THE EFFECT OF ENVIRONMENTAL STRESS ON PROLINE ACCUMULATION IN BARLEY AND RADISH

by

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SUMMARY

The effects of water, temperature and salinity stress on plant water status and metabolism, in particular proline accumulation, were investigated with barley (*Hordeum distichum* L., cv. Prior) and radish (*Raphanus sativus* L., cv. Long White Icicle). The induction of proline accumulation by water stress had been established previously for barley (Singh, 1970) and was here shown also to occur in radish. The responses to leaf water potential, time course of accumulation and distribution between plant organs were similar in all respects to that of barley, but the potential rate of accumulation was higher in radish.

Proline also accumulated in plants of either species exposed to elevated temperature (greater than 30°C); this effect, however, was related to the water status of the plant tissues. Barley and radish plants were subjected to heat stress (39°C) for 1-5 days when growing in either a high (90-95%) or low (50%) relative humidity environment. Although plants were watered daily, the leaf water potential (ψ) in those at the low relative humidity fell whereas this did not occur at the higher humidity. Growth in height was inhibited and leaf chlorophyll concentration decreased by the high temperature, these effects being accentuated by an accompanying water-stress in the leaves. On the other hand, proline only accumulated when leaf water potential was depressed, there being no direct response to elevated temperature.

These effects were confirmed for a range of temperatures extending to a maximum of 41°C.

Proline also accumulated rapidly, after an initial lag phase, in both barley and radish plants exposed to a low temperature (5°C). This accumulation, in contrast to that occurring at high temperature, was unrelated to any changes in leaf water status. Neither leaf water potential, osmotic potential nor turgor changed sufficiently with low temperature so as to account for the accumulation of proline. These effects also were confirmed for a range of temperatures from 20 to 4°C in barley. The characteristics of this cold-induced accumulation of proline were compared with those of water stress-induced accumulation. It was found that light was a pre-requisite for proline accumulation in response to low temperature but not for water stress-induced accumulation. This response to light was not apparently due to any shortage of substrates for proline synthesis.

Proline also accumulated in plants of both species exposed to salinity stress, and an attempt was made here to separate the effects of the several components of leaf water potential from each other and from any effects of absorbed ions. The water balance of leaf tissue was unfavourably affected by increasing salinity in the substrate. A transitional water deficit with a loss of turgor potential occurred when plants were transferred directly to NaCl solutions of -10 bars osmotic potential, but this effect was not permanent and turgor potential

increased as the plant adapted to saline conditions. On the other hand, a gradual increase in the external salinity allowed adjustment to changing conditions without any dramatic change in plant water status. Despite these differences in response of water status, proline accumulated in both situations. This accumulation of proline was apparently dependent on the osmotic potential of the tissue rather than on turgor or salt concentration within the tissue. A similar accumulation of proline also occurred in plants exposed to KCl, $MgCl_2$, $CaCl_2$ or Na_2SO_4 but the rates of accumulation varied. Compared with polyethylene glycol treatment it was found that high concentrations of the various salts in the leaf tissue inhibited proline accumulation to varying degrees, Mg^{++} ions being least inhibitory and $SO_4^{=}$ most.

Apart from this large change in proline content, there were much smaller changes in the concentrations of a number of other amino acids. Although a number of parallel changes were observed with some of these amino acids in the various stress situations, there was no characteristic pattern of change in any but proline, which accounted for 50-80% of the total variation in amino acid content. Studies with ^{14}C-glutamic acid and ^{14}C-proline fed to stress tissue revealed that proline accumulation in the various environmental stress situations appeared to result from a common response. Incorporation of radioactivity from ^{14}C-glutamic acid into proline was substantially increased in all stress situations, and, conversion of ^{14}C-proline to a variety of other amino acids was considerably reduced. It was concluded that the further metabolism of proline through glutamic acid was the most likely site of response to stress.

STATEMENT

I hereby declare that the thesis here presented is my own work, that it contains no material previously published, except where due reference is made in the text, and that no part of it has been submitted for any other degree.

(Teh-Ming Chu)

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PREFACE

A portion of the subject matter of this thesis has been published under the title "Stress Metabolism. VI. Temperature Stress and the Accumulation of Proline in Barley and Radish" by T.M. Chu, D. Aspinall and L.G. Paleg, Aust. J. Plant Physiol. (1974) 1 : 87-97.

ABBREVIATIONS AND SYMBOLS USED IN THIS THESIS

Abscisic acid	ABA
Adenosine diphosphate	ADP
Adenosine monophosphate	AMP
Adenosine triphosphate	ATP
Bovine serum albumin	BSA
Centigrade	^{o}C
Centimeter(s)	cm
Counts per minute	cpm
Deoxyribonucleic acid	DNA
Ethylenediamine tetra-acetic acid	EDTA
Gibberellin A_3	GA_3
Gram(s)	g
Gravity	g
Gravitational potential	ψ_g
Hour(s)	hr
In a test tube, beaker, flask	in vitro
In a living organism	in vivo
Kilowatt-hour	KWh
Litre(s)	l

Matric potential	ψ_m
Messenger ribonucleic acid	m-RNA
metre	m
Methanol : Chloroform : Water(12:5:3 v/v)	MCW
Microcurie(s)	µCi
Microgram(s)	µg
Microlitre(s)	µl
Micromolar	µM
Milligram(s)	mg
Millilitre(s)	ml
Millimetre(s)	mm
Minute(s)	min
Molar	M
Normal (concentration)	N
Normal (in trivial names in organic compounds)	*n*-
Number	No.
Optical density	OD
Osmotic potential	ψ_π
Part(s) per million	ppm
Percent	%
Polyethylene glycol	PEG
Relative turgidity	RT

Ribonucleic acid	RNA
Transfer ribonucleic acid	t-RNA
Turgor potential	ψ_p
Volume/volume (concentration)	v/v
Water potential	ψ
Weight/volume (concentration)	w/v

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I INTRODUCTION

Water is the most abundant constituent of plant tissues. Large amounts of water are continuously being taken from the soil, translocated through the plant, and evaporated into the atmosphere. As the plant transpires, the water loss process depends on the balance between water absorption, the rate of movement in the plant, and the evaporation from exposed surfaces. Under steady state conditions, these processes proceed at comparable rates, such that both the absolute water content and the relative distribution of water in the plant remain constant (Kramer 1969). A change in any factor of the plant environment may directly or indirectly affect this balance such that the internal water status of the plant tissues change to reach a new state of balance after a period of adjustment. A water deficit in the plant tissues will be induced by any environmental factor which causes a water imbalance in the plant by influencing transpiration or absorption or both. It is not surprising, therefore, that plant water deficits may be produced by several different environmental stresses (Levitt, 1972). Chilling-induced water deficit is thought to be due to inadequate absorption (Kramer, 1969); freezing-induced water deficit to intercellular ice formation (Levitt, 1972); salt-induced water

deficit to osmotic adjustment (Bernstein and Hayward, 1958); and heat-induced water deficit to excessive transpiration.

Some investigators claim that injuries from drought, heat and freezing are similar in nature (Kramer, 1969), and further, it has been suggested that plant resistances to cold, heat and water stress are interrelated (Levitt, 1972). This could follow from the common factor of reduction in tissue water potential and, according to Strogonov (1964), the mechanism for adaptation, in the face of different sources of environmental dehydration (drought, low temperature, high temperature and salinity) is the same at all phases of plant development.

Recently, in investigations of the metabolic consequences of plant water deficit, increasing attention has been paid to the change in nitrogen metabolism. There is much evidence to show that water deficit induces a characteristic change in the level of free amino acids in many plants, especially a great increase in free proline. Proline has been found to increase with increasing severity of drought, up to five to ten times in rice, spinach and bean (Stewart *et al.*,1966), and up to ten to 100 times in sunflower, peas and tobacco (Palfi, 1969), barley, wheat (Singh *et al.*, 1973 a,c) and Bermuda grass (Barnett and Naylor, 1966). Since it has been postulated that the physiological changes in plants during various environmental stress situations may

be due, at least partially, to a water deficit in the plant tissues, it is possible that these other environmental stresses can also induce proline accumulation in plant tissue and that the accumulated proline plays a significant role in metabolism during these stresses. This thesis aims, therefore, to investigate directly whether various stress conditions affect the internal water relations of the plant tissues and, if so, whether this leads to changes in nitrogen metabolism resulting in proline accumulation.

Environmental stresses are of two main types— biotic and physicochemical. The former belongs to the field of pathology and ecology and will not be considered in this thesis. Among the physicochemical stresses, at least eight stresses have been delineated (Levitt, 1972). In this thesis only water deficit, chilling, heat, and salinity stress will be considered.

II LITERATURE REVIEW

1. The relationship between environmental stress and plant water status

1.1 Water Deficit

Plant water deficit can be caused by excessive rates of water loss or slow absorption or both. The magnitude of plant water deficit depends on the soil moisture stress, atmospheric evaporative demand and resistance to water flow in plants (Slatyer, 1967). Internal water deficits caused by excessive transpiration are usually shorter and less severe than those caused by deficient absorption (Vaadia *et al.*, 1961). It has been observed that practically all plants undergo a diurnal variation of plant water status. Even plants growing under near optimum moisture conditions undergo a range of variation of water status depending upon the lag of absorption behind transpiration (Slatyer, 1967). If this lag continues for a long period, visible or permanent wilting may occur. This tendency for the absorption rate to lag behind the transpiration rate suggests the existence of considerable resistances to water movement within the plant (Kramer, 1969). The resistance to water transport is nearly twice as high in the roots as in the leaves of several crop plants (Tomar and Ghildyal, 1973), but the size of these resistances varies with plant water status (Stevenson and Shaw, 1971).

Under equilibrium conditions, the state of the water in a plant leaf cell may be written in terms of the various components of the

potential energy.

$$\psi = \psi_\pi + \psi_p + \psi_m \ldots\ldots (1)$$

Where ψ is the total water potential, ψ_π is the osmotic potential component, ψ_p is the pressure (turgor) potential component and ψ_m is the component due to adsorption forces. Partitioning of the energy between osmotic and adsorption components is somewhat arbitrary since a proportion of the water in the leaf tissue will be subject to both osmotic and adsorption forces, particularly at low leaf-water contents (Gardner and Ehlig, 1965). In the vacuole the osmotic component will predominate and, as a consequence, ψ_m is usually neglected and ψ of the leaf tissue expressed as simply the sum of osmotic and pressure potential.

$$\psi = \psi_\pi + \psi_p \ldots\ldots (2)$$

With initially fully turgid tissue, a decrease in tissue water content causes a large decrease in ψ. In this phase of dehydration, decreases in ψ_p are usually much more marked than decreases in ψ_π and account for the major part of the diminution in ψ. After more water is lost and ψ_p falls to a negligible level, decreases in ψ_π alone account for most of the further decrease in ψ (Gardner and Ehlig, 1965). It has been generally assumed (Slatyer, 1967) that the wilting point corresponds to zero turgor potential in leaves when $\psi = \psi_\pi$. The first value of water potential at which $\psi = \psi_\pi$

(when $\psi_p = 0$) varies from species to species. It has been reported that for barley it is -32 bars (Millar *et al.*, 1970); for cotton, -19 bars; for sunflower, -22.4 bars (Gardner and Ehlig, 1965). However, it has been reported that the value of ψ_p is always greater than zero at the first sign of wilting; for dicotyledons, visible wilting symptoms have been reported to occur at + 2 or 3 bars ψ_p (Gardner and Ehlig, 1965), and for rice plants it was found to be approximately + 3 bars (Tomor and Ghildyal, 1973). In this case it is associated with a marked change in the elastic property of leaf tissue.

During the past half-decade, water potential (ψ) has gained wide acceptance as a fundamental measurement of plant and soil water status. However, the ψ value *per se* may not be as crucial in determining plant response as the effects of a reduction in ψ_p on growth (Ordin, 1960). In addition, some metabolic processes (Greenway and Leahy, 1970) were minimized or eliminated if turgor was maintained by providing the tissue with an easily absorbable solute, although tissue ψ remained at a reduced level. Greenway and Leahy (1970) suggested that a full description of plant response to water stress requires a measure of cell hydration rather than of cell water potential, the latter being more relevant to water movement. It has been suggested that "suction potential" should be retained as an acceptable alternative to water potential (Spanner, 1973). Alternatively, a fuller delineation of

the forces affecting water availability in the tissue, including osmotic (ψ_π), pressure (ψ_p) and possibly matric (ψ_m) forces, allows a better appreciation of the relationship between plant response and internal water status.

1.2 Salinity Stress

For many years it has been accepted that the deleterious effect of salinity is due to the decreased osmotic potential of the soil solution and that the plants are, in fact, exposed to a physiological drought (Bernstein and Hayward, 1958). However, it is now well established that when the osmotic potential of the soil solution around the plant roots decreases, the osmotic potential of the plant tissue also decreases proportionally. Such osmotic adjustment occurs whether the osmotically active substance in the growth medium permeates the plant tissues or not (Slatyer, 1961; Lagerwerff and Eagle, 1961; Ruf *et al.*, 1963; Cooper and Dumbroff, 1972). Consequently, the water potential gradient driving water into the plant is maintained under saline conditions and water availability to the plant is not reduced (Bernstein, 1963). In some cases, plants growing under conditions of increasing salinity suffer from a disturbed water balance initially. This effect is not permanent and is at least partially negated when adaptation to salinity occurs (Meiri and Poljakoff-Mayber, 1969). Moreover, plants grown in high salinity are often more succulent than control plants (Boyer, 1965), indicating that they are not suffering

from dehydration. Slatyer (1967) concluded that the main effects of salinity are associated with ion accumulation in the plant, rather than with reduced water availability.

Not withstanding these considerations, however, salinity toxicity symptoms often resemble drought symptoms (Bernstein,1961) and growth inhibition has been found to be proportional to the osmotic potential of the growth solution. It has been suggested that a high proportion of ions in the tissues of saline-treated plants is present in the cell wall, and the protoplasts of at least some cells within the tissues are actually under water stress, even when the plant appears to be at osmotic equilibriation (Oertli, 1966). O'leary (1969) found that the permeability decreased considerably in osmotically adjusted plants and indicated that an increase in resistance in the water flow pathway from the external solution to the leaves can result in the leaves experiencing a physiological drought even if osmotic adjustment has occurred. Klepper (1967) found evidence that a small decrease in osmotic potential of the substrate causes a measureable decrease in root permeability and, in fact, a reduction in water uptake through roots in the concentrated solution has been observed (Long, 1943). Nevertheless, reduced absorption of water is not the principal cause of reduced growth in a saline substrate (Kramer, 1969; Slatyer, 1967).

1.3 Temperature Extremes

Several temperature-sensitive plants respond to low temperature

by the leaves losing water and wilting, which may lead, eventually, to death by desiccation. Cucumber seedlings exhibit this chilling response at 5°C, where the leaves begin to droop and lose water within a few hours (Wright and Simon, 1973). Wheat plants grown at 10°C had a significantly lower water potential than those at 18°C or 27°C (Frank *et al.*, 1973) and cotton plants wilt when their roots are cooled quickly, the amount of water lost from the leaves being dependent on the length of the chilling period (Amin, 1969). In some cases, some of the symptoms of chilling injury are precisely the same as those that have been recognized as being the result of drought (Kramer, 1969).

This loss of water from the leaves of a chilled plant may be an indirect result of root cooling or the direct outcome of the chilling of the leaves. Kleinendorst and Brouwer (1972) have demonstrated that cooling the root medium reduced the water potential of maize plants by a primary effect on water permeability of the root tissue. According to Kramer (1942), low temperature reduced water absorption by the roots of all species studied, but more in the chilling-sensitive warm-season crops than in cool-season crops. Watermelon and cotton, for instance, absorbed only 20% as much water at 10°C as at 25°C. He concluded that reduced water uptake was caused by an increased viscosity of water and a decreased permeability in response to low temperature. Low temperature also increases the resistance

to water flux in cotton bark and affects the lateral movement of water across the xylem-phloem boundary in intact cotton stem (Klepper *et al.*, 1973).

In addition to these effects of chilling on water uptake and transport located perhaps primarily in the roots, there is also evidence for effects of chilling on membrane permeability (Lyons, 1973). Studies in which permeability changes have been measured by solute leakage or ion accumulation have provided some direct evidence for an increased membrane permeability in response to chilling. Increased permeability could result in greater water loss from the cells which would lead to a situation in which symptoms of drought would occur as well as symptoms of chilling (Wright and Simon, 1973).

Plant response to supra-optimal temperature, heat stress, is frequently associated with water deficit, as the elevated temperature of the leaf results in an enhanced transpiration rate. Lahiri and Singh (1969) exposed *Pennisetum typhoides* plants to 48°C and found that tissue moisture content decreased only slightly within 12 hours but thereafter tissue dehydration accelerated. Similarly, when young corn seedlings were exposed to a temperature of 38°C, leaf relative turgidity remained constant for the first two days of exposure, but thereafter, a consistent daily decrease occurred (Mattas and Pauli, 1965). Separation of the direct effects of high temperature on growth and development from the concomitant effects of water deficit presents

difficulties. The water deficit undoubtedly results mainly from enhanced transpiration, although root temperature above 30° reduced water absorption by lemon (Bialoglowski, 1936) and grapefruit (Haas, 1936). Growing plants in conditions of high humidity could, therefore, presumably allow investigation of the effects of high temperature uncomplicated by tissue water deficit. When Petinov and Razmaev (1961) investigated the effect of a brief exposure to high temperature (40°C) on wheat and corn plants under conditions of high relative humidity (85-90%), there were no substantial changes in the total water content of the leaves during heating or recovery. Moreover, the protoplasmic colloids did not lose their water-retaining capacity (Petinov and Razmaev, 1961). Onwueme *et al.* (1971) measured water potential and relative water content of leaves of barley plants briefly exposed to a supra-optimal air temperature with continuing sub-irrigation, and found that stressed plants had a slightly lower relative water content than the unstressed, while the water potential of the stressed plants averaged 0.7 bars lower than that of the unstressed. They did not attempt to control the relative humidity of the atmosphere, however. Such studies suggest that heat-induced tissue dehydration can be controlled by manipulating the external environment. Other changes in internal water relationships may occur as a result of high temperature, however. Recently, it has been suggested that high temperature could change the composition of the

leaf cell membranes, resulting in increased membrane leakage (Itai, *et al.*, 1973). Increased membrane leakage could cause greater water loss from the cells to intercellular spaces during heat stress, an effect which would not necessarily be evident from a gross measure of leaf water status.

In summary, a tissue water deficit may be produced by several different environmental stresses through different effects. Chilling stress will induce water deficit mainly due to changes in cell membrane permeability with consequently reduced water uptake and transport or increased membrane leakage. Similarly, it has been suggested that high temperature may also change the cell membrane, resulting in increased membrane leakage and greater water loss from the cell. In salt stress, despite the argument concerning the role of osmotic adjustment in regulating water uptake and transport, the ψ_π is reduced which could have a pronounced effect on metabolic processes in the cell. For example, the presence of enhanced quantities of electrolytes in the plant cell is likely to have a direct effect on protein hydration (Klotz, 1958). This in turn is likely to affect protoplasmic viscosity and volume relationships and lead to some internal water status changes in the plant cell.

2. Metabolism and environmental stress

2.1 Photosynthesis

2.1.1 Water Deficit

It is known that a decrease in plant water potential is followed by a decrease in the rate of photosynthesis. In general, the rate of apparent photosynthesis begins to decrease at a water deficit of only a few bars and reaches zero at zero turgor or even falls below the compensation point with respiration exceeding photosynthesis (Brix, 1962). The detailed contribution of the various components to this decrease in net photosynthesis is unknown. Numerous studies have shown a close parallel between CO_2 assimilation and stomatal resistance in the time course of development and release of water deficit (Brix, 1962; Boyer, 1970b) and, consequently, stomatal closure resulting from the increased water stress was usually considered to be the main cause of reduced photosynthesis.

In some circumstances, however, it was claimed that an increase in mesophyll resistance to CO_2 diffusion in the liquid phase was a factor controlling photosynthesis under water stress conditions (Troughton, 1969; Redshaw and Meidner, 1972). However, there is now increasing evidence that the mesophyll resistance is unaffected by water stress over a wide range of leaf water potential (Moidau, 1973). Other investigators have demonstrated reductions in either the photochemical or the biochemical activities of the chloroplast itself

when leaf water potentials are lowered (Fry, 1970). The chloroplast changes appear to involve reduced activity for electron transport (Boyer and Bowen, 1970), altered levels of photosynthetic intermediates (Santarius, 1967) and CO_2 fixation (Plaut and Bravdo, 1973). Recently, Boyer and Potter (1973) indicated that about 80% of the photochemical activity of the chloroplast remained even after turgor had decreased to zero. What is more, photoreduction and photophosphorylation may still be measured *in vivo* in a tissue which has lost 85% of its water content (Livne, 1971). In an attempt to determine the relative importance of these various effects, Jones (1973b) applied limitation analysis to water-stressed cotton plants and showed that stomatal resistance, intracellular resistance and the corresponding photosynthetic limitation were greater in the stressed plant than in the control. Similar changes in the activities of carbonic anhydrase, ribulosediphosphate carboxylase and $^{14}CO_2$ fixation by leaf slices were less significant, however, and although the activities of all the processes showed changes in the same direction, the evidence indicated that the major factor causing reduced photosynthesis in the stressed plant was stomatal closure.

2.1.2 Salinity Stess

The effect of salinity on photosynthesis appears to be variable. Thus, Nieman (1962) in investigating the salt tolerance of twelve crop species, showed that the rate of photosynthesis was little affected by NaCl and was not correlated with salt tolerance. On the other hand,

Boyer (1965) reported that the photosynthesis of cotton was reduced under saline conditions, despite the fact that the stomata remained open and CO_2 movement into the leaves was unaffected. In other cases, it has been suggested that CO_2 diffusion through the stomata could be limited (Gale *et al.*, 1967), or that diffusion across the plasma membrane of the mesophyll cell was affected (Gale *et al.*, 1966). An increase in the stomatal resistance to water vapour loss and CO_2 uptake, accompanied by an increase in mesophyll resistance to CO_2 uptake was also found in halophytes grown in a salt medium (Gale and Poljakoff-Mayber, 1970).

Apart from these effects on CO_2 movement to the chloroplast, there is evidence that the structure and function of the chloroplast changes with salinity stress. This change in chloroplast structure and function appears to be determined by both the ions involved and by their concentration. In an isotonic solution of NaCl, the structure of the maize chloroplast was disturbed, whereas in a comparable Na_2SO_4 solution, substantial structural changes were not observed (Lapina *et al.*, 1968). These differences in maize chloroplast structure induced by NaCl have been associated with changes in functional activity, a sharp reduction in assimilating area, reduction in photochemical activity and the Hill reaction. Significantly, the rate of photosynthesis is not inhibited by salinization of the soil with sodium sulphate (Lapina and Bikmukhametova, 1969).

These effects of salinity on chlorplasts have also been observed at the ultrastructural level (Lapina and Popov, 1970), and are accompanied by changes in chemical composition. Ponomareva *et al.* (1971), for instance, found that there was a reduction in chlorophyll content under salinization which was correlated with the stability of the bond of chlorophylls to the protein-lipid complex. Recently, Sivtsev *et al.* (1973) pointed out that an increase in the activity of chlorophyllase may be one of the factors responsible for the decrease in chlorophyll in tomato under salinization. This breakdown of chlorophyll leads to a salinity-induced chlorosis, but Vlasyuk and Zorya (1970) also found a reduction in protein content during this chlorosis and asserted that chlorosis during salinization is determined by disintegration of the chloroplast protein as well as loss of chlorophyll.

2.1.3 Temperature extremes

Chilling causes a progressive reduction in the photosynthetic capacity of the leaves of several plants of tropical or subtropical origin (Taylor and Craig, 1971). Drake and Raschke (1973) found that net photosynthesis and stomatal conductance decreased in plants exposed to a chilling temperature but that the reduction in net photosynthesis was not a consequence of the decreased stomatal conductance as intercellular CO_2 concentration in the chilled leaves was equal to or greater than that in the control. The effect of chilling on photo-

synthesis appears to be due mainly to ultrastructural changes in the chloroplast. Kimball and Salisbury (1973) pointed out that chilling modified chloroplast ultrastructure more than that of any other organelle. The chloroplast undergoes marked and quite rapid ultrastructural changes prior to the development of necrotic lesions (Taylor and Craig, 1971). The pattern of ultrastructural changes was similar in both C_4 and C_3- pathway species, despite some differences in the initial sites of chilling temperature action on photosynthesis. The most striking changes included stromal swelling, a decrease in thylakoid interspace widths and reduction in granal stacking.

These changes in chloroplast structure occur with changes in chloroplast function. Chloroplasts isolated from leaves cooled under light exhibit a sharply reduced capacity for cyclical and non-cyclical phosphorylation (Kislyuk and Vaśkovskii, 1972), and also a reduced rate of Hill reaction (Margulies and Jagendorf, 1960). Wright and Simon (1973) consider that change in the permeability of the chloroplast membrane may result in a reduction in photosynthesis at chilling temperatures. Gallopin and Jolliffe (1973) found that chilling can induce chlorosis in the leaves of corn, and they attributed this both to chlorophyll photo-oxidation and impaired chloroplast development. A spray application of kinetin was found to prevent this net chlorophyll loss from the chilled corn leaves. A similar response to chilling is the yellow-brown discoloration which develops in Bermuda grass and

Zoysia (Sachs *et al.*,1971).

An inhibition of the removal of assimilated products has also been suggested as a factor affecting photysynthesis and growth. In the case of the tropical grass, *Digitaria decumbens*, the high starch content accumulated in the leaves during the day disappears at night at normal temperatures but remains when the plant is exposed to a chilling temperature (Hilliard and West, 1970). This inhibition of translocation of carbohydrates away from the chloroplast was postulated to account for the reduced photosynthesis and growth.

As with low temperatures, supra-optimal temperatures also cause a temporary or permanent impairment of the photosynthetic system. The CO_2 fixation of detached tobacco leaves exposed for 2 minutes to temperatures above 45^{o}C was sharply decreased (Ben-Zioni and Itai, 1972). This decreased CO_2 fixation rate was not due to increased stomatal resistance but, rather, to ultrastructural changes in the chloroplast, and damaged membrane integrity was postulated to be the primary factor. Similarly, Daniell *et al.* (1969) exposed soybean and *Elodea* to high but sub-lethal temperatures which caused a loss of chlorophyll and swelling of chloroplasts. At the thermal death point disorganization of the chloroplast membrane occurred and it was concluded that the primary cause of the reduced photosynthesis was attributable to this disintegration of the chloroplast. Ben-Zioni and Itai (1972) also found that high temperatures caused chloroplast swelling.

Reasons other than structural alterations in the chloroplast have been advanced to explain the effects of high temperature on photosynthesis. Thus, Chernov *et al.* (1967) suggested that the reduced rate of CO_2 fixation is due to a limiting rate of ATP formation in photophosphorylation. Similarly, Langridge (1963) suggested that thermal inactivation of enzymes appeared to be the main reason for the progressive decline and ultimate cessation of photosynthesis. Even normally optimal temperature can cause a temporary inhibition of photosynthesis at certain rates of leaf heating (Belikov and Melekhov, 1972).

Heat stress may not only reduce photosynthesis but also change the photosynthetic products. With isolated chloroplasts and leaves exposed to high temperatures, less assimilated ^{14}C was detected in free sugar, starch and protein, while more was detected in the amino acids, alanine, aspartate and glutamate when compared to the same systems at lower temperatures (Ouellet, 1951). Similar changes occurred in the distribution pattern of ^{14}C in detached tobacco leaves exposed to temperatues above $45^{o}C$, the changes being associated with the impaired CO_2 fixation (Ben-Zioni and Itai, 1972). The basic cause of these changes in the distribution of ^{14}C among different compounds may be either an ATP deficit at high temperatures (Khlyustova and Tarchevskii, 1972) or differences in the thermal stability of the various enzymes acting at different stages of the photosynthetic metabolism of carbon (Shcherbakova, 1969).

In summary, all the environmental stresses considered (water, salinity and extreme temperature stress) could lower water potential in the leaves of the plant and consequently decrease photosynthesis. Lower water potential may affect photosynthesis in several ways: (1) It may affect the diffusion of CO_2; (2) alter the structure and function of organelles; (3) inhibit the dark reaction, and (4) inhibit removal of assimilated products. Most frequently, all these processes are affected by a low water potential simultaneously, although not to the same extent. As a consequence, the rate-limiting process differs in different circumstances (Waisel, 1972), and the nature and degree of rate-limitation depends upon both the environment and the plant species (Gale *et al.*, 1967). Generally speaking, the major factor causing reduced photosynthesis in water-stressed plants is stomatal closure which affects the diffusion of CO_2. In salinity or extreme changes in temperatures, however, the structure and function of the chloroplast could be the main factor to explain the reduced photosynthesis.

2.2 Respiration

2.2.1 Water deficit

It is generally agreed that under severe water stress respiration is drastically reduced (Hsiao, 1973). There is no consensus on the respiratory response of plants under less severe stress, however. In some experiments there has been a temporary increase in respiration, followed by a decrease, as more severe water stress develops (Brix,1962), whereas others have observed a continuous reduction in respiration as water potential becomes more negative (Boyer, 1970a; Flowers and Hanson, 1969). The reason for these changes in respiratory metabolism under conditions of stress has remained largely unexplored. Kursanov (1960) reasoned from the decrease in phosphorylated compounds and the reports of stimulated respiration during drought that oxidation and phosphorylation become uncoupled. More recently, however, studies with mitochondria have indicated that this is unlikely (Bell *et al.*, 1971). A lowering of tissue water potential by more than 4 or 5 bars resulted in a reduction in state III and state IV mitochondrial respiration from etiolated maize shoots, and at water potentials less than -35 bars, all respiration ceased. Phosphorylation was not uncoupled by tissue water stress. Osmotically stressing isolated plant mitochondria also reduced state III respiration and phosphorylation,though not state IV respiration (Flowers and Hanson, 1969). Mitochondria isolated from water-stressed corn shoots were found to show altered

swelling and ion transport characteristics when compared with mitochondria from non-stressed plants (Miller *et al.*, 1971), suggesting that water stress had a marked effect on the membrane integrity of the mitochondrion. Similar *in vitro* mitochondrion responses to water stress have been correlated with the respiration processes of the whole plant by Koeppe *et al.* (1973), who suggest that the decrease in the rate of whole plant respiration with increased stress may be due to membrane alterations that affect the ability of the mitochondria to oxidize substrate.

Structural damage to mitochondria has been observed in the meristematic cells of maize and pea roots during atmospheric desiccation (Nir *et al.*,1969). The structure of these organelles from slightly vacuolated tissue was restored following rehydration of the tissue, except when desiccation had been extremely severe. Exposure of plant cells to osmotica which permeate the cell slowly has often been shown to decrease respiration and, in contrast, osmotica which permeate the cell rapidly have little effect on the respiration of *Zea mays* (Greenway and Leahy, 1970). They postulated that reduced respiration during osmotic stress is due to damage to the mitochondria as a result of a sharp increase in the osmotic potential of the cell fluids (Greenway and West, 1973).

2.2.2 Salinity stress

It is also difficult to generalize on the effects of salinity on respiration. Nieman (1962) reported that the leaves of salt-treated plants showed an increased respiration rate and Livne and Levin (1967) found that NaCl enhanced the respiration of the leaves, roots and stems of pea seedlings, stimulation of respiration being found in both etiolated and green plants. In contrast, Hasson-Porath and Poljakoff-Mayber (1964) report that NaCl reduced the respiration of pea root tips and salinization reduced respiration in wheat and gram seedlings (Sarin and Rao, 1958). The response of respiration to salinity of the growth medium may vary between different groups of plants as, according to Takaoki (1957), an increase in the salinity of the medium for halophytes caused an increase in their respiration rate, whereas it caused a decrease in the respiration of glycophytes. Nevertheless, information is only available for very few species and such generalizations can be misleading. Differences in response to salinity not only appear to exist between species, but also between different organs on the plant as salinization usually affects leaves more than stems and roots (Livne and Levin, 1967).

The ionic species used to induce salinity will also affect the respiration response. Whilst most studies have been concerned with the response to NaCl, there are comparative studies that suggest that the response to isosmotic concentrations of Na_2SO_4 may be less (Hasson-Porath and Poljakoff-Mayber, 1964, 1971; Lapina and Bikmukhametova,

1972). This difference may be related to the effects on the ATP/ ADP ratio (Livne and Levin, 1967) but this suggestion has been challenged (Samukawa and Harada, 1971).

2.2.3 Temperature extremes

The relationship between respiratory activity and chilling has been investigated in tissue slices, excised roots and intact plants. In the detached organ, except during the ripening of fruit of the climacteric type, the normal pattern of respiration is a gradual decline with time after harvest (Lyons, 1973), but with a chilling-sensitive organ, for example a cucumber fruit, exposed to low temperature, the respiratory rate increases to a plateau and then decreases (Eaks and Morris, 1956). Similar reports of accelerated respiration have been made for sweet potato roots (Lewis and Morris, 1956), tomatoes (Lewis, 1956), and a number of plants, (Lyons, 1973). This rapid respiration during storage at a chilling temperature usually precedes any external visible symptoms of injury. The response of leaf respiration to chilling temperatures is so similar to that reported for isolated mitochondria (Wright and Simon, 1973) as to indicate strongly that it is the mitochondria that are especially sensitive to chilling. Lyons and Raison (1970) point out that the mitochondrial membranes from chilling-resistant plants have a higher proportion of unsaturated fatty acids than those of sensitive plants. They found that mitochondria from chilling-sensitive species exhibited a thermal transition in

response. Similarly, mitochondria from different apply cultivars undergo phase transitions at temperatures ranging from 3° to 10°C (McGlasson and Raison, 1973). Lyons and Raison (1970) concluded that in chilling-sensitive plants the hightened Q_{10} of mitochondrial activity at chilling temperatures may reflect a consequence of this phase change, which also results in a change in the permeability of the mitochondrion and increased Arrhenius activation energy (E_a) of membrane-bound enzyme systems, leading to a suppression of the reaction rate. A greatly reduced energy supply may accompany the suppressed mitochondrial respiration, due possibly to altered activity of the membrane-bound ATPase system (Lyons, 1973). These effects may be related to structural changes observed with the electron microscope. Thus, mitochondria derived from chilled sweet potato roots showed a large proportion in an extremely swollen form (Yamaki and Uritani, 1972). This swollen appearance resulted from degradation of mitochondria and the release of phospholipid from both the inner and outer membranes. Kushnirenko *et al.* (1969) also found changes in the ultrastructure of the mitochondria from cucumber at chilling temperatures, including clarification of the matrix, shortening of the cristae and a decrease in their number, these ultrastructural changes being accompanied by a loss of phosphorylating activity.

As the temperature rises, the respiration rate increases, reaching a certain maximum value characteristic for the given organs, and then starts to drop with further increase in temperature (Goodman and

Wedding, 1956). The maximum respiration rate at any supra-optimal temperature is only maintained for a short period of time (5-6 hours) and then starts to decrease. The drop in respiration rate is thought to be caused by the inactivation of enzymes (Petinov and Razmaev, 1962). The respiration rates of leaf discs and root tips of pea are sharply reduced on exposure to high temperature, the reduction being proportional to exposure time, and the activity of mitochondria closely parallels this reduction in respiration on heating (Cole and Steponkus, 1969). Kurkova and Andreeva (1966) found that there was an alteration in oxidative and phosphorylation activities of corn mitochondria with increasing temperature; coupling between oxidation and phosphorylation began to decrease at 30-35^{o}C and at 40^{o}C uncoupling was complete.

As with cold stress, the alteration in biochemical activity was accompanied by a disorientation of internal mitochondrial structure (Andreeva, 1969). Heat stress results in irreversible damage to mitochondrial ultrastructure, the symptoms of this damage including shape changes, decrease in the electronic density of the matrix, vesiculation of cristae, and destruction of the internal membranous structure (Andreeva, 1969). Similarly, heat stress damaged membrane integrity of mitochondria of tobacco leaves (Ben-Zioni and Itai, 1973), although this was accompanied by an increase in respiration rate.

It is generally agreed that respiration is reduced under environmental stress although the respiration response is variable in some circumstances. The main reason for reduced respiration under environmental stress appears to be damage to mitochondrial ultrastructure. The symptoms of this damage include shape changes, vesiculation of cristae and destruction of the internal membranous structure. These changes consequently affect the ability of the mitochondria to oxidize substrate. The degree of respiration depression and the extent of alteration of mitochondrial structure varies both with different types of stress and different species.

2.3 Carbohydrate

2.3.1 Water deficit

Water stress produces important changes in the kinds and amounts of carbohydrates in the plants. The starch content of the leaves generally has been observed to decrease during water stress, which is usually accompanied by an increase in sugar content (Iljin,1957; Stewart, 1971). The changes in proportions of sugars and starch are presumably related to changes in enzyme activity. Stimulated amylase activity (Spoehr and Malner, 1939) and decreased invertase activity (Maranville and Paulsen, 1970) in plants under water stress appeared responsible for these changes. However, the sugar content does not increase in all species (Wadleigh and Ayer, 1945), and changes in starch content were not compensated for completely by changes in sugar content (Woodhams and Kozlowski, 1954); they attributed the differences to high respiration in the stressed plant. Evidently, there are important differences among species in the effects of water stress on carbohydrate metabolism. The situation is complicated by the fact that respiration often decreases more slowly than photosynthesis, causing depletion of food reserves and changes in the proportions of various carbohydrates.

Decreases in starch or increases in sugar synthesis has also been observed by several investigators (Iljin, 1957; Nezgovorov, 1957).

Recently, Greenway and Leahy (1972) suggested that effect of water deficit on carbohydrate metabolic processes are due to reduced turgor and hydration and not to low water potential *per se*. They observed that stimulated sucrose synthesis and reduced starch synthesis in root tissues of *Zea mays* by exposure to low water potential (ψ = -20 atm) induced by mannitol which slowly permeates cells. In contrast, carbohydrate metabolism changes little during exposure to isosmotic concentation of ethylene glycol, which rapidly permeates cells.

In addition to these alterations in carbohydrate composition, water stress also affects the pathway of glucose metabolism. Glycolytic activity was suppressed almost completely in droughted leaves of corn and fodder bean, while at the same time the rate of oxidation via the pentose phosphate pathway doubled (Abrarov, 1969).

2.3.2 Salinity stress

The metabolism of carbohydrates in plants is affected by a general increase in salinity, as well as by the type of ion present. Boiko and Matukhin (1960) have shown that in barley and millet, the synthesis of sucrose is stimulated in leaves by exposure to NaCl, sucrose metabolism is reduced, and starch synthesis is inhibited. Similar salt stress-induced inhibition of the synthesis of starch and sucrose accumulation has also been found in pea root tip tissue

(Hasson-Porath and Poljakoff-Mayber, 1973), the accumulation of sucrose being due to promotion of the activity of hydrolytic enzymes. Marked increases in the activity of amylase have been shown to occur on exposure to NaCl or Na_2SO_4 salinity, (Dixon and Webb, 1957; Strogonov, 1964).

It has also been reported that carbohydrate metabolism may be altered by salt stress. Pea roots grown in a medium containing NaCl exhibited depressed glucose absorption and increased metabolism of glucose through the pentose phosphate pathway, the activity of the soluble glucose-6-phosphate dehydrogenase coupled to NADP also increased considerably (Hasson-Porath and Poljakoff-Mayber, 1970). They suggested that the chloride type of salinity induced a shift in carbohydrate metabolism from the energy-yielding Embden-Meyerhof-Parnas pathway to the pentose phosphate pathway and thus interfered with the energy supply to the normal processes occurring in the cells and tissues. On the other hand sulphate type of salinity has a different fundamental effect even though the respiratory activity in root tips grown in sulphate salinized media is depressed to the same extent as that in roots grown in chloride-salinized media (Hasson-Porath and Poljakoff-Mayber, 1964). No clear shift from Embden-Meyerhof-Parnas pathway to the pentose phosphate pathway is observed, nor any increase in the activity of soluble NADP coupled to glucose-6-phosphate dehydrogenase. It has also been suggested that under chloride salinization, the first stage

of glycolysis for barley and sunflower is regulated by phospho-fructokinase, whereas under sulphate salinization glucose phosphate isomerase is involved (Zhukouskaya *et al.* 1969).

2.3.3 Temperature extremes

The sugar content of plants almost always increases during exposure to low temperature, and sucrose is frequently the major sugar to accumulate (Guinn and Hunter, 1968). The accumulation of sugar is not always accompanied by a decrease in starch content (Guinn, 1971). In contrast, Guinn and Hunter (1968) found that sugar as well as starch accumulated in all plant parts as the young cotton plant was subjected to 10°C (low) temperature. Increases in the concentration of all forms of soluble sugar and starch were also to be found in melon, cucumber, pumpkin and tobacco grown in low temperature (Nezgovorov *et al.*, 1966). Taylor, Jepsen and Christeller (1972) indicated that the changes in sugar and starch seemed to correlate with the degree of chilling tolerance of various species. The carbohydrate content rose in stress-tolerant ryegrass, with sugars showing the sharpest increase, while they fell in the stress-sensitive sorghum, with starch being most affected. Paspalum, which is semi-tolerant, showed an intermediate response with levels rising initially, then falling. Starch level in these plants is dependent on the time that the temperature is lowered and seems to

be related to rates of CO_2 assimilation under stress conditions. Since starch as well as sugar increases at chilling temperatures, the accumulation of sugar is not likely to be caused by starch breakdown (Guinn and Hunter, 1968); it has also been suggested (Moe and Wickstorm, 1973) that low temperature increased carbohydrate synthesis.

In contrast to the effects of low temperature on carbohydrate content, exposure to high temperatures leads to progressive depletion of the carbohydrate pool. Such responses have been observed with ryegrass (Sullivan *et al.*,1949) and Kentucky blue grass (Watschke *et al.*, 1972) amongst other species

These responses need to be distinguished from the effects of a brief period of overheating on carbohydrate metabolism. Following an exposure at 40°C the total suger content in both wheat and corn leaves increased (Petinov and Razamer, 1962). In wheat this is associated with an increase of glucose and fructose and in corn of sucrose. This difference, apparently, is associated with the resistance of wheat and corn to the injurious effect of high temperature. It was suggested by Rustambekov (1948) that under condition of elevated temperature, invertase activity shifts towards synthesis in resistant plants and towards hydrolysis in non-resistant. Itai and Ben-Zioni (1973) also found the amylolytic activity increased in tobacco leaf discs after exposure to short intervals of high temperature (47-49°C). In recent

years, the notion has become popular that the relation between the glycolytic and pentose phosphate pathway can be altered by a number of compounds and also by external factors. Semikhatova and Yudina (1964) showed that the relation between these two pathways does not change under high or low non-damaging temperatures. Further, Yudina *et al.* (1969) confirmed that high non-lethal temperature affects the rate of respiratory metabolism but does not change the pathway of breakdown of glucose.

Environmental stress affects many aspects of carbohydrate metabolism apart from photosynthesis. The changes in concentrations of the carbohydrate constituents during periods of environmental stress are generally explicable in terms of changes in enzymatic activities. An increase in the sucrose concentration in a stressed plant is a general phenomenon and apparently is due to depressed hydrolysis rather than to accelerated synthesis. Similarly, the general decrease in starch concentration caused by stress is due to accelerated hydrolysis rather than depressed synthesis. A general stimulation of hydrolytic activity, such as an effect on starch hydrolase, is a common phenomenon during stress; sugar concentration did not increase so as to compensate for the decreased starch concentration in stressed plants in some circumstances. Utilization of starch breakdown products by respiration, at rates faster than their synthesis, would deplete the starch content but may have no effect on the sugar content.

Irreversible changes in the structure of membranes constitute one of the primary causes of injury to the living cell in the course of temperature or water stress (Parker, 1972), and probably in salinity stress (Levitt, 1972). These changes can be prevented when sugars are present, and sugar itself, might be able to stabilize protein and, therefore, the membrane, against different kinds of stress.

2.4 Nucleic acid

2.4.1 Water deficit

Water stress has been stated to lead to an initial rise in RNA content followed by a drop over a 20 day period in the tomato plant (Kessler, 1961). As the RNA content decreased, RNase activity increased. The rise in RNase activity coupled with a failure of water stress to affect uracil incorporation suggested that the reported lowering of RNA content during drought resulted from RNA hydrolysis. Similar results were obtained by Gates and Bonner (1959).

On the other hand, in sugar beet, total RNA and protein content were affected before visible wilting occurred (Shah and Loomis, 1965). During moisture stress, soluble RNA increased and the amount of RNA in other fractions, especially in ribosomes, decreased. In this case, uracil-^{14}C turnover in the water-stressed plants was reduced, suggesting inhibition of RNA synthesis under water stress, in contrast to the situation reported by Kessler (1961).

Notwithstanding the apparent lack of agreement on the effect of water stress on RNA synthesis, it has been shown by several workers that RNase activity increases in tissues subjected to water stress (Todd, 1972). Increased RNase activity has been suggested as a cause of the shift from polysomes to monosomes (Bewley, 1973). Genkel *et al.* (1967) exposed maize and bean plants to drought. Although incorporation of ^{15}N into protein decreased, RNA content was not affected.

Polysomes, however, disappeared from the droughted plants. They concluded that RNase destroyed mRNA which binds ribosomes into polysomes. In agreement with this conclusion, Chen *et al.* (1968) found that dehydration of wheat embryos inactivated mRNA and arrested protein synthesis. However, in black locust, a drought hardy species, there is no drastic loss of polysomes nor any drastic increase in RNase activity during water stress (Brandle *et al.*, 1973). Moreover, the relationship between RNase activity and the dispersion of polysomes has been questioned (Hsiao, 1970). In early stages of stress, the polysome level has been shown to decline substantially, whereas tissue RNase level showed no change. Further, the shape of the polysome profile obtained from stressed tissue was not consistent with a random cleavage of mRNA linking ribosomes. As random cleavage would be expected if RNase activity were the cause of the low proportion of polysomes, this evidence also suggests that the early stress effect is not mediated by RNase (Hsiao, 1970).

Apart from these gross changes in RNA metabolism, water stress has also been shown to alter the base ratio in the nucleic acid (Kessler and Frank-Tishel, 1962). There was an increase in the $(G+C)/(A+U)$ base ratio when drought-tolerant olive leaves, *Olea europea*, were subjected to water stress, whereas the ratio in *Ligustrum sinensis*, which is drought-sensitive, did not change. A similar increased base ratio was also recorded in corn seedlings that were subjected to water

stress (West, 1962). Many of these and other early reports of dramatic changes in base composition caused by water stress now seem implausible in the light of recent evidence, however, and might possibly be attributed to poor analytic techniques (Hsiao,1973).

2.4.2 Salinity stress

Salinity stress, like water stress, reduces nucleic acid content, particularly when the salinity treatments are first imposed. The magnitude of nucleic acid degradation depends on the ion and plant species. It has been shown that sodium ions are more effective than potassium in accelerating the endogenous reduction of RNA in soybean roots (Rauser and Hanson, 1966). The amount of nucleic acid degradation may be a result of intensified decomposition or retarded synthesis. The decrease in RNA has been attributed to an intensification of RNase activity and the decrease in DNA to impaired synthesis (Kessler *et al.*, 1964). However, from isotope incorporation studies, some authors consider that retarded RNA synthesis under salinity stress may be the primary factor (Rauser and Hanson, 1966). For example, sodium sulphate drastically reduces ^{32}p incorporation into nucleic acid in soybean roots (Rauser and Hanson, 1966). A high concentration (1.6%) of NaCl decreased both low and high molecular weight RNA content in tomato leaf tissue and inhibited 2-C^{14}-orotic acid incorporation into all nucleic acid fractions, including 4s RNA, 5s RNA, rRNA and mRNA (Tsenov *et al.*,

1973). Similarly, salinity suppression of the synthesis of RNA has also been confirmed for the soybean leaf (Nieman, 1965). In contrast to these reports of inhibition of RNA accumulation by salinity stress, Morozovskii and Kabanov (1968) found that 0.2-0.4% NaCl did not interfere with nucleic acid synthesis in pea leaves and the incorporation of ^{14}C-adenine into total RNA, 5s RNA and rRNA did not decrease at a high concentration (0.8%) of NaCl Kabanov *et al.*, 1973)

In halophytic plant, Catarino and Frewavas (1970) found that the incorporation of adenine-H^3 into all ribonucleic acid fractions in the leaves of the halophytic plant, *Labularia*, was considerably accelerated by spraying with a 2% NaCl solution. Synthesis of 4s RNA increased to the greatest extent in the process. This may be the reason why the halophytes can adapt to wide variations in soil salinity.

2.4.3 Temperature extremes

Several workers have found an increase in RNA in winter rye, wheat and barley during chilling (Konarev, 1954; Shvedskaya and Kruzhilin, 1968; Shiomi and Hori, 1973a). For instance, barley seedling grown at 2-3^{o}C for four weeks had a greater DNA and RNA content than plants grown at 20^{o}C (Shiomi and Hori, 1973a). Such an increase is not invariable, however, and various authors have reported no change in

the content of RNA, DNA or both in chilled plants. Finch and Carr (1956) found no change in the DNA and RNA content in chilled plants of Petkus rye. Similarly, Guinn (1970) did not find any change in the RNA content of cotton leaves exposed to $5^{o}C$.

Where changes in RNA content occur, specific nucleic acid synthesis may be involved (Salisbury and Bonner, 1960). Shiomi and Hori (1973a) found a rapidly labelled nucleic acid in barley coleoptiles grown at cold temperatures. Much thymidine-^{3}H was incorporated into DNA and uridine-^{3}H into RNA fractions, but the distribution pattern was not distinctly different from that of plants grown at a higher temperature. Evidence has also shown that low temperatures bring about increased levelling of RNA in potato leaves (Li and Weiser, 1969). Although there was a marked quantitative increase in RNA following the low temperature, the nucleotide composition of all types of RNA did not change appreciably (Oslund and Li, 1972).

More recently it has been suggested that tRNA has a regulatory function in the translation of mRNA (Stent, 1964). Differential synthesis of protein in response to environmental stimuli may depend on the functional concentration of specific aminoacyl-tRNA (Anderson, 1969). Brown and Yang (1973) studied the changes in the iso-accepting tRNA pattern during a chilling stress in soybean and found that the total change was greater for all three aminoacyl-tRNA species examined from the non-chilled source.

The decrease in top growth in perennial grasses caused by high temperature has been found to be accompanied by a decrease in RNA concentration and a slight decrease in DNA content (Baker and Jung, 1970). Also, thermal hardening of corn plants could lead to the creation of better conditions for the conservation of the RNA content (Satarova and Tvorus, 1966). It was proposed, in view of these results, that the influence of high temperature on growth may occur in part through an effect on nucleic acid. High temperature not only reduces the nucleic acid content but also has been demonstrated to change the nucleotide composition of nucleic acid (Bozhenko *et al.*, 1972). There is a distinct decrease in the content of G-C pairs of DNA bases in both the stem growing point and the roots of sunflower plants subjected to 46-47°C for 48 hours (Bozhenko *et al.*, 1972). The mechanism of this effect of high temperature on nucleic acid metabolism is unknown so far. Bernheim (1955) suggested that heat destroys RNA and results in an inhibition of enzyme formation. Tvorus (1970) ascribed the decrease in RNA content during heat stress to high RNase activity and found that RNase activity in the cytoplasm of leaves of horse bean plants subjected to a high temperature was increased by 66%. RNase is also fairly resistant to high temperature; the optimum temperature for pancreatic RNase action is 65°C and for that from citrus leaves is 70°C (Kessler and Monselise, 1959). However, Tvorus (1970) in his experiment did not separate the effects of heat stress from those

of a water deficit. The increase in RNase activity which he reported could have been caused by water stress rather than by heat stress.

In general, RNA, particularly mRNA, is much more affected by environmental stress than is DNA. A decrease in RNA, which is often found, is probably linked to a decrease in the rate of protein synthesis. This decrease in RNA has been frequently attributed to an increase in the level of potency of ribonuclease. However, the early stress effect seems not to be mediated by ribonuclease (Hsiao, 1973). Changes in ribosomal pattern have also been described but, in general, were obtained with tissue from young plants at a stage of development when stress resistance is at its lowest (Levitt, 1972). It is difficult, therefore, to draw broad generalizations from the limited number of species at a limited range of development with different past environmental histories.

In the light of current knowledge on the complexity of the controls operating in the cell at both the transcription and translation levels, information on total or bulk fractions of nucleic acids as related to environmental stresses is of minimal value.

2.5 Nitrogen compounds

2.5.2 Water deficit

It has been considered for some time that water stress reduces the protein content of the plant despite some results showing an apparent increase (Chen *et al.* 1964). Such a decrease in protein content may result from either a retardation of protein synthesis or an acceleration of protein degradation. Leaf protein had been shown to undergo accelerated hydrolysis as water stress develops (Mothes, 1956; Petrie and Wood, 1938). However, inhibition of amino acid incorporation has also been observed in several studies (Kozlowski, 1972), and it has been suggested that protein synthesis is readily inhibited, even by a very mild water stress. Further, Barnett and Naylor (1966) demonstrated that water stress causes inhibition of protein synthesis but that amino acids are continually synthesized during water stress. In the initial stage of moisture deficit an inhibition of the synthesis of protein may be the primary effect, and protein hydrolysis, if promoted, may be associated only with extreme conditions of wilting (Lahiri and Singh, 1969).

Associated with these effects on protein metabolism, water stress induces a characteristic change in the levels of free amino acids; particularly, in many species, a great increase in free proline and amides (Crafts, 1968). The concentration of proline accumulated can amount to more than 1% of the total dry matter in some species. The

accumulation takes place only if there are adequate amounts of carbohydrates in the tissue (Stewart *et al.*, 1966) and it is readily reversed by rewatering. However, proline does not accumulate in dehydrated discs of storage root and the phenomenon appears to be restricted to green tissue (Stewart *et al.* 1966; Singh *et al.*,1973b).

Stewart (1972a) demonstrated that primary effects of wilting that lead to an accumulation of proline, were a decrease in utilization, and an increase in proline formation. Proline formation occurs primarily by proteolysis and by *de novo* synthesis. Since the increase in proline during wilting exceeds the proline released from protein (Kemble and MacPherson, 1954), the accumulated proline apparently derives primarily from *de novo* synthesis (Singh *et al.*, 1973c) with glutamic acid as a precursor (Barnett and Naylor, 1966). In some circumstances, it may still, however, be an indirect result of protein breakdown in the same way as the accumulation of amides, *i.e.*, by conversion into proline of other amino acids which are primary products of protein hydrolysis (Stewart, 1972a). On the other hand, some evidence indicates that carbohydrates are the ultimate source of the carbon skeleton (Stewart *et al.*, 1966).

Several workers (Barnett and Naylor, 1966; Routley, 1966) suggested that proline may serve as a storage compound for reduced carbon and nitrogen during stress. This suggests that proline accumulation is possibly beneficial to the plant under stress and free proline has been

ascribed a protective function during the advent of unfavourable conditions (Savitskaya, 1967). This function has been linked with the high hydrophilicity of proline and its ability to stabilize colloids (Protsenko *et al.*, 1968). Singh *et al.* (1972) found that the ability of 10 barley varieties to accumulate proline under severe stress was positively correlated with their drought resistance rating. Moreover, the growth rate of barley seedlings following release from a period of stress was positively correlated with the amount of proline accumulated during the stress (Singh *et al.*, 1973d). Further, it has been suggested as an evaluating parameter for irrigation scheduling and for selecting drought-resistant varieties (Palfi *et al.*, 1973).

2.5.2 Salinity stress

There can be no doubt that the nitrogen metabolism of a plant growing in saline conditions undergoes considerable modification. The protein content of various plant tissues declines, apparently due to increased proteolysis and decreased protein synthesis (Vaadia and Waisel, 1967). Such conditions also inhibit transamination processes (Zholkevitch and Koretskaya, 1959) and cause the accumulation of ammonia (Strogonov, 1964). It was reported that the protein synthesis potential of leaves was decreased upon the addition of NaCl to the root media (Ben-Zioni *et al.*, 1967). Exposing the roots to a saline medium of several bars for days depressed the subsequent incorporation of amino

acids into protein in leaf discs (Itai *et al.*, 1968). A similar depressive effect on amino acid incorporation in pea root apices was much more severe with Na_2SO_4 than with NaCl at the same ψ (Kahane and Poljakoff-Mayber, 1968) attesting that salinity has effects other than those of water stress.

An amino acid-incorporating microsomal fraction has been isolated from the leaves of the halophyte *Suaeda maritima* (Halls and Flowers, 1973), and no differences were found in the properties of the microsomes isolated from plants grown in saline and non-saline conditions. The incorporation was severely inhibited by high concentrations of sodium or potassium ions. In addition, enzymes isolated from halophytes do not appear to show any enhanced tolerance to salinity, being inhibited by concentrations of sodium chloride which also inhibit similar enzymes from salt-sensitive species (Flowers, 1972; Greenway and Osmond, 1972). This is presumably related to the spatial separation of the salt from the cytoplasmic components by sequesterization within the vacuole and argues for a low cytoplasmic sodium content. However, this is not supported by results obtained with the electron probe microanalyser (Waisel and Eshel, 1971) which were interpreted in terms of a high, cytoplasmic sodium content.

Changes in nitrogen metabolism in plants growing in saline conditions also leads to a marked increase in the content of a number of free amino acids (Strogonov, 1964). The accumulation of these amino acids depends

not only on the concentration of salts, but also on their biological properties. For example, high concentrations of Na_2SO_4 promote the accumulation of S-amino acids, sulfoxides of l-methionine and S-methyl-l-cysteine (Shevyakova and Strogonov, 1968).

Salinity stress also induces proline accumulation in plant tissues. (Palfi and Juhasz (1970) found that in paprika and sunflower plants grown in a 2% salt solution, the proline content increased, respectively, 60 or 53 times above that of the control. In peas and beans, the increase was less marked (x 6.3 and x 4.3). In addition to this increase in the proline content, the total amount of free amino acids also increased by 30-90% in plants grown in highly saline soil.

2.5.3 Temperature extremes

Many workers have observed an increase in the total soluble nitrogen content of plants grown at low temperatures (Pauli and Mitchell, 1960; Zech and Pauli, 1960). In some circumstances, the total nitrogen and total soluble nitrogen content were both found to decrease (Omran and Powell, 1969) or to remain unchanged (Guinn,1971). However, several reports indicate that chilling temperatures induce amino acid accumulation in the leaf (Trione, Young and Yamamoto,1967), seedlings (Trione,1966) and grains (Sparmann, 1961). Again, the source of the accumulated amino acids may be increased proteolytic activity or a decreased rate of protein synthesis from the amino acid pool.

Protein breakdown at chilling temperatures, without an equally rapid resynthesis, has been suggested as a cause of the observed injury, either due to a deficiency of protein or to a toxicity from the products of hydrolysis. Wilhelm (1935) produced evidence of such hydrolysis in the case of beans and tomato plants exposed to chilling temperature stress and demonstrated a close relation between the degree of injury and the rate of protein decomposition.

Similarly, Razmaev(1965), observed proteolysis in chilling-sensitive plants, but not in those resistant to chilling injury. Minamikawa *et al.* (1961), on the other hand, failed to detect any hydrolysis of protein in the mitochondria of chilled sweet potatoes and concluded that proteins are not likely to be degraded during chilling injury. Recently, Rodchenko and Maricheva (1973) studied changes in protein metabolism and the rate of root cell growth in corn at $10^{o}C$ and found that the rate of protein synthesis at chilling temperatures ($10^{o}C$) was significantly reduced. The reason for reducing protein synthesis in higher plants at chilling temperature is unknown, but evidence from bacteria has indicated that regulatory enzymes may become too sensitive to feedback effectors so that they cannot function at low temperature. In other cases, the component proteins of essential aggregates lose their ability to form such aggregates. In *E. coli*, ribosomal subunits accumulate at chilling temperatures due to a block in the formation of the initiation complex, and therefore, protein synthesis (Friedman *et al.*,1969).

The level of all major free amino acids and several minor amino acids is changed significantly by chilling treatments (Srivastava and Fowden, 1972). Exposure of ryegrass, soybean and paspalum to a chilling temperature resulted in a general increase in the level of all the free amino acids. Amide tended to accumulate in C_3 more than in C_4 pathway species; but there was a sharp, though sometimes transitory, fall in the level of many amino acids associated with intermediates of the C_4 pathway (Taylor, Jepsen and Christeller,1972). Chilling temperatues also induce proline accumulation in sunflower bean and paprika (Palfi and Juhasz, 1970) wheat (Scheffer and Lorenz,1968) oat (Kinbacher, 1960) and *Stylo* (Gates *et al.*, 1971). Palfi and Juhasz (1970) ascribed this proline accumulation to water deficit in the plant, since the chilling temperature reduced water absorption and resulted in a physiological drought. A close correlation has been demonstrated between the accumulation of free proline and tissue resistance to chilling temperatures (Bokarev and Ivanova, 1971) which has also been suggested to depend upon the degree of hydration of the protoplast.

Heat stress causes the hydrolysis of complex proteins; during the heating period the proteolysis process is active, but soon after the removal of heat stress, proteolysis ceases and gives way to vigorous protein synthesis (Petinov, 1960). The increased proteolysis caused by heat stress leads to the accumulation of soluble nitrogen as well

as amino acids and amides (Henckel, 1964). Although an accumulation of soluble nitrogen is the resultant effect of both temperature and water stress, hyperthermia appears primarily to trigger off proteolysis while dehydration causes an inhibition of protein synthesis. With further dehydration, protein degradation may occur when the plants have wilted (Lahiri and Singh, 1969). Thus, accumulation of amino acids under the two types of stress appears to be due to different causes. This conclusion seems quite doubtful, however, since in their experiment they did not separate water deficit from heat stress and tissue dehydration was greatly aggravated after a 24 hour exposure of three-week-old *Pennisetum typhoides* to 48 $\pm$ 1^{o}C.

High temperature death may occur when the speed of resynthesis of an indispensable component is unable to compensate for its degradation (Allen, 1950) but there is little evidence indicating that rapid resynthesis of native protein is important in enhancing survival at high temperature. In corn mitochondria, phosphorylation was decreased during exposure to high temperature (Kurkova and Andreeva, 1966) and the decreased phosphorylation would certainly have lead to decreased protein synthesis. When the alga *Physarum polycephalum* was subjected to heat shocks at 40^{o}C for 30 minutes, the incorporation of amino acid into protein was decreased by approximately 70%. There was also a decrease in polyribosomes of more than 50%, which was the cause of the decreased protein synthesis (Schiebel *et al.*, 1969). Similarly, in

the case of a thermal blue-green alga, high temperatures which had a strong growth-inhibitory effect, inhibited both amino acid and protein synthesis (Moyse and Guyon, 1963).

Death from exposure to high temperature has usually been explained as the result of the denaturation of protein. Heat-denatured protein may aggregate or coagulate, with the coagulation being a secondary phenomenon (Putnam, 1953). In 1938, Maximov stated that the action of supraoptimal temperature in this way is not often sufficient to account for the response, since death may begin at 40°C, a level which is far below the coagulation point of proteins. Recently, Levitt (1972) has pointed out that heat injury is due to protein denaturation at high temperature followed by aggregation. The first effect of high temperature on protein is denaturation which is reversible, but as the temperature rises, aggregation occurs. Many proteins are converted so rapidly to the irreversibly-aggregated state that the reversible denaturation is difficult to detect and has been overlooked previously.

A common characteristic of plants under different environmental stress is an increase in the concentration of free amino acids. The source of these accumulated amino acids may be an increase in proteolytic activity or a decreased rate of protein synthesis from the amino acid pool. Although increased protein hydrolysis is a common response of plants to many different environmental stresses, the relationship of environmental stress to protein hydrolysis is not fully clarified. At

least some evidence indicates that free amino acids could be synthesized during stress, particularly proline. The changes in amino acid metabolism may play a significant physiological role in the plant and may be concerned in the adaptation of the plant to different environmental conditions. It has been suggested (Slatyer, 1967) that if stress is gradually imposed over a period of days, slow changes in protein and protoplasmic structure and function occur which are associated with a gradual inactivation of nucleic acid synthesis and an accelerated breakdown of metabolites in less active cells and tissues. As stress becomes more severe, synthesis is probably completely inhibited, and protein breaks down.

3. Plant growth and environmental stress

3.1 Water deficit

Water deficit depresses plant growth and yield. It is logical to assume that the depression in growth and dry weight is due to an effect of the water deficit on photosynthesis, and it has been demonstrated that low leaf water potential reduces photosynthetic activity per unit of leaf area (Brix, 1962). Thus, it is probable that at least one reason for yield reduction during drought is a reduction in the rate of photosynthesis for the crop.

Studies (Boyer, 1970a,b) on the comparative effects of low leaf water potential on photosynthetic activity and leaf growth, however, have shown that leaf growth is more sensitive than photosynthetic activity. When leaf ψ dropped from -2 to -4 bars, leaf expansion was inhibited by at least 75% in corn, soybean and sunflower. Photosynthesis per unit leaf area was inhibited by only 10% in corn and unaffected in the other two species. Since leaf growth was affected before photosynthesis, reductions in photosynthetic activity could not account for the reduction in growth. In these cases, leaf growth was largely a function of cell enlargement and the inhibition was most likely due to reduced turgor, which reduces the rate of cell enlargement (Green,1968). Cell turgor has to exceed a minimum value before irreversible enlargement can occur (Green *et al.*,1971). These studies suggest that for a rapidly growing vegetative plant, one of the first effects of drought

is a reduced rate of cell enlargement. During reproductive growth, however, vegetative development is often practically complete, and it is at this stage that plant yield is most vulnerable to inhibition by dessication. It is most likely, therefore, that although leaf development is an important factor during vegetative growth, photosynthetic activity or some other factor becomes limiting during reproductive growth.

3.2 Salinity stress

Soil salinity depresses plant growth, the growth suppression being more or less proportional to the salt concentration and varying with the composition of the salts and the plant species. Greenway (1962) compared the growth and yield, under saline conditions, of a relatively salt-tolerant variety of barley with that of a salt-sensitive variety. Not only was total dry weight increment lowered by salinity treatments to a different extent in the two varieties, but also various components of grain yield were reduced differently. In general, the degree of growth suppression has been more pronounced when a non-electrolyte substrate containing a slowly permeating solute has been used rather than a rapidly permeating electrolyte solute (Jarvis and Jarvis, 1963).

Various modes of action of salinity in affecting plant growth have been suggested from time to time. These include osmotically reduced water availability in the substrate and an excessive ion accumulation in

the plant tissue. A considerable difference of opinion exists as to the relative importance of these factors. Hayward and Wadleigh (1949) emphasized the reduced water availability in the substrate which they termed a "physiological drought". It was found, however, that plants can adapt to the saline condition by decreasing cell osmotic potential (Bernstein, 1961). Reduced water availability, *per se*, cannot, therefore, explain the reduction in growth since plants undergo this osmotic adaptation. In general, some osmotic adaptibility is observed, but the damage due to salinity is not completely overcome (Bernstein,1961). O'Leary (1971) collected the information on plant growth inhibition due to salinity from several sources, and integrated these observations into a unified theory. He proposed that the root resistance to water movement increases in plants grown in a saline solution, which results in the development of a water deficit in the leaves when plants are grown in an environment with a high evaporative demand. He also suggested that hormone transport from the roots to the leaves is reduced in plants grown in a saline solution and that this possibly causes a decrease in cell-wall extensibility in the leaf cells. This factor may be of importance when plants are grown in an environment with a low evaporative demand so that water stress does not develop. Although evidence suggests that a primary effect of salinity in the root environment is to decrease the supply of cytokinins and/or other hormones to the leaves (Itai and Vaadia, 1965), the mechanism of action

of cytokinin in controlling plant growth is not understood and, consequently, the relationship between such an effect and plant growth is unknown.

Despite these proposals, the reduction in plant growth caused by a saline substrate appears to be related more closely to the uptake of abnormal amounts of salt than to a reduced absorption of water (Kramer, 1969). According to Greenway (1968), the rate of plant growth under saline conditions is affected by the concentration of salt inside the cell, and the salt tolerance of the plant is related to its ability to regulate tissue ion content. In reality, it is extremely difficult to draw a distinction between the osmotic and the ionic effects of absorbed salt since plant reactions to these two factors occur simultaneously. It has been assumed that, under natural conditions, it is only in cases of extreme salinity that the osmotic pressure of the soil solution is an important factor; on less saline soils, where the plants are able to absorb water, the decisive factor is without doubt the degree of toxicity of the salts (Strogonov, 1964). Other workers, on the other hand, considered that the relative importance of each factor depends largely on the species, the climatic conditions (Berg, 1952) and on the salt composition of the substrate (Kelley, 1951). It follows that a general theory combining the effects of osmotic pressure and salt accumulation, and explaining the divergent effects of salinity on plants cannot be set up as yet.

3.3 Temperature extremes

Chilling temperatures will retard plant growth and produce a variety of visual injury symptoms on susceptible plants. The most apparent symptoms of greater generality are surface pitting, necrotic areas and external discoloration. With young corn seedlings exposed to 0.3±0.3°C, visual leaf injury appears in 36 hours and becomes more intense as chilling is continued. After 48 hours, some seedlings collapsed and were presumably dead (Creencia and Bramlage, 1971). The chilling temperature at which injury occurs varies with the species and can be as high as 12.5°C (Pentzer and Heinze, 1954). The lower the temperature and the longer the time of exposure, the more severely a plant will be injured. Extended periods of chilling are usually lethal, whereas shorter periods delay and can permanently modify growth and development. A period of chilling during germination in cotton can alter the growth and fruit pattern throughout the season (Christiansen and Thomas, 1969). However, some effects of chilling injury are reversible following transfer to a non-chilling temperature. Cacao seed viability is normally destroyed in 10 minutes at 4°C, but it will survive this exposure if quickly immersed in water at 37°C (Casas *et al.*,1965). Creencia and Bramlage (1971) demonstrated a considerable capacity of the growing corn plant to recover from a short chilling treatment even though significant physiological changes occurred at the low temperature.

The mechanism of chilling injury encompasses several elements operating independently and simultaneously: these include imbalances in metabolism, accumulation of toxic compounds and increased permeability (Lyons, 1973). Levitt (1972) proposed that all types of chilling injury can be the result of a change in cell permeability, and Christiansen *et al.* (1970) concluded that the initial impact of chilling on cotton is a rapid physical change in the membranes to a freely permeable state. Alternative proposals for the basic mechanism of injury have been reduced ATP supply (Stewart and Guinn, 1969) and a possible breakdown in the ATP-ADP transfer system leading to an accumulation of unavailable energy (Vakis *et al.*,1970). However, these concepts can all be accommodated by the common event of a temperature-induced phase transition in the cellular membrane as the primary response in chilling injury (Lyons, 1973). The cause of this change in state may be a simple physical phenomenon, due to a high melting point of the lipids, or chemical, due to their peroxidation.

The growth of plants is reduced by either a brief exposure to extreme heat or continuous exposure to an above-optimum temperature. Mitchell (1958) compared the daily increase in dry weight of the shoots of four grasses and three legumes at a constant temperature and found the maximum rate of dry weight increase in the 65-75^{o}F range. Above 85^{o}F the growth rate declined and at 95^{o}F the rate was negligible for some species. Similarly, Jones (1947) found that all heat-treated corn

seedlings were shorter in height, less vigorous in growth and later to flower. Although normal tassels were produced, the anthers were small and shrivelled and the pollen sterile.

Responses to a short exposure to high temperature can also be considerable. A significant reduction in plant height, in number of leaves and in number of tillers and a delay in head emergence resulted from a 5 hour heat stress of 130°F at the 2 leaf stage in Red Brome (Laude and Chaugule, 1953). In addition to growth reductions, exposure to extreme temperatures often produces a characteristic leaf tip burn in grass and leads to the development of yellow or brown spots ("scorch") in many plants. Wheat subjected to 130°F for $4^{3}/4$ hours on the fifth day after planting developed a chlorotic band and constriction in the leaf within a few days, some of the leaves breaking at the constriction (Bukharin, 1958). Red Brome, on the other hand, produced a characteristic leaf tip burn, due to browning and twisting of leaves, one week after heat stress. The principal visible effect of such treatments gradually disappears if the plants are placed in a favourable environment, although subsequent development is affected for some time (Laude and Chaugule, 1953).

The cause of death from heat stress usually has been explained as the result of denaturation and irreversible coagulation of cellular protein (Henckel, 1964). However, Daniell *et al.*, (1969) considered that the primary effect of lethal temperatures is the disintegration of

membranes. Levitt (1972) pointed out that there appear to be three zones of heat injury, each controlled by a different mechanism. In the lowest heat zone, the injury is commonly metabolic or indirect; the metabolism becomes disrupted and toxins may be accumulated. In the intermediate heat zone, injury is due to protein denaturation, whilst, in the highest heat zone, injury is due to chemical reactions. Alternatively, there are several lines of evidence that indicate that direct injury is initiated in the cell membranes and is most likely due to denaturation of membrane proteins (Chapman, 1967).

All types of environmental stress considered produce some growth depression, resulting in death of the plant in the most extreme conditions. It is apparent that the relationship between environmental stress and growth is very complex, involving a multiplicity of physiological processes. The stress in itself is complex and often has not been expressed in terms which are both comparative and quantitative. There are innumerable combinations of intensity, frequency and duration of stress discussed in the literature. Moreover, the condition of the plant when the stress is imposed varies, and many different species with different responses have been used. Results also differ in the criterion of growth or metabolism measured. It is not remarkable, therefore, that there has been considerable difficulty in establishing a basis for generalization on this extensive and diverse collection of data. However, it is generally agreed that the progressive reduction

in growth rate caused by increasing salinity appears to be caused primarily by the effect of excessive ion accumulation in the affected plant. Whilst a direct osmotic effect on water availability appears to be of secondary importance, a temperature-induced phase transition in cellular membranes is regarded as the primary response in chilling injury, and disintegration of cell membranes could be the primary effect of heat injury. Any damage to membranes will affect the activity of bound membrane enzymes as well as compartmentation of metabolites in the tissue. These may in turn cause a disturbance in metabolism, leading to 'starvation', protein breakdown and biochemical lesions (Levitt, 1972).

4. The interrelationships between environmental stresses

There is some evidence that an internal water deficit can be induced in plants by several unfavourable environmental stresses, including chilling, freezing, heat and salinity stress (Levitt, 1972). In other words, many different environmental stress situations may induce the same kind of primary effect, possibly mediated through a loss of membrane semipermeability due to membrane damage (Levitt, 1972). Chilling injury may be due to lipid solidification at chilling temperature, leading to damage and, therefore, to loss of semipermeability (Lyons, 1973). In freezing tissue, membrane laceration by the expansion of intracellular ice crystals or lipid solidification may produce similar effects (Levitt, 1956). Lipid solidification can also be induced by oxidation of the unsaturated, low-melting point lipids to saturated, high-melting point

lipids. It has been suggested by Hatefi and Hanstein (1970) that some salt or ion stresses may cause injury by means of such an oxidation. Heat stress-induced membrane injury may be by protein denaturation (Ben-Zioni and Itai, 1973). Research on protein hydration has demonstrated that the hydration shell consists of an ice-like sheath of several layers of water molecules surrounding and linking the protein molecules (Klotz, 1958). The effect of heat is thought to reduce the extent of ice-like structure. An excess accumulation of salt in plant cells will result in progressive changes in protein hydration. Although there is little evidence as to the effect of reduced hydration, *per se*, on the ice-cage structure, the observed effects of water stress on protoplasmic viscosity suggest that, in the range of water potential, ψ = -50 to -10 bars, marked changes in structure may occur.

Different unfavourable environmental stress situations may also produce a common indirect injury due to a disturbance in protein metabolism commonly due to increased protolysis and decreased protein synthesis (Levitt, 1972).

Although the direct evidence for a common factor in plant response to a variety of environmental stress situations is meagre, indirect support for the concept is gathered from a consideration of plant resistance to such stresses. Levitt(1956) pointed out that frost, drought and heat resistance are all basically similar, and that any resistance to

one of these factors carries with it a resistance to the other. When plants harden in autumn, their hardiness towards desiccation and heat stress rises to a maximum in many cases in parallel with the development of frost resistance (Parker, 1972). It has also been found that cryoprotective compounds stabilize membranes against three types of stress, i.e. freezing, desiccation and heat (Santarius, 1973). However, there have been very few attempts to correlate the resistance to the effects of freezing, drought and heat with that of the other stresses, particularly chilling and salinity. *Gloeococcum bavaricus*, an alga with much higher heat tolerance than other algae, showed an extraordinary tolerance for Na, V and Cr sulphates (Url and Fetzman, 1959). Sergejev and Lebedev (1936) concluded that the resistance of wheat and rye seedlings to salinity paralleled their frost hardiness, and halophytes seem to have at least some resistance to drought (Waisel, 1972). There is insufficient evidence to show that a general correlation exists between resistance to freezing, drought, heat and the remaining stresses that appear to induce a similar kind of primary injury.

The evidence, both direct and indirect, although scanty is sufficient to suggest that a common factor exists in both injury and resistance to injury of plants exposed to a variety of environmental stress situations.

III MATERIALS AND GENERAL METHODS

The general cultural practices and the design of the experiments are described here but further details of the individual experiments are given in the Results section.

1. Materials

The barley (*Hordeum distichum* L.,cv. Prior) seed used throughout the experimental programme was obtained from the South Australian Department of Agriculture. Radish (*Raphanus sativus* L., c.v. Long White Icicle) seed was obtained initially from local sources; later experiments utilized seed obtained from plants grown from the original sample.

2. General Methods

2.1 Environmental control

Controlled environmental growth cabinets (Zankel) were used for growing the plants. Each cabinet has space (1.1 x 1.1 m) for 100 pots (10 cm diameter) arranged in 10 rows of 10. The light source was a bank of 32/80 watt 'Cool White' fluorescent tubes (Philips TLF 80/33) supplemented with eight incandescent strip tubes (75 watt). The ~~light~~ radiant flux intensity was 4.1 mW cm^{-2} [illegible], with a 16 hour photoperiod, in all experiments. Temperature was maintained at 20 ± 1°C and, generally, the relative humidity of the cabinet was not controlled. As there was a slight temperature gradient in one direction across the cabinets,

blocks consisting of a number of rows of pots were arranged at right angles to this gradient and treatments were randomized within the blocks. In order to minimize variation within the blocks, pots were re-randomized daily within the blocks.

2.2 Plant culture

2.2.1 Pot culture

Seeds were germinated on wet filter paper in petri dishes for 24 hours at 20°C in the dark before they were planted in pots. Ten pre-germinated seeds were planted in each 10 cm plastic pot, and five uniform barley plants or four radish plants were retained after an initial thinning soon after emergence. The plants were grown either in fertile soil (Urrbrae loam 4: Plympton sand 1 by volume), sand alone or perlite, depending upon the type of stress to be imposed. Each pot was irrigated every day with 50 ml of nutrient solution for the first 10 days and 100 ml thereafter when the plants were rooted in sand or perlite. No nutrient solution was supplied when the plants were grown in soil, but they were watered daily until ready for treatment.

2.2.2 Water culture

Seeds of uniform size were allowed to germinate on wet filter paper in a 9 cm petri dish in the dark at 20°C. After 24 hours the pre-germinated seeds were transferred to a 19 cm large petri dish with two sheets of wet filter paper for 4 days in the dark at 25°C before transplanting to nutrient culture solution. The composition

of the nutrient solution is given in the following table (Singh,1970):

Ion	ppm
N	266
P	62
K	273
Mg	48
S	64
Ca	160
Fe	0.50
Mn	0.25
B	0.25
Zn	0.25
Cu	0.02
Mo	0.02

All solutions were adjusted initially to pH 5.5 with 0.1 M KOH. Twenty-four uniform barley or eight radish plants were grown in a 2.5 litre opaque polyethylene container. The plants were supported by a 20 x 20 cm PVC lid placed on the top of the container. The seedling was grown through a 1.5 cm diameter hole and was supported with a strip of polyurethane foam wrapped around the stem. The holes in the PVC lid were arranged in a circle with 2.5 cm distance between two holes for growing barley and 5 cm for growing radish plants. Cultures were aerated by blowing air through capillary tubes (diameter 5 mm) into the solution, using a small electric pump. The pump delivered 300 l/hour at 1 meter water gauge. All nutrient solutions were renewed weekly and the nutrient solution in each container was

adjusted daily to maintain the same level.

2.3 Imposition of stress

2.3.1. Water stress

Plants grown in soil were subjected to a water stress by withholding further water supply. The plants grown in Perlite were subjected to a water deficit by flooding the rooting medium with polyethylene glycol solution (mol. wt. 4000) of the desired osmotic potential. Water stress was relieved by thoroughly washing out the solution with six changes of 200 ml distilled water. In water culture, plants were subjected to a water deficit by replacing the nutrient culture solution with a solution of polyethylene glycol of known osmotic potential (including -0.7 bars osmotic potential of the Hoagland's solution). Polyethylene glycol has been claimed to be the most suitable osmotic agent for water stress investigations (Barrs, 1966; Singh *et al.*, 1973c), and, although it has been claimed that contaminants can produce toxic effects (Le Shem, 1966), no evidence of such toxicity has been found with the sample (I.C.I. Australia Ltd.) used in this laboratory or in previous (Husain and Aspinall, 1970; Singh *et al.*, 1973c) investigations.

2.3.2 Temperature stress

Cold stress was imposed by transferring plants growing in pots of sand to a cold room where the temperature was maintained at 5 ± 1°C for 3 to 5 days. The plants were exposed to light continuously at a similar

intensity to those remaining at 20°C. The plants were irrigated daily with Hoagland's solution maintained at the temperature of the environment in which they were growing. Plants were subjected to heat stress in a cabinet fitted with humidity control but similar in other respects to that used to grow plants. The temperature was maintained within 0.5°C of that intended, and the relative humidity was controlled at 90-95%.

2.3.3 Salinity stress

Before treatment, plants were grown in aerated water culture for 5 to 10 days, depending on which salt stress was used. Salinity stress was imposed either abruptly or gradually. In the abrupt salinization treatment, Hoagland's nutrient solution in the root medium was replaced with a known osmotic potential of salt solution. In slow salinization treatments, the osmotic potential of the rooting medium was gradually decreased by the addition of NaCl to produce a decrease of one bar in the osmotic potential every 24 hours, down to a final osmotic potential of -10 bars. This process ensured time for osmotic adjustment of barley under these growing conditions. The osmotic substrates were prepared with A.R. reagents to provide the desired osmotic potentials. The concentrations appropriate to each solution were computed from data on freezing-point depression and osmotic coefficients tabulated by Robinson and Stokes (1955) and Hodgman (1955). All solutions were checked with a micro-osmometer (Knauer). When stress was imposed,

plants growing in culture solution were rapidly transferred to identical containers filled with the appropriate salt solution.

2.4 Measurement of water status

2.4.1 Water potential

The leaf potential was measured with a Spanner thermocouple psychrometer (Barrs, 1968). Three to four barley leaves or one radish leaf were wrapped around a wire mesh insert protecting the thermocouple and pushed into the psychrometer chamber which was then stoppered. The chamber was then placed in a water bath ($25^{o} \pm 0.001$) and allowed to equilibrate for at least two hours before reading the thermocouple output. The water potential was calculated by comparing the recorded deflection with the deflections obtained from a graded series of NaCl solutions.

2.4.2 Osmotic potential

ψ (Water potential) is usually considered to be the sum of solute and turgor potential, $\psi = \psi_{\pi} + \psi_{p}$.

It is assumed that in dead tissue $\psi_{p} = 0$; therefore, the water potential of the dead tissue will be equal to the osmotic potential (Barrs, 1968). Accepting this assumption, osmotic potential was measured as follows. After measuring the water potential, the chambers containing the leaf tissue were stoppered with aluminium foil and frozen in liquid nitrogen for 1 minute to kill the tissue. The leaf tissue

was then allowed to thaw in a closed space and the osmotic potential was measured by the same procedure used to measure the water potential.

2.4.3 Turgor potential

The turgor potential was assumed to be the difference between the water potential and the osmotic potential for any leaf sample.

2.4.4 Relative turgidity

The term relative turgidity was introduced by Weatherley (1950) and defined as the amount of water in a measured quantity of tissue expressed as a percentage of the amount of water retained by the same tissue when fully turgid. The value and repeatability of the relative turgidity method of assessing leaf water deficit in barley and radish in the present study was tested by the methods recommended by Barrs and Weatherley (1962).

Barley leaves were cut into approximately 1 cm sections, or discs from radish leaves were cut as rapidly as possible with a sharp wad punch supported on a rubber bung. These sections or discs were immediately transferred to a gas-tight weighing bottle and weighed (WS). After fresh weight determination, the leaf sections or discs were floated on distilled water in a 4 cm closed petri dish and left for 4 hours at 20°C in the dark. The leaf sections or discs were then removed and placed on a layer of four sheets of No.1 Whatman filter paper. They were covered with another four-sheet layer and surface-dried by applying

a 500 g weight to the top layer for 20 seconds. The samples were then returned to the weighing bottle and the turgid weight recorded (WT). Finally, the leaf sections or leaf discs were placed in a forced-draught oven at 80°C and dried for at least 48 hours, and re-weighed (WD). The relative turgidity (RT) was calculated as follows (Weatherley, 1950):

$$RT = \frac{WS-WD}{WT-WD} \times 100$$

2.5 Plant growth

2.5.1 Plant height

Plant height was measured from the point of root initiation to the tip of the leaf blade. In order to diminish variation, the plants used for measuring plant height were marked with Indian ink at the point of root initiation and the same plants were measured each time.

2.5.2 Plant weight

Fresh plant material was placed in a 2 x 1 cm weighing bottle, weighed immediately and then dried in an oven at 80°C for 48 hours before the dry weight was measured.

2.6 Preservation of plant material

All samples intended for proline, chlorophyll and amino acids assay were wrapped in aluminium foil, immediately frozen in liquid nitrogen, and then stored at -20°C until assay.

2.7 Chemical procedures

2.7.1 Extraction and measurement of free proline

A rapid method for estimating free proline has been developed (Singh *et al.*, 1973c), based on the method of Troll and Lindsley (1955) for animal tissues. 150-200 mg frozen tissue and 1.5 g DeCalso resin (Singh *et al.*, 1973c) were placed in 5 ml of MCW (methanol:chloroform:water 12:5:3 v/v) and homogenized in a Duall conical glass homogenizer at room temperature. The homogenate was centrifuged, and the clean supernatant collected. The pellet was resuspended in a further 5 ml MCW and shaken for 5 minutes and then centrifuged. 3 ml chloroform and 5 ml water were added to the combined supernatants to break the stable emulsion formed during extraction. The mixture was then shaken and centrifuged. The upper aqueous layer was removed with big pipettes into a boiling tube and 5 ml glacial acetic acid and 5 ml fresh acidic ninhydrin reagent (125 mg ninhydrin: 3 ml glacial acetic acid: 2 ml 6 M orthophosphoric acid) were added. The mixture was then boiled for 45 minutes, cooled at room temperature, and shaken with a known amount of benzene (5 - 20 ml depending upon proline concentration). The optical density of the ninhydrin product dissolved in the benzene was measured at 515 nm and the proline concentration estimated from a standard curve.

2.7.2 Total amino acids determination

2.7.2.1 Extraction

Amino acids were extracted from the frozen tissues by homogenizing a sample (wt. 150-200 mg) with 5 ml MCW at room temperature. The homogenate was briefly centrifuged, and the supernatant collected. The residue was re-extracted by shaking it for 5 minutes with a further 5 ml MCW and then centrifuged. The supernatants were combined and separated into a lower chlorophyll-containing chloroform layer and an upper methanol-water phase by adding 5 ml chloroform and 7.5 ml H_2O. The upper aqueous phase was then dried under reduced pressure at 35°C on a rotary evaporator. The dried extract was taken up in 10 ml H_2O (Bieleski and Turner, 1966).

2.7.2.2 Purification of plant extract

Plant extract solutions were allowed to run in a Dowex 50W x 8(H^+) column (Splittstoesser, 1969). The non-adhering substances (neutral and acidic material) were washed through the column with 35 ml of water. Elution with 50 ml of 2N HCl removed the acidic and neutral amino acids; elution with 10 ml of 10N HCl then removed the basic amino acids (Wang, 1960).

2.7.2.3 Thin layer chromatography

The plant extract obtained following separation on the Dowex column was evaporated under reduced pressure to dryness. Water was added

to the dry extract and evaporated repeatedly until no trace of HCl remained. The purified plant extract was then made up to a known volume with water and was spotted on a thin layer plate of cellulose (MW 300, Macherey, Nagel & Co.) with capillary pipettes. The spots were dried in a stream of warm air (Heathcote and Haworth, 1969). Plates (20 x 20 cm) were spread with washed cellulose, using the equipment supplied by Shandon (Haworth and Heathcote, 1969).

Amino acids in the extracts were separated by two-dimensional chromatography. The solvent used for the first dimension was iso-propyl alcohol: methyl ethyl ketone: 1N HCl (60:15:25 v/v). The time for the solvent front to ascend to the finishing line was about 2.5 hours at 22°C. The plates were then dried in a stream of cold air for 15 minutes and heated in a convection oven at 60°C for a further 15 minutes to remove the final trace of HCl. The solvent used for the second dimension was *n*-propyl alcohol: methylethyl ketone: acetone: methanol: water: ammonia (40:20:20:1:14:5 v/v). The plate was developed at right angles to the first dimension by ascending technique until the solvent front was 13 cm above the origin. At 22°C temperature this occurred over a period of about 2.5 hours.

After the plates had been run in the second solvent, they were dried thoroughly, dipped in a 10% solution of ninhydrin-cadmium acetate and developed in a dessicator at 30°C for 24 hours.

2.7.2.4 Quantitative estimation of amino acids

The developed plates were coated with a film of cellulose acetate solution (6% cellulose acetate + 3% diethylene glycol + 2% camphor in acetone/propanol 3/1 v/v) by pouring on at one end and spreading over the entire surface with the aid of a glass rod. After the cellulose acetate film had dried completely, each coloured spot was cut out, lifted and put into a cetrifuge tube with 3 ml eluting solution (methanol: ethyl acetate: water, 1:1:1 v/v). The tube was shaken for 10 minutes and centrifuged at 1,000 *g* for 5 minutes. The optical densities of the coloured supernatants were measured at 505 nm. All the amino acids were identified by comparison of R_f with mixtures of known amino acids subjected to the same separation procedure and their concentrations were estimated from standard curves constructed for each amino acid (Heathcote and Haworth, 1969).

2.7.3 Chlorphyll determination

Chlorophyll was extracted in 80% acetone and measured by the method of MacKinney (1941). Two leaves of the same age were excised, cut into small pieces and mixed thoroughly for each determination. Equal fresh weights (approximately 15 mg dry weight) from the leaf were twice homogenized in a Duall conical glass homogenizer with 12.5 ml 80% acetone. The extracts were centrifuged each time, and the supernatants were pooled. The optical density of the pooled

supernatant was recorded at 663 nm (chlorophyll a) and 645 nm (chlorophyll b), and the amount of chlorophyll was presented as the sum of chlorophyll a and b.

IV RESULTS AND DISCUSSION

Section I. Water Deficit

1. Introduction

It was known from previous experiments (Singh *et al.* 1973C) that the barley plant (cv. Prior) exhibited a rapid and considerable accumulation of free proline as plants were subjected to a water deficit, either by withholding the water supply or supplying polyethylene glycol solution in the root zone. Palfi *et al.* (1973) have investigated more than 70 different species belonging to six families and found that all the plants except four species are able to accumulate proline in the plant tissues during a water deficit. Although proline accumulation is a general phenomenon in most water-stressed plants, the response of water-stressed radish plants was unknown. Therefore, these preliminary experiments were conducted to ascertain whether a similar accumulation of free proline occurred in this species during water deficit.

2. Water deficit and proline accumulation

2.1 Method

The experiments were carried out on radish plants (cv. Long White Icicle) grown for 18 days in 10 cm plastic pots. The plants were grown either in fertile soil (Urrbrae loam 4: Plympton sand 1 v/v) or perlite alone. Hoagland's nutrient solution was supplied daily to plants rooted

in perlite. Four uniform radish plants per pot were retained after an initial thinning. The environment was maintained at 20 ± 1°C with a 16 hour photoperiod of 4.1 mW cm^{-2} [redacted]. Water stress was imposed by withholding water from soil-grown plants. The plants grown in perlite were subjected to water stress by flooding the root zone with 250 ml of polyethylene glycol (molecular weight 4,000) solution of -10 or -20 bars osmotic potential. The extent of the stress treatment was assessed from leaf water potential measurements. Water potential of the leaf tissue was measured with a Spanner psychrometer at 25°C. Plants were sampled for proline content at intervals following imposition of stress; the samples were immediately plunged into liquid nitrogen, and stored at -20°C before assay. In these experiments the whole of the leaf laminae were taken from the first pair of leaves.

2.2 Results

(1) Water deficit imposed by withholding water supply

In the first 24 hours, the leaf water potential of the radish plants did not show any change and the plants remained almost turgid (Figure 1). The leaf water potential declined to -9.4 bars at 48 hours and the plants appeared to wilt slightly. Thereafter, the decline in water potential with time became rapid. After 72 hours, the leaf water potential had dropped to -33.9 bars and the plants were severely wilted.

As leaf water potential declined, there was a concurrent accumulation of free proline in the leaf tissue (Figure 2). This accumulation of

FIGURE 1

Water potential of the first pair of leaves of 18 day old radish plants (cv. Long White Icicle) subjected to a water deficit by withholding the water supply. Each value is the mean of four replicates.

▲ Control

■ Water stress

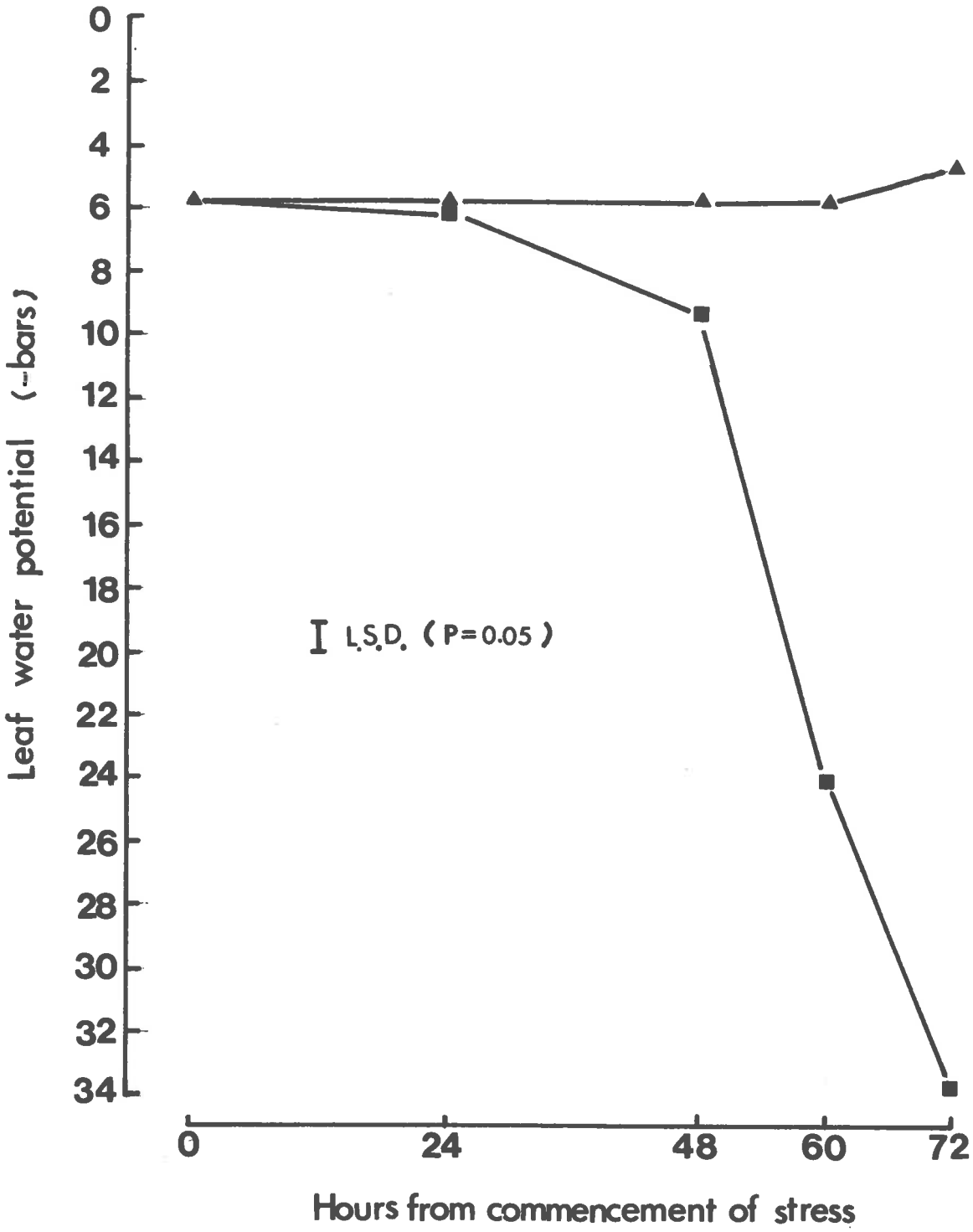
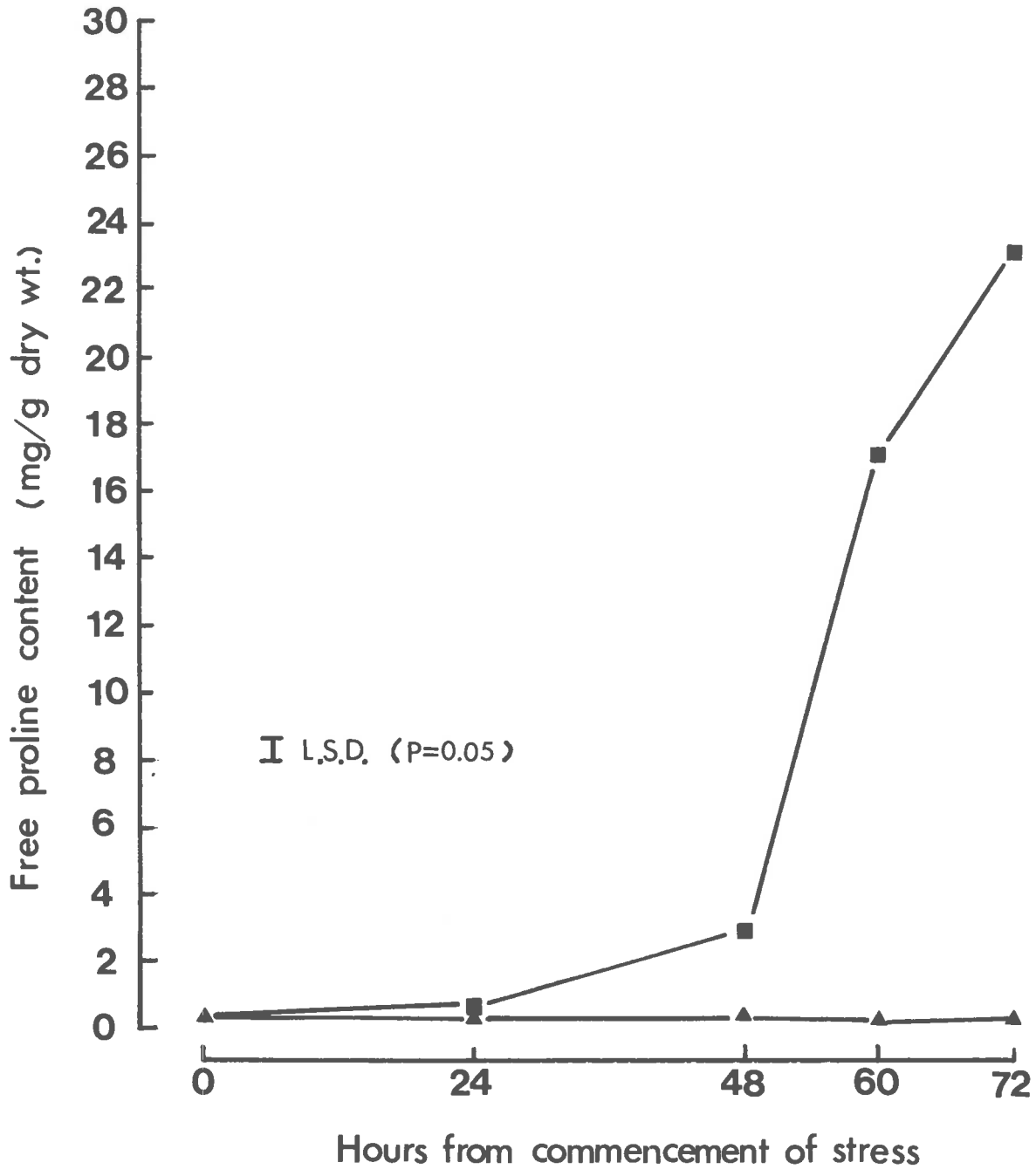

Leaf water potential (-bars)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
I L.S.D. (P=0.05)
0
24
48
60
72
Hours from commencement of stress

FIGURE 2

Accumulation of free proline in the first pair of leaves of 18 day old radish plants subjected to a water deficit by withholding water supply. Each value is the mean of four replicates.

▲ Control

■ Water stress


30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Free proline content (mg/g dry wt.)
I L.S.D. (P=0.05)
0
24
48
60
72
Hours from commencement of stress

proline followed a similar pattern of change with time as did leaf water potential. During the 72 hours of the experiment, free proline accumulated to a concentration of 23.2 mg per gram dry weight and leaf water potential declined to -33.9 bars.

(2) Water deficit imposed by supplying polyethylene glycol (PEG) solution

Plants were subjected to a water deficit by replacing the solution around the roots with a solution of PEG of -10 or -20 bars osmotic potential. The leaf water potential declined rapidly in both treatments and wilting appeared within half an hour (Figure 3). Thereafter, leaf water potential continued to decline rapidly in both treatments, the rate of decline being less in the -10 bars treatment. At 72 hours after stress was imposed, the leaf water potential had declined to -22.6 and -29.8 bars respectively in the -10 bars and -20 bars treatments.

The water deficit again caused a rapid accumulation of proline in the leaves (Figure 4). The accumulation was considerably more rapid and extensive at -20 than at -10 bars osmotic potential. At the end of 72 hours, the leaves had accumulated more than 10.6 mg per gram dry wt (147.2 $\mu g^{-1}h^{-1}$) under the -10 bars treatment, and 16.7 mg per gram dry wt (231.9 $\mu g^{-1}h^{-1}$) under the -20 bars treatment.

(3) The relationship between leaf water potential and proline accumulation in radish leaf tissue

A plot of proline concentration (per gram dry weight) against leaf water potential, shown in Figure 5, yields a straight line whose equation is: $Y = 6.123 + 0.777\ (x - 13.79)$. The correlation coefficient of this

FIGURE 3

Water potential of the first pair of leaves of 18 day old radish plants subjected to a water deficit by flooding the rooting medium with polyethylene glycol solution (Mwt. 4,000). Each value is the mean of three replicates.

▲ Control

○ Water stress ($\psi\pi$ = -10 bars)

■ Water stress ($\psi\pi$ = -20 bars)

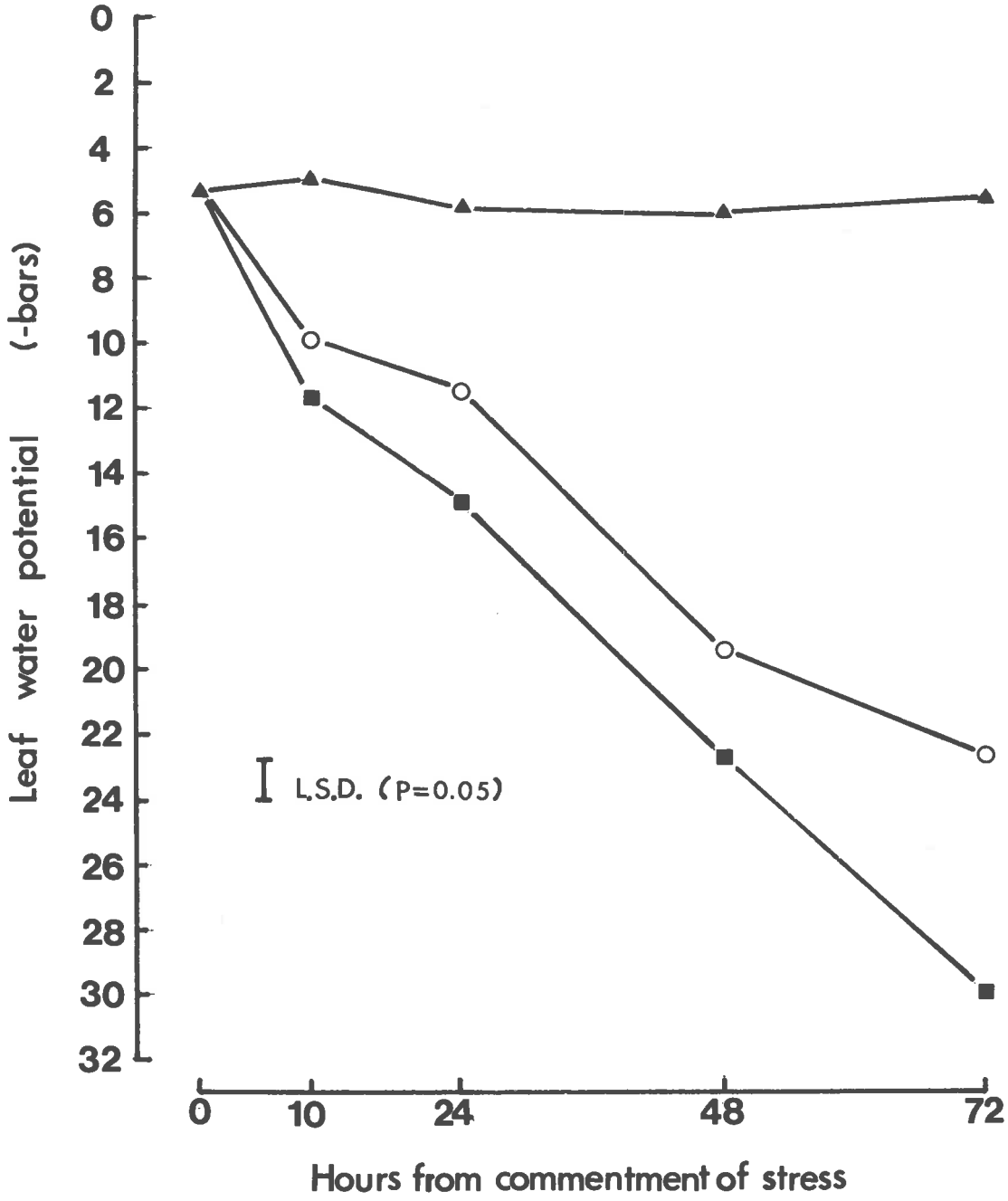

Leaf water potential (-bars)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
L.S.D. (P=0.05)
0
10
24
48
72
Hours from commentment of stress

FIGURE 4

Accumulation of free proline in the first pair of leaves of radish plants subjected to a water deficit by flooding the rooting medium with polyethylene glycol solution. Each value is the mean of three replicates.

▲ Control

○ Water stress ($\psi\pi$ = -10 bars)

■ Water stress ($\psi\pi$ = -20 bars)

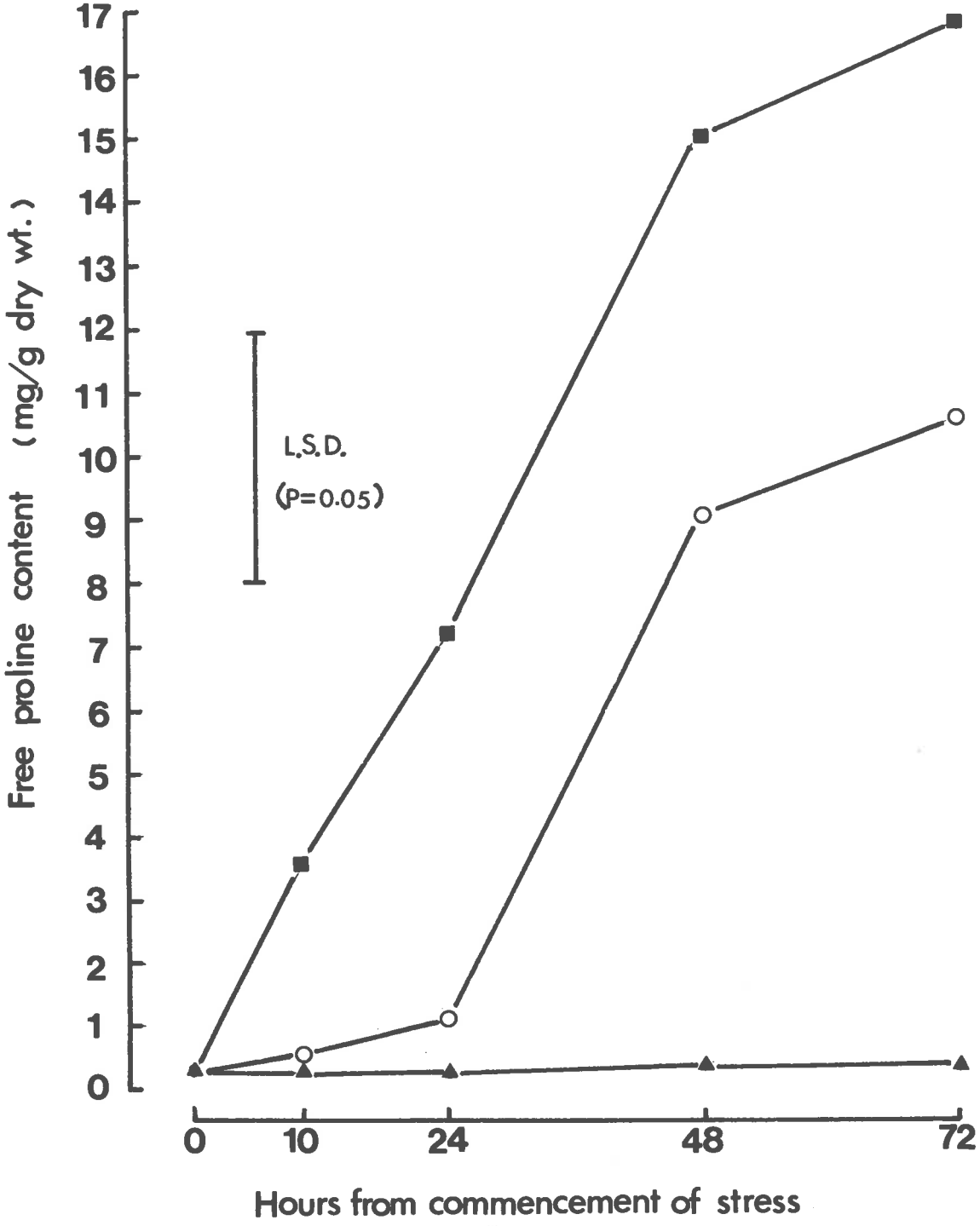
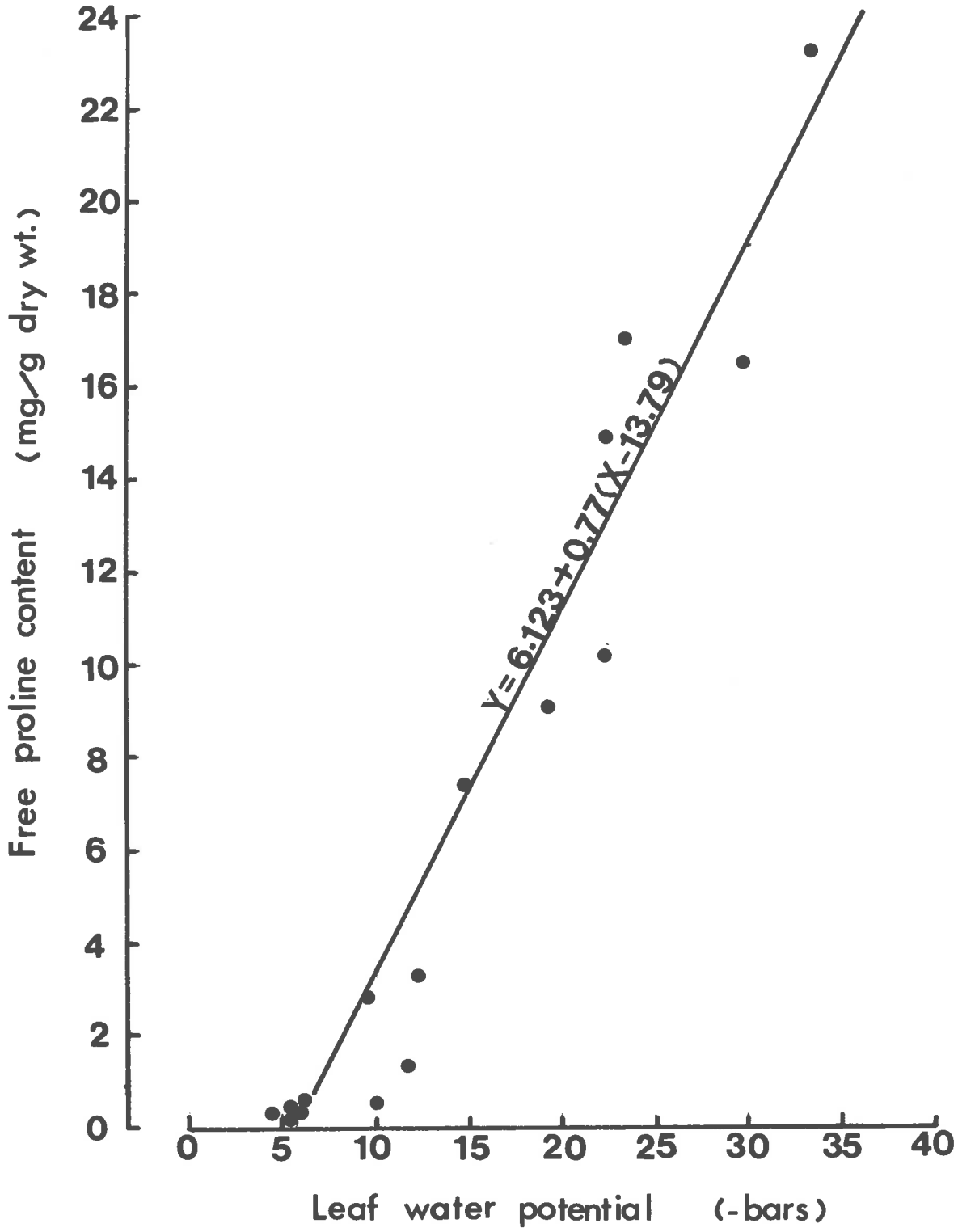

Free proline content (mg/g dry wt.)
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
L.S.D.
(P=0.05)
0
10
24
48
72
Hours from commencement of stress

FIGURE 5

Relationship between proline accumulation and leaf water potential in radish plants.
(Values for proline content and water potential were obtained from Figures 1, 2, 3 and 4 respectively).

Correlation coefficient = 0.946


Free proline content (mg/g dry wt.)
Leaf water potential (-bars)
Y=6.123+0.77(X-13.79)
0
2
4
6
8
10
12
14
16
18
20
22
24
0
5
10
15
20
25
30
35
40

equation is 0.946. The accumulation of proline in the leaf tissue paralleled the reduction in water potential in that tissue. The water potential in the control plants was between -4 and -6 bars and proline commenced to accumulate when leaf water potential declined to -7 or -8 bars. This correlation between leaf water potential and proline accumulation in radish leaves is similar to that found in barley leaves (Singh, 1970).

3. Distribution of accumulated proline in the radish plant

3.1 Methods

Radish plants (cv. Long White Icicle) were grown in a controlled environment at 20 ± 1°C in a 16 hour photoperiod, at a ~~light~~ radiant flux intensity of 4.1 mW cm^{-2}. Ten pre-germinated seeds were planted in each 10 cm pot and the emerged seedling were thinned to four after one week's growth. They were planted in perlite and irrigated with half-strength Hoagland's solution. 16 days from planting, the first pair of leaves was completely developed, and the second pair of leaves had reached about one third of the mature size. The plants were subjected to a water deficit by flooding the root medium with 250 ml of polyethylene glycol solution (molecular weight 4,000) of -10 bars or -20 bars osmotic potential. Plants were sampled 10, 24, 48 or 72 hours following imposition of this osmotic stress, and proline was estimated in various organs.

3.2 Results

(1) The first pair of leaves

Proline accumulation in the lamina from first leaf followed a pattern similar to that already described, but it is noteworthy that accumulation was considerably more rapid and extensive at -20 than at -10 bars osmotic potential (Figure 6.A.).

(2) The second pair of leaves

A similar pattern of proline accumulation in response to stress occurred in the second pair of leaves, but the accumulation was more rapid than that in the first pair of leaves. At the end of 72 hours, the leaves had accumulated more than 14.7 mg/gram dry weight in -10 bars treatment and 22.7 mg per gram dry weight in -20 bars treatment (Figure 6.B.).

(3) Cotyledons

Cotyledons also accumulated considerable amounts of proline during the period of water deficit, but the rate of accumulation was considerably less than the corresponding rate in both pairs of leaves. By the end of the experiment, cotyledons had accumulated 6.8 mg per gram under -10 bars treatment and 9.8 mg per gram under -20 bars treatment (Figure 6.C.).

(4) Roots

The roots also accumulated proline during stress, but to a lesser extent than any other organ of the plant (Figure 6.D), and the concentration was also less, although still increasing when the experiment

FIGURE 6

Accumulation of free proline in the leaf, cotyledon and root of radish plants subjected to a water deficit by flooding the rooting medium with polyethylene glycol solution. Each value is the mean of three replicates.

A. The first pair of leaves

B. The second pair of leaves

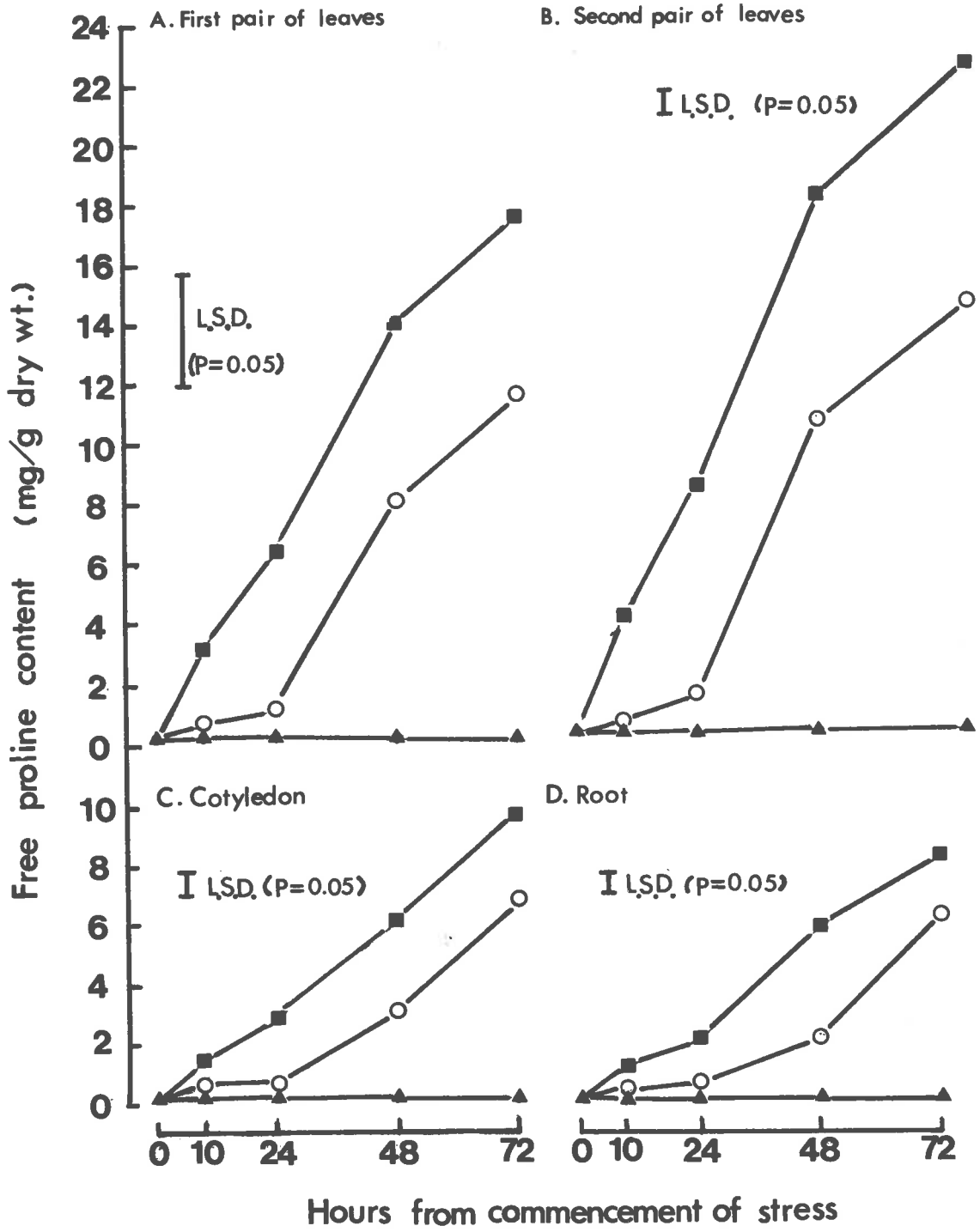
C. Cotyledon

D. Root

▲ Control

○ Water stress ($\psi\pi$ = -10 bars)

■ Water stress ($\psi\pi$ = -20 bars)


A. First pair of leaves
B. Second pair of leaves
C. Cotyledon
D. Root
L.S.D. (P=0.05)
Free proline content (mg/g dry wt.)
Hours from commencement of stress
0 10 24 48 72
0 2 4 6 8 10 12 14 16 18 20 22 24

was terminated.

Proline accumulated in all organs of radish plants during water stress. Accumulation was most rapid in the second pair of leaves, the younger tissue of the plant, and the least rapid in the root. In each case, the concentration of proline at any time in any organ was considerably higher at -20 bars than -10 bars.

4. Accumulation of proline in isolated organs of the radish plant

4.1 Methods

Radish seedlings were grown in perlite with nutrient solution for 18 days in a 16 hour photoperiod at 20°C. After this the plants were removed from the pots and washed free of perlite with tap water and the first pair of leaves was excised. Leaf discs, 10 mm in diameter, were obtained, using a cork borer, 20 discs being taken from each leaf. Roots were cut into 0.5 cm sections after excision. Each type of plant tissue was floated on distilled water as soon as it was excised, one sample of each tissue being frozen immediately as an initial sample. Twenty-five excised leaf discs or root sections were then transferred to a 9 cm diameter petri dish containing 5 ml of either distilled water or -20 bars polyethylene glycol solution (molecular weight 4,000). These were then incubated in the dark at 20°C. Samples were removed and frozen 10, 24 and 48 hours after beginning the treatment. The samples were thoroughly washed, frozen in liquid nitrogen and maintained at -20°C for proline assay.

4.2 Results

There was only a low concentration of free proline present in the leaf and root tissues of radish when excised from the plant (Figure 7). Leaf lamina discs floated upon -20 bars polyethylene glycol solution commenced to accumulate free proline within 4 hours and continued to accumulate for the 48 hour period of the experiment,reaching a concentration of 6 mg per gram dry weight when the experiment was terminated (Figure 7). For comparison, the leaves of an intact radish plant subjected to -20 bars polyethylene glycol water stress accumulated 15.0 mg per gram dry weight in the same period (Figure 4). Free proline also accumulated in leaf lamina discs floated on distilled water alone. There was an initial lag phase of some 24 hours during which the proline content remained low and constant, after which it gradually increased and reached 1.8 mg per gram dry weight at 48 hours. Excised root systems were not capable of accumulating free proline, although significant quantities of proline accumulated in the roots of intact water-stressed plants (Figure 6). In fact, proline gradually decreased in excised roots incubated either in water or PEG, only trace amounts being found after 48 hours incubation.

FIGURE 7

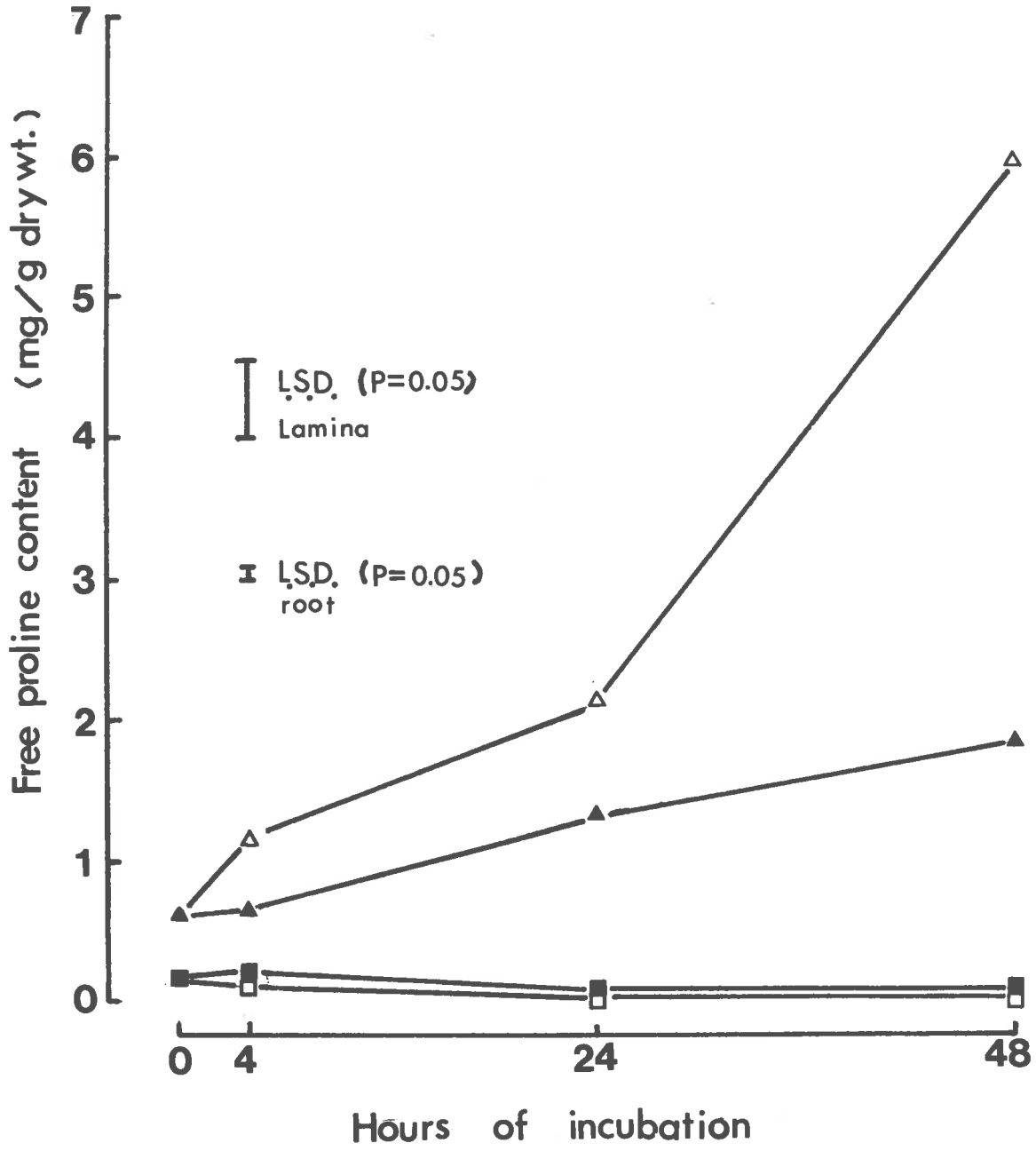
Accumulation of free proline in excised leaf lamina discs and excised root system of radish when floated on distilled water or polyethylene glycol solution. Each value is the mean of three replicates.

Leaves: ▲ Water

△ polyethylene glycol solution ($\psi\pi$ = -20 bars)

Roots: ■ Water

□ polyethylene glycol solution ($\psi\pi$ = -20 bars)


Free proline content (mg/g dry wt.)
7
6
5
4
3
2
1
0
L.S.D. (P=0.05)
Lamina
L.S.D. (P=0.05)
root
0
4
24
48
Hours of incubation

5. Discussion

The radish plant was chosen as a suitable experimental dicotyledonous plant to use in these studies along with the monocotyledon, barley. As much was known of the response of barley to water deficit, it was important initially to compare the response of radish with that of barley to a water deficit before exploring the responses of both species to other environmental stress situations. In the following discussion, the response of barley to water stress are those reported by Singh (1970), Singh *et al.*(1973, a-d) and Aspinall *et al.*(1973).

In both species, there was a striking proline accumulation in response to water deficit, as measured by leaf water potential, induced either by withholding the water supply or by flooding polyethylene glycol solution around the roots. Proline has also been found to increase with increasing severity of drought to 5 - 10 times the original concentration in corn, spinach and bean (Stewart *et al.*, 1966), and to 10 - 100 times in sunflower, peas, rice, tobacco (Palfi, 1969), rye grass (Kemble and MacPherson, 1954), ladino clover (Routley, 1966), wheat (Singh *et al.*, 1973a), Bermuda grass (Barnett and Naylor, 1965), creosote bush (Saunier *et al.*, 1968) and stylo (Gates *et al.*, 1971).

No toxic symptoms from the PEG treatment were found in either species, indicating that polyethylene glycol (Mwt. 4,000) is a satisfactory osmotic agent for studies on water deficit in both species. Proline accumulated in all parts of the plant in both species, but, again in both species,

accumulation was most rapid in the leaf laminae and least rapid in the roots. Similar results have been observed in winter wheat (Pretsenko *et al.*, 1968), in which a high content was primarily accumulated in the leaves and ears.

Where a water deficit was induced by PEG treatment, proline accumulation was considerably more rapid and extensive at -20 bars than at -10 bars osmotic potential in both species. This resulted in a correlation between proline accumulation and leaf water potential. The equation of the regression line between proline concentration [mg per gram dry wt. (Y)] and leaf water potential (X) was Y = 6.123+0.777 (X-13.79) in radish and Y = 5.300 + 0.645(X-13.60) in barley (Singh,1970). In both cases, of course, this relationship is confounded with the effect of time of exposure to water stress and a time-independent relationship cannot be derived from the data available.

Proline accumulated rapidly in isolated leaf lamina sections from barley or leaf lamina discs from radish subjected to osmotic stress, and less rapidly in those floated on water. Isolated root systems of both species did not accumulate proline, although the roots accumulated significant quantities of proline in intact, water-stressed plants of both species. The lack of proline accumulation in isolated root tissue appears to be a general phenomenon, as similar results have been reported by Stewart *et al.*(1966) in that proline did not accumulate in isolated, dehydrated discs of storage tissue from potato, carrot or turnip, nor

in excised fibrous roots of corn seedlings. The amount of proline accumulated by isolated leaf tissue was small when compared with that accumulated by an intact plant. Intact radish plants subjected to -20 bars PEG osmotic stress accumulated 15 mg per gram dry wt. proline 48 hours after stress, but only 6 mg per gram in isolated leaf discs floated in -20 bars PEG solution for the same period. In barley, intact plants accumulated 14 mg per gram dry wt. and isolated leaf sections only 5 mg per gram in the same period.

From the above comparison, it is clear that the accumulation of large quantities of free proline is a characteristic feature of the tissues of plants subjected to drought, and this accumulation is a general phenomenon in higher plants. Moreover, many features of the accumulation, including distribution, translocation and the response of isolated organs, are comparable. Among the species which accumulate proline, the only apparent difference is that each species has a different proline accumulation potential in response to water deficit. In these experiments, radish exhibited a higher proline accumulation potential than barley. This difference may be more apparent than real as it has been demonstrated that the capacity to accumulate free proline is dependent upon the genotype within a species (Singh *et al.*, 1972). Of course, environmental and other factors may also affect the response.

Section II. Temperature Extremes

1. Introduction

The inter-relationships between the effects of temperature extremes and of tissue water deficit on the growth and metabolism of the intact plant are difficult to disentangle. Thus, supra-optimal temperature can lead to rapid transpiration and a consequent lowering of tissue water potential in the leaves. Furthermore, low environmental temperature can lower the availability of water in the soil and its movement to the plant roots, also resulting in a lowering of leaf ψ. In both cases, it is difficult to separate the direct effects of temperature on metabolism from those mediated through the concomitant changes in ψ. It has been suggested that plant resistance to cold, heat and water stress are inter-related (Levitt, 1956) which is easily understood if each is a manifestation of response to a similar change in tissue environment. On the other hand, more direct metabolic responses to temperature extremes are obviously also involved.

The most striking metabolic consequence of lowered ψ in many plants is a rapid and extensive accumulation of the imino acid proline (Singh *et al.*, 1973c). Accumulation of proline has been reported to occur in plants subjected to low temperatures (Shvedskaya and Kruzhilin, 1964; Benko, 1968; Palfi and Juhasz, 1970; Gates *et al.*, 1971), and in desert plants exposed to high temperature (Oshanina, 1972). In neither case

is it known whether the accumulation of proline was a consequence of the temperature regime or due to a correlated change in tissue water potential. The present investigation was initiated to explore the changes in proline content with temperature stress and to examine whether any of the changes observed, and other manifestations of temperature stress, were derived from variations in plant water potential rather than from more direct temperature responses.

2. High temperature stress

2.1 The response of barley to high temperature (39°C) at two levels of relative humidity

2.1.1 Methods

Barley plants (cv. Prior) were grown in 10 cm plastic pots filled with fertile soil (Urrbrae loam 4: Plympton sand 1 v/v) in a constant environment (20 ± 1°C, 16-h photoperiod and 4.1 mW cm^{-2} h^{-1} light intensity). Ten pre-germinated seeds were planted in each pot and emerged seedlings were thinned to five after three days growth. After ten days, plants were exposed to 39 ± 1°C (high temperature). In order to distinguish between the effects of tissue water deficit and of high temperature on the growth and metabolism of the intact plant, one group of plants was grown in a high-humidity cabinet and others were grown in a low-humidity cabinet. The high-humidity growth cabinet was programmed to maintain a constant relative humidity of 90 to 95%, while the low-humidity growth cabinet

maintained relative humidity at 50%. These high temperature-treated plants were compared with plants maintained at 20°C, without control of relative humidity in the growth cabinet. The field capacity of the soil in the pots was about 27 to 28%. These plants were irrigated every 24 hours so that the soil water content was returned to field capacity daily in all treatments. The plants were sampled at 0, 24, 72 and 120 hours after stress was initiated and all samples were taken before the daily irrigation. The first leaf of barley plants was taken on each occasion for leaf water potential, proline and chlorophyll determinations. Plant height was measured as the distance between the point of root initiation and the tip of the terminal fully-emerged leaf.

2.1.2 Results

(1) Plant height

Growth of the barley seedlings, as measured by plant height, was rapidly inhibited by exposure to a combination of high temperature and low humidity (Figure 8). Elongation continued at a diminished rate for some three days at 39°C with a high humidity, but eventually ceased. The inhibition of elongation at the high temperature was thus not solely due to water stress, although water stress accentuated the inhibition.

(2) Chlorophyll concentration

The chlorophyll concentration in the first leaf of plants growing at 20°C remained effectively constant over the five day experimental period (Figure 9), whereas there was a continuous, approximately linear

FIGURE 8

The effect of high temperature (39°C) at two levels of relative humidity on plant height in barley (cv. prior). Each value is the mean of three replicates.
[10 day old plants growing in soil were subjected to a high temperature (39°C) for 1-5 days in a low (50%) or high (90-95%) relative humidity environment. Plants were watered daily, the plant height measurements being made immediately prior to re-watering.]

▲ 20°C

○ 39°C, high relative humidity

■ 39°C, low relative humidity

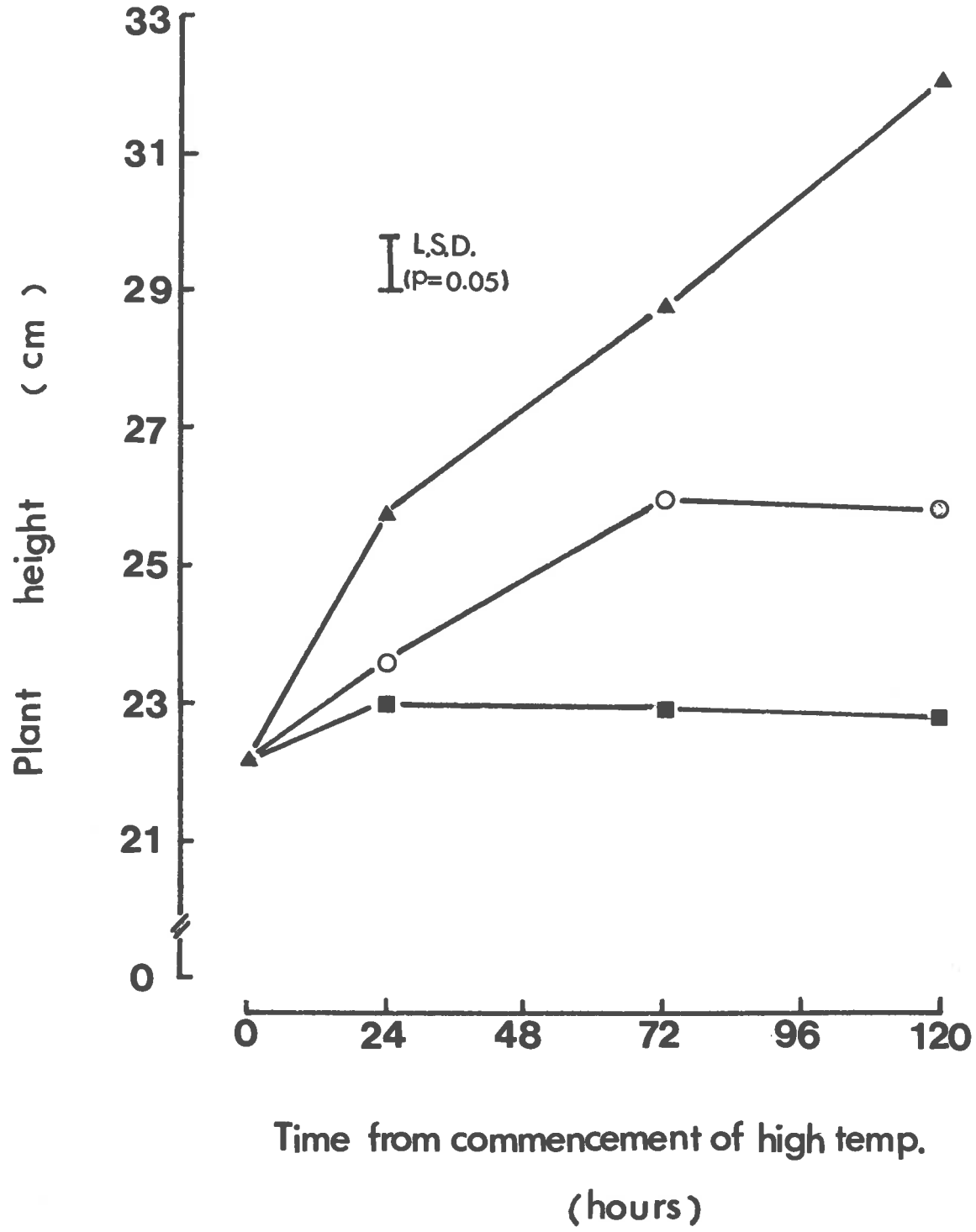

33
31
29
27
25
23
21
0
Plant height (cm)
L.S.D.
(P=0.05)
0
24
48
72
96
120
Time from commencement of high temp.
(hours)

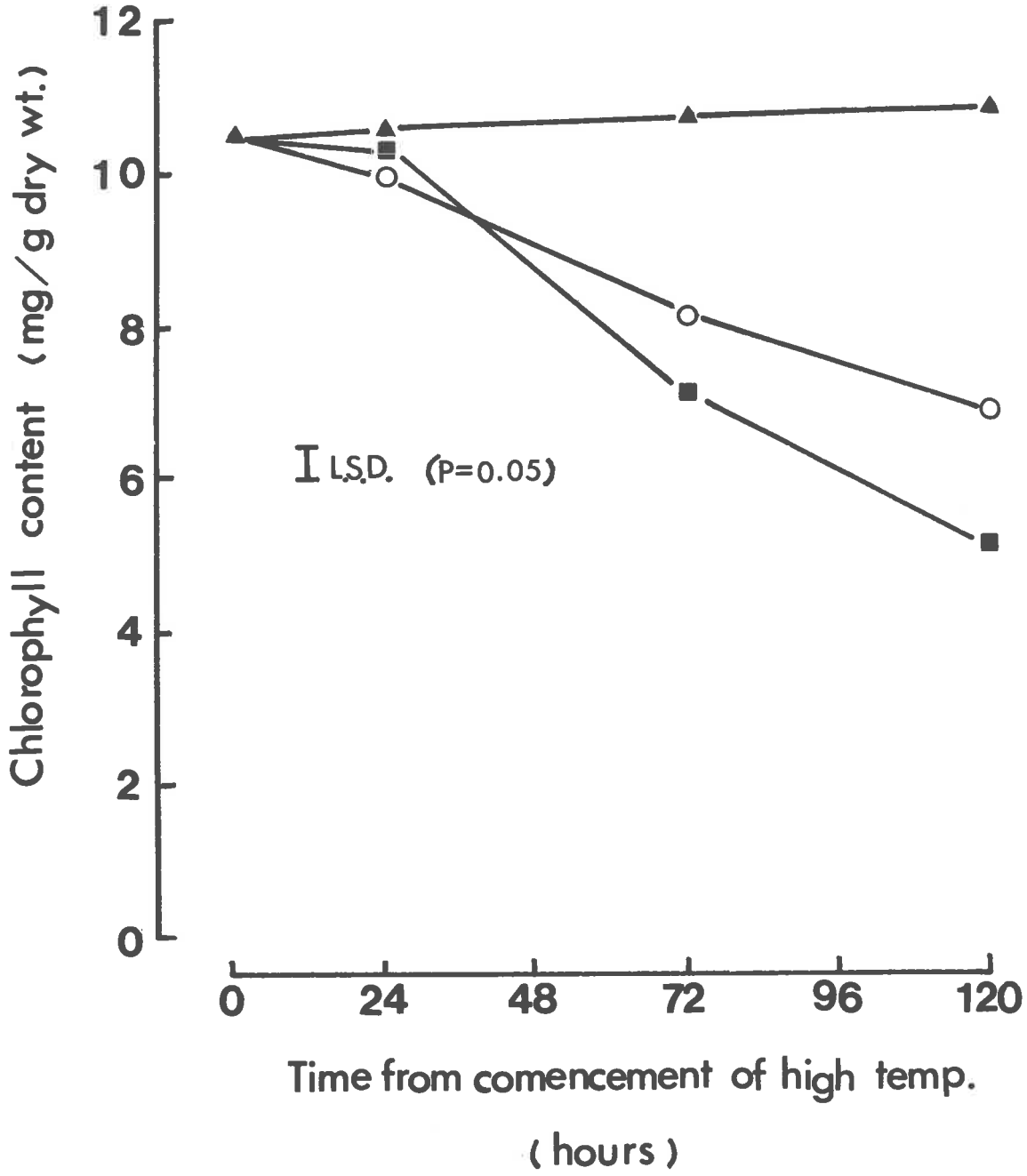
FIGURE 9

The effect of high temperature (39°C) at two levels of relative humidity on chlorophyll content of the first leaf of barley. Each value is the mean of three replicates. [10 day old plants growing in soil were subjected to a high temperature (39°C) for 1-5 days in a low (50%) or high (90-95%) relative humidity environment. Plants were watered daily, the chlorophyll content measurements being made immediately prior to re-watering.]

▲ 20°C

○ 39°C, high relative humidity

■ 39°C, low relative humidity


Chlorophyll content (mg/g dry wt.)
12
10
8
6
4
2
0
I L.S.D. (P=0.05)
0
24
48
72
96
120
Time from comencement of high temp.
(hours)

decline in the chlorophyll content of the first leaves of plants exposed to 39°C. This decline in chlorophyll concentration was accelerated slightly by water stress, such that 50% of the chlorophyll in the first leaf had disappeared in five days.

(3) Soil water content

The soil water content in the pots was returned to field capacity (27-28%) daily in all treatments. In the low-humidity, high-temperature treatment, extremely rapid transpiration occurred and the water content of the soil was depleted more extensively in the 24 hours after returning the soil to a 28% water content the longer the plants had been exposed to stress. In the high-humidity, high-temperature treatment, the water content of the soil was only reduced by 6-9% in the 24 hours before watering (Table 1).

(4) Leaf water potential

In the low-humidity, high-temperature treatment, ψ fell to a low level each day before watering (Table 1). At 120 hours, leaf water potential fell to -29.8 bars. However, controlling the atmospheric relative humidity at 90% or above, prevented any decline in ψ, although transpiration, as measured by soil water content, was more rapid than at 20°C.

(5) Free proline content

In contrast to plant height and leaf chlorophyll concentration, where the deleterious effects of heat stress were merely accentuated by accompany-

TABLE 1

Water potential (ψ) of the first leaf and soil water content(S) when barley plants (cv.Prior) were subjected to heat stress.

[10 day old plants growing in soil were subjected to a high temperature (39°C) for 1-5 days in a low (50%) or high (90-95%) relative humidity environment. Plants were watered daily, the water status measurements being made immediately prior to re-watering.]

Period of heat stress (hr)	20°C, low humidity: ψ (-bars)	S (%)	39°C, high humidity: ψ (-bars)	S (%)	39°C, low humidity: ψ (-bars)	S (%)	L.S.D.(p=0.05) ψ (-bars)	S (%)
0	4.0	28	4.0	28	4.0	28		
24	4.4	28	4.7	22	5.1	8	0.6	1.6
72	4.2	27	4.7	19	5.0	4	0.7	1.6
120	4.2	27	4.6	20	29.8	2	0.7	1.6

ing water stress, the accumulation of free proline in the leaf tissue appeared to be determined by water stress alone (Figure 10). Where plants were subjected to heat stress with no accompanying decline in leaf water potential, there was no increase in the concentration of free proline in the leaves. Water stress at 39°C led to rapid accumulation of proline, however, as during water stress at lower temperatures. Proline accumulated to a concentration of 4 mg per gram dry weight 120 hours after stress commenced.

2.2 The response of barley and radish plants to a range of high temperatures at high humidity

2.2.1 Methods

The effect of a range of high temperatures in a high humidity environment on plant height, chlorophyll concentration and proline accumulation was examined further with both barley and radish plants. Five barley plants (cv. Prior) grown for ten days, and four radish plants (cv. Long White Icicle) grown for 18 days in 10 cm plastic pots containing fertile soil in a controlled environment, were subjected to heat stress in a cabinet fitted with humidity control. The temperature was maintained within 0.5°C of that intended; the relative humidity was controlled at 90-95%, and the light environment was identical to that in the early growth phase. These plants were irrigated every eight hours to prevent the development of a water deficit. Plants were sampled at 0, 24, 72 and 120 hours. In addition to measurements of plant height, the first leaf

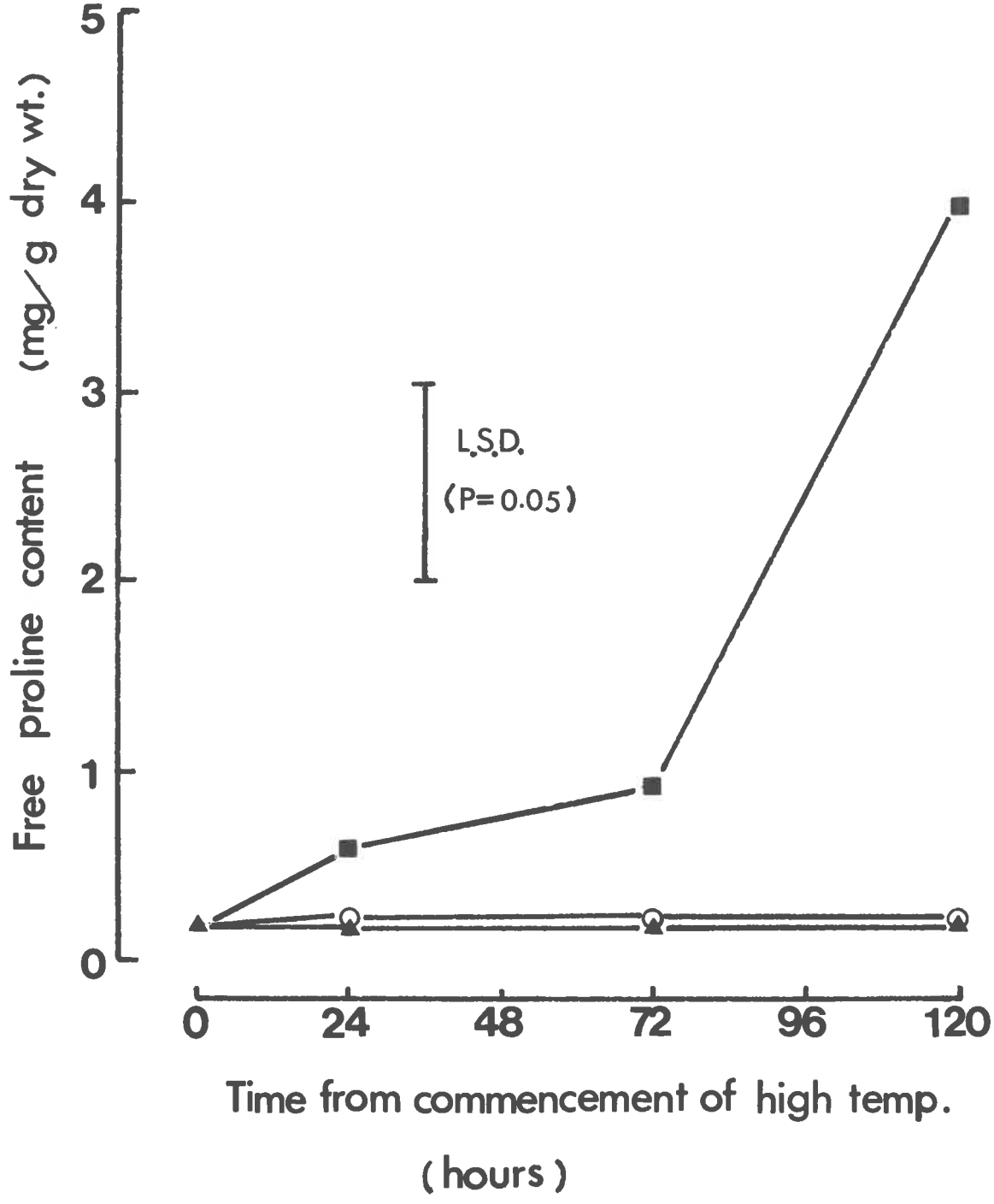
FIGURE 10

Accumulation of free proline in the first leaf of barley in response to high temperature (39°C) at two levels of relative humidity. Each value is the mean of three replicates.

▲ 20°C

○ 39°C, high relative humidity

■ 39°C, low relative humidity


Free proline content (mg/g dry wt.)
5
4
3
2
1
0
L.S.D.
(P=0.05)
0
24
48
72
96
120
Time from commencement of high temp.
(hours)

of barley or the first pair of leaves of radish were taken for measurement of leaf water potential, chlorophyll concentration and proline content.

2.2.2 Results

(1) Plant height

Plant height increase in barley was inhibited by temperatures above 30°C, although plants grown at 30°C elongated as rapidly as those grown at 20°C. Above 30°C, the inhibition of elongation increased with increasing temperature and duration of stress. At 41°C, growth was completely inhibited 24 hours after stress. However, at 39°C, growth was completely inhibited at 72 hours (Figure 11A). Radish plants responded similarly, although elongation was not as rapid in this species (Figure 11B). Above 36°C, growth was completely inhibited 24 hours after stress began.

(2) Chlorophyll concentration

In both species, there was a decline in the chlorophyll content of the leaves at temperatures above 30°C, barley exhibiting some loss of chlorophyll even at 30°C (Figure 12). When the seedling was subjected to 36, 39 and 41°C for 5 days, the magnitude of decline in chlorophyll increased with increasing temperature and duration of the stress. The higher temperature resulted in a considerable loss of chlorophyll in both species. At 41°C, most of the chlorophyll was lost between 24 and 72 hours after initial exposure to the high temperature. The effect of

FIGURE 11

Plant height of barley and radish subjected to a range of temperatures at a high relative humidity. Each value is the mean of three replicates.
[10 day old barley or 18 day old radish plants growing in soil were subjected to a range of temperatures (20° - 41°C) for 1-5 days at a high relative humidity (90-95%). Plants were watered daily.]

⊖ 0 hour

▲ 24 hours

O 72 hours

■ 120 hours

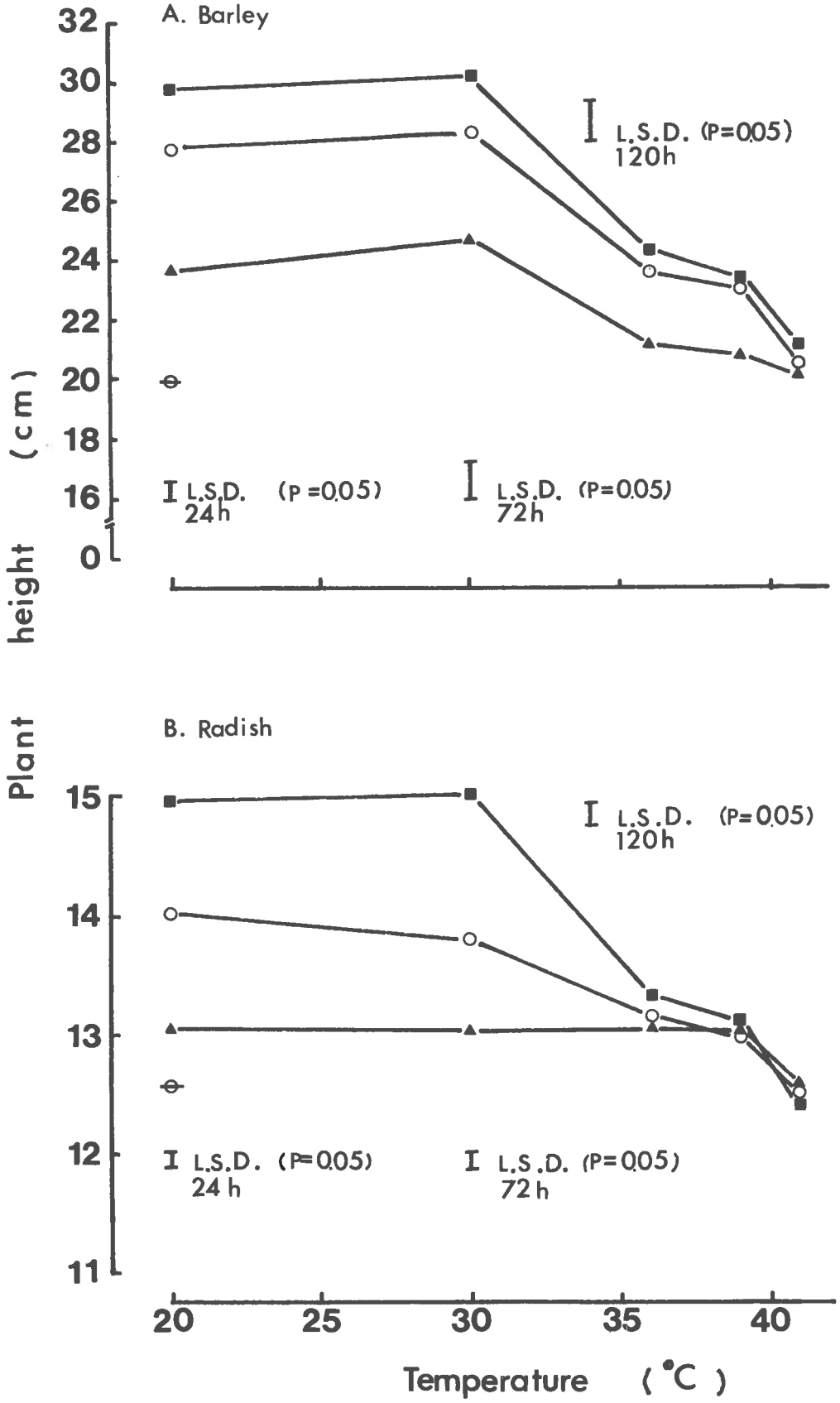

A. Barley
32
30
28
26
24
22
20
18
16
0
Plant height (cm)
L.S.D. (P=0.05) 120h
L.S.D. (P=0.05) 24h
L.S.D. (P=0.05) 72h
B. Radish
15
14
13
12
11
L.S.D. (P=0.05) 120h
L.S.D. (P=0.05) 24 h
L.S.D. (P=0.05) 72 h
20
25
30
35
40
Temperature (°C)

FIGURE 12

Change in chlorophyll concentration of the first leaf in barley and radish plants subjected to a range of temperatures at a high relative humidity. Each value is the mean of three replicates.

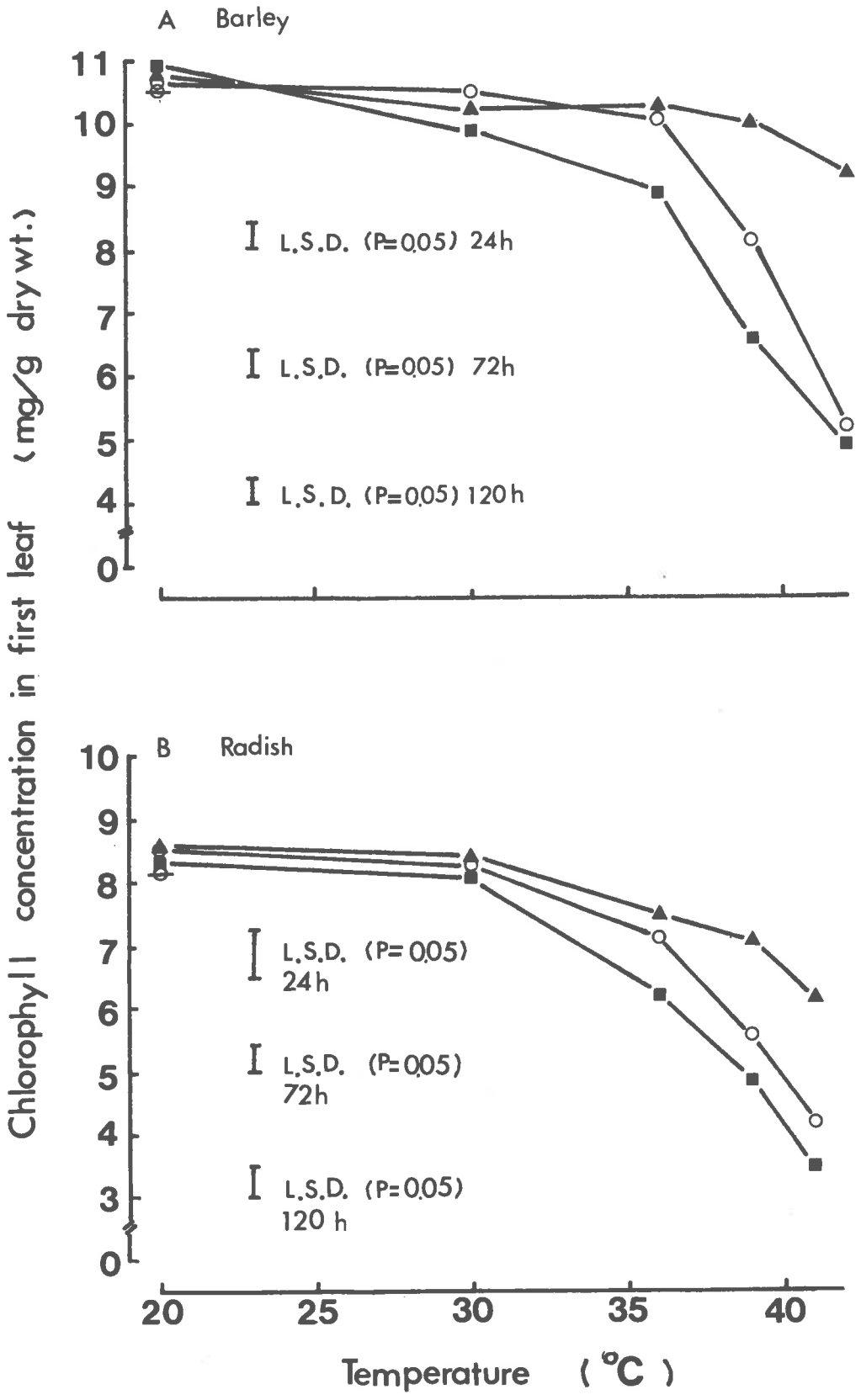
[10 day old barley or 18 day old radish plants growing in soil were subjected to a range of temperatures (20°-41°C) for 1-5 days at a high relative humidity (90-95%). Plants were watered daily.]

⊖ 0 hour

▲ 24 hours

○ 72 hours

■ 120 hours


A Barley
11
10
9
8
7
6
5
4
0
L.S.D. (P=0.05) 24h
L.S.D. (P=0.05) 72h
L.S.D. (P=0.05) 120h
B Radish
10
9
8
7
6
5
4
3
0
L.S.D. (P=0.05) 24h
L.S.D. (P=0.05) 72h
L.S.D. (P=0.05) 120 h
20
25
30
35
40
Temperature (°C)
Chlorophyll concentration in first leaf (mg/g dry wt.)

a 39°C stress for the same duration was similar to the 41°C stress but slightly less pronounced.

(3) Leaf water potential

At temperatures of 20, 30, 36 and 39°C, maintenance of the cabinet relative humidity at 90% or above prevented a decline in leaf water potential throughout the whole experimental period (Figure 13). Small fluctuations in leaf water potential at different temperatures can be seen, but these small variations were not significant. At 40°C, high humidity still prevented a decline in leaf water potential in the first 3 days, but there was a slight decrease in leaf water potential after 3-5 days in both species (maximum reduction -2 bars), suggesting that transpiration at this temperature was rapid despite the high relative humidity.

(4) Proline content

The free proline concentration in the first leaves changed very little over the 5 days of the experiment at temperatures from 20°C to 39°C (Figure 14). There was a slight increase in the concentration of proline at 41°C after 3-5 days in both species, but the change was tenfold less than that associated with water stress (Figure 10) and may have been due to the slight decline in leaf water potential which occurred at that temperature.

FIGURE 13

Water potential of the first leaf of barley or radish plants subjected to a range of temperatures at a high relative humidity. Each value is the mean of three replicates. [10 day old barley or 18 day old radish plants growing in soil were subjected to a range of temperatures (20^{o}-41^{o}C) for 1-5 days at a high relative humidity (90-95%). Plants were watered daily.]

⊖ 0 hour

▲ 24 hours

○ 72 hours

■ 120 hours

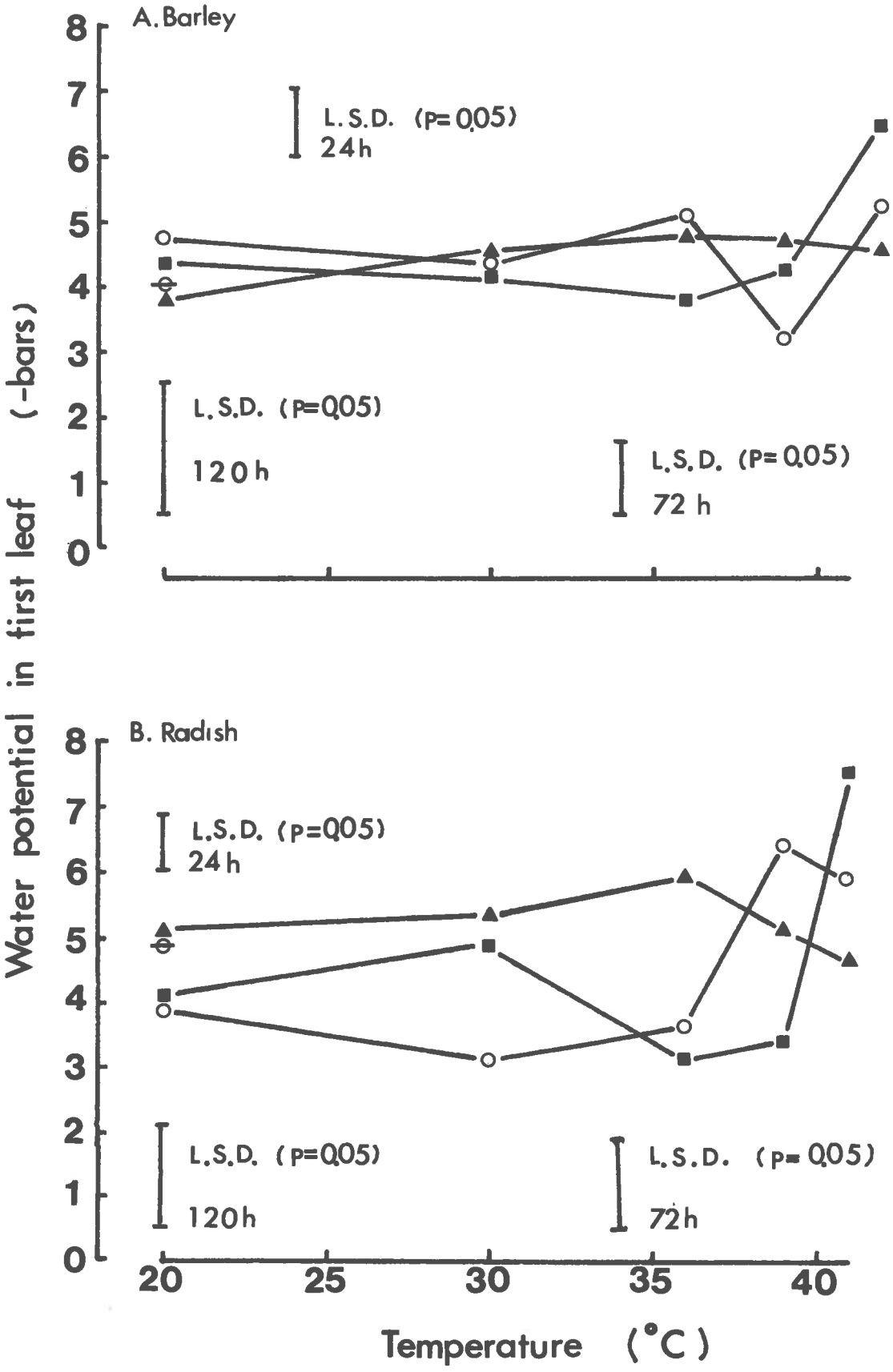

A. Barley
L.S.D. (P=0.05)
24h
L.S.D. (P=0.05)
120h
L.S.D. (P=0.05)
72 h
B. Radish
L.S.D. (P=0.05)
24h
L.S.D. (P=0.05)
120h
L.S.D. (P=0.05)
72h
Water potential in first leaf (-bars)
0
1
2
3
4
5
6
7
8
20
25
30
35
40
Temperature (°C)

FIGURE 14

Free proline concentration in the first leaf of barley or radish plants subjected to a range of temperatures at a high relative humidity. Each value is the mean of three replicates.

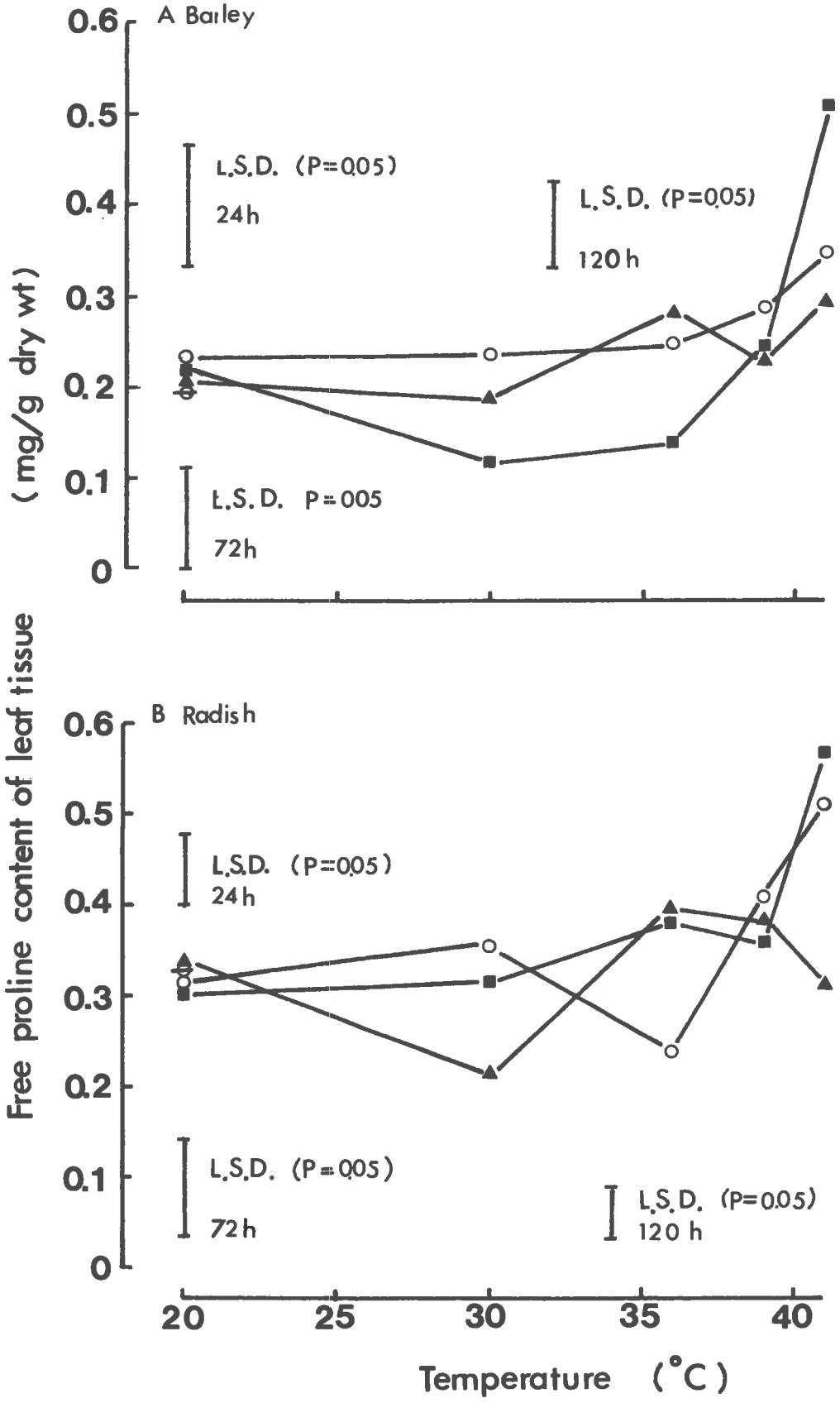
[10 day old barley or 18 day old radish growing in soil subjected to a range of temperatures (20^{o}-41^{o}C) for 1-5 days at a high relative humidity (90-95%). Plants were watered daily.]

⊖ 0 hour

▲ 24 hours

○ 72 hours

■ 120 hours


A Barley
B Radish
Free proline content of leaf tissue
(mg/g dry wt)
0.6
0.5
0.4
0.3
0.2
0.1
0
L.S.D. (P=0.05)
24h
L.S.D. (P=0.05)
120h
L.S.D. P=005
72h
L.S.D. (P=0.05)
24h
L.S.D. (P=0.05)
72h
L.S.D. (P=0.05)
120 h
20
25
30
35
40
Temperature (°C)

2.3. Discussion

In the initial experiments, the plants were exposed to high temperature (39°C) without control of the relative humidity in the growth cabinet. This was 50% or lower, the plants transpired rapidly and leaf water potential also fell despite frequent watering of the plants. As a consequence, any metabolic effects of high temperature were confounded with those of lowered leaf water potential. Therefore a high relative humidity (90-95%) has been used in order to separate water deficit from heat stress.

It is evident that at least some of the metabolic effects of exposure to supra-optimal temperatures are due wholly or in part to the unavoidable changes in leaf water balance which occur. In particular, the accumulation of proline appears to be solely controlled by tissue water status and unaffected by elevated tissue temperature, at least up to a temperature of 39°C. Additionally, other effects such as the inhibition of extension growth and decline in leaf chlorophyll content which occur at higher temperature are magnified by concurrent water stress in the plant tissues.

The decline in ψ at higher temperatures is not entirely prevented by increasing the supply of water to the roots, as the potential gradient between the root system and the leaves is steep under these conditions of extremely rapid transpiration. The association between high ambient temperature and low leaf water potential is broken by conditions which reduce transpiration, and under these conditions the effects of high

temperature *per se* become apparent. Thus, in the field, it is rare that plants will experience heat stress uncomplicated by accompanying water stress.

Heat stress is frequently associated with moisture stress and separation of direct effects of high temperature on growth and development from the concomitant effects of water deficit, is a difficult problem not entirely resolved. Often, a measurement of water potential or relative water content is employed to distinguish these two stresses. The thermocouple psychrometer method of measuring water potential has been considered to be the most accurate (Barrs, 1968). However, this method is expensive, often difficult to maintain and unsuitable for studies where many determinations are needed. It has also recently been shown that the method underestimates the true water potential value in the 0 to -5 bars range in a number of species (Klepper and Barrs, 1968). Although the technique is simple and a minimum amount of equipment is involved for measurement of relative water content, difficulties arise in interpreting relative water content measurements since they may change in meaning with plant age, tissue, part or season (Knipling, 1967). Errors may arise from infiltration of water into the cut edges and intercellular spaces, cell growth may occur during the saturation time and weight may be lost due to respiration (Weatherley, 1950; Barrs and Weatherley, 1962). Thorough blotting of the wet discs or leaf pieces may also be difficult in some cases. Proline accumulation in water-stressed leaf tissue is a

common phenomenon and there is a repeatable correlation between the accumulation of free proline and the degree of tissue dehydration. Moreover, proline does not accumulate in leaf tissue when the plant is subjected to heat stress without concomitant water deficit. These considerations suggest that it may be possible to use proline accumulation as a reliable indicator of tissue water deficit so as to differentiate between water deficit and heat stress.

3. Low temperature stress

3.1 The response of the barley and radish plant to low temperature.

3.3.3 Methods

The experiments were carried out on barley plants (cv. Prior) grown for 10 days or radish plants (cv. Long White Icicle) grown for 18 days in 10 cm plastic pots filled with sand. Plants were irrigated daily with Hoagland's solution and were maintained in a controlled environment at 20 ± 1°C with a 16 hour photoperiod of 4.1 mW cm^{-2} radiant flux intensity. Cold stress was imposed by transferring sand-grown plants to a cold room where the temperature was maintained at 5 ± 1°C and the plants were exposed to light continuously at a similar intensity to those remaining at 20°C. The effect on proline accumulation of continuous exposure to light, as opposed to a 16 hour photoperiod, was examined with plants growing at 20°C, and found to be negligible. The plants were irrigated daily with Hoagland's solution maintained at the temperature of the environment in which they were growing. Plants were sampled 0, 1, 3 and 5 days after stress and the water potential and free proline content of the first leaf of both species were assayed.

3.1.2 Results

(1) Water potential

The temperature regime was without any consistent effect on leaf water potential in either species throughout the experimental period

(Table 2), although there was a tendency for slightly lower ψ in the leaves at the lower temperature, this was only significant in two cases. Leaf water potential varied between -4.1 and -6.9 bars in barley and between -3.2 and -6.4 bars in radish. Neither species showed any visible wilting nor any other chilling symptom throughout the period of the experiment.

(2) Plant growth

Changes in fresh weight and dry weight of five barley plants or one radish plant is shown in Figure 15. Exposure to the low temperature ($5^{o}C$) immediately inhibited fresh weight increase in both species and this fresh weight did not significantly increase during the following 5 days. In contrast, the dry weight of both species continued to increase at the low temperature, although at a considerably reduced rate. As low temperature inhibited the increase in fresh weight more effectively than the increase in dry weight there was a consequent increase in the dry weight to fresh weight ratio. The ratio increased from 7.8 to 13.0 in barley and from 10.3 to 11.7 in radish during the experimental period. This change is significant when compared with the control plants grown at $20^{o}C$ where the ratio varied between 7.8 and 9.1 for barley and between 7.7 and 10.3 for radish.

(3) Proline content

Although low temperature had only a small and inconsistent effect on leaf water potential, there was a marked accumulation of free proline

TABLE 2

Water potential of the first leaf of barley (cv. Prior, 10 day old) or radish (cv. Long White Icicle, 18 day old) plants subjected to 20°C or 5°C for 1-5 days. Each value is the mean of four replicates.

A. Barley

Time (hr)	20°C ψ (- bars)	5°C ψ (- bars)	L.S.D. (p=0.05)
0	4.1	4.1	
24	4.3	5.0	1.4
72	4.9	6.7	1.1
120	4.7	6.9	2.4

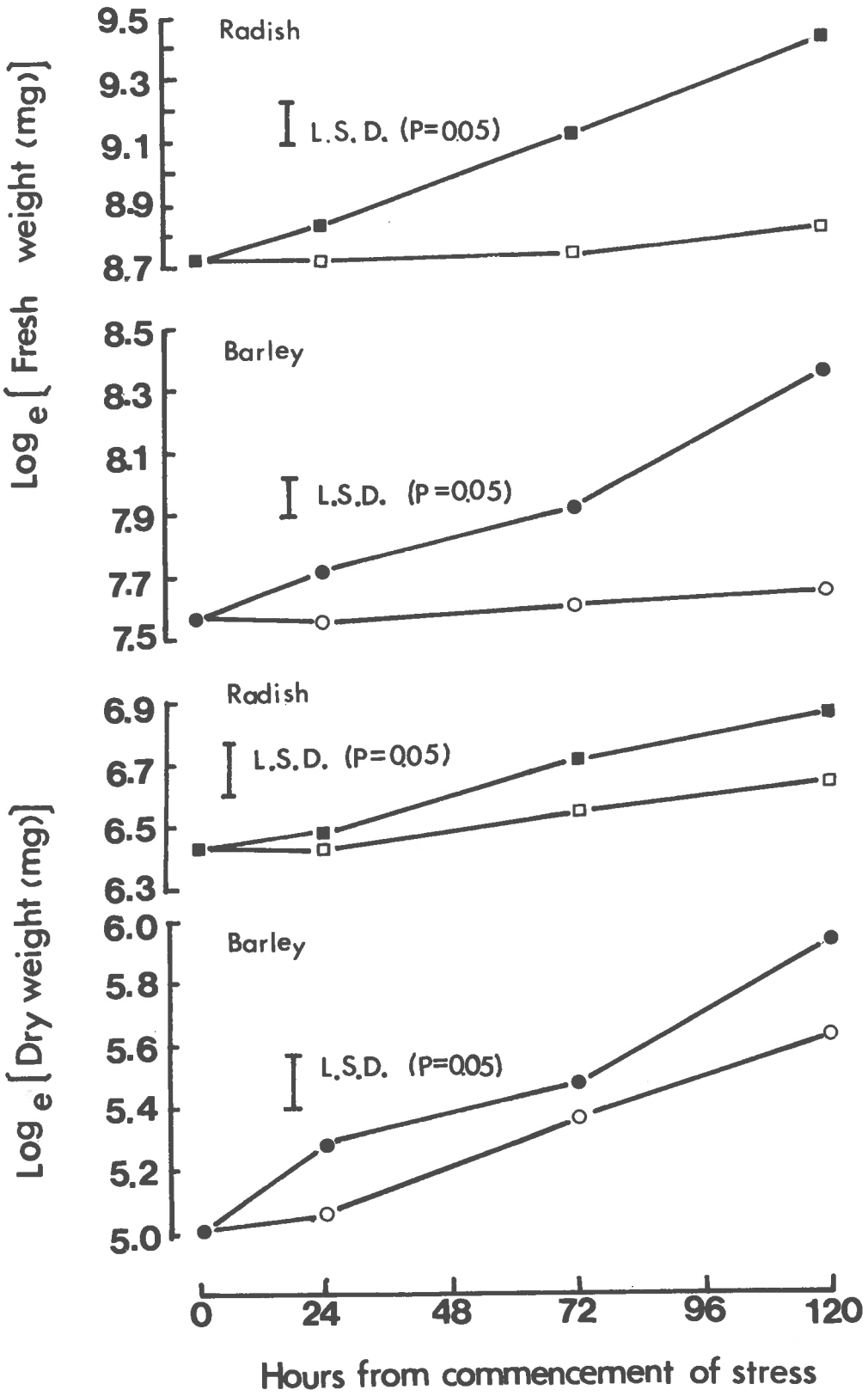
B. Radish

Time (hr)	20°C ψ (- bars)	5°C ψ (- bars)	L.S.D. (p=0.05)
0	3.2	3.2	
24	3.8	4.5	1.6
72	3.9	5.1	1.4
120	3.5	6.4	2.0

FIGURE 15

Fresh weight and dry weight changes in barley and radish plants subjected to 5°C for 5 days.
[Barley plants were grown for 10 days and radish plants for 18 days at 20°C before transfer to 5°C.]

Barley : ● 20°C
○ 5°C
Radish : ■ 20°C
□ 5°C


Radish
L.S.D. (P=0.05)
Barley
L.S.D. (P=0.05)
Radish
L.S.D. (P=0.05)
Barley
L.S.D. (P=0.05)
Log e [Fresh weight (mg)]
Log e [Dry weight (mg)]
9.5
9.3
9.1
8.9
8.7
8.5
8.3
8.1
7.9
7.7
7.5
6.9
6.7
6.5
6.3
6.0
5.8
5.6
5.4
5.2
5.0
0
24
48
72
96
120
Hours from commencement of stress

in the leaves of both species (Figure 16). In barley, accumulation of proline commenced after 24 hours at 5°C and continued for at least the following 4 days at a rate of 74 μg (g dry wt.)$^{-1}$ h^{-1}. This compares with a rate of accumulation of proline in barley due to water stress of 247 μg (g dry wt.)$^{-1}$ h^{-1} previously reported (Singh *et al.*, 1973c). Proline was also accumulated continuously at a steady rate in radish leaf, although the accumulation of free proline was less rapid. The rate of proline accumulation was estimated to be 28 μg (g dry wt.)$^{-1}$h^{-1} in the low temperature as compared with a rate of 231.9 μg (g dry wt.)$^{-1}$h^{-1} induced by water deficit (Figure 4).

3.2 Distribution of proline in barley and radish plant exposed to low temperature

3.2.1. Methods

Barley (cv. Prior) and radish (cv. Long White Icicle) plants were grown in sand for 10 days (barley) or 18 days (radish) at constant environment before transferring to 5°C for a further 3 days. Plants were harvested 0, 6, 12, 24, 48 and 72 hours after initial exposure to cold stress and the free proline content of the lamina and sheath of the first leaf and the roots of the barley together with the lamina of the first and second leaf, cotyledons and the root of the radish were determined.

3.2.2 Results

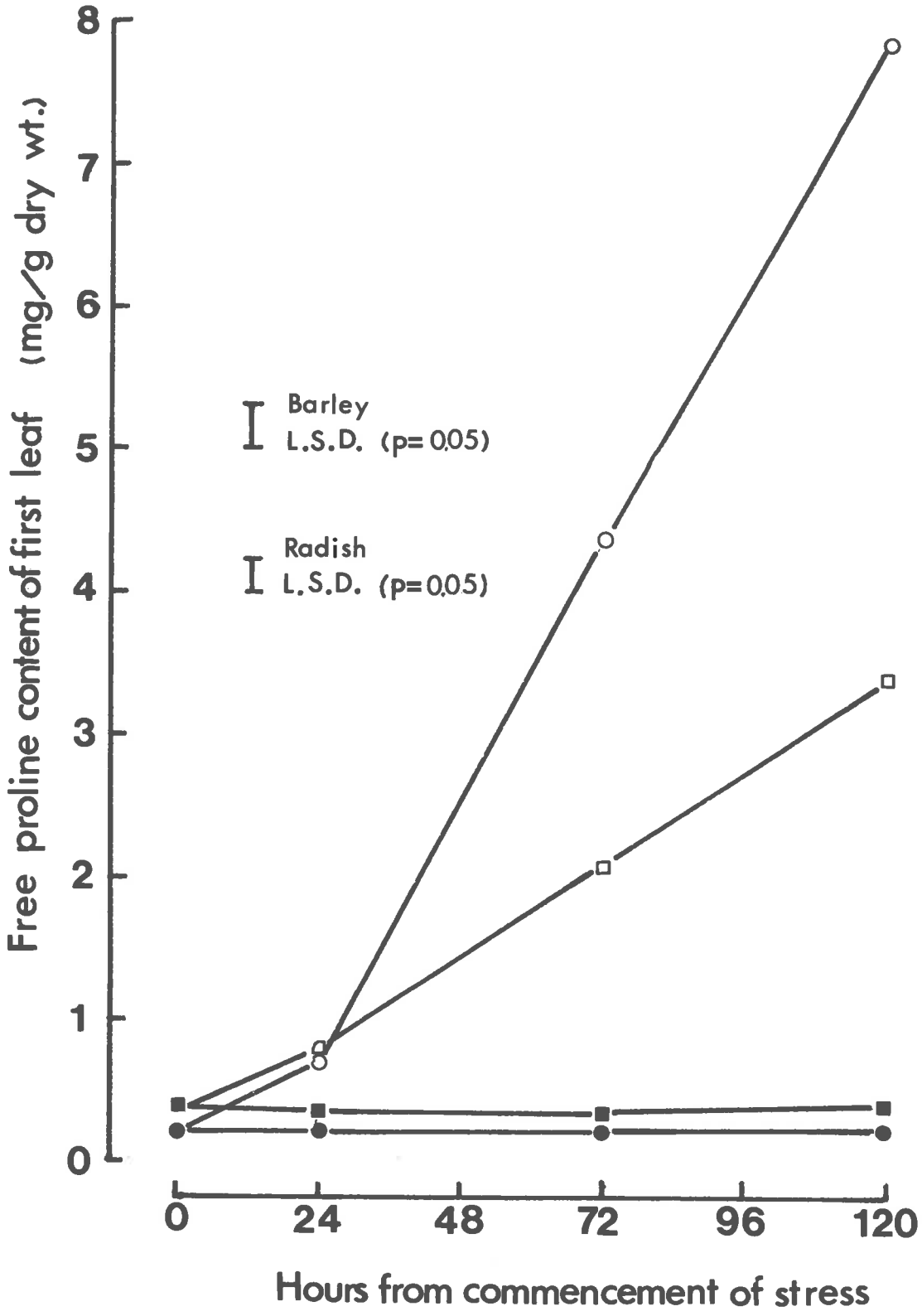
(1) Barley

As in response to a water deficit (Singh *et al.*, 1973c), proline

FIGURE 16

Accumulation of free proline in the leaves of barley and radish plants exposed to low temperature (5°C). Each value is the mean of four replicates.
[10 day old barley or 18 day old radish plants growing in sand were subjected to 5°C low temperature stress for 1-5 days.]

Barley: ● 20°C
○ 5°C
Radish: ■ 20°C
□ 5°C


Free proline content of first leaf (mg/g dry wt.)
8
7
6
5
4
3
2
1
0
Barley
L.S.D. (p=0.05)
Radish
L.S.D. (p=0.05)
0
24
48
72
96
120
Hours from commencement of stress

accumulated in all organs of the barley plant when the temperature was reduced (Figure 17). The rate of accumulation was most rapid in the leaves. In the first leaf lamina there was an initial lag phase of some 24 hours, during which the proline content remained low and constant but, subsequently, proline accumulated relatively rapidly. 72 hours after exposure to the low temperature, the free proline content in the lamina of the first leaf had reached 4.76 mg per gram dry weight. A similar pattern of proline accumulation occurred in the second leaf, although the response was less rapid than that in the first leaf. Free proline also accumulated in the leaf sheath and in root tissue during the period of cold stress, the rate of accumulation in both these situations was considerably less than that in the leaf lamina. At 72 hours after commencement of stress, the proline concentration was five times higher in the leaf lamina than in the roots or the leaf sheath.

(2) Radish

In contrast to barley, proline accumulated only in the leaf tissues of radish plants subjected to low temperature (Figure 18). Free proline accumulated rapidly in the leaves commencing 12 hours after the temperature was lowered. The younger leaf lamina had a higher proline accumulation potential than the older lamina. Following 72 hours exposure to low temperature, the free proline content of the young lamina had reached 3.8 mg per gram dry weight but only 2.4 mg per gram dry weight in the older lamina. Neither the cotyledons nor the root system accumulated

FIGURE 17

Accumulation of free proline in the various organs of intact barley plants exposed to low temperature (5°C). Each value is the mean of three replicates.
[10 day old barley plants growing in sand were subjected to 5°C low temperature for 1-3 days.]

●	All organs	20°C
○	Lamina of the first leaf	5°C
△	Lamina of the second leaf	5°C
▽	Leaf sheath of the first leaf	5°C
□	Root	5°C

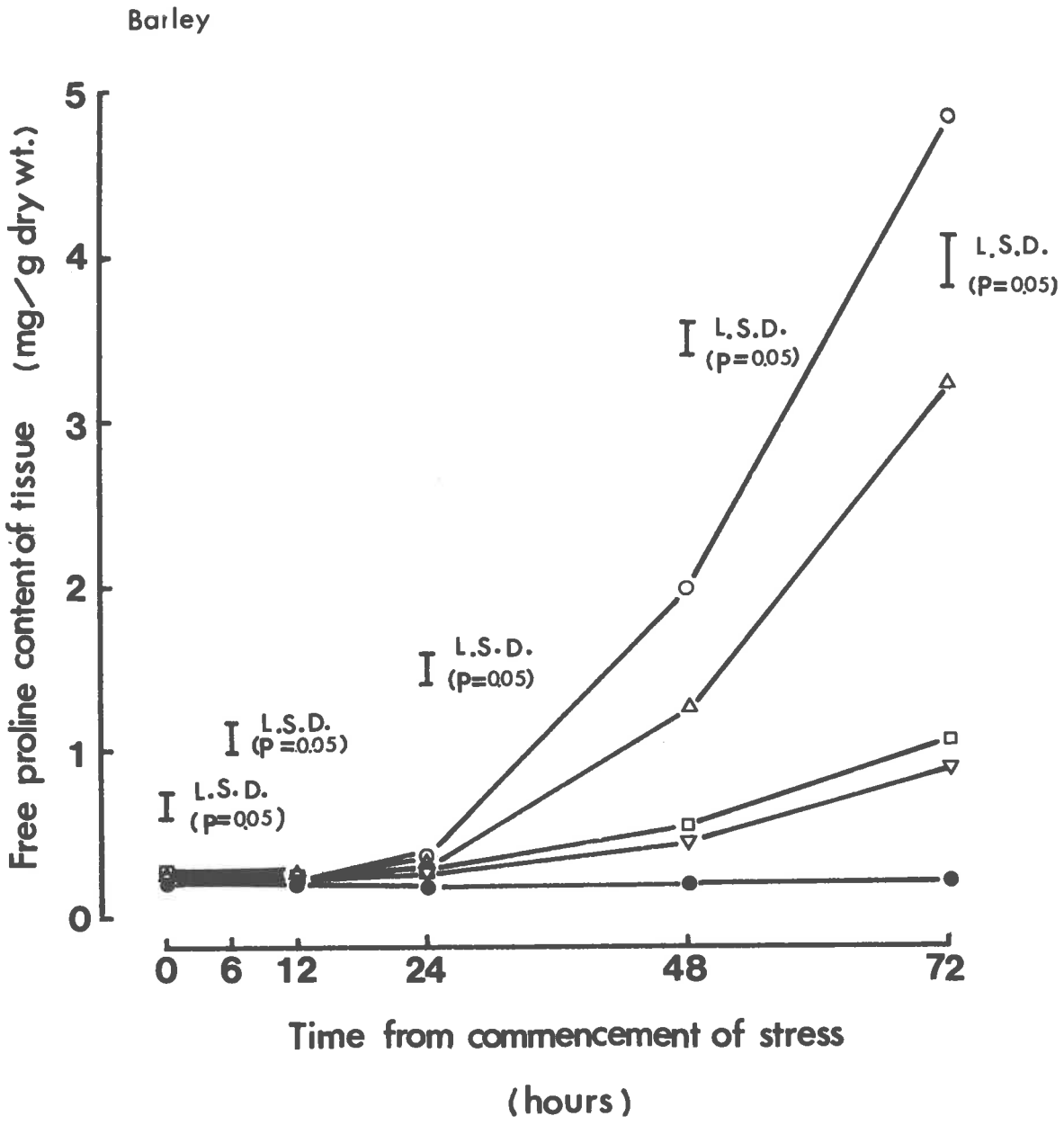
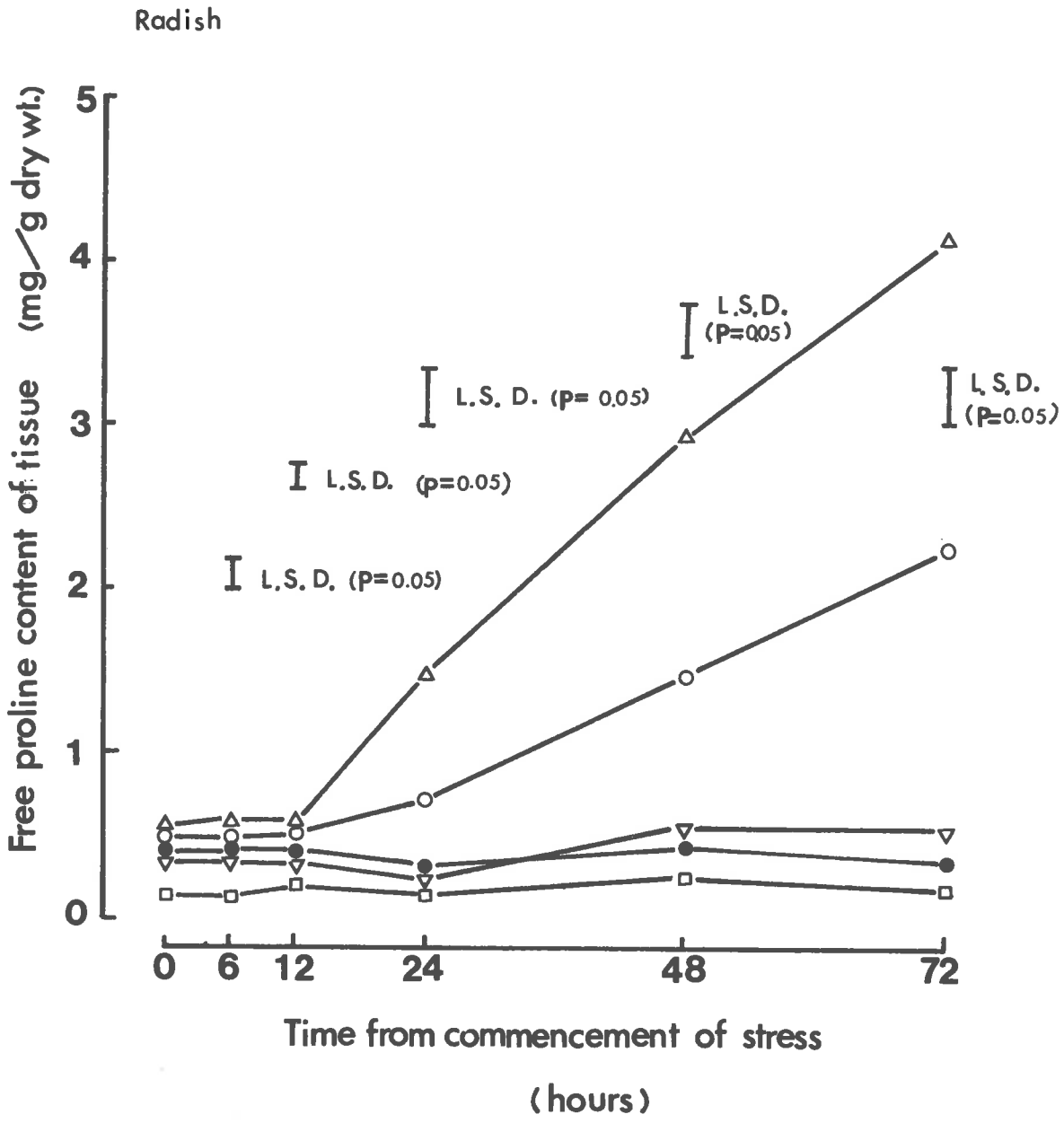

Barley
Free proline content of tissue (mg/g dry wt.)
0
1
2
3
4
5
L.S.D. (P=0.05)
L.S.D. (P=0.05)
L.S.D. (P=0.05)
L.S.D. (P=0.05)
L.S.D. (P=0.05)
0
6
12
24
48
72
Time from commencement of stress
(hours)

FIGURE 18

Accumulation of free proline in the various organs of intact radish plants exposed to low temperature ($5^{o}C$) for 72 hours. Each value is the mean of three replicates. [Radish plants were grown in sand for 18 days at $20^{o}C$ before transfer to $5^{o}C$.]

●	All organs	$20^{o}C$
○	The first pair of leaves	$5^{o}C$
△	The second pair of leaves	$5^{o}C$
▽	Cotyledons	$5^{o}C$
□	Root	$5^{o}C$


Radish
Free proline content of tissue (mg/g dry wt.)
5
4
3
2
1
0
L.S.D. (P=0.05)
L.S. D. (P= 0.05)
L.S. D. (p=0.05)
L.S. D. (P=0.05)
L. S. D. (P=0.05)
0
6
12
24
48
72
Time from commencement of stress
(hours)

free proline throughout the experimental period, although both cotyledon and root accumulated significant quantities of proline in intact, water-stressed plants (Figure 6).

3.3 The effect of low temperature on leaf water status

3.3.1 Introduction

The increase in the free proline concentration in cold-stressed barley and radish appeared to occur without any accompanying marked or consistent decrease in leaf water potential (Table 2), but it is possible that internal adjustment in the components of ψ during cold stress could initiate the proline accumulation. This possibility was tested in this experiment. In the leaf, ψ is determined by the components:

$$\psi = \psi_p + \psi_\pi + \psi_m + \psi_g \qquad (1)$$

where p, π, m and g refer to pressure (turgor), solute, matric, and gravitational components. Gravitational potential is usually insignificant and was neglected and no attempt was made to separate ψ_m and ψ_π. Thus ψ of the tissue became effectively the sum of solute and turgor potential:

$$\psi = \psi_\pi + \psi_p \qquad (2)$$

Direct measurement of turgor potential of plant cells is difficult, and turgor potential is usually calculated as the difference between

the measured water and osmotic potentials. Osmotic potential is estimated on tissue that has been killed. It is assumed that in dead tissue $\psi_p = 0$, whence from equation (2) the water potential of the dead tissue is identical to the osmotic potential.

3.3.2 Methods

Free proline, leaf water potential, osmotic potential and relative turgidity were estimated independently on the first leaves of 10 day old barley and 18 day old radish plants subjected to a low temperature (5°C) for 5 days. Some plants were moved from 5 to 20°C after 3 days exposure to the low temperature to observe recovery. Plant tissues were harvested every 24 hours following the initial exposure to the low temperature. Leaf water potential was measured with a psychrometer operating at 25°C and leaf osmotic potential was determined after the same sample had been frozen (with liquid nitrogen for 1 minute in the covered psychrometer chamber) and thawed. The freezing and thawing treatments ruptured cell membranes and reduced ψ_p of the tissue to zero. Relative turgidity was also estimated using the method of Barrs and Weatherley (1962).

3.3.3 Results

(1) Proline content

As in the previous experiments, free proline accumulated to a high concentration in the leaves of cold-exposed plants of both species (Figure 19). In barley, accumulation was relatively slow during the first 2 days

FIGURE 19

Effect of transfer from low (5°C) to high (20°C) temperature on proline concentration in the first leaf of 10 day old barley or 18 day old radish plants. Each value is the mean of three replicates.

Barley: ● 20°C

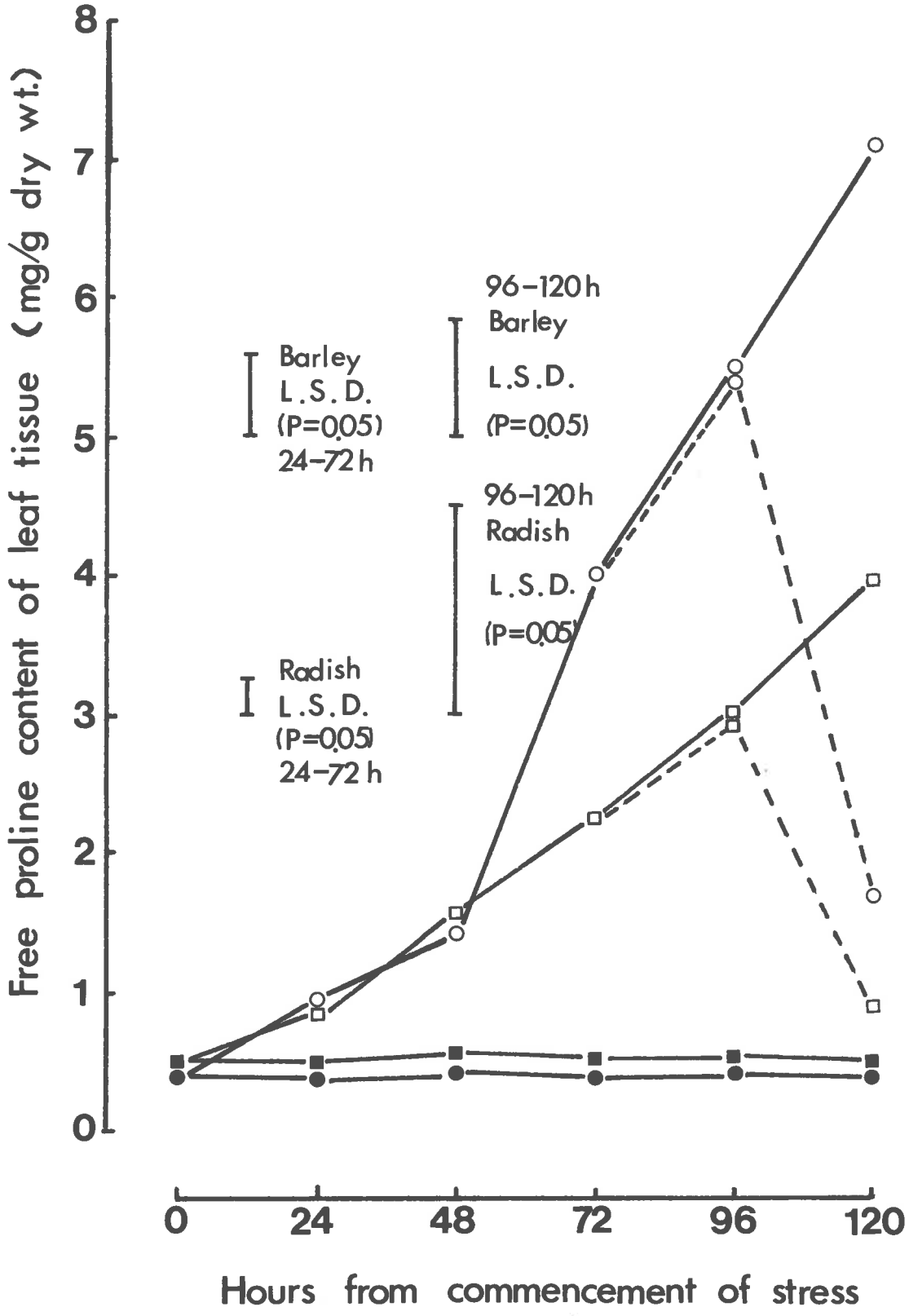
○ 5°C

○---○ Transferred from 5°C to 20°C at 72 hrs.

Radish: ■ 20°C

□ 5°C

□---□ Transferred from 5°C to 20°C at 72 hrs.


Free proline content of leaf tissue (mg/g dry wt.)
8
7
6
5
4
3
2
1
0
Barley
L.S.D.
(P=0.05)
24–72 h
96–120 h
Barley
L.S.D.
(P=0.05)
96–120 h
Radish
L.S.D.
(P=0.05)
Radish
L.S.D.
(P=0.05)
24–72 h
0
24
48
72
96
120
Hours from commencement of stress

of low temperature, but then accelerated to a rapid rate over the remaining 3 days. When plants were returned to 20°C after 3 days at 5°C, proline continued to accumulate for 24 hours at the same rate as in plants maintained at 5°C. This was not due to a persistence of low temperature in any part of the plant following transfer, as the root systems were flooded with nutrient solution at 20°C when the plants were transferred to that temperature. Following this 24 hours of continued accumulation, the free proline concentration in the leaves declined rapidly.

In radish, accumulation of proline was less rapid. Proline accumulated continuously at a steady rate over the experimental period (Figure 19). Again, the radish plants also continued to accumulate proline at the same rate as did plants maintained at 5°C for 24 hours when plants were returned to 20°C. The pattern of change in proline accumulation in radish was very similar to that in barley although the species had different proline accumulation potential.

(2) Water potential

As before (Table 2), the accumulation of free proline in the cold-stressed plants was not accompanied by a marked decline in the leaf water potential of either species (Figures 20, 21). Leaf water potential decreased by approximately 2 bars in barley and 3 bars in radish 5 days after exposure to the low temperature. The leaf water potential increased slightly as the plants were released from cold stress, the leaf water

FIGURE 20

Effect of low temperature (5°C) on leaf water relationships in barley. Each value is the mean of three replicates. [10 day old barley plants growing in sand were subjected to 5°C low temperature for 3 days when half of the stressed plants were transferred back to 20°C.]

● Water potential (ψ), 20°C

○ Water potential (ψ), 5°C

○- - -○ ψ , transferred from 5 to 20°C

■ Osmotic potential (ψ_π), 20°C

□ Osmotic potential (ψ_π), 5°C

□- - -□ ψ_π , transferred from 5 to 20°C

▲ Relative turgidity (RT), 20°C

△ Relative turgidity (RT), 5°C

△- - -△ RT , transferred from 5 to 20°C

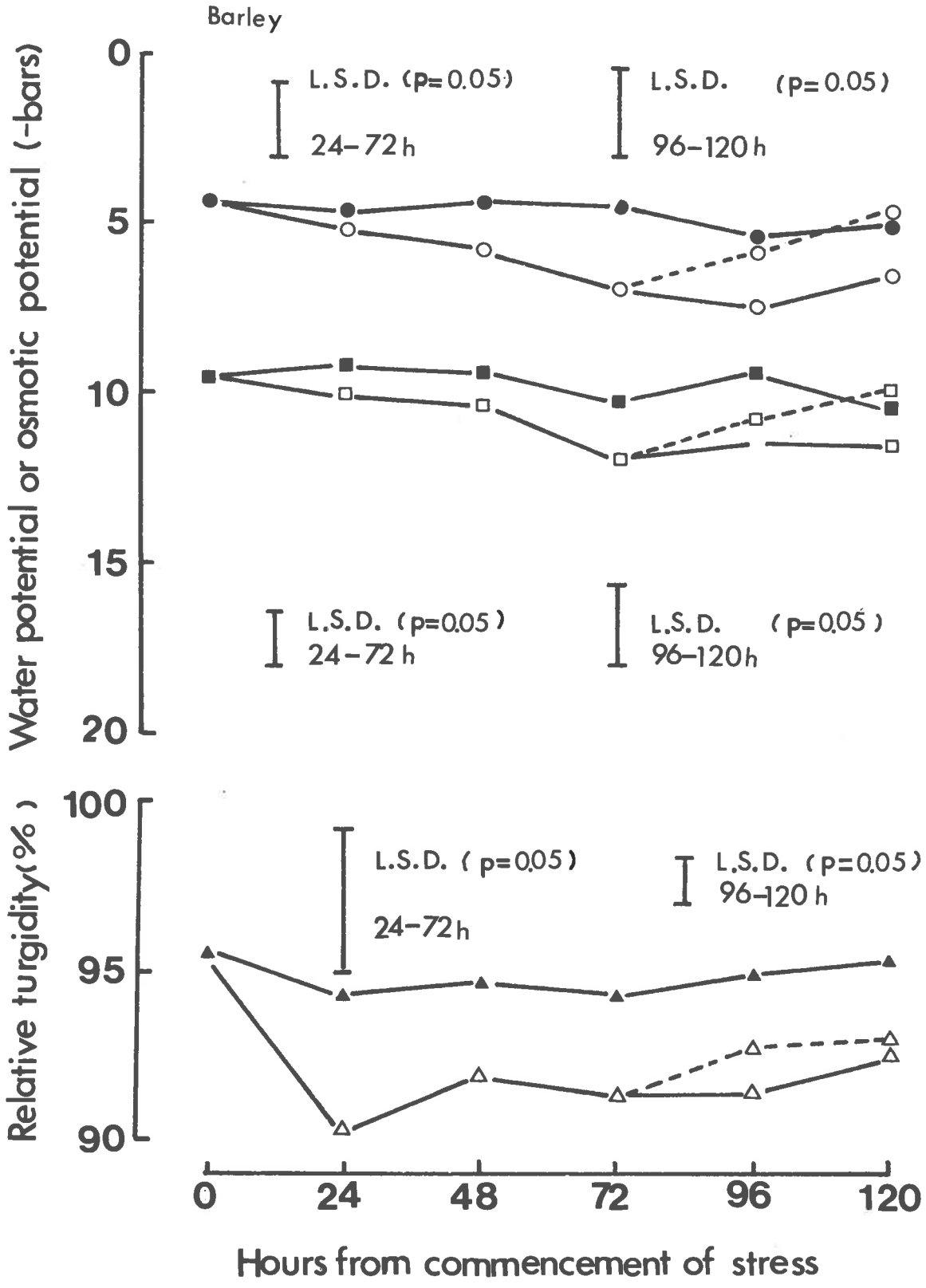

Barley
Water potential or osmotic potential (-bars)
0
5
10
15
20
L.S.D. (p=0.05)
24–72 h
L.S.D. (p=0.05)
96–120 h
L.S.D. (p=0.05)
24–72 h
L.S.D. (p=0.05)
96–120 h
Relative turgidity (%)
100
95
90
L.S.D. (p=0.05)
24–72 h
L.S.D. (p=0.05)
96–120 h
0
24
48
72
96
120
Hours from commencement of stress

FIGURE 21

Effect of low temperature (5°C) on leaf water relationships in radish. Each value is the mean of three replicates. [18 day old radish plants growing in sand were subjected to 5°C for 3 days when half of the stressed plants were transferred back to 20°C.]

● Water potential (ψ), 20°C

○ Water potential (ψ), 5°C

○---○ ψ , transferred from 5 to 20°C

■ Osmotic potential (ψ_π), 20°C

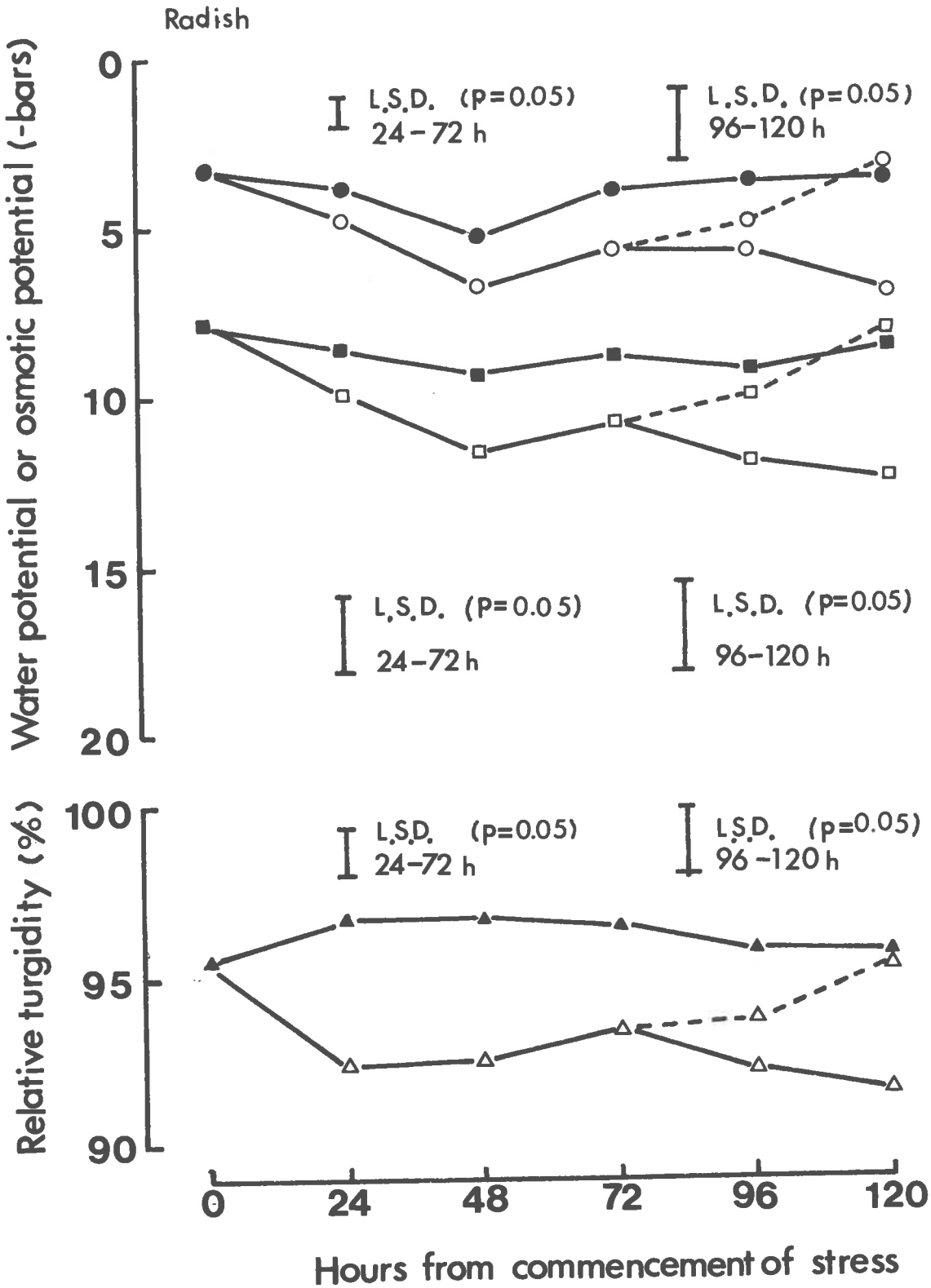
□ Osmotic potential (ψ_π), 5°C

□---□ ψ_π , transferred from 5 to 20°C

▲ Relative turgidity (RT), 20°C

△ Relative turgidity (RT), 5°C

△---△ RT , transferred from 5 to 20°C


Radish
Water potential or osmotic potential (-bars)
0
5
10
15
20
L.S.D. (P=0.05)
24-72 h
L.S.D. (p=0.05)
96-120 h
L.S.D. (P=0.05)
24-72 h
L.S.D. (p=0.05)
96-120 h
Relative turgidity (%)
100
95
90
L.S.D. (p=0.05)
24-72 h
L.S.D. (p=0.05)
96-120 h
0
24
48
72
96
120
Hours from commencement of stress

potentials in both species being restored to the original values when the experiment was terminated.

(3) Osmotic potential and relative turgidity

In both species, cell osmotic potential also showed a slight decrease in the cold-stressed plants (Figures 20, 21). The cell osmotic potential had decreased by approximately 2 bars in barley and 3 bars in radish 5 days after exposure to the low temperature. The change in osmotic potential was identical to that in leaf water potential and, therefore, turgor potential, calculated from the difference between these two measurements was, 4.8 ± 0.3 bars in barley and 5.3 ± 0.5 bars in radish. These changed with neither treatment nor time. On the other hand, the relative turgidity determined by the method of Barrs and Weatherley (1962) decreased by approximately 3% in both species following cold stress.

Each of these changes in the parameters of the water balance of the leaf tissue were insignificant when compared with those induced by exposure of the plants to water stress (Singh *et al.*, 1973c and Figures 1,3) and yet the rate of proline accumulation was comparable to that in water stressed tissues at much lower values of ψ. This confirms that proline accumulation in these cold-stressed tissues cannot be attributed to any accompanying effects on tissue water balance.

3.4 The response of the barley plant to a range of low temperatures

3.4.1 Methods

Barley plants (cv. Prior) were sown at intervals and grown in sand

supplied daily with half-strength Hoagland's solution in a controlled environment at 20°C with a 16 hour photoperiod of 4.1 mW cm^{-2} radiant flux intensity. After 10 days, plants were transferred to one of a range of low temperatures (± 0.5°C) in a second growth cabinet. These plants were also irrigated daily with half-strength Hoagland's solution maintained at the temperature of the environment within which they were growing. Total plant height, leaf water potential and proline content of the first leaf were measured 24, 72 and 120 hours after transfer. As only two growth cabinets were available, the responses to the different temperatures were examined with successive batches of plants.

3.4.2 Results

(1) Plant height

Growth of the barley seedlings, as measured by plant height, decreased with decrease in temperature below 20°C (Figure 22). Growth was immediately and completely inhibited by the onset of a low temperature when the plants were exposed to 4 or 6°C.

(2) Water potential

The effect of the range of temperatures on leaf water potential was examined. There was no appreciable change in ψ with time in any temperature (Table 3). The water potential fluctuated between -3.5 and -4.9 bars. Similarly, there was no significant change in leaf water

FIGURE 22

Height of barley plants subjected to a range of temperatures for 1-5 days. Each value is the mean of five replicates. [Barley plants were grown in sand for 10 days at 20°C and were then transferred to a range of temperatures (20°- 4°C) for 1-5 days.]

⊖ 0 hour

▲ 24 hours

○ 72 hours

■ 120 hours

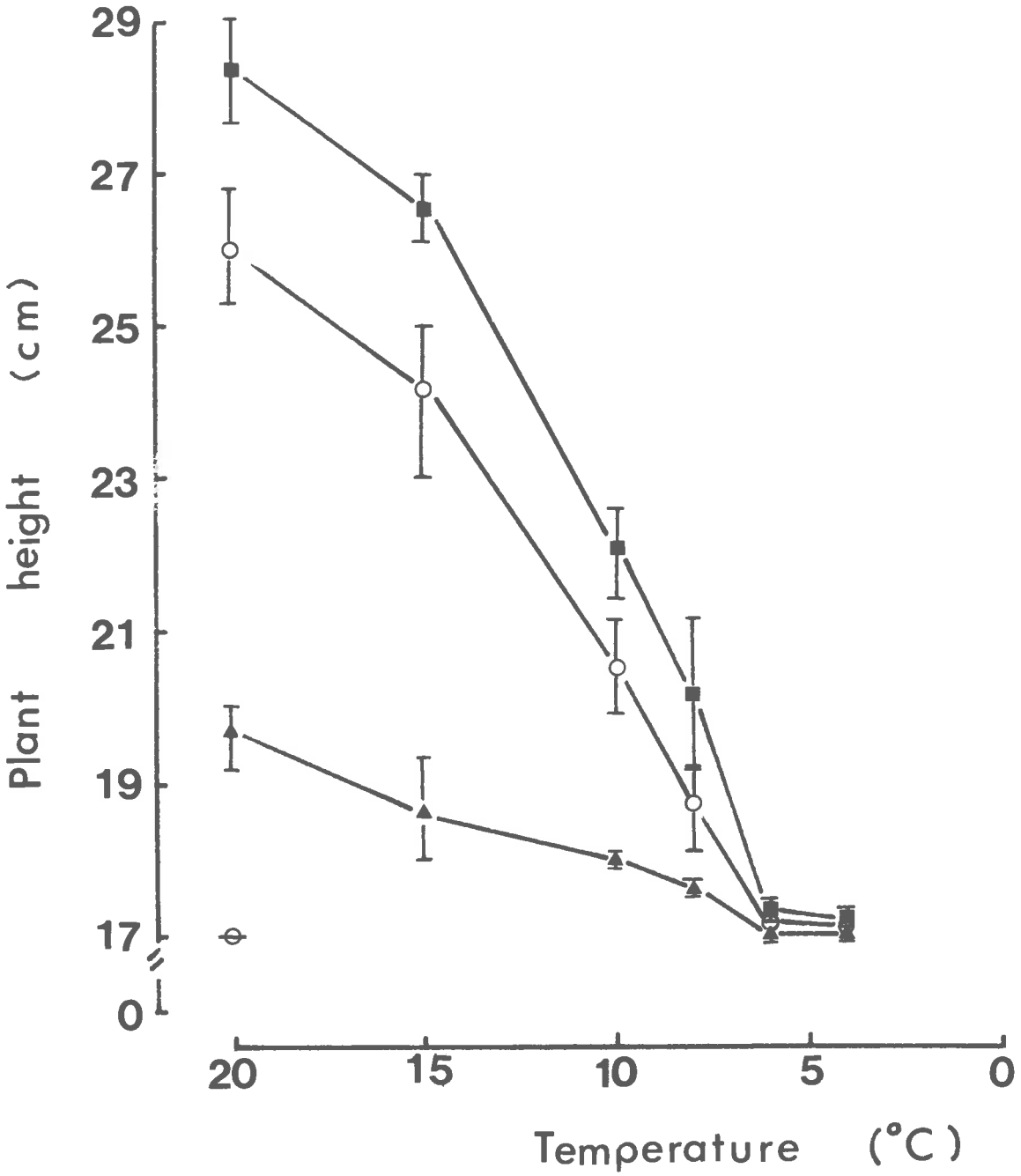

29
27
25
23
21
19
17
0
Plant height (cm)
20
15
10
5
0
Temperature (°C)

TABLE 3

Water potential of the first leaf of barley plants subjected to a range of low temperatures.

[10 day old barley plants growing in sand were subjected to a range of temperatures (20°- 4°C) for 1-5 days.]

Time (hr)	20°C ψ (-bars)	15°C ψ (-bars)	10°C ψ (-bars)	8°C ψ (-bars)	6°C ψ (-bars)	4°C ψ (-bars)
0	3.5±0.6	3.7±0.9	3.4±0.2	3.7±0.2	3.7±0.6	3.5±0.2
24	3.7±0.2	3.7±0.4	3.7±0.1	3.9±0.2	4.1±0.6	4.2±0.1
72	3.6±0.1	3.9±0.4	3.8±0.5	4.1±0.5	4.3±0.2	4.7±0.3
120	4.2±0.4	3.6±0.3	4.2±0.2	4.3±0.1	4.7±0.3	4.9±0.1

potential with temperature and it can be concluded that the effects of this range of temperatures on growth and metabolism were unrelated to changes in ψ.

(3) Proline content

There was no significant accumulation of proline in the first leaf at temperatures above 8°C (Figure 23) but accumulation was initiated when the temperature fell below this temperature. The rate of accumulation at both 6 and 4°C was slow at first but accelerated after the first 24 hours (Figure 24). Accumulation was more rapid at 4°C than at 6°C. At 72 hours, free proline content had reached 7.1 mg per gram dry weight at 4°C.

3.5 Distribution of proline in subcellular fractions of barley leaf tissue

3.5.1 Introduction

The previous results show that decreasing the temperature below 8°C induces a marked increase in the concentration of free proline in both barley and radish, independent of any effect on water potential. As a fall in water potential at higher temperatures can also induce proline accumulation, it is possible that a similar cellular response is invoked by both types of environmental stress. It has been proposed that both cold injury (Lyons, 1973) and water stress response (Levitt,1972) are both due to changes in membrane permeability, possibly similar to the

FIGURE 23

Change in free proline concentration in the lamina of the first leaf of barley plants subjected to a range of temperatures (20°C- 4°C). Each value is the mean of three replicates.

⊖ 0 hour

▲ 24 hours

○ 72 hours

■ 120 hours

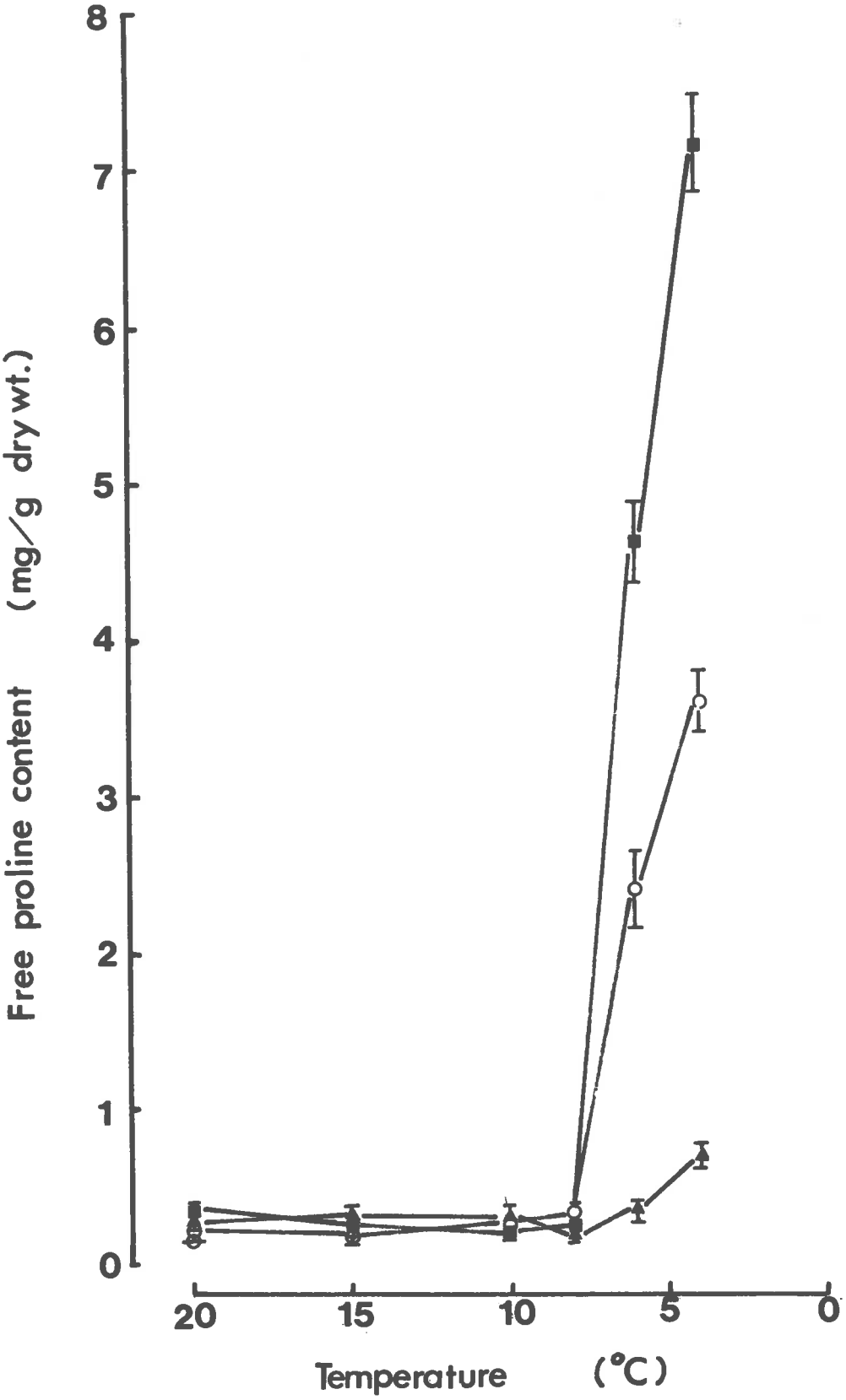

Free proline content (mg/g dry wt.)
8
7
6
5
4
3
2
1
0
20
15
10
5
0
Temperature (°C)

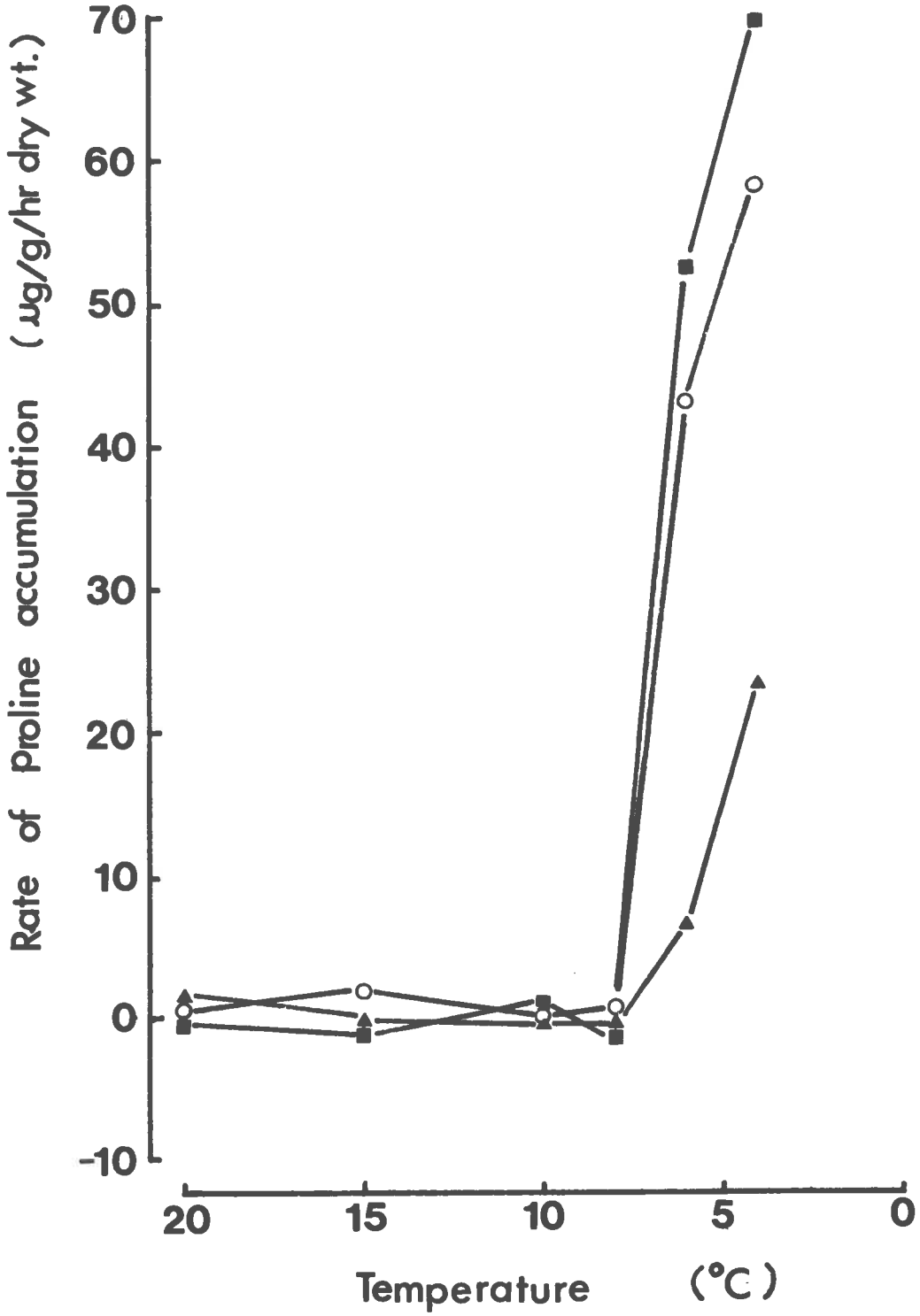
FIGURE 24

The effect of a range of temperatures on the rate of free proline accumulation in the first leaf of barley plant.

▲ 0 - 24 hours

○ 24 - 72 hours

■ 72 -120 hours


Rate of Proline accumulation (μg/g/hr dry wt.)
70
60
50
40
30
20
10
0
-10
20
15
10
5
0
Temperature (°C)

temperature-induced phase transitions described by Lyons (1973). Such changes in membrane permeability may affect the movement of proline between sub-cellular organelles and the cytoplasm, possibly leading to accumulation of proline in particular organelles. This possibility was examined.

3.5.2 Methods

Barley (cv. Prior) plants were grown in controlled environmental conditions at 20°C for 10 days. Some seedlings were then transferred to 5°C, some were subjected to a water deficit by flooding polyethylene glycol solution (ψ_π = -20 bars) on the rooting medium and the remainder were retained at 20°C without water stress. 5 days after the initiation of cold stress or 1 day after the initiation of water stress, the first leaf of the plants was sampled to determine the proline content in the different sub-cellular fractions. Leaf tissue (5g) was placed in a pre-chilled mortar (6 cm diameter) with 10 ml of grinding medium (without sand). The tissue was ground by rotating the pestle with a steady motion for 60 seconds with frequent checks to see that all the tissue was being homogenized. The sides of the mortar were then washed with 10 ml of grinding medium and the homogenate filtered through 4 layers of cheesecloth into clean centrifuge tubes to produce the cell-free homogenate. This cell-free homogenate was centrifuged at 1,000 *g* to produce the 1,000 *g* pellet and the 1,000 *g* supernatant fraction. The latter was recentrifuged at 20,000 *g* to produce a 20,000 *g* pellet and a 20,000 *g* supernatant fraction.

This supernatant fraction was again centrifuged at 100,000 *g* producing a 100,000 *g* pellet and a final supernatant. Low speed centrifugation (1,000 *g*, 20,000 *g*) was carried out in an MSE MK1 refrigerated centrifuge using an 8 x 50 ml rotor and plastic centrifuge tubes. High speeds (100,000 *g*) were attained in a Beckman Model L refrigerated ultracentrifuge using a 50 Ti rotor and 13.5 ml polyallomer tubes. All centrifugation was carried out at 0 - 5°C. Centrifugation times were 10 minutes at 1,000 *g*, 20 minutes at 20,000 *g* and 60 minutes at 100,000 *g*. Immediately after centrifugation, the resulting pellets were suspended in 15 ml of distilled water and the free proline contents of the various pellets and the final superanatant were measured as before and expressed on the basis of the fresh weight of the tissue sampled.

The grinding medium used was based on that developed by Breidenbach and Beevers (1967) and consisted of 0.4 M sucrose, 0.05 M Tris-HCl (PH. 7.0), 0.1% bovine serum albumin (BSA), 0.01 M KCl, 1 mM EDTA, 0.1 mM $MgCl_2$ and 0.1% glutaraldehyde.

3.5.3 Results

In the tissue of the plants grown at 20°C without any stress, the majority (80-90%) of the free proline was located in the final supernatant fraction (Table 4), only minor amounts of free proline were detected in other fractions. A large amount of free proline had accumulated in the cold- and the water-stressed plants, there being approximately a 17 fold increase over the non-stressed plants; but again this was located

TABLE 4

Distribution of proline in fractions obtained by differential centrifugation of homogenates of the first leaf of barley plants subjected to 5°C low temperature for 5 days or to a water deficit by flooding the rooting medium with polyethylene glycol solution for 1 day.

Cell fraction	Control		Cold stress		Water stress		L.S.D.(p=0.5)	
	%	µg	%	µg	%	µg	%	µg
1,000 *g*	5.7	(5.5)	8.1	(140.5)	3.8	(64.5)	2.5	18.4
20,000 *g*	4.1	(3.9)	6.2	(107.5)	1.4	(25.0)	1.2	14.7
100,000 *g*	3.7	(3.5)	4.3	(74.0)	4.3	(74.5)	1.1	11.2
Supernatant	86.5	(83.6)	81.4	(1410.0)	90.5	(1582.0)	2.1	275.2
Total	100	(96.5)	100	(1736.0)	100	(1746.0)		

mainly in the supernatant fraction. The results suggest either that water stress or cold stress could induce a great amount of free proline accumulation in the cell but either it did not change the pattern of distribution of proline between the cell organelles or, proline was highly mobile in the separation procedure.

3.6 Proline accumulation in isolated organs.

3.6.1 Methods

The experiments were performed with tissue excised from barley seedlings (cv. Prior) 10 day old and grown as before. The first leaves of the seedlings were excised and cut into 1 cm sections which were pooled on water before distribution to the various treatments. Batches of 15 leaf sections were transferred to 9 cm petri dishes containing 5 ml of distilled water or polyethylene glycol solution of -20 bars osmotic potential. Replication was 3 fold. These petri dishes were then incubated at 5°C or 20°C in the dark. Samples were taken after 0, 4, 24 and 48 hours incubation and the free proline content of each sample was determined and expressed on a tissue dry weight basis.

3.6.2 Results

(1) The effect of low temperature on proline accumulation in isolated organs

Barley leaf sections accumulated a considerable amount of free proline when incubated on a polyethylene glycol solution at 20°C and

free proline also accumulated in leaf lamina sections floated on distilled water alone at that temperature (Figure 25). In contrast, there was little accumulation of proline in sections on water or polyethylene glycol solution incubated at 5°C. For comparison, intact barley plants, subjected to this temperature (5°C) accumulated 2.0 mg per gram dry weight in the same period (Figure 17). Excised leaf tissue incubated at 5°C accumulated very little free proline in response to an osmotic stress whereas leaf tissue incubated at 20°C accumulated a substantial amount of proline. It is evident that proline accumulation in isolated leaf tissue is dependent upon the temperature of incubation. Leaf sections floated on polyethylene glycol accumulated proline at 20°C but not at 5°C, and it was of interest to ascertain whether sections which had initiated accumulation at 20°C would continue to do so if transferred to 5°C.

Leaf sections obtained as described before, were floated on distilled water or -20 bars PEG solution. Half the sections were incubated at 5°C whereas the remainder were incubated at 20°C. After 24 hours, some sections were transferred from 5°C to 20°C or vice versa. Sections were harvested 0, 24 and 48 hours after the experiment commenced. Again the initial incubation temperature had a pronounced effect on proline accumulation (Figure 26). At the 20°C incubation temperature, leaf tissue accumulated proline rapidly, reaching a concentration of 5.1 mg per gram dry weight after 48 hours when floated on PEG solution. In contrast, leaf tissue incubated at 5°C throughout accumulated virtually

FIGURE 25

Effect of incubation temperature on proline accumulation in excised barley leaf laminae sections when floated on water or polyethylene glycol solution (ψ_π = -20 bars). Each value is the mean of three replicates.
[Leaf sections were floated separately on water or polyethylene glycol solution of -20 bars osmotic potential; these were then incubated in the dark at 20°C or 5°C for 48 hours.]

- ● Water 20°C
- ○ Water 5°C
- ■ Polyethylene glycol solution (ψ_π = -20 bars) 20°C
- □ Polyethylene glycol solution (ψ_π = -20 bars) 5°C

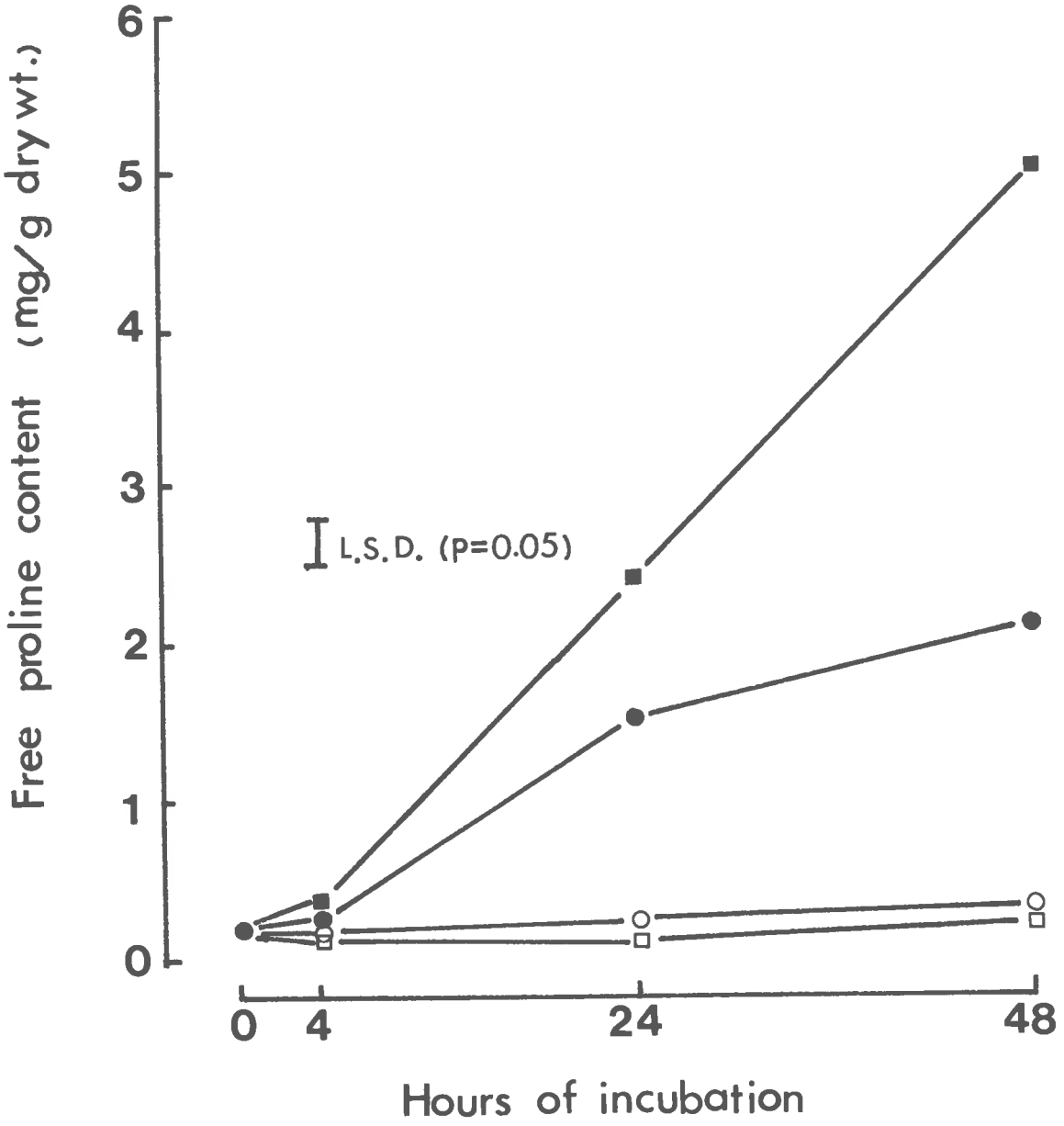
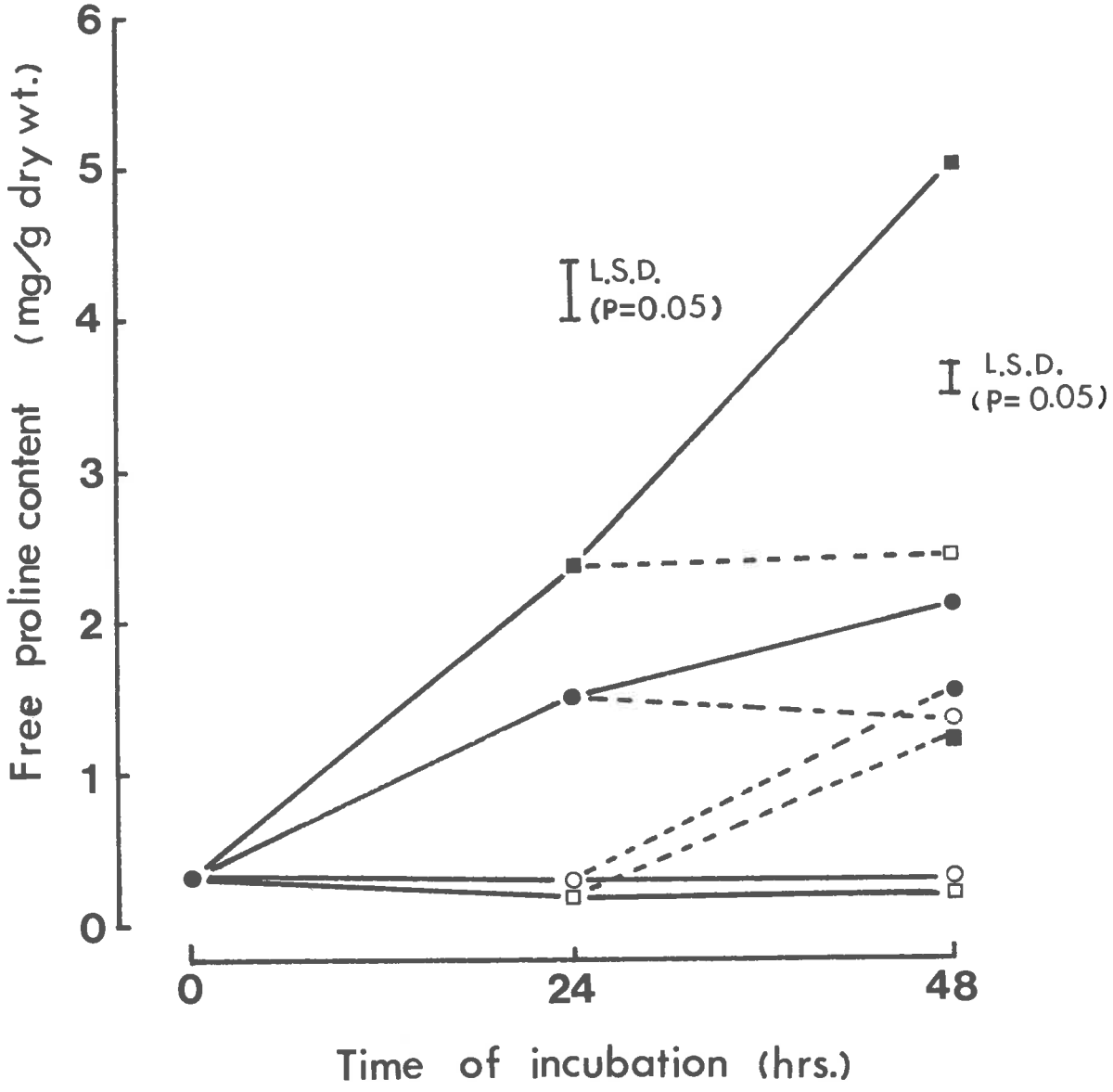

Free proline content (mg/g dry wt.)
6
5
4
3
2
1
0
L.S.D. (P=0.05)
0
4
24
48
Hours of incubation

FIGURE 26

Effect of incubation temperature on the accumulation of proline in excised leaf laminae sections from barley plants when floated on distilled water or polyethylene glycol solution (ψ_π = -20 bars) in the dark. Each value is the mean of three replicates.

[Leaf sections were floated on water or polyethylene glycol solution (ψ_π = -20 bars) 20°C or 5°C initially, half the remaining cultures were transferred from 5°C to 20°C or vice versa after 24 hours.]

- ● H_2O, 20°C
- ○ H_2O, 5°C
- ■ Polyethylene glycol solution (ψ_π = -20 bars), 20°C
- □ Polyethylene glycol solution (ψ_π = -20 bars), 5°C


Free proline content (mg/g dry wt.)
6
5
4
3
2
1
0
L.S.D.
(P=0.05)
L.S.D.
(P= 0.05)
0
24
48
Time of incubation (hrs.)

no proline. Proline accumulation ceased abruptly when leaf tissues were transferred from 20° to 5°C after 24 hours, but the proline previously accumulated remained in the leaf tissue. Conversely, leaf tissue removed from 5°C to 20°C commenced to accumulate proline immediately but not as rapidly in the first 24 hours as the tissue which had been at 20°C throughout. Similar responses were found when the leaf sections were incubated on distilled water (Figure 26), although proline accumulation was less rapid than in the presence of polyethylene glycol.

One possibility to account for this difference is that the supply of proline precursors, such as amino acids and carbohydrate, was limited in excised leaf tissue incubated at 5°C but not in the intact plant at that temperature. This suggestion was tested by supplying sucrose and glutamic acid hydrochloride alone or in combinations to excised leaf lamina sections.

Leaf sections obtained as before were pre-incubated for 2.5 hours in the dark at 20°C on 0.5M sucrose, 0.1M glutamic acid hydrochloride or a combination of the two solutions. They were gently stirred at 30 minute intervals. These leaf sections were then blotted dry and transferred to polyethylene glycol solution (ψ_π = -20 bars) or distilled water and incubated at 5°C in the dark. Although there were minor variations in free proline content, the data (Table 5) strongly suggest that supplying proline precursors does not lead to proline accumulation in excised barley leaf sections at 5°C.

TABLE 5

Effect of sucrose and glutamic acid on the accumulation of proline in excised barley leaf laminae sections floated on water or polyethylene glycol solution.

(The leaf sections were incubated in the dark at 20°C for 2½ hours in beakers containing solutions of either sucrose or glutamic acid or a combination thereof before they were transferred to water or a polyethylene glycol solution of -20 bars. These were then incubated in the dark at 5°C for up to 48 hours.)

Pretreatment	Incubation medium	0 hr proline (mg/g dry wt)	4 hrs proline (mg/g dry wt)	24 hrs proline (mg/g dry wt)	48 hrs proline (mg/g dry wt)
Sucrose(0.5M)	H_2O	0.21	0.26	0.23	0.31
	PEG(-20 bars)	0.21	0.16	0.11	0.07
glutamic acid (0.1M)	H_2O	0.21	0.15	0.18	0.14
	PEG(-20 bars)	0.21	0.12	0.08	0.06
Sucrose(0.5M) + glutamic acid (0.1M)	H_2O	0.21	0.21	0.21	0.19
	PEG(-20 bars)	0.21	0.16	0.15	0.08
L.S.D. (p=0.05)			0.11	0.12	0.20

(2) Light and proline accumulation in isolated organs.

Although intact plants accumulate proline in response to low temperature, excised leaf sections do not do so. Indeed the accumulation induced by water stress in such sections at 20°C is inhibited at 5°C. This inhibition was not removed by supplying the plants with precursors of proline biosynthesis. One further difference between experimental conditions in the two types of experiment remains, however. In all previous experiments, isolated organs were incubated in complete darkness but proline accumulation was detected in intact plants when grown under continuous illumination of 4.1 mW cm^{-2} radiant flux intensity at 5°C. It is possible that light might play some important role in proline accumulation in leaf tissue. In the next experiment intact barley plants and isolated leaf sections were incubated at 5°C in light or dark. Sand-grown barley seedlings, after 10 days growth at 20°C in the light were transferred to 5°C. Some of these plants were grown thereafter under continuous illumination (4.1 mW cm^{-2} [redacted]) and some were grown in completely dark conditions. Similarly, 1 cm leaf sections excised from the first leaves were floated in 9 cm petri dishes on 5 ml distilled water, and were incubated at 5°C in the dark or under top illumination of 4.1 mW cm^{-2} [redacted] at the level of the dishes.

Intact plants grown at 5°C under continuous illumination again accumulated a considerable amount of proline in the leaf tissue. In contrast there was no significant increase in free proline in plants

grown at 5°C in the dark (Figure 27). Moreover, whereas there was again no proline accumulation in leaf sections floated on distilled water at 5°C in the dark there was a considerable accumulation in leaf sections incubated at 5°C under continuous illumination (Figure 27). After 72 hours, proline had accumulated to a concentration of 2.2 mg per gram dry weight in these isolated leaf tissues. It is apparent that light is required to enable proline accumulation to proceed at 5°C in both leaf sections and intact plants.

In view of this response, it was of interest to examine whether light is required to enable proline accumulation to proceed in response to water stress. Leaf sections floating on polyethylene glycol solution in the dark at 20°C accumulate proline, but there is no information for intact plants (Singh *et al.*, 1973b). This point was briefly explored.

Barley plants grown for 10 days at 20°C in sand in a constant environment were subjected to water stress by flooding the rooting medium with polyethylene glycol solution of -20 bars osmotic potential. Some of these plants were grown under illumination consisting of a 16 hour photoperiod with 4.1 mW cm^{-2} radiant flux intensity, and others were transferred to complete darkness at the same temperature. Under illumination, water stress produced a rapid accumulation of free proline (Figure 28). In this case, proline accumulation was most rapid between 24 and 72 hours after initiation of the stress. Proline accumulated at a considerably reduced rate following exposure to -20 bars PEG treatment in darkness

FIGURE 27

Effect of illumination (4.1mW cm^{-2} [illegible]) on proline accumulation in the first leaf of intact barley plants subjected to 5°C and in excised barley leaf laminae sections floated on distilled water at 5°C. Each value is the mean of three replicates.

Leaf sections: ○ Illumination

● Without illumination

Intact plants: □ Illumination

■ Without illumination

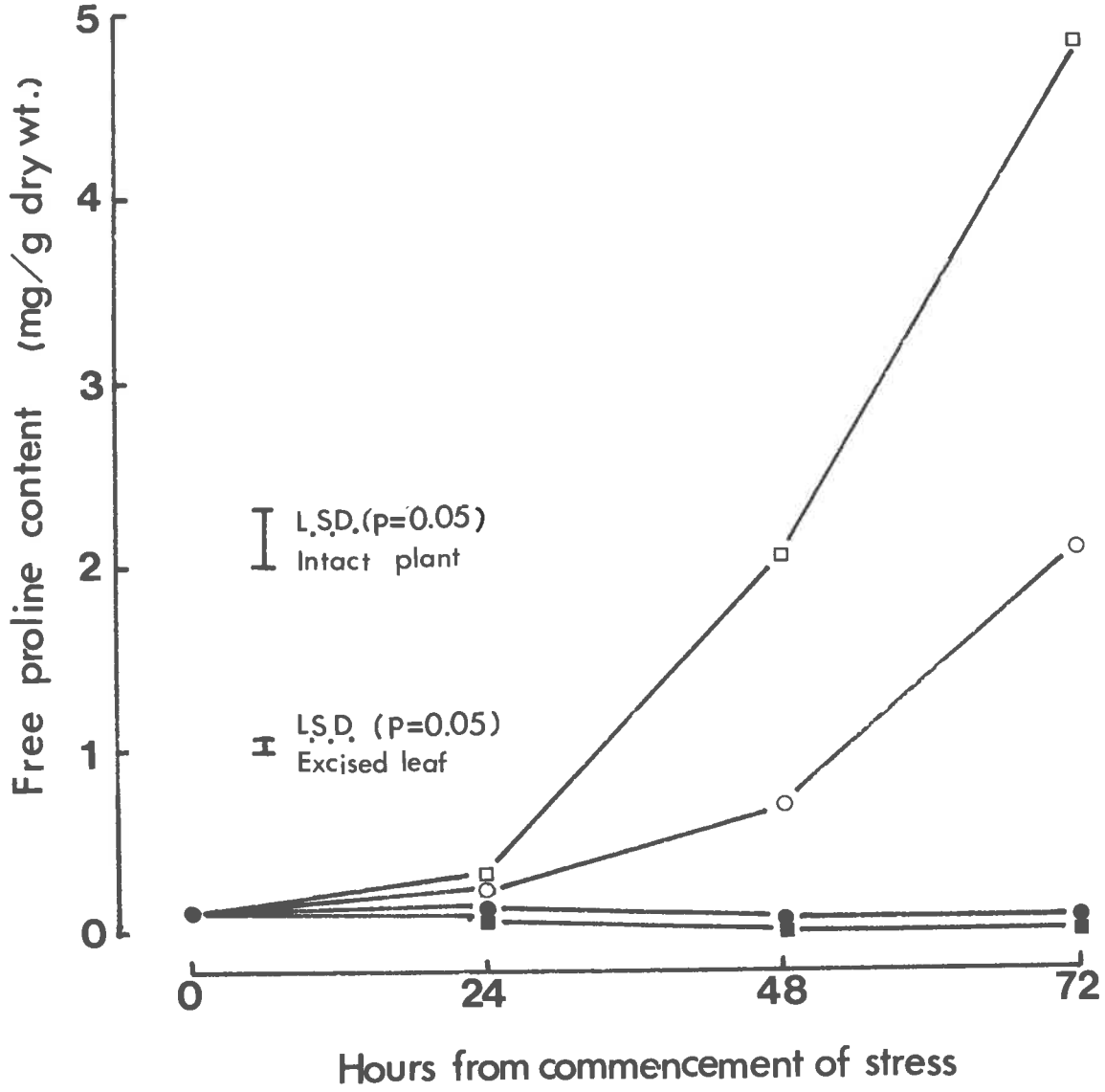
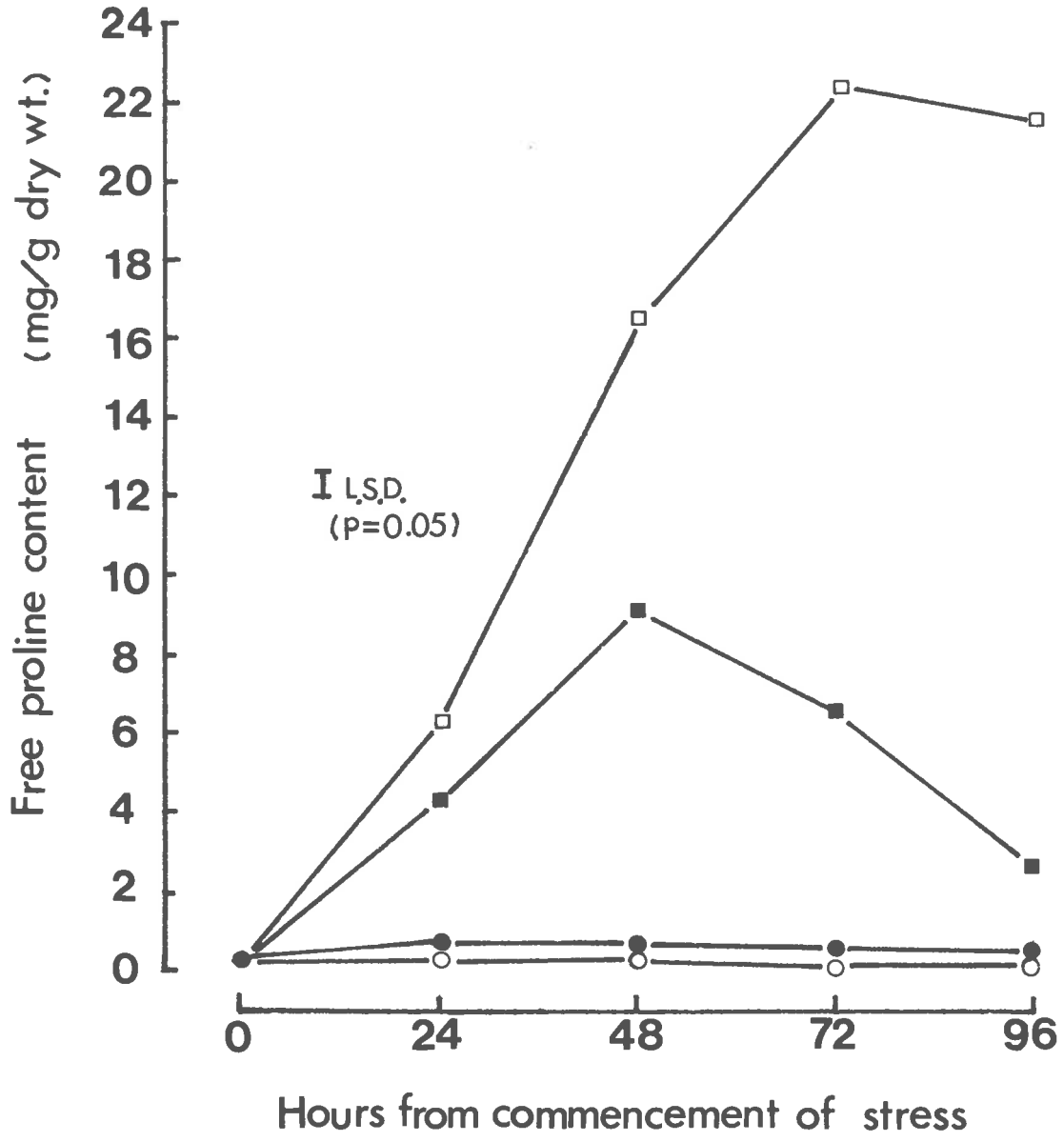

Free proline content (mg/g dry wt.)
5
4
3
2
1
0
L.S.D.(p=0.05)
Intact plant
L.S.D. (P=0.05)
Excised leaf
0
24
48
72
Hours from commencement of stress

FIGURE 28

Effect of illumination on proline accumulation in the first leaf of barley plant subjected to water stress by flooding the rooting medium with polyethylene glycol solution of -20 bars osmotic potential. Each value is the mean of three replicates.

Control:	○	Illumination
	●	Without illumination
Water stress:	□	Illumination
	■	Without illumination


Free proline content (mg/g dry wt.)
24
22
20
18
16
14
12
10
8
6
4
2
0
I L.S.D. (P=0.05)
0
24
48
72
96
Hours from commencement of stress

but, nevertheless, accumulated to a considerable extent in the first 48 hours. The free proline content increased to a maximum of 9.1 mg per gram dry weight at 48 hours, but subsequently fell. The results apparently indicate that leaves wilting in the dark synthesize proline rapidly, with the subsequent fall in proline content possibly being due to exhaustion of the carbohydrate reserves within the plant.

3.7 Discussion

It has been suggested that low temperature, and, in particular, low root temperature, can result in a water deficit and a reduction in leaf water potential (Kleinendorst and Brouwer, 1972). If this were so, then at least some of the metabolic and growth effects of the low temperature could be ascribed to the reduction in leaf water potential rather than to the low temperature *per se*. This view has been disputed, however, and work with barley (Power *et al.*, 1970) and corn (Watts, 1970; 1972) suggests that water deficit associated with low root temperature is not the major factor restricting plant growth. Watts (1970) produced evidence that cooling of the meristematic regions of the shoot by water in the transpiration stream may be responsible for the inhibitory effect of low root temperature on corn growth, and Kramer (1956) also suggested that the effects of low temperature on growth were direct. In the present investigation, although the growth of barley decreased with a decrease in temperature below 20°C and was completely suppressed at 6°C (Figure 22), leaf water potential did not change significantly within

this range (Table 3). This also suggests that the effects of low temperature on growth and metabolism which were recorded were not mediated indirectly through changes in leaf water status. The marginal effect of the low temperature on leaf water status which occurred in the present experiments was due almost entirely to a reduction in the osmotic potential (Figure 20,21). This reduction was unlikely to have been the cause of the effects on metabolism, particularly proline accumulation, as proline accumulation appears to be insensitive to a mild water deficit although the increase during severe stress is dramatic (Stewart *et al.*, 1966). It is difficult to establish the water potential at which proline accumulation commences (Singh *et al.*, 1973c), but rates of accumulation comparable to those recorded here at low temperature are associated with considerably lower water potential (-20 to -30 bars) when induced by water deficit (Singh *et al.*, 1973c).

The reduction in leaf osmotic potential upon exposure to low temperature was presumably due to a release of osmotically active compounds. It is unlikely that the decrease was caused by a loss of water from the leaf as turgor potential, as measured by difference between water and osmotic potential, was unaltered. Relative turgidity, measured independently by the method of Barrs and Weatherley (1962) fell, however, the discrepancy between those two estimates of very similar parameters is probably due to the method of estimation of relative

turgidity. Thus, tissue obtained from plants growing at 5°C was floated on water at 20°C and the water uptake measured. Under these conditions, water uptake may be due to both satisfaction of an internal deficit and expansion of the cells by active growth following the change in temperature. If the latter occurred, the relative turgidity would be underestimated and the apparent response of relative turgidity to temperature would be an artefact. If it is accepted that leaf osmotic potential fell but turgor potential was unaltered when plants were placed at a low temperature, then Kleinendorst and Brouwer's (1972) conclusion that low root temperature increased resistance to water uptake could not be operating in this system. Such a response should result, at least initially, in a decline in turgor potential rather than osmotic potential.

If one accepts that low ambient temperature causes proline accumulation, independent of any effect on water potential, then one is drawn to conclude that two independent and dissimilar environmental factors can both cause the same metabolic response. This could conceivably occur through quite distinct effects on metabolism with the same ultimate effect, or because both environmental stress conditions affect the same basic metabolic process. A distinction between these alternatives cannot be drawn on the present evidence, but it is pertinent to compare the induction of proline accumulation by water stress and by low temperature. The pattern of proline accumulation in the first leaf of the

barley plant following exposure to low temperature (Figures 17,18) was very similar to that following exposure to water stress. Accumulation commenced more rapidly with induction of a water deficit (24 hours) than with exposure to low temperature (48 hours) but the rate of accumulation thereafter was comparable in the two stress environments. With water stress, relief of the stress led to an immediate and rapid loss of proline from the leaves. When plants were moved from a low temperature to a higher, however, proline continued to accumulate for 24 hours at the same rate as before. Subsequently, the concentration of proline in the tissue also fell. This suggests that the metabolic event influenced by low temperature is not as rapidly reversed as that influenced by water stress.

Further evidence that the metabolic controls leading to proline accumulation, which are affected by the two stress situations, may differ, is provided by the response to illumination. Light was a prerequisite for proline accumulation in response to low temperature but not for water stress-induced accumulation. Although the rate at which proline accumulated in the first leaf of intact barley plants following exposure to -20 bars polyethylene glycol solution was reduced in the dark (Figure 28), proline still accumulated in response to the water deficit. After 48 hours, stressed plants had accumulated 9.1 mg per gram dry weight, about 40 times higher than plants maintained on water. In contrast, there was no increase in proline concentration in the leaves of intact plants exposed to a low temperature in the dark (Figure 27). Similar phenomena were observed with isolated leaf sections. When leaf sections

were floated on a -20 bars polyethylene glycol solution at 20°C in the dark there was a rapid accumulation of proline in 48 hours. In contrast, there was no proline accumulated in leaf sections floated on water or even on polyethylene glycol (-20 bars) solution incubated at 5°C in the dark. Increasing the incubation temperature from 5°C to 20°C with sections floating on polyethylene glycol, or moving sections into light at 5°C resulted in proline accumulation. These data indicate that illumination is a prerequisite for proline accumulation in response to low temperature, but not in response to water stress.

The stimulation of accumulation during water stress, produced by illumination, might be caused by either increased wilting or an increase in carbohydrate supply. When barley plants were subjected to a -20 bars polyethylene glycol solution without illumination, free proline accumulated up to a maximum concentration of 9.1 mg per gram dry weight in 48 hours, but the concentration subsequently fell almost as rapidly. This suggests that leaves wilting in the dark, synthesize proline only until their carbohydrate reserves are exhausted. After the sugar has been exhausted, the large concentration of proline produced in the first 48 hours also undergoes decomposition. If this is so, the effect of illumination may be to maintain the carbohydrate of the leaves at a higher level (Yemm, 1949). The availability of carbohydrate as a substrate for proline biosynthesis is clearly important (Singh *et al.*, 1973c), although Stewart (1972b) has recently pointed out that the effect of wilting on

proline accumulation is not necessarily dependent on a large supply of carbohydrate in the leaf, the role of carbohydrate being to prevent the loss of accumulating proline by oxidation. The stimulation to proline accumulation due to illumination may be due to the maintenance of a high level of carbohydrate in leaves, which may serve either as a substrate or an inhibitor of proline oxidation. Alternatively, Noguchi *et al.*(1968) suggested that light itself may regulate the biosynthesis of proline from glutamate, possibly through photoreduction and photophosphorylation. In contrast, plants fail to accumulate proline in dark at 5°C and this is not due to a lack of substrate.

During water stress, considerable amounts of proline have been detected in the root tissue of barley (Singh *et al.*,1973c) and radish (Figure 6). As excised roots are not capable of proline accumulation, it was postulated that the accumulated proline originated in the leaves whence it was translocated to the root (Singh *et al.*, 1973b). At a low temperature, however, although proline accumulated in the leaves to a high concentration, there was little proline accumulated in barley roots and none in radish roots (Figures 17, 18). This suggests first that, as with water deficit, roots are unable to accumulate proline, independently, at low temperature and secondly, there was little translocation of the accumulated proline in the leaves to the roots at low temperature. Although the translocation of proline in higher plants has not been examined, it has been demonstrated that the translocation

of some amino acids and amides is mainly downwards toward the root, and that chilling the roots decreases the velocity of downwards translocation (Nelson and Gorham, 1959).

Proline accumulation in response to a low temperature is probably as common a phenomenon in higher plants as is proline accumulation in response to water deficit. Apart from barley and radish, proline accumulation in response to low temperature has been found in wheat (Teltscherova, 1957; Scheffer and Lorenz, 1968), sunflower, bean, paprika and pea (Palfi and Juhasz, 1970) and Townsville stylo (Gates *et al.*, 1971). Moreover, proline normally tends to increase with increasing cold-hardiness (Wilding *et al.*, 1960). The significance of this accumulation of proline in low temperature is unknown. Three possibilities can be considered; firstly, net proline synthesis is promoted, secondly the synthesis of proline-rich proteins is suppressed, or thirdly protein hydrolysis is promoted. When ^{14}C-proline was fed to cold-stressed plants, both the turnover of free proline and ^{14}C incorporation into protein was lower than in control plants (Shiomi and Hori, 1973b). So far, however, there is no direct evidence to show that the increase in proline during low-temperature stress, exceeds the proline simply released from protein due to accelerated proteolysis, as has been demonstrated for water stress (Thompson *et al.*,1966). Moreover, several authors have suggested that protein breakdown is promoted by low temperature (Wilding *et al.*,1960).

Section III. Salinity Stress

1. Introduction

Dissolving a salt in the water surrounding the roots of a plant has the effect of lowering the Gibbs free energy of that water, and hence decreasing the potential gradient along which water flows into the plant. This is equivalent to what occurs to the free energy of water surrounding the roots of a plant in a particulate medium as the water is depleted and many of the responses of the plant to the two phenomena are identical. Thus the plants suffer a loss of turgor, stomata close and, more slowly, the osmotic potential of cell solutions decreases restoring the potential gradient. Eventually, in the ideal state, turgor is restored, stomata re-open and the plant resumes transpiration albeit at a lower internal osmotic potential (Slatyer, 1961; Bernstein, 1963). It is therefore not unexpected that many of the growth responses to the two situations are also identical (Bernstein and Hayward, 1958).

The major difference between the two situations stems from the uptake of solute by the roots of the plant. In a simple salt solution, this absorption may be rapid and growth effects due to toxicity of the absorbed ions may confound the responses to the concomitant change in plant water status (Slatyer, 1961; Bernstein, 1961).

It has been established that in many plants a striking accumulation of the imino-acid proline occurs when plant water potential is lowered,

either by depleting the water in a particulate rooting medium or by bathing the roots in a solution of an osmoticum which does not enter the plant (Singh *et al.*, 1973c). A similar accumulation of proline has also been reported to occur in plants subjected to salinity stress (Palfi and Juhasz, 1970). Although it has been established that the rate of proline accumulation varies with plant water potential (Singh *et al.*, 1973c), it is not known whether this accumulation is responsive to cell osmotic potential (ψ_π) or to turgor potential (ψ_p) or both. In order to understand the role of water in proline accumulation, it is necessary to examine not only the relationship between accumulation and leaf water potential, but also between accumulation and osmotic potential. Manipulation of the salinity of the rooting medium allows these factors to be examined, and in addition the effects of individual absorbed ions can be assessed.

2. Abrupt salinization

2.1 Methods

Barley (cv. Prior) seeds were germinated at 20°C for 24 hours and were then grown in darkness for a further 4 days at 25°C before they were transferred to nutrient solution. The plants were grown in full-strength modified Hoagland's solution (III Materials and Methods, page 64), 25 uniform barley seedlings being grown in each 2.5 litre polyethylene container. The plants were supported in holes in a P.V.C. lid with strips of polyurethane foam wrapped around the stems. The solution was aerated continuously and completely replaced weekly. The growth

cabinet was maintained at 20 ± 1°C with a 16 hour photoperiod and light intensity of 4.1 mW cm^{-2} h^{-1}.

When these barley seedlings were 12 days old they were subjected to an abrupt salinity stress by replacing the nutrient solution with one containing NaCl at an osmotic potential of -5 or -10 bars (also containing Hoagland's solution -0.7 bars). The plants were grown for a further 72 hours, sample plants being removed every 12 hours. After 48 hours, the stress was removed from some plants of both treatments by washing the salt solution from the rooting medium with 2,000 ml distilled water; these plants were thereafter grown on Hoagland's solution alone. All plant samples were plunged into liquid nitrogen immediately on excision and stored at -20°C before assay. The free proline content of the first leaf was estimated. The water status of the plant was determined by measuring the water and osmotic potential of first leaf tissue with a Spanner psychrometer at 25°C. The turgor potential was assumed to be the difference between leaf water potential and osmotic potential.

2.2 Results

(1) Water status

Following imposition of the osmotic stress with -10 bars NaCl, the water potential of the first leaf declined rapidly for 24 hours and fell to a low level (-17 bars) within 36 hours. Thereafter, leaf water potential did not change significantly (Figure 29). When the osmotic

FIGURE 29

Effect of abrupt salinization on water potential and osmotic potential in the first leaf of barley. Each value is the mean of four replicates.
[12 day old barley plants growing in water culture were subjected abruptly to salt stress with NaCl of -5 or -10 bars osmotic potential.]

Water potential

● Nutrient solution

▲ -5 bars NaCl solution

■ -10 bars NaCl solution

--- Stress relieved

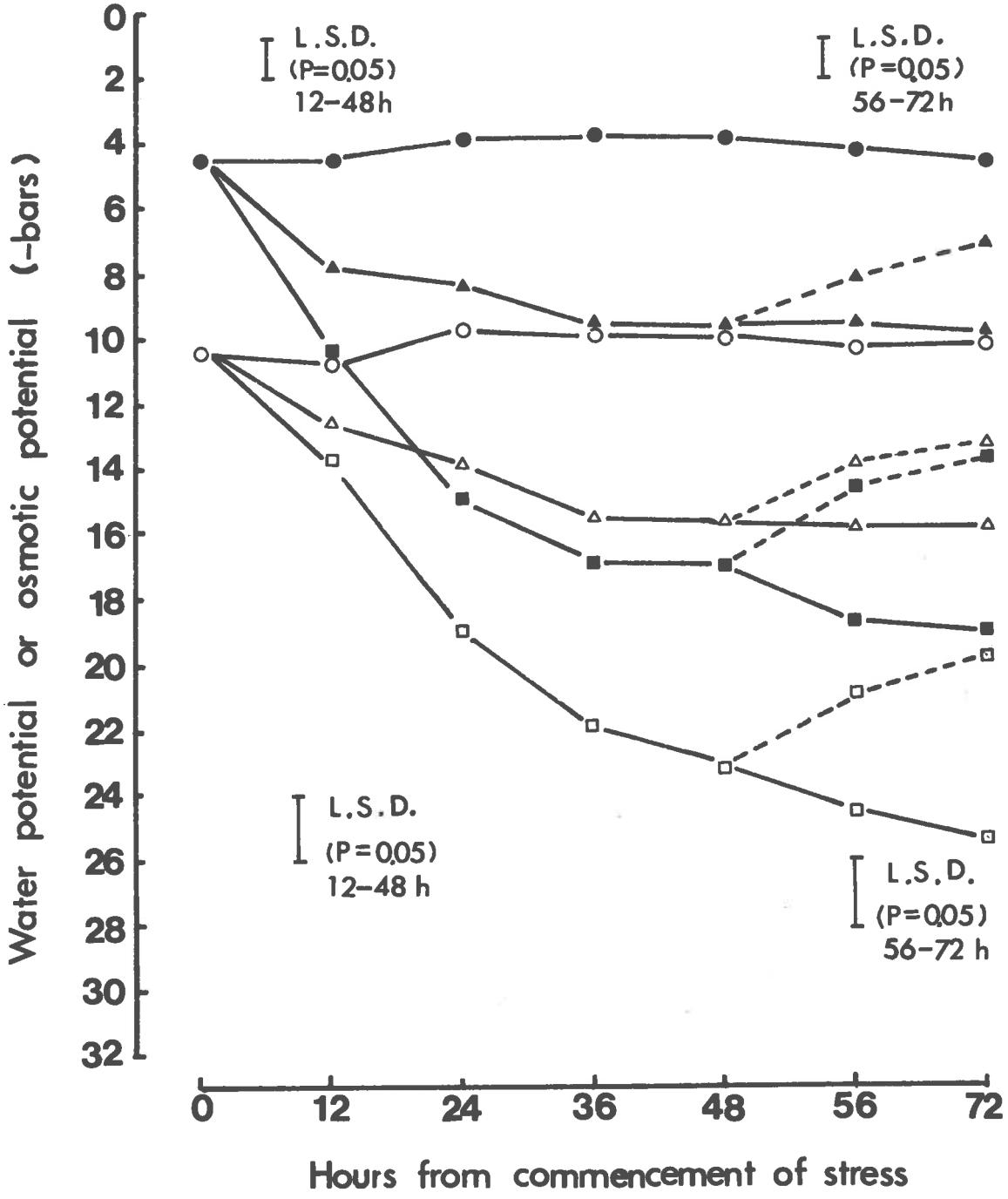
Osmotic potential

○ Nutrient solution

△ -5 bars NaCl solution

□ -10 bars NaCl solution

--- Stress relieved


L.S.D.
(P=0.05)
12–48h
L.S.D.
(P=0.05)
56–72h
L.S.D.
(P=0.05)
12–48 h
L.S.D.
(P=0.05)
56–72 h
Water potential or osmotic potential (-bars)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
0
12
24
36
48
56
72
Hours from commencement of stress

stress was relieved 48 hours after imposition, the water potential recovered slowly, rising 3 bars in 24 hours. Plants exposed to -5 bars NaCl solution showed a similar, although less extreme, pattern of fall in leaf water potential. The osmotic potential of the first leaf also declined rapidly in the first 24 hours after exposure to -10 bars salinity stress, and then more slowly for the remainder of the experiment (Figure 29). In the first 12 hours, however, this reduction in osmotic potential was less than that in water potential and there was a consequent reduction in turgor potential (Figure 30) which was evident as severe wilting in the plants. The same response was found with -5 bars NaCl but the changes in water, osmotic and turgor potentials were smaller than with -10 bars NaCl, and only slight wilting occurred. Following this initial phase of wilting, osmotic potential fell more rapidly than water potential with the consequence that turgor recovered (Figure 30). Within 36 hours in the -5 bars treatments and 48 hours in the -10 bars treatments, turgor potential had returned to the value for plants grown without salinity stress. These changes in turgor potential were parallelled by visible changes in the plants, the initial severe wilting disappearing as turgor was recovered.

Where the salinity stress was relieved after 48 hours, both osmotic and water potential rose in parallel (Figure 29), and there was no significant effect on turgor, which had returned to the original value before the stress was relieved. This recovery in turgor in the continued presence of salt could be attributed to the fall in osmotic potential.

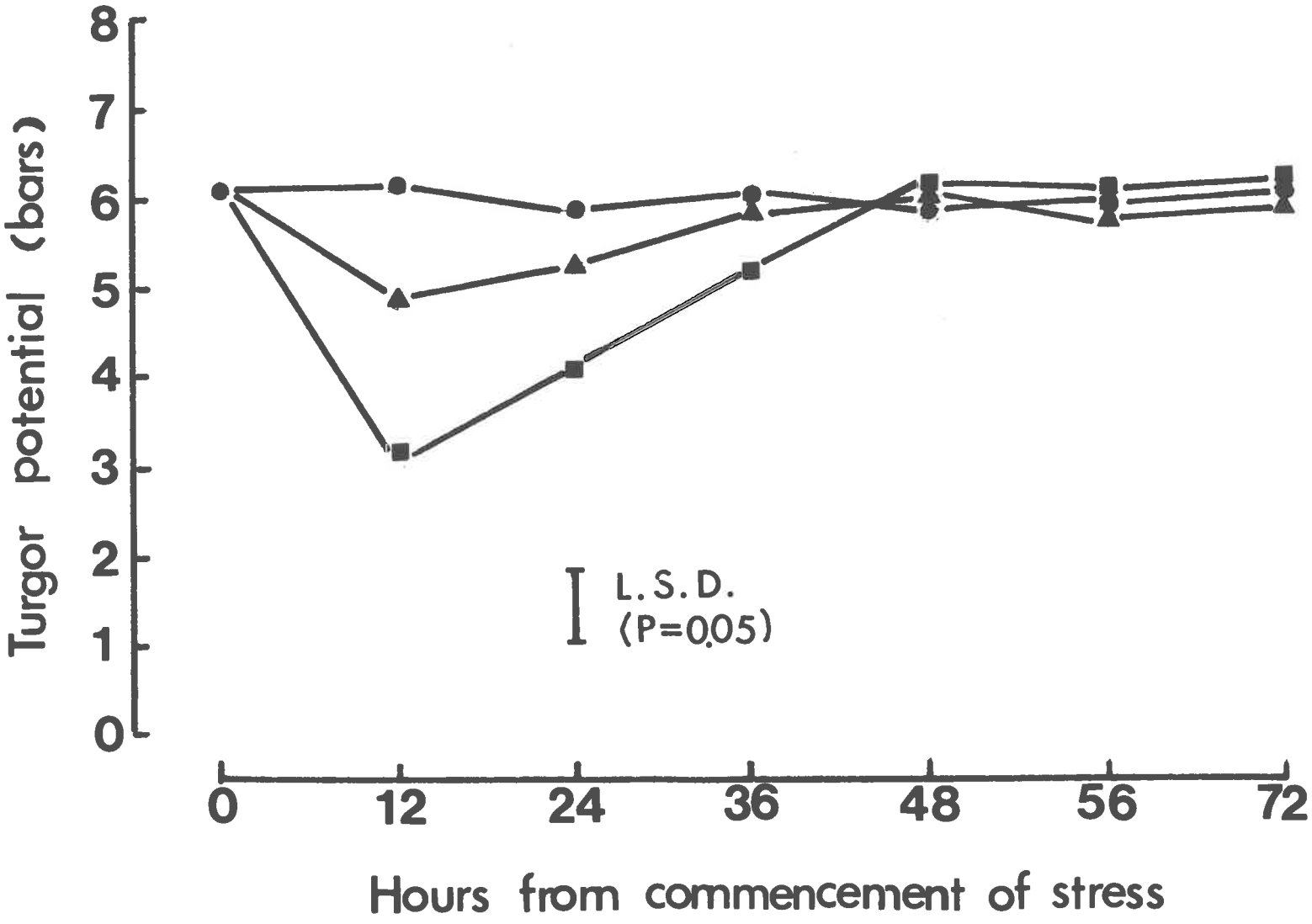
FIGURE 30

The change in turgor potential of the first leaf of 12 day old barley plants subjected abruptly to -5 or -10 bars NaCl salt stress.
[Turgor potential of the first leaf of barley plant calculated from Figure 29 as the difference between water potential and osmotic potential.]

● Nutrient solution

▲ -5 bars NaCl solution

■ -10 bars NaCl solution


Turgor potential (bars)
8
7
6
5
4
3
2
1
0
L.S.D.
(P=0.05)
0
12
24
36
48
56
72
Hours from commencement of stress

This has been suggested to be due to solute uptake (Slatyer, 1961; Meiri and Poljakoff-Mayber, 1969) from the root medium.

It seems from these results that although plants grown under saline conditions are capable of osmotic adjustment they do suffer from, at least a transitional, drop in turgor. Their water balance may, therefore, be disturbed. The ability and rate of adjustment depend, to a great extent, on the rate of the concentration change in the external medium.

(2) Proline accumulation

In the mild salt stress (-5 bars), there was no proline accumulation in the plant tissue throughout the whole experimental period (Figure 31). However, in the moderate (-10 bars) salt stress, proline accumulation commenced after 12 hours exposure to salinity and continued throughout the experimental period, even after 48 hours when leaf turgor potential recovered completely from the initial fall. Plants removed from salt stress at 48 hours and returned to standard Hoagland's solution, continued for 12 hours to accumulate proline at the same rate as plants remaining in the -10 bars salt stress solution. Following this 12 hours of continued accumulation, however, the free proline concentration declined rapidly.

3. Progressive decrease in osmotic potential

3.1 Methods

Barley plants (cv. Prior) were grown in the standard water culture

FIGURE 31

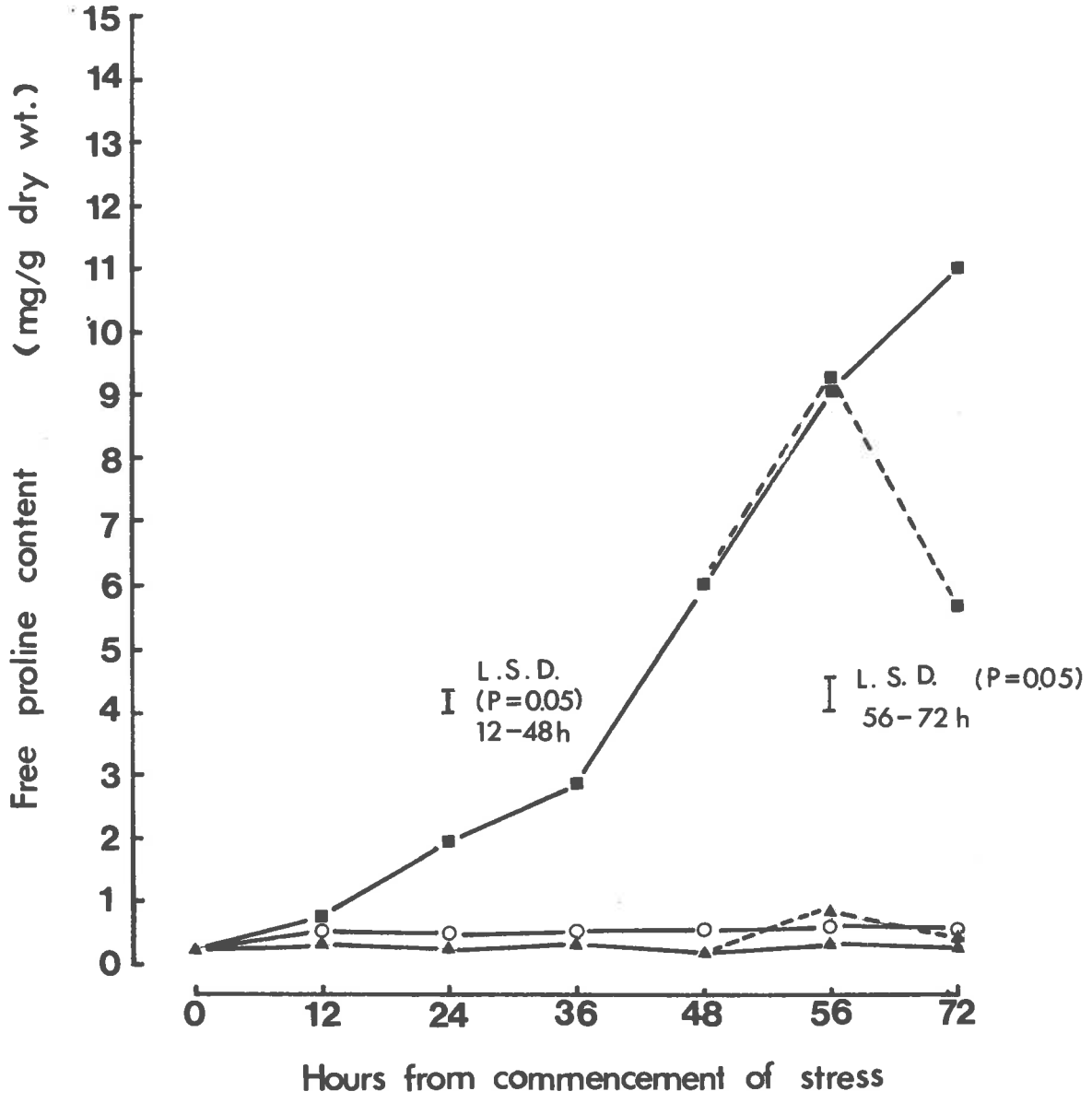
Proline accumulation in the first leaf of barley plants subjected abruptly to -5 or -10 bars NaCl salt stress. Each value is the mean of four replicates.
[12 day old barley seedlings growing in water culture were subjected abruptly to NaCl solution of -5 or -10 bars osmotic potential.]

○ Nutrient solution

▲ -5 bars NaCl solution

■ -10 bars NaCl solution

--- Release from stress


Free proline content (mg/g dry wt.)
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
L.S.D. (P=0.05) 12-48h
L. S. D. (P=0.05) 56-72 h
0
12
24
36
48
56
72
Hours from commencement of stress

system as described before (III Materials and Methods, page 64). NaCl was first added to the nutrient solution when these barley seedlings were 10 days old, the osmotic potential of the nutrient solution was progressively reduced by the addition of NaCl so as to reduce the osmotic potential by one bar every 24 hours until it had reached -10 bars. Throughout the experiment the nutrient solution also contained Hoagland's solution with an osmotic potential of -0.7 bars. The total time from the first addition of saline solution to the end of the experiment was 10 days. Samples of the first and second leaves were taken every 24 hours and leaf water and osmotic potentials were measured with a Spanner psychrometer on samples taken immediately before adjusting the solution daily. Turgor potential was again estimated from the difference between these two measurements.

3.2 Results

(1) Water status

In plants grown in nutrient solution alone, the water potential of the first leaf fluctuated between extremes of -3.7 and -5.2 bars while the osmotic potential varied between -9 and -10.5 bars (Figure 32). As a result, turgor potential was virtually constant at +5.5 bars throughout the whole experimental period. A similar situation was found with the second leaf, although both leaf and osmotic potential were approximately 1 bar more positive (Figure 33).

As the osmotic potential of the root medium was decreased from 0 to -10 bars, leaf water potential decreased proportionally from -4.5 to -14.2

FIGURE 32

The effect of gradually decreasing the osmotic potential of the root medium on the water and osmotic potentials of the first leaf of barley. Each value is the mean of four replicates.

[10 day old barley plants growing in water culture were subjected to osmotic stress by adding NaCl to lower the osmotic potential of the nutrient solutions one bar every 24 hours. Water and osmotic potentials were measured daily immediately before changing the solutions.]

Water potential	▲	Nutrient solution
	■	NaCl solution
Osmotic potential	△	Nutrient solution
	□	NaCl solution

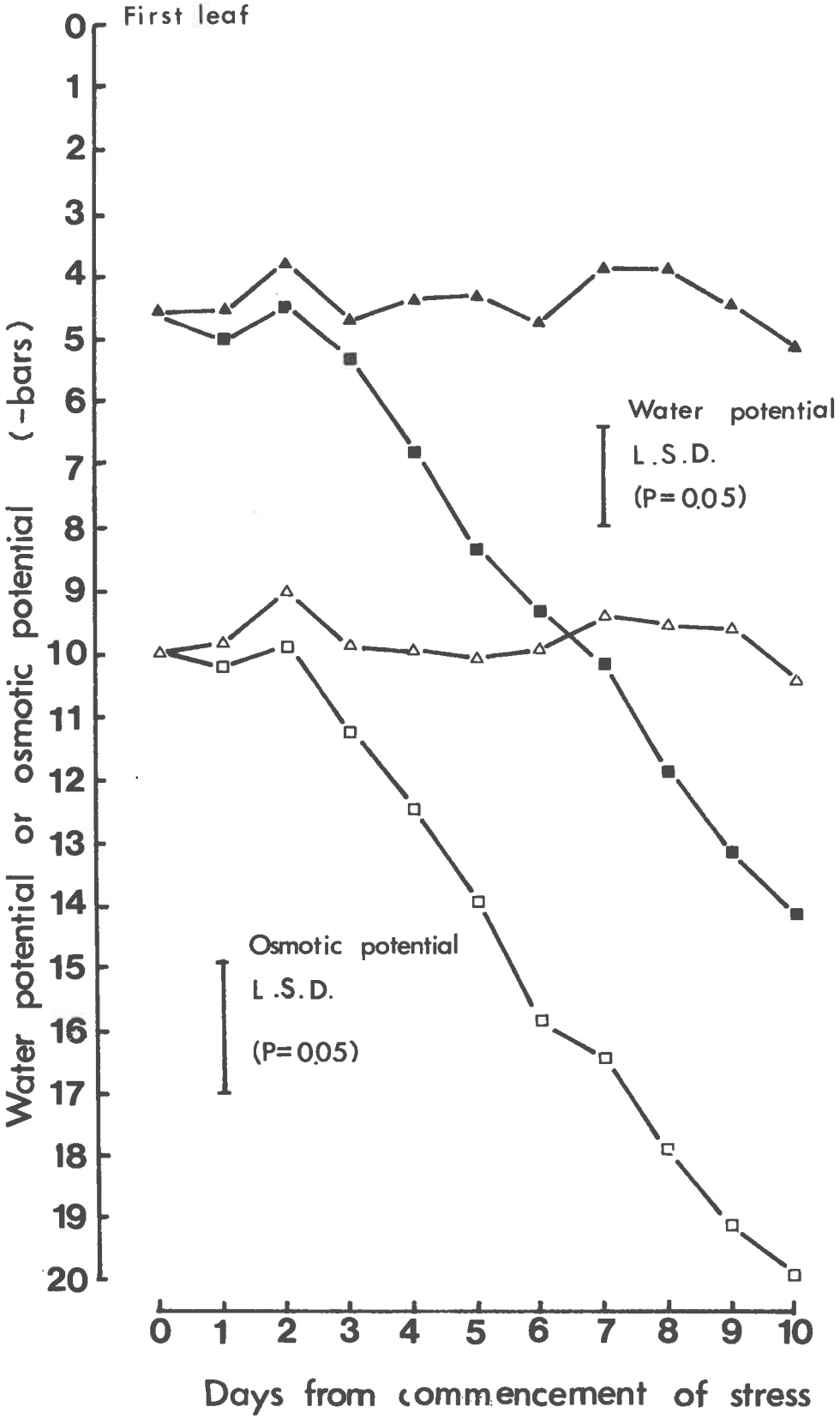
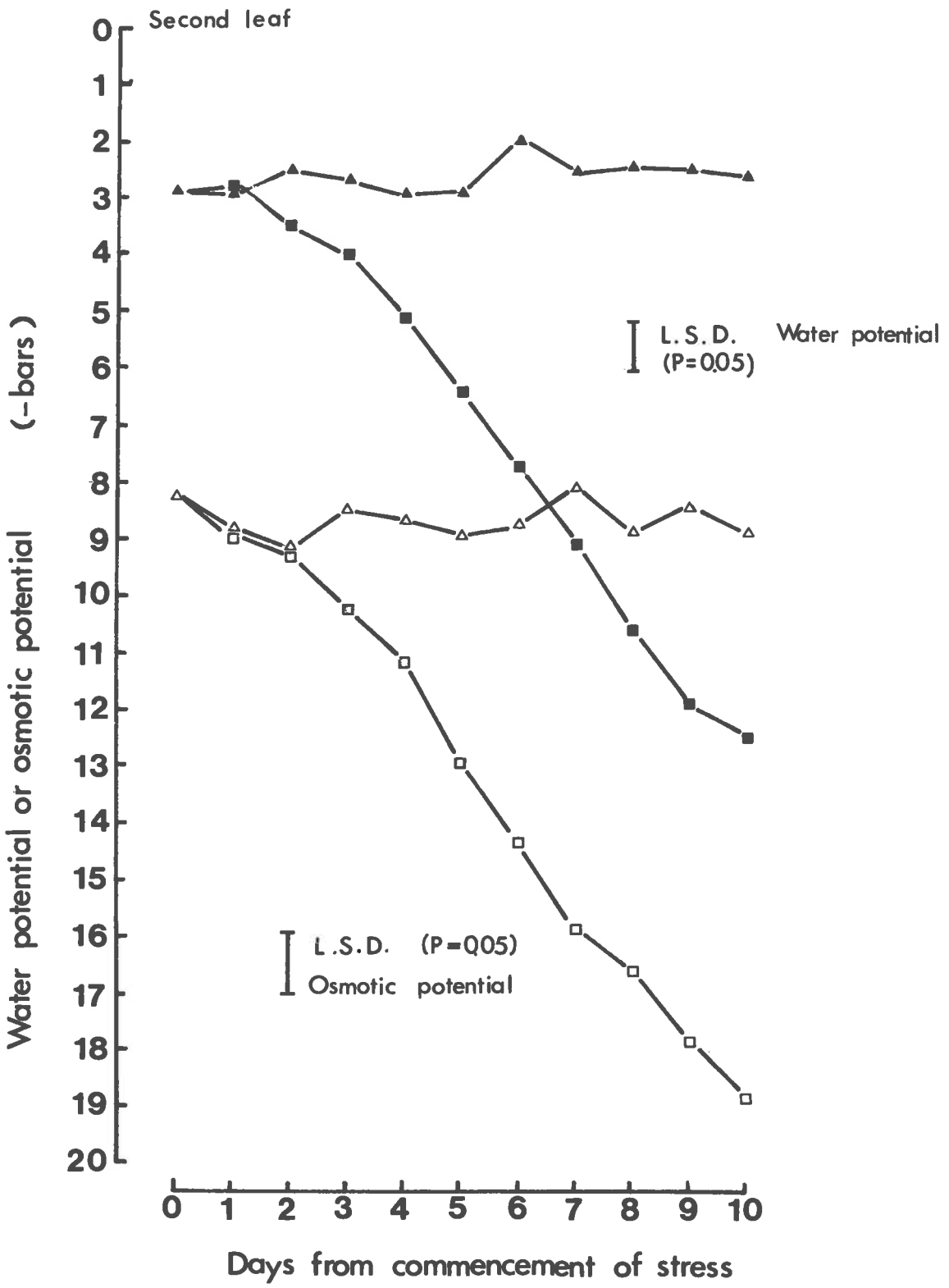

First leaf
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Water potential or osmotic potential (-bars)
Water potential
L.S.D.
(P=0.05)
Osmotic potential
L.S.D.
(P=0.05)
0 1 2 3 4 5 6 7 8 9 10
Days from commencement of stress

FIGURE 33

The effect of gradually decreasing the osmotic potential of the root medium on the water and osmotic potentials of the second leaf of barley. Each value is the mean of four replicates.

[10 day old barley plants growing in water culture were subjected to osmotic stress by adding NaCl to lower the osmotic potential of the nutrient solution one bar every 24 hours. Water and osmotic potentials were measured daily immediately before changing the solutions.]

Water potential	▲	Nutrient solution
	■	NaCl solution
Osmotic potential	△	Nutrient solution
	□	NaCl solution


Second leaf
Water potential or osmotic potential (-bars)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
L.S.D. (P=0.05)
Water potential
L.S.D. (P=0.05)
Osmotic potential
0 1 2 3 4 5 6 7 8 9 10
Days from commencement of stress

bars (Figure 32). Over the whole range of decline, the differential in potential between the rooting medium and the first leaf remained constant at -5 to -6 bars. This was due entirely to a parallel decline in leaf osmotic potential during this period. As a result, leaf turgor potential was almost constant and the same as in plants not exposed to salinity (+5.5 bars), throughout the decline in osmotic potential in the nutrient solution. There was no apparent wilting, although the growth of the salt-stressed plants was retarded. The water status of the second leaves responded identically (Figure 33).

(2) Proline concentration

There was no proline accumulation in either the first or second leaves during the time in which the osmotic potential of the root medium was lowered to -5 bars (Figure 34). When the osmotic potential of the root medium decreased to -6 bars however, the tissue commenced to accumulate proline and the proline concentration in the leaves continued to increase proportionally as the osmotic potential of the root medium was decreased to -10 bars, in the following 5 days. At the end of the experiment, the proline concentration had reached 4.4 mg per gram dry weight in the first leaf and 5.6 mg per gram dry weight in the second leaf. As in all previous experiments, plants grown in nutrient solution alone did not accumulate proline.

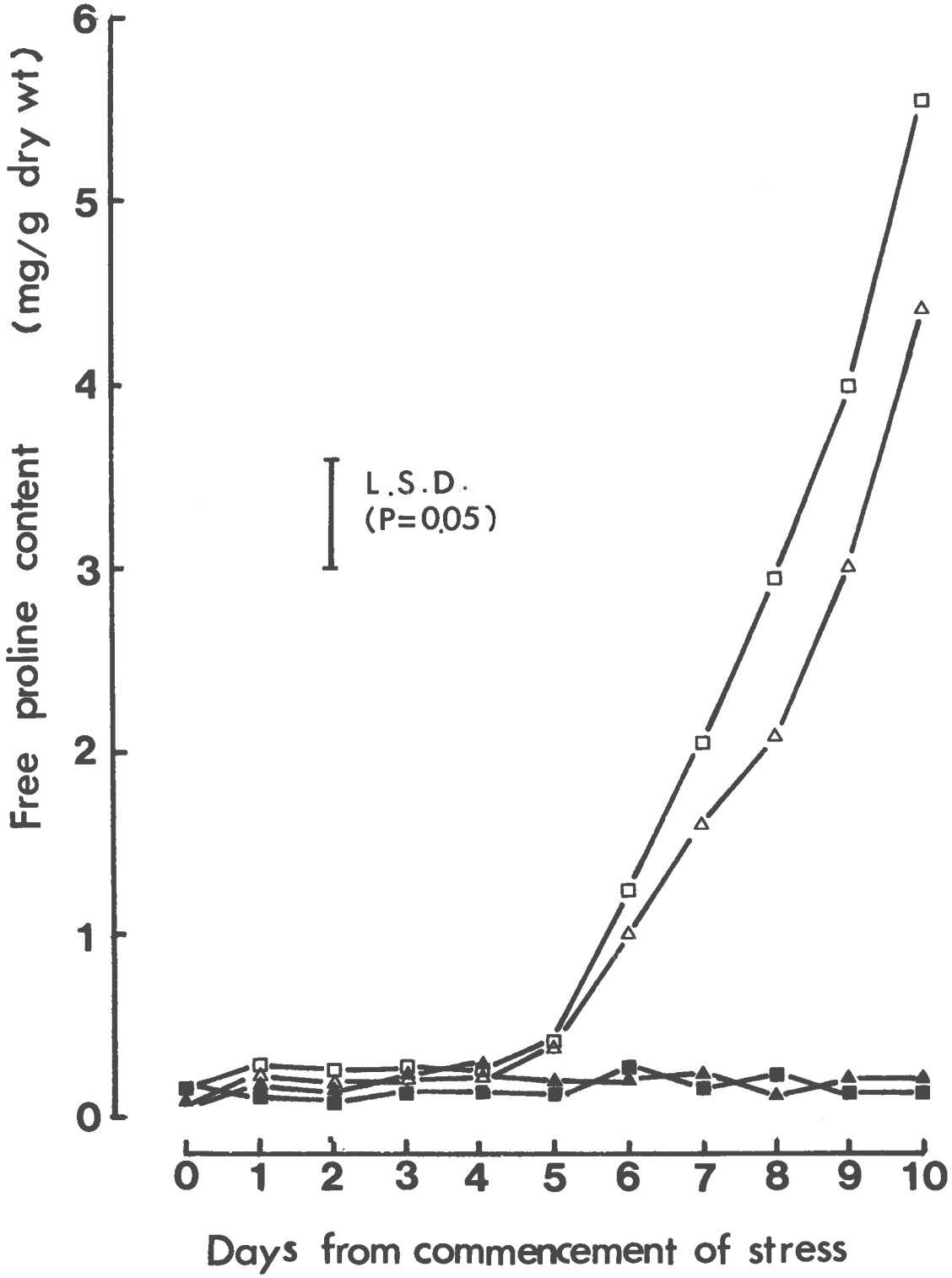
(3) Growth

There was no significant difference in the fresh weights of plants subjected to the increasing saline nutrient solution and those grown in

FIGURE 34

Proline accumulation in the first and second leaves of barley plants subjected to a gradual decline in osmotic potential in the nutrient solution by adding NaCl to decrease osmotic potential by one bar every 24 hours.

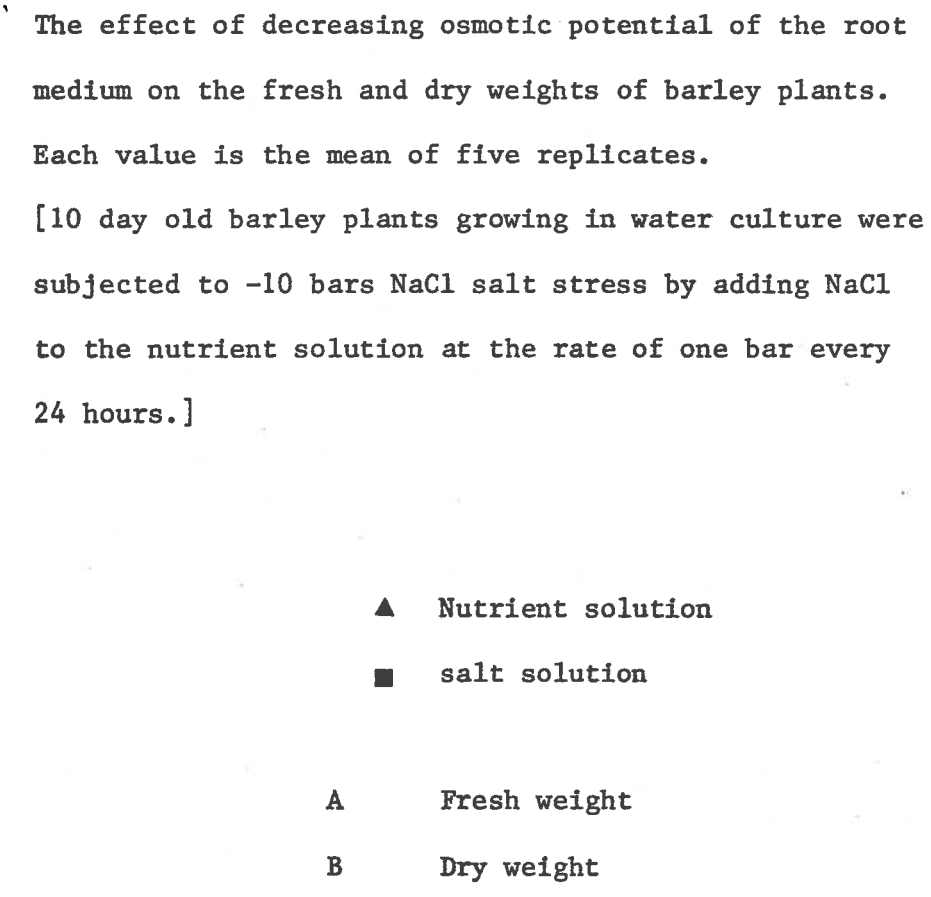
First leaf:	▲	Nutrient solution
	△	NaCl solution
Second leaf:	■	Nutrient solution
	□	NaCl solution


Free proline content (mg/g dry wt)
6
5
4
3
2
1
0
L.S.D.
(P=0.05)
0 1 2 3 4 5 6 7 8 9 10
Days from commencement of stress

nutrient solution until day 6 when the osmotic potential of the nutrient solution had declined to -6 bars; thereafter growth in fresh weight was entirely inhibited (Figure 35A). An inhibition of dry weight increase did not become evident until day 7 when the nutrient solution osmotic potential was -7 bars, and even then growth in dry weight was not completely suppressed as a slow but significant increase in plant dry weight occurred in the following 3 days (Figure 35B).

Salt stress also inhibited leaf emergence and expansion. The lengths of the first three leaves on each plant were measured following dissection but as the first leaf had completely expanded when the first measurement was made there was no further change during the experimental period and the data for these leaves are omitted (Table 6). The elongation of the second leaf was affected before day 2, well before any effect on growth in weight or on proline accumulation. At this time the water and osmotic potentials of this leaf had declined by no more than 1 bar and turgor potential was unaffected (Figure 33). Although elongation was inhibited at this early stage, it continued, albeit at a much reduced rate until day 9 when a final length of 15.6 cm was attained. In comparison, the second leaves of plants growing on nutrient solution elongated rapidly but reached a final size (18.8 cm) on day 7. Slow leaf elongation appeared to continue after increase in plant fresh weight was suppressed (Figure 35) and concurrently with a rapid accumulation of proline in those leaves (Figure 34). Emergence of the third leaf was slightly delayed

FIGURE 35



The effect of decreasing osmotic potential of the root medium on the fresh and dry weights of barley plants. Each value is the mean of five replicates.
[10 day old barley plants growing in water culture were subjected to -10 bars NaCl salt stress by adding NaCl to the nutrient solution at the rate of one bar every 24 hours.]

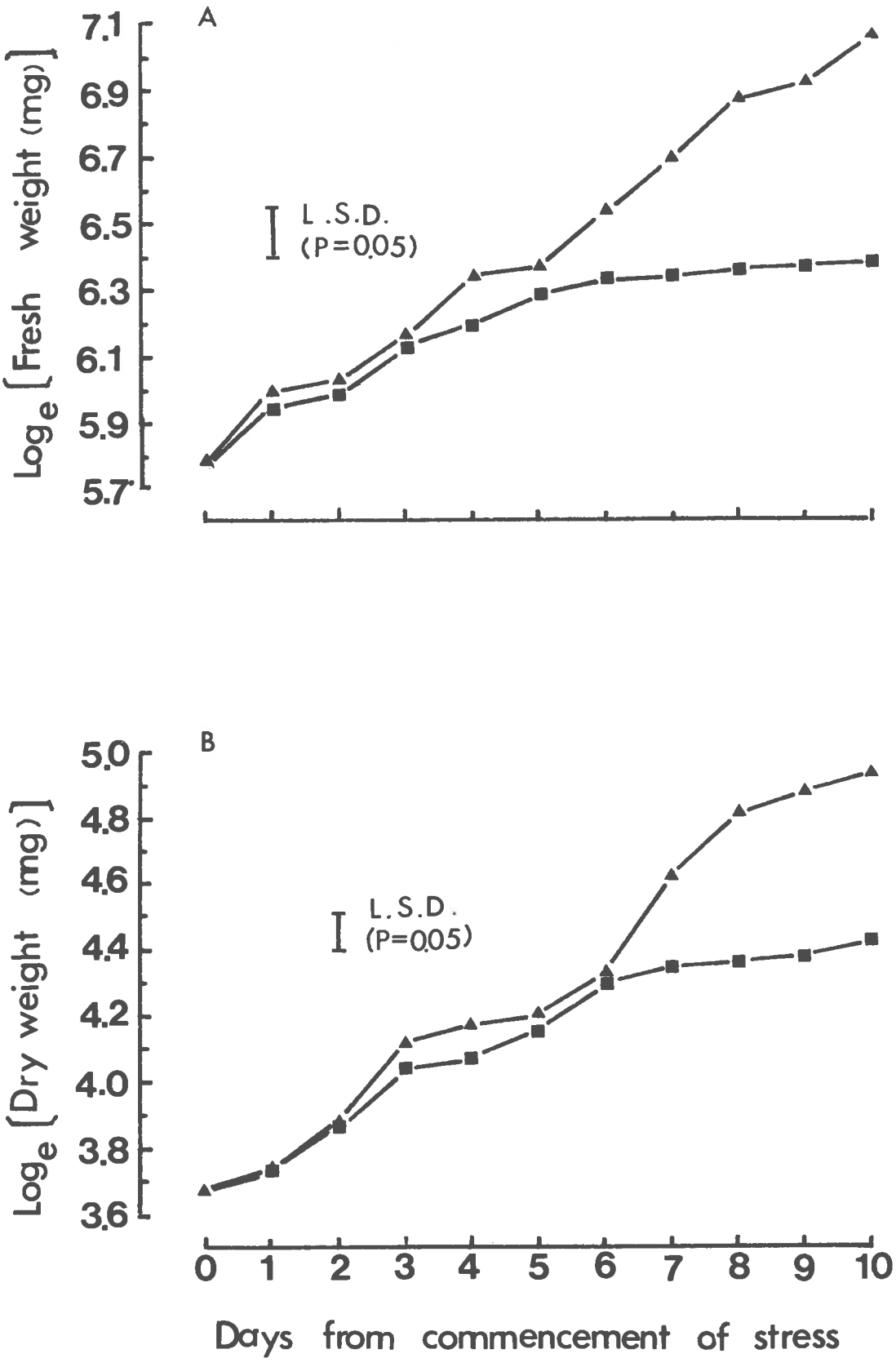

A
7.1
6.9
6.7
6.5
6.3
6.1
5.9
5.7
Log$_e$ [Fresh weight (mg)]
L.S.D.
(P=0.05)
B
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
Log$_e$ [Dry weight (mg)]
L.S.D.
(P=0.05)
0 1 2 3 4 5 6 7 8 9 10
Days from commencement of stress

TABLE 6

The effect of decreasing osmotic potential of the root medium on the increment of leaf length and leaf emergence. [The osmotic potential of the root medium was decreased by adding NaCl at the rate of one bar every 24 hours.]

Time (Days)	Non-stressed 2nd leaf (cm)	Stressed 2nd leaf (cm)	Non-stressed 3rd leaf (cm)	Stressed 3rd leaf (cm)	L.S.D. (p = 0.05) 2nd leaf	3rd leaf
0	0	0	-	-	-	-
1	2.2	2.4	-	-	1.29	-
2	8.1	5.4	-	-	0.78	-
3	11.7	7.9	-	-	0.71	-
4	14.8	10.0	0	-	0.63	-
5	17.4	12.0	3.1	0	1.53	-
6	18.2	13.5	7.5	1.4	1.57	0.58
7	18.8	14.3	11.9	2.6	1.72	0.32
8	18.8	15.0	16.4	4.1	1.89	0.90
9	18.8	15.6	20.2	5.7	1.60	1.97
10	18.8	15.6	20.2	7.1	1.60	1.88

Each value is the mean of five replicates.

(Table 6), but it also continued to elongate slowly to the end of the experiment when the osmotic potential of the nutrient solution had been reduced to -10 bars.

4. Prolonged exposure to salinity

4.1 Methods

Barley seedlings (cv. Prior) were grown in the standard water-culture system (III. Materials and Methods, page 64) until they were 10 days old. NaCl solution was then added to the nutrient solution to lower the osmotic potential of the solution by 1 bar each day to a final potential of -10 bars. Plants were then held at this osmotic potential for a further 6 days. Control plants were grown in non-saline Hoagland's solution. Plants were sampled daily once the osmotic potential of the nutrient solution had been lowered to -10 bars. Proline content determinations were made on the first and second leaves; three replicate samples were taken in each case.

4.2 Results

(1) Water status and proline accumulation

As in the previous experiment, the gradual decline in osmotic potential in the nutrient solution resulted in an equivalent reduction in both water and osmotic potentials in the leaves so that turgor potential was virtually unaffected (Table 7 and 8). While the plants continued to be held in a nutrient solution containing salt at -10 bars osmotic potential, both water and osmotic potentials of the leaves continued to decline slowly, although turgor remained apparently unaffected. Relative turgidity, measured independently, did decline slightly in this period; however, proline had

TABLE 7

The effect of prolonged exposure to salinity stress on water status of the first leaf of barley.

[10 day old barley plants growing in water culture were subjected to NaCl salinization treatment. Slow salinization occurred at the rate of -1 bar per day for 9 days, then continued exposure to -10 bars for 6 days. The measurements were made after osmotic potential of the solution had been lowered to -10 bars.]

Time (Days)	Water potential (-Bars)	Osmotic potential (-Bars)	Turgor potential (-Bars)	Relative Turgidity (%)
0	13.3	19.2	5.9	96.5
1	14.2	19.9	5.7	94.1
2	14.8	20.3	5.5	93.7
3	15.5	21.3	5.8	93.5
4	15.8	21.7	5.9	93.3
5	17.1	22.8	5.7	89.2
6	18.2	24.1	5.9	91.5
Control average	3.5-4.6	9.5-10.7	6.0-6.1	96.9-99.1
L.S.D. (p=0.05)	0.83	1.10	1.74	1.75

Each value is the mean of three replicates.

TABLE 8

The effect of prolonged exposure to salinity stress on water status of the second leaf of barley.

[10 day old barley plants growing in water culture were subjected to NaCl salinization treatment. Slow salinization occurred at the rate of -1 bar per day for 9 days, then continued exposure to -10 bars for 6 days. The measurements were made after osmotic potential of the solution had been lowered to -10 bars.]

Time (Days)	Water potential (-Bars)	Osmotic potential (-Bars)	Turgor potential (-Bars)	Relative turgidity (%)
0	11.3	17.8	6.5	94.6
1	12.3	18.6	6.3	91.0
2	13.3	19.6	6.3	92.9
3	13.9	20.2	6.3	93.2
4	14.6	21.1	6.5	88.1
5	15.6	22.3	6.7	85.6
6	16.5	23.3	6.8	91.0
Control average	2.3-3.3	8.6-9.7	6.3-6.4	96.4-98.3
L.S.D. (p=0.05)	0.94	0.98	1.52	1.52

Each value is the mean of three replicates

accumulated to a concentration of 3 - 4 mg per gram dry weight in the leaves when the nutrient solution had been brought to an osmotic potential of -10 bars (Figure 36). As the plants continued to be held in the nutrient solution at that salinity, proline accumulated at a rate of 50 - 70 $\mu g\ g^{-1}\ h^{-1}$ for 3 days in both leaves. Thereafter accumulation ceased in the first leaf but continued at a reduced rate in the second leaf.

(2) Plant growth

In the non-stressed plant, plant growth measured by fresh and dry weights, was exponential over the period of the experiment (Figure 37). The mean relative growth rate in this period was 0.13 mg $mg^{-1}day^{-1}$ and there was little change in the fresh weight/dry weight ratio. Lowering the osmotic potential of the nutrient solution with NaCl reduced the relative growth rate to 0.09 mg $mg^{-1}\ day^{-1}$, this decrease being apparent within 24 hours and remaining constant for the whole 6 day period. Growth thus continued at a reduced rate when salt was added to the rooting medium. The effect on fresh weight was more pronounced, however, and as a result there was a decrease in the fresh weight/dry weight ratio in plants subjected to salinity stress. After 6 days, the fresh weight/dry weight ratio was 10.37 in plants growing in nutrient solution and 5.64 in plants in the saline medium.

Salt stress again inhibited leaf emergence and expansion. Since the first and second leaves had completely expanded when the first measurement

FIGURE 36

Proline accumulation in the first and second leaves of barley plants subjected to prolonged exposure to salinity.

Each value is the mean of three replicates.

[10 day old barley plants growing in water culture were subjected to NaCl salinization treatment. Slow salinization occurred at the rate of -1 bar per day for 9 days, then continued exposure to -10 bars for 6 days. The measurements were made after osmotic potential of the solution had been lowered to -10 bars.]

First leaf:	▲	Nutrient solution
	△	NaCl solution (ψ_π = -10 bars)
Second leaf:	■	Nutrient solution
	□	NaCl solution (ψ_π = -10 bars)

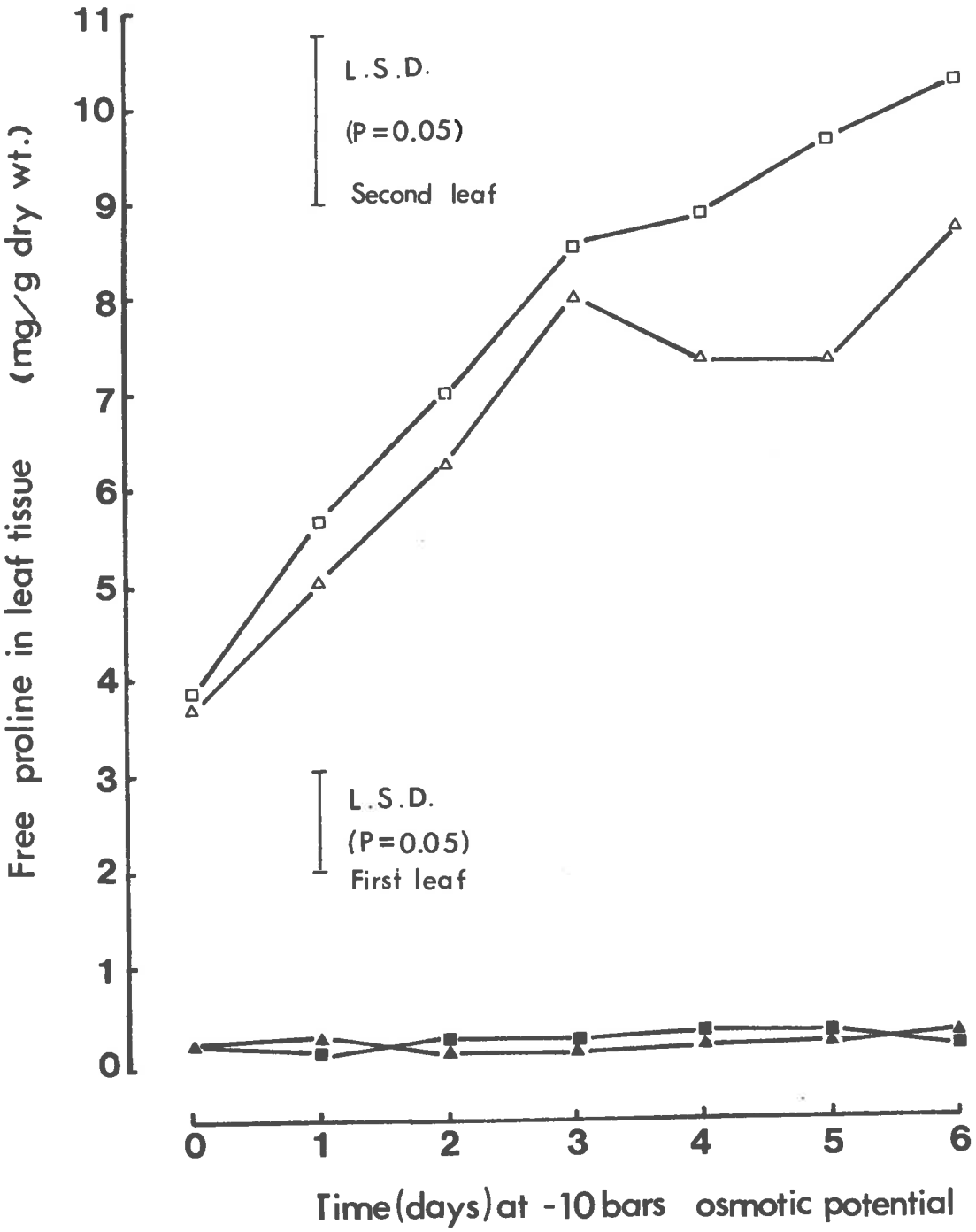
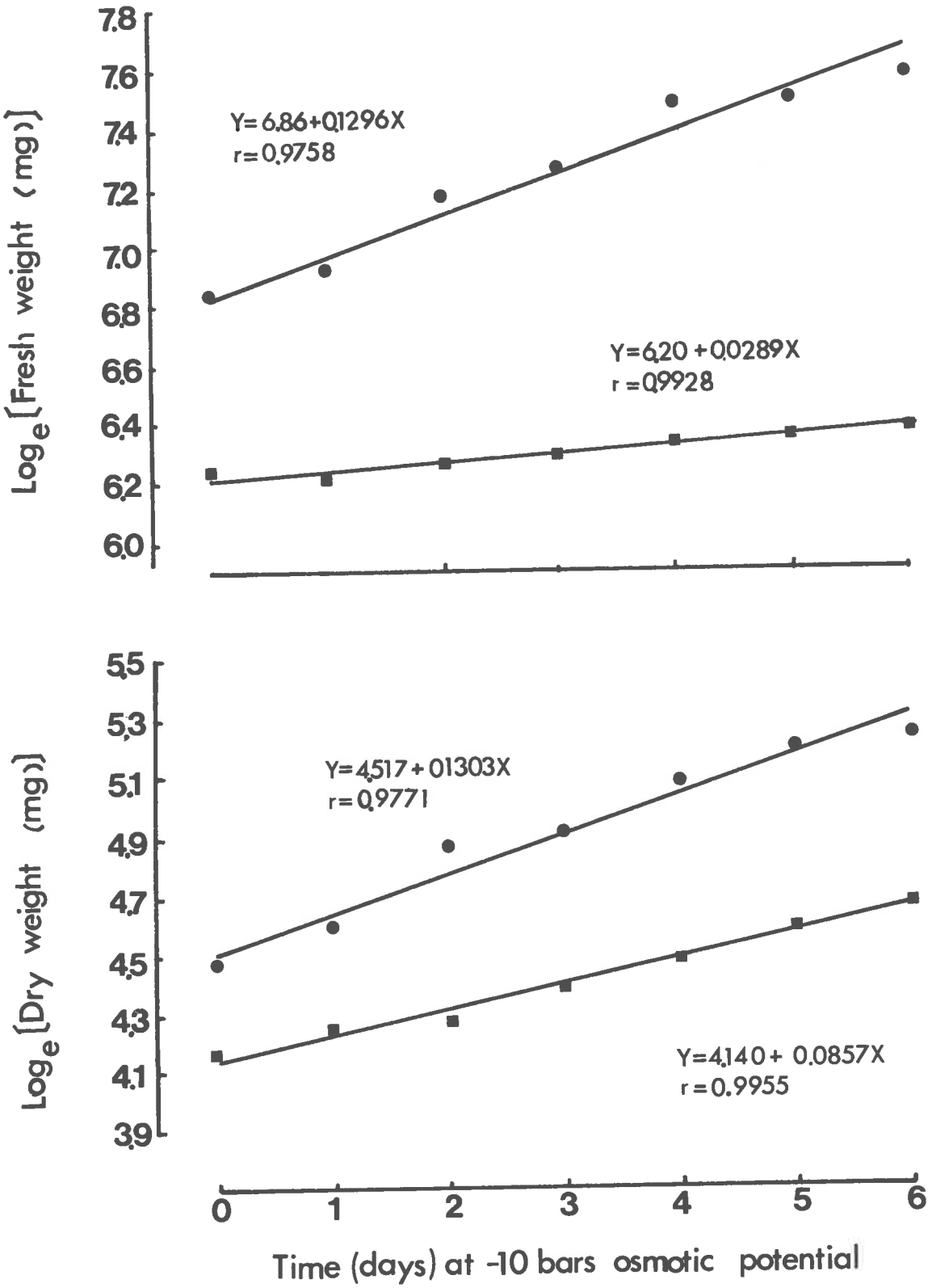

Free proline in leaf tissue (mg/g dry wt.)
11
10
9
8
7
6
5
4
3
2
1
0
L.S.D.
(P=0.05)
Second leaf
L.S.D.
(P=0.05)
First leaf
0
1
2
3
4
5
6
Time (days) at -10 bars osmotic potential

FIGURE 37

The effect of prolonged exposure to salinity stress on fresh weight and dry weight of the barley plant. Each value is the mean of five replicates.
[10 day old barley plants grown in water culture were subjected to NaCl salinization treatment. The slow salinization occurred at the rate of -1 bar per day for 9 days, then continued exposure to -10 bars for 6 days. Measurements were made after osmotic potential of the solution had been lowered to -10 bars.]

● Nutrient solution

■ NaCl solution (ψ_π = -10 bars)


7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
Log$_e$ [Fresh weight (mg)]
Y= 6.86+0.1296X
r=0.9758
Y=6.20 +0.0289X
r =0.9928
5.5
5.3
5.1
4.9
4.7
4.5
4.3
4.1
3.9
Log$_e$ [Dry weight (mg)]
Y=4.517 + 01303X
r=0.9771
Y=4.140 + 0.0857X
r=0.9955
0
1
2
3
4
5
6
Time (days) at -10 bars osmotic potential

was made there was no further change during the experimental period. The data for these leaves are not included here. The third leaf continued to elongate slowly in plants subjected to salinity, although the rate of elongation was inhibited (Table 9). The emergence of the fourth leaf was delayed, but this leaf also continued to elongate slowly.

5. Proline accumulation in the presence of various salts

5.1 Methods

The effect of a range of salts (NaCl, KCl, $MgCl_2$, $CaCl_2$ and Na_2SO_4) at four osmotic potentials (-5.7, -10.7, -15.7 and -20.7 bars) was assessed with barley. Barley (cv.Prior) plants were grown in a standard nutrient solution (III Materials and Methods, page 64). When these seedlings were 12 days old, various salts were added to the nutrient solutions to achieve the range of osmotic potential required. The plants were exposed to the required solution immediately and not gradually as in the previous two experiments. The salt solutions were compared with an iso-osmotic solution of polyethylene glycol (Mwt. 4,000) in an attempt to estimate the effect of the specific ions involved. All the plants were sampled 24 hours after first exposing them to salinity stress. The water and osmotic potential together with the proline content of the first leaf was measured.

5.2 Results

(1) Water status

After 24 hours exposure to the various salts, leaf water potential declined proportionally to the osmotic potential of the root solution

TABLE 9

Leaf length increment of 3rd and 4th leaves of 10 day old barley plants subjected to prolonged exposure to salinity.
[10 day old barley plants growing in water culture were subjected to NaCl salinization treatment. Slow salinization occurred at the rate of -1 bar per day for 9 days, then continued exposure to -10 bars for 6 days. Measurements were made after osmotic potential had been lowered to -10 bars.]

Time (days)	Non-stressed 3rd leaf (cm)	Stressed 3rd leaf (cm)	Non-stressed 4th leaf (cm)	Stressed 4th leaf (cm)	L.S.D.(p = 0.05) 3rd leaf	4th leaf
0	0	0	0	-	-	-
1	7.5	1.4	9.9	-	0.54	-
2	12.0	3.7	13.6	-	0.69	-
3	12.8	4.7	17.6	-	0.58	-
4	14.4	6.2	23.6	-	1.57	-
5	14.6	7.4	25.6	1.3	1.07	1.41
6	14.7	8.0	25.6	1.5	0.82	1.39

Each value is the mean of five replicates.

(Figure 38). Leaf water potential was highly correlated with the osmotic potential of solution. Within the range -0.7 to -20.7 bars osmotic potential of solution, the relationship was linear. Moreover, there was no significant difference between any of the various solutes, including polyethylene glycol.

Leaf osmotic potential also decreased with decrease in the osmotic potential of the root medium. The response was linear over the range of -0.7 bars to -20.7 bars in the root medium and again there was no significant difference between the various salt treatments. In this case, however, the decline in leaf osmotic potential was less with plants grown in polyethylene glycol solution than in plants grown in iso-osmotic solutions of salts. This difference was more pronounced at the lower osmotic potentials of the root medium.

In all treatments, the leaf water potential fell more than leaf osmotic potential with a decrease in osmotic potential in the root medium. At high salt concentrations this difference was pronounced. As a result, the leaf turgor potential was reduced by each increase in salt concentration (Figure 39). At -5.7 bars osmotic potential, there was a slight wilting of the plants, this wilting was more severe in those plants exposed to the high concentrations of salts, but, there were no significant differences between the various salt treatments at any one osmotic potential in the nutrient solution. In comparison with the salt solutions, turgor potential fell more and wilting was more marked

FIGURE 38

The water potential and osmotic potential of the first leaf of 12 day old barley plants grown in solution maintained at various osmotic potentials with different osmotic substrates. Each value is the mean of four replicates.

[Sodium chloride, potassium chloride, sodium sulphate, magnesium chloride, calcium chloride and polyethylene glycol were added to base nutrient solution (-0.7 bars) as single osmotic substrates to produce isosmotic potentials of -5.7, -10.7, -15.7 and -20.7 bars. Measurements were made 24 hours after exposure to stress.]

- ● NaCl solution
- □ KCl solution
- ▼ $CaCl_2$ solution
- ■ $MgCl_2$ solution
- ▲ Na_2SO_4 solution
- ○ Polyethylene glycol solution (Mwt. 4,000)

Water potential:

NaCl	: Y = -3.23 + 1.004x	r = -0.9975
KCl	: Y = -3.08 + 1.004x	r = -0.9970
$CaCl_2$	: Y = -3.54 + 1.028x	r = -0.9934
$MgCl_2$	: Y = -2.68 + 1.026x	r = -0.9910
Na_2SO_4	: Y = -3.31 + 1.042x	r = -0.9976
PEG	: Y = -3.16 + 1.024x	r = -0.9969

Osmotic potential:

NaCl	: Y = -9.39 + 0.892x	r = -0.9978
KCl	: Y = -9.15 + 0.926x	r = -0.9969
$CaCl_2$	: Y = -9.59 + 0.896x	r = -0.9917
$MgCl_2$	: Y = -8.90 + 0.884x	r = -0.9910
Na_2SO_4	: Y = -9.42 + 0.910x	r = -0.9972
PEG	: Y = -9.31 + 0.781x	r = -0.9890

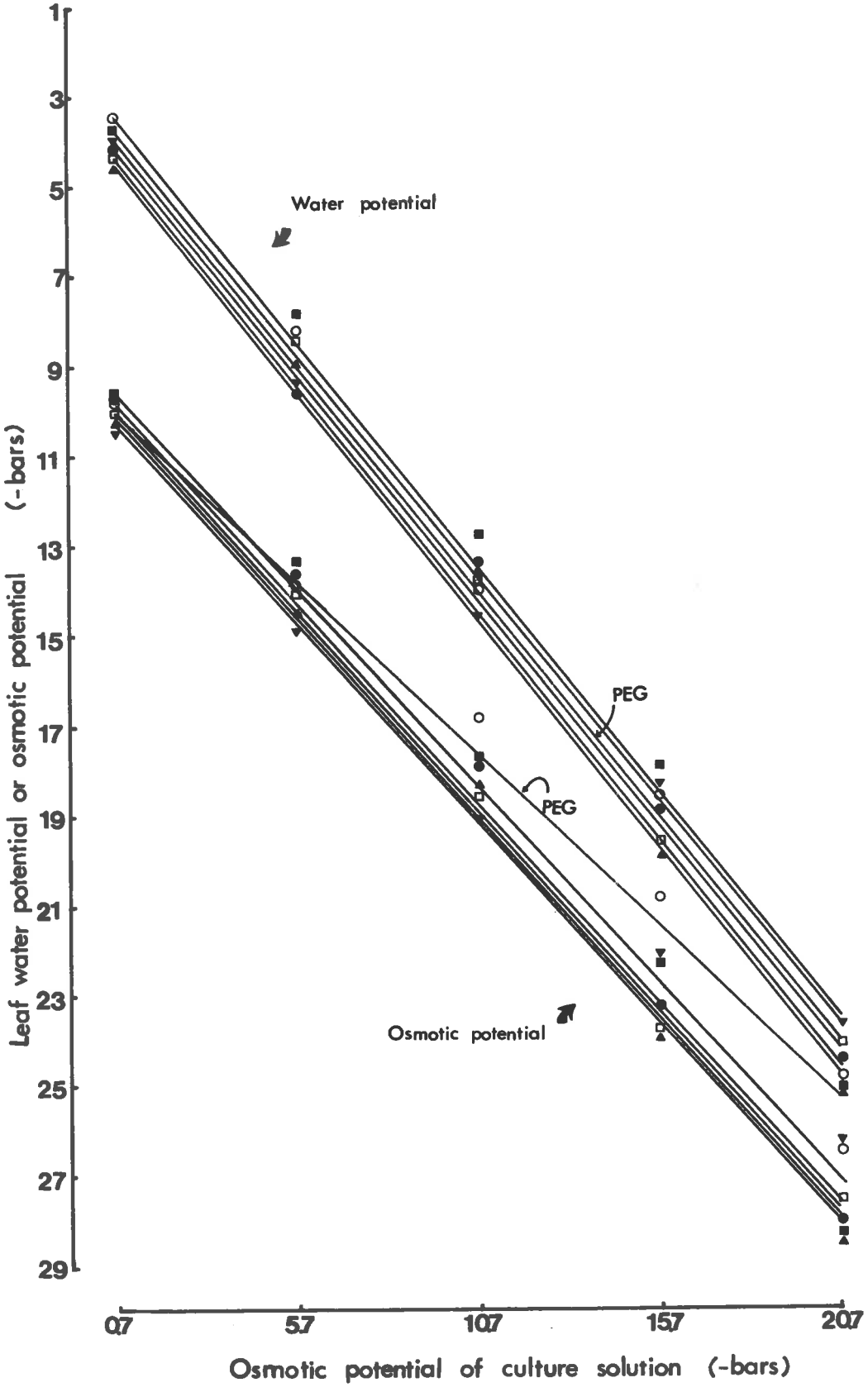
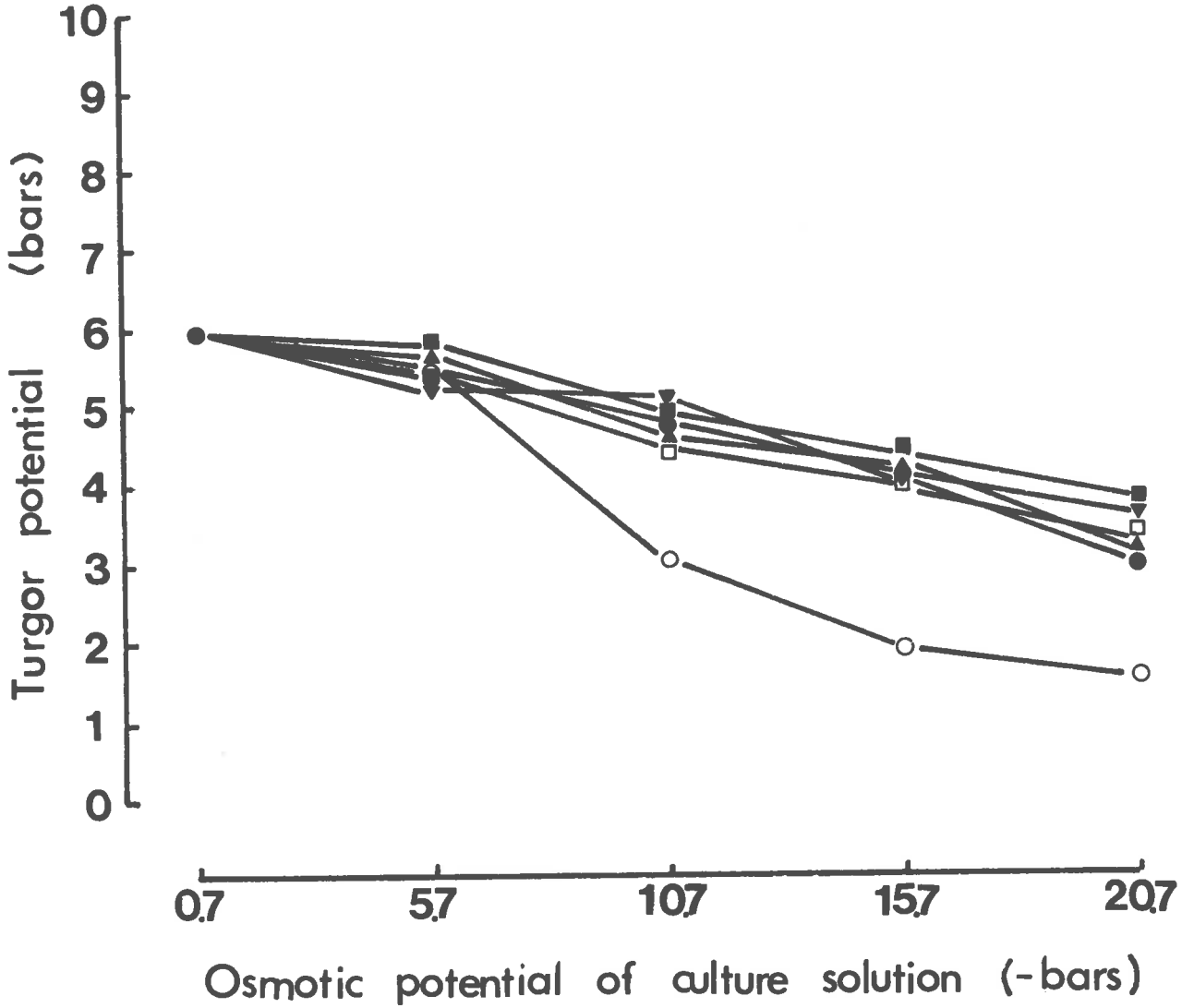

Water potential
PEG
PEG
Osmotic potential
Leaf water potential or osmotic potential (-bars)
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
0.7
5.7
10.7
15.7
20.7
Osmotic potential of culture solution (-bars)

FIGURE 39

Turgor potential change of the first leaf of 12 day old barley plants grown in solutions maintained at various osmotic potentials with different osmotic substrates. Each value is the mean of four replicates. [Calculated from Figure 38, as the difference between osmotic potential and water potential.]

- ● NaCl solution
- □ KCl solution
- ▼ $CaCl_2$ solution
- ■ $MgCl_2$ solution
- ▲ Na_2SO_4 solution
- ○ Polyethylene glycol solution (Mwt. 4,000)


Turgor potential (bars)
10
9
8
7
6
5
4
3
2
1
0
0.7
5.7
10.7
15.7
20.7
Osmotic potential of culture solution (-bars)

in the polyethylene glycol treatments. For instance, at -20.7 bars osmotic potential of root medium, the turgor potential of plants grown in polyethylene glycol solution had fallen to 1.5 bars while the turgor potential of plants exposed to the iso-osmotic concentration of salts had reached between 3 and 4 bars. This was due to the smaller effect of polyethylene glycol treatment on leaf osmotic potential.

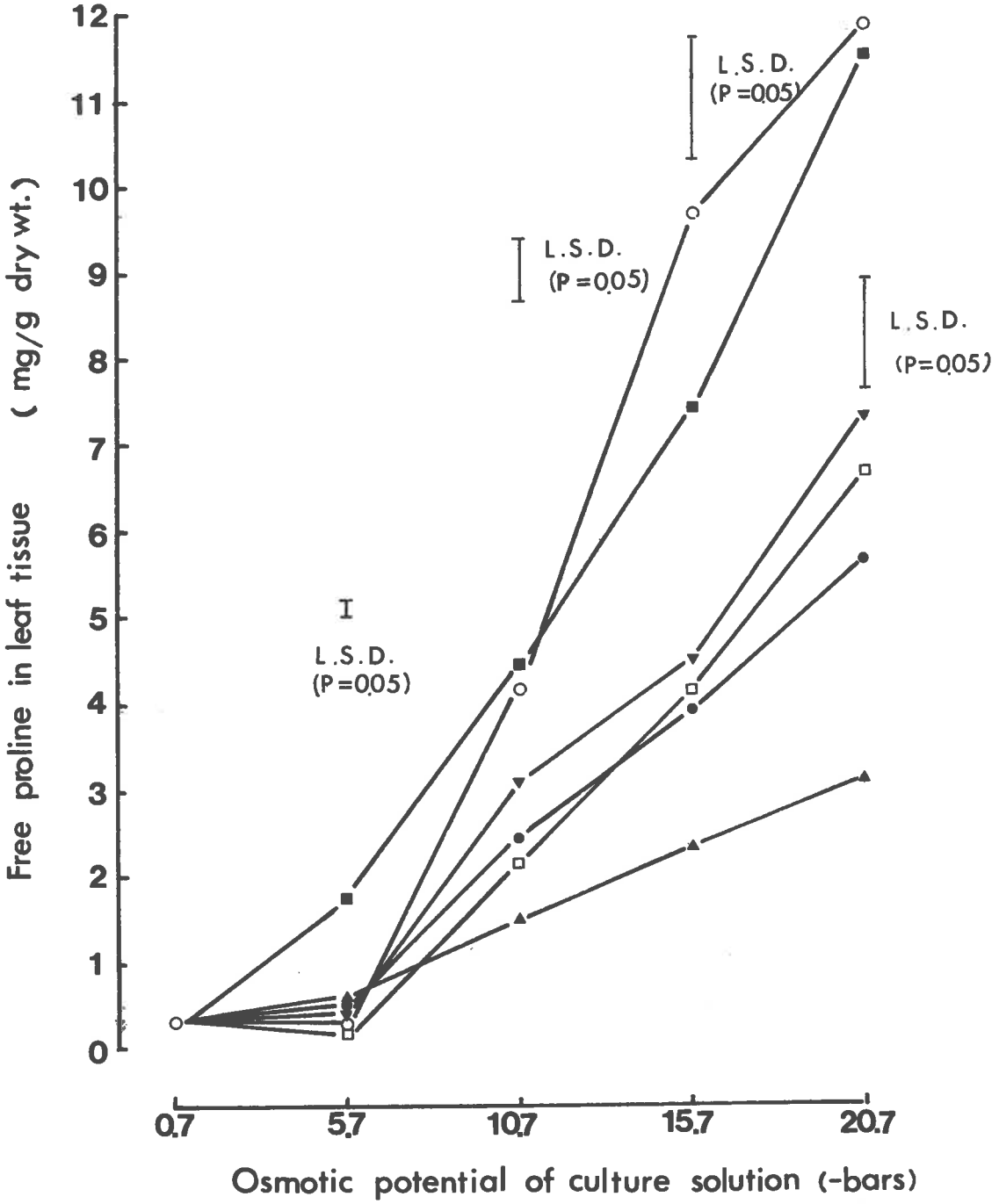
(2) Proline content

It is clear that lowering the osmotic potential of the rooting medium irrespective of the osmoticum used, resulted in an accumulation of proline (Figure 40). It is also clear, however, that the proline accumulation potential was considerably affected by the osmoticum. At the lowest concentration of osmoticum, only the plants exposed to $MgCl_2$ accumulated a significant amount of proline. At higher concentrations plants exposed to either $MgCl_2$ or polyethylene glycol in iso-osmotic solution, accumulated the highest concentration of proline, there being no consistent significant differences between these two treatments. If the accumulation in polyethylene glycol is taken as a model, then it is apparent that neither Mg^{++} ions nor Cl^- ions inhibited proline accumulation. On the other hand, the data indicate that Na^+, K^+, Ca^{++} and $SO_4^=$ ions all inhibit proline accumulation to varying degrees as lowering the osmotic potential with $CaCl_2$, KCl, NaCl or Na_2SO_4 resulted in a lesser accumulation of proline than in an iso-

FIGURE 40

Proline accumulation in the first leaf of 12 day old barley plants grown in solutions maintained at various osmotic potentials with different osmotic substrates. Each value is the mean of four replicates. [NaCl, KCl, Na_2SO_4, $MgCl_2$, $CaCl_2$ and polyethylene glycol were added to base nutrient solution (-0.7 bars) as single osmotic substrates to produce isosmotic potentials of -5.7, -10.7, -15.7 and -20.7 bars. Measurements were maed 24 hours after exposure to stress.]

- ● NaCl solution
- □ KCl solution
- ▼ $CaCl_2$ solution
- ■ $MgCl_2$ solution
- ▲ Na_2SO_4 solution
- ○ Polyethylene glycol solution (Mwt.4,000)


Free proline in leaf tissue (mg/g dry wt.)
12
11
10
9
8
7
6
5
4
3
2
1
0
L.S.D. (P=0.05)
L.S.D. (P=0.05)
L.S.D. (P=0.05)
I
L.S.D. (P=0.05)
0.7
5.7
10.7
15.7
20.7
Osmotic potential of culture solution (-bars)

osmotic solution of polyethylene glycol or $MgCl_2$. This was despite these ions having similar effects on plant water potential.

6. Proline accumulation in isolated organs

6.1 Method

Barley (cv. Prior) seedlings were grown as described previously. The first leaves were excised and cut into 1 cm sections which were pooled on distilled water before distribution to the various treatments. 25 leaf sections were placed on solutions (5 ml) of various salts or polyethylene glycol (Mwt. 4,000) of 0, -5, -10, -15 or -20 bars osmotic potential. These were contained in 9 cm petri dishes and were incubated at 20°C in the dark for 24 hours. Each treatment was replicated three-fold.

6.2 Results

Two experiments are reported. In the first, leaf sections were floated on solutions of PEG, NaCl, KCl, Na_2SO_4, $CaCl_2$ and $MgCl_2$. At the end of 24 hours the proline content of leaf sections floated on PEG had only increased in solutions with an osmotic potential of -15 bars or lower (Figure 41). At -20 bars proline had accumulated to a concentration of greater than 7 mg per gram dry weight. There was no proline accumulation at any tested osmotic potential solution of NaCl, KCl or Na_2SO_4. Interestingly, however, there was an extensive accumulation of proline in sections exposed to $MgCl_2$ or $CaCl_2$ solutions even at osmotic potentials of -5 and -10 bars. The accumulation appeared to be more

FIGURE 41

Accumulation of proline in excised leaf laminae sections from barley plants when floated on various salt solutions and polyethyleneglycol solution at 20°C in the dark for 24 hours. Each value is the mean of three replicates.

▲ NaCl solution

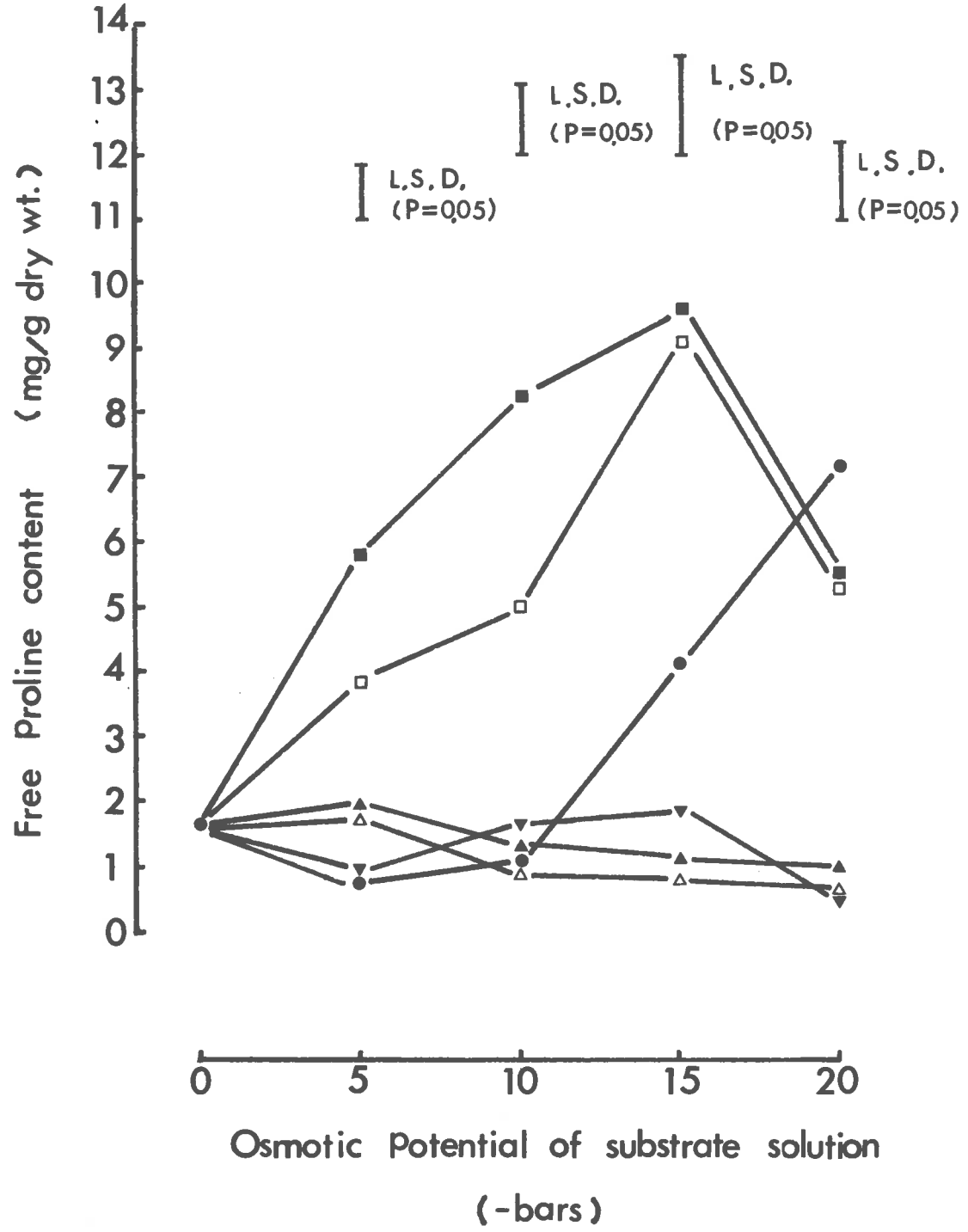
△ KCl solution

□ $CaCl_2$ solution

■ $MgCl_2$ solution

▼ Na_2SO_4 solution

● Polyethylene glycol solution (Mwt. 4,000)


L.S.D. (P=0.05)
L.S.D. (P=0.05)
L.S.D. (P=0.05)
L.S.D. (P=0.05)
Free Proline content (mg/g dry wt.)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
5
10
15
20
Osmotic Potential of substrate solution
(-bars)

extensive in $MgCl_2$ than in $CaCl_2$ solution, as in the intact plants, but in both solutions maximum accumulation occurred at an osmotic potential of -15 bars and was reduced at -20 bars. At -15 bars osmotic potential, between 9 and 10 mg per gram dry weight of proline accumulated in the leaf sections exposed to $MgCl_2$ or $CaCl_2$.

This remarkable response to Mg^{++} and Ca^{++} salts was further explored in a second, similar experiment in which leaf sections were incubated in solutions of $MgSO_4$, $MgCl_2$, $Mg(NO_3)_2$, $CaCl_2$ and $Ca(NO_3)_2$. Similar patterns of proline accumulation were recorded with maximum accumulation again occurring at -15 bars osmotic potential (Figure 42). Again the Ca^{++} salts tended to promote the accumulation of less proline than the equivalent Mg^{++} salts, at least at the lower osmotic potential. The accumulation potential of the anions fell in the order Cl^-, NO_3^-, $SO_4^=$ for both cations, although these differences also tended to be lost at the higher osmotic potentials. Clearly the response of both intact plants and leaf sections to the divalent cations Mg^{++} and Ca^{++} differs from the response to the monovalent cations Na^+ and K^+, although the response in the two systems differed in detail.

7. Proline distribution in salt-stressed plants

7.1 Method

The effects of two levels of NaCl salinity stress of -10 bars and -20 bars on the accumulation of proline in the lamina, leaf sheath and roots were investigated. Barley plants (cv. Prior) were grown in

FIGURE 42

Accumulation of proline in excised leaf laminae sections from barley plants when floated on $MgCl_2$, $Mg(NO_3)_2$, $MgSO_4$, $CaCl_2$ and $Ca(NO_3)_2$ solutions at $20^{\circ}C$ in the dark for 24 hours. Each value is the mean of three replicates.

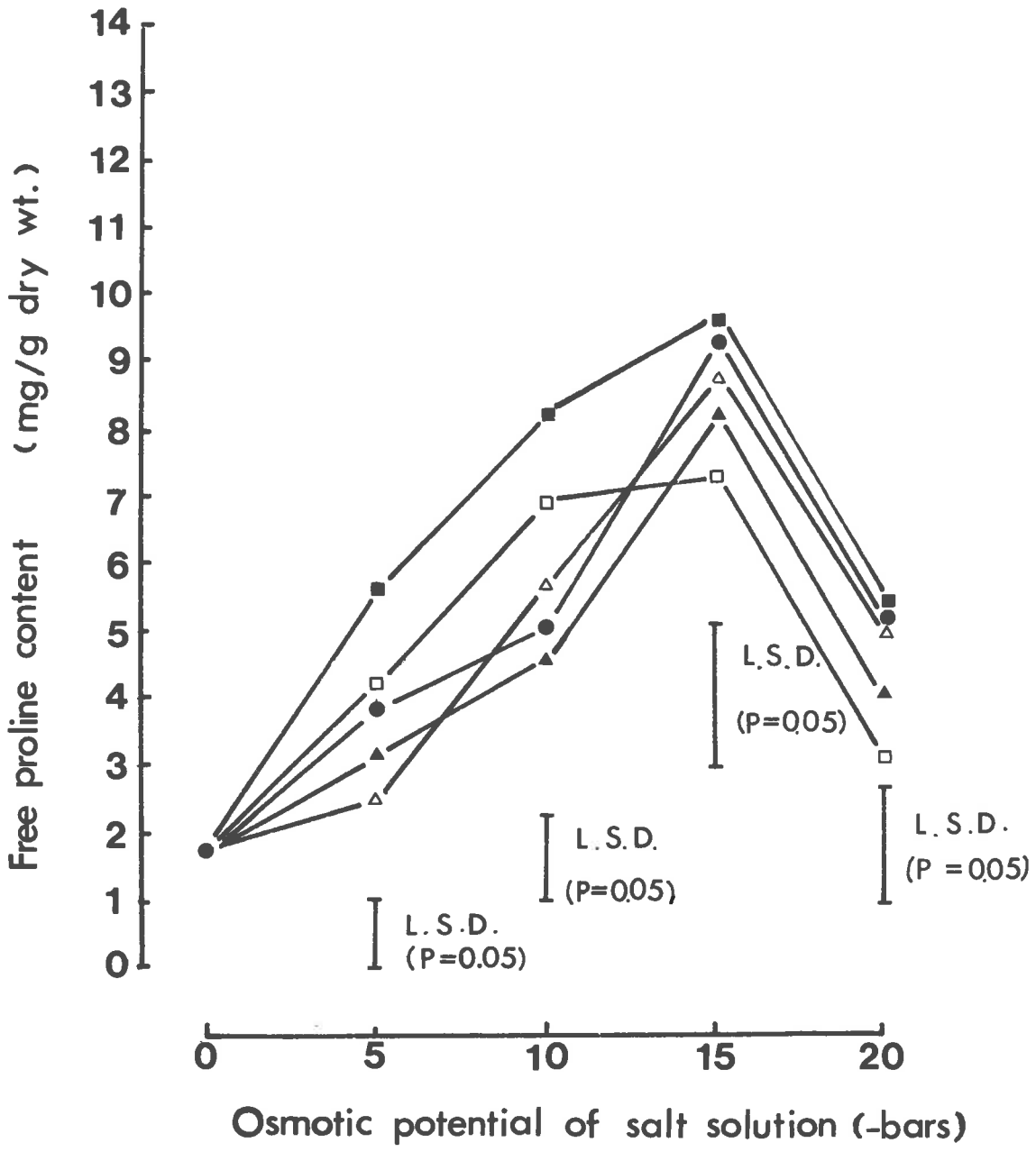
△ $MgSO_4$

■ $MgCl_2$

□ $Mg(NO_3)_2$

● $CaCl_2$

▲ $Ca(NO_3)_2$


Free proline content (mg/g dry wt.)
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
L.S.D. (P=0.05)
L.S.D. (P=0.05)
L.S.D. (P=0.05)
L.S.D. (P=0.05)
0
5
10
15
20
Osmotic potential of salt solution (-bars)

standard nutrient solution (III Materials and Methods, page 64). When these barley seedlings were 12 days old they were then subjected to salinity stress by adding the appropriate concentration of NaCl to the nutrient solution. The plants were sampled 0, 10, 24, 48, 56 and 72 hours after stress commencement. The water potential of the first leaf was measured and the proline content of various organs was estimated.

7.2 Results

(1) Water potential

In the -10 bars NaCl treatment, the water potential of the first leaf declined rapidly during the first 10 hours of stress. Following this initial decline, water potential continued to fall, reaching -21 bars at 48 hours. Thereafter the water potential did not show any further significant change (Figure 43).

In the severe (-20 bars) treatment the water potential also fell rapidly initially; following this initial decline, however, the water potential continued to fall almost as rapidly as before throughout all the experimental period. In this treatment, leaf water potential had reached -33.8 bars at the end of 72 hours. In the -10 bars treatment, the plants had completely recovered turgidity 48 hours after treatment, whereas, as judged by the plant appearance, those subjected to -20 bars NaCl salinity were still wilted at 72 hours.

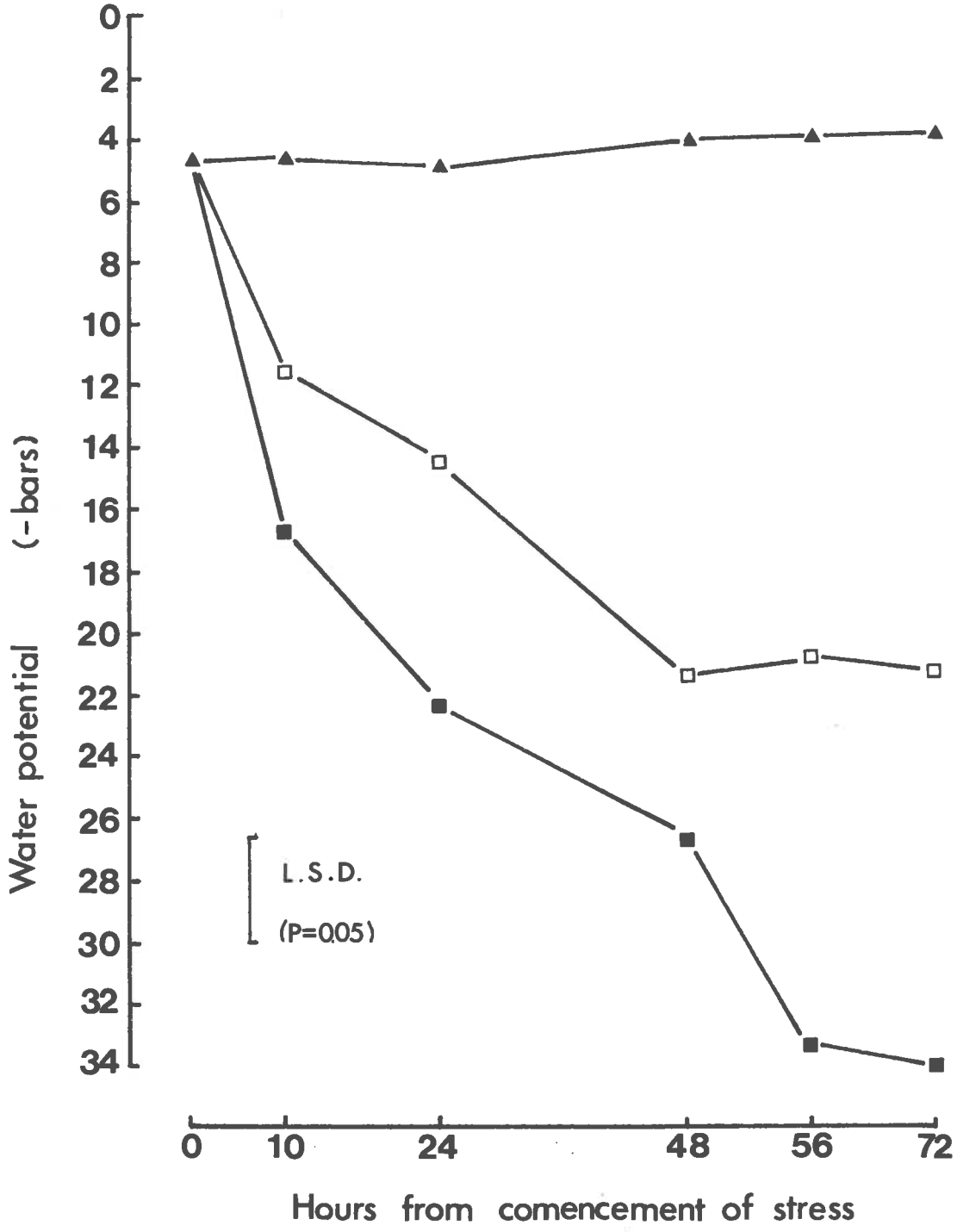
FIGURE 43

Water potential of the first leaf when intact 12 day old barley plants were subjected to NaCl stress of -10 or -20 bars osmotic potential. Each value is the mean of three replicates.

▲ Nutrient solution

□ NaCl solution (ψ_π = -10 bars)

■ NaCl solution (ψ_π = -20 bars)


0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Water potential (-bars)
L.S.D.
(P=0.05)
0
10
24
48
56
72
Hours from comencement of stress

(2) Proline accumulation

(A) First leaf lamina

The rate of proline accumulation was slow for the first 24 hours after subjecting plants to -10 bars NaCl salinity stress, but, thereafter, proline continued to accumulate at an almost constant rate at least to 72 hours after stress was imposed (Figure 44A). Plants subjected to the more severe stress (-20 bars) on the other hand, accumulated proline rapidly for the first 24 hours but then the rate of accumulation declined. After 48 hours the amount of proline accumulated in response to -20 bars NaCl stress was less than that in response to -10 bars NaCl stress and at 72 hours, the leaf had accumulated 10.7 mg per gram dry weight in the -10 bars treatment and 8.4 mg per gram dry weight in the -20 bars stress.

(B) Second leaf lamina

The second leaf laminae responded identically to the first leaf laminae except that slightly more proline accumulated in both treatments (Figure 44B).

(C) First leaf sheath

The leaf sheath of stressed plants accumulated considerably less proline than the leaf laminae (Figure 44C). In the severe treatment (-20 bars, NaCl) proline showed a linear increase in the first 48 hours, but, thereafter, there was no further increase in the concentration in the sheath but a significant loss in the final 16 hours. In contrast,

FIGURE 44

Accumulation of free proline in the various organs of intact barley plants subjected to NaCl stress of -10 or -20 bars osmotic potential. Each value is the mean of three replicates.

[12 day old barley plants growing in water culture were subjected to NaCl stress by adding NaCl of -10 or -20 bars osmotic potential to the rooting medium.]

A. leaf lamina of first leaf

B. leaf lamina of second leaf

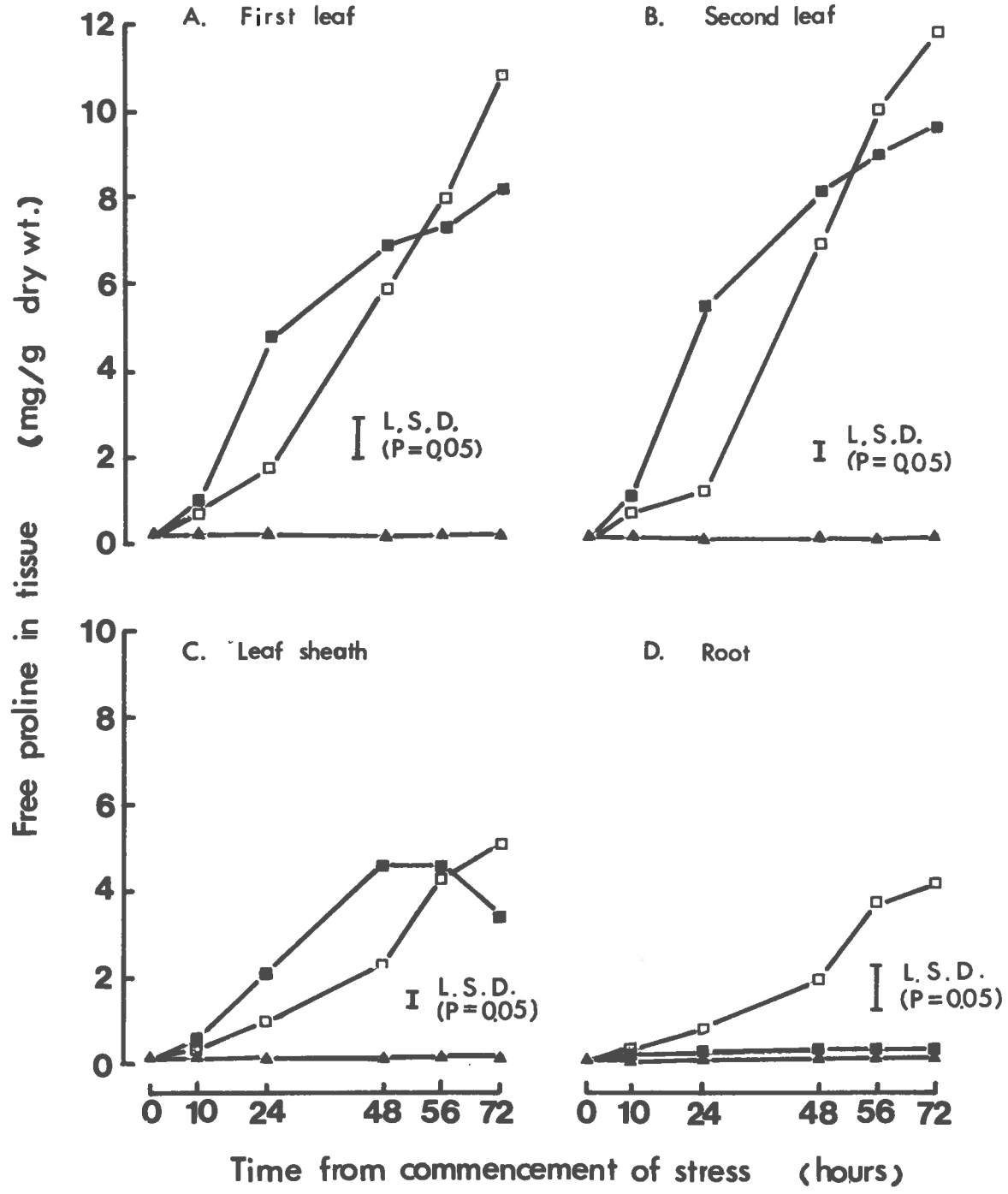
C. leaf sheath of first leaf

D. roots

▲ Nutrient solution

□ NaCl solution (ψ_π = -10 bars)

■ NaCl solution (ψ_π = -20 bars)


A. First leaf
B. Second leaf
C. Leaf sheath
D. Root
L.S.D. (P=0.05)
Free proline in tissue (mg/g dry wt.)
Time from commencement of stress (hours)
0
2
4
6
8
10
12
0 10 24 48 56 72

equivalent leaf sheaths in the -10 bars treatment accumulated proline throughout the experimental period to an eventual concentration of 5 mg per gram dry weight.

(D) Root

The root also accumulated proline when the plants were subjected to -10 bars NaCl stress, but to a lesser extent than any other organ of the plant (Figure 44D). In the severe (-20 bars, NaCl) treatment there was no proline accumulation in the root tissues throughout the experimental period.

8. Proline accumulation in different species

8.1 Method

The effects of salinity on proline accumulation was further examined with 12 crop species covering a wide range of reputed salt tolerance. The plants were grown in a growth cabinet in a controlled environment, and sown in 1 litre containers filled with "pea" gravel and provided with a drainage port at the bottom. The base nutrient solution had an osmotic potential of -0.7 bars and was supplied to plants daily. When the first leaves had completely expanded, from 1 week to 1 month after germination depending on the species, plants were subjected to salt stress by adding -10 bars NaCl to the solution. After 48 hours, the first leaf of each species was excised and then frozen in liquid nitrogen and preserved at -20^{o}C for proline assay. The species employed, the number of plants per culture and age of plant when NaCl was applied are listed below:

Species	No. of plants per culture	Age of plant when NaCl applied (days)
Beet (*Beta vulgaris* L.,c.v. Early Wonder)	2	35
Cotton (*Gossypium hirsutum* L., c.v. Empire)	2	35
Barley (*Hordeum distichum* L., cv. Proctor)	10	14
Tomato (*Lycopersicum esculentum* Mill. cv South Aust. Dwarf red)	2	32
Corn (*Zea mays* L., cv. Hybrid 10 Chief)	4	20
Cucumber (*Cucumis sativus* L.,cv. Marketer)	4	20
Radish (*Raphanus sativus* L.,cv. Long White Icicle	4	20
Rape (*Brassica napus* L., cv. Oro)	4	20
Wheat (*Triticum aestivum* L.,cv. Olympic)	10	14
Oats (*Avena sativa* L.,cv. Victory)	10	14
Broad Bean (*Vicia faba* L.,cv. South Aust. Dwarf bean)	4	18
Peas (*Pisum satirum* L.,cv. Maori King)	4	18

There were two cultures of each species, one for control and the other for salt stress. Each treatment was replicated threefold.

7.2 Results

Crop plants differ greatly in their tolerance to salinity. Garden beet and spinach are highly tolerant and often show a stimulation of growth by levels of NaCl that would be lethal for the pea (Hayward and Bernstein, 1958). The majority of crop plants fall between these two extremes. The effect of NaCl salinity on proline accumulation in 12 species are shown in Table 10. In this table, the species are arranged in the approximate order of decreasing salt tolerance, suggested by Nieman (1962) who investigated some effects of NaCl on growth, photosynthesis and respiration of 12 crop plants. NaCl induced proline accumulation in 11 of the 12 species tested, the sole exception being cotton. It is apparent that proline accumulation is a common plant response to a high concentration of NaCl. However, the proline accumulation which was initiated by NaCl stress varied between species. Among these species, the greatest increase occurred in barley and oats, in which proline accumulated to a concentration of 4.47 and 6.54 mg per gram dry weight respectively after 48 hours exposure to salt stress, being 17.9 and 16.0 fold increases over the concentrations of proline in plants growing in a non-saline solution. Rape and wheat increased 9 fold, tomato, bean, pea and radish 5-6 fold and corn and cucumber 3-4 fold. Since the rate of proline accumulation was not consistently higher in reputedly tolerant species and, in fact, the rate in some sensitive plants was consistently higher than in the very tolerant beet, the rate of proline accumulation bore no apparent relationship to the salt tolerance of that species.

TABLE 10

Effect of salinity on proline accumulation in several plant species

Plant	Ave. initial total proline content (mg/g dry weight)	Total proline content after 48 hr. exposure to -10 bars NaCl (mg/g dry weight)	
		Untreated	Treated
Beet	0.46	0.46	1.43*
Cotton	0.50	0.49	0.50
Barley	0.25	0.25	4.47***
Tomato	0.15	0.16	0.92**
Corn	0.13	0.14	0.54*
Cucumber	0.22	0.20	0.66*
Radish	0.56	0.54	3.01***
Rape	0.72	0.73	6.78***
Wheat	0.43	0.45	4.06***
Oats	0.41	0.41	6.54***
Broad bean	0.40	0.41	2.22**
Pea	0.18	0.19	1.21**

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability

9. Discussion

The present work has demonstrated that there is a striking accumulation of proline in response to salt stress in both barley and radish plants. A similar accumulation of proline as a result of salt stress has been reported in leaves of paprika, sunflower, peas, bean (Palfi and Juhasz, 1970), tomato and cotton plants (Strogonov,1964). In the present investigation, it has been shown, with a further 12 crop species covering a wide range of salt tolerance, that proline accumulation in response to salt stress is a general phenomenon in higher plants. The degree of proline accumulation varied greatly between species and, moreover, there seemed to be no correlation between proline accumulation potential and the salt tolerance of the species. Since the relative salt tolerance for each species was judged from the work of Nieman (1962) and was not measured in the present experiment, this lack of correlation should be viewed with caution. Moreover, it has been shown that there exists considerable intraspecific variation in proline accumulation potential with water stress (Singh *et al.*, 1972). It is apparent that conclusions about the relationship between proline accumulation and plant resistance or tolerance to salinity should await further experimentation.

Exposure of plants to elevated levels of salt in the medium surrounding the roots results in at least two situations potentially inhibitory to plant growth. Firstly, dissolved salt lowers the osmotic

potential of the solution surrounding the roots and reduces the potential gradient for water transfer from the medium into the cells of the plant. This results in changes in the water status of the plant tissue. Secondly, nonselective ion absorption from a high external salt concentration results in a build-up of ions within the cell with both metabolic and osmotic consequences. These changes in both plant water status and ion absorption depend on the external salt concentration, the specific ion involved, the rate of change in ionic concentration in the external medium as well as the plant species.

When the potential gradient which controls water movement from the rooting medium to the plant is modified by the addition of salt, a variety of changes in the internal water status of the plant occur. If the osmotic potential of the rooting medium is decreased abruptly by adding NaCl to the solution, the leaf water potential, in this case of barley seedling, falls rapidly but leaf osmotic potential decreases at a slower rate (Figure 29). As a consequence turgor potential of the leaves falls rapidly and the leaves wilt severely. After 12 hours exposure to the saline medium, however, the rate of decline in leaf osmotic potential increases to exceed the rate of decline in water potential so that there is a recovery of leaf turgor. 48 hours after the initial exposure to the saline medium, the turgor potential is apparently restored to that of plants grown without salinity stress

(Figure 30). The decline in internal osmotic potential may be due in part to internal osmotic adjustment, but undoubtedly uptake of Na^+ and Cl^- ions is also involved (Slatyer, 1961).

In contrast to this response to a rapid decline in the osmotic potential of the rooting medium, where the potential of the medium bathing the roots was reduced gradually by -1 bar per day for 9 days, leaf osmotic and water potentials fell in parallel with each other and the changes in the medium (Figures 32, 33). During this slow decline, and a following 6 days when the osmotic potential of the medium was held at -10 bars, leaf turgor potential was unchanged and plants remained fully turgid (Plate 1).

It appears that a gradual increase in the external salinity allows internal adjustment without dramatic changes in plant water status.

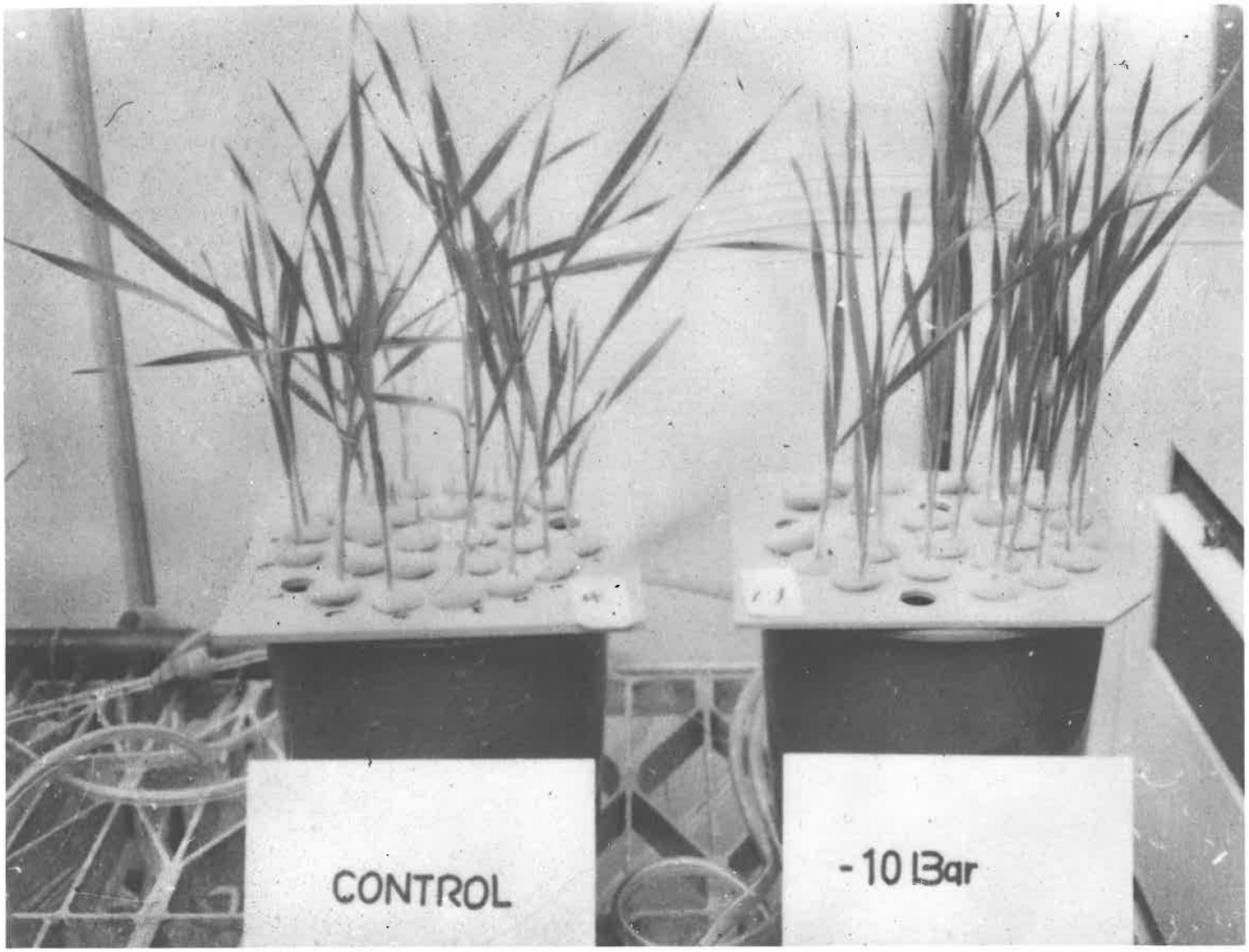
In both these cases, the estimates of leaf osmotic and turgor potential were derived from psychrometric measurements of frozen and re-thawed tissues (Ehlig, 1961). It has been suggested that such measurements are erroneous for tissue from plants exposed to salinity as they do not take account of salt located in cell walls (Oertli, 1966); such cell wall salt, external to the protoplast, would not take part in osmotic regulations. This would mean that the osmotic potential of the tissue estimated in this way would be more negative than the actual osmotic potential of the protoplast. Consequently the turgor potential would be less than that estimated by this means. It is conceded that such

PLATE 1

The effect of gradually decreasing the osmotic potential of the rooting medium on plant turgor.

Control - grown in Hoagland's solution

-10 bars - grown in -10 bars NaCl solution for 3 days.


CONTROL
-10 Bar

consequences would follow from any accumulation of salts in the cell walls, but the effect was unlikely to be large enough to alter the overall conclusions as visible evidence of lack of wilting (Plate 1) confirmed the conclusions drawn from the psychrometric estimation in every particular.

Although the effects of salinity on tissue water status appeared to be dependent on the rate at which the plant was exposed to the increase in salinity, proline accumulated in the plants in all circumstances. The threshold osmotic potential of NaCl in the rooting medium for the induction of proline accumulation was lower than -5 bars as no accumulation occurred in plants exposed to that potential (Figure 31). It was higher than -10 bars, however, as a significant concentration of proline accumulated in leaf tissue 12 hours after exposure to a NaCl solution of -10 bars, and proline continued to accumulate for at least 72 hours. Similarly, when ψ_π of the external medium was gradually reduced at a rate of -1 bar per day by adding NaCl, the plant commenced to accumulate proline when ψ_π of the rooting medium decreased to -6 bars. Proline continued to accumulate as the osmotic potential of the rooting medium was further reduced (Figure 34). In this situation, as with water stress, it is difficult to disentangle the effects of the continuing decline in tissue water status from those of the time of exposure to osmotic stress. It is evident, however, that in treatments in which the salinity stress was imposed gradually, a rapid rate of proline accumulation was maintained for a considerable period (Table 11).

TABLE 11

Rate of proline accumulation as a result of salinity

[Data obtained from Figures 31, 34 and 36.]

Osmotic potential Root medium (Bars)	Time interval (Days)	Gradual decline in osmotic potential		Rapid decline in osmotic potential
		1st leaf	2nd leaf	1st leaf
		Rate of proline accumulation (µg/g/hr)		
0	0-1	-	-	
-1	1-2	1.0	0.4	
-2	2-3	9.2	4.6	
-3	3-4	-	0.8	
-4	4-5	7.1	5.8	
-5	5-6	18.3	31.7	
-6	6-7	28.8	40.0	
-7	7-8	17.9	39.6	
-8	8-9	37.5	37.5	
-9	9-10	66.7	64.6	
-10	10-11	52.5	73.3	68.3
-10	11-12	53.3	56.6	168.3
-10	12-13	73.3	62.8	210.0
-10	13-14	-	11.2	
-10	14-15	-	34.6	
-10	15-16	54.6	24.6	

Indeed, in the second leaf accumulation continued for some 10 - 11 days with only a moderate decline in rate towards the end of the period. With the first leaf, accumulation virtually ceased after 7 days, a response which appeared to be related to the rapid visible senescence of the leaf at that time. The rates of proline accumulation associated with a gradual decline in osmotic potential of the rooting medium were not maximal for the tissue, however. Plants which were exposed suddenly to a salinity of -10 bars osmotic potential, accumulated proline at a maximal rate of at least 3 times higher than plants exposed to salinity stress gradually. Several explanations are possible for this difference in rate in the two situations. Firstly, the slow induction of salinity stress may allow time for osmotic and metabolic adjustment within the plant tissues which would reduce the metabolic impact of the stress. Secondly, it has been shown (Figure 40) that Na^+ or Cl^- ions inhibit proline accumulation; where stress was induced gradually, NaCl uptake might occur over an extended period until the ions within the plant tissues reached inhibitory concentrations. In plants exposed to -10 bars osmotic potential NaCl solution directly, however, the consequent change in tissue water status may initiate proline accumulation before any substantial NaCl uptake had taken place (Meiri and Poljkoff-Mayber, 1969).

The data from these experiments on the effects of salinity may be used to explore the more general question of what factor in the tissue

initiates proline accumulation in both water and salinity stress. It has already been suggested that this cannot be a specific effect of the ions (Na^+ of Cl^-) involved, and indeed these are more probably inhibitory. At comparable osmotic potentials, plants exposed to NaCl invariably accumulated proline less rapidly than plants exposed to polyethylene glycol (Figure 40), which does not penetrate the plant as readily.

The alternative explanation for these effects of salinity is that the accumulation of proline is a consequence of the change in water potential of the solution bathing the roots, rather than a response to the ions producing the change in potential. A change in external water potential will result in a change in plant tissue water potential, which presumably induces proline accumulation. Tissue water potential can be partitioned into the component forces which together determine the Gibbs free energy of water in the tissue (Warren Wilson, 1967):

$$\psi = (\psi_\pi + \psi_m) + \psi_p$$

It is difficult to separate $\Psi_\pi + \psi_m$ (Barrs, 1968) and this is not attempted in this study, the method of measurement effectively combined the two parameters; but ψ_p can be distinguished from the other two. In plants subjected to a major change in the salinity of the rooting medium without gradual acclimatization, turgor decreased rapidly at first but then recovered, presumably as osmotic adjustment took place (Figure 30).

Proline accumulation, however, accelerated with time and the rate was maximal when turgor had recovered. Similarly, plants introduced to salinity in a gradual manner maintained turgor throughout, as judged both visually and by the psychrometric measurements and yet proline accumulated rapidly (Figures 31, 32). It appears, therefore, that here proline accumulation is not controlled by turgor.

This conclusion is in conflict with several reports in which it has been suggested that turgor is the major determinant of metabolism. In particular, Greenway and Leahy (1972) concluded that a decrease of -10 bars in either ψ or ψ_π without a change in turgor had little effect on several metabolic processes in corn roots when ethylene glycol was used as the osmoticum. This apparent discrepancy may reflect a fundamental difference in the location or activity of the enzyme systems concerned in proline synthesis as compared with those considered by Greenway and Leahy (1972). Alternatively, the difference may be due primarily to differences in technique. Greenway and Leahy (1970, 1972) immersed tissue in ethylene glycol solutions. The σ (reflection coefficient) for ethylene glycol is less than 0.5 and it is regarded as a rapidly permeating osmoticum which quickly penetrates cell membranes. However, the water status of the tissues in Greenway and Leahy's experiment was not measured and the values of ψ and ψ_π in the cells might have been higher than expected. Furthermore, the influence of ethylene glycol itself on metabolism is not known, and it is conceivable that metabolism was influenced by this molecule.

Clearly, proline accumulation was uninfluenced by turgor (Figure 45) and other reasons for the response must be sought.

If specific ion effects and turgor changes are eliminated as causes of proline accumulation, tissue osmotic (together with matric) potential remains. Despite inconsistencies due to sampling, previous history of the tissue and the passage of time there is a general relationship between the rate of proline accumulation and the osmotic potential of the leaf concerned (Figure 46), which suggests that for barley plants there is a threshold value of -11 to -12 bars, above which proline does not accumulate, and a negative relationship between accumulation rate and tissue osmotic potential down to -20 bars at least.

Kramer (1969) has pointed out that metabolism might be affected directly by the free energy of water, and that changes in the Gibbs free energy of water within the cell could act directly on membranes or enzyme systems and result in changes in certain aspects of metabolism. Changes in the free energy of water in the cell could result in changes in the aqueous environment immediately surrounding enzymes or next to membranes (Klotz, 1958), or relatively small changes in osmotic potential could cause marked changes in protein structure and enzyme activity (Chen *et al.*, 1964).

Proline accumulation in the salt-stressed plant thus appears to be dependent on changes in $(\psi_\pi + \psi_m)$ rather than ψ_p or salt accumulation.

FIGURE 45

Relationship between proline accumulation rate and turgor potential of salt stressed plant. [Data obtained from slow salinization experiments, Figure 34 and Figure 36.]

● First leaf of barley plant

○ Second leaf of barley plant

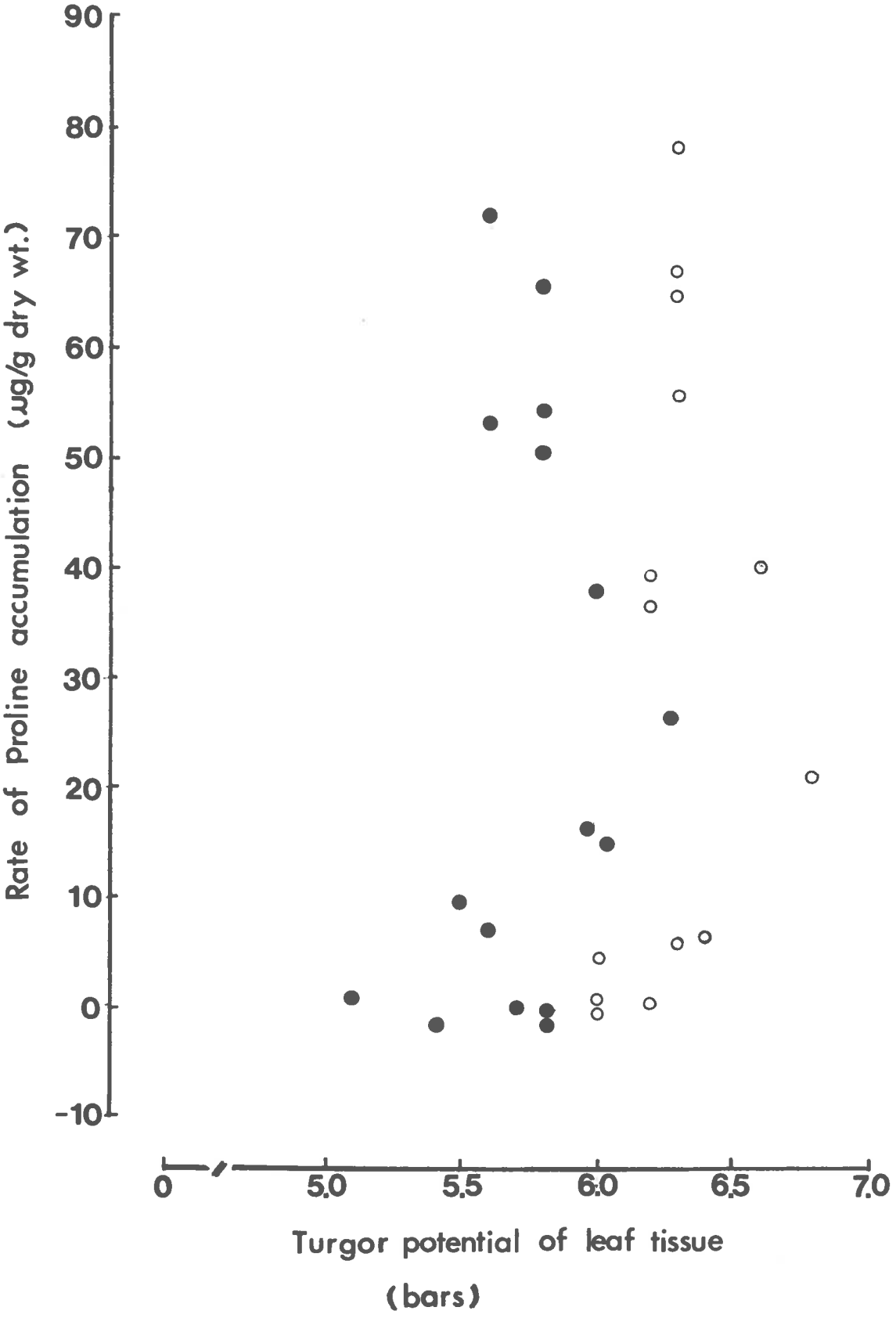
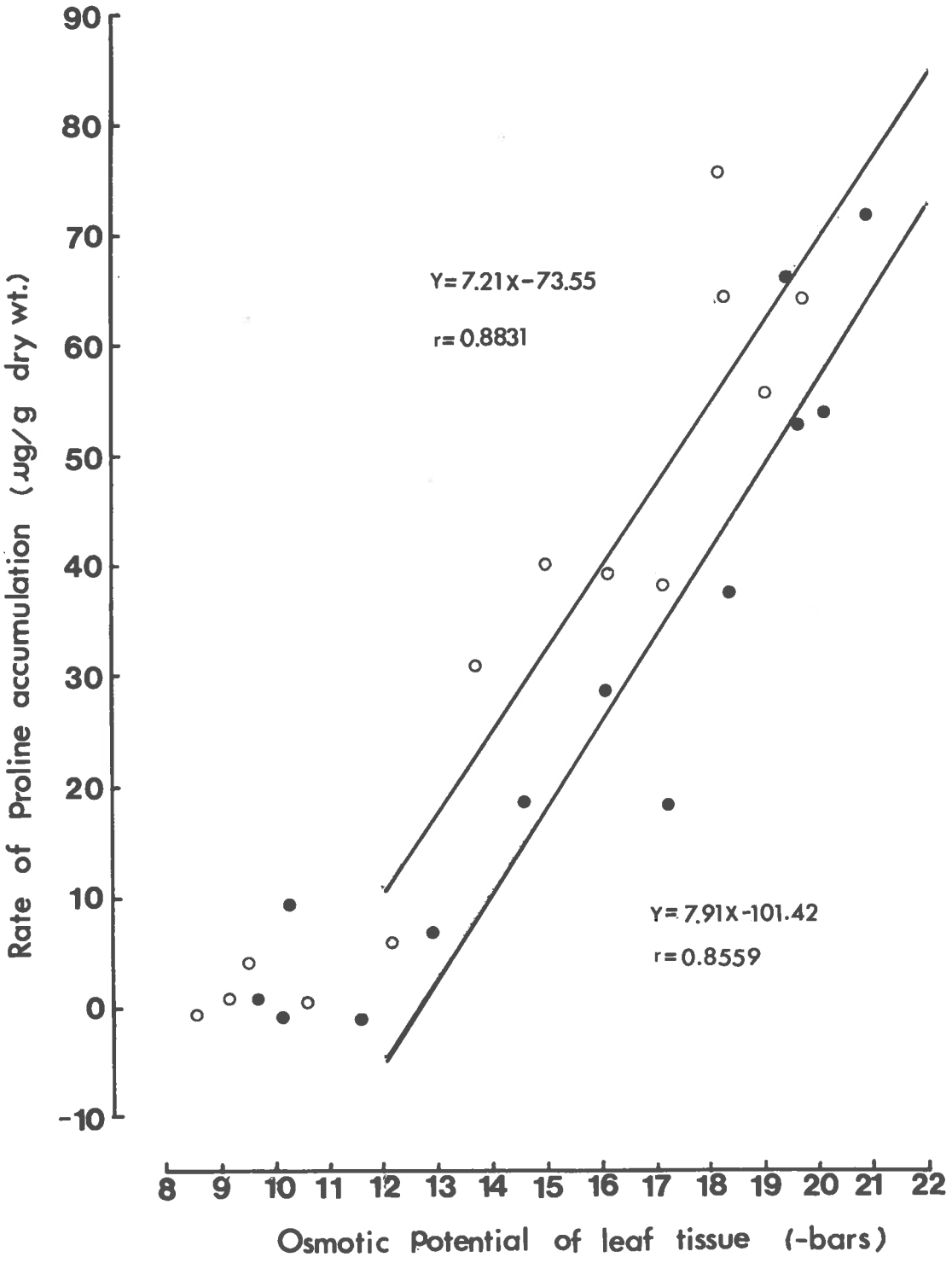

90
80
70
60
50
40
30
20
10
0
-10
Rate of Proline accumulation (µg/g dry wt.)
0
5.0
5.5
6.0
6.5
7.0
Turgor potential of leaf tissue
(bars)

FIGURE 46

Relationship between proline accumulation rate and osmotic potential of salt stressed plant.
[Data obtained from slow salinization experiments, Figure 34 and Figure 36. Regression line calculated only between -12 bars and -20 bars of ψ_π.]

● First leaf Y = -101.42 + 7.91x r = 0.8559

○ Second leaf Y = - 73.55 + 7.21x r = 0.8831


Rate of Proline accumulation (µg/g dry wt.)
90
80
70
60
50
40
30
20
10
0
-10
Y= 7.21X-73.55
r= 0.8831
Y= 7.91X-101.42
r= 0.8559
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Osmotic Potential of leaf tissue (-bars)

The plant absorbs excess ions from the bathing solutions in these circumstances but the elevated Na^+ and Cl^- concentration in the plant appears to inhibit rather than enhance proline accumulation (Figure 40). This inhibition becomes more evident when the plant is exposed to a severe salinity stress. Plants transferred directly to -20 bars NaCl solution, initially accumulated much more proline than those transferred to -10 bars, but after 48 hours the accumulation of proline slowed and the final concentration of proline attained in plants at -20 bars was less than at -10 bars (Figure 44), although the water potential of the plant at -20 bars was lower than that at -10 bars (Figure 43).

Other salts can also be used to lower the osmotic potential of the external solution and hence induce proline accumulation, but the rate of accumulation was considerably affected by the specific ions used. If the accumulation of proline in barley growing in polyethylene glycol solution is used as a measure of potential accumulation rate, it is apparent that K^+, Ca^{++}, and $SO_4^=$ ions also inhibit proline accumulation to varying degrees, as lowering the osmotic potential of the rooting medium with KCl, $CaCl_2$ or Na_2SO_4 results in less proline accumulation. On the other hand, there was no significant difference between $MgCl_2$ and PEG treatment except at the lowest concentration of osmoticum (-5.7 bars) where plants exposed to $MgCl_2$ accumulated more proline. This response to divalent cations rather than monovalent cations was more evident in the results of experiments with excised leaves, where

both Mg^{++} and Ca^{++} salts consistently allowed more proline accumulation than equivalent monovalent cationic salts (Figures 41, 42). These differences in response to stress induced by different ions were not due to any difference in internal water status (Figure 38) and were presumably due to specific ion effects, which directly or indirectly affect proline formation or utilization processes. Proline accumulation occurs primarily as a result of *de novo* synthesis and proteolysis, and proline is utilized in protein synthesis and oxidation reactions (Stewart, 1972a). Salinity stress has been reported to enhance proteolysis (Strogonov, 1964) and inhibit protein synthesis (Ben-Zioni, Itai and Vaadia, 1967; Itai, Richmond and Vaadia, 1968). The enzymic pathways of proline synthesis and degradation in higher plants has not been elucidated in detail but it is possible that certain enzyme steps are sensitive to specific ions. High concentrations of Na^{+} or NH_4^{+} reduced the incorporation of ^{14}C-glutamic acid into proline in the tobacco leaf (Noguchi *et al.*, 1966) but, on the other hand, one of the enzymes which convert proline to glutamic acid, Δ^{1}-pyrroline-5-carboxylate (P-5-C) dehydrogenase is strongly inhibited by 0.2 M NaCl or KCl, but not by 0.2 M K_2SO_4 or Na_2SO_4 (Boggess and Stewart, 1974). As inhibition of this enzyme would favour proline accumulation it would not appear that this may be the step responsible for the ionic inhibition of accumulation. Increased concentrations of specific ions may affect many enzyme systems, several of which have been shown to be sensitive

to ψ_π, salt concentration or specific ions (Miller and Evans, 1956; Hackett, 1961; Honda and Muenster, 1961). Elucidation of the mechanism of the ion inhibition of proline accumulation reported here must await further experimentation.

Section IV. Comparison of Plant Responses to Different Environmental Stresses

1. Introduction

In the previous sections, it has been shown that barley and radish plants exhibit a common metabolic response, proline accumulation, to a variety of environmental stress situations. It has been demonstrated for one of these stress situations, low temperature, that the response is not due to an effect on plant water relations. Accordingly, it is of interest to explore the features of metabolism, including proline metabolism in various stress situations, to determine how closely the metabolic changes elicited by disparate stress situations are related. Such comparisons are possibly hampered in the experiments reported so far in that different cultural conditions were employed for the different types of stress (i.e. Perlite medium for water stress, sand for low temperature stress and water culture for salinity stress). Initially, the plant growth, water status, chlorophyll content and proline content of plants exposed to water, cold or salt-stress was re-examined using a standard water culture technique. The comparison was then extended further by examining changes in free amino acid concentrations in response to the stress and by exploring the metabolism of supplied ^{14}C-glutamate and ^{14}C-proline. Heat stress was omitted from the comparisons as it was demonstrated earlier (page 94) that in this case proline accumulation was related to leaf water status.

2. Response of plant growth, water status, chlorophyll and free proline content of barley and radish to different environmental stresses.

2.1 Methods

Barley (cv. Prior) or radish (cv. Long White Icicle) seeds were germinated in a 9 cm petri dish for 24 hours at 20°C in the dark. 24 uniform pre-germinated seeds were then transferred to a 19 cm petri dish with two sheets of wet filter paper and grown for a further 4 days at 25°C in the dark before transplanting into the water culture. The general water culture practices and the design of the experiments have been described earlier (III. Material and Methods, page 64). The seedlings were raised in a controlled environment cabinet at 20 ± 1°C with a fluorescent radiant flux intensity of 4.1 mW cm^{-2}, using a 16 hours photoperiod. Barley plants were grown in water culture for one week and radish plants for 13 days before treatments were applied.

At the start of the treatment, in the salinity or water stress, the culture solution in the root medium was immediately replaced with NaCl solution or polyethylene glycol solution of -10 bars osmotic potential (including base nutrient solution of -0.7 bars). In the chilling stress, the seedlings were transferred to a cold room at 5 ± 1°C. All other environmental factors remained constant.

The first leaf of barley or radish was sampled at 12 hour intervals following imposition of different environmental stresses, immediately

plunged into liquid nitrogen and stored at -20°C for chlorophyll and proline content assay. Water and osmotic potential of the first leaf of both species were measured by a Spanner thermocouple psychrometer (Barrs, 1968). ψ was measured on the fresh tissue, which was then placed in liquid nitrogen for 1 minute to rupture the cells. Water in the tissue was then affected only by solute potential which was measured by returning the frozen and thawed tissue to the psychrometer. Turgor potential was estimated from the difference in magnitude between ψ and ψ_π. Plant growth was determined from elongation and fresh and dry weight measurements on the total plant. The technique used is described earlier in III. Material and Methods (see page 63). The replications for each parameter measurement were changed from one to another and are shown in the figures.

2.2 Results

(1) Total plant height

Elongation of the plant, as measured by the total plant height, was rapidly and completely inhibited when the plant was subjected to each of the different environmental stresses, chilling, water and salinity (Figure 47). This response was identical in both barley and radish and in contrast, the non-stressed plants grew 9 cm (barley) and 2 cm (radish) in the 72 hours of the experiment.

(2) Fresh and dry weight

Although each of the environmental stress situations immediately and completely inhibited further increase in plant height, the response

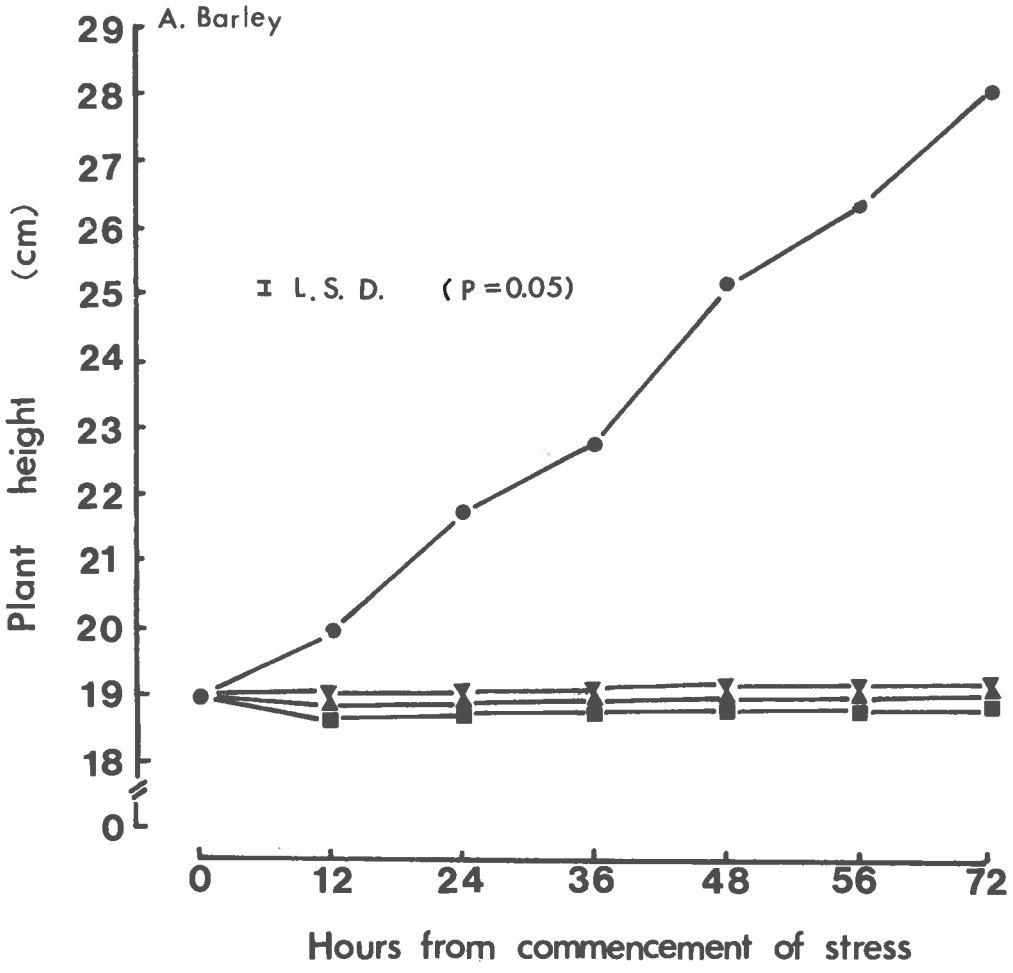
FIGURE 47

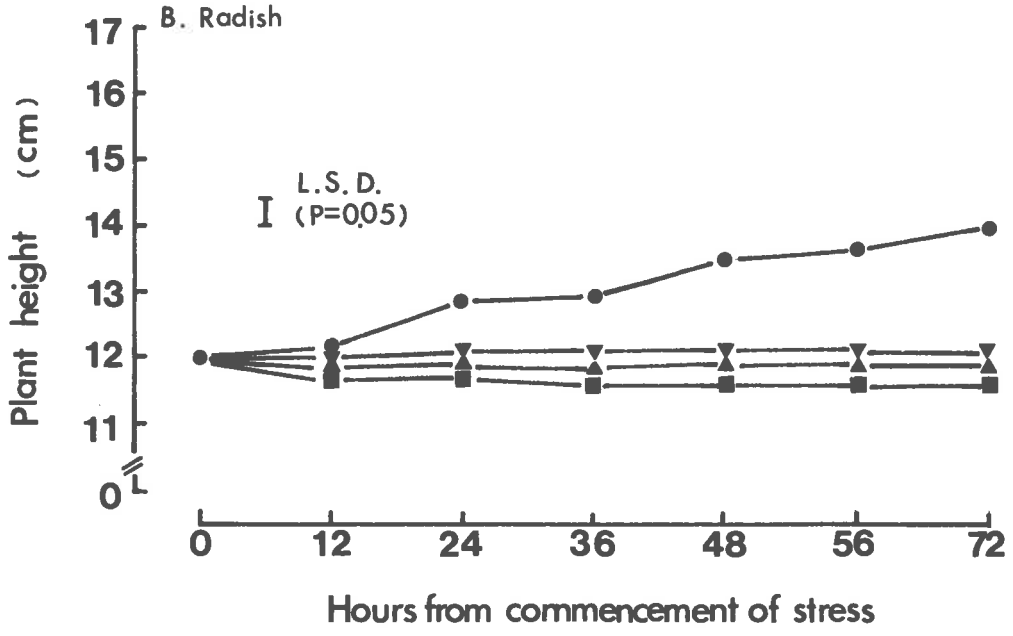
Height of barley and radish plants in different environmental stresses. Each value is the mean of five replicates.
[12 day old barley or 18 day old radish plants growing in standard water culture were subjected to salinity stress (NaCl, -10 bars osmotic potential), water stress (PEG, -10 bars osmotic potential) or low temperature stress (5°C).]

A. Barley

B. Radish

●	Nutrient solution	(ψ_π = -0.7 bars, 20°C)
▼	Low temperature stress	(ψ_π = -0.7 bars, 5°C)
▲	NaCl solution	(ψ_π = -10 bars, 20°C)
■	Polyethylene glycol solution	(ψ_π = -10 bars, 20°C)


A. Barley
Plant height (cm)
29
28
27
26
25
24
23
22
21
20
19
18
0
I L. S. D. (P = 0.05)
0
12
24
36
48
56
72
Hours from commencement of stress


B. Radish
Plant height (cm)
17
16
15
14
13
12
11
0
I L.S.D. (P=0.05)
0
12
24
36
48
56
72
Hours from commencement of stress

of plant fresh and dry weight increase differed between the various stresses (Figure 48). Fresh weight increase in the barley plant was immediately inhibited by the onset of a water deficit and there was a progressive decrease in the fresh weight as the stress continued for 48 hours; thereafter, the fresh weight of the plant remained constant. In neither of the other stress situations, salinity and low temperature, did the fresh weight of the plant actually decrease. In both cases, however, there was no significant further increase in fresh weight after the stress commenced.

The dry weight of plants grown in nutrient solution increased exponentially throughout the experiment with a mean relative growth rate of 0.29 mg $mg^{-1}day^{-1}$ (Figure 48B). Plants transferred to a temperature of $5^{o}C$, continued to grow at the same relative growth rate for the first 12-24 hours but thereafter the relative growth rate declined to 0.10 mg mg^{-1} day^{-1} in the last 24 hours. In the salt-stressed plants production of dry weight was considerably reduced immediately upon imposition of the stress. During the first 48 hours the mean relative growth rate was 0.04 mg mg^{-1} day^{-1} but then it increased rapidly to a rate of 0.18 mg mg^{-1} day^{-1}. This renewal of growth in salt-stressed plants coincided with their regaining full turgor (Figure 52). Water deficit, on the other hand, completely inhibited dry weight increase, there being no significant increase in dry weight in the 72 hours of the experiment. These plants did not regain turgor (Figure 52). It is apparent that plant response

FIGURE 48

Changes in the fresh weight and dry weight of barley plants subjected to different environmental stresses. Each value is the mean of five replicates.
[Barley plants growing in a standard water culture for 7 days were transferred to -10 bars NaCl solution, -10 bars PEG solution or 5°C low temperature for 1-3 days.]

A. Fresh weight

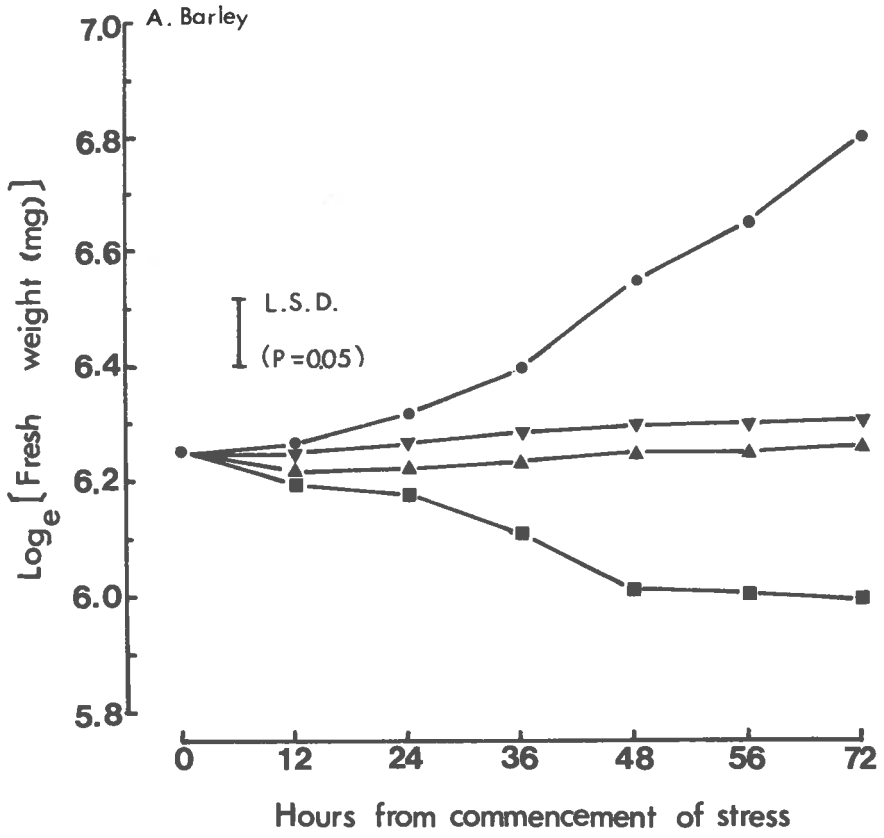
B. Dry weight

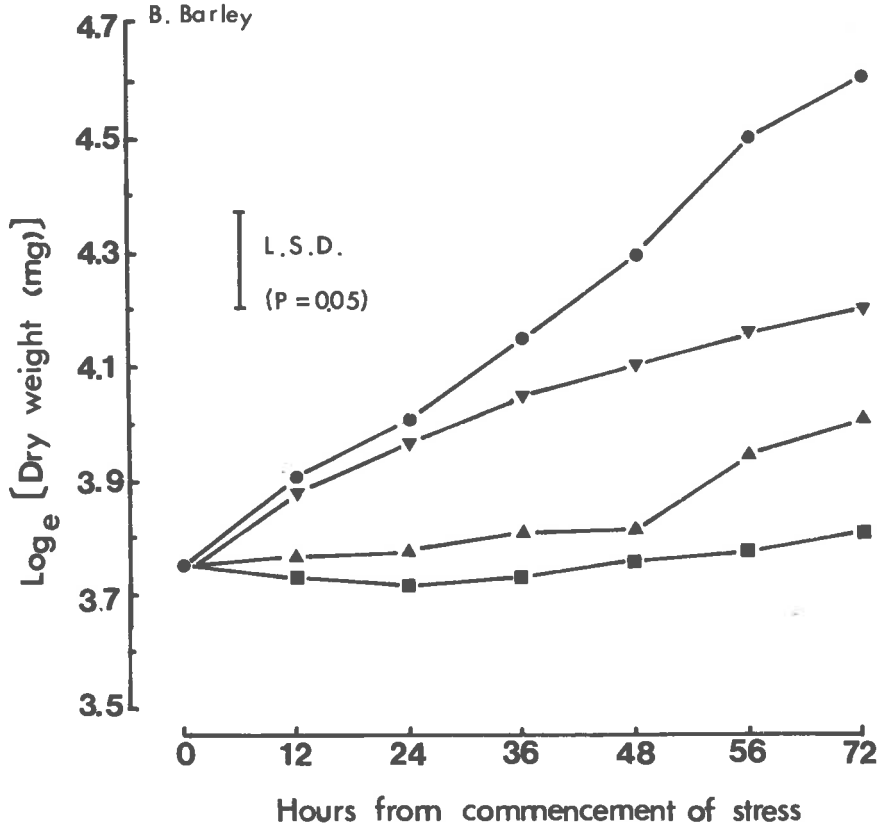
● Nutrient solution (ψ_π = -0.7 bars, 20°C)

▼ Low temperature stress (ψ_π = -0.7 bars, 5°C)

▲ NaCl solution (ψ_π = -10 bars, 20°C)

■ Polyethylene glycol solution (ψ_π = -10 bars, 20°C)


A. Barley
7.0
6.8
6.6
6.4
6.2
6.0
5.8
Log e [Fresh weight (mg)]
L.S.D.
(P = 0.05)
0
12
24
36
48
56
72
Hours from commencement of stress


B. Barley
4.7
4.5
4.3
4.1
3.9
3.7
3.5
Log e [Dry weight (mg)]
L.S.D.
(P = 0.05)
0
12
24
36
48
56
72
Hours from commencement of stress

in these growth parameters varied considerably between the different stress situations. Fresh weight and plant height appeared more sensitive to stress than was growth in dry weight, but even there water deficit produced a more profound effect than either of the other stress situations.

(3) Chlorophyll content

The chlorophyll concentration in the first leaf of barley and radish plants growing at 20°C remained constant for the 72 hour experimental period (Figure 49). Exposure to a chilling temperature (5 ± 1°C) did not affect the chlorophyll concentration in either species, but there was a decline in the chlorophyll content of the first leaves of plants exposed to a water deficit or to a salinity stress. With barley, the decrease was greater in plants grown in polyethylene glycol solution than in those grown in an isosmotic solution of NaCl (Figure 49A). In the salt solution there was little change in chlorophyll concentration in the first 24 hours but, thereafter, the chlorophyll concentration declined at a constant rate. In water-stressed plants, on the other hand, the chlorophyll concentration declined from initiation of the stress, a significant change being observed in the first 24 hours. 72 hours after the water deficit was imposed, about 50% of the chlorophyll had disappeared from the first leaf. Radish plants responded similarly, although the magnitude of the changes was less than the barley plants (Figure 49B).

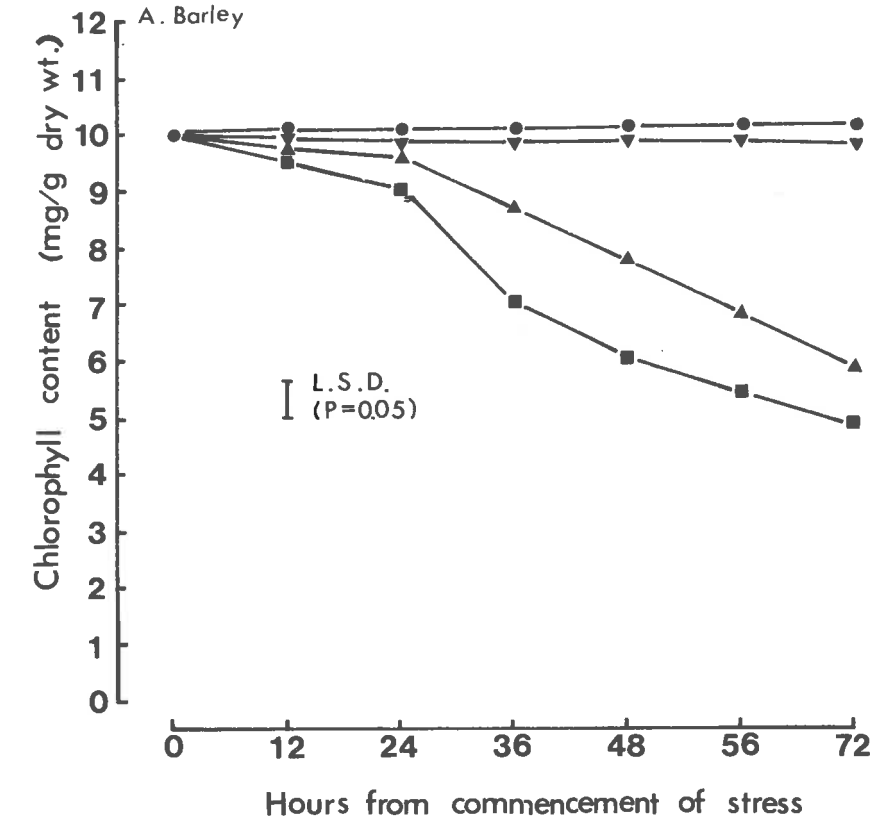
FIGURE 49

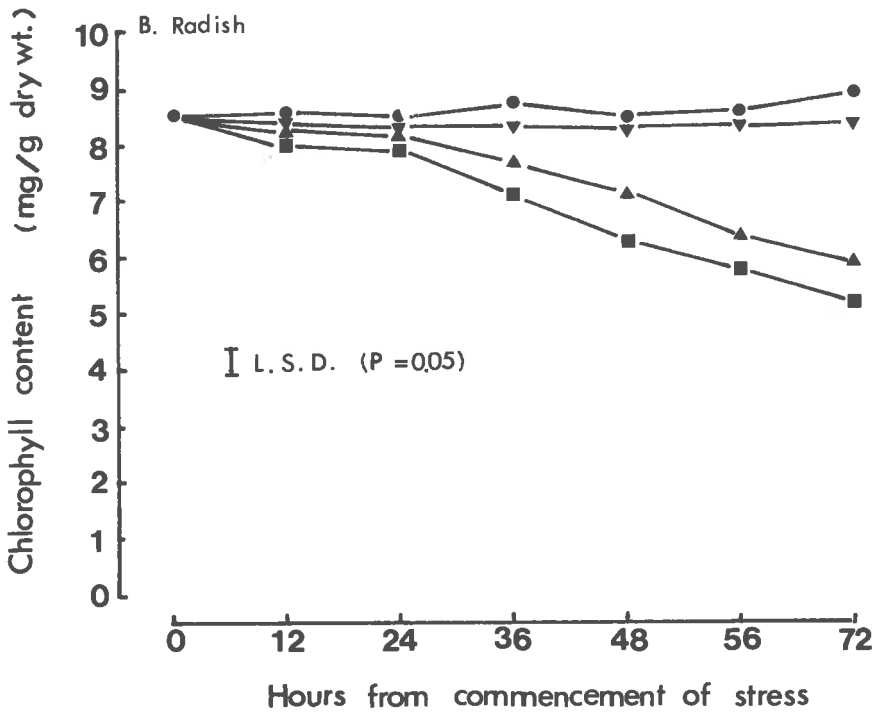
Changes in chlorophyll concentration in barley and radish plants subjected to different environmental stresses. Each value is the mean of three replicates. [Barley or radish plants were grown in a standard water culture for 7 or 13 days before being transferred to NaCl solution (ψ_π = -10 bars), PEG solution (ψ_π = -10 bars) or 5°C for 1-3 days.]

A. Barley

B. Radish

●	Nutrient solution	(ψ_π = -0.7 bars, 20°C)
▼	Low temperature stress	(ψ_π = -0.7 bars, 5°C)
▲	NaCl solution	(ψ_π = -10 bars, 20°C)
■	Polyethylene glycol solution	(ψ_π = -10 bars, 20°C)


A. Barley
Chlorophyll content (mg/g dry wt.)
0
1
2
3
4
5
6
7
8
9
10
11
12
L.S.D.
(P=0.05)
0
12
24
36
48
56
72
Hours from commencement of stress


B. Radish
Chlorophyll content (mg/g dry wt.)
0
1
2
3
4
5
6
7
8
9
10
L. S. D. (P =0.05)
0
12
24
36
48
56
72
Hours from commencement of stress

(4) Water potential

In barley, the water potential of the first leaf of plants growing at 20 or 5°C fluctuated between -3 and -5 bars with no consistent effect of the low temperature (Figure 50A). This confirms the previous observation (see page 120). A similar situation existed in the radish plants where temperature also had no effect on water potential (Figure 50B).

Leaf water potential declined rapidly in the 24 hours following exposure to water or salinity stress but thereafter, there was little further decline in leaf water potential in the salt-stressed plants. At the end of the experiment (72 hours) leaf water potential had declined to -19 bars in barley and -16 bars in radish in the salinity treatments. In contrast, the leaf water potential continued to decline uniformly over the whole period in plants subjected to polyethylene glycol solution in the rooting medium; leaf water potential was -30 bars in barley and -36 bars in radish at 72 hours.

(5) Osmotic potential

Chilling stress had little effect on the osmotic potential of the first leaf of either species (Figure 51). In water or salinity stress, however, leaf osmotic potential declined with time. In barley, the rate of change of osmotic potential in the salt-stressed plant was similar to that in the water-stressed plant in the first 48 hours, but thereafter the rate of decline in the salt-stressed decreased whereas the osmotic potential of the water-stressed plant continued to decline at the same

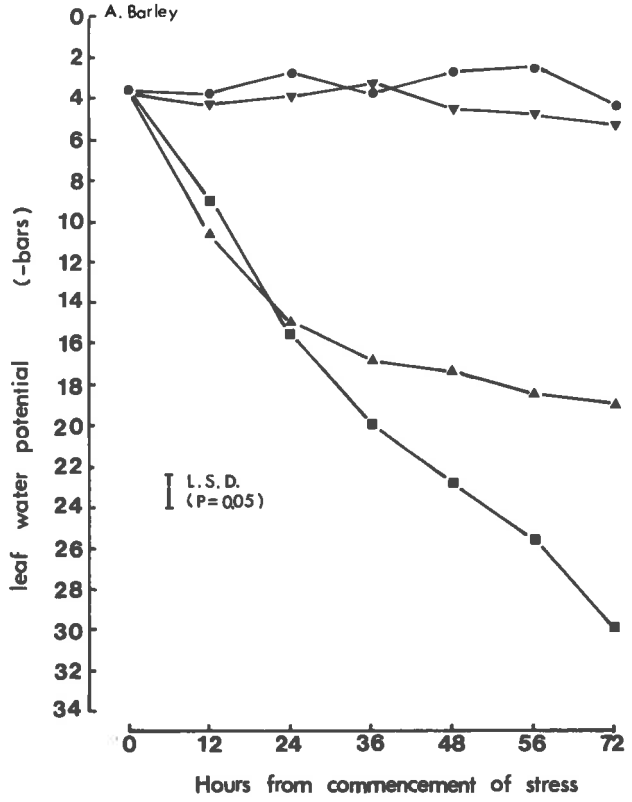
FIGURE 50

The water potential of the first leaf of barley or radish plants subjected to different environmental stresses. Each value is the mean of three replicates.

A. Barley

B. Radish

●	Nutrient solution	(ψ_π = -0.7 bars, 20°C)
▼	Low temperature stress	(ψ_π = -0.7 bars, 5°C)
▲	NaCl solution	(ψ_π = -10 bars, 20°C)
■	Polyethylene glycol solution	(ψ_π = -10 bars, 20°C)


A. Barley
leaf water potential (-bars)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
L.S.D.
(P=0.05)
0
12
24
36
48
56
72
Hours from commencement of stress

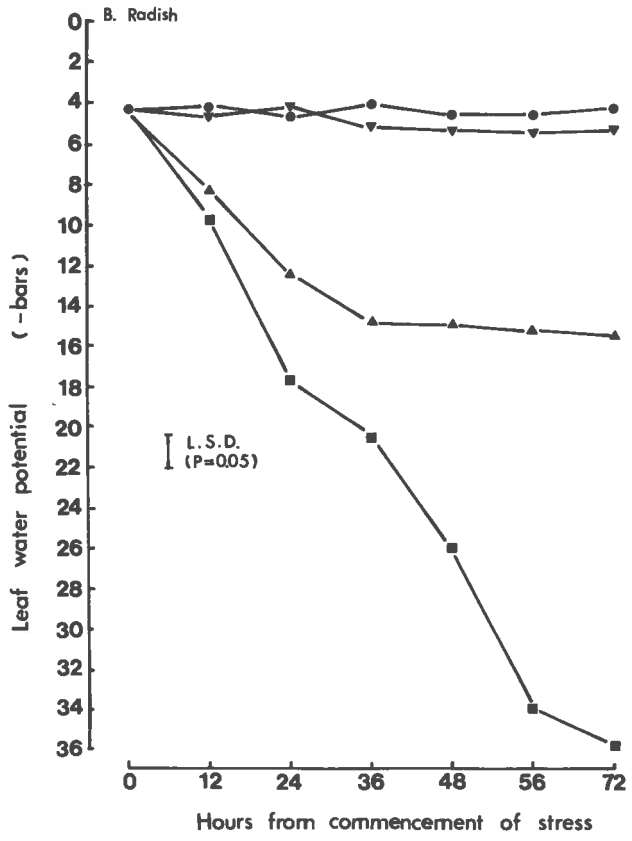

B. Radish
Leaf water potential (-bars)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
L.S.D.
(P=0.05)
0
12
24
36
48
56
72
Hours from commencement of stress

FIGURE 51

The osmotic potential of the first leaf of barley or radish plants subjected to different environmental stresses. Each value is the mean of three replicates.

A. Barley

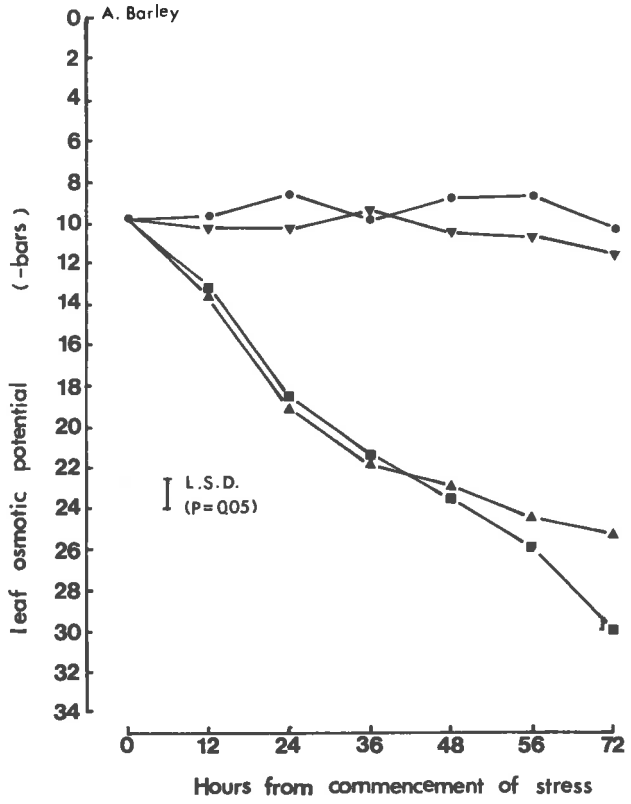
B. Radish

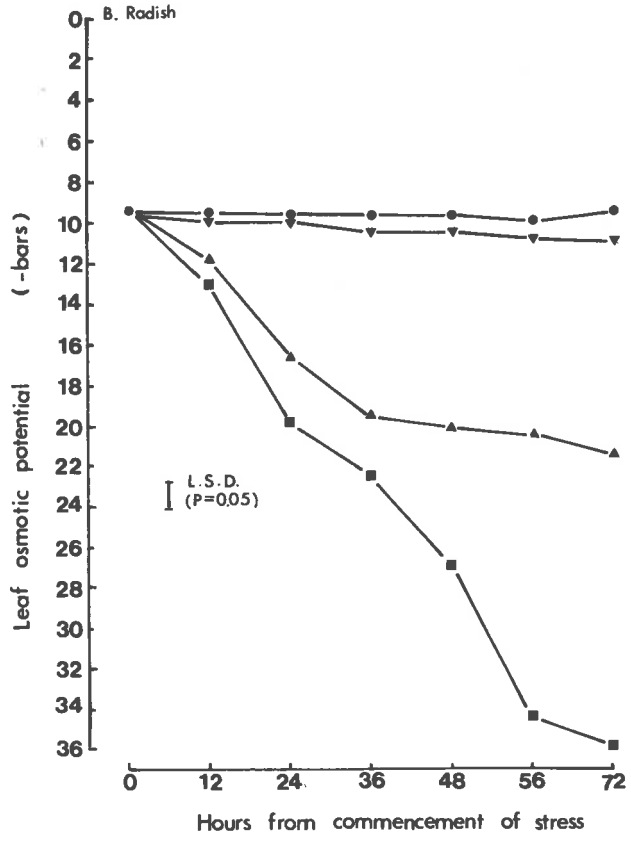
● Nutrient solution (ψ_π = -0.7 bars, 20°C)

▼ Low temperature stress (ψ_π = -0.7 bars, 5°C)

▲ NaCl solution (ψ_π = -10 bars, 20°C)

■ Polyethylene glycol solution (ψ_π = -10 bars, 20°C)


A. Barley
Leaf osmotic potential (-bars)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
L.S.D.
(P=0.05)
0
12
24
36
48
56
72
Hours from commencement of stress


B. Radish
Leaf osmotic potential (-bars)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
L.S.D.
(P=0.05)
0
12
24
36
48
56
72
Hours from commencement of stress

rate. In the radish plant (Figure 51B), this difference in response was more evident, the rate of decline in osmotic potential in the water-stressed plant was always greater than that in salt-stressed plants. After 36 hours, however, the osmotic potential in the salt-stressed plants reached equilibrium.

(6) Turgor potential

Chilling stress again had little effect on the turgor potential of both species (Figure 52), the turgor potential of chilled-plants fluctuated between 5.5 and 6.5 bars in barley and 4.5 and 5.5 bars in radish. In the salt-stressed plants, in the first 12 hours the decline in osmotic potential was less than that in water potential and consequently there was a reduction in the turgor potential. Turgor potential fell by 3 bars in barley and 2 bars in radish in this period, but subsequently the leaf osmotic potential fell more rapidly than did water potential with a consequent recovery of leaf turgor. Within 48 hours the turgor potential had returned to the original value in both species. These changes in turgor potential were paralleled by visible changes in wilting in the plants.

In contrast, the turgor potential of water-stressed plants decreased progressively as water stress continued in both species. Zero turgor was reached in the barley leaf at 56 hours after stress initiation and at 36 hours in radish. Zero turgor occurred at a ψ of -30 bars and of -26 bars in barley and radish respectively. This suggests that the leaves of radish wilt at a higher ψ than leaves of barley.

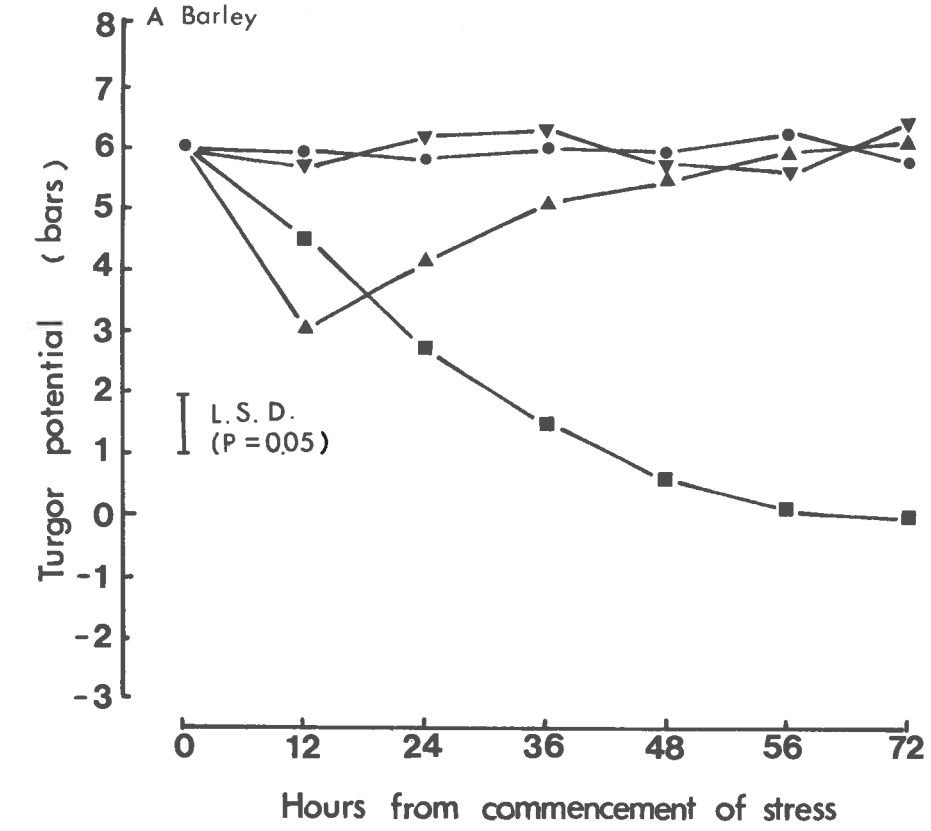
FIGURE 52

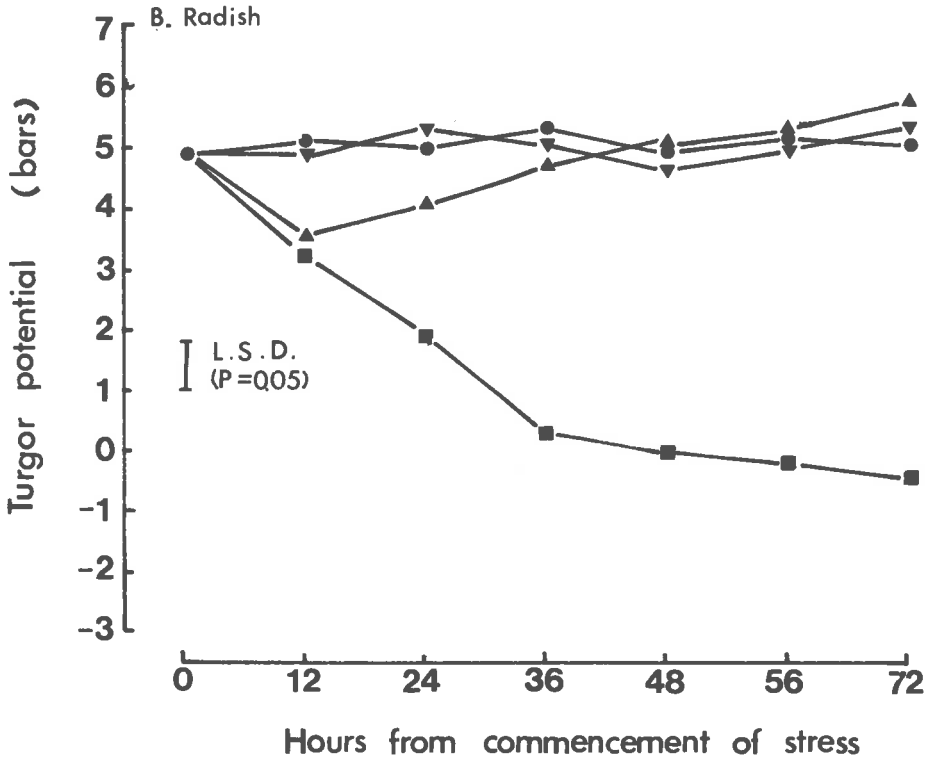
Change of turgor potential in the first leaf of barley and radish plants subjected to different environmental stresses. Each value is the mean of three replicates. [Turgor potential of the first leaf of barley and radish calculated from the difference between water and osmotic potentials, data taken from Figure 50 and Figure 51.]

A. Barley

B. Radish

●	Nutrient solution	(ψ_π = -0.7 bars, 20°C)
▼	Low temperature stress	(ψ_π = -0.7 bars, 5°C)
▲	NaCl solution	(ψ_π = -10 bars, 20°C)
■	Polyethylene glycol solution	(ψ_π = -10 bars, 20°C)


A Barley
Turgor potential (bars)
8
7
6
5
4
3
2
1
0
-1
-2
-3
L.S.D.
(P = 0.05)
0
12
24
36
48
56
72
Hours from commencement of stress


B. Radish
Turgor potential (bars)
7
6
5
4
3
2
1
0
-1
-2
-3
L.S.D.
(P = 0.05)
0
12
24
36
48
56
72
Hours from commencement of stress

(7) Proline accumulation

All environmental stresses used in this experiment, i.e., chilling, water and salinity stress, induced proline accumulation in both species (Figure 53). However, the same plant exhibited considerable differential proline accumulation potential in response to various environmental stresses. In both species, water stressed plants accumulated the greatest amount of proline and chilling stressed plants the least, salinity stressed plants being intermediate between the two extremes. 72 hours after initiation of stress, the free proline content in barley leaf tissue had reached 4.9 mg per gram dry weight in the chilling treatment, 11.0 mg in the salinity treatment and 15.8 mg in the water stress treatment. This suggested a mean rate of proline accumulation over the period of 68, 153 and 219 µg per gram dry weight per hour in the chilling, salinity and water stress treatments respectively. In radish, proline had accumulated to 2.2, 15.0 and 21.5 mg per gram dry weight in the chilling, salinity and water stress treatments respectively, giving mean rates of accumulation of 31, 208 and 299 µg per gram dry weight per hour in these treatments. In spite of the fact that the rate of proline accumulation differed considerably between the two species, the free proline accumulation increased with time in response to all these environmental stresses, and the relative effectiveness of the individual stresses on each species was similar.

A comparison of the proline accumulation potential of these two species in response to water stress (see page 92) has clearly shown that the radish plant has a higher proline accumulation potential than barley.

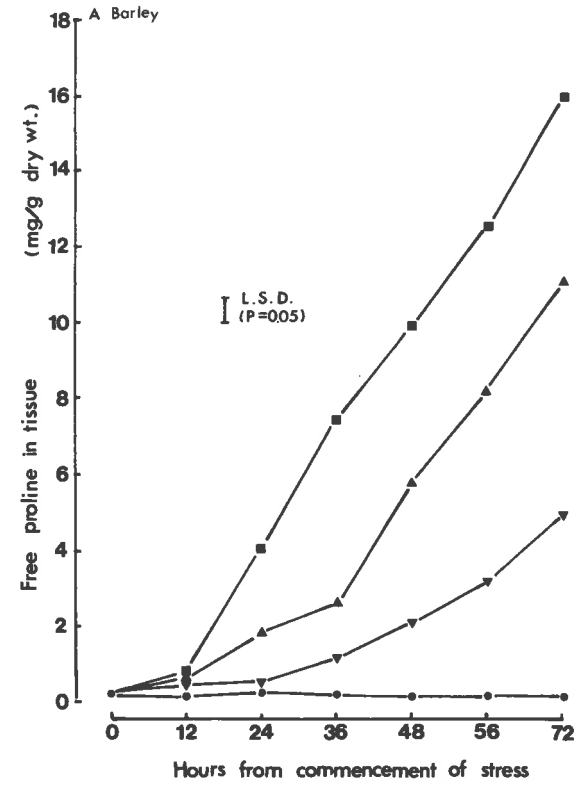
FIGURE 53

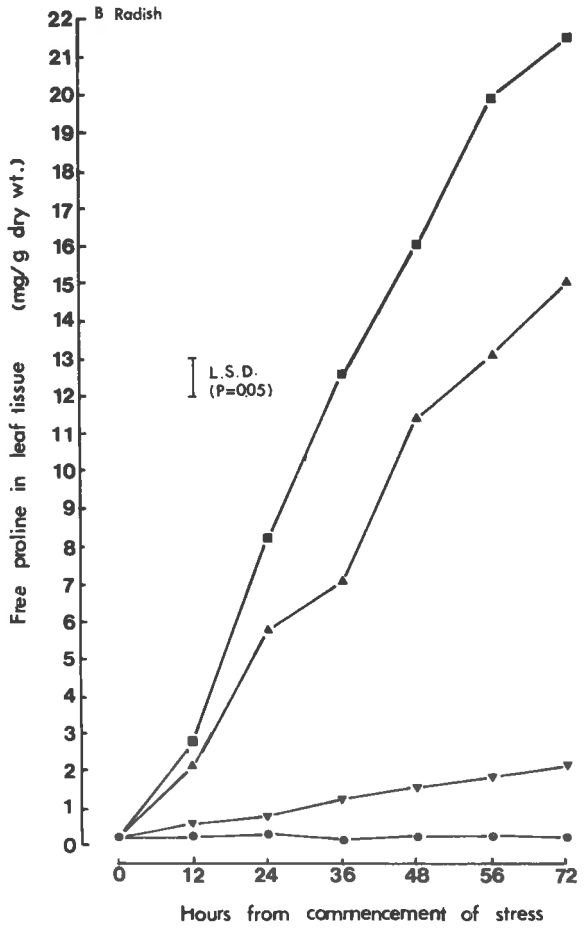
Accumulation of free proline in the first leaf of barley and radish plants exposed to different environmental stresses. Each value is the mean of three replicates.

A. Barley

B. Radish

●	Nutrient solution	(ψ_π = -0.7 bars, 20°C)
▼	Low temperature stress	(ψ_π = -0.7 bars, 5°C)
▲	NaCl solution	(ψ_π = -10 bars, 20°C)
■	Polyethylene glycol solution	(ψ_π = -10 bars, 20°C)


A Barley
Free proline in tissue (mg/g dry wt.)
L.S.D. (P=0.05)
0
2
4
6
8
10
12
14
16
18
0
12
24
36
48
56
72
Hours from commencement of stress


B Radish
Free proline in leaf tissue (mg/g dry wt.)
L.S.D. (P=0.05)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
0
12
24
36
48
56
72
Hours from commencement of stress

Radish again exhibited a higher proline accumulation potential than barley in response to both water and salinity stress in this experiment but was less responsive to chilling. 72 hours after initiation of a chilling stress, the free proline concentration in the leaves of barley was double that in the leaves of radish. This result indicates that proline accumulation potential not only depends on genetic factors by also varies with the external environment.

3. Free amino acid content of barley and radish in response to different environmental stresses

3.1 Methods

The materials used in this experiment for the assay of the amino acid content were different from those in the previous experiment. In this case the plants were grown in a particular medium, perlite, rather than in water culture for the water deficit, and in sand culture for the cold stress. 10 day old barley and 18 day old radish plants were subjected to stresses for 72 hours; water deficit was imposed by flooding the rooting medium with polyethylene glycol solution (Mwt. 4,000) of -10 bars osmotic potential. Chilling stress was imposed by transferring plants growing in sand culture at 20^{o}C to a 5^{o}C cold room and salinity stress was initiated by replacing the nutrient solution with a NaCl solution of -10 bars osmotic potential with plants growing in standard water culture. The procedure for assay free amino acid content has

been described in III. Materials and Methods (see page 72). The content of free amino acids in control plants is the mean of plants growing in three different cultural mediums.

3.2 Results

The concentrations of the various free amino acids present in barley and radish seedlings subjected to different environmental stresses are shown in Tables 12 and 13. The concentrations of the major free amino acids and of several minor amino acids were changed significantly by the environmental stress treatments. The total free amino acid content of barley leaves was increased 2.1, 4.4 and 2.5 folds as compared with control plants by chilling, salinity or water stress respectively. This increase was due almost entirely to increases in the free proline concentration (Table 12). Similarly, the total free amino acid content of radish leaves increased with stress, being 1.9, 2.6 and 2.3 times higher in plants subjected to chilling, salinity or water stress than in control plants. This increase was also due mainly to proline accumulation (Table 13).

The major change in proline concentration induced by stress was consistent in both species and all three stress situations, and, in comparison, changes in other amino acids were only minor. Proline was identified both by its characteristic R_f in the solvent system used and the distinctive colour reaction with ninhydrin.

Apart from these changes in proline concentration, there were much

TABLE 12

Free amino acids in the first leaf of barley plants subjected to different environmental stresses for 72 hours.

Amino acid	Control	Water stress	Cold stress	Salinity stress
		(µg g-1 dry weight)		
Alanine	205.1	190.2	884.3***	775.3***
Serine	340.0	189.6**	235.2*	557.9*
Glutamic acid	530.0	260.6***	385.7***	453.2**
Aspartic acid	366.6	152.8**	177.6**	181.6**
Arginine	83.7	123.6**	86.7	106.8*
Histidine	46.9	63.6	41.0	51.6
Lysine	52.0	67.8	46.1	58.3
Glutamine	311.7	395.9	284.9	436.4**
Asparagine	207.9	312.0	205.0	504.9***
Threonine	126.9	194.0*	203.7**	284.9**
Valine	151.4	218.0**	181.5	325.8**
Tyrosine	53.2	64.7	49.6	66.9*
Phenylalanine	34.2	103.4**	22.2*	121.9***
Methionine	32.9	39.2	30.7	37.4
Leucine	176.3	244.3	122.1	227.9**
Isoleucine	113.6	124.8	108.5	113.4
Ethanolamine	-	206.8***	-	323.0***
γ-Aminobutyric acid	32.5	55.5	37.9	42.5
½ Cysteine	190.4	186.5	196.4	220.7
Glycine	210.9	292.5**	237.3	312.7**
Proline	210.9	5102.3***	4099.9***	10232.5***
Total	3465.5	8588.1	7436.3	15444.6

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability
} Differences from control

TABLE 13

Free amino acids in the first pair of leaves of radish plants subjected to different environmental stresses for 72 hours.

Amino acid	Control	Water stress	Cold stress	Salinity stress
			(µg g-1 dry weight)	
Alanine	477.1	640.1***	791.3***	609.2***
Serine	774.4	708.0	849.5	667.9*
Glutamic acid	1278.8	887.1***	975.4*	891.5***
Aspartic acid	965.2	831.9*	830.7**	807.2**
Arginine	360.6	419.8	303.9*	447.0
Histidine	219.3	180.5	168.8**	189.2
Lysine	413.4	256.4	327.5**	251.9***
Glutamine	256.0	386.0	304.7	359.3
Asparagine	845.9	1096.6	947.0	1016.2
Threonine	538.3	713.3***	721.0**	752.1**
Valine	403.2	552.7**	586.1**	537.0**
Tyrosine	137.0	201.3	178.6*	174.8
Phenylalanine	117.0	238.8***	204.4***	215.7
Methionine	148.6	64.6**	70.8**	77.9
Leucine	411.0	477.9*	616.3**	467.1*
Isoleucine	156.3	236.9*	243.4***	236.2***
Ethanolamine	86.0	130.7**	126.3**	138.4***
γ-Aminobutyric acid	75.1	74.7	75.9	75.4
½ Cysteine	125.5	174.1	113.9	148.9
Glycine	345.8	387.9	264.7	433.4
Proline	351.1	10705.6***	1255.0***	13526.5***
Total	8535.6	19364.9	9855.2	22022.8

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability
} Differences from control

smaller changes in the concentrations of a number of other amino acids. Of these, it is significant that the concentrations of glutamic acid and aspartic acid fell in both species and all three stress situations. The concentrations of the corresponding amines, glutamine and asparagine, were not affected except in salt-stressed barley plants where their concentrations rose. Of the other amino acids, only threonine showed a consistent effect, its concentration increasing slightly in all situations. Valine and alanine concentrations also generally rose, although the increase was not always statistically significant. Leucine, isoleucine and ethanolamine concentrations also increased significantly in all three stress situations in radish but not as consistently in barley. Finally, serine and phenylalanine concentrations were environmentally labile in barley and, to a lesser extent, radish. Both the percentage and absolute changes in the pool sizes of the amino acids other than proline were relatively minor. The increase in proline concentration accounted for between 80 and 98% of the increase in free amino acids in all but radish plants at low temperature where it accounted for 68% of the increase.

4. Metabolism of ^{14}C-glutamic acid and ^{14}C-proline in barley leaf tissue subjected to different environmental stresses

4.1 Methods

Barley plants (cv. Prior) were grown in water culture in controlled environmental conditions until the first leaf was fully expanded (12 days).

The plants were then subjected to water, salinity or chilling stress. Water stress was imposed by replacing the culture solution with polyethylene glycol solution of -10 or -15 bars osmotic potential. In a parallel experiment, plants were subjected to chilling or salinity stress by transfer to a 5°C cold room with fluorescent lighting (4.1 mW cm^{-2} [illegible]) or by replacing the culture solution with a NaCl solution of -10 bars osmotic potential. All experiments were conducted on the first leaf of these plants; the feeding of ^{14}C compounds was carried out after 72 hours chilling or 36 hours in water or salinity stress conditions.

Uniformly labelled L-glutamic acid (glutamic acid -^{14}C(U)) and L-proline (proline -^{14}C(U)) was purchased from the Radiochemical Centre, Amersham, Bucks., England. Labelled glutamic acid was purified on Dowex-1-acetate before use (Morris and Thompson, 1962). All labelling experiments except the chilling stress were conducted in an isotope hood. The first leaf was carefully cut from the plant and placed in a small vial containing a 5 µl aliquot of tracer solution, containing 20 µCi of ^{14}C-glutamic acid or of ^{14}C-proline.

Leaves from plants subjected to water or salinity stress were maintained at 20°C for 2 hours. In general, the tracer solution was almost entirely absorbed into the leaf section within 10 minutes, and additional 100 µl aliquots of salt or PEG solution were then supplied to each leaf. In the case of the chilling stress, the leaf was incubated

at 5°C in the cold room under illumination of 4.1 mW cm^{-2} [illegible] light intensity for 2 hours. The tracer solution was completely absorbed into the leaf section in about 1.5 hours and, after that, 50 µl aliquots of distilled water were supplied.

Following incubation, the plant tissue was killed by immersion in liquid nitrogen for 1 minute. Amino acids were extracted by homogenizing 1.5 g wet weight of tissue in 5 ml of MCW (methanol: chloroform: water 12: 5: 3 v/v) at room temperature. The homogenate was centrifuged for 5 minutes and the supernatant was collected into another tube. The residue was shaken with a further 5 ml MCW for 5 minutes and centrifuged. The supernatants were combined and 3 ml water and 1 ml chloroform were added to break the stable emulsion formed during extraction. The supernatant then separated into two layers, the upper (methanol-water) phase was then removed and dried under pressure at 35°C.

The dry extract was taken up into 0.5 ml distilled water and spotted onto a thin-layer plate (20 x 20 cm) of cellulose (mixture of 15 mg cellulose and 90 ml of water, Macherey Nagel Co.). The amino acids were separated by two-dimensional descending chromatography. The semi-stench solvent (EDTA 1.2 g, 17 N ammonia solution 100 ml, water 950 ml, *n*-propanol 350 ml, isopropanol 75 ml, *n*-butanol 75 ml and isobutyric acid 2500 ml) was used for the first dimension. The second dimension was developed with *n*-butanol-acetic acid-water, 12: 3: 5 v/v (Crowley

et al., 1963). Both solvents were allowed to run to the edge of the plate, this taking 4 hours (20°C) for the first dimension (ca. 18 cm) and 4.5 hours for the second dimension (ca. 16 cm). The plate was then placed on Kodak X-ray film and exposed for 2 weeks at 20°C before developing. Labelled compounds including the amino acids produced noticeable darkening of X-ray sheets. Amino acids were identified by R_f and autoradiography. The ^{14}C in each labelled compound visible on the chromatograph was determined by cutting the spot from the plate, placing it in a scintillation vial, and adding 1 ml of scintillation solution [3.5 g 2.5-Diphenyloxazole: 1.5 g 1,4-bis-2(5-diphenyloxazolyl)- benzene: 1000 ml Toluene w/v] followed by standing for 30 minutes. The radioactivity of each compound was determined with a Packard liquid scintillation spectrometer.

4.2 Results

The distribution of radioactivity in the various free amino acids in barley leaves incubated with ^{14}C-glutamic acid is summarised in Table 14. Labelling with ^{14}C-glutamic acid took place for 2 hours before the tissues were sampled and the distribution of activity determined. Total incorporation of ^{14}C into recovered compounds was higher in non-stressed plants than in those subjected to any of the environmental stress situations, although the differences were not great. Approximately 30-45% of the ^{14}C was recovered in the glutamic acid spot, suggesting considerable metabolism of this compound in all treatments. The remaining

TABLE 14

^{14}C distribution in the amino acids from leaf tissue from barley plants supplied with ^{14}C-glutamic acid and subjected to different environmental stresses

Amino acid	Control (20°C, ψ_π = -0.7 bars)		Cold stress (5°C, ψ_π = -0.7 bars)		Water stress (20°C, ψ_π = -10 bars)		Water stress (20°C, ψ_π = -15 bars)		Salinity stress (20°C, ψ_π = -10 bars)	
	%	cpm	%	cpm	%	cpm	%	cpm	%	cpm
Glutamic acid	36.3	8018	46.0	8493	28.2	5274	29.1	4814	42.3	8359
Proline	0.3	66	3.0	554	9.7	1814	12.4	2051	9.2	1818
Glutamine	17.2	3786	25.7	4745	9.5	1777	4.6	761	8.2	1620
Asparagine	0.3	66	0.3	55	0.3	56	1.9	314	0.3	59
Aspartic acid	5.3	1160	1.9	351	4.3	804	1.8	298	4.5	889
Alanine	0.6	143	0.4	74	1.5	280	1.5	248	0.5	99
γ-Aminobutyric acid	9.9	2197	13.6	2511	33.6	6285	25.6	4235	4.9	968
Unknown compounds	30.0	6621	9.1	1680	12.9	2413	23.1	3821	30.1	5948
Total	100	22065	100	18464	100	18705	100	16543	100	19761

activity was distributed between compounds identified as glutamine, asparagine, aspartic acid, alanine, proline and γ-aminobutyric acid together with ninhydrin-negative unknown compounds which were probably sugars and organic acids. There was negligible recovery of radioactivity in the basic amino acids including ornithine and arginine. ^{14}C-glutamic acid disappeared to about the same extent in water-stressed leaves and turgid leaves although rather less disappeared from the leaves excised from chilling stress treatment. The largest amount of metabolized ^{14}C was recovered in the unknown ninhydrin-negative compounds (30%) in the turgid and salinity-stressed leaves, whereas in water-stressed leaves more was present in γ-aminobutyric acid (25-34%), and glutamine (26%) accumulated most label in the chilling-stressed leaves. Much less ^{14}C was recovered in glutamine in turgid leaves (17.2%) and even less in water- and salinity-stressed leaves (less than 10%). Some ^{14}C was recovered in aspartic acid, asparagine and alanine but the total was small and there was little between the various stress treatments and the control leaves. Proline was very slightly labelled in the control leaves, only 0.3% of the total activity being recovered in proline after 2 hours infiltration. However, the activity of ^{14}C in proline was about 10 fold higher in the leaf excised from plants exposed to 5^{o}C low temperature for 72 hours and about 30-40 fold higher in the leaf excised from plants growing in PEG or NaCl solution for 36 hours prior to infiltration with ^{14}C-glutamic acid. All results indicate that glutamic

acid is rapidly converted into other amino acids in barley in both turgid and stressed conditions, but the pattern of conversion of glutamic acid to other amino acids was considerably varied by the environmental stresses, and incorporation into proline was substantially increased in all stress situations.

When ^{14}C-proline was supplied to barley leaves in a similar manner, there was a rapid conversion of ^{14}C-proline to other amino acids in the control plants, about 75% of the ^{14}C-proline being converted into other amino acids after 2 hours infiltration (Table 15). Most of the label from proline was found in glutamic acid (22.7%), glutamine (12.8%) and γ-aminobutyric acid (8%) and the unknown ninhydrin-negative compounds (30%). Total recovery of ^{14}C was unaffected by prior water stress, but slightly decreased by prior chilling or salinity stress. In the stress situations, ^{14}C-proline conversion to other compounds was severely inhibited. Less than 5% of ^{14}C-proline was converted into other amino acids in the leaves excised from plants subjected to chilling or salinity stress.

On the other hand, about 40% of the label in the leaves from water-stressed plants was recovered in compounds other than proline following 2 hours incubation. In summary, the incorporation of ^{14}C from proline into other compounds was considerably reduced following stress in any of the treatments considered. This appears to be the most pronounced common metabolic effect of the stress treatments compared.

TABLE 15

^{14}C distribution in the amino acids from leaf tissue from barley plants supplied with ^{14}C-proline and subjected to different environmental stresses.

Amino acid	Control(20°C, ψ_π = -0.7 bars)		Cold stress(5°C, ψ_π = -0.7 bars)		Water stress(20°C, ψ_π = -10 bars)		Water stress(20°C, ψ_π = -15 bars)		Salinity stress (20°C, ψ_π =-10 bars)	
	%	cpm	%	cpm	%	cpm	%	cpm	%	cpm
Glutamic acid	22.7	7566	1.1	284	9.0	2567	7.9	2486	3.8	896
Proline	24.5	8318	95.7	25066	56.1	15951	79.6	24904	92.7	21874
Glutamine	12.8	4250	0.5	131	10.6	3006	1.9	611	0.7	165
Asparagine	0.6	212	0.1	26	0.8	225	0.2	57	0.1	23
Aspartic acid	0.5	169	0.2	52	2.3	676	0.7	211	0.3	69
Alanine	0.7	235	0.3	79	0.1	285	0.3	94	0.4	91
γ-Aminobutyric acid	8.1	2691	0.8	209	11.3	3202	7.5	2369	0.7	165
Unknown compounds	30.0	9969	1.1	288	8.9	2523	1.7	544	1.3	306
Total	100	33232	100	26200	100	28435	100	31276	100	23589

5. Discussion

One of the potential areas in which different types of environmental stress might exert a common effect on plant growth and metabolism is through changes in tissue water status. In this case, however, it has again been demonstrated that low temperature has no effect on plant water status in both barley and radish. Water potential, osmotic potential and turgor potential of the leaves of both species did not depart significantly from those of plants growing at 20°C. It follows that the effects of low temperature on plant growth and metabolism observed were not mediated through changes in internal water status and the effect of low temperature is more direct. Although the metabolic response to water and salinity stress may well be mediated through changes in internal water status, the changes in internal water status are considerably different in these two stress situations. An apparent osmotic adjustment occurs in salt-stressed plants of both species and there was a consequent recovery in leaf turgor potential after the initial decrease (Figure 52). Previous reports indicate that changes in the osmotic potential of plant tissue are similar in magnitude to changes in the concentration of the medium (Bernstein, 1963; Black, 1960). In the present case, the osmotic potential of radish leaf tissue from plants in a saline medium was lower than the control by an amount equal to the concentration of medium (Figure 51B), which is in agreement with the previous reports. The change in the osmotic potential of barley leaf

tissue, however, exceeded changes in the concentration of the medium (Figure 51A). Similar, over-adjustment has been observed in cotton (Boyer, 1965).

The osmotic potential of the leaf tissue of both species decreased rapidly when plants were exposed to polyethylene glycol solution. Although the rate of decline in osmotic potential was more rapid in such water-stressed plants than in salt-stressed plants, the decrease in water potential was more extensive than the decrease in osmotic potential in the water-stressed plants and, consequently, led to a loss of turgor and the plant wilted. The leaf turgor potential continued to decrease and eventually fell to a negligible level as water stress proceeded.

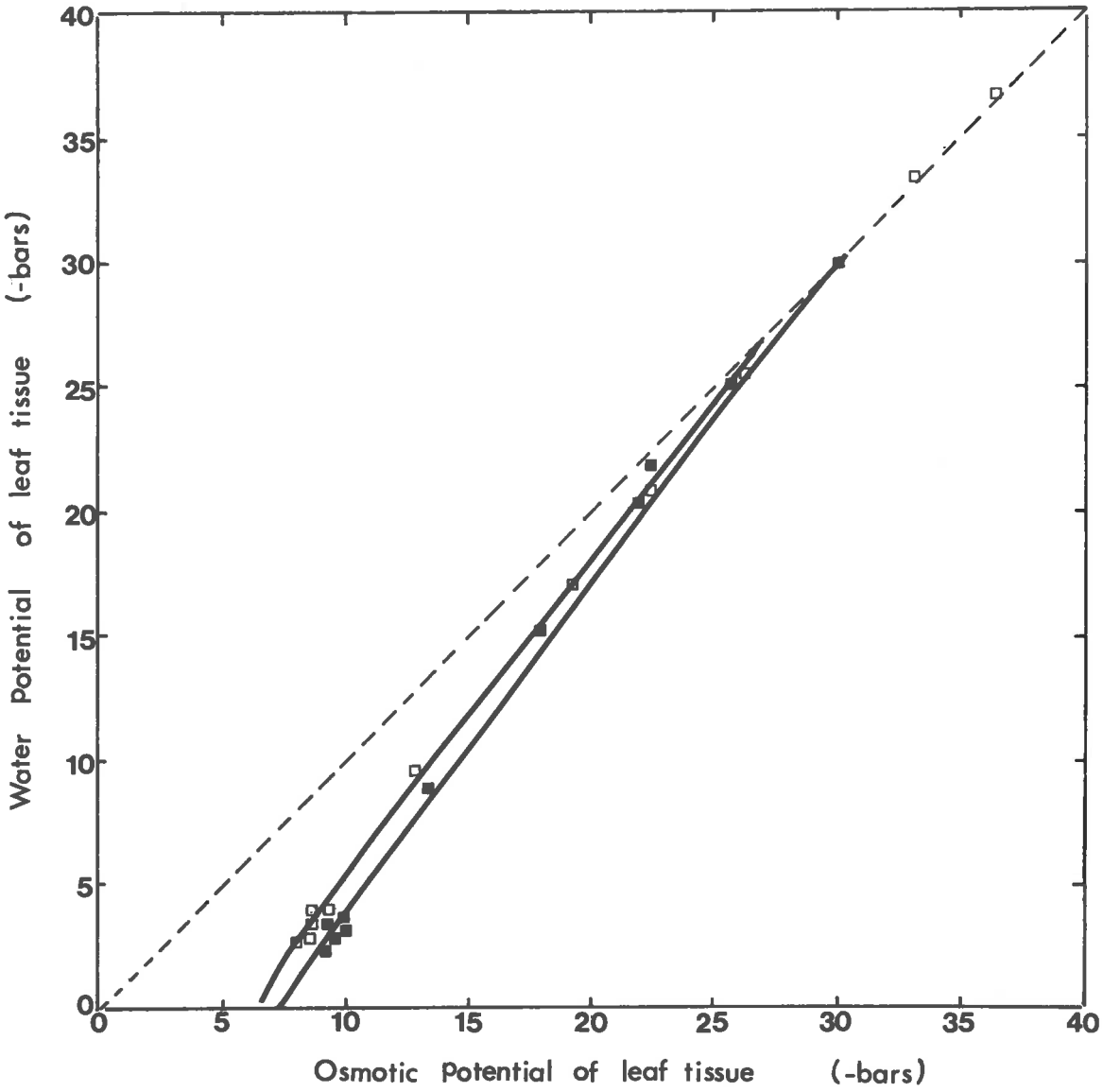
The water potential has been reported to vary from species to species when turgor = 0 (Tomar and Ghildyal, 1973). It has been reported that for barley this occurs at a water potential of -32 bars (Millar *et al.*, 1970) and for several different dicotyledonous plants it is approximately -22 bars (Gardner and Ehlig, 1965). The relationship between water potential and osmotic potential is plotted for both species in Figure 54. Turgor potential decreased with decreasing osmotic potential, eventually becoming zero. In the case of radish, the last two values indicate a lower osmotic than water potential corresponding to a negative turgor potential. Although Slatyer (1957) has also reported a negative potential for cotton, their existence has been questioned for other than xerophytic

FIGURE 54

Relationship between total water potential and osmotic potential of barley and radish leaves. [Data obtained from Figure 50 and Figure 51.]

■ Barley

□ Radish


Water Potential of leaf tissue (-bars)
Osmotic Potential of leaf tissue (-bars)
0
5
10
15
20
25
30
35
40

plants (Noy-Meir and Ginzburg, 1969). However, it has been clearly indicated that the relationship between ψ and ψ_π are similar in the two species. The value for barley at which $\psi = \psi_\pi$ is about -30 bars, a value in close agreement with that reported by Millar *et al.* (1970), but the value for radish at which $\psi = \psi_\pi$ is about -27 bars which is approximately 5 bars lower than other dicotyledons including cotton, trefoil and pepper (Gardner and Ehlig, 1965).

Elongation in either species was rapidly and completely inhibited when the plant was subjected to any environmental stress (Figure 47). Plant and cell elongation is very sensitive to the turgor potential of the tissue (Hsiao, 1973) and turgor may be the major controlling influence on elongation (Ordin, 1960; Hsiao, 1973). Leaf turgor potential is compared with the contemporary rate of plant elongation in Table 16. In the case of low temperature stress, the inhibition of elongation is not apparently related to turgor potential as in both species, turgor was maintained at between 4 and 5 bars whilst elongation was initially completely inhibited but later showed limited recovery. Low temperature itself, thus appears to exert a strong influence on plant elongation, possibly acting on the meristematic regions (Beauchamp and Lathwell, 1966; Watts, 1972). With water stress, elongation was rapidly inhibited in both species and remained virtually completely inhibited for the 3 days of the stress. During this period, turgor potential fell from 5-6 bars to zero, and these data are consistent with a requirement for full turgor

TABLE 16

The relationship between turgor potential and plant elongation

[Data obtained from Figure 47 and Figure 52.]

Time interval after stress (h)	Control		Cold stress		Water stress		Salinity stress	
	mean ψ_p (bars)	elongation rate $(mmh^{-1})\times10^{-2}$	mean ψ_p (bars)	elongation rate $(mmh^{-1})\times10^{-2}$	mean ψ_p (bars)	elongation rate $(mmh^{-1})\times10^{-2}$	mean ψ_p (bars)	elongation rate $(mmh^{-1})\times10^{-2}$
Barley								
0 - 12	5.8	69.0	5.6	0.6	5.0	6.6	4.4	0
12 - 24	5.8	275.0	5.4	0.8	3.5	2.5	3.5	1.6
24 - 36	5.9	119.0	5.5	3.3	2.1	1.6	4.6	5.8
36 - 48	5.9	121.0	5.4	8.3	1.0	0.8	5.1	6.6
48 - 56	5.9	30.0	5.4	14.1	0.3	0.8	5.6	6.6
56 - 72	5.8	127.0	5.7	16.0	0.1	0.6	5.9	9.2
Radish								
0 - 12	5.0	16	4.9	0	4.0	-25	4.2	-8
12 - 24	5.1	50	5.1	0	2.5	0	3.7	0
24 - 36	5.2	8	5.2	0	1.8	-8	4.4	-8
36 - 48	5.2	50	4.9	8	1.0	-8	4.9	0
48 - 56	5.1	16	4.9	8	0.1	0	5.2	0
56 - 72	5.2	25	5.2	8	-0.1	0	5.5	-8

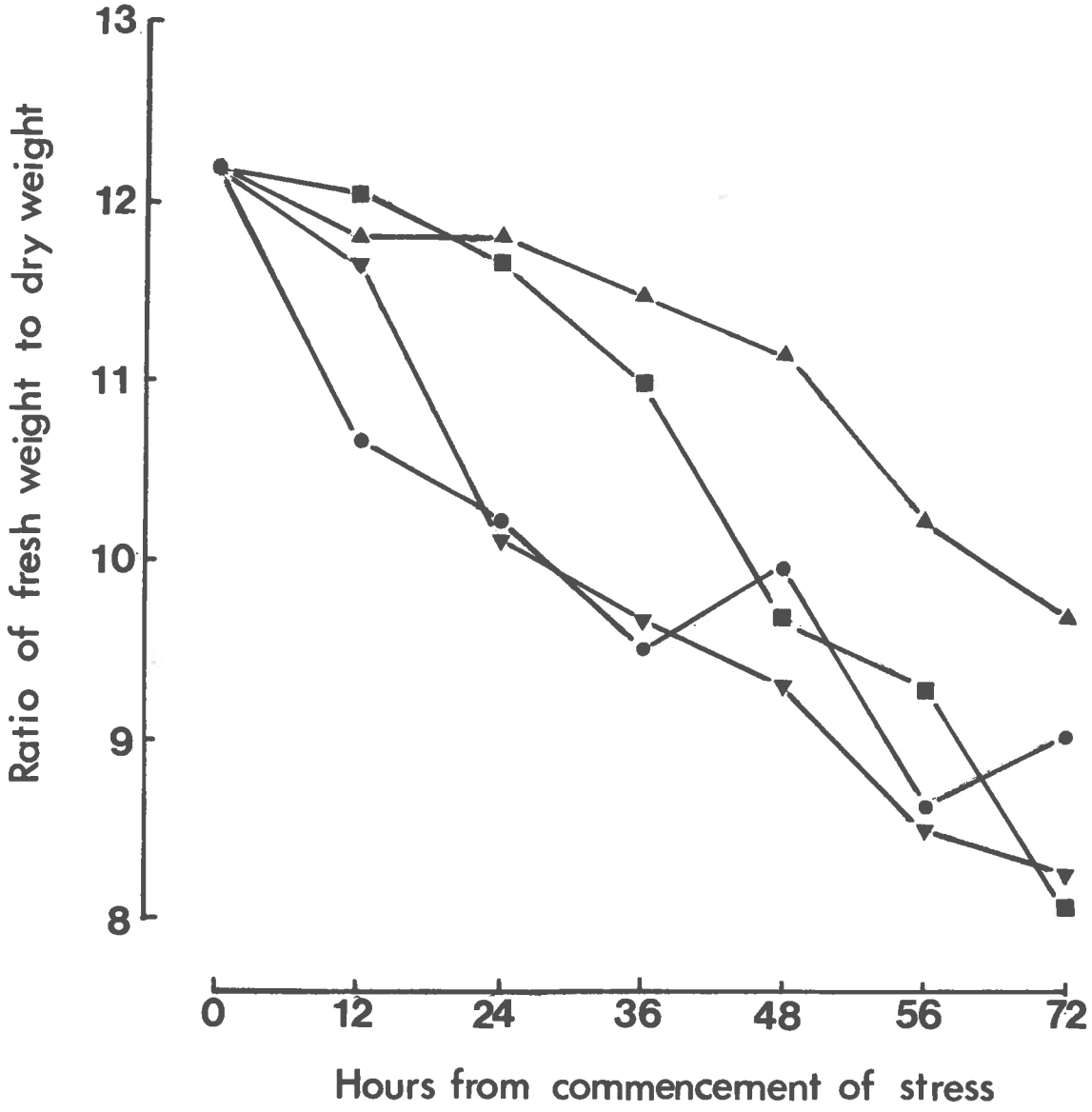
to allow elongation (Lockhart, 1965). In the case of salinity stress, however, elongation also remained strongly inhibited throughout, even though the turgor potential apparently recovered fully after an initial decline (Table 16). Although some question must be attached to the absolute values of turgor potential reported (see earlier discussion), this response does raise the possibility that elongation was here controlled not only by turgor but also by some other factors such as tissue osmotic potential, specific ion effects, even plant hormone effects. A growth stimulation from GA_3 applied to wheat plants during a period of water stress (Singh *et al.*, 1973a) or to bean plants during a period of salinity stress (O'Leary and Prisco, 1968) has been reported.

As well as the inhibition of plant elongation, each of the environmental stress situations also decreased the rate of increase in fresh and dry weight, but the magnitude of the effect differed. Generally speaking, fresh weight increase was more sensitive to stress than was growth in dry weight. This was reflected in changes in the fresh to dry weight ratio which can be taken as a measure of succulence. In all situations, including control plants, this ratio decreased with age (Figure 55). However, the fall in the ratio was much less in salt-stressed plants than in any others. Such a change in the succulence of leaves has been noted for many different species (Nieman, 1962) and it has been suggested that it is a fairly common plant response to a high level of chloride in the environment.

FIGURE 55

Changes in the fresh and dry weight ratio of barley plants subjected to different environmental stresses. Data obtained from Figure 48.

●	Nutrient solution	(ψ_π = -0.7 bars, 20°C)
▼	Low temperature stress	(ψ_π = -0.7 bars, 5°C)
▲	NaCl solution	(ψ_π = -10 bars, 20°C)
■	Polyethylene glycol solution	(ψ_π = -10 bars, 20°C)


Ratio of fresh weight to dry weight
13
12
11
10
9
8
0
12
24
36
48
56
72
Hours from commencement of stress

Plant growth, as measured by the relative growth rate, was also decreased by environmental stress (Table 17). Low temperature did not completely inhibit dry weight increase, but the relative growth rate fell with time of exposure to the low temperature. The relative growth rate was rapidly and completely inhibited by both salinity and water stress, however. In the case of water stress there was no recovery during the period of the experiment but the relative growth rate recovered to approximately 50% of the control value after 48 hours of salinity stress. The reasons for these influences of environmental stress on dry weight production are likely to be complex. Stress may affect photosynthesis, respiration, dry matter distribution and transport amongst other factors and the effect on relative growth rate would most likely result from the interaction of a number of factors. Of these factors, probably the process most directly and severely affected is photosynthesis (Crafts, 1968). Pronounced effects of a variety of environmental stress conditions on photosynthesis have been frequently reported (Crafts, 1968; Gale *et al.*, 1967; Taylor and Craig, 1971; Ben-Zioni and Itai, 1972). Reduced photosynthesis may stem from closure of stomata, decreased photosynthetic activity per unit of leaf or a reduction in leaf expansion (Gale *et al.*, 1967; Taylor and Craig, 1971). Whatever the basic reason for the reduction in relative growth rate, it is likely that, in the case of water and salinity stress the response arises from a change in leaf water status, either water potential, osmotic potential or turgor. It is apparent that the decrease in relative growth

TABLE 17

The relationship between relative growth rate and internal water status of barley plants

[Data obtained from Figures 48, 50, 51 and 52.]

Time interval after stress (h)	Control ψ	Control ψ_π (bars)	Control ψ_p	Control Relative growth rate (mg $mg^{-1}h^{-1}$)$\times10^{-3}$	Cold stress ψ	Cold stress ψ_π (bars)	Cold stress ψ_p	Cold stress Relative growth rate (mg $mg^{-1}h^{-1}$)$\times10^{-3}$
0 - 12	-3.5	-9.3	5.8	13.3	-3.7	-9.3	5.6	4.1
12 - 24	-3.6	-9.4	5.8	9.1	-4.1	-9.5	5.4	18.3
24 - 36	-3.6	-9.5	5.9	10.8	-4.1	-9.6	5.5	7.5
36 - 48	-3.5	-9.4	5.9	10.8	-4.5	-9.9	5.4	5.0
48 - 56	-3.2	-9.1	5.9	18.0	-5.1	-10.6	5.4	4.2
56 - 72	-3.4	-9.2	5.8	10.0	-5.4	-11.1	5.7	4.2

Time interval after stress (h)	Water stress ψ	Water stress ψ_π (bars)	Water stress ψ_p	Water stress Relative growth rate (mg $mg^{-1}h^{-1}$)$\times10^{-3}$	Salinity stress ψ	Salinity stress ψ_π (bars)	Salinity stress ψ_p	Salinity stress Relative growth rate (mg $mg^{-1}h^{-1}$)$\times10^{-3}$
0 - 12	-6.2	-11.2	5.0	-1.6	-7.1	-11.5	4.4	0.8
12 - 24	-12.2	-15.7	3.5	-0.8	-12.8	-16.3	3.5	0.8
24 - 36	-17.7	-19.8	2.1	1.6	-15.8	-20.4	4.6	3.3
36 - 48	-21.2	-22.2	1.0	1.6	-17.0	-22.1	5.1	2.5
48 - 56	-23.9	-24.2	0.3	0.8	-17.9	-23.5	5.6	9.1
56 - 72	-27.9	-28.0	0.1	0.8	-18.8	-24.7	5.9	5.8

rate occasioned by low temperature is not related to any components of water balance (Table 17). With water stress, on the other hand, a slight change in water potential or its components was associated with a considerable reduction in relative growth rate. It is not possible to distinguish which component of water status is responsible for this change in relative growth rate, although it is significant that Boyer (1970a) found that leaf enlargement was largely inhibited when water potential fell to -4 bars. The response to salinity stress is also relevant, again there was a rapid reduction in relative growth rate initially with a fall in all components of water potential. However, there was a renewal of growth later when the turgor potential had apparently recovered to the initial value. Such a renewal of growth with return to full turgor has also been reported for several plants (Greenway and Thomas, 1965; Oertli, 1968). This resumption of growth whilst the plants remain exposed to salinity may be due to one of several possibilities. Firstly, it may be that following cell osmotic adjustment and the recovery of turgor potential that plants have excess energy which could be used for growth (Bernstein, 1963). A second possibility, is that the inhibited photosynthesis might recover when turgor potential increases (Jones, 1973a) despite the fact that the water and osmotic potential of tissue remain at a lower level. Net photosynthesis of onion plants is reduced by salinity, but it can be rapidly raised to the level of the control by increasing leaf turgor (Gale *et al.*, 1966).

In this study, however, the relative growth rate of plants under salinity stress did not recover to the level of the control when turgor potential increased. This continuing inhibition might be related to some specific ion effect or a response to internal osmotic potential, such as partial stomatal closure with a consequent increase in the resistance to CO_2 movement into the plant (Gale *et al.*, 1966).

Apart from the general effect of stress on metabolism which is characterized by a decrease in the growth rate,a more specific response can be identified as an increase in the amino acid and amine pool sizes in plants in response to unfavourable environmental situations as has been frequently reported (Crafts, 1968; Trione, 1966; Strogonov, 1964). In the present study, the total free amino acid pool also increased by 2 to 4 fold in the leaf tissue of both species following exposure to low temperature, water or salinity stress, and this increase was mainly due to a much more pronounced relative accumulation of proline. This increase in proline accounted for 85-98% of the total increase in all free amino acids in each of the stress situations with the exception of radish at low temperature where it accounted for 68% of the increase. The rate of proline accumulation differed considerably between the two species and the three environmental stress situations. Radish appear to have a higher proline accumulation potential than barley in response to chilling stress (Figure 53). In comparing the three environmental stress situations, it is apparent that water stress here induced the

greatest concentration of proline in the leaf tissue of both species, whilst chilling stress induced the least and salinity stress was intermediate.

It has been suggested that the increase in the amount of free amino acids observed as a result of stress is due to an inhibition of protein synthesis and an increase in the decomposition of existing protein (Gates and Bonner, 1959; Trione, 1966; Strogonov, 1964). Since the increase in free proline concentration in the tissues during wilting exceeds the potential proline release from protein (Kemble and MacPherson, 1954), at least some of the accumulated proline is apparently derived primarily from *de novo* synthesis (Stewart *et al.*, 1966). Furthermore, studies with ^{14}C-proline have suggested that proline utilization, mainly in protein synthesis (Stewart, 1972a) and oxidation (Boggess *et al.*, 1974) is inhibited in wilted leaves. Although enhanced proteolysis and inhibited protein synthesis during chilling and salinity stress have been reported (Levitt, 1972; Strogonov, 1964), it is not known whether the accumulated proline is derived from *de novo* synthesis. In the present case, however, the results of supplying ^{14}C-glutamic acid and ^{14}C-proline suggest the response to chilling, water and salinity stress is very similar despite the fact that the rate of proline accumulation differed considerably. Firstly, incorporation of label from ^{14}C-glutamic acid into proline was substantially increased in all three stress situations. Secondly, the conversion of ^{14}C-proline to a variety of other compounds

was considerably reduced following any of the stress treatments. The effect on proline metabolism was, in each case, more pronounced than the effect on glutamic acid metabolism. This can be interpreted as suggesting that the stress effect is primarily on proline utilization, as the enhanced incorporation of ^{14}C into proline from labelled glutamic acid could follow from such an effect whereas the reverse is less likely.

Apart from this large change in proline concentration, there were much smaller changes in the concentrations of a number of other amino acids, but the percentage and absolute change in pool size were relatively minor. Similar and parallel changes were observed in some of the amino acids during stress, but there appears to be little or no relation between these patterns of change and any biosynthetic relationships between the amino acids. The concentration of glutamic acid and aspartic acid fell in both species and all three stress situations. Such a decline in glutamic and aspartic acids may have been suggested to be due to conversion to the corresponding amide (Chibnall, 1939; Singh *et al.*,1973c) but in the present study the concentrations of glutamine and asparagine were not affected except in the salt-stressed barley plants where their concentrations rose (Table 12). The absence of any accumulation of glutamine and asparagine in the leaves suggests that proline may substitute for both amines and serve as a primary storage compound during stress. Of the other amino acids, only threonine showed a consistent increase in both species and all three stress situations. Valine and alanine also generally rose although the increase was not always significant. In

radish, leucine, isoleucine and ethanolamine increased in all three stress situations but did not do so consistently in barley. An increase in valine, alanine, leucine and isoleucine under stress conditions can be interpreted as a reflection of an increase in pyruvate availability resulting from accelerated glycolysis (Stewart *et al.*1966). Both valine and alanine may also have an $\dot{N}H_3^+$ storage function during stress (Tarchevskii and Siyanova, 1963). In comparing proline accumulation with the changes in these amino acids, there seemed to be no clear relationship. Except for proline accumulation, there is no common pattern of change in these amino acids between species and stress situations. The appearance of increased pool sizes of amino acids with the exception of proline, under environmental stress conditions, may simply be a result of protein breakdown.

Where ^{14}C-glutamic acid was fed to control leaves, the largest amount of ^{14}C was recovered in unidentified ninhydrin-negative compounds. These unknown ninhydrin-negative compounds are probably sugars and organic acids (Boggess *et al.*,1974). This labelling of these compounds indicated that the pathway of metabolism for glutamic acid through α-ketoglutaric acid into the Krebs cycle was functioning rapidly. Since glutamic acid quickly penetrates the membrances of cells and is metabolized readily when supplied to plant tissues (Najjar and Gale, 1950), the 2 hour infiltration appears to have been sufficient for equilibrium conditions to be established between the added glutamic acid and those substances deriving label from it. Naylor and Tolbert

(1950) calculated the percentage of ^{14}C-glutamic acid utilized up to a given time and found that equilibrium conditions were quickly established, and Naylor *et al.* (1958) indicated that equilibrium between added ^{14}C-aspartic acid and product was established within 30 - 60 minutes.

In addition to these unknown compounds, the major product from ^{14}C-glutamic acid in the control leaves was glutamine (17.2%). Environmental stress drastically altered glutamic acid metabolism. Formation of glutamine from glutamic acid was reduced under both water and salt stress conditions, probably because its synthesis requires high-energy phosphate from oxidative phosphorylation (Elliott, 1951). With water stress, glutamic acid was decarboxylated and yielded γ-aminobutyric acid as the major product (25-35%). Such an increase in γ-aminobutyric acid on wilting has also been found in silage and microbe-free grass (MacPherson and Slater, 1959). γ-Aminobutyric acid is generally accepted as the decarboxylation product of the action of glutamic acid decarbohydrase (Dixon and Fowden, 1961). This enzyme has a wide distribution in barley leaf and is highly active in the normal green barley leaf (Beevers, 1951). It follows that the accumulation of γ-aminobutyric acid observed under stress may be due either to acceleration of the rate of γ-aminobutyric acid synthesis from glutamic acid or to inhibition of the further metabolism of γ-aminobutyric acid or both. The γ-aminobutyric acid content of plants exposed to stress for a longer period (72 hours) was not increased above the control (Table 12), however, and it is conceivable that water stress

initially stimulated γ-aminobutyric acid synthesis, but eventually this accumulated γ-aminobutyric acid was metabolized further. Inatomi and Slaughter (1971) have suggested that the γ-aminobutyric acid content of a tissue probably depends more on the utilization rate of γ-aminobutyric acid than on the availability of the substrate and the enzyme activity.

Following feeding of ^{14}C-proline into control leaf tissue, there was considerable conversion into a variety of compounds. After 2 hours infiltration, 30% of ^{14}C was recovered in unidentified ninhydrin-negative compounds and 24.5% in glutamic acid. The amount of label in glutamic acid suggests a major portion of the proline is metabolized via that compound. Stewart (1972a) also examined the rate of ^{14}C-proline and indicated that proline was first oxidized to glutamic acid which was subsequently metabolized through the Krebs cycle to produce CO_2. However, all the environmental stresses inhibited ^{14}C-proline conversion to other amino acids and compounds. This appeared to be the most pronounced common metabolic effect of the stress treatments compared. It is conceivable that this apparent inhibition of proline metabolism was due to a change in the permeability of the tissue or cell organelles to the added proline such that the exogenous proline did not reach the sites of metabolism in stressed tissue. The observed accumulation of endogenous proline under stress condition, however, suggests that this response is real rather than an artefact of the experimental procedures.

V GENERAL DISCUSSION

Plant growth and survival depend on water availability as much as on any other single environmental factor, yet a failure of the environment to provide an optimum supply of water is a frequent occurrence. In these circumstances, a water deficit arises within the plant tissues whenever water is used or lost from the plant faster than it is absorbed by the roots. The term "plant water deficit" usually refers to situations where the cells and tissues are less than fully turgid. It is characterized by a decrease in water content, a decrease in water potential accompanied by loss of turgor and a decrease in plant growth. If the disturbance of many metabolic processes is severe, cessation of growth and finally death from dessication occurs. Apart from a simple water deficiency, resulting from a lack of water supply, an internal water deficit can be produced by several other unfavourable environmental factors. For example, a supra-optimal temperature can lead to rapid transpiration beyond the capacity of the roots to take up water, and a consequent lowering of the tissue water potential in the leaves. Low temperature can lower movement of water to the plant root and increase the resistance to water passage through the plant also resulting in a lowering of leaf water potential. Increasing salt concentration in the solution around the roots of a plant will lower the Gibbs free energy of that water, and hence decrease the

potential gradient along which water flows into the plant resulting in a limitation in the rate of water uptake. In all these situations, the inter-relationships between the direct effects of the environmental factor, and its effects on tissue water deficit in influencing the growth and metabolism of the plant, are difficult to disentangle. It is particularly difficult to separate the direct effects of these environmental factors on metabolism from those mediated through the concomitant change in ψ. It is possible that this separation could be made if a metabolic response was discovered which was sensitive to tissue water deficit alone and not to the other environmental factors.

The effects of lowered water potential on plant growth and metabolism have been studied for more than a century and it can only be concluded that it does not have a uniform effect on the various aspects of plant growth and functioning. However, it has been frequently reported that major effects of water deficit on plant metabolism include reduced photosynthesis, respiration, translocation and nutrient uptake, changes in the tissue content of polysaccharides and nucleic acid and alterations in protein metabolism (Vaadia *et al.*, 1961; Slatyer, 1967; Crafts, 1968; Hsiao, 1973). Amongst these metabolic changes, the most noticeable consequence of lowered ψ in many plants is a modification of amino acid metabolism leading to a rapid and extensive accumulation of the imino acid proline (Barnett and Naylor, 1966; Singh *et al.*, 1973c). It has been established that plants with adequate water supply generally have

leaves with rather low free proline content and that the proline content rapidly increases with induction of a water deficit giving up to several hundred or even a thousand per cent increase in the proline content in many species (Singh *et al.*, 1973c). Proline accumulates within a few hours of the induction of stress and disappears as rapidly when water deficit is relieved (Singh *et al.*, 1973c). The innate potential of the plant to accumulate proline appears to vary directly with the ability of the genotype to yield under drought conditions (Singh *et al.*, 1972) and it has been suggested that proline accumulation may serve as a sensitive index of metabolism during water stress (Singh *et al.*, 1973a). Proline accumulation may, therefore, be an example of a metabolic process sensitive only to tissue water deficit and not to other changes in the environment. If this is so, rapid and extensive proline accumulation would be expected to occur in plant tissues when plants are subjected to environmental changes causing a lowering of tissue water potential but not when this does not occur. It would be expected then, that if plants subjected to high temperature, low temperature, salinity stress or other stress situations accumulated proline, this accumulation would be derived from variations in plant water potential rather than from a more direct metabolic response to the environment. The present study was an attempt to investigate this possibility.

As anticipated, a water deficit accompanied by a lowering of water potential developed within an hour when barley or radish plants were

exposed to water stress by immersing the roots in a solution of polyethylene glycol. When plants were allowed to transpire water from soil without replacement, a similar tissue water deficit developed over a period of days. In both situations, the lowered water potential was accompanied by a marked increase in the free proline content of the leaf tissue of both species, and this accumulation of proline followed a similar pattern of change with time as did leaf water potential. Accordingly, an apparent correlation between leaf proline content and leaf water potential existed in both species. These results suggest that the proline accumulation induced by water stress is mediated through the change in water potential.

An increase in the ambient temperature can lead to excessive transpiration and results in a rapid water deficit accompanied by a lowering of the leaf water potential. This water deficit induced by high temperature can be prevented by increasing the relative humidity of the immediate environment of the leaves or by frequent irrigation. In these circumstances, proline only accumulated in the leaves when ψ fell; there was no increase in the concentration of proline when plants were subjected to heat stress with no accompanying decline in leaf water potential, although plant growth was inhibited and leaf chlorophyll content was decreased by the high temperature. It is evident that the accumulation of proline was controlled by tissue water status alone and was unaffected by elevated tissue temperature.

The effect of salinity stress on water status is more complicated, mainly depending on the salt concentration and the rate of salinization. Abrupt salinization caused a transitional water deficit with a loss of turgor, but this effect was not permanent and turgor potential increased as the plant adapted to the saline conditions. Gradual increase in the external salt concentration, on the other hand, allowed adjustment to the changing conditions without any dramatic change in plant water status. Despite the fact that the changes in water status were different, a large amount of proline accumulated in the leaves of both species in both types of salinity stress. This proline accumulation in the salt-stressed plant is apparently mediated through the change in leaf ψ, since these changes corresponded closely to changes in the potential of the solution surrounding the roots. Again, with salinity stress, proline accumulation appeared to be mediated through a change in leaf water status and the direct effect of the ions involved, if anything, tended to inhibit accumulation. Proline also accumulated rapidly, after an initial lag phase, in both species exposed to low temperature, but in this case the accumulation occurred without any change in leaf water status. It is evident, therefore that proline accumulation is not solely dependent on leaf water status but that low temperature can independently produce the same response.

Nevertheless, significant changes in proline content have frequently been suggested to be associated with changes in water equilibrium in the

cell. Perdrizet (1972) summarised all cases known to him of parallelism between changes in proline content and changes in water content of tissues. This view is shared by many authors who believe that some degree of desiccation would appear to be essential for proline accumulation (Kemble and MacPherson, 1954; Savitskaya, 1967) and that the degree of dehydration is of significance in determining the rate of proline formation (Savitskaya, 1967). From the results presented here, it is apparent that proline accumulation is also triggered by low temperature. An initiation of proline accumulation induced by chilling has been reported in several other species (Benko, 1968; Palfi and Juhasz, 1970; Gates *et al.*, 1971).

The water potential at any point in a tissue can be partitioned into three components, osmotic potential (ψ_π), matric potential (ψ_m) and turgor potential (ψ_p) (Warren Wilson, 1967). ψ_π and ψ_m are usually considered together, so that total water potential (ψ) is effectively $\psi = (\psi_\pi + \psi_m) + \psi_p$. The change in water potential induced by unfavourable environmental factors may be due to a change in $(\psi_\pi + \psi_m)$, or in ψ_p or both. In water or heat stress, the changes in water potential are always greater than the changes in $(\psi_\pi + \psi_m)$, and there is a turgor potential loss. In these circumstances it is difficult to distinguish the effects of the components of water potential from each other. In salinity stress, however, plant turgor potential can be maintained by a gradual increase in the external salt concentration. In this case, the change in leaf

water potential results from a change in $\psi_\pi + \psi_m$ rather than in ψ_p. As significant amounts of proline accumulated in plants treated in this way, proline accumulation would appear to be controlled by $\psi_\pi + \psi_m$ rather than by ψ_p. Therefore, we can conclude that proline accumulation in stressed tissue may be triggered by $\psi_\pi + \psi_m$ or by low temperature.

Despite the fact that proline accumulation may have been triggered by either lowered osmotic potential or by low temperature, the resulting proline accumulation exhibited several common features. Firstly,proline did not only accumulate in the leaf but also accumulated in all organs of the plant. A considerable concentration of proline was detected in the leaf sheath of barley, the cotyledons of radish and the roots of both species. Secondly, there was a considerable difference in the proline accumulation potential of the various plant organs. The leaf laminae accumulated most proline, the leaf sheaths of barley and the cotyledons of radish rather less and the roots of both species accumulated the least. This occurred whether accumulation was induced by a water deficit or by low temperature. Thirdly, the roots of plants of either species were unable to independently accumulate proline under conditions of either water deficit or low temperature. The considerable amounts of proline in roots attached to the plant during stress were translocated there from the leaves (Singh *et al.*, 1973b). Fourthly, feeding labelled ^{14}C-glutamic acid and ^{14}C-proline into barley tissue indicated that incorporation of ^{14}C from ^{14}C-glutamic acid into proline was substantially

increased and conversion of ^{14}C-proline to a variety of other compounds was considerably reduced in all environmental stress situations.

In contrast to these common features, there are several differences between the various stresses:

(1) Accumulation potential varied with environmental stress, water stress induced the greatest accumulation, chilling stress the least and salinity stress was intermediate in both species.

(2) Proline accumulation induced by low temperature required illumination, whereas accumulation induced by water deficit was independent of illumination.

(3) A high concentration of salt in the leaf tissue had an inhibitory effect on proline accumulation.

From these comparisons, we can conclude that at least two independent and dissimilar factors cause the same metabolic response. At present we cannot distinguish whether this response occurs through distinct effects on different aspects of metabolism which have the same ultimate effect, or whether both factors affect the same basic metabolic process.

It has frequently been suggested that heat, chilling,water and salinity stress produce the same kind of injury - a loss of semipermeability due to membrane damage, and the same indirect metabolic disturbances - enhanced proteolysis, inhibited protein synthesis, a change in the proportions of sugar and starch, reduced photosynthesis and increased toxic compounds (Levitt, 1972). Amongst these changes, enhanced proteolysis

and inhibited protein synthesis may result in an increase in proline concentration in all stress situations. However, it has been reported that the increase in proline during wilting exceeds the proline released from protein and accordingly, *de novo* synthesis from glutamic acid must at least contribute to the increase in proline (Stewart, 1972a). The rate attained by any particular metabolic reaction in a cell depends both on the presence and amount of reactants and enzymes, and on their activity. The activity of the enzymes will depend upon local environmental factors, and there are, therefore, two possibilities which can be postulated. One possibility is that in all the environmental stresses considered the same kind of injury to the cell membranes occurred. Such changes in membranes may break a barrier between proline synthetic enzymes and their substrate which existed in the normal plant, and result in proline accumulation. Another possibility is that both osmotic potential and low temperature directly or indirectly change the local environment, including the pH, inhibitors, coeffectors, ion concentration or temperature and produce the same response. Any further elucidation of the similarities between the two systems, however, must await elucidation of the enzyme pathway and the points of rate regulation.

Once stress, of any type, is removed, proline content of the tissue declines. This decline in proline concentration when a period of water deficit is ended, or following upon the application of GA_3 to the plant, occurred simultaneously with a stimulation of growth (Singh *et al.*, 1973a).

A direct relationship between the proline accumulated and the growth rate following upon a period of water stress has been established (Singh *et al.*, 1973a) and it has been suggested that proline may be the major source of energy and nitrogen during immediate post-stress metabolism (Stewart *et al.*, 1966). On the other hand, there does not appear to be a close relationship between the inhibition of growth and the accumulation of proline during the induction of stress (Figure 56). In particular, with salinity stress, high relative growth rates occurred with high proline contents in the plant. The reduced plant growth rate, as previously indicated, is more related to a change in water status (Table 17). In water or salinity stress, a small change in water potential or its components probably caused greater reduction of relative growth rates than increases in proline. Proline accumulation appeared to be related to chlorophyll degradation (Figure 57), at least in response to a water deficit. In water or salinity stress, there was a relationship between proline accumulation and chlorophyll concentration in that the more proline that was accumulated the less chlorophyll that was present. This relationship did not occur with chilling stress.

It has been reported that changes in chlorophyll content are often accompanied by an accumulation of proline and arginine in plant tissue (Diener and Dekker, 1954), and a direct metabolic connection between proline and chlorophyll has been recorded in germinating potato tubers

FIGURE 56

Relationship between free proline concentration in the leaves and the relative growth rate of barley plants subjected to different environmental stresses.

[Data obtained from Figure 48 and Figure 53.]

▲ chilling stress

■ salinity stress

○ water stress

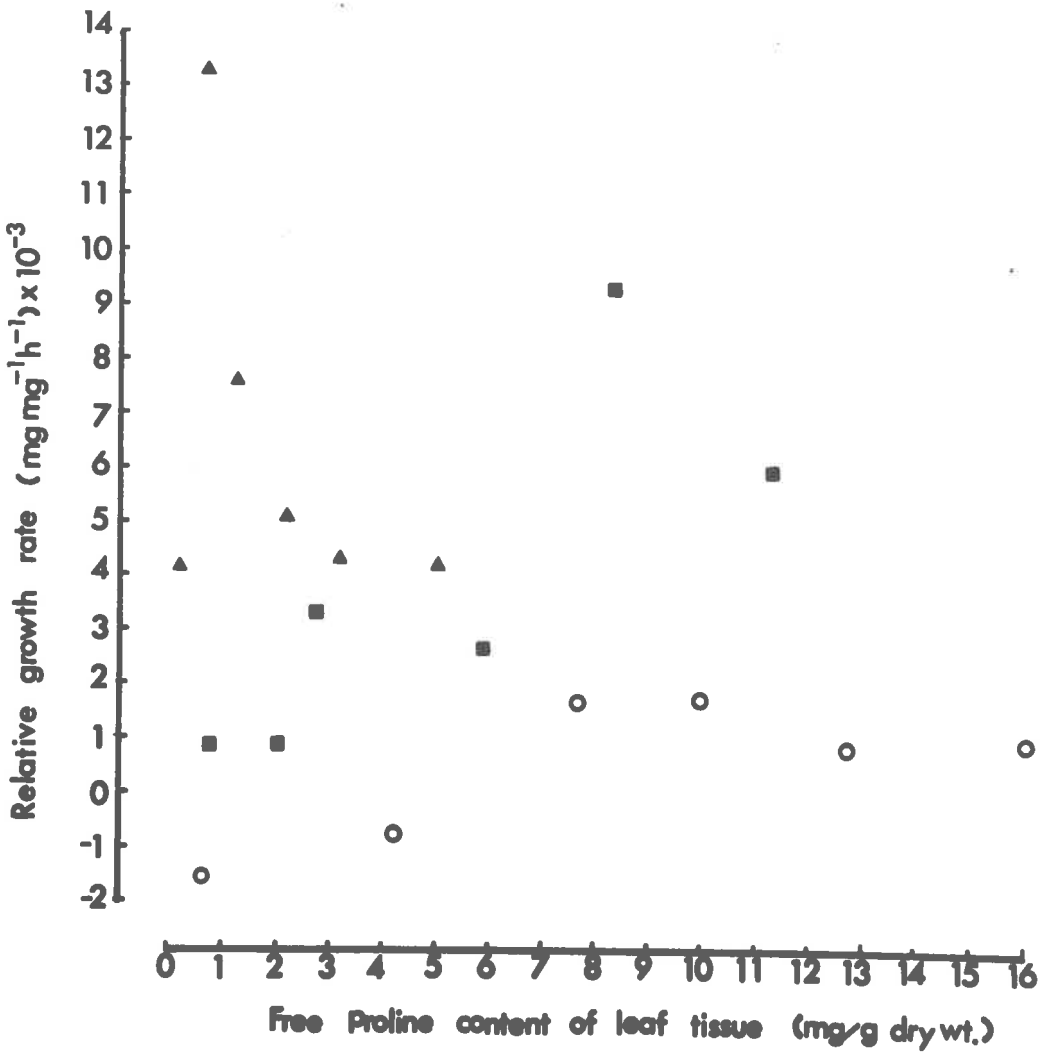

Relative growth rate (mg mg⁻¹ h⁻¹) x 10⁻³
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Free Proline content of leaf tissue (mg/g dry wt.)

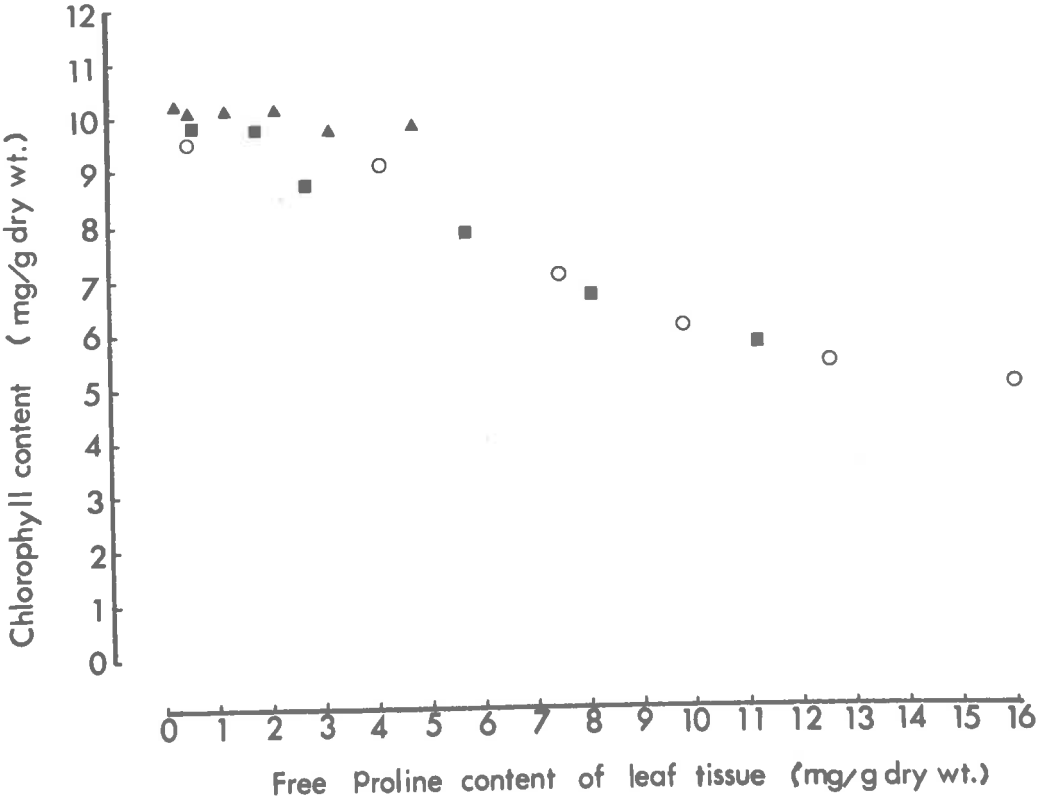
FIGURE 57

Relationship between free proline and chlorophyll concentration in the leaves of barley plants subjected to different environmental stresses.
[Data obtained from Figure 49 and Figure 53.]

▲ chilling stress

■ salinity stress

○ water stress


Chlorophyll content (mg/g dry wt.)
12
11
10
9
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Free Proline content of leaf tissue (mg/g dry wt.)

and Jerusalem artichoke seedlings (Duranton and Maille, 1962). The evidence from present results, however, in which proline accumulation was not related to chlorophyll degradation in chilling-stressed plants, together with the finding that proline is accumulated rapidly by etiolated leaf tissue in the presence of proline precursors (Singh *et al.*, 1973b), leads to the conclusion that chlorophyll degradation and proline accumulation are unrelated. Environmental stress probably acts more directly on chlorophyll synthesis or degradation rather than through effects on proline accumulation.

Notwithstanding the differences in causation of proline accumulation, it is possible that proline can be used as a stress index when the plant is subjected to various environmental stresses or even in the field situation. Since proline accumulation is a sensitive index of metabolism during water stress, and there is an apparent correlationship between proline accumulation and tissue dehydration, Palfi and Juhasz (1970) suggested using proline as the sole parameter to measure water stress. Proline accumulation is not sensitive to mild stress (Stewart *et al.*, 1966; Routley, 1966), however, and in the present study no proline accumulated in barley or radish leaf tissue when plants were exposed to solutions with osmotic potentials higher than -5 bars, whether they were of PEG or NaCl. In some species and in some circumstances, proline did not accumulate markedly until plants were severely stressed and visibly wilting (Waldren and Teare, 1974). This would have been sufficient to

adversely affect yield (Waldren *et al.*, 1974), and the authors concluded that proline cannot be used as an early sign of potential damage. Accumulation is also modified by a number of environmental factors (Routley, 1966; Stewart *et al.*, 1966). If proline is to serve as a water stress index then the effect of additional factors would have to be considered carefully. In studying drought resistance problems, it has been suggested that the amount of proline accumulated is related to drought resistance (Protsenko *et al.*, 1968). Under comparable water deficit conditions, drought resistant varieties synthesize more proline than less resistant varieties (Palfi and Juhasz, 1971), and the ability of 10 barley varieties to accumulate proline under severe stress has been positively correlated with their drought resistance rating (Singh *et al.*, 1972). This evidence suggests that the amount of proline accumulated under standard conditions may be a good indicator of drought resistance or susceptibility.

In an attempt to extend this correlation between genotypic resistance to water stress and proline accumulation to salinity stress, we compared 12 different species. The results indicated that there was no correlation between proline accumulation potential and the salt tolerance of the species (Table 10). Since, however, the relative salt tolerance for each species was judged from the work of Nieman (1962), and was not measured in the present experiment, the difference may have resulted from working with different varieties of the same species. Alternatively,

genetic differences in stress tolerance within species may be correlated with proline accumulation potential but the correlation may not extend across inter-specific boundaries.

Apart from water stress, proline accumulation may be used as a reliable index of tissue water deficit so as to differentiate between water and heat stress, since proline did not accumulate in leaf tissue when the plant was subjected to heat stress without concomitant water deficit. This use cannot be extended to chilling stressed plants, however. It has been demonstrated that neither leaf water potential, osmotic potential nor turgor changes occuring at low temperature are sufficient to account for the accumulation of proline. It is possible, however, that proline accumulation may be as useful an index of cold resistance as it is of drought resistance. Although a close correlation has been demonstrated between the accumulation of free proline and tissue resistance to low temperature (Lebedev and Komarnitskii, 1971) this possibility requires further investigation.

The effect of salinity on water uptake by, and the internal water status of the plant is still under dispute. Many plants compensate for the lower osmotic potential of the growth media by a decrease in their internal osmotic potential particularly when the plants are subjected to a gradual increase in the salt concentration in the root environment. The osmotic gradient for water absorption is thereby apparently maintained. However, in this condition, plants still accumulated large amounts of

proline in the tissues. This proline accumulation seems to be mediated through a lowering of the osmotic potential in the plant. This accumulation demonstrates that even though plant water status has returned apparently to normal, and turgor has been regained, and transpiration has resumed, plants exposed to a saline substrate show persistent metabolic responses.

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