Evidence Against an Involvement of Cyclic Nucleotides in the Induction of Betacyanin Synthesis by Cytokinins

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1. A wide range of purine bases, nucleosides and cyclic nucleotides were shown to induce betacyanin synthesis in *Amaranthus* seedlings. 2. The induction of pigment by benzyladenine, dibutyl cyclic AMP or cyclic AMP was not potentiated by aminophylline. Aminophylline was shown to inhibit *Amaranthus* cyclic AMP phosphodiesterase activity. 4. Incubation of seedlings with aminophylline inhibited the conversion of 6-[G-³H]benzyladenine into presumed 9- and 7-glucosylbenzyladenine. 5. Induction of betacyanin synthesis by 6-benzyladenine or by exposure to red light was not accompanied by changes in the total cyclic AMP content in seedlings. 6. It is concluded that the inducers tested act as cytokinin analogues; no evidence was obtained to support cyclic AMP as an intermediate in the induction process.

Wood & Braun (1973) have demonstrated that exogenously supplied 8-bromo cyclic AMP can completely replace cytokinins as a cell-division-promoting factor in excised tobacco-pith parenchyma tissue. In the present paper we have investigated whether cyclic nucleotides will replace the requirement for cytokinins in another bioassay system for cytokinins, the induction of betacyanin synthesis in *Amaranthus* seedlings. Our results suggest that, although cyclic adenine nucleotides promote the pigment formation, they act as cytokinin analogues rather than as cyclic nucleotides. Further, no significant increase in cyclic AMP content was found in the seedlings under conditions where induction was taking place. In view of these results, it cannot be assumed from the work of Wood & Braun (1973) that cytokinin effects are mediated by cyclic AMP. Rast *et al.* (1973) have claimed that cyclic AMP will replace phytochrome in betacyanin induction. We suggest that their results obtained with dibutyl cyclic AMP are due to the cytokinin activity of the butyl group in the N-6 position of the adenine ring. A preliminary account of these findings has been given (Elliott, 1974).

Materials and Methods

Betacyanin assay

The experimental procedure was based on methods developed by Kohler & Conrad (1966) and Bigot (1968). Seeds of *Amaranthus tricolor* var. Flying Colours (Yates and Co., Sydney, Australia) were germinated in the dark at 37°C on moist plastic foam in Petri dishes. After 46h batches were transferred to

filter-paper discs in 7cm Petri dishes containing 5ml of 10mM-Na₂HPO₄-KH₂PO₄ buffer, pH6.3, to which 0.1% L-tyrosine had been added. After a further 24h in the dark at 25°C the seedlings were harvested in dim green light supplied by a Sylvana Gro-Lux green fluorescent tube (G.T.E. Australia Pty. Ltd., Adelaide, Australia), 40W, covered with two layers of green cellophan and suspended 40cm above the seedlings. Batches of 40 seedlings were added to 2.9ml of water and then frozen in liquid N₂. Subsequent homogenization in a glass-Teflon homogenizer was carried out in the light, 0.1ml of 0.1M-acetic acid added and cell debris removed by centrifugation at 38000g for 20min. Amaranthin was determined spectrophotometrically at 537nm by using a molar extinction coefficient (ϵ) of 5.66×10^4 (Piatelli *et al.*, 1969).

Cyclic AMP phosphodiesterase assay

Amaranthus seedlings germinated in the dark at 37°C for 46h were homogenized in the cold in a glass-Teflon homogenizer with 3vol. of 50mM-Tris-HCl buffer (pH7.5). The extract was squeezed through two layers of muslin and centrifuged at 14000g for 15min. The standard assay contained 4 μ mol of Tris-HCl (pH8.0), 0.5 μ mol of MgCl₂, 0.3 μ mol of β -mercaptoethanol, either 0.25 or 50nmol of cyclic [8-³H]AMP (specific radioactivities 43 and 0.22mCi/mmol respectively) and 50 μ l of plant extract (14000g supernatant) in a total volume of 100 μ l. After incubation for 15 (low cyclic AMP) or 30min (high cyclic AMP) at 30°C (cyclic AMP hydrolysis being linear over these times) the reaction was stopped by incubating for 3min in a boiling-water bath. Assays

were then incubated for a further 10 min at 30°C with 10 μ l of snake venom 5'-nucleotidase (Sigma Chemical Co., St. Louis, Mo., U.S.A.; *Crotalus adamanteus*; 1 mg/ml). Portions (50 μ l) were chromatographed on Whatman 3MM paper in 1M-ammonium acetate-95% (v/v) ethanol (3:7, v/v). Radioactivity associated with adenosine markers was measured after elution of the paper in 0.1M-EDTA (3 $\times$ 3 ml) and freeze-drying. Controls were stopped at zero time. Proteins were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Cyclic AMP determinations

Seedlings were blotted, frozen in liquid N₂ and homogenized in 20 vol. of 5% (w/v) trichloroacetic acid. The trichloroacetic acid extract was made 0.1 M with respect to HCl and extracted three times with 2 vol. of water-saturated diethyl ether. The samples were fractionated on Dowex 50 (H⁺ form) columns as described by Krishna *et al.* (1968) and cyclic AMP was assayed by the binding-protein method of Gilman (1970). DNA in the acid-insoluble material was measured by the method of Burton (1956), after washing the pellet twice with 2 vol. of ethanol-ether (1:1, v/v).

Uptake and metabolism of labelled 6-benzyladenine

Seedlings were grown as described above, the radioactive compound being added to the phosphate buffer-tyrosine solution and present for the whole of the induction period. 6-[G-³H]Benzyladenine was prepared at The Radiochemical Centre (Amersham, Bucks., U.K.) by catalytic exchange in an aqueous medium. It was purified as previously described (Elliott & Murray, 1972).

Aqueous extracts of gently blotted seedlings, after measurement of betacyanin, were evaporated to dryness and chromatographed on Whatman 3MM paper. The residue from aqueous extraction was further extracted with 5 ml of boiling ethanol and similarly analysed chromatographically. Solvents used were either butan-1-ol-acetic acid-water (20:3:7, by vol.) or butan-1-ol-conc. NH₃ (sp.gr. 0.88)-water (85:5:9, by vol.). Benzyladenine and benzyladenosine were identified by co-chromatography with authentic samples. Glucosylbenzyladenines were identified by *R_F* values reported in the literature (Dyson *et al.*, 1972; Deleuze *et al.*, 1972; Parker *et al.*, 1973). Extracts were counted for radioactivity in Bray's scintillation solution (1960) with an efficiency of 35%. Radioactivity associated with segments cut from the chromatograms was counted directly in toluene scintillator [5 g of 2,5-diphenyl-oxazole and 0.5 g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/litre of toluene] with an efficiency of 8.5%.

In the results reported in Table 4, the majority of the radioactivity in the aqueous extract was in the area *R_F* 0.45-0.50 [9-glucosylbenzyladenine (Parker *et al.*, 1973); 7-glucosylbenzyladenine (Parker *et al.*, 1973); however, see Dyson *et al.* (1972), where a much lower *R_F* value is given for the 7-glucosyl derivative]. On elution and rechromatography in butan-1-ol-acetic acid-water (20:3:7, by vol.) the radioactive material had *R_F* 0.55-0.58 [7-glucosylbenzyladenine (Dyson *et al.*, 1972)]. The material eluted from this chromatogram showed spectral characteristics which indicated the presence of both 9- and 7-glucosylbenzyladenine (Deleuze *et al.*, 1972; Parker *et al.*, 1973).

Results

The results in Table 1 have been compiled from a number of experiments and are expressed as percentages of the maximum induction found with 5 μ M-benzyladenine. Since variations in seed batches were found, controls with benzyladenine were always included.

Table 1. Induction of betacyanin synthesis in *Amaranthus* seedlings

Betacyanin production was assayed as described in the Materials and Methods section and is related to the amount produced in the presence of 5 μ M-benzyladenine, taken as 100%. Values for amaranthin concentration ranged from 0.4 to 1.0 nmol/seedling. Controls with buffer-tyrosine solution alone (ranging from 0.02 to 0.1 nmol/seedling) were subtracted in each case. All treatments were assayed in triplicate.

Inducing compound	Concentration	Induction (%)
Benzyladenine	0.05 μ M	5
	0.5 μ M	46
	1.0 μ M	63
	5.0 μ M	100
	0.01 mM	81
Dibutyl cyclic AMP	0.01 mM	7
	0.1 mM	41
	1.0 mM	89
Sodium butyrate	0.1 mM	2
	1.0 mM	-2
Cyclic AMP	0.1 mM	19
	1.0 mM	21
Adenine	1.0 mM	9
Adenosine	0.1 mM	13
	1.0 mM	18
8-Bromo cyclic AMP	0.01 mM	23
8-Bromoadenine	0.01 mM	25
8-Bromoadenosine	0.01 mM	22
Cyclic GMP	1.0 mM	20
Guanosine	1.0 mM	17
6-Methylmercaptapurine	0.1 mM	10
	1.0 mM	26
Aminophylline	0.5 mM	21
	5.0 mM	8

It is clear that a wide range of purine bases, nucleosides and cyclic nucleotides are capable of inducing betacyanin synthesis. Although 8-bromo cyclic AMP and, to a lesser extent, cyclic AMP promote betacyanin development they are no more active than a range of purine and purine riboside derivatives. 6-*N*,2'-*O*-Dibutyryl cyclic AMP was an excellent inducer. This compound is known to break down to cyclic AMP, 6-*N*-butyryl cyclic AMP and sodium butyrate (Swislocki, 1970); sodium butyrate had no inducing activity. It is likely that the activity of dibutyryl cyclic AMP is due to the butyryl group in the N-6 position of the adenine ring, since *N*⁶-butyryladenine is active in soya-bean callus (Dekhuijzen & Overeem, 1972), tobacco callus (Martin *et al.*, 1973) and cabbage leaf disc (Berridge *et al.*, 1970) bioassays for cytokinins. Berridge *et al.* (1970) found *N*⁶-butyryladenine to be as active as *N*⁶-benzyladenine in the leaf-disc assay, and at 23 μ M dibutyryl cyclic AMP had an effect comparable with *N*⁶-benzyladenine or kinetin. They considered the activity of dibutyryl cyclic AMP could be due to *N*⁶-butyryladenine produced by degradation *in vivo*. Dekhuijzen & Overeem (1972) found *N*⁶-butyryladenine and dibutyryl cyclic AMP to be equally active in soya-bean callus (optimum concentration 50 μ M), this being 50 times the optimum concentration for kinetin (1 μ M). They concluded that the cytokinin activity of dibutyryl cyclic AMP apparently does not depend on the ribosyl 3':5'-cyclic monophosphate moiety of the molecule but is determined by the *N*⁶-butyryl side chain.

Effects of aminophylline

Aminophylline itself had some inducing activity (Table 1), and this type of result is normally interpreted as resulting from an inhibition of cyclic AMP phosphodiesterase leading to increased cyclic AMP concentrations (see Wood *et al.*, 1972). Although aminophylline was found to inhibit the phosphodiesterase present in the seedlings, especially at low concentrations of substrate (Table 2), it was not effective in potentiating the action of a number of inducers of pigment formation tested at suboptimal concentrations (Table 3). In fact combination of inducers with aminophylline resulted in less induction than would be expected even on a simple additive basis. These results do not agree with those of Rast *et al.* (1973), who found a potentiating effect of theophylline on induction by dibutyryl cyclic AMP. The induction period in the latter case was 84h, compared with 24h in the present experiments.

The addition of aminophylline to seedlings grown on 6-[G-³H]benzyladenine had a dramatic effect on the metabolism of the cytokinin (Table 4). Although aminophylline caused about a 50% inhibition of the total uptake of label over the 24h inducing period, radioactivity associated with benzyladenine plus

Table 2. *Inhibition of Amaranthus phosphodiesterase by aminophylline*

The enzyme assay is described in the Materials and Methods section. The specific activity of the enzyme in the 14000g supernatant was 3.68 (with 2.5 μ M-cyclic AMP) and 41.0 (with 0.5 mM-cyclic AMP) pmol hydrolysed/min per mg of protein. Values are the mean of duplicate assays.

Cyclic AMP concn.	Aminophylline concn. ...	Inhibition (%)	
		0.5 mM	5 mM
2.5 μ M		32	74
0.5 mM		5	16

benzyladenosine was only decreased by 25%. However, the amount of label associated with the metabolites presumed to be 9- and 7-glucosylbenzyladenine was decreased by 86%. This result was confirmed qualitatively in a separate experiment with a lower concentration of benzyladenine and raises the possibility that the formation of these metabolites is intimately connected with the mode of action of benzyladenine. These results stress the complicated and multiple effects obtained with aminophylline in this system and preclude its use as a simple inhibitor of phosphodiesterase activity.

Content of cyclic AMP in *Amaranthus* seedlings

A direct analysis of the cyclic AMP content under conditions where betacyanin induction was taking place showed that there was no significant difference in the endogenous cyclic AMP (Table 5). The results also show that incubation with aminophylline does not increase cyclic AMP content of seedlings. The validity of the cyclic AMP assay was confirmed by recovery of authentic cyclic AMP added to *Amaranthus* extracts and by digestion of extracts with cyclic AMP phosphodiesterase (Table 6). Cytokinin [*N*⁶-(Δ^2 -isopentenyl)adenosine] in concentrations from 50 nM to 5 μ M did not compete with cyclic AMP in the assay.

Discussion

There is some indirect and rather diffuse evidence indicating a connexion between phytochrome-mediated responses and cyclic AMP, which might support a role for cyclic AMP (as distinct from dibutyryl cyclic AMP) in betacyanin synthesis. The light-requiring synthesis of betacyanins in *Amaranthus* seedlings (Bamberger & Mayer, 1960) has been shown to be phytochrome-controlled (Piatelli *et al.*, 1969). Kinetin will completely replace the light requirement (Bamberger & Mayer, 1960). Cytokinins can replace light or decrease the requirement for light in a number

Table 3. *Effect of aminophylline on betacyanin induction by other compounds*

See Table 1 for experimental details.

Inducing compound	Concn.	Aminophylline concn.	...	Induction (%)		
				0	0.5 mM	5 mM
Benzyladenine	0.5 μ M			46	36	24
Dibutyl cyclic AMP	0.1 mM			41	58	29
Cyclic AMP	1.0 mM			21	37	19
Aminophylline				0	21	8

Table 4. *Effect of aminophylline on the uptake and metabolism of 6-[G-³H]benzyladenine supplied to Amaranthus seedlings*

See the Materials and Methods section for experimental details. Seedlings were incubated with 5 μ M-6-[G-³H]benzyladenine (specific radioactivity 22.8 mCi/mmol). Chromatograms for this experiment were run in butan-1-ol-conc. NH₃-water (85:5:9, by vol.) and the values given are for the combined aqueous plus ethanolic extracts. Results are the mean of duplicate experiments.

Area of chromatograms	10 ⁻⁴ × Radioactivity (c.p.m./g fresh wt.)		
	Control	+5 mM-Aminophylline	Inhibition (%)
Total	5.130	2.465	52
Eluate at R _F 0.45–0.50	1.882	0.265	86
Benzyladenine plus benzyladenosine (R _F 0.82; 0.72)	2.535	1.898	25

Table 5. *Effect of various betacyanin-inducing treatments on cyclic AMP content of Amaranthus seedlings*

The source of red light was a Philips 40 W red fluorescent tube (TL 15) suspended 50 cm from the seedlings. Otherwise experimental details are described in the Materials and Methods section. Betacyanin production was observed in the 6 h extracts after red light or benzyladenine treatment. Values are means $\pm$ S.E.M.

Treatment	Time	Cyclic AMP (fmol/ μ g of DNA)	
		Control	Treatment
Red light	10 min	91 $\pm$ 17	77 $\pm$ 16
	2 h	70 $\pm$ 18	90 $\pm$ 7
	4 h	85 $\pm$ 17	50 $\pm$ 4
	6 h	93 $\pm$ 9	148 $\pm$ 38
	8 h	80 $\pm$ 20	50 $\pm$ 10
5 μ M-Benzyladenine	1 h	95 $\pm$ 23	81 $\pm$ 15
	2 h	65 $\pm$ 20	78 $\pm$ 15
	4 h	79 $\pm$ 10	113 $\pm$ 14
	6 h	120 $\pm$ 17	113 $\pm$ 18
0.5 mM-Aminophylline	8 h	144 $\pm$ 24	82 $\pm$ 9
	3 h	61 $\pm$ 13	56 $\pm$ 11
	6 h	51 $\pm$ 7	46 $\pm$ 6
5.0 mM-Aminophylline	3 h	61 $\pm$ 13	46 $\pm$ 3
	6 h	51 $\pm$ 7	52 $\pm$ 9

Tesuka, 1973). In at least one of the physiological responses where cyclic AMP has been shown to have an effect [germination of lettuce seed (Kamisaka & Masuda, 1971; Hall & Galsky, 1973)], a similar effect can be achieved with red light (Khan, 1960). The action of light on frog retinal-rod outer segments results in changes in sodium conductance and membrane potential, and this has been shown to be mediated by cyclic AMP (Bitensky *et al.*, 1971). These changes are reminiscent of the action of red light on phytochrome, which rapidly leads to alterations in membrane permeability and in polarization of membranes (see Briggs & Rice, 1972).

The above considerations therefore make it necessary to ask whether the promotion of betacyanin synthesis by cyclic AMP indicates an involvement similar to that found in lettuce seeds. The fact that adenosine and adenine have no effect in lettuce-seed germination (Hall & Galsky, 1973), whereas they do induce pigment formation in *Amaranthus*, makes this interpretation unlikely.

Wood & Braun (1973) considered that the activity of 8-bromo-5'-AMP in tobacco pith could be explained by it serving as a phosphodiesterase-resistant precursor for 8-bromo cyclic AMP, which is known to be more resistant to cyclic AMP phosphodiesterases than is cyclic AMP. It seems unlikely that conversion into cyclic AMP could explain pigment induction by the wide range of purines tested in the present experiments. This is particularly so with the unsubstituted purines, unless they enter a

of other biological systems (Miller, 1956, 1958; Hillman, 1957; Skinner *et al.*, 1956; Scott & Liverman, 1956), and have been shown to mediate a cell-free reaction involving phytochrome (Yamamoto &

Table 6. *Validity of cyclic AMP assay*

Assay conditions are described in the Materials and Methods section.

	Amount of cyclic AMP (pmol)		
	Expected	Observed	Amount after phosphodiesterase
Standard	3.5	3.6	0
<i>Amaranthus</i> extract	—	0.34	0.04
<i>Amaranthus</i> extract+0.5 pmol of cyclic AMP	0.84	1.05	—

compartment specifically utilized for the synthesis of cyclic AMP. In addition conversion of 6-methylthioinosine into cyclic AMP is unlikely. It remains possible that all of these compounds raise intracellular cyclic AMP concentration indirectly by interacting with cyclic AMP synthesis or breakdown. The results with aminophylline do not support this possibility, and direct measurements of cyclic AMP content of seedlings provide no evidence that the responses to cytokinin and red light are mediated via this nucleotide.

The present results do not support the concept that cytokinin effects are mediated via cyclic AMP. The results can be explained most simply by assuming that the purine derivatives act as cytokinin analogues, and that the bromo group in the 8-position enhances this activity.

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