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KINETINLIKE FACTORS IN THE ROOT EXUDATE OF SUNFLOWERS

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The existence of hormones which originate in the root and act in the shoot was postulated many years ago. Went and Bonner found a drastic reduction of stem growth when the roots of tomato plants were removed.[1] This inhibition could be partly reversed by supplying the plant with coconut milk, now known to be rich in kinetinlike compounds.[2] Went suggested that a hormone (caulocaline) is produced in the root and translocated to the shoot where it is essential for stem growth.[3] A different approach led Chibnall to the conclusion that root hormones play an important role in plant metabolism.[4] He found that the protein content of detached leaves declines, leading to their rapid senescence and death. Normal protein metabolism, however, could be re-established if adventitious roots developed on the leaf cuttings. Chibnall therefore concluded that a root factor is necessary to maintain proper protein levels in leaves. Richmond and Lang showed that application of 6-furfurylaminopurine (kinetin) delays protein and chlorophyll loss in detached primary leaves of *Xanthium pennsylvanicum*.[5] Osborne suggested that kinetin might act like Chibnall's root hormone,[6] while Skoog and Miller observed that kinetin induced bud elongation in tissue culture in the absence of roots, thus substituting for the required hypothetic caulocaline.[7] Kulaeva presented circumstantial evidence that a kinetinlike root factor is translocated to the shoot through the xylem.[8] She also tested crude root exudate of tobacco plants for its capacity to retard senescence of isolated leaves. The response obtained was marginal but sufficient to encourage the search for kinetinlike factors in xylem sap. Substances retarding chlorophyll degradation were recently found in the bleeding sap of vine and root exudate of sunflowers.[2, 9]

Materials and Methods.—Plants: Sunflower plants (*Helianthus annuus*, local commercial variety) were grown to 50–60 cm in vermiculite under natural greenhouse conditions. Half-concentrated Hoagland solution was given twice a week and deionized water when additional irrigation was needed. Barley (*Hordeum distichum*, var. Delta) was soaked overnight and sown in vermiculite. The plants were kept at 20°C under continuous illumination for 13 days and watered every other day alternately with deionized water and half-strength Hoagland solution.

Collection of exudate: Sunflower plants were topped just below the cotyledonary node, and the stumps were fitted with rubber tubes. Exudate was collected into vials which were emptied 3–4 times daily. The sap was frozen immediately after each collection and subsequently lyophilized.

Paper chromatography: The lyophilized material was dissolved in small volumes of water and streaked on Whatman no. 3 paper. The following solvents, either of chromatographic grade or

redistilled, were used: solvent A: n-butanol-acetic acid-water (4:1:1, v/v), ascending over 20 cm; solvent B: sec. butanol-acetic acid-water (70:2:28, v/v), descending over 30 cm.[10]

Bioassays: A bioassay using the capacity of kinetin to delay chlorophyll degradation was described previously.[9] One-cm sections of fully expanded first leaves of barley were floated on test solutions for 48 hr and then extracted in 80% ethanol. Chlorophyll content was determined by measuring the optical density of the extracts at 665 mμ. In this assay kinetin gave a linear response at concentrations ranging from 0.003 to 3 μg/ml if plotted on a logarithmic scale (Fig. 1). Sucrose and glucose (0.1–20 gm/l), casein hydrolyzate (1–10 gm/l), inorganic nitrogen sources (ammonium nitrate, ammonium sulfate, potassium nitrate, all up to 0.1 *M*), and White's medium did not retard chlorophyll degradation. Adenine (3 and 30 mg/l), indole-3-acetic acid (3 and 30 mg/l), and gibberellic acid (1–100 mg/l) were also inactive. High salt concentrations, however, retarded chlorophyll degradation, as observed by Sachs and Thimann.[11] This effect always appears above a concentration of 0.15 *M* irrespective of the solute's nature. Cryoscopic measurements showed that solute concentrations were well below the critical level in all chromatographic fractions, thus excluding the possibility of an artifact caused by salts of the xylem exudate.

Each fraction also was tested for substances which stimulate cell division in tissue cultures of soybean callus (*Glycine max*, var. Lee or Acme). This assay specifically detects kinetin-like compounds, as described by Miller.[12] Test solutions were added to 30 ml of nutrient medium either before or after autoclaving; in the latter case, small volumes of the test solutions were sterilized by Seitz filtration. Autoclaving neither destroyed nor enhanced the activity of the fractions which stimulated cell division. All fractions were tested in two or three replicates on four calluses cultured in 100-ml Erlenmeyer flasks.

Cell counts: Callus tissue was left for 24 hr in a mixture of 10% nitric acid and 10% chromic acid (1:1, v/v), and macerated by gentle agitation with a glass rod. The washed cells were stained with orcein and counted on a haemocytometer.

Results.—Root exudate chromatographed with solvent A yielded two zones capable of retarding chlorophyll

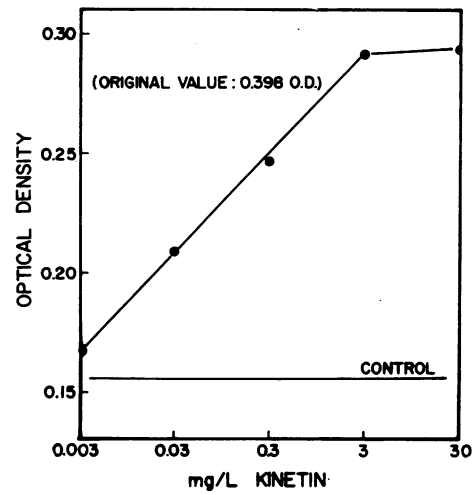

FIG. 1.—Dose response curve of kinetin in the chlorophyll-retention assay using barley leaf sections. Each point represents the average value of four replicates.

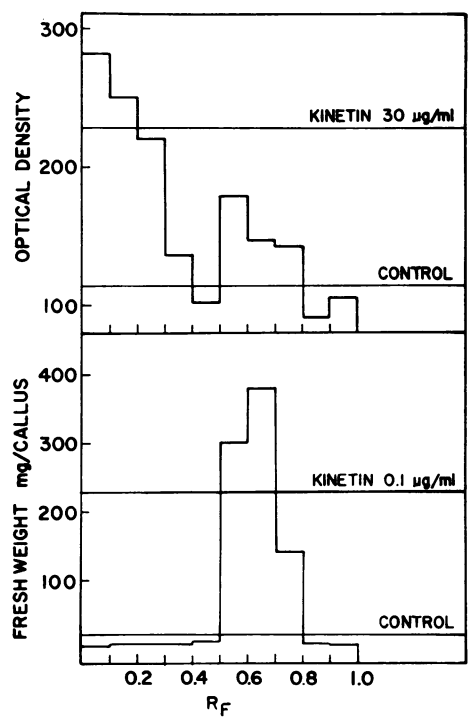

FIG. 2.—Paper chromatogram of root exudate developed with solvent A. Each fraction was tested in duplicate. *Top*: Chlorophyll assay, 88 ml of exudate. *Bottom*: Soybean-callus assay, 85 ml of exudate.

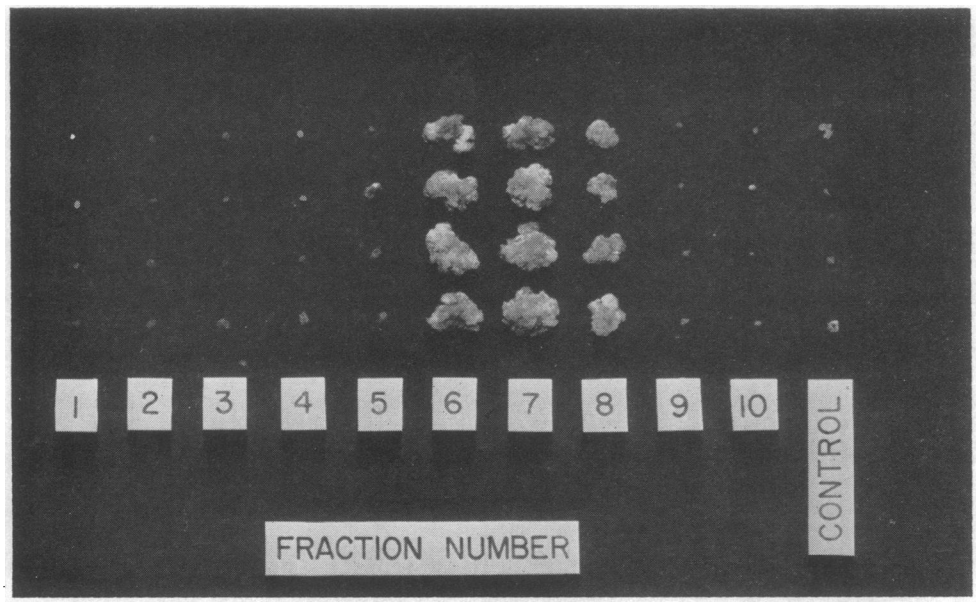

FIG. 3.—Cell division in soybean calluses induced by factor II obtained from 42 ml of exudate. The chromatogram was developed with solvent A. Fraction *1* corresponds to R_F 0.0–0.1, fraction *2* to R_F 0.1–0.2, etc. Calluses were grown for five weeks at 25°C.

degradation and senescence in barley leaf sections (Fig. 2, top). The active substance at R_F 0.0–0.3 will be called factor I, the active substance at R_F 0.5–0.8 factor II. After chromatography with solvent A, only factor II showed kinetinlike activity in the cell-division assay (Fig. 2, bottom; Fig. 3); with solvent B, factor I moved to R_F 0.2–0.4, and factor II to R_F 0.6–0.9. Eluates from both zones of the chromatogram retarded chlorophyll degradation. In the cell-division assay, factor II showed high activity but factor I consistently induced some callus growth as well (Table 1).

After chromatography with solvent A, factor I never promoted cell division. Rechromatography of factor I with solvent B, however, always yielded cell division activity in the region where factor II used to be (R_F 0.6–0.9), as well as a much smaller peak of activity at R_F 0.2–0.4. Factor II could apparently arise from factor I during repeated chromatography, elution, and prolonged handling. The possibility of factor I being converted to factor II by hydrolysis was tested: 210 ml of exudate from 20 plants were chromatographed with solvent A. The eluate containing factor I was dried, taken up in 3 ml of *N* hydrochloric acid, and heated at

TABLE 1

CONCENTRATION OF THE ROOT FACTORS IN THE EXUDATE*

	Kinetic Equivalents (μg/l)			
	Chlorophyll Assay		Cell-division Assay	
Day	Factor I	Factor II	Factor I	Factor II
1	4.4	5.2	3.6	13.2
2	3.4	2.8	1.8	7.2
3	3.0	4.4	2.4	11.4
4	3.4	3.0	3.6	10.2

* On the first through the fourth day after topping of the plant. Estimates were made by means of the chlorophyll-retention and the cell-division assay. Exudate for the chlorophyll assay was chromatographed with solvent A, for the cell-division assay with solvent B.

90°C for one hr. The hydrolyzate was neutralized, rechromatographed with solvent A, and cell division promoting activity was detected at R_F 0.5–0.8. The histogram of this chromatogram was like the one shown in Figure 2 (bottom) which was obtained after chromatographing the original xylem exudate. These results suggest that factor I is a "bound form" of factor II. Conversion of factor I to factor II was repeated several times, as were all experiments described here. Cell counts of macerated tissue confirmed that callus growth was due to cell division rather than to cell enlargement.

In time-course experiments root exudate was collected for four days, and the concentration of factors I and II was determined for each day's collection. The concentration of both factors appears to be fairly constant over this interval whether estimated by the chlorophyll-retention or cell-division assay (Table 1). Fluctuations such as recorded in Table 1 are within the range of experimental error and are mainly due to the semiquantitative nature of the bioassays. Since the rate of exudation did not decrease over four days, the absolute amounts of factors I and II in the sap stayed constant as well. The concentration of factor II in the exudate as estimated by the chlorophyll assay was fairly low (0.003–0.005 mg/l kinetin equivalents). Values derived from the cell-division assay were usually to the order of 0.1–0.3 mg/l kinetin equivalents (Fig. 2), but lower values were occasionally obtained (Table 1). The sensitivity of the soybean-callus assay to factor II was higher than that of the chlorophyll-retention test. The activity of factor I in the cell-division assay was comparable to a kinetin concentration of 0.003–0.005 mg/l (Table 1).

In all experiments, root exudate was collected frequently and, at least during the first 24 hr, the sap was clear and apparently uncontaminated. Nevertheless, it was important to prove that neither factor I nor II was produced by microorganisms developing in the exudate after the plants were topped. For this purpose, exudate was collected continuously during the first two hr after topping and immediately frozen. This exudate, in which microbial growth was prevented, contained both active fractions at high concentration.

Factors I and II are translocated in the xylem. Removal of the phloem at the cut end of the stump and fitting the rubber tube over the secondary xylem and pith did not reduce the amount of factors I and II in the exudate.

Discussion.—The capacity of factor I to induce cell division was very low and only detectable after chromatography with solvent B. Lack of activity after chromatography with solvent A might have been due to the presence of inhibiting substances. After hydrolysis, factor I was converted to a substance with high cell division stimulating activity which, chromatographically, behaved like factor II. Factor I might thus be a bound form of factor II. This would be in accord with similar findings by Loeffler and van Overbeek for the kinins of coconut milk,[2] the formation of benzyl adenosine in *Xanthium* leaves,[13] and the presence of kinin glycosides in crown gall tumors.[14]

Jablonsky and Skoog reported that a compound diffused from isolated phloem bundles into agar which induced cell division in adjacent callus tissue.[15] In the girdling experiments described above, leaching of any active substances from the sieve tubes into the sap was prevented, but the exudate contained both active fractions at unreduced concentrations.

Haberlandt attributed the development of wound callus to the formation of a "wound hormone" in injured cells.[16] Washing the cut surface removed this cell division stimulating substance and no callus growth took place. Since root exudate was obtained from cut stems, the possibility remained that its cell division factors were formed in response to wounding. Two arguments speak against this assumption. When the cut surface of the stem was washed for four days by the exudate, the concentration of factors I and II did not decrease (Table 1), while Haberlandt removed the "wound hormone" by rinsing the injured surface, after which no hormone formation took place. Moreover, factors I and II were demonstrated in extracts of freeze-killed sunflower roots, by chromatography and bioassays.[17] This proves that root factors are contained in the roots and are not produced in response to injury. All available evidence favors the assumption that factors I and II are synthesized in the root and not simply recirculated from the shoot via the root system. Removal of the roots causes a deficiency of "root factors" in the leaves and leads to aging processes.[4, 18] This indicates that the root is the original source of hormones needed to maintain protein metabolism. It is improbable that factors I and II are originally formed in the shoot and simply stored in the roots. Parthier found that the capacity of detached leaves to synthesize proteins increased again as soon as the first root primordia were visible on the petioles.[19] These very young roots are not storage organs but most probably supply the leaves with the factors needed for protein synthesis. The fact that the concentration of the root factors in the exudate remains constant over four days (Table 1) suggests that they are continuously formed in the root.

Since factors I and II delay senescence in leaf sections, they very likely fulfill the function of Chibnall's root hormone in regulating protein metabolism of leaves. While the role of root kinins as growth hormones (caulocaline) has not been studied yet, indirect evidence[1, 7] suggests that they are needed for stem growth as well. The importance of the root as a hormone-supplying organ is further stressed by recent reports on the presence of gibberellins in root exudate.[20, 21] If roots are exposed to unfavorable environmental conditions such as salinity, water stress, lack of aeration, and high temperature, shoot growth is retarded. One possible reason for this inhibition might be a reduced supply of root hormones to the shoot. Went already suggested that insufficient aeration of the roots lowers "caulocaline" formation which in turn retards stem growth.[3] Weiss and Vaadia found that the concentration of kinetinlike factors in the root exudate of sunflowers was markedly lowered if the plants were water-stressed prior to reirrigation.[22] This fits the observation of Shah and Loomis that the kinin 6-benzylaminopurine (benzyladenine) preserves RNA and protein synthesis in water-stressed plants.[23] Livne and Vaadia recently showed that kinetin and gibberellic acid given through the vascular system induce opening of stomata.[24] It is thus possible that the supply of root hormones in the transpiration stream plays some role in regulating stomatal movements.

Further purification of the kinetinlike root factors and studies on their physiological action are currently under way.

Summary.—Two kinetinlike factors (I and II) were obtained from root exudate of sunflowers by means of paper chromatography. Their biological activity was estimated with a chlorophyll-preservation and cell-division test. Available evidence suggests that both factors are synthesized in the root and translocated via xylem

to the shoot. Factor I apparently can be converted to factor II by hydrolysis. The significance of these root factors to shoot metabolism is discussed, especially with regard to the influence of the root environment on the supply of these hormones.

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EVIDENCE FOR THE LOCALIZATION OF PLASTOCYANIN IN THE ELECTRON-TRANSPORT CHAIN OF PHOTOSYNTHESIS

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A reversible light-induced absorbance change near 600 mμ was observed recently in green plants and attributed to the functioning of the copper protein plastocyanin.[1] This light-induced increase of absorbance was linked to oxidation of plastocyanin. Since red and far-red actinic illumination had antagonistic effects on this change, it was suggested that plastocyanin served as a link between the two photochemical systems of photosynthesis. Far-red light (713 mμ) absorbed