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EFFECT OF EXOGENOUS DOPA AND TYROSINE ON AMARANTHIN SYNTHESIS AND PIGMENT TYPE IN *AMARANTHUS**

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Key Word Index—*Amaranthus caudatus*, Amaranthaceae; love-lies-bleeding; amaranthin, effects of DOPA and tyrosine, biosynthesis, betalains

Abstract—The effects of exogenously applied DOPA (L-3,4-dihydroxy-phenylalanine) and tyrosine on amaranthin synthesis under a variety of light conditions are described. A possible explanation for the light-sensitive period in development is suggested to be limitation of substrate in the less sensitive stages of development A yellow pigment tentatively identified as a betaxanthin becomes apparent under exogenous DOPA feeding.

INTRODUCTION

ALTHOUGH light stimulation of betalain synthesis has attracted increasing attention in recent years,[1–4] the biosynthetic pathway of the pigments remains obscure. Tyrosine and DOPA are now regarded as likely precursors[5,6] but the way in which they are incorporated into the pigments is still not known. It seems probable[7] that light acts at two (or more) points on the pathway, namely the conversion of tyrosine to DOPA and the incorporation of DOPA into amaranthin.

A previous analysis of the pigments in *Amaranthus caudatus*[8] reported that amaranthin and isoamaranthin are the only betalains present in this species. The results now presented indicate that a yellow pigment, probably a betaxanthin, is formed in the tissues under the influence of exogenous DOPA. Previous results[6,9] have shown that exogenous tyrosine and DOPA cause a stimulation of light induced amaranthin synthesis. The present work investigates in some detail the effects of DOPA and tyrosine with a view to understanding their roles as precursors of betacyanins.

[1] PIATTELLI, M., GUIDICI DE NICOLA, M and CASTROGIOVANNI, V. (1969) *Phytochemistry* **6,** 731
[2] KOEHLER, K (1972) *Phytochemistry* **11,** 127
[3] GUIDICI DE NICOLA, M, PIATTELLI, M, CASTROGIOVANNI, V and MOLINA, C (1972) *Phytochemistry* **11,** 1005
[4] GUIDICI DE NICLOA, M, PIATTELLI, M and AMICO, V (1973) *Phytochemistry* **12,** 353
[5] HOERHAMMER, L., WAGNER, H and FRITZCHE, W (1964) *Biochem Z* **339,** 398
[6] GARAY, A S and TOWERS, G H N (1966) *Canad J Bot* **44,** 231
[7] FRENCH, C J, PECKET, R C and SMITH, H (1974) *Phytochemistry* (in Press)
[8] PIATTELLI, M and MINALE, L (1964) *Phytochemistry* **3,** 547.
[9] KOEHLER, K (1965) *Naturwissenschaften* **52,** 561

* Part II in the series "Betalain synthesis in *Amaranthus caudatus* var *viridis*, for Part I, see ref 7

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RESULTS AND DISCUSSION

The stimulation of amaranthin synthesis by DOPA appeared to be consistent over a period of 48 hr in continuous light (Fig. 1). In addition the lag phase of amaranthin synthesis was reduced from 8–10 hr to approximately 4 hr in the presence of exogenous DOPA. This may imply that part of the lag phase is concerned with the production of endogenous DOPA. More pronounced effects of DOPA were observed when it was supplied to plants given a 4 hr period of white light followed by darkness (Fig. 2). Synthesis of amaranthin terminated at the same point whether or not exogenous DOPA was supplied. The comparison between synthesis of amaranthin in the dark and light under exogenous DOPA feeding indicates that light stimulates the mechanism of DOPA incorporation into amaranthin.

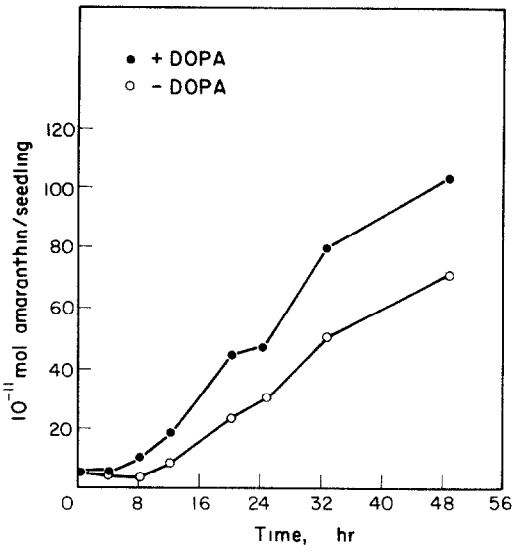

FIG. 1. EFFECT OF EXOGENOUS DOPA (5 mM) ON AMARANTHIN SYNTHESIS IN CONTINUOUS LIGHT. 24 hr dark grown material exposed to continuous light in the presence and absence of DOPA.

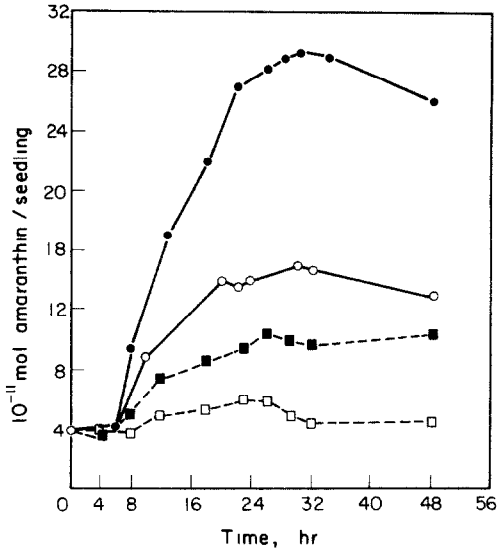

FIG. 2. EFFECT OF EXOGENOUS DOPA ON (a) SEEDLINGS GIVEN 4 hr LIGHT AND RETURNED TO DARKNESS (b) DARK GROWN SEEDLINGS. 24 hr dark grown material at T_0. Solid circles 4 hr light + DOPA, open circles 4 hr light minus DOPA. Solid squares dark + DOPA, open squares dark minus DOPA.

In order to determine whether DOPA had to be present at the onset of irradiation to cause a stimulation of synthesis, the time at which DOPA was applied was varied with respect to the light period, and the synthesis followed in the subsequent dark period (Fig. 3). The rates of synthesis and final pigment contents did not appear to vary according to the time at which DOPA was applied. This implies that the light effect on the DOPA incorporation mechanism is stable in the dark for at least 4 hr. The synthesis of amaranthin in the three treatments stopped at approximately the same time after the onset of irradiation.

The instability of DOPA in aqueous solution complicates its use in extended feeding experiments. It has been reported[6] that seedlings of *A. caudatus* var. Molten Fire turned black on exposure to exogenous DOPA and a similar blackening of seedlings was

observed in the present material. This blackening is due to the auto-oxidation of DOPA a reaction which is light stimulated. Consequently, the possibility arose that the amount of exogenous DOPA available to the plants after a period of feeding might vary according to the light treatment, which might contribute to the observed differences in response to

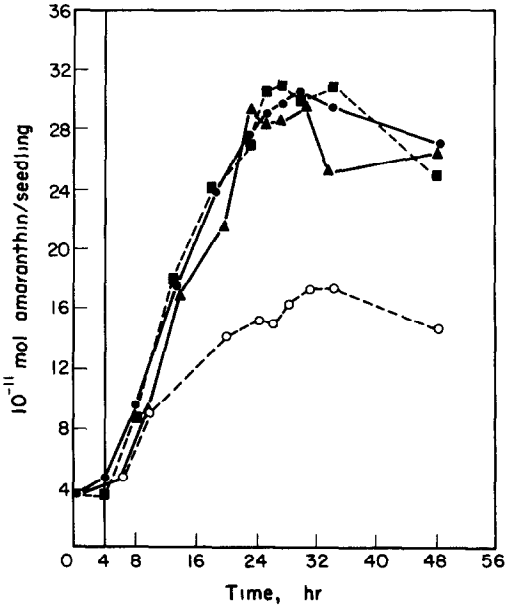

FIG 3 EFFECT OF FEEDING DOPA (5 mM) AT (a) T_0, (b) T_4, (c) T_8 ON DARK AMARANTHIN SYNTHESIS STIMULATED BY A 4 hr LIGHT PERIOD
24 hr dark grown material at T_0 Open circles untreated controls. Solid circles DOPA feeding from T_0, solid squares DOPA feeding from T_4, solid triangles DOPA feeding from T_8

DOPA. Accordingly the concentration of DOPA in the Petri dishes was determined at intervals under different light conditions (Table 1) After 24 hr the concentration of DOPA in all treatments was still sufficient to cause stimulation of synthesis.[7] As an additional

TABLE 1 EFFECT OF VARIOUS LIGHT TREATMENTS ON CONCENTRATION OF DOPA IN PETRI DISH

Hr	DOPA concn (mM) A	B	C
0	2 50	2 50	2 50
4	2 20	1 70	1 70
8	2 20	1 80	1 30
12	2 00	1 00	0·90
24	0 90	0 90	0 90

24 hr dark grown material transferred into a 5 mM soln at TO Light treatments started at same time A = continuous dark, B = continuous light, C = 4 hr light followed by darkness

TABLE 2 EFFECT OF ADDING EXTRA 5 mM EXOGENOUS DOPA AT T24 ON SUBSEQUENT AMARANTHIN SYNTHESIS

Hr	10^{-11} mol amaranthin/seedling +DOPA	−DOPA
24	28 20	28 00
30	32 20	34 00
36	37 00	34 00
48	36 00	32 00

24 hr material exposed to 4 hr white light and 5 mM DOPA feeding (continuously from the start of light period), returned to darkness for 24 hr One set of seedlings received a fresh supply of DOPA (+ DOPA), the other set were left in the original solution (−DOPA)

experiment, extra exogenous DOPA was supplied 4 hr before the cessation of synthesis to determine whether DOPA was the factor limiting synthesis 32 hr after the onset of irradiation (Table 2). A small stimulation of synthesis was observed but it appears that the concentration of exogenous (or endogenous) DOPA does not limit synthesis beyond this point. One interpretation of this finding is that decay in the dark of the light effect on DOPA incorporation is the limiting factor on synthesis.

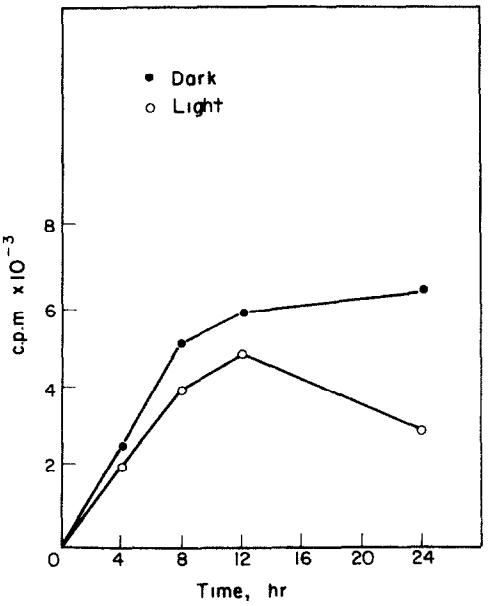

FIG 4 UPTAKE OF ^{14}C-DOPA UNDER CONDITIONS OF (a) CONTINUOUS LIGHT, (b) CONTINUOUS DARK
Seedlings were transferred into ^{14}C-DOPA at T_0 One set were exposed to continuous light (open circles), the other maintained in darkness (solid circles)

The results shown above could be modified by an effect of light on the uptake of DOPA; for example the uptake of phenylalanine has been shown to be light-stimulated.[10] In the present experiments radioactive ^{14}C-DOPA was supplied to the seedlings grown under different light conditions and the uptake into the tissue measured (Fig 4). More radioactive DOPA was taken up by dark-grown seedlings than those exposed to continuous light. The explanation for the differences in response to DOPA between light- and dark-grown material is therefore unlikely to be related to a limitation of DOPA uptake in the dark

A light-sensitive stage in development has been reported in several species of *Amaranthus*[6,9,11] In order to determine the effect of pigment precursor on the light-sensitive stage, DOPA was supplied to seedlings given a 4 hr light period at different stages of development and compared with seedlings untreated with DOPA (Fig. 5). The consequence of the DOPA treatment was that maximum light sensitivity was increased to 24–48 hr compared with the untreated seedlings' 24–32 hr The reduced light sensitivity in the period 32–48 hr

[10] HARPER, D B, AUSTIN, D J and SMITH, H (1970) *Phytochemistry* **9**, 497
[11] GIMISI N GARAY A, POZSAR, B and FOKAS, G (1951) *Agrokemia es Talajtan* **1**, 339

therefore appears to be due to a limitation of DOPA since the effect of light on the incorporation of DOPA is apparently undiminished between 32–48 hr. Feeding DOPA to 16 and 64 hr material produced some stimulation of synthesis but in these seedlings there is clearly some additional limiting factor(s) since the pigment level remained below that observed with 24–48 hr seedlings. In 16 hr material treated with DOPA there was little difference between those exposed to light and those maintained in darkness. This indicates that the effect of light on DOPA incorporation is low at this developmental stage.

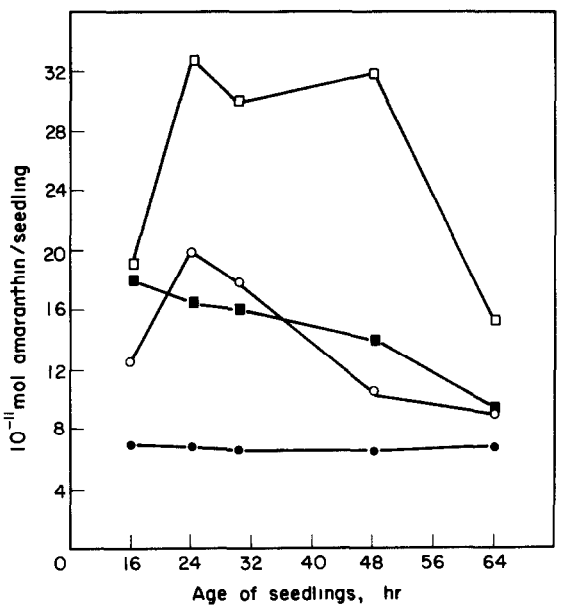

FIG 5. EFFECT ON AMARANTHIN SYNTHESIS OF FEEDING 5 mM DOPA TO SEEDLINGS OF DIFFERENT AGES EITHER (a) MAINTAINED IN DARKNESS OR (b) EXPOSED TO 4 hr LIGHT AND RETURNED TO DARKNESS
Open circles: material given 4 hr light at the appropriate age and returned to darkness for a further 44 hr. Open squares as above but fed DOPA continuously from the start of the light period. Solid squares material fed DOPA in the dark, solid circles dark controls in absence of DOPA.

In general the effects of exogenous tyrosine were similar (Fig. 6) to those observed with DOPA with the exception that there was little effect on dark-grown seedlings.[7] A very similar effect on the light-sensitive stage in development was observed indicating that limited conversion of tyrosine to DOPA is unlikely to result in the apparent substrate deficiency, reversible with exogenous DOPA (Fig. 5). Measurements of the uptake of ^{14}C-tyrosine in dark-grown material gave no indication that low tyrosine uptake was responsible for its small effects in the dark compared with DOPA. .

In the course of these experiments, a change in the colour of the extracted pigments from purple to orange was observed. In addition, the seedlings had orange/red hypocotyls compared with the typical red–purple coloration of the controls. An absorption spectrum of the crude extract revealed a second peak at approximately 480 nm in addition to that of

amaranthin (547 nm) The peak of absorption corresponded to those of betaxanthins isolated from other members of the Centrospermae, e.g. of indicaxanthin[12] (487 nm) and a betaxanthin from *Pleiosphos dekenahi*[13] (478 nm). The second pigment was separated from amaranthin by ion exchange chromatography and was yellow with an absorption maximum at 475–480 nm. A comparison of the spectrum with those of known betaxanthins revealed similarities in maxima but differences in the absorption in the blue region. One of the diagnostic features of betalains is their anionic electrophoretic mobility at low pH's[8] The R_f of the pigment in low pH buffer was found to be 0 35 with respect to amaranthin Attempts to hydrolyse the molecule and identify the products failed due to the small amounts available However, the colour of the pigment, its anionic electrophoretic mobility at low pH, water solubility and absorption spectrum strongly suggest that the pigment is a betaxanthin not previously reported

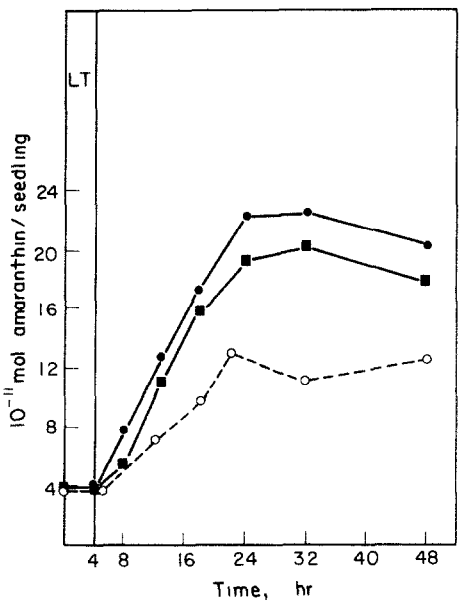

FIG 6 EFFECT OF FEEDING TYROSINE (5 mM) AT (a) T_0 OR (b) T_4 ON DARK AMARANTHIN SYNTHESIS STIMULATED BY A 4 hr LIGHT TREATMENT
24 hr material at T_0 Solid circles tyrosine feeding from T_0, solid squares tyrosine feeding from T_4 Open circles untreated controls

No obvious stimulation of yellow pigment synthesis was observed in the experiments involving exogenous tyrosine. This may imply that the conditions favouring the synthesis of the yellow pigment include a large pool of available DOPA Presumably the regulation of the conversion of tyrosine to DOPA prevents this occurring during the application of exogenous tyrosine

[12] PIATTELLI, M , MINALE, L and PROTA, G (1964) *Tetrahedron* **20**, 2325
[13] REZNIK, H (1957) *Planta* **49**, 406

EXPERIMENTAL

Plant material, light sources, estimation of amaranthin and transfer of plant material into cold DOPA and tyrosine was as described previously [7]

Radioactivity was estimated in a Packard Tricarb Liquid Scintillation Spectrometer Composition of scintillant was as follows 1 l toluene, 500 ml Triton X-100 (octylphenoxypolyethoxy ethanol), 5 g PPO (2,5-diphenyloxazole) A 1 ml sample was added to 9 ml of scintillant Amaranthin was found to have negligible quenching effect in the quantities found in the samples

Uptake of ^{14}C-DOPA and ^{14}C-tyrosine plant material was transferred into 1 ml of distilled H_2O containing 2 μCi of DL-3 (3,4-dihydroxy phenylalanine)-1-C14 (sp act 52 μCi/mmol) or DL-tyrosine-2-C14 (sp. act 45·4 μCi/mmol). At suitable intervals 50 seedlings were removed and after washing homogenized in 5 ml of distilled H_2O One ml aliquots were used for estimation of radioactivity

Paper electrophoresis was carried out using a vertical apparatus The sample was spot loaded on to Whatman No. 1 paper and run at a potential of 15 V/cm for a period of 2 hr in formic acid buffer 0·1 M pH 2·4 (as used previously[7])

Estimation of DOPA employed the method of Arnow [15] One ml of sample was added to 1 ml of 0·5 N HCl To this mixture 1 ml of nitrite/molybdate reagent (10 g sodium nitrite + 10 g sodium molybdate in 100 ml) was added One ml of 1 N NaOH was added and the total volume made up to 5 ml Absorption maximum was 500 nm and calibration showed a linear relationship between OD and DOPA concentration over the range 0·05–0·5 μmol DOPA

Separation of yellow pigment and amaranthin was achieved using a modification of the procedure of Lewis[14] for fractionation of amino acids on ion exchange resin. A crude extract of the pigments was centrifuged at 18000 *g* for 10 min and the supernatant adjusted to pH 3 with 1 N HCl and recentrifuged at 18000 *g* for 10 min The supernatant was adjusted to pH 2·35 with 1 N citrate and added to a 5 × 1 cm column of Dowex 50 W H^+ from resin (50 × 8–400; 8%; 200–400) The column was washed with 20 ml of 2N NaOH. The following buffers were used in the separation Buffer I pH 3·40 (1 l.) citrate 16·0 g, NaOH 2·60 g, NaCl 4·10 g Buffer II pH 4·80 (1 l) citrate 6·90 g, NaOH 2·60 g, NaCl 5·0 g Twenty ml of Buffer I was run through the column and the pigment extract added Under the above conditions the yellow pigment was adsorbed on to the column whilst amaranthin passed freely through The column was washed with 20 ml of Buffer I and the yellow pigment subsequently eluted with 10 ml of Buffer II

Acknowledgement—C F thanks the University of Manchester for a postgraduate award for the period of research

[14] LEWIS, D K. (1963) Ph D Thesis, University of Bristol
[15] ARNOW, L E (1937) *J Biol Chem* **118,** 531