CHANGES IN RIBONUCLEASE ACTIVITY OF WHEAT PLANTS DURING WATER STRESS

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ABBREVIATIONS

A_{260} and A_{280}:	absorbance change at 260 nm and 280 nm
3'-AMP:	adenosine-3'-monophosphate
atm:	atmosphere
ATP:	adenosine-5'-triphosphate
ATPase:	ATP phosphohydrolase
BTEE:	N-benzoyl-L-tryosine ethyl ester
Cellex D:	diethylaminoethyl cellulose
pCMB:	para-chloromercuribenzoate
CM-sephadex:	carboxymethyl sephadex
DEAE-cellulose:	diethylaminoethyl cellulose
DNA:	deoxyribonucleic acid
DNase:	deoxyribonuclease
d.p.m.:	disintegration per minute
EDTA:	(ethylenedinitrilo)tetraacetic acid
IAA:	3'-indoleacetic acid
IAA-oxidase:	3'-indoleacetic acid oxidase
NAD:	nicotinamide-adenine dinucleotide
NADP:	nicotinamide-adenine dinucleotide phosphate
PPO:	2,5-diphenyloxazole
RNA:	ribonucleic acid
RNase:	ribonuclease
mRNA:	messenger RNA

tRNA:	transfer RNA
RPM:	revolution per minute
RWC:	relative water content
TAME:	p-toluenesulfonyl-L-arginine methyl ester
TCA:	trichloroacetic acid
Triton-X-100:	alkylarylpolyethoxyethanol

CHAPTER I

INTRODUCTION

Water is the major component in most plant cells. The actual cellular water content varies with cell types and physiological conditions. When any entity of a plant body becomes metabolically active, a sufficient amount of water is essential for the transformation. Thus, the lack of water or water deficit can be regarded as a stress factor.

There are many phases of plant responses to water stress. An increase in rates of non-synthetic metabolism is probably the most common characteristic, when the water status in the cell is changed. Important physiological processes altered by water stress include reduced rates of transpiration (Salim and Todd, 1965), translocation (Basler, et al., 1961), respiration (Flowers and Hanson, 1969), photosynthesis (Todd and Basler, 1965), and growth (Chen, et al., 1968).

These metabolic changes may lead to changes in enzymatic capacities. Severe water deficits generally cause an overall decrease in enzyme levels (Todd, 1972); levels of the enzymes involving hydrolysis tend to increase and levels of the enzymes involved in synthesis tend to decrease. Qualitative changes as well as quantitative changes in enzymes or protein occur during water stress treatment. Peroxidase (Stutte and Todd, 1969) and malic dehydrogenase (Tsai, 1972) appeared to change qualitatively since different isoenzymes appeared after water

stress. These consequences of water stress and the senescence that follows may not be a random process, but an orderly one, if based on the protein structure. The protein will assume the thermodynamically most stable conformation under a given environmental condition (Anfinsen, 1973). Thus, if the physiological condition is changed around a protein molecule, resulting in a conformational change, some enzymes are active, or even activated, while others are not changed or may be inactivated.

Since it is already known that the total RNA content of wheat plants was reduced under water stress (Stutte and Todd, 1968), it seems possible that the reduction in RNA content is caused by the increase in RNA-hydrolyzing enzyme, RNase, during the water stress. The molecular structure and properties of pancreatic RNase are well known. Its molecular weight is relatively small (ranging from 14000 to 40000) and the molecule is very stable. Characteristics of RNases from plant sources have been studied very little, but many assume that plant RNase has the same properties as the pancreatic RNase. The isolation procedures are relatively straightforward.

However, the changes in RNase level along with environmental stress or senescence have been studied only in crude extracts in many cases. There are many conflicting data on the causes of the increased level of RNase activity after water stress: some are suggesting de novo synthesis and others the activation of the pre-existing enzyme. Disappearance of inhibitors present in the homogenate could lead to the observed results. Therefore, this study was initiated to determine RNase changes occurring during water stress using purified enzyme

preparations in an attempt to clarify the nature of these changes and determine the underlying causes for the changes in activity.

CHAPTER II

LITERATURE REVIEW

Any enzyme that breaks down RNA has been called a ribonuclease. Three major groups of enzymes are known that contain some members having that capability: phosphotransferases, phosphodiesterases, and phosphorylases. Phosphorylases are polynucleotide phosphorylase and polynucleotide pyrophosphorylase, and these are not discussed in the present study.

The basic feature of phosphotransferases is the use of the 2'-OH group in an intramolecular attack at the adjacent phosphodiester bond, in addition to some device that confers specificity for one or more of the nucleic acid bases. The nucleoside 2'-:3'-cyclic phosphates are, therefore, obligate intermediate products. In phosphodiesterases, a direct attack of water on the 3'-:5'-phosphodiester bond is catalyzed. This group includes, thus, enzymes that hydrolyze both DNA and RNA, and those that cleave diester bridges in RNA to form only 5'-nucleotide products.

According to Barnard (1969), enzymes depolymerizing RNA are classified into two groups.

1. RNA phosphotransferases (cyclizing RNases) are 3'- or 2'-phosphate formers and have specificities towards bases adjacent to the bond cleaved and for the macromolecular form of RNA.

2. Phosphodiesterases (noncyclizing RNases and nucleases) include two groups of enzymes: polynucleotide phosphodiesterases and nonspecific phosphodiesterases. Polynucleotide phosphodiesterases are ribose-specific, sugar-nonspecific or deoxyribose-specific. These are 3'- or 5'-phosphate monoester formers and include endo- or exonucleases. They have specificities towards bases and macromolecular form of RNA. Nonspecific phosphodiesterases hydrolyze both RNA and diesters of phosphates other than nucleotides.

Ribonucleases mentioned in the literature sometimes are referring to an enzyme specific for RNA and nucleases refer to a sugar-nonspecific enzyme. The crystalline RNase obtained from the bovine pancreas contains at least two catalytically indistinguishable forms, RNase A and RNase B. Ribonuclease B is a glycoprotein form of RNase A with five mannose and two glucosamine residues attached to the protein at residue 34 (asparagine) (Plummer and Hirs, 1964). Bovine pancreatic RNase A, the form that has been most extensively studied, is a single polypeptide chain free of carbohydrates with a molecular weight of 13,600; the complete sequence of its 124 component amino acids is known (Moore and Stein, 1973). A preparation with 70% of the activity of native RNase has been synthesized (Gutte and Merrifields, 1971), and the preparation includes folding of the reduced chain with 8 SH groups to give the proper pairs of SS bonds for the active conformation of the protein. The intramolecular forces that guide such a folding and the similar forces that contribute to the specific aggregation of the chains form a continuing subject of the research.

Study of stereo structure shows that starting from the N-terminal end of RNase chain there are two short lengths of alpha helix, residues from 2 to 12 and residues from 26 to 33, packed closely to one another (Dickerson and Geis, 1969). At residue 42, the chain starts a beta-sheet structure, and the first strand of the sheet is laid down by 42 to 49, before the third and last length of alpha helix, residues from 59 to 58. The chain then doubles back to begin a double-stranded V of beta sheet which runs the entire length of the molecule, first with residues 71 to 92 and then back down with 94 to 110, with 80 to 86 lying alongside the earlier strand 42 to 49. This V framework essentially defines the shape of the molecule and its crevice active site. The last act of the folding process is to bring the tail, 116 to 124, across the inside of the crevice and to position the catalytically important histidine 119 at the active site.

Pancreatic RNase has a hydrophobic core on one side of the active site crevice and a less substantial second wing. A full 34% of its residues are the potentially hydrogen-bridging serine, threonine, asparagine or glutamic acid. These 4 residues are distributed along the extended beta chain except where a chain is surrounded by the other chains and become hydrophobic (Dickerson and Geis, 1969).

The active site has been located by binding an inhibitor to the enzyme and locating the inhibitor molecule in the map of the crystal. The inhibitor used was an analogue of a monomer RNA, uridine 2',3'-phosphate (Moore and Stein, 1973). The chemical study has implicated histidine 12, histidine 119 and lysine 41 in the catalytic action, and all three show up close to one another at the active site (Roberts, et al., 1969). Furthermore, a P^{31} nuclear magnetic resonance study

indicated that the phosphate group of uridine-3'-phosphate was simultaneously bound to two positively charged amino acid residues at the active site of the enzyme (Lee and Chan, 1971).

The RNases so far characterized from flowering plants are all cyclizing enzymes of acid pH optimum. Sugar-nonspecific nucleases also occur. These enzymes all appear to cleave next to all four bases, although at different rates (Wilson, 1968-a). Otherwise, they show properties similar to those of the corresponding types of pancreatic RNases. Partially purified RNases have been prepared from corn (Wilson, 1963), mung bean (Sung and Laskowski, 1962, and Johnson and Laskowski, 1968), rye grass (Shuster, et al., 1959, and Freeman, 1964), spinach (Tuve and Anfinsen, 1960), and a number of other plant species.

Plant RNases fall into 3 groups, according to Wilson (1968-a). The best known plant enzyme is a ribonuclease (ribonucleate nucleotido-2'-transferase (cyclizing), E. C. 2.7.7.17) with a pH optimum near 5.0 and which liberates all 4 cyclic nucleotides from RNA. It has been obtained in pure form from corn (Wilson, 1967), and was originally named RNase A. A second RNase, with a pH optimum near 6.0, has been obtained from the microsomes of tobacco leaf (Reddi, 1965) and the microsomes of corn root (Wilson, 1968-b). Reddi (1965) named the first type of enzyme RNase I, and distinguished it by the ability to hydrolyze only cyclic purine nucleotides, while RNase II from microsomes would hydrolyze both purine and pyrimidine cyclic nucleotides. A third plant enzyme from corn roots hydrolyzes both RNA and DNA with the release of 5'-nucleotides, and it was suggested that it might be a 3'-nucleotidase (Wilson, 1968-b).

The molecular weights found are 20,000 in garlic RNase (Carlsson and Frick, 1964), 12,600 in cucumber RNase (Kado, 1968) and 23,000 in corn RNase (RNase A) (Wilson, 1967). The RNase from microsomes of corn root had a molecular weight of approximately 17,000 (Wilson, 1968-b). RNases from mung bean (Johnson and Laskowski, 1968) and potato tubers (Bjork, 1965) require Mg^{++} ions, while others are Mg^{++} or Ca^{++} ion-independent.

In vertebrate animals, extracellular RNases are secreted by the pancreatic and salivary glands. In plants and microorganisms, the existence of extracellular RNase is less obvious. Many RNases of microorganisms are known to be intracellular, but in some cases this distinction may not yet have been clearly made. Apparent intracellular RNase in microorganisms may sometimes be actually extracellular (Heppel, 1967), because the enzyme is outside the cell membrane, confined by the cell wall.

A considerable effort has been invested in studies of the functional aspects of RNases. These studies have uncovered a number of interesting phenomena, but thus far no clear picture has emerged of the function of the various RNases in cells.

One of the biological functions is a general intracellular digestion. In this case, RNases are released from lysosomes or equivalent situation for the digestion of cytoplasmic macromolecules. Lysosomal RNase differs from the better known pancreatic alkaline RNase in several respects (Barrett, 1969). Lysosomal RNase (ribonucleate pyrimidinenucleotide-2'-transferase (cyclizing), E. C. 2.7.7.16) has a molecular weight of 18,500, is acidic, and metal-independent. It is assumed that the lysosomal RNase has a mode of action similar to that

elucidated for the pancreatic enzyme. Also, the lysosomal enzyme does not exhibit the exceptional thermal stability of pancreatic RNase. In flowering plants, parenchymatous cells contain central vacuoles which might be suspected of being lysosomes (Matile, 1969). Lysosomal enzymes including RNase originate from the induced synthesis at the tonoplast or the transport of cellular components from the cytoplasm into the vacuole (Matile, et al., 1971). This plant lysosomal RNase increased over several hours in response to wounding (Tappel, 1966).

Antiviral function has been ascribed to the RNA-depolymerase action at the surface or the interior of the plant cell when infected by virus, but the overall content of RNase in the leaf was not correlated with susceptibility to infection by intact virus (Hamilton, 1974, and Takebe, 1975).

A synthetic role for cellular RNases has not been seriously suggested. Much attention, however, has been paid to their relation or influence on protein synthesis. While all RNAs (ribosomal, messenger, transfer and nuclear) are likely to be competent substrates for any one enzyme in various appropriately manipulated in vitro conditions, the intracellular location of the RNA of the enzyme may introduce considerable restrictions in vivo (Barnard, 1969). The restriction, or even the total prevention of any attack, could arise from the complexing of the RNA to form a resistant structure or complexing of the enzyme with an inhibitor.

Complexing of the RNA to form a resistant structure is illustrated by the case of the ribosomal nucleoprotein. Intracellular RNase in the liver cell is attached to ribosomal subunits and does not attack mRNA, intact 80 S ribosomes and polysomes (Utsunomiya and Roth, 1966, and

Brewer, et al., 1969). Occasionally, a ribosome was reformed from a subunit which contains bound RNase and the RNase in the 80 S ribosome formed was prevented from attacking the ribosome, probably through stereochemical hindrance (Utsunomiya and Roth, 1966). When the artifactual ribosome becomes attached to an mRNA strand, the strand was cut by the enzyme. Thus, when the cell contains a higher proportion of artifactual ribosomes or lower amount of inhibitors, as seen in hepatomas, there will be more rapid destruction and turnover of its messenger RNA. The susceptibility of ribosome-bound aminoacyl-tRNA to hydrolysis by RNase decreased, when a peptidyl transferase caused physical changes (closing or tightening of the ribosome) on the ribosome during protein synthesis (Culp, et al., 1973). This resistance, therefore, is presumably due to some steric inaccessibility, and involved in the regulation of a single species of RNA.

Complexing of the enzyme with a soluble inhibitor appears to be the device generally used in mammalian tissue cells to control the alkaline RNase (Shortman, 1961 and 1962). The single stranded mRNA could be protected from hydrolysis by endogenous and pancreatic RNase, when a protein from postmicrosomal supernatant of rat liver cells was added to the reaction mixture, and the inhibition of RNase was inactivated by the addition of 1.7×10^{-3} M pCMB (Blobel and Potter, 1966). The rat adrenal cell also had the same kind of inhibitor present in liver cells (Girija and Sreenivasan, 1966). There was production inside the cell of an inhibitor which was capable of inactivating the extracellular RNase during the culture of Bacillus subtilis (Smeaton and Elliot, 1967). This inhibitor was purified and shown to have the properties of a protein with molecular weight of

12,500. The RNase of Salmonella typhimurium was essential for overall structure of ribosomes, and its activity was inhibited by 70 S ribosomes and 30 S subunits, but not by 50 S subunits (Datta and Burma, 1972). The 70 S ribosomes and 30 S subunits contained an inhibitor with properties of a protein of RNase and so RNase became latent on association with the ribosome.

In plant cells, a comparable inhibitor has not been found. The RNase was active under conditions favoring the maintenance of integrity of the ribosomes of corn seedlings (Hsiao, 1968-a), and it was found that no latent RNase was present in the ribosomes. The RNase activity was studied in resting and growing cells of tobacco pith blocks cultured on White's basic medium (Gagnon and de Lamirande, 1972). It revealed that the ribosomal RNase controlled the stability of ribosomes and the enzyme, active in resting cells, favored a rapid turnover of ribosomes.

Because of many years of study on pancreatic RNase molecule (Moore and Stein, 1973), RNase became one of the best defined enzymes in terms of both structure and function. Structural and functional models of RNases are now compared by the complete three-dimensional structure determined from unfolding and refolding processes in vitro. The reduction of pancreatic RNase in 8 M urea usually causes cleavage of disulfide bonds and destruction of its native structure, so that the enzyme becomes inactive. Reactivation of the reduced enzymes by air oxidation had been successful in the cases of pancreatic RNase (White, 1960) and RNase T1 (Kasai, 1965). When the fully reduced RNase, with 8 SH groups, was allowed to reoxidize under denaturing conditions such as exist in a solution of 8 M urea, the mixture of products was

obtained containing many or all of the possible 105 isomeric disulfide bonded forms (Anfinsen, 1973). This mixture was essentially inactive, having on the order of 1% activity of the native enzyme. When the urea was removed and the "scrambled" protein was exposed to a small amount of a compound containing a sulfhydryl group such as mercaptoethanol, disulfide interchange took place, and the mixture eventually was converted into a homogeneous product, indistinguishable from the native RNase. This process was driven entirely by the free energy of conformation that was gained in going to the stable, native structure. The transition from incomplete enzymes, with random structure, to competent enzymes, with unique and stable structure, is a delicately balanced one. The transition of denaturing and renaturing took place during transfers from solution at pH 6.7 to 3.2 and from solution at pH 3.2 to 6.7, respectively. The conformational change appeared over a very narrow range of pH, centered at pH 3.9 for a very short time period (less than 1 second). Both the refolding kinetics and the unfolding kinetics of pancreatic RNase were measured at pH 3.9 (Tsong, et al., 1972). The refolding followed the first order kinetics and was essentially temperature-independent. The unfolding kinetics were biphasic, in which the first phase was a fast, temperature-independent reaction and the second phase was a slow, temperature-dependent reaction.

It was suggested that the carbohydrate moiety served to stabilize the tertiary structure of the protein moiety in pancreatic RNase (Pazur, et al., 1970). In order to estimate stabilization of the glycoprotein due to the oligosaccharide, the effect of denaturants on the reversible unfolding of RNase B (glycoprotein form of pancreatic RNase A) was determined and compared with RNase A (Puett, 1973). It

revealed that the carbohydrate moiety had little effect on the conformation of the protein during denaturation, suggesting no physiological significance of stabilizing this particular structure of RNase.

There is a strong noncovalent interaction between the two peptide components of the RNase molecule (Richards and Vithayathil, 1959), and it was suggested that this interaction helped to maintain the intact protein in its proper conformation. The proteolytic enzyme, subtilisin, catalyzes the hydrolysis of a single peptide bond between residues 20 and 21 in pancreatic RNase. The product, designated RNase S, was still active, because the two component peptides were strongly associated and could only be separated by acid treatment, gel filtration with 50% acetic acid as the solvent gave a separation of the two peptides, a peptide containing residues 21 through 124 of the original and was designated S-protein and a second, smaller component of N-terminal peptide containing residues 1 through 20, designated S-peptide. Neither of the separated components had any biological activity. However, when S-peptide was added back to S-protein there was a complete recovery of the activity and a titration curve could be obtained, showing a sharp end point at an S-peptide:S-protein ratio of 1:1.

The reconstruction of RNase leads to dimerization and hybridization of the molecule (Fruchter and Crestfield, 1965). Lyophilization of pancreatic RNase from 50% acetic acid resulted in a 25% yield of a stable, enzymatically active dimer. Lyophilization from neutral buffers did not yield dimers. When the dimers were separated by gel filtration, the dimers had the same specific activity as the monomer; there were two active sites per dimer. Heating at 65 C for 10 minutes dissociated the dimer into active monomers. The hybrid dimer with partially restored

activity was formed from inactive RNase which had been carboxylated at histidine 119 and another inactive RNase which had been carboxylated at histidine 12. Conversely, carboxymethylation of the dimer gave a predictable amount of dicarboxymethylated RNase upon dissociation, a derivative that was not obtained from the monomer. Thus, the complementation phenomenon is possible in the case of RNase molecule, and so the active RNase will be formed from the inactive source.

There has been no study on the dehydration effect of RNase molecules. Most enzymes in general are inactivated when either pure or partially purified preparations are subjected to drying at room temperature (Todd, 1972). This dehydration effect was casually observed during the enzyme purification. Dehydration of wheat RNase caused a decrease in its activity (Hanson and Fairley, 1969).

Aspergillus niger was grown in varying sugar concentrations, the osmotic pressure of which ranged from 7.7 to 132.2 atm, and it was found that the bound water of proteins increased with an increase in osmotic pressure (Todd and Levitt, 1951). This bound water was also measured in calf thymus DNA, in which the water uptake by the DNA molecule in the presence of NaCl was greater than that in the absence of the salt (Chattoraj and Bull, 1971).

When water molecules were extracted from the matrix space of rat liver mitochondria by increasing the molarity of nonpenetrating solutes such as sucrose, ATPase activity induced by 2,4-dinitrophenol decreased (Cerejio-Santalo, 1972). This inhibition effect of nonpenetrating solutes could be released by the addition of permeant salts such as KCl. Alkaline phosphates activity also changed in KB cells from human nasopharyngeal carcinoma with a higher osmotic pressure in the culture

medium (Hertz, 1973). Increasing osmolality by addition of NaCl resulted in a reduction in heat-labile isozyme and an increase in the heat-stable isozyme of the phosphatase.

The freezing injury of plant tissues also causes dehydration inside the cell, and imposes water deficits on the protein and enzymes. During the freezing process, protein molecules are forced into closer proximity, or the distance between adjoining strands of the same protein molecule may become less, therefore, increasing the possibility of linkage occurring through disulfide bonds (Levitt, 1965). It was suggested that if a protein having an SH group was frozen, intra-molecular SS formation would be accelerated, leading to denaturation of the protein. On the other hand, the heat stable proteins with SS bonds should show a strong resistance to freezing injury or desiccation inside the cell

Changes in RNase levels in plants have been associated with injury, growth inhibition, senescence, germination, water stress, and so on. Many attempts were made to establish if the increased enzyme activity following such damage and senescence was due to the synthesis of new protein or to activation of pre-formed enzyme protein.

Earlier works of Kessler (Kessler and Monselis, 1959; Kessler, 1961; Kessler and Engelberg, 1962; and Kessler and Frank-Tishel, 1962) suggested that RNase was activated by removal of enzyme inhibitors such as zinc ions during water stress and that the enzyme was released from the microsomes into cytoplasm during the aging process. From the study of animal cells and tissues, Tappel (1966) hypothesized that since a high latency of RNase existed, freezing and thawing and concurrent osmotic and solute fluctuations in tissues and cells might cause the

unit phospholipid-protein membrane of the lysosome to rupture and thereby release hydrolytic enzymes including RNase, resulting in a high increase in RNase activity following freezing damage. Matile (Matile, 1968; Matile, 1969; and Matile, et al., 1971) demonstrated the presence of a lysosome-like structure in plant cells and found hydrolytic enzymes such as RNase inside lysosomes. In animal systems, the properties of lysosomal RNase is different from pancreatic enzyme, with respect to pH optimum and heat stability (Barrett, 1969). The lysosomal RNase from plant sources has not yet been characterized. However, there was a report that RNase with the characteristics of animal lysosomal RNase disappeared and another kind of RNase appeared during germination of wheat (Vold and Sypherd, 1968). No work has been done on the structural change of lysosomes during water stress or other environmental stresses. Instead, some workers showed that decreases in aluminum and cobalt ions, RNase inhibitor (Bozhenkov, 1968), and increases in potassium ions, RNase stimulator (Hsiao, 1968-b), caused by water deficits resulted in increases in RNase activity. Thus, definite evidence regarding the activation of RNases by water stress has not been provided.

Considerable attention has been devoted towards de novo synthesis of RNase during senescence and following tissue damage. In many cases, plant tissues were immersed in a solution containing cycloheximide or actinomycin D immediately after cutting of tissues or before the start of senescence. This is followed by preparation of crude extract for the enzyme assay. The inhibition of an increase in RNase activity in damaged tissues and during senescence by the treatment with cycloheximide or actinomycin D was regarded as evidence of de novo synthesis

of RNase. This kind of experiment was performed on many plants; damaged tobacco leaf (Bagi and Farkas, 1967), ripening banana (de Leo and Sacher, 1970-a and 1970-b), germinating lettuce seeds (Meyer, et al., 1971), potato tuber and leaf tissues (Pitt and Galpin, 1971), detached wheat leaves (Sodek and Wright, 1969), and excised Avena leaf (Udvardy, et al., 1967, and Udvardy, et al., 1969). When the enzyme was partially purified by using ion exchange chromatography, it appeared that some components of RNases were produced during water stress of wheat plants (Henkel, et al., 1974) and following mechanical damage of potato tubers (Pitt, 1974).

Recent investigations have used isopycnic density gradient centrifugation of RNases to prove the de novo synthesis. Although some were unsuccessful in showing such synthesis (Pitt and Galpin, 1971), plant tissues could incorporate deuterium oxide into RNase molecules during senescence (Sacher and Davies, 1974; Baumgartner, et al., 1975; and Sacher, et al., 1975), demonstrating de novo synthesis of RNase. During the senescence of morning glory, there was a definite time period of de novo synthesis of RNase 20 hours after the start of senescence (Baumgartner, et al., 1975). Furthermore, the period of sensitivity to actinomycin D inhibition of RNase synthesis was only within the initial 45 minutes after cutting of turnip tissues (Sacher, et al., 1975). This definite time period of actinomycin D effectiveness in RNase synthesis as a result of cutting was explained as a stimulation of transcription of a structural gene for RNase which was translated for synthesis of RNase.

There are reports that growth substances affect RNase activity during senescence. During Rhoeo leaf senescence, IAA inhibited the

increase in RNase activity and overcame the effect of abscisic acid on the promotion of RNase activity (de Leo and Sacher, 1970-b). The production of reducing sugar promoted by gibberellic acid was inhibited by treatments with abscisic acid and pancreatic RNase during germination of barley seeds (Leshem, 1971). Cytokinin inhibited an increase in RNase activity in detached tomato leaves (McHale and Dove, 1969). IAA treatment increased the RNA content, but decreased the RNase activity in Lens roots (Pilet and Brown, 1970). Auxin suppressed the increase in RNase activity during senescence of bean endocarp, and it was suggested that auxin increased RNase turnover at the level of either transcription or translation (Sacher, 1969). With excised mesocotyl tissues of corn plants, a low concentration of 2,4-dichlorophenoxyacetic acid accelerated growth and RNase activity in parallel (Shannon, et al., 1964). Kinetin inhibited the increase in RNase activity in detached wheat leaves (Sodek and Wright, 1969). Kinetin repressed the increase in RNase activity due to leaf excision of Avena (Udvardy, et al., 1967). However, no widely accepted function of these growth substances in regulating of RNase activity has yet been found. In mammalian tissues, a steroid acts as an initiator of a modifier molecule which interacts with alkaline phosphatase to produce an enzyme with enhanced catalytic efficiency (Herz, 1973).

CHAPTER III

MATERIALS AND METHODS

Chemicals

Alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, and hexokinase were purchased from Sigma Chemical Co., St. Louis, Mo. Lactate dehydrogenase, glutamate dehydrogenase, pancreatic RNase, pepsin, trypsin, chymotrypsin, papain, and lipase were purchased from Cal Biochem, La Jolla, Calif. Glucose oxidase, xanthine oxidase, catalase, and peroxidase were purchased from Worthington Biochemical Co., Freehold, N. J.

Dithiothreitol, homovanillic acid, ethidium bromide, TAME, BTEE, 3'-AMP, and acrylamide were purchased from Cal Biochem, La Jolla, Calif. N,N'-methylene bisacrylamide was purchased from Eastman Organic Chemicals, Rochester, N. Y. Sephadex G-75 was purchased from Pharmacia Fine Chemicals, Piscataway, N. J.

Enzyme Assay

The fluorometric and spectrometric methods for measuring enzyme activities are described below.

Fluorometric Methods

The appearance or disappearance of fluorescence after addition of enzymes was measured in a quartz tube (8 X 100 mm) with a Turner Fluorometer with a standard UV lamp. The filters used for the enzyme assay are given in Table I. The rate of change of the fluorescence was observed every 30 seconds for the first 5 minutes. All fluorometric enzyme assays were performed at 25 C, unless otherwise indicated.

The assay of alcohol dehydrogenase was carried out according to the method of Yonetani and Theorell (1964). The reaction mixture contained 0.2 ml of enzyme (100 μg/ml), 0.2 ml of 1 mM NAD and 3 ml of 1% ethanol in 0.1 M phosphate buffer, pH 7.0.

The assay of glucose-6-phosphate dehydrogenase and lactate dehydrogenase were carried out following the method of Goldberg, et al. (1965). The reaction mixture for glucose-6-phosphate dehydrogenase contained 1 mM glucose-6-phosphate, 0.05 M NAD, and 0.1 ml of enzyme (0.25 μg/ml) and 50 mM Tris-HCl buffer, pH 8.5 to give a total volume of 3 ml. The reaction mixture for lactate dehydrogenase contained 3 ml of 1% (v/v) pyruvate in 20 mM imidazole buffer, pH 7.0, 0.2 ml of 1 mM NADPH and 0.3 ml of enzyme (0.25 μg/ml).

The assay of isocitrate dehydrogenase was carried out according to the method of Cleland, et al. (1969). The reaction mixture contained 1 ml of a solution (containing 1 mM EDTA, 0.3 mM dithiothreitol and 100 mM Tris-HCl buffer, pH 7.4), 0.2 ml of 20 mM $MnSO_4$, 0.2 ml of 1.5 mM NAD, 0.05 ml of threo-D, L-isocitrate and 0.1 ml of enzyme (0.25 μg/ml).

Glucose oxidase and xanthine oxidase were assayed according to the method of Guilbault, et al. (1968). The reaction mixture of glucose

oxidase contained 2.7 ml of 0.1 M Tris-HCl buffer, pH 8.1, 0.1 ml of 0.1 M glucose, 0.1 ml of 2.5 mg/ml homovanillic acid, 0.1 ml of 0.75 mg/ml peroxidase and 0.1 ml of glucose oxidase (15 µg/ml). The reaction mixture for xanthine oxidase was the same as described for glucose oxidase, except that 0.1 ml of xanthine oxidase (15 µg/ml), instead of glucose oxidase, and 0.1 ml of 10 mg/ml hypoxanthine were added.

Glutamate dehydrogenase was assayed according to the method of Graham and Aprison (1966). The reaction mixture contained 10 mM NAD, 100 mM glutamate, 0.2 ml of enzyme (25 µg/ml) and 50 mM glycine-hydrazine buffer, pH 8.6, to give a total volume of 3 ml.

The hexokinase assay was carried out according to the method of Joshi and Jagannathan (1964). The reaction mixture contained 0.15 M glucose, 0.2 M Tris-HCl buffer, pH 7.6, 1 mM EDTA, 1.3 mM NADP, 0.3 M ATP(pH 7.6), glucose-6-phosphate dehydrogenase (2 units/ml) of hexokinase (10 µg/ml) to give a total volume of 3 ml.

The assay of lipase was carried out according to the method of Sapira, et al. (1966). The reaction mixture contained 3 ml of 5.1 mM fluorescein dibutyrate dissolved in methylcellosolve, 0.05 M citrate buffer, pH 6.5, and 0.05 ml of the enzyme (500 µg/ml) to give a total volume of 3 ml.

A fluorometric method for assay of RNase was performed according to the method of Kamm, et al. (1970). The assay measures the formation of a fluoroscent complex of the dye ethidium bromide (2,7-diamino-9-phenylphenanthridine 10-acetyl bromide) with nonhydrolyzed RNA. The reaction mixture contained 2 ml of ethidium bromide (15 µg/ml), 1 ml of 0.085% NaCl, 1 ml of RNA (0.1 mg/ml) dissolved in 5 mM succinate buffer, pH 6.95, and 0.03 ml of pancreatic RNase (5 µg/ml) or wheat RNase.

TABLE I

FILTER SYSTEMS USED FOR ASSAYS WITH THE TURNER FLUOROMETER

Enzyme	Primary Filter	Secondary Filter
alcohol dehydrogenase	110-811(7-60)	110-816(2A)
glucose-6-phosphate dehydrogenase and lactate dehydrogenase	110-811(7-60)	110-816(2A)
isocitrate dehydrogenase	110-811(7-60)	110-816(2A) plus 110-831(48)
glucose oxidase and xanthine oxidase	110-816(2A) plus 110-813(47B)	110-818(2A-12)
glutamate dehydrogenase	110-811(7-70)	110-816(2A) plus 110-831(48)
hexokinase	110-811(7-60)	110-817(8)
lipase	110-816(2A) plus 110-813(47B)	110-818(2A-12) plus 110-824(23A)
RNase	110-811(7-60)	110-824(23A)

Spectrophotometric Methods

Absorbance changes were measured with either a Perkin-Elmer Spectrophotometer Model 202 or a Beckman Spectrophotometer, DU, Model 2400. Corrections were made for a blank without enzyme or zero time blank containing enzyme.

Catalase was assayed according to the method of Chance and Maehly (1955). The reaction mixture contained 0.01 M phosphate buffer, pH 7, 3 ml of peroxide (2000-fold dilution of 30% solution with the buffer solution) and 0.2 ml of enzyme (25 μg/ml). The absorbance of 240 nm was measured with a Perkin-Elmer Spectrophotometer and readings made every 30 seconds for 5 minutes at 25 C.

Pepsin was assayed according to the method of Ryle (1968). The reaction mixture contained 1 ml of dialyzed hemoglobin (2.5%), acidified by addition of 0.25 volume of 0.3 N HCl, and 0.2 ml of enzyme (0.2 mg/ml). After 10 minutes at 37 C, the reaction was stopped by the addition of 5 ml of 4% TCA. The A_{280} was measured with a Beckman Spectrophotometer.

Trypsin and chymotrypsin were assayed according to the method of Walsh and Wilcox (1970), and Walsh (1970). The reaction mixture for trypsin contained 3 ml of 1.04 mM p-toluenesulfonyl-L-arginine methyl ester (TAME) dissolved in 40 mM Tris-HCl buffer, pH 8.1, containing 10 mM $CaCl_2$ and 0.05 ml of enzyme (50 μg/ml). The reaction mixture for chymotrypsin was exactly analogus to that for the assay of trypsin, except that N-benzoyl-L-tyrosine ethyl ester (BTEE) at a concentration of 1.04 mM was used as a substrate. The A_{247} of the two enzyme assays was determined in a Beckman Spectrophotometer.

The assay of papain was carried out according to the method of Arnon (1970). The reaction mixture contained 0.3 ml of 50 mM Tris-HCl buffer, pH 8, 0.2 ml of activating agent (0.05 M cysteine plus 0.02 M EDTA adjusted to pH 8), 1 ml of 1% casein and 0.5 ml of papain (0.05 mg/ml). Three ml of 5% TCA was used to terminate the reaction. The A_{280} was determined in a Perkin-Elmer Spectrophotometer.

DNase and 3'-nucleotidase were assayed according to the method by Hanson and Fairley (1969). The assay measures the conversion of DNA (denatured) to fragments which are soluble in a lanthanum nitrate-HCl reagent. The reaction mixture contained, in the order of mixing, 0.05 ml of 30 mM dithiothreitol, 1 ml of denatured DNA (10 minutes at 100 C, followed by quick cooling) at a concentration of 0.1 mg/ml, an aliquot of enzyme up to 0.1 ml and sodium acetate buffer (0.05 M, pH 5) to give total volume of 1.5 ml. The mixture was incubated at 37 C for 10 minutes and then placed on ice for 3 to 4 minutes. The next step was the addition of 1.5 ml of lanthanum nitrate-HCl reagent (0.02 M lanthanum nitrate in 0.2 M HCl). The mixture was stirred vigorously, allowed to stand on ice for 10 minutes, and centrifuged at 1100 X g for 20 minutes at 4 C. The A_{260} of the supernatant fluid was determined in a Perkin-Elmer Spectrophotometer. A unit of enzyme is defined as that amount which in 10 minutes catalyzes the formation of lanthanum-acid-soluble material with A_{260} of 0.01 per 1.5 ml of the incubation mixture. This assay was used in purification studies, and used for all experiments bearing on the properties of DNase.

The assay used for 3'-nucleotidase activity measures the release of Pi from 3'-AMP. The reaction mixture contained, in the order of mixing, 0.05 ml of 30 mM dithiothreitol, 1 ml of 2 mM 3'-AMP, an aliquot

of enzyme up to 0.1 ml, and sodium acetate buffer (0.05 M, pH 5) to give a total volume of 2.5 ml. The mixture was incubated at 37 C for 15 minutes and then placed in a screw-cap culture tube (1 X 10 cm) that contained 3 ml of 1 N H_2SO_4 and 0.4 ml of 8% ammonium molybdate. To each tube, 2 ml of xylene-isobutyl alcohol (65:35, v/v) was added. The tubes were shaken for 20 seconds, and centrifuged at 1100 X g for 10 minutes at 4 C. The A_{310} of the supernatant fluid was determined against a zero time blank in a Perkin-Elmer Spectrophotometer. A unit of 3'-nucleotidase is defined as that amount which catalyzes the release of Pi with A_{310} of 0.01 per 2.5 ml of incubation mixture in 15 minutes.

Spectrophotometeric assays for RNases were performed according to two methods by Hanson and Fairley (1969), and Gray (1974).

RNase Assay No. 1. This enzyme assay (Hanson and Fairley, 1969) was essentially the same as that described above for the DNase activity with the following exceptions. The reaction mixture contained 1 ml of yeast RNA at a concentration of 0.1 mg/ml, and the incubation was carried out at 37 C for 30 minutes. A unit of RNase is defined as that amount which in 30 minutes catalyzes the formation of lanthanum-acid-soluble material with A_{260} of 0.01 per 1.5 ml of the incubation mixture. For the inhibition study, components of the reaction mixture were doubled and 0.1 ml of properly diluted crude extracts was added: 0.1 ml of 30 mM dithiothreitol, 2 ml of 0.1 mg/ml RNA, 0.1 ml of enzyme, 0.1 ml of crude extract, and 0.05 M acetate buffer to give a total volume of 3 ml. After 30 minutes, the reaction was stopped by adding 3 ml of lanthanum nitrate-HCl reagent.

RNase Assay No. 2. The incubation mixture contained 0.5 ml of MacIlvaine's citrate-phosphate buffer (pH 3 to pH 7.6), 2 mg yeast RNA,

and enzyme in a total volume of 1.0 ml (Gray, 1974). After 15 minutes at 37 C, the reaction was stopped by the addition of 0.25 ml of 0.75% (w/v) uranyl acetate in 25% aqueous perchloric acid at 0 C. The precipitated protein and nucleic acid were removed by the centrifugation at 5000 X g for 10 minutes and A_{260} of the supernatant was measured after suitable dilution with water by using a Perkin-Elmer Spectrophotometer. A unit of the enzyme is defined as 0.01 reading at A_{260} per ml of enzyme used.

Protein Estimation

Protein concentrations were determined by the method of Lowry, et al. (1951), with bovine serum albumin as a reference standard. The determination was made at A_{500} in a Perkin-Elmer Spectrophotometer.

Culture of Wheat Plants and Water Stress Treatment

Wheat, Triticum aestivum L., cultivar KanKing, was used throughout this study. In a preliminary test, wheat seeds germinated at more than 95%. In order to harvest the same number of plants, equal amounts of seeds were used in both control and water stress treatments. Ten to forty grams (about 400 to 1,600 seeds) were at the first day usually planted on 8 layers of cheese cloth supported by a rack in a tray (50 cm X 30 cm X 6 cm) filled with water. After germination (4 days), the water was replaced with Hoagland's solution (half strength), and plants were grown for 6 days after supplying the nutrient solution. At the 11th day plants with cheese cloth supported by the rack were transferred to pots (19 cm X 28 cm X 5 cm) filled with vermiculite and

with holes in the bottom. The pots were transferred to a tray filled with nutrient solution. After 2 days (at the 13th day), one pot was removed from the watering tray to another without water and nutrient for 4 to 7 days to provide the water stress treatment. The control pot was maintained with a continuous supply of nutrient solution. After 4 to 7 days of water stress, the plant tops, including the crown portions, were removed and subjected to enzyme extraction. At the same time, the control plants were removed and subjected to the same analysis.

The relative water content (RWC) was used to estimate the internal severity of drought stress (Todd, et al., 1962) and determined as follows: a 2 cm segment of the first leaf was quickly weighed (fresh weight) followed by floating 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 the dry weight measurement. The relative water content was calculated as:

$$RWC = \frac{\text{fresh weight - dry weight}}{\text{turgid weight - dry weight}} \times 100 \quad .$$

RWC of control plants was about 95%. RWC of wheat leaves after 4 days of water stress was about 60% to 70%. After 6 days of water stress the RWC was about 30% to 50%. The treatment of water stress for 4 days usually caused wilting of the leaves, and water stress for 6 days resulted in severe wilting where even the root portion was dried up.

For calculating the protein content per gm dry weight and RNase activity per mg protein per gm dry weight, the dry weight was measured on a representative plant by drying the plant top, including the first leaf, the second leaf, the third leaf if any, and crown portion, at 80 C for 24 hours. The fresh weight of 2 plants from the control treatment was about 430 mg and the dry weight was about 50 mg.

Incubation of Wheat Plants with C^{14}-leucine

The L-leucine-C^{14}(U)(240 mCi/mM) with purity of 99.1% was purchased from New England Nuclear, Boston, Mass. For incubation with C^{14}-leucine, plants grown on cheese cloth supported by the rack were transferred from the nutrient solution to the 4 liters of nutrient solution containing 5.8×10^6 d.p.m. of C^{14}-leucine 5 days after germination. After 24 hours of incorporation under light, the plants were removed and the root portions were washed with distilled water. Then, the plants were carefully transferred to a pot as before and the nutrient solution was supplied for 2 days. A total radioactivity of 4×10^6 d.p.m. was left in the nutrient solution after transferring. Plants were subjected to water stress and harvested as previously described. Incorporation of radioactive label was measured by addition of plant extract samples into 15 ml of a scintillation mixture consisting of 240 g naphthalene, 15 g PPO, 696 ml of ethanol (95%), 1155 ml of xylene, and 1155 ml of dioxane to 3 liters. The counting was carried out in a Packard Tri-Carb 3000 series liquid scintillation spectrometer with the measured radioactivity being corrected for background and quenching. All results are expressed as disintegrations per minute (d.p.m.) per fraction.

Purification of Wheat RNase

Two methods of purification procedures were adopted in this study, according to Hanson and Fairly (1969), and Tuve and Anfinsen (1960). All operations were performed in a cold room at 4 C, unless otherwise indicated.

Purification Procedure No. 1

This procedure, according to Tuve and Anfinsen (1960), was used for most of the work in this study.

Step 1: Preparation of Homogenates. After collecting plants, they were frozen for 1 hour, and then the material was homogenized for 2 minutes in the cold room in a 1.5 liter Waring Blendor with about 100 ml of 0.1 M potassium phosphate buffer, pH 5.7. The homogenates were stirred for 2 hours at 4 C. Insoluble material was centrifuged down at 2500 RPM for 20 minutes and discarded. The supernatant was adjusted to pH 5.1 (cold) with 2 N HCl and allowed to stand overnight. The green precipitate which formed on standing was removed by centrifugation, as above, and discarded. The clear brown supernatant (Fraction I) (Table VI) was then readjusted in pH 5.1 when necessary.

Step 2: Fractionation with Ammonium Sulfate. To each 100 ml of homogenate, 14.4 g of ammonium sulfate were added. The precipitate which formed was collected by centrifugation and discarded. The enzyme was precipitated by the addition of an additional 37 g of ammonium sulfate per 100 ml of supernatant solution. The solution was stirred overnight and then centrifuged at 20,000 X g for 40 minutes. The precipitates were suspended in water (about 25 ml) and dialyzed for 24 hours against several changes of distilled water. It was then dialyzed, with stirring, for 1 hour against 2 liters of 0.01 M sodium phosphate buffer, pH 7. The insoluble precipitate which formed during dialysis was removed by centrifugation and discarded. The supernatant (Fraction II) was subjected to Cellex D column chromatography.

Step 3: Chromatography on Cellex D. About 30 ml of fraction II was allowed to pass through a Cellex D column, 2.2 X 30 cm, equilibrated to pH 7 with the 0.01 M buffer. After initial charge and brief wash with the 0.01 M buffer, the column was washed with 0.04 M sodium acetate buffer, pH 7, until the A_{280} of theeeffluent was about 0.05. The enzyme was eluted by the gradient technique, with a mixing chamber volume of 500 ml of 0.04 M buffer into which flowed 0.1 M sodium phosphate buffer, pH 7, containing 0.3 M NaCl. The enzyme was eluted in a sharp peak at approximately 0.09 M of NaCl. This fraction was condensed by a dialysis for 8 hours against 40% sucrose solution. The volume of the fraction was reduced by a factor of 10. This enzyme preparation (Fraction IV) was used for disc gel electrophoresis and gel filtration.

Chromatography on Sephadex G-75

Chromatography on Sephadex G-75 was done by the method of Tomoyeda, et al. (1969). A column, 2.2 X 25 cm, was packed with 10 g of sephadex G-75, medium grade, and equilibrated overnight with 0.05 M sodium acetate buffer (pH 5). The elution was performed with 0.1 M buffer at 50 cm of operating pressure and 10 ml fractions were collected.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to the method of Ornstein (1964) and Davis (1964). A column, 0.5 cm in diameter, composed of a 4-cm layer of 10% running gel and 1.5-cm layer of stocking gel, was routinely employed. A sample of enzyme (0.05 to 0.2 ml) containing 50 to 70 μg of protein was applied to the gel column. A potential of 340 volts and a current of 5 mamp were applied for a period of 2 hours at 4 C. The pH 4.3 buffer system was acetate-alanine

buffer and acetate-KOH buffer. The protein bands were located by staining the gel with Coomassie blue by the method of Chrambach, et al. (1967). For the detection of RNase, the gel was cut into 2.5 mm segments. Each segment was placed in 1 ml of Tris-HCl buffer, pH 8, and allowed to stand overnight at 4 C.

Purification Procedure No. 2

Another purification procedure was attempted according to the method by Hanson and Fairley (1969). In this method, fresh plant material was homogenized with 100 ml of sodium acetate buffer, 0.05 M, pH 4.5, for 1 minute in a Waring Blendor. The homogenate was squeezed through cheese cloth to remove the bulk of the insoluble material. The filtrate was centrifuged at 5,800 X g for 10 minutes at 4 C and the clear supernatant fluid was decanted. The freshly prepared crude extract was brought to 57% of saturation by addition of ammonium sulfate. The solution was stirred for 30 minutes and then was centrifuged at 15,000 X g for 20 minutes at 4 C. The precipitate was discarded and the supernatant solution was adjusted to 75% of saturation with ammonium sulfate. The suspension was stirred for 30 minutes and centrifuged at 15,000 X g for 30 minutes at 4 C. The precipitate was dissolved in about 25 ml of sodium acetate buffer, 0.05 M, pH 4.5.

To 100 ml of the ammonium sulfate fraction were added 150 ml of 95% ethanol at 4 C. The ethanol was added from a separatory funnel over a period of about 15 minutes into the vortex of the stirred solution. The suspension was stirred for another 10 minutes and centrifuged at 15,000 X g for 20 minutes. The precipitate was discarded and 60 ml of 95% ethanol were added as above to the clear

supernatant solution. After stirring for 10 minutes the solution was centrifuged at 15,000 X g for 30 minutes at 4 C. The excess ethanol was removed from the precipate with nitrogen gas until the aroma could no longer be detected.

The precipitate was dissolved in 30 ml of 0.05 M sodium acetate buffer, pH 4.5, containing 1 mM zinc acetate and 2 mM cysteine. The ethanol fraction was transferred to a 75 C water bath and maintained at this temperature for 10 minutes with shaking. The material was then chilled in ice water until the temperature decreased to below 10 C. The solution was centrifuged at 25,000 X g for 45 minutes at 4 C. The supernatant was subjected to phosphocellulose chromatography.

A column, 2.2 X 25 cm, was packed with 6.5 g of phosphocellulose resin, which had been equilibrated with 500 ml of 0.05 M sodium acetate buffer, pH 4.5, with 1 mM zinc acetate and 2 mM cysteine. The column was extensively washed with this buffer and the fraction from the heat step was applied. The column was washed with 200 ml of the same buffer, followed by 200 ml of the buffer containing 0.1 M NaCl. This was followed by a linear salt gradient from 0.1 to 0.3 M NaCl in the same buffer, the total volume of the gradient being 1200 ml. The fraction of 20 ml were collected at a flow rate of 0.5 ml/minute.

Preparation of Soluble and Particulate RNases

This preparation was obtained by the method of Gray (1974). Plant material was homogenized in 100 ml of 0.08 M phosphate buffer, pH 7.0, containing 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol in a Waring Blendor for 1 minute at 4 C. The homogenate was squeezed through cheese cloth and centrifuged at 105,000 X g for 2 hours. The supernatant was used as

the source of the soluble enzyme preparation whereas the particulate enzyme preparation was obtained from the green pellet. All operations were carried out at 4 C.

Soluble Enzyme Preparation

The supernatant was adjusted to pH 4.5 with 2 M acetic acid and the resulting precipitate was removed by centrifugation at 500 X g for 20 minutes. The supernatant was readjusted to pH 5.2 with 2 M NaOH. This was used as the soluble enzyme preparation.

Particulate Enzyme Preparation

The green pellets were suspended in 0.08 M phosphate buffer, pH 7, containing 5 mM $MgCl_2$ and 5 mM mercaptoethanol, and 5% (v/v) Triton-X-100 to solubilize chloroplast membranes. The suspension was centrifuged at 1000 X g for 10 minutes to remove large particulate matter and then at 105,000 X g for 2 hours to pellet particulate fraction. The pale-green pellets were suspended in 0.1 M Tris-HCl buffer, pH 7.5, and dialyzed overnight against the same buffer to give the particulate preparation.

Drying of Enzymes

The enzymes were purchased from commercial sources such as Cal Biochem, Sigma, and Worthington companies and were highly purified (more than 95% purity). Desiccation of enzymes was performed at 25 C, 4 C, and -4 C. Calcium chloride was used as the desiccant at 25 C and 4 C and calcium sulfate was used at -4 C, because calcium chloride did not effectively remove water portion after ice crystal formation.

Drying of 1 ml of enzyme solution required one day at 25 C, about 3 days at 4 C, and 7 days at -4 C.

After measuring the initial activity of the enzyme, 1 ml of diluted enzyme was spread on the bottom surface of each of six 100-ml beakers (4.5 cm in diameter). The beakers were then placed into 3 desiccators which had been kept at 25 C, 4 C, and -4 C for several hours. Desiccators, each containing 2 beakers, were kept at 25 C, 4 C, and -4 C for 7 days. Another portion of enzyme solution was kept for 7 days at 4 C without desiccation. After 7 days of desiccation, the beakers were taken out and 1 ml of water was added to each beaker, dissolving the dried enzyme. The initial activity of the enzyme from 3 treatments was determined. Three enzyme assays were performed with the enzyme from each beaker. Two mean values from two beakers were averaged, and the treatment at different temperatures was performed at least two times. The values were compared with the value of the standard enzyme assay.

CHAPTER IV

RESULTS

The Effect of Water Stress on Wheat RNase Activity

Previous work (Yi, 1972) showed that RNase activity per leaf and per plant increased during water stress treatment with wheat plants. It is necessary to keep the ratio of material to buffer solution constant in control and water stress treatments, in order to determine the amount of RNase activity increased by water stress. It was attempted in this study, therefore, to use equal amounts or numbers of wheat plants from both treatments for the enzyme extraction. The same weight of wheat seeds were planted and grown in control and water stress treatments of a single experiment, even though different amounts of seeds were planted in various experiments.

The effect of water stress on RNase activity was at first investigated by using crude extracts. The enzyme was extracted by purification procedure No. 2 and RNase activity was measured by spectrometric assay No. 1. Changes in RNase activity were followed during water stress induced by withholding water (Table II). The potential for RNA hydrolysis was enhanced after water stress. Generally, the total RNase activity reached a peak after 4 days of water stress with a slight decline thereafter. The total protein content decreased more after

TABLE II

RNASE ACTIVITY OF WHEAT SEEDLINGS FOLLOWING WATER STRESS

Treatment	RWC %	Fresh Weight g	Dry Weight g	Total Protein mg	Total RNase units	Specific Activity*
Experiment No. 1						
Control	96	57.1	8.5	238	5060	21
4 days water stress	67	31.5	6.0	206	5600	27
Control	95	54.0	8.9	253	5060	20
6 days water stress	39	19.5	5.9	194	5290	27
Experiment No. 2						
Control	92	57	7.8	206	3744	18
4 days water stress	72	43.3	7.6	175	4576	26
Control	91	68.4	8.8	270	3870	14
6 days water stress	43	42.5	4.1	128	2870	22

*Specific activity is RNase activity in units per mg protein.

RNase was extracted by Purification Procedure No. 2 and measured by Spectrometric assay No. 1.

6 days of water stress, resulting in a higher value of the enzyme activity per mg protein. In the control treatment, the total RNase activity was at the same level as the harvest 2 days earlier, although the protein content increased slightly.

The increase in RNA-hydrolyzing capacity of the crude preparations from wheat plants could be due to the activity of phosphomonoesterase, phosphodiesterase and RNase either singly or in combination. RNase can be separated from phosphomonoesterase and phosphodiesterase by gel filtration using Sephadex G-100 (Pitt and Galpin, 1971). When partially purified through phosphocellulose chromatography (Hanson and Fairley, 1969), RNase could be separated from phosphomonoesterase, phosphodiesterase and 5'-nucleotidase. After DEAE-cellulose chromatography, spinach RNase was separated from phosphomonoesterase (Tuve and Anfinsen, 1960). Thus, the present work sought to eliminate the contributions by other enzymes to the total phosphoester hydrolyzing activity in the control and water stress treatments. It is also possible that RNases from control plants and water stressed plants behave differently during enzyme purification. To obtain evidence for such an effect of water stress, parallel purification of the enzyme was attempted from the two sources.

A purification procedure of RNase through the chromatography of phosphocellulose was performed according to Hanson and Fairley (1969), (Purification Procedure No. 2). In this experiment, the sensitive fluorometric method of the enzyme assay was used for the determination of the activity, since very low enzyme activity could be expected in chromatographic fractions. Sodium acetate buffer, pH 5 (0.05 M), was

used for the enzyme assay, replacing the succinate buffer for the assay of pancreatic RNase (See Materials and Methods).

As shown in Table III, higher specific activity (defined here as Kunitz unit per mg protein) was maintained throughout the purification procedure. It seems that RNases from control and water-stressed plants have similar properties of solubility in ethanol and heat stability. The activity of RNases from control and water-stressed plants following chromatography on phosphocellulose showed a similar pattern of RNase distribution (Figures 1 and 2). Peaks of RNase activity appeared at about 0.2 M NaCl in the linear salt gradient in both control and water stress treatments. This agrees with the previously published value for wheat plants (Hanson and Fairley, 1969).

Phosphomonoesterase and phosphodiesterase did not increase following mechanical damage to potato slices, while RNase increased more than 200% (Pitt and Galpin, 1971), when enzyme activities were measured after Sephadex G-100 chromatography. If these two enzymes (phosphoesterases) did not increase during water stress in wheat plants and if purification procedure (Table III) could separate RNase from other enzymes in this study, the total activity of RNase after phosphocellulose chromatography would be, then, the true RNase activity, which increased about 8% by water stress treatment. In crude extract (Table III), the total RNase activity increased less than 2% by the water stress treatment. This may be due to the presence of other interfering enzymes such as phosphoesterases. The higher increase after ion exchange chromatography also is shown in another purification method (see Tables IV and V). Table III also shows that a definite ratio of specific activities of control and water stress treatments are not

TABLE III

PARALLEL PURIFICATION OF RNASES FROM CONTROL AND WATER-STRESSED WHEAT SEEDLINGS

	CONTROL fresh weight = 78.56 g dry weight = 9.8 g RWC = 97%			WATER STRESS fresh weight = 38.24 g dry weight = 7.3 g RWC = 67%		
Purification Step	Total Protein mg	Total RNase units	Specific** Activity	Total Protein mg	Total RNase units	Specific** Activity
Crude extract	193	2277	12	180	2310	13
Ammonium sulfate*	25	479	20	25	627	25
Ethanol	5	1144	207	4	847	210
75 C treatment	0.75	331	441	0.75	362	483
Phosphocellulose	-	170	-	-	184	-

Enzyme activity are Kunitz units, and 23/min of Turner Fluorometer reading corresponded to 0.03 ml containing 14.2 units of pancreatic RNase per ml in water.

The enzyme was extracted by purification procedure No. 2.

*The values for this step of the purification are thought to be low, and vary among different preparations, because of the presence of ammonium sulfate which is inhibitory to the enzyme activity.

**Specific activities are Kunitz units per mg protein.

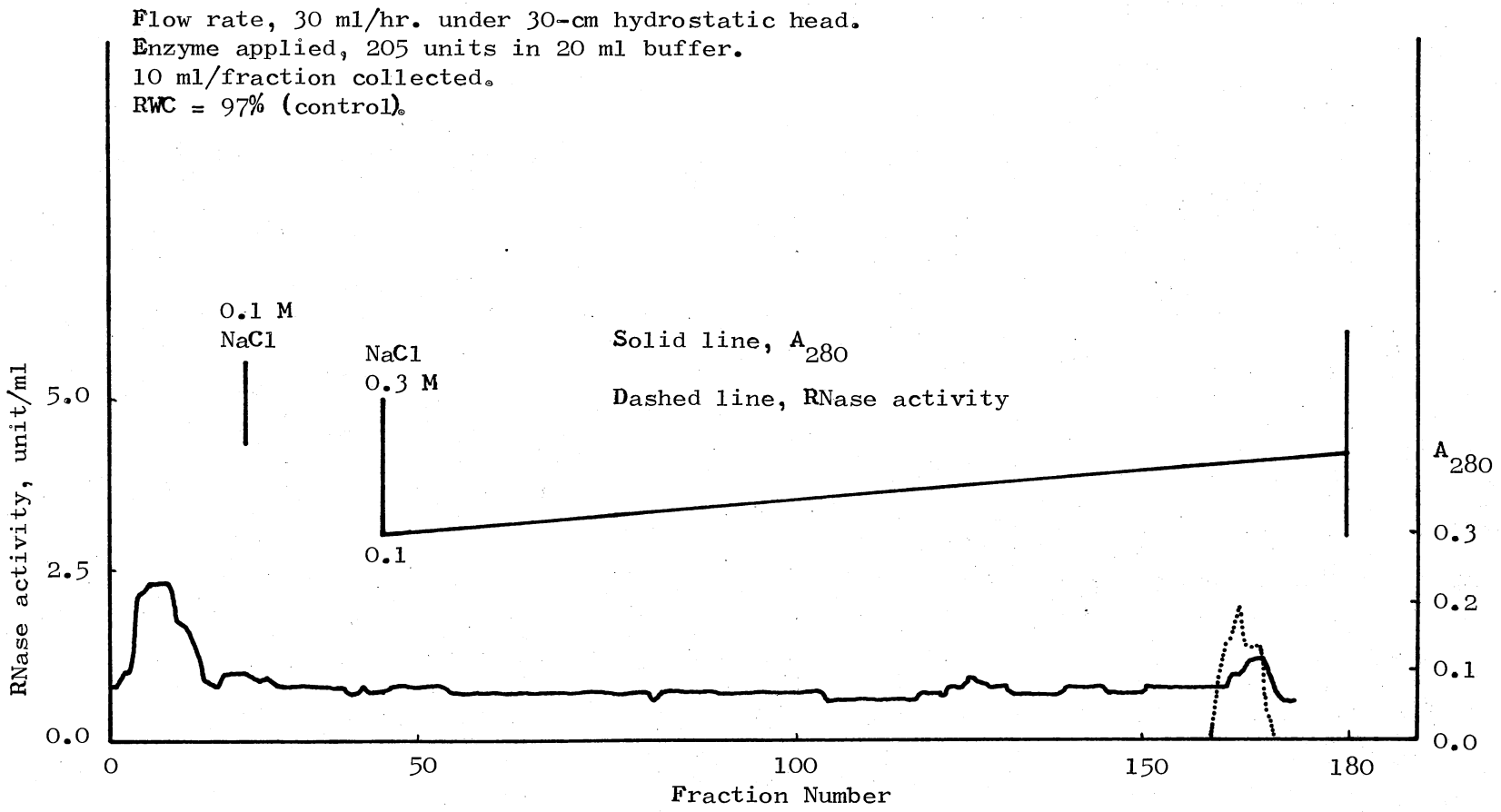

Figure 1. Chromatography of Wheat Plant RNase on Phosphocellulose

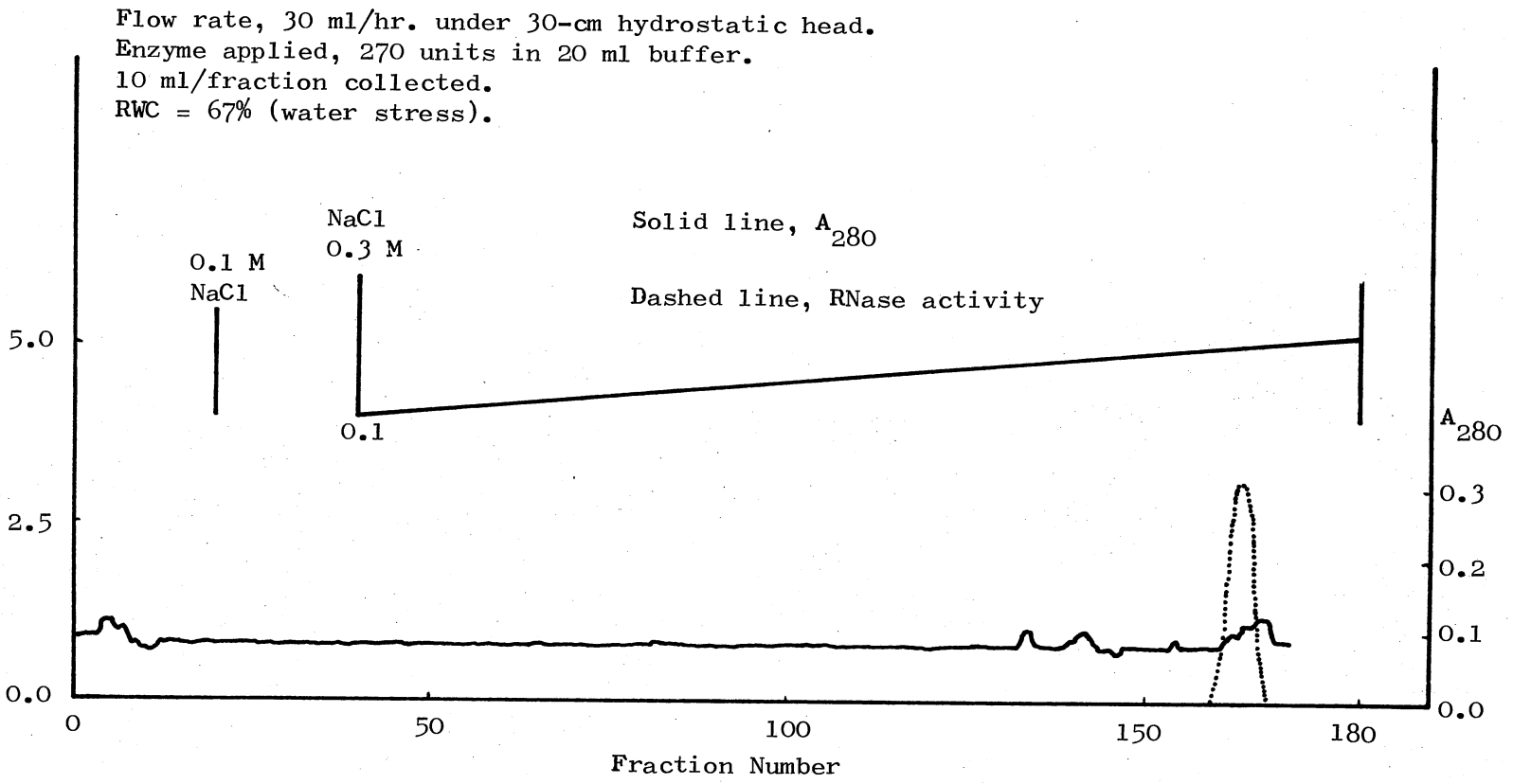

Figure 2. Chromatography of Wheat Plant RNase on Phosphocellulose

maintained at a constant level throughout the purification. In this purification method (Table III), half of the original RNase activity of the homogenate was found in the fraction precipitated by the 57% saturation of ammonium sulfate. The RNase recovered following phosphocellulose chromatography was low and was not sufficient to use for further purification.

The increase in RNase activity could be due to the appearance of different kinds of RNases (isoenzymes). Disc gel electrophoresis and gel filtration were utilized to examine this possibility. Partial purification of RNases was necessary before running disc gel electrophoresis and gel filtration. The purification procedure No. 1 was used, and RNase activity was determined by spectrophotometric assay No. 1. During the purification, dialysis sacks, used after ammonium sulfate fractionation, were greatly weakened after 24 hours of exposure to the extract, which suggests the presence of a cellulase. No RNase activity was found in the water during dialysis. The yield of the enzyme was better than that of the procedure No. 2, and more than 10% of the homogenate RNase was recovered after Cellex D chromatography. The distribution of RNase activity following chromatography on Cellex D is shown in Figures 3 and 4, control and water stress treatment, respectively. Some RNase activity was found early in the elution with 0.04 M sodium acetate buffer, pH 7.0. It appeared very irregularly, not showing in other experiments (Figures 5 and 6). This kind of RNase distribution was also reported for spinach RNase (Tuve and Anfinsen, 1960). The optimum pH of this enzyme was about pH 5.2, the same as the one major peak RNase, and it might be the same kind of RNase protein which appeared in the major peak on the elution with the salt. The RNase

TABLE IV

PARALLEL PURIFICATION OF RNASES FROM CONTROL AND WATER STRESS TREATMENTS

			CONTROL fresh weight = 140.2 g dry weight = 14.91 g RWC = 97%			WATER STRESS fresh weight = 107.7 g dry weight = 11.99 g RWC = 81%		
Step		Fraction	Total Protein mg	Total RNase units	Specific Activity	Total Protein mg	Total RNase units	Specific Activity
1	I.	Initial homogenate	380	8300	18	396	11410	29
2	II.	Saturated ammonium sulfate precipitate	97	5600	58	87	7350	84
3	III.	Combined peak tube from Cellex-D column	-	2335	-	-	3600	-
4	IV.	Peak tubes, after dialysis	4	1465	366	4	2439	610

Specific activity is RNase activity in units per mg protein.

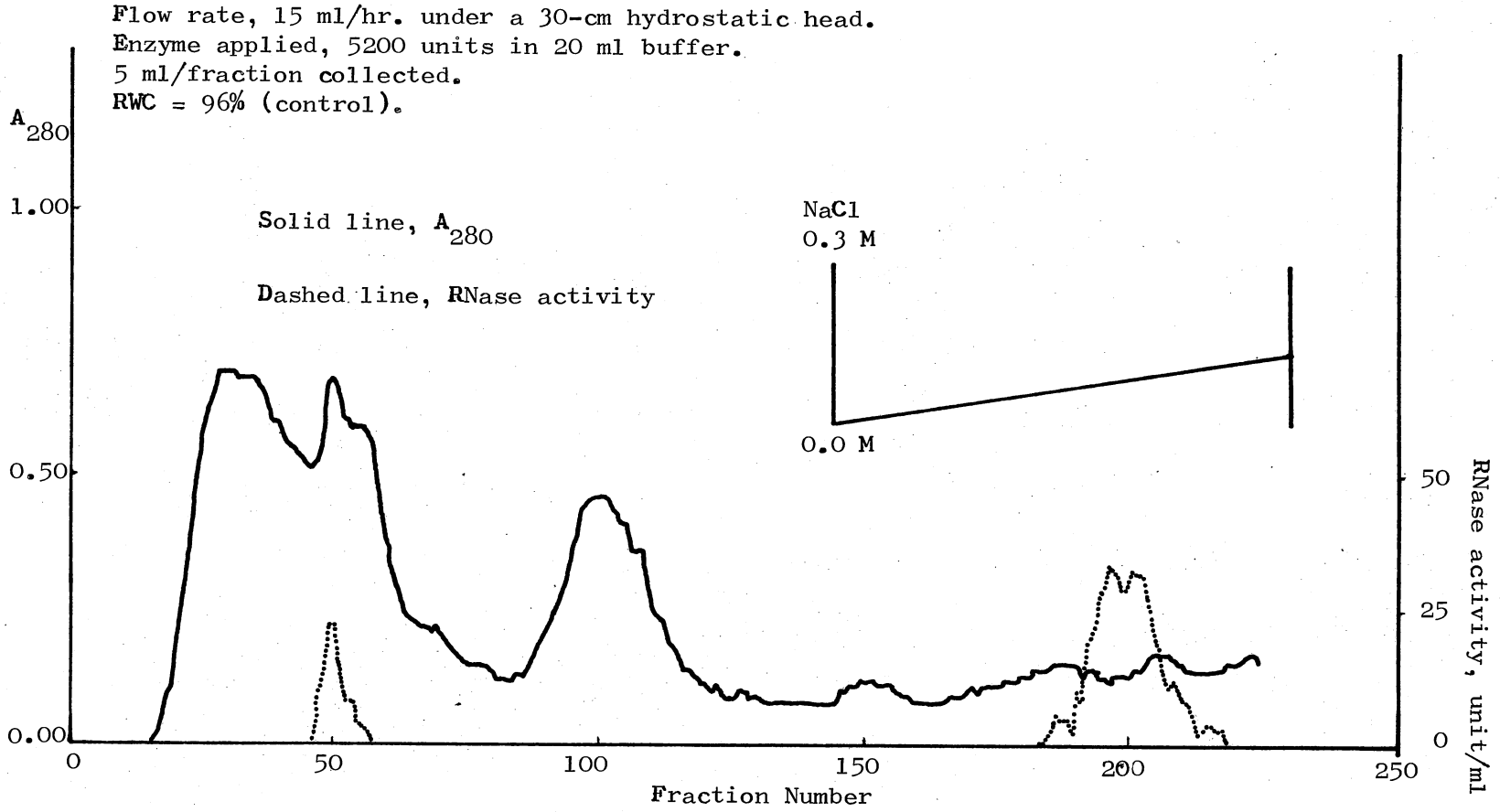

Figure 3. Chromatography of Wheat RNase on Cellex-D

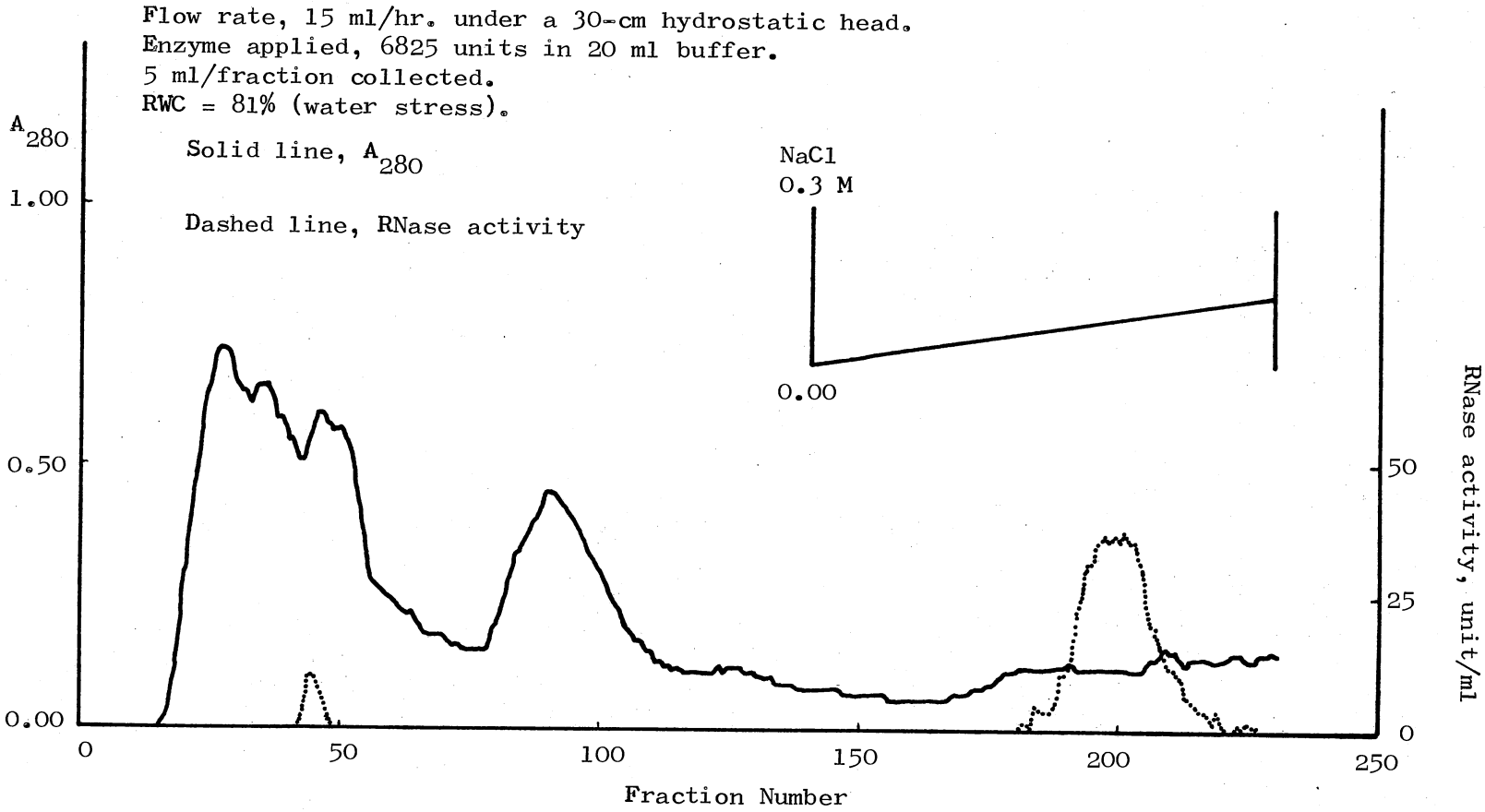

Figure 4. Chromatography of Wheat RNase on Cellex-D

appearing earlier in the elution was not used for further purification. As shown in Figures 3 and 4, the RNase from the water stress treatment was eluted in the same volume at approximately 0.08 M of NaCl as the RNase from the control treatment.

The enzyme was extracted from the severe water stress treatment (39% of relative water content), and eluted by Cellex D chromatography. The elution pattern of the protein and of the enzymatic activity were the same as in the mild water stress (Figure 4). The pattern of RNase elution was not changed by the degree of water stress, and the RNase activity was eluted by about 0.08 M NaCl.

The eluate from Cellex D chromatography was lyophilized, but most of the enzyme was inactivated during freeze-drying. The concentration method with a rotary evaporator, described by Hanson and Fairley (1969), did not work. The pooled material from Cellex D chromatography was then dialysed against distilled water and then 40% commercial sucrose solution (about 4 liters) for 8 hours. The prolonged period of dialysis (more than 8 hours) resulted in a considerable loss of enzyme activity. The volume of the fraction was reduced by a factor of 10. This concentrated fraction was used in disc gel electrophoresis and gel filtration.

In this purification procedure, the specific activity of RNase (defined as RNase activity in units per mg protein) from the water stress treatment was higher than that from the control treatment and the difference persisted to the final purification stage (Table IV). This is the same result as the experiment reported in Table III. After the final step of the purification (Table IV) the total RNase activity was higher in control treatment than that in water stress treatment (more than 50%). A constant ratio of the specific activities of about

1.6 between two treatments was maintained throughout the purification procedure, and purification and yield were practically identical at all stages. However, in further experiments (Tables V and VI) constant ratios of specific activities were not noted as they were shown to be ranging between 1.1 to 1.5. If a constant ratio appeared throughout experiments, it would indicate an evidence of de novo synthesis, as proved by the induced RNase synthesis of cancer cells (Schlaeger and Hiltz, 1969).

The activities of DNase were measured at every step of the purification procedure (Table V). The purification procedure is the same as shown in Table IV. DNase activity in the initial crude extract of the control plants was only about 25% as great as total RNase activity. DNase activity increased during water stress, and specific activity (DNase activity in units per mg protein) was higher in water-stressed plants than that in control plants. The condensed eluate of Cellex D chromatrography with RNase activity (see Figures 3 and 4) had DNase activity (Table V). The ratio of two enzymes was about 10 to 1. This same peak of Cellex D chromatography also had 3-nucleotidase activity: 114 units in water stressed plants and 86 units in control plants. Therefore, it is possible that the peak with high RNase activity on the elution pattern has DNase activity and 3'-nucleotidase activity. DNase and 3-nucleotidase were separated on Sephadex G-75 (Figures 8 and 9).

Further separation using polyacrylamide disc gel electrophoresis was attempted. A portion containing about 70 μg of protein was examined by electrophoresis with a 10% polyacrylamide gel at pH 4.3, as described in Materials and Methods. Figure 5 shows the staining pattern of the protein and the distribution of RNase activity within the gel. The band

TABLE V

PARALLEL PURIFICATION OF DNASE FROM CONTROL AND WATER STRESS TREATMENTS

	CONTROL fresh weight = 140.3 g dry weight = 15.43 g RWC = 94%			WATER STRESS fresh weight = 81.9 g dry weight = 14.64 g RWC = 47%		
Fraction	Total Protein mg	Total RNase units	Total DNase units	Total Protein mg	Total RNase units	Total DNase units
Initial homogenate	420	9600	2400	413	11900	3400
Ammonium sulfate	105	6600	1100	101	7000	1120
Cellex-D	4.25	2342	52	3.90	3600	101

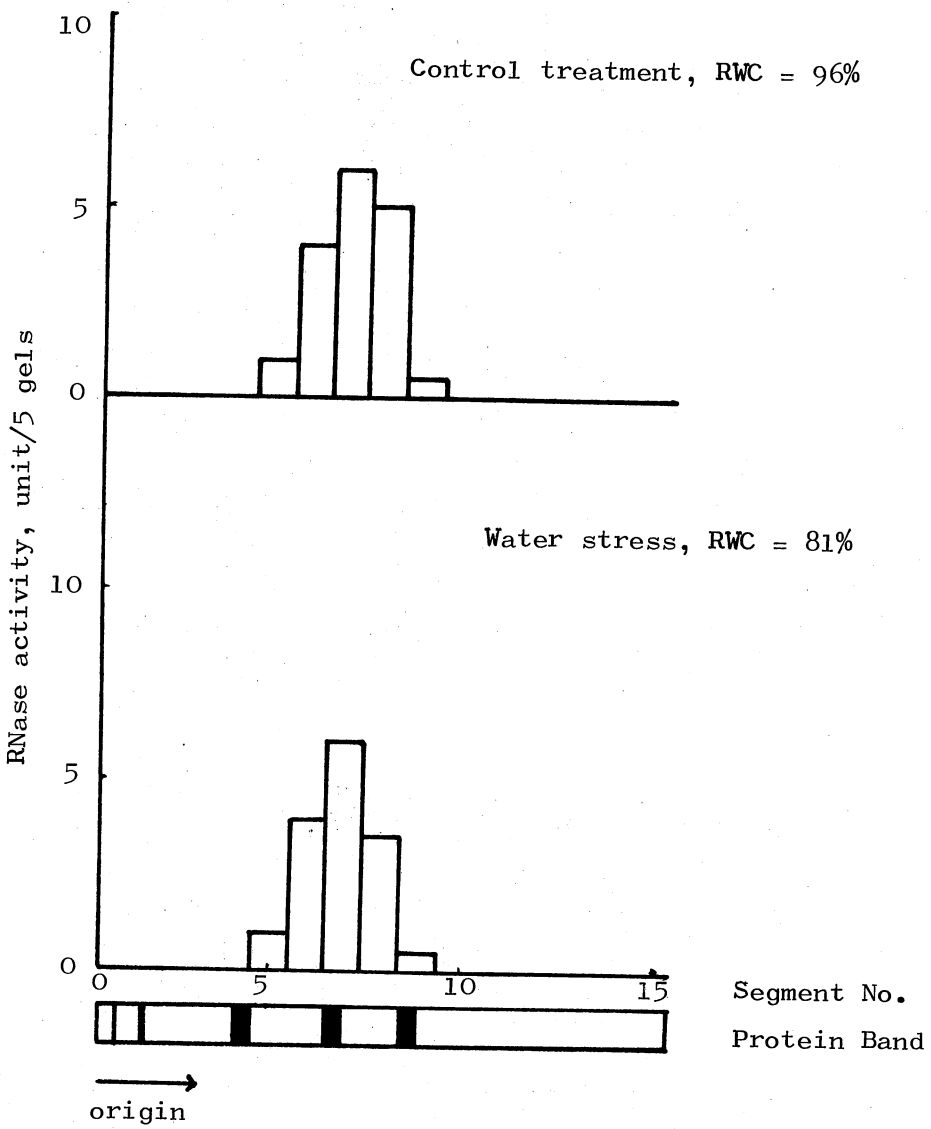

Figure 5. Staining Pattern of Protein and Distribution of Wheat Seedling RNase Activity Within a Polyacrylamide Gel Electrophoresis

distribution and RNase distribution were essentially identical in both control and water stress treatments. A similar result was obtained with 7.5% polyacrylamide gel on the electrophoresis. Lower current of 2 ma resulted in unsatisfactory separation of wheat proteins, in which the RNase band appeared close to the boundary of the stocking and running gels. Pancreatic RNase moved faster than wheat RNase during electrophoresis at pH 4.3 and 10% gel and 2 ma: pancreatic RNase appeared on segment No. 10 and wheat RNase on segment No. 1 of the gel such as shown in Figure 5. Electrophoresis at pH 9.5 was not successful, because the pancreatic RNase used as a marker could not enter the running gel.

Partially purified RNase through Cellex D chromatography was obtained from severe water stress treatment (RWC = 47%), and run on electrophoresis under the same conditions as shown in Figure 5 (at pH 4.3 and 5 ma with 10% polyacrylamide gel). The same pattern of RNase distribution was found. This indicates that RNases with different properties or differences in charge were not produced by the water stress treatment.

To obtain additional evidence on the origin of the increase in RNase activity during water stress, plants were incubated with C^{14}-leucine, as described in Materials and Methods. The plant material was subsequently subjected to enzyme extraction which is essentially that in Table IV. Steps of purification and radioactivity determination in each fraction are given in Table VI. Radioactivities in initial crude extract were 28130 d.p.m. in the control treatment and 44696 d.p.m. in the water stress treatment. The higher radioactivity of homogenate from the water stress treatment does not necessarily mean that this

TABLE VI

C^{14}-LEUCINE INCORPORATION INTO RNASE

	CONTROL fresh weight = 122.1 g dry weight = 14.5 g RWC = 97%			WATER STRESS fresh weight = 87.3 g dry weight = 14.0 g RWC = 53%		
Fraction	Total Protein mg	Total RNase units	Total dpm	Total Protein mg	Total RNase units	Total dpm
Initial homogenate	384	8700	28130	372	10570	44696
Ammonium sulfate	81	5000	2200	81	7000	2100
Cellex-D	7	3535	344*	5	3465	107*

*Radioactivity found in combined peak tubes of RNase on Cellex-D chromatography.

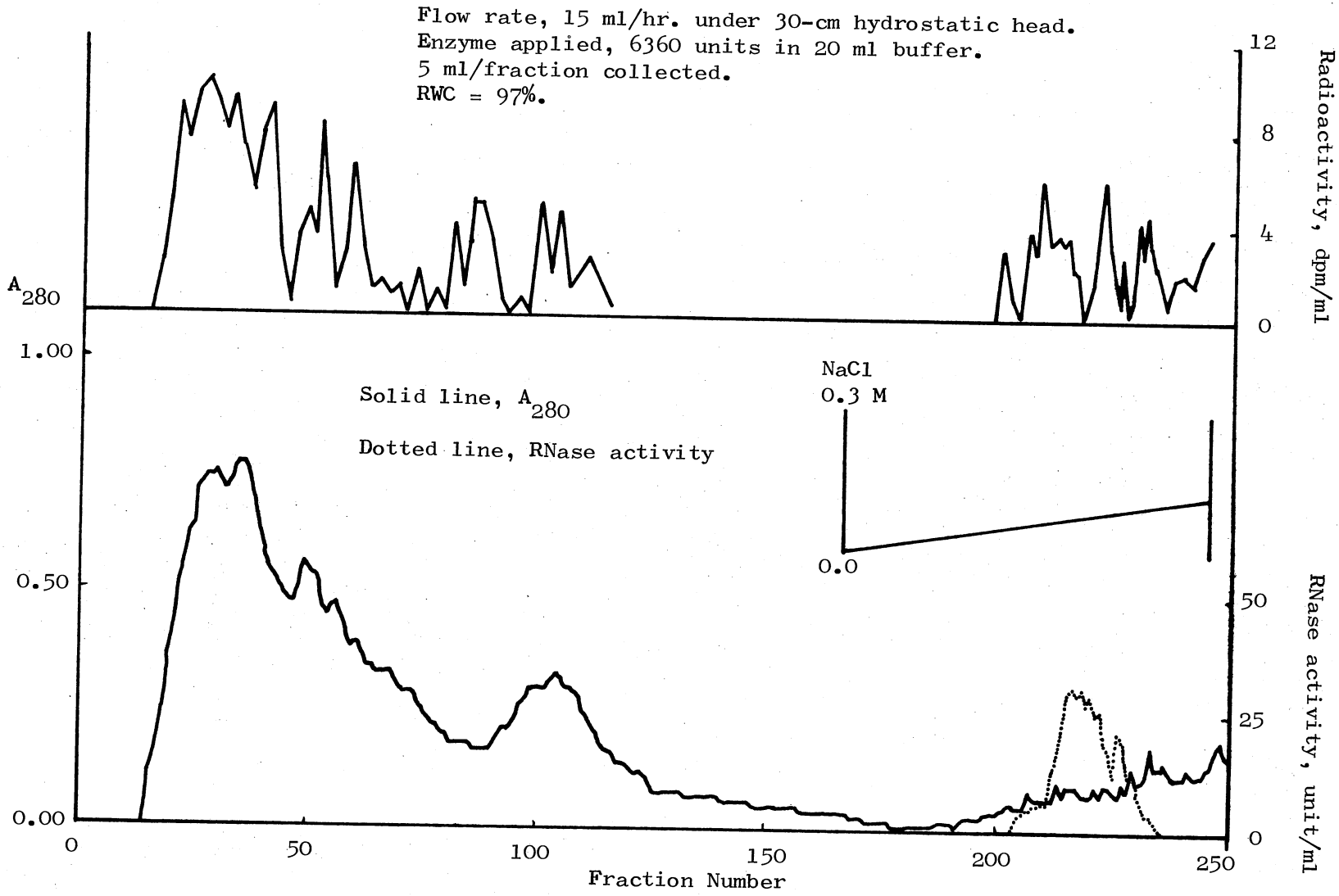

Figure 6. Chromatography of Wheat RNase on Cellex-D

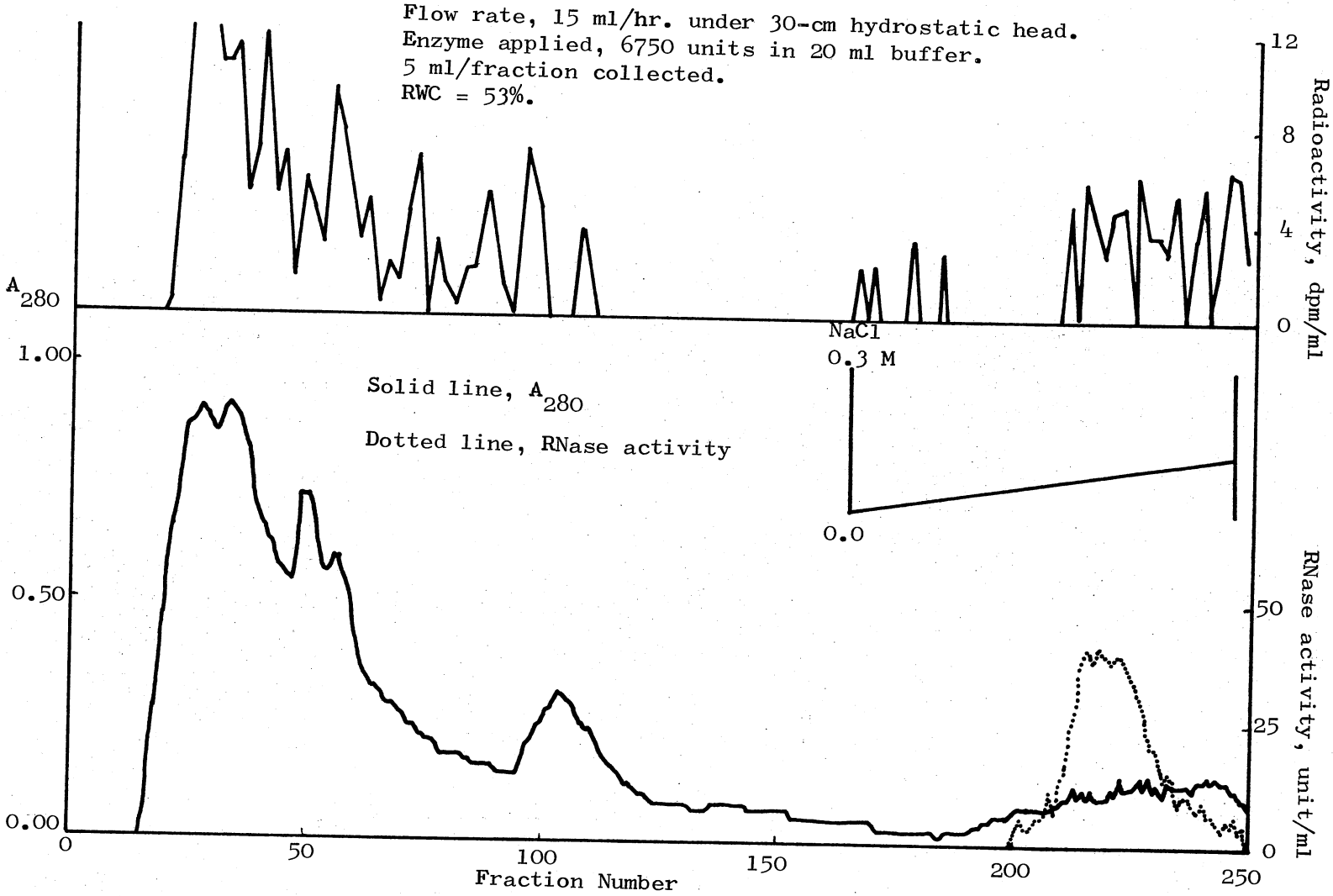

Figure 7. Chromatography of Wheat RNase on Cellex-D

treatment resulted in higher incorporation. Rather, ammonium sulfate fraction revealed slightly higher radioactivity level in the control treatment. During Cellex D chromatography, the column was extensively washed with 0.04 M buffer, until the A_{280} of the effluent was less than 0.5.

The elution pattern of RNase with radioactivity measurement is shown in Figures 6 and 7. The enzyme was eluted in a sharp peak at approximately 0.08 M of NaCl, and the peak had measurable radioactivity of C^{14}. It seems that the incorporation rate of the water stress treatment is the same as the rate of the control treatment. The most active fractions (about 200 ml) were pooled and concentrated by a dialysis against 40% sucrose solution. The volume of the fraction was reduced to 30 ml. When the radioactivity of this concentrated Cellex D eluate was measured, it was 1.41 d.p.m./0.5 ml of sample in the water stress treatment, and 4.05 d.p.m./0.5 ml of sample from the control treatment. The standard deviation is ± 5 d.p.m. Thus, incorporation rates are not significantly different between the two treatments.

The concentrated enzyme was eluted on Sephadex G-75 chromatography for further purification (Figures 8 and 9). Using blue dextran (molecular weight = 2×10^6) which was assumed to move in the void volume, the elution volume of the given substance was determined. The RNase from the water stress treatment as well as the RNase from the control treatment was eluted at about 100 ml of the elution volume. It is likely that the molecular weight of RNases from both treatments are similar. The activities of DNase and 3'-nucleotidase were not detected on the peak of RNase following gel filtration.

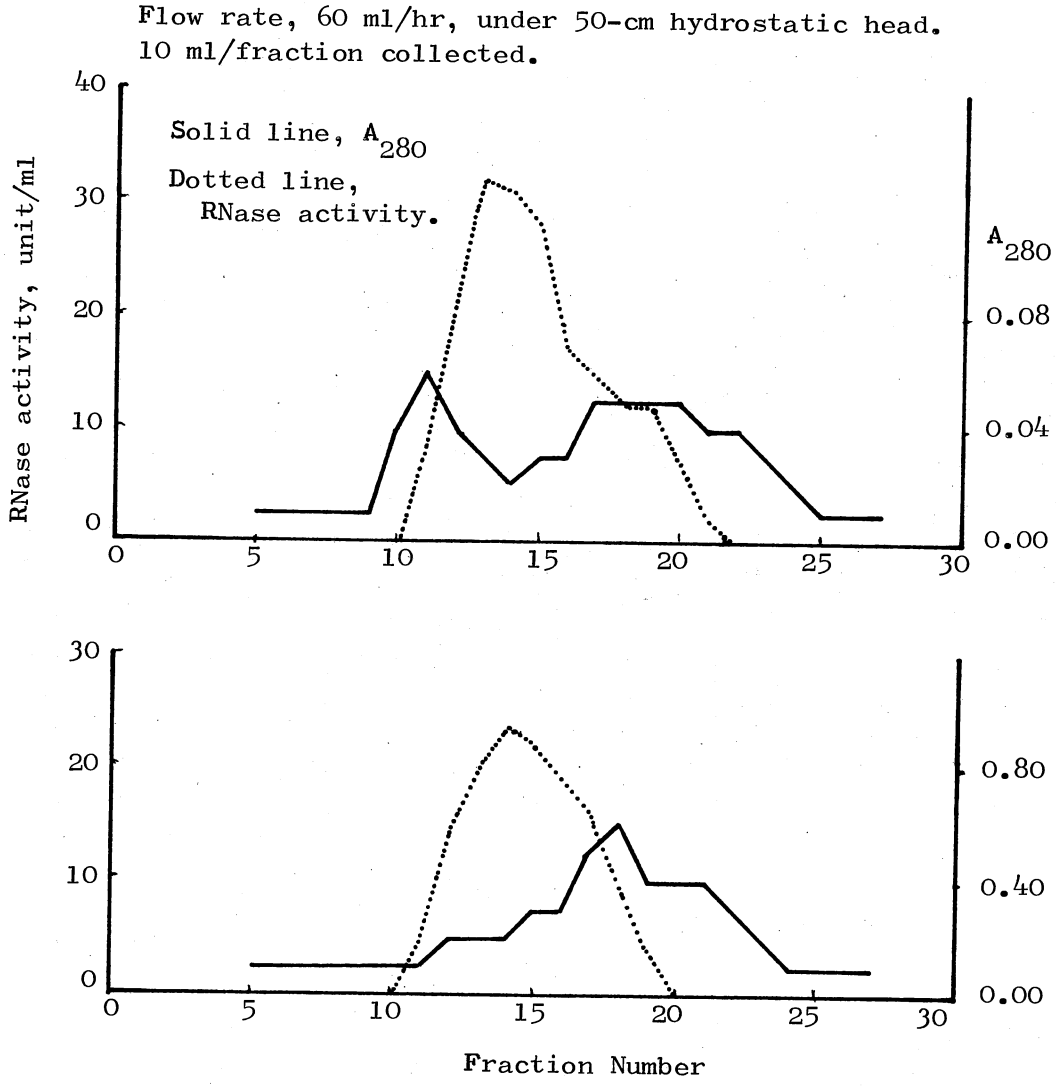

Figure 8.
Control treatment.
RWC = 96%.
800 units applied.

Figure 9.
Water stress.
RWC = 53%.
800 units applied.

Figures 8 and 9. Purification of Wheat RNase by Gel Filtration on Sephadex G-75

Five ml of each fraction of gel filtration were freeze dried, and 15 ml of scintillation counting mixture were added to determine the radioactivity. Radioactivity was found in fractions Nos. 8, 9, and 10, but was not found on the peak of RNase, fractions Nos. 12, 13, 14, 15, 16, 17, and 18. The radioactivity found on the peak of RNase on the chromatography of Cellex D (Figures 5 and 6) was presumably caused by the contamination with other labeled protein, and this contamination was probably removed by gel filtration.

Plants contain a number of RNases which degrade RNA molecules. To study the effect of water stress on the individual RNases, both soluble and particulate fractions were examined. The mixture of RNases could be separated by differential centrifugation at 105,000 X g for 2 hours (Gray, 1974; Kessler, et al., 1962; and Wilson, 1968-a). The fractionation, enzyme preparation, and enzyme assay (spectrophotometric assay No. 2) are described in the Materials and Methods.

As shown in Table VII, more than 70% of the total RNase activity was present in the soluble fraction of control plants. The water stress treatment (relative water content = 73%) increased RNase activity by 10% in the soluble fraction and 13% in the particulate fraction. Protein content in the particulate fraction decreased more than that in the soluble fraction during water stress, resulting in increased specific activity. Particulate and soluble RNases were also extracted from wheat plants with 64% of relative water content. The result (Table VII) was essentially identical with regard to RNase distribution and effect of water stress on enzyme distribution.

The effect of pH on particulate and soluble RNases are shown in Figures 10 and 11. The soluble RNase of control plants has optimum

TABLE VII

EFFECT OF WATER STRESS ON SOLUBLE AND PARTICULATE RNASES

EXPERIMENT NO. 1

	CONTROL			WATER STRESS		
	fresh weight = 73.7 g dry weight = 9.53 g RWC = 91%			fresh weight = 57.7 g dry weight = 7.0 g RWC = 73%		
Fraction	Total RNase units	Total Protein mg	Specific Activity	Total RNase units	Total Protein mg	Specific Activity
Soluble	2814	56	50	3105	53	58
Particulate	536	17	31	607	14	43

EXPERIMENT NO. 2

	CONTROL			WATER STRESS		
	fresh weight = 105 g dry weight = 15.0 g RWC = 93%			fresh weight = 64.1 g dry weight = 12.4 g RWC = 64%		
Fraction	Total RNase units	Total Protein mg	Specific Activity	Total RNase units	Total Protein mg	Specific Activity
Soluble	2015	37	68	3214	36	90
Particulate	509	21	24	608	18	35

Specific activity is RNase activity in units per mg protein.

Soluble RNase was determined at pH 5.2 and particulate RNase measured at pH 6.2.

RNase activity was measured by Spectrophometeric assay No. 2.

RWC = 91% in control plants and 73% in drought plants.

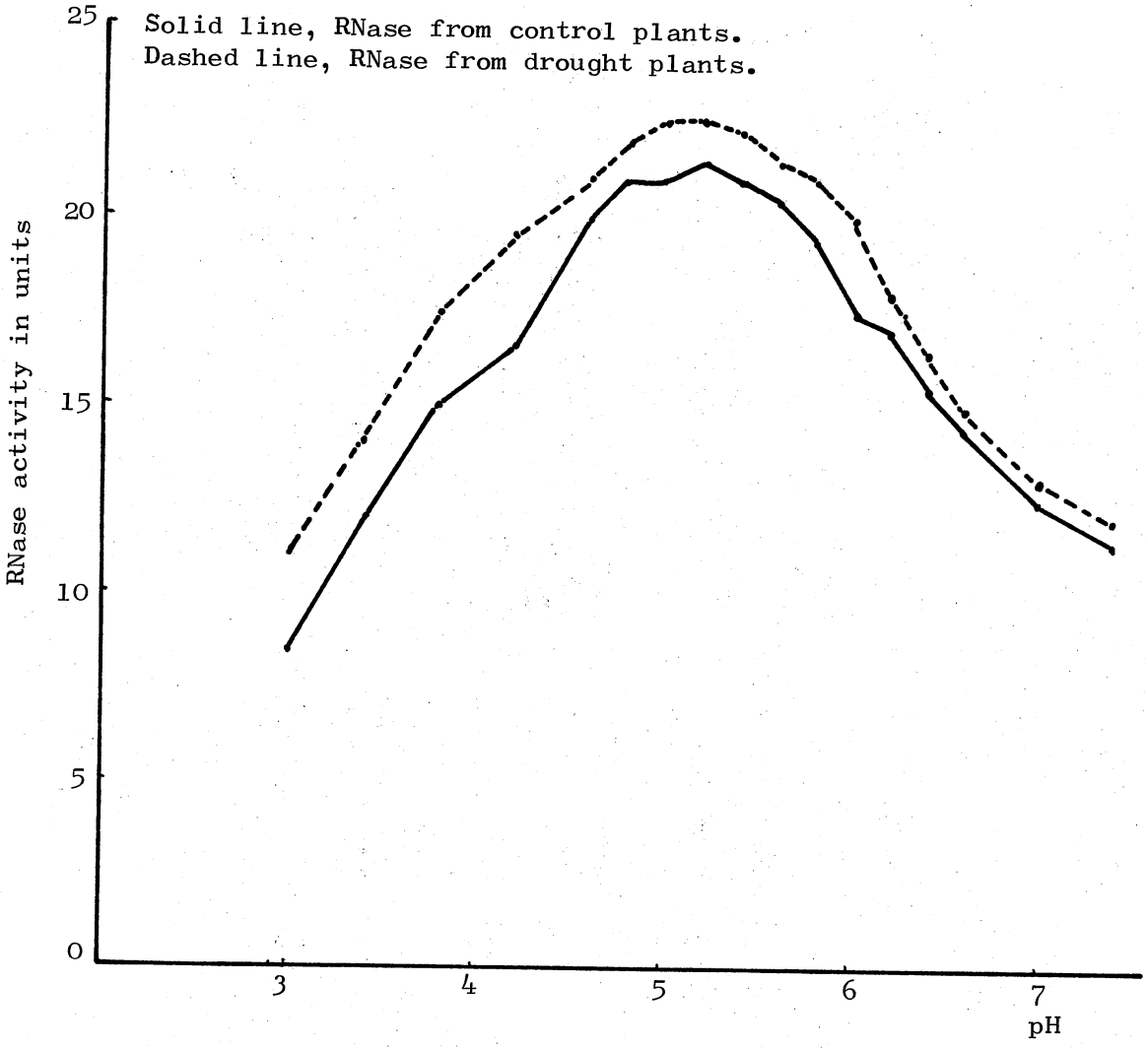

Figure 10. Effects of pH on RNase Activity of Soluble Fraction

(See Table VIII for the reaction mixture.)

RWC = 91% in control plants and 73% in drought plants.

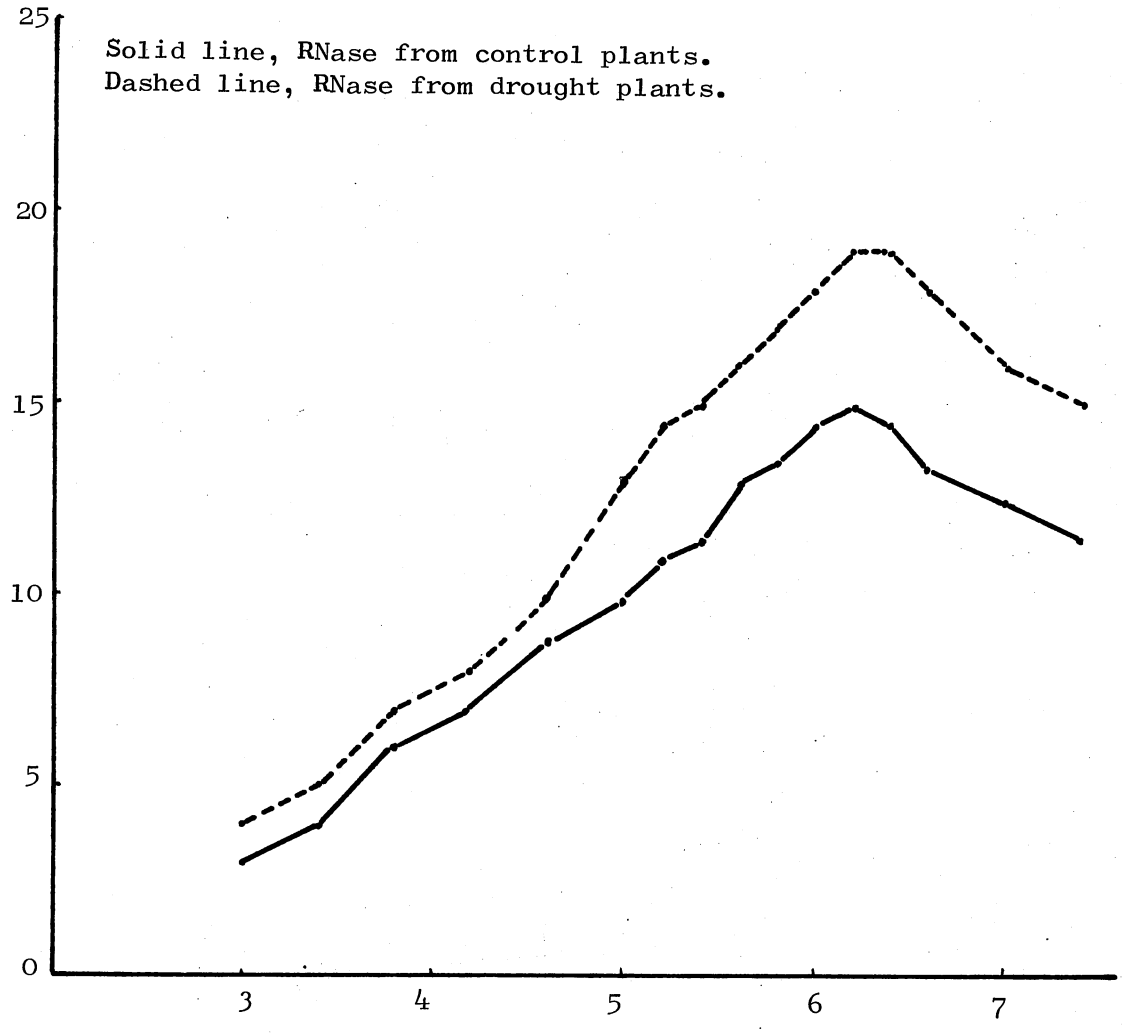

Figure 11. Effects of pH on RNase of Microsomal Fraction

(See Table VIII for the reaction mixture.)

activity at about pH 5.2 and the particulate at pH about 6.2. These values were not changed by the water stress treatment and fractions from water-stressed plants had higher RNase activity over the whole pH range tested.

Cations such as Cu^{++} and Zn^{++} ions did not inhibit RNases of either fraction (Table VIII). Zinc ion slightly increased enzyme activity. It seems that RNases from wheat plants are different from those of bean plants which were inhibited more than 90% by cations (Gray, 1974). This is supported by the fact that zinc ions were essential for stabilizing wheat RNase at pH 4.5 (Hanson and Fairley, 1969).

It is possible that RNases may undergo chemical modification of molecular structure if exposed to organic and inorganic reagents (Barnard, 1968) during water stress. This may be expressed as an inhibition of partially purified RNase by crude homogenates. The effect of inhibitors in crude extracts was examined according to the method of Morgan et al. (1966). The crude extracts (such as Fraction I in Table IV) was boiled at 100 C for 10 minutes and cooled on ice water. The enzyme came from Cellex D eluates. The reaction mixture is shown in Table IX-a and -b.

The crude extract from control plants tended to inhibit RNase activity slightly (Table IX-a). The inhibition of RNase from control plants by the crude extract from water-stressed plants in Experiment No. 1 does not seem to agree with the result of Experiment No. 2, and this may be due to differences in concentration of inhibitors in the extract used for the enzyme assay. To obtain higher concentration of inhibitors or homogenates, greater amounts of

TABLE VIII

EFFECT OF CATIONS ON SOLUBLE AND PARTICULATE WHEAT SEEDLING RNASE ACTIVITY

EXPERIMENT NO. 1	CONTROL RWC = 91%		WATER STRESS RWC = 73%	
	activity*	% of no treatment	activity*	% of no treatment
Soluble RNase at pH 5.2				
none	0.20	100	0.20	100
Cu	0.21	105	0.20	100
Zn	0.21	105	0.21	100
Particulate RNase at pH 6.2				
none	0.13	100	0.14	100
Cu	0.13	100	0.14	100
Zn	0.13	100	0.16	114

TABLE VIII (Continued)

EXPERIMENT NO. 2

	CONTROL RWC = 93%		WATER STRESS RWC = 64%	
	activity*	% of no treatment	activity*	% of no treatment
Soluble RNase at pH 5.2				
none	0.29	100	0.32	100
Cu	0.28	97	0.30	95
Zn	0.28	97	0.31	98
Particulate RNase at pH 6.2				
none	0.30	100	0.32	100
Cu	0.30	100	0.31	98
Zn	0.30	100	0.33	101

*activity is absorbance change at 260 nm.

Incubation contained 1 mg RNA, MacIlvaine's citrate-phosphate buffer, and enzyme. Enzymes were 38 µg of protein in water stress and 41 µg of protein in control for soluble RNase in Experiment No. 1. 123 µg of protein in water stress and 150 µg of protein in control for particulate RNase in Experiment No. 1. 49 µg of protein in water stress and 50 µg of protein in control for soluble RNase in Experiment No. 2. 250 µg of protein in water stress and 328 µg of protein in control for particulate RNase in Experiment No. 2.

All cations were tested at 10 mM, with SO_4 ions as the counter ion.

RNase activity was measured by Spectrophotometeric assay No. 2.

TABLE IX-a

EFFECTS OF BOILED, CRUDE EXTRACTS ON ACTIVITY OF PURIFIED RNASE

RNase from	Crude extract from	Activity*	% of no treatment
EXPERIMENT NO. 1			
control	-	0.25	100%
control	control	0.23	92%
control	water stress	0.23	92%
water stress	-	0.27	100%
water stress	control	0.25	93%
water stress	water stress	0.27	100%
EXPERIMENT NO. 2			
control	-	0.31	100%
control	control	0.29	94%
control	water stress	0.31	100%
water stress	-	0.32	100%
water stress	control	0.31	97%
water stress	water stress	0.32	100%

*Activity is absorbance change at 260 nm.

Relative water contents were 96%, control plant, and 53%, water stress plant in Experiment No. 1 and 94%, control plant, and 47%, water stress plant in Experiment No. 2.

RNase was extracted by Purification Procedure No. 1 and measured by Spectrophotometric assay No. 1.

TABLE IX-b

EFFECTS OF BOILED, CRUDE EXTRACTS ON ACTIVITY OF PURIFIED RNASE

Source of extract	Dilution of extracts	Activity*	% of no treatment
-	-	0.26	100%
control	5 times	0.21	80%
control	2 times	0.12	46%
water stress	5 times	0.24	92%
water stress	2 times	0.16	61%

*Activity is absorbance change at 260 nm.

Crude extracts were from control plant with RWC = 93% and from water stress plant with RWC = 74%.

RNase was extracted by Purification Procedure No. 1 and measured by Spectrophotometric assay No. 1.

plant material were homogenized: 221 g of plants (fresh weight) in control treatment and 128 g of water-stressed plants. When these higher concentrations were added to the reaction mixture for RNase, the inhibition was clearly shown (Table IX-b). The greater amounts of extracts used for the enzyme assay, the greater inhibition of RNase activity that occurred. The crude extract from control plants showed stronger inhibition than that from water-stressed plants. The data indicate that control plant extracts have RNase inhibitors which may disappear during water stress. The difference between the inhibitions by extracts from the two treatments accounts for most of tne increase in RNase activity by water stress treatment, when measured in crude homogenates (see Tables IV and V).

Desiccation of Purified Enzymes

For the experiment of drying enzymes (Tables X, XI, and XII), the initial velocity of the catalytic activity was determined under fixed conditions of pH, temperature and added ions. To obtain the initial velocity, various states of relative enzyme and substrate concentrations were attempted for the determination: high concentrations of substrates and properly diluted concentrations of the enzymes were usually employed. After establishing conditions that allow accurate determination of the initial velocities, the initial velocity is directly proportional to enzyme concentration. Therefore, careful measurement of the initial velocity would allow an estimation of the enzyme concentration.

The enzymes tested here were selected from enzyme groups listed by J. Levitt (1965): pepsin, pancreatic RNase, and trypsin are heat stable proteins with SS bonds, whereas glutamate dehydrogenase,

TABLE X

EFFECT OF DRYING TEMPERATURE ON
ENZYMES HAVING SS GROUPS

Enzyme	Activity as % of initial activity*			
	no drying at 4 C	drying temperature		
		-4 C	4 C	25 C
pepsin, animal+	91	91	81	81
	100	100	90	90
trypsin, animal+	95	83	91	62
	95	95	77	68
chymotrypsin, animal	100	100	89	67
	100	88	88	63
RNase, pancreatic** +	60	31	25	23
	56	27	24	21
papain	50	27	23	19
	48	28	24	16
lipase, wheat germ	97	88	88	96
	97	97	88	88

*Enzyme activity based on initial velocity.

**RNase activity was measured by Fluorometric assay.

+Heat stable enzymes (Levitt, 1965).

TABLE XI

EFFECT OF DRYING TEMPERATURE ON
ENZYMES HAVING SH GROUPS

Enzyme	Activity of % of initial activity*			
	no drying at 4 C	drying temperature		
		-4 C	4 C	25 C
glutamate dehydrogenase** +	0	0	0	0
	0	0	0	0
xanthine oxidase**	8	0	0	0
	10	0	0	0
glucose oxidase**	100	16	16	0
	100	0	0	0
alcohol dehydrogenase	0	0	0	0
	0	0	0	0
hexokinase	71	7	7	7
	61	14	11	11
lactic dehydrogenase+	94	89	89	89
	68	68	68	68
catalase+	10	0	0	0
	0	0	0	0
glucose-6-phosphate dehydrogenase	35	0	0	0
	40	1	0	0

*Enzyme activity based on initial velocity.

**Heat labile enzymes (Levitt, 1965).

+Enzymes inactivated by freezing (Levitt, 1965).

TABLE XII

EFFECTS OF SUCROSE AND MANNITOL ON DRYING ENZYMES AT 4 C

Enzyme	Agent	Activity as % of no treatment	
		no drying	dried
Glucose-6-phosphate dehydrogenase			
	no sucrose	65	0
	sucrose	15	0
	no sucrose	57	0
	sucrose	12	0
Pancreatic RNase*			
	no sucrose	70	17
	sucrose	70	26
	no sucrose	70	25
	sucrose	70	38
	no mannitol	70	22
	mannitol	70	22
	no mannitol	70	26
	mannitol	70	26

*Pancreatic RNase was measured by the spectrophotometric method (spectrophotometric assay No. 1).

xanthine oxidase, and glucose oxidase are heat labile proteins with SH group. Lactate dehydrogenase, glutamate dehydrogenase and catalase are SH-containing enzymes and are inactivated by freezing. In addition, other enzymes such as chymotrypsin, papain, and lipase with SS bonds and alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, and hexokinase with SH groups were studied.

Many enzymes lost activity in the solution state when kept at 4 C, except pepsin, trypsin, chymotrypsin, lipase, and glucose oxidase (Tables X and XI). Heat stable enzymes having SS groups resisted desiccation deactivation at different temperatures: lipase and pepsin were very resistant and pancreatic RNase and papain were least resistant. The desiccation caused severe loss of activity of heat labile enzymes with SH groups. Enzymes with SS groups showed more resistance at the lower temperature (-4 C) compared to the higher temperature. The major exception in loss of activity by desiccation of enzymes with SH groups was lactic dehydrogenase which was not inactivated by the treatment.

To investigate the protective effect of organic substances on the desiccation of enzymes, sucrose and mannitol were selected from the group of protective agents listed by Todd (1972), which increased the resistance to freezing injury. One ml of enzyme solution containing 0.5 M sucrose or 0.5 M mannitol was desiccated at 4 C as described earlier. After 7 days of desiccation, the initial velocity was measured. As shown in Table XII, sucrose accelerated the loss of activity of glucose-6-phosphate dehydrogenase in the solution state at 4 C for 7 days. Sucrose had no protective effect during desiccation of

glucose-6-phosphate dehydrogenase, as all enzyme activity was lost in both treatments.

On the contrary, sucrose did not accelerate the activity loss of RNase in solution state at 4 C for 7 days. The sucrose contained in the desiccated solution slightly protected RNase activity from loss caused by desiccation. Mannitol did not protect RNase from the activity loss during desiccation.

CHAPTER V

DISCUSSION

The present study dealt with changes in RNase activity between the normal intact plants and droughted plants. Water stress treatments result in increased RNase activity in wheat plants. The experiments were designed to determine how the enzyme increases in wheat plants. Possible causes of RNase activity increase are discussed in this chapter, based on the results obtained from this study.

If RNases were extracted from the same numbers of plants in each treatment, one could estimate the true change in RNase activity due to water stress treatment. Thus, the same number of wheat seeds were planted, throughout all experiments, for both treatments, assuming the same rate of germination, and grown under the same conditions until the water supply was withheld in the water stress treatment. The plants from the control and the water stress treatments were harvested at the same time, and the same amount of buffer solution was used for the homogenation. This should make a direct comparison possible between the watered and droughted plants.

At first, RNase activity was studied by using crude extracts, in order to estimate changes in RNase activity due to water stress. Tables II, IV, V, and VI show that the water stress caused about 10% to 37% increase in total RNase activity. Assuming that the same number of plants from both treatments were used for the enzyme extraction,

RNase activity per plant or cell was not dramatically increased by the water stress treatment. In true de novo synthesis of an alkaline RNase caused by Trenimon treatment of HeLa cells, cell proliferation ceased but protein content per cell exceeded the increase due to normal growth in the control culture (3 times higher) and RNase activity per cell increased more than 4 times (Schlaeger and Hiltz, 1969). Previous work with plant materials has revealed that protein synthesis is a prerequisite for the increased RNase activity resulting from 2,4-D treatment. With excised mesocotyl tissues of corn seedlings, low concentrations of 2,4-D increased protein content per gram tissue (less than 10%) and RNase activity per gram tissue (30%) in parallel (Shannon, et al., 1964). On the contrary, the water stress treatment in this study caused a decrease in total protein content per plant or per cell. It would appear, therefore, that the effect of the water stress treatment did not reflect de novo synthesis of RNase.

The increase in RNase activity following damage to tobacco leaves (Bagi and Farkas, 1967), after detachment of wheat leaves (Sodek and Wright, 1969), in senescence of banana slices (de Leo and Sacher, 1970-a and -b), and water stress (Kessler and Engelberg, 1962) has been examined in considerable detail. Practically all previous work in this field has been done with crude extracts. Thus, the possibility that enzymes other than RNase (such as phosphoesterases) might be involved in hydrolyzing RNA molecules has been overlooked. Recently, some attempts have been made to purify RNase following injury and senescence in order to separate RNase from phosphoesterases, but they resulted in poor separations by ion exchange chromatography (Pitt, 1974; and Henkel, et al., 1974) and low yields after gel filtrations (Udvardy, et al.,

1969). To differentiate the increase in free enzyme activity from the water stress treatment and to exclude other enzymes, parallel purification of the enzyme from the control and water stress treatments was carried out.

Several plant RNases have been partially purified (Merola and Davis, 1962; Reddi, 1965; and Tuve and Anfinsen, 1960), and some were purified to a high degree (Kado, 1968; and Tomoyeda, et al., 1969). The ultimate purification of RNases such as cucumber RNase (Kado, 1968) was considered undesirable in this study, because it is accompanied by a substantial loss of enzyme activity. Many distinguishable properties could be detected in the partially purified state of the enzyme (Gray, 1974; Tuve and Anfinsen, 1960; and Wilson, 1971).

As shown in Table IV, wheat RNase was purified more than 200 times by purification procedures adopted in this study. Specific activities (RNase activity in units per mg protein) are higher in the water stress treatment throughout the purification procedure than that in the control treatment. The yield is practically identical in both treatments. A very similar behavior was observed under severe water stress (Table V). Higher specific activity was observed during the purification of alkaline RNase from HeLa cells when treated with Trenimon, and this was an indication of de novo synthesis of the enzyme (Schlaeger and Hiltz, 1969). This concept of an induced synthesis is not clearly supported in the present study, because the water stress treatment caused a decrease in total protein content whereas RNase activity of HeLa cells was exceeded by the increase in protein synthesis. When RNases were purified following mechanical damage, whether the specific activity of RNase from damaged tissues was higher during the purification procedure

or whether the increased activity was followed by the increase in protein content was not investigated (Pitt and Galpin, 1971; Pitt, 1971; Pitt, 1974; Udvardy, et al., 1969; and Henkel, et al., 1974).

Dehydration of wheat leaves at 38 C caused 4 subfractions of RNases which were eluted by 0.05 M, 0.10 M, 0.15 M, and 0.20 M of Tris-HCl buffer, pH 7.0, on DEAE-cellulose chromatography, suggesting that 4 different isoenzymes were produced by water deficit (Henkel, et al., 1974). In contrast, RNases isolated from water-stressed and control plants in this study were eluted as a single peak at 0.08 M of NaCl and 0.08 M of phosphate buffer, pH 7.0 from a Cellex D (commercial name of DEAE-cellulose by Bio-Rad Laboratories) chromatographic column. As indicated in the Results, additional activity was found during the elution with 0.04 M buffer (Figures 3 and 4), but this was not found in all experiments (see Figures 5 and 6). No further RNase activity was found after the peak of RNase activity appeared at 0.08 M NaCl on the linear salt gradient. Alkaline RNase of HeLa cells tended to have a division into 4 fractions at about the same salt concentration on CM-cellulose chromatography. The subfractions all exhibited the same optimum at pH 7.7 (Schlaeger and Hiltz, 1969) and were a similar kind of enzyme. The subfraction of RNase in this study which was eluted by 0.04 M buffer had the same optimum pH at pH 5.2 as the subfraction eluted by 0.08 M NaCl (Figures 3 and 4), suggesting that the two subfractions might be similar in nature. This is supported by the fact that two subfractions of spinach RNase showed the same stability to heat treatment and produced the same product during the hydrolysis of RNA (Tuve and Anfinsen, 1960). The result

from ion exchange chromatography, thus, indicates that RNases from both treatments behave the same in the acid-base partition.

It was suggested that there were two kinds of RNases in wheat plants and that the RNase insensitive in EDTA inactivation increased through new synthesis and the RNase sensitive to EDTA inactivation increased through the activation from the latent forms of RNase during senescence (Sodek and Wright, 1969). These isoenzymes of RNases were detected after polyacrylamide gel electrophoresis (Wilson, 1971). Drying wheat seedlings at 38 C resulted in a qualitative change in the isoenzyme spectrum on polyacrylamide gel electrophoresis (Henkel, et al., 1974). The newly formed RNase following mechanical damage and the one present originally before the injury were eluted at the same salt concentration of DEAE-cellulose chromatography (Pitt, 1974). The RNase shown in Figure 4 might consist of the enzyme originally present and the newly formed one during water stress, which might have different properties and be distinguished by further purification. Thus, an attempt was made in this study to determine whether the water stress might be accompanied by qualitative changes in RNase molecules. The gel electrophoresis used by Wilson (1971) was sensitive enough to detect 0.1 unit of RNase and was suitable for crude extracts from plant materials. The RNase band was located by staining the products of the RNA-RNase reaction with toluidine blue. One difficulty was that large amounts of enzyme or protein caused poor separation of RNases. Improved sensitivity was achieved with proteins by using Coomassie blue in 15% trichloroacetic acid, enabling detection of 1 μg of protein on the gel (Chrambach, et al., 1967), but a prior requisite for such an approach is to purify the enzyme to some extent. In this study, the method of

Davis (1964) followed by the staining method of Chrambach, et al. (1967), was adopted for electrophoresis, since the character of RNase protein was the major concern. The partially purified enzyme from the Cellex D eluates was used for electrophoresis. As shown in Figure 5, the pattern of RNase distribution on disc gel electrophoresis was the same in the water stressed and control treatments. RNase shown in Figures 3, 4, and 5 corresponds approximately to the RNase which was located in the supernatant after centrifugation at 20,000 X g, and may correspond to the fraction which was insensitive to EDTA inactivation and was increased by de novo synthesis during senescence of wheat plant as described by Sodek and Wright (1969). Even if this fraction of RNase was synthesized during water stress in this experiment, the properties of the enzyme from water-stressed plants seems to be identical to the RNase which had been present in un-stressed plants. The similarities also appeared after Sephadex G-75 chromatography (Figures 8 and 9).

When the partially purified enzyme from mung bean sprouts was passed through a Sephadex G-100 column, activities of DNase, RNase, and 3'-nucleotidase were located in the same peak and were always found in the same proportions (Johnson and Laskowski, 1968). Nuclease from germinating wheat plants also appeared to possess DNase, RNase, and 3'-nucleotidase activities (Hanson and Fairley, 1969), and it was suggested that wheat nuclease consisted of one protein with enzyme activity for all of the three substrates. The possible biological significance of such a unit of related enzyme activities is not yet known. Table V shows that the peak of RNase activity on Cellex D chromatography had DNase and 3'-nucleotidase activities. After chromatography on phosphocellulose, germinating wheat nuclease had a ratio of

DNase : RNase : 3'-nucleotidase = 1.3 : 1.0 : 1.2, based on the total activity (Hanson and Fairley, 1969). However, the ratios of the three enzyme activities after Cellex D chromatography in this study was DNase : RNase : 3'-nucleotidase = 1 : 45 : 1.7 in the control plants and 1 : 36 : 1.1 in the water-stressed plants. The reason that the ratio of the three enzymes is quite different in this study from that reported by Hanson and Fairley (1969) may be that highly polymerized DNA and highly purified adenylic acid were used as substrates for DNase and 3'-nucleotidase assays, whereas partially polymerized RNA was used for the RNase enzyme assay. With partially purified enzyme from mung bean, DNase activity decreased during storage, whereas RNase activity remained unchanged (Johnson and Laskowski, 1968). It is not known how growth can affect the ratio of the three enzymes. DNase and 3'-nucleotidase activities were not detected on the peak of RNase activity following Sephadex G-75 chromatography (Figures 8 and 9). The two enzymes were probably separated from the RNase by gel filtration. It is also possible that the two enzyme activities were so low that they could not be detected on the peak of RNase activity.

Several workers have provided evidence from a number of tissues that increased levels of RNase activity following mechanical damage and during senescence may be due to extensive de novo synthesis of this enzyme. Pretreatment of tobacco leaf tissue with actinomycin D before injury completely prevented the rise in RNase activity (Bagi and Farkas, 1967). The increase in RNase activity was greater in light than that in darkness (McHale and Dove, 1969; and Sodek and Wright, 1969) during senescence. Fragments of Avena leaves were incubated in Petri dishes in the presence of cycloheximide (50 μg/ml), and the increase in RNase

activity following excision of leaf tissues was completely inhibited by cycloheximide (Udvardy, et al., 1969). Short term studies of banana slices during senescence utilizing cycloheximide and actinomycin D indicated that the increase in RNase activity was a result of new synthesis of RNA (de Leo and Sacher, 1970-a). When wheat leaves were sprayed with cycloheximide during water stress, the increase in RNase activity due to water stress was supressed (Yi, 1972). The increase in RNase activity during senescence was reportedly due to de novo synthesis, since the buoyant density of the RNase from deuterium labelled tissue was significantly higher than that of the control (Baumgartner, et al., 1975; Sacher and Davies, 1974; and Sacher, et al., 1975). However, an endogenous D_2O concentration of 50% inhibited more than 60% of the RNase activity increase caused by senescence (Sacher and Davies, 1974). No density shift could be detected by isopycnic equilibrium centrifugation using deuterium oxide in the increase in RNase activity following mechanical damage (Pitt and Galpin, 1971). Relatively small density shifts may be involved during de novo synthesis of the enzyme in the presence of deuterium oxide and these may be difficult to detect. In the present study C^{14}-leucine incorporation was attempted in order to obtain further evidence on the origin of the increase in RNase activity which occurs during water stress. Table V shows that C^{14}-leucine was incorporated into wheat plants. The plant root presumably would absorb the amino acid by means of membrane transport mechanisms that may be similar to the transport of inorganic ions, as indicated by leucine absorption in duckweed root tips (Newton, 1974). When potato slices were incubated over 3 hours in 150 μCi of L-(1-C^{14})-leucine, the increase in total RNase activity due to mechanical damage was lowered

by the leucine treatment, when compared with no treatment (Pitt, 1974). In the present study, however, the decline of RNase activity in the treatment with radioactive amino acid was not observed in assays of the homogenate (see Tables IV, V, and VI).

When potato RNase was isolated from the damaged tissue, two peaks of RNase activity were eluted on DEAE-cellulose chromatography (Pitt, 1974). The first peak represented a form of the enzyme that occurred in the undamaged tissue and the second one represented the enzyme which occurred in the damaged tissue and possessed radioactive label after further purification using DEAE-cellulose column and gel filtration. As shown in Figures 6 and 7, the distribution of RNase activity on Cellex D chromatography was same in the control and water stress treatments. Furthermore, no radioactivity was detected on the RNase peak in gel filtration (Figures 8 and 9). It is possible that the two peaks of RNase activity from potato slices (Pitt, 1974) may represent the same enzyme, since they were eluted at about the same concentration of the salt gradient of DEAE-cellulose chromatography. It is also possible that the radioactivity of the second peak was caused by contamination. The DEAE-cellulose (Pitt, 1974) column was washed with less than 100 ml of buffer solution at the first and DEAE-cellulose column for the further purification was washed with another 100 ml of buffer solution, whereas more than 800 ml of buffer solution was used for the washing of the Cellex D column before running the linear salt gradient in this study. It appears possible that the radioactivity in the RNase peak after gel filtration using Sephadex G-100 (Pitt, 1974) would not have been detected during further purification if the DEAE-cellulose column at the earlier purification step had been washed

extensively with a large volume of buffer solution. Figures 6, 7, and Table VI give the incorporation rate during the 6-day period (including 4 days of water stress treatment) after incubation with C^{14}-leucine. Enzyme extraction and purification at the first day after incubation was not performed in the present study. Comparison of the incorporation rate during the first day with that at the sixth day could determine whether RNase synthesized during the 24 hours following the incubation was lost due to turnover during the longer incubation periods. It is, thus, not possible to ascertain if the labelled compound was incorporated into RNase molecules early and the labelled protein was lost during the period of water stress, resulting in no radioactivity persisting through gel filtration. Since the incorporated label did not persist through gel filtration in both treatments, control and water stress, one can presume that de novo synthesis of RNase protein did not occur during the period of 6 days or that the increase in RNase activity due to de novo synthesis during water stress could not be detected by the system adopted in this study. Some RNase molecules may be produced as a result of water stress treatment, but the contribution of this source to total RNase activity must be very small.

Gel filtration has the capability of separating substances according to molecular size. The elution volumes of globular protein are largely determined by their molecular weight in gel filtration. Over a considerable range, the elution volume is approximately a linear function of the logarithm of the molecular weight. It is possible to estimate the molecular weight of RNase from the wheat plant in this study. The elution volume of pancreatic RNase was more than 200 ml on Sephadex G-75 chromatography (Moore and Stein, 1973) and that of RNase

from Rhizopus Sp. was about 200 ml (Tomoveda, et al., 1969). Wheat plant RNase was eluted at about 100 ml on Sephadex G-75 columns (Figures 8 and 9). This lower elution volume as well as the slower movement in the electric field during disc gel electrophoresis, when compared with pancreatic RNase (Figure 5), implies that the molecular weight of wheat RNase may be larger than that of pancreatic RNase. The molecular weight of pancreatic RNase is about 12,500, and it varies in flowering plants: 20,000 in garlic plants (Carlsson and Frick, 1964), 12,600 in cucumber (Kado, 1968), and 23,000 in corn plant (Wilson, 1967). The molecular weight of wheat RNase in this study is estimated to be about 23,000.

If the increase in RNase activity during water stress is not due to de novo synthesis, then the activation of the enzyme already present must be responsible for the increase in RNase activity. Activation as used here may include a reduction in concentrations of inhibitors, both organic and inorganic, which are removed from the vicinity of the RNase molecules in the protoplast by the water stress treatment.

When cotton plants were grown on a nutrient solution containing manganese at the toxic level, the leaf extract contained very high amounts of manganese (Morgan, et al., 1966). It was suggested that the manganese toxicity symptom in cotton plants was an expression of auxin deficiency caused by IAA-oxidase activity increase due to the abnormal tissue levels of manganese. In zinc-deficient leaves of Citrus, RNase activity was higher than in normal leaves which contained sufficient zinc ions, and RNase extract from zinc-deficient leaves hydrolyzed about 10% more substrate (RNA) than the extracts from healthy leaves (Kessler and Monselise, 1959). Presowing treatment of sunflower seeds

with aluminum nitrate and cobalt nitrate raised RNA and DNA content in the growing points under water deficiency conditions, but it lowered RNase activity (Bozhenkov, 1968). A protein isolated from rat liver inhibited bovine pancreatic RNase, but did not affect the activity of RNase from bean plants (Shortman, 1962). A protein isolated from a bacterial source (Bacillus subtilis) could be selectively released by cooling the cells. It then formed a complex with bacterial RNase, resulting in the inhibition of RNase activity (Smeaton and Elliott, 1967). If the inhibitor is still present in crude extracts from the control plants, it should inhibit the partially purified RNase which has been eluted from a Cellex D column. The results shown in Table IX-a and -b strongly indicate that homogenate from control plants does contain a factor inhibiting RNase activity. Furthermore, the increase in RNase activity during water stress can be explained by the decrease in inhibition. The term inhibition is used here because the net effect of an inhibition is a decrease in the observed enzyme activity. However, the effect of crude extracts on RNase activity may be postulated as operating in several ways.

1. The enzyme inhibitors, competitive, noncompetitive, and uncompetitive, might be decreased by water stress treatment.
2. Ligands, activating the enzyme instead of inhibition, might increase during water stress.
3. A specific chemical modification of RNase molecule occurs to decrease the enzyme activity in control plants. In this case, a chemical reagent specifically and covalently modifies one specific amino acid residue out of many hundreds in a

protein, and then this change leads to a measurable change in enzyme activity.

As indicated in Table IX-a and -b, the factor inhibiting the partially purified RNase from the crude extract of the control plant may not be a protein, because it should be denatured by boiling. This wheat RNase is not inhibited by zinc and copper ions (Table VIII) (Hanson and Fairley, 1969), and so the inhibiting factors present in the crude extract of the control plants should not be these metal ions. The inhibiting substances may be other cations such as Co, Fe, Ni, and Cd ions, and organic substances such as EDTA (Hanson and Fairley, 1969), diethylpyrocarbonate and guanosine-2'(3')-monophosphate (Gray, 1974). The inorganic ions were required in high concentrations (more than 10 mM), but 10 μM EDTA and 100 μM guanosine-2'(3')-monophosphate inhibited more than 50% of the RNase activity. Potassium ion is known to be an activator of corn RNase (Wilson, 1963). Bromoacetate and iodoacetate modifies pancreatic RNase at pH 5.5 by carboxymethylation of histidine residue, resulting in RNase inactivation (Crestfield, et al., 1962).

RNase molecules undergo a dramatic change from the native globular structure to the random disoriented polypeptide, when subjected to low pH (lower than pH 3.9) and with 8 M urea. However, the enzyme may return to the original structure, if put in more normal physiological conditions (Anfinsen, 1973). Even wrong combinations of S-S pairs along the RNase molecule are interchanged until the thermodynamically stable combination is reached; this is also the catalytically active (native) form. When pancreatic RNase was hydrolyzed by subtilisin, it produced two single peptide chains without enzyme activity. The active

enzyme was reconstructed when the two inactive components were combined at neutral pH (Richards, 1959). The reactivation by air oxidation of reduced, inactive RNase T1 was poor at pH 9, but was better at pH 6.2 (Kasai, 1965). The unfolded, inactive A could be refolded at acidic pH (to less than pH 4) and be active (Tsong, et al., 1972). Since dehydration was postulated to lower the pH down to 4.5 in the plant cytoplasm (Todd, 1972), the inactive forms of RNase could undergo reactivations by oxidation, refolding (aided by a sulfhydryl) or reconstruction to become active during water stress. This is supported by the evidence that the reduced, latent RNase was transformed into active enzymes in rat liver ribosomes by hypophysectomy (Brewer, et al., 1969). The amount of inhibitors and inactivators may also disappear during water stress, but the reactivation process may be the prime mechanism, because the water stress would directly affect the RNase molecule itself. The pancreatic RNase B, glycoprotein form of pancreatic RNase A, was unfolded in 3 M guanidine hydrochloride at neutral pH and the free energy was 15.3 Cal per mole (Puett, 1973), and so the unfolding process (yielding the inactive form) may not happen during water stress. It is not clear that the formation of inter- and intramolecular SS bonds caused by drought (Levitt, 1966) could lead to dimerization and hybridization of inactive RNases to produce the active form of the enzyme (Crestfield, et al., 1962; and Fruchter and Crestfield, 1965). It is not known whether these types of repair, complementation of RNase, molecules, can be induced by changes in physiological conditions in animal and plant tissues.

Kessler (1961) postulated the existence of nucleoprotein particles with which RNase is associated and which are surrounded by and bound to

compounds of lipoidal character. The liponucleoprotein structure would disappear during water stress, setting free RNases. The microsomal and soluble RNases could be separated by a centrifugation at 105,000 X g for 60 minutes from apple leaves, and the microsomal RNase decreased with age, but the soluble RNase increased (Kessler and Engelberg, 1962). RNase from wheat embryo had an optimum activity at pH 5.0 and was heat stable, whereas RNase from seedlings had the optimum activity at pH 5.5 and was inactivated at a temperature of 50 C (Vold and Sypherd, 1968). The embryo RNase was lost during the first 10 hours, followed by the appearance of a second seedling RNase after 15 hours of germination. The latent acid phosphatase of tobacco chloroplast was activated by heating at 37 C in the presence of 0.15% of Triton X-100, and the enzyme remained only partially structure bound (Ragetli, et al., 1966). Triton X-100 could desorb acid phosphatase from membrane or cell wall surfaces of banana fruits (de Leo and Sacher, 1970-b). The microsomal acid phosphatase was extracted from the precipitate after centrifugation at 30,000 X g for 20 minutes by using 1% Triton X-100 and the soluble enzyme was obtained from the supernatant of the centrifugation. During ripening of banana, the soluble acid phosphatase increased about 3 times, but the microsomal enzyme was increased more than 13 times. The distributional change in acid phosphatase activity was also observed during germination of lettuce seeds, in which the microsomal enzyme decreased, followed by a rise of the new soluble acid phosphatase (Meyers, et al., 1971). As shown in Figures 10 and 11, the soluble preparation showed optimal activity at pH 5.2 and the particulate preparation showed optimal activity at pH 6.2. These pH optima are in agreement with those for soluble and microsomal preparations from bean

plants. However, the metal salt treatment with enzymes did not inhibit wheat RNases (Table VIII). If assuming that the microsomal and soluble RNases were properly separated by the centrifugation and the treatments with Triton X-100, it is clear that the distributional changes suggested by Kessler (1961) and Meyers, et al. (1971), are not occurring in wheat plants during water stress. The RNase activity per mg protein is lower in the particulate fraction than that in the soluble fraction and both RNases in the microsomal and soluble fractions increased during water stress (Table VII). The particulate and the soluble RNases differ in their pH optima and in their response to metallic salts (Wilson, 1971). They also differ in their ability to hydrolyze the substrate, producing different kinds of products (Reddi, 1965). During the water stress, the soluble RNase may not be simply liberated from the particulate fractions in wheat plants, as suggested by Meyers, et al. (1971).

It is well known that RNases, in general, from plant sources are more stable than pancreatic RNase (Johnson and Laskowski, 1968; Reddi, 1965; and Tuve and Anfinsen, 1960). The ATPase activity induced by uncouplers was inhibited by impermeant solutes such as sucrose when their concentrations increased above isotonicity in the rat liver mitochondria (Cereijo-Santalo, 1972). It was suggested, thus, that the inhibition of ATPase by impermeant solutes was due to the loss of water from the matrix space in the mitochondria, and the inhibition was in the order of

$$\text{arabinose} < \text{mannitol} < \text{sucrose} \quad .$$

However, little or nothing is known about the chemical changes in the proteins which leads to the denaturation during the desiccation. Levitt (1966) postulated that the cryoresistance of plants was

correlated with desiccation and heat resistance and that the cryostability of enzymes was correlated with heat stability. He separated proteins into two categories: heat stable enzymes with low molecular weight and without SH groups and heat labile enzymes with high molecular weight and with SH groups. It is obvious from the consideration above that the desiccation can affect differently the two groups of proteins. If the enzyme survives the desiccation or dehydration, it will give the capacity of the enzyme to operate at low moisture contents during the water stress. For this experiment, the purified enzyme must be used, because the contaminants can protect proteins from the desiccation or unnecessarily injure them. As shown in Tables X and XI, all results agree well with the postulation of Levitt (1966), except lactate dehydrogenase. Proteins with SS groups resisted desiccation more strongly than the protein with SH groups. The desiccation at higher temperature at 25 C caused more damage to the protein in both groups of proteins.

According to Wold (1971), the native state of proteins or enzymes refers to any biologically active molecule in terms of both structure and activity, and the denatured state represents any change in either native structure or native activity and thus does not require that enzyme activity is lost. Although only the enzyme activity (biological function) was measured, rather than determining physical and chemical modifications, the perturbation from the native state (standard enzyme assay) could lead to observable loss of or change in any of the native characteristics. Therefore, it is safe to say that the desiccation of the proteins (Tables X, XI, and XII) represents the removal of water from around the protein to cause changes in structure and activity of

proteins (Todd, 1972). The process responsible for the change from the native to denatured forms during desiccation, especially in Table XI, may be the intermolecular or intramolecular SS bonds formed between protein molecules during injurious dehydration (Levitt, 1966). When SS bonds are present, they can maintain the protein conformation unaltered even after the hydrogen bonds have been weakened or broken. The SS protein would, therefore, withstand the desiccation even without denaturation (Table X). When the protein with SH groups is dehydrated, the intermolecular SS formation would be greatly accelerated at high temperature (25 C). More than 70% of pancreatic RNase and papain activities were lost during the desiccation at 25 C and their activity losses were higher than other enzymes shown on Table X. The case of lactate dehydrogenase is different from other proteins (Table X). All models of lactate dehydrogenase isoenzymes require a free SH group in the active site of the enzyme, involved in thioacetal formation with the carbonyl function of the substrate (Pfleiderer, 1957). If it is possible to reduce SS bonds formed at the active site during the desiccation when the dried enzyme was dissolved in the water, thus, the dehydration itself may not give any damage to the enzyme activity. Or the SH group in the active site of lactate dehydrogenase may not be involved in SH = SS interaction.

Sugar and other organic compounds were found to protect proteins from desiccation injury (Todd, 1972). As shown in Table X, RNase with SS groups was protected slightly by 0.5 M sucrose from desiccation damage, whereas glucose-6-phosphate dehydrogenase was not. The mannitol treatment was without effect. It is not known why the sucrose was inhibitory on the glucose-6-phosphate dehydrogenase activity when kept

together with the enzyme in the solution state. According to Levitt (1966), RNase should have an interaction or coagulation during the desiccation, because the enzyme has 4 SS groups. If removal of water from the protein allowed for new interactions of chemical groups either within the same protein molecule or between different molecules, forming hydrophillic or hydrophobic bonds (Todd, 1972), this coagulation or inactivation caused by the desiccation could be prevented by the presence of sucrose molecules in the medium. Sucrose would associate with hydrophillic groups of RNase molecules, thereby preventing close spatial juxtaposition of hydrophillic or hydrophobic bonds of the same or different macromolecules.

The relation between SS = SH interaction theory and the stability of RNases in general strongly indicate that RNase molecules in the plant cell are not destroyed or denatured easily during water stress, while other proteins and enzymes can be inactivated. Even if the molecules of RNase are severely dehydrated, the enzyme will be prevented from the denaturation or inactivation in the presence of the organic compounds such as sucrose in the cell. Without de novo synthesis, RNase activity will rise during water stress, because of the disappearance of inhibitors.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. The crude homogenate from wheat plants grown under water deficit hydrolyzed RNA slightly more rapidly than that from the normal, watered plant. The hydrolyzing capacity per mg protein of the preparation was also higher in the droughted plant than that in the normal plant.

2. The ribonuclease from the control and water stress treatments were partially purified by using ion exchange chromatography (phosphocellulose and Cellex D) to exclude factors other than RNase. The degree of purification was estimated by comparing the specific activity of RNase before and after each step of the purification procedure. The specific activity, enzyme activity per mg protein, increased during the purification. Specific activities of RNases from the control and water stress treatments were compared at each step, and this parallel purification revealed higher specific activities at every step, when the enzyme was extracted from the droughted plant.

3. The ribonuclease from droughted plants and normal plants exhibited a similarity with respect to their affinity to ion exchange chromatography. This similarity was also shown during polyacrylamide gel electrophoresis and gel filtration using Sephadex G-75. These facts indicate that RNase from the droughted plant has the

same molecular characteristics as the enzyme from the intact wheat plant and that the water stress treatment does not produce different kinds of isoenzymes in the wheat plant tested.

4. Particulate and soluble RNases were separated by using ultracentrifugation at 105,000 X g. The optimum activity of soluble RNase was at pH 5.2 and that of particulate RNase was at pH 6.2. The total RNase activity and the specific RNase activity of particulate and soluble fractions were higher in the droughted plant than that in the control plant. Thus, both the particulate and soluble RNase activities increase during water stress, and the water stress treatment does not cause a distributional change in RNase activity.

5. Wheat plants were incubated with C^{14}-leucine before starting water stress to investigate the possibility of de novo synthesis of RNase during water stress. The radioactivity could not be detected on the peak of RNases from the control and water stress treatments, when the enzyme was extensively purified by gel filtration on Sephadex G-75. This incorporation study implies that water stress treatment does not cause de novo synthesis of RNase different from the normal growth.

6. Activities of partially purified RNase, which were eluates of Cellex D chromatography, were measured in the presence of crude boiled extracts from control and droughted plants. The crude extract from the control plant inhibited RNase activity more than that from the water-stress plant. It is possible that the increase in RNase activity during water stress is partially due to the disappearance of substances inhibiting RNase activity.

7. Highly purified enzymes, purchased from commercial sources, were dried at 25 C, 4 C, and -4 C. Enzymes such as dehydrogenases completely lost their activities after 7 days of desiccation, regardless of drying temperature. Enzymes involved in hydrolysis, such as papain and pancreatic RNase, did not lose all activity. The desiccation at a higher temperature (25 C) promoted the loss of activity. In the case of pancreatic RNase, more than 20% of the original activity remained after the desiccation. Furthermore, the loss of RNase activity during desiccation could be prevented to some extent by the presence of sucrose in the medium.

8. It is, therefore, concluded from this study that RNases from the normal and droughted wheat plants are homogeneous and that the increase in RNase activity during water stress is due to the disappearance of inhibitors and/or the activation of pre-formed enzyme. Without de novo synthesis, the enhanced RNase activity can be maintained during water stress, because of its stability to the desiccation.

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