THE EFFECT OF WATER STRESS ON RIBONUCLEASE ACTIVITY OF WHEAT PLANTS

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ABBREVIATIONS

A:	adenine
ΔA_{260}:	absorbance change
AMP:	adenosine monophosphate
C:	cytosine
p-CMB:	p-chloromercuribenzoate
2,4-D:	2,4-dichlorophenoxyacetic acid
EDTA:	(ethylenedinitrilo)tetraacetic acid
G:	guanine
GA:	gibberellic acid
GMP:	guanosine monophosphate
m-RNA:	messenger ribonucleic acid
RNA:	ribonucleic acid
RNase:	ribonuclease
U:	uracil
UMP:	uridine monophosphate

CHAPTER I

INTRODUCTION

A reduction in protein and RNA content are well established features of water stressed leaves. High osmotic stress caused tomato leaves to lose RNA although the plant retained the ability to incorporate labeled phosphate into RNA and hence to synthesize RNA (Gates and Bonner, 1959). The rate of labeled uracil incorporation into RNA was similar in the control and water stressed leaves of tomato, suggesting that water stress enhanced RNA hydrolysis (Kessler, 1961). Water stress resulted in a greater reduction of total RNA content in the leaves of a non-resistant variety of wheat plant than that in a resistant variety (Stutte and Todd, 1968).

The harmful effect of drought on protein content may be the result of defective synthesis and/or accelerated proteolysis. The total soluble protein decreased with increasing stress in wheat plants (Todd and Basler, 1965, and Stutte and Todd, 1969) while specific activity of peroxidase increased (Stutte and Todd, 1969).

The effect of drought on the nucleic acid content referred to above suggests that a RNA hydrolyzing enzyme is involved in the decreased RNA content. RNases, however, have not been studied extensively with regard to water stress, and existing reports appear to be conflicting. An initial decrease in RNase activity occurred following four days of water stress of tomato leaves (Kessler, 1961), but

desiccation of detached tomato leaflets caused increased RNase activity (Dove, 1967). Nevertheless, RNA content decreased after 4 days of water stress in wheat plants (Stutte and Todd, 1968).

Increases in RNase activity accompanied by yellowing in plant leaves (Eilam, et al., 1969) and mechanical damage of leaf tissues (Udvardy, 1969).

There may be more than one RNase present; an active form and a latent, enzymatically inactive RNase have been reported (Shortman, 1961). Moreover, different kinds of RNases having different properties appeared during germination (Vold, et al., 1968) and senescence (Sodek, et al., 1969) of wheat plants.

The mechanism for the increase in RNase activity during water stress is thought to involve the release of enzyme from lysosomes (Kessler, 1961, and Tappel, 1966). Enhanced synthesis is also suggested as an explanation for the increase in RNase activity during senescence (Sodek, et al., 1969).

In this study, the changes in levels of RNases in water stressed wheat plants are presented. The experiments described herein establish RNase activity as a reliable, early indicator of water stress in both leaf and crown tissues of wheat plants. The increase in RNase activity in water stressed plants was studied from viewpoints of de novo synthesis and conversion of enzyme to an active state during the exposure to drought stress.

CHAPTER II

LITERATURE REVIEW

Water Stress and RNase Activity

Water stress may have both qualitative and quantitative effects on physiological processes of plants. Under the influence of brief periods of water stress, net accumulations of RNA ceased in young tomato leaves. The incorporation of P^{32} labeled phosphorus showed that the block to net RNA accumulation under conditions of moisture stress primarily was due to the accelerated destruction of RNA (Gates and Bonner, 1959). After 3 days of water stress in tomato and pea plants grown in the field or in mannitol, there was a decrease in RNA accumulation although the rate of uracil-C^{14} incorporation into RNA was similar in control and water stressed plants, suggesting an enhenced hydrolysis provoked by water stress (Kesller, 1961). Although drought-resistant olive leaves accumulated RNA on dehydration, plants synthesized RNA molecules of different types as judged by an increasing $\frac{G + C}{A + U}$ ratio (Kessler, et al., 1962). Water stress of wheat plants resulted in a reduction of total RNA content in which the drought-sensitive variety showed a greater decrease in RNA content per plant than the resistant variety, and more severe drought resulted in a higher ratio of $\frac{\text{purine}}{\text{pyrimidine}}$ in the RNA (Stutte and Todd, 1968). West (1962) found a decreased RNA content in water stressed corn seedlings that was accompanied by increased GMP and UMP concentrations in the

remaining RNA. He also found elevated levels of free mononucleotides in the water stressed seedlings.

In mature leaf blades of sugar beet, 13% of the RNA was found in the soluble fraction, 20% in ribosomal fraction and 67% in other fractions. During moisture stress, soluble RNA increased and the amount of RNA in the other fractions, especially in ribosomes, decreased, suggesting that the character of the protein-synthesizing machinery does undergo considerable alteration (Shah, et al., 1965). There is other evidence that lethal effects due to water stress in wheat leaves may be due to the breakdown of the various components and loss of the synthetic machinery. Todd and Basler (1965) found a greater destruction of nucleic acids than that of proteins in the supernatant fraction and compositional study of cell fractions showed that the nucleic acid and protein changes in the crowns of the same plants were different from that for the leaves. Dehydration of wheat embryos during germination resulted in the inactivation of m-RNA, and rehydration caused the transcription of the complementary RNA which was inactive even though it resembled, superficially, the normal m-RNA of control plants (Chen, et al., 1968). In the case of dehydration at a later stage of germination, rehydration caused the inactivation of m-RNA. Therefore, it might be expected that water stress during development of wheat plants affects protein biosynthesis through the effects of dehydration on the transcription and translation mechanism. Detachment of wheat leaves caused the protein content to decrease rapidly whether they kept turgid or dried following detachment (Todd and Yoo, 1964). By using acrylamide gel electrophoresis, it was shown that certain peroxidase bands disappeared and new ones appeared

with increasing water stress. In this experiment, the total peroxidase activity per unit of protein increased with water stress while total soluble protein decreased drastically with increasing water stress (Stutte and Todd, 1969). It was found that RNase activity was sensitive to water stress of tomato leaflets (Kessler, 1961). It was suggested that detachment of tomato leaves increased RNase activity (Dove, 1967).

On the other hand, increased RNase activity results from various physiological disturbances in plant leaves. Greater activity was found in homogenates of leaves stressed by zinc deficiency (Kessler, et al., 1959), and the RNase level in corn shoots was increased as much as 3 fold by K^+ deficiency (Hsiao, 1968). With excised tissue of corn, a low concentration of 2,4-D accelerated growth and RNase activity in parallel (Shannon, et al., 1964). RNase activity was found to increase in the developing leaves of apple (Kessler, et al., 1962), and following mechanical damage of tobacco leaf tissue (Bagi, et al., 1967), and of Avena leaves (Udvardy, et al., 1967). Kinetin treatment of tobacco tissue subjected to mechanical damage reduced the rise in RNase level (Udvardy, 1969). Phytokinins decreased RNase synthesis after detachment of tomato leaves (McHale, et al., 1969). The specific activity of RNase increased as senescence progressed in detached wheat leaves (Sodek, et al., 1969). In bean endosperm, auxin affects RNase turnover at the level of either transcription or translation (Sacher, 1969). A direct proportionality between RNA and auxin levels was found in the growth of intact Lens root tip, but an inverse proportionality between RNA content and RNase activity was found in the excised root tip (Pilet, et al., 1970). Abscisic acid inhibition of

GA_3 induced production of reducing sugar was stimulated by RNase but not by the specific RNA polymerase inhibitor (rifamicin) in barley endosperm (Leshem, 1971).

Several explanations have been offered to explain the increase in RNase activity with senescence and water stress. Suppression of increased RNase activity by actinomycin D suggests that enzyme synthesis was responsible for the observed additional activity during senescence of tomato leaves (McHale, et al., 1968). The increase in RNase activity induced by excision of Avena leaves was inhibited by cycloheximide (Udvardy, et al., 1969). The increase in activity of RNase during Rhoeo leaf senescence was inhibited by inhibitors of RNA synthesis and protein synthesis (De Leo, et al., 1970-b). The short term studies of banana tissue slices treated with cycloheximide and actinomycin D showed that the increase in RNase activity during senescence was due to new synthesis, which was dependent upon synthesis of RNA (De Leo, et al., 1970-a). The observed increase in RNase occurs at a time when protein synthesis is declining and less rather than more RNase would have been expected. For example, a reduction in protein content was correlated with a reduced number of polysomes in sunflower leaves (Genkel, 1967). Sucrose gradient profiles of polysomes from the coleoptile node region of maize showed that water stress caused a shift of ribosomes from polymeric to the monomeric form (Hsiao, 1970). Another explanation for the observed increased RNase was that several kinds of RNase could exist in the plant system. The breakdown of RNA at the substrate level was accelerated by addition of reduced gluathione in cotton plant extracts (Basler, 1963), but RNase from soybean was not affected by reduced glutathione (Merola, et al., 1962). The

properties of RNase from non-germinating embryos, as compared to seedlings of wheat, differed with respect to pH optimum, heat stability and intercellular location, suggesting that the differences in RNase activity during germination were due to varying proportions of 2 RNases (Vold, et al., 1968). Two kinds of RNases were found in detached leaves of wheat. The increase of RNase during senescence was accounted for in terms of an EDTA-sensitive form, while the EDTA-insensitive enzyme remained more or less constant (Sodek, et al., 1969). Several isozymes of corn RNase II from microsomes were detected by polyacrylamide gel electrophoresis (Wilson, 1971). Although the activation of an inactive form of RNase referred to above was responsible for the increase in RNase, the release of RNase from some subcellular structures might be partially responsible for the increase in RNase. Kessler (1961) suggested the existence of nucleoprotein particles with which RNase was associated and which was surrounded by a membrane of lipid character. The dictyosomes of aleurone cells of barley treated with GA_3 proliferated many vesicles. This proliferation was associated with the phase of rapid RNase release from the aleurone cell (Jones, et al., 1970). Electron microscopy of cells from cucumber leaves during senescence showed that spherosome RNase might be released into the cell during later stages of senescence (Eilam, et al., 1971).

Properties of RNase

Ribonuclease (E. C. 2.7.7.16, ribonucleate pyrimidineucleotide-2'-transferase cyclizing) appears to be a normal component of plant cells. Purification of RNases present in plants has been achieved (Bernard, 1969). The activity of a purified enzyme preparation from

spinach reached a maximum in 0.1 M buffers at pH 5.0 to 6.0 in which a double maximum was observed in the pH optimum curve, and it was possible that this indicated the presence of two RNases differing slightly in pH optima (Tuve and Anfinsen, 1960). In soybean RNase, the activity reached a maximum at pH 5.2 when an insoluble fraction of yeast RNA was the substrate (Merola, et al., 1962). Two Rnases have been isolated from corn seedlings which had pH optima at 5.0 and 6.2 (Wilson, 1963). Ribonuclease from rye grass had a pH optimum at 6.5 without the addition of NaCl (Freeman, 1964). In RNase from tobacco leaves, the pH optimum in two buffer systems, sodium acetate and citrate buffers (0.1 M), was 5.1 (Reddi, 1965). Optimum activity of cucumber RNase occurred between pH 5 and 6 in 0.1 M acetate buffer (Kado, 1968). In Rhizopus sp., maximum activity of the most highly purified enzyme solution was obtained at pH 5.0 in 0.2 M sodium acetate buffer (Tomoyeda, et al., 1969). In wheat seedlings, the maximum activity for RNA hydrolysis was between pH 4.8 and 5.5 in 0.05 M acetate buffer (Hanson, et al., 1969).

The activity of purified RNase from spinach was not affected by the addition of any of the following: p-CMB (0.01M), mercaptoethanol (0.01 M), Mg^{++} (0.001 M), Ca^{++} (0.001 M), or Versene (0.001 M) (Tuve and Anfinsen, 1960). In rat liver, inactive RNase was released by p-CMB (1.7×10^{-3} M) at pH 5.8, although p-CMB did not release full activity in crude preparations and acid RNase was depressed under these conditions (Shortman, 1961). The activity of rat liver RNase was increased by the addition of NaCl to the buffer solutions. In soybean RNase, the ionic strength of the reaction mixture was varied by the addition of NaCl, and optimum enzyme activity was found at a concen-

tration of about 0.1 M NaCl (Merola, et al., 1962). Cu^{++} and Zn^{++} inhibited the activity of soybean RNase. There was a shift in optimum pH for RNase A from corn seedlings from 5.8 to 5.0 in the presence of KCl (Wilson, 1963). It was demonstrated in rye grass that in the range of ionic strengths from 10^{-3} to 3 x 10^{-2} M KCl, MgSO4, or NaCl produced an apparent increase in RNase activity (Freeman, 1964). For tobacco leaf RNase, all cations tested had an inhibitory effects: $Cu^{++} > Zn^{++} > Cd^{++} > Mg^{++} > Ca^{++}$ (Reddi, 1965). The ribosome-associated RNase of Zea mays required KCl for maximal activity, and excluding KCl from the assay medium (pH 5.8) reduced the activity at 37°C by about half (Hsiao, 1968). RNase activity of Rhizopus sp. was inhibited with Zn^{++}, Cu^{++}, and Hg^{++}, but EDTA and sodium pyrophosphate showed no effect (Tomoyeda, et al., 1969). The RNase of wheat seedlings was found to require the presence of sulfhyryl compounds for maintenance of maximum activity at pH 4.5. However, the presence of such compounds at pH 8 caused complete loss of activity in 24 hours at 4°C (Hanson, et al., 1969). In same report, it was indicated that KH_2PO_4 (5 x 10^{-3} M), sodium fluoride (3 x 10^{-3} M), EDTA (10^{-5} M to 10^{-2} M), Mn^{++}, Co^{++}, Fe^{++}, NI^{++}, and Cd^{++} (all 10^{-2} M) inhibited the activity of wheat RNase, but KCl (10^{-2} M), NaCl (10^{-2} M) and NH_4Cl (10^{-2} M) stimulated the activity slightly. The stimulatory effect of Ca^{++} and Mg^{++} on the RNase activity might be the result of an effect of these metal ions on the substrate and not necessarily an effect on the enzyme itself (Hanson, et al., 1969).

Spinach RNase was incubated at 24°C for 25 minutes (Tuve and Anfinsen, 1960). Most RNases were assayed at 37°C for 30 minutes to 1 hour (Shortman, 1961, Merola, et al., 1962, Wilson, 1963, Freeman,

1964, Reddi, 1965, Kado, 1968, and Hanson, et al., 1969). In the assay of RNase from Rhizopus sp., the reaction was carried out 50°C Tomoyeda, et al., 1969).

In contrast to pancreatic RNase, which gave rise only to pyrimidine mononucleoside cyclic phosphate, all of the RNases obtained from plants in purified form were capable of more extensively hydrolyzing RNA to mononucleoside-2', 3'-cyclic phosphates. In each case, guanosine 2',3'-cyclic phosphate was the earliest detectable mononucleotide. In addition, spinach RNases further catalyzed the hydrolysis of purine and pyrimidine nucleoside cyclic phosphates to nucleoside 3'-phosphates (Tuve and Anfinsen, 1960). Purine and pyrimidine nucleoside 2',3'-cyclic phosphates and 3-mononucleotides were produced by action of soybean RNase on RNA. Adenosine 2',3'-cyclic phosphate was cleaved to the 3'-nucleotide but cyclic pyrimidine nucleotides were not acted upon by the soybean RNase-catalyzed hydrolysis of RNA led to the formation of large oligonucleotides, dinucleotides, 3'-uridylic and 3'-guanylic acids, and 2',3'-cyclic guanylic acids (Kado, 1968). Wheat RNase catalyzed the hydrolysis of RNA to yield purine and pyrimidine ribonucleoside 2',3'-cyclic phosphate intermediates followed by the formation of 3'-purine and pyrimidine nucleoside phosphates as the final products (Kado, 1968). The RNase of Rhizopus sp. did not have absolute base specificity contrary to other RNases (Tomoyeda, et al., 1969).

The possible mechanisms of action of RNase have been discussed in the light of the detailed knowledge of the geometry of the active site that has been derived from studies of inhibitor binding by X-ray diffraction and nuclear magnetic resonance. Bovine pancreatic RNase

catalyzed the hydrolysis of 3',5'-phosphodiester linkage of RNA at the 5'-ester bond in a two step reaction. The first step was a transphosphorylation to give an oligonucleotide terminating in a pyrimidine 2', 3'-cyclic phosphate. The second was the hydrolysis of cyclic phosphate to give terminal 3'-phosphate. Numerous chemical studies have suggested that histidine 12, histidine 119 and lysine 41 were involved in the active site of the enzyme (Roberts, et al., 1969). According to Roberts, et al., two types of mechanisms in bovine pancreatic RNase A could be distinguished, the linear and pseudo rotation. The linear mechanism included a catalytic role of both histidine residues at the active site and did not involve psudorotation of intermediates. In contrast, one histidine residue performed all the catalytic functions in the pseudorotation mechanism, while the other served only to bind the phosphate anion; this necessarily involved pseudorotation of intermediates and specific protonation of the mononucleotide leaving group by the enzyme.

Lysosomes of liver and several other organs have been shown to contain RNase (de Duve, 1969). It was assumed in the animal system that the lysosomal acid phosphatase had a mode of action similar to that elucidated for the pancreatic enzyme (Barrett, 1969). The lysosomal RNase of animal systems differed from the better known pancreatic RNase in several respects. The pH optimum of lysosomal acid RNase varied with the nature of substrate and assay conditions (Barrett, 1969). The acid RNase had a molecular weight of approximately 18,500 as compared with 13,500 for the pancreatic enzyme and a substantially different amino acid composition (Barrett, 1969). Although catabolic enzymes such as RNase, esterase, and protease seemed

to be localized in lysosomal compartment exclusively (Matile, 1968, and 1969, Matile, et al., 1971, and Tappel, 1966), there is no evidence of difference between lysosomal RNase and non-lysosomal RNase in plants.

CHAPTER III

METHODS AND MATERIALS

The procedure for growing plants and exposure to water stress were similar to those of Stutte and Todd (1969). Winter wheat, (Triticum aestivum L.) variety Kan King, was grown in 50 cm x 30 cm x 6 cm trays with perlite as the root medium in controlled environment chambers at 22° to 25° C. Light intensity from Grolux fluorescent lamps was 11,000 lux with a 12-hour light and dark regime. The plants were watered daily with 1000 ml of water until they were 10 days old at which time the water stress periods were started. Relative humidity was not controlled but was in the range of 40 to 60% during the light period and 70 to 80% during the dark period. Water stress was imposed by withholding water for 3 to 8 days. Relative water content was used to estimate the internal severity of drought stress (Todd, et al., 1962) and was determined as follows: a 2 to 3 cm segment of the first leaf was quickly weighed (fresh weight) followed by floatation on water at 25° C for 24 hours. The turgid weight was then determined and followed by drying at 80° C for 24 hours for dry weight measurement. Relative water content was calculated as: $\frac{\text{fresh weight-dry weight}}{\text{turgid weight-dry weight}} \times 100$.

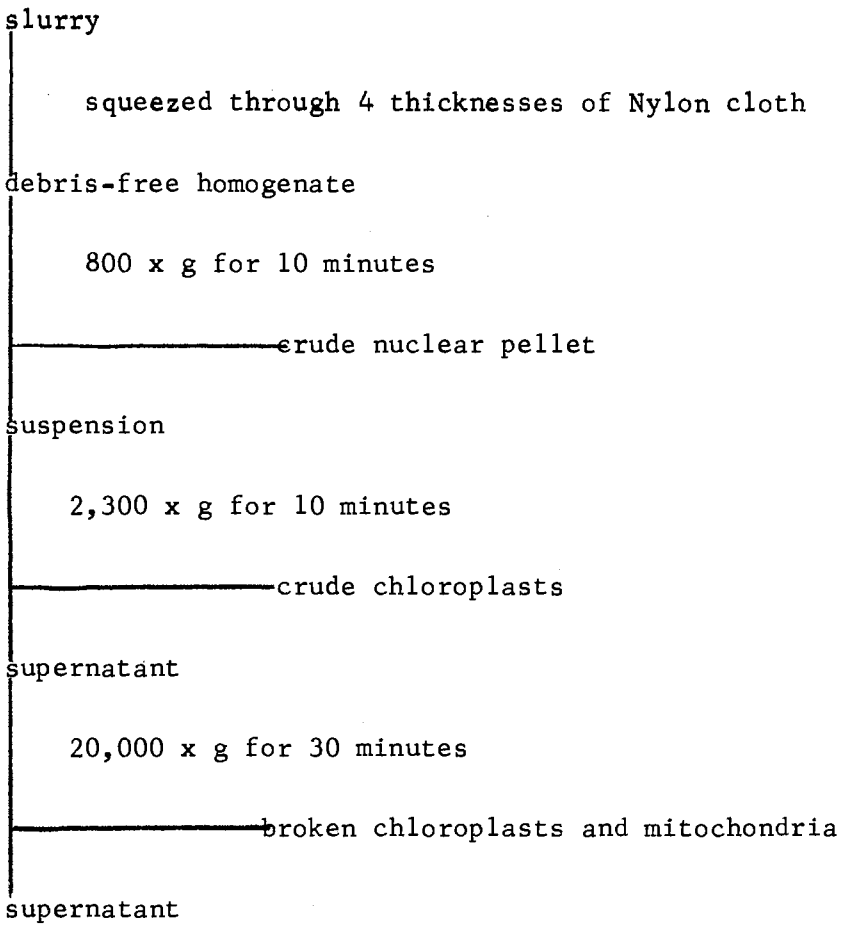
The first mature leaves about 10 cm in length were selected for enzyme assay. The fresh leaf material from 100 plants, about 4 g in fresh weight and 500 to 800 mg in dry weight, was ground in a mortar

with 10 ml of 25 mM Tris-HCl buffer (pH 7.8) containing 0.4 M sucrose, 10 mM $MgCl_2$, and 4 mM mercaptoethonol according to the method of Hadziyev, et al., (1969). The ground slurry was filtered through 4 thicknesses of nylon cloth and made up to a volume of 30 ml. For the fractionation experiment, the resultant slurry was treated as shown in Scheme 1 according to the method of Hadziyev, et al., (1969). This same slurry was also centrifuged at 20,000 x g for 30 minutes and the pellet fraction was suspended in 20 ml of extraction buffer for another experiment. All operations referred to above were performed in a cold room at 4° C, unless otherwise indicated. Supernatant and pellet solutions (0.5 ml) were used immediately for enzyme assay, and 0.1 ml of supernatant and pellet for estimation of protein concentration. Protein was determined by the method of Lowry, et al., (1951), on 5% trichloroacetic acid precipitates from crude homogenates, using crystalline egg albumin as a standard. Determinations were also carried out on the stem and non-leaf portions of the plant which is called crown in this experiment according to the terminology of Todd and Basler (1965). The 100 crowns, about 1.8 g in fresh weight and about 200 mg in dry weight, of the same plants were homogenized in 10 ml of the extraction buffer referred to above and made up to a volume of 30 ml. Then, they were treated in the same manner as the leaves. RNase assays on the fractions of leaves and crowns were carried out at pH 5 and pH 5.8 by the method of Tuve Anfinsen (1960) as modified by Wilson (1963). The reaction mixture consisted of 1 ml of substrate-buffer solution (0.125 M cacodylic acid, 5 mg/ml yeast RNA in 0.1 M acetate buffer, and imidazole to the desired pH), 0.4 ml of KCl, and 1.5 ml of water in a 15 ml-cetrifuge tube. This reaction mixture was equilibrated at room

temperature. Aliquots (0.5 ml) of the individual preparations containing 80 to 100 ug protein were added following equilibration and incubated at 37°C for 60 minutes. The enzyme reaction was terminated by adding 1 ml of 25% $HClO_4$ containing 0.75% uranylacetate. The tuves were kept at 4°C for 30 minutes, centrifuged at 6000 x g for 20 minutes, and then the absorbance of the clear supernatant diluted 30 fold with water was measured against water at 260 nm by using a Perkin-Elmer 202 Spectrophotometer. Corrections were made for an appropriate blanks containing no enzyme, or zero-time blanks containing enzyme because of the crude enzyme preparations used in this study (Tuve and Anfinsen, 1960).

The absorbance change (ΔA_{260}) used as a measure of activity was determined by (absorbance after 60-minutes reaction minus absorbancy at zero-time) — (absorbance after 60-minutes reaction without enzyme minus absorbance at zero-time without enzyme). The specific activity was defined as ΔA_{260} per 100 ug protein, and the total activity as ΔA_{260} x the volume of solution obtained after centrifugation according to Scheme 1 or after 20,000 x g for 30 minutes per 500 mg dry weight of plant material used for the enzyme assay.

In the assay for bound RNase, p-CMB sodium salt at concentrations of 0.16 mM to 0.28 mM (Hadziyev, et al., 1969) was used in attempt to release from its supposed inhibitor complex (Kessler, 1961, and Brewer, et al., 1969). A 0.01 M solution of p-CMB (Sigma Chem. Co.) was prepared by the addition of a minimum amount of NaOH according to the method of Shortman (1961). The final pH was 8.5 to 9.0. The pH of the solution was adjusted to the pH of the assay system by addition of Tris[(hydroxymethyl) aminomethane] before use. The substrate RNA was



Scheme 1. Separation of subcellular particles

dialyzed with 20 volumes of 10 mM EDTA sodium salt, then with several changes of 0.15 M NaCl, and finally with six changes of water according to the methods of Shortman (1961) and Hadziyev (1969). A 4 mg/ml RNA was prepared and stored at -4°C. Mercaptoethanol was exluded from the extracting buffer system, and cacodylic acid and KCl were excluded from the reaction mixture according to the method of Hadzlyev, et al., (1969). The incubation mixture for these assays contained 0.1 ml of dialyzed RNA as substrate, 0.9 ml of 0.1 M acetate buffer (pH 5 and pH 5.8), and 1.5 ml of water containing p-CMB.

In assays for oxidation and reduction of RNase, mercaptoethanol was excluded from the extracting buffer, because mercaptoethanol also decreased the enzyme activity after storage for 15 hours at 4°C (Hanson, et al., 1969). Methods used here were those of Basler (1963) and Hanson, et al. (1969) in which supernatant fractions were kept at 4°C for 6 hours in the presence of reduced or oxidized agents. Reduced and oxidized glutathione at a final concentration of 10^{-2} M and 5 x 10^{-3} M were used in the K^{+}-indued RNA hydrolysis of cotton cotyledons (Basler, 1963). Enzyme assays then were performed as referred to above.

Twelve and one-half mg of cycloheximide was dissolved in 250 ml of water (50 ug/ml). This cycloheximide solution was sprayed on the leaf surfaces of about 300 plants at the beginning of water stress period.

CHAPTER IV

RESULTS

An investigation of relation between RNA hydrolysis and enzyme concentration yielded the results presented in Figure 1. For the estimation of activity in this study, aliquots were chosen to give the observed ΔA_{260} values within the linear portion of the curve, i.e., less than 100 ug protein. Figure 1 also shows the effect of KCl used in the reaction mixture on hydrolysis. The addition of KCl to the reaction mixture increases the ΔA_{260} at a given level of enzyme, especially below a concentration of 100 ug protein. The effect of KCl at different concentrations on the activity was not studied because the concentration of KCl used in this study was adopted from the method by Hadziyev, et al. (1969) and extensively studied by Wilson (1963). Figure 2 shows that the rate of RNA hydrolysis was linear up to 90 minutes. Therefore, 60 minutes for the reaction time was chosen and used thereafter in this study. The activity of the crude enzyme preparation of the supernatant fraction after centrifugation was greatest at pH 5 and pH 5.8 (Figure 3). The double maximum in the pH optimum curve was observed consistently. This indicates the presence of two RNases differing slightly in pH optima as was found for spinach RNase (Tuve and Anfinsen, 1962). The enzyme activity was slightly lower in 0.1 M citrate buffer at pH 5.8 than that in acetate buffer. No activity at alkaline pH values was observed in crude preparations.

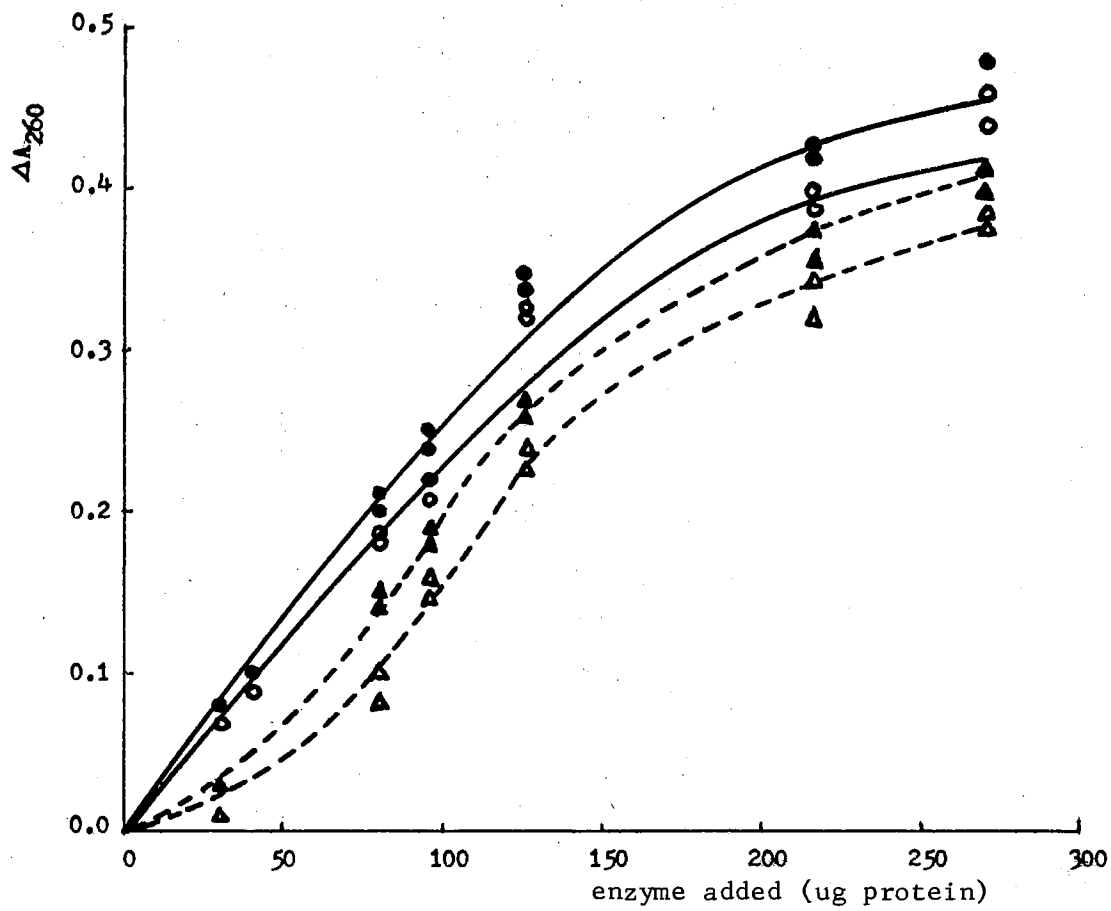


Figure 1. Relationship between the amount of supernatant fraction added to the reaction mixture and RNA hydrolysis.

pH 5.0 with KCl ——O——O——

pH 5.8 with KCl ——●——●——

pH 5.0 without KCl – – –Δ– – –Δ– – –

pH 5.8 without KCl – – – ▲ – – – ▲ – – –

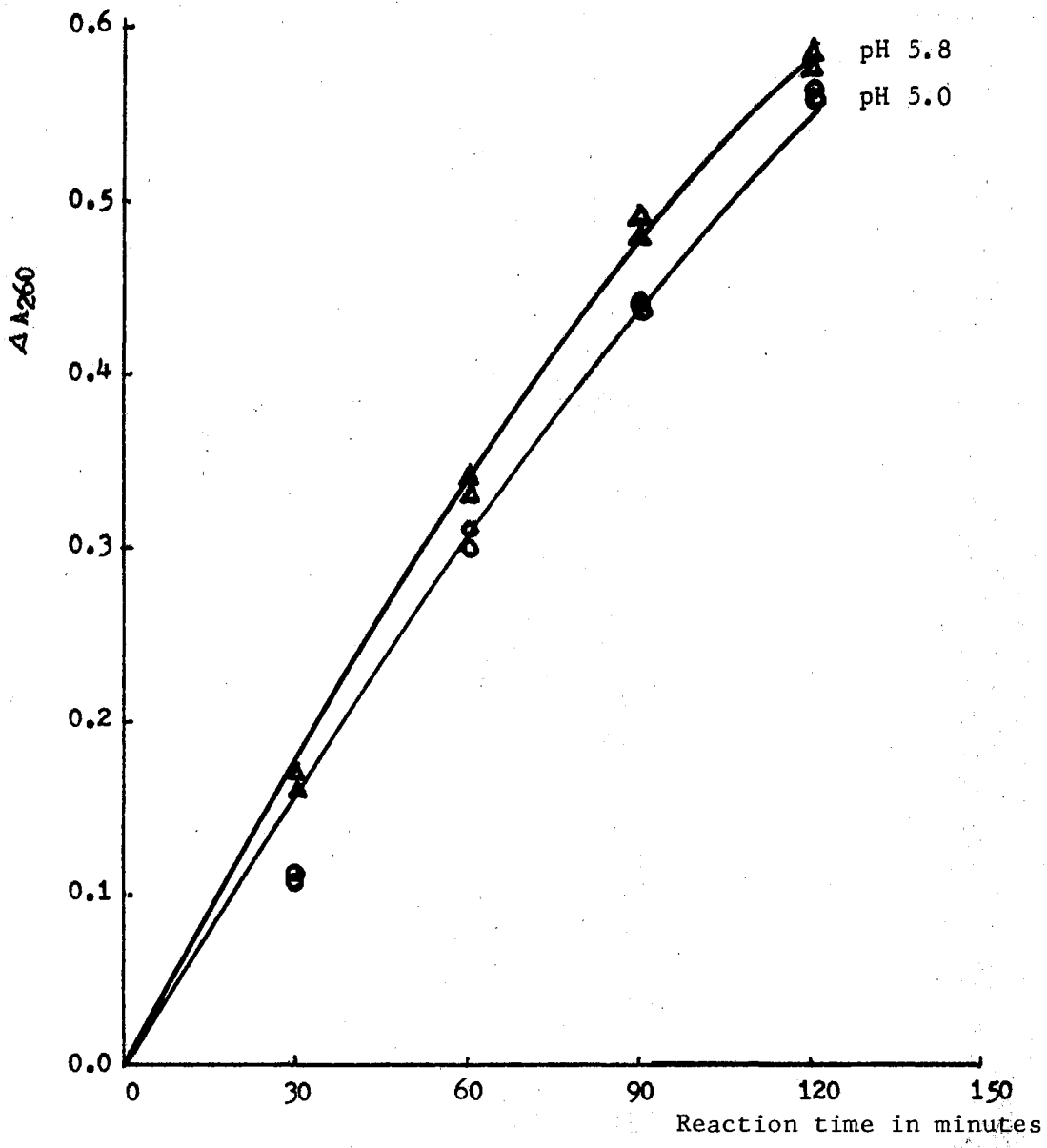


Figure 2. Time course of RNA hydrolysis
One-half ml of supernatant (135 ug protein) was incubated with 5 mg of RNA in acetate buffer.

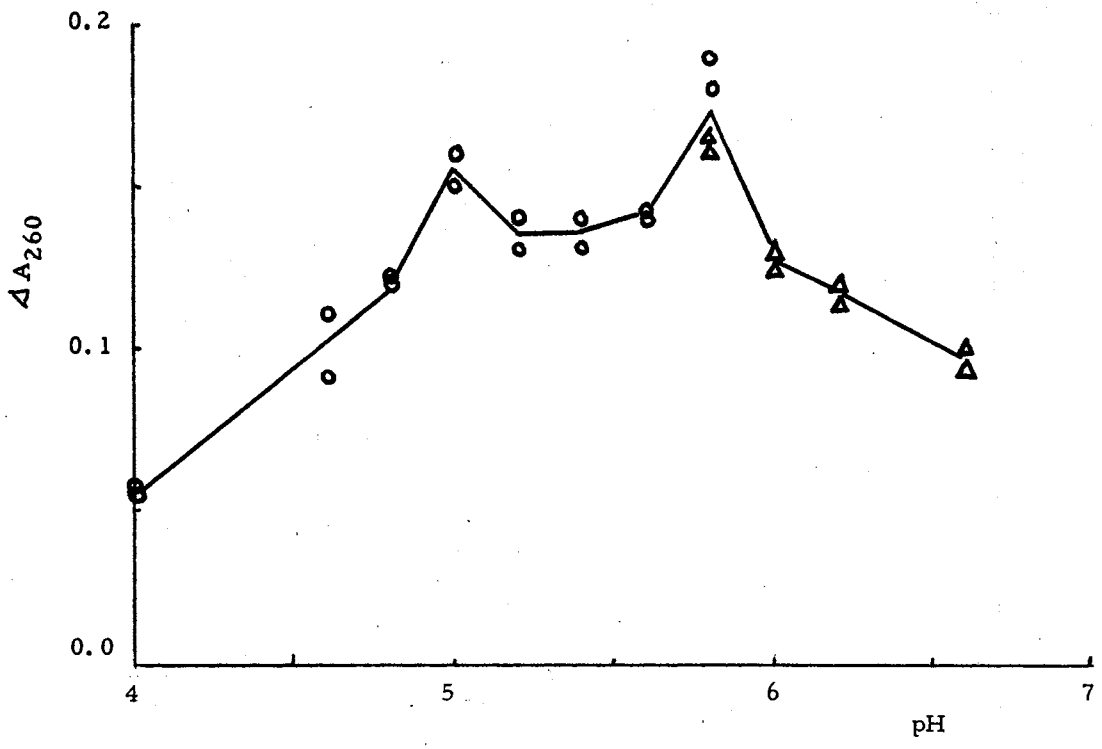


Figure 3. Influence of pH on wheat RNase activity
One-half ml of supernatant (80 ug protein) was incubated with 5 mg of RNA in buffer solutions.
acetate buffer (o). citrate buffer (Δ).

It can be noted from Figure 4 that at low substrate concentration (below 3 mg/ml RNA), the reaction was first order with respect to substrate. Substrate concentrations of 4 or 5 mg/ml were chosen in this study. As seen in Figure 4, the rate of reaction at 4 or 5 mg/ml should be within 5% of each other.

In an initial experiment to determine the changes in RNase content with time, plants were grown in the growth chamber for 14 days. No leaf senescence was apparent at this time. The control plants were kept at a relative water content of 95% throughout. The specific activity and total activity as described in the methods and materials were measured in both pellet and supernatant fractions obtained after centrifugation at 20,000 x g for 30 minutes. The values from both fractions were combined because the pellet fraction had very little activity as shown in Figure 12, and the combined values are shown in Figure 5. The protein content was also measured in both supernatant and pellet fractions, combined and plotted as shown in Figure 6. Dry weight of 100 leaves is shown in Figure 6. Protein content and total activity at pH 5 and pH 5.8 from wheat leaves increased continuously over the experimental period, and dry weight also increased steadily.

When plants were subjected to water stress, the relative water content gradually decreased during the 6 days of the experiment (Figure 7). After 3 days of water stress, the leaves showed no visible signs of water stress although leaf rolling occurred after the 6 days of water stress. The effect of water stress on the specific and total activities are shown in Figures 8 and 9. The total protein content of the combined supernatant and pellet fractions and the dry weight of 100 leaves are shown in Figure 10. In the plant leaves subjected to water

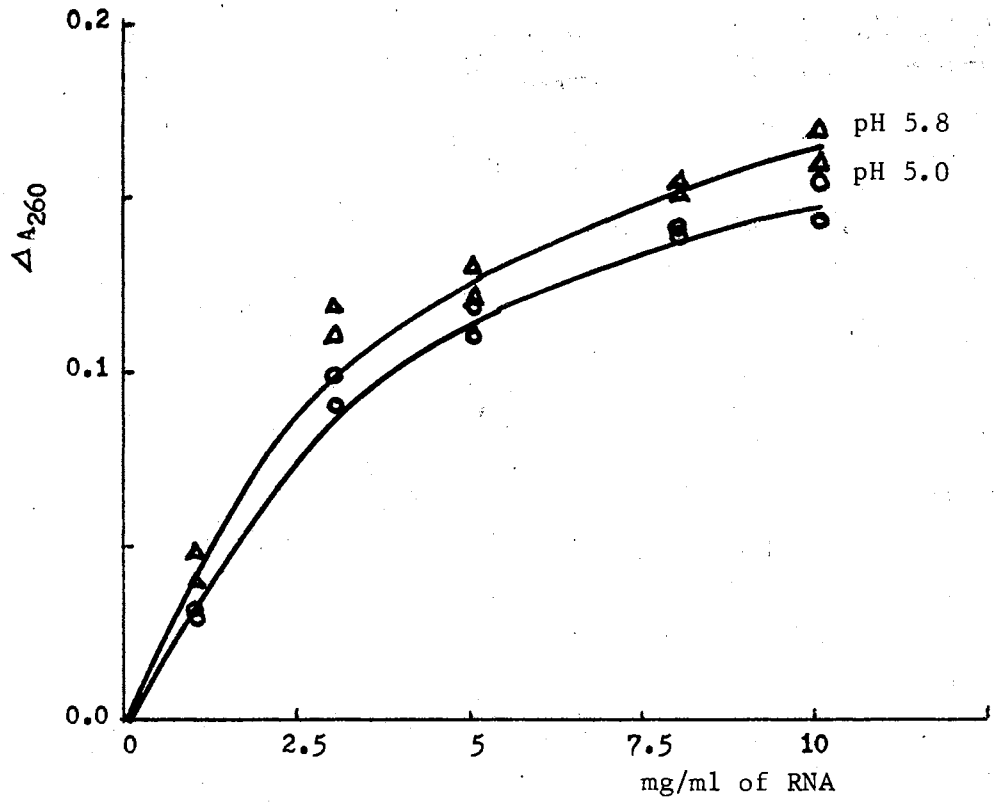


Figure 4. Plot of substrate concentration versus RNA hydrolysis One-half ml of supernatant (60 ug protein) was incubated at different RNA concentrations in acetate buffer.

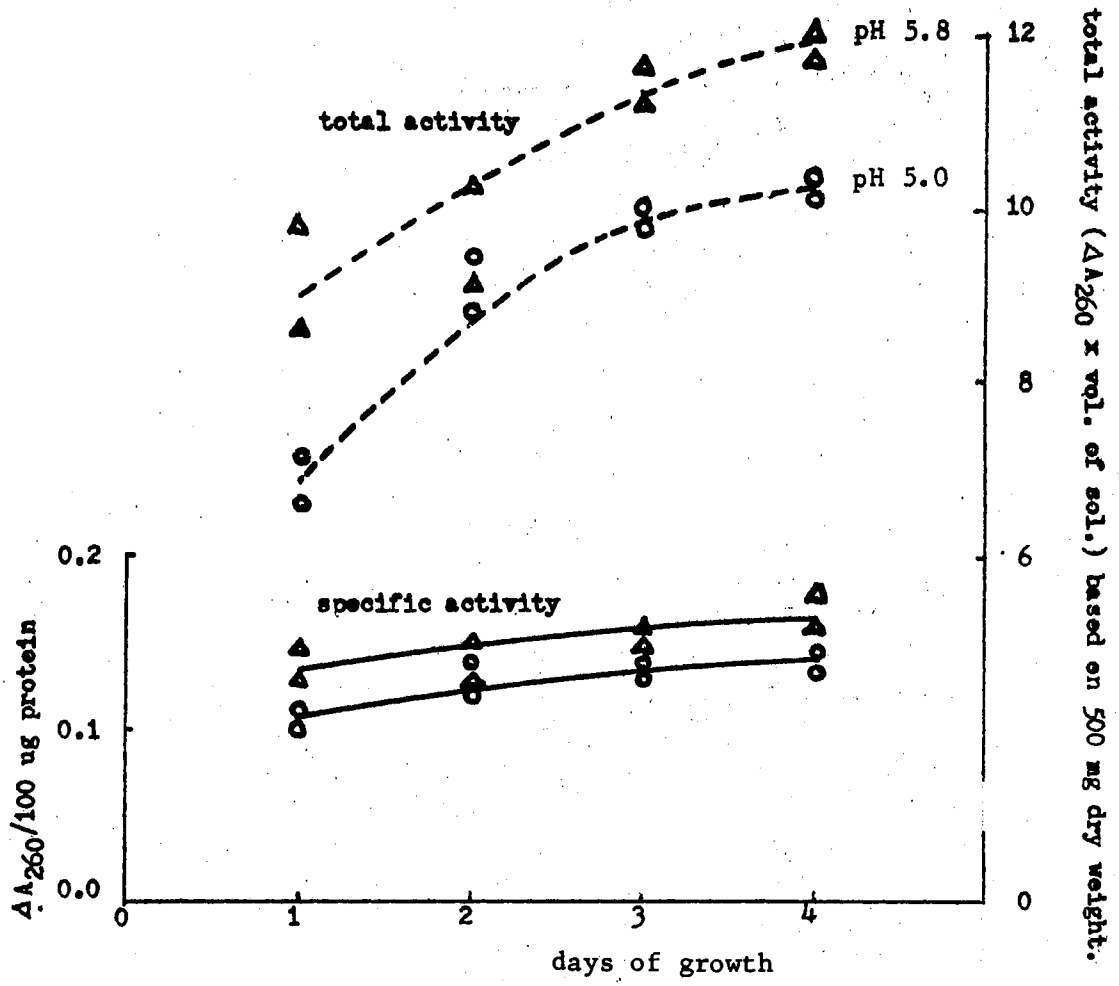


Figure 5. Effect of growth on total activity and specific activity of combined supernatant and pellet fractions of 100 leaves.

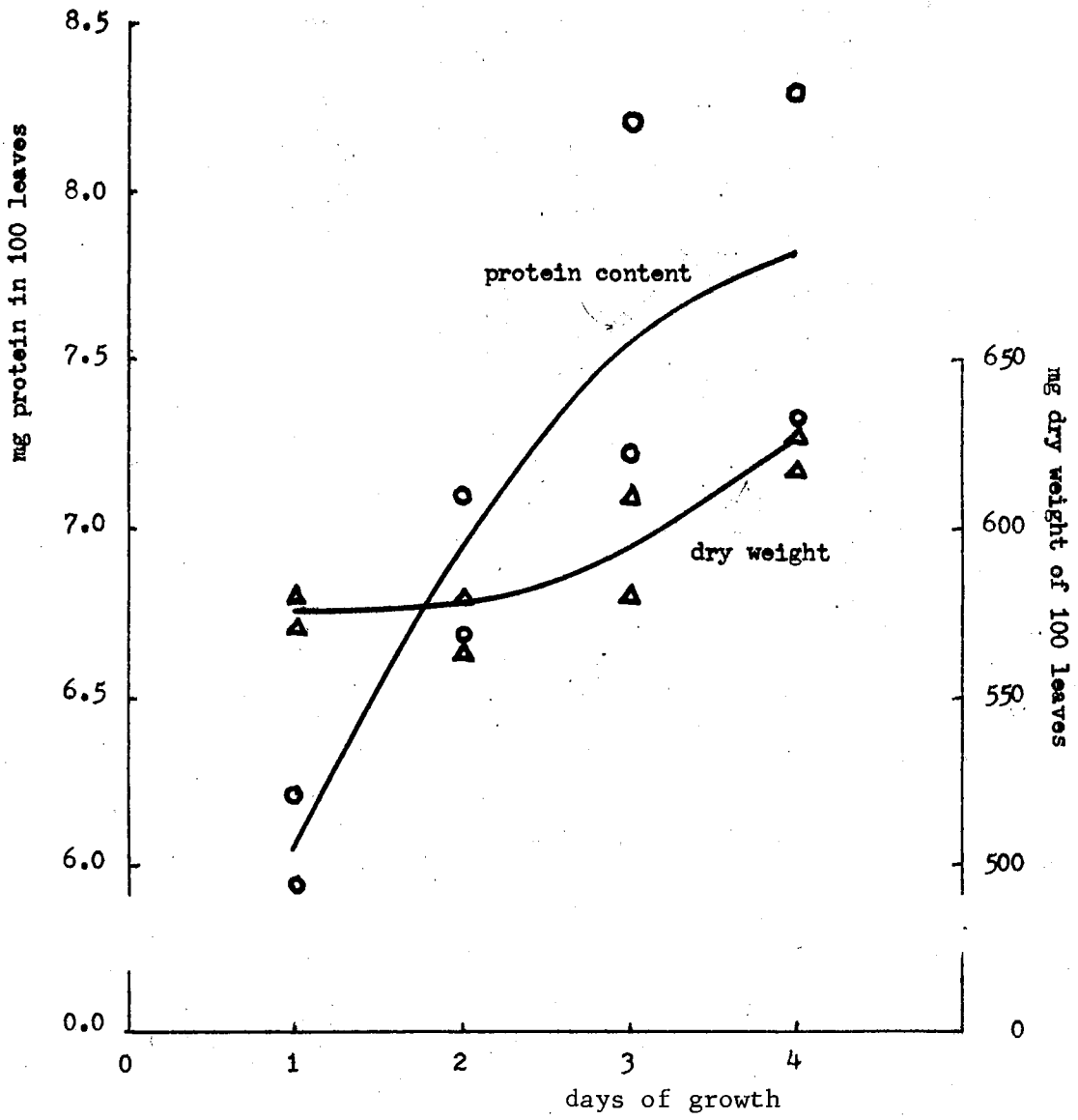


Figure 6. Effect of growth on protein content and dry weight of 100 leaves. Protein was measured in combined supernatant and pellet fractions.

protein content (—O—— O——)

dry weight (—Δ——Δ——)

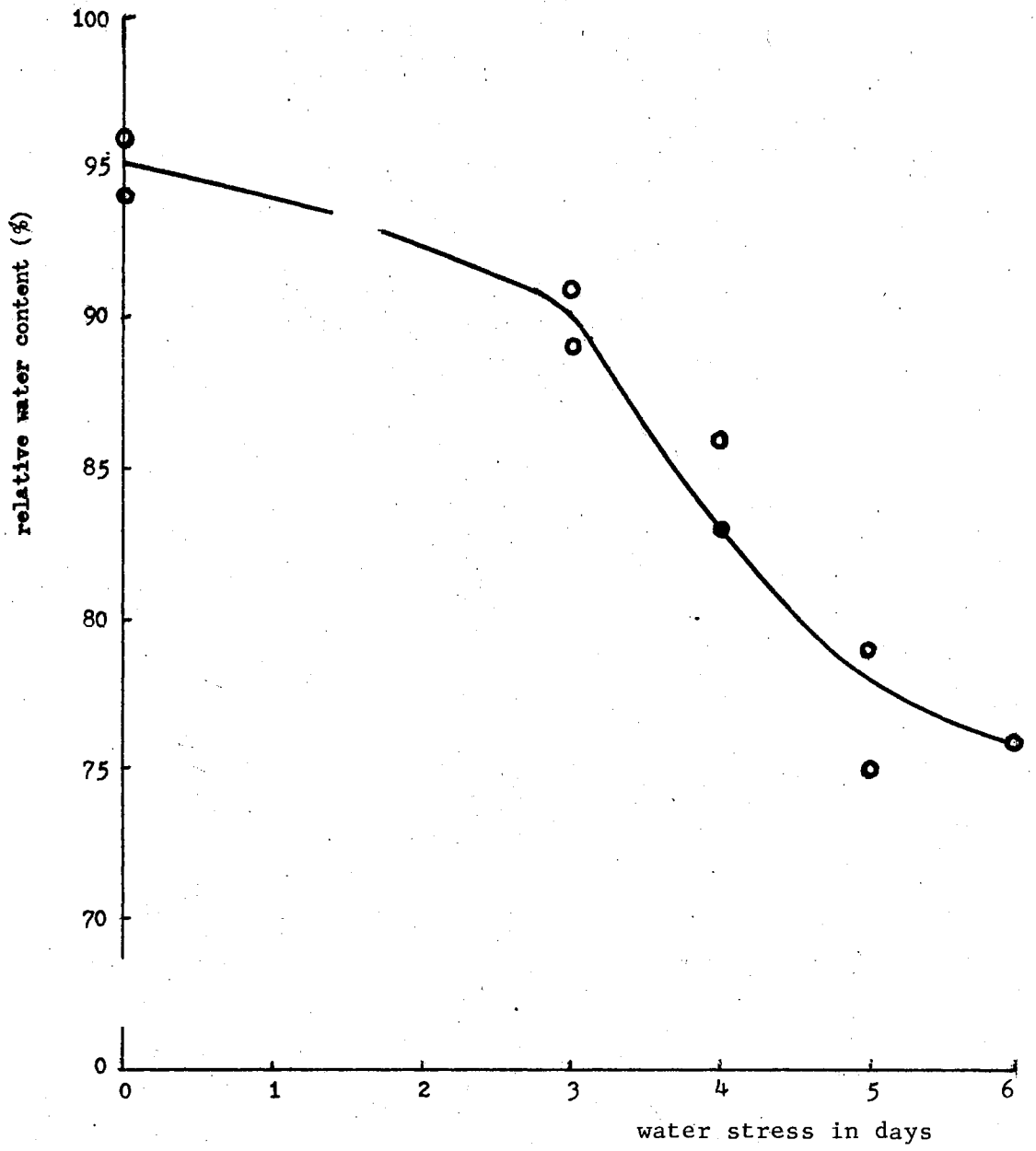


Figure 7. Changes in relative water content with water stress

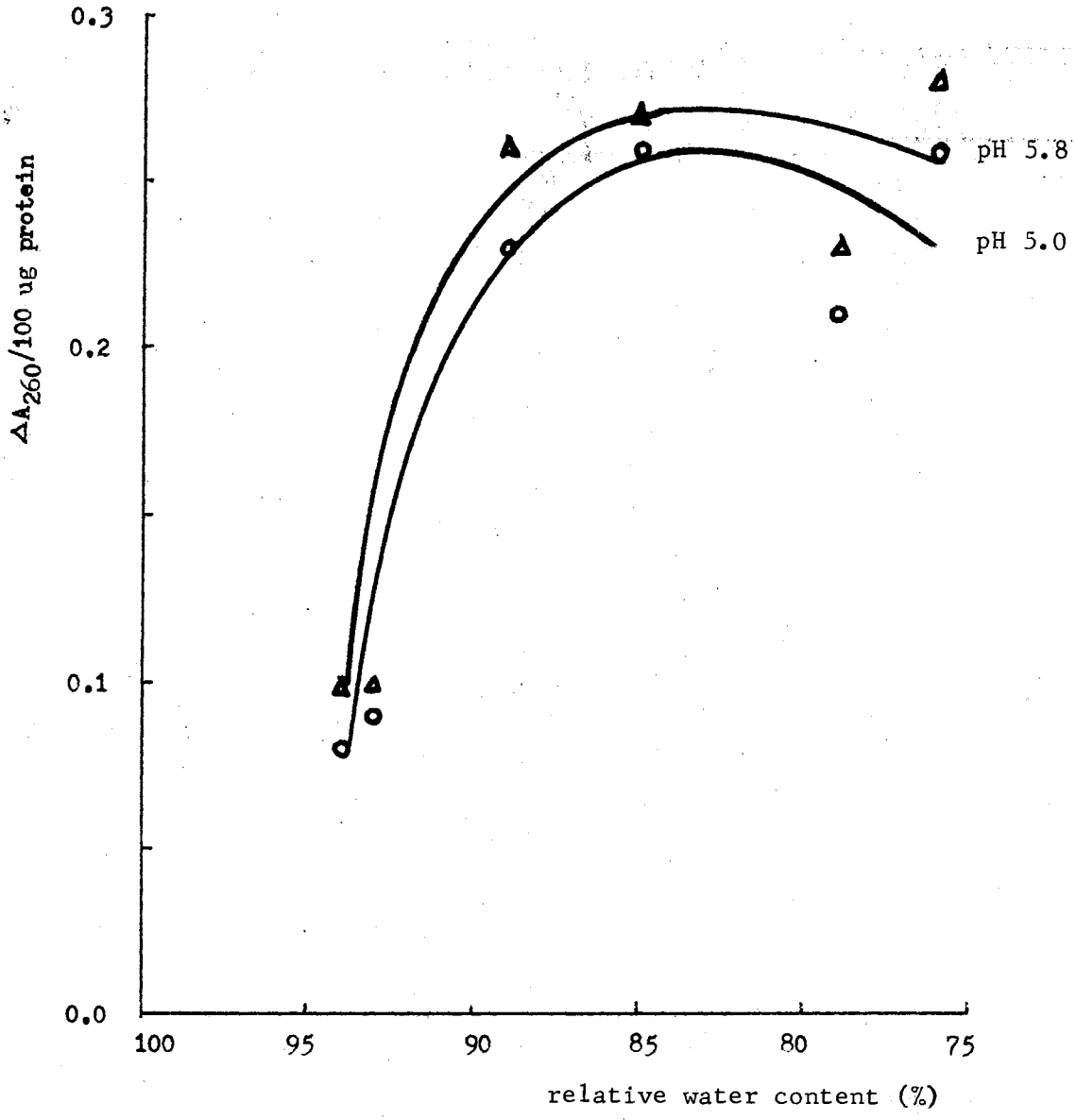


Figure 8. Specific activities at pH 5.0 and pH 5.8 of combined supernatant and pellet fractions from 100 leaves of wheat plants at various stages of water stress

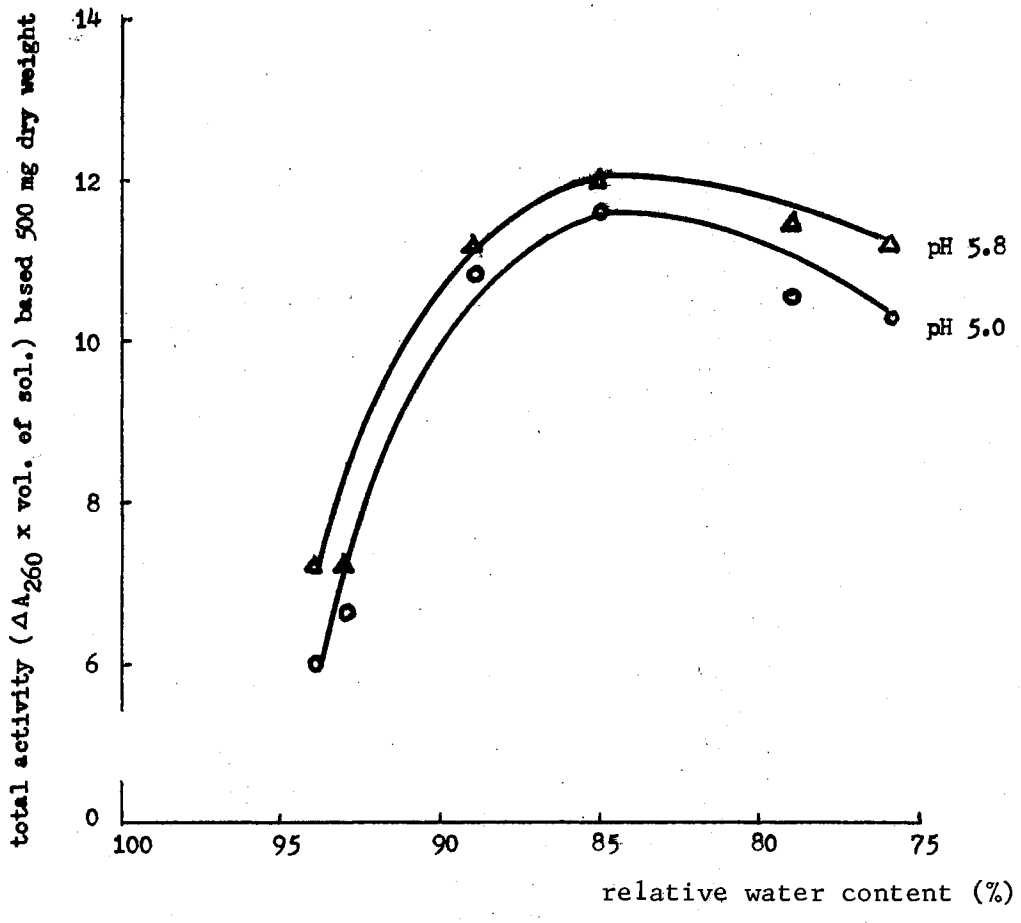


Figure 9. Total activity in combined supernatant and pellet fractions from 100 leaves of wheat plants at various stages of water stress

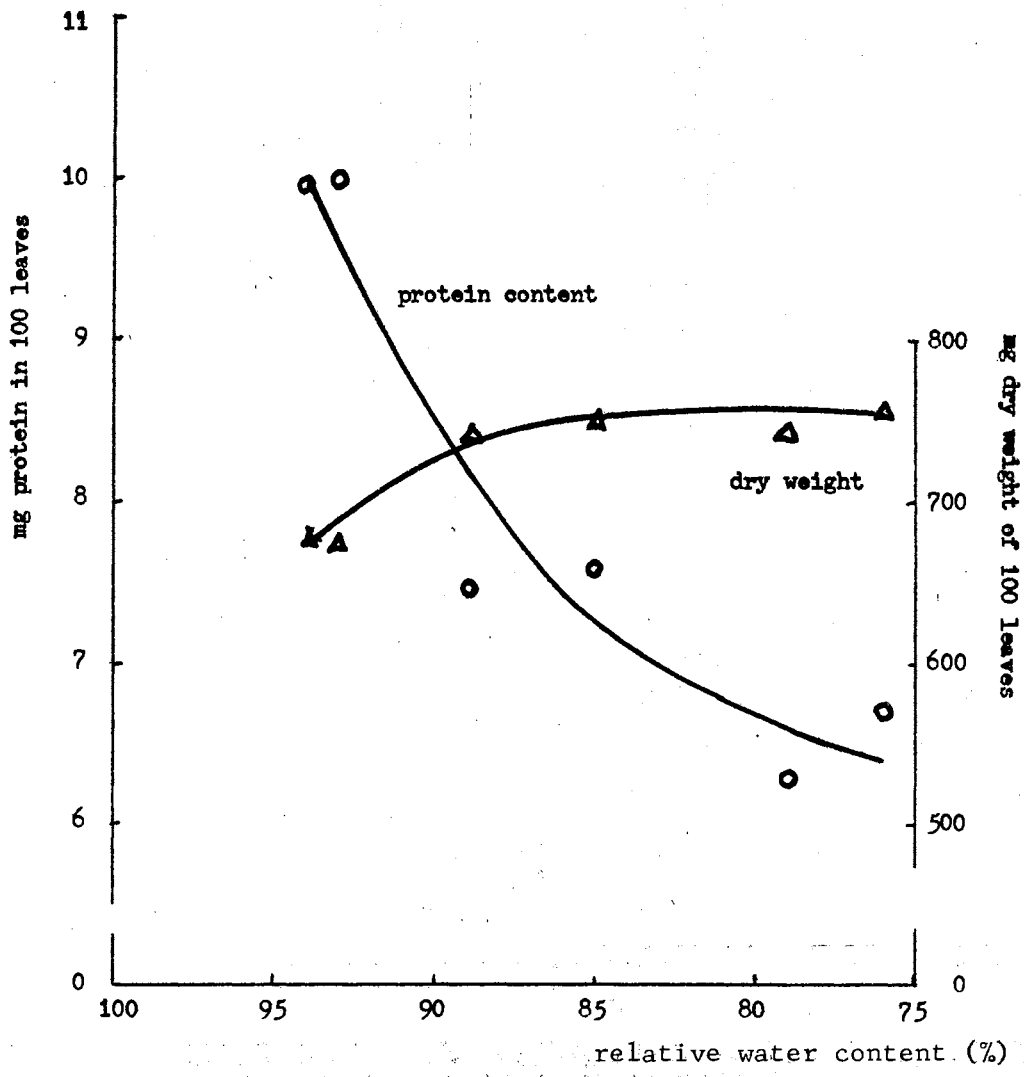


Figure 10. Total protein content and dry weight of 100 leaves of wheat plants at various stages of water stress. Protein was measured in combined supernatant and pellet fractions.

stress, the protein content was decreased markedly during the 6-day period of water stress, but leaves maintained about the same values of dry weight after water stress (Figure 10). Specific activity (Figure 8) and total activity (Figure 9) of RNases at pH 5 and pH 5.8 increased several fold during the first 3 to 4 days of water stress (89% to 85% of relative water content) as water stress developed and then gradually decreased as the water stress became more severe (below 80% of relative water content). Changes in total RNA content of control and water stressed wheat leaves are plotted from the results by Stutte and Todd (1968), and changes in nucleic acid content of wheat leaves are plotted from the results obtained by Todd and Basler (1965) in order to show whether the decreased RNA content with water stress is correlated with the increased activity of RNase (Figure 11). With water stress, the nucleic acid and RNA content dropped rapidly in leaves. If the RNA or nucleic acid content dropped rapidly in response to water stress in the Kan King variety of wheat used in present study as it did in Cheyenne, one would expect that the increase in RNA loss might be accompanied by an increased RNase activity. The total activity of RNases shown in Figures 5 and 9 increased during 3- to 4-day period of growth and water stress. The total activity of RNases in water stressed plants (Figure 9) shows higher activity than that of growth experiment (Figure 5), although two experiments were not performed at the same time. As shown in Figures 8 and 9, the specific activity and the total activity at pH 5.8 were higher than those activities at pH 5.0 which was predicted from pH versus activity curve (Figure 3). Thus, the growth experiment (Figure 5) and the water stress experiment (Figures 8 and 9) show that the pH optima of the RNases were not

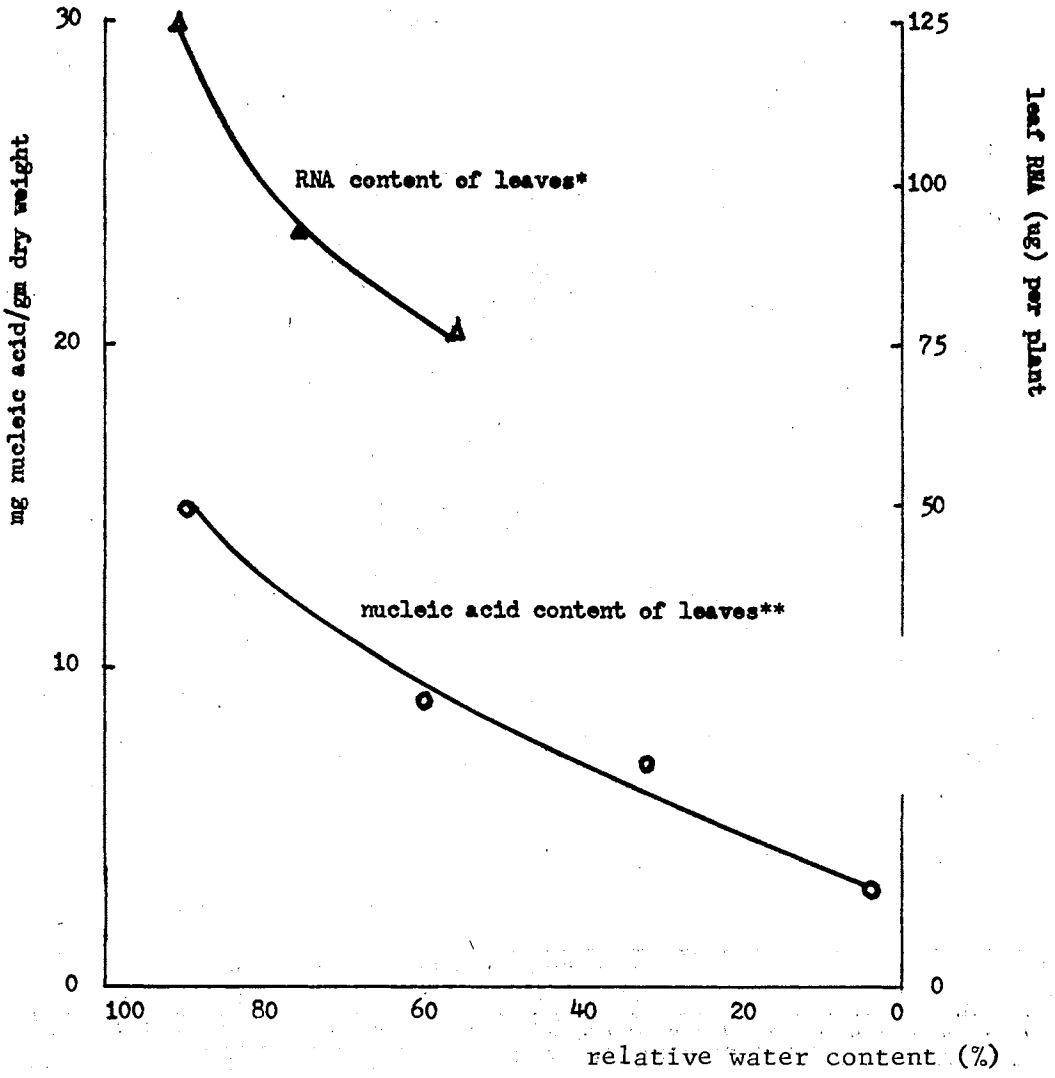


Figure 11. Effect of water stress on RNA content * and nucleic acid content**.
*cited from Stutte and Todd (1968)
**cited from Todd and Basler (1965)

changed by water stress. This result is comparable to RNase activities in germination of wheat, in which the optimum pH of embryo RNase was at pH 5 and that of seedling RNase was at pH 5.5 (Vold, et al., 1968).

The distribution of RNase activity in subcellular fractions from control and water stressed plants was studied two times, and is shown in Table I-a and b. The highest total RNase activity was always associated with the supernatant fractions of control and water stressed plants. Small amounts of RNases were associated with the nuclear, chloroplast, and mitochondrial fractions. Distributional changes in RNase activity did not take place as a result of water stress, in contrast to another report which showed more than 200% increase in RNase activity of microsomal fraction and 100% decrease in the enzyme activity of cytoplasmic fraction with increase in age of apple leaves (Kessler, et al., 1962).

Another experiment was performed in order to show any differences of RNase content between the leaf and the crown portion of the same plant, since the nucleic acid content of crown tissue was much less affected by drought stress (Todd and Basler, 1965). The relative water content of the crown was not as sensitive as the leaves of the same plant to water stress, since the relative water content of crowns from 4-day water stressed plants were about the same as in control plants, e.g., 84% and 85% respectively. In this experiment (Figures 12 and 13), the specific activity of RNase and total protein content of the supernatant and pellet from crowns were plotted against relative water content determined for the leaves. Activities of RNases were estimated by using the dialyzed RNA as substrate. This resulted in a lower absorbance of the blank cuvette, but the same magnitude

TABLE I-a

DISTRIBUTION OF TOTAL RNase ACTIVITY IN SUBCELLULAR FRACTIONS OF 100 WHEAT LEAVES AT VARIOUS STAGES OF WATER STRESS

	Control RWC,94%		3-day stress RWC,89%		5-day stress RWC, 79%	
Experiment No. 1	pH 5	pH 5.8	pH 5	pH 5.8	pH 5	pH 5.8
Crude nuclear pellet	0.5 (6%)*	0.9 (9%)	0.9 (6%)	1.9 (12%)	1.3 (11%)	1.5 (12%)
crude chloroplast	0.2 (2%)	0.4 (4%)	0.4 (2%)	0.8 (5%)	0.6 (5%)	0.6 (5%)
broken chloroplast and mitochondria	0.5 (6%)	0.6 (6%)	0.4 (3%)	0.9 (5%)	0.7 (6%)	0.8 (6%)
supernatant	6.0 (84%)	7.5 (81%)	11.7 (89%)	15.0 (78%)	8.7 (78%)	9.2 (79%)

* per cent distributions at pH5 and pH5.8.

TABLE I-b

DISTRIBUTION OF TOTAL RNase ACTIVITY IN SUBCELLULAR FRACTIONS OF 100 WHEAT LEAVES AT VARIOUS STAGES OF WATER STRESS

	Control RWC, 94%		3-day stress RWC, 89%		5-day stress RWC, 79%	
Experiment No. 2	pH 5	pH 5.8	pH 5	pH 5.8	pH 5	pH 5.8
crude nuclear pellet	0.5 (5%)*	0.7 (7%)	1.0 (7%)	0.9 (6%)	0.6 (5%)	0.8 (6%)
crude chloroplast	0.4 (4%)	0.5 (5%)	0.3 (3%)	0.3 (2%)	0.5 (4%)	0.6 (4%)
broken chloroplast and mitochondria	0.6 (6%)	0.5 (5%)	1.0 (7%)	1.2 (8%)	0.3 (3%)	0.3 (4%)
supernatant	8.0 (84%)	7.7 (82%)	11.5 (83%)	11.5 (81%)	10.0 (86%)	12.0 (85%)

* per cent distributions at pH5 and pH5.8.

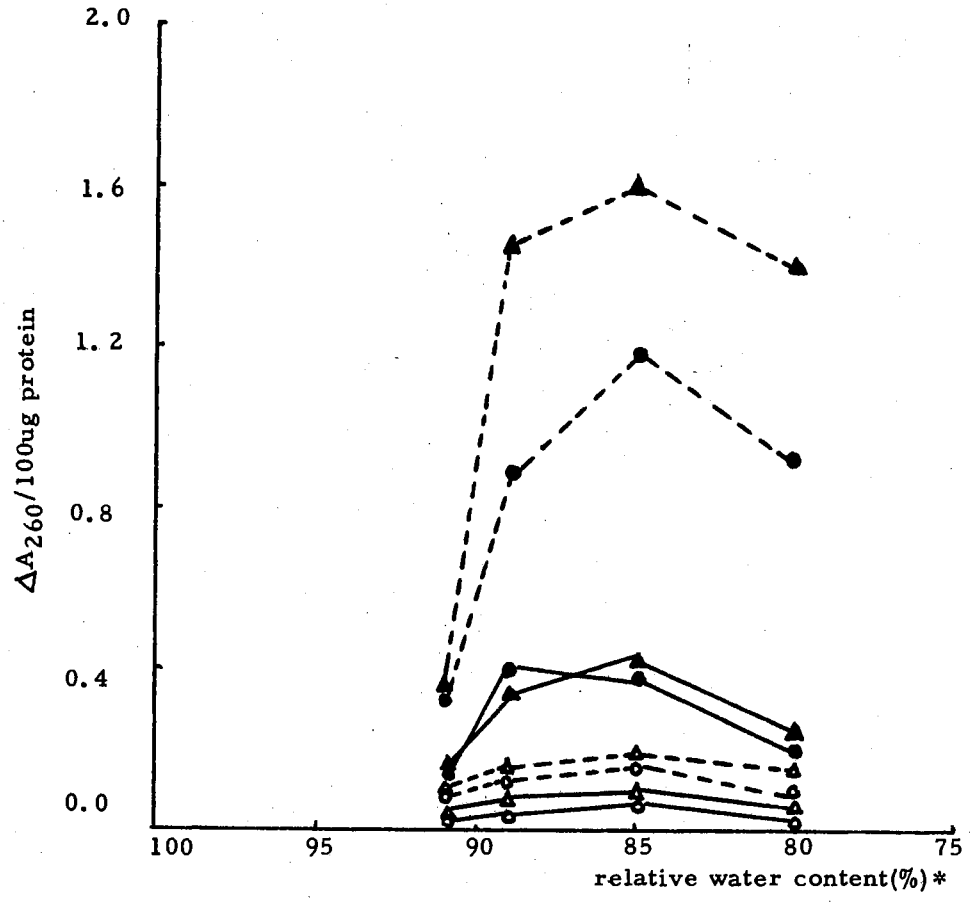


Fig. 12. Specific activities of RNase at pH5 and pH5.8 of supernatant and pellet fractions from wheat leaves and crowns at various stages of water stress.

Crown supernatant at pH5
Crown supernatant at pH5.8
Crown pellet at pH5
Crown pellet at pH5.8
Leaf supernatant at pH5
Leaf supernatant at pH5.8
Leaf pellet at pH5
Leaf pellet at pH5.8

* based on the value of leaves.

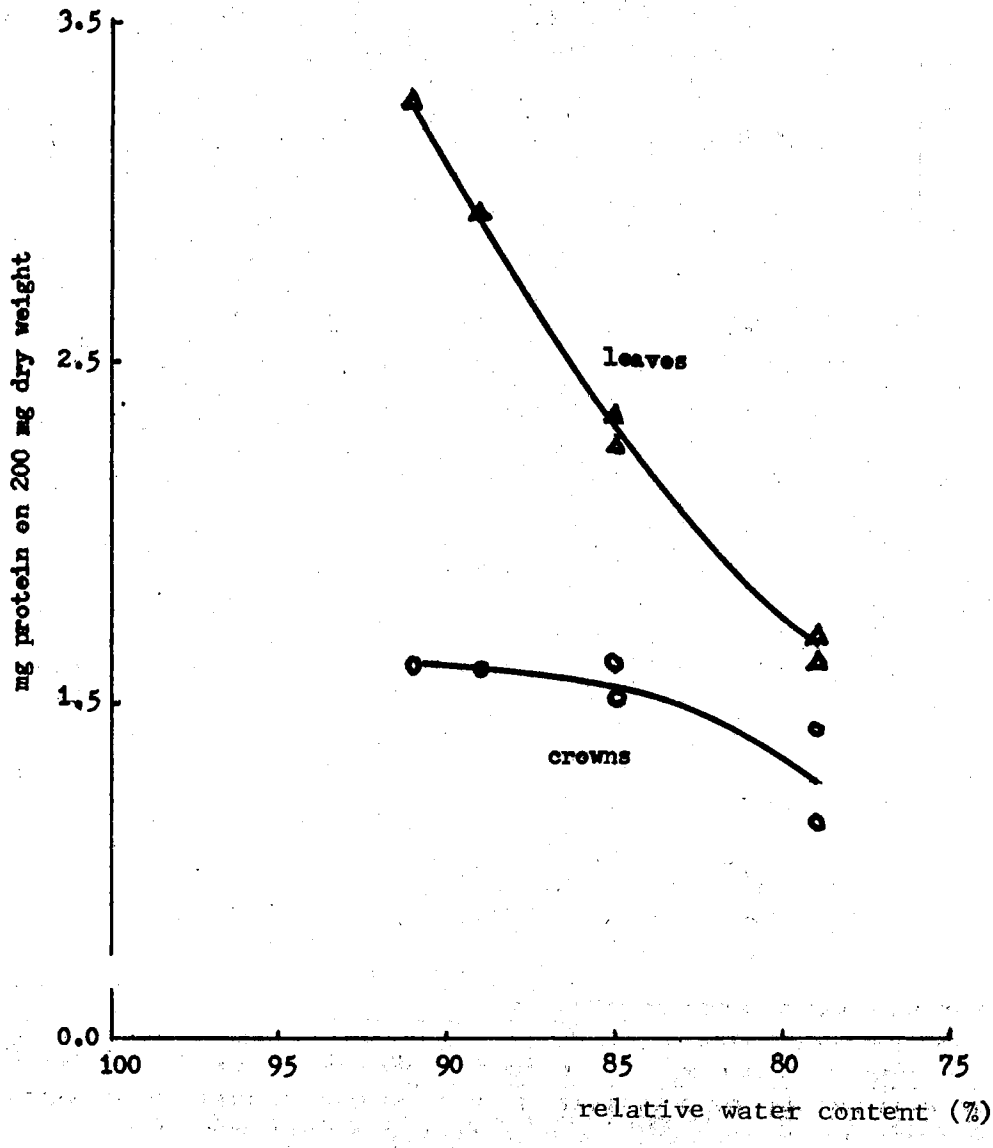


Figure 13. Protein content of combined supernatant and pellet fractions from leaves and crowns of wheat plants at various stages of water stress.
*based on the value of leaves

in ΔA_{260} was caused by the enzyme. The substrate concentration was lower (4 mg/ml) than that of the experiments of Figures 5, 8, and 9 in which 5 mg/ml of substrate concentration was used, because of the final concentration of RNA after dialysis. The rate of RNA hydrolysis should not be changed significantly judging from the plot against substrate concentration in Figure 4, even though a lower substrate concentration (4 mg/ml) was used. The total dry weight of 100 crowns used here was in the range of about 200 mg and the total dry weight of 100 leaves was in the range of about 500 mg.

To compare the protein content of leaves and crowns from the same plant, the protein contents of leaves and crowns in Figure 13 were calculated on the basis of 200 mg dry weight, although the leaf dry matter per plant was much greater than the crown portion (e.g. 33 mg versus 14 mg, respectively). The specific activities of RNases at pH 5 and pH 5.8 from supernatant fractions of crowns were much higher than that from both crowns and leaves of the same plant increased during the first 3 to 4 days of water stress (89% to 84% of relative water content) and decreased after 5 to 6 days of water stress (80% to 79% of relative water content). RNase activities at pH 5 and pH 5.8 were about the same in leaves of water stressed plants, but the enzyme activity at pH 5.8 was consistently much higher than that at pH 5.0 in crowns of water stressed plants. The pellet fraction from crowns had much higher specific activities of RNases than that from leaves. Specific activities at a given relative water content of leaf RNase in this experiment (Figure 12) were lower than those of the first experiment (Figure 8).

The total protein content on a dry weight basis from crowns followed a different trend from that of the leaf during the water stress

period (Figure 13): the decrease caused by water stress in the leaf was very rapid, but changes in the crown were slight.

The increase in RNase activity from water stressed plants might be due either to the activation of an inactive form of enzyme during water stress or to the de novo synthesis of new enzyme during water stress. In order to distinguish between these two possibilities, experiments were carried out in which p-CMB, glutathione, and cycloheximide were applied to both control and water stressed plants.

p-CMB can cause the release of otherwise inactive alkaline RNases (Shortman, 1961). "Active" RNase (RNase activity in the absence of p-CMB) and "total" RNase (RNase activity in the presence of added p-CMB which inactivates the RNase inhibitor) may be determined. "Latent" RNase equals "total" RNase minus "active" RNase. Since disulfide bonds were reduced by sulfhydryl compounds like dithiothreitol at pH 8 for 3 to 6 hours of storage at $4^{o}C$ (Hanson, et al., 1969), the rate of enzyme destruction by glutathione compounds at pH 7.8 was measured. Thus, it may be found that the sensitivity of enzymes to glutathione is changed during water stress as shown by the different sensitivities of RNases to EDTA during senescence of wheat leaves (Sodek, et al., 1969). The de novo synthesis of RNase could be prevented by cycloheximide (Schlaeger, et al., 1969). If the increase in RNase activity after initiation of water stress is responsible for de novo synthesis of RNase, this may be suppressed by cycloheximide.

The purity of RNA used as substrate was found to be factor of major importance in the assay of acid and alkaline RNases with reference to the inactive RNase and RNase-inhibitor complex (Shortman, 1961). As a result of metal contaminants, some RNA preparations inactivated

the cellular inhibitor in animal systems and caused a concomitant release of otherwise inactive alkaline RNase. Treatment of the RNA preparations with the chelating agent EDTA allowed a satisfactory assay to be made. The activity was measured in supernatant fractions from leaves and crowns in which p-CMB was added to the reaction mixture. Enzyme activity of pellet fractions is not shown becase of the small amounts of RNase activity before and after addition of p-CMB. At higher concentrations of p-CMB (above 0.24 mM), the treatment resulted in the slight inhibition of enzyme activity. However, p-CMB treatments were not effective on increasing activity of enzymes from either control or water stressed plants (Table II), indicating that the release of RNases from an inactive-bound complex was not involved in the increase in RNases after water stress.

Reduced glutathione inhibited the K^+-induced breakdown of RNA by 2,4-D in cotton cotyledons (Basler, 1963) but was not effective on soybean RNase (Merola, et al., 1962). EDTA-sensitive RNase showed a larger increase than EDTA-insensitive RNase after senescence of wheat leaves (Sodek, et al., 1969). It was, therefore, reasonable to check whether this differential sensitivity to glutathione was present during water stress or not. The enzyme in the supernatant fractions from leaves was kept for 6 hours at $4^{\circ}C$ in the presence of reduced glutathione (10^{-2} M) or oxidized glutathione (5×10^{-3} M) at pH 7.8, because a 76% loss of original RNase activity was caused by keeping the enzyme at pH 8 for 6 hours at $4^{\circ}C$ in the presence of sulfhydryl compounds like cysteine and dithiothreitol (Hanson, et al., 1969). As shown in Table III, the reduced glutathione (GSH) treatments inhibited RNases from control leaves but caused a slight increase in activity of the enzyme

TABLE II

EFFECT OF p-CMB ON SPECIFIC ACTIVITY OF RNase AT pH5.8 IN SUPERNATANT FRACTIONS* FROM 100 CROWNS AND 100 LEAVES OF WHEAT PLANTS AT VARIOUS STAGES OF WATER STRESS

	p-CMB added	Control RWC, 96%	3-day stress RWC, 88%	5-day stress RWC, 78%
leaves	0.00 mM	0.16	0.31	0.26
	0.16 mM	0.16	0.30	0.26
	0.20 mM	0.16	0.31	0.27
	0.24 mM	0.15	0.30	0.25
	0.28 mM	0.14	0.29	0.25
crowns	0.00 mM	0.52	1.46	1.36
	0.16 mM	0.52	1.46	1.36
	0.20 mM	0.52	1.47	1.40
	0.24 mM	0.51	1.45	1.38
	0.28 mM	0.50	1.44	1.36

*after centrifugation at 20,000 x g for 30 minutes

TABLE III

EFFECT OF GLUTATHIONE ON THE SPECIFIC ACTIVITY OF RNases IN SUPERNATANT FRACTIONS* OF WHEAT LEAVES AT VARIOUS STAGES OF WATER STRESS

		Control RWC, 91%		3-day water stress RWC, 87%	
	glutathione added	pH 5	pH 5.8	pH 5	pH 5.8
Experiment 1.	none	0.16 (100%)	0.16 (100%)**	0.22 (100%)	0.24 (100%)
	GSH(0.001 M)	0.08 (53%)	0.11 (68%)	0.23 (106%)	0.25 (104%)
	GSSG(0.0005 M)	0.07 (44%)	0.11 (70%)	0.19 (88%)	0.18 (77%)
		Control RWC, 93%		3-day water stress RWC, 85%	
Experiment 2.	none	0.12 (100%)	0.15 (100%)	0.20 (100%)	0.23 (100%)
	GSH(0.001 M)	0.08 (64%)	0.11 (70%)	0.22 (110%)	0.23 (100%)
	GSSG(0.0005 M)	0.07 (62%)	0.09 (57%)	0.16 (80%)	0.19 (82%)

*after centrifugation at 20,000 x g for 30 minutes

**ability of glutathione-treated sample as a per cent of non-added glutathione

from water stressed leaves. However, the oxidized glutathione (GSSG) treatments decreased the RNase activity of control an water stressed leaves. It was reported that the oxidized molecule of RNase assumed a random coil conformation, in contrast to the folded conformation of native molecules (Sheraga, et al., 1962). It was also indicated that reduced-carboxymethylated RNase possessed properties similar to those of oxidized RNase (Sheraga, et al., 1962, and Kasai, 1965).

Thus, a decrease in RNase activity by the glutathione treatment could presumably caused by the extensive changes in RNase conformation either by oxidation with GSSG or reduction with GSH.

As a further test of the hypothesis that water stressed leaves continue, for a time at least, to synthesize RNase, a further type of experiment was done. Cycloheximide is known to inhibit protein synthesis. The experiment included 3 treatments, treatment A plants were water-stressed for 3 days in the absence of cycloheximide; treatment B plants were water-stressed for 3 days after being sprayed with cycloheximide; treatment C plants were sprayed but not subjected to water stress. When wheat plants were sprayed with cycloheximide and not subjected to water stress, the total activity of RNase was slightly increased (treatment C of Table V), contrasted with the greater increase in the growth experiment (Figure 5). The specific activity of RNases (treatment C of Table IV) did not change which was also found in the growth experiment (Figure 5). Dry weight and protein content of 100 leaves were slightly increased after 3 days from the start of the experiment (treatment C of Table VI), but the protein content per dry weight remained almost at the same level during the 3-day experiment (treatment C of Table VI). Cycloheximide caused the total activity

TABLE IV

SPECIFIC ACTIVITIES OF RNases FROM WHEAT LEAVES AFTER TREATMENT WITH OR WITHOUT CYCLOHEXIMIDE FOR 3 DAYS WITH AND WITHOUT WATER STRESS

Treatment		zero day	3-day stress	3-day water
A-1	RWC	93%	85%	
	pH 5	0.06	0.20	
	pH 5.8	0.08	0.25	
A-2	RWC	94%	89%	
	pH 5	0.12	0.15	
	pH 5.8	0.13	0.20	
A-3	RWC	91%	89%	
	pH 5	0.09	0.16	
	pH 5.8	0.10	0.22	
B-1	RWC	92%	83%	
	pH 5	0.07	0.13	
	pH 5.8	0.08	0.18	
B-2	RWC	95%	87%	
	pH 5	0.10	0.12	
	pH 5.8	0.10	0.12	
B-3	RWC	92%	89%	
	pH 5	0.07	0.15	
	pH 5.8	0.08	0.13	
C-1	RWC	95%		87%
	pH 5	0.06		0.07
	pH 5.8	0.05		0.07
C-2	RWC	95%		96%
	pH 5	0.09		0.11
	pH 5.8	0.11		0.13

RNase activity was measured in combined supernatant and pellet fractions after centrifugation at 20,000 x g for 30 minutes.

A indicates treatment without cycloheximide. B and C indicate treatment with cycloheximide.

TABLE V

TOTAL ACTIVITIES OF RNases FROM 100 LEAVES AFTER TREATMENT WITH OR WITHOUT CYCLOHEXIMIDE FOR 3 DAYS WITH AND WITHOUT WATER STRESS

Treatment		zero day	3-day stress	3-day water
A-1	pH 5	7.2	14.4	
	pH 5.8	9.0	18.4	
A-2	pH 5	9.7	12.2	
	pH 5.8	10.6	15.4	
A-3	pH 5	9.4	13.2	
	pH 5.8	10.6	18.2	
B-1	pH 5	5.5	5.8	
	pH 5.8	6.3	5.8	
B-2	pH 5	6.4	5.8	
	pH 5.8	6.3	5.8	
B-3	pH 5	4.1	3.6	
	pH 5.8	4.5	3.7	
C-1	pH 5	4.1		4.9
	pH 5.8	3.7		4.9
C-2	pH 5	4.2		4.3
	pH 5.8	4.9		5.3

RNase activity was measured in combined supernatant and pellet fractions after centrifugation at 20,000 x g for 30 minutes.

A indicates treatment without cycloheximide. B and C indicate treatment with cycloheximide.

See Table IV for relative water content.

TABLE VI

DRY WEIGHT (mg), PROTEIN CONTENT (ug), AND PROTEIN CONTENT PER DRY WEIGHT (ug/mg) OF 100 LEAVES AFTER TREATMENT WITH OR WITHOUT CYCLOHEXIMIDE FOR 3 DAYS WITH AND WITHOUT WATER STRESS

Treatment		zero day	3-day stress	3-day water
A-1	dry weight	651	724	
	protein	10595	7134	
	protein/dry weight	16.3	9.8	
A-2	dry weight	646	700	
	protein	7950	7700	
	protein/dry weight	12.3	11.0	
A-3	dry weight	691	761	
	protein	9700	8150	
	protein/dry weight	14.0	10.7	
B-1	dry weight	574	725	
	protein	8432	6150	
	protein/dryweight	14.6	8.0	
B-2	dry weight	662	763	
	protein	8374	7050	
	protein/dry weight	12.6	9.2	
B-3	dry weight	732	812	
	protein	8222	5710	
	protein/dry weight	11.2	8.5	
C-1	dry weight	603		688
	protein	7680		8743
	protein/dry weight	12.7		12.7
C-2	dry weight	567		697
	protein	5020		5460
	protein/dry weight	8.8		7.8

Protein content was measured in combined supernatant and pellet fractions after centrifugation at 20,000 x g for 30 minutes.

A indicates treatment without cycloheximide. B and C indicate treatment with cycloheximide.

See Table IV for relative water content.

of RNase to decrease (treatment B of Table V), contrasted with no cycloheximide treatment where there was an increase in RNase levels (treatment A of Table V). This indicates that cycloheximide suppressed the additional increase in RNase levels due to water stress. The specific activities of both treatments A and B slightly increased. The dry weight increased but protein content and protein content per dry weight decreased in both treatments A and B, although they remained at about the same in treatment C (Table VI). The value of 87% relative water content for the cycloheximide treated leaves without water stress (treatment C-1) is probably in error since the plants had not been subjected to any water stress. A relative water content of about 95% would have been expected.

CHAPTER V

DISCUSSION

Attempts were made to study the early stages of water stress, since RNases are stable enzymes and they appear to be a biochemical indicator of early alterations of leaf metabolism during water stress. The maximum activities of the crude enzyme in the preparations were obtained at pH 5 and pH 5.8 (Figure 3). There were two types of RNases having different optimum pH values in spinach leaves (Tuve and Anfinsen, 1960), and other works found on the basis of studies of wheat leaves (Hadziyev, et al., 1969) and corn leaves (Wilson, 1963) that RNase A, soluble or cytoplasmic RNase, had an optimum at pH 5 and RNase B, particulate or bound RNase, at pH 5.8. The normal growth of wheat leaves during the 4-day experimental period was accompanied by an increase in total protein content (Figure 6) and total activities of RNases at pH 5 and pH 5.8 (Figure 5). The increase in RNase activity with increasing age of leaves extends the more general data of tobacco leaves (Reddi, 1965), barley coleoptiles (Ledoux, et al., 1962), corn leaves (Wilson, 1963), and tomato leaves (McHale, et al., 1963) in which the increased activity was associated with the increased synthetic activity. Because RNA levels were not measured in the present study, it was not possible to determine in the growth experiment (Figure 5) whether maximum RNase activity coincided with maximum RNA content.

The higher RNase level in older leaves (Figure 5) was the result

of the normal growth, because morphological changes caused by senescence did not occur during the experiment. The yellow of tomato leaves during senescence was shown to be accompanied by increased RNase levels (McHale, et al., 1968).

When plants were water-stressed by withholding water after 10 days of growth, the specific activities of RNases at pH 5 and pH 5.8 were increased nearly two to three times (Figures 8 and 12) while protein content was decreased during the first 3- to 4-day period of water stress (Figures 10 and 13). The increase in RNase content in control plants that were grown over the same time period (14 days) was about 30%. Thus, the observed 300% increase must be mostly a direct consequence of the water stress imposed.

The result of the increased specific activity of RNase with water stress in this study parallels that of peroxidase of detached wheat leaves (Todd and Yoo, 1964), peroxidase activity of water stressed leaves of intact wheat leaves (Stutte and Todd, 1969) and RNase activity of detached leaves of tomato (Dove, 1967, and Kessler, 1961). Because of the increased RNase activity, a decline in RNA in the tissue might be anticipated. Such a decrease was observed in the RNA content of wheat leaves after 4-day water stress (Stutte and Todd, 1968), although an initial increase in RNA content was followed by a decrease in tomato leaves with increasing moisture stress (Gates and Bonner, 1959).

Hadziyev, et al., (1969) found gradual increase in the ratio of activities of the two RNases (RNase pH 6.0/RNase pH 5.2) with increasing age of wheat leaves indicating a greater accumulation of particulate or bound enzyme relative to the soluble or cytoplasmic enzyme. If it is assumed that the pH 5.8 enzyme is the particulate RNase and

pH 5.0 enzyme is soluble RNase in this study, it is likely that the particulate enzyme is present in larger amounts than the soluble enzyme in water stressed leaves as well as in growing leaves; the relative proportions of these two enzymes were not changed by water stress. This was also observed in supernatant and pellet fractions from crowns from the same wheat plants (Figure 12).

Earlier it was found that there were smaller nucleic acid and protein changes in the crowns of wheat plants than those in the leaves, and postulated that there was a movement of intact nucleic acids from the leaves to the crowns replacing the nucleic acids which might be destroyed in the crown tissue (Todd and Basler, 1965). If the loss of nucleic acid including RNA at the substrate level was associated with increased RNase levels, there should have been more extensive breakdown of RNA in the crowns during the early stages of water stress because of higher specific activities of RNases in crown tissues of the plant as shown in Figure 12. However, the RNA content of the crown portions was reported to remain at about the same level during water stress (Todd and Basler, 1965).

The changes in the nucleic acid content of debris-nuclear fraction were much smaller than the other fractions of wheat leaves killed by drought (Todd and Basler, 1965). In the same report it was shown that the supernatant fractions had the greatest destruction (80%) of nucleic acid while there was a 50% of loss of nucleic acid in the chloroplast and mitochondrial fractions. As shown in Table I-a and b, however, the fractional distribution of RNase levels among the nuclear pellet, the chloroplast, the mitochondrial, and the supernatant fraction was not changed sufficiently in the early stages of water stress to account

for the observed changes of nucleic acid content in the various fractions mentioned above. RNase levels in different fractions remained more or less constant during water stress. About 80% of RNases are present in the supernatant fraction in control and water stressed leaves of wheat. This agrees with the results of Hadziyev, et al., (1969) in which two-thirds of the total RNase activity was present in the supernatant fraction of wheat leaves in the normal state regardless of the variety assayed while the rest was distributed among the subcellular particles.

Some explanations have been given for the increased RNase activity during water stress and senescence. The first suggestion was that the gradual increase in RNase activity with increasing senescence as well as water stress might be attributed to a decrease in enzyme inhibitors. Since zinc in citrus trees (Kessler, et al., 1959), and Al and Co in sunflower (Bozhenkov, 1968) are inhibitors of RNases, accumulation of these ions in young leaves was thought to be responsible for the lowered enzyme activity in homogenates from those plants as compared with the activity of older leaves. Another worker suggested that loss of mineral inhibitors was not crucial to the increase of RNase activity during senescence of detached tomato leaves, since an increase in enzyme activity was observed before appreciable changes in mineral content would have occurred in the leaves (McHale, et al., 1969).

Another hypothesis that has been suggested is that liponucleoprotein structure, which might bind RNase in the inactive form, was disruted during stress and thus liberated free RNase (Kessler, 1961, and Tappel, 1966). The structure suggested may have been the lysosome-like bodies described as being present in plant tissues (Cocking, 1960,

Matile, 1968, 1969, and 1971, and Jones, et al., 1970). If lysosomes were present in control and water stressed leaves and crowns of wheat plants, these probably could have been rutured during grinding since the lysosomal structure was found to be very susceptible to grinding of leaves and other plant parts (Matile, 1969) or by the long time incubation at 37°C since plant lysosomes were ruptured by 5 minutes of incubation at 37.5°C with recovery of latent enzyme activity (Gahn, 1965). The lysis of coleoptile protoplasts of Avena showed that enzymatically inactive RNase was bound to sites on the plasma-membrane normally occupied by divalent cations weakening the attraction between wall and plasamembrane and making the subsequent plasmolysis-form more concave (Ruesink, 1971). RNase extracted from the precipitate after addition of Triton X-100 to the solution indicated also that the increase in enzyme activity during banana ripening was due to the breakdown of weak bonding on the membrane or cell wall, rather than being released from membrane-enclosed particles (De Leo, et al., 1970). It was apparent, therefore, that some RNases might be released from lysosomes into the cell only during the final stages of cucumber leaf senescence, but not in the early stages (Eilam, et al., 1971).

After excision of leaves from Avena seedlings, kinetin treatment prevented the rise in RNase activity in the supernatant fraction caused by illumination of sucrose supplied in darkness (Udvardy, et al., 1967). The treatment of cytoplasmic supernatant fraction from wheat stem (probably crown tissue) with p-CMB should have released the enzyme that was believed to be an enzyme-bound complex (Hadziyev, et al., 1969). The possible presence of an enzyme-bound complex in the cyto-

plasmic supernatant fraction from leaves and crowns was investigated by adding p-CMB to the reaction mixture at pH 5.8 (Table II). No additional enzyme activity was found indicating either no enzyme-bound complex or that the contaminants indicated by Shortman (1961) in the crude homogenates nullified the effect of p-CMB.

Another possible mechanism for the observed increase in RNase activity with water stress could be that the mode of existence of the enzyme in its normal state would be changed during water stress, since the oxidation of reduced, enzymatically inactive RNase resulted in the regeneration of the activity similar in magnitude to that of native enzyme (White, 1960). It was found that 2,4-D prevented RNA breakdown in the intact tissue of cotton cotyledons by effecting a reduced state of sulfhydryl components in the tissue (Basler, 1963). Some isozymes of RNase were found in corn plants (Wilson, 1971). Certain peroxidase isozyme disappeared and new ones appeared with increasing water stress (Stutte and Todd, 1969). In addition to this, properties of the RNase from non-germinated embryos and seedlings of wheat differed, and the proportions of these two RNases varied during germination (Vold, et al., 1968). In spite of the reported harmful effects of extensive oxidation and reduction on the RNases (Kasai, 1965, and Hanson, et al., 1969), the wheat leaf RNases, both at pH 5 and pH5.8, were insensitive to the reduced glutathione after 3 days of water stress (Table III). This result agrees with the idea of Sodek, et al., (1969) who found that EDTA-sensitive RNase increased but the level of EDTA-insensitive RNase remained constant throughout senescence. RNase from soybean was also insensitive to reduced glutathione, and the enzyme had unique specificity to substrate in which

cyclic pyrimidine nucleotides were not acted upon by the enzyme and soluble RNA was hydrolyzed to a less extent than more highly polymerized RNA (Merola, et al., 1962). However, no definite conclusion can be drawn as to the significance of the changes in RNase activity described here with glutathion treatments. With the exception of reduced glutathione treatment of the supernatant fraction from water stressed leaves, the oxidized glutathione treatment with supernatants from control and water stressed leaves and the reduced glutathione treatment with supernatant fraction from control leaves inhibit the enzyme. Nevertheless, the possibility that more than one nuclease or other phosphate-ester-hydrolyzing enzymes (Udvardy, et al., 1969) might be affected by glutathione treatments has not been studied.

The idea that the enhanced synthesis of RNase is caused by water stress is in line with the recent results of senescence in detached leaves. The decreased protein content and the increased activity of some enzymes (Stutte and Todd, 1969) also suggested the enhanced synthesis of some enzymes is involved in water stress. Actinimycin D suppressed the enhanced RNase activity during senescence (McHale, et al., 1969) and after mechanical damage (Bagi, et al., 1967) of tomato leaves. The total inhibition by chlorophenicol would suggest that the enzyme was synthesized during senescence of wheat leaves (Sodek, et al., 1969). The formation of new phosphatases in germinating lettuce seed (Meyer, et al., 1971), in the excised Avena leaf (Udvardy, et al., 1969), in senescence of Rhoeo leaf tissue (De Leo, et al., 1970-b) and banana ripening (De Leo, et al., 1970-a) were inhibited by cycloheximide, since cycloheximide inhibited the peptide chain termination or release in protein synthesis (Glasziou, 1969,

and Rajalakshmi, 1971). Suppression of the increase in RNase activity during water stress by cycloheximide (Table V) suggests that enzyme synthesis was at least partially responsible for the additional activity caused by water stress in wheat leaves. If de novo synthesis of enzyme was responsible for the increase in RNase activity with water stress, then one might consider the polysome level with regard to the new synthesis of enzyme in water stressed plants, because protein synthesis takes place at polysome level, but not at monosome level of ribisomes (Lin, et al., 1967). But it is known that RNase inhibits protein synthesis since ribosomal RNA was hydrolyzed by RNase (Lehninger, 1970). In fact, sucrose gradient profiles showed that water stress of maize caused a shift of ribosomes in coleoptile node region from the polymeric form to monomeric form after water stress initiation when the water potential of tissue began to decrease measurably (Hsiao, 1970). The leaf, however, had not completely lost the power to synthesize protein (Simmon, 1967), although the quality of protein declined at the time of senescence of the cucumber leaf. In addition, the polysomal ribosome might be present in later period of senescence of cucumber leaves, and new m-RNA might have been present or a very long-lived m-RNA was present even though the leaves were yellowing (Eilam, et al., 1971). In the same experiments on cucumber leaf senescence, the quantative study of the changes in the various classes of ribosomes showed that the main change was in the free ribosome fractions during growth and senescence, but not in membrane bound ribosomes, and that all of the acid soluble phosphate was available for RNA synthesis, eventhough incorporation of P^{32} into the ribosomal fraction RNA decreased with the age of cucumber leaves. It can be

said, therefore, that the ability to synthesize protein in a leaf is not completely lost, although polysomal ribosomes responded rapidly to small changes in water stress as indicated in the coleoptile node region of maize (Hsiao, 1970). Compared to the non-cycloheximide treated plant (Tables IV, V, and VI), however, the suppressed total activity of RNases in cycloheximide-treated plants (Table V) suggests that new protein synthesis or enzyme synthesis still proceeds, even though protein breakdown is accelerated after the initiation of water stress.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. The maximum activity of crude enzyme preparations of the supernatant fraction from wheat leaves was obtained at pH 5 and pH 5.8 in 0.1 M buffers, indicating the presence of two RNases differing in pH optima.

2. The protein content and the total activity of RNases at pH 5 and pH 5.8 increased in wheat leaves over a 4-day growth period with 10-day old plants.

3. When the plant was subjected to water stress, RNase levels increased nearly two to three fold while the total protein content was decreased about 30% during a 3-day period of water stress.

4. Crown tissues had higher specific activities of RNases than leaf tissues, and RNase activities at pH 5 and pH 5.8 were about the same in leaves of water stressed plants, but the enzyme activities at pH 5.8 was much higher than that at pH 5 in crowns of water stressed plants. The relative proportions of these two RNases at pH 5 and pH 5.8 from both leaves and crowns were not changed by water stress.

5. The highest total RNase activity was associated with supernatant fractions from control and water stressed leaves. p-CMB had no effect on the RNase activity in the supernatant fraction when added to the reaction mixture at pH 5.8.

6. The RNase in the supernatant fraction from leaves of water

stressed plants was insensitive to reduced glutathione treatment, while the enzyme from leaves not subjected to water stress was inhibited by this treatment.

7. Cycloheximide spray suppressed the additional increase in RNase levels normally caused by water stress.

8. It is concluded, therefore, that there is normally more than one RNase present in wheat plants. A RNase insensitive to reduced glutathione is probably synthesized after the initiation of water stress in spite of the overall degradation of proteins caused by water stress.

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