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THE EFFECT OF 6-BENZYLAMINOPURINE ON PROTEIN METABOLISM IN SENESCING CORN LEAVES

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Abstract—The incorporation of ^{14}C-leucine into senescing disks from corn leaves was measured in the presence and absence of 6-benzylaminopurine (benzyladenine). Differences in the amount of incorporation were detected as early as 90 min after start of the hormone treatment. Tissue treated for 24 hr with benzyladenine incorporated up to twice as much ^{14}C-leucine as untreated tissue. This difference may reflect an effect of the hormone on the size of the protein precursor amino acid pool resulting in differences in the isotopic dilution of the added ^{14}C-leucine, rather than an effect of benzyladenine on the rate of protein synthesis. Leaf disks prelabeled with ^{14}C-leucine for 4·5 hr and subsequently transferred to media containing unlabeled leucine with or without benzyladenine showed a slower breakdown of the pre-labeled protein in the presence of the cytokinin. Since the specific activity of the protein of leaf disks incubated with or without benzyladenine did not change over 48 hr, it is suggested that cytokinins retard senescence of corn leaves primarily through inhibiting protein degradation.

INTRODUCTION

THE ABILITY of cytokinins to retard the loss of protein in senescing leaf tissue offers the opportunity to study the action of this hormone at a biochemical level. One of the first questions to be answered is whether cytokinins affect the protein level in aging leaves by stimulating protein synthesis or, conversely, by retarding protein breakdown.

A number of investigators[1-3] have shown that the incorporation of a labeled amino acid into the protein of a leaf is increased by cytokinin treatment. Such results were interpreted as demonstrating a stimulatory effect of cytokinins on protein synthesis. However, the rate of incorporation of a radioactive amino acid into protein cannot be taken as a measure of the rate of protein synthesis. Three parameters determine how much of an exogenously supplied, labeled amino acid is found in protein: (i) the rate of protein synthesis, (ii) the rate of protein degradation, and (iii) the size of the protein precursor amino acid pool. Only if the amount of protein breakdown is negligible and if the amino acid pool sizes do not change, can the rate of amino acid incorporation serve as an estimate for the rate of protein synthesis. Such simplified assumptions cannot be made in the case of senescing leaf tissue in the presence and absence of a cytokinin since the hormone treatment may cause differences in the rate of proteolysis and in amino acid pool sizes.

The free amino acids of a plant cell exist at least in two separate pools[4,5] which can be designated as protein precursor amino acid pool and metabolically inactive amino acid pool. Because there is more than one amino acid pool, the specific radioactivity that an exogeneously supplied, labeled amino acid attains within the protein precursor pool cannot be determined

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directly from an amino acid analysis of the whole tissue. Different experimental treatments can affect the size of the protein precursor pool, thus changing the specific activity of a labeled amino acid in this pool. An expansion of the protein precursor pool will increase the isotopic dilution of an applied labeled amino acid and result in a reduced incorporation of the label into protein although the rate of protein synthesis may remain unchanged. A direct estimate of protein precursor pool sizes according to Oaks[4] and Holleman and Key[5] has not been possible in corn leaves treated with and without a cytokinin. The first method is only applicable if protein breakdown is negligible while the second method requires considerably faster rates of amino acid uptake and equilibration with the tissue than could be observed in corn leaves.

Inhibition of protein degradation by a cytokinin has been demonstrated by Kuraishi[6] who showed that kinetin (6-furfurylaminopurine) retarded the loss of ^{14}C-leucine from prelabeled protein in disks of senescing leaves of *Brassica rapa* L. However, his data did not rule out the possibility that bulk protein synthesis was also stimulated by the applied cytokinin. In our experiments we attempted to measure whether the effect of cytokinins on the protein level in corn leaves is primarily mediated through retarded protein breakdown, or also through increased protein synthesis.

The chloroplast has been implicated as a possible site of cytokinin action in delaying senescence of leaves. Srivastava,[7] and Shaw and Srivastava[8] reported that kinetin was not effective in retarding aging of albino and variegated barley leaves; the criterion for aging in this case was loss of turgidity by the detached leaves. However, Goldthwaite[9] demonstrated that kinetin retarded protein loss in excised leaves of etiolated, normal barley which "contained no chlorophyll and only rudimentary plastids". We reinvestigated the question whether the cytokinin effect is restricted to chlorophyll-containing leaf tissue, by using an albino mutant of corn.

RESULTS AND DISCUSSION

The senescing first leaf of corn exhibits a rapid response to applied cytokinin; the uniformity of this response is improved by using leaf disks in which differences in chlorophyll retention between hormone-treated and control tissue can be measured within 48 hr (Fig. 1). In disks from senescing corn leaves, cytokinin retarded but did not prevent yellowing, and in no case did it induce re-greening of leaf disks that had lost their chlorophyll. The rapidity and irreversibility of the reaction make corn an especially suitable material for the study of senescence. Aging corn leaves have been used earlier by Müller and Leopold[10] to study the effect of kinetin on the transport of phosphate.

The cytokinin-induced retardation of protein loss in corn leaf disks paralleled the retardation of chlorophyll loss (Fig. 2) and the effective concentration range for the synthetic cytokinin 6-benzylaminopurine (benzyladenine) extended over three orders of magnitude. It is interesting to note that low concentrations of benzyladenine (10^{-10} M) seemed to promote senescence. This phenomenon was observed consistently, and has also been observed in barley leaves.

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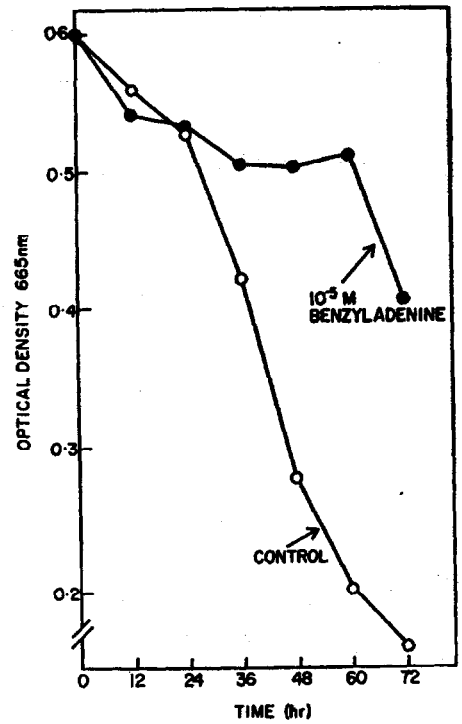

FIG. 1. CHANGES IN CHLOROPHYLL LEVEL IN CONTROL AND BENZYLADENINE-TREATED CORN LEAF DISKS. EACH POINT IS THE AVERAGE OF SIX REPLICATES OF FIVE DISKS EACH.

Retardation of senescence by cytokinins was not restricted to tissue with functional chloroplasts. Benzyladenine also retarded protein loss in senescing leaf disks of light-grown albino corn seedlings. Results obtained in experiments with disks from the first leaf of the albino mutant strain wh_{8896} are given in Table 1. Plastids in leaves of this albino mutant lose their internal structure when grown in the light.[11]

Cytokinin treatment of senescing leaf disks increased the incorporation of ^{14}C-leucine into protein. This agrees with earlier work using *Xanthium*,[1] tobacco[2,3] and radish.[6] Table 2 shows that benzyladenine treatment approximately doubles the incorporation of ^{14}C-leucine into protein. When incorporation was measured as per cent of total uptake, the effect was slightly greater. The data in Table 3 demonstrate that this effect could be measured within 90 min. after the start of the cytokinin treatment. However, from these data it is impossible to determine which of the three factors—(i) leucine pool size, (ii) rate of protein synthesis, or (iii) rate of protein degradation—has changed under the influence of the cytokinin. For example, a doubling of the leucine concentration in the protein precursor pool of the control tissue would account for the 2-fold difference in incorporation of exogenously supplied ^{14}C-leucine.

Anderson and Rowan[12] reported an increase of free amino acids in detached senescing tobacco leaves. In corn the concentration of free amino acids, measured as soluble α-amino nitrogen, increased during senescence and benzyladenine retarded this increase along with

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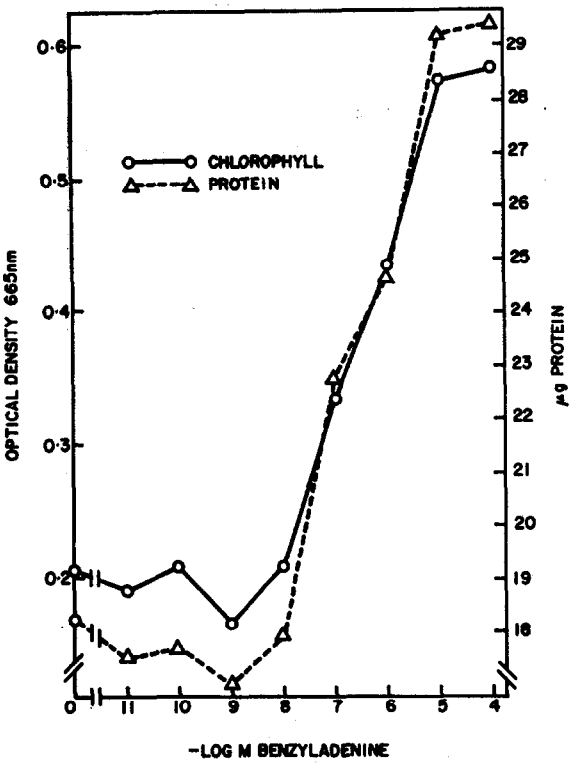

FIG. 2. THE EFFECT OF BENZYLADENINE CONCENTRATION ON THE CHLOROPHYLL AND PROTEIN LEVEL IN SENESCING CORN LEAF DISKS 48 hr AFTER START OF TREATMENT. EACH POINT IS THE AVERAGE OF SIX REPLICATES OF FIVE DISKS EACH.

TABLE 1. THE EFFECT OF BENZYLADENINE ON PROTEIN LOSS IN DISKS FROM SENESCING ALBINO CORN LEAF

Incubation time (hr)	Protein (μg)*	
	Benzyladenine	Control
0	—	293
24	230	181
48	199	159

* The protein was measured as α-amino nitrogen per six disks.

Each value represents the average of ten replicates of six disks each.

Benzyladenine was given at 10^{-5} M.

the loss of protein (Table 4). These results may be indicative for an expanded protein precursor amino acid pool, but nothing definite can be concluded since the data reflect the concentration of the total free amino acids in the cell.

The simultaneous effect of cytokinins on protein degradation and protein synthesis was studied by pre-labeling the leaf protein and determining the specific activity of the protein and

TABLE 2. THE EFFECT OF BENZYLADENINE ON THE INCORPORATION OF ^{14}C-LEUCINE INTO CORN LEAF DISKS

Labelling period (min)	Incorporation (counts/min)		Incorporation as % uptake*	
	Benzyladenine	Control	Benzyladenine	Control
8	56·0	19·3	8·7	2·7
15	112·1	48·7	11·1	3·6
30	339·4	143·3	18·8	5·9
45	614·7	281·1	21·8	8·0
60	905·8	357·0	24·7	8·4

* Uptake = total radioactivity (counts/min) in soluble and protein fractions.

Each value represents the average of three replicates of six disks each. Leaf disks were incubated for 24 hr with or without benzyladenine (5×10^{-6} M).

After this pre-treatment, ^{14}C-leucine (1 μc/ml) was given for 8–60 min.

TABLE 3. THE TIME COURSE OF THE BENZYLADINE EFFECT ON THE INCORPORATION OF ^{14}C-LEUCINE INTO CORN LEAF DISKS

Pre-treatment (min)	Incorporation (counts/min)	
	Benzyladenine	Control
0	594	610
30	744	566
60	886	618
120	988	699
240	799	503

Each value represents the average of three replicates of five disks each. Disks were pre-treated with or without benzyladenine (10^{-4} M) for 0–240 min. They were then transferred to medium with or without 5×10^{-6} M benzyladenine and labelled for 60 min with ^{14}C-leucine (1 μc/ml).

TABLE 4. THE EFFECT OF BENZYLADENINE ON PROTEIN AND SOLUBLE α-AMINO NITROGEN IN FRESH AND SENESCING CORN LEAF DISKS

Treatment	α-Amino nitrogen (μg/mg fr. wt.)	
	Protein	Soluble
Freshly cut tissue	1·66	0·08
24 hr Benzyladenine	0·94	0·12
24 hr Control	0·73	0·39
48 hr Benzyladenine	0·84	0·30
48 hr Control	0·40	0·42

Each value represents the average of two determinations. Benzyladenine was given at a concentration of 10^{-5} M.

its rate of degradation after different times of cytokinin treatment. When leaf disks were incubated for 4·5 hr in ^{14}C-leucine ($1·5 \times 10^{-6}$ M) and subsequently rinsed and transferred to a medium containing ^{12}C-leucine (10^{-4} M) with and without benzyladenine, the breakdown of pre-labeled protein was slower in the cytokinin-treated disks than in the controls. This agrees with the data obtained by Kuraishi using *Brassica* leaves.[6] The per cent loss of radioactivity and the per cent loss of total protein during leaf senescence were remarkably similar (Table 5). This indicates that the small amount of labeled protein, synthesized during the

TABLE 5. THE EFFECT OF BENZYLADENINE ON PROTEIN DEGRADATION IN SENESCING CORN LEAF DISKS

		Time after pre-labeling (hr)			
		24		48	
	0	Benzyladenine	Control	Benzyladenine	Control
Experiment I					
% Loss of radioactivity from protein	—	9·5	17·3		
% Loss of total protein	—	10·4	19·5		
Specific activity of protein (counts/min/μg)	55·5	56·1	57·1		
Experiment II					
% Loss of radioactivity from protein	—	24·5	28·4	36·6	49·6
% Loss of total protein	—	24·5	33·3	34·7	47·4
Specific activity of protein (counts/min/μg)	53·3	53·3	57·2	51·8	51·0

Each value represents the average of three replicates of five disks each. Leaf disks were pre-labeled for 4·5 hr with ^{14}C-leucine ($1·5 \times 10^{-6}$ M, 0·27 μc/ml), washed thereafter three times with 10^{-4} M ^{12}C-leucine and incubated in 10^{-4} M ^{12}C-leucine with or without benzyladenine (5×10^{-6} M).

4·5 hr of the ^{14}C-leucine pulse, was degraded at the same rate as the bulk of the protein. Turnover of the ^{14}C-leucine itself during senescence should be negligible in the presence of ^{12}C-leucine at a 70-fold concentration.

Does cytokinin, apart from its clear effect in retarding protein degradation, also enhance protein synthesis in aging leaf disks? An appreciable rate of protein synthesis in leaf disks during the 24 and 48 hr of senescence would have produced unlabeled protein, and this would have lowered the specific activity of the total protein. However, as Table 5 shows, the specific activity of the total protein of the leaf disks did not change significantly during 48 hr of aging. Thus, we must conclude that the differences in the bulk protein level of benzyladenine-treated and control leaf disks are due to a retardation of protein breakdown under the influence of the cytokinin. These results do not exclude the possibility, however, that cytokinins stimulate the synthesis of a small protein fraction which does not measurably alter the specific activity of the bulk protein. Our conclusions are supported by experiments of Balz[13] and Atkin and Srivastava[14] who demonstrated that protease and RNase activities increased in detached, senescing leaves and that this increase could be counteracted by cytokinin treatment.

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EXPERIMENTAL

Plant Material

Corn seed, *Zea mays* L. hybrid WF9 × Bear 38 (Bear Hybrid Corn Co., Decatur, Illinois), was soaked overnight in running tap water and sown in vermiculite. The seedlings were grown in a growth chamber at 97% humidity and a 12 hr photoperiod provided by fluorescent and incandescent lamps with an intensity at plant height of 44,000 lx. The temp. was 23° during the dark period and 27° during the light period. The plants were watered daily with distilled water and were ready for use 10 days after sowing.

Albino corn seed, mutant wh_{8896}, was germinated and grown in the same manner but the seedlings were used 7 days after sowing since the first leaf of albino corn seedlings began to senesce on the plant by the tenth day.

Preparation of Leaf Disks

Leaf disks, 7 mm dia., were cut with a cork borer from the first seedling leaf of 10-day-old corn. Disks, 5 mm dia., were used in the case of 7-day-old albino mutants. Care was taken to select uniform leaves and to avoid including midrib tissue in the disks. Four to six disks were cut from each leaf. The disks were randomized and floated, adaxial surface up, on 4 ml buffered test solution in a sterile 5 cm dia. petri dish. The dishes were placed on a reciprocal shaker, 1 oscillation per sec, in darkness at 20°. At the end of the experiment, the disks were rinsed and rapidly frozen on dry ice. The buffered test solution, which was filter-sterilized, contained 0·05 M N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES) at pH 7·55 as buffer and 2×10^{-4} M chloramphenicol to suppress bacterial growth. The presence of chloramphenicol at this low concentration did not affect the course of senescence and the response of the leaf disks to benzyladenine.

Every petri dish in each experiment was tested for bacterial contamination by plating 0·2 ml of the incubation medium onto nutrient agar. Dishes showing contamination were discarded. Although the corn leaves were not surface sterilized, they were handled using sterile techniques and, as a result, contamination was very low.

Chemicals

Solutions of benzyladenine were sterilized by autoclaving and ^{14}C-leucine (U. L.) was obtained in sterile solution (specific activity 180 mc/mM, Tracerlab, Waltham, Mass., U.S.A.).

Assay Methods

Chlorophyll. Frozen disks were extracted in 80% ethanol (1 ml per disk) for 20 min at 80° and the absorbance of the extracted chlorophyll was measured at 665 nm.

Protein. Protein was measured as α-amino nitrogen in acid-hydrolysed samples following the method of Yemm and Cocking.[15] This method proved more reliable for measuring total protein than the Lowry assay. The leaf disks were extracted three times with 80% ethanol to remove all soluble α-amino nitrogen before being hydrolysed with 6 N HCl in sealed test tubes at 105° for 18 hr. No protein was extracted with 80% ethanol for identical results were obtained with 7% trichloroacetic acid. A factor of 10 for converting μg α-amino nitrogen to μg protein was determined using a purified protein extract of corn leaves.

Radioactivity. Replicates of five frozen leaf disks were homogenized in a glass Duall Tissue Grinder in 4 ml of 80% ethanol and allowed to precipitate on ice for 1 hr before centrifugation. The pellet was washed with 2 ml ethanol and the supernatants combined to yield the soluble extract. The precipitate was decolorized with a 1 ml rinse of diethyl ether and subsequently washed with 0·5 ml absolute ethanol to remove traces of ether which quench in scintillation counting. This ether wash did not remove any radioactivity. The precipitated sample was suspended in Bray's[16] solution containing 3·5% cabosil and counted in a Packard 3375 liquid scintillation spectrometer.

The soluble fraction was evaporated to dryness and the residue was decolorized by two 1-ml rinses of diethyl ether before being dissolved in 0·2 ml of 80% ethanol and counted using Bray's solution as scintillator.

Chromatography of acid-hydrolized protein and of the soluble amino acids from ^{14}C-leucine-labelled disks confirmed that all the radioactivity remained associated with leucine.

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Note added in proof—The increase in the incorporation of ^{14}C-leucine into protein of benzyladenine-treated leaf disks above the zero-time value (Table 3) may very likely not be due to stimulation of protein synthesis. Recent experiments with similarly treated material (J. McClements, unpublished data) showed that proteolytic activity is reduced below the zero-time level during the first hours of benzyladenine treatment. Reduction of proteolysis would result in higher apparent incorporation of ^{14}C-leucine into protein.