form, 1 × 5 cm). After washing of the column with 0·1% HCl (50 ml), the pigments were eluted with water. The eluate was concentrated under reduced pressure at 30° (bath temp) to a volume of about 0·2 ml. Paper electrophoresis (Whatman 3MM paper in phosphate buffer 0·025 M, pH 6·8) of the concentrated solution resulted in the separation of indicaxanthin and betanin which were eluted with water from the corresponding bands. Upon desalting by resin treatment, the eluates were each made up to a known volume and the absorptions at 538 mμ (betanin, $E^{1\,cm}_{1\%} = 1120$) and 485 mμ (indicaxanthin, $E^{1\,cm}_{1\%} = 1355$) were determined. Aliquots of these solutions, containing about 200 μg of pigment, were used for ^{14}C counting.

Degradation of Indicaxanthin

Indicaxanthin (2 mg) obtained from experiments with labelled proline or DOPA was added to a 1 ml boiling solution of 70% NaOH (w/w) under nitrogen. Immediately after the addition of the pigment, the mixture was cooled and taken up in water (5 ml). The resulting solution was freed from alkali by passing it through a column of Amberlite IRC-50 (H^+ form) and the eluate was continuously extracted with ether. The ether extract, containing 4-methylpyridine-2,6-dicarboxylic acid in too small a quantity for satisfactory purification, was discarded. The remaining aqueous solution after concentration *in vacuo* to a small volume was streaked on Whatman no. 1 paper, which was developed in *n*-butanol: acetic acid: water (12:3:5). The band corresponding to a proline marker, revealed by spraying with 0·1% isatin in acetone, was cut out and eluted with water. The amino acid was further chromatographed on paper with ethanol: 33% ammonia: water (18:1:1) as developing solvent, and the purified proline zone was excised and eluted with water. The eluate was made to volume (50 ml) and the solution used for estimation of proline and radioactivity. Estimation of proline was carried out by paper chromatography (spray reagent: isatin 0·1% in acetone; the intensity of the spots was measured with the Beckman Analytrol apparatus).