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Antagonisms between Kinetin and Amino Acids

EXPERIMENTS ON THE MODE OF ACTION OF CYTOKININS

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ABSTRACT

The maintenance of chlorophyll in darkened first leaves of oats was used as a bioassay for cytokinins in pea (*Pisum sativum*) roots. No cytokinin was found (in contrast with earlier reports on sunflower roots); however, the extracts contained two or more substances antagonistic to cytokinin, *i. e.*, promoting the yellowing in this test. Because the most active of these appeared to be an amino acid, individual amino acids were examined for their ability to modify the greening reaction. As a result, L-serine was found to have these properties. It promotes yellowing whether the greening agent is kinetin, indoleacetic acid, or adenine; it is, therefore, not functioning as a specific cytokinin antagonist. Its action is due to promoting proteolysis. Its D-isomer is inactive. L-Arginine, which alone does not cause chlorophyll retention and only weakly inhibits proteolysis, strongly antagonizes the action of L-serine, and thus prevents the yellowing; this effect is specific, and the only other effective serine antagonist found, although much weaker, is L-threonine. The action of arginine is not due to its preventing serine uptake, but rather the action parallels the serine-arginine antagonism previously described for nitrate reductase induction. A novel interpretation of the effect of amino acids on this process is therefore put forward. In studies of the RNase in darkened oat leaves, serine was found to have no effect; however, kinetin strongly inhibits the normal rise in the level of RNase which occurs in the isolated leaf. Kinetin also maintains the integrity of the cell membranes. A variety of evidence leads to the conclusion that the primary action of kinetin on the leaf is to inhibit proteolysis, rather than to promote protein synthesis.

Unlike auxin, the cytokinins which operate in normal growth do not yet appear to be synthesized in specific organs. The one rich source identified so far, *i.e.*, the immature seed, has not yet been shown to influence growth of adjacent parts of the plant. Evidence from the growth of lateral buds suggests that the biosynthesis of cytokinins may be promoted by light (16). However, several reports that cytokinins are present in the bleeding sap of root exudate of decapitated plants suggest that it may be produced in important quantities in roots (4, 5, 8, 19; cf.1). Since roots are known to form amino acids, this would certainly seem, *a priori*, a reasonable location for cytokinin synthesis. Accordingly, the present research was initiated with the intention of examining pea root tips for their content of cytokinins. Instead of cytokinin, however, only antagonists of cytokinin action have been obtained. This paper will describe some of these antagonists and will use their action to draw some conclusions concerning the possible mode of action of cytokinins.

MATERIALS AND METHODS

Extraction and Fractionation. Pea seeds (*Pisum sativum* cv. 'Alaska') were soaked, laid out on wet tissue paper, and kept in darkness at 20 C for 2 days. Approximately 1000 root tips, 4 mm in length, were cut from the seedlings and were put immediately into ice-cold absolute methanol. The root tips were extracted in sequence with 100 ml of ice-cold absolute methanol (2 hr), 100 ml of ice-cold 80% methanol (4 hr) and 100 ml of 80% methanol at room temperature (15 hr). The methanol was evaporated from the combined extracts under reduced pressure, and the extract was fractionated by shaking successively with chloroform (50 ml × 3), ethyl acetate (50 ml × 5), and *n*-butanol (50 ml × 3).

Paper Chromatography. The fractions were subjected to ascending chromatography on 12-cm-wide strips of Whatman No. 1 filter paper, with the use of *n*-butanol-acetic acid-water (4:1:1, v/v/v) as developing solvent, which was allowed to run 19.5 cm from the starting line. The chromatogram, 20 cm in length, (from 5 mm below the starting line up to the solvent front), was divided into 10 2-cm pieces, each of which was cut into small pieces and soaked in 5 ml of 80% ethanol for 6 hr, with occasional vigorous shaking. The eluates were concentrated to small volume, were dissolved in pH 4.7 McIlvain buffer solution diluted 1:10 containing 0.2% Tween-80 with or without 0.1 mg/liter of kinetin, and were examined by the oat leaf assay for their activities in promoting or inhibiting yellowing.

Oat Leaf Assay. The oat leaf assay was performed as described by Thimann and Sachs (15). Oat seedlings (*Avena sativa*, cv. Victory) were grown in vermiculite under continuous light at 25 C. The first leaves, about 12 cm in length, were harvested from 7-day-old seedlings, and the upper 5.0-cm segments were taken. About 10 of these leaf segments were placed on a microscope slide and kept in a Petri dish, the bottom of which was covered with a circle of filter paper moistened with 2.5 ml of water. Test materials were dissolved in McIlvain citrate-phosphate buffer solution diluted 1:10 (pH 4.7) containing 0.2% Tween 80, and were applied as a small droplet (10 μl) on the leaf segments, at 2 cm from the tip. The Petri dishes with leaf segments were kept in darkness at 25 C for 3 days (occasionally, 4 days), and thereafter, the lengths of the green zones superimposed on the yellow background, or the amounts of chlorophyll in the green zones, were measured. For the chlorophyll estimations, the green zones were cut out and extracted with boiling 80% ethanol, and the absorbance of the ethanolic extract (made up to 10 ml) was measured at 665 nm in a Bausch & Lomb 20 spectrophotometer. When kinetin alone was applied, the length of the green zone closely

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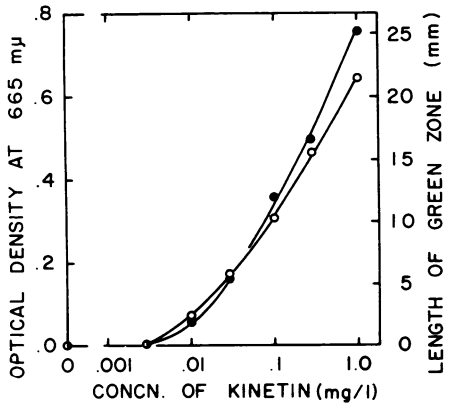


FIG. 1. Dose response curves of kinetin in the oat leaf assay. Open circles: Length of green zone (average of 12 leaves); closed circles: absorbance at 665 nm (per six leaves).

paralleled the amount of chlorophyll (Fig. 1). Figure 1 also shows that 10 μl of 0.01 mg/liter of kinetin caused an appreciable retention of chlorophyll; in other words, this assay can detect 1×10^{-4} μg of kinetin. This is comparable to the results of Kende (5) and Thimann and Sachs (15).

Estimation of Ribonuclease Activity. On the center of the uppermost 2.5 cm of the first leaf, cut from 7-day-old light-grown oat seedlings as above, 10 μl of test solution were placed. The segments were kept in darkness for 3 days, after which time both ends (5 mm each) of each leaf segment were removed and 30 of the remaining 1.5-cm portions were homogenized in a glass homogenizer with 4 ml of ice-cold 0.1 M sodium acetate buffer (pH 5.5). One milliliter of homogenate was mixed with 1.0 ml of 4 mg/liter of RNA in 0.1 M acetate buffer (pH 5.5) and was incubated for 60 min at 30 C. The reaction was stopped, and the undigested RNA was precipitated by adding 2.0 ml of McFadyen's reagent (0.25% uranyl acetate in 2.5% trichloroacetic acid). After being centrifuged, the absorbance of the supernatant was read at 260 nm in a Beckman DU spectrophotometer. For comparison, 1 ml of the homogenate was mixed with 1 ml of the acetate buffer and was incubated for 60 min at 30 C; 2 ml of McFadyen's reagent (9) was added and the mixture then centrifuged. The resulting supernatant was used as the comparison blank. An additional 1 ml of homogenate was mixed with 4.0 ml of acetone kept in the refrigerator overnight and then centrifuged. The supernatant from this was used for the determination of chlorophyll.

Measurement of Uptake and Incorporation of ^{14}C-Amino Acids. At the center of the uppermost 2.5 cm of the first leaf, obtained as above, was placed 10 μl of test solution (0.2% Tween-80, pH 4.7) which contained ^{14}C-L-leucine (about 15,000 cpm) or ^{14}C-L-serine (about 20,000 cpm) and 0.2% Tween-80, pH 4.7. The leaf segments were incubated in darkness at 25 C. Each lot of 20 leaf segments was then washed with running water, homogenized with 5 ml of ice-cold 80% ethanol, and centrifuged. The precipitate was washed twice with 5 ml of ice-cold 80% ethanol each time. The ethanol was evaporated from the combined homogenate and washings, and the aqueous residue was shaken three times with benzene to remove chlorophyll, which acts as a powerful quenching agent. The aqueous layer was concentrated to 0.5 ml and transferred to a scintillation vial with 15 ml of scintillation solution (PPO[3] 5, naphthalene 10, dioxane to 1 liter), and then the radioactivity was read in a Beckman CPM-100 liquid scintillation counter. The losses of radioactivity from the aqueous solution caused by the shaking with benzene were estimated by using ^{14}C-leucine or ^{14}C-serine and by correcting the measured counts. The ethanol-insoluble precipitate was washed with a mixture of ethanol and ether (1:1, v/v) to remove yellow pigments (inasmuch as the loss of radioactivity in this procedure was always less than 1%, no correction was made), and it was transferred with 0.5 ml of water to a scintillation vial with 15 ml of the above scintillation solution for counting. The quenching effect was estimated by an internal and external standard.

In the experiment shown in Figure 8, the protein fraction was obtained as described by Kuraishi (6): the tissues were homogenized with McIlvain buffer, pH 5.5, the homogenate was centrifuged, and the protein in the supernatant was precipitated with 5% trichloroacetic acid.

Ten microliters of U-^{14}C-L-leucine (31 mc/mmole, 50 μc in 0.5 ml), from New England Nuclear Corporation, Boston, Massachusetts, or of U-^{14}C-L-serine (105 mc/mmole, 50 μc in 0.5 ml) from CalBiochem, Los Angeles, California, were diluted to a volume of 1 ml for all experiments, unless otherwise stated. It is recognized that the incorporation of a ^{14}C-amino acid into an alcohol-insoluble precipitate does not rigorously prove the synthesis of protein. In the description of incorporation experiments, therefore, the word "protein" may be considered an abbreviation for "the twice-washed and pigment-free precipitate produced by ice-cold 80% ethanol."

RESULTS

Antagonists to Kinetin. The fractions extracted from pea root tips, as described under Materials and Methods, were chromatographed, and the successive zones of the chromatograms were tested in the oat leaf bioassay. Surprisingly, none caused appreciable retention of chlorophyll, showing that there is very little extractable cytokinin in this material. Tests in presence of kinetin, 0.1 μg/ml, however, revealed very great decreases in chlorophyll (Table I). This evidence of substances inhibiting the maintenance of chlorophyll was the main starting point of the present investigation. Chromatograms of four fractions are shown in Figure 2, where the length of the green zone is plotted as a percentage of that in the low kinetin controls. There are evidently two inhibitors. One, which moves to the solvent front in butanol-acetic acid-water, is somewhat soluble in ethyl acetate, but more so in butanol. The other, evidently much larger in amount, is only moderately soluble in butanol, but more so in water. On the other hand, Table I shows that the total butanol fraction decreased the length of the green zone from 8.6 mm (in kinetin alone) to 0.9 mm—a drastic effect. The inhibitor which moves to the solvent front was not found to give any color reactions and has not been studied further. The larger fraction, soluble in butanol and water, gave a blue color with ninhydrin in both

Table I. *Effect of Methanolic Extract from Pea Root Tips on the Oat Leaf Assay*

Extract of 1080 4-mm pea root tips was fractionated successively with chloroform, ethyl acetate, and 1-butanol. Fractions were tested on the oat leaf by using extract of two and one-half tips per leaf. (In this test 1×10^{-4} μg of kinetin equivalent per leaf would have been detectable.)

Test Solution	Length of Green Zone
	mm
Kinetin, 0.1 mg/liter	8.6
+ chloroform fraction	7.2
+ ethyl acetate fraction	6.2
+ *n*-butanol fraction	0.9
+ water fraction	7.8
All four fractions without kinetin (separately)	0.0

[3] Abbreviation: PPO: 2,5-diphenyloxazole.

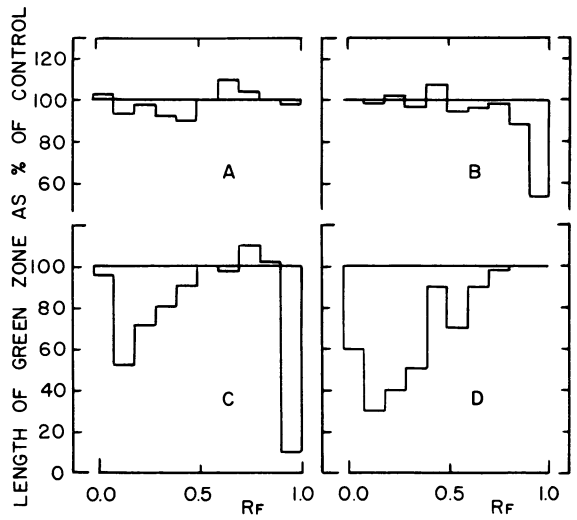


FIG. 2. Chromatograms of chloroform (A), ethyl acetate (B), *n*-butanol (C), and water (D) fractions of methanolic extract from 1025 4-mm Alaska pea root tips, developed with a mixture of *n*-butanol, acetic acid, and water (4:1:1, v/v). Eluate from each divided chromatogram dissolved in 0.1 mg/liter of kinetin solution and tested on oat leaves with extract of 25 tips per leaf. Eluate from corresponding part of blank chromatogram, dissolved in 0.1 mg/liter of kinetin, used as control.

None of the eluates alone (at a concentration of 25 tips per leaf) from extract chromatograms produced a green zone on the oat leaf segments.

Table II. *Interaction between Amino Acids and Kinetin in the Oat Leaf Assay*

	Concentration of Amino Acids[2]			
	3×10^{-3} M		3×10^{-2} M	
	Length of green zone[1] (in % of control)	Inhibition is significant at:	Length of green zone[1] (in % of control)	Inhibition is significant[2] at:
		%		%
Kinetin, 0.3 mg/liter (control)	100		100	
+ L-Alanine	95	—[3]	45	0.1
+ D-Alanine	100	—	96	—
+ β-Alanine	104	—	97	—
+ L-Arginine	96	—	98	—
+ L-Aspargine	101	—	101	—
+ L-Aspartic acid	102	—	99	—
+ L-Citrulline	101	—	101	—
+ L-Cysteine	90	10	37	0.1
+ L-Glutamic acid	97	—	76	0.1
+ Glycine	99	—	79	0.1
+ L-Histidine	93	20	97	—
+ L-Isoleucine	96	—	99	—
+ L-Leucine	101	—	98	—
+ L-Lysine	96	—	97	—
+ L-Methionine	104	—	97	—
+ L-Phenylalanine	95	—	72	0.1
+ L-Proline	100	—	101	—
+ L-Serine	82	1	26	0.1
+ D-Serine	99	—	93	20
+ L-Threonine	101	—	89	5
+ L-Tryptophan	105	—	100	—
+ L-Valine	96	—	95	—

[1] Average of 20 leaves; zone length in controls was 10 mm.
[2] By Student's t-test.
[3] Not significant at 20% level.

butanol and water fractions, and the ninhydrin color coincided with the decreases in green color. This evidence for functional α-amino groups, together with the rather characteristic solubilities, showed that the inhibitor fraction probably consisted of one or more amino acids.

Pure amino acids were therefore examined for their inhibiting power, and the results are shown in Table II. Of 22 amino acids tested, 14 showed green zones within 4% of the control length, but 6 showed significant inhibitions of chlorophyll retention. Of these, only L-alanine, L-cysteine, and L-serine gave inhibition greater than 50% at 3×10^{-2}M; at one-tenth this concentration L-serine was the most active. It will be shown below that glycine and L-threonine also have real, although smaller, inhibiting effects. On the other hand, D-serine was barely active, and D-alanine and β-alanine were inactive. Thus, the effect is limited to those mono-amino acids which compose proteins.

Since L-serine is clearly the most active, further work was centered on this amino acid.

Interactions between Serine and Other Amino Acids. A peculiarity, mentioned above, is that the chromatograms show the inhibitor in pea roots to be present evidently in much greater concentration in the aqueous fraction than in the butanol fraction; however, when the whole (unchromatographed) fractions were tested, the aqueous fraction was only weakly active. This suggested that other constituents in the latter fraction might be antagonizing the inhibition. Amino acids were therefore tested directly for their ability to prevent the action of L-serine. Kinetin 3×10^{-7} M and L-serine 3×10^{-2} M were applied in the droplet,

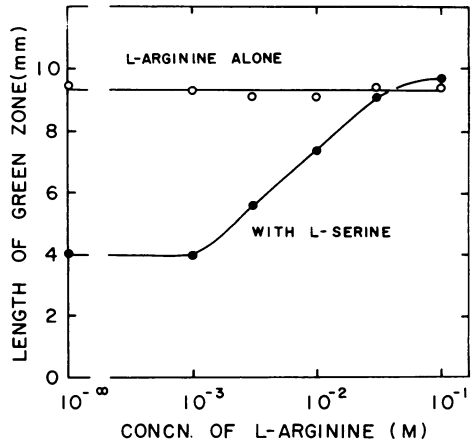


FIG. 3. Interaction between L-serine (3×10^{-2} M) and L-arginine in the oat leaf assay. All leaf segments received kinetin at 0.1 mg/liter, resulting in a 9.3-mm green zone in the controls.

Table III. *Interaction between L-Arginine and L-Serine, L-Alanine, Glycine, or L-Phenylalanine in the Oat Leaf Assay*

Each lot of 10 segments was extracted for determination of chlorophyll. Absorbance of the extract was read against that of the extract from water-treated leaf segments.

Treatment	Absorbance at 665 nm	
	Without L-arginine	With L-arginine
		3×10^{-2} M
Kinetin, 0.03 mg/liter	0.315	0.338
+ L-Serine, 3×10^{-2} M	0.073	0.303
+ L-Alanine, 3×10^{-2} M	0.101	0.336
+ Glycine, 3×10^{-2} M	0.147	0.332
+ L-Phenylalanine, 3×10^{-2} M	0.213	0.241

together with serial concentrations of each amino acid in turn. Most had no effect; however, L-arginine, L-citrulline, L-histidine, and even, surprisingly, L-threonine clearly increased the length of the green zone. L-Arginine could even bring it up to that of the serine-free controls (Fig. 3). L-Threonine was about one-tenth as effective.

The action of arginine was found not to be wholly specific for serine, because arginine also reverses the effects of the weaker inhibitors alanine and glycine (although not that of phenylalanine). Table III shows examples of these reversals, and also shows that arginine alone has no significant effect.

Threonine was also found to reverse the effects of alanine and glycine, and even, partially, that of serine, in spite of the fact (cf. Table IV) that by itself it promotes yellowing somewhat. Reversals by threonine at 0.1 M are shown in Table IV.

An obvious explanation for this type of antagonism would be that one amino acid interferes with the uptake of another. In order to test this, ^{14}C-L-serine was applied to leaves in the usual way, with or without arginine in the droplet. After 24 hr, the ethanol-soluble and -insoluble radioactivities were determined as described under Materials and Methods. Table V shows two such experiments with different amounts of ^{14}C; it is clear that arginine does not decrease the total uptake, and therefore, it cannot be antagonizing serine simply by excluding it from the leaf.

It is to be noted that the incorporation of serine into protein is far lower than that of leucine, as seen in Figures 4 to 6, and in Tables 9 and 11. In addition, the effect of arginine, which increases chlorophyll retention, is actually to decrease slightly the incorporation of serine into protein—a paradoxical result, the explanation of which has not yet been found.

Thus the mutual antagonism of arginine and serine is not wholly specific for either one. It is evident that both act on some

Table IV. *Interaction between L-Threonine and L-Serine, L-Alanine, or Glycine (in Presence of Minimal Kinetin) in the Oat Leaf Assay*

Each lot of 10 leaf segments was extracted with acetone for determination of chlorophyll. Absorbance of extract was read against that of the extract from the water-treated segments.

Treatment	Absorbance at 665 nm	
	Without L-threonine	With L-threonine
		1×10^{-1} M
Kinetin, 0.03 mg/liter	0.324	0.288
+ L-Serine 3×10^{-2} M	0.095	0.125
+ L-Alanine 3×10^{-2} M	0.157	0.264
+ Glycine 3×10^{-2} M	0.237	0.300

Table V. *Effect of ^{12}C-L-Arginine (1.5×10^{-2} M) on Net Uptake of ^{14}C-L-Serine and Its Incorporation into the Ethanol-soluble Fraction*

Leaf segments were treated with ^{14}C-serine (1.0 μc/ml) or ^{14}C-serine with ^{12}C-arginine (1.5×10^{-2} M) for 24 hr.

	Radioactivity as cpm in 20 Leaves		
	Ethanol-soluble fraction	Ethanol-insoluble fraction	Total
Experiment 1 (plants 7 days old)			
Without arginine	39550	7830	47380
With arginine	43950	7085	51035
Experiment 2 (plants 8 days old)			
Without arginine	20620	7950	28570
With arginine	22670	7480	30150

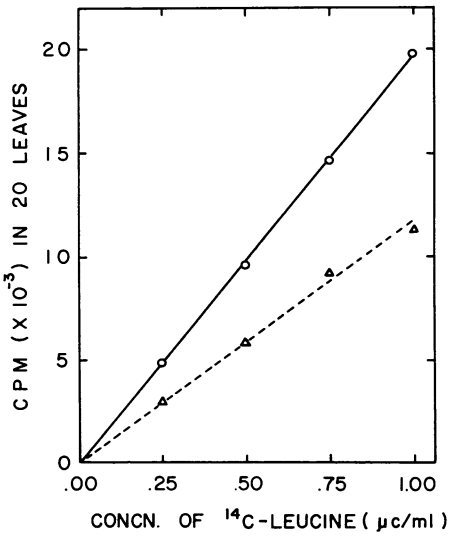


FIG. 4. Effect of the amount of ^{14}C-L-leucine applied in the droplet on the total uptake and on incorporation into the ethanol-insoluble fraction. Each droplet (10 μl) at 1.00 μc/ml contained 15,200 cpm. Solid line: uptake, total cpm in ethanol-soluble and -insoluble fractions; dashed line: incorporation into protein, as cpm in ethanol insoluble fraction.

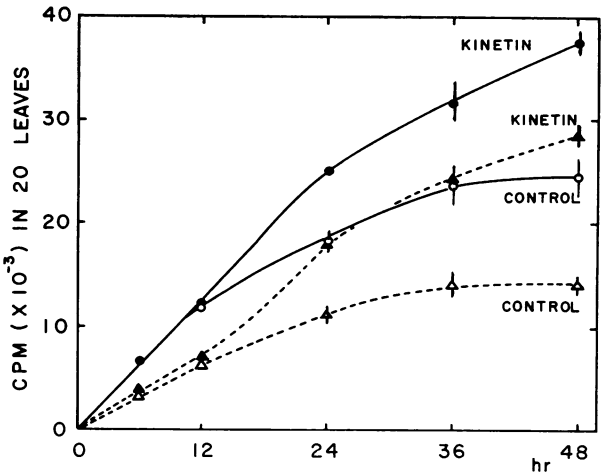


FIG. 5. Effect of kinetin (3.0 mg/liter) on net uptake and incorporation into ethanol-insoluble fraction of ^{14}C-L-leucine (1.0 μc/ml). Solid lines: uptake; dashed lines: incorporation. The vertical lines in this and the following figure show the standard deviations of the mean values plotted.

step in the processes of protein synthesis or breakdown; nevertheless, the antagonism does not as yet help to explain the mode of action of serine. Because serine is, however, producing an effect essentially opposite to that of kinetin in the oat leaf assay, it is first necessary to show more precisely what action kinetin has in this system.

The Action of Kinetin on the Isolated Leaf. Certain properties of the assay system should first be made clear.

The dependence of the uptake and incorporation of ^{14}C-leucine after 24 hr on the amount applied in the droplet is shown in Figure 4. Clearly, both the uptake and incorporation show linear proportionality to the leucine concentration. This means that the percentage incorporated into the ethanol-insoluble fraction (which we shall regard as "protein"), is independent of leucine concentration, and amounts to about 60% of the total taken up. A simple calculation shows that the amount of leucine is not a limiting factor, because at the highest concentration shown each droplet contained 15,200 cpm, and 20 leaves were used; of this

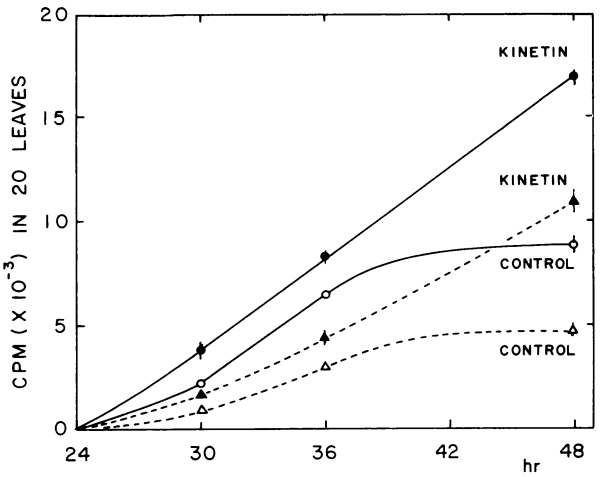


FIG. 6. Effect of kinetin (3.0 mg/liter) on total uptake and incorporation into ethanol-insoluble fraction of ^{14}C-L-leucine. Leaf segments were treated with water for the first 24 hr, then with ^{14}C-leucine (1.0 μc/ml) or ^{14}C-leucine with kinetin. Solid lines: Uptake; dashed lines: incorporation.

Table VI. *Effect of Kinetin on Incorporation of ^{14}C-L-Leucine into Ethanol-insoluble Fraction*

Leaf segments were treated with ^{14}C-leucine or ^{14}C-leucine with kinetin (3.0 mg/liter).

Time (hr)	Ratio[1]		Promotion by Kinetin
	Control	With kinetin	
	%		%
6	47.5	54.0	13.5
12	52.0	58.0	11.5
24	61.0	73.0	20.0
36	60.0	76.0	27.0
48	57.5	75.5	31.0

[1] Ratio of cpm incorporated into ethanol-insoluble fraction to cpm taken up. Data are calculated from Figure 5.

304,000 cpm, the leaves absorbed (in 24 hr) only 19,700 cpm in all, or 6.5% of the amount applied. After the first 24 hr the rate of uptake falls off somewhat (cf. Fig. 5) so that even in 72 hr (when the experiments were usually terminated) less than 20% of the applied radioactivity had been taken up.

The effect of kinetin is shown very characteristically in Figure 5 and Table VI; it is not a simple one. At 12 hr there is little or no effect on the total uptake of ^{14}C, but at 24 hr the kinetin has increased the ^{14}C uptake by 36%. At about 40 hr the control leaves have ceased to take up leucine, whereas those given kinetin continue to absorb it at a steady rate. (Note that the 10-μl droplets in Fig. 5 contained about the same counts as those at 1μc/ml in Fig. 4.) As expected, kinetin also affects the incorporation into protein (dashed lines in Fig. 5); at 6 hr the increase found was only 13% but this has been confirmed in three separate experiments with ethanol and in one experiment by using trichloracetic acid. At 24 hr the percentage incorporated was increased from 61 in the controls to 73 with kinetin, and at 48 hr from 58 in the controls to 76 in kinetin.

It is interesting to compare this effect of kinetin on protein formation with its effect on the chlorophyll. In Table XII it will be seen that kinetin, 0.03 mg/liter, increases the chlorophyll retention after 72 hr from 21.6 to 38.9%, *i.e.*, almost double. In another experiment at 3 mg/liter the chlorophyll content at 72 hr was more than 3 times that of the controls. The comparison is rough, but evidently the influence of kinetin on the maintenance of chlorophyll-protein is at least as great as its influence on the neoformation of protein, and probably somewhat greater. We shall return to this point below.

Why does 12 hr or so have to elapse before an effect of kinetin becomes evident? From the fact that the uptake of leucine begins at once and proceeds linearly with time, it seems unlikely that the uptake of kinetin would not do likewise. The experiment of Figure 6 is an attempt to answer this question. Here, the leaves were given water droplets for 24 hr, and then ^{14}C-leucine (with or without kinetin 3 mg/liter) for the second period of 24 hr. There is now, unlike the situation in Figure 5, a clear effect of kinetin in 6 hr; both uptake and incorporation are increased by 70%. Kinetin must, therefore, have entered in physiologically active amounts in this time. During the next 18 hr, uptake and incorporation taper off in the controls but continue at an undiminished rate in presence of kinetin. The percentage of the leucine taken up, which is incorporated into "protein," however (see Table VIII), shows only a small kinetin effect; in 6 hr (*i.e.*, 30 hr total) there is no difference; in 12 hr (*i.e.*, 36 hr total) controls have incorporated 47.5% of the counts, and the kinetin-treated, 53.5%. Even after 24 hr (48 hr total) controls have incorporated 53.5%, and the kinetin-treated have incorporated 64.5%, an increase of only one-fifth. At this point the total uptake has been increased from 8800 cpm in control to 17000 cpm with kinetin, *i.e.*, 93%.

We conclude: (*a*) that the response of the leaf to kinetin has become more rapid after 24 hr with water, and therefore, that its very small response in the first 12 to 24 hr is due to a change in the leaf itself; (*b*) that enough kinetin enters the leaf tissue to have physiological effects within 6 hr; (*c*) that the primary effect of kinetin is probably not being exerted on protein formation as such.

Table VII. *Interaction between L-Serine and Kinetin Supplied Separately in the Oat Leaf Assay*

Leaf segments were treated with kinetin for 24 hr, then with L-serine for a second period of 72 hr (*i.e.*, 4 days total).

1st Period (0–24 hr)	2nd Period (24–96 hr)	Length of Green Zone[1]
		mm
Kinetin, 0.1 mg/liter	Water	9.0
	L-Serine 3×10^{-2} M	3.2
Kinetin, 0.3 mg/liter	Water	13.4
	L-Serine 3×10^{-2} M	8.0

[1] Average of 20 leaves.

Table VIII. *Effect of Kinetin on Incorporation of ^{14}C-L-Leucine into Ethanol-insoluble Fraction*

Leaf segments were treated with water for 24 hr, then ^{14}C-leucine or ^{14}C-leucine with kinetin (3.0 mg/liter).

Time (hr)	Ratio[1]		Promotion by kinetin
	Control	With kinetin	
			%
30	47.0	45.5	−3.0
36	47.5	53.5	12.5
48	53.5	64.5	20.5

[1] Ratio of cpm incorporated into ethanol-insoluble fraction to cpm taken up. Data are calculated from Figure 6.

Table IX. *Effect of Kinetin on Breakdown of Protein and Leakage of Free Amino Acid out of the leaf*

^{14}C-L-Leucine (1.0 μc/ml) was applied for the first 24 hr to three groups of leaves. It was then washed off, and one group of 20 leaves was analyzed, and water or kinetin (3.0 mg/liter) was applied to the other two groups. Both were analyzed 24 hr later.

Analyzed after:	Radioactivity in cpm per 20 Leaves			
	Ethanol-soluble fraction	Ethanol-insoluble fraction	Total	Loss in second 24 hr
24 hr	7160 ± 210	10300 ± 1160	17460 ± 1170	
48 hr				
Without kinetin	6980 ± 350	8460 ± 200	15440 ± 405	2020 ± 1240
With kinetin	7100 ± 170	10130 ± 1120	17230 ± 1130	230 ± 1630

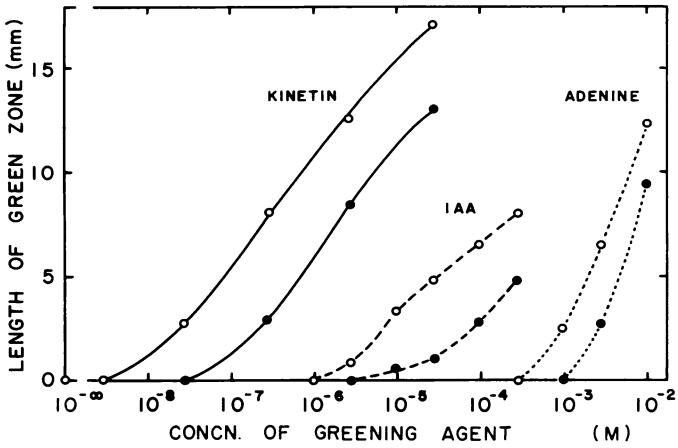


FIG. 7. Effect of L-serine (3×10^{-2} M) on length of green zones caused by kinetin, IAA, or adenine. Open circles: Without serine; closed circles: with serine.

The last conclusion is supported by the experiment of Table IX. Here, leucine alone was applied for the first 24 hr to three groups of leaves. It was then washed off, and one group of 20 leaves was analyzed; water or kinetin was applied to the other two groups. Both of the latter were analyzed 24 hr later. We can see that during the second 24 hr, the protein initially formed has broken down by 2000 counts which have leaked out of the leaf; kinetin has prevented this. Kinetin has not increased the percentage incorporation. Thus the action of kinetin (when uptake cannot be promoted) is mainly to *prevent proteolysis*. It also prevents leakage (*cf.* note to Table XIII).

These results with kinetin and leucine open up some additional modes of attack on the problem of the action of serine. Obviously, an understanding of the action of serine in promoting the loss of chlorophyll may shed light on the action of kinetin in maintaining the chlorophyll.

Mode of Action of L-Serine. The first possibility tested was that serine might inhibit the entry of kinetin into the leaf. Kinetin was therefore applied alone for 24 hr, and then water or serine was applied for the next 72 hr (Table VII). Evidently, serine promotes the loss of chlorophyll, even when the kinetin has already been absorbed. Indeed, in other experiments, serine actually lowers the chlorophyll content below that of water controls (*cf.* Table XII).

The next possibility is that serine is directly an anticytokinin in the molecular sense. To test this, use was made of the previous observation that both adenine and IAA have very weak chlorophyll-maintaining effects in the leaf bioassay. The concentration of IAA needs to be about 300 times that of kinetin, and that of adenine, some 30,000 times. Nevertheless, these are within practical limits, and serine was therefore tested with both compounds. Figure 7 shows that serine, 3×10^{-2} M, decreases the chlorophyll content, whichever is the chlorophyll-maintaining agent. Although the slopes of the curves for kinetin, IAA, and adenine are different, the effectiveness of serine is quantitatively very similar for all three; when the green zone is 6 mm long, serine reduces it to:

1.6 mm with kinetin
2.0 mm with IAA
2.2 mm with adenine.

It is evident that serine is not a specific anticytokinin.

A third possibility is that serine might prevent the movement of amino acids, *e.g.*, from cells rich in amino acids to others in which protein synthesis would be occurring. This would be a direct antagonism to cytokinins, inasmuch as an opposite effect has been ascribed to kinetin by Mothes (10). In the case of externally applied leucine, such an action would imply also that serine would inhibit the uptake of ^{14}C-leucine into the leaf. To test this, ^{14}C-leucine was applied with and without L-serine, and leaves were analyzed after 24 and 48 hr in the dark. In view of the antagonizing effect of arginine, both arginine alone, as well as the combination of serine and arginine, were included. The results (Table X) show that L-serine does, in fact, strongly inhibit total leucine uptake. Arginine has no real effect on the uptake,

Table X. *Interaction between L-Serine and L-Arginine in Net Uptake of ^{14}C-L-Leucine and Its Incorporation into the Ethanol-insoluble Fraction*

Concentration of ^{14}C-L-leucine (1.0 μc/ml); concentrations of both L-serine and L-arginine 1.5×10^{-2} M.

Time and Treatment	Ethanol-insoluble Fraction	Total in Ethanol-soluble and insoluble Fractions	Incorporation Ratio[1]
	cpm/20 leaves		%
After 24 hr			
Kinetin, 0.03 mg/liter	14780	27340	54.1
+ L-Serine	8780	14860	59.1
+ L-Arginine	13820	24580	56.3
+ L-Serine + L-arginine	9020	15840	56.9
After 48 hr			
Kinetin, 0.03 mg/liter	14700	32580	45.2
+ L-Serine	11020	20860	52.8
+ L-Arginine	16300	32720	49.8
+ L-Serine + L-arginine	12860	27200	47.3

[1] Ratio of cpm incorporated into ethanol-insoluble fraction to cpm taken up by the leaf segments.

Table XI. *Effect of ^{12}C-L-Leucine on Net Uptake and Incorporation into Ethanol-insoluble Fraction of ^{14}C-L-Serine*

Leaf segments were treated with ^{14}C-serine (1.0 μc/ml) or ^{14}C-serine plus ^{12}C-leucine (1.5×10^{-2} M) for 24 hr.

Treament	Radioactivity as cpm in 20 Leaves		
	Ethanol-soluble fraction	Ethanol-insoluble fraction	Total
Without leucine	39550	7830	47380
With leucine	33700	5640	39340

Table XII. *Interaction between Kinetin and L-Serine in Change of Level of RNase Activity*

Treatment	Chlorophyll Amount	RNase Activity
	% of initial	
Control	21.6	182
L-Serine, 3×10^{-2} M	13.8	180
Kinetin, 0.03 mg/liter	38.9	126
Kinetin + serine	21.5	185

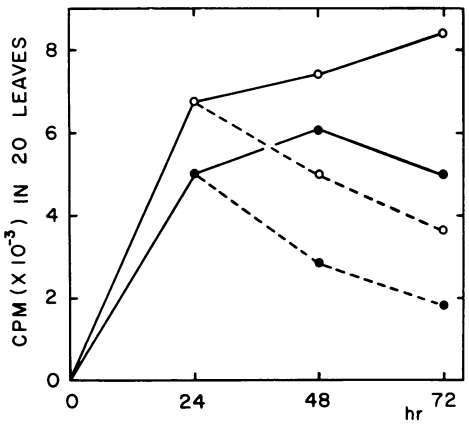


FIG. 8. Effects of L-serine on incorporation of ^{14}C-L-leucine into protein fraction and on breakdown of protein. The fraction, soluble in buffer and insoluble in trichloroacetic acid, was regarded as the protein fraction. L-Serine and ^{14}C-L-leucine were used at 1.5×10^{-2} M and 1.0 μc/ml, respectively. Open circles: ^{14}C-Leucine alone; closed circles: ^{14}C-leucine plus ^{12}C-serine; dashed lines: leucine removed after 24 hr; all points refer only to protein fraction.

but at 48 hr it partially reverses the inhibition. Whether this effect is sufficient to explain its antagonism to serine in the chlorophyll retention test remains to be seen. Neither of the amino acids greatly modifies the incorporation of the leucine into protein, which remains nearly a fixed fraction of the amount taken up (as seen in Figs. 4 to 6.)

It must be noted that the inhibition of amino acid uptake could not explain the action of serine in promoting yellowing in the absence of added amino acids.

The inhibition of leucine uptake by serine is to a slight extent mutual. If ^{14}C-serine is applied to the leaf with and without ^{12}C-leucine, the leucine decreases the uptake of ^{14}C (Table XI). The decrease in uptake is only 17%, whereas serine decreases leucine uptake (in 24 hr) by almost 50% (46% in Table X); nevertheless, the demonstration that there is some mutualism in the relationship is important. The relatively small incorporation of serine into protein confirms the data of Table V.

Another possible area of serine action would be on nucleic acid metabolism. Since leaves contain considerable ribonuclease activity, serine could act by promoting the breakdown of nucleic acids. The RNase of *Avena* leaves was determined as described in Materials and Methods; the activity is readily detectable. In these tests, whole leaves, 2.5 cm long, were treated with L-serine or kinetin, or both, and then after 72 hr in the dark the most apical and basal 5 mm were cut off, and the rest was homogenized in 0.1 M pH 5.5 acetate buffer. The RNase activity and the chlorophyll content were then determined.

Table XIII. *Interaction between L-Serine and L-Arginine (in Presence of Minimal Kinetin) in Uptake of Leucine and in Breakdown of Protein*

For each treatment, four groups of leaves were used. ^{14}C-L-Leucine (1.0 μc/ml) alone or with test agent (*i.e.*, serine, arginine, or serine plus arginine) was applied to all the groups; then it was washed off, and one group of 20 leaves was analyzed. The test solution without ^{14}C-leucine was applied to the remaining groups for a second period of 24 hr. Both L-serine and L-leucine were used at 1.5×10^{-2} M. All leaves received kinetin at 0.03 mg/liter throughout. Data refer to "protein-^{14}C" only.

Treatment	cpm per 20 Leaves		Decrease in 24 hr after Leucine Removal	
	After 24 hr	After 48 hr	in cpm	as % of initial cpm
Control	10160	7800	2360	23
Serine	7880	4460	3420	43
Arginine	9740	7850	1890	19
Serine + arginine	7340	5960	1380	19

Table XII shows that although serine decreases the chlorophyll content by about one-third, it has no effect whatever on the leaf RNase activity. However, kinetin, which nearly doubles the chlorophyll, does inhibit the RNase, and serine completely reverses this inhibition. The inhibition of RNase activity may well be the prime basis for the action of kinetin. Even though serine does not promote the RNase, the fact that it reverses the inhibition suggests that it may have the same action in the untreated leaf by way of endogenous cytokinin.

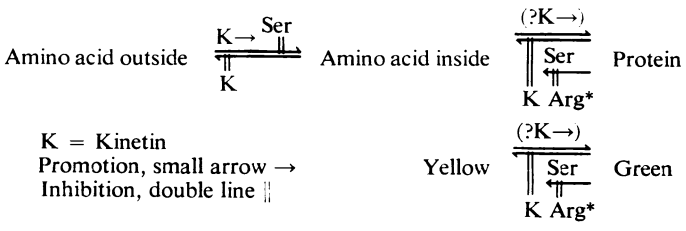
The above experiments focus attention on protein breakdown, rather than protein synthesis. The validity of this approach is supported by a simple variant of the procedure. ^{14}C-leucine, with or without serine, was applied for 24 hr, and then in half of the leaves it was removed and replaced by water or serine. Samples were taken at 24, 48, and 72 hr and were assayed for trichloroacetic acid-insoluble radioactivity. The results are summarized in Figure 8. Firstly, serine inhibits incorporation by one-third, due to the decrease in ^{14}C-uptake. Secondly, and more important, when the leucine is removed, the protein-^{14}C very rapidly decreases. If the leucine remains on, then protein-^{14}C does increase, although more slowly. If serine is present, net proteolysis sets in after 48 hr, at a rate almost equal to that of controls without leucine. Thus, there is indeed vigorous proteolysis in these leaves, and serine promotes it.

A similar experiment, but with kinetin (0.03 μg/ml) present, is shown in Table XIII. In this case, arginine was added as well. After ^{14}C-leucine is removed, the "protein-^{14}C" decreases 23%; but in presence of serine, the percentage of decrease is nearly doubled.[4] Although the total ^{14}C incorporated is, as expected, decreased by the serine, the absolute decrease in "protein-^{14}C" is considerably larger than in the controls. Arginine totally prevents this influence of serine on proteolysis. This table confirms the preceding data and appears to justify the conclusion that the serine-kinetin antagonism is primarily due to the promotion of proteolysis by serine and its inhibition (perhaps via the inhibition of ribonuclease) by kinetin.

[4] In the low concentration of kinetin used here, there was definite leakage of counts out of the leaf, resembling to a lesser degree the leakage noted in Table IX.

DISCUSSION

The reactions can be summarized as follows:



* Arginine counteracts the serine promotion of proteolysis (and yellowing) but does not inhibit the proteolysis (or the yellowing) in the controls; kinetin counteracts the *control* proteolysis.

Serine is evidently a kind of "senescence substance," in that it promotes proteolysis and yellowing. If it were effective in very much lower concentrations, serine could be regarded as a "senescence hormone," because it is certainly transportable; however, the idea of plant hormones has always been associated with concentrations below 0.001 M. Nevertheless, serine could be responsible for some of the many senescence effects reported in the literature.

The absence of detectable cytokinin in pea roots was unexpected, inasmuch as Kende (5) and Weiss and Vaadia (19) had no difficulty with *Helianthus*. The latter workers obtained 0.15 mg from 1200 1-mm root tips, which means that the sunflower must yield some 30,000 times as much cytokinin as the pea. Our use of 4-mm instead of 1-mm root tips cannot explain the discrepancy. Possibly, the cytokinin of pea roots is in a methanol-insoluble form. In any event, the high content of inhibitors in pea roots means that instead of the root system contributing to lateral bud development, or to other functions of cytokinin in the shoot, it will, if anything, work in the opposite direction. Perhaps this is why isolated pea stems give as good growth of lateral buds in response to kinetin as do intact plants, for the first days (20).

Why should serine promote proteolysis? The answer is perhaps to be found in the frequent presence of serine at the active center of proteolytic enzymes (see 18). Only a small part of the serine supplied in Table V was incorporated into leaf proteins; but if these included a proteinase for whose activity serine is essential this might explain the facts. Only one molecule of serine appears to be required per molecule of protein to form the active center of a proteinase enzyme.

A more direct interaction of serine with cytokinin might be visualized from the presence of cytokinin in the tRNA for L-serine (11). However, the presence of cytokinin in other tRNA's makes this mode of action unlikely.

The role of arginine in antagonizing serine is less easy to understand. That citrulline, histidine, and threonine all act in the same way is peculiar, because threonine with its OH group resembles serine and indeed, threonine alone was found to promote proteolysis. L-Threonine also reversed the weaker inhibitions by L-alanine and glycine.

Arginine has been recorded to exert certain other effects antagonistic to amino acids; it reverses the inhibiting action of glycine on the growth of roots of *Senecio* (13) and roots of *Avena* (2); however, the biochemical basis of these effects is not known. As to the action of threonine, it is adjacent to the serine in subtilopeptidase A, but not in any other proteinases that have been sequenced. Perhaps it specifically interferes with the incorporation of serine into proteinase through its structural similarity, whereas the three basic amino acids act in some quite different ways. Arginine, indeed, reverses the weaker yellowing effects of alanine and glycine as well as that of serine, which clearly points to a more nonspecific site of action; nonetheless, since it has no cytokinin-like effect by itself, it cannot be causing a general inhibition of proteolysis.

There is a close similarity between the roles of amino acids in the retention of chlorophyll and their action in the Heimer and Filner experiments on the induction of nitrate reductase (3). This induction (by nitrate) is inhibited to varying extents by all the monoamino acids, but threonine and serine are the most effective. It is likely, then, that at least these two latter do not inhibit the enzyme induction by being the end products of the nitrate reduction process, as might be thought (by analogy with bacterial enzymes), but rather by promoting hydrolysis of the newly formed protein. Indeed, it was specifically noted that, "if threonine is added to a culture after the steady-state nitrate level has been attained, the nitrate reductase decays rapidly" (3). The parallel is completed by the observation that arginine antagonizes this action of threonine. Arginine in such experiments is again evidently inhibiting the proteolysis promoted by serine and threonine.

These considerations bring us to the critical problem of the mode of action of kinetin.

It is evident that in the leaf assay proteolysis is the dominant reaction. The liberation of ^{14}C from the trichloroacetic acid-insoluble fraction (Fig. 8) is particularly clear evidence of this. It is also evident that serine promotes the proteolysis, along with its effect in promoting the yellowing, and we can conclude, with a high degree of probability, that yellowing is *due* to proteolysis. Kinetin does promote the incorporation of leucine into protein, but only to a small degree. In Table IX kinetin did not increase the incorporation, but it did clearly prevent the protein breakdown. The logical conclusion is that the increased incorporation so frequently observed is a secondary effect, and the prevention of protein breakdown is the primary one. The increased uptake of leucine might also be secondary, since kinetin prevents the leakage of amino acids and thus, probably prevents the breakdown of membranes. As a result, it would prevent the uptake of leucine from decreasing with time. We conclude that the primary effect of kinetin, therefore, is to inhibit proteolysis. Kuraishi (6), in comparable experiments on *Brassica* leaf disks, has arrived at the same conclusion. Since some proteinases are —SH-activated, the cytokinin-enhancing effect of —SH-reagents (7) may be explicable in this way.

It was shown in Table XII that kinetin at 0.03 μg/ml strongly inhibits the increase of RNase activity. This had previously been shown (12, 17), but with a kinetin concentration 100 to 300 times as high. The question is, could the inhibition of proteolysis be due to inhibiting the rise in RNase, or are they independent? In the former case, the proteinase might be normally inhibited by a nucleic acid, or the RNase by a protein. In the latter case, kinetin would be seen as a generalized inhibitor of hydrolytic processes. A decision between these two alternatives will require further research. It is suggested that if cytokinins were to inhibit the hydrolysis of starch, this would explain Tasseron-de Jong's unexpected finding (14) that benzylaminopurine causes the accumulation of starch in *Lemna*. Furthermore, an important corollary is that the apparent effect of cytokinins on membranes may well be due to the prevention of hydrolysis of one of the polymers of which membranes are composed.

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