CYTOKININS IN ETIOLATED BARLEY LEAVES

By R. MENHENETT*† and D. J. CARR*

[Manuscript received 3 April 1973]

Abstract

By the use of solvent partition, ion-exchange column chromatography, and the soybean callus bioassay, cytokinin-like substances were detected in extracts of etiolated whole leaves and leaf segments of barley, *Hordeum vulgare* L. cv. Pallas. The active material was retained by a cellulose phosphate column at pH 3·1 (cation exchanger) but not by a DEAE-cellulose column at about pH 6 (anion exchanger). This is consistent with the view that the active substance(s) is an N^6-substituted purine derivative in the free base or nucleoside form. Preliminary attempts at characterization of the cytokinin(s) are reported.

Similar amounts of cytokinin(s) were found in extracts from subapical leaf segments or from whole leaves. No significant change in cytokinin level was apparent in segments extracted after 2, 4, or 8 hr in the dark following 10 min red light compared to those maintained in darkness throughout.

I. INTRODUCTION

The stimulus for the present investigation was the discovery (Menhenett 1972) that brief application of cytokinins to segments of first leaves of dark-grown barley seedlings was as effective in causing leaf unrolling as red light (Carr *et al.* 1972). Under the same conditions gibberellin was far less effective in substituting for red light although in other leaf unrolling systems gibberellic acid was fully effective (Carr and Reid 1966; Reid and Clements 1968; Reid *et al.* 1968; Carr *et al.* 1972). The effectiveness of cytokinins in the response led to the expectation that irradiation might increase the endogenous level of cytokinins in the leaves.

While cytokinins have been detected in leaves of dicotyledonous plants (Heide and Skoog 1967; Nitsch 1967; Engelbrecht *et al.* 1969; Letham and Williams 1969; Engelbrecht 1971), their presence has not yet been reported in leaves of a graminaceous species. A preliminary account of this work has been given by Carr *et al.* (1972).

We here show that cytokinins are natural constituents of etiolated barley leaves and leaf segments and present preliminary information on their possible identity. The effects of red light on the level of endogenous cytokinins in whole leaves or in leaf segments were also investigated.

* Department of Developmental Biology, Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra City, A.C.T. 2600.

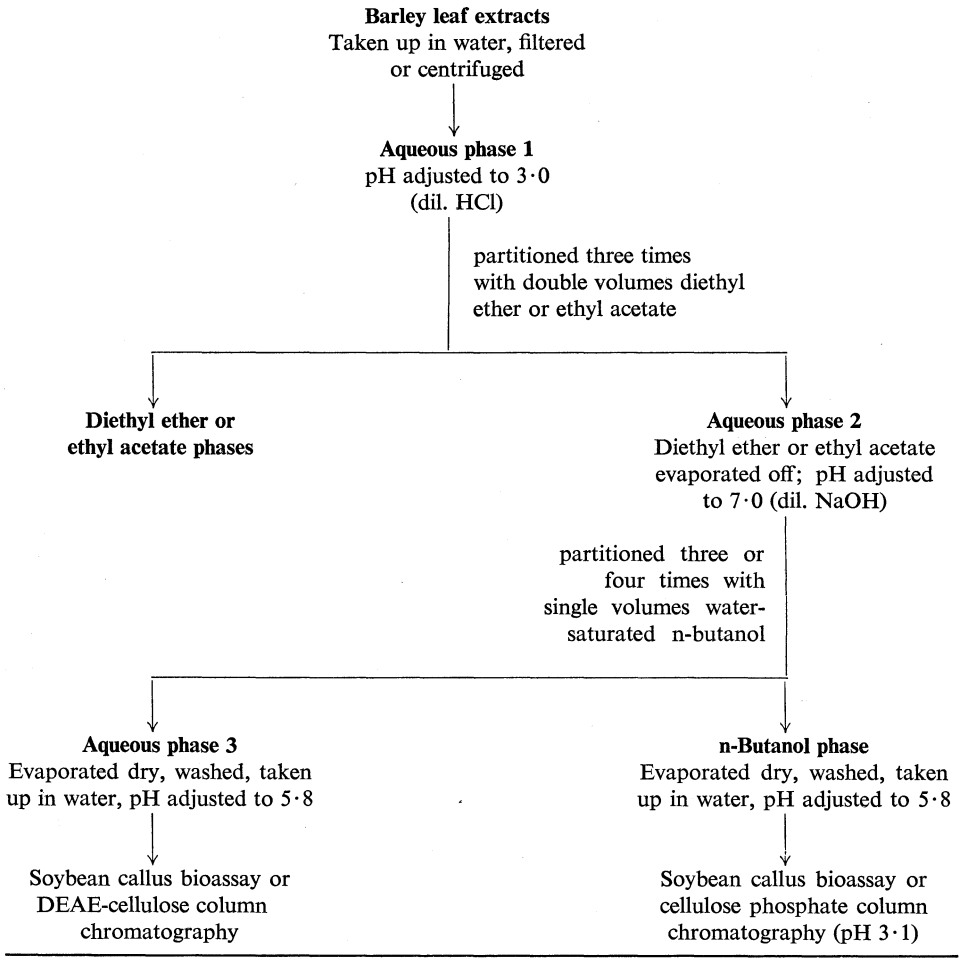
† Present address: Department of Botany and Microbiology, University College of Wales, Aberystwyth, Cards., SY23 3DA, U.K.

II. Materials and Methods

Barley seed (*Hordeum vulgare* L. cv. Pallas) was sown in plastic trays, about 1 cm deep in coarse river sand that had been washed with tap water and autoclaved for 1 hr. The sand was saturated with distilled water after sowing and the excess allowed to drain away. The trays were maintained at high humidity in a darkroom maintained at $25 \pm 1°C$ and the leaves harvested after 7 days. Harvesting was done with the aid of a dim green "safelight", consisting of a Philips 40-W MCFE "daylight" fluorescent tube with a filter of four sheets of primary green Cinemoid No. 39 and one of primary deep blue Cinemoid No. 20. These combined filters transmit maximally at 520 nm. About 10 cm above the bench the total energy level was $1 \cdot 7$ $\mu W/cm^2$.

Table 1

Flow sheet outlining the solvent partition procedures used in the preliminary purification of barley extracts



Red light was supplied by two Philips TL 20W/15 red fluorescent tubes with a filter of one sheet of Plexiglas 501 (red) 3 mm thick. At the level of the leaves the energy was $6 \cdot 5$ $\mu W/cm^2$.

Where whole leaves were used they were harvested by pulling them from the coleoptiles. Leaf segments were cut 3 cm long and 1 cm back from the tip. Whole leaves or segments were

stored on moist filter paper in large Petri dishes and maintained at full turgor. Leaves were given red light (10 min) in the dishes and then returned to darkness. After varying periods in the dark the leaves (or segments) were cut into approximately $\frac{1}{2}$-in. lengths and homogenized with ethanol in a 200 ml chamber of a "Sorvall" Omnimixer at 0°C for 10 min at speed setting 8. About 200 ml cold ethanol (70 or 85%) were used for 350 whole leaves (21·8 g fresh wt.). After homogenization extra ethanol was added and the whole left to stand for 22–24 hr at 25°C. Extracts were filtered, insoluble material washed, and the filtrates evaporated to dryness and stored at −15°C in the dark. Up to this point all procedures were carried out in the darkroom at 25°C. Subsequent procedures were carried out in the laboratory at room temperature, with full light excluded by keeping flasks wrapped in aluminium foil.

Extracts were subjected to solvent partitions (Table 1) and column and thin-layer chromatography.

Cellulose phosphate (Whatman, coarse fibrous) columns were prepared so that they were in the ammonium form at pH 3·1 and the DEAE-cellulose column so that it was in the bicarbonate form at about pH 6. The amounts of cellulose phosphate and DEAE-cellulose (medium fibrous) were calculated from a consideration of likely amino acid equivalents in the extracts and the nominal capacities of the celluloses (7·4 and 1·0 m-equiv/g respectively).

The cellulose phosphate column technique resembles that used by Letham and Williams (1969) to purify cytokinins from apple fruits. Adenine and adenine-like derivatives, such as nucleosides, in aqueous solution at pH 3·1 would be retained on the column and subsequently eluted by 0·2N NH_4OH. Nucleotides in aqueous solution at about pH 6 would be retained by the DEAE-cellulose column and eluted by 15% NH_4HCO_3.

Thin-layer chromatography was carried out on 20 by 20 cm plates coated with a layer of silica gel PF_{254} 1 mm thick. Before use all plates were twice run in ascending fashion in redistilled absolute methanol. Material to be chromatographed, usually in 85% ethanol, was streaked onto the origin. A blank plate was also chromatographed, to provide "blanks" for the kinetin calibration flasks. The solvent front was allowed to advance 15 cm from the origin before the plates were removed, dried in a stream of air, and viewed under u.v. light. Particular zones of silica gel were removed to conical flasks for elution. Elution techniques are described under particular experiments. After elution and removal of solvent the eluates were incorporated in bioassay medium. The soybean callus bioassay (Miller 1968) was used to test cytokinin activity. Three cubic pieces of callus (about 4 mm side) were placed on 30 ml of medium in each of three 50-ml conical flasks. The flasks were incubated in a constant temperature darkroom at 25°C. After 3–5 weeks the callus was weighed fresh.

III. Experimental Details and Results

(*a*) *Cytokinin-like Activity in Extracts from Whole Leaves Purified by Solvent Partitions and Ion-exchange Column Chromatography*

In preliminary experiments cytokinin activity was sometimes detected in the n-butanol fractions of extracts of whole leaves but not in aqueous phase 2 fractions (see Table 1). Cytokinin activity in these aqueous fractions was therefore masked by the presence of inhibitors and moved preferentially into n-butanol.

Two batches of whole 7-day-old etiolated barley leaves (395 in each, 24·57 g fresh weight) were harvested. One batch was given 10 min red light and returned to darkness, the other being kept as a control in darkness. After 2 hr each was extracted by homogenization in cold 70% ethanol. The crude extracts were subjected to solvent partitions (Table 1) and the n-butanol fractions (in water) adjusted to pH 3·1 and each run through a 1·5-g cellulose phosphate column in the ammonium form.

The fraction eluted from the column with 0·2N NH_4OH was bioassayed after drying and washing. Figure 1 shows that extracts prepared from leaves promoted growth of the callus. Irrespective of red light treatment the response was less than that produced by 10 μg/l kinetin. The fractions not retained by the cellulose phosphate

columns were also tested for their ability to induce callus growth. No promotion was observed at the concentrations tested.

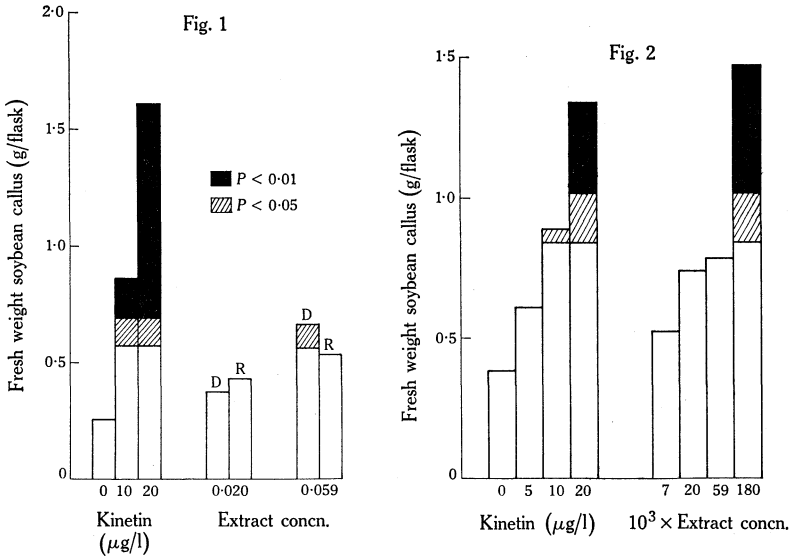


Fig. 1.—Cytokinin activity in the soybean callus bioassay, after 33 days, of the 0·2N NH_4OH eluate fractions from cellulose phosphate columns. Extract concentrations are expressed as grams fresh weight of tissue per millilitre of bioassay medium. *R*, whole leaves extracted after 2 hr in the dark following 10 min red light. *D*, whole leaves extracted after 2 hr in the dark. Significant differences from the control are indicated by shading. On a separate *t*-test the treatment 0·059*R* was significantly different from control at $P \leqslant 0{\cdot}05$.

Fig. 2.—Promotion of soybean callus growth, after 31 days, induced by the 0·2N NH_4OH eluate from a cellulose phosphate column. Whole leaves were extracted after 2 hr in the dark following 10 min red light. Extract concentration and statistical significance are expressed as in Figure 1.

A large-scale extraction of whole barley leaves was made. Leaves were harvested, exposed to red light as before, and macerated in 85% ethanol. In this experiment the initial solvent partition was made between the aqueous phase (pH 3·0) and diethyl ether. It was considered possible that some cytokinin-like material might partition into ethyl acetate (D.S. Letham, personal communication). The 0·2N NH_4OH eluate from the cellulose phosphate column induced considerable callus growth, the highest concentration giving a slightly larger growth increment than 20 μg/l kinetin (Fig. 2). It was apparent, since activity could occasionally be detected in the n-butanol fraction tested directly, but not in aqueous phase 2, that the latter contained inhibitory material, much of which would remain in aqueous phase 3. Purification of aqueous phase 3 (from the experiment presented in Fig. 2) was therefore attempted by DEAE-cellulose chromatography. The NH_4HCO_3 eluate and fraction not retained by the column were tested in the callus assay. No significant promotion of callus growth was observed (data not presented).

The question whether cytokinin activity was an artifact of heat treatment during autoclaving of the medium was answered in the negative by filter sterilization of some of the 0·2N NH_4OH eluate from the cellulose phosphate column experiment described

above. A "Kimax" sintered glass 20UF filter was used and the following results were obtained:

Extract concn. (g fresh wt. tissue/ml medium)	Fresh wt. soybean callus (g) after 31 days
0	0·144
0·171	0·409

This response of soybean callus to the filter-sterilized extract was highly significant ($P < 0{\cdot}01$).

Information on the chromatographic behaviour of the cytokinin-like substance(s) was sought. Material eluted from a cellulose phosphate column and equivalent to 28·38 g fresh weight of etiolated barley leaves was chromatographed as described earlier. Zeatin and zeatin riboside were co-chromatographed with the extract on a 3-cm strip at the edge of the plate. The plate was then resolved with n-butanol : conc. NH_4OH : water (300 : 50 : 100 v/v) (top phase only) as solvent. A blank plate was run in the same tank. None of the u.v.-absorbing zones visible after resolution corresponded to the positions of zeatin or its riboside. On the basis of this examination zones were cut out (allowing a 2-cm margin of safety from the position on the origin where zeatin and its riboside were co-chromatographed) and eluted as follows. The powdered silica gel from each zone was transferred to a flask with 30 ml of redistilled absolute methanol, shaken, and left overnight at room temperature. Then each flask was re-shaken, filtered, and washed with two successive 10-ml aliquots of methanol. After filtration and evaporation the material in each flask was taken up into a hot soybean callus medium (minus agar), shaken, left overnight, and then distributed to the treatment flasks. Zones from the blank plate were treated in the same way. The treatment flasks were then inoculated with callus. The results (Fig. 3) show that the eluate from only one zone, R_F 0·62–0·72, significantly promoted soybean callus growth, to about the level of 10 μg/l kinetin. This zone did not correspond to zeatin, zeatin riboside, or to any zone exhibiting u.v. absorbance. In a later experiment N^6-(Δ^2-isopentylamino)purine (2iP) ran at R_F 0·64–0·73 when chromatographed under the same conditions. The positions of 2iP and its riboside (2iPA) are shown in Figure 3.

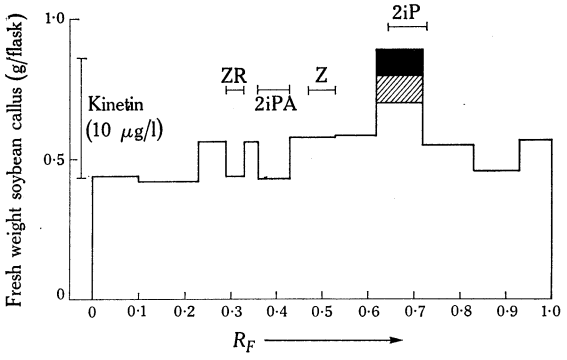


Fig. 3.—Response of soybean callus, after 31 days, to eluates from a thin-layer chromatogram (1 mm silica gel PF_{254}). Material loaded onto the plate was the NH_4OH eluate from a cellulose phosphate column. Whole leaves were extracted after 2 hr in the dark following 10 min red light. ZR = zeatin ribonucleoside; Z = zeatin; 2iP = N^6-(Δ^2-isopentylamino)purine; 2iPA = ribonucleoside of 2iP. ZR, Z, and 2iP were co-chromatographed with the extract; the R_F of 2iPA was determined in another experiment. Statistical significance indicated as in Figure 1.

In the next experiment leaves (70·53 g fresh weight) were treated (i.e. given 10 min red light and extracted after 2 hr more in the dark) and the extracts purified

exactly as before. A portion of the 0·2N NH_4OH eluate fraction was chromatographed. Zeatin and 2iP were run from a 3-cm strip of the origin. After chromatography in the same solvent only four zones were marked and removed for elution. These were R_F 0·48–0·55 (zone A), 0·55–0·64 (zone B), 0·64–0·73 (zone C), and 0·73–0·81 (zone D). Zeatin and 2iP corresponded to zones A and C respectively. It was expected that the activity would occur at or near the position of 2iP. The elution technique was as follows. Each zone was placed in a conical flask with 50 ml of 80% methanol and stirred with heating overnight. Then the methanol was decanted off into filter funnels (Whatman No. 42 paper), and a further 10 ml methanol added to the flasks, which were shaken and the liquid decanted off. After addition of another 50 ml of methanol the flasks were stirred and warmed for about 6 hr. A further decantation and washing and a final wash of the filter funnels with warm 80% methanol completed the elution procedure. The eluates were taken up in a bioassay medium.

Zones from a blank plate were treated similarly to provide a series of flasks containing 0, 5, 10, and 20 μg/l of kinetin.

The main feature of the experiment was that the significant cytokinin-like activity was now located at the same zone to which zeatin co-chromatographed (Fig. 4). None was detected at the position of 2iP (zone C).

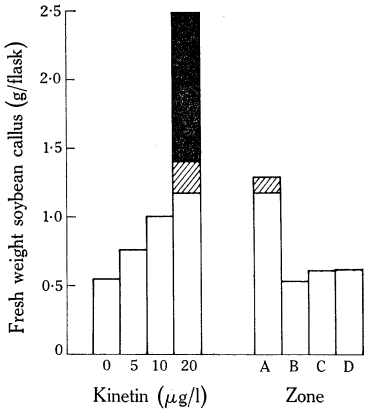


Fig. 4.—Response of soybean callus, after 32 days, to eluates from four zones [see Section III(*a*)] of a thin-layer chromatogram (1 mm silica gel PF_{254}); the extract chromatographed corresponded to 27·29 g fresh weight of leaves. Other details as for Figure 3.

(*b*) *Evidence for Cytokinin-like Substances in Etiolated Barley Leaf Segments and Attempts to Detect Changes in Cytokinin Content after Red Light*

Since the young leaf of a monocotyledon such as barley has a basal (intercalary) meristem active in cell division (Esau 1959), it appeared possible that the extracted cytokinin-like material might be derived predominantly from this region of the leaf. Thus a demonstration of cytokinins in subapical leaf segments was attempted. Information on whether the level of extractable cytokinins changed at times after red light was also sought.

Unrolling of segments of etiolated barley leaves is phytochrome-dependent. In concurrent experiments, unrolling in the absence of red light was maximally induced by cytokinins but not by gibberellins. Thus red light could conceivably cause leaf unrolling by increasing the endogenous cytokinin level.

Segments were cut from the subapical regions of 7-day-old dark-grown barley leaves and given 10 min red light. They were then placed in darkness and extracted with 85% ethanol 2, 4, and 8 hr after the red light treatment. Batches of leaf segments which had not received red light were similarly extracted. Leaf segments 3 cm long unroll after brief exposure to red light (Menhenett 1972) although the response measured at 25 hr is not as great as that exhibited by 1-cm segments (Carr *et al.* 1972).

After partitioning (initially with diethyl ether) and cellulose phosphate chromatography of the n-butanol fraction, the dried NH_4OH eluates were tested on soybean callus. All the extracts strongly enhanced growth of the callus but the promotion by extracts from red light-treated leaf segments was not significantly different from that of the corresponding leaf segments receiving no red light (Fig. 5). Nevertheless, the cytokinin activity per gram fresh weight of leaf segment was about the same as that of whole leaves.

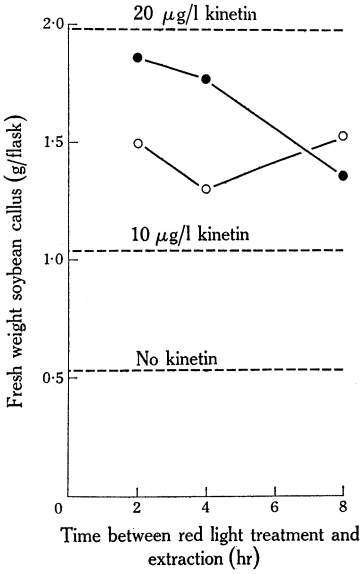


Fig. 5.—Promotion of soybean callus growth, after 35 days, by extracts of barley leaf segments. Leaf segments were extracted after 2, 4, or 8 hr in the dark following 10 min red light (○) or after the same periods in the dark (●). Material tested was the NH_4OH eluates from cellulose phosphate columns. Extract concentration for each treatment was 0·149 g fresh weight of tissue per millilitre of bioassay medium.

IV. Discussion

Cytokinin-like substances have been detected in extracts from etiolated whole barley leaves and leaf segments. This may be the first report of cytokinin activity in leaves of a monocotyledonous species. Miller and Witham (1964) found activity in combined stem and leaf tissue of maize.

It seems unlikely that the active substance in the experiments described was an artefact produced by the extraction procedures. These were in fact very mild and the activity was also clearly detected when an extract was filter-sterilized rather than autoclaved.

The behaviour of the barley leaf cytokinin-like activity in solvent partitioning and column and thin-layer chromatography is consistent with the idea that it was due to an N^6-substituted adenine derivative. The lack of cytokinin activity in the fraction

eluted from the DEAE-cellulose column suggests the absence of a nucleotide cytokinin in the aqueous phase fraction of the barley leaf extract. It is possible, of course, that any cytokinin activity present is masked by inhibitory material.

In one experiment the cytokinin activity had an R_F similar to that of 2iP, while in the second it was detected in the zone to which zeatin was resolved. These results suggest that the cytokinin(s) in barley leaves may be similar to zeatin or 2iP.

No evidence was obtained that the level of extractable cytokinin differed in leaf segments given red light compared to those kept in the dark. There is certainly nothing resembling the considerable change in gibberellins demonstrated by others in barley and wheat leaves (Reid *et al.* 1968; Loveys and Wareing 1971). More recently, however, it has been shown that in seeds of *Rumex* cytokinin levels were markedly increased following exposure to red light (Staden *et al.* 1972).

V. Acknowledgments

We thank Dr. D. S. Letham for advice on the purification of plant extracts, for useful discussion, and gifts of natural cytokinins. Dr. K. G. M. Skene, Division of Horticultural Research, CSIRO, Adelaide, kindly gave us the strain of soybean callus used. One of us (R. M.) gratefully acknowledges a research scholarship from the Australian National University.

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