POLYRIBOSOME CONTENT AND IN VITRO PROTEIN SYNTHESIS STUDIES OF EXTRACTS FROM PLANT TISSUES UNDERGOING WATER STRESS

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I hereby recommend that this dissertation prepared under my direction by Patsy Ruth Rhodes

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be accepted as fulfilling the dissertation requirement for the degree of DOCTOR OF PHILOSOPHY

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As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

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ABSTRACT

Water status measurements of green tissues from a variety of plants were made using the Campbell J-14 press after 30 to 60 minutes exposure of the roots to nutrient media containing different levels of salt. In all species tested, values obtained with the press were proportional to the solution water potential and correlated well with relative water content. Studies with cotyledons of 3-day-old pumpkin seedlings indicated that changes in press value could be detected within 5 minutes of exposure to salt- or desiccation-induced stress and that the press values continued to increase for 20 to 30 minutes before a plateau value was reached.

The polyribosome contents of tissues from a number of plant species were obtained following short duration water stress. Slight reductions in the polyribosome content of 3-day-old pumpkin cotyledons were detected after 10 minutes exposure to saline nutrient media with an osmotic potential of -4 bars but more pronounced changes were found after 30 minutes. In experiments with pumpkin and pea seedlings, the polyribosome contents of cotyledon and shoot tissues, respectively, were significantly lower in tissues allowed to dry for 30 minutes than in control tissues. Reduced polyribosome percentages were also noted

in salt-stressed tissues of some corn and barley varieties. Occasionally little or no change in polyribosome percentage was found in salt-stressed tissues and reasons for this are discussed.

Growth rates of light-grown tissues from several plant species subjected to salt-induced water stress were determined. Experiments with pumpkin and pea tissues showed that both polyribosome percentages and growth rates (measured over a 24 hour period) were reduced in proportion to the increasing press values. Finding a 0.18 and 0.20 cm/day reduction in growth rate per 1% decrease in polyribosome content of pumpkin and pea, respectively, suggested that a constant proportion of the polyribosome population in rapidly expanding tissues may be committed to growth-related processes but preliminary evidence from other species did not support this hypothesis. Peas were more sensitive to stress than pumpkins in that polyribosome levels and growth rates of pea tissues were reduced twice as much per unit of water loss as those of pumpkin; and the implication that drought-sensitivity may be related to polyribosome reduction was discussed. To more closely relate growth rates to polyribosome percentages a photographic method which detected changes in growth over 10 to 15 minute periods was devised.

In order to understand how polyribosome levels may be reduced by water stress, polyribosome profiles were

carefully analyzed for evidence of a preferential loss of a particular class of polyribosome but the data were inconclusive. A wheat germ *in vitro* protein synthesis system was then utilized to identify any soluble supernate or ribosome-bound factor which was sensitive to water stress. In studies which recombined ribosome-free wheat germ supernate with ribosomes extracted from control and stressed pumpkin or pea tissues, the protein synthetic activity of the ribosomes was usually low showing a variable response to added messenger RNA and no consistent differences between control and stress ribosomes. Reconstitution experiments with supernatant fractions from control and stressed plants were also inconclusive. Suggestions for improving the reconstitution studies as well as an analysis of their potential were presented.

INTRODUCTION

The detrimental effects of plant water deficits on growth rates and crop yield are well known (Hsiao 1973, Hsiao et al. 1976), but an integrated explanation of the processes responsible for reduced growth is lacking. In many previous studies, measurements of metabolic parameters were made without concern for the duration or degree of stress imposed on the plant tissue. Consequently, a variety of metabolic processes have been found to be affected in some way by water stress, and a number of explanations about how water deficits affect growth have been proposed. Few attempts, however, have been made to test the validity of the various proposals.

Hsiao (1973) was able to classify certain metabolic effects of water stress in terms of their sensitivity to water deficits. He identified three degrees of water stress: mild stress in which the plant water potential (ψ) was reduced by a few bars or where there is as much as a 10% reduction in relative water content (RWC), moderate stress in which ψ was reduced by more than several but less than 12 or 15 bars or where there is a 10% to 20% reduction in RWC, and severe stress where ψ or RWC are reduced by more than 15 bars or 20%, respectively. With brief periods of mild stress, Hsiao argued that altered

turgor pressure appears to be the controlling component of ψ; thus metabolic processes affected by these mild conditions must be most sensitive to turgor. A second component, osmotic potential, ψ_π, may become important with severe stress or with long periods of exposure to water deficits. Under these conditions ψ_π adjustments (Hsiao et al., 1976) of plant tissues may in turn regulate extensive secondary alterations in metabolism. In order to understand how growth may be regulated and to avoid problems of interpretation due to osmotic adjustment, studies should be made of the sequence in which metabolic events are altered during development of mild to moderate water stress. Presumably metabolic processes which are altered most rapidly by mild stress in many plant species may be correlated into one unified hypothesis in which water stress acts through one or more primary steps to regulate growth.

Changes in growth rate are known to occur very rapidly upon alteration of plant water status. Acevedo, Hsiao, and Henderson (1971) found that when corn seedlings were first stressed, then relieved of stress, an increase in growth rate of leaves occurred within seconds. Maintenance of a new growth rate must be accompanied by rapid adjustments in metabolism, and the most rapidly detected alteration in metabolism in response to water stress has been that of protein synthesis. Detectable

changes in polyribosome content of etiolated corn leaves were obtained by Hsiao (1970) within 35 minutes of exposure to mild stress. Subsequently, Hsiao (1973) and Dhindsa and Bewley (1976) reported altered polyribosome percentages within 15 minutes following exposure to water stress conditions. Results with tissues of corn (Morilla, Boyer, and Hageman 1973; Nir, Poljakoff-Mayber, and Klein 1970), black locust (Brandle et al. 1973), squash (Cocucci, Cocucci, and Treccani 1976), and barley aleurone layers (Armstrong and Jones 1973) also show that water stress reduces the level of polyribosomes but none of these studies were designed to test for rapid changes in polyribosome percentage.

This study was initiated to establish whether polyribosome levels in a variety of higher plants would be reduced by water stress and to determine how quickly such a reduction could be detected. Since little is known about how growth is reduced by water stress, efforts were made to obtain measurements of polyribosomes, growth, and plant water status during the first 30 minutes of exposure to mild stress conditions and to examine possible relationships among these parameters.

It seems reasonable that when changes in a complex metabolic process like protein synthesis are detected this rapidly in response to water stress, protein synthesis may be closely linked to rapid changes in growth or in other

metabolic processes through any of a number of components which regulate protein synthesis. Therefore examinations were made of extracts from stressed and unstressed tissues to see if ribosome-bound or soluble supernatant factors could be recognized in terms of their sensitivity to water stress. For these studies, a wheat germ *in vitro* protein synthesis system was developed and plant tissue extracts were tested for their effect on *in vitro* protein synthesis.

LITERATURE REVIEW

Cell Growth

Cell elongation appears to be the plant process that is most susceptible to water deficits (Hsiao et al. 1976). Acevedo and others (1971) found that corn leaf elongation rates could be rapidly reduced with as little as a 1 bar reduction in the ψ of the rooting medium. Also within seconds after water stress was relieved, the growth rates were found to equal or surpass the prestress rate. More recently, Sands and Correll (1976) demonstrated rapid growth responses to water deficits in pine needles and wheat leaves. Although the magnitude of the growth rate reduction was less than that of corn, these tissues responded to small ψ changes. Similar results using 0.7 atm increments in cell turgor pressure (Green, Erickson, and Buggy 1971) were obtained from an *in vivo* study of cell elongation in a coenocytic green alga, *Nitella*. Based upon a complex analysis of cell extension in this organism, the authors concluded that two parameters--a mechanical stretch due to turgor and an undefined metabolic process--were involved in growth. Whatever the metabolic process may be, it somehow readjusts the cell so that altered turgor pressures are translated into new rates of growth.

Identifying this metabolic process, determining how it is altered, and how its alteration can be linked to other metabolic changes occurring in water-stressed plants has been the goal of plant physiologists for some time. This search has been guided to some extent by the ranking (Hsiao 1973, Hsiao et al. 1976) of various metabolic processes in terms of their sensitivity and rate of response to water stress conditions. On this basis, new rates of cell elongation, established in response to changes in plant water status, appear to be followed by rapid alterations in the synthesis of cell wall material and protein. Though little work has been done on the effects of water stress on cell wall synthesis, protein synthesis has been examined in a wide variety of plant systems undergoing water stress.

Protein Synthesis

Water stress has been reported to inhibit the incorporation of radioactive amino acids into proteins of excised corn root tips (Nir et al. 1970) and tobacco leaves (Ben-Zioni, Itai, and Vaadia 1967) and to cause a decrease in the total protein content of sugar beet, wheat, and tobacco leaves (Shah and Loomis 1965, Stutte and Todd 1969, Ben-Zioni et al. 1967). The protein population of any tissue, however, is a dynamic one in which synthesis and degradation of most proteins is a continuous process,

and studies which measure net protein synthesis or net protein content cannot distinguish between these two processes. Furthermore, these studies did not look for changes in protein metabolism occurring rapidly after stress was imposed.

Although unable to distinguish between synthetic and degradative processes, Dhindsa and Cleland (1975) demonstrated that differences in radioactive amino acid incorporation into proteins could be detected soon after stress was imposed. In these studies Avena coleoptile sections were floated on solutions of ^{3}H-leucine with or without 0.3 M mannitol for various periods of time. Coleoptiles harvested 30 minutes after exposure to these solutions showed that unstressed tissues incorporated more amino acid into protein than tissues exposed to the mannitol solution, and these differences became more apparent as time passed. These workers were also able to demonstrate that water stress affected the synthesis of some proteins more than others. By using a double label technique in which the solution without mannitol contained ^{14}C-leucine while the one with mannitol contained ^{3}H-leucine, tissues were again floated on the solutions and harvests were made 3 hours later. Proteins from each treatment were combined and separated on polyacrylamide gels which were then sliced, and the ^{14}C:^{3}H ratio of each section was determined. These ratios varied widely from

slice to slice whereas ratios from double label studies in which only unstressed conditions (and therefore the same relative proportions of protein) were used did not change appreciably throughout the gel. Had all proteins been equally affected by water loss, the ratios would have been constant and higher than the unstressed ratio due to reduced net incorporation of ^{3}H-leucine in the stressed tissue.

Nitrate Reductase

The studies by Dhindsa and Cleland (1975) were not the first to suggest that there were qualitative differences in the protein content of stressed and unstressed tissues. Differential effects of water stress on enzyme activity have been known for some time and results of several studies were compiled by Todd (1972). Differences in enzymatic activity, however, cannot distinguish between synthesis and degradation of proteins nor resolve the possibility that effects of water stress on substrate concentrations could account for the reduced activity. One enzyme that has been singled out for study, nitrate reductase, is inducible by nitrate (Beevers and Hageman 1969). The activity of nitrate reductase is also known to be sensitive to mild stress (Morilla et al. 1973) and correlates well with leaf water potential (Huffaker et al. 1970). Thus this enzyme should be a useful tool for

studying the effects of water stress on protein synthesis and substrate supply.

Using shoots of desiccated corn seedlings or well-watered corn seedlings treated with cycloheximide, Morilla et al. (1973) could find no differences in the rate of decay of nitrate reductase. They did find, however, that the polyribosome content of desiccated corn shoots decreased to 58% of the polyribosome content in well-watered seedlings after 1 hour of stress. Since both ribonuclease and nitrate reductase activities had not changed after even 1.5 hours of desiccation, the polyribosome content was considered to be reflective of decreased protein synthesis activity and to have preceded changes in the enzyme activity. The recovery of nitrate reductase activity during rewatering was also shown to be dependent on protein synthesis (Morilla et al. 1973, Shaner and Boyer 1976, Plaut 1974).

More recently, Shaner and Boyer (1976) found that increasing the supply of nitrate to the leaf by tripling the amount present in the rooting medium, increased the amount of nitrate reductase in corn leaves undergoing water stress. These results are not surprising, since this is an inducible enzyme, and experiments conducted over a number of days like this may be expected to involve interactions of nitrate supply, protein synthesis, age, and environmental factors. Since Morilla et al. (1973) found that

nitrate levels in leaves were not correlated with the reduction in nitrate reductase activity, these results cannot be used to indicate that water loss alters nitrate reductase synthesis by reducing the level of nitrate as opposed to reducing the level of protein synthesis. Finding that protein synthesis may be reduced prior to detectable changes in enzyme activity (Morilla et al. 1973) suggests that the components involved in synthesis of enzymes rather than the activity of those enzymes should be investigated.

Polyribosome Content

Studying the effect of water stress on the polyribosome content of etiolated corn shoots, Hsiao (1970) determined that reductions in polyribosome levels were rapid and sensitive to very small changes in tissue water potential. With mild stress he was able to detect reductions in polyribosome percentages within 35 minutes and, in subsequent studies, within 15 minutes of exposure to the stress conditions. Rapid changes in polyribosome levels have also been noted for the moss Tortula ruralis (Bewley 1973, Dhindsa and Bewley 1976, Gwozdz and Bewley 1975), and like Morilla et al. (1973), Dhindsa and Bewley (1976) were able to show that these changes occurred at least 45 minutes before increases in ribonuclease activity were detected. A number of studies conducted with higher

plants such as black locust (Brandle et al. 1973), corn (Morilla et al. 1973, Nir et al. 1970), and squash (Cocucci et al. 1976) also demonstrated that water stress reduced the polyribosome level in these tissues, but unfortunately none of these studies were designed to determine how quickly such changes occurred.

Lacking a quantitative or temporal relationship between alterations in polyribosome content and ribonuclease activity, these reductions in polyribosome percentage were thought to reflect events occurring within stressed tissues. In studies with barley aleurone layers imbibed for 14 hours in the presence of gibberellic acid, Armstrong and Jones (1973) were able to show that exposure to 0.6 M polyethylene glycol for 2.5 or 5 hours resulted in decreased polyribosome percentages. In addition they obtained electron micrographs which suggested that the reductions in polyribosome percentage were accompanied by a lower concentration of ribosomes along the rough endoplasmic reticulum. Counting the number of ribosomes per given length of endoplasmic reticulum, they were able to quantitate this relationship so that fewer ribosomes per unit length were associated with smaller polyribosome percentages. Effects of stress on the attachment of ribosomes to the rough endoplasmic reticulum were also noted by Nir et al. (1970) but attempts to quantitate the loss of membrane-bound ribosomes by these authors were not

made. With evidence that these altered polyribosome percentages are indicative of events within the cell, reasons for this effect on the polyribosome population should be determined so that this metabolic response may be related to other metabolic effects and growth responses of water stressed plants.

HeLa Cell Response to Hypertonic Media

The mechanism by which water stress reduces the polyribosome content of plant tissues is unknown, but evidence that water loss may reduce the rate of initiation in mammalian cells has been provided by Saborio, Pong, and Koch (1974). For this study HeLa cells grown in culture media containing 11 mM NaCl were exposed to ^{35}S-methionine and an additional 100 mM NaCl. Under these conditions amino acid incorporation into cellular proteins was reduced by 100% while incorporation into poliovirus proteins of poliovirus-infected cells was reduced by only 40%. With even greater levels of NaCl the synthesis of viral proteins could be reduced further, and this differential sensitivity to stress was used to investigate the suppression of viral protein synthesis *in vivo*.

The poliovirus mRNA is polycistronic, and its primary protein product is cleaved to yield 7 peptides. Knowing the sequence of these peptides with respect to the 5' end of the mRNA from mapping studies, Saborio et al.

(1974) reasoned that if peptide-chain initiation were selectively inhibited by exposure to 150 mM NaCl, then the effect should be seen in the peptide coded by sequences near the 5' end and proceed toward the 3' end of the message. To test this, HeLa cells were pulse-labelled at three minute intervals and the polypeptides were examined after a 30 minute chase in unlabelled media. The incorporation of ^{35}S-methionine was reduced in the 5'-terminal peptides before all other peptides and the reduction in amino acid incorporation into the other peptides was found to progress toward the 3' end with subsequent pulse-labeled intervals. Similar pulse-labelling studies in which HeLa cells were incubated for 40 minutes in 150 mM NaCl before transfer to 100 mM NaCl, showed that the incorporation of radioactive amino acid appeared first in the 5' peptide and progressed toward the 3' terminal peptide. The exposure of HeLa cells to these saline conditions also resulted in a decrease in the polyribosome content while decreasing the level of NaCl produced an increase in polyribosomes.

Having established that hypertonicity inhibits initiation in cultured mammalian cells, the work of Saborio et al. (1974) also showed that hypertonicity selectively suppresses initiation complex formation of host proteins more than viral proteins. In subsequent studies, Nuss and Koch (1976a) demonstrated that light and

heavy chains of immunoglobulin G (IgG) were more resistant than non-IgG proteins to as much as 140 mM NaCl. Differential effects of hypertonicity among host proteins were also noted by England, Howett, and Tan (1975) who found at least two histones coded by the host genome to be synthesized along with viral proteins under hypertonic conditions. Similarly, selective inhibition of two of the five proteins synthesized from vesicular stomatitis virus mRNA at 100 mM NaCl was detected when salt levels were increased to 250 mM NaCl (Nuss and Koch 1976b).

The mechanism of selective suppression of host and viral protein synthesis by NaCl is unknown. Nuss, Oppermann, and Koch (1975) have suggested that viral and certain host polyribosomes may be more efficient than other polyribosomes in forming initiation complexes, but whether this reflects differences in species of mRNA or in intermediate initiation factors remains to be tested. Although it may be tempting to speculate that initiation is the process affected by water stress in plant tissues, inferences about the mechanism of polyribosome reduction in water-stressed plants based on these findings would be tentative at best.

Mechanism of Water Stress-Induced Polyribosome Reduction in Plants

Dhindsa and Cleland (1975) were able to demonstrate qualitative differences in protein synthesis of water-stressed plants; but as with mammalian cells, the mechanism of selective effects of water stress on various proteins is unknown. Dhindsa and Bewley (1976) have suggested that polyribosomes of rapidly and slowly dried moss decrease because ribosomes fail to reinitiate. In support of this idea, they offer a correlation between decreased polyribosome levels and decreased ability of the ribosomes to incorporate ^{3}H-puromycin into peptides *in vitro* implying that fewer ribosomes were associated with mRNAs.

One factor which should be a good candidate for regulation of protein synthesis under water stress conditions is ATP (adenosine triphosphate) levels or energy charge (EC) defined by Chapman, Fall, and Atkinson (1971) as:

$$EC = \frac{ATP + 1/2\ ADP}{ATP + ADP + AMP}$$

where AMP and ADP are adenosine mono- and di-phosphates respectively. ATP levels as well as EC values are known to be reduced in stressed pea seedlings (Hassan-Porath and Poljakoff-Mayber 1971) and in proportion to the leaf water potential of corn seedlings (Barlow, Ching, and Boersma

1976). Furthermore EC has been related to growth in a number of organisms (Chapman et al. 1971), and would provide a ready link to other metabolic processes affected by stress. While all these factors look promising, the only study in which stressed tissues were analyzed for EC values within 30 minutes of stress (Wu 1973) and which showed fluctuations in the ATP content within 10 minutes of exposure to stress was not repeatable (Matsuda 1977). And since Bewley and Gwozdz (1975) concluded that ATP and EC values were correlated with protein synthesis only during rehydration of desiccated moss, the ATP content does not seem to be regulating the reduction of polyribosomes in stressed plants.

The content of GTP in stressed tissues has not been measured, however, and may well be sensitive to water stress. In vitro assays have shown that though GTP (guanosine triphosphate) is required for both initiation and elongation processes, the formation of the initiation complex is more sensitive to changes in GTP:GDP (guanosine diphosphate) ratios than was the elongation reaction (Walton and Gill 1976). EC also seemed to modify the GTP:GDP regulation of initiation in vitro. It is not difficult to see that altered nucleotide pools could regulate total protein synthesis, but how such a mechanism could result in the differential effects on protein synthesis noted in water

stressed Avena coleoptiles (Dhindsa and Cleland 1975) is difficult to envision.

The philosophy, which has evolved since the initial determination that protein synthesis was reduced during drought, is aimed at discovering why polyribosomes, and hence protein synthesis, are reduced by water loss. Workers have begun to realize that a meaningful approach to this problem must involve the study of rapidly altered processes which are sensitive to slight changes in plant water status. These alterations must occur consistently in many plants and must be proportional to altered growth rates if such data are to be useful in understanding how growth is reduced by water stress.

MATERIALS AND METHODS

Plant Material

Seedlings of various plants were used in these studies. Polyribosomes and water status determinations were obtained from cotyledons of 3- and 4-day-old pumpkin, Cucurbita pepo L. var. Small sugar; shoots of 5- to 8-day-old peas, Pisum sativum L. var. Alaska; and leaves of 5-day-old corn, Zea mays L. var. Double dent and Golden cross bantam, and barley, Hordeum vulgare L. var. Hi Proly, N-70, Arivat, Chevron, and California Mariot. Growth rate determinations were also made by measuring changes in shoot heights of the above seedlings. Barley varieties were supplied by Dr. Robert T. Ramage (The University of Arizona, Tucson); all other seeds were obtained from W. Atlee Burpee Co. (Riverside, California).

Pumpkin seedlings were grown in vermiculite at 36 C $\pm$ 1 C with a 12-hour photoperiod from 8:00 a.m. to 8:00 p.m. (800 ft-c, Sylvania Gro Lux). All other seedlings were grown at 26 C with a 13-hour photoperiod from 6:45 a.m. to 7:45 p.m. (2000 ft-c, fluorescent and incandescent lamps). Two to 4 hours prior to each experiment vermiculite was washed from the roots and the seedlings were transferred to aerated modified Hoagland nutrient solution

(Hoagland and Arnon 1938) consisting of 1 ml 1 M $MgSO_4 \cdot 7H_2O$, 1 ml 1 M KNO_3, 1 ml 1.5 M $Ca(NO_3)_2$, 1 ml 1 M KH_2PO_4:1 M K_2HPO_4 (1:1), 1 ml of micromix (3.75 g H_3PO_3, 2.25 g $MnCl_2 \cdot 2H_2O$, 75 mg $CuCl_2 \cdot 2H_2O$, 75 mg MnO_3, 0.33 g $ZnSO_4$, made to a volume of 3 liters), and 15 mg Chel 138 (Geigy) for each liter of solution.

Determination of Plant Water Status

Measurements of tissue water status were made using the Campbell J-14 press (Campbell Scientific, Logan, Utah, 84321). For this procedure, a single cotyledon or leaf was cut at its base and placed on a flexible membrane situated atop a hydraulic jack. Pressure was then applied quickly until water was exuded from the uncut edges of the tissue. Normally as many as 10 press values could be obtained per minute and all water status measurements accompanying the polyribosome studies were completed within 3 minutes of the time of harvest.

Since the Campbell J-14 press is a relatively recent device, tests were conducted to insure that press values could be correlated to another measurable expression of plant water status, the relative water content (RWC). For these studies plants were exposed in light to nutrient solutions containing NaCl equivalent to 0, -2, -4, and -8 bars osmotic potential (Lang 1967). After 1 hour, press values were obtained for six separate tissues.

Next, fresh weights (FW) of four replicate populations of plants were measured. The weighed tissues were then allowed to imbibe water from wet Kimtowels (Kimberly-Clark) for 3 hours, blotted, and reweighed to determine saturated weights (SW). These tissues were later dried at 70 C overnight for dry weight (DW) determinations. The RWC was calculated as

$$RWC = \frac{FW - DW}{SW - DW} \times 100.$$

Growth Rate Measurements

The height of the hypocotyl (pumpkin), epicotyl (pea), or first leaf (corn and barley) was measured with a ruler just prior to adding NaCl to the nutrient solution and again 3 to 4 hours or 24 hours later. In order to detect more rapid growth changes, barley seedlings were aligned against a black background with grid markings spaced 1 cm apart for reference, and the roots were bathed in 500 ml of aerated nutrient solution in a container which could be drained and refilled in 1 minute. A Honeywell Pentax Spotmatic camera equipped with Vivitar No. 1, 2, and 3 close-up lens and mounted on a tripod was used to take a series of 4 photographs at 10 to 15 minute intervals to establish growth rates for seedlings in the control medium. After draining and refilling the tank with saline nutrient solution, another series of photographs was

taken to measure growth rates during stress. Finally, the saline solutions were replaced with control solutions and photographs were taken to determine growth rates during recovery from water stress. The Kodak Plus-X or Tri-X film was developed according to specifications (Eastman Kodak) and the image was focused onto a rigid, white screen where changes in plant height were measured in relation to the markings on the grid paper. In this manner the seedling could be magnified 20 to 40 times making it relatively easy to detect changes in growth within 10 minutes after stress was imposed.

Polyribosome Preparation and Analysis

Plants equilibrated for 2 to 4 hours in nutrient solution were stressed by adding sodium chloride (Lang 1967) to the nutrient solution or by blotting the roots and holding the seedlings in their lighted growth chambers. Tissues were harvested, frozen immediately in liquid nitrogen, and stored at -45 C. Usually the polyribosomes were extracted and analyzed on the same day; but when there were many replications, or when the ribosomes were needed for *in vitro* protein synthesis, polyribosome pellets were frozen and stored in liquid nitrogen until the next day. Polyribosomes could be stored in this fashion for several weeks without affecting the ribosome percentages or protein synthetic activity.

Extraction from Tissue

For higher yields, tissues were ground to a powder with liquid nitrogen in a mortar and pestle just prior to extracting polyribosomes. The buffers used for homogenization (buffer 1: 0.25 M sucrose, 0.2 M tris-HCl, 60 mM KCl, 30 mM $MgCl_2$, pH 8.5) and gradient preparation (buffer 2: 40 mM tris-HCl, 20 mM KCl, 10 mM $MgCl_2$, pH 8.5) were those of Davies and Larkins (1972). Approximately 1 g of powdered or whole tissue was homogenized in an ice-cold mortar and pestle with a total of 10 ml buffer 1 containing 3% (v/v) Triton X-100 (Rohm and Haas). When polyribosomes were to be used for in vitro protein synthesis, ribosomes were extracted with and without adding 3% (v/v) Triton X-100 to the grinding medium. The homogenate was centrifuged at 30,000 g (max) in a Sorvall SS-34 rotor for 10 minutes at 0 to 4 C and the ribosomes present in the supernatant were fractionated in various ways. The polyribosomes were usually partially purified by layering the 30,000 g supernatant over a 3 ml cushion of 1 M sucrose in buffer 2 and centrifuging at 200,000 g for 2 hours in a Beckman Ti-50 rotor held at 2 to 4 C. Initially sedimentations were conducted at 100,000 g for 2 hours but this resulted in excessively high polyribosome percentages due to partial recovery of the monoribosomes (Leaver and Dyer 1974). A second isolation technique was employed in which the ribosomes were precipitated from the

30,000 g supernatant by rapidly adding 1 M acetic acid to pH 5.1 (Rhodes and Matsuda 1976), centrifuged quickly, and resuspended in 4 ml buffer 1. This technique produced profiles comparable to the centrifuged preparations only from pumpkin cotyledons, and the acid-precipitated ribosomes were not used for in vitro protein synthesis. In early experiments, unpurified crude polyribosome preparations were layered directly onto a sucrose gradient for analysis, but contamination by UV-absorbing material at the top of the gradient often obscured the resolution of the monoribosome peak.

Resuspension of the Ribosome Pellet

Unless ribosomes were to be used for in vitro protein synthesis, the polyribosomes were resuspended in 1 to 3 ml of homogenization buffer (Rhodes and Matsuda 1976). For use in the wheat germ protein synthesizing system, the polyribosomes, sedimented through a 1 M sucrose pad, were resuspended in: (1) HM buffer (70 mM HEPES-KOH, 5 mM MgAc (magnesium acetate), pH 7.4); (2) HMK buffer (70 mM HEPES-KOH, 5 mM MgAc, 0.277 M KCl, pH 7.4); (3) H2MK buffer (70 mM HEPES-KOH, 10 mM MgAc, 0.277 M KCl, pH 7.4); or (4) H2M2K buffer (70 mM HEPES-KOH, 10 mM MgAc, 0.554 M KCl, pH 7.4). The A_{260} units per ml were determined for each sample before the ribosomes were dropped from 30 µl capillary pipets into

liquid nitrogen. The frozen beads were gathered into a 0.5 ml polystyrene beaker (Scientific Products), capped with aluminum foil, and stored in liquid nitrogen.

Analysis of Polyribosome Percentages

Polyribosome profiles were obtained from discontinuous sucrose gradients as described by Rhodes and Matsuda (1976), and monitored at 254 nm with an ISCO model UA-2 UV analyzer. Polyribosome percentages were calculated as 100 times the area of the polyribosome region divided by the total area of the ribosome profile.

Wheat Germ Cell-Free System for Protein Synthesis

Preparation of the wheat germ extract was as described by Marcu and Dudock (1974). Among several commercial sources of refrigerated, raw wheat germ tested for their ability to transcribe TMV RNA (tobacco mosaic virus ribonucleic acid), only two were found to be active: (1) Health Best, San Juan Capistrano, Escondido, California, 92025, and (2) Bonnie Brand, Niblach Natural Foods, 3282 Monroe Avenue, Rochester, New York, 14618. Using the storage method of Roberts and Paterson (1973), the wheat germ extracts were dispensed from sterile pasteur pipets into petri dishes containing liquid nitrogen. The frozen drops were scooped with sterile spatulas into polypropylene scintillation vials; the vials were then capped and

suspended by a string into a Dewar flask filled with liquid nitrogen. With this means of storage, individual drops could be removed as needed for months without detectable loss of activity.

Required Assay Solutions

The following solutions were based on the reaction mixtures of Marcu and Dudock (1974) and Zaitlin (1976). Because of strict ion requirements for these assays, the amount of K^+ used for pH adjustment was recorded. The lyophilized powders of ATP (adenosine triphosphate), GTP (guanosine triphosphate), creatine phosphokinase, creatine phosphate, and DTT (dithiothreitol) were preweighed into 1 to 2 dram vials and stored at -20 C with desiccant. The amino acids (Nutritional Biochemicals Corporation) were provided by Dr. Konrad Keck, Department of Cellular and Developmental Biology, The University of Arizona; all other biochemicals were obtained from Sigma Chemical Company.

1. A stock solution of 140 mM HEPES-KOH reaction buffer, pH 7.4, was stored at -20 C until needed.
2. A solution of 10 mM ATP, 200 µM GTP, and 400 µg/ml creatine kinase was prepared immediately before use as follows: (a) 1.76 ml deionized water were added to 12.3 mg ATP and a miniature pH electrode (Leeds and Northrup) was used to monitor the

addition of approximately 40 µl 1.0 N KOH to pH 7.4, (b) 2.00 ml distilled water were added to 2.2 mg GTP, (c) 0.20 ml GTP (step b) were added to the ATP solution, and (d) 1.0 ml of the ATP, GTP solution was added to 0.4 mg creatine phosphokinase.

3. A solution of 80 mM creatine phosphate and 20 mM DTT was also prepared immediately before use as follows: (a) 0.76 ml distilled water were added to 4 mg DTT, and (b) 0.11 ml 40 mM DTT (step a) plus 0.11 ml distilled water were added to 4.4 mg creatine phosphate.

4. A concentrated stock solution was prepared containing 250 µmoles/liter distilled water of each of the following L-amino acids: serine, glutamic acid, histidine, valine, tryptophan, phenylalanine, tyrosine, threonine, methionine, isoleucine, lysine, cysteine, aspartate, arginine, asparagine, alanine, glycine, cystine, and proline or leucine. The labelled amino acid (proline or leucine) was not included in the stock mixture.

5. When necessary, enough unlabelled amino acid was added to ^{3}H-leucine, ^{14}C-leucine, or ^{3}H-proline to yield approximately 170 µmole/liter of that amino acid with 0.1 mCi or 0.05 mCi ^{3}H or ^{14}C per ml, respectively.

6. A stock solution of TMV RNA was prepared by extracting a suspension of TMV (the gift of Dr. Don Bourque, Department of Nutrition and Food Science, The University of Arizona) three times with an equal volume of water-saturated, redistilled phenol. The aqueous layer was mixed with 2 volumes 95% ethanol and kept at -20 C overnight. The precipitated RNA was then washed twice with ethanol and dissolved in 15 mM Sorenson's phosphate buffer, pH 7.0, at a concentration of 0.9 mg TMV RNA per ml.
7. A solution of 20 mM MgAc and 0.554 M KCl satisfied the ion requirements of wheat germ ribosomes. Ion solutions (alone or in combination with the HEPES reaction buffer) were used instead of water to make the ATP-GTP-creatine phosphokinase or creatine phosphate-DTT solutions when additional components were to be included in the reaction mixture.

Complete Assay System

The addition of 5 µl of each of the above seven solutions to 15 µl of wheat germ extract produced, in a final volume of 50 µl, the complete system containing the concentration of reactants used by Marcu and Dudock (1974) with the exception that 3.5 mM MgAc was found to be

optimal for these wheat germ preparations. The assays were incubated in 5 ml test tubes (8 x 75 mm) at 30 C and the reaction was essentially complete in 60 minutes. Ten µl aliquots of the reaction mixture were applied to glass fiber filter disks (Whatman GFA), and the disks were dropped into 30 ml test tubes containing 5 ml of 5% (w/v) TCA (trichloroacetic acid) at 90 C in order to deacylate transfer RNA. Fifteen minutes later the disks were washed twice with cold 5% TCA and once with ether:95% ethanol (2:1, v/v). The filters were dried and counted in 2.5 ml of toluene fluor [10 g PPO (2,5-diphenyloxazole), 0.5 g POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene), and 2 liters toluene] using a Packard 3320 Tri-carb scintillation counter.

Reconstituted Assay System

The wheat germ supernate was prepared by centrifugation of wheat germ extracts at 200,000 g for 2 hours in a Beckman Ti-50 rotor to pellet all ribosome monosomes and subunits. The ribosomes were resuspended in HM buffer (70 mM HEPES, 5 mM MgAc, pH 7.4) to a final volume that was 1/3 that of the original extract. The A_{260} units per ml were determined and the supernate and ribosome fractions were stored in liquid nitrogen as described. The reconstituted assay mixture was then composed of 15 µl wheat germ supernate, 5 µl ribosomes (either wheat germ

or seedling), and the remaining ingredients adjusted to yield 3.5 mM MgAc, 100 mM KCl, and 20 mM HEPES in a final volume of 50 µl for the assay mixture.

RESULTS AND DISCUSSION

In order to study biochemical alterations that occur during development of water stress, it was necessary to acquire a reliable method for rapidly measuring plant water status. The recently introduced Campbell J-14 press appeared to be ideally suited for these determinations; however, because the press was a fairly recent innovation, tests were conducted to assure that the values obtained were truly indicative of tissue water status. For these tests, several plants were exposed to nutrient solutions containing NaCl equivalent to 0, -2, -4, or -8 bars osmotic potential for one hour, then press values were obtained and compared with the RWC of the plant tissue and the solution water potential. In all species tested, the press values of green tissues were inversely related to RWC (Figure 1A) values and to the solution water potentials (Figure 1B). In a later study a similar inverse relationship was found for press values and solution water potentials when corn and barley seedlings were stressed for 1/2 hour (Table 1). Occasionally, relatively minor changes in the press values were found for tissues exposed to increasing solution osmotic potentials (e.g., the second Alaska pea experiment b', Figure 1B), but the RWC values for these same tissues were all quite high

Figure 1. The relationship of Campbell J-14 press value to RWC (A) and solution water potential (B) -- Seedlings of barley (a), pea (b), pumpkin (c), sunflower (d), and safflower (e) were in nutrient solutions containing NaCl at osmotic potentials of 0, -2, -4, or -8 bars for 1 hour prior to determination of press values and RWC as outlined in Materials and Methods. Statistical analyses of pea and pumpkin data are from two experiments except in B where pea experiments were treated separately (b, b'). Each point represents the mean of 4 replications for RWC and 6 replications for press value determinations. Except for curve b' in Figure 1B, correlation coefficients ranged from 0.83 to 0.98.

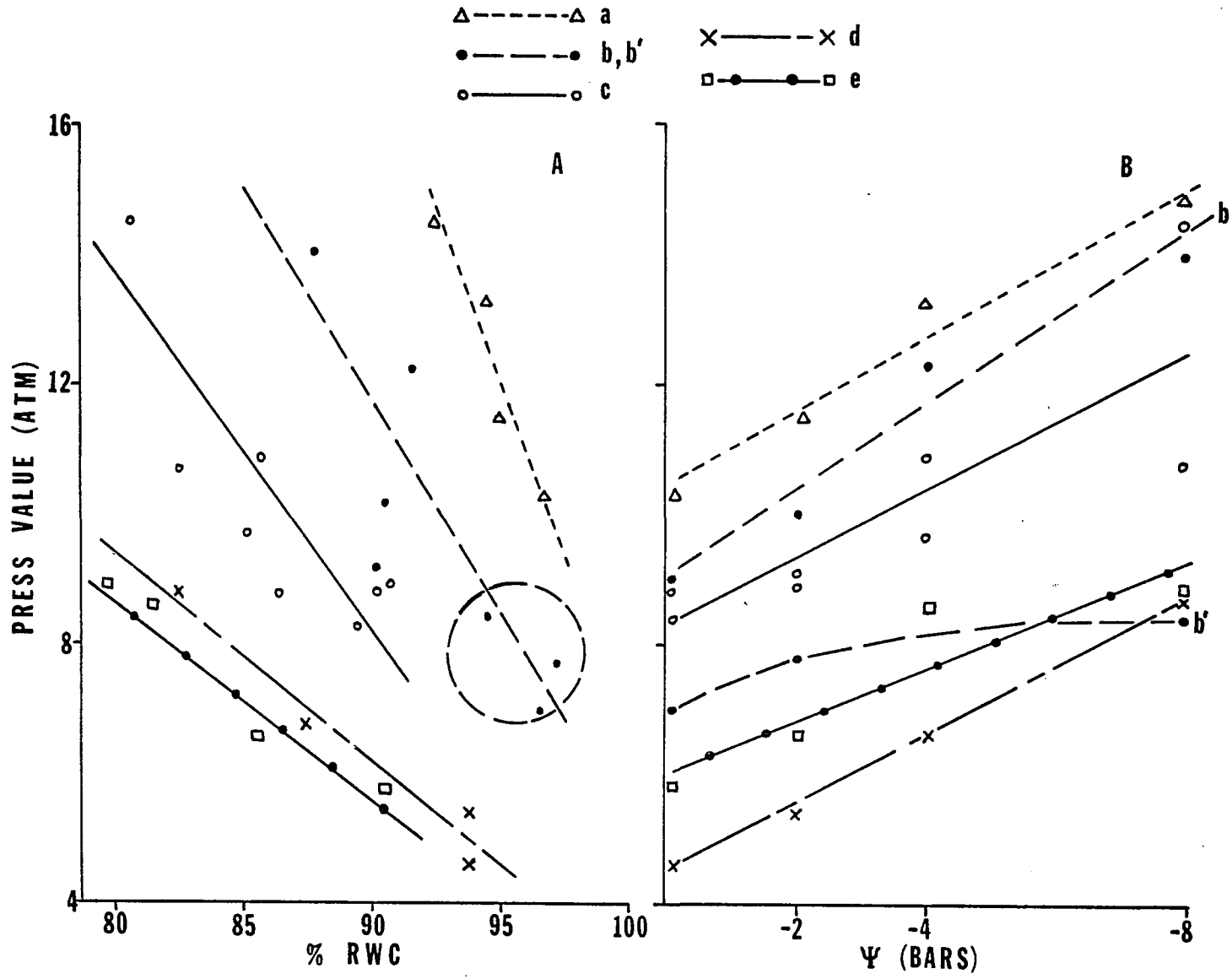

Figure 1. The relationship of Campbell J-14 press values to RWC (A) and solution water potential (B).

Table 1. The leaf water status of various plants exposed to saline nutrient media at the indicated osmotic potentials for 30 minutes -- Press values are the mean of 6 replications. Numbers in a row with the same subscript letters have overlapping mean ± standard deviation values.

Plant	Age (Days)	Solution Water Potential			
		0 bars	-2 bars	-4 bars	-8 bars
		Press Value (Atmospheres)			
Corn Varieties:					
Double Dent	7	6.70 a	--	9.06 b	11.77 c
Golden Cross	7	6.76 a	7.50 a	9.57 b	10.60 b
Barley Varieties:					
Hi Proly	5	7.03 a	8.00 b	8.90 b	9.27 b
	7	6.81 a	7.67 b	9.74 c	10.76 c
N-70	5	10.80 a	11.29 a	11.20 ab	12.76 b
Arivat	6	9.05 a	--	11.72 ab	12.06 b
Mariot	6	7.93 a	--	10.77 b	13.09 b
Chevron	6	8.44 a	--	9.13 a	13.87 b

(circled points, Figure 1A) further indicating that the press values reflect the tissue water status. Press values were not only simpler to obtain than RWC, but for some species (e.g., barley, Figure 1A) they were a much more sensitive indication of plant water status. Since values obtained with the Campbell J-14 press are also known to correlate well with water potential values measured by the pressure bomb (Campbell, 1975) when light-grown tissues are used, it was used for all further determinations of plant water status.

Once the degree of water stress could be quantified, experiments were performed to determine how quickly changes in tissue water status would occur in plants subjected to salt- or desiccation-induced stress. In Figure 2A, pumpkin roots were immersed in various NaCl solutions and press values were obtained from cotyledons harvested at the indicated times. With all treatments, the press values were found to increase over a 20 to 30 minute period reaching a plateau value after about 30 minutes. Both the rate of increase (0.1, 0.13, and 0.2 atm/minute for the -2, -4, and -8 bar solutions, respectively) and the press value at which the plants were equilibrated (-9.5, -10.5, and -15 atm, respectively) appear to be an exponential function of the solution osmotic potential.

Rapid increases in the press value were also seen when pumpkin roots were blotted and allowed to dry

Figure 2. Water status of pumpkin cotyledons during development of stress -- Roots of intact seedlings were immersed in nutrient solutions containing NaCl (A) or they were blotted and allowed to dry (B). Cotyledons were randomly selected from seedlings and press values were obtained at the indicated times. The -2 bar treatment of (A) was begun at 11:00 a.m.; all other treatments were initiated at 3:00 p.m. Each point and its respective vertical bar represents the mean ± standard deviation of 6 replications. Individual determinations of press values after 22 and 26 minutes drying are also shown for (B).

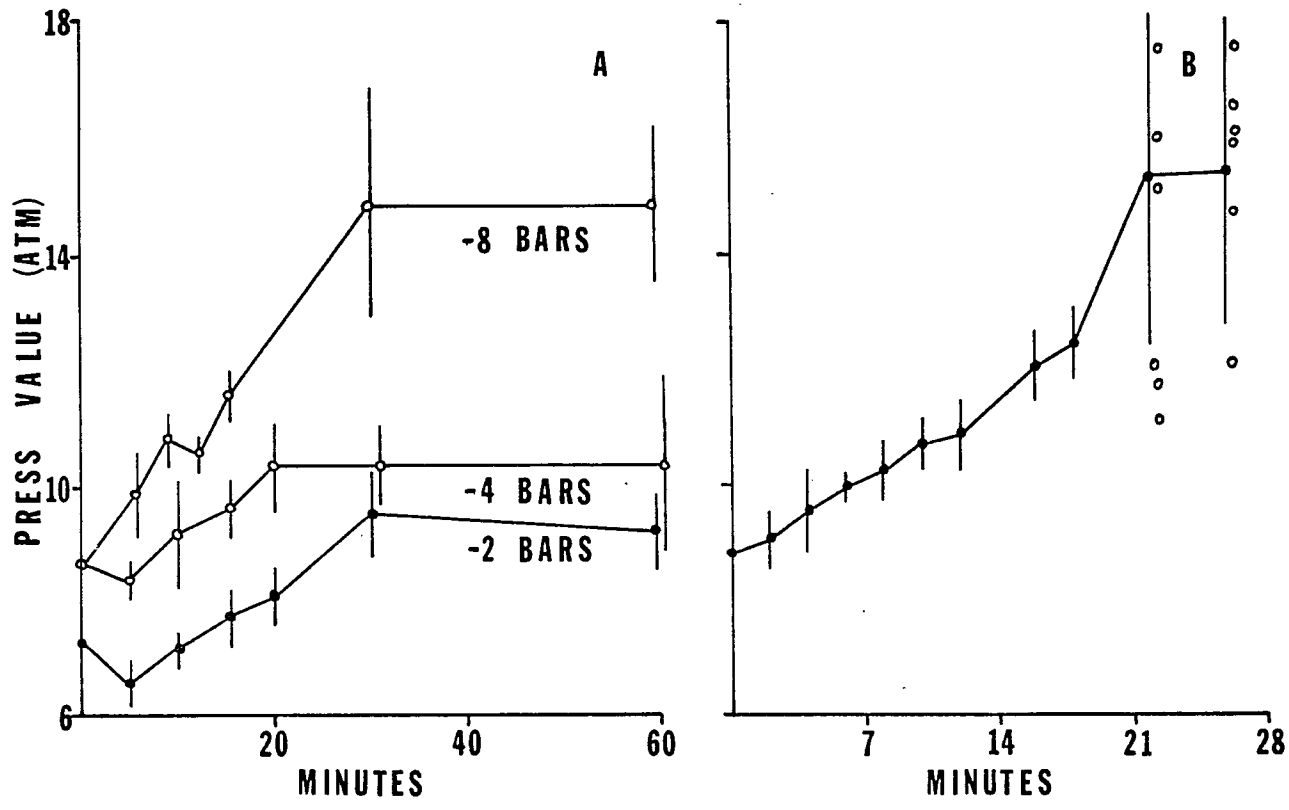

Figure 2. Water status of pumpkin cotyledons during development of stress.

(Figure 2B). Interestingly, in both Figures 2A and 2B the variances were quite large when mean press values reached about 15 atm. The individual values represented by open circles in Figure 2B suggest that sub-populations may exist which differ in their response to water deficits.

Polyribosome Content of Control and Stressed Plant Tissues

When plants are subjected to water deficits, growth rates are known to be reduced in proportion to plant water status (Hsiao 1973, Hsiao et al. 1976) and to be altered rapidly when plant tissues are stressed or when stressed tissues are rewatered (Green et al. 1971, Acevedo et al. 1971). To sustain a slower rate of growth in stressed plants, one would expect that a metabolic process such as protein synthesis must also be reduced rapidly and in proportion to the degree of stress. Evidence suggesting that protein synthesis may be altered quickly was presented by Hsiao (1970) in which polyribosome percentages were reduced in etiolated corn shoots within 35 minutes following mild stress. Rapid changes in polyribosome levels are also known to occur in mosses (Bewley 1973, Dhindsa and Bewley 1976), but other studies with higher plants (Brandle et al. 1973, Cocucci et al. 1976, Morilla et al. 1973, Nir et al. 1970) in which polyribosome percentages were reduced following stress were not designed to determine how quickly these changes occurred. Consequently, it was felt

that a variety of plants should be examined to see if water stress would consistently and quickly reduce polyribosome levels.

To demonstrate that polyribosome preparations were composed of ribosome-mRNA (messenger RNA) complexes and not some other UV-absorbing material, samples from the two species which comprised the bulk of the polyribosome work were tested for their sensitivity to RNase. The polyribosome profiles shown in Figures 3A and 3C from pea shoots and pumpkin cotyledons, respectively, have a monoribosome region, designated as m, and a polyribosome region, designated as p. Upon exposure of duplicate samples to pancreatic RNase (Figures 3B and 3D), the p region of both profiles was shifted to the m region indicating that contamination of the polyribosome region with membranous material was negligible.

Representative studies showing the effect of short duration water stress on polyribosome percentages are presented in Figure 4. For studies with pumpkin plants (Table 2 and Figure 4, bottom) roots were immersed in nutrient solution containing NaCl at an osmotic potential of -4 bars, and cotyledons were harvested after 0, 10, and 30 minutes. The polyribosome percentages were found to be 2.5% to 8.8% below that of the control samples after 30 minutes of salt-induced water stress; and except for experiment 4, the bulk of this reduction was attained

Figure 3. Sedimentation profiles of ribosomes from unstressed pea and pumpkin tissues -- Homogenates from 6-day-old pea shoots and 3-day-old pumpkin cotyledons were layered over 3.5 ml of 1 M sucrose and centrifuged at 200,000 g for 2 hours. The pelleted ribosomes were resuspended and a portion of the ribosome suspension was mixed with an equal volume of pancreatic ribonuclease to yield a final ribonuclease concentration of 1 µg/ml. Sedimentation profiles of pea and pumpkin ribosomes were obtained after incubation of the undiluted, control ribosome suspensions (A and C, respectively) and the diluted ribonuclease-treated ribosome suspensions (B and D, respectively) at 20 C for 2 minutes.

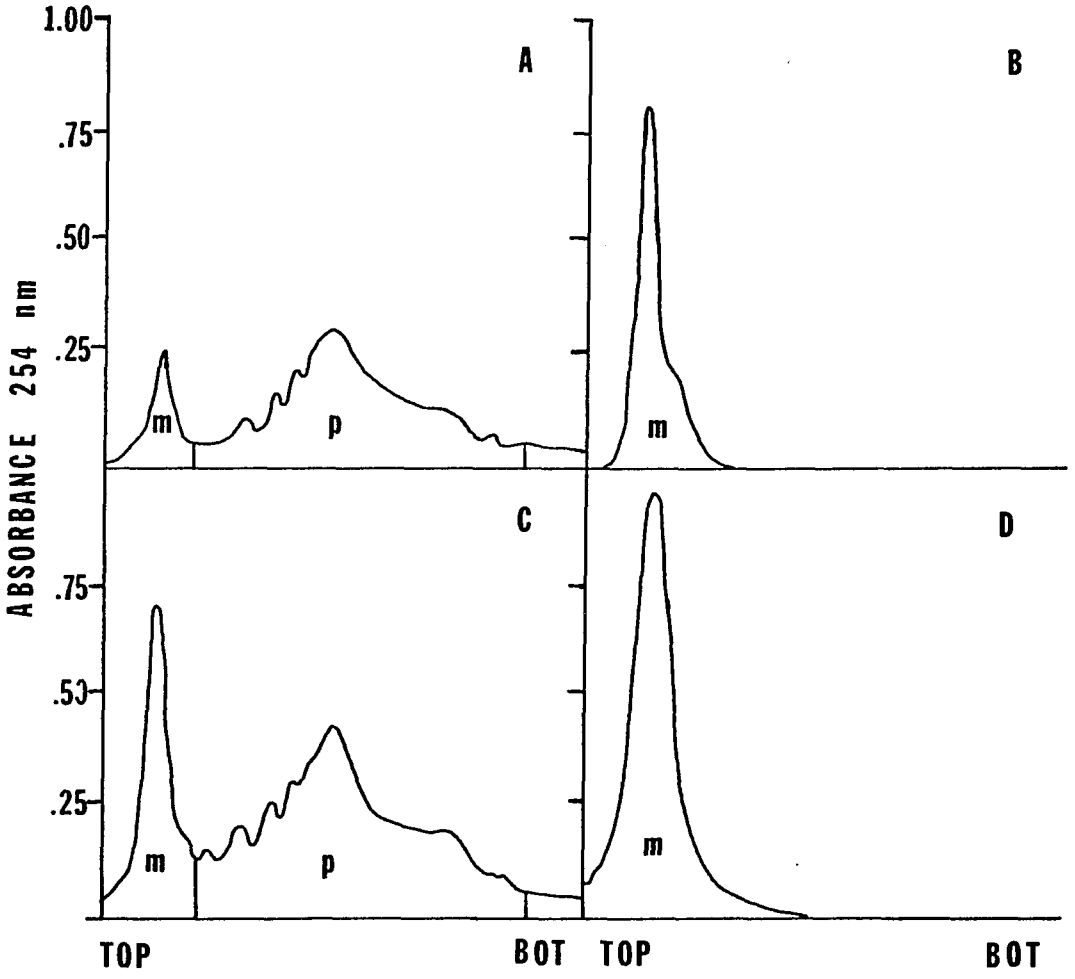

Figure 3. Sedimentation profiles of ribosomes from unstressed pea and pumpkin tissues.

Figure 4. Effect of short duration stress on polyribosome profiles from 6-day-old pea shoots (top row) and 3-day-old pumpkin cotyledons (bottom row) -- Seedlings were stressed by transferring from control nutrient solution to a solution with NaCl added to produce -4 bars osmotic potential (20 minutes for pea and 30 minutes for pumpkin). Polyribosomes from peas were isolated by centrifugation at 200,000 g through a 1 M sucrose pad and those from pumpkin were obtained by precipitation with 1 M acetic acid. Areas used for polyribosome percentage determinations are indicated.

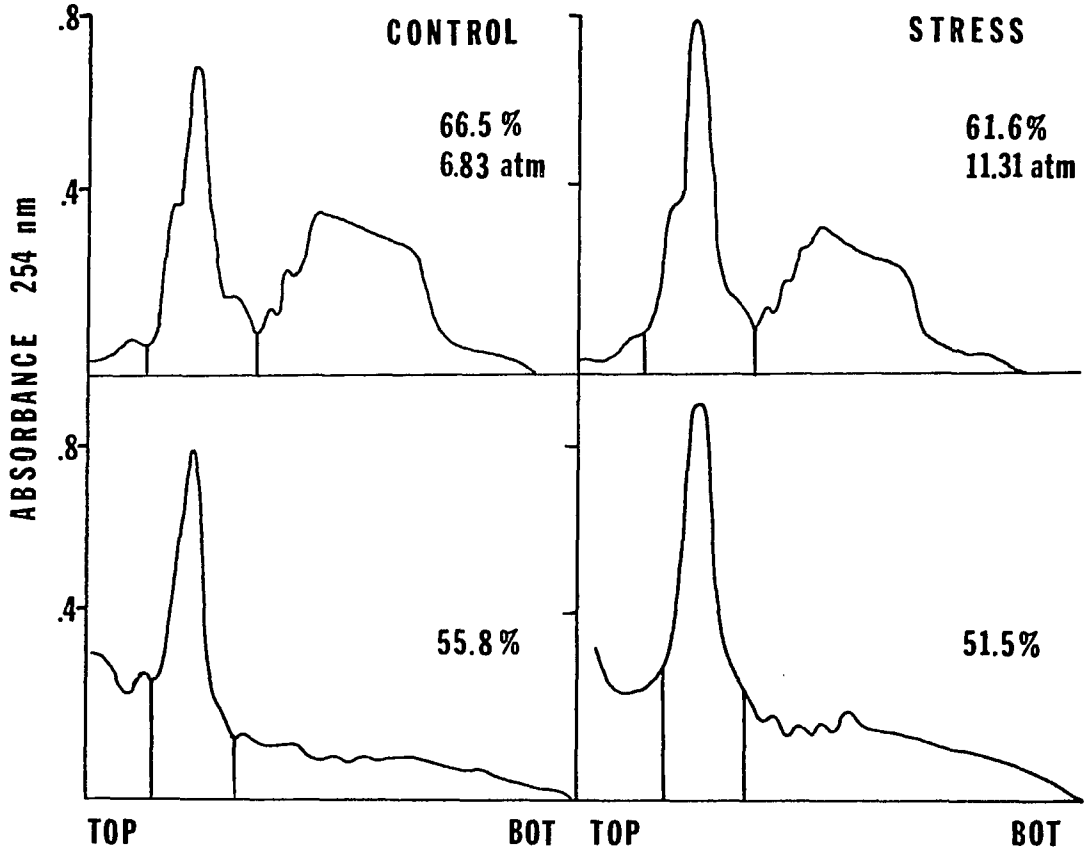

Figure 4. Effect of short duration stress on polyribosome profiles from 6-day-old pea shoots (top row) and 3-day-old pumpkin cotyledons (bottom row).

Table 2. The effect of short duration stress on the polyribosome content of pumpkin cotyledons -- Three-day-old plants were stressed by transferring to nutrient solutions containing NaCl equivalent to -4 bars osmotic potential for the indicated periods. Polyribosomes were prepared by extraction with 3% Triton-X-100 followed by precipitation with 1 M acetic acid and were resolved on discontinuous gradients as described in Materials and Methods.

Experiment Number	Duration of Stress (minutes)	Polyribosome Percentages		
		Total	2-,3-,4-mers	5-mer and Larger
1	0	46.2	19.5	26.6
	10	44.4	18.1	26.3
	30	43.7	18.1	25.7
2	0	66.7	27.5	39.3
	10	62.5	26.0	36.4
	30	61.6	28.5	33.1
3	0	55.8	31.5	24.2
	10	50.6	20.5	30.1
	30	51.5	19.2	32.2
4	0	48.8	27.8	21.0
	10	44.0	22.5	21.4
	30	40.0	19.8	20.2

within 10 minutes. Pea shoots (Figure 4, top) were treated in a similar manner and also showed rapid reductions in polyribosome content but after 20 minutes of stress. Since the reductions reported in Table 2 and Figure 4 were quite small, experiments were performed (Table 3) in which replicate samples were collected from pumpkin cotyledons and pea shoots whose roots had been blotted and allowed to dry for 30 minutes. In all experiments the total polyribosome content (region s+1) of the stressed tissue was found to be significantly less than that of the control.

In addition to determining how stress might affect polyribosome percentages of green tissues, experiments were conducted to test how polyribosome levels might change following relief from salt-induced stress. The results presented in Table 4 show that (as noted in Figure 4 and Table 2) the polyribosome content of pumpkin cotyledons was reduced when the plants were exposed to saline media for 30 minutes. When the seedlings were returned to control media for another 30 minutes, the polyribosome level increased and in all cases surpassed the polyribosome level in unstressed tissues. If this surge in polyribosome content is significant, it may be related in some way to the transient burst in growth rate of tissues in which stress has been relieved (Acevedo et al. 1971, Green et al. 1971).

Table 3. Polyribosome content of control and stressed pumpkin cotyledons and pea shoots -- Plants were stressed by blotting the roots and allowing the tissues to dry in the growth chamber at 36 C and 26 C for pumpkin and pea, respectively. Polyribosomes were extracted with Triton-X-100 as described in Materials and Methods, and the unpurified 30,000 g supernate was layered directly onto a sucrose gradient. The polyribosome profile region designated as s consists of 2-mer through 4-mer size classes of polyribosomes while that designated as l consists of 5-mer and larger polyribosomes. Water status values are the means of 6 replications and the polyribosome percentages were determined from 3 (experiment 1) or 4 (experiments 2 and 3) replicate extractions.

Experiment Number	Press Value (atm)		Profile Region	Mean Polyribosome Percentage		
	Control	Stress		Control	Stress	Difference ± $t_{0.05}(s_{\bar{d}})$
Pumpkin cotyledons:						
1	9.04	20.00	s+l	62.5	45.6	16.9 ± 2.776(0.6) **
			s	24.7	22.5	n.s.
			l	37.8	24.7	13.1 ± 2.776(0.4) **
2	8.14	13.72	s+l	71.2	61.0	10.2 ± 2.447(2.8) *
			s	26.7	30.8	n.s.
			l	45.8	31.8	14.0 ± 2.447(4.3) *
Alaska pea shoots:						
3	8.07	10.62	s+l	64.1	58.9	5.1 ± 2.447(4.0) *
			s	28.2	31.3	n.s.
			l	35.9	27.7	8.2 ± 2.447(3.4) *

*Significant at 0.05 level, t-test.
**Significant at 0.01 level, t-test.

Table 4. Reversible reductions in polyribosome content of pumpkin cotyledons from seedlings exposed to saline nutrient media with an osmotic potential of -4 bars -- Seedlings were stressed for 30 minutes or, following 30 minutes stress, were transferred back to control media and allowed to recover for 30 minutes so that tissues could be harvested at the same time. Polyribosomes were prepared by precipitation with 1 M acetic acid as described in Materials and Methods.

	Polyribosome Percentages		
Experiment Number	Control	-4 bar NaCl, 30 min	-4 bar NaCl, 30 min Control, 30 min
1	59.8	46.0	60.7
2	48.6	34.7	54.8
3	57.8	44.6	60.6

Since differences in the polyribosome content of control and stressed pumpkin and pea seedlings were quite distinct after 30 minutes of stress, this time interval was used routinely in subsequent studies to see if stress would reduce the polyribosome content of other plant types. Representative studies using two varieties of corn and Hi Proly barley are shown in Figure 5 and Table 5. Except for 7-day-old Golden Cross (Table 5), salt-induced water stress resulted in reduced polyribosome contents of both corn varieties and Hi Proly barley. These data taken together with those reported for other plants (Hsiao 1970, Rhodes and Matsuda 1976) show that rapid, stress-induced polyribosome reductions are a common feature of many plants.

Although the vast majority of studies indicated that polyribosome levels were reduced rapidly when plants were stressed for water, results such as those found for 7-day-old Golden Cross corn in Figure 5 and Table 5 (where no change in polyribosome percentage occurred during salt-treatment) were occasionally obtained. This variability in results from apparently similarly treated plants is not understood, but a number of causes might be suggested. Some differences undoubtedly exist because of difficulties in extracting polyribosomes and estimating their percentages. While artifacts in the preparation of polyribosome extracts cannot be discounted, exposure to identical saline solutions may not result in similar

Figure 5. Polyribosome profiles from leaves of two varieties of corn and one variety of barley -- Seven-day-old Double Dent corn (A), 6- and 7-day-old Golden Cross corn (B and C, respectively), and 7-day-old Hi Proly barley (D) seedlings were stressed for 30 minutes by transferring from control nutrient solution to one with NaCl added to produce -4 bars osmotic potential. Polyribosomes were extracted with Triton-X-100 as described in Materials and Methods and isolated by centrifugation at 200,000 g through a 1 M sucrose pad. Areas used for determination of the polyribosome percentage are indicated.

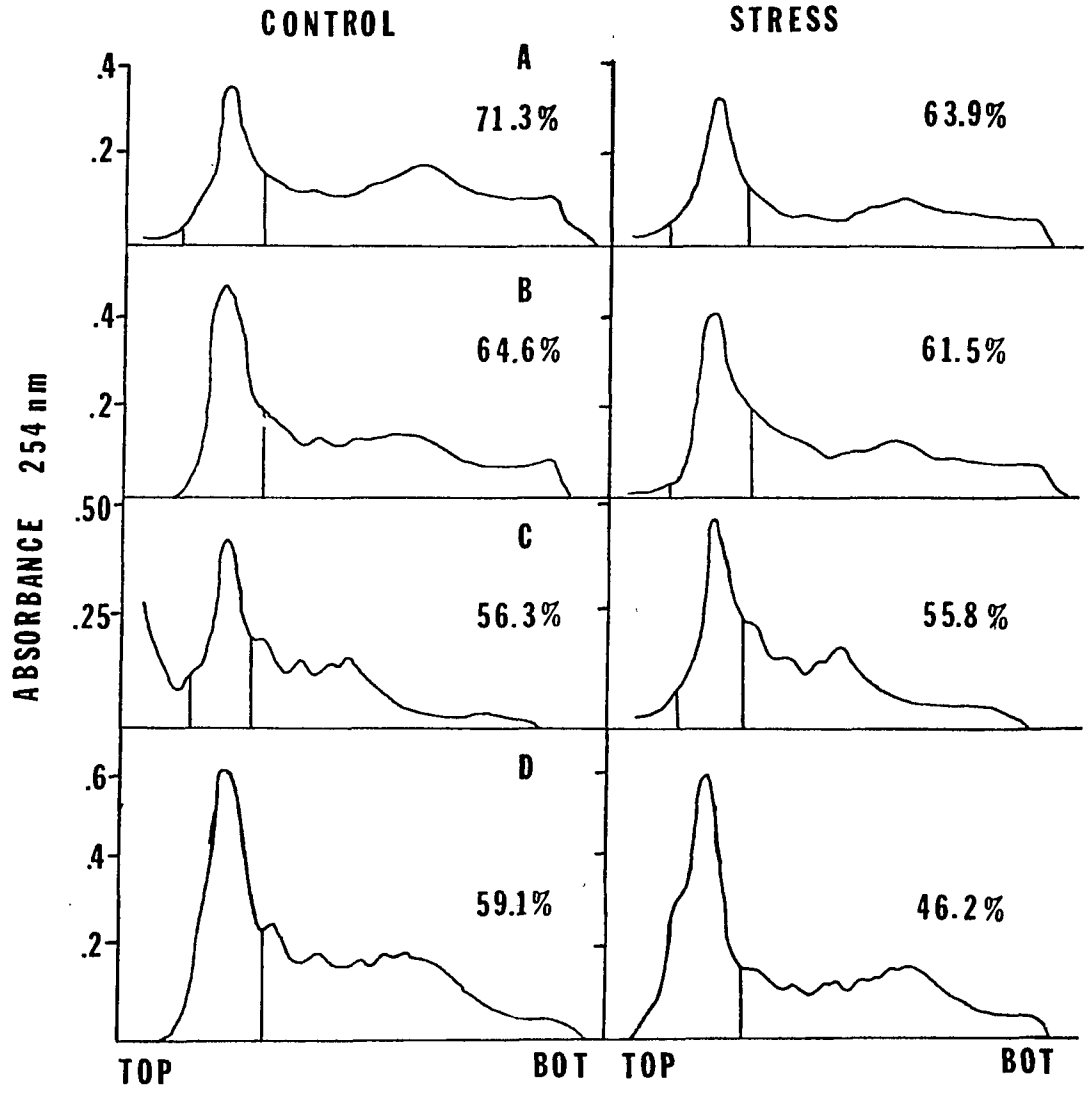

Figure 5. Polyribosome profiles from leaves of two varieties of corn and one variety of barley.

Table 5. Growth rate and polyribosome content of various plants exposed to saline nutrient solutions of the indicated osmotic potentials -- Growth rates were measured with a ruler over a 24 hour period and are the mean of 15 to 21 replications. In each row numbers with the same subscript letter have deviation ranges that overlap. Values in parentheses represent one determination of the polyribosome percentage in extracts from the group of plants used for growth measurements. Polyribosomes were extracted with Triton-X-100 30 minutes after seedlings were transferred to the indicated nutrient solutions and sedimented through a 1 M sucrose pad at 200,000 g as described in Materials and Methods.

Plant	Age (Days)	Growth Rate in mm/day (Polyribosome %) 0 bars	-2 bars	-4 bars	-8 bars
Corn Varieties:					
Double Dent	7	19.83 a (71.3)	--	18.00 a (63.9)	14.86 a (63.7)
Golden Cross Bantam	6	16.43 a (64.6)	--	12.33 ab (61.5)	8.71 b (59.1)
	7	15.36 a (60.4)	15.00 a (61.4)	10.92 ab (58.9)	7.33 b (59.7)
Barley Varieties:					
Hi Proly	5	24.95 a (52.5)	21.14 a (45.5)	18.14 a (--)	8.50 b (--)
	7	18.65 a (59.1)	13.47 ab (61.5)	11.40 b (46.2)	3.33 c (45.9)
N-70	5	30.00 a	24.30 ab	17.80 b	2.90 c
Arivat	5	18.17 a	--	12.83 b	4.33 c
	6	29.50 a	--	22.67 a	2.50 b
Mariot	5	32.00 a	--	19.33 b	6.33 b
Chevron	5	28.00 a	--	22.83 a	12.00 b

degrees of stress due to uncontrolled variations in relative humidity. In addition, evidence that the type of mRNA present in tissues may vary widely was provided by Rhodes and Matsuda (1976). These authors found that polyribosome percentages of whole pumpkin and cotton seedling roots were not reduced in salt-treated plants in contrast to data obtained for all shoot tissues examined and to data obtained with corn root tips (Nir et al. 1970). Interestingly, the polyribosome profiles obtained with root extracts (Matsuda, 1977) and with extracts from other experiments which showed little response to stress were similar to those found for 7-day-old Golden Cross corn leaves. Compared to other preparations (Figures 4 and 5), these were enriched in small (2 to 4 ribosomes per message) polyribosomes. Stress-sensitive mRNAs associated with cell growth may be found in relatively high concentrations only on larger polyribosomes of rapidly growing tissues.

Correlation of Polyribosome Content to Tissue Water Status and Plant Growth Rates

The occurrence of rapid reductions in polyribosome levels in various plants suggests that a metabolic link may exist between the initial physical loss of water and protein synthesis. Further support for this can be provided if reductions in polyribosome percentage can be related quantitatively to the degree of stress. This test can be made by measuring polyribosome percentages in plants

exposed to nutrient solutions of various osmotic potentials. Large day to day variations, however, were obtained for both press values and polyribosome percentages of unstressed 3-day-old pumpkin cotyledons (Tables 2 and 3). This fact, together with the knowledge that decreasing relative humidity reduces both tissue water potential and chlorophyll accumulation in jack beans (Bourque and Naylor 1971) suggests that relative humidity is a large factor in these results. Thus it was felt that comparisons should be made between polyribosome levels and press values rather than solution water potentials. Accordingly, data from several experiments with pumpkin cotyledons and pea shoots were compared and the results presented in Figure 6A. These data and their respective regression analyses show a linear reduction in polyribosome levels with increasing degrees of stress in which a 1 atmosphere increase in press value was accompanied by a 1.8% and 3.3% reduction in the polyribosome percentage of 3-day-old pumpkin cotyledons and 7-day-old pea shoots, respectively. This demonstrated relationship of plant water status to a rapidly altered, stress-sensitive metabolic parameter provides a promising approach toward understanding the relationship between stress-induced metabolic and growth responses.

Since it was found that polyribosome levels of pumpkin cotyledons and pea shoots were reduced by different amounts for a 1 atmosphere increase in press value, an

Figure 6. The relation of polyribosome content (A) and growth rate (B) of pumpkin and pea to tissue water status -- A: Data are from several experiments in which polyribosomes were extracted from cotyledons of 3-day-old pumpkin (a) and shoots of 7-day-old pea (b) seedlings following 30 minutes exposure to solutions containing various amounts of NaCl. Press values were obtained from representative tissues within 3 minutes of time of harvest. Regression equations for (a) and (b) are, respectively: $Y = -1.8\ X + 82.45$, $r = 0.92$; $Y = -3.30\ X + 86.15$, $r = 0.77$. B: Water press measurements were made using representative cotyledons of shoot tissues 30 minutes after exposure of seedlings to salinized solutions. Other seedlings were allowed to grow a total of 24 hours and growth rates of pumpkin hypocotyls (a) and pea shoots (b) were then determined. Regression equations for (a) and (b) are, respectively: $Y = -0.32\ X + 3.81$, $r = 0.89$; $Y = -0.65\ X + 8.81$, $r = 0.97$.

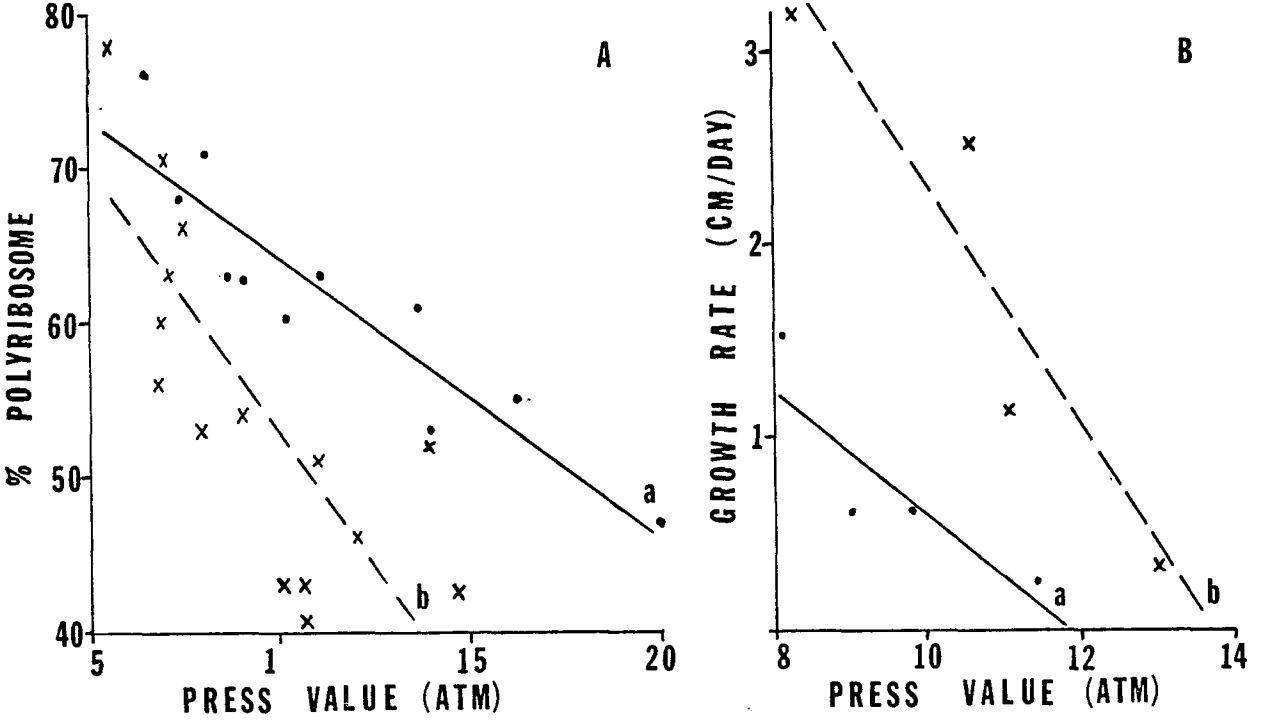

Figure 6. The relation of polyribosome content (A) and growth rate (B) of pumpkin and pea to tissue water status.

investigation was made to see how a specific change in press value would affect the growth rates of these two plants. Because it was not feasible initially to measure detectable growth changes within the 30 minute period used for polyribosome studies, seedlings were transferred to saline nutrient solutions of various osmotic potentials and measurements of pumpkin hypocotyl and pea shoot lengths were made after 24 hours. Growth rates for these tissues and press values for pumpkin cotyledons and pea leaves are presented in Figure 6B. These data show a linear relationship in which a 1 atmosphere increase in press value resulted in a 0.32 and 0.65 cm/day reduction in the growth rate of pumpkin hypocotyls and pea shoots, respectively.

It was intriguing to note that when the polyribosome response to stress was directly compared to the growth rate response, a parallel association was found. Using the data in Figure 6, a 1% reduction in polyribosome percentage was accompanied by a 0.18 and 0.20 cm/day reduction in the growth rate of pumpkin and pea seedlings, respectively. Since these strikingly similar values were derived from studies using different but rapidly growing tissues, it is tempting to speculate that different plants use a constant fraction fo their polyribosome population for growth.

Two approaches were used to test the hypothesis that a given reduction in polyribosome percentage will be

accompanied by similar reductions in growth rate in different plants. Initially, comparisons were made using data in which stress effects on polyribosome levels (Hsiao 1970) and growth (Acevedo et al. 1971) were measured for the same variety of corn. Calculations from these data show that a decrease of 1 bar in water potential produced a 6.54% reduction in the polyribosome content of 3-day-old coleoptile nodes and a 2.73 cm/day reduction in the growth rate of the youngest leaf of 10-day-old seedlings. Combining these results, a 1% decrease in polyribosome content was associated with a 0.42 cm/day reduction in the growth rate of this variety of corn. Because corn tissues of different ages were used, these figures should be viewed with caution; however, it appears that these corn seedlings may utilize a larger portion of their polyribosomes for growth-related processes than pumpkin or pea.

As a further test of the hypothesis, 2 varieties of corn and 5 varieties of barley were placed in solutions containing NaCl of various osmotic potentials and growth over a 24 hour period was measured (Table 5). In some cases, also, estimates of polyribosome percentages (parenthetically indicated in Table 5) and press values (Table 1) were obtained 30 minutes after first exposure to stress. A comparison of plant water status (Table 1) with the growth data (Table 5) shows that a linear relationship

exists between press values and growth rates and that these plants differ widely in their response to a given change in tissue water potential. A 1 atmosphere increase in press value is accompanied by a 0.10 and 0.21 cm/day reduction in the growth rates of 7-day-old Double Dent and Golden Cross corn and 0.33, 0.58, 0.65, and 1.3 cm/day reductions in growth rates of 7- and 5-day-old Hi Proly, 6-day-old Arivat, and 5-day-old N-70 barley, respectively.

Since altered polyribosome percentages were detected in seedlings of 7-day-old Doubld Dent and 6-day-old Golden Cross corn as well as 7-day-old Hi Proly barley (parentheses, Table 5), these data were used to test the hypothesis that a rapid reduction in polyribosome content would be accompanied by a similar reduction in growth rates among these species. Since the polyribosome percentages listed in Table 5 were obtained in conjunction with their respective growth studies, direct comparison of these data were made. It appears that 0.05, 0.14, and 0.06 cm/day reductions in growth rate accompany a 1% decline in the polyribosome content of Double Dent, Golden Cross, and Hi Proly, respectively. Although these values should be supported with more extensive polyribosome percentage data, they do not thus far indicate that a constant proportion of the polyribosome population is involved in growth.

In addition to showing a relationship between polyribosome content and growth, the results presented in

Figure 6 indicate that pumpkin polyribosome levels as well as growth rate are less affected by water deficits than the peas. In a related study, Brandle et al. (1973) found that large reductions in leaf water potential were required to produce detectable changes in polyribosome levels of a drought-hardy plant, black locust. Brandle et al. concluded that polyribosome levels were not very responsive to altered plant water status; in contrast, Hsiao (1970) detected changes in the polyribosome content of corn, a drought-sensitive plant, with only small changes in leaf water potential. With the data presented in Figure 6, the findings of Brandle et al. and Hsiao suggest that polyribosome levels as well as growth rates of drought-tolerant plants may decrease less per unit loss of water than drought-sensitive plants. Changes in the polyribosome percentage may also provide a basis for determining how a stress-sensitive metabolic response is translated into reduced growth rates.

Rapid Growth Rate Changes in Stressed and Unstressed Barley Leaves

The suggested relationship between polyribosome percentages and growth rates prompted an attempt to devise a method of measuring growth rates over time intervals comparable to those used in the polyribosome studies. The method employed a camera equipped with a close-up lens with which photographs were taken at 10 to 15 minute intervals

as described in Materials and Methods. Measurements obtained in this manner from leaves of two Chevron and two Arivat seedlings are illustrated in Figure 7, and growth rates from such data for these and other varieties of barley are presented in Table 6. The initially determined growth rates of Arivat, Mariot, and Hi Proly barley plants grown in control nutrient solutions during the 0 to 36 minute interval (Table 6) were very similar to growth rates of identically treated plants measured with a ruler over a period of 4.5 hours. However, for reasons that are still not clear, growth rates for Chevron plants were about 50% higher in Figure 7 than those determined with a ruler. It is felt that this discrepancy can be resolved with further studies and that short term growth measurements will accurately reflect growth changes.

Although the results shown in Figure 7 and Table 6 are preliminary, they emphasize a number of points worthy of further investigation. Growth rates of all barley varieties responded rapidly to changes in solution osmolarity, and measurements made at 10 to 15 minute time intervals were usually suitable for studies with these seedlings. When plants were transferred to saline solutions, growth was initially severely inhibited; but after 15 to 45 minutes, growth rates increased but not to the rate found in the controls (see Figure 7). The extent of inhibition in both of these phases appears to be directly

Figure 7. Effect of alternating nutrient solutions containing various levels of NaCl on the growth of the first leaf of two Chevron and two Arivat barley seedlings -- Four-day-old seedlings were equilibrated in control media for 3 hours before growth was recorded with a series of 4 photographs taken at the indicated time intervals as described in Materials and Methods. The arrows indicate when nutrient solutions were changed to control or to solutions with added NaCl equivalent to -1, -2, or -4 bars osmotic potential. The heights of the leaves at 0 minutes were 2.21 mm (●——●) and 2.02 mm (●---●) for the two Chevron seedlings and 1.74 mm (x——x) and 1.43 mm (o---o) for the two Arivat seedlings.

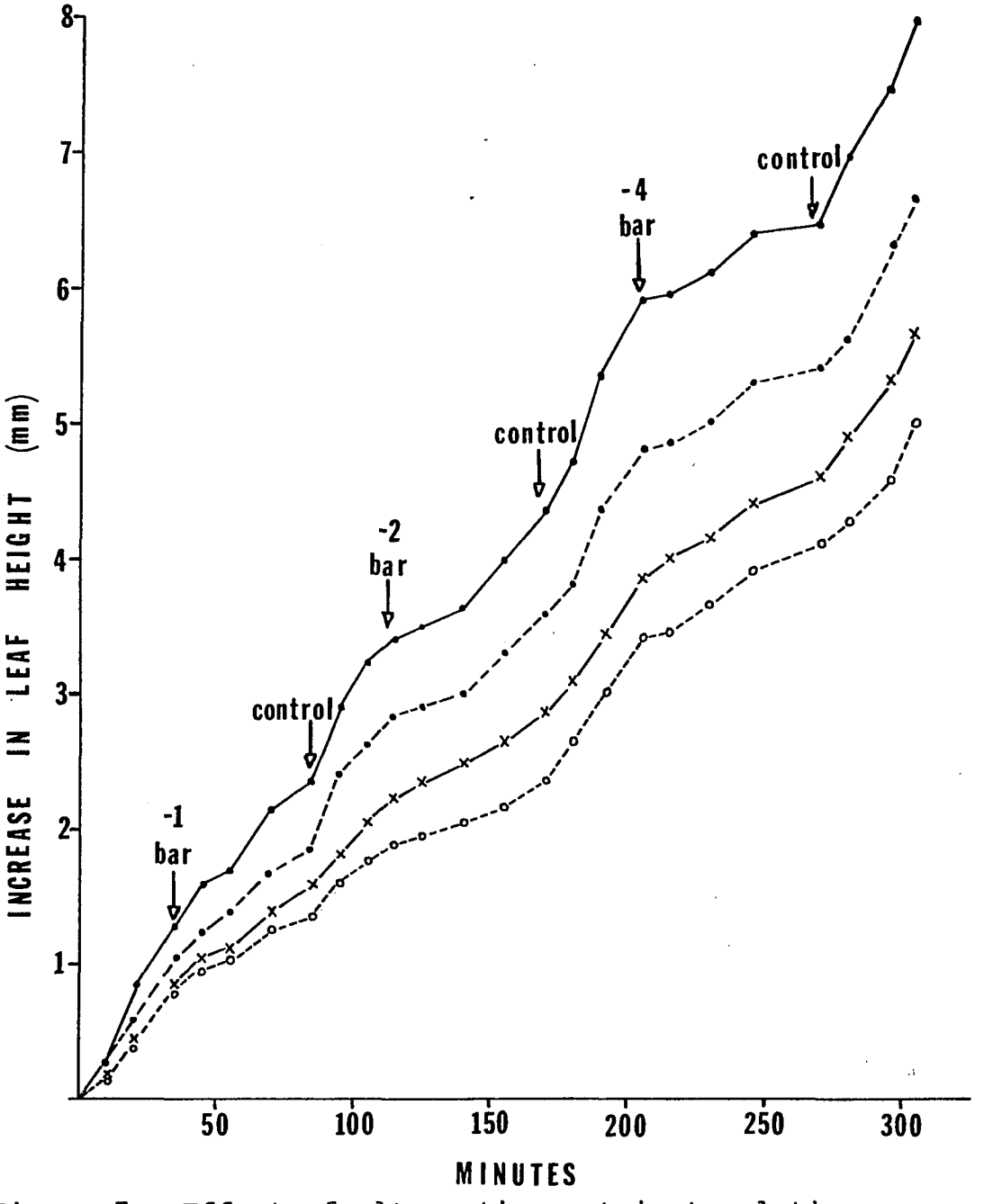

Figure 7. Effect of alternating nutrient solutions containing various levels of NaCl on the growth of the first leaf of two Chevron and two Arivat barley seedlings.

Table 6. Growth rates for the first leaf of four varieties of barley -- Two 4-day-old seedlings of each variety were exposed to nutrient solutions containing NaCl equivalent to the indicated osmotic potentials. The saline solutions were alternated with control media at the time intervals and sequence indicated in Figure 7. Growth was recorded with a camera as described in Materials and Methods and measurements of the projected image were used to calculate these growth rates. Values indicated parenthetically are the mean growth rates expressed as a percentage of the unstressed growth rate established during the 0 to 36 minute interval.

Variety and Plant Number		Growth Rate in μm/minute						
		Control	-1 bar	Control	-2 bars	Control	-4 bars	Control
Time Interval (minutes)		0-36	37-85	86-115	116-170	171-205	206-269	270-305
Hi Proly	#1	18.31	14.83	15.75	13.67	24.30	11.44	27.76
	#2	23.85	20.00	20.25	15.33	29.07	10.53	27.76
	Mean	21.08	17.42	18.00	14.50	26.69	10.99	27.76
		(100%)	(82.6%)		(68.8%)		(52.1%)	
Mariot	#1	18.05	16.84	21.00	12.50	28.50	6.67	26.05
	#2	26.29	18.71	26.50	15.83	38.83	10.00	35.38
	Mean	22.17	17.78	23.75	14.17	33.67	8.34	30.72
		(100%)	(80.2%)		(63.9%)		(37.6%)	
Arivat	#1	23.37	14.39	21.75	11.00	29.07	11.21	29.48
	#2	22.90	10.34	17.25	8.67	30.89	11.97	29.21
	Mean	23.14	12.37	19.50	9.84	29.98	11.59	29.35
		(100%)	(53.5%)		(42.5%)		(50.1%)	
Chevron	#1	29.40	15.58	32.00	13.76	35.98	12.62	35.86
	#2	37.54	20.14	34.75	17.13	45.79	12.45	41.38
	Mean	33.47	17.86	33.38	15.45	40.89	12.54	38.62
		(100%)	(53.4%)		(46.1%)		(37.5%)	

related to the concentration of NaCl used in the nutrient solutions. Furthermore, these varieties differed in their response to stress as, for example, when the growth of Chevron, Mariot, and Hi Proly was severely inhibited for about 30 minutes following the -2 bar treatment while that of Arivat was strongly inhibited for about 45 minutes.

The existence of an apparent 2-step growth inhibition following transfer to salinized solutions raises a question about which phase should be considered in assessing how plants respond to stress. Growth rates listed in Table 6 were calculated where possible from the second phase when growth had increased but not to control levels. While these data are not yet adequate for ranking these varieties in terms of drought-sensitivity, additional studies of this type should help select for stress-resistant varieties. Additionally, coupling this kind of study with polyribosome percentage determinations should aid in understanding how water stress affects growth. As an example, except for recovery from very mild stress in the -1 bar solution, the growth rates upon return to control media were much higher than the initial control rates of growth (Table 6) in agreement with Acevedo et al. (1971) and Green et al. (1971). Considering the rise in polyribosome content found in pumpkin cotyledons recovering from stress (Table 4), one could ask whether this

burst in growth rate can be correlated with a surge in polyribosome level.

Drought-Sensitive Factors Involved in Polyribosome Formation

Most studies reported thus far (Hsiao 1970, Bewley 1973, Rhodes and Matsuda 1976) show that stress effects rapid reductions in polyribosome percentage. Polyribosome formation, however, involves the participation of many factors, each of which may be the stress-sensitive step in altering the polyribosome content. The unresponsiveness of polyribosome percentages in leaves of some plants (noted in Table 5), in whole roots (Rhodes and Matsuda 1976), and in drought-tolerant species like black locust (Brandle et al. 1973) may be related to a condition where a stress-sensitive factor is absent or is less susceptible to water loss. Identification of this stress-sensitive factor and the reasons behind changes in polyribosome levels may lead ultimately to an understanding of how water stress affects growth.

Initially it was thought that information about the preferential loss of certain classes of polyribosomes might prove useful in assessing how stress may be acting to reduce protein synthesis. Saborio et al. (1974) demonstrated that a reversible inhibition of initiation occurs *in vivo* in mammalian cell cultures exposed to mild salt-induced water stress. These workers showed that

radioactive amino acid incorporation into viral proteins near the initiation site of a polycistronic messenger RNA molecule was reduced by water stress before proteins coded further along the message were affected. If one speculates that initiation is also the process affected by water stress in plants, then a reduction in the proportion of large polyribosomes would be expected as fewer ribosomes became attached to the messenger.

To provide at least circumstantial evidence to support the hypothesis that initiation is the stress-sensitive step in protein synthesis, attempts were made to determine whether a preferential loss of large polyribosomes occurred when plants were stressed for water. Polyribosome regions were divided into small polyribosomes, those with 2 to 4 ribosomes per message, and large polyribosomes, those with 5 or more ribosomes per message. No consistent trend in the loss of large or small polyribosomes was found in the 4 experiments presented in Table 2, but all 3 studies in Table 3 show that the reduced polyribosome content was almost entirely accounted for by reductions in the proportion of large polyribosomes. The amount of small polyribosomes appears to be unaffected by water loss (Table 3). With the conflicting data of Table 2, firm conclusions that large polyribosomes are preferentially lost are difficult to make. While the available data do not detract from the suspicion that initiation may

be the stress-sensitive process in protein synthesis, a more direct means of determining how polyribosome percentages can be reduced by water loss must be employed.

To study how stress affects protein synthesis, an approach was sought in which factors present on ribosomes or in the cytoplasm could be partially separated, identified, and measured for their sensitivity to water stress. To help select for these ribosome-bound or soluble supernatant factors a wheat germ *in vitro* protein synthesizing system was developed. In this system protein synthesis was measured as the incorporation of radioactive amino acids into TCA-precipitable material; and experiments of the following type were performed:

1. Ribosomes obtained from control and stressed plant tissues were recombined with ribosome-free wheat germ supernatant containing nearly all the soluble factors necessary for protein synthesis. Comparisons were made of the activity of ribosomes from control and stressed plants and any differences found would be taken as an indication that some alteration in the ribosome or any bound component had been induced *in vivo* by water stress.
2. Homogenates from control and stressed seedlings were treated in various ways to remove inhibitory substances before adding these to the wheat germ

reaction mixture. The effect of these additives on wheat germ protein synthesis activity was compared in hopes that if soluble factors were produced by or altered in stressed plants, they would act to reduce protein synthesis *in vitro*.

The Wheat Germ System of *In Vitro* Protein Synthesis

Before attempts were made to study extracts from control and stressed tissues, a number of studies were performed to determine the optimal concentrations of ions and other factors needed to obtain high rates of protein synthesis by the wheat germ system. In early experiments 86 µM spermine (Marcu and Dudock 1974) or 0.34 mM spermidine (Zaitlin 1976) were included in the assay because these polyamines were reported to enhance radioactive amino acid incorporation in cell-free protein synthesis systems (Marcu and Dudock 1974, Atkins et al. 1975). Reasons for this reported enhancement, which varied with the mRNA used, are not known though involvement in a variety of reactions summarized by Cohen (1971) have been postulated. Figure 8A illustrates the profound effect spermine and spermidine have on the magnesium requirement of the wheat germ system. In the absence of polyamines, the optimal magnesium ion concentration required for the assay was 3.5 mM MgAc; but upon addition of 86 µM spermine or 0.34 mM spermidine, this optimum shifted to 1.5 and 2.5 mM MgAc, respectively.

Figure 8. Magnesium (A) and potassium (B) ion requirements of the wheat germ *in vitro* protein synthesis system -- Wheat germ extracts were obtained from Niblach brand raw wheat germ and reaction mixtures were prepared as described in Materials and Methods. In (A) the reactions (50 µl) contained 100 mM KCl and either 0.34 mM spermidine (o——o), 86 µM spermine (●---●), or no added polyamine (●——●), in addition to the indicated MgAc concentrations. In (B) the reactions contained 3.5 mM MgAc and the potassium ions were adjusted with KCl (●——●) or with 100 mM KCl alone or in combination with 28 and 55 mM KAc (o---o). All reactions contained 4.5 µg TMV RNA and were incubated at 30 C for 60 minutes. Incorporation of ^{3}H-leucine in reactions without TMV RNA were subtracted as background, and all values are the mean of 2 to 3 determinations.

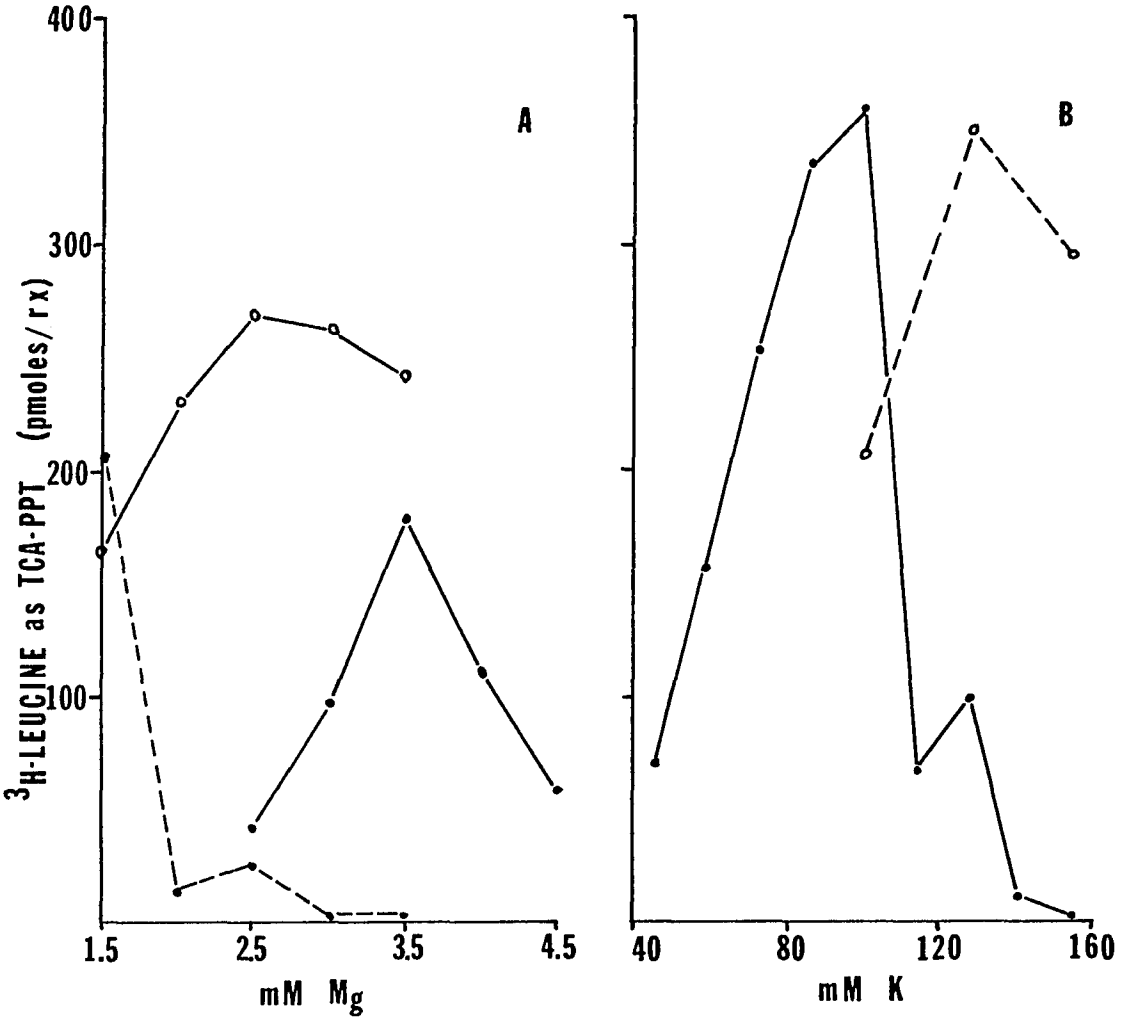

Figure 8. Magnesium (A) and potassium (B) ion requirements of the wheat germ <u>in vitro</u> protein synthesis system.

Because the wheat germ extract itself added 1.5 mM of magnesium to the assay, the action of spermine at lower magnesium concentrations was not studied. When the efficiency of these wheat germ extracts was expressed as picomoles leucine incorporated per A_{260} unit of extract in the reaction mixture, the activity was found to be 128, 170, and 140 picomoles leucine per A_{260} unit with spermine, spermidine, or no added polyamine, respectively. This is only a 21% increase in the presence of spermidine. Since the wheat germ system was highly active without polyamines and since spermidine often unpredictably inhibited the wheat germ system, polyamines were not utilized in further studies.

Determination of the potassium ion concentration required for maximum protein synthetic activity of the wheat germ system were performed, then, in the presence of 3.5 mM MgAc and without added polyamines. When KCl was used to create the 45 to 155 mM range in potassium ion concentrations (solid line, Figure 8B), the ability of the wheat germ extract to incorporate amino acids into protein steadily increased up to 100 mM KCl, and at concentrations greater than 100 mM this activity dropped sharply. The possibility that chloride ions were responsible for this inhibition was suggested by Rose (1977); and in contrast, when KAc (potassium acetate) was used to raise the potassium levels above 100 mM (dashed line, Figure 8B),

the protein synthesis activity remained high even when 155 mM potassium ions were present. Similar studies to determine the optimum magnesium and potassium ion concentrations for the limited quantities of ribosomes extracted from control and stress plants were attempted. With these ribosomes there was very little response to potassium levels ranging from 72 mM to 200 mM and responses to magnesium ranging from 1.5 to 4.5 mM seemed to vary from one preparation to the next. Since supplies of ribosomes obtained from green tissues were usually limited, the ion concentrations used for wheat germ ribosomes, 3.5 mM magnesium and 100 mM potassium, were also utilized with ribosomes from pea shoots and pumpkin cotyledons.

The sensitivity of the in vitro wheat germ system to ion concentrations suggests the possibility that in vivo reductions in protein synthesis during water stress may result from inter- or intra-cellular movement of magnesium or potassium ions. Inter-cellular movement of potassium is known to occur in connection with the opening and closing of stomates (Humble and Hsiao 1970) and one can conceive of a compartmentation phenomena which would allow for rapid intra-cellular adjustment of ions in response to water stress. It would be difficult, however, to create a means of testing this hypothesis.

Reconstitution Studies with Ribosome Fractions from Control and Stressed Plants

Once suitable conditions had been determined for the *in vitro* assay, a search was begun for ribosome-bound factors which would be sensitive to stress. The hope was that ribosomes from control and stressed tissues would behave *in vitro* in a way that could be related to the reduction in polyribosome content. Initially the ability to reconstitute supernatant and ribosome fractions was tested using the wheat germ system. Freshly prepared wheat germ extracts were fractionated into a supernatant fraction and a ribosome pellet. The two fractions were then examined to see if separation was complete. Sucrose gradient centrifugation of these fractions (Figure 9) shows that the supernatant was free of 80s ribosomes and 60s ribosome subunits but may have contained some 40s subunits. Using these fractions, two groups of studies (Table 7) were conducted to test the effect of fractionation on the protein synthesis activity recovered when ribosome and supernatant fractions were recombined.

Initially studies were performed in which wheat germ ribosome and supernatant fractions were recombined so that the proportion of each in the unfractionated wheat germ system was carefully restored (experiment #1, Table 7). For this study ribosome and supernatant fractions were obtained from a portion of a single wheat germ extract,

Figure 9. Sucrose gradient profiles of whole wheat germ extract (A), supernatant (B), and isolated ribosomes (C) -- The wheat germ extract was prepared from raw wheat germ and a portion was set aside for fracionation into supernatant and ribosomes by centrifugation at 200,000 g (max) for 2 hours as described in Materials and Methods. Approximately 3.4 A_{260} units each were layered onto freshly prepared sucrose gradients (0.6, 1.2, 1.2, and 0.6 ml of 1.75, 1.31, 0.88, and 0.44 M sucrose in buffer 2, respectively), and the gradients were then centrifuged at 175,000 g in a Spinco SW50 rotor at 2 to 4 C for 3 hours. The arrows indicate the position of the 40s ribosome subunit in each gradient.

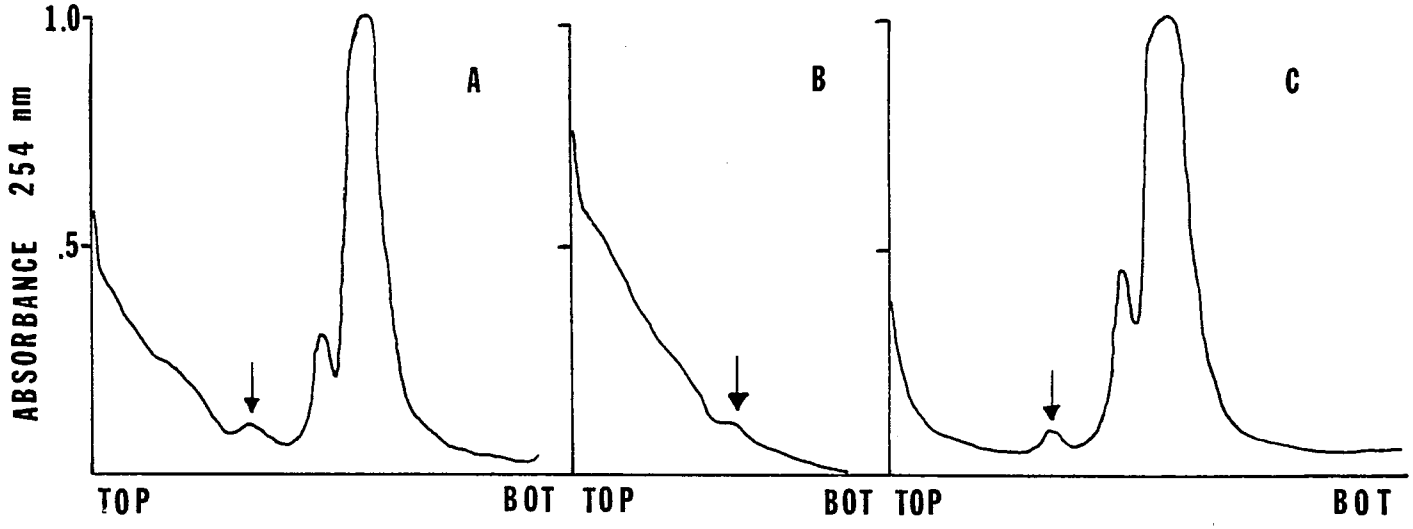

Figure 9. Sucrose gradient profiles of whole wheat germ extract (A), supernatant (B), and isolated ribosomes (C).

Table 7. Protein synthetic activity of whole and reconstituted wheat germ preparations -- Two separate extracts of wheat germ were prepared and a portion of each was fractionated into supernatant and ribosomes as described in Materials and Methods. Ribosomes were resuspended and diluted with 70 mM HEPES, 5 mM MgAc, pH 7.4, and the A_{260} values were determined. All reactions contained 3.5 mM MgAc, 100 mM KCl, and the indicated amounts of unfractionated or fractionated wheat germ in a final volume of 50 µl. The mixtures were incubated for 60 minutes at 30 C and backgrounds of 5 to 7 picomoles leucine incorporated without TMV RNA were subtracted. Each value is the mean of 2 replications.

Experiment and Reaction Number		Wheat Germ Preparation	µl/ Assay	A_{260} Units/ Assay	Picomoles ^{3}H-leucine Incorporated	
					Per Assay	Per A_{260} Unit
1	a	Whole #1	15	1.30	289	222
		Reconstituted:				
	b	Whole #1	10	0.87		
		Supernate	5	0.08		
				0.95	144	152
	c	Whole #1	10	0.87		
		Supernate	5	0.08		
		Ribosomes	10	0.35		
				1.30	241	185
2	a	Whole #2	15	1.35	169	125
		Reconstituted:				
	b	Supernate	15	0.24		
		Ribosomes	10	0.35		
				0.59	45	--
	c	Supernate	15	0.24		
		Ribosomes	10	0.66		
				0.90	79	--
	d	Supernate	15	0.24		
		Ribosomes	10	1.39		
				1.63	135	--

Table 7.--Continued

Experiment and Reaction Number	Wheat Germ Preparation	µl/ Assay	A_{260} Units/ Assay	Picomoles ^{3}H-leucine Incorporated	
				Per Assay	Per A_{260} Unit
e	Supernate	15	0.24		
	Ribosomes	10	2.51		
			2.75	146	--
f	Supernate	15	0.24		
	Ribosomes	10	5.00		
			5.24	135	--

whole #1. When a 10 µl portion of the whole extract was recombined with the supernatant fraction in reaction b, 68% of the protein synthesis activity per A_{260} unit of reaction a was obtained. When ribosomes, however, were also included in the reaction mixture (reaction c) only 83% of the activity was recovered. If the wheat germ ribosome fraction had been damaged during the isolation procedure, excess amounts of ribosomes in the assay should compensate for this lost activity. Consequently, a second group of studies (experiment 2, Table 7) was conducted in which increasing amounts of wheat germ ribosomes were added to the reaction mixture in an attempt to increase the protein synthetic activity per assay to control levels. Even when the concentration of wheat germ ribosomes was more than twice as great as the unfractionated extract (reaction 3, experiment 2, Table 7), only 86% of the activity could be recovered. These results suggest, then, that the fractionation procedure had affected the activity of the wheat germ supernatant fraction. With ribosomes contributing 1.05 and 1.11 A_{260} units to the whole #1 and #2 extracts (Table 7), respectively, wide variations in activity of similarly prepared extracts may not be due to differences in ribosome concentration but to altered supernatant activity. Thus factors in the wheat germ supernate seem to be less stable than the ribosome fractions. Experiment 2 (Table 7) also shows that when

fewer ribosomes are present in the reaction mixture than are found in the whole extract reaction mixture, the protein synthetic activity was dependent on the concentration of ribosomes.

Although wheat germ supernatant activities were reduced by 15% when prepared for use in reconstitution studies, this slight reduction was not considered to affect the ability to detect differences in ribosomes obtained from control and stressed plants. Data from several studies with ribosomes extracted from green tissues using homogenization buffers with and without 3% Triton-X-100 are presented in Table 8. Unlike the wheat germ ribosome fraction, the activity of these ribosomes was quite low regardless of the extraction or resuspension media used. The use of Triton-X-100, however, seemed to yield ribosomes with lower protein synthesis activities than those obtained without detergent.

The studies presented in Table 8 indicate two properties of the pea and pumpkin ribosome fractions which should be considered in assessing the activity of fractions from control and stressed plants. First of all, the incorporation of radioactive amino acid per absorbance unit is dependent on the ribosome concentration (also noted for wheat germ ribosomes in Table 7). When activities of both control and stress ribosomes prepared without detergent were compared with the A_{260} units of ribosomes

Table 8. Protein synthetic activity of ribosomes extracted from control and stressed pea shoots and pumpkin cotyledons -- After 2 hours equilibration in control nutrient solution, half of the seedlings were transferred to saline nutrient solution at -4 bars osmotic potential; and shoot or cotyledon material were harvested 30 minutes later. Ribosomes were extracted from these tissues using the indicated buffers and were isolated by centrifugation through a 1 M sucrose pad as described in Materials and Methods. Ribosomes were resuspended in the indicated buffers, the A_{260} of each sample was determined, and *in vitro* protein synthesis assays were performed on the same day. The reaction mixtures contained 15 µl ribosome-free wheat germ supernate, 5 µl pea or pumpkin ribosomes, 3.5 mM MgAc, 100 mM KCl, and either 5 µl TMV RNA (4.5 µg/5 µl) or 5 µl 15 mM Sorenson's phosphate buffer. Each value is the mean of 2 replications.

Extraction Method: Ribosome Source and Experiment #		Resuspension Buffer	A_{260} Units of Ribosomes Per Assay		Picomoles ^{3}H-leucine Incorporated per A_{260} Unit			
					- TMV RNA		+ TMV RNA	
			Control	Stress	Control	Stress	Control	Stress
Buffer 1 + 3% Triton-X-100:								
Alaska pea	#1	HM	1.08	1.97	3	8	20	22
	#2	H2MK	2.10	2.09	30	29	25	24
	#3	H2MK	1.93	1.90	27	27	31	16
Pumpkin	#1	HM	1.39	1.42	--	--	16	17
	#2	HM	3.54	3.58	13	27	15	31
Buffer 1:								
Alaska pea	#1	HM	1.64	1.64	12	11	10	11
	#2	HM	1.72	0.87	60	77	82	107
	#3	HMK	1.09	0.69	84	95	82	110

Table 8.--Continued

Extraction Method: Ribosome Source and Experiment #	Resuspension Buffer	A_{260} Units of Ribosomes Per Assay		Picomoles ^{3}H-leucine Incorporated per A_{260} Unit			
				- TMV RNA		+ TMV RNA	
		Control	Stress	Control	Stress	Control	Stress
#4	H2M2K	2.10	1.23	53	79	41	61
#5	HMK	3.01	3.07	41	34	39	29
Pumpkin	HMK	3.17	3.01	18	28	27	28

included in the reaction mixture, an inverse relationship was found in which the activity was greatest in assays with the most dilute sample. If differences between control and stress ribosomes are to be detected, this ribosome dilution phenomenon suggests that a series of dilutions of a given ribosome preparation will have to be used. The measured protein synthesis activity of each dilution should then accurately determine the activity per A_{260} unit of that ribosome preparation.

In addition to the problem of ribosome concentration, data in Table 8 also show that the ability of pea or pumpkin ribosomes to respond to added TMV mRNA varied widely. When kinetic studies were done using pea ribosome fractions which did not appear to accept TMV RNA (Figure 10A), protein synthesis appears to have stopped after 30 minutes of incubation. The different plateau values shown here for control and stressed ribosomes are a function of the ribosome concentration. When a ribosome fraction from control pea plants which responded to TMV mRNA was examined (Figure 10B), the reaction continued for 60 minutes in the presence of TMV RNA but ceased after 30 minutes when TMV was omitted. For comparison, a kinetic study of assay mixtures containing wheat germ extract (Figure 10A) show that these reactions were 90% complete after 60 minutes of incubation. These data seem to indicate that some pea ribosome preparations have lost the

Figure 10. In vitro incorporation of ^{3}H-leucine into TCA-precipitable material during the incubation of reaction mixtures containing wheat germ or pea ribosomes -- Unfractionated wheat germ extracts were prepared as described in Materials and Methods. Pea seedlings were equilibrated in control nutrient solution and after 2 hours half were transferred to a nutrient solution containing NaCl at an osmotic potential of -4 bars. After 30 minutes shoots were harvested from control and stressed plants; and ribosomes were extracted using homogenization buffer 1 without Triton-X-100, isolated, and resuspended in H2M2K buffer as described in Materials and Methods. For each study a large reaction mixture was prepared from which aliquots were withdrawn at the indicated times. All points are the mean of 2 replicates and have had the incorporation of 3 to 4 picomoles leucine at 0 minutes subtracted as background. In A: Each 50 µl reaction volume contained 4.5 µg TMV and 1.27, 2.10, or 1.23 A_{260} units of wheat germ extract (●——●), control pea ribosomes (o---o), or stress pea ribosomes (▲---▲), respectively. The pea ribosome reactions also contained 15 µl of wheat germ supernate. In B: Each 50 µl assay contained 1.93 A_{260} units of control pea ribosomes, 15 µl of wheat germ supernate and either 5 µl TMV RNA (4.5 µg) (●——●) or an equivalent volume of 15 mM Sorenson's phosphate buffer (o---o).

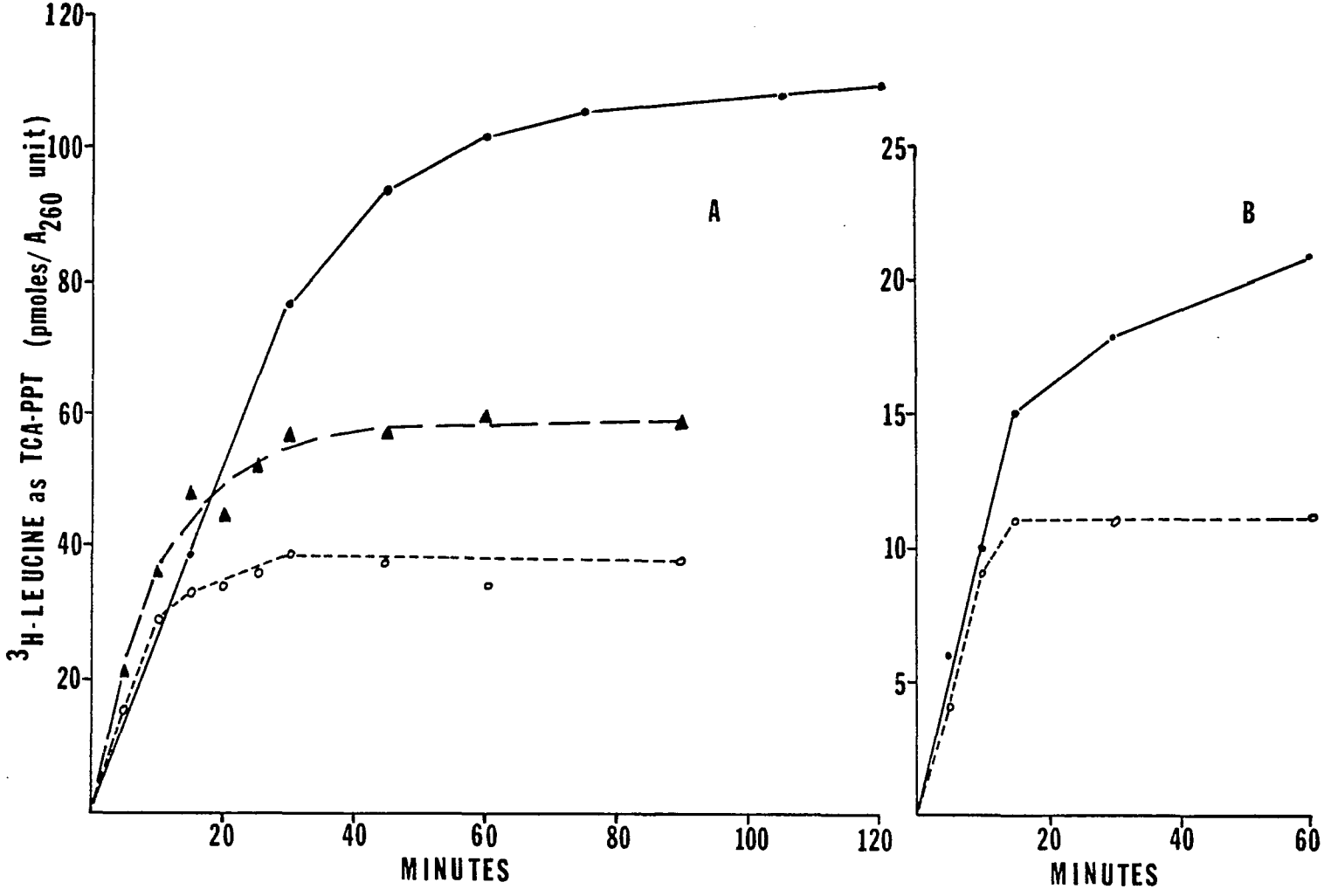

Figure 10. In vitro incorporation of ^{3}H-leucine into TCA-precipitable material during the incubation of reaction mixtures containing wheat germ or pea ribosomes.

ability to initiate protein synthesis using added TMV RNA, but an examination of protein products using polyacrylamide gel electrophoresis is needed to demonstrate that TMV RNA is not being transcribed in these reactions. The question of whether these ribosomes are able to reinitiate their endogenous messengers has not yet been tested; the inconsistencies in activity of these pea ribosome preparations will have to be resolved before meaningful conclusions can be drawn about the affect of stress on ribosomes.

Reconstitution Studies with Supernatant Fractions from Control and Stressed Plants

The second objective in developing an *in vitro* protein synthesizing system was to see if supernatant fractions from control and stressed plants would have an effect on the activity of wheat germ in relation to the degree of water stress experienced by the plant tissues. Factors comparable to those in the wheat germ supernatant fraction would have to compete with the wheat germ supernate for protein synthesis activity; but if components like GTP or ATP could be left out of the mixture, then the *in vitro* protein synthesis reaction might serve to indicate differences in levels of these components in control and stressed supernatant fractions. To test the feasibility of such an approach, studies such as those shown in Table 9 were conducted. All of the reaction mixtures presented in Table 9 required the addition of ATP; but omitting GTP

Table 9. The *in vitro* protein synthesis requirements for added mRNA, GTP, and ATP in reaction mixtures containing whole wheat germ extracts or reconstituted pea or pumpkin ribosomes -- All 3 wheat germ extracts were prepared from Niblach's brand raw wheat germ as described in Materials and Methods. Pumpkin seedlings were stressed for 30 minutes in saline nutrient solution with an osmotic potential of -4 bars. Ribosomes from pumpkin cotyledons and pea shoots were extracted with homogenization buffer 1 containing 3% Triton-X-100 and were resuspended in HM or H2MK buffer, respectively. Complete reaction mixtures included 15 µl wheat germ extract or ribosome-free wheat germ supernate with 5 µl pumpkin or pea ribosomes, 4.5 µg TMV RNA, 1 mM ATP, 20 µM GTP, 3.5 mM MgAc, and 100 mM KCl. When TMV RNA was omitted, an equivalent volume of 15 mM Sorenson's phosphate buffer was used. All values are the mean of 2 replications and background values were not subtracted.

System	Additives	A_{260} Units/ Assay	Picomoles ^{3}H-leucine Incorporated	
			Per Assay	Per A_{260} Unit
Wheat germ (whole)				
#1	complete	--	130	--
	- GTP	--	126	--
	- TMV RNA	--	14	--
#2	complete	1.40	226	161
	- GTP	1.40	141	101
	- TMV RNA	1.40	1	1
#3	complete	--	122	--
	- ATP	--	1	--
Reconstituted:				
Alaska pea ribosomes, control	complete	1.93	44	23
	- GTP	1.93	39	20
	- ATP	1.93	3	2
	- TMV RNA	1.93	25	13
Pumpkin ribosomes, control	- TMV RNA	3.54	38	11
	- TMV RNA, - ATP	3.54	3	1
Pumpkin ribosomes, stress	- TMV RNA	3.58	100	28
	- TMV RNA, - ATP	3.58	3	1

resulted in protein synthesis activity ranging from 63% to 100% of the activity when GTP was included in the reaction mixture. With these endogenous levels of GTP, the wheat germ system would not be useful in measuring GTP levels of control or stress supernatant fractions.

Many studies were conducted using plant supernatant extracts treated in a variety of ways. In some studies extracts were dialyzed for various periods of time; in others fractions were collected from Sephadex G-25 columns and either dialyzed or added directly to assay mixtures. In all cases, even when extensively dialyzed for up to 3 hours or heated for 10 minutes at 90 C, supernatant fractions from pumpkin cotyledons always inhibited the activity of wheat germ extracts. The reason for this is not known but a possible source of inhibition may be the high levels of carbohydrates in this tissue (Pegelow 1971). The addition of 0.1 mM glucose or fructose or of 10 mM sucrose or rhamnose is known to reduce wheat germ activity by 30 to 35% (Sullivan, 1977). Considering how sensitive the reaction mixture is to ATP levels (Table 9), phosphorylation of carbohydrates present in the pumpkin cotyledon extract may be depleting the reaction mixture of the ATP required for protein synthesis.

Although pumpkin extracts greatly inhibited the wheat germ, pea supernatant fractions were not usually inhibitory. Two studies with extracts from this plant are

presented in Table 10; and in both of these, the supernatant fractions appear to stimulate the activity of the wheat germ system. Furthermore, in experiment 2 (Table 10) this stimulation decreased with the degree of stress experienced by the pea seedlings. The basis for this stimulation has not yet been investigated.

In searching for a basis for the reduced levels of polyribosomes found in stressed tissues (Tables 2, 3, and 5), tests such as those conducted in Table 10 were designed in hopes of recognizing alterations in supernatant factors of stressed plants. However, water stress also reduces the chemical activity of water, and experiments of the type found in Table 10 would not test for effects of altered chemical activity of water on protein synthesis. Consequently, attempts were made to use the wheat germ system to see if an altered chemical potential of water in the reaction mixture would alter <u>in vitro</u> protein synthesis. Several studies were conducted in which 100, 200, or 300 mM sorbitol (equivalent to a 2.5, 5.0, or 7.5 bar reduction in the osmotic potential of the reaction mixture) were added to the assay and found to reduce the protein synthesis activity by 4%, 21%, and 34% respectively. Calculating the chemical activity of water using the equation:

$$\psi = (RT/\overline{v}_w) \ln a_w$$

Table 10. The effect of supernatant fractions from control and stressed tissues of peas on the protein synthetic activity of wheat germ extracts -- Seedlings were stressed by transferring to saline nutrient solutions at an osmotic potential of -4 bars (-2 and -8 bars were also used in experiment 2). Tissues were harvested from control and stressed plants 30 minutes later. Experiment 1: Fifteen pea shoots were ground in HEB (HEPES elution buffer: 20 mM HEPES, 120 mM KCl, 5 mM MgAc, 1 mM DTT, pH 7.6) which was the buffer used to elute the wheat germ extract from Sephadex G-25 columns (Marcu and Dudock 1974). The slurry was then centrifuged at 30900 g for 12 minutes in a Sorvall SS-34 rotor, and the supernatant fraction was then dialyzed against HEB for 70 minutes. The material passing through the dialysis bag was adjusted to equal volumes and a portion was heated to 90 C for 10 minutes. Experiment 2: Fifteen pea shoots were homogenized in 5 ml buffer 1 without Triton-X-100 and centrifuged as described in experiment 1. The 30900 g supernate was then made to pH 5 with 1 M acetic acid as outlined in Materials and Methods, and the precipitate was removed by centrifugation at 30900 g for 3 minutes. The supernate was then adjusted to pH 7.4 with 1N NaOH, and the ion content was reduced to 1/8 the original concentration by adjusting the volume with water and concentrating this supernatant fraction with solid polyethylene glycol. Reactions containing the indicated additives were prepared with 15 µl wheat germ extract as described in Materials and Methods. All values are the mean of 2 replications with incorporation in the absence of TMV RNA subtracted as background.

Experiment Number	Additives	Picomoles ^{3}H-leucine Incorporated Per Assay
1	5 µl HEB	177
	5 µl pea dialysate:	
	control, unheated	205
	control, heated	225
	stress, unheated	187
	stress, heated	195

Table 10.--Continued

Experiment Number	Additives	Picomoles ^{3}H-leucine Incorporated Per Assay
2	5 µl water	37
	5 µl pea supernatant:	
	control	90
	-2 bars	58
	-4 bars	58
	-8 bars	45

where ψ, R, T, $\bar{v}_w$, and a_w are the water potential, the gas constant, absolute temperature, partial molal volume of water, and the chemical activity of water, a 7.5 bar reduction in water potential would reduce the activity of water by only 0.5%. With such small changes in the chemical activity of water, it may be that increased viscosity rather than activity of water plays a larger role in studies with sorbitol.

General Discussion and Suggestions for Future Work

From the time Hsiao (1970) first reported that polyribosome reductions occurred rapidly in water-stressed corn seedlings, a number of studies (Nir et al. 1970, Morilla et al. 1973, Brandle et al. 1973, Cocucci et al. 1976) have reported stress-induced reductions in polyribosome contents of higher plants but did not attempt to detect rapid alterations in polyribosome levels. Work showing how quickly such changes could occur in other plants was confined to mosses (Bewley 1973, Dhindsa and Bewley 1976) until Rhodes and Matsuda (1976) expanded the data to include rapidly growing tissues of pea, pumpkin, wheat, barley, and safflower. The work presented here (Tables 2, 3, and 5; and Figures 4 and 5) together with that of Rhodes and Matsuda (1976) and Hsiao (1970) establishes that rapid polyribosome reductions are a common feature in growing tissues of many plants undergoing

water stress and that these reductions are reversible (Table 4).

Exceptions were noted in which polyribosome levels did not change when 7-day-old Golden Cross corn (Figure 5) or whole root tissues (Rhodes and Matsuda 1976) were stressed for water. Among the reasons suggested for these differences in similarly treated plants was the possibility that different populations of polyribosomes were affected by water stress. Differential effects of water stress on a number of proteins synthesized in vivo were demonstrated by Dhindsa and Cleland (1975) using a double-label technique over a period of 3 hours. Similar studies should be conducted within the 30 minute interval used for the polyribosome work. Should selective effects on the protein population be detected, the polyacrylamide gel profiles of proteins synthesized in vivo can be compared with those synthesized in vitro using ribosome extracts from tissues which respond and those which did not respond to stress conditions. If responsiveness to water loss is a function of the type of proteins being synthesized, it may be possible to show an association of those proteins with a particular size class of polyribosomes.

It was possible with the Campbell J-14 press to relate polyribosome reductions and growth rates to the degree of stress undergone by the tissue. In the studies presented in Figure 6, a 1 atmosphere increase in the

press value (corresponding to increasing degrees of water stress) was accompanied by a 1.8% and 3.3% reduction in the polyribosome content of 3-day-old pumpkin cotyledons and 7-day-old pea shoots, respectively. Growth rates determined over a 24 hour period were reduced by 0.32 and 0.65 cm/day for each 1 atmosphere increase in press value for pumpkin hypocotyls and pea epicotyls, respectively. In addition to the relationship of these two parameters to plant water status, these data indicated that remarkably similar reductions in growth (0.18 and 0.20 cm/day for pumpkin and pea, respectively) accompanied a 1% decrease in polyribosome content.

It was interesting to note that similar values relating polyribosome content to growth could be obtained when different but rapidly growing tissues were used. This suggested that plants may use a constant fraction of their polyribosome population for growth. This hypothesis was tested using published data from a single variety of corn (Hsiao 1970, Acevedo et al. 1971) and the data in Table 5 for two other varieties of corn and one of barley. The values obtained with the published data (0.42 cm/day per 1% decrease in polyribosome content) and those from Table 5 (0.05, 0.14, and 0.06 cm/day per 1% decrease in polyribosome content for the two varieties of corn and the barley, respectively) did not tend to support this hypothesis. In order to feel confident of these values,

however, more data need to be gathered on the polyribosome content of these varieties as well as other monocot and dicot species.

Data presented in Figure 6 also indicated that pumpkin polyribosome content and growth rate are less affected by water loss than peas. In comparable studies, Brandle et al. (1973) demonstrated that a drought-hardy plant, black locust, required large reductions in leaf water potential before reductions in polyribosomes were noted. In contrast, Hsiao (1970) found that relatively small reductions in tissue water potential resulted in large changes in polyribosome content in corn seedlings, a drought-sensitive plant. These findings suggested that polyribosome levels as well as growth rates may be less responsive to water stress in drought-resistant than drought-sensitive species. This hypothesis should be tested with a variety of stress resistant and sensitive plants.

The rapid growth detection method of Figure 7 and Table 6 should be useful in detailing relationships between growth, polyribosome content, and water status since all of these parameters can now be measured over relatively short periods of time. More studies should also be conducted to verify the biphasic nature of the growth curves for plants exposed to -2 and -4 bar osmotic potentials. In addition data of this type may provide a basis for

selecting stress resistant and sensitive plants for comparison of their polyribosome levels under a given degree of stress.

In an effort to understand how water stress reduces polyribosome levels, a wheat germ *in vitro* protein synthesis system was used to test for ribosome-bound or soluble supernatant factors which had been altered by water loss. In studies where wheat germ supernatant fractions were combined with ribosome extracts, the protein synthetic activity and the response to added TMV RNA was variable among ribosome preparations. In other studies seedling supernate preparations were treated in a variety of ways and added to wheat germ reaction mixtures. Since supernatant fractions from pumpkin cotyledons were extremely inhibitory, pea supernatant fractions were utilized; and, as with ribosome preparations, the results were highly variable from one experiment to the next. If there are ribosome-bound or soluble supernatant factors sensitive to water stress, these assays did not detect them. Unless techniques of obtaining consistently active preparations improve, I do not think that a cell-free protein synthesis system is the approach to use in studying water stress effects on protein synthesis. It may be more fruitful to measure the GTP content of stressed and unstressed tissues using an appropriate assay procedure. Or alternatively, a modified Edman procedure (Oleinick and

Salengo 1976) may be adapted to plant material in order to show *in vivo* whether initiation or elongation are affected by water stress. This procedure distinguishes between N-terminal and internal positions of radioactive methionine and would avoid justifying *in vitro* work in terms of how accurately it reflects *in vivo* events. Thirdly, differential extraction of free and membrane-bound polyribosomes may be useful in suggesting whether or not a membrane effect is involved in the reduction of protein synthesis during water stress. And finally, ion compartmentation studies should be initiated to investigate the distinct possibility that *in vivo* protein synthesis may be regulated by a delicate ionic environment. Such studies might begin with analyses of ion contents in various cell fractions, such as vacuoles, other organelles, and the cytoplasm, but isolation of these fractions without altering their ion content would be difficult.

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