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Modification of the Cytokinin Promotion of Deoxyisoflavone Synthesis in Soybean Tissue[1]

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ABSTRACT

Soybean tissue incubated in liquid media formed daidzin in response to cytokinin when the media contained 0.1 M sucrose but formed another, unidentified compound when the media contained 0.6 M sucrose or mannitol. The cytokinin effect in either setup was detectable only after a lag period of several hours. Addition of possible precursors of the compounds being synthesized or of related compounds did not alter the lag period to any great extent. *Trans*-cinnamic acid did perhaps shorten it a little as did preincubation of the tissue in the basal medium before the addition of kinetin. Several inhibitors of RNA synthesis substantially reduced the production of daidzin and the other compound. Protein synthesis inhibitors were also effective except that cycloheximide and puromycin actually promoted synthesis of the unknown compound while inhibiting that of daidzin. The results do not give a clear impression as to how the cytokinins are involved in the synthesis of the compounds, but are at least suggestive that cytokinin is involved in RNA or protein synthesis.

Certain soybean callus tissue cultures are very responsive to cytokinins and may require exogenous supplies of the compounds in order to continue growth or even to stay alive (6). These tissues therefore should be useful in studying the mechanism(s) of cytokinin action. One approach in such studies is to examine metabolic effects of the cytokinins in the hope of finally pinpointing the exact site(s) of action. The promotion of deoxyisoflavone synthesis, especially that of the glucoside daidzin, by cytokinins in soybean tissue has been reported (7). This promotion was apparent only after an incubation period of several hours and seemed to be accompanied by a small amount of growth. These observations were suggestive of a dependence on growth and perhaps on an essential involvement of a nucleic acid-protein synthesis sequence. Experiments to test these and other possibilities have been performed, and the results are presented in this paper.

MATERIALS AND METHODS

The procedures used were essentially those outlined previously (7). Briefly, callus tissue cultures of *Glycine max* (L.) Merrill, cv. Acme, were chopped into fine pieces with a scalpel and 1 g of the tissue was added to 20 ml of the incubating medium. The basal incubating medium (pH 5.8) contained (mg/liter): KH_2PO_4, 300; KI, 0.8; α-naphthalene acetic acid, 2; and sucrose, 30,000. The tissue and media were kept sterile throughout the experiment. The 125-ml flasks containing the medium and the tissue were shaken on a gyrotory shaker for 44 to 48 hr at 29 C, and then 80 ml of 95% ethanol were added to each flask. The synthesized compounds, which had remained to a large extent inside the cells during incubation, now reached equal concentrations inside and outside the cells. After standing overnight in the refrigerator, the tissue was filtered off and the remaining debris was removed by centrifugation. Absorbance was read at 232 and 260 nm. The reading at 260 nm serves as an indicator (not absolute) of the amount of daidzin present and that at 232 nm as an indicator of another compound discussed below. To make certain that these indices were valid in each experiment, difference spectra were obtained for appropriate pairs of extracts by use of a Cary 14 spectrophotometer.

RESULTS

High Sucrose. In attempts to divorce the stimulation of daidzin formation from promotion of growth, the concentration of sucrose in the incubation medium was increased from 0.1 M to higher values. In 0.6 or even 1.1 M sucrose, stimulation of daidzin formation by kinetin (6-furfurylaminopurine) was very much reduced or completely eliminated, but the kinetin promoted the formation of another compound. The difference spectrum obtained by comparing an extract of a culture containing 0.1 M sucrose in the incubating medium with an extract of another culture differing only by having 2 μM kinetin in addition showed the expected peak of daidzin at 262 nm (Fig. 1). When 0.6 M sucrose was used, however, the difference spectrum showed peaks at 232, 281, and 285 nm. Essentially the same results were obtained when mannitol was used to increase the molarity (with 0.1 M sucrose still present). An absorption spectrum for a band of material obtained by chromatography of the alcoholic extract of the kinetin-treated culture had features identical to those of the difference spectrum; the main features of the difference spectrum therefore represent a distinct type of compound. Making the alcohol extract alkaline by addition of sodium hydroxide caused the appearance of a soluble pink substance with absorption peaks at 498 and 530 nm; the chromatographed material showed the same reaction. This reaction was much weaker with extracts from cultures in 0.1 M sucrose. The intensity of the pink color is strong enough to permit one to use it as a quantitative indicator of cytokinin action. The new compound has not been identified, but its presence has been looked for in all experiments by examining the difference spectra. Even with low molarities of sucrose a little of the compound seemed to be present in most experiments. An important point at the mo-

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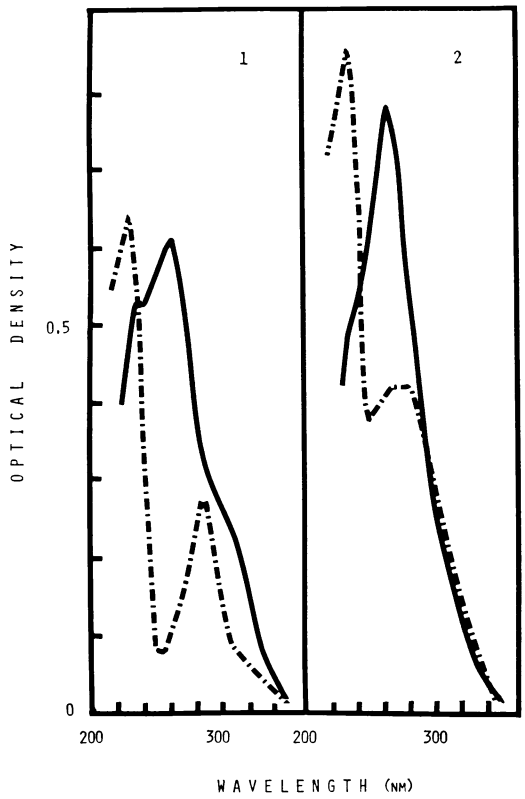

FIG. 1. Difference spectra between ethanolic extracts of control cultures and those with 2 μM kinetin. Solid line: media contained 0.1 M sucrose; broken line: media contained 0.6 M sucrose.

FIG. 2. Difference spectra between ethanolic extracts of control soybean cultures and those with 2 μM kinetin. All media contained 0.1 M sucrose. Solid line: usual media; broken line: media contained 0.2 mM cycloheximide.

ment and for the purposes of this investigation is that kinetin promoted synthesis of a compound even though conditions were quite unfavorable to growth. It is highly unlikely that the formation of the compound, and therefore the kinetin effect, is dependent upon growth.

The Lag Period. A lag period of several hours before the kinetin effect could be detected has already been reported (7). This lag is obtained with either 0.1 or 0.6 M sucrose. I have tried to shorten this lag period in various ways. Since the delay could be due to an inadequate supply of precursors of the compounds being synthesized, several possible daidzin precursors (some admittedly remote) or related compounds have been tested by adding them to the incubation media. These include L-phenylalanine; L-tyrosine; shikimic acid; caffeic acid; chalcone; *trans*-cinnamic acid; *p*-hydroxybenzoic acid; *o*- and *p*-hydroxycinnamic acids; quinic acid; ribose; xylose; L-tryptophan; and citric, fumaric, malic, α-ketoglutaric, and succinic acids. Malonic acid, which is quite promotive when the tissue is cultured on the surface of an agar gel (7), did not consistently increase synthesis in liquid media either with or without kinetin but did give some increase in a few experiments. The only compound which usually seemed to promote synthesis in liquid cultures (with either 0.1 or 0.6 M sucrose) was *trans*-cinnamic acid (10 or 20 mg/liter), and in some experiments it may have shortened the lag a little. This may have been more apparent than real, however, since the time of beginning of synthesis may not have changed but rather the rate of synthesis once started may have been sufficiently greater to allow detection at an earlier time than in experiments not using cinnamic acid.

The delay in appearance of the kinetin effect could depend on other events which must occur in the tissue after it has been chopped up and switched from a semisolid medium to a liquid one. With this in mind, the tissue was incubated in the liquid medium for several hours before the kinetin was added; this seemed to shorten the lag a little. By combining the use of cinnamic acid and the preincubation procedure and by reading the absorption of the extracts at 232 nm (at which wavelength the kinetin effect could be seen a little earlier than at 260 nm), lag periods as short as 6 hr have been obtained. This is, of course, long enough for many events to occur.

Protein and RNA Synthesis Inhibitors. An ever present question in cytokinin studies is whether or not the cytokinins are directly involved in some step of nucleic acid or protein synthesis. The long lag period observed in the present experiments is suggestive of this, and the failure of high solute concentrations to stop the cytokinin effects does not necessarily mean that the possibility can be eliminated (see Ref. 4, for example). Furthermore, in another publication from this laboratory, evidence will be presented that kinetin does stimulate protein synthesis in the soybean cells during the lag period. For these reasons, inhibitors of both nucleic acid and protein synthesis have been added to control and kinetin-containing media. A summary of many of the results is given in the two tables. As may be seen in Table I, actinomycin D was quite effective in

Table I. *Kinetin Stimulation of Formation of Ultraviolet-absorbing Compounds and Inhibition by Nucleic Acid Synthesis Inhibitors*

Ethanolic extracts of soybean suspensions cultured for 44 to 48 hr in media with or without 2 μM kinetin were read at the indicated wavelengths; the differences in absorbance provided the control values for the kinetin effect. A similar set of values was obtained for media with the inhibitor present, and the percentage inhibition was computed.

Inhibitor	Experiment[1]	Concn	Inhibition of Kinetin Effect	
			232 nm	260 nm
		μM	%	
Actinomycin D	A	0.08	17	18
		0.8	30	21
	B	8	19	74
		16	100	101
8-Aza-guanine	A	33	24	73
	B[2]	33	87	...
6-Aza-uracil	A	9	10	17
		45	11	27
		89	15	37
5-Fluorouracil	A	2000	86	89
5-Fluorodeoxyuridine	A	100	83[3]	83[3]
	A[2]	100	47	...
	B	100	71[4]	69[4]
6-Methylpurine	A	0.001	−9	−5
		0.01	−3	−1
		0.1	−2	20
		1	63	76
		10	109	100
Abscisic acid	A	1.5	5	34
		7.6	23	41

[1] Only one inhibitor was tested in any one experiment.

[2] In 0.6 M sucrose; 0.1 M sucrose was used in all others.

[3] Reduced to 30 and 61 by inclusion of 1 mM uridine.

[4] Reduced to 39 and 37 by inclusion of 0.1 mM uridine and to 53 and 52 by inclusion of 0.1 mM thymidine.

Table II. *Kinetin Stimulation of Formation of Ultraviolet-absorbing Compounds and Inhibition by Protein Synthesis Inhibitors*

Ethanolic extracts of soybean tissue suspensions cultured for 44 to 48 hr in media with or without 2 μM kinetin were read at indicated wavelengths; the differences in absorbance provided the control values for the kinetin effect. A similar set of values was obtained for media with the inhibitor present, and the percentage inhibition was computed.

Inhibitor	Experiment[1]	Concn	Inhibition of Kinetin Effect	
			232 nm	260 nm
		μM	%	
Chloramphenicol	A	62	32	42
		310	46	58
	A[2]	62	30	...
		310	84	...
Cycloheximide	A	200	...	29
	B	200	−87	38
DL-Ethionine	A	310	...	72
p-Fluorophenylalanine	A	550	83	88
	B[2]	550	63	...
L-Methionine	A	340	...	94
	B	340	58	89
Puromycin	A	42	3	41
	B	42	−50	16
D-Serine	A	950	...	36
Streptomycin	A	3.4	−1	−4
		34	14	−3

[1] Only one inhibitor was tested in any one experiment.

[2] In 0.6 M sucrose; 0.1 M sucrose was used in all others.

decreasing the response to kinetin, as were 8-aza-guanine, 6-methylpurine, 5-fluorouracil, and 5-fluorodeoxyuridine, and 6-aza-uracil was at least somewhat effective. The inhibition by 5-fluorodeoxyuridine was partially reversed by thymidine but also by uridine; the inhibition therefore probably is due to a suppression of RNA rather than DNA synthesis. Abscisic acid possibly also is capable of inhibiting RNA synthesis (8). The inhibition by all of these compounds may therefore result from a deficiency of some sort of RNA. Several inhibitors of normal protein synthesis, including *p*-fluorophenylalanine (phenylalanine was not effective), D-serine, DL-ethionine, chloramphenicol, and cycloheximide (Actidione), were at least partially effective, although puromycin was only slightly and streptomycin was not at all effective (Table II). In some cases, the inhibitor reduced the production of daidzin (as indicated by absorption at 260 nm) much more than that of the new compound previously mentioned (absorption at 232 nm). In fact, formation of the new compound was actually promoted in some instances. This was observed consistently with cycloheximide and sometimes with puromycin. This effect of cycloheximide is depicted in Figure 2. Both inhibitors decreased the final absorbance at 232 nm when added to the control incubating media lacking kinetin. They therefore seem not to promote formation of the new compound unless kinetin is present.

DISCUSSION

The compound with peaks at 232, 281, and 285 nm has not been identified but does strongly resemble—with peaks slightly displaced—that reported by Klarman and Sanford (5) to be produced by soybean cotyledons in the presence of copper salts or of certain fungi. Although I did not observe an increase in the amount of the compound when copper salts were added, mercuric chloride (at 10 μM) did cause some production even in the absence of kinetin. As indicated earlier in this paper, sucrose or mannitol at high molarities, cycloheximide, and perhaps puromycin all seem to promote the synthesis of the compound when a cytokinin is present. This might mean that the compound is a precursor of daidzin or that the two syntheses are competitive for a common substrate. A rather attractive idea is that all these effects should be grouped together, in the sense that they cause stress conditions in the cells, and that the cells respond by producing the compound. Hadwiger and Schwochau (3) have recently presented evidence that, and proposed how, a response of this type may occur at the RNA synthesis level. Klarman and Sanford (5) found their compound to be toxic to fungi so the production of such compounds may be part of a defensive mechanism which is activated by many conditions which do not kill the cells outright. The soybean tissue seems to hold promise for use in studying this type of mechanism.

Synthesis of the compound and the synthesis of daidzin were clearly lessened by the inhibitors of RNA production. The story with respect to protein synthesis is not so simple. The differential effect of puromycin was striking and warrants further investigation. The ability of cycloheximide to consistently increase formation of the unknown compound while reducing that of daidzin is especially interesting, since this inhibitor is considered to be effective against protein synthesis in the cytoplasm but not against that in organelles such as mitochondria (1). This observation and the data obtained with chloramphenicol, which is most effective in organelles, suggest that daidzin formation requires protein synthesis at two sites with the formation of the new compound requiring protein synthesis at only one site. However, since puromycin may not be so specific as to locus of inhibition, this may not be the explanation of the differential effects. The data in Table I indicate that even actinomycin D may have a differential effect when used at the appropriate concentration. This was not always apparent at the low level of sucrose and never was observed at the high concentration of sucrose. Nevertheless, an attempt to detect compartmentalization of RNA synthesis was made by adding the selective RNA inhibitor rifampicin (200 mg/liter) (2) to the incubation media. No effect of the inhibitor was noted, but perhaps the rifampicin simply was not effective in stopping any RNA synthesis in the setup used.

One should not neglect the possibility that some of the inhibitors were acting in ways not directly concerned with macromolecular syntheses. For example, methionine did not reverse the inhibition caused by ethionine and actually inhibited just as well as ethionine: therefore, the ethionine may not have been acting directly on protein synthesis. Such caution may apply to cycloheximide, chloramphenicol, and puromycin. Nevertheless, the present results generally do indicate a close relationship between cytokinin action and RNA and protein synthesis. Possibly kinetin promotes the formation of daidzin and the other compound by directly stimulating RNA or protein synthesis, or perhaps the expression of kinetin action requires the operation of the sequence without the kinetin being involved in it. A choice of one of these possibilities does not seem justified at this time although there have been many reports of apparent stimulation of the sequence by cytokinins (9). The results bear no direct evidence for or against the idea that cytokinins are in some way involved in the roles of tRNA (9), but are consistent with it.

Work with the soybean tissue is being continued in other directions in the hope of finding simpler phenomena detectably influenced by cytokinins in shorter times.

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LITERATURE CITED

1. Ashwell, M. and T. S. Work. 1970. The biogenesis of mitochondria. Annu. Rev. Biochem. 39: 251–290.
2. Goldberg, I. H. and P. A. Friedman. 1971. Antibiotics and nucleic acids. Annu. Rev. Biochem. 40: 775–810.
3. Hadwiger, L. A. and M. E. Schwochau. 1971. Specificity of deoxyribonucleic acid intercalating compounds in the control of phenylalanine ammonia lyase and pisatin levels. Plant Physiol. 47: 346–351.
4. Jones, R. L. 1969. Inhibition of gibberellic acid-induced α-amylase formation by polyethylene glycol and mannitol. Plant Physiol. 44: 101–104.
5. Klarman, W. L. and J. B. Sanford. 1968. Isolation and purification of an antifungal principle from infected soybeans. Life Sci. 7: 1095–1103.
6. Miller, C. O. 1963. Kinetin and kinetin-like compounds. *In:* H. F. Linskens and M. V. Tracey, eds., Modern Methods of Plant Analysis, Vol. 6. Springer-Verlag, Berlin. pp. 194–202.
7. Miller, C. O. 1969. Control of deoxyisoflavone synthesis in soybean tissue. Planta 87: 26–35.
8. Pearson, J. A. and P. F. Wareing. 1969. Effect of abscisic acid on activity of chromatin. Nature 221: 672–673.
9. Skoog, F. and D. J. Armstrong. 1970. Cytokinins. Annu. Rev. Plant Physiol. 21: 359–384.