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INFLUENCE OF LIGHT ON CYCLIC NUCLEOTIDE METABOLISM IN PLANTS; EFFECT OF DIBUTYRYL CYCLIC NUCLEOTIDES ON CHLOROPLAST COMPONENTS

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Key Word Index—*Phaseolus vulgaris*, Leguminosae, *Spinacea oleracea*; Chenopodiaceae; adenosine 3′,5′-cyclic monophosphate, cyclic AMP, guanosine 3′,5′-cyclic monophosphate; cyclic GMP, cyclic nucleotides; terpenoids, phosphodiesterase; nucleotide cyclase; chloroplasts; light

Abstract—Dark-grown *Phaseolus* seedlings exposed to light for 18 hr contain more than × 3 the concentration of cyclic AMP in controls kept in an 18 hr light/6 hr dark cycle. Chloroplast cyclic AMP concentrations behaved similarly. Cyclic AMP phosphodiesterase activity of spinach chloroplasts showed a light-dependent response. The phosphodiesterase activity of chloroplasts from light-grown and dark-grown spinach seedlings exhibit similar V_{max} but different K_m values The K_m of the enzyme from light-exposed plants was more than × 30 that of the corresponding activity from dark-treated plants. The two enzymes showed different responses to the combined addition of Ca^{2+} and calmodulin; a × 3 stimulation of the enzymic activity from light-treated plants was observed. The light-effects were not directly related to general photosynthetic effects, reflected in protein and chlorophyll concentrations. Light regimes also markedly modified the effects of dibutyryl cyclic AMP and dibutyryl cyclic GMP on terpenoid concentrations. Dibutyryl cyclic AMP doubled the α-tocopherol content and reduced by 40% the γ-tocopherol and chlorophyll concentrations of chloroplasts from light-grown plants; simultaneously their ubiquinone content increased by 48%. The main effect on dark-treated plants was that of dibutyryl cyclic AMP which gave a 107% increase in ubiquinone concentration. The main response to dibutyryl cyclic GMP was seen in dark-grown plants with substantial increases in the concentration of α-tocopherol, γ-tocopherol, and chlorophyll Plants grown in darkness and then given 15 min illumination showed an 83% increase in α-tocopherol concentration and a 168% increase in ubiquinone concentration following treatment with dibutyryl cyclic GMP. Under these conditions the cyclic AMP analogue increased chlorophyll and ubiquinone concentrations by 57 and 78%, respectively.

INTRODUCTION

It is established that adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) play key roles in the regulation of mammalian [1, 2], bacterial [3, 4], and lower plant metabolism [5]. The presence in higher plants of both cyclic AMP [6] and cyclic GMP [7, 8] has also been unambigously demonstrated by mass spectrometry and there are an increasing number of indications that they are involved in the control of higher plant metabolism. As yet however, no precise function has been demonstrated for them [9, 10]. Attempts to investigate the role of cyclic AMP in plants have frequently been based upon the known function of this compound in mammals as a hormone mediator, and have therefore involved studies of possible relationships between phytohormones and cyclic AMP. While the function of cAMP as a mammalian second messenger is well established, it is only comparatively recently that the functions of cyclic GMP have been elucidated Cyclic GMP has been shown to be a component of the mammalian visual cycle [11] and an intermediary in the effect of atrial natiuretic factor on smooth muscle [12]. The earlier suggestion that many of the actions of cyclic GMP are antagonistic to the actions of cyclic AMP, described as the 'Yin-Yang' hypothesis [13], seems to be largely unsubstantiated.

As has been discussed previously [10], most of the hormone effects mediated by cyclic nucleotides in animal tissues are short-term, sudden effects. It is therefore reasonable to infer that this is the type of rapid physiological response in which the role of cyclic nucleotides may reside in plants. Consequently, demonstration of the involvement of cyclic GMP in a rapid, light response mechanism in animals may be of relevance to the plant problem. A role for cyclic AMP in connexion with phytochrome activity has previously been suggested [14, 15] but others have argued that the cyclic nucleotide exerted its effect merely by acting as a cytokinin analogue [16, 17]. Although other evidence for an interaction between cyclic nucleotides and light has been reported, e.g. the concentration of cyclic AMP in *Avena* etioplasts greatly increased following exposure to light [18], no relationship has been elaborated. To examine this possibility further, the present paper describes and considers the effects of different light/dark regimes on the concentrations of cyclic AMP and cyclic GMP, and on the

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respective cyclase and phosphodiesterase activities Also analysed are the effects of cyclic nucleotide derivatives on the terpenoid composition of higher plant plastids.

RESULTS AND DISCUSSION

During our earlier studies on the biochemistry of cyclic nucleotides in the tissues of higher plants, we observed [10] that etiolated seedlings of *Phaseolus vulgaris* consistently had substantially lower concentrations of adenosine 3',5'-cyclic monophosphate (cyclic AMP) than their light-grown counterparts (Table 1). However, as Table 1 shows, dark-grown seedlings, extracted immediately after an 18 hr period of constant illumination, contained more than $\times 3$ the concentration of cyclic AMP in control seedlings grown in an 18 hr light/6 hr dark cycle Examination of the chloroplast cyclic AMP concentrations (Table 1) revealed similar trends

Subsequent investigations of the cyclic AMP phosphodiesterase activity of spinach chloroplasts also indicated a light-dependent response (Table 2) Whereas the phosphodiesterase activity extracted from chloroplasts of spinach seedlings kept in the dark for 18 hr showed a similar V_{max} to that of the corresponding activity obtained from spinach seedlings kept under constant illumination for 18 hr, their respective K_m values were significantly different. The K_m of the enzyme from the light-treated plants was more than $\times 30$ that of the corresponding activity extracted from the dark-treated plants A further difference was in the *in vitro* response of the two enzymic activities to the combined addition of Ca^{2+} and calmodulin. Simultaneous addition of these two factors effected a three-fold stimulation of the activity extracted from light-treated seedlings whereas in a parallel experiment the enzymic activity extracted from the dark-treated plants was not significantly affected

Studies with the chloroplasts of light-grown and dark-grown seedlings of *Phaseolus vulgaris* showed (Table 3) that light affected the total enzymic activities and the concentrations of a number of components of cyclic nucleotide systems in chloroplasts and that these responses were not directly related to generalized photosynthetic effects on total protein or chlorophyll content For example, in chloroplasts of dark grown plants, whereas the protein content fell to half the control value, and the chlorophyll content decreased to a similar extent, there was a concomitant three-fold increase in adenylate cyclase activity and a smaller but significant increase in that of cyclic AMP phosphodiesterase activity. On the other hand, the same dark-grown conditions produced an almost complete depletion of chloroplast cyclic GMP phosphodiesterase and guanylate cyclase activities, with the total chloroplast cyclic GMP concentration falling by 50% With all the chloroplast parameters examined, exposure of the plants to 1 hr of light immediately following the 12 hr dark period caused a reversal of the trends with, in most cases, a considerable overshoot For the chloroplast enzymes investigated, this overshoot had

Table 1 Cyclic AMP concentrations in shoots and isolated chloroplasts from light-grown and dark-grown seedlings of *Phaseolus vulgaris*

Material	Growth conditions*	Cyclic AMP (pmol/mg dry wt)	%
Shoot	light	4.6	100
Shoot	dark	0 6	13
Shoot	dark/18 hr light	15 2	330
		(pmol/mg protein)	
Chloroplast	light	38 3	100
Etioplast	dark	11 2	29
Chloroplast	dark/18 hr light	52 9	138

*Details of the light regimes are given in the Experimental.

Table 2 Properties of cyclic AMP phosphodiesterase activity in isolated chloroplasts from spinach plants kept in light or dark conditions

Property	Light (18 hr)*	Dark (18 hr)*
K_m	$8\,7 \times 10^{-4}$ M	$2\,7 \times 10^{-5}$ M
V_{max}	1 1 nmol/min/mg protein	1 1 nmol/min/mg protein
Response to Ca^{2+} and/or calmodulin		
Control	45 pmol/min/μl	47 pmol/min/μl
Control +calmodulin	90 pmol/min/μl	nd
Control +calmodulin + Ca^{2+}	151 pmol/min/μl	52 pmol/min/μl

*Plants were grown as described in the Experimental section but exposed to 18 hr continuous light (6 klx) or 18 hr dark immediately before chloroplast isolation, as indicated
nd = Not determined

largely disappeared after a further 5 hr in the light. It was of interest that the cyclic GMP concentration showed a 400% overshoot after 1 hr in the light but that this, too, had returned to the control level by the end of the 5 hr illumination period.

Despite their magnitude, there appears to be no simple, direct correlation between light-induced changes in the corresponding cyclase or cyclic nucleotide phosphodiesterase activities For example, relative to controls, the cyclic GMP concentration in the chloroplast decreases by 50% in the dark, increases by 500% in 1 hr in the light, and after 5 hr is still elevated by 250%. However, the 30-fold depression of guanylate cyclase activity in the dark is still apparent after 1 hr illumination and only 65% of the original activity is restored after 5 hr, while cyclic GMP phosphodiesterase is decreased 50-fold in the dark and is slightly above the control value after illumination (Table 3). A similar situation exists for cyclic AMP and its associated enzymes (Tables 1 and 3). It would thus appear that other factors such as cyclic nucleotide interaction are involved, e g. cyclic AMP and cyclic GMP competing for the same phosphodiesterase site. Equally, it has to be borne in mind that the chloroplast is not a completely closed system and any change in membrane permeation by cyclic nucleotides, induced by changes in light conditions, may be significant. The earlier suggestion that cyclic AMP is transported from root to shoot in a light-dependent process [19] and the presence of cyclic AMP in plant transport vessels [20] may also be relevant. As reviewed in ref. [10], cells of green and blue-green algae, bacteria, and slime moulds, all permit physiological passage of cyclic AMP and the same may be true of chloroplasts.

In addition to the responses described, the light regime under which *Phaseolus* seedlings were grown markedly influenced the effects that the dibutyryl derivatives of cyclic AMP and cyclic GMP had on terpenoid concentrations (Table 4). Whereas dibutyryl cyclic GMP had little significant effect on the concentration of the major terpenoid constituents in light-grown plants, dibutyryl cyclic AMP doubled the concentration of α-tocopherol and reduced the concentration of γ-tocopherol and chlorophyll by 40% in the same group of plants; simultaneously their concentration of ubiquinone increased by 48%. If the light-grown plants were put in the dark for 15 hr before similar treatment with dibutyryl cyclic nucleotides, the main effect was that of dibutyryl cyclic AMP on ubiquinone concentration, which increased by 107%. Again, dibutyryl GMP had little significant effect. The main responses to dibutyryl cyclic GMP were seen with the terpenoid constituents of dark-grown plants with 62, 81 and 78% increases, respectively, in α-tocopherol, γ-tocopherol, and chlorophyll concentrations. After 15 min illumination, plants grown in the dark showed an 83% increase in α-tocopherol content and a 168% increase in that of ubiquinone after treatment with dibutyryl cyclic GMP. The main effect of dibutyryl cyclic AMP on the concentration of the terpenoids in these plants was to increase the concentration of chlorophyll and ubiquinone by 57 and 78%, respectively.

In mammals, the primary process in light perception involves excitation of rhodopsin which then induces changes in cyclic GMP concentrations, and these in turn affect cytoplasmic membrane permeability. In higher plants, phytochrome has been identified as the light-receptor for a number of light-sensitive processes but the subsequent biochemistry is largely unknown; analogy suggests that cyclic nucleotides may be involved. It can be seen from the data presented that changes elicited by dibutyryl cyclic nucleotides are not solely ascribable to

Table 3. Enzymes of cyclic nucleotide metabolism and other components of chloroplasts isolated from light-grown and dark-grown *Phaseolus* seedlings

	Light conditions			
	Control*	Dark (12 hr)†	Light (1 hr)‡	Light (5 hr)§
Protein (mg/ml)	6	3	14	15
Chlorophyll (mg/ml)	6.5	3 2	11.8	12.5
NADP-glyceraldehyde dehydrogenase (nmol/min/mg protein)	500	100	1000	600
Cyclic AMP phosphodiesterase (nmol/min/mg protein)	1.13	1.51	1.47	1.61
Cyclic GMP phosphodiesterase (nmol/min/mg protein)	4 9	0.1	6.9	5.3
Adenylate cyclase (nmol/min/mg protein)	1.0	2.8	1.0	0.8
Guanylate cyclase (pmol/min/mg protein)	294	9.9	7.24	190
Cyclic GMP (pmol/g fresh wt)	20	10	100	50

*14 day seedlings grown in 16 hr light (6 klx)/8 hr dark cycle Leaves taken 2 hr after light phase began

†As for control but plants then placed in dark for 12 hr

‡As for † but then exposed to 1 hr in the light.

§As for ‡ but final light phase was 5 hr instead of 1 hr.

Table 4 Effect of cyclic AMP and cyclic GMP derivatives upon terpenoid concentrations in *Phaseolus vulgaris* grown under different light regimes

	α-Tocopherol	γ-Tocopherol	Chlorophyll	β-Carotene	PQ_{A_9}	UQ_9
*Light grown						
+dbcAMP	+105	−40	+41	−3	−28	+48
+dbcGMP	+2	−3	−12	−5	−12	−4
Light grown + 15 hr dark						
+dbcAMP	+31	+23	+38	+47	−1	+107
+dbcGMP	+3	−1	+23	+23	+31	−7
Dark grown						
+dbcAMP	+47	+32	+48	−3	+30	+45
+dbcGMP	+62	+81	+78	−5	+43	+28
Dark grown + 15 hr light						
+dbcAMP	−13	−3	+57	−4	+31	+78
+dbcGMP	+83	−37	−15	+15	−28	+168
*Light cycle of 18 hr light + 6 hr dark						

Each datum is expressed as % change relative to a light-grown butyric acid control, actual control values for the terpenoids per g tissue (fresh wt) were α-tocopherol 10 2 μg, γ-tocopherol 4 3 μg, chlorophyll 1 5 mg, β-carotene 0 7 mg, PQ_{A_9} 1 0 μg, UQ_9 1 1 μg

butyrate residues and that the differences in response to the two cyclic nucleotides, and changes in these responses dependent upon light regimes, are consistent with a hypothesis involving cyclic AMP and cyclic GMP as mediators in light-regulated metabolic events The present observations that the concentrations of cyclic AMP and cyclic GMP in the chloroplast and the activities of the associated plastidic cyclase and phosphodiesterase vary in response to change in light conditions are also consistent with such a hypothesis The light-related changes in sensitivity of the chloroplastic cyclic AMP phosphodiesterase to calmodulin are of especial significance As yet, however, these findings can only be considered as indicators of a possible role for cyclic AMP and cyclic GMP in light-induced or light-regulated events in higher plants Even in the absence of such a role *in vivo*, the data confirm the complexity of the plant cyclic nucleotide system as previously discussed [10] Further, the apparent sensitivity of the system to changes in light conditions may explain earlier contradictory reports on the physiological effects of cyclic nucleotides in higher plants

EXPERIMENTAL

Plant material Seeds of *Phaseolus vulgaris* cv The Prince (Bees Seeds Ltd, Sealand, U K) were germinated in moist vermiculite and seedlings grown at 23° in a light cycle of 16 hr light (6 klx)/8 hr dark except where otherwise indicated Seeds of *Spinacea oleracea* cv Viroflay (Hurst, Gunson, Cooper and Taber Ltd. Witham, U K.) were also germinated in moist vermiculite but seedlings were grown at 20° Light grown plants were cultivated in a 20 hr light (6 klx)/4 hr dark cycle, dark grown plants were cultivated in 20 hr dark/4 hr light (6 klx) cycle

Determination of cyclic AMP and cyclic GMP concentrations Cyclic nucleotides were extracted from whole leaf tissue and from isolated plastids and partially purified as previously described [21] Cyclic AMP concentrations were determined by saturation binding analysis as described earlier [21] Cyclic GMP concentrations were determined by radioimmunoassay (RIA) using a commercially available RIA kit [7] from Amersham International plc, Amersham, U.K

Isolation and extraction of plastids Leaves (160–200 g samples) were harvested from 8-day *Spinacea* or 8-day *Phaseolus* seedlings a green safelight was used as appropriate Chloroplast preparations were obtained by the procedure of ref [22] with minor modifications For enzymic assay purposes, isolated plastids were either broken by a rapid cycle of freezing and thawing, repeated six times, or by ultrasonication for 90 sec at 20 kHz (cycle of 15 sec on/15 sec off) During ultrasonication, the sample was kept on ice

Assay of plastid enzymes The adenylate cyclase, guanylate cyclase, cyclic nucleotide phosphodiesterase, and NADP-linked glyceraldehyde 3-phosphate dehydrogenase activities of isolated chloroplasts were assayed by the following procedures adenylate cyclase [23], guanylate cyclase [24], cyclic AMP- and cyclic GMP- phosphodiesterase [7, 25], and NADP-linked glyceraldehyde 3-phosphate dehydrogenase [26]

Partial purification of chloroplast phosphodiesterase for examination of effect of Ca^{2+}*/calmodulin.* Sonicated chloroplast preparations obtained from *Spinacea* leaves, as described above, were dialysed exhaustively against 40 mM Tris–HCl buffer at pH 7 5 and samples (2 ml) applied to a Sephacryl-200 column (26 × 1 5 cm) at a flow rate of 1 ml/min of the same buffer The fraction eluting between 70 and 95 ml was retained and used as the cyclic AMP phosphodiesterase preparation Its activity was determined by the standard assay procedure [7, 25] in the absence and presence of 20 μM Ca^{2+} and 0 4 units calmodulin/assay tube

Administration of dibutyryl cyclic nucleotides Seeds of *Phaseolus vulgaris* were soaked overnight in running water and germinated in trays of moist vermiculite Each batch of plants comprised 4 trays (*ca* 100 seedlings/tray) One batch was grown for 15 days at 25° in a light cycle of 18 hr light (6 klx)/6 hr dark Another batch was kept under the same conditions but on days 13 and 14 one tray was sprayed with dibutyryl cyclic AMP (10 ml, 0 4 M) a second tray with *n*-butyric acid (10 ml, 0 8 mM) a third tray with N^4,2′-*O*-dibutyryl cyclic GMP (10 ml, 0 2 mM) and the fourth tray with *n*-butyric acid (10 ml, 0 4 mM) The butyryl compounds were all in aq soln, the concentrations of the butyric acid solutions were chosen to provide controls against possible effects of the dibutyryl cyclic nucleotides being due to the butyrate residues alone A further eight trays of seedlings were used to duplicate all the above treatments but after 15 days they were placed in the dark for 15 hr Finally, eight trays of

seedlings were grown for 15 days in the dark at 25°, subjected to the same chemical treatments to those described, and on day 15 placed in a light cycle (6 klx) of 18 hr light/6 hr dark for 15 hr. The entire experiment was repeated on a separate occasion

Lipid extraction and analysis Leaves (60–80 g) were washed in H_2O, blotted dry with paper tissues, and macerated in aq. Me_2CO (10% w/v) using a Polytron homogenizer (Northern Media Supplies) The homogenate was filtered under vacuum through a sintered glass Buchner funnel (porosity 3) then mixed with an equal vol of petrol (bp 40–60°) Two phases were obtained, the upper (petrol) fraction was taken, washed × 3 with H_2O, then dried over Na_2SO_4 After evapn to dryness under O_2-free nitrogen, the sample was redissolved in the minimum vol. of cyclohexane and applied to a column of Al_2O_3 (20 g, Brockmann grade III) Lipids were eluted in a series of petrol mixtures containing increasing concentrations of Et_2O [27] The concns of individual tocopherols and chlorophylls were determined as previously described [27] Quinones were estimated by reducing them with $NaBH_4$ to the corresponding quinols and measuring the decrease in $A_{\lambda max}$ (255 and 275 nm for PQ_{A9} and UQ_9, respectively) β-Carotene was determined from its $A^{1\%}_{1cm}$ value at λ_{max}.

REFERENCES

1. Rall, T. W , Sutherland, E W and Berthet, J (1975) *J Biol Chem.* 224, 463
2. Jost, J-P and Rickenberg, H. V. (1971) *Ann Rev. Biochem.* **40**, 741
3 Makman, R S and Sutherland, E W (1965) *J. Biol. Chem.* **240**, 1309
4 Pastan, I. and Perlman, R. L (1972) *Adv Cyclic Nucleotide Res* **1**, 11.
5. Gerisch, G. and Malchow, D. (1976) *Adv. Cyclic Nucleotide Res.* **7**, 49
6. Newton, R P., Gibbs, N., Moyse, C D , Wiebers, J L and Brown, E G (1980) *Phytochemistry* **19**, 1909.
7. Newton, R P , Kingston, E. E , Evans, D E., Younis, L M and Brown, E. G (1984) *Phytochemistry* **23**, 1367
8 Janistyn, B (1983) *Planta* **159**, 382
9. Brown, E. G and Newton, R P (1981) *Phytochemistry* **20**, 2453
10. Newton, R. P and Brown, E. G (1987) in *Hormones, Receptors and Cellular Interactions in Plants* (Chadwick, C M. and Garrod, D. R , eds) pp. 115–153 Cambridge University Press, Cambridge
11. Bitensky, M. W., Wheeler, M. A., Rasenick, M M., Yamazaki, A., Stein, P. J , Halliday, K. R and Wheeler, G. L (1984) *Proc Natl Acad Sci* U S.A **79**, 3408
12. Heim, J M , Gottman, K , Weil, J , Haufe, M C and Gerzer, R (1988) *Z Kardiol.* **77**, 41
13 Goldberg, N D., Haddox, M. K , Nicol, S E , Glass, D B., Sanford, C M., Kuehl, F. A and Estensen, B (1975) *Adv Cyclic Nucl. Res.* **5**, 307.
14. Janistyn, B. and Drumm, H (1975) *Planta* **125**, 81
15. Rast, D., Skrivanova, R and Bachofen, R (1973) *Phytochemistry* **12**, 2669.
16 DeNicola, M. G , Amico, V. and Piatteli, M (1975) *Phytochemistry* **14**, 989.
17 Elliot, D C. and Muray, A W. (1975) *Biochem. J.* **146**, 333.
18 Wellburn, A R., Ashby, J. P. and Wellburn, F A. M (1973) *Biochim Biophys. Acta* **320**, 363
19. Bachofen, R (1973) *Plant Sci. Letters* **1**, 447
20. Becker, D and Ziegler, H (1973) *Planta* **110**, 85.
21 Brown, E G , Al-Najafi, T. and Newton, R P (1979) *Phytochemistry* **18**, 9.
22. Lominski, A. and Reinerts, K. G. (1981) *Phytochemistry* **20**, 993.
23 Salomon, Y (1979) *Adv. Cyclic Nucl Res* **10**, 35.
24. Garbers, D L and Murad, F (1979) *Adv Cyclic Nucl. Res.* **10**, 57.
25 Thompson, W J., Terasaki, W L., Epstein, P M. and Strada, S J. (1979) *Adv. Cyclic Nucl. Res* **10**, 69
26. Brown, E G., Edwards, M. J , Newton, R P and Smith, C J. (1979) *Phytochemistry.* **18**, 1943
27 Newton, R. P and Pennock, J F (1971) *Phytochemistry* **10**, 2323