CYTOKININS AS ENDOGENOUS GROWTH REGULATORS IN THE ALGAE *ECKLONIA* (PHAEOPHYTA) AND *HYPNEA* (RHODOPHYTA)

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Summary

Cytokinin activity was detected in extracts prepared from *Ecklonia radiata* (Phaeophyta) and *Hypnea musciformis* (Rhodophyta), and was associated with chromatogram bands which appeared to contain purine. Kinetin, a cytokinin with a purine ring system, stimulated growth in cultured plants of the same species. It is concluded that cytokinins are normally involved in regulating growth in these algae. On the basis of this and other evidence, it is suggested that plant growth regulators of the auxin, gibberellin, and cytokinin type may have arisen early in plant evolution.

I. Introduction

During the last 15 years a great deal has been learned concerning the occurrence and role of cytokinins in higher plants (for review see Letham 1967), but similar information is almost completely lacking for the algae. Kinetin has been reported to stimulate growth in *Porphyra tenera* of the Rhodophyta (Iwasaki 1965), *Ectocarpus fasciculatus* and *Pylaiella litoralis* of the Phaeophyta (Pedersen 1968), *Ulva lactuca* and *Acetabularia mediterranea* of the Chlorophyta (Provasoli 1958; Zetsche 1963; Spencer 1968), and in several species of Charophyta (Imahori and Iwasa 1965). Bentley-Mowat and Reid (1968) reported evidence for the presence of cytokinins in extracts prepared from a diatom and a dinoflagellate, but these results must be viewed with some caution because of the probable impurity of the extract and the lack of specificity of the bioassay employed.

The present work sought information on the possibility that cytokinins are implicated in controlling growth in an alga belonging to the Phaeophyta and in one belonging to the Rhodophyta. Attempts were made to detect materials which possessed cytokinin activity, and tests were carried out to determine if these materials might be similar chemically to the known cytokinins in possessing a purine ring system. Kinetin was applied to cultures of the species from which the extracts had been made in order to determine any effects on growth.

II. Materials and Methods

(*a*) *Plant Materials*

Plants of *Hypnea musciformis* (Wulf.) Lamour (Rhodophyta) and *Ecklonia radiata* (C. Ag.) J. Ag. (Phaeophyta) were collected from shallow sublittoral reefs at Cottesloe ocean beach. Culture experiments were performed with *H. musciformis* collected during April and May, while *E. radiata* was collected for this purpose during February and March. Extraction experiments

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were performed with *H. musciformis* (400 g fresh weight) which was collected during May. Care was taken to select plants free from macroscopic epiphytes. Contamination by microorganisms was found to be slight, presumably because of the cleansing action of an early winter storm. Blade tissue of *E. radiata* (500 g fresh weight) was collected for extraction experiments during April. The upper half of the blade of each plant was discarded, as this, being the oldest region, was frequently highly contaminated. Only those regions of the lower half which appeared free from epiphytes were used. All plant material was thoroughly wiped with sheets of blotting paper prior to weighing, and this also served to reduce the level of contamination by microorganisms.

(*b*) *Extraction and Assay Technique*

The extraction procedure was based on methods used by Letham and Bollard (1961) for the detection of cytokinins in the fruits of some higher plants, and which lead to the isolation of zeatin by Letham (1964). The plant material was initially homogenized in 2 litres of 95% ethanol. The homogenate was placed in the cold overnight and was then filtered. The filtrate was dried *in vacuo* at 40°C, and the residue shaken with 200 ml of water. This aqueous extract was then adjusted to pH 3·0–3·3 with HCl and shaken three times with ethyl acetate. The aqueous phase was passed through a cation exchange resin [Amberlite IR-120 (H)] which was washed in turn with water, 70% ethanol, and water. These washings were bulked and will be referred to as the ethanol fraction. The resin was then eluted successively with 1·5N NH_4OH, 1·5N HCl, 3·0N HCl, and 6·0N HCl. These fractions were designated the NH_4OH fraction, the 1·5N HCl fraction, the 3·0N HCl fraction, and the 6·0N HCl fraction respectively. Each fraction was dried *in vacuo* at 40°C and the residue was taken up in a small volume of water and neutralized ready for bioassay (see below). The NH_4OH fraction was neutralized with HCl. A weak anion exchange resin [Bio-Rad AG 3-X4 (OH)] was added to the acidic eluates to pH 3·0. The resin was subsequently removed and NaOH added to pH 7·0.

Aliquots of fractions which appeared to be of interest were dried, taken up in a small volume of absolute ethanol, and streaked across strips of Whatman No. 3 chromatography paper. Except where indicated all chromatograms were prepared from material derived from 100 g of plant tissue. They were developed with n-butanol–acetic acid–water (12 : 3 : 5) as a descending solvent.

The known natural cytokinins possess a purine ring system, and it seems probable that this structure is necessary for at least some of the biological activity of these compounds in higher plants (Fox and Chen 1967; Letham and Ralph 1967). For this reason attempts were made to detect purine on the chromatograms by inspection under a short-wave ultraviolet lamp, and after spraying with the silver nitrate–bromophenol blue reagent of Wood (1955). Compounds which yield a colour reation with this test form a water-insoluble complex with the silver ions of the reagent. This step therefore involves a similar principle to the silver nitrate precipitation technique, frequently employed to effect a purification of cytokinins from higher plant extracts (e.g. Milles 1963; Maheshwari and Prakash 1967; Nitsch 1967). Chromatogram zones associated with compounds which gave a positive purine response were eluted with water and the eluates reduced in volume ready for bioassay. The remaining regions of the chromatograms were arbitrarily divided up and prepared in a similar fashion for bioassay.

The bioassay used was the barley leaf senescence test developed by Kende (1964), and the procedures employed were similar to those described by that author. This assay is based on a cytokinin-induced chlorophyll retention in barley leaf segments, when they are incubated in the dark. In all assays 10 leaf segments of uniform size were used. Chlorophyll was extracted in absolute ethanol and measured at 665 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer. Owing to variations between assays performed at different times, the results are expressed as percentages of the control, when the control is taken as 100%. Maximum responses obtained under the conditions employed here were approximately 150% of the control, and were brought about by 2 mg/l of kinetin. In order to ascertain when the response of an assay differed significantly from the control, 10 cultures, each of 10 barley leaf segments, were incubated under the assay conditions and the chlorophyll was subsequently measured. The maximum difference between any two cultures was 10%, and so assay values in excess of 110% of the control were taken to indicate cytokinin activity.

Letham (1967) reported that this assay is not affected by indole-3-acetic acid, gibberellic acid, sugars, adenine, or other common constituents of plant extracts. We have found that it is not influenced by the non-cyclic monophosphates of adenine, guanine, uracil, and cytosine, at concentrations even in excess of 400 mg/l, and so the assay appears to be highly specific. High concentrations of inorganic salts, however, are able to mimic cytokinins in delaying chlorophyll breakdown (Letham 1967), and salts were present in some of the unchromatographed fractions. Positive results from these assays were therefore viewed with caution. Cytokinins were only considered present if activity could subsequently be demonstrated on chromatograms prepared in the manner described above, which involved solubility in absolute ethanol. To minimize the possibility of errors due to contamination by microorganisms, the cultures were maintained at least 1 week after the termination of the assay, and then examined. Mould developed in several cultures and those assays were repeated.

(c) *Culture of Algae*

(i) E. radiata.—Gametophytes of *E. radiata* were cultured, and their growth analysed, in the manner described by Jennings (1967). Kinetin, dissolved in distilled water, was added over a concentration range of 0·01–1·0 mg/l.

(ii) H. musciformis.—Branch apices of *H. musciformis*, each 2 cm in length, were cut from freshly collected plants. These were shaken vigorously in filtered sea water in order to dislodge contaminating organisms, and then placed in Petri dishes containing 100 ml of filtered sea water. Five segments were placed in each dish. Kinetin was added to cultures over a concentration range of 0·01–1·0 mg/l. Experiments were conducted for periods of between 2 and 4 days, during which time the cultures were maintained at 22°C and exposed to a light intensity in the region of 50 f.c., provided by "warm white" fluorescent tubes (Philips).

Table 1

Cytokinin activity of fractions prepared from *E. radiata* blade tissue and *H. musciformis* compared with that of kinetin

Biological assays with barley leaf segments were carried out as described in Section II. All fractions were assayed with material derived from 100 g of plant tissue added to 40 ml of culture solution. Values are expressed as percentage of control, with control = 100% (see Section II)

Alga	Cytokinin Activity of						
	Ethanol Fraction	Ethanol Fraction + Kinetin (1·0 mg/l)	NH_4OH Fraction	1·5N HCl Fraction	3·0N HCl Fraction	6·0N HCl Fraction	Kinetin (1·0 mg/l)
E. radiata	85	107	92	130	120	97	147
H. musciformis	91		98	126	70	90	

III. Results

(a) *Assay of Extracts from* E. radiata

It can be seen from Table 1 that the only fractions possessing cytokinin activity were the 1·5N and the 3·0N HCl fractions. The ethanol fraction contained material inhibitory to kinetin.

Chromatography of the 1·5N HCl fraction (Table 2) indicated that a band of R_F 0·1–0·5, which gave a positive purine reaction, could account for the activity of this fraction; eluates from the remainder of the chromatogram were inactive. Cytokinin activity was evinced by material from two regions of the 3·0N HCl chromatogram, these having R_F values of 0·1–0·3 and 0·7–1·0. Only the zone with R_F 0·1–0·3 appeared to contain purine.

A number of workers have detected cytokinins in the NH_4OH fraction from higher plants (Letham and Bollard 1961; Letham 1964; Maheshwari and Prakash 1967). It therefore seemed possible that inhibitors were masking the activity of cytokinins in the NH_4OH fraction from *E. radiata*. Eluates were prepared from five ultraviolet-light-absorbing bands and the non-absorbing bands between them on chromatograms of this fraction. Cytokinin activity could not be detected in them, and so it is reasonable to conclude that the algal NH_4OH fraction did not contain cytokinins.

Table 2

CYTOKININ ACTIVITY OF MATERIAL ELUTED FROM ZONES OF PAPER CHROMATOGRAMS PREPARED FROM THE 1·5N HCl AND 3·0N HCl FRACTIONS OF *E. RADIATA* AND THE 1·5N HCl FRACTION OF *H. MUSCIFORMIS*

Material was derived from 100 g fresh weight of *E. radiata* and 200 g fresh weight of *H. musciformis*. Biological assays carried out as described in Section II. Boundaries of zones eluted are indicated by horizontal lines. Values expressed as in Table 1

R_F	Cytokinin Activity of		
	1·5N HCl Fraction (*E. radiata*)	3·0N HCl Fraction (*E. radiata*)	1·5N HCl Fraction (*H. musciformis*)
0–0·1	No assay	No assay	No assay
0·1	Purine present Assay 124%	Purine present Assay 115%	Purine present Assay 107%
0·3		Purine absent Assay 103%	Purine present Assay 114%
0·5	Purine absent Assay 106%	Purine absent Assay 104%	Purine absent Assay 96%
			Purine absent Assay 99%
	Purine absent Assay 98%	Purine absent Assay 118%	Purine absent Assay 102%
1·0			

(*b*) *Assay of Extracts from* H. musciformis

From Table 1 it can be seen that only the 1·5N HCl fraction from *H. musciformis* possessed cytokinin activity. Chromatography indicated that this was associated with a single band of R_F 0·25–0·35 (Table 2), which gave a positive purine test. Activity detected was of a low level and could only be demonstrated with eluates from chromatograms prepared with 200 g of tissue, twice that used with *E. radiata*.

(*c*) *Effect of Kinetin on the Growth of Gametophytes of* E. radiata

Growth of gametophytes of this species occurs in four distinct phases, consisting of cell extension during zoospore germination and followed in turn by cell division,

cell extension, and finally further cell division (Jennings 1967). This situation is also clearly evident in Figure 1. Kinetin, particularly at the lower concentrations, stimulated cell division during the first division period, and 0·05 mg/l was the most

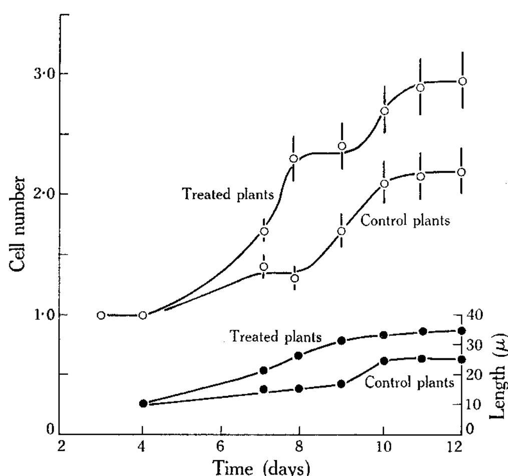

Fig. 1.—Effect of 0·05 mg/l of kinetin on the growth of *E. radiata* gametophytes. Vertical bars represent standard errors. ○ Cell numbers. ● Total lengths.

effective concentration (Fig. 1 and Table 3). Higher concentrations of kinetin were progressively less stimulatory and 1·0 mg/l was sometimes inhibitory. Cell length was not significantly affected by any concentration tested.

Table 3

EFFECT OF VARIOUS CONCENTRATIONS OF KINETIN ON THE TOTAL LENGTH, CELL NUMBER, AND CELL SIZE OF 12-DAY-OLD *E. RADIATA* GAMETOPHYTES

Values in parentheses are standard errors

Measurement	Control	Concentration of Kinetin			
		0·01 mg/l	0·05 mg/l	0·1 mg/l	1·0 mg/l
Length (μ)	25·1(3·1)	30·4(4·3)	33·0(5·0)	28·6(3·1)	23·8(2·5)
Percentage of control	100	121	131	114	95
Cell number	2·1(0·3)	2·6(0·5)	2·7(0·5)	2·2(0·2)	2·0(0·3)
Percentage of control	100	124	129	106	95
Cell length (μ)	12·0	11·7	12·2	13·0	11·9
Percentage of control	100	97	102	108	100

(*d*) *Effect of Kinetin on the growth of* H. musciformis

It is clear from Table 4 that kinetin stimulated growth in excised branch apices of *H. musciformis*. Usually this was most marked at the lower concentrations, but in some experiments 1·0 mg/l proved to be the most effective. It is not known to what extent cell division and cell elongation contributed to this response.

IV. Discussion

From the extraction experiments it appears that *E. radiata* contains at least two free cytokinins, while *H. musciformis* contains at least one. These cytokinins resemble the higher plant cytokinins in being basic and polar, as indicated by their partitioning properties in ethyl acetate–water at pH 3·0–3·3, and their ion-exchange properties. One of the active chromatogram zones from the 3·0N HCl fraction of *E. radiata*, and the single active zone from the 1·5N HCl fraction chromatogram of *E. radiata* and *H. musciformis*, was associated with material which forms a water-insoluble complex with silver ions and which yields a colour reaction similar to a purine. Material in these zones also absorbs ultraviolet light, as would be expected if purine was present. While conclusive evidence would involve chemical purification techniques, these data suggest that these algal cytokinins resemble, chemically, the cytokinins usually extracted from higher plants. Inability to detect purine associated

Table 4

EFFECT OF VARIOUS CONCENTRATIONS OF KINETIN ON THE GROWTH INCREMENTS OF BRANCH APICES OF *H. MUSCIFORMIS*

Standard errors are given in parentheses

Measurement	Control	Concentration of Kinetin		
		0·05 mg/l	0·50 mg/l	1·0 mg/l
Mean growth increment (mm)	0·89(0·15)	1·42(0·14)	1·20(0·17)	1·15(0·22)
Percentage of control	100	159	134	129

with one active eluate from the 3·0N HCl fraction chromatogram of *E. radiata* may indicate the presence of a cytokinin with a markedly different structure, though of course it is possible that concentrations of a purine on the chromatogram were too low to be detected by either the ultraviolet light or the silver nitrate–bromophenol blue reagent. The barley leaf bioassay is sensitive to concentrations of kinetin which may not be detected on chromatograms by either of these tests.

Bioassay data suggests that the levels of cytokinin in *H. musciformis* were substantially lower than in *E. radiata*, and this also appears to be the case for gibberellin-like materials extracted from these species (Jennings and McComb 1967; Jennings 1968).

The evidence that kinetin, a cytokinin with a purine ring system, is capable of stimulating growth in *E. radiata* gametophytes, and in branch apices of *H. musciformis*, suggests that endogenous cytokinins of similar chemistry are capable of growth regulation in these plants. In 1958, Provasoli raised the possibility that those metabolic regulators which are commonly associated with higher plants, the auxins, gibberellins, and cytokinins, may have played a role in the development of multicellular plants from more primitive forms. This hypothesis implies that these groups of regulators arose early in plant evolution. Evidence has now accumulated that they are involved in the growth and development of plants belonging to diverse groups, including the algae (e.g. van Overbeek 1940; Davidson 1950; Jacobs 1951;

Mowat 1965; Jennings and McComb 1967; Schiewer 1967; Jennings 1968; and the present paper). This suggests that they may, in fact, have arisen early in plant evolution, and lends support to Provasoli's hypothesis.

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