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EFFECT OF KINETIN ON THE PHYCOERYTHRIN AND CHLOROPHYLL CONTENT OF A RED ALGA

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Abstract—A decline in R-phycoerythrin occurs in cultured branch apices of the red alga *Hypnea musciformis*. This decline is offset by kinetin, and studies with chloramphenicol suggest that chloroplast protein synthesis is a requirement for the kinetin effect. δ-Aminolevulinic acid also stimulates chloramphenicol-sensitive phycoerythrin synthesis. Kinetin treatment increases the levels of chlorophyll-*a* and soluble protein.

INTRODUCTION

DURING investigations into the culture of excised apical segments from the red alga *Hypnea musciformis*,[1] it was observed that segments tend to remain red for longer periods in the presence of kinetin. This suggested that kinetin may exert a stabilizing effect on the level of the chloroplast biliprotein, phycoerythrin (PE), an observation of particular interest in view of reports that implicate cytokinin in the regulation of chloroplast protein metabolism in higher plants.[2–4] The present work was concerned with obtaining information about the effect of kinetin on phycoerythrin levels in *Hypnea*.

RESULTS

Partial Characterisation of Phycoerythrin

Comparison of absorption spectra[5] identified R-phycoerythrin as the major biliprotein present. Zone sedimentation of the crude NaCl-soluble PE revealed a major component with a MW of 297 000, which closely matches the value of 290 000 estimated for *Ceramium* PE.[6] A minor red component was also present, with a MW of about 70 000. Isoelectric focusing of the crude PE extract also produced two distinct red bands. The major band was broad, the isoelectric points ranging from 4·18 to 4·38, with the main peak at 4·25 ± 0·1. This compares favourably with the value of 'approx. 4·5' quoted for *Ceramium* PE.[7] The minor band had an isoelectric point of 5·22 ± 0·1, and is presumed to correspond with the low MW peak detected during zone sedimentation. It is suggested that the major PE

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[4] J. FIEIRABEND, *Planta* **84**, 11 (1969).
[5] C. Ó hEOCHA, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. 1, p. 407, Academic Press, London (1966).
[6] I. B. ERIKSSON-QUENSEL, *Biochem. J.* **32**, 585 (1938).
[7] P. O. CARRA, *Biochem. J.* **94**, 171 (1965).

fractions from *Hypnea* and *Ceramium* are similar molecules. The minor, low molecular weight PE fraction from *Hypnea*, detected by zone sedimentation and isoelectric focusing, could be a sub-unit of the larger molecule, as has been suggested for other low MW phycobiliproteins.[8] However, the larger molecule was apparently stable under the various experimental conditions used, and had an isoelectric point which was one unit different from the smaller molecule. This suggests that the one molecule may not be derived from the other, though more definitive evidence is required. In the following work, PE was estimated using the extinction coefficient calculated from *Ceramium*.[7]

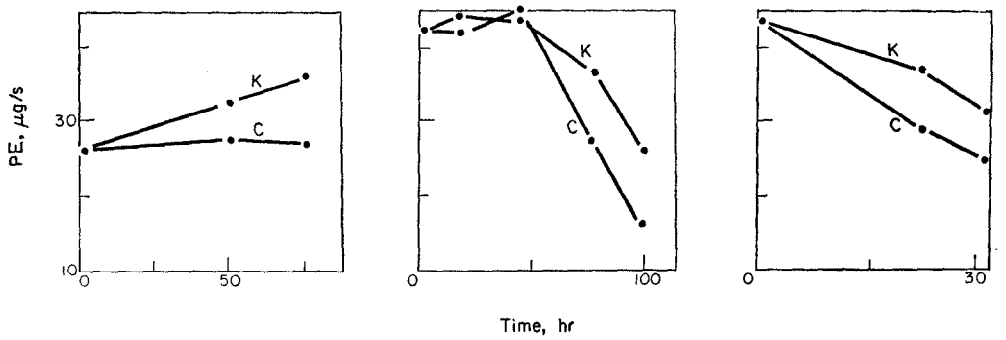

FIG. 1. EFFECT OF KINETIN (0·05 mg/l.) ON THE CONTENT OF PHYCOERYTHRIN (PE) IN CULTURED BRANCH APICES OF *Hypnea* COLLECTED FROM THE OCEAN AT DIFFERENT TIMES DURING THE YEAR.
Values are expressed as μg/segment (μg/s). K-kinetin treated; C-control.

Phycoerythrin Stability and the Effect of Kinetin

When apices of *Hypnea* are cultured under the conditions of these experiments the level of PE declines. Sometimes the decline begins almost immediately, while on other occasions the pigment may remain at an approximately constant level for a period of two to more than seven days before the decline commences (Fig. 1). The decline is largely attributable to photodestruction, as levels of pigment fall much more rapidly in the light than in the dark, an effect which can be observed *in vitro* as well as *in vivo* (Table 1).

TABLE 1. EFFECT OF LIGHT (50 lm/ft²) ON LEVELS OF PHYCOERYTHRIN

Phycoerythrin	(μg/segment) Time 0	Dark	Light
In vivo	9·3	7·0	3·5
In vitro	9·2	8·1	5·3

Phycoerythrin was measured after culture of isolated branch apices in filtered sea water (*in vivo*), or after isolation and incubation of phycoerythrin in 1% NaCl (*in vitro*). The experiments were conducted for 48 and 28 hr respectively.

[8] G. A. MIERAS and R. A. WALL, *Biochem. J.* **107**, 127 (1968).

Incubation of segments with kinetin, applied at levels which are effective in controlling growth, frequently though not invariably resulted in higher levels of PE. Sometimes there was a stabilisation during PE decline, while on other occasions there was a net increase in PE (Fig. 1). Variability in response between different experiments was attributed to differing physiological states, as plants were collected from the ocean at different times of the year. Kinetin at 0·05 mg/l. was more effective than at 1 mg/l. (Table 2). As kinetin has very little effect on fresh weight, the effect on PE was similar whether considered on a per segment basis or on a fresh weight basis.

TABLE 2. EFFECT OF TWO CONCENTRATIONS OF KINETIN ON THE PHYCOERYTHRIN CONTENT OF APICAL SEGMENTS OF *Hypnea*

Phycoerythrin (μg/segment)			
Time 0	Control	Kinetin (0·05 mg/l.)	Kinetin (1 mg/l.)
9·3	3·5	6·0	4·3
6·3	5·7	8·6	7·5

Results are from two experiments, each of 48 hr.

Synthesis of Protein and Phycoerythrin

To ascertain if the effect on level of PE might reflect a more general influence of kinetin on protein and tetrapyrrole content, NaCl-soluble protein, chlorophyll-*a* and PE were estimated (Table 3). Kinetin-treated plants contained higher concentrations of salt-soluble protein and chlorophyll-*a*, as well as PE, than control plants, and the increase in soluble protein paralleled the increase in PE.

TABLE 3. EFFECT OF KINETIN (0·05 mg/l.) ON THE LEVELS OF PHYCOERYTHRIN, NaCl-SOLUBLE PROTEIN AND CHLOROPHYLL-*a* IN EXCISED APICAL SEGMENTS OF *Hypnea*

		μg/segment		
		Time 0	Control	Kinetin
A	Phycoerythrin	8·0	8·5	12·8
	Protein	82	79	123
B	Phycoerythrin	16·8	7·6	10·9
	Chlorophyll-*a*	0·19	0·37	0·48

Experiments were conducted for 120 hr (A) and 31 hr (B).

The effect of kinetin on the incorporation of label from ^{14}C-amino acids into PE and a high MW fraction was next investigated. The high MW fraction included salt-soluble compounds with MWs in excess of 30 000 (calculated for globular proteins), and would have

contained little nucleic acid, as only the *s*RNA fraction is soluble in 1 % NaCl.[9] Radioactive amino acids were included in the cultures when it was visually apparent that the kinetin effect was being exerted. Incubation was continued for a further 40 hr, when segments were homogenised in 1 % NaCl, and the PE purified. Radioactivity of an aliquot of the high MW fraction from the Sephadex G-75 column was measured. Both controls and kinetin-treated plants showed incorporation of label, and kinetin-treated plants incorporated more than twice as much label into the high MW fraction and PE than did the controls (Table 4).

TABLE 4. EFFECT OF KINETIN TREATMENT (0·05 mg/l.) ON PHYCOERYTHRIN CONTENT, RADIOACTIVITY OF PHYCOERYTHRIN, AND RADIOACTIVITY OF A HIGH MOLECULAR WEIGHT FRACTION AFTER INCUBATION FOR 40 hr WITH A ^{14}C-AMINO ACID MIXTURE

	Time 0	Control	Kinetin
Phycoerythrin (μg/segment)	11·0	8·3	10·7
Radioactivity in phycoerythrin (cpm)	—	223 (±35)	570 (±29)
Radioactivity in high MW fraction (cpm)	—	11 850	30 000

Interactions Between Kinetin and Chloramphenicol

The antibiotic chloramphenicol was used in a further analysis of the relation between PE and general protein synthesis. This compound is known to inhibit protein synthesis on chloroplast ribosomes of algae and higher plants at low concentrations, while cytoplasmic protein synthesis is unaffected.[10–12] Experiments were therefore designed to determine if PE synthesis is chloramphenicol sensitive. From Table 5 it can be seen that chloramphenicol-treated plants contained considerably less PE than the controls, and in the presence of the

TABLE 5. EFFECT OF CHLORAMPHENICOL (100 mg/l.) ON THE LEVELS OF PHYCOERYTHRIN, NaCl-SOLUBLE PROTEIN, AND ^{14}C-INCORPORATION INTO PHYCOERYTHRIN

		Time 0	Control	Chloramphenicol
A	Phycoerythrin (μg/segment)	7·9	9·7	6·2
	Protein (μg/segment)	82	75	96
B	Phycoerythrin (μg/segment)	8·3	5·0	2·0
	Radioactivity (cpm)	—	6·4 ± 1·3	0

One-twentieth of the sample was dissolved in a Triton-X-toluene scintillation fluid and 20 000 counts accumulated. Experiments were conducted for 80 hr (A) and 32 hr (B).

[9] G. ZUBEY, *J. Molec. Biol.* **4**, 347 (1962).
[10] J. M. EISENSTADT and J. BRAWERMAN, *J. Molec. Biol.* **10**, 392 (1964).
[11] D. SPENCER, *Arch. Biochem. Biophys.* **111**, 381 (1965).
[12] L. A. ANDERSON and R. M. SMILLIE, *Biochem. Biophys. Res. Commun.* **23**, 535 (1966).

inhibitor there was reduced incorporation into PE of label from ^{14}C-amino acids. There was, however, no general inhibition of protein synthesis, as indicated by the results for NaCl-soluble protein. As PE synthesis is more chloramphenicol-sensitive than is total protein synthesis, the antibiotic was used in an interaction experiment with kinetin. Results are presented in Table 6, where it can be seen that kinetin did not influence the level of PE in the presence of chloramphenicol. In a subsequent experiment, a similar physiological interaction between these two compounds was found in the control of chlorophyll-*a* content (Table 6), suggesting a similarity between the effect of kinetin on PE and chlorophyll.

TABLE 6. INTERACTION BETWEEN KINETIN (0·05 mg/l.) AND CHLORAMPHENICOL (100 mg/l.) ON THE LEVELS OF PHYCOERYTHRIN AND CHLOROPHYLL-*a* IN APICAL SEGMENTS OF *Hypnea*

		μg/segment				
		Time 0	Control	Chloramphenicol	Kinetin	Chloramphenicol plus kinetin
A	Phycoerythrin	7·9	8·8	3·9	12·9	3·8
B	Chlorophyll-*a*	0·39	0·29	0·29	0·45	0·29

Experiments were conducted for 115 hr (A) and 80 hr (B).

Effects of δ-Aminolevulinic Acid

To further investigate the possibility that the effect of kinetin on PE might resemble its effect on chlorophyll, experiments were conducted with δ-aminolevulinic acid (ALA). This compound is an early precursor in tetrapyrrole biosynthesis, and has been shown to be involved in the synthesis of bilin chromophore.[13]

From Table 7 it is apparent that ALA stimulated PE synthesis, and 85 mg/l. (0·5 mM) was the most effective concentration. Response to this compound was slightly reduced at 850 mg/l. (5·0 mM), and this was observed repeatedly. The visible absorption spectrum for PE from ALA-treated plants was identical to the usual PE spectrum and there was no loss of optical density after 8 hr dialysis, indicating that it was PE and not free chromophore which was being measured. This was confirmed by chromatography on Sephadex G-75, where only a single coloured band, corresponding to PE, could be observed. The influence of ALA on PE resembled that of kinetin in being sensitive to chloramphenicol (Table 7).

TABLE 7. A. EFFECT OF δ-AMINOLEVULINIC ACID (ALA) ON THE PHYCOERYTHRIN CONTENT OF APICAL SEGMENTS OF *Hypnea*

		μg/segment				
				ALA		
		Time 0	Control	(8·5 mg/l.)	(85 mg/l.)	(850 mg/l.)
A	Phycoerythrin	4·2	3·2	4·4	6·0	5·2
					Chloram-phenicol	ALA plus chloram-phenicol
B	Phycoerythrin	7·4	4·8	6·3	2·7	2·2

A. The experiment was conducted for 60 hr. B. Interaction between δ-amino-levulinic acid (ALA) (85 mg/l.) and chloramphenicol (200 mg/l.) on the levels of phycoerythrin in apical segments of *Hypnea*. The experiment was conducted for 85 hr.

[13] R. F. TROXLER and L. BOGARAD, *Pl. Physiol.* **41**, 491 (1966).

DISCUSSION

The information presented here shows that treatment of cultured branch apices of *Hypnea* with kinetin offsets a decline in the levels of PE, an effect which is accompanied by enhanced levels of chlorophyll-*a* and soluble protein. Incorporation of label from amino acids into soluble protein and PE is observed in untreated plants and this incorporation is enhanced by kinetin. The effect of kinetin might therefore result from increased rates of synthesis, decreased rates of destruction, or both. Chloramphenicol blocks PE synthesis without reducing the level of total protein, presumably because of its effect in blocking protein synthesis in the chloroplasts,[10–12] and so preventing PE synthesis. In the presence of the antiobiotic, kinetin does not prevent a decline in PE, suggesting that PE synthesis in the chloroplasts is essential for the response to kinetin, and that the effect of kinetin is to stimulate PE synthesis.

Experiments in which δ-aminolevulinic acid resembled the effect of kinetin in stimulating PE synthesis, a response which was also chloramphenicol-sensitive, suggest the possibility that the primary effect of kinetin may have been via the enhanced synthesis of the tetrapyrrole chromophore. That tetrapyrrole synthesis is kinetin sensitive is also indicated by the influence of this hormone on chlorophyll levels. In this respect it is interesting to note that chloramphenicol has been shown to inhibit the synthesis of bilin-chromophore and chlorophyll in greening cells of *Cyanidium caldarum*,[13] and an interaction between chloramphenicol and kinetin, similar to that reported here for *Hypnea*, has been recorded for chlorophyll levels in *Cucurbita* cotyledons.[14] Thus the biochemical basis of the kinetin response may well be identical in plastids of algae and higher plants.

EXPERIMENTAL

Plant material and culture technique. Plants of *Hypnea musciformis* (Wulf.) Lamour were collected from shallow sublittoral reefs at Cottesloe ocean beach from April to July over three consecutive years. Branch apices, each 2 cm long, were excised and shaken vigorously in filtered sea water to dislodge contaminating organisms. Between 6 and 10 segments were placed in Petri dishes containing 40 ml of filtered sea water. Additions of kinetin (Sigma Chemicals) chloramphenicol (Parke Davis & Co.), and a mixture of ^{14}C-amino acids, specific activity 2 mc/mg (Radiochemical Centre, Amersham) were made in aq. soln, and culture solns were changed every 2 or 3 days. Lighting was provided by 'warm white' fluorescent tubes (*ca.* 50 ft-c).

Extraction and estimation of phycoerythrin. Plant material was homogenised in 3 ml of 1% NaCl with a ten-Broek glass homogenizer, and particulate material removed by centrifugation at 40 000 *g* for 20 min. The pH remained constant at 6·5. PE was estimated at 570 nm using the extinction coefficient calculated for PE from *Ceramium rubrum*.[7]

Estimation of ^{14}C-amino and incorporation into PE. Purification of the biliprotein was achieved by a combination of gel filtration and gel electrophoresis. Carrier, non-labelled extract was first added to the labelled extracts, which were then concentrated by ultra-filtration and subjected to gel filtration on a 25 × 1·5 cm column of Sephadex G-75. The red biliprotein was eluted with approximately 1 void volume of 1% NaCl, concentrated by ultrafiltration, and subjected to further gel filtration on a 25 × 1·5 cm column of Sepharose 4B with 1% NaCl. PE was eluted with between 2 and 3 void volumes. Fractions containing PE were bulked, dialysed against water for 12 hr to remove NaCl and concentrated to 0·25 ml. These chromatographic techniques removed 80–90% of protein from the original extract. Aliquots of 4·8 μl were subjected to electrophoresis on strips of cellulose acetate (Gelman Instrument Co.) using a Tris-barbital buffer (ionic strength 0·05; pH 8·8) for 30 min at 1·5 mA/gel. Staining was effected with Ponceau S (Gelman) dissolved in 5% CCl_3COOH. Two bands were detected with the protein stain, one coinciding with the red pigmentation. The same number of bands was detected after disc electrophoresis on 7·5% polyacrylamide in a Tris-glycine buffer at pH 8·5 and staining with Coomassie Blue B. The simpler cellulose acetate method was adopted as standard procedure.

Estimation of MW and isoelectric point of PE. The MW of PE was determined by zone sedimentation in a 5–20% linear sucrose gradient (in 0·1 M phosphate buffer, pH 6·5) at 39 000 rpm for 5 hr at 2° in the SW 39 rotor of the Beckman L2 ultracentrifuge. MWs were calculated by the method of Martin and Ames[15] using catalase (MW 254 000; Calibiochem) as the standard.

[14] D. S. BERNS, H. L. CRESPI and J. F. KATZ, *J. Am. Chem. Soc.* **85**, 8 (1963).
[15] R. G. MARTIN and B. M. AMES, *J. Biol. Chem.* **236**, 1372 (1961).

Isoelectric points were determined by isoelectric-focusing in a 5% polyacrylamide gel with pH 4–6 carrier ampholytes (LKB Products) for 5 hr at 4°, after the method of Catsimpoulas.[16] Gels were sliced into 33 segments and the pH of each segment measured in deionized H_2O. A regression equation relating distance with pH was used in determining the isoelectric points.

Estimation of chlorophyll-a. Chlorophyll was extracted from the 1% NaCl pellet in absolute EtOH and estimated at 663 nm using the extinction coefficient of MacKinney.[17]

Estimation of soluble protein. Extracts made in 1% NaCl were dialysed overnight against water, and protein estimated with the Folin–Ciocalteu reagent.[18]

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Key Word Index—Hypnea musciformis; Algae; biosynthesis; phycoerythrin; chloramphenicol; kinetin; aminolevulinic acid.