The Effect of Kinetin on Nucleic Acids and Nucleases of Excised Barley Leaves [1, 2]

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Introduction

Recent work from several laboratories (6, 7, 9, 11, 12, 13) has shown that in excised green leaves floated on water there are rapid declines in the level of RNA, DNA, protein and chlorophyll which are retarded if the leaves are floated on kinetin solution. Furthermore, the local application of kinetin on detached tobacco leaf causes the maintenance of the protein level and color of the treated area which has been suggested to act as a metabolic sink (6). The stimulation of both RNA and protein synthesis by kinetin in tobacco (8) and *Xanthium* (7) leaves has been reported, and McCalla, Moore and Osborne (5) using another kinin, the benzyladenine-C^{14}, have obtained tentative evidence that label was incorporated into RNA although at a very low rate. Wollgiehn and Parthier (13) have recently shown that chloramphenicol and thiouracil accelerated the yellowing of detached tobacco leaves and the breakdown of RNA and protein which was prevented by kinetin. All these findings have indicated that kinetin in some way maintains the level of RNA and thus keeps protein synthesis going in detached leaves. Since the level of RNA and DNA in detached leaves may depend not only on the rate of synthesis, but also on the rate of breakdown, it was thought desirable to investigate the effect of kinetin on the amount of RNA, DNA and chlorophyll, on the rate of incorporation of P^{32} into RNA and DNA, and on the activity of soluble ribonuclease and deoxyribonuclease in excised barley leaves. The present paper describes the results of this investigation.

Materials and Methods

Barley (*Hordeum vulgare* L. var. Wolfe) plants were grown in the greenhouse in pots and given 16 hours of light per day. When the plants were 9 days old the first seedling leaves were excised, washed with deionized distilled water and floated in glass petri dishes on deionized water or 10 mg/liter kinetin solution in the dark and at 23 ± 1°. The duplicate samples for analysis were taken on the day the experiment was started and each subsequent day over a period of 7 to 8 days. The leaves were always washed with deionized water, blotted dry and weighed before analysis. Each experiment was repeated at least 3 times and the mean values were plotted.

Extraction and Estimation of RNA and DNA. For the extraction of RNA and DNA the procedure of Smillie and Krotkov (10) was followed. One g of barley leaves was homogenized and extracted successively with cold ethanol, cold ethanol containing 0.05 M formic acid (twice), cold 5 % perchloric acid, 95 % boiling ethanol (twice), boiling ethanol-ether (2: 1) and boiling ether. The insoluble residue was extracted with 5 % perchloric acid for 15 minutes at 90°. This perchloric acid extract was used for the estimation of RNA as ribose by the orcinol procedure (4) and of DNA by the diphenylamine procedure (1).

Extraction and Estimation of Chlorophyll. For the estimation of chlorophyll 1 g of barley leaves was homogenized and extracted thoroughly with hot 80 % ethanol. The ethanol extract was made up to 25 ml with ethanol and its OD at 600 mμ was read in a Beckman (model DU) spectrophotometer. The amount of chlorophyll in mg per g fresh weight of leaves was calculated from the OD readings using the specific absorption coefficient of 9.95 ($\alpha = 9.95$ when concentration expressed as mg/ml).

Assay of Ribonuclease and Deoxyribonuclease. One g of leaves was homogenized with 0.05 M potassium acetate pH 6.5 and acid-washed sand using a chilled pestle and mortar and the homogenate was centrifuged at 20,000 × *g* for 20 minutes at 0°. The supernatant fluid was made up to 50 ml with buffer and aliquots were used for the assay of ribonuclease and deoxyribonuclease activity.

For the estimation of ribonuclease 5 ml of enzyme preparation was incubated with 5 ml of 5 mg/ml yeast RNA in 0.05 M potassium acetate pH 6.5 for 2 hours at 37°. For the estimation of deoxyribonuclease 5 ml of enzyme preparation was incubated with 2 ml of 2 mg/ml sodium deoxyribonucleate (highly polymerized) in 0.05 M potassium acetate pH 6.5 and 1 ml 3 mg/ml $MgCl_2$ for 4 hours at 37°. At the end of incubation time the undigested nucleic acid was precipitated with 20 ml of chilled $HClO_4$-ethanol mixture (5 % $HClO_4$ in absolute ethanol) (3). The contents were chilled at −10° for 1 hour and then centrifuged at 20,000 × *g* for 20 minutes at 0°. The supernatant

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Table I. *Incorporation of P^{32} into RNA and DNA of the Barley Leaf Segments (1 g fr wt) in 1 Hour*

Sample		RNA		DNA	
		mg (ribose)/g tissue	cpm/g tissue	μg/g tissue	cpm/g tissue
Fresh leaves		12.6	980	429	430
Four days after floating	Water	7.8	300	198	230
	Kinetin	11.4	900	235	410

solution was diluted to 100 ml and its OD at 260 mμ was read against blank (for zero time). Ribonuclease or deoxyribonuclease activity was expressed as enzyme units per g fresh weight of leaves. One enzyme unit was defined as an increase of OD 1.0 in 1 ml of solution for an incubation period of 1 hour.

P^{32} Incorporation into RNA and DNA. The incorporation of P^{32} into RNA and DNA was studied for fresh detached leaves and for leaves which had been floating on water or on 10 mg/liter kinetin solution for 4 days in the dark. The leaves (ca. 1 g fr wt) were washed with deionized water and cut into 0.5 cm broad segments with a razor blade. Segments of fresh leaves or of leaves floated on water were suspended in 50 ml of water containing 0.5 μc P^{32}/ml whereas the segments of leaves floated on kinetin solution were suspended in 50 ml of 10 mg/liter kinetin solution containing 0.5 μc P^{32}/ml. The incubation was carried out by putting the contents in 250 ml flask on a shaker at 25° in usual laboratory light. After an incubation period of 1 hour the leaf segments were poured in a Buchner funnel and quickly washed with a large amount of deionized water. The nucleic acids from the leaf segments were extracted by the procedure of Smillie and Krotkov (10). The RNA from the residue containing RNA, DNA and protein was extracted as nucleotides by digesting the residue at 37° with 0.3 N KOH for 16 hours. The DNA was subsequently extracted by digesting the DNA-protein precipitate left after KOH digestion with 5 % $HClO_4$ at 90° for 15 minutes. Aliquots of RNA hydrolysate and of perchloric acid extract, after neutralization and removal of potassium perchlorate, were transferred to steel planchets, dried under an IR lamp and the amount of P^{32} counted. Aliquots from the RNA and DNA samples were also used for the estimation of RNA by the orcinol procedure (4) and of DNA by the diphenylamine procedure (1).

Results and Discussion

The data presented in figure 1 represent the mean value of 3 to 4 separate determinations made on duplicate samples. The SE of the mean was ±3.8 % for RNA, ±2 % for DNA, ±1.1 % for ribonuclease, ±1.4 % for deoxyribonuclease and ±4 % for chlorophyll. The chlorophyll content of the leaves floated on water declined rapidly whereas this decline was greatly retarded in leaves floated on kinetin solution (figure 1). There was a decline in the amount of DNA both in leaves floated on water and on kinetin solution. Again, this decline was slower in leaves floated on kinetin. The decline in the level of DNA in leaves floated on water was accompanied by a consistent rise in the activity of deoxyribonuclease starting 2 days after floating. The activity of deoxyribonuclease in leaves floated on kinetin solution changed very little until late and a rise in deoxyribonuclease activity occurred only after 6 days of floating on kinetin solution. As found by earlier workers (7, 11, 13) there was an initial rise in the amount of RNA (greater in leaves floated on kinetin solution) followed by a con-

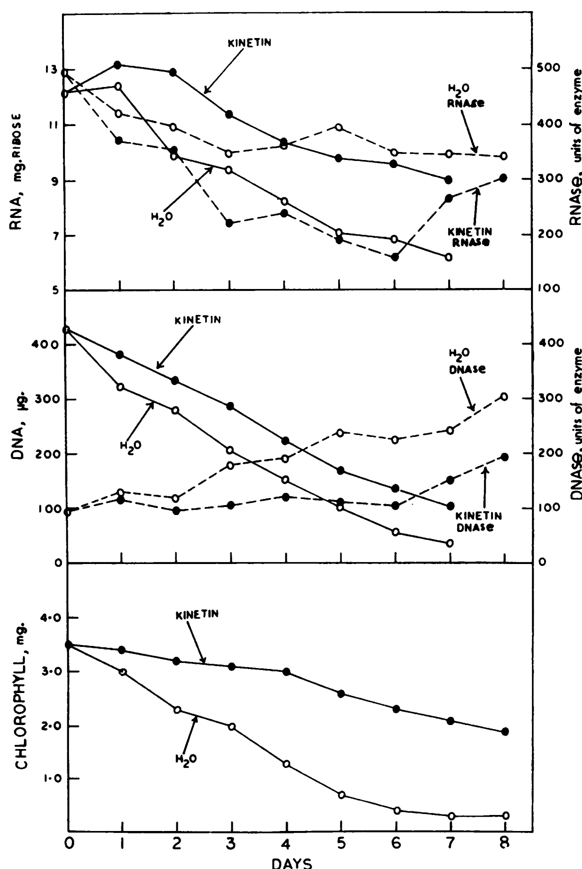

FIG. 1. The changes in the amount of chlorophyll, RNA, DNA and the activity of ribonuclease and deoxyribonuclease in excised first seedling leaves (1 g ft wt) of barley floated in the dark on water or on 10 mg/liter kinetin solution.

sistent decline both in leaves floated on water and on kinetin solution, with the amount of RNA remaining higher, throughout the duration of the experiment, in leaves floated on kinetin solution as compared to leaves floated on water. There was a decline in the activity of ribonuclease until the third day of floating in leaves floated on water after which the ribonuclease activity remained more or less constant. In leaves floated in kinetin the ribonuclease activity declined more rapidly and continued to decline until the sixth day and increased subsequently parallel with the rise of deoxyribonuclease activity. The inhibition of ribonuclease and deoxyribonuclease activity by kinetin observed here appears to be deep seated since 1 ml of 10 or 20 mg/liter kinetin added to the incubation mixture had no effect on the activity of ribonuclease or deoxyribonuclease.

The data presented in table I show that P^{32} was incorporated both into RNA and DNA of leaf segments. Osborne (7) could not observe the incorporation of C^{14} orotic acid into DNA of *Xanthium* leaf discs, and suggested that DNA synthesis did not take place during incubation. Although interference from bacteria in the present studies is not ruled out since no penicillin was used, it is, nevertheless, hard to imagine that any significant amount of P^{32} incorporated by bacteria would appear in DNA from leaf segments especially since the leaf segments were washed thoroughly with deionized water both before and after incubation with P^{32}. In addition, a short incubation period of 1 hour was used. In the absence of cell division the incorporation of P^{32} into DNA in the present study may suggest the existence of a metabolically active DNA fraction as reported to be present in rat liver (2). The data presented in table I show that in leaves floated on water the rate of synthesis of both RNA and DNA was decreased, whereas in leaves floated on kinetin solution the rate of synthesis of both RNA and DNA remained almost unaltered. It is suggested that kinetin by maintaining synthesis and suppressing destruction of nucleic acids, may preserve the integrity of the nucleic acid apparatus, especially of polyribosomes, and thus maintain protein synthesis, which is essential for the survival of the detached leaves.

Summary

The effect of kinetin on changes in the amount of chlorophyll, RNA, DNA, the rate of incorporation of P^{32} into RNA and DNA, and the activity of ribonuclease and deoxyribonuclease in detached barley leaves was examined.

Kinetin retarded the decline in the amount of chlorophyll, RNA and DNA, maintained the synthesis of RNA and DNA at almost the same rate as in fresh leaves and suppressed the activity of ribonuclease and deoxyribonuclease.

It is suggested, that kinetin, by maintaining synthesis and by suppressing destruction of nucleic acids may preserve the integrity of the nucleic acid apparatus, especially of polyribosomes, and thus maintain protein synthesis which is essential for the survival of the detached leaves.

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