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THE EFFECT OF KINETIN ON RIBONUCLEASE, ACID PHOSPHATASE, LIPASE AND ESTERASE LEVELS IN DETACHED WHEAT LEAVES

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Abstract—Detached wheat leaves gradually undergo senescence when they are floated on water in darkness and this process can be retarded by kinetin. Using this system the fluctuations in the activity of ribonuclease, lipase, esterase, and acid phosphatase have been followed. The changes in RNase levels were resolved into two activities corresponding to WL-RNase I and WL-RNase II. In detached leaves floating on solutions in the dark, kinetin depressed the level of RNase apparently through WL-RNase II. The level of RNase increased in leaves senescing on water in the dark, due to increases in both WL-RNase I and II. Illumination of the detached leaves further enhanced this increase in RNase, mainly through WL-RNase I, and this additional increase was abolished by chloramphenicol; WL-RNase I is apparently synthesized during the senescence of detached leaves under illumination. Kinetin suppressed the increase in the levels of both RNases under illumination. Lipase and esterase activities were found to decline during the senescence of detached wheat leaves, but the levels were substantially maintained in leaves floated on 10^{-4} M kinetin. By contrast, acid phosphatase activity in detached wheat leaves was not maintained at near the original levels by kinetin. Indeed the initial fall in activity was greater for leaves floating on kinetin solutions than for the water controls. However, after this rapid decline there was a period of 7 days in illuminated detached leaves in which the fall in activity was checked by kinetin at about 50 per cent of the original level, before the final period of decline. There may be more than one acid phosphatase present; one form is probably activated (or synthesized) during the degradation of the intracellular structure during senescence and it is the activation of this enzyme that is retarded by kinetin.

INTRODUCTION

IT IS well established that kinetin retards the net degradation of nucleic acids, proteins, phospholipids and chlorophyll, in detached leaves and leaf disks.[1–9] The degradation of macromolecules in these tissues may be a reflection of an increase in the activity of hydrolases. In recent years, several workers have studied the role of hydrolytic enzymes in leaf senescence and have examined the effect of kinetin on these enzymes. Although there are problems in relating *in vitro* to *in vivo* activities, the activities of several hydrolases have been reported to increase during the senescence of isolated leaves and these increases were suppressed or retarded by kinetin. Such hydrolases include protease,[1,10] DNase,[6] RNase and esterase.[10]

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In senescing barley leaves, however, RNase levels have been reported to decline, though the level was further depressed by kinetin.[6]

In this paper the changes in levels of RNase, acid phosphatase, lipase and esterase in detached wheat leaves floated on water and 10^{-4} M kinetin are reported. In a previous study[11] the separation of two RNases, designated WL-RNase I and II, from wheat leaves was described. A method was presented for estimating the relative amounts of WL-RNase I and II in a leaf homogenate, based on the differential sensitivity of the two enzymes to EDTA. This method was used in the work described here in an attempt to resolve the changes of RNase activity in terms of WL-RNase I and II.

RESULTS AND DISCUSSION

The Effect of Kinetin on Enzyme Levels in Detached Leaves

(i) *RNase.* Leaves were detached and floated on water or 10^{-4} M kinetin in the dark and analysed at daily intervals for changes in extractable RNase activity. The results show that RNase levels increased in detached leaves floated on water of both wheat [Fig. 1(*a*)] and barley [Fig. 1(*b*)]. The increase of RNase in barley was completely accounted for in terms of BL-RNase II activity, while the level of BL-RNase I remained more or less constant (assuming barley leaves contain RNases with the same characteristics as wheat). Both WL-RNase I and II accounted for the increase in RNase levels in wheat, though WL-RNase II showed the larger increase.

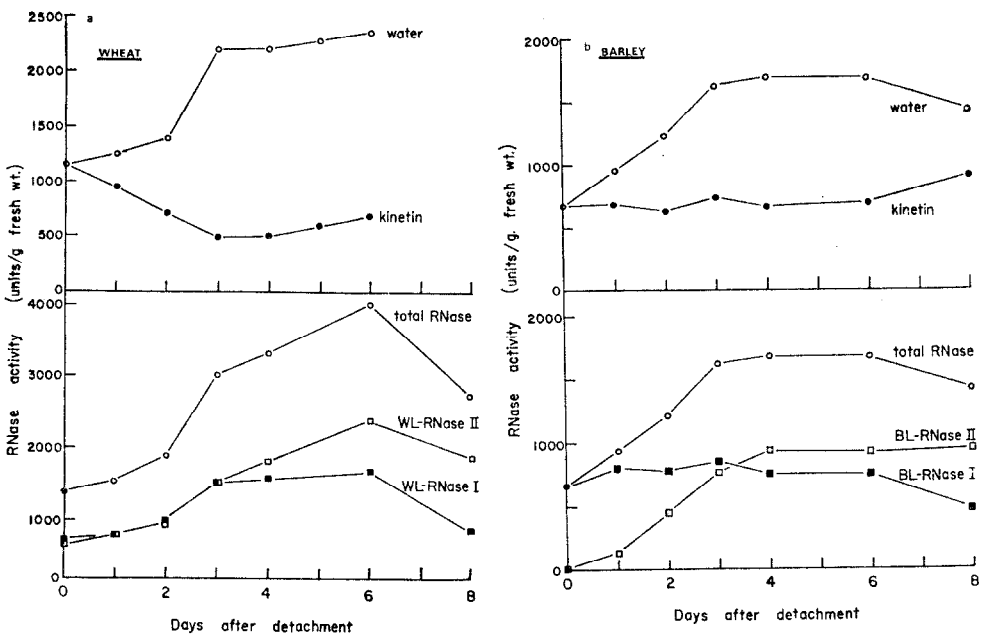

FIG. 1. UPPER FIGURES SHOW THE CHANGES IN THE LEVELS OF TOTAL RNase IN DETACHED LEAVES FLOATED ON WATER (○) AND 10^{-4} M KINETIN (●).

The figures below show the analysis of the total RNase (○) of leaves floated on water into the RNase I (■) and RNase II (□) components.

(*a*) Wheat leaves (*b*) Barley leaves

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Kinetin was found to depress the levels of RNase in wheat, whereas in barley it had little effect, maintaining the initial level of the fresh leaves [cf. Figs. 1(*a*) and 1(*b*)]. In detached wheat leaves kinetin depressed the level of WL-RNase II (Table 1), while the level of WL-RNase I remained constant. Zeatin, the natural cytokinin of maize,[12] had a similar effect (Table 1).

TABLE 1. RNase LEVELS IN DETACHED WHEAT LEAVES AFTER TREATMENT WITH 10^{-4} M KINETIN AND 10^{-5} ZEATIN FOR 3 DAYS IN THE DARK

Treatment	RNase activity (units/g fresh wt.)		
	Total RNase	WL-RNase I	WL-RNase II
Fresh levels	1800	435	1365
Kinetin	1390	435	955
Zeatin	1350	445	905

Illumination of detached leaves floated on water resulted in a substantial increase (i.e. approx. two-fold) in RNase activity over and above the level found in non-illuminated leaves [Fig. 2(*b*)] and a four-fold increase over the initial level. The changes in levels were very similar in both illuminated and non-illuminated leaves up to about 4 days after detachment [Fig. 2(*b*)]. Thereafter the RNase level continued to increase in the illuminated leaves only, and this further increase was due entirely to WL-RNase I [Fig. 2(*c*)]. The increase in RNase levels appears to be closely correlated with chlorophyll breakdown in illuminated leaves [Fig. 2(*b*)]. In the non-illuminated experiments, kinetin depressed the RNase levels through its effect on WL-RNase II (Table 1), whereas in the illuminated experiments the increase in the levels of both enzymes was suppressed [Fig. 2(*a*)]. Preliminary experiments indicated that illumination similarly increased the level of BL-RNase I in detached barley leaves.

(ii) *Lipase and esterase.* Lipase and esterase activities were differentiated on the basis of Desnuelle's definition that a lipase attacks emulsified substrates whereas an esterase attacks dissolved substrates.[13] Thus lipase was assayed using an emulsion of tributyrin and esterase by using a solution of *p*-nitrophenyl acetate. Since an emulsion of tributyrin contains a saturated solution of tributyrin, it is amenable to attack by an esterase by Desnelle's definition. However, the activity measured by this substrate was entirely lypolytic since no activity was recorded when a saturated solution of tributyrin was substituted for the emulsion.

TABLE 2. THE SUBCELLULAR DISTRIBUTION OF ESTERASE AND LIPASE ACTIVITIES IN FRESH WHEAT LEAVES

Subcellular fraction	Esterase activity (% total activity)	Lipase activity (% total activity)
Muslin filtrate	100	100
1000 *g* supt.	90	62
20,000 *g* supt.	88	16

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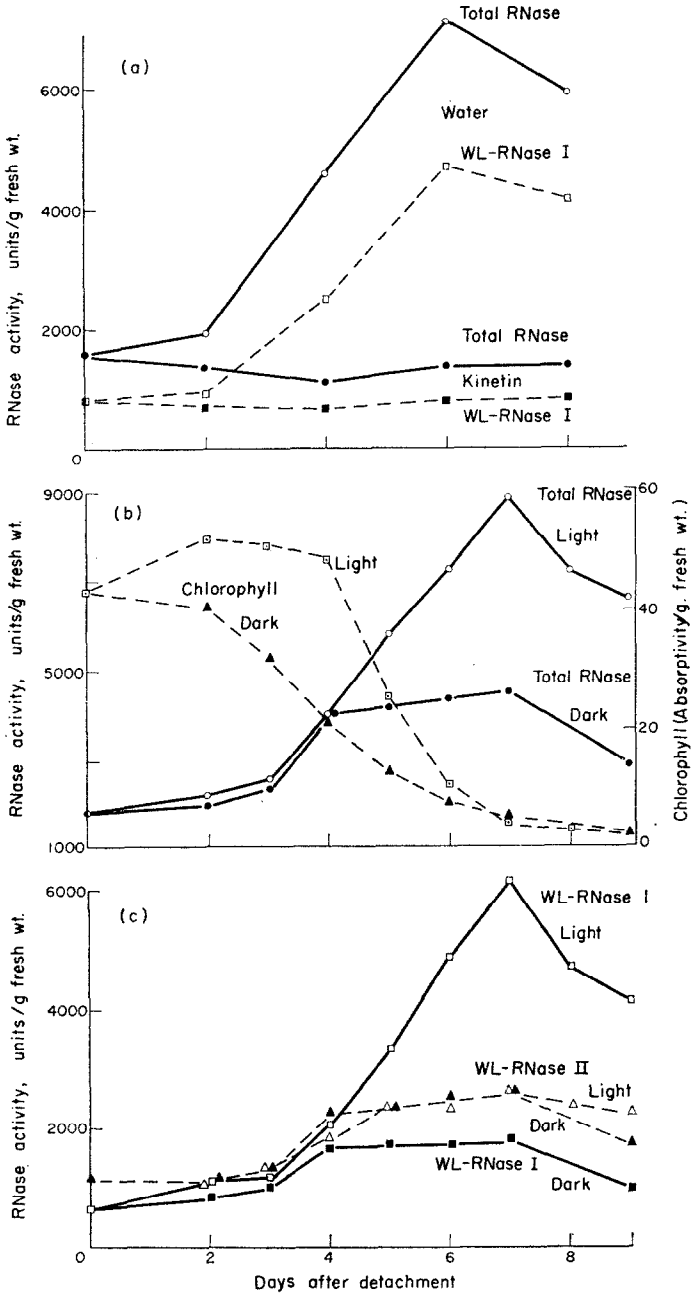

FIG. 2. (*a*) CHANGES IN RNase LEVELS UNDER ILLUMINATION OF DETACHED WHEAT LEAVES FLOATED ON WATER [total RNase (○); WL-RNase I (□)], and on 10^{-4} M kinetin [total RNase (●); WL-RNase I (■)].

(*b*) AND (*c*) CHANGES IN RNase AND CHLOROPHYLL LEVELS IN DETACHED WHEAT LEAVES FLOATED ON WATER

under Illumination [total RNase (○), WL-RNase I (□), WL-RNase II (△) and chlorophyll (⊡)] and in the dark [total RNase (●), WL-RNase I (■), WL-RNase II (▲) and chlorophyll (▲)].

Furthermore, the subcellular distribution of the enzymes in the same extraction medium (0·1 M NaCl) was completely different (Table 2). Esterase activity was mostly located in the 20,000 × *g* supernatant fraction whereas lipase was mostly particle bound. After leaf detachment, both lipase and esterase activities were found to decline in the controls whereas kinetin almost maintained the fresh-leaf level (Fig. 3). Thus the maintenance of phospholipids[2] or membrane integrity[14] in detached leaves by kinetin does not appear to be mediated through the suppression of lipase and esterase activities.

(iii) *Acid phosphatase.* The fluctuations in acid phosphatase levels (during the week following detachment) of detached leaves floated on water and kinetin solutions in the dark are shown in Fig. 4(*a*). The initial fall in acid phosphatase level was greater in the kinetin-

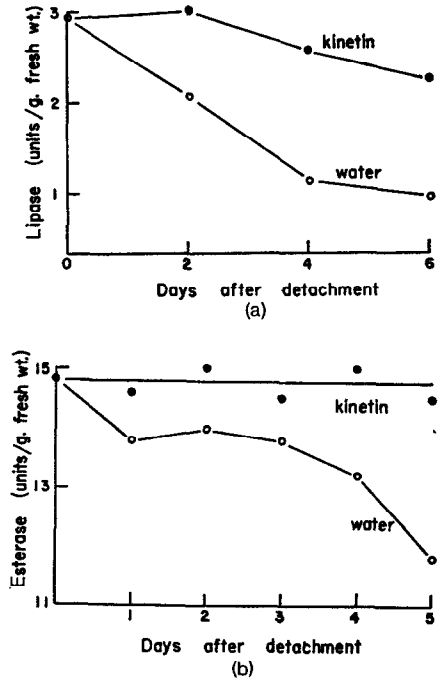

FIG. 3. CHANGES IN LIPASE (*a*) AND ESTERASE (*b*) LEVELS IN DETACHED WHEAT LEAVES FLOATED ON WATER (○) AND 10^{-4} M KINETIN (●).

treated leaves than in the water controls. Later, however, the control levels fell below those of the kinetin-treated leaves and remained so during the period of the experiment. When a similar experiment was carried out on leaves illuminated for 12 hr each day, the same kind of pattern emerged except the rates of decline were slower. In the kinetin-treated leaves the enzyme activity remained constant at about the 50 per cent level for about a week (between 2nd and 9th day after detachment) except for an increase in acid phosphatase activity between the 6th and 7th day [Fig. 4(*c*)].

The fluctuations in the levels of acid phosphatase in these two experiments suggest the curves may represent the summation of the activities of two enzymes. This supposition can be deduced more easily from the second experiment [Fig. 4(*c*)]. Thus one acid phosphatase could decline in activity from the time of detachment in both treatments (i.e. C and

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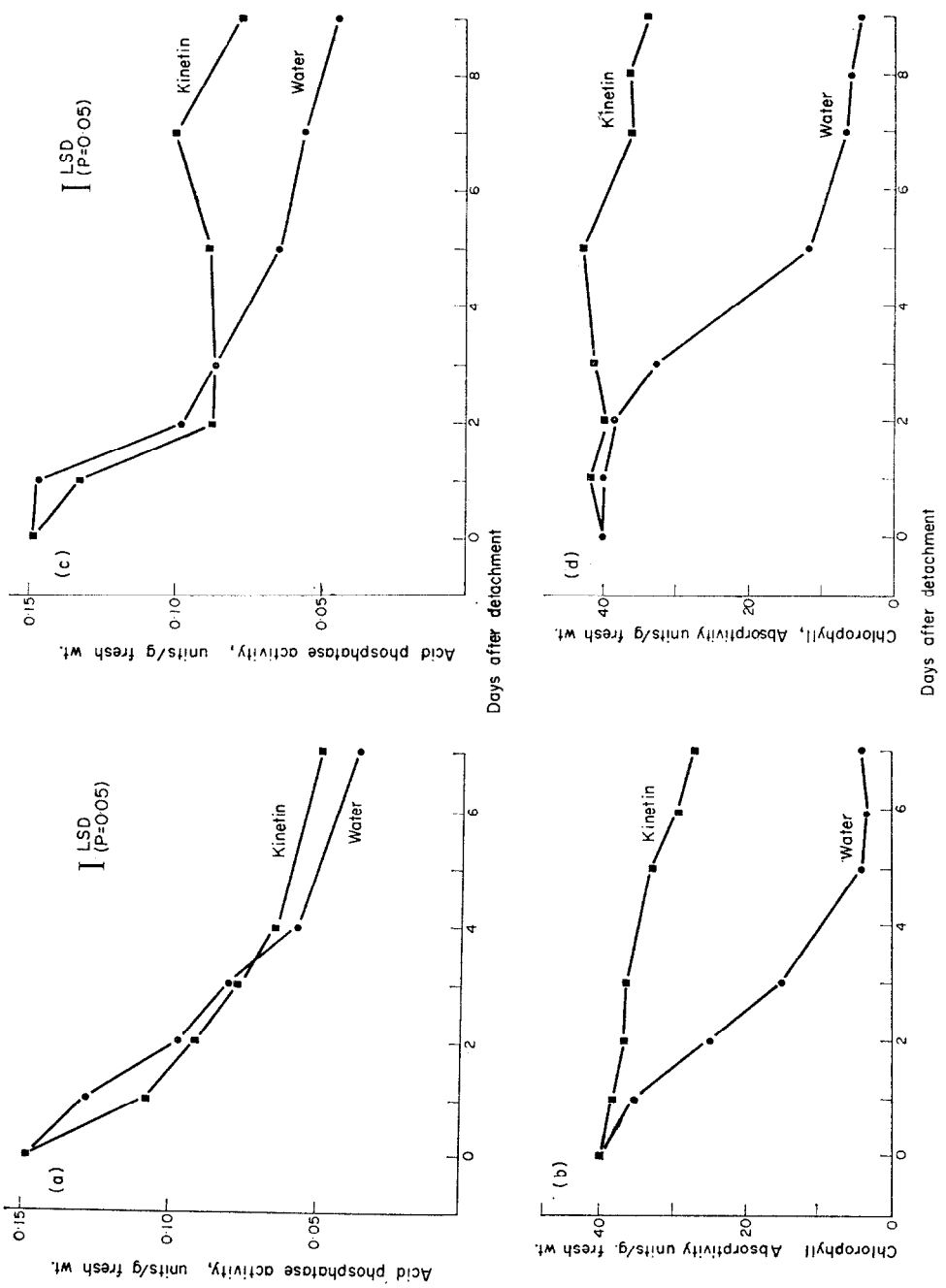

FIG. 4. CHANGES IN THE ACID PHOSPHATASE (*a* AND *c*) AND CHLOROPHYLL (*b* AND *d*) LEVELS OF DETACHED WHEAT LEAVES IN DARKNESS (*a* AND *b*) AND UNDER ILLUMINATION (*c* AND *d*) ON WATER (●) AND 10^{-4} M KINETIN (■).

K), whereas the other phosphatase might be released in the water controls soon after detachment; note the high control values at this stage, and in the kinetin-treated leaves between the 3rd and 7th day after detachment.

The early high activity in the controls [Fig. 4(*c*)] compared to the kinetin-treated leaves might result from degradation of the ER, possibly by the unfolding of the membranes and the exposure of acid phosphatase sites; alternatively one could postulate lysosome-like particles (Balz[10]) which degenerate at this time. Chlorophyll levels are beginning to decline after 1 day in the controls and after 5 days in the kinetin-treated illuminated leaves [Fig. 4(*d*)], and could reflect similar stages in intracellular breakdown.

The Effect of Chloramphenicol on RNase Levels in Detached Wheat Leaves

The increase in WL-RNase I in senescing leaves exposed to light might be due to an actual synthesis of this enzyme. To investigate this possibility, the effect of floating leaves on chloramphenical (CAP), an inhibitor of protein synthesis, was studied. The results show that CAP totally inhibited the increase in RNase levels due to illumination, that is WL-RNase I [Fig. 5(*b*) and (*c*)]. CAP had no effect on the increase occurring in non-illuminated leaves, nor in a similar increase in illuminated leaves. Because CAP retarded the loss of chlorophyll in illuminated leaves [Fig. 5(*a*)], measurements of RNase levels were continued until the chlorophyll level had declined to a low level, as in the controls. However, RNase levels in leaves floating on CAP never rose above the levels obtained in non-illuminated leaves floating on water [Fig. 5(*b*) and (*c*)]. Thus it appears that WL-RNase I is synthesized in detached leaves under illumination. The effect of salt on the pH-activity curve of an extract of illuminated detached leaves suggested that the enzyme synthesized is WL-RNase I rather than a second RNase insensitive to EDTA.[11]

It is of interest that CAP retards the decline of chlorophyll in detached leaves senescing under illumination, whereas it has no visible effect on the decline of chlorophyll in non-illuminated leaves. These results are in complete contrast to those of Srivastava,[14] who found that CAP accelerated the breakdown of chlorophyll in detached barley leaves under illumination.

Changes in the Subcellular Distribution of RNase Activity in Detached Leaves under Illumination

Balz[10] found that during the senescence of detached tobacco leaves there was an increase in RNase activity associated with the particles, and later this activity appeared in the high-speed supernatant fraction as the activity on the particles declined. Similarly, we studied the changes in the subcellular distribution of RNase activity during the senescence of detached leaves under illumination. The results are shown in Fig. 6. In the fresh leaf most of the activity was associated with the particulate matter, especially WL-RNase II. During the course of senescence the largest increases occurred in the high-speed supernatant fraction, and this activity was almost entirely WL-RNase I. Activity also increased in the particulate fractions, mostly due to WL-RNase II. The subcellular distribution of WL-RNase I and II is in agreement with Wilson's[16] finding that RNase A of corn roots is largely associated with the high-speed supernatant whereas RNase B is mostly particle-bound. In a previous study[11] evidence was presented that WL-RNase I and II may be similar to RNase A and B respectively. It is, however, surprising that RNase activity associated with the particles continues to increase throughout senescence since the particles break down during senescence.

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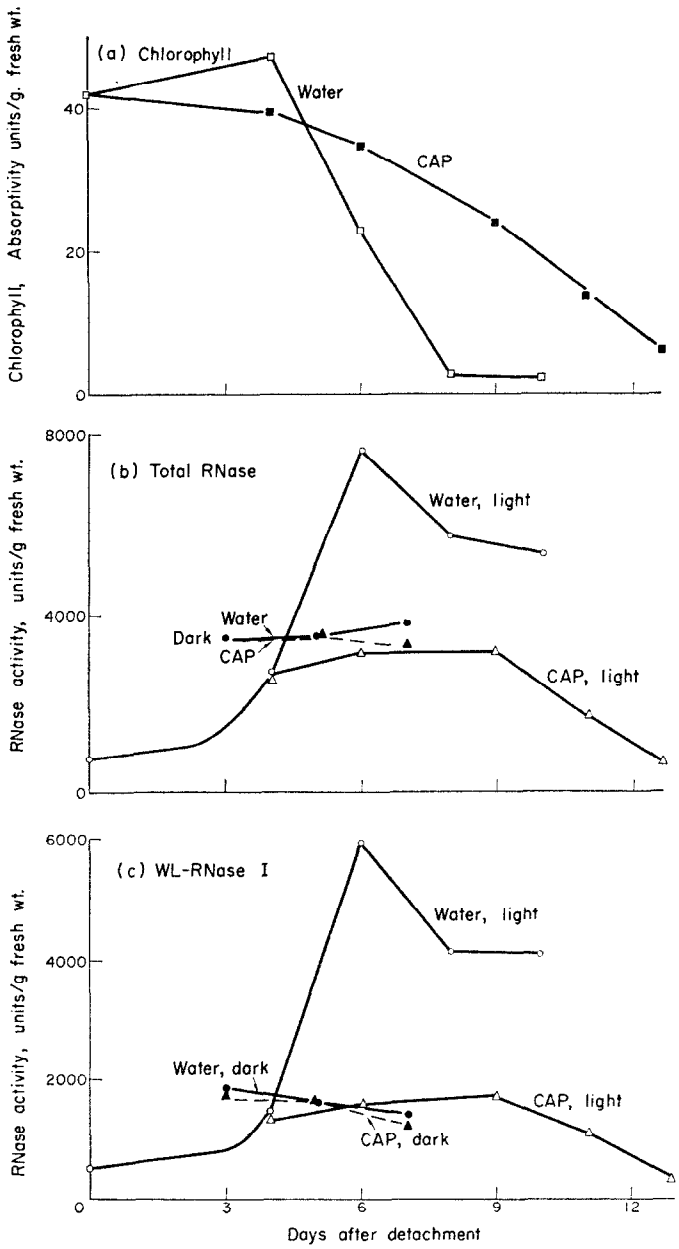

FIG. 5. THE EFFECT OF CAP ON CHLOROPHYLL AND RNase LEVELS DURING THE SENESCENCE OF DETACHED WHEAT LEAVES.

(*a*) Chlorophyll levels of leaves floated under illumination on water (□) and 1 mg/ml CAP (■).

(*b*) Total RNase levels of leaves under illumination on water (○) and 1 mg/ml CAP (△), and in the dark on water (●) and CAP(▲).

(*c*) WL-RNase I levels; legend as (*b*).

Perhaps the association of RNase with the particles arises by adsorption of the enzyme on to the particles.

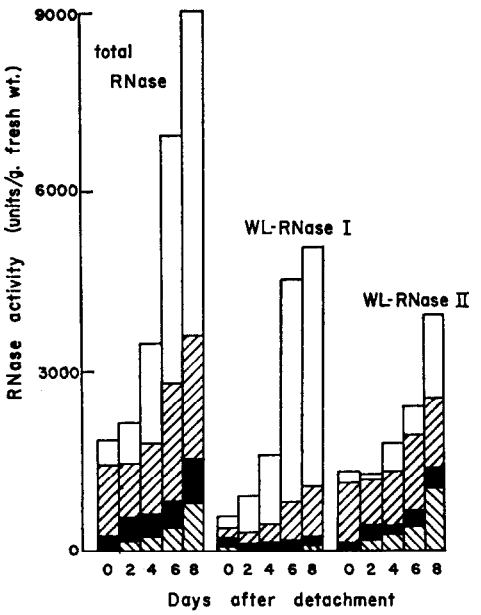

FIG. 6. CHANGES IN THE SUBCELLULAR DISTRIBUTION OF RNase DURING THE SENESCENCE OF DETACHED WHEAT LEAVES AFTER FLOATING ON WATER UNDER ILLUMINATION.

□ 100,000 *g* supt. ▨ 20,000–100,000 g frn.
■ 1000–20,000 g frn. ▧ 1000 g pellet.

DISCUSSION

RNase activity was found to increase in detached leaves during senescence in the dark. Under illumination there was a much greater increase in RNase activity, and all the extra RNase appeared to be WL-RNase I. The total inhibition by CAP of the increase in RNase activity due to illumination (WL-RNase I) would suggest that the enzyme was synthesized in illuminated leaves. CAP is known to inhibit the synthesis of chloroplast proteins,[17] but protein synthesis in the cytoplasm of photosynthesizing cells is comparatively insensitive to CAP.[18] Consistent with these results, Anderson and Smillie[19] demonstrated that more ^{14}C-CAP was bound per unit RNA by chloroplast ribosomes than cytoplasmic ribosomes. Stutz and Noll[20] observed that CAP inhibits protein synthesis in systems containing the 70 s ribosome (e.g. chloroplasts) but not in systems containing the 80 s type (e.g. plant cytoplasm). Thus, in view of the inhibition of WL-RNase I synthesis by CAP, it is possible that the synthesis of the enzyme is associated with protein synthesis in the chloroplast.

The results in Fig. 2(*b*) and (*c*), suggest that RNase (WL-RNase I) is also synthesized in the dark. Here it appears that light is replacing a factor limiting in the production of RNase in leaves senescing in the dark. This factor may be chemical energy derived from photo-

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[18] J. M. EISENTADT and G. BRAWERMAN, *J. Molec. Biol.* **10**, 392 (1964).
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synthesis. Thus it is possible that the increase in WL-RNase I in the dark was also due to synthesis, and this proceeded until the energy supply was depleted. Since CAP had no effect on the increase of RNase independent of light (Fig. 5), it seems unlikely that CAP is inhibiting RNase synthesis directly. A possible explanation is that CAP may in some way block the production of photosynthetic energy. This possibility receives support from the results of Parthier *et al.*,[21] who found that CAP at 1 mg/ml strongly inhibited the incorporation of ^{14}C-amino acids and $^{14}CO_2$ into protein of leaf disks when the disks were illuminated, but had little effect on incorporation in the dark.

The idea that the synthesis of RNase is supported by photosynthetic energy is in line with the recent results of Udvardy *et al.*[21] They also found that illumination increased the level of RNase activity in detached *Avena* leaves, and that the effect of illumination could be partially replaced by sucrose and reduced by dichlorophenyldimethylurea, an inhibitor of photosynthetic CO_2 fixation. The authors concluded that the light effect was explained at least in part by the photosynthetic production of sugars. Udvardy *et al.*[22] also found that illumination increased the RNase activity of the $20{,}000 \times g$ supernatant of detached *Avena* leaves, but not the activity of the particulate fraction. This is in agreement with the findings reported here where only the activity of WL-RNase I, located chiefly in the $20{,}000 \times g$ supernatant, was increased by illumination, whereas the predominantly particle-bound WL-RNase II was not.

The increase of WL-RNase II in detached leaves was unaffected by illumination and CAP, whereas WL-RNase I was affected. This suggests that the increase of WL-RNase II is not due to synthesis, or at least that the nature of its production is distinct from that of WL-RNase I. Perhaps the enzyme is released from a latent form during senescence, though it was not possible to demonstrate latency using either detergents, high salt concentrations, or 3 M urea.[23]

No definite conclusions can be drawn on the *in vivo* significance of the changes in activities of the various hydrolases reported here. With the exception of acid phosphatase, kinetin more or less maintained the levels of these enzymes existing at the time of detachment. Only RNase levels could be correlated with the changes in RNA levels known to occur in detached leaves. The fact that RNase (WL-RNase I) is synthesized during senescence, a time when protein in general is breaking down, strengthens the possibility that its increase in activity has *in vivo* significance.

EXPERIMENTAL

Plant Material

Wheat (var. Eclipse) and barley (var. Proctor) grains were soaked in distilled water at room temp. for 2 hr and 1 day respectively. Undamaged grains of average size were sown with their embryos orientated uppermost in trays of vermiculite and grown for 7 days in a growth room with a light period of 14 hr per day at 800 lm/ft^2 from "natural daylight" Atlas (5 ft, 80 W) fluorescent tubes, a temperature of 23° and a minimum relative humidity of 80 per cent.

Experiments with Detached Leaves

(i) *RNase, lipase and esterase experiments.* The first leaves were excised and weighed in batches of about 500 mg and floated abaxial side down on water, 10^{-4} M kinetin, or 1 mg/ml CAP in closed Petri dishes. The dishes were left at 23° either in the dark or in the growth room described above (i.e. in a light period of 14 hr per day). Duplicate batches were set up for each treatment.

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(ii) *Acid phosphatase experiments.* Wheat first leaves were floated on 150 ml of water or 10^{-4} M kinetin in open plastic trays (18 × 10 × 4 cm). Each tray contained forty leaves (approx. 1·6 g), enough for two replicate extracts. The trays were placed in an incubator at 25° for the dark-treatment experiments or in a growth room as described above (except the light period was 12 hr and the temperature 25°) for the light-treatment experiments.

Determination of Enzyme Activity

Extraction of leaves. Each batch of detached leaves was rinsed in sterile distilled water, blotted dry and ground up in the extraction medium using a chilled pestle and mortar. Sand was not used for grinding since it strongly absorbed RNase in the absence of a high salt concentration. For RNase determinations, each batch of leaves (500 mg) was extracted in 20 ml of 0·1 M NaOAc, pH 6, the extract filtered through muslin and the filtrate centrifuged at 1000 × *g* for 15 min; the supernatant was assayed. Lipase determinations were carried out on the muslin filtrate of leaves (500 mg) extracted in 20 ml of 0·1 M NaCl. Each batch of leaves was extracted in 40 ml of 0·067 M sodium phosphate buffer, pH 7·0, for esterase determinations. The muslin filtrate was centrifuged at 20,000 × *g* for 20 min and the superantant was assayed. In the acid phosphatase experiments twenty leaves (800 mg approx.) were extracted in 20 ml acetate buffer, pH 5·2, and the extract filtered through nylon gauze ("Nytal", 25-μ pore size) under reduced pressure; the filtrate was assayed.

Subcellular distribution. The subcellular distribution of RNase was determined by extracting 500-mg batches of leaves in 20 ml 0·4 M sucrose and centrifuging the muslin filtrate at 1000 × *g* for 15 min followed by 20,000 × *g* for 20 min and 100,000 × *g* for 90 min.

Enzyme Assay

(i) *RNase.* RNA (B.D.H. Ltd. ex. yeast) was purified by precipitating RNA as the sodium salt from ethanol. The substrate solution was made by dissolving the purified RNA at 4·8 mg/ml in 0·1 M NaOAc buffer, pH 6·0, and the substrate stored frozen until required. RNase was assayed by the method of Tuve and Anfinsen[24] with modifications. The assay mixture consisted of 1 ml of the substrate solution, enzyme (up to 0·4 ml), and 0·1 M NaOAc buffer, pH 6·0, to give a final volume of 2 ml. The assay mixture was incubated at 37°, and 0·5 ml samples withdrawn 10, 40 and 70 min after the addition of the enzyme and pipetted into a 0·5 ml 5% perchloric acid containing 0·25% uranyl acetate. The tubes were chilled and the precipitates spun down. A 0·5 ml sample of the supernatant was made up to 5·0 ml with water, and the absorptivity at 260 nm measured. Activity was linear with time and proportional to the amount of enzyme added. One unit is equivalent to the production of 0·1 μmole of acid-soluble nucleotides per hour, assuming an average $A^{1\,cm}_{260}$ of 10 for a μmole/ml mixture of nucleotides.

WL-RNase I and II were assayed by their differential sensitivity to EDTA.[11] The method essentially involves the pre-incubation of the leaf extract with EDTA prior to the RNase assay, a process which inactivates one of the enzymes. The EDTA-insensitive enzyme, WL-RNase I, can be assayed directly as described here, while the activity of the inactivated enzyme, WL-RNase II, may be calculated by difference from the total activity. The enzyme sample was incubated for 30 min at 37° with 4×10^{-3} M EDTA and 0·1 M NaOAc buffer, pH 6·0, in a total volume of 1 ml. 1 ml of the substrate was then added, to give an assay mixture the same as that described above, apart from the presence of EDTA. 0·5 ml samples were removed 5, 35 and 65 min after the addition of the substrate and treated as described above.

(ii) *Esterase.* Esterase activity was assayed by measuring the hydrolysis of *p*-nitrophenyl acetate. The method of Huggins and Lapides[25] was modified to suit the leaf esterase. A solution of *p*-nitrophenyl acetate was made up and adjusted to 0·65 μmole/ml immediately before use.[25] 4·0 ml of the adjusted substrate was mixed with the enzyme sample (usually 1·0 ml) and 0·067 M sodium phosphate buffer, pH 7·0, at 25° to give a final volume of 5 ml. The intensity of the colour was measured 1 min later at 400 nm against water, and again after incubation at 25° for 20 min. The substrate blank value was subtracted and activity expressed as that amount of enzyme which liberates 1 μmole of NP per hour under the above conditions.

(iii) *Lipase.* The method adopted was essentially that of Downey and Andrews,[26] whereby butyric acid produced by the hydrolysis of a tributyrin emulsion is titrated automatically. 12 g of gum arabic was dissolved in 100 ml of glass-distilled water and cooled. 12 ml of tributyrin was added and the mixture emulsified by whipping for 5 min at top speed in an M.S.E. homogenizer. The pH was adjusted to 7·5 with a few drops of N NaOH and the volume made up to 120 ml. The emulsion was prepared fresh daily. In performing the assays, 3·0 ml of the tributyrin emulsion, 0·5 ml of 1·0 M NaCl and 0·033 M $MgCl_2$, and enzyme, were mixed in the reaction vessel. The pH was adjusted to 7·7 with alkali and recording commenced when the pH dropped to 7·5 (the pH optimum of the enzyme). The titrator was set to maintain this pH by the addition of 5 mM NaOH from the micro-syringe. Enzyme activity was calculated from the slope of the trace and expressed as μmoles of acid liberated per minute at 25°.

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[25] C. Huggins and J. Lapides, *J. Biol. Chem.* **170**, 467 (1947).
[26] W. K. Downey and P. Andrews, *Biochem. J.* **94**, 642 (1965).

(iv) *Acid phosphatase.* The assay was based on the method described by Torriani.[27] To 3 ml of 0·2 M acetate buffer at pH 5·5 and 25° were added 1 ml 0·04 M NPP (*p*-nitrophenyl phosphate, disodium salt) and 1 ml of leaf extract. The reaction mixture was maintained at 25° in a water bath. 1 ml samples were withdrawn at 10, 15, 20 and 25 min and added to 2 ml Tris buffer, pH 8·5, containing 0·4 M phosphate. The amount of NP liberated was measured at 420 nm using 1 cm glass cells and plotted against time. The activity was calculated from the slope of the resulting linear curve. A unit of enzyme activity was defined as the amount of enzyme which liberates 1 μmole of NP per minute under the prescribed conditions.

Chlorophyll. Chlorophyll determinations were made concurrently with RNase and acid phosphatase extractions on batches of three leaves. Triplicate batches were set up for each treatment. Each batch was extracted with 10 ml of 80% ethanol for 10 min and the absorptivity measured at 645 nm, which is the absorption maximum in red light of chlorophyll b.[28] Chlorophyll levels were expressed as absorptivity units per g fresh weight.

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