# THE ROLE OF LIGHT IN STRESS-STIMULATED PROLINE ACCUMULATION IN BARLEY

by

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To

Amma and Appa

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## SUMMARY

The influence of light on stress-stimulated proline accumulation has been examined in barley leaf tissue. Light increased the accumulation of proline in water-stressed tissue, independent of changes in tissue water status; while in tissue exposed to low temperatures there appeared to be an absolute requirement for light for proline accumulation. This response to light could conceivably be mediated through a phytochrome response or be due to photosynthesis. The present results showed that neither a medium intensity light exposure for a brief period, nor continuous illumination at very low irradiances (<1 $\mu E\ m^{-2}s^{-1}$ during water stress or <15 $\mu E\ m^{-2}s^{-1}$ during cold stress) increased proline accumulation; thus rendering it unlikely that proline accumulation was controlled by a low-energy phytochrome response. The wavelengths of maximum effectiveness for proline accumulation were in the blue and red regions of the spectrum, similar to those for photosynthesis, while irradiation with far red did not increase proline accumulation above accumulation in the dark. These data were consistent with the hypothesis that light acts on proline accumulation through photosynthesis and that a phytochrome response was not involved. Evidence for the involvement of photosynthesis was obtained from experiments with photosynthetic inhibitors which showed an inhibition of proline accumulation concurrent with inhibited photosynthesis. Further experiments with various $CO_2$ concentrations, levels of irradiance and supply of radioactive $CO_2$ support the conclusion that the light energy trapped was the prime requirement for proline accumulation during water stress, whilst, during exposure to the

cold, both the carbon fixed and the energy trapped were required. The trapped energy appeared to influence proline metabolism through both proline synthesis and proline utilization. Apart from these effects of light, the presence of carbohydrates also influenced the accumulation of proline. Tissue which was high in carbohydrates synthesised more proline from glutamate (in water stressed samples only) and metabolized less proline (in both water stressed and turgid samples).

It was concluded that proline accumulation was increased by light during both water and cold stress. An increase in carbohydrate concentration in the tissue was not an obligatory prerequisite for proline accumulation during water stress, but was for accumulation during exposure to low temperatures. Thus, no single unique role for light in proline accumulation during stress has been demonstrated, but various mechanisms contribute to the response.

## STATEMENT

This thesis has not been previously submitted for a degree at this or any other University, and is the original work of the writer except where due reference is made in the text.

(Priya Sreenivasan Aiyar)

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ABA | Abscisic acid |
| Alc | Alcoholic |
| Amm. chloride ($NH_4Cl$) | Ammonium chloride |
| ATP | Adenosine triphosphate |
| $^oC$ | Centigrade (in degrees) |
| CCCP | Carbonylcyanide m-chloro phenylhydrazone |
| CHO | Carbohydrates |
| Ci | Curie |
| cm | Centimetre |
| CMU | 3-(4-chlorophenyl)-1,1-dimethylurea |
| Conc. | Concentration |
| $CO_2$ | Carbon dioxide |
| cpm | Counts per minute |
| DCMU | 3-(3',4'-dichlorophenyl)-1,1-dimethylurea |
| df | Degrees of freedom |
| DW | Dry weight |
| $Em^{-2}s^{-1}$ | Einsteins per metre$^2$ per second |
| g | Gram |
| GA | Gibberellic acid |
| h | Hour |
| IRGA | Infra red gas analyser |
| LSD (I) | Least significant difference |
| l | Litre |
| m | Milli |
| MCW | Methanol:chloroform:water |
| mg | Milligram |
| ml | Millilitre |

| | |
|---|---|
| mol | Molecule |
| mol wt | Molecular weight |
| m.volt | Millivolt |
| NAD(H) | Nicotinamide adenine dinucleotide (reduced) |
| NADP(H) | Nicotinamide adenine dinucleotide phosphate (reduced) |
| nm | Nanometre |
| $O_2$ | Oxygen |
| OD | Optical density |
| P | Probability |
| PEG | Polyethylene glycol |
| pH | Negative logarithm of the hydrogen ion activity |
| POPOP | 1,4-Bis-[2-(5-phenyl-2-oxazolyl)]Benzene |
| PPO | 2,5-Diphenyloxazole |
| ppm | Parts per million |
| Rf | (In TLC) = $\frac{\text{distance of the solute from the origin}}{\text{distance of the solvent from the origin}}$ |
| RWC | Relative water content |
| sec | Second |
| Sat | Saturated |
| TLC | Thin layer chromatography |
| VR | Variance Ratio |

SYMBOLS

| | |
|---|---|
| $\gamma$ | Gamma |
| $\mu$ | Micro |
| | Osmotic potential |
| % | Percent |
| $\therefore$ | Therefore |
| $\psi$ | Water potential |

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# I INTRODUCTION

# I. INTRODUCTION

It is now well established that proline accumulates under unfavourable environmental conditions. Proline is known to accumulate in response to water deficit (Stewart et al, 1966; Palfi et al, 1973; Singh et al, 1973a), salinity (Chu et al, 1974; Stewart and Lee 1974), temperature extremes (Trione et al, 1967; Chu et al, 1976, 1978) and insect and pathological infection (Meon et al, 1978). The trigger for proline accumulation is still not known, although a change in proline concentration has been observed to be correlated with a change in water status of the plant. This imbalance in the water status of the plant is brought about either by excessive transpiration or reduced water uptake or both. The significance of this increased accumulation of proline is still not clear. Some workers (Palfi et al, 1972; Singh et al, 1972) have shown a positive correlation between the potential to accumulate proline and drought resistance while there are others (Hanson et al, 1977) who hold the view that the highest proline accumulators are the least resistant plants to drought.

Several external factors which alter plant metabolism also seem to alter proline accumulation. One such factor is light. Proline accumulation can be substantially increased by light under water stress conditions (Routley, 1966; Palfi, 1971; Stewart, 1972; Hanson and Tully, 1979) and is dependant on light at low temperatures (Chu et al, 1978). Investigation on the mechanism of light stimulation of proline accumulation is lacking. This thesis is an attempt at elucidating this aspect of proline accumulation.

# II LITERATURE REVIEW

# II LITERATURE REVIEW

## 1. Accumulation of proline during stress

### 1.1 Water stress

Plants respond differently to a reduction in the available water. Apart from physical adaptation, a frequent response is the accumulation of low molecular weight organic solutes. Proline, glycinebetaine, sucrose, sorbitol and some amides constitute the majority of the solutes known to accumulate in plants. An increase in the concentration of free proline in response to water deficit is, however, the most frequent and dramatic. Proline has been shown to accumulate to a concentration as high as 10% of the dry weight of the tissue (Stewart and Lee, 1974) under stress conditions. Not only does proline accumulation occur in mesophytes, but also in halophytes (Stewart and Lee, 1974), resurrection plants (Tymms and Gaff, 1979) and lower plants (Schobert, 1977b) Brown and Hellebust, 1978; Setter and Greenway, 1979). Schobert (1977b) observed a ten-fold increase in proline concentration in response to osmotic stress of 2000 milliosmols in the diatom Phaedactylum tricornutum and other diatom species. The proline concentration in Chlorella emersonii increased hundred-fold in response to a -16 bars osmotic stress (Setter and Greenway, 1979), and similar accumulation occurred in the green alga Stichococcus bacillaris (Brown and Hellebust, 1978).

In higher plants, proline accumulation has been investigated in a number of different species. When Ladino clover lost 15% of the water content of the leaves due to a depletion of soil moisture, proline accumulated in the leaves (Routley, 1966). A reduction in soil water content from 70% to 13% induced a 10% decrease in leaf

water content and a five-fold increase in proline in barley leaves (Saviskaya, 1967). Similar results, where reduced soil water caused a reduction in water content and an increase in proline levels in intact plants, include radish (Chu, 1974 ), sorghum and soybean (Waldren et al, 1974), rice (Mali and Mehta, 1977) wheat (Rajgopal et al, 1977; Bengston et al, 1978; Munns et al, 1979) and many others. The addition of polyethylene glycol (PEG) to the root medium, simulates a water stress condition by lowering the water potential and also produces an increase in free proline in intact plants. For example, Singh et al (1973a) have shown a linear increase over several days in proline concentration upto 15 mg $g^{-1}$ DW in intact barley plants in response to -20 bars PEG; and experiments with intact Pennisetum typhoides plants showed a five-fold increase in proline concentration when stressed with -20 bars PEG for 24 h (Huber and Schmidt, 1978).

Water stressing excised tissue also induces proline accumulation in plants such as bean (Stewart et al, 1966), turnip (Thompson et al, 1966), bermuda grass (Barnett and Naylor, 1966), wheat (Tyankova, 1967), capsicum (Palfi et al, 1974) and tobacco (Boggess et al, 1975).

Proline also accumulated in excised leaves floated on PEG solutions. Singh et al (1973b) observed a twenty-fold increase in proline in leaves of barley stressed with -20 bars PEG and a similar increase was noted by Chu (1974) in radish leaves. Floating the isolated organs of barley on -20 bars PEG for 48 h induced maximum proline accumulation in the leaves, some in leaf sheaths and none in the roots or shoot apices (Singh et al, 1973b). However,

since proline accumulated in the roots of stressed, intact barley (Singh et al, 1973a) and radish (Chu, 1974) it would seem that proline or its precursors may be synthesized in the green tissue and then transported to the roots. This hypothesis is supported by experiments on two wheat varieties where the plants were dehydrated either intact or excised just above the roots or the tillering nodes where the leaf accumulated maximum proline (Tyankova, 1967). Similar results were obtained with isolated, dehydrated organs of wheat, paprika and sunflower where there was no proline accumulation in the roots and stem, but accumulation to a very high concentration in the lamina (Palfi, 1971). Provision of precursors of proline synthesis to excised stressed barley roots could not induce proline accumulation, although similar provision to etiolated or chlorotic leaves initiated accumulation (Singh et al, 1973b). It can be concluded that tap roots and fibrous roots are not capable of accumulating proline independent of the aerial parts of the plant. Storage roots, however, may be different (Wright et al, 1977).

The subcellular location of the proline in leaf cells has still not been defined, although most of the evidence would suggest a cytoplasmic location. If proline is localized in a similar region to betaine, which also accumulates under environmental stress, then it is not in the vacuole. Work by Wyn Jones et al (1977) clearly excluded the vacuole as a possible site for proline

accumulation. Using electron microscopic techniques, Flowers et al (1978) have shown a cytoplasmic distribution of betaine. Work on maize roots indicates that proline also may be located in the cytoplasm (Goring et al, 1977) although isolation techniques have not been successful in localizing proline to particular compartments due to the extreme solubility of proline in most solvents.

A consequence of the dehydration of the cell during stress is the increase in concentration of the various ions present in the cell. This increase may exert some influence on proline accumulation and the effects of various ions has been assessed by applying salts containing these ions to the growing medium. An increased accumulation of proline in excised tobacco leaves in the presence of 0.25 M $Mg^{++}$ has been reported by Tyankova and Stoyanova (1977). In stressed maize leaf discs and intact cucumber, cowpea and bean leaves, $K^{+}$ was found to enhance proline accumulation (Mukherjee, 1974; Udaykumar et al, 1976; Arcay and Rena, 1977). This difference in enhancement by different ions could be due to differences between species. By altering the pH of the medium, it was shown that the differences between the ions was not due to pH changes. Proline accumulation was greatest at a pH of 7. Both an increase and decrease in pH led to a decrease in the proline concentration (Arcay and Rena, 1977; Tyankova and Stoyanova, 1977).

Thus, proline accumulates in response to an environmental stress, in most plants, predominantly accumulates in the leaf, has a pH optimum of 7 and is influenced by specific ions.

## 1.2 Low temperature stress

The temperature of the environment influences metabolism and there have been differing reports on the effects of lowered temperature on the nitrogen status of the plant. Gates et al (1971) reported a reduction in total nitrogen with a gradually decreasing growth temperature, down to 13°C day and 9°C night temperatures in Stylosanthes humilis. Soluble protein, however, has been reported to increase in many plants at low temperatures; including perennial ryegrass (Draper and Watson, 1971), live bark of black locust tree (Siminovitch and Briggs, 1949), corn roots (Rodchenko and Maridera 1973) and winter wheat (Trione, 1966). There are a few reports (e.g. Minimikawa et al, 1961; Young, 1969) of a lack of change in the soluble proteins in response to chilling temperature. However, it is likely that differences exist in the metabolic response of different plants to low temperature stress resulting in varied effects on the different metabolic components. Even closely related species show a difference in response to chilling temperature. Winter wheat showed a three-fold increase in soluble proteins when exposed to 2°C while soluble protein concentration in spring wheat did not change (Trione, 1966).

Reports of an increase in total free amino acids have, however, been unanimous. Winter wheat plants showed an increase in free amino acid from 5% to 12% of the total nitrogen when exposed to 2°C for two weeks (Pauli and Mitchell, 1960). Total free amino acid increases reported in other plants include spring and winter rye seedlings exposed to 4°C for 3 weeks (Thomson and Zalik, 1974) and Lolium plants at 2°C for 2 weeks (Draper, 1972).

Proline was invariably the main amino acid contributing to this increased free amino acid pool. In wheat it increased ten-fold in the winter and four-fold in the spring variety (Trione et al, 1966). Increases in proline concentration have also been reported in young apple shoots (Benko, 1968), and in stylo (Gates et al, 1971). Palfi and Bito (1970) reported increases in the leaves of paprika, sunflower, bean and wheat in response to lowered root temperatures, and, in Lolium plants, proline increased from 2.1% to 14.4% of the total amino acids, which had increased three-fold with cold treatment (resulting in a twenty-fold increase in proline) (Draper, 1972).

As in the case of water stress, proline accumulated in all parts of the intact plant, with the maximum in the aerial organs and minimum in the roots. In intact barley plants exposed to 5°C, proline concentration was greater in the leaves, somewhat less in the shoot and still less in the leaf sheath and roots (Chu et al, 1974). The young growing tissues are also capable of accumulating massive amounts of proline when exposed to low temperatures, e.g. apices of wheat and rape (Stefl et al, 1978), cabbage buds (Shvedskaya and Kruzhilin, 1964, 1966) and the buds of chicory (Bouinols and Margara, 1969). While the aerial parts of carrot and table beet showed twelve-fold increase in proline concentration with cold treatment, the roots showed no increase (Shvedskaya and Kruzhilin, 1964, 1966). In paprika, sunflower, bean and wheat, although the aerial organs accumulated proline, the roots did not (Palfi and Bito, 1970), and winter wheat and rape showed only slight accumulation of proline in the roots (Stefl et al, 1978). Proline accumulation has also been reported in the imbibed grains of wheat after vernalization (Jones and Weinberger, 1970). This accumulated proline was localized in the embryo, with no proline in the endosperm of the grain.

Proline accumulation in excised tissue has been reported only for barley (Chu et al, 1978). Leaf sections were capable of accumulating proline when floated on water at 5°C in the presence of light. The amount of proline accumulated, however, was not as much as in intact barley plants.

## 2. Endogenous factors influencing proline accumulation

### 2.1 Water stress

Water deficit is marked by a reduction in the water potential of the tissue. This decrease in water potential may be due primarily to a decrease in the osmotic potential of the cell or to a decrease in the turgor pressure, or, more commonly, to a combination of both. Where water deficit is induced by the exposure of the plant to permeable solutes or where stress has been induced very gradually and osmotic adjustment has occurred, turgor may be maintained during the stress period. This has been shown to occur in barley and radish in response to sodium chloride stress (Chu, 1974b) and in sorghum which was stressed very gradually (plants remained turgid at a water potential of -20 bars (Fereres et al, 1978)). The stomata remained open in these sorghum plants, so turgor was not maintained as a result of reduced transpiration, and hence increased leaf water potential, but by osmotic adjustment. This reduction in water potential is almost always followed by an increase in proline concentration in the tissue.

The tissue water potential threshold for proline accumulation has been measured in several plants including excised barley leaf segments which accumulate proline at -10 bars external osmotic potential of the PEG medium but not at -5 bars (Singh et al, 1973b). In intact barley plants accumulation commenced at -8 bars water potential (Singh et al, 1973a) in radish at -11 bars (Chu et al, 1976), Pennisetum typhoides leaves at -5 bars (Huber and Schmidt, 1978), Phaseolus vulgaris -10 bars (Jager and Mayer, 1977), cotton leaves between -15 and -17 bars (McMichael and Elmore, 1978), field grown

sorghum -24 bars and field-grown soybean at -20 bars, and -12 bars for soybean grown in a controlled environment (Waldren et al, 1974; Waldren and Teare, 1974). It is evident from these results that the threshold for proline accumulation varies considerably between plants. However, the differences may rest largely on technical grounds e.g. the same authors reported a different threshold for proline accumulation in soybean under field conditions to that in a controlled environment. On going through their data it is clear that under controlled environment conditions water stress was imposed rapidly, the rate of decline in water potential was rapid, stomatal closure was rapid and the threshold for proline accumulation was -12 bars. In the field situation, however, the stress was imposed over a three week period, the decline in water potential was gradual, stomates closed at a lower water potential and proline accumulation began only at -20 bars. It is highly likely that under field conditions, due to imposition of a gradual stress, the plants were able to osmoregulate. This explanation would favour proline accumulation in response to a reduction in turgor potential rather than a decrease in water potential.

As proline concentrations are well correlated with the reduction in plant water potential, it is not surprising that proline undergoes a diurnal fluctuation in its concentration in parallel with the diurnal variation in plant water potential. Field-grown wheat plants experiencing water stress showed a minimum relative water content (RWC) at 1200 hours which increased thereafter. The proline concentration in these plants was inversely related to RWC, maximum proline amounts occurring at 1200 hours, decreasing thereafter. In turgid plants, however, the proline concentration was greatest at night, indicating

that the controls over proline accumulation under stress conditions are different to those in turgid plants (Rajgopal et al, 1977). Triglochin maritima shoots, showed a seasonal variation in proline concentration; the highest levels occurred at the time of maximum growth and the concentration declined towards the end of the growing season (Stewart and Lee, 1974).

Age and activity of the tissue also seem to determine the amount of proline accumulated. Young actively growing tissue has been reported to contain higher concentrations of proline than old tissue. The second leaves of barley contained more proline than the first leaves (Singh et al, 1973a) and stressed plants of radish and wheat respond similarly (Chu et al, 1976; Munns et al, 1979). The greater accumulation of proline in the younger tissue may be due to lower water potential in these tissue (Munns, et al, 1979) or to less vacuolation. In corn roots, the non-vacuolated meristematic cells of the tip accumulated a higher concentration of proline than do the cells of the more mature region (Goring, 1977).

On re-watering, proline disappears from the tissue very rapidly, mainly through the oxidation of proline to glutamic acid (Stewart, 1972; Singh et al, 1973c). Younger leaf tissue loses its proline more rapidly than older tissue (Singh et al, 1973c) and this may be due to less leaf death in the younger tissue in response to water stress, as suggested by Hanson et al (1977). The time taken for proline levels to drop to non-stressed levels after removal of stress varies with the plant, and is probably determined by its water stress history. Chlorella attained pre-stress levels of proline within one hour after transfer to non-stressing medium (Greenway and Setter, 1979). In higher plants the time for recovery varied between 10 hours for

wheat (Bengston et al, 1978), to 4 days in cotton (McMichael and Elmore, 1978) and bean (Jager and Meyer, 1977).

Although many of the factors which influence proline accumulation are well known, the factor(s) which triggers it off is still a subject of controversy. Greenway and Setter (1979) believe that, in Chlorella, the decrease in turgor is the switch for proline accumulation. They argue that proline accumulation increased at a linear rate in response to the increase in external osmotic pressure as long as the turgor potential was positive. At incipient plasmolysis, when turgor was zero, the proline accumulation rate was reduced. Moreover, when the cells were exposed to a permeating, non-metabolisable osmoticum, methyl-glucose, proline accumulation did not occur. In this case, the turgor of the cells was not reduced, although there was a reduction in internal osmotic potential. Chu et al, (1976) found that proline continued to accumulate in barley plants exposed to NaCl stress and which had regained turgor after an initial decrease. They proposed that proline accumulation was linked to osmotic potential reduction rather than to turgor pressure. However, it is possible that, as the turgor potential measurements were based on the difference method (TP = WP - OP), small changes in turgor pressure were not detectable. Whatever might be the initial sensor, however, it is clear that the change in water potential is correlated with proline accumulation.

Not only is reduced water potential capable of inducing proline accumulation, but a similar effect can frequently be brought about by the application of abscisic acid. Aspinall et al (1973) induced proline accumulation in both intact and excised leaves of turgid

barley by supplying exogenous abscisic acid. Similar results were obtained by Rajgopal and Anderson (1978) where $10^{-7}$ M ABA caused proline accumulation within 2 to 3 hours; a similar increase in proline with ABA treatment was observed in Lolium temulentum (Aspinall et al, 1973) and in Lemma (Andres and Smith, 1976). In no case did ABA lower plant water status. This response is not universal, however, as ABA did not increase the proline concentration in tobacco (Aspinall, 1980) nor in sunflower (Wample and Bewley, 1975).

## 2.2 Low temperature stress

It is important to consider the manner in which the water status of a plant may be altered by chilling temperatures prior to discussing consequential changes in proline concentrations.

The two main processes which significantly affect the water status of the plant are the uptake of water by the roots and water loss due to transpiration from the leaves. In well watered conditions an equilibrium between the two is maintained, resulting in a steady water potential in the cell. If there is an imbalance between these two processes the water status of the plant is changed, and this results in an altered water potential.

According to Kramer (1942) there is a reduction in the uptake of water by the roots under cold temperatures. This decrease is due to decreased root growth and a reduction in the hydraulic conductivity of the cell membrane, especially at the soil-root zone, coupled with

an increase in the viscosity of water. In short term experiments, however, where the effect of low temperatures on root growth is small, the main change in water status is due to a combination of reduced water uptake and increased viscosity of water (Kramer, 1942). Comparing different plant types with respect to water uptake at low temperature, Kramer (1942) concluded that plants growing in a warm environment (e.g. cotton and watermelon) showed a greater reduction (80%) in water uptake at 10°C compared to 25°C, than temperate crops (e.g. cabbage and white pine) which experienced only a 25% reduction in the uptake of water at 10°C compared to 20°C.

Due to the increased viscosity of water at low temperatures, the movement of water within the plant may also be reduced. A decrease in the lateral movement of water across the xylem-phloem boundary in response to reduced temperatures has been reported in cotton stems (Klepper et al, 1973). This, according to Klepper et al (1973) was a consequence of a reduction in the effective diffusion coefficient, with an associated increase in resistance to water flow at low temperatures.

Water status is also greatly influenced by transpiration of the plant. In Phaseolus vulgaris, there was an increase in stomatal aperture when plants were transferred to 5°C (Wilson, 1976) while cotton showed a 50% reduction in the transpiration rate at 10°C when compared with that at 25°C (Grobbelaar, 1963). It is possible that such varied effects are due to differences in the optimum growth temperatures of the two species; cotton being a tropical crop may be more severely affected than Phaseolus vulgaris which is temperate.

Studying the response to reduced temperatures in only one of the above processes is of no great significance as the ratio between the two processes is decisive in establishing the water status of the plant.

This point has been demonstrated by Kramer (1940) who showed that the decrease in water uptake due to cold root environments leads to a concomitant reduction in the transpiration rate of the plant, such that the ratio between the two processes remains constant. Such a 1:1 relationship was found to be the case in tomatoes, collards watermelon, sunflower and pines (Kramer, 1940, 1942). Recently, Aston and Lawlor (1979) have also shown a 1:1 relationship between water uptake and transpiration, in sunflower, maize and barley. They too reported a cooling-induced reduction in uptake and a proportional decrease in transpiration of the plant; such that the ratio remained constant.

It is not unexpected, therefore, that the effects of reduced temperature on leaf water potential are absent or transient. In maize (Kleinendorst and Brouwers, 1972), barley and radish (Chu et al, 1974), water potential was reduced initially, but increased to the original level within a short period. Chilling sensitive tropical species (cotton, stylo, tobacco and Rhodes grass) developed a lower water potential at 10°C than did temperate crops (peas, radish, lentils and lettuce) Chu et al, 1978). However, there was no correlation between proline accumulation and tissue water potential.

In response to cold temperatures, barley and radish accumulated a considerable amount of proline although the plant water potential showed little initial change (Chu et al, 1974). Proline accumulated

in intact and excised tissues of barley in response to cold temperatures, only in the presence of light (Chu et al, 1978). Accumulation of proline showed an initial lag of 24 hours after which it accumulated rapidly at the rate of 74 μg $g^{-1}$ DW $h^{-1}$.

The existence of a critical temperature for proline accumulation was investigated and in barley, there was no evidence of proline accumulation within 72 hours at or above 8°C, but rapid accumulation at 6.5°C or lower. This was strong evidence of a precise, critical temperature above which accumulation does not occur (Chu et al, 1978). The initial rate of accumulation during the first 24 hours of cold treatment was greater at 4°C than at 5.6°C, suggesting the initiating responses occur more quickly at lower ambient temperatures than at higher ones. The critical temperature appears to differ for different species (Chu et al, 1978).

Once initiated, proline continued to accumulate in barley plants exposed to cold for 27 days. The rate was rapid for the first 11 days (818 μg $g^{-1}$ DW $day^{-1}$) after which it was reduced to 280 μg $g^{-1}$ DW $day^{-1}$; accumulation continued at this rate to a final concentration of 14.7 mg $g^{-1}$ DW by day 27 (Chu et al, 1978). Unlike water stress, the transfer of barley plants from 5°C to 20°C did not result in an immediate decline in proline levels. Instead, proline continued to accumulate for 24 hours at the same rate as in plants maintained at 5°C. The possibility of this being due to the persistence of low temperatures in any part of the plant following transfer was eliminated by flooding the root systems with nutrient solution at 20°C (Chu et al, 1974). After 24 hours, however, proline concentration declined rapidly.

As with water stress responses, the age of the tissue also influences cold-induced proline accumulation. In barley, the first leaf accumulated more proline than the developing second leaf, while in radish, the younger second leaves accumulated more proline than the first leaves. In the results with barley, it may well be that the second leaf had not expanded fully when given the treatment (Chu, 1974 ). Young leaves of wheat and rape were also found to have greater proline concentrations in response to cold treatment (Stefl et al, 1978).

Thus like water stress, there is a threshold condition which must be reached before proline accumulation can occur. In water stress the water potential must fall below a critical level, in cold stress water potential does not seem to be the controlling factor, but the temperature must fall below a critical level.

3. Consequence of proline accumulation

There exist different speculations on the consequences of the high proline content accumulated by many plants in response to environmental stress conditions. Some reports assign a special beneficial consequence, while others do not attribute any special significance to this phenomenon; other than as a metabolic breakdown product.

Proline accumulates up to very high concentrations and is extremely soluble in water (162 g/100g $H_2O$ at 25°C). It behaves like a hydrophilic colloid, forming strong hydrogen bonds with water, especially at high concentrations. From NMR studies, Schobert and Tschesche (1978) proposed that proline forms aggregates by stepwise stacking and hydrophobic interaction of the pyrrolidine ring. Thus, the resulting multimer of proline is pictured as containing a hydrophobic backbone with hydrophilic groups on the surface exposed to water, making it extremely hydrophilic.

Being a neutral imino acid, the accumulation of proline does not result in major pH changes, necessitating the simultaneous accumulation of neutralizing ions. By virtue of its solubility and neutrality proline is able to accumulate upto high concentrations within the plant without deleterious effects.

Speculation on the consequences of proline accumulation has touched on osmoregulation, protection, and function as a non-toxic reserve for carbon, nitrogen and energy. Also there are a few reports which consider the phenomenon as having no important metabolic or physiological consequences.

## 3.1 Osmoregulation

When plants are stressed gradually, there is an initial loss of turgor which is regained in time (the time required varying with the type of stress and the species). This recovery of turgor has often been attributed to increased osmotic concentration within the plant (by the synthesis of osmotically active substances) but could also be due to the increase in membrane elasticity, and is known as turgor regulation or osmotic adjustment. In Chlorella, proline accumulation increased with an increase in the concentration of the external non-permeating medium (Setter and Greenway, 1979). Proline contributed substantially (66% to 80% of the internal osmotic potential) to the osmoregulation of Chlorella adapted to 335 mM NaCl for 2 to 3 days. Rapidly permeating ethylene glycol at similar osmotic potential, however, had relatively little effect on growth (due probably to the lack of effect on turgor potential) and did not lead to accumulation of proline or any other organic solute. Thus, lower water potential per se, had no large effects on the metabolism of Chlorella, but a reduction in the turgor potential induced rapid proline accumulation.

In wheat, proline accumulation commenced at the stage when extension growth had almost ceased (Munns et al, 1979). The authors suggest that proline accumulation is associated with and perhaps dependant on, a reduction in growth or protein synthesis; and the concomitant build up of precursors.

In the halophytes, however, although high external osmotic concentrations increased proline concentrations, growth was not retarded (Stewart & Lee, 1974). In three different species of halophytes, the amount of proline accumulated was very well correlated with the changes in osmotic potential, again suggesting the involvement of

proline in osmoregulation. In diatoms and Chlorella as well, changes in the osmotic concentrations of the external solution resulted in proportional changes in the internal concentrations of proline (Schobert, 1974; Setter and Greenway, 1979).

## 3.2 Protection

In addition to the probable function of proline in osmoregulation, proline may maintain cellular integrity by protecting the cell constituents from dehydration and other adverse effects during environmental stress. Freezing the chloroplasts of spinach for 4 hours at 25°C, inactivated the photophosphorylation of thylakoids, by irreversibly altering the essential membrane properties (Heber et al, 1971). Washed thylakoids, frozen in the presence of proline, however, were protected against damage. Further, increasing the concentration of proline in the medium, increased the photophosphorylation until frozen samples had the same activity as unfrozen controls. This increased stability of membranes was suggested to be due to the protection by proline of the membranes against the toxic organic and inorganic cellular substances concentrated during stress (Heber et al, 1971). Similarly, mitochondria isolated from etiolated leaves of oats showed reduced activity in the presence of 0.1 M NaCl. The simultaneous addition of 1 M proline, however, completely compensated this inhibitory effect of salt (Schobert, 1977b). Also, the freezing of maize cells in the presence of 10% proline solution conferred an increased post-thaw viability and growth (Withers and King, 1979). Not only does proline protect organelle integrity during stress, but has also been reported to protect several cytoplasmic, mitochondrial and chloroplastic enzymes against inactivation by environmental stress. The addition of 0.4 M proline solution to the enzyme prior to heating at 50°C

for 30 minutes, protected PEP carboxylase, fumarase and isocitrate dehydrogenase (both chloroplastic and mitochondrial) of peas against heat-inactivation (Paleg, 1980; Nash et al, 1980). Proline showed similar protection against NaCl inhibition of the enzyme malate dehydrogenase (Pollard and Wyn Jones, 1979). This protection provided by proline is perhaps due to its unusual properties, and its interactions with proteins.

It was found that proline solutions were able to increase the solubility of sparingly soluble proteins (Schobert and Tschesche, 1978). The enhancement effect depended on the nature of the protein and the proline concentration. The authors postulated that a hydrophobic interaction of proline with the hydrophobic surface residues of protein, increased the hydrophilic areas of the complex. The presence of proline in solutions of bovine serum albumin reduced the precipitation of this protein by ethanol and ammonium sulphate, presumably by an increased water binding capacity of the proline-protein solution.

Not only is free proline reported to increase under environmental stress conditions, but protein-bound proline is increased significantly. In barley leaves, the amount of proline in protein increased from 3.03% to 4.56% during stress. A similar increase was observed in the roots of barley (Singh, 1970). Similar results were obtained by Boggess et al (personal comm) with tobacco. Stefl et al (1978) have also reported a significant increase in protein-bound proline in winter wheat and rape plants exposed to low temperatures. This increase in proline in the protein can influence the configuration of the proteins considerably, as has been shown by Brandts et al (1977). The presence of proline influenced the rate of folding and unfolding of the secondary structure of proteins.

### 3.3 Other roles

Apart from the physical properties of proline which may be advantageous to the plant during unfavourable conditions, proline may have a metabolic role as a storage compound; rapidly utilized on the release of stress (Barnett and Naylor, 1966; Stewart et al, 1966). Proline could function as an effective reserve for nitrogen and carbon skeletons by virtue of its metabolic proximity to glutamic acid and ready conversion to that key compound in amino acid synthesis. It could also serve as a ready source of energy, as the oxidation of proline to glutamic acid produces two equivalents of reduced nucleotides. In Myxomycetes, cell division was well correlated with proline concentration. Proline levels were high prior to the $G_2$ phase, and fell during the $G_2$ phase, presumably due to its oxidation to provide energy. Prevention of proline oxidation by anaerobiosis resulted in decreased mitosis (Threlfall and Thomas, 1979). In the cockroach, Periplanata americana, proline and glutamine were the major amino acids in resting animals. Subjecting the insects to forced activity or electrical stimulation, resulted in a 50% to 60% drop in the proline level, indicating that proline was used as a reserve energy store and as a possible source of glutamic acid (Holden, 1973). In sorghum varieties, proline accumulation was significantly correlated with dark respiration rate and free ammonia generated during recovery from water stress (Blum and Ebercon, 1976). In addition to being accumulated rapidly in response to stress, proline is distributed to the other parts of the plant. It is thus conceivable that proline may play a major role as a storage compound, being rapidly metabolised with the onset of favourable conditions to yield energy and glutamic acid, akin to its role in pollen grains (Britikov et al, 1965).

Contrary to the interpretation of proline accumulation as being advantageous to the plant during unfavourable conditions, there are some workers who feel that proline accumulation is of no consequence to the plant other than it being the end result of metabolic disruption. By girdling (steaming and using a cold jacket) experiments, Tully et al (1979) have demonstrated that proline accumulation commenced under stress conditions when transport out of the leaf was inhibited. Water deficit resulted in the accumulation of amino acids and was thought to be due to reduced protein synthesis (Hsiao, 1973) though unaffected by hydrolysis of proteins (Shah and Loomis, 1965). This correlation between cessation of translocation and the commencement of rapid proline accumulation was confirmed using $^{14}C$ photosynthates. Much of the proline which accumulated was located in the severely wilted portion of the leaf, which did not recover when stress was relieved. (Hanson, et al, 1977). Quantitatively, proline would thus seem not to be an important nitrogen reserve within stressed leaves, especially because of the localization of a major proportion of it within killed tissue, which precludes its re-use upon relief of stress. However, Singh et al (1973c) and Blum and Ebercon (1976) have shown a positive correlation between leaf survival and proline accumulation under stress, in which plants accumulating higher proline concentrations recovered better than low accumulators.

Accumulated proline may be advantageous not only in one or more of the possible ways mentioned above, but it seems to be one of the least toxic compounds to plant growth and metabolism. The

germination of wheat seeds treated with glutamine, asparagine or proline in the medium, showed proline to be least inhibitory (Palfi *et al*, 1974). The activity of several enzymes of carbohydrate and nitrogen metabolism were unaffected by proline concentration up to 700 mM in the halophyte *Triglochin maritima* (Stewart and Lee, 1974) while in barley, not only was proline not inhibitory upto 500 mM concentration, but also protected the enzyme malate dehydrogenase against inhibition by 100 mM NaCl and KCl (Wyn Jones *et al*, 1977).

Thus, of the various organic compounds present within the plant, proline may be one of the most suitable to accumulate, with the least deleterious effects and, perhaps, several advantageous attributes.

## 4. Proline accumulation and resistance to stress

### 4.1 Water stress

Proline accumulation in response to water deficit is almost universal in higher plants. Of 80 different cultivated plants, only four (corn, spinach, sorrel and lettuce) did not accumulate proline in response to water deficit (Palfi *et al*, 1972). There was no consistency in variation even amongst the plants in the same family. Sunflower and lettuce belong to the same family, yet sunflower accumulated high amounts of proline, while lettuce accumulated none in response to stress. On the other hand, plants of the family Solanaceae (potato, tomato, pepper, tobacco, *Solanum laciniatum*, etc) accumulated very similar amounts of proline in response to stress. Considering the widespread occurrence of proline accumulation and the variation between different species in the amount accumulated it is not surprising that attempts have been made to correlate proline accumulation with resistance to water stress. A positive correlation between resistance and proline concentration in the roots of *Tamarix* and peas was reported by Bar Nun and Polyakoff-Mayber (1977), in which *Tamarix* roots, which were more resistant to environmental stress, contained higher proline levels. A comparison of soybean and sorghum, stressed under both laboratory and field conditions, however, showed an inverse relationship, where soybean, the less resistant plant, accumulated more proline than sorghum, (Waldren and Teare, 1974). Such confusing results are to be expected when comparing two or more unrelated species, as the strategies for survival under unfavourable

conditions vary in different plants. This has been well documented in the report by Pourrat and Hubac (1974), in which two desert plants, Artemisia herba alba and Carex pachystylis were compared. Although both were extremely drought resistant in their natural habitat, their mechanisms for resistance seemed different and C. pachystylis accumulated high concentrations of proline during drought, i.e. cellular and metabolic changes took place, while in A. herba alba physiological adaptation occurred to avoid dehydration (i.e. reduced transpiration, well developed root systems, etc). Similarly, when diverse species of marsh halophytes were compared, (2-$C_3$ species, 3-$C_4$ species and 3 succulents), although all the species were resistant to drought, the $C_3$ grasses showed massive proline accumulation (63.6 µmoles $g^{-1}$ FW) commencing at a higher water potential ( -10 bars) the $C_4$ grasses showed less proline (max 27.4 µmoles $g^{-1}$ FW), accumulation commencing at a lower water potential (-20 bars) while the succulents accumulated the least proline (less than 20 µmoles $g^{-1}$ FW) and also had the lowest water potential threshold (> -25 bars) (Cavalieri and Huang, 1979). Thus, although comparisons between broad taxonomic groups may not be useful some interspecific comparisons of proline accumulation showed a good correlation with drought resistance.

In two carex species, C. pachystylis from the Negev desert, and C. setifolia from the wetter region of Europe, proline accumulated more in the species which was more drought resistant (C. pachystylis), than in C. setifolia which accumulated proline for the first 12 days, and then metabolized it paralleling the loss of resistance (Pourrat and Hubac, 1974).

Correlations between intraspecific variations in proline accumulation and drought resistance have been made by several workers, with different plants. Such comparisons may be more meaningful and may show an even closer relationship between resistance and proline accumulation. Barnett and Naylor (1966) compared proline accumulation capacity in two Bermuda grass varieties - 'coastal' and 'common' in which the former was more drought resistant. Proline accumulation was positively correlated with resistance. Similar relationships, where the more resistant varieties accumulated more proline, were reported in two populations of the halophyte Armeria maritima (Stewart and Lee, 1974) and in different varieties of wheat (Protsenko et al, 1968).

Due to the high correlation between water potential and proline accumulation, it is important to ensure that the water status of the samples which are to be compared experience similar changes in the internal water potential. Inherent differences in rates of transpiration and water uptake make it difficult to obtain and maintain such ideal conditions. However, by the use of methods which minimise such problems, comparisons between varieties with respect to resistance and accumulation potential have been made. Singh et al(1972) induced stress by substituting polyethylene glycol (mol wt 4000) solutions of the required osmotic potential for the nutrient culture. They compared ten cultivars of barley, stressed with -20 bars polyethylene glycol for 3 days. There was no genotypic variation in leaf water potential, but proline accumulation showed striking differences between varieties. The concentration of proline accumulated was linearly related to the stability index of the varieties, (where stability index was calculated as a ratio between the yield of the cultivar and the mean yield of all the cultivars at each site in each season) with the unstable

varieties accumulating the lowest amount of proline. Two of these cultivars of barley were used by Hanson et al (1977) to evaluate proline as a measure for drought resistance. They were unable to obtain uniformity in water potential between the two varieties, the less resistant variety having lower water potential and higher proline. The proline accumulated by the two varieties when water potentials were similar (albeit after different days of stress) was not significantly different. As the method for determining water potential was different to that used by Singh et al (1972) they questioned the accuracy of the water potential reported for the different cultivars by Singh et al (1972) in which bulk leaf water potential was measured.

In other studies where precautions were taken to ensure uniform water deficit in all cultivars, proline accumulation was highly correlated with resistance in safflower (Reddy and Shastry, 1977) and rice seedlings (Mali and Mehta,(1977). On the other hand, in two cotton genotypes, the less resistant variety accumulated more proline than the resistant variety but also experienced lower relative water content than the resistant variety, making it difficult to comment on the relevance of these results (Ferriera et al, 1979).

In sorghum, Blum and Ebercon (1976) found high correlations between proline accumulation and 'recovery' resistance. Recovery, 24 hours after re-watering, was evaluated by a visual estimate of the amount of leaf that regained turgor in the plant, measured on an arbitrary scale. Although proline accumulation capacity was highly correlated with recovery after stress, there was no correlation between dessication tolerance (measured using a more severe treatment) and proline accumulation, or any of the other parameters studied (recovery, respiration, ammonia evolution). Singh et al (1973c) reported similar correlations between proline accumulation and plant growth

following stress (i.e. recovery) in 14 barley cultivars, where those cultivars demonstrating high proline accumulation also showed higher leaf survival and recovery following the release of stress.

In experiments using barley cultivars given one, two or three cycles of water deficit, interspersed with adequate water supply, proline concentration increased, despite water potential in the different stress treatments remaining unaffected (Singh et al, 1973c). This hardening treatment also reduced the decline in chlorophyll content in response to stress. Free proline increased to 14.3 $mg\,g^{-1}$ DW in the first leaf after 2 previous stress episodes, while those plants experiencing stress for the first time, accumulated less proline (9.0 $mg\,g^{-1}$ DW).

Finally, if drought resistance is correlated with proline accumulation, it should be possible to induce resistance by the addition of exogenous proline to the tissue. When 500 µM proline was added to the rooting medium of wheat plants there was a greater maintenance of green colour and also quicker resumption of growth after re-watering (Tyankova, 1966). A similar increase in resistance was observed in Brassica campestris treated with 3% proline solution (Hubac, 1967) and in drought sensitive Carex sertifolia (Hubac and Guerrier, 1972). Also, red pepper plants sprayed with 1.5% proline solution lost their turgor more slowly in response to stress than those sprayed with water (Palfi et al, 1972). However, in all three reports mentioned above, the changes in the internal proline concentration following the external addition of proline was not measured.

The evidence presented so far seems to indicate an innate variation in proline accumulation capacity between cultivars experiencing identical water potentials (manipulatable to some

extent). However, before it can be concluded that proline exerts a positive role in drought resistance, there are several points which require clarification.

### 4.2 Low temperature stress

An increase in proline concentration in plants exposed to low temperature has been reported in several plants (Section 2.2). As with water stress, proline accumulation in response to low temperature showed no correlation with the geographical distribution of the plant (Chu *et al*, 1978). In the 14 different species examined from both temperate and tropical habitats, there was considerable variation in the proline accumulation at 10°C within both groups, showing no influence of the optimum growth temperature on proline accumulation potential. Similarly, proline accumulated to varying concentrations in $C_3$ and $C_4$ species, in response to low temperature treatment (Taylor *et al*, 1972). While ryegrass, soybean and paspalum accumulated proline, sorghum did not.

Variation within species with regard to proline accumulation potential was considerable. Within species which accumulated low amounts of proline in response to cold treatment, the individual varieties did not vary significantly in their potential to accumulate (e.g. *Cucumis sativus* and *Trifolium subterranean*). Barley, on the other hand, accumulated significant amounts of proline in response to reduced temperatures (5°C) and cultivars showed a more than two-fold difference in concentration of proline accumulated (Chu *et al*, 1978). When the potential for proline accumulation at 5°C was compared with accumulation in response to water stress, cultivars ranked differently (Chu *et al*, 1978). However, it may well be that, as in water stress, proline accumulation in response to low temperatures was correlated with the resistance of the cultivars, but this was not tested.

The causes of all increase in cold hardiness has long been sought in changes in extractable chemical compounds. Of the compounds which change in hardened plants, proline and total sugars are the most consistent and frequently reported. In Robinia pseudo-acacia, the proline concentration increased markedly in hardened plants (Parker, 1958). Similarly, in 3 alfalfa varieties there was a 20% increase in total amino acids in the roots, with the induction most hardy varieties accumulating the highest proline concentrations (Wilding et al, 1960). Lolium perenne leaves showed a three-fold increase in total amino acids and a two-fold increase in free proline with cold hardening (Draper, 1972). The most cold resistant varieties have been reported to have the highest increases in proline concentrations with apple (Benko, 1968), potato (Bokharev and Ivanova,1971 ), grape varieties (Bozhinova, 1972) and Halimione-Portulacoides (Kappen et al, 1978). Proline accumulated in the leaves of citrus trees (Orange and grapefruit) during cold hardening (Yelenosky, 1979). In general, proline increases were greater in the rootstocks rated cold hardy than those rated cold tender in orange trees. However, this association was not evident for leaves of grapefruit trees (Yelenosky, 1979). Although the trend in concentration of proline did not parallel resistance to cold, grapefruit trees kept in the dark during cold hardening, accumulated neither proline nor sugars, and the degree of hardiness was also less than in trees exposed to light. A similar requirement for light in the development of cold resistance has been reported in Hedera helix (Steponkus, 1971) and Savoy cabbage (Le Saint and Hubac, 1978).

Under cold conditions, light is essential for proline accumulation. In barley, both in intact plants and excised leaf sections, proline accumulation at low temperature was shown to be light dependant (Chu et al, 1978). A requirement for light for proline accumulation in response to a cold environment has also been reported in wheat (Stefl et al, 1978) and intact and excised leaves of Savoy cabbage (Le Saint 1969a; Le Saint and Hubac 1978).

Earlier workers attributed this increase in resistance in the presence of light to the synthesis and accumulation of sugars (Li et al, 1965). However, reports by Le Saint (1969b) favour proline as the metabolite influencing cold resistance in cabbage. Proline increased in response to low temperatures, from 2-4% of the total amino acid in unhardened plants to 60% in the hardened plants. The basal leaves which were rich in sugars but low in proline showed little freezing tolerance while the terminal part of the shoot which was richest in proline and not particularly rich in sugar, showed very high tolerance. When hardiness was transmitted from old illuminated leaves to darkened younger leaves during exposure to hardening temperatures (4°C), proline accumulated in the darkened leaves. Conversely, there was no transmission of proline from young illuminated leaves to old darkened leaves, and there was no increase in hardiness either (Le Saint 1969a). The authors concluded that the chemical effector responsible for the transmission of hardening was either proline itself or a substance closely related to its metabolism. Some hardiness could be induced by allowing cabbage shoots to absorb proline from a solution ($5\ gl^{-1}$) at a non-hardening temperature (Le Saint, 1966). Also, with Jerusalem artichoke

tissue the additon of proline to the medium increased the resistance of the tissue to sub zero temperatures, 0.1 M proline increased the survival of cells by 50%. In other work increasing the concentration of proline resulted in increased tolerance to lower temperatures; so that 0.5 M proline treated tissue could tolerate -7°C temperatures while untreated control cells did not survive below -3°C (Leddet and Schaeverbeke, 1975). In maize cells, the provision of 10% proline for 3 days prior to freezing produced post-thaw growth and greater viability of the callus cells (Withers and King, 1979).

Apart from the increase in cold resistance following gradual conditioning to low temperatures, there are reports of resistance being induced by other treatments; especially water stress treatment. Water stress has been reported to induce cold hardiness in Red Osier dogwood tissue (Chen et al, 1975) and young orange and grapefruit trees (Yelenosky, 1979), and chilling resistance in Phaseolus valgaris (Wilson, 1976) and cucumber seedlings (Rikin et al, 1976).

Although reports of close correlations between proline accumulating potential and stress resistance are few, the evidence presented so far seems to favour proline as having a significant role in the development of resistance to both water and low temperature stress. The fact that proline accumulates under both stresses and that water stress can induce resistance to low temperature stress, strongly suggests that proline is the likely chemical effector responsible for the transmission of resistance and hardening.

## 5. Proline Metabolism

### 5.1 Proline metabolism in turgid tissue

The metabolic interactions of proline described by Steeten (1955) still completely define the metabolism of proline; shown in detail in Fig. 1.

#### 5.1.1 Proline biosynthesis

Glutamic acid and ornithine are the two main amino acids which have been reported to be the direct precursors responsible for proline synthesis in both lower organisms and higher plants, including Neurospora crassa (Vogel and Bonner, 1954) E. coli (Strecker, 1957; Baich, 1969), and Cyclotella cryptica (Liu and Hellebust, 1976) among the lower organisms and tobacco (Misuzaki et al, 1964), Swiss chard (Morris et al, 1969), and barley and tobacco (Boggess et al, 1976).

##### 5.1.1.1 The glutamate pathway for proline biosynthesis

Proline synthesis from glutamic acid occurs via 4 steps involving 3 enzymic reactions.

γ glutamyl kinase (EC 2.7.2.11) the first enzyme in the pathway, catalyzes the activation of the γ-carboxyl group of glutamate to produce an intermediate acyl phosphate, the instability of which has precluded its direct determination in any system. The measurement and detection of the enzyme can only be achieved by assays developed for glutamate activation via the production of γ-glutamyl hydroxamate, or by the release of Pi from ATP in the presence of hydroxylamine in E. coli (Baich, 1969) or Pseudomonas aeruginosa (Krishna and Liesinger, 1979).

Fig. 1

Steps in the pathways of proline metabolism in animals, plants and bacteria.

Enzyme 1 is γ-glutamyl kinase

2, γ-glutamyl phosphate reductase

3, P5C reductase

4, Ornithine transaminase in the ornithine catabolic direction

5, Ornithine cyclase (deaminating), clostridial enzyme

6, Proline oxidase

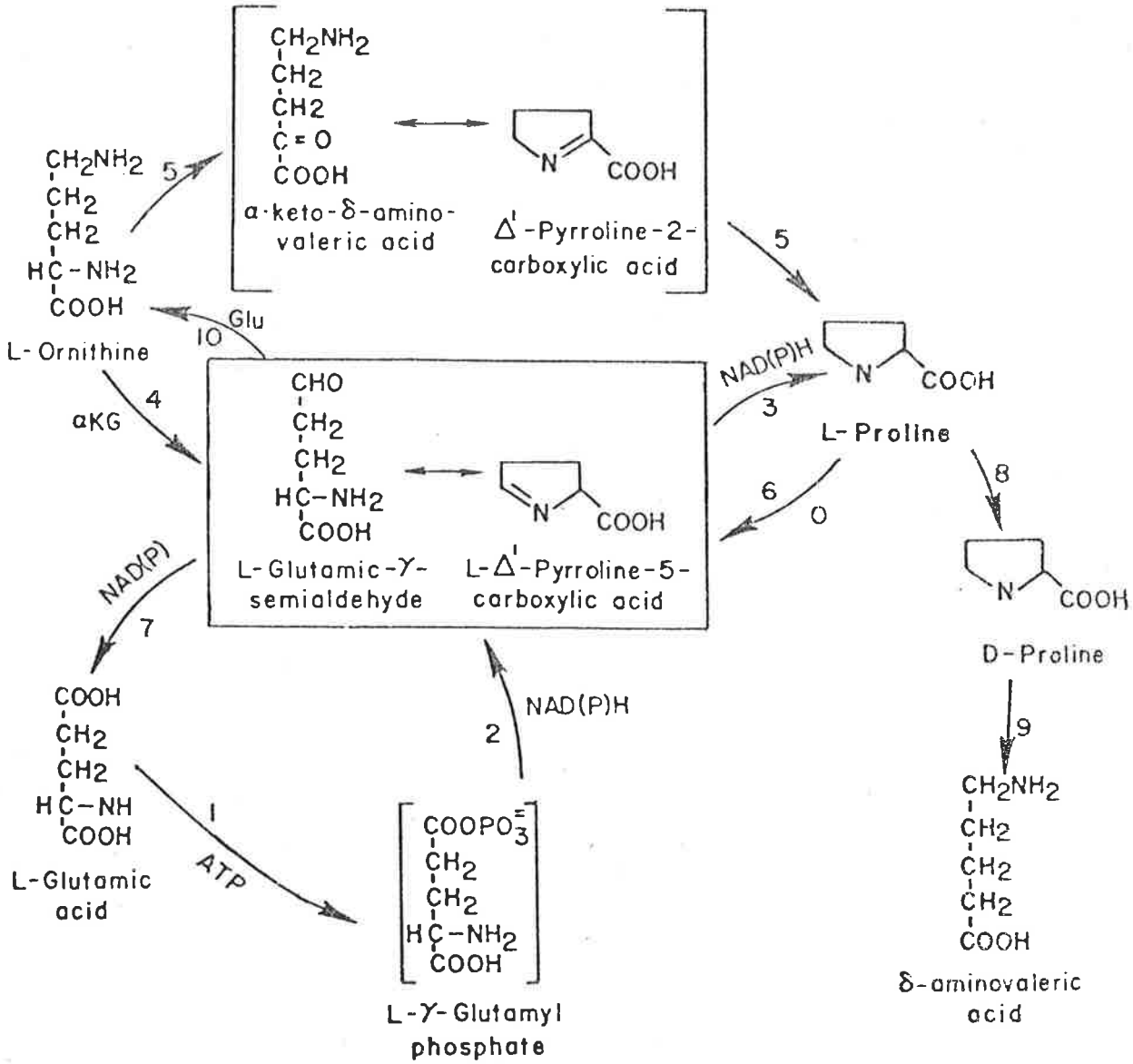
7, P5C dehydrogenase

8, Proline racemase, Clostridial enzymes

9, D-Proline reductase, Clostridial enzyme

10, Ornithine transaminase in the direction of ornithine synthesis from P5C

(Adapted from Adams and Frank, 1980)


α-keto-δ-amino-valeric acid
Δ¹-Pyrroline-2-carboxylic acid
L-Ornithine
Glu
αKG
L-Glutamic-γ-semialdehyde
L-Δ¹-Pyrroline-5-carboxylic acid
NAD(P)H
L-Proline
NAD(P)
L-Glutamic acid
ATP
NAD(P)H
L-γ-Glutamyl phosphate
D-Proline
δ-aminovaleric acid
1
2
3
4
5
6
7
8
9
10

The first reaction can thus be formulated as -

$$\text{Glutamate} + \text{ATP} \xrightarrow{Mg^{++}} \gamma\text{-glutamyl phosphate} + \text{ADP}$$

γ glutamyl P reductase, the second enzyme in the pathway, catalyzed the NADPH-dependent reduction of γ-glutamyl phosphate to yield glutamyl-γ-semialdehyde (Baich, 1969; Krishna et al, 1979). It was purified 150-fold but its activity has been studied only in the reverse direction because of the unavailability of γ-glutamyl phosphate as substrate.

$$\gamma \text{ glutamyl phosphate} + \text{NAD(P)H} \longrightarrow \text{glutamyl-semialdehyde} + \text{NAD(P)} + \text{Pi}$$

γ glutamyl-semialdehyde, formed by this reaction, exists in rapid non-enzymatic equilibrium with Δ'-pyrroline-5-carboxylate which is formed by cyclisation and the liberation of one water molecule.

$$\gamma \text{ glutamyl semialdehyde} \rightleftharpoons \Delta'\text{-pyrroline-5-carboxylate} + H_2O$$

P-5-C reductase (EC 1.5.1.2), the third enzyme, catalyzes the final step in the biosynthesis of proline. Pyrroline-5-carboxylate yields proline by the reduction of the double bond in the presence of the enzyme. The co-factor for this reaction may be NADH or NAD(P)H -

$$\text{Pyrroline-5-carboxylate} + \text{NAD(P)H} + H^{+} \longrightarrow \text{Proline} + \text{NAD(P)}$$

The preference for NADH or NAD(P)H varies in different tissue; for example, in rat liver (Piesach and Strecker, 1962), blowfly

(Balboni, 1978), Clostridia (Costilow and Cooper, 1978) and Pseudomonas (Krishna et al, 1979), NADH was the preferred reductant, while NAD(P)H was preferred in calf liver (Piesach and Strecker, 1962), tobacco (Noguchi et al, 1966) and E. coli (Baich, 1969). In Cyclotella cryptica, both NADH and NAD(P)H were equally effective (Liu and Hellebust, 1976).

Localization of synthetic enzymes in higher plants.

The first two enzymes described, catalyzing the conversion of glutamate to P5C have not been described from any plant tissue. The third enzyme, P5C reductase, has been reported to be localized in the chloroplast fraction in tobacco leaves (Noguchi et al, 1968) and the reduction was closely associated with non-cyclic photophosphorylation.

5.1.1.2 Biosynthesis of proline from ornithine

Synthesis of proline from ornithine is catalyzed by ornithine transaminase to yield glutamyl-γ-semialdehyde which cyclizes to P5C which is then converted to proline via P5C reductase.

Ornithine + α2-oxoglutarate $\xrightarrow{\text{NADH}}$ glutamyl-γ-semialdehyde

By examining the transfer of radioactivity from L-U-$C^{14}$ arginine to proline in tubers of Jerusalem artichoke, Wrench et al (1977) concluded that the conversion occurred via ornithine and glutamyl-γ-semialdehyde, without passage through a glutamate pool. That ornithine can serve as a precursor for proline synthesis, has also been shown in Neurospora crassa (Vogel and Bonner, 1954), peanut cotyledons (Mazelis and Fowden, 1969), Cyclotella cryptica (Liu and Hellebust, 1976) and bean leaves (Stewart and Boggess, 1977). Using $^{15}N$ ornithine, Duranton and Wurtz (1965) obtained a similar conversion of ornithine to proline in Jerusalem artichoke tubers.

Recently Mestichelli et al (1979) questioned the acceptance of δ transmination of ornithine as a route to proline and suggested a re-examination, on the basis of their results. Studying the incorporation of 3H-ornithine labelled in the 2 position or 5 position, along with $5^{14}$C-ornithine, in tobacco, Datura and Lupinus, the authors concluded that α-deamination of ornithine occurred implicating the intermediate α-keto δ-amino valeric acid, whose cyclic form is pyrroline 2-carboxylate. However, Adams and Frank (1980) list several shortcomings in the work of Mestichelli and colleagues and conclude that additional criteria must be used as in the case of other systems (Meister, 1954). The conversion of P5C to proline has already been detailed (Section 5.1.1.1).

Since the pathway for the synthesis of proline from either precursor is identical from Δ'pyrroline-5-carboxylate, this compound serves as a link between the citric acid cycle and its products (via glutamic acid) and the urea cycle and its components (via ornithine).

5.1.2 Proline degradation

The main pathway for proline oxidation is a two step process, in which proline is first oxidized to pyrroline-5-carboxylate, and then further oxidized to glutamic acid, or transaminated to ornithine (Strecker 1960; Frank and Ranhand, 1964). Synthesis of glutamate from glutamyl-semialdehyde directly is not possible, as the reduction specifically requires the ring-closed condensation product Δ'-pyrroline-5-carboxylate (Bender, 1975). During the oxidation of $U^{14}$C proline by the roots of corn seedlings (Bernard and Oaks, 1970), 3 hours after feeding, the tissue converted 66% of the proline into glutamic acid, malate and Kreb's cycle intermediates,

whilst 34% was incorporated into proteins. In wheat mitochondria, $U^{14}C$ proline was converted to $^{14}C$ glutamate and further metabolised to $^{14}CO_2$ (Boggess et al, 1977). A similar metabolism of radioactive proline has been reported for insect flight muscle (Sacktor and Childers, 1967), corn leaves (Wang, 1968), barley (Sane and Zalik, 1968), spruce buds (Durzan, 1973) and pumpkin cotyledons (Rena and Splittstoesser, 1974).

The pathway proposed for the oxidation of proline is conversion to Δ'pyrroline-5-carboxylate by the enzyme proline oxidase (EC 1.5.1.2) which is closely associated with the electron transport chain and interacts directly with cytochromes. Unlike Δ'P5C reductase, which is the NAD(P)-linked enzyme associated with the formation of proline from glutamate, proline oxidase is an oxygenase. This enzyme has been reported in plant tissue only recently (Boggess et al, 1978; Huang and Cavalieri, 1979). Earlier reports for plant tissue suggested that the enzyme proline dehydrogenase (EC 1.5.1.2) was responsible for the oxidation of proline and required $NAD^+$ as the electron acceptor. This enzyme has been reported in the cotyledons of pumpkin (Rena and Splittstoesser, 1974), peanuts (Mazelis and Fowden, 1971), and the leaves of Triticum vulgare (Mazelis and Creveling, 1974) and barley (Boggess et al, 1975). Doubt as to the role of proline dehydrogenase in the in vivo oxidation of proline had arisen from the necessity to assay it at high pH (>pH 10) and from the observation that this enzyme co-purifies with P5C reductase In addition, this enzyme has not been proven to catalyse P5C formation in vivo . Also Boggess et al (1977) showed that although mitochondria isolated from 3 day old wheat shoots showed no proline dehydrogenase activity, they were able to oxidize proline taken up by the

mitochondria, suggesting the presence of a proline oxidising system similar to that observed in mitochondria isolated from mammals and other organisms. A later report of these authors (Boggess et al, 1978), demonstrated oxidation of proline by mitochondria isolated from etiolated shoots of corn, wheat, barley, soybean and mung bean. In addition, this oxidation was dependant on oxygen, but not on NAD, and the mitochondria lacked the enzyme proline dehydrogenase. A recent report of Huang and Cavalieri (1979) supports the indirect evidence for a proline oxidase enzyme obtained by Boggess et al (1978) in that they have clearly shown that an oxygen-dependant proline oxidase was detected in the mitochondrial fraction of spinach leaves and castor bean endosperm. The enzyme had an optimal activity at pH 8.0 to 8.5 and an apparent $K_m$ value of 0.28 molar for proline. The enzyme was apparently linked to the mitochondrial electron transport system. It was unable to reduce NAD to NADH and NAD was not inhibitory to the enzyme activity. The enzyme preparation reduced cytochrome in the presence of KCN.

Nevertheless, none of the evidence obtained by Boggess et al (1978) negates the possibility that proline dehydrogenase may catalyze proline metabolism in other tissues or in the cytoplasm of the species examined. The reaction can be formulated :

$$\text{Proline} \xrightarrow{\text{Cyt C}} \Delta'\text{-pyrroline-5-carboxylate}$$

The second step in the oxidation of proline to glutamate is the conversion of P5C to glutamate, catalyzed by the enzyme Δ'P5C dehydrogenase (EC 1.5.1.12). P2C was not formed since a) the enzyme P5CDH cannot utilise P2C (Stewart and Lai, 1974) and b) when 5H-proline was fed, there was no label in the resulting glutamic acid (Stewart and Boggess, 1978). The enzyme was reported (Frank and

Ranhand, 1964) in cell-free extracts of E. coli and in various tissues of different plants, for example in mitochondria from root and shoot tissue of pea and corn, castor bean endosperm and pumpkin cotyledons (Stewart and Lai, 1974) and barley shoots (Boggess et al, 1975). The reaction was unrelated to, and clearly distinct from, the enzyme which catalyzes the reduction of glutamate to the semialdehyde (Frank and Ranhand, 1964). This was shown by imposing a mutational loss in the auxotroph which removed P5C reductase but not proline oxidase activity. The reaction has a pH optimum of 7.6 and $NAD^+$ is the preferred electron acceptor, but $NAD(P)^+$ will serve, yielding lower rates. The activity when $NAD^+$ was the acceptor was not inhibited by $NAD(P)^+$ (Stewart and Lai, 1974; Boggess et al, 1975). The enzymic reaction can be written as :

$$\Delta'\text{- pyrroline-5-carboxylate} \xrightarrow{NAD^+} \text{glutamic acid}$$

### 5.1.3 Regulation of proline synthesis and breakdown

#### 5.1.3.1 Proline synthesis : Regulation

In E. coli, feedback inhibition of proline synthesis has been localized in the first of the two reduction steps i.e. the conversion of glutamate to γ-glutamyl semialdehyde. This reaction is strongly inhibited by small amounts of proline in both resting and growing cells of E. coli (Strecker, 1957; Baich and Pierson, 1965). When the reaction was catalyzed by the enzyme from a strain known to lack feedback control over proline biosynthesis, it was not inhibited by proline (Baich and Pierson, 1965). Recently Krishna and Leisinger (1979) have shown a 40% inhibition by 5 mM proline of the enzymes responsible for conversion of glutamate to glutamyl-γ-semialdehyde, and complete inhibition by 30 mM proline. Other similar reports of

regulation of proline synthesis at this step of synthesis include the diatom Cyclotella cryptica (Liu and Hellebust, 1976c), maize roots (Oaks et al, 1970) and leaves of barley and tobacco (Boggess et al, 1976).

The second reduction step in the pathway, the conversion of glutamyl-γ-semialdehyde to proline proceeds unrestrained in E. coli (Baich and Pierson, 1965) and Pseudomonas auriginosa (Krishna et al, 1979) in the presence or absence of proline. Similarly in tobacco leaves, the enzyme was not inhibited by 10 mM proline concentration (Noguchi et al, 1966). Thus the rate limiting step in the biosynthesis of proline is from glutamate to glutamyl-γ-carboxylate formation; after which the reduction continues unrestrained.

5.1.3.2 Regulation of proline degradation

A stimulation of the oxidation of proline to glutamic acid in the presence of added proline has been reported in bean leaves (Stewart, 1972), maize root tips (Oaks et al, 1970), tobacco leaf discs (Boggess et al, 1976) and barley leaf discs (Boggess et al, 1976; Stewart et al, 1977). The enzyme proline oxidase, which catalyzes the oxidation of proline to Δ'-pyrroline-5-carboxylate, showed increased activity with proline reaching a maximum at 20 to 30 mM proline in plant mitochondria (Boggess et al, 1978). Similarly the enzyme from spinach leaves continued to reduce cytochrome C in the presence of exogenous proline (Huang and Cavalieri, 1979) indicating that proline content did not inhibit the enzyme activity. Thus, Stewart et al, (1977) conclude that the proline oxidizing system of barley leaves seems not to be saturated by normal proline concentrations. Proline oxidation may therefore serve a regulatory function, acting in concert with control of synthesis in maintaining free proline at a low level in turgid tissue. The enzyme, P5C

dehydrogenase was not inhibited significantly by 19 different amino acids tested, including proline and glutamic acid (Lundgren and Ogur, 1973), in yeast. In higher plants the effects of proline on the enzyme have not been reported.

## 5.2 Mechanism of proline accumulation during stress

The accumulation of any compound may occur by an increase in its synthesis and/or a reduction in its breakdown and proline accumulation could result from either category. Apart from de novo synthesis of proline from other amino acids via glutamic acid, there are a few reports which suggest a release of proline from the bound to the free form (Kudrev, 1967). In order that de novo synthesis of proline may continue, a constant supply of precursors is necessary. It can be speculated that such an increase may result from a reduced utilization of amino acids for protein synthesis or from an increased breakdown of proteins during stress treatment.

### 5.2.1 Precursors for proline

#### 5.2.1.1 Protein changes associated with stress

Water stress frequently has been reported to reduce either the ratio of protein to free amino acids in the plants or the total protein content (Vaadia et al, 1961), although some results show an apparent increase in protein content (Wadleigh and Ayers, 1945). Both the soluble and total protein content of sugar beet leaves declined progressively over several days when water was withheld, falling to as low as half the protein content of the well-watered control (Shah and Loomis, 1965). Other species in which a marked

reduction in soluble protein content with stress has been reported, include wheat (Stutte and Todd, 1969) and Townsville stylo (Gates et al, 1971). In wheat, soluble protein content also fell in response to chilling temperatures (Srivastava and Fowden, 1972). No significant change in the soluble protein content was observed in sorrel, cabbage, spinach, maize, lucerne and sunflower under water stress conditions (Palfi, 1971), while in tomato (Dove, 1968) and barley (Singh et al, 1973) under water stress conditions and in wheat and rape plants exposed to chilling temperatures (Stefl et al, 1978), an increase in the total soluble protein content has been reported.

A decrease in the total protein content of the plant may result from a retardation in the rate of protein synthesis or an acceleration of the degradation process. The effect of stress on synthesis has been examined by measuring the ability of tissue to incorporate labelled amino acids into proteins. For instance, using radioactive proline, Stewart (1972) reported a decrease in the rate of incorporation of proline into protein in excised bean leaves. When $^{14}CO_2$ was supplied and its incorporation into firstly amino acids, and ultimately protein was studied, Barnett and Naylor (1966) showed an 80% reduction in protein synthesis under mild stress (-15 bars) and a total inhibition of protein synthesis at severe stress (-30 bars) in Bermuda grass varieties. Their data support the conclusion that free amino acids are synthesized during water stress, but that their incorporation into proteins is drastically reduced. Similar reductions in the rate of protein synthesis have been reported by Goas et al (1970) and Goas and Gautheret (1971) in Aster trifolium, using radioactive glutamic acid and proline.

These measurements of protein synthesis during stress are complicated by problems associated with the technique, including possible effects of uptake differences and pool size. An independant estimate of effects on protein synthesis can be inferred from studies on the polyribosome complement of the tissue. Supposedly the more protein synthesis in a cell, the larger will be the proportion of ribosomes in the polymeric form and the larger will be the polymers. Detailed study carried out by Hsiao (1970) in etiolated maize seedlings, showed that water stress caused a shift from the polymeric to the monomeric form of the ribosomes in rapidly growing meristematic tissue. Decreases in polysomes have also been reported in maize root tips ( Nir et al, 1971), moss (Dhindsa and Bewley, 1976) and black locust seedlings (Brandts et al, 1977) in response to water stress. A similar effect of low temperature stress has been reported in E. coli (Friedman et al, 1969).

That this reduction in the rate of protein synthesis could not alone cause proline accumulation has recently been shown by Boggess and Stewart (1980) with tobacco leaves. Cycloheximide-treated leaves accumulated little proline in contrast to wilted leaves, in spite of comparable or greater inhibition of leucine incorporation into protein. The incorporation of radioactive leucine into protein was reduced as the concentration of cycloheximide was increased but although the proline content increased somewhat with inhibitor concentration, the highest concentration attained was only approximately equal to the proline concentration in turgid tissue. Cycloheximide did not inhibit the enzymes responsible for proline accumulation as tissue treated with cycloheximide and then stressed accumulated significant amounts of proline. Thus an inhibition of protein synthesis alone is inadequate and will not induce proline accumulation in turgid tissue.

The increased amino acid pool in stressed tissue may also result from accelerated degradation. Thompson et al (1966) reported an increase in the total amino acid content of turnip leaves during water stress which resulted from extensive proteolysis. A stimulation of proteolysis by water stress has also been reported in perennial ryegrass (Kemble and MacPherson, 1954) and in leaves of wheat and pumpkin (Kudrev, 1967). Barnett and Naylor (1966) reported an increased proteolysis of arginine - rich proteins in Bermuda grass under water stress conditions. It has been suggested by Kudrev (1967) that the hydrolysis of proteins results in the release of free proline. However, in wilting ryegrass (Kemble and MacPherson, 1954) and wilted turnip leaves (Thompson et al, 1966) the changes in various amino acids formed by proteolysis were estimated and it was concluded that the amounts of free proline accumulated under stress were greatly in excess of those expected from protein breakdown. It is also noteworthy that Singh et al (1973a) reported an increase in protein-bound proline in water stressed barley leaves and Stefl et al (1978) also reported such an increase in the leaves of winter wheat and rape plants exposed to chilling temperatures. In lower organisms, protein breakdown may have no role in proline accumulation. For example, in Cyclotella cryptica, stressed cells showed no proteolysis, and moreover, pre-labelled proteins in cells transferred to stress conditions showed no loss of radioactivity, yet the cells accumulated significant amounts of proline (Liu and Hellebust, 1976). Similarly, stressed Chlorella cells were unable to accumulate proline when treated with DCMU or transferred to the dark, indicating that protein changes during stress are insufficient to induce proline accumulation (Greenway and Setter, 1979). Rye grass plants allowed to wilt accumulated massive amounts of proline, but when similar plants were kept under high humidity conditions, the

proteins were hydrolysed, and the amino acids increased as in plants which were allowed to wilt, except for proline content which remained low (Kemble and MacPherson, 1954). Therefore, in spite of protein breakdown, wilting seems to have been necessary for proline to accumulate.

5.2.1.2 Reduced translocation

Apart from reduced utilization, total free amino acids can also increase following a decrease in transport out of the organ to other parts of the plant. Dove (1968) has shown significant translocation of nitrogen compounds in water stressed tomatoes, even at fairly low relative water contents, while Tully et al (1979) maintain that the translocation is not reduced during the first two days of stress in barley, but is significantly reduced after this period. This reduction in translocation of nitrogenous compounds out of the leaf coincides with the commencement of proline accumulation. When the transport system in wilted plants was interrupted by a cold jacket or steam, proline accumulation was accelerated. The total reduced nitrogen exported out of the leaf fell from 203 µg $day^{-1}$ in the turgid leaf to 16 µg $day^{-1}$ in the stressed leaf at -30 bars water potential and at the same time, the proline content increased from 0.3 µg to 67 µg. This accumulated proline is probably synthesized de novo from other amino acids (Hanson and Tully, 1979). The contribution of proline to the exported nitrogen pool was only 9 µg $day^{-1}$ (3% of the total amino acids exported) while glutamic acid and glutamine together carried 76 µg $day^{-1}$ (30% of the total amino acids exported). At the same time about 64.5 µg N, as proline, accumulated within 3 days when nitrogen export from the tissue was reduced to 10% of that from turgid tissue.

Here, also, although proline accumulated in response to an inhibition of translocation from the leaf, a simultaneous stress condition was required for proline to accumulate. Massive proline accumulation does not occur in turgid tissue despite the fact that the transport system is severed (Singh et al, 1973b).

5.2.1.3 Amino acid precursors

The two main amino acids closely related to proline are glutamic acid and ornithine (Section 5.1). Using $^{15}N$ labelled ornithine, proline synthesis by α desamination was demonstrated by Duranton and Wurtz (1965) in the tubers of Jerusalem artichoke during germination. Wright et al (1977) reported an increase in proline concentration which was preceded by a loss of arginine during water stress in Jerusalem artichoke tubers. The treatment of slices with indospicine (a competitive inhibitor of arginase and other aspects of arginine metabolism), not only severely limited arginine loss upon stress, but nearly completely abolished proline accumulation. Using labelled arginine, Wrench et al (1977) established that proline was synthesized from arginine directly via ornithine without passage through a glutamate pool in osmotically stressed Jerusalem artichoke tuber slices. Calculations based on specific activity of precursors, the pool sizes of arginine and glutamate, and the transfer rates of radioactivity to proline, indicated that arginine was quantitatively the most important precursor for proline synthesis in this tissue. Similar results showing a decrease in arginine content prior to an increase in proline under stress conditions have been reported in the buds of spruce (Durzan, 1973) and in the subterranean parts of Carex pachystylis (Hubac et al, 1969), while in the leaves, the precursor for proline was not arginine but glutamic and aspartic acid.

In barley plants, glutamic acid was the major source of proline (Boggess and Stewart, 1976). The synthesis of proline from ornithine was not stimulated by wilting unless the leaves were pre-wilted to allow proline accumulation to commence. In this system, in which the conversion of arginine and ornithine to proline occurred 9 hours after the imposition of stress, it seems likely to be a result rather than a cause of proline accumulation. Glutamic acid, on the other hand, was converted to proline within 3 to 6 hours at which time proline accumulation was barely detectable. Similar studies in bean leaves showed a rapid conversion of ornithine and arginine within 3 to 4 hours; which could account for some but not all of the accumulated proline suggesting that both precursors (glutamic acid and arginine) are utilized for proline accumulation. Moreover, in the marine diatom Cyclotella cryptica, proline accumulation under stress conditions was derived from both glutamic acid and arginine (Liu and Hellebust, 1976).

Other amino acids which have been shown to decrease with concomitant increase in proline following stress, include alanine, in response to chilling temperatures in rape (Sosinska and Maleszewski, 1978) and γ aminobutyrate in stressed Phaseolus vulgaris plants ( Jager and Meyer, 1977).

When turgid turnip leaf tissue was infiltrated with various amino acids, no proline accumulated (Stewart et al, 1966), suggesting that the presence of increased precursors alone was not sufficient to stimulate proline accumulation, and that concomitant effects of water stress are essential.

## 5.2.2 Metabolism of proline during stress

### 5.2.2.1 Increased synthesis

Reports of a stimulation of the synthesis of proline as a result of water stress exist for both lower organisms and higher plants. In the marine diatom Cyclotella cryptica, increased osmotic stress led to the accumulation of proline (Liu and Hellebust, 1976 b). The synthesis of proline from all 3 precursors (glutamic acid, arginine and ornithine) was significantly increased in stressed cells. However, whether this was due to increased synthesis alone or in part to reduced oxidation was not established. In the higher plants several workers have reported increased synthesis of proline during stress but have failed to eliminate a contribution to the increase in proline content as a consequence of reduced oxidation (Morris et al, 1969; Barnett and Naylor, 1966; Boggess et al, 1976).

An increased synthesis from glutamic acid during stress of bean leaves was reported by Stewart (1973). The rate of proline formation was calculated from an equation which took into consideration the rate of utilization of proline and the rate of change of the proline content. Proline synthesis was increased two to four-fold under stress conditions. Stewart et al (1977) have again showed an increase in proline due to increased synthesis by deducting the increase in proline attributable to reduced oxidation (calculated from oxidation rates of turgid tissue) from the total proline accumulated. This stimulation of proline biosynthesis was not observed when ornithine or pyrroline 5-carboxylate were used as precursors for proline, unless the proline level within the tissue was already high as a result of water stress induced prior to the addition of precursor (Boggess et al, 1976). This led to the conclusion that the rate of formation of pyrroline 5-carboxylate was increased by water stress, and not its reduction to proline. Similar observations have been made in turgid tobacco leaves (Noguchi et al,

1968) and E. coli (Baich and Pierson, 1965).

5.2.2.2 Reduced oxidation

Loss of proline from stressed tissue was slower than that from turgid tissue in bean leaves (Stewart, 1973). Turgid leaves utilized 0.09 µmoles proline $h^{-1}g^{-1}$ FW, while stressed tissue metabolized proline at a third of this rate (0.03 µmoles $h^{-1}g^{-1}$ FW). In pyrroline-5-carboxylate feeding studies in barley leaves, Boggess et al (1976) observed very quick conversion of pyrroline-5-carboxylate to proline so that 30 minutes after introducing the compound, the data described proline disappearance rather than its synthesis. Proline disappeared rapidly from turgid leaves, but more slowly from stressed leaves. Using tritiated proline, Stewart and Boggess (1978) have that the oxidation of proline is reduced under stress conditions in barley. Comparing re-hydrated tobacco tissue with wilted tissue, Iwai et al (1979) concluded that proline oxidation in stressed tissue was about half that in re-hydrated tissue. The authors justify the comparison of re-hydrated with stressed tissue by drawing attention to the similarities between the two systems with respect to the proline content, at the commencement of re-hydration, the rates of protein synthesis and $CO_2$ evolution.

Few reports on enzymatic activity changes during stress exist. Jager and Meyer (1977) and Huber and Schmidt (1978) reported an increase in the activity of the enzyme proline dehydrogenase, but this enzyme is probably unimportant (Section 5.2). The enzyme proline oxidase has been recently isolated from spinach leaves (Huang and Cavalieri, 1979). The activity of this enzyme was not altered by stress, and was high enough to prevent the accumulation of proline

that occurs under water stress conditions. As this enzyme seems to be mitochondrial in location, it may well be that the proline accumulated during stress is located in a different compartment in the cell and is not accessible for oxidation. Further, the measured *in vitro* activity of the enzyme may not reflect the actual *in vivo* activity. The site of synthesis is apparently different to the site of oxidation in both turgid tobacco and barley leaves (Boggess *et al*, 1976b). Experiments using a double labelling technique led to the conclusion that the synthesis and the oxidation of proline involved two different cellular pools of glutamic acid. However, it may be that such compartmentalization of precursors away from products may break down during stress (Stewart and Boggess, 1978).

The second enzyme in the oxidation pathway was unaffected by lowered water potential in barley leaf tissue (Stewart *et al*, 1977). The observed inhibition of proline oxidation might result from a compartmentational change which prevents proline from reaching the enzymes of proline oxidation during water stress. However, further research is necessary to determine whether such changes are involved, and if so, how they are triggered by the relatively small water losses that stimulate proline accumulation.

The inhibition of oxidation and increased synthesis of proline are both essential for proline accumulation at the rate observed in many plants. Since the level of proline is regulated within the turgid cell, it is essential that there is also a simultaneous loss of these regulatory mechanisms, thus permitting accumulation. A breakdown of end product inhibition and a lack of the stimulation of oxidation of proline by high proline content has been reported in stressed tissue.

In diatoms, a breakdown of end product inhibition during stress has been well documented (Liu and Hellebust, 1976). The synthesis of proline from all three precursors was greatly inhibited by the presence of exogenous proline in turgid cells, but not in stressed cells. Thus, proline synthesis from these precursors continued in spite of increasing proline content. In higher plants, there is also evidence for a breakdown of feedback inhibition. The conversion of $^{14}C$ glutamic acid into proline was inhibited in the presence of exogenous proline in turgid barley and tobacco tissue, while in stressed tissue this did not occur (Boggess et al, 1976).

Not only is feedback inhibition of proline synthesis lost during stress, but the stimulation of proline oxidation by high concentration of proline is also absent. Increasing the proline concentration from 2 mM to 25 mM resulted in increased oxidation of proline from 2 to 14 µg/leaf.hour in turgid barley leaves (Stewart et al, 1977). Also, the inhibition of oxidation in stressed tissue, can be inferred from the rapid loss of proline upon rehydration of stressed barley leaves (Singh et al, 1973) and stressed bean leaves (Stewart 1972c).

It can be concluded that proline accumulates due to the combined effect of many different processes occurring simultaneously; any one of these metabolic changes acting singly would not be able to induce an accumulation of the same magnitude.

### 5.2.3 Factors influencing proline accumulation

#### 5.2.3.1 Hormones

Proline levels are influenced by the level of hormones within the cell. $GA_3$ reduced the proline content of wheat plants when applied at 10 and 100 g per plant (Singh et al, 1973d). The decline in free proline content in response to $GA_3$ application resembled that

during rehydration, except that $GA_3$ treatment did not increase the water potential. Kinetin, like $GA_3$, has been reported to reduce the content of proline accumulated in stressed sunflower plants (Wample and Bewley, 1975) and in stressed cucumber cotyledons (Uday Kumar et al, 1976). The hormone, abscisic acid (ABA), increases during stress and treatment of barley plants and peas with ABA caused an increase in proline content (Aspinall et al, 1973; Rajagopal and Anderson, 1978). The mechanism of the action of ABA in proline accumulation has recently been elucidated (Stewart, 1980). ABA stimulated the synthesis of proline from glutamic acid in the presence of carbohydrates. Although this response of turgid tissue to ABA is similar to that of a stress treatment in invoking proline accumulation, the effects of ABA differed from those of stress in that the oxidation of proline was not inhibited.

5.2.3.2 Specific ion effects

An effect of K on proline accumulation has been reported in maize, lucerne and cucumber cotyledons. In maize leaf discs, pre-treatment with high levels of K caused markedly greater proline accumulation in stressed tissue (Mukherjee, 1974) compared with untreated stressed ones. Such an enhancement by treatment with K ions has also been reported for lucerne (Palfi, 1976) and cucumber cotyledons (Uday Kumar et al, 1976). When KCl was used as an osmotic agent to stress barley plants, an enhancement of accumulation by KCl was not observed. Instead, divalent ions seemed to stimulate maximum proline accumulation (Chu et al, 1976a, b ). The mechanism by which these ions enhanced proline accumulation is little studied. These ions may be influencing enzyme activity as has been shown by Boggess et al

(1975). The enzyme P5C dehydrogenase, responsible for the conversion of P5C to glutamic acid, was inhibited by KCl. Such an inhibition would induce proline to accumulate as a result of reduced oxidation. It is difficult to assess the effects of ions on proline accumulation, due to the lack of sufficient data.

5.2.3.3 Light

Reports of an increased accumulation of proline in light as compared with dark are many. Increased accumulation in the presence of light in lower organisms include Cyclotella cryptica (Liu and Hellebust, 1976c), Phaedactylum tricornutum (Schobert 1977a) and Chlorella (Greenway and Setter, 1979). In higher plants, light is essential for proline accumulation at chilling temperatures (Chu et al, 1978), while it stimulates enhanced proline accumulation under water stress conditions (Palfi, 1971; Palfi et al, 1974; Hanson and Tully, 1979). If this increase is due to photosynthesis it could depend upon either the increased supply of necessary energy-rich compounds (reduced nucleotides and phosphorylated compounds) or the provision of carbohydrates which may serve as precursors for proline synthesis. These products of photosynthesis may have an indirect effect on proline accumulation by influencing one or more of the metabolic steps.

A. Effect of energy-rich compounds

In the biosynthetic pathway of proline, two reduced nucleotides and one ATP molecule are required for the synthesis of one molecule of proline from glutamic acid. It is possible that at a high light intensity, photosynthetic assimilatory power could be available to meet the reductant and ATP demands for proline accumulation

in water stressed leaves. This possibility is strengthened by the report that turgid tobacco leaves converted $^{14}C$ glutamic acid to proline far more actively during the day than the night (Mizusaki et al, 1964) and that $^{14}C$ proline synthesis in the light was inhibited by CMU. This inhibition could not be reversed by the addition of NADP, ascorbate, 2,6-dichlorophenol or ATP. Uncouplers of photophosphorylation, such as DNP, also suppressed the formation of proline from glutamic acid (Noguchi et al, 1968). In addition, broken chloroplasts (containing the enzyme P5C reductase) supplied with NADP, precursor P5C, and ferredoxin were able to synthesize proline in the presence of light. In the dark, on the other hand, the amount of proline produced was only 10% of that in the light (Noguchi et al, 1966). The oxidation of proline in this tissue was not affected by light, occurring at similar slow rates during the day and night. The authors concluded that in this tissue light affects the reduction of P5C to proline by reducing NADP to NADPH via the non-cyclic electron flow system of photosynthesis.

In stressed diatoms, the requirement of light for proline accumulation could not be replaced by sucrose or by any amino acid (Schobert 1977b). The carbohydrate supply was not limiting and exogenous glucose was not effective in enhancing proline levels. The author concluded that light was involved through photophosphorylation and the production of reductants. Light may have a similar role in proline synthesis in stressed barley leaves. Hanson and Tully (1979) studied the conversion of $^{14}C$ glutamic acid to proline at a range of water potentials. This was done to eliminate the indirect effect of light on proline accumulation mediated via the drop in leaf water potential that occurs as an inevitable consequence of illumination of the leaf. These results showed that $^{14}C$ glutamic acid was converted to proline in both light and darkness, but at any given water potential

in the range of -12 to -20 bars illuminated leaves converted twice as much $^{14}C$ glutamic acid to proline.

Although light had a significant effect on the synthesis of proline it had no effect on proline oxidation or utilization in stressed barley leaves (Hanson and Tully, 1979). Stressed leaves incubated in both light and darkness for upto 6 hours, incorporated almost no $^{14}C$ proline label into insoluble products and over 90% in the aqueous phase remained as proline. In turgid tissue in the light, 84% of the label was recovered in the insoluble fraction after a 6 hour incubation period.

B. Effect of carbohydrates

In addition to chemical energy, photosynthesis also supplies photosynthates-predominantly carbohydrates. In turgid roots of maize, increasing the concentration of glucose resulted in an increased synthesis of proline (Oaks et al, 1970).

In wilted tissue a close correlation between carbohydrate changes and extent of proline accumulation has been reported. In wilted ladino clover, Routley (1966) reported a higher proline content in plants receiving high light treatment; these plants also contained greater amounts of carbohydrates. When excised leaves were pre-incubated on glucose solution for several hours and then wilted, there was an increase in proline accumulation. Similarly, using wilted turnip leaves, Thompson et al (1966) showed proline accumulation paralleled the changes in carbohydrate content (resulting from variations in pre-illumination conditions or infiltrating with sucrose) and Stewart et al (1966) showed a greater and more pronounced accumulation of proline in wilted turnip leaves with a high carbohydrate content.

Tissue that had been kept in the dark for 48 hours prior to wilting accumulated very little proline. However, infiltration with carbohydrates substantially increased the proline accumulated. Other such reports where proline accumulation was increased by increasing the carbohydrate content include bean leaves (Stewart, 1973) and etiolated barley leaves (Singh et al, 1973 b).Several workers have shown an inhibition of proline accumulation in the presence of inhibitors of the Kreb's cycle and oxidative phosphorylation (Palfi, 1966; Routley, 1966; Singh et al, 1973; Thompson et al, 1966). Such results, however, do not confirm that a direct link between carbohydrate supply and proline accumulation exists, as it is complicated by other effects of these inhibitors. Stewart (1978) has shown that of the metabolic processes causing proline to accumulate in barley leaves (Section 5.2.2) only the stimulation of proline synthesis from glutamic acid requires the presence of a high level of carbohydrates in the leaves. Wilting caused a forty-fold stimulation of proline synthesis from glutamic acid in non-starved leaves, but had very little effect in starved leaves (from plants held in the dark for 48 hours). The author concluded that the role of carbohydrates in proline accumulation was to supply precursors for the stimulated proline synthesis. In starved leaves, which lacked sufficient carbohydrates, 40% of the $^{14}C$ still remained in glutamic acid after 5 hours suggesting that carbohydrates were necessary to supply the protons or reducing power for proline synthesis. Similar observations of increased synthesis of proline from glutamic acid in the presence of carbohydrates have been reported for Chlorella (Greenway and Setter, 1979) where supplying glucose alone did not stimulate proline formation, but resulted in a stimulation of proline synthesis when glutamic acid was supplied as the substrate. In Cyclotella also,

glutamic acid and arginine conversion to proline increased when starved cells were supplied with glucose (Liu and Hellebust, 1976c). In cells which were not starved, however, exogenous glucose had little effect.

In contrast to these experiments, the presence of carbohydrates in stressed bean plants seemed more to inhibit proline oxidation rather than to stimulate proline synthesis. Both starved and non-starved stressed plants synthesized proline at similar rates, but starved plants utilized proline at a five-fold greater rate than non-starved plants (Stewart, 1972b). Moreover, the addition of sucrose to starved leaves together with labelled proline inhibited the oxidation of proline under stress conditions (Stewart, 1973). Thus, it appears that some carbohydrates or intermediate of carbohydrate metabolism may inhibit the oxidation of proline in bean leaves. A similar inhibition of the oxidation of proline in the presence of glucose has been reported for turgid maize roots (Oaks et al, 1970).

The difference in the effect of carbohydrates in bean and barley leaves which were stressed was probably due to the differences in the mechanism of proline accumulation (Section 5.2.5). However, it is evident that carbohydrates play a significant role in proline accumulation, though the manner of their action may differ in different plant species.

It can be concluded that proline accumulation during stress is a complex phenomenon, controlled by the simultaneous occurrence of several metabolic alterations, in addition to possible hormonal changes.

# III MATERIALS AND METHODS

# III MATERIALS AND METHODS

## 1. Materials

Barley seed (Hordeum distichum cv Prior) used throughout the experimental programme was obtained from the Department of Agronomy at the Waite Agricultural Research Institute.

## 2. General methods

### 2.1 Environmental control

Controlled environmental growth cabinets (Zankel) were used for growing the plants. In all the experiments, during the growing period, the photon flux density at the pot level was maintained at approximately 300 $\mu E\ m^{-2}s^{-1}$ . The light source was a bank of 32/80 watt 'cool white' fluorescent tubes (Philips TLF 80/33) supplemented with 8 incandescent bulbs (60 watts). In all experiments, plants were grown in a 16 hour photoperiod, and the cabinet temperature was maintained at 20 ± 1°C during the day and the night. The relative humidity of the cabinet was not controlled. The position of the pots within the cabinet were changed regularly in order to minimise the gradient effects of light and temperature across the cabinet. The experimental design adopted in most of the experiments was a split-plot design.

### 2.2 Plant culture

Seeds were germinated on wet filter paper in petri dishes for 48 hours at 20°C in the dark before they were planted in 10 cm plastic pots. Ten such seeds were planted in each pot and 6 uniform plants were maintained after an initial thinning soon after emergence. The plants were grown in river sand, well washed prior to sowing. Each pot was

watered with rain water for the first four days, after which they were watered daily with full strength Hoagland's solution (Hoagland and Arnon, 1938). The volume supplied daily was always greater than the field capacity of the sand in the pot which ensured against accumulation of any of the ions.

## 2.3 Pre-stress treatment

The intact plants were exposed to 48 hours of continuous light or darkness, commencing on day 13 . Except for the illumination , all other conditions were maintained the same as that during the earlier (2-12 day) period. Continuous light treatment was attained in the same cabinet whilst the continuous dark treatment was given either a) by transferring plants to a separate growth cabinet under identical conditions except that it was darkened; or b) by placing the plant in a 'Dark Box' in the illuminated cabinet. The 'Dark Box' consisted of a double layered black cotton cloth (Blackout cloth) placed on a metal frame. This arrangement ensured free movement of air but light could not penetrate it (checked with a LI-COR Quantum sensor).

## 2.4 Stress treatment

### 2.4.1 Leaf excision

At the end of the 48 hour pre-stress period, the first leaves were cut into 1.5 cm segments with a sharp razor blade and floated on water in a large petri dish, after discarding the top and basal ends of the lamina.

2.4.2 Imposition of water stress

Ten leaf segments were floated on 10 ml of polyethylene glycol (mol wt 4000) solution (in a 10 cm petri dish) of the desired osmotic potential (concentrations were calculated using a Spanner psychrometer). The petri dish was then exposed to 24 hours light or darkness in the growth cabinet. Turgid controls were obtained by floating segments on distilled water. Polyethylene glycol has been claimed to be the most suitable osmotic agent for water stress investigations (Barrs, 1966; Manohar, 1966; Singh et al, 1973) and although it has been suggested that contaminants can produce toxic effects (Le Shem, 1966), no evidence of such toxicity has been found with the samples of PEG (I.C.I. Australia) used during these experiments or in previous investigations (Husain and Aspinall, 1970; Singh et al, 1973) in this Department.

2.4.3 Cold stress

Cold stress was imposed by placing ten segments from the 'segment pool' into a 10 cm petri dish containing 10 ml of distilled water at 5°C. The tissue was maintained at 5 ±1°C for three days in the presence or absence of continuous light provided by a bank of fluorescent tubes (distance of the petri dishes from the light was adjusted to obtain an irradiance of 300 $\mu E\ m^{-2}s^{-1}$).

2.5 Harvest after treatment

At the end of the treatment period, tissue was removed from the petri dish, rinsed in distilled water for a few seconds (to remove PEG which introduces error during weighing) and blotted dry between Whatman no. 1 filter papers. The segments were placed in a numbered vial, frozen in liquid nitrogen and stored at -20°C, until all replicates had

been harvested. The frozen tissue was then freeze dried, weighed (after equilibration at room temperature in a desiccator) and stored at -20°C in capped vials until required for chemical analysis.

## 2.6 Measurement of water status of the plant

These measurements were confined to experiments with intact plants described in the Results and Discussion (Section I).

### 2.6.1 Water potential

The leaf water potential was measured with a Spanner thermocouple psychrometer (Barrs, 1968). The freshly harvested leaf was placed in the chamber enclosing the thermocouple and left to equilibrate for at least two hours before recording the thermocouple output. The water potential was calculated by comparing the readings with those obtained from a graded series of NaCl solution.

### 2.6.2 Relative water content

The relative water content (RWC) was measured by the method of Barrs and Weatherley (1962). The first leaf was harvested, cut into 1.5 cm segments and sealed in pre-weighed glass vials which were then weighed (FW). The leaf segments were then transferred to petri dishes containing distilled water and left in a dark cabinet for four hours. The segments were removed, dried between Whatman no. 1 filter papers and weighed to yield the saturation weight (SW). These segments were then oven dried for 48 hours at 80°C and their dry weight recorded (DW). The relative water content was calculated as follows (Weatherley, 1950).

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

2.6.3 Total water content

This was calculated from the difference between the fresh and dry weights and was expressed as follows :

$$\text{Water content} = \frac{FW - DW}{DW}$$

2.7 Stomatal resistance

The stomatal resistance was measured with LI-COR diffusive resistance meter (Model LI-60) possessing a horizontal sensor. It was calibrated at the humidity-2 position as outlined by Kanemasu et al (1969).

2.8 Chemical Procedures

2.8.1 Extraction and measurement of proline

A rapid method for estimating free proline developed by Singh et al (1973c) based on the method of Troll and Lindsley (1955) for animal tissue was used. 1.5 g of Zerolit SF Decalso resin was placed in a Kontes Duall glass homogenizer. To this was added about 0.05 g of freeze dried sample and 5 ml of methanol:chloroform:water (MCW 12:5:3) solution which was then homogenized at room temperature. The mixture was decanted into a 50 ml centrifuge of MCW solution. 8 ml water (in two 4 ml volumes) was then added to the homogenate to break the stable emulsion formed during extraction. The mixture was then shaken and centrifuged. The supernatant was transferred to a measuring cylinder, and its volume noted. An aliquot of this solution was then transferred to a boiling tube containing glass beads (to reduce bumping) and a glass marble covering the mouth of the tube (to prevent evaporation). To the sample was added 5 ml of fresh ninhydrin solution (3 ml glacial acetic acid:2 ml of 5 M orthophosphoric acid and 125 mg ninhydrin) and 5 ml of glacial acetic acid. The mixture was boiled in a water bath for 45 minutes, cooled to room temperature

and shaken with a known volume of toluene (5 to 20 ml depending upon the concentration of proline). The optical density of the ninhydrin product dissolved in the toluene was measured at 520 nm, and the proline concentration estimated from a standard curve (graphed from OD of standard proline solutions run simultaneously alongside the samples). In the original method described by Singh et al (1973c), the colour was taken up in benzene instead of toluene, and OD read at 515 nm.

### 2.8.2 Total amino acid determination

#### 2.8.2.1 Extraction

Amino acids were extracted from freeze dried tissue by boiling the tissue with 10 ml of 80% ethanol on a sand bath and leaving in the dark at 2°C overnight. The next day, the supernatant was decanted and the residue was extracted with 80% ethanol in a similar manner and the procedure repeated two more times. The combined supernatants were dried under reduced pressure in a rotary evaporator (Buchi Rotavapor) at 35°C. The dried extract was then taken up in 0.5 ml or 1 ml of 10% isopropanol and constituted the 'alcohol soluble extract'.

#### 2.8.2.2 Preparation of thin layer plates

Thin layer plates (20 x 20 cm) were made using cellulose Mn 300 powder. 20 g of cellulose powder was slurried with 130 ml of water in an electric blender. From this quantity of slurry, five 20 x 20 cm plates were coated with layers of slurry each 400 microns in thickness when wet (150 microns when dry), using a Desaga spreader. The plates were left to dry overnight at room temperature.

2.8.2.3 Application of sample

Sample spots were placed at a position of 1.5 cm above the lower edge of the layer and 1.5 cm inside from the left hand edge of the layer. The sample was applied to the layer using capillary pipettes (Microcaps) and the spots were dried in a stream of warm air. The solvent front in each dimension (13 cm from the origin) was also marked.

2.8.2.4 Development of plates

Two dimensional chromatography of these amino acids was performed using the solvent systems of Haworth and Heathcote (1969). The solvent for development in the first dimension consisted of isopropyl alcohol: Methyl ethyl ketone: 1N HCl (60:15:25 v/v). Ascending development of the chromatogram was allowed to continue at 22°C until the solvent front had travelled a distance of 12 to 13 cm above the origin. This took place over a period of approximately 3 hours. The plates were then removed and allowed to thoroughly dry before running in the second direction. The second dimension solvent consisted of n-propyl alcohol. Methyl ethyl ketone:acetone:methanol:water:ammonia (40:20:20:1:15:5 v/v). The plate was developed at right angles to the first dimension by the ascending technique until the solvent front was 13 cm above the origin. This occurred over a period of about 2 hours at 22°C temperature. The plates were allowed to dry thoroughly before detection.

2.8.2.5 Detection of amino acids

The amino acids and amines were located using the ninhydrin-cadmium acetate reagent. The reagent was prepared by dissolving 0.5 g ninhydrin and 0.1 g cadmium acetate in 100 ml of solution containing 0.5 ml glacial acetic acid, 2.0 ml of distilled water and 97.5 ml of ethanol. After spraying the plates with the reagent, the plates were left in a

40°C oven overnight, to develop colour.

2.8.2.6 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/N v/v) by pouring one at one end and spreading a thin film evenly over the entire surface with the aid of a glass rod. After the cellulose acetate film had completely dried, each coloured spot was cut out, lifted and put into a centrifuge tube with 3 ml eluting solution (1% acetic acid and 1% cadmium acetate in methanol/ethyl acetate/water 1/1/1 v/v mixture). The tube was shaken for 10 minutes and centrifuged at 1000 g for 5 minutes. The optical densities of the coloured supernatants were measured at 505 nm except proline which was read at 345 mm. All the amino acids were identified by comparison of Rf with mixtures of known amino acid subjected to the same separation procedure and their concentrations were estimated from standard curves constructed for each amino acid (Heathcote and Haworth, 1969).

2.8.3 Total ethanol soluble carbohydrate determination

The total ethanol soluble carbohydrate content was estimated in a 10 µl aliquot of the 'Ethanol Soluble Extract' described in Section 2.8.2.1. The volume of the sample was made to 1 ml by diluting with distilled water before being assayed for total soluble carbohydrates by the Anthrone test.

2.8.3.1 Preparation of Anthrone reagent

200 mg of anthrone was dissolved in 100 ml of $H_2SO_4$ solution. The $H_2SO_4$ solution was prepared by gradually adding 2 litres of conc $H_2SO_4$ to 400 ml of dist $H_2O$. The reagent was prepared just before use, and stored in a dark bottle in the cold until required.

2.8.3.2 Measurement of total ethanol soluble carbohydrates

5 ml of the anthrone reagent was placed in a 6" x 1" tube in an ice bath. To this, 1 ml of sample was gently layered. A hot marble (80°C) was placed in the tube which was then vigorously shaken for 5 seconds. These tubes were then plunged in a boiling water bath and boiled for 10 minutes. They were then removed, cooled in cold running water, and the absorbance at 630 nm determined when the tubes were at room temperature. The optical density values were converted to sugar content from a standard curve constructed from sucrose standards run with each set of samples.

2.8.4 Hot water soluble carbohydrates

The hot water soluble carbohydrates were extracted by boiling the alcohol insoluble residue in water, three times, and pooling the supernatants. This was then evaporated to dryness under reduced pressure, taken up in 10 ml of water, and total carbohydrate content estimated by the Anthrone method (Sections 2.8.3.1 and 2.8.3.2).

2.8.5 Total chlorophyll content

The chlorophyll content was estimated in the chloroform layer of plant tissue homogenized with MCW (Section 2.8.1) and shaken with 3 ml of chloroform and 5 ml of water. The optical density at 663 nm and 645 nm was read, and the chlorophyll content calculated using the following formula

$$\text{Chl a} = 0.01272\ A663 - 0.002582\ A645$$

$$\text{Chl b} = 0.02288\ A645 - 0.004671\ A663$$

where A645 is the absorption at 645 nm and

A663 is the absorption at 663 nm.

The total chlorophyll content expressed in mg $g^{-1}$ leaf DW

$$= \frac{\text{Chl (a + b) x dilution x vol of chloroform}}{\text{dry weight}}$$

### 2.8.6 Radiotracer studies

#### 2.8.6.1 $^{14}CO_2$ feeding

Ten segments were floated on 10 ml of PEG (-15 bars) in a 125 ml conical flask. The flask was sealed with a one-holed rubber stopper through which passed a glass tube, attached to a small vial at one end (which was floated on the PEG solution) and connected to a pinch cock via a rubber tubing at the other end (fig 1). Ten µl of radioactive $NaH^{14}CO_3$ (1 µCi) was placed at the bottom of the vial which was then gently lowered into the flask. Fifty per cent lactic acid was introduced through the top end of the glass tube which would react with the $NaH^{14}CO_3$ to liberate $^{14}CO_2$ into the flask.

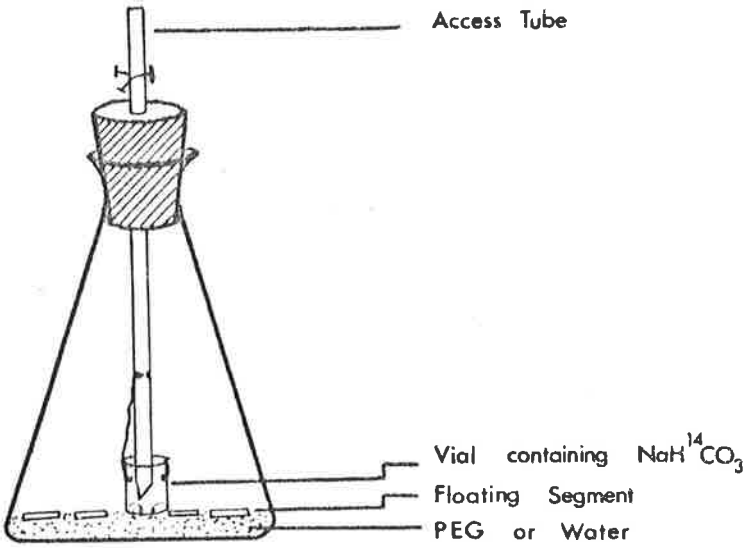
#### 2.8.6.2 Harvesting

The flask was unsealed and the vial was gently removed (to avoid spilling the lactic acid into the medium) and the segments were preserved as described in section 2.5.

#### 2.8.6.3 Determination of radioactivity in the ethanol soluble fraction

The alcohol soluble fraction was obtained as detailed in section 2.6.2.1. Ten µl of this extract was placed in a scintillation vial to which 5 ml of ACS:toluene (2:1) scintillation fluid was added (ACS as a Amersham product which is a complete xylene surfactant based liquid scintillation cocktail for aqueous sample counting). The vials were shaken thoroughly on a 'Vortex' mixer, resulting in a clear solution which was then counted in a Packard Tri-carb scintillation counter.

Fig. 1 METHOD FOR EXPOSING SEGMENTS TO $^{14}CO_2$



2.8.6.4 Determination of counts in amino acids

A. Autoradiography

The sample was chromatographed on TLC plates as described in section 2.8.2.1 to 2.8.2.4. However, after developing the plates in both directions, they were allowed to completely dry before placing them on Kodak x-ray film in the dark. The films were exposed to the plates for 4 weeks at 20°C in the dark. Labelled amino acids produced noticeable darkening of the x-ray sheets. The amino acids were identified by their Rf.

B. Counting of radioactive amino acids and specific activity

The amino acid spots were scraped from the plate and placed in a scintillation vial. To these vials was added 5 ml of toluene based scintillant (3 g PPO and 0.3 g POPOP per litre of toluene). The vials were then counted in a Packard scintillation counter; the ratio of the counts per g dry weight of sample against the total content of amino acid per g dry weight (section 2.8.2.6) gave the specific activity.

2.8.6.5 Detection of radioactive sugars

A. Separation of sugars

The reducing sugars were separated by one dimensional descending paper chromatography. The solvent system used was ethyl acetate: pyridine:water (10:4:3) and papers were run for approximately 16 hours in a Shandon paper chromatography tank, under a fume hood. A 10 µl sample of the ethanol-soluble extract was applied across a 4 cm x 0.5 cm band and was allowed to dry well before the paper was chromatographed. At the end of this period, the papers were removed from the tanks and allowed to dry until the smell of pyridine had completely gone.

B. Detection and identification

Sugars were detected in the marker strips by dipping in $AgNO_3$ reagent (Anet and Reynolds, 1954). The reagent consisted of two solutions - 1 g $AgNO_3$ in 10 ml water to which was added 500 ml acetone; and a solution of alcoholic NaOH made by dissolving 0.5 g NaOH in 25 ml water and adding 250 ml of ethanol to this.

The marker strip was first dipped in the $AgNO_3$ solution and allowed to dry. It was then dipped in the alcoholic NaOH solution and allowed to dry. The spots of reducing sugars appeared brown when this paper was heated in an oven for a few minutes. The position of the sugars in the sample was outlined by comparison with the marker strip and the corresponding areas were then cut out. These were eluted with 2 ml of distilled water.

C. Counting and calculation of specific activity

Of the 2 ml of eluted sugar, 1 ml was used for counting in the liquid scintillation counter using an ACS : Toluene (2:1 v/v) scintillant. The other 1 ml was used to estimate the quantity of sugar by the Anthrone test (section 2.8.3). The specific activity was calculated as the ratio of the counts in sugar in 1 g of sample dry weight to the content of sugar in the same weight of sample.

# IV RESULTS AND DISCUSSION

# SECTION I WATER STRESS

## 1. Proline accumulation in intact plants

# IV RESULTS AND DISCUSSION

## Section I Water Stress

### 1. Proline accumulation in intact plants

#### 1.1 Introduction

A promotion of proline accumulation by light has been reported in both turgid (Noguchi et al, 1966) and stressed tissue from higher plants (Palfi 1971; Stewart,1973, 1978 and Hanson and Tully, 1979) and in lower organisms (Liu and Hellebust, 1976; Schobert, 1977; Setter and Greenway, 1979). It has been proposed that light acts through photosynthesis in all these tissues - either due to an increased supply of carbohydrates or by the production of energy-rich reductants and ATP.

This effect of light on stress-induced proline accumulation has not been explored in detail and the preliminary experiments reported here were designed to examine the nature of this light production of proline accumulation and to ascertain whether it has correlated with any of the other parameters measured.

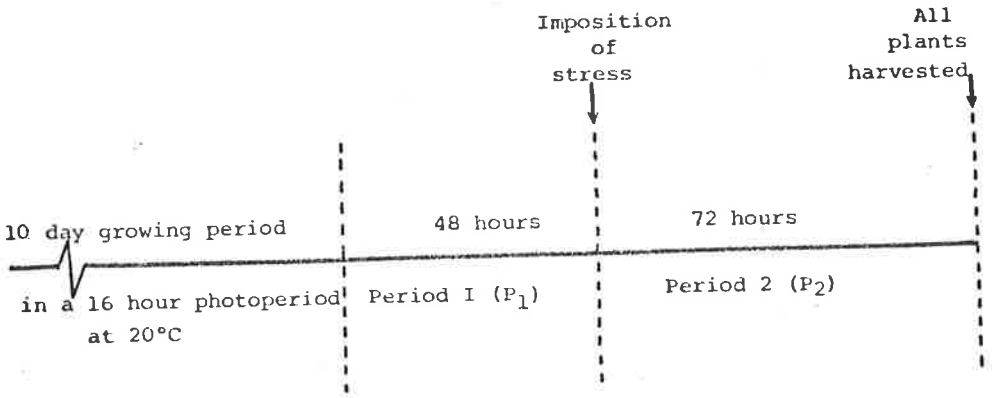
#### 1.2 Effect of light or darkness prior to and during the stress treatment

##### 1.2.1 Methods

Barley plants were grown for 10 days on sand at 20°C in a 16 hour photoperiod as described in the Materials and Methods (Section 2.2). The plants were then exposed to continuous light or continuous darkness for 48 hours, commencing 4 hours after the beginning of the photoperiod ($P_1$) followed by 72 hours of continuous darkness or continuous light ($P_2$). Water stress, where given, was imposed from the beginning of this second period (Fig. 1.1 - experimental design for experiment 1.2). The experimental design was a split plot and there were 4 replicates

Fig 1.1

EXPERIMENTAL DESIGN FOR EXPERIMENT 1.2



for each treatment. The stress treatment consisted of supplying the pot daily with 200 ml of -10 bar polyethylene glycol solution (PEG) made up in Hoagland's solution, while control plants were supplied with an equal quantity of Hoagland's solution. At the end of this period, the first leaves were harvested and immediately frozen in liquid nitrogen. When all the plants were harvested, they were freeze dried, weighed and left at -20°C until analysed for proline, total carbohydrates and chlorophyl content. Another set of first leaves of plants treated in an identical manner, were taken for the measurement of the water status parameters as described in Materials and Methods (Section 2.6).

### 1.2.2 Results

#### 1.2.2.1 Water potential

Control plants were at a water potential ranging between -5 and -7 bars in all light combinations (Fig. 1.2). In stress treatments the water potential was considerably lower than in the control plants, especially in plants which were exposed to light during $P_2$ (Table 1.1). Of the plants maintained in darkness during $P_2$, the plants which were also in the dark during $P_1$ had the highest water potential.

Higher transpiration rates in the presence of light is probably responsible for the lower water status of stressed plants exposed to light, as the water potential was lowest in stressed plants exposed to light during $P_2$. The lack of a significant effect of $P_1$ may be due to the duration of $P_2$ (3 days) and the magnitude of the $P_2$ effect, which could have masked any effects of $P_1$.

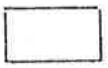
Relative water content and total water content were also measured and gave similar results to water potential.

Fig 1.2

The effect of water stress and light on the water potential of the first leaves of intact barley seedlings.

[10 day old barley plants were given 48 hours pre-stress light or dark treatment, followed by 72 hours light of dark treatment during stress.]

Each value is the mean of 2 replicates



Turgid (watered with Hoagland's solution)

Stress (rooting medium flooded with -10 bars PEG solution during period 2)

| | $P_1$ (48 hours) | $P_2$ (72 hours) |
|---|---|---|
| L-L | Light | Light |
| L-D | Light | Dark |
| D-L | Dark | Light |
| D-D | Dark | Dark |

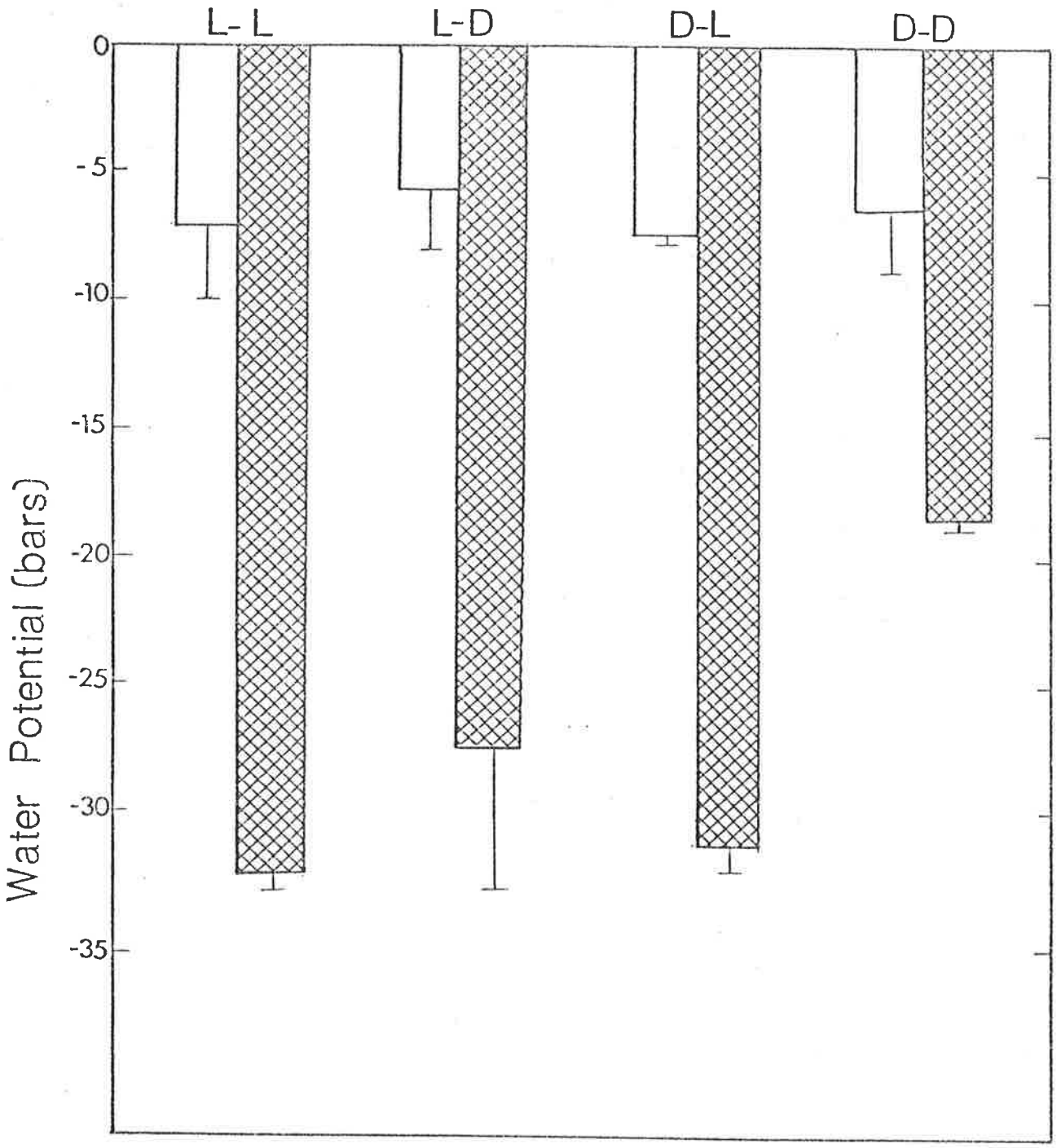
(Bars indicate standard error of the means)

Table 1.1 F values for results in Fig 1.2

| Variate | Water potential |
|---|---|
| Source of variation | VR |
| $P_1$ | 3.94 |
| $P_2$ | 7.13* |
| $P_1 \times P_2$ | 0.99 |
| Stress | 330.24** |
| $P_1$ x Stress | 4.64 |
| $P_2$ x Stress | 9.05* |
| $P_1 \times P_2$ x Stress | 2.35 |

Significance level * $P \leq 0.05$

** $P \leq 0.01$


L-L
L-D
D-L
D-D
0
-5
-10
-15
-20
-25
-30
-35
Water Potential (bars)

1.2.2.2 Proline content

The free proline content of the first leaves of all stressed plants was significantly higher than that of the corresponding turgid plants (Fig. 1.3; Table 1.2). The increase in stressed plants compared to control plants was greatest in those exposed to light during $P_2$ (more than a hundred-fold), irrespective of the treatment received during $P_1$. Plants in continuous darkness during $P_1$ and $P_2$ (D-D) accumulated the least amount of proline (ca. 4 mg $g^{-1}$ dry wt), while the plants in light during $P_1$ and in darkness during $P_2$ accumulated an intermediate level of proline (ca. five fold increase compared with turgid controls). It is also interesting to note that control plants in light during $P_2$ consistently showed a reduced proline content when compared with similar plants in darkness during $P_2$. The cause of this response is not clear.

1.2.2.3 Total soluble carbohydrates

A. Ethanol soluble carbohydrates

Plants exposed to light during $P_2$ contained the highest concentration of soluble carbohydrates (Fig 1.4A) in both turgid and stress treatments, irrespective of the $P_1$ treatment (Table 1.3). Stressed plants exposed to darkness during $P_2$ (i.e. L-D and D-D) showed the lowest carbohydrate content. It is interesting that the turgid plants exposed to darkness in $P_1$ and to light in $P_2$ (i.e. D-L) showed the highest content, while stressed plants subjected to the same sequence of light regimes were not different from plants which were exposed to light during $P_1$ and $P_2$. Exposure to light in $P_2$ after darkness during $P_1$ had a considerable effect on carbohydrate content, which increased from 49.5 mg $g^{-1}$ dry wt (level at the end of 2 days darkness) to 169 mg $g^{-1}$

Fig 1.3

The effect of water stress and light on proline accumulation in the first leaves of intact barley seedlings.

Plant culture and treatments were as described in Fig 1.2

Each value is the mean of 4 replicates.


Turgid (watered with Hoagland's solution)


Stress (rooting medium flooded with -10 bar PEG solution during period 2)

| | $P_1$ (48 hours) | $P_2$ (72 hours) |
|---|---|---|
| L-L | Light | Light |
| L-D | Light | Dark |
| D-L | Dark | Light |
| D-D | Dark | Dark |

(Bars indicate standard error of the means)

Table 1.2 F values for results in Fig 1.3

| Variate | |
|---|---|
| Source of variation | VR |
| $P_1$ | 0.64 |
| $P_2$ | 47.06** |
| $P_1 \times P_2$ | 1.36 |
| Stress | 146.82** |
| $P_1$ x Stress | 0.98 |
| $P_2$ x Stress | 73.61** |
| $P_1 \times P_2$ x Stress | 1.36 |

Significance levels * $P \leq 0.05$

** $P \leq 0.01$

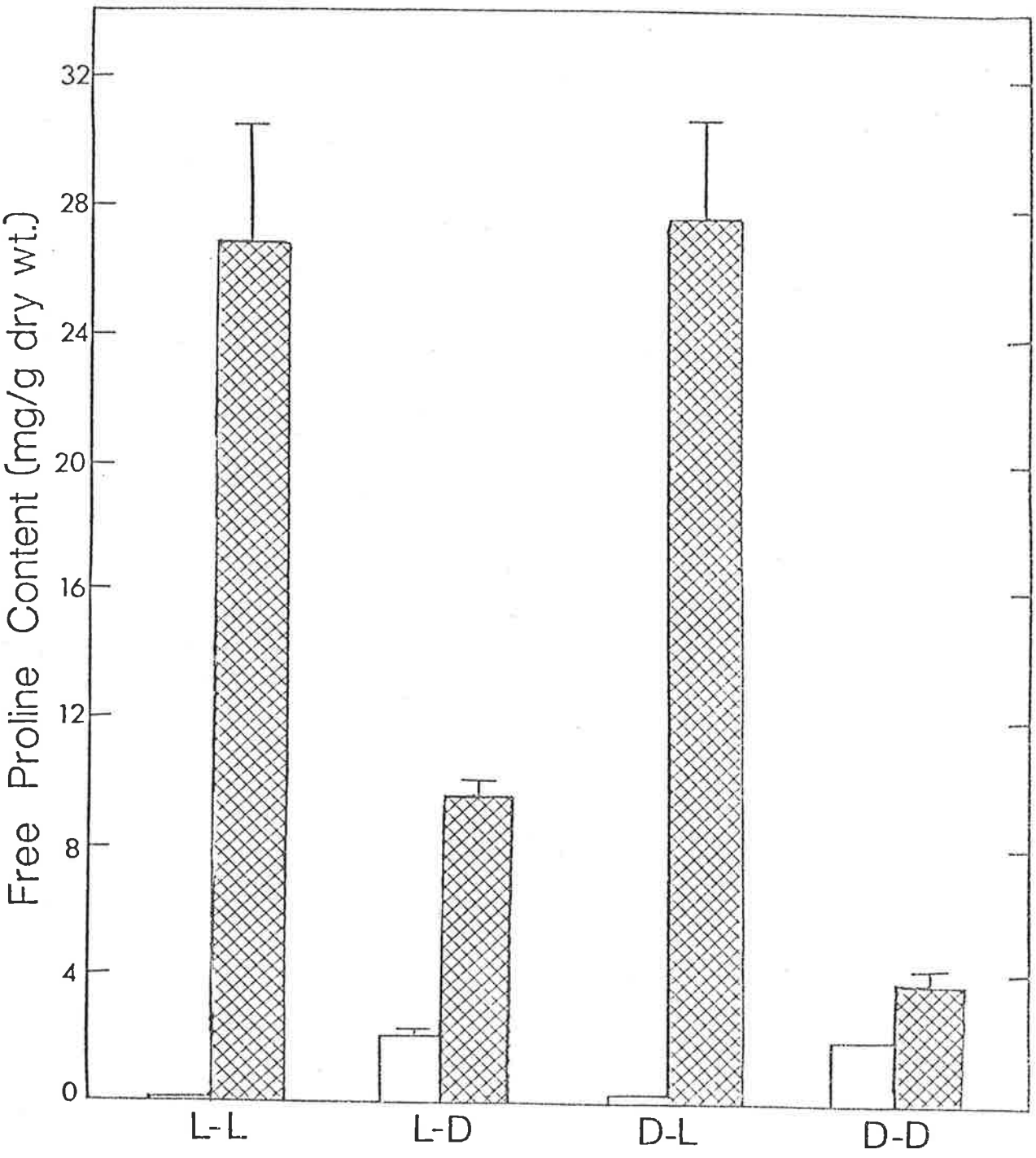

Free Proline Content (mg/g dry wt.)
32
28
24
20
16
12
8
4
0
L-L
L-D
D-L
D-D

Fig. 1.4

The effect of water stress and light on carbohydrate and chlorophyll content in the first leaves of intact barley seedlings.

Plant culture and treatments were as described in Fig 1.2.

Each value is the mean of 4 replicates

□ Turgid (watered with Hoagland's solution)

▨ Stress (rooting medium flooded with -10 bar PEG solution during period 2)

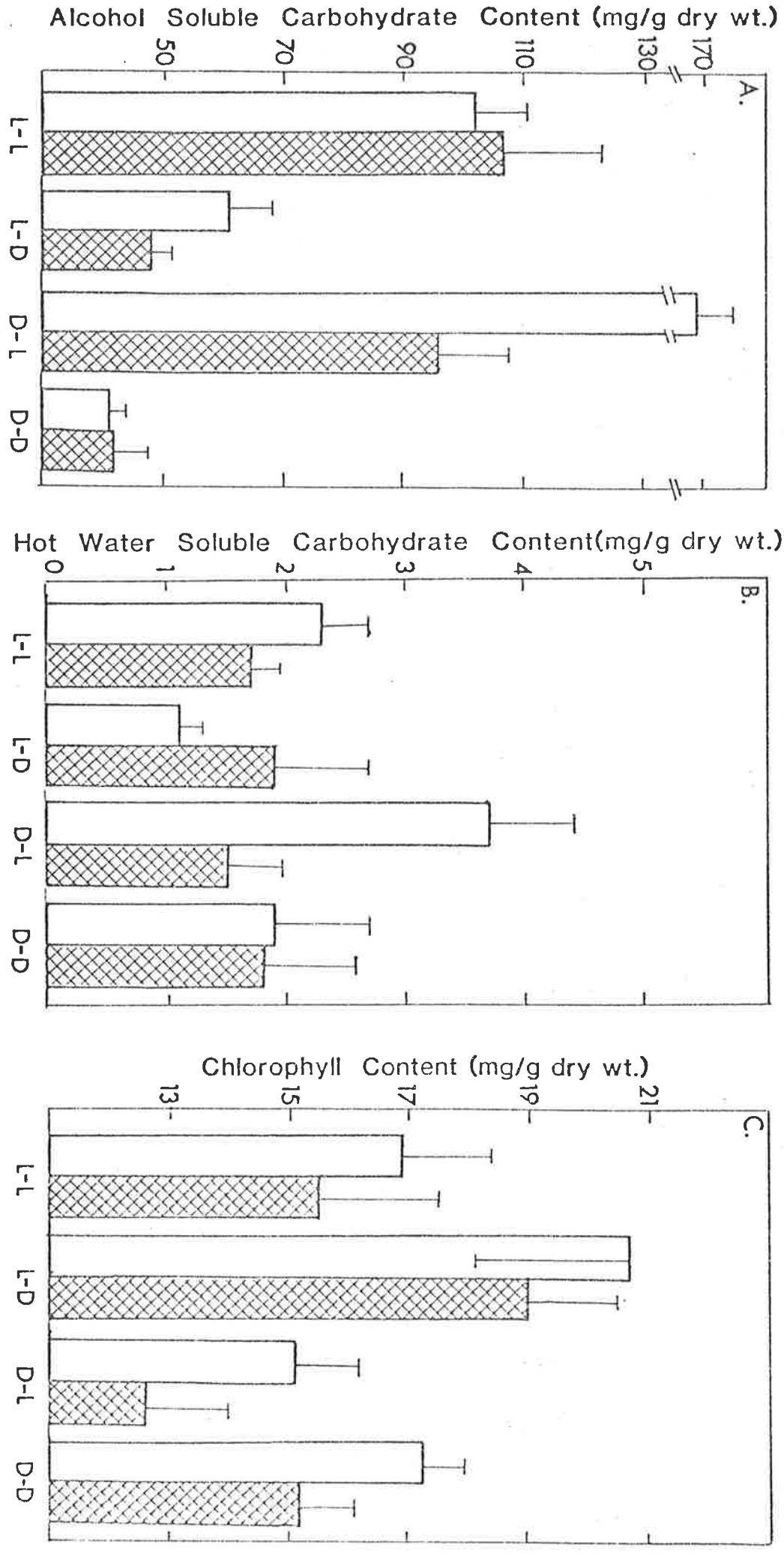
| | $P_1$ (48 hours) | $P_2$ (72 hours) |
|---|---|---|
| L-L | Light | Light |
| L-D | Light | Dark |
| D-L | Dark | Light |
| D-D | Dark | Dark |

Table 1.3 F values for results in Fig 1.4

| Variate | Carbohydrates | Chlorophyll |
|---|---|---|
| Source of variation | | |
| $P_1$ | 0.68 | 9.66 |
| $P_2$ | 152.62** | 9.57* |
| $P_1 \times P_2$ | 12.78* | 0.43 |
| Stress | 5.40* | 6.40* |
| $P_1$ x Stress | 3.49 | 0.21 |
| $P_2$ x Stress | 2.57 | 0.00 |
| $P_1 \times P_2$ x Stress | 7.10* | 0.07 |

Significance levels * $P \leq 0.05$

** $P \leq 0.01$


Alcohol Soluble Carbohydrate Content (mg/g dry wt.)
50
70
90
110
130
170
A.
L-L
L-D
D-L
D-D
Hot Water Soluble Carbohydrate Content(mg/g dry wt.)
0
1
2
3
4
5
B.
L-L
L-D
D-L
D-D
Chlorophyll Content (mg/g dry wt.)
13
15
17
19
21
C.
L-L
L-D
D-L
D-D

dry wt. Exposure to light after 2 days light on the other hand showed no significant enhancement (from 93.0 mg $g^{-1}$ dry weight to 102 mg $g^{-1}$ dry wt).

B. Hot water soluble carbohydrates

This fraction, mainly composed of fructans and glucans, was also the highest in turgid plants which were in the light during $P_2$ (Fig 1.4B). Turgid plants in the dark during $P_1$ and in light during $P_2$ (D-L) showed the greatest amount of this fraction, as was the case with the total soluble carbohydrates. However, this hot water soluble fraction constituted less than 5% of the total soluble carbohydrate content and therefore its estimation was considered unwarranted in future experiments.

1.2.2.4 Chlorophyll content

Plants in darkness during $P_2$ had a higher chlorophyll content than similar plants maintained in the light, in both stressed and turgid tissue. In addition, the stress treatment reduced the leaf chlorophyll content in all treatments (Fig 1.4C; Table 1.3).

The correlation matrix (Table 1.4A) showed a high negative correlation between proline accumulation and the water status parameters, while no correlation existed between chlorophyll and proline content. The carbohydrate content was not correlated with proline when tested between all samples. However, when compared in stress treatments only (Table 1.4B), there was a significant correlation between the two. The reason for this is obvious - as control turgid plants do not accumulate proline yet have high levels of carbohydrates.

Table 1.4

A. CORRELATION MATRIX (28 df)

| | Proline | CHO | RWC | Chlorophyll | Water content |
|---|---|---|---|---|---|
| Proline | 1.00 | | | | |
| Total CHO | 0.08 | 1.00 | | | |
| RWC | -0.91** | -0.06* | 1.00 | | |
| Chlorophyll | -0.27 | -0.37* | 0.43* | 1.00 | |
| $H_2O$ content | -0.72** | -0.44* | 0.82** | 0.53** | 1.00 |

B. STRESS ONLY CORRELATION MATRIX (12 df)

| | Proline | CHO | RWC | Chlorophyll | Water content |
|---|---|---|---|---|---|
| Proline | 1.00 | | | | |
| Total CHO | 0.62** | 1.00 | | | |
| RWC | -0.88** | -0.60* | 1.00 | | |
| Chlorophyll | -0.17 | -0.39 | 0.36 | 1.00 | |
| $H_2O$ content | -0.87** | -0.61** | 0.91** | 0.35 | 1.00 |

Significance level *P≤0.05

**P≤0.01

## 1.3 Effect of a short duration of light during stress on proline accumulation

The previous experiment confirmed reports that proline accumulation is markedly enhanced by light. Although light in the period immediately before stress had little effect on subsequent proline accumulation, it was possible that light during stress may have induced proline accumulation through a triggering response rather than by a continuous effect such as the supply of photosynthetic precursors (energy-rich compounds and carbon skeletons).

The next experiment was designed to test the hypothesis that light acts to induce proline accumulation rather than to sustain increased accumulation and it was assumed that, if this were so, a brief exposure to light during stress would markedly increase proline accumulation.

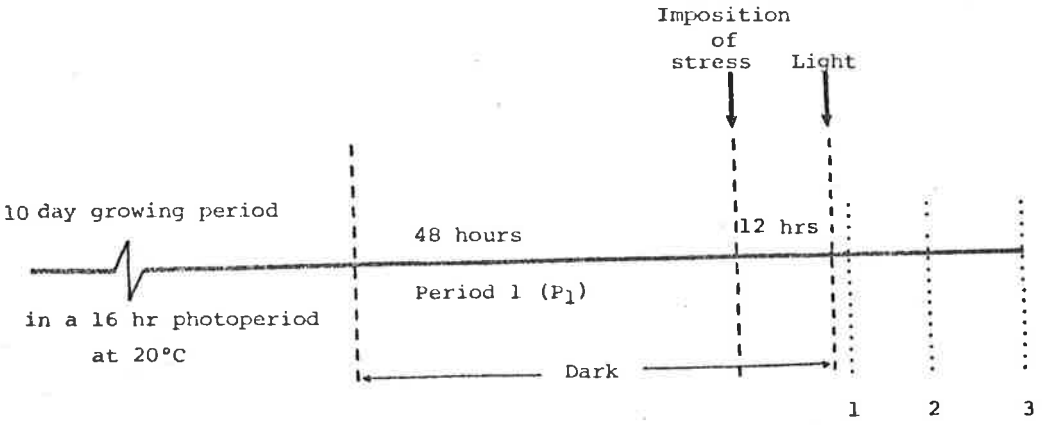
### 1.3.1 Methods

Plants were grown as detailed in Materials and Methods (Section 2.1). The plan of the experiment is detailed in Fig. 1.5 - experimental design for experiment 1.3. Treatments given were stress (by flooding the rooting medium with 200 ml of -10 bars PEG solution prepared in Hoagland's solution) or turgid (watered with 200 ml of Hoagland's solution). The light source was a bank of fluorescent tubes supplemented with 8 incandescent bulbs (Materials and Methods, Section 2.1) at an irradiance of 300 $\mu E\ m^{-2}\ s^{-1}$.

At the end of the 10 day growing period, a 60 hour continuous dark treatment was given. This consisted of an initial period of 48 hours of continuous darkness (commencing 4 hours after the beginning of the photoperiod), before the stress treatment was imposed

Fig 1.5

EXPERIMENTAL DESIGN FOR EXPERIMENT 1.3




1 - 2 hr harvest

2 - 12 hr harvest

3 - 24 hr harvest

and a further 12 hours in darkness after the stress treatment was given. This sequence was chosen as the results of experiment 1.2 showed that the enhancement of proline accumulation by light was greatest when the plants were maintained in darkness initially. In these circumstances, it was anticipated that even a small stimulation of proline accumulation by light was likely to be observable. Plants were also maintained in darkness for 12 hours after the imposition of stress, in order to minimise any effect of light on the water status of the plant. This expectation was based on the results of Singh et al, (1973a) who showed that the drop in water potential was rapid during the first 10 hours after imposition of stress and that the subsequent decline was gradual. The plants were exposed for a period of 5 or 30 minutes to light at the end of this 60 hour dark period, other plants were exposed to either light or darkness continuously from that time until all the remaining plants were harvested 84 hours after the plants were initially placed in the darkness. Plants from each treatment were also harvested 0, 2 and 12 hours after commencement of the light treatment. First leaves were excised, frozen in liquid nitrogen, lyphilized, weighed and assayed for proline.

### 1.3.2 Results

Neither the 5 minute nor 30 minute interruption of the dark period with light induced more proline accumulation in stressed or in turgid plants than in plants in continuous darkness (Fig 1.6; Table 1.5). Stressed plants maintained in continuous light had accumulated more proline than any other treatment within 2 hours, this difference

Fig 1.6

Effect of exposure to light for different periods (0, 5, 30 min or continuous) on proline content of barley plants.

10 day old barley plants were transferred to 48 hours continuous darkness. They were then left turgid or stressed with -10 bars PEG added to the rooting medium. They were left in the dark for 12 hours at the end of which, the light treatment was given. Plants were harvested after 2, 12, 24 hours after light treatment.

Each point is the mean of 4 replicates.

A. Stress B. Turgid

● ○ Darkness

▼ ▽ 5' Light

★ ☆ 30' Light

■ □ Continuous Light

↓ Commencement of Light treatment

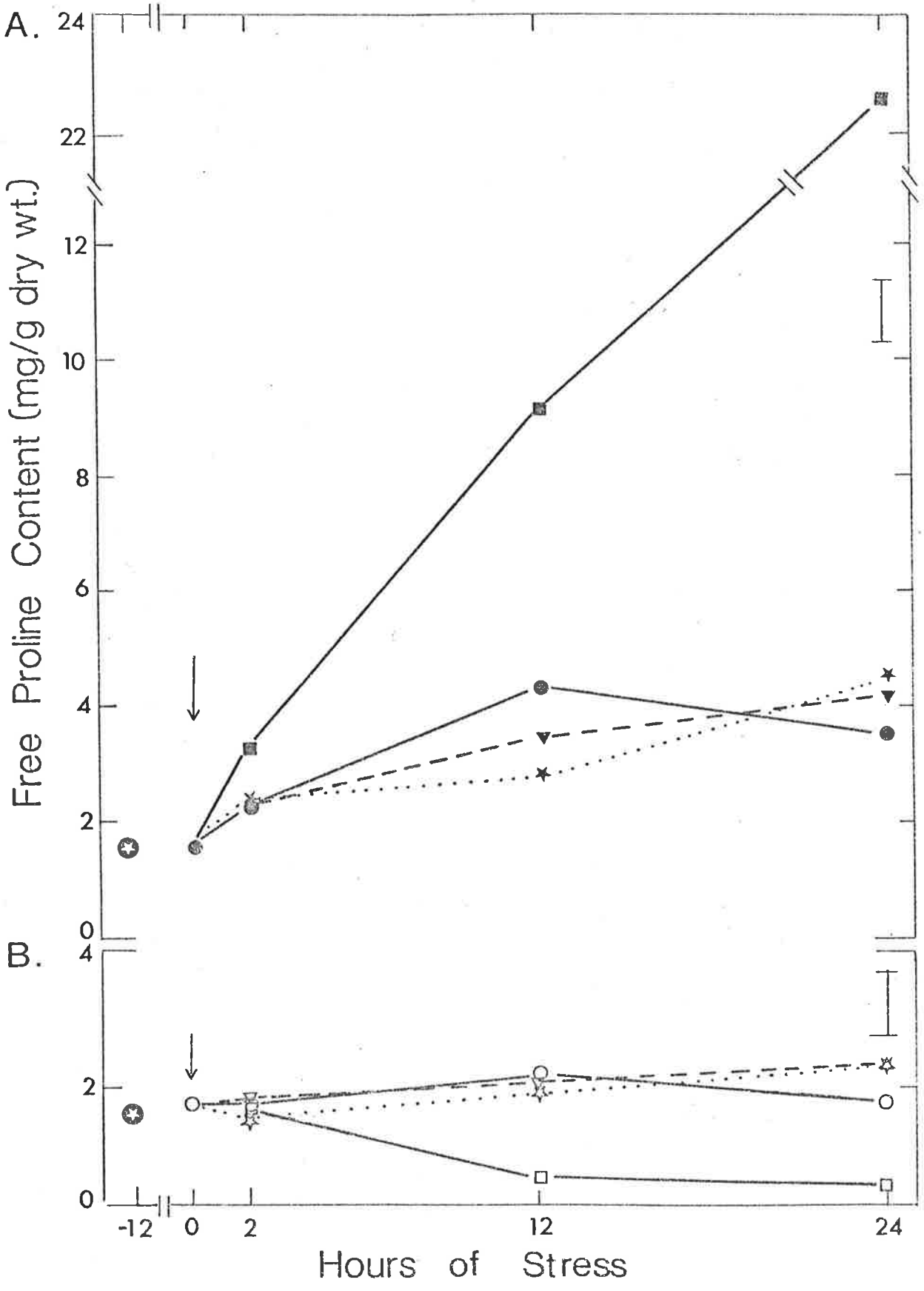
✪ Prestress Proline content

Table 1.5 F values for results in Fig 1.6

| Source of variation | VR |
|---|---|
| Duration of light | 130.57** |
| Harvest | 129.06** |
| Stress | 556.60** |
| Duration x Harvest | 59.02** |
| Duration x Stress | 230.75** |
| Harvest x Stress | 125.92** |
| Duration x Harvest x Stress | 82.29** |

Significance level * $P \leq 0.05$

** $P \leq 0.01$


A.
B.
Free Proline Content (mg/g dry wt.)
24
22
12
10
8
6
4
2
0
4
2
0
-12
0
2
12
24
Hours of Stress

increasing greatly with further exposure to light (Fig 1.6A). Turgid plants on the other hand showed some decline in proline content in those plants exposed to continuous light compared to other treatments, although the initial concentrations were low (Fig 1.6B). Although, the water potential was not measured, the plants from the continuous light treatment appeared less turgid than all the other treatments. It is highly likely that the plants exposed to continuous light had a lower water potential and that this was associated with the accumulation of proline.

## 1.4 Effect of different irradiance on proline accumulation

Stressed plants do not show massive accumulation of proline in response to a brief light period which suggests that light does not trigger a response, but it is required continuously. Light may induce proline accumulation via the photosynthates produced which may serve as precursors for proline or may act indirectly by activating enzymes or enzyme systems closely linked to proline metabolism. It is also possible that light may act indirectly by influencing the water status of the plant which initiates enhanced proline accumulation.

The next experiment was conducted to test the effect of different irradiances and to see if the increases in proline accumulation was linear, independent of water potential changes.

### 1.4.1 Methods

Plants were grown for 10 days in a 16 hour photoperiod (Materials and Methods, Section 2.2) after which the treatment commenced. All the plants were transferred to continuous darkness for 48 hours,

commencing 4 hours after the light cycle had begun. At the end of this period, the plants were stressed by flooding the rooting medium of each pot with 200 ml of -10 bar PEG solution (made up in Hoagland's solution) and then were maintained in darkness for a further 12 hours. The plants were then exposed to light of 3 irradiances viz. 235 (high), 70 (intermediate) and 20 (low) μE $m^{-2}$ $s^{-1}$ or were maintained in the dark (Fig. 1.7 - experimental design for experiment 1.4). The irradiances were selected such that the one was above the compensation point (235 μE $m^{-2}$ $s^{-1}$) one below it (20 μE $m^{-2}s^{-1}$) and the third approximately at the compensation point (70 μE $m^{-2}$ $s^{-1}$). The light compensation point was determined with a differential infra red carbon dioxide gas analyser (Beckman Model 215). The details of estimation of the light compensation point are given in Appendix I.

The first leaves were harvested after 12, 24 or 48 hours of light treatment. Fresh leaf samples were used for RWC and water potential measurements, while proline was analysed in freeze dried tissue. In addition, stomatal resistance was measured 8 hours and 28 hours after commencement of the light treatment. Turgid plants were analysed for proline only on one occasion after 24 hours light (Fig. 1.7).

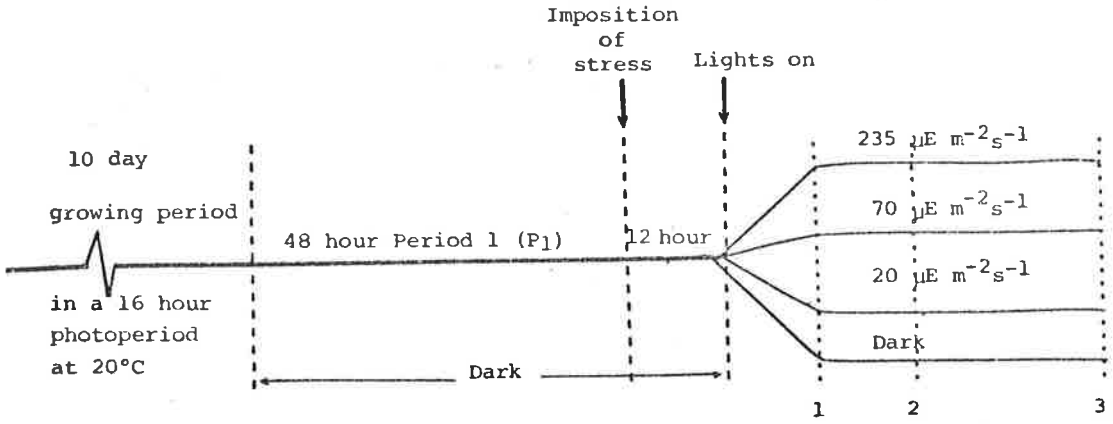
### 1.4.2 Results

#### 1.4.2. Water potential

Plants exposed to high light showed a linear reduction in water potential with time; their water potential was significantly lower than that of the other 3 treatments at all harvest periods (Fig 1.8). The difference in water potential between the dark and low light treatments was not significant at any harvest while the intermediate light was

Fig 1.7

EXPERIMENTAL DESIGN FOR EXPERIMENT 1.4




1 - 12 hr harvest

2 - 24 hr harvest

3 - 48 hr harvest

Fig 1.8

Effect of irradiance on water potential of stressed barley plants. [Barley plants were grown in sand in a 16 hour photoperiod at 20°C until they were 10 days old. They were then transferred to 48 hours continuous darkness, after which the plants were stressed by flooding the rooting medium with -10 bar PEG solution and illuminated at different irradiances or left in the dark.]

Each value is the mean of 2 replicates.

● Dark

Δ 20 μE $m^{-2}s^{-1}$

∇ 70 μE $m^{-2}s^{-1}$

□ 235 μE $m^{-2}s^{-1}$

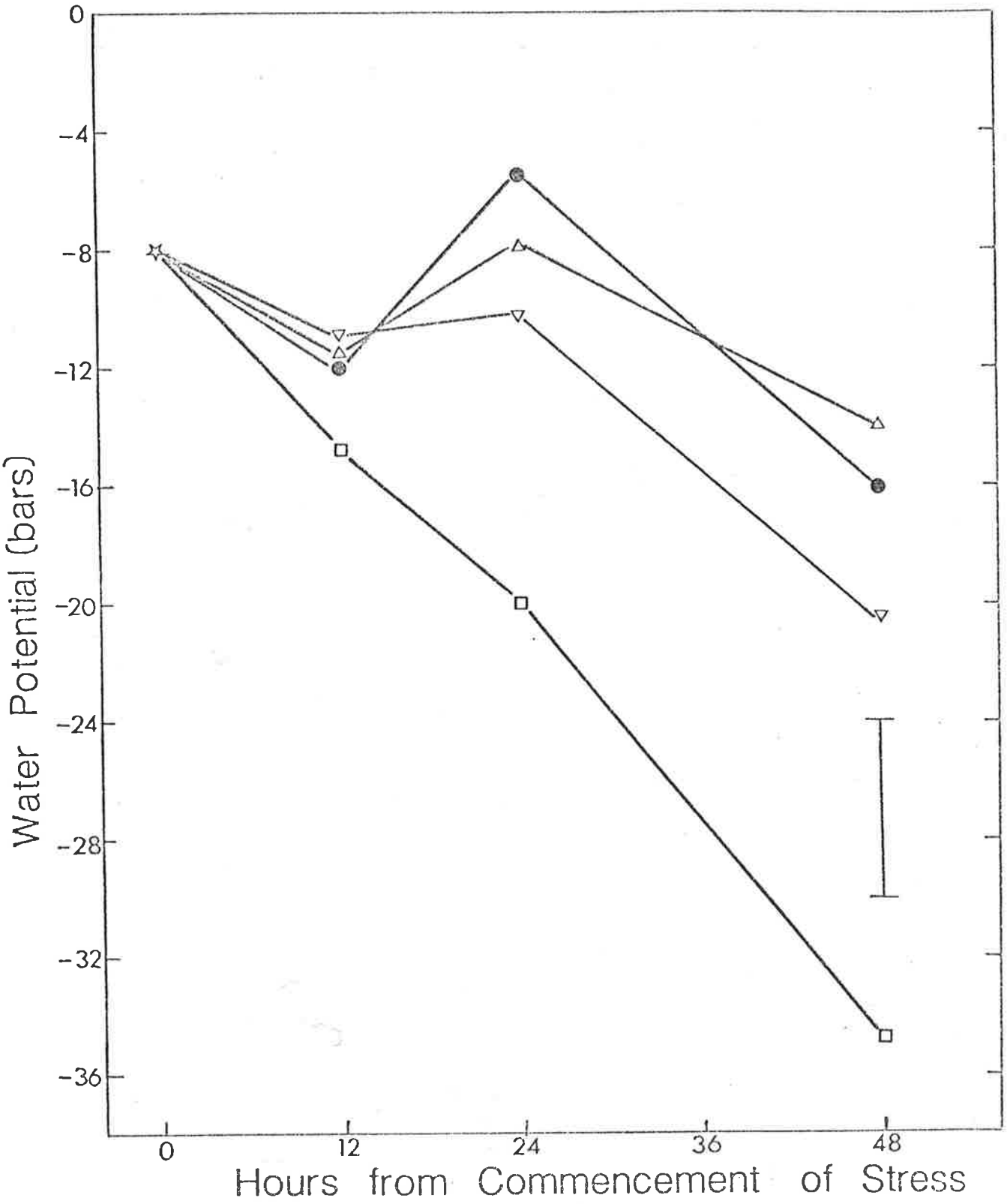
Table 1.6 F values for results in Fig 1.8

| Source of variation | VR |
|---|---|
| Irradiance | $16.43^{**}$ |
| Duration | $20.74^{**}$ |
| Irradiance x Duration | 2.85 |

Significance level $^{*}P\leq0.05$

$^{**}P\leq0.01$

N.B. : As the interaction of variance ratio was only just not significant at $P\leq0.05$, the interaction LSD has been presented in Fig 1.8.


0
-4
-8
-12
-16
-20
-24
-28
-32
-36
Water Potential (bars)
0
12
24
36
48
Hours from Commencement of Stress

significantly different from low light treatment only in the 48 hour harvest. Relative water content was also measured and gave similar results to water potential.

1.4.2.2 Stomatal resistance

Leaves were sampled for stomatal resistance 8 and 28 hours after the light treatment commenced, in stressed and turgid plants. The stomatal resistance of the dark turgid leaves was considered to be that of closed stomates. In the 8 hour sample, only leaves of high and low light were examined. Results (Table 1.7) show that in all the treatments (stressed and turgid) at high and low light, the resistance of the lower epidermis was always less than that of the upper epidermis. The leaves of plants at high light showed lower resistance of both lower and upper epidermis than leaves at low light, in stressed and turgid leaves (Table 1.7). The stomates of plants stressed in low light were closed within 8 hours of light, unlike the plants stressed at high light, which still showed some conductance at that time.

After 28 hours, however, all stressed plants showed closed stomata (both upper and lower) while resistance of turgid leaves was still low. It is noteworthy that turgid plants at a low irradiance showed a greatly reduced diffusion resistance of the upper epidermis after 28 hours of light compared to the 8 hour resistance, while turgid plants at high light showed the reverse effect, i.e. greater resistance at 28 hours. The intermediate light leaves showed an intermediate resistance compared to either high or low light, in both upper and lower epidermal stomata; while the dark treated ones had closed stomata.

Table 1.7 Stomatal resistance (sec.cm$^{-1}$) of the first leaf of barley

Lower Epidermis

| Irradiance | High | Intermediate | Low | Dark |
|---|---|---|---|---|
| Turgid | | | | |
| 8 hr Turgid | 6.6 | | 22.8 | |
| 28 hr Turgid | 10.4 | 11.5 | 15.6 | 92.6 |
| Stressed | | | | |
| 8 hr Stressed | 44.9 | | 92.8 | |
| 28 hr Stressed | 750.6 | 259.9 | 158.6 | 185.3 |

Upper Epidermis

| Irradiance | High | Intermediate | Low | Dark |
|---|---|---|---|---|
| Turgid | | | | |
| 8 hr | 11.7 | | 36.4 | |
| 28 hr | 45.6 | 21.4 | 16.2 | closed |
| Stressed | | | | |
| 8 hr | 58.1 | | closed | |
| 28 hr | closed | closed | closed | closed |

10 day old barley plants were transferred to 48 hours continuous darkness after which they were stressed with -10 bars PEG solution which was added to the rooting medium. The plants were left in the dark for a further 12 hours and then exposed to light of different irradiances. Stomatal resistance was measured 8 and 28 hours after the commencement of light treatment.

1.4.2.3 Proline content

Leaf proline increased with time in all three light treatments, the rate of accumulation increasing with irradiance, whilst the plants in darkness accumulated little proline even after 64 hours of stress. (Fig 1.9). High irradiance induced rapid proline accumulation with significant accumulation occurring within 12 hours, whilst the intermediate irradiance showed significant accumulation after 24 hours and the low irradiance caused significant accumulation only after 48 hours.

Leaves from non-stressed plants were harvested only on one occasion after 24 hours of light. In these plants, the dark grown leaves contained 2.05 mg proline $g^{-1}$ dry weight and the low irradiance 0.39 the intermediate 0.51 and the high 0.35. As in the previous experiment there was a reduced concentration of proline in the leaves of turgid plants grown in the light. Statistical analysis showed a significant interaction between the duration (period) of light and the irradiance level (Table 1.8).

It is of interest that when the water potential and proline were plotted against irradiance, within 12 hours the water potential at 70 or 235 μE $m^{-2}$ $s^{-1}$ was not significantly different, yet free proline was significantly greater at 235 than at 70 μE $m^{-2}$ $s^{-1}$ (Fig. 1.10). Similarly after 48 hours plants stressed at 20 μE $m^{-2}$ $s^{-1}$ showed higher proline content but had similar water potential as the dark-treated plants.

Fig 1.9

Effect of irradiance during stress on proline accumulation in the first leaves of intact barley seedlings.

10 day old barley plants grown in a 16 hour photoperiod at 20°C were transferred to 48 hours continuous darkness after which they were stressed by flooding the rooting medium with -10 bar PEG solution. The plants were illuminated at different irradiances or left in darkness during the stress period.

Each value is the mean of 4 replicates.

- ● Dark
- △ 20 μE $m^{-2}s^{-1}$
- ▽ 70 μE $m^{-2}s^{-1}$
- □ 235 μE $m^{-2}s^{-1}$

Table 1.8 F values for results in Fig 1.9

| Source of variation | VR |
|---|---|
| Irradiance | 41.66** |
| Duration | 42.22** |
| Irradiance x Duration | 7.55** |

Significance level * $P \leq 0.05$

** $P \leq 0.01$

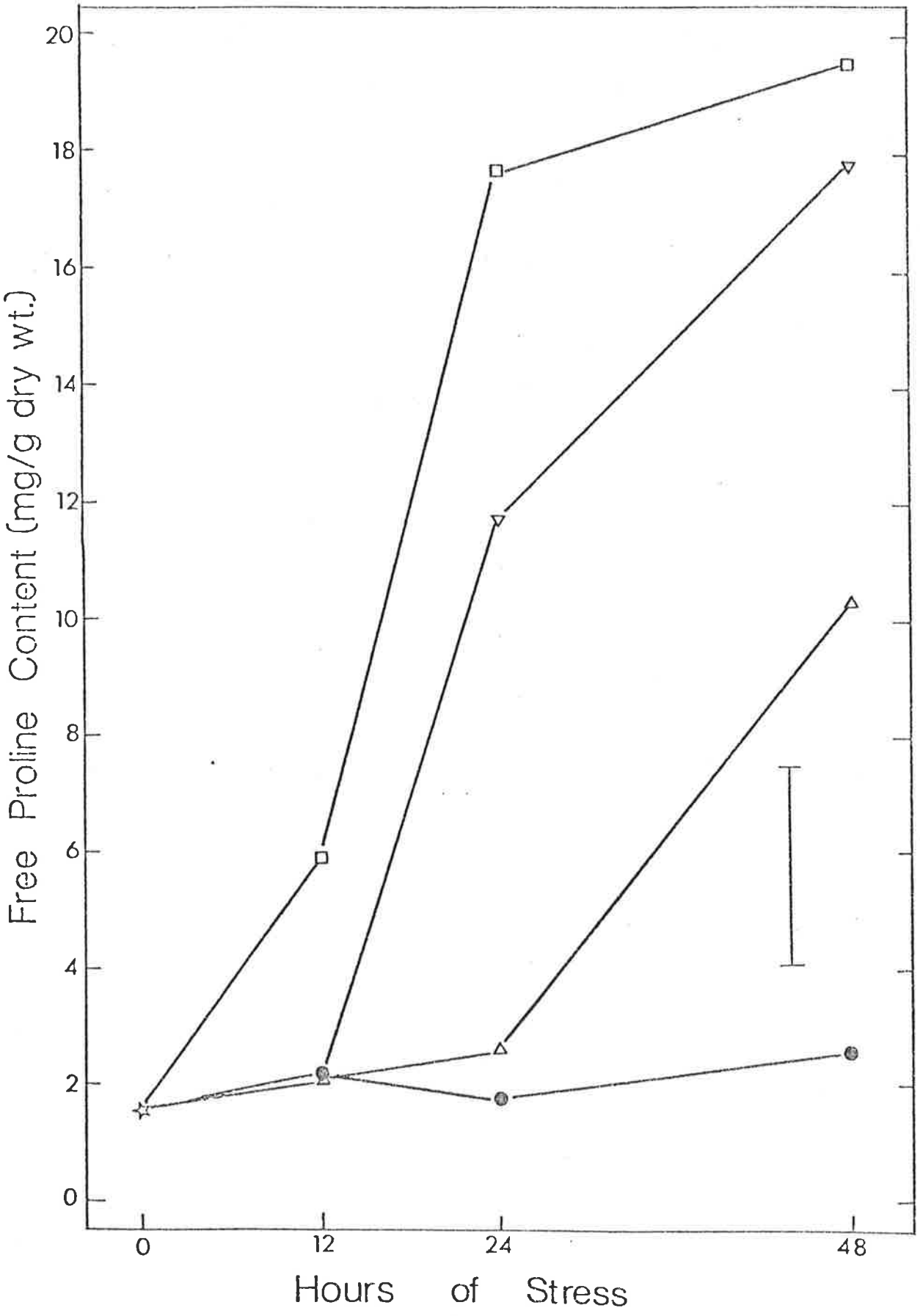

Free Proline Content (mg/g dry wt.)
20
18
16
14
12
10
8
6
4
2
0
0
12
24
48
Hours of Stress

Fig 1.10

Effect of irradiance on

water potential (A) and proline content (B)

in stressed barley plants.

The values are the same as those plotted in Figs 1.8, 1.9

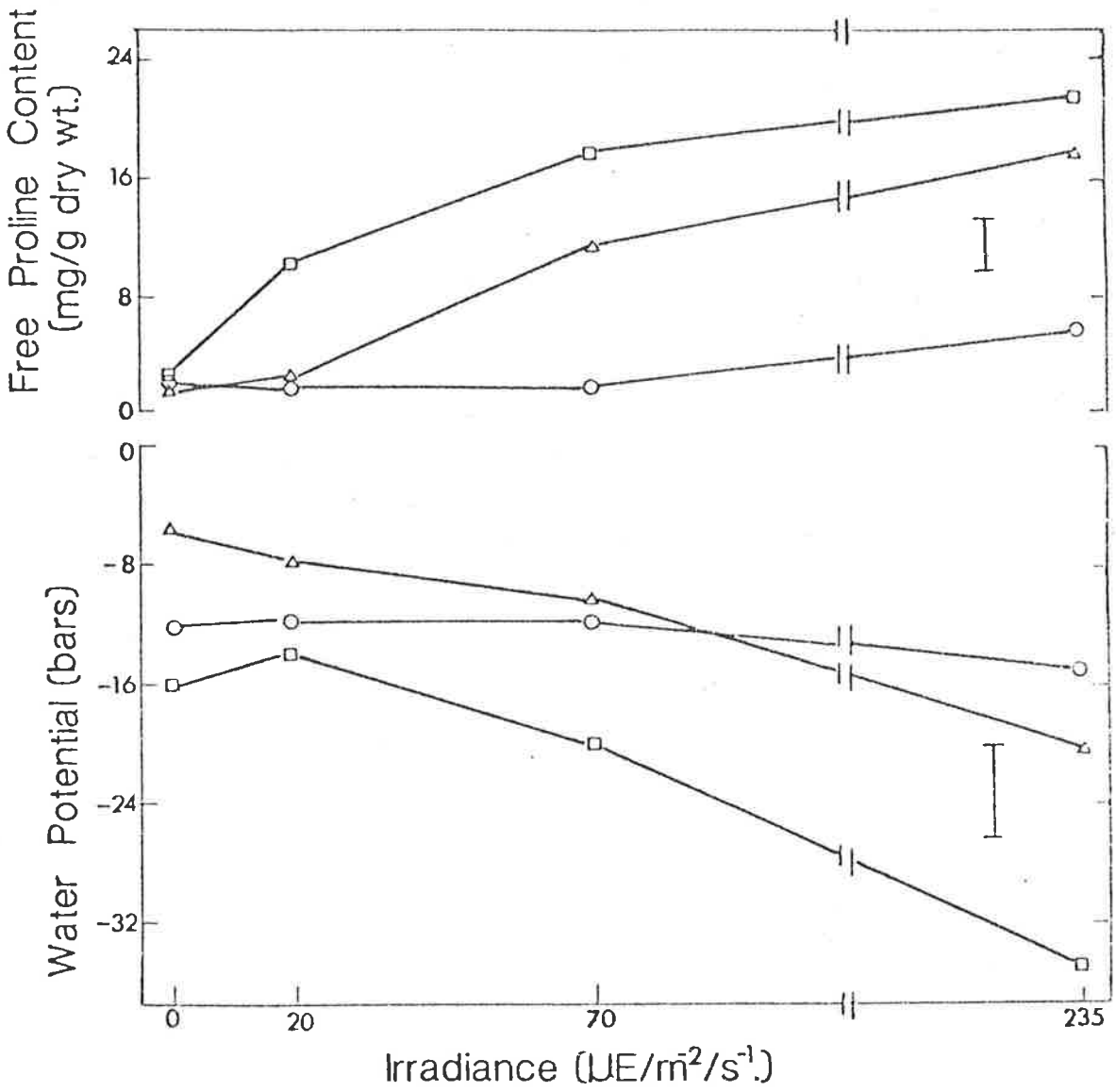
but are plotted against an irradiance level rather than duration

of irradiance.

○ 12 hour harvest

△ 24 hour harvest

□ 48 hour harvest


Free Proline Content
(mg/g dry wt.)
24
16
8
0
Water Potential (bars)
0
-8
-16
-24
-32
0
20
70
235
Irradiance (μE/m²/s⁻¹.)

## 1.5 Discussion

Light enhancement of proline accumulation is evident. It is also clear that the enhancement in light is not a short term trigger response but requires continuous illumination, which is effective even at a low irradiance of 20 μE $m^{-2}$ $s^{-1}$ (Figs 1.6 and 1.10). In addition, light reduces the water potential which seems to be associated with increased proline accumulation. The stomatal resistance data provide one possible explanation for the reduction in water potential (Table 1.7). Plants stressed at a higher irradiance (235 μE $m^{-2}$ $s^{-1}$) showed lower diffusive resistance than plants stressed at 20 μE $m^{-2}$ $s^{-1}$, which would induce a greater loss of water and consequently result in lower water potential in the plants in high light. It is of course possible that stomatal resistance may influence proline accumulation more directly through its control on photosynthesis as higher diffusive resistance would inhibit $CO_2$ exchange more severely, resulting in reduced photosynthesis and ultimately resulting in less proline accumulation.

The relationship between water potential and proline accumulation in the two experiments (1.2 and 1.4) appears to be linear and the regression coefficient was highly significant (Fig. 1.11).

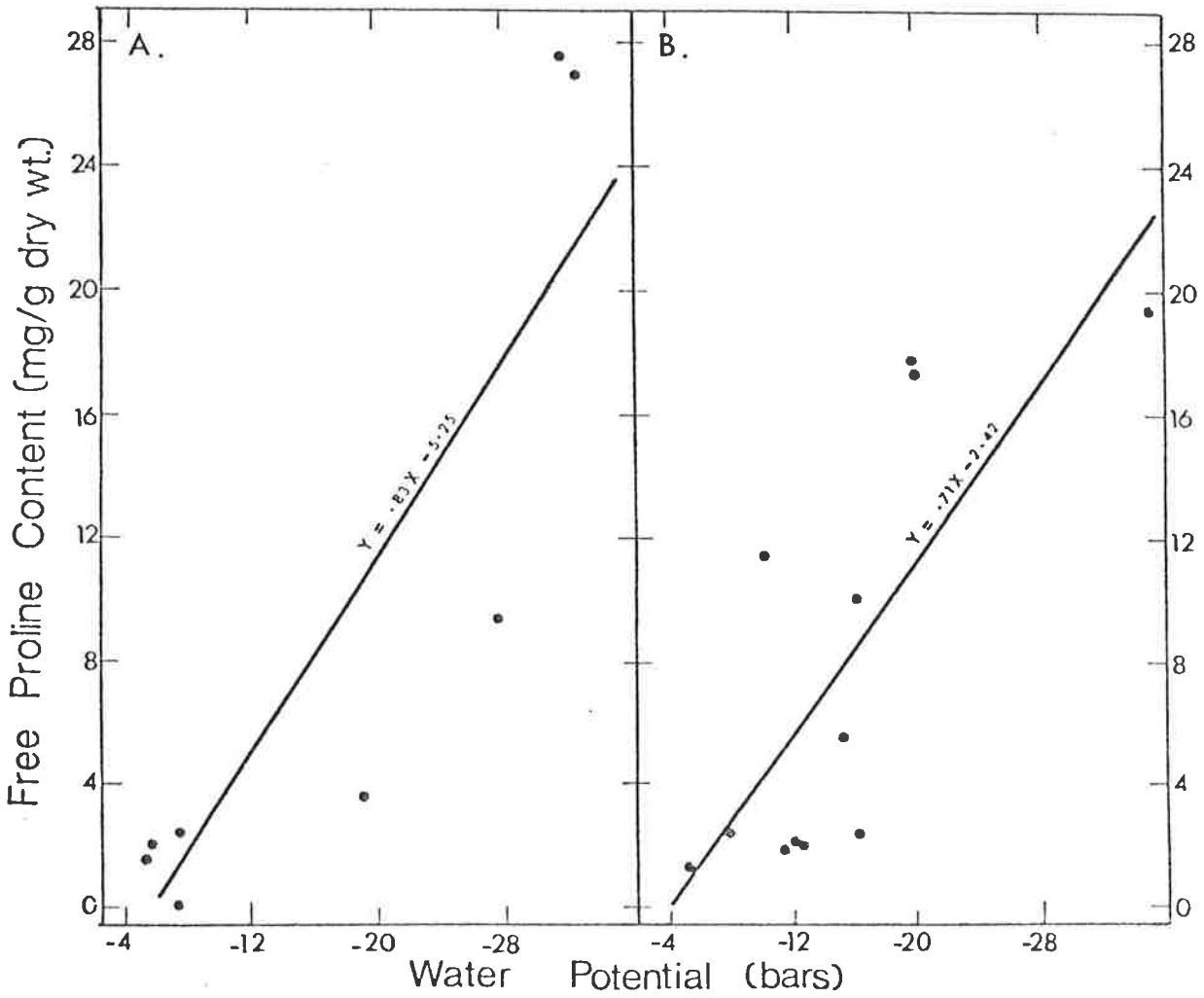
Although the results with whole plants clearly indicate an involvement of light in proline accumulation, it is difficult with this tissue to separate the direct effects of light (i.e. photosynthesis and other light-induced reactions) from indirect effects (water potential). In addition, although stress was imposed on the whole plant, only the first leaf was analysed and the role played by different parts of the plant is unknown. It was therefore decided to examine the role of light in proline accumulation with a more clearly defined system using leaf sections.

Fig. 1.11

Relationship between water potential and proline accumulation

A. Experiment 1.2 - Linear regression coefficient = 0.87**

B. Experiment 1.4 - Linear regression coefficient = 0.77**


A.
B.
Free Proline Content (mg/g dry wt.)
Water Potential (bars)
28
24
20
16
12
8
4
0
-4
-12
-20
-28
Y = .83X - 5.25
Y = .71X - 2.47

# SECTION I WATER STRESS

## 2. Proline accumulation in excised leaf segments

## 2. Proline accumulation in excised leaf systems

### 2.1 Introduction

Results obtained with excised leaves demonstrated that it was an appropriate system to use for water stress studies (Appendix II). The major advantage of such a tissue system over the previously-used intact plant system was that the effects of light could be examined independently of confounding effects on leaf water potential. Before using the system to explore the response, it was first necessary to confirm that an enhancement of proline accumulation by exposure to light took place, and to characterize this response in terms of rate and extent of proline accumulation.

### 2.2 Effect of light and darkness, prior to and during the stress treatment

Excised leaves have been previously reported to accumulate proline when subjected to a water stress by floating on PEG solutions in the dark (Singh et al, 1973b).

It was not known, however, whether exposure to light enhanced the accumulation of proline in such systems. This experiment was designed to test this point, together with the response to exposure to light prior to or during stress.

#### 2.2.1 Methods

Barley plants were grown in sand for 12 days in a 16 hour photoperiod at 20°C and an irradiance of 300 $\mu E\ m^{-2}\ s^{-1}$. On the 13th day, 4 hours after the beginning of the light cycle, plants were either transferred to the dark or maintained in continuous light for a further 48 hours.

The first leaves from plants in these two treatments were then excised, cut into 1.5 cm segments, floated on 10 ml of -15 bar PEG or water in 9.0 cm petri dishes and left in the dark or light for 24 hours (Fig 2.1 experimental design for experiment 2.2). At the end of this period the segments were harvested, rinsed quickly in water, dried on filter paper, frozen in liquid nitrogen and freeze dried. The dry samples were weighed and then analysed for proline.

### 2.2.2 Results

It is apparent from the results (Fig 2.2) that light during a period of stress enhanced proline accumulation irrespective of whether the tissue was exposed to light or darkness prior to the stress period. Although stressed tissue which was maintained in light throughout periods 1 and 2 (L-L) accumulated the most proline, leaves maintained in darkness during period 1 demonstrated the greater response to light in period 2 (L-L - LD = 5.5 mg $g^{-1}$ DW, DL - DD = 8.5 mg $g^{-1}$ DW). Tissue which was in darkness during both periods 1 and 2 (D-D) accumulated the least proline of all the stressed treatments. Tissue floated on water accumulated some proline compared to the zero hour control plants, but the amount accumulated was significantly less than in stressed tissue in all treatments. The interactions between the effects of light in $P_1$ and $P_2$ with the effect of stress were highly significant while that between the effect of light in $P_1$ and in $P_2$ was not (Table 2.1, 2.2 i and ii). However, the effects of light during $P_1$ or during $P_2$ were highly significant when taken separately (Table 2.2 iii and iv). Tissue which was maintained in darkness during $P_2$ accumulated significantly more proline when exposed to light in $P_1$ rather than being kept in darkness during that period.

Fig 2.1

EXPERIMENTAL DESIGN FOR EXPERIMENT 2.2

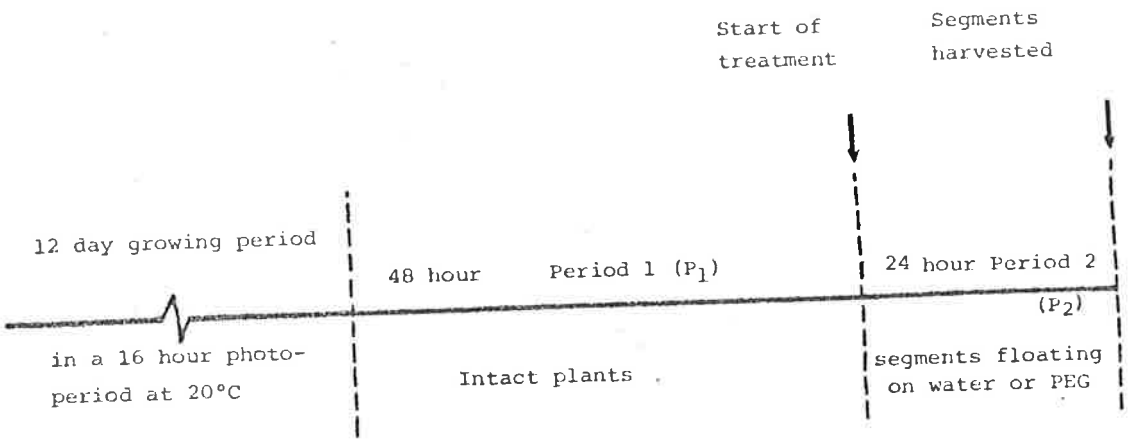


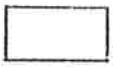
Fig 2.2

Effect of light prior to and during stress treatment on proline accumulation in first leaf segments of barley floated on water or PEG (-15 bars) for 24 hours.

Barley plants were grown in sand for 12 days in a 16 hour photoperiod at 20°C and were then exposed to 48 hours continuous light or continuous darkness, following which the first leaves were excised, cut into 1.5 cm segments and floated as 10 ml water or PEG (10 segments per petri dish) for 24 hours in light or darkness. Each value is the mean of 4 replicates.

● 0 hours light

x 0 hours dark



Turgid (floated on water for 24 hours)

Stress (floated on PEG for 24 hours)

| | Period 1 (48 hrs) | Period 2 (24 hrs) |
|---|---|---|
| L-L | Light | Light |
| L-D | Light | Dark |
| D-L | Dark | Light |
| D-D | Dark | Dark |

Bars indicate standard error of means.

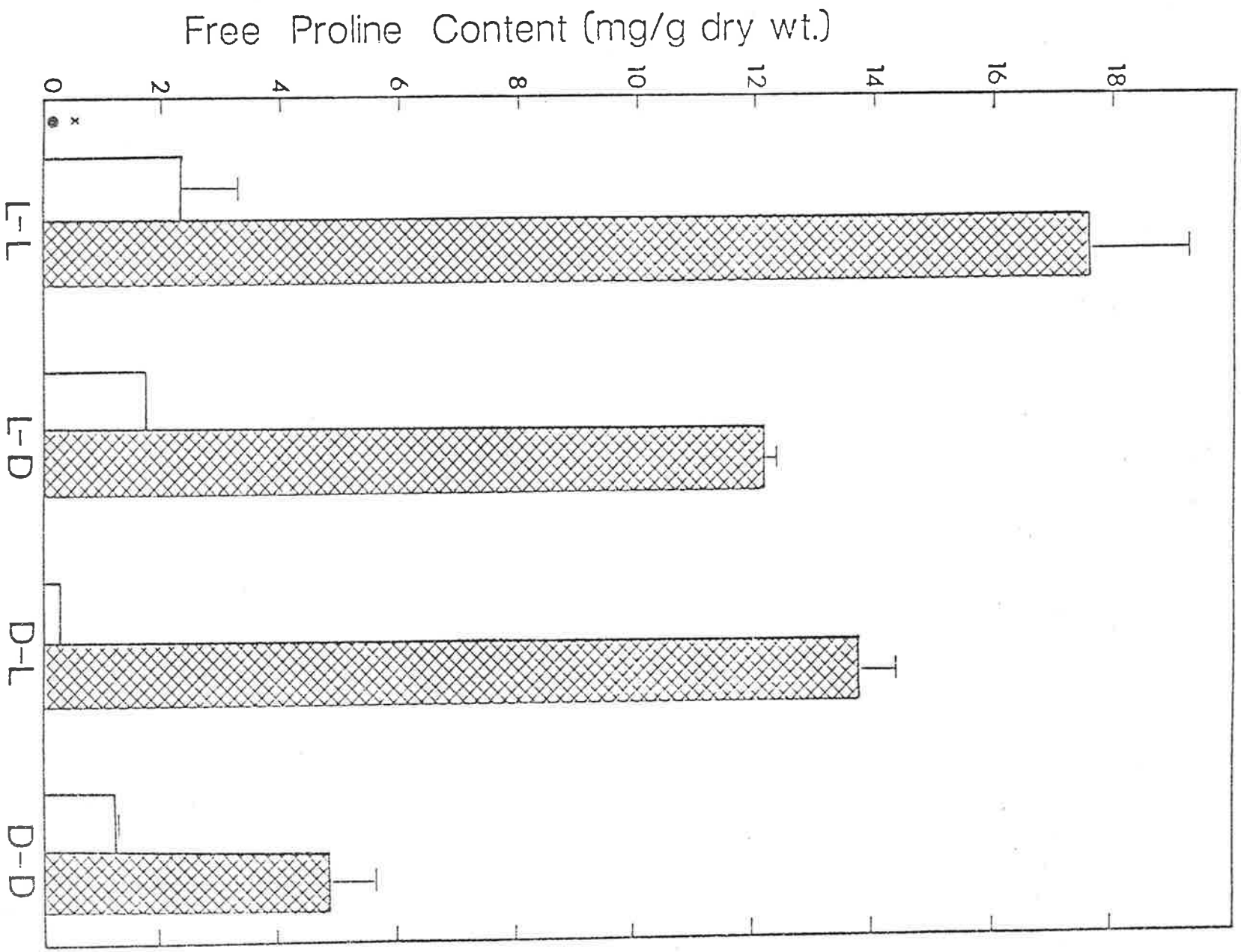

Free Proline Content (mg/g dry wt.)
0
2
4
6
8
10
12
14
16
18
L-L
L-D
D-L
D-D

Table 2.1 F values for results in Fig 2.2

| Source of variation | VR |
|---|---|
| $P_1$ | 26.88** |
| $P_2$ | 27.66** |
| Stress | 255.31** |
| $P_1 \times P_2$ | 0.49 |
| $P_1$ x Stress | 10.54** |
| $P_2$ x Stress | 30.33** |
| $P_1 \times P_2$ x Stress | 3.49 |

Table 2.2 Table of Means of results in Fig 2.2

(i) $P_1$ x Stress

| | Turgid | Stress |
|---|---|---|
| Light | 2.03 | 14.86 |
| Dark | 0.73 | 9.23 |
| LSD | 1.95 | |

(ii) $P_2$ x Stress

| | Turgid | Stress |
|---|---|---|
| Light | 1.30 | 15.64 |
| Dark | 1.46 | 8.45 |
| LSD | 1.95 | |

(iii) $P_1$

| Light | Dark |
|---|---|
| 8.44 | 4.98 |
| LSD 1.38 | |

(iv) $P_2$

| Light | Dark |
|---|---|
| 8.47 | 4.96 |
| LSD 1.38 | |

## 2.3 Brief period of illumination at two different irradiances and proline accumulation

Results in the previous section have shown that light provided during stress enhances proline accumulation. Light may have induced proline accumulation through a triggering response rather than by a continuous effect e.g. via photosynthesis.

The next experiment was designed to test the hypothesis that light acts to induce proline accumulation (i.e. short term response) rather than to sustain increased synthesis.

### 2.3.1 Methods

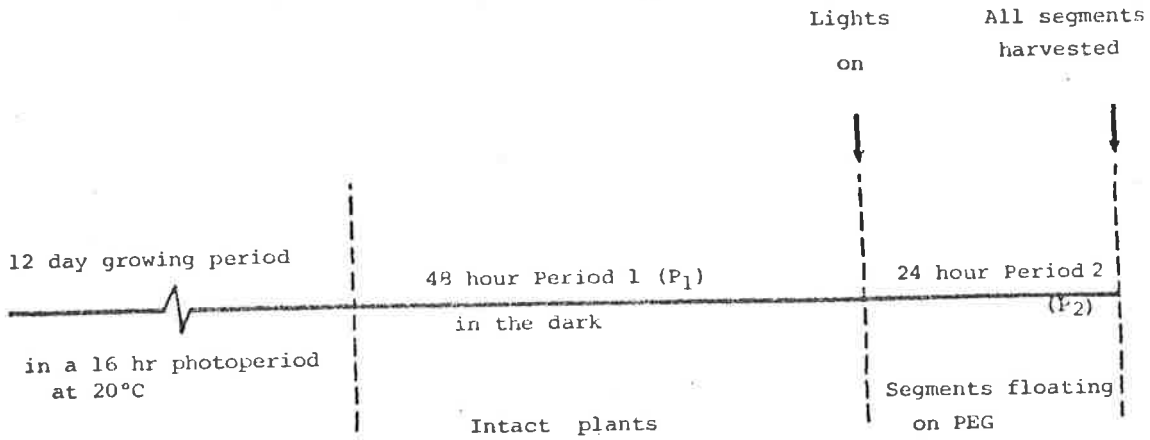
This experiment was conducted with plants exposed to 48 hours continuous darkness prior to excision and exposure to stress, as the results of the previous experiment suggested that the light stimulation of proline accumulation during stress was greater in this treatment. Treatments given are detailed in the Fig 2.3 experimental design for experiment 2.3. The segments were harvested 24 hours after commencement of stress, frozen in liquid nitrogen, lyophilized and analysed for proline. Four replicates of each treatment were taken and results were analysed and tested for significant differences by performing an analysis of variance on the data.

### 2.3.2 Results

Light given for 5 min or 30 min duration at the commencement of the stress treatment, was insufficient to induce proline accumulation. There was no significant difference between these treatments and the dark treatment, while segments exposed to continuous light showed a significant increase at both irradiance levels (Fig 2.4). Thus, it appears that the continued presence of light may be required to significantly enhance proline accumulation and that irradiance as low as 1·5 $\mu E\ m^{-2}s^{-1}$ is effective in promoting proline accumulation.

Fig 2.3

EXPERIMENTAL DESIGN FOR EXPERIMENT 2.3




Light treatment of 15 or 250 μE $m^{-2}s^{-1}$

a) 5 minutes light

b) 30 minutes light

c) continuous light (24 hours)

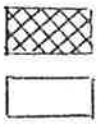
d) darkness (24 hours)

Fig 2.4

Effect of exposure to light for different periods (0, 5, 30 min or continuous) on proline content of barley leaf segments floated on PEG solution (-15 bar).

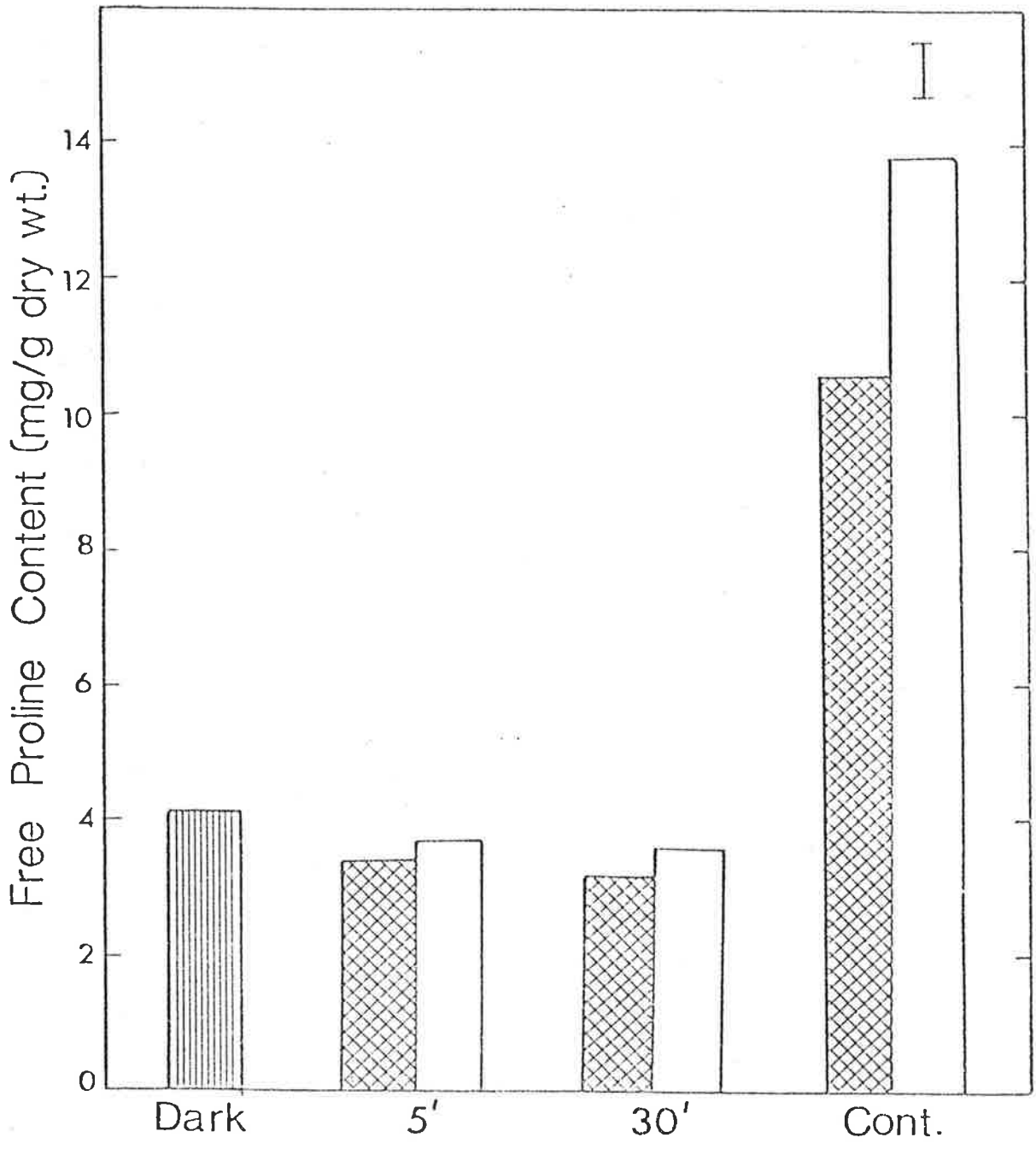
Twelve day old barley plants were transferred to 48 hours continuous darkness prior to excision and segmentation of the first leaves. The segments were floated on PEG solution (-15 bars) for 24 hours in darkness or continuous (cont) irradiance of 15 μE $m^{-2}s^{-1}$ or 250 μE $m^{-2}s^{-1}$ or transferred to darkness following a brief period of exposure to either irradiance (see Fig 2.3).

Each point is the mean of 4 replicates.



Illuminated at 15 μE $m^{-2}s^{-1}$

Illuminated at 250 μE $m^{-2}s^{-1}$


Free Proline Content (mg/g dry wt.)
0
2
4
6
8
10
12
14
Dark
5'
30'
Cont.

## 2.4 Proline accumulation - capacity and rate

### 2.4.1 Severity of stress and proline accumulation

The results described earlier (Section 2.2) were obtained in segments exposed to -15 bar PEG solution. The nature of the response of tissue given different light/dark treatments (both prior to and during the stress treatment) with regard to proline accumulation at varying external osmotic potentials ($\pi$ ext) was not known. Of special interest was whether proline accumulation was influenced by $\pi$ ext irrespective of the light.

#### 2.4.1.1 Methods

The growth of plants and the conditions of the light treatment during both periods 1 and 2 were as described in Results and Discussion Section 2.2.1. The stress treatments consisted of 5 different concentrations of PEG (-5, -10, -15, -20, -27 bars) and were imposed by floating 10 segments on 10 ml of PEG solution of the appropriate osmotic potential; commencing simultaneously with the imposition of the $P_2$ treatment. There were 3 replicates for each treatment and the results were tested by conventional analysis of variance test.

#### 2.4.1.2 Results

Segments stressed at -5 and -10 bars did not accumulate large amounts of proline in any treatment except where exposed to continuous light in both $P_1$ and $P_2$ (L-L) (Fig 2.5). These segments accumulated comparatively high concentrations of proline even at the lowest stress level of -5 bars, and the amount accumulated increased with the intensity of stress (Table 2.3). The amounts accumulated were significantly greater than in other light treatments at every level of stress. The

Fig 2.5

Effect of intensity of stress on proline accumulation in barley leaf segments floated on PEG (-15 bars).

Barley plants were grown in sand for 12 days in a 16 hour photoperiod at 20°C, and were then exposed to 48 hours continuous light or continuous darkness, following which the first leaves were excised, cut into 1.5 cm segments and floated on PEG (10 segments/petri dish) in continuous light or continuous darkness for 24 hours . Each value is the mean of 3 replicates.

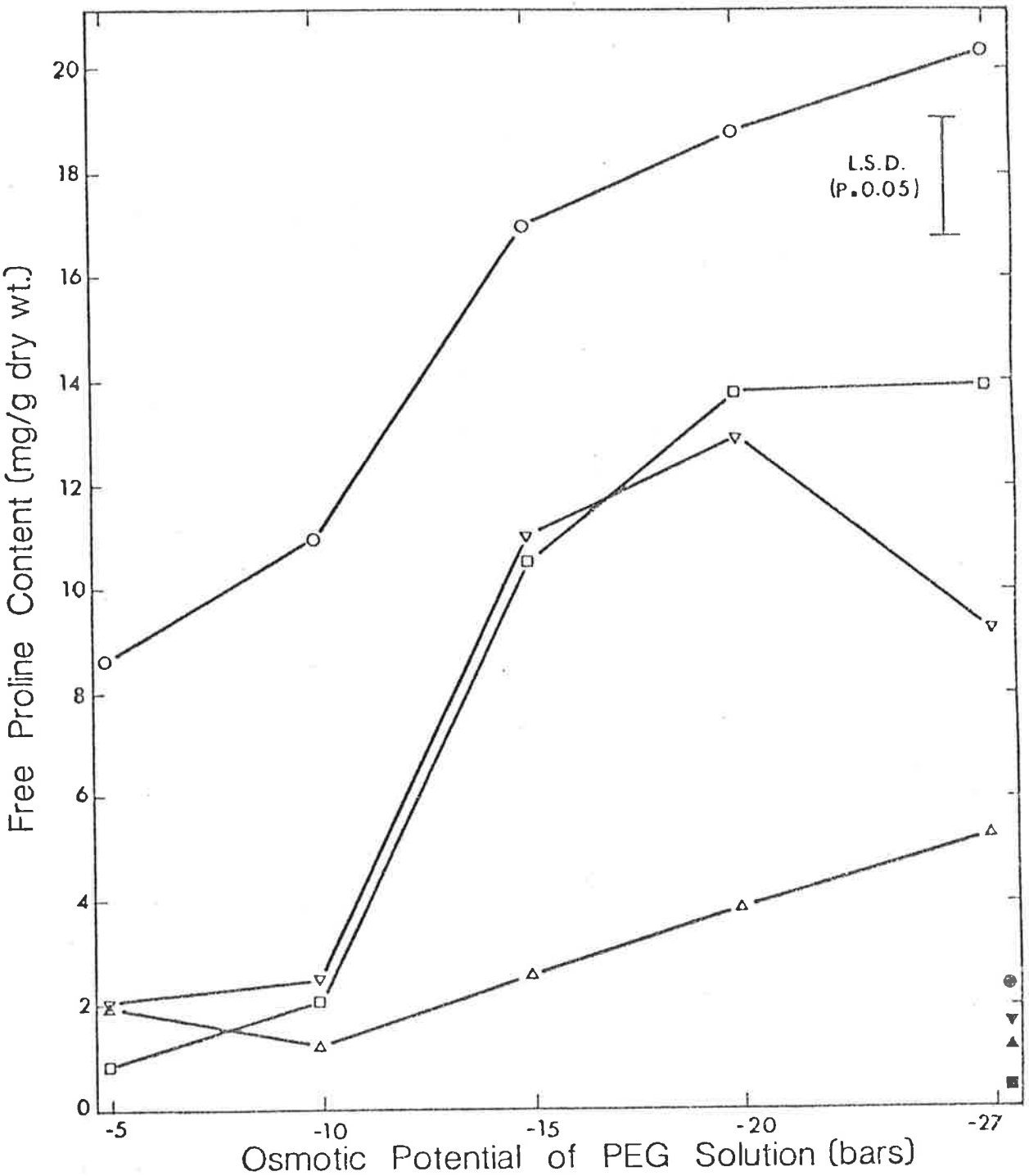
| Turgid | Stress | | Period 1 | Period 2 |
|---|---|---|---|---|
| ● | ○ | L-L | Light | Light |
| ▼ | ▽ | L-D | Light | Dark |
| ■ | □ | D-L | Dark | Light |
| ▲ | △ | D-D | Dark | Dark |

Table 2.3 F values for results in Fig 2.5

| Source of variation | VR |
|---|---|
| Stress | 119.71** |
| Light | 197.34** |
| Stress x Light | 9.01** |

Significance level * $P \leq 0.05$

** $P \leq 0.01$


Free Proline Content (mg/g dry wt.)
0
2
4
6
8
10
12
14
16
18
20
L.S.D.
(P.0.05)
-5
-10
-15
-20
-27
Osmotic Potential of PEG Solution (bars)

increase in proline content with level of stress in segments maintained in constant darkness (D-D) was less but consistent, with the highest proline concentration being accumulated at -27 bars. This differed from all the other treatments (i.e. L-L, D-L and L-D) where there was a greater increase in accumulation between -10 and -15 bars than between any other interval of stress level. In only one case did the proline content decline in response to a decrease in osmotic potential (L-D, -20 to -27 bars); the reason for this is not immediately apparent (Fig 2.5). In summary, however, in terms of the response of proline concentration to osmotic potential (>10 bars), treatments in which the tissue was exposed to light at some stage more closely resemble each other than they do tissue maintained in darkness throughout.

### 2.4.2 Duration of stress and proline accumulation

Results of the previous experiment clearly reveal that tissue exposed to light throughout (L-L) accumulated significantly more proline than did other treatments at all stress levels. The differences under different light treatments may be due either to differenences in rates of proline accumulation or to differences in the capacity to accumulate proline, as proline accumulation was measured at one time only.

The following experiments were conducted to distinguish between these possibilities.

#### 2.4.2.1 Methods

The conditions under which the plants were grown (to 12 days) were the same as in 2.2.1 of this section. Plants were given 48 hours continuous light or darkness, the first leaves were then excised and floated on -15 bar PEG solution in continuous light or darkness. The leaf segments were harvested 0, 12, 24, 36 or 48 hours later and

analysed for proline. There were 3 replicates for each treatment and an F test was performed to test for significance.

2.4.2.2 Results

Proline accumulated at a comparatively slow rate in the first 12 hours in all treatments, although the difference between segments floating in the light and those in darkness was already apparent (Fig 2.6). Accumulation in darkness by segments from plants maintained in darkness prior to leaf excision (D-D) continued at the same slow rate for the following 36 hours. In all other cases, accumulation accelerated after 12 hours, even where the segments were maintained in darkness after (but not before) excision. Segments exposed to light during incubation ($P_2$) appeared to reach a maximum proline content after 24-36 hours incubation but those in darkness during this period continued to accumulate proline up to termination of the experiment at 48 hours.

2.4.3 Transfer of segments between light and darkness during stress

In the previous experiment, segments in light during stress accumulated more proline than those in darkness, the difference becoming evident after 12 hours. It is not known whether the first 12 hours of light is sufficient to stimulate proline accumulation over the entire duration of stress (i.e. the following 36 hours) or alternatively, whether the continued presence of light is required. Also the response to the provision of light during the first 24 hours compared to the response following an initial 12 hour dark period is unknown. It was envisaged that transfer of segments between light and darkness 12 hours after stress commenced might provide some additional information on this light stimulation of proline accumulation.

Fig 2.6

Effect of duration of stress on proline accumulation in barley leaf segments.

Barley plants were grown in sand for 12 days in a 16 hour photoperiod at 20°C and were then exposed to 48 hours continuous light or continuous darkness, following which the first leaves were excised, cut into 1.5 cm segments and floated on -15 bar PEG (in the light or the dark).

Each value is the mean of 3 replicates.

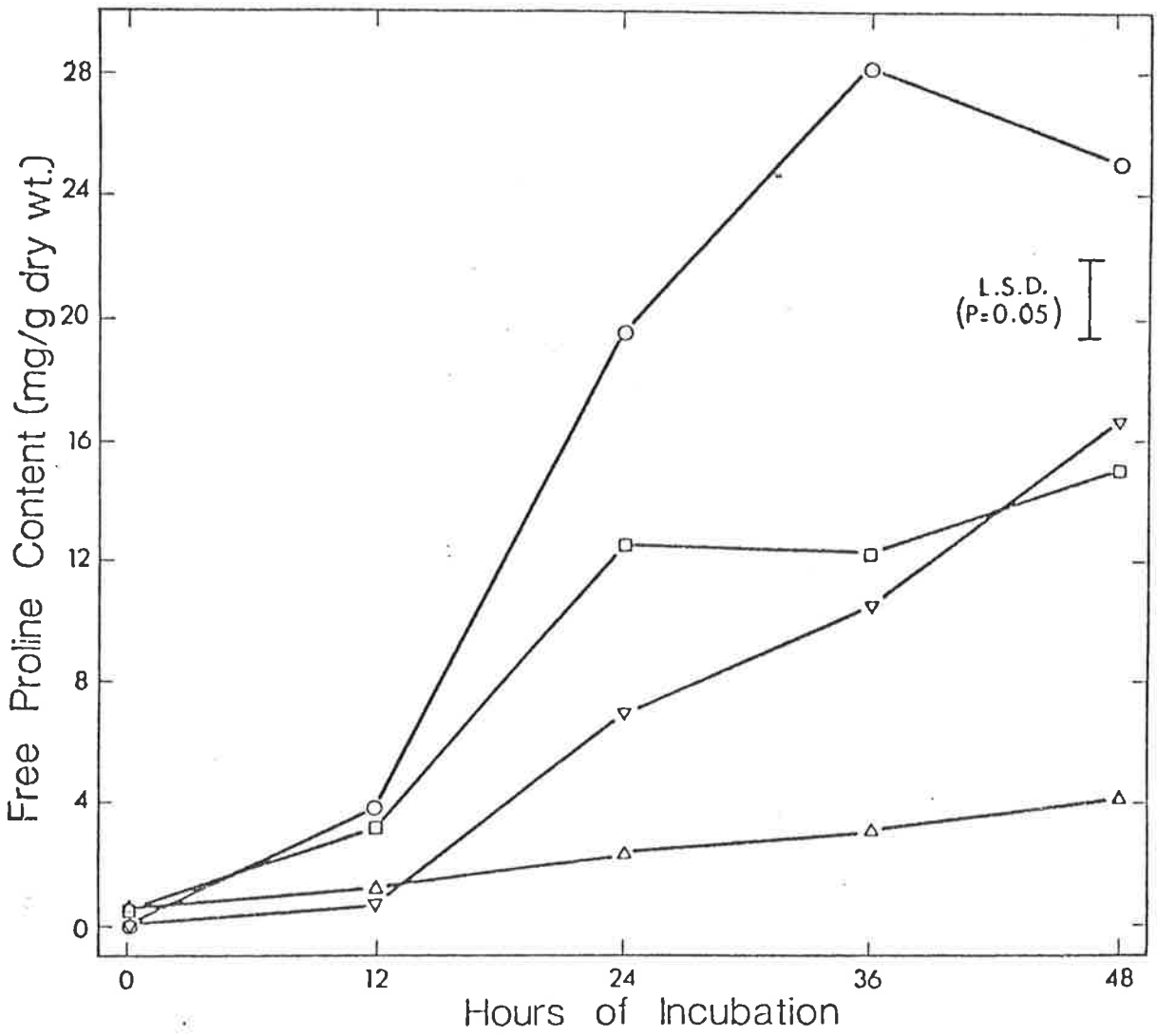
| | | Period 1 | Period 2 |
|---|---|---|---|
| ○——○ | L-L | Light | Light |
| ▽——▽ | L-D | Light | Dark |
| □——□ | D-L | Dark | Light |
| △——△ | D-D | Dark | Dark |

Table 2.4 F values for results in Fig 2.6

| Source of variation | VR |
|---|---|
| Duration | 216.16** |
| Light | 178.67** |
| Duration x Light | 29.32** |

Significance levels * $P \leq 0.05$

** $P \leq 0.01$


Free Proline Content (mg/g dry wt.)
28
24
20
16
12
8
4
0
0
12
24
36
48
Hours of Incubation
L.S.D.
(P=0.05)

2.4.3.1 Methods

This experiment was carried out in conjunction with experiment 2.2 and therefore as the plant culture technique and prestress treatments have been explained in 2.4.2.1, it will not be repeated here. Stress was imposed in light or darkness by floating the segments on -15 bar PEG solution (10 segments per sample). Half the samples of each treatment was transferred to the opposite $P_2$ treatment (i.e. light to dark and vice versa) 12 hours after the commencement of stress treatment. There were 3 replicates of each treatment, and 3 sampling i.e. at 12, 24 and 36 hours after transfer. The segments were rinsed quickly in water, dried with filter paper and frozen in liquid nitrogen. Samples were analysed for proline after they were freeze dried and weighed.

2.4.3.2 Results

The data from experiment 2.2 are presented along with the results of this experiment to facilitate comparisons (Fig 2.7). Transfer to light from darkness at 12 hours stimulated proline accumulation and transfer to darkness from light depressed it irrespective of exposure of segments to light or darkness during $P_1$. All plants initially in light (0-12 hours) seemed to reach a ceiling proline content as did those transferred to light at 12 hours, while those in dark throughout or after 0 hours did not. The continued presence of light was required for maximum proline accumulation. It is interesting to note that light provided during 12 to 36 hours after commencement of incubation on PEG was as effective in enhancing proline accumulation as when segments were exposed to light from 0 to 24 hours of imposition of stress (Difference between O——O (0 to 24 hours) in Fig 2.7 A compared with ▼- - -▼ (12 to 36 hours)and □——□ (0 to 24 hours)

Fig 2.7

Effect of transferring segments between light and darkness on proline accumulation in segments floated on -15 bar PEG for 12 hours.

Barley plants were grown in sand for 12 days in a 16 hour photoperiod at 20°C, and were then exposed to 48 hours continuous light or continuous darkness, following which the first leaves were excised, cut into 1.5 cm segments and floated on PEG (10 segments/petri dish) in continuous light or continuous darkness for 12 hours. At the end of this period, half the samples which were in the light were transferred to the dark, and those in the dark to light; and were harvested 12, 24 and 36 hours after transfer were made.

Each value is the mean of 3 replicates

| | | |
|---|---|---|
| Fig A | ○ | L-L |
| | ● | L-L transferred to D after 12 hours light (L-L-D) |
| Fig B | ▽ | L-D |
| | ▼ | L-D transferred to L after 12 hours darkness (L-D-L) |
| Fig C | □ | D-L |
| | ■ | D-L transferred to D after 12 hours light (D-L-D) |
| Fig D | △ | D-D |
| | ▲ | D-D transferred to L after 12 hours dark (D-D-L) |

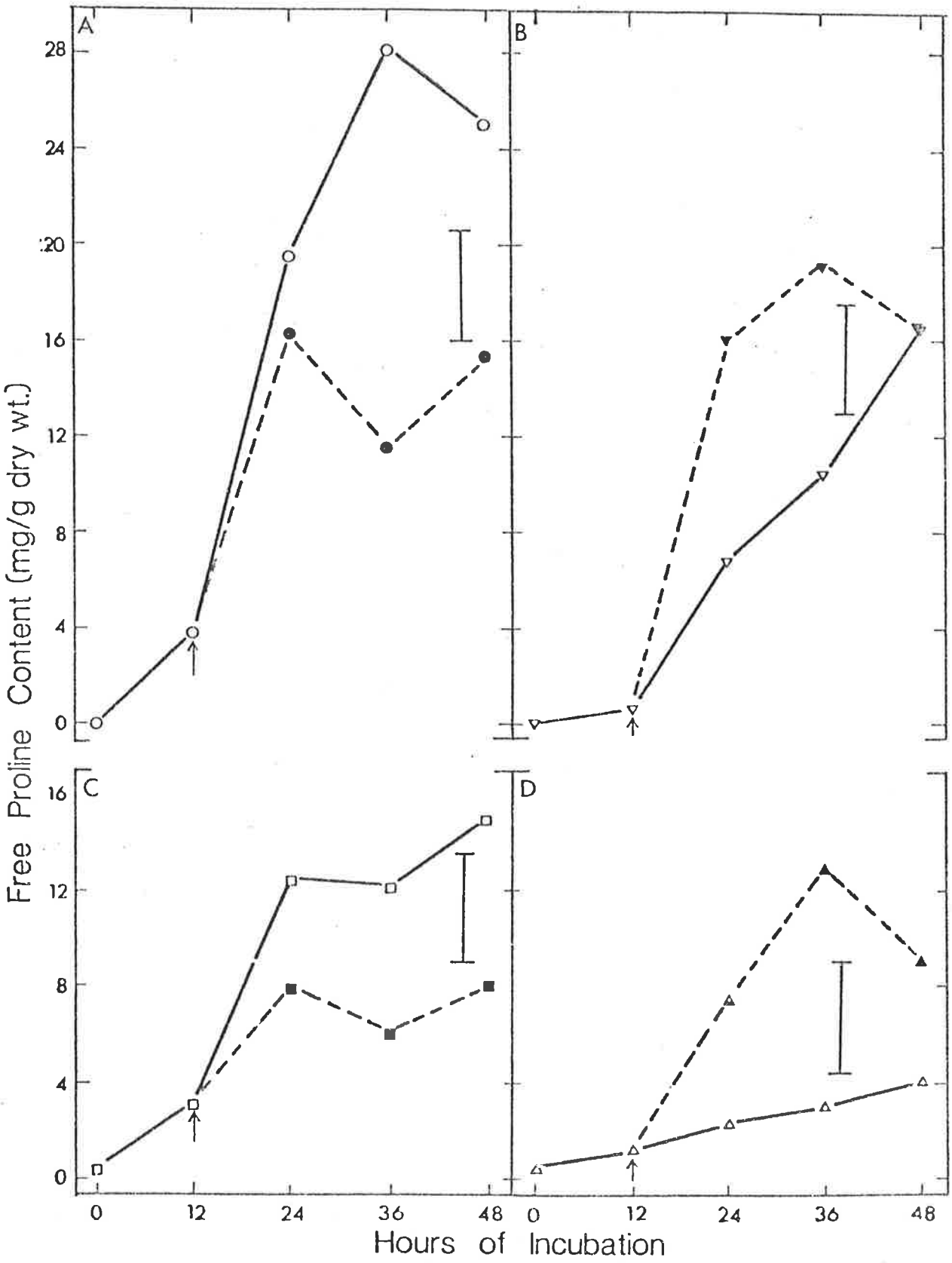

A
B
C
D
Free Proline Content (mg/g dry wt.)
Hours of Incubation
0
4
8
12
16
20
24
28
0
12
24
36
48

Table 2.5 F values for results in Fig 2.7

| Source of variation | VR |
|---|---|
| Period 1 ($P_1$) | 163.85** |
| Period 2 ($P_2$) | 45.26** |
| Period 3 (P3) | 118.75** |
| Time | 5.34** |
| $P_1 \times P_2$ | 0.78 |
| $P_1 \times P_3$ | 1.69 |
| $P_2 \times P_3$ | 1.63 |
| $P_1$ x Time | 0.92 |
| $P_2$ x Time | 1.57 |
| $P_3$ x Time | 5.85* |
| $P_1 \times P_2 \times P_3$ | 3.59 |
| $P_1 \times P_2$ x Time | 1.49 |
| $P_1 \times P_3$ x Time | 1.47 |
| $P_2 \times P_3$ x Time | 4.36* |
| $P_1 \times P_2 \times P_3$ x Time | 3.87* |

Significance level * $P \leq 0.05$

** $P \leq 0.01$

in Fig 2.5C compared with ▲-----▲ (12 to 36 hours).

Segments in light from 0-12 hours showed a lag phase which was eliminated when segments were transferred from darkness to light after 12 hours. This could occur if the lag phase represents the period during which the metabolic base (of either the precursors or enzymes) is being built up. Once this base is achieved, it does not decay readily and therefore transfer to light after 12 hours of dark treatment showed an absence of the lag phase. If this is the case, then it must mean that segments in dark follow a similar sequence of responses evoked by stress as those in light and have a potential to accumulate more proline if provided with light. Experimental results obtained here confirm this expectation (Table 2.5).

### 2.4.4 Exposing different sections of the entire leaf to light or darkness during stress, and proline accumulation

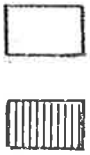
It is evident from the previous experiment that the continued presence of light was necessary to obtain maximum proline accumulation. It is not known whether light effect can be translocated to other parts of the leaf or whether it is immobile, and only affects the area exposed to light. This experiment was conducted to elucidate this point.

#### 2.4.4.1 Methods

Barley plants were grown for 12 days as described in 2.2.1 of this section, and given 48 hours continuous light before the first leaf was excised and floated on -15 bar PEG solution for 24 hours. Along the length of the leaf, 1.5 cm apart, alternate light and dark treatments were given (Fig 2.8A). The dark treatment consisted of enclosing the

Fig 2.8A

Method for exposing sections of an entire leaf to light or darkness during stress.



Exposed to light

Exposed to darkness

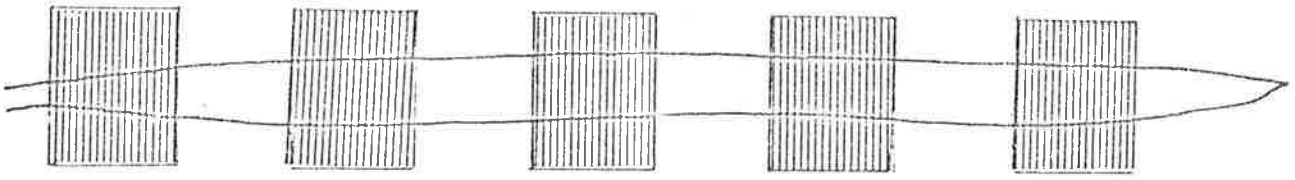
Fig 2. B

Effect of exposing entire intact first leaves to continuous light or darkness or sections of the intact leaf to light or darkness during stress. Stress was imposed by floating the intact leaves on -15 bar PEG for 24 hours.

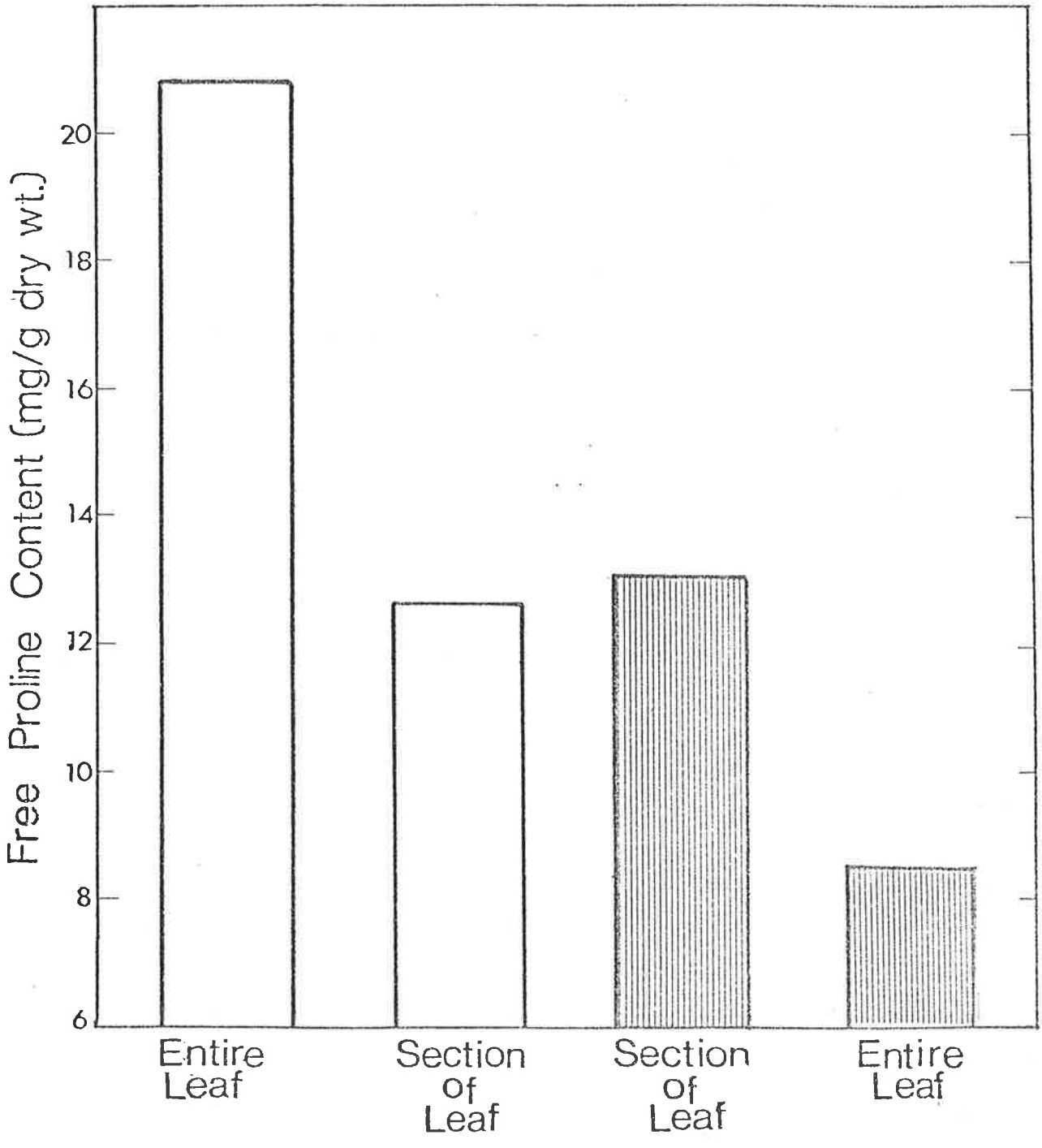
Exposed to light

Exposed to darkness

A.



B.


Free Proline Content (mg/g dry wt.)
20
18
16
14
12
10
8
6
Entire Leaf
Section of Leaf
Section of Leaf
Entire Leaf

area in strips of aluminium foil (1.5 cm long) gently placed over the leaf surface, while the light-treated section was exposed to continuous light (Fig 2.8A). In addition to this treatment, intact leaves floated on PEG (-15 bars) were exposed entirely to continuous light or to continuous darkness. After 24 hours, the leaves were harvested, divided into light and dark segments where necessary and preserved for proline analysis.

#### 2.4.4.2 Results

The leaves exposed entirely to continuous light accumulated the most proline and those in continuous darkness accumulated the least (Fig 2.8B). In those leaves partially exposed to light, both light and dark segments accumulated an intermediate level of proline and there was no difference in the amount accumulated by segments exposed to light or to darkness.

## 2.5 Discussion

Results obtained with excised segments floated on PEG (-15 bars) clearly showed that light stimulates proline accumulation (Fig 2.2). The response was not one in which exposure to light for short duration triggered accumulation, but the continued presence of light was necessary to elicit maximum proline accumulation (Fig 2.4). Light may act via photosynthesis either by supplying energy rich reductants, NAD(P)H and ATP or via the carbon compounds produced. Alternatively, light may induce membrane changes consequent upon synthesis of metabolites, enzymes or hormones. Results from the entire leaves (experiment 2.4.4) suggested that the light effect is translocatable, but whether the end product (proline) or the stimulant (hormones or precursors, etc) was translocated is unknown. The presence of light increased the rate of proline accumulation as well as the eventual extent of accumulation

(Fig 2.5). The greatest amount or proline accumulated in tissue which was in continuous light in both $P_1$ and $P_2$. After 36 hours of light, proline accumulation appeared to level off in the L-L treatment while in D-L and the dark treatments, accumulation appeared to continue until 48 hours when the experiment was concluded. Singh et al (1973), Chu (1974) and Schobert (1977a) have reported continued proline accumulation in detached Barley and radish leaves and diatoms respectively, while in excised turnip leaves, in the dark a decline in proline content after the first 24 hours has been reported (Stewart et al, 1966). These latter authors suggested that this phenomenon was due to insufficient supply of carbohydrates. In barley segments floated in the dark, however, proline continued to accumulate albeit at a slower rate than in light treated tissue (Fig 2.6). Stored reserves may influence the amount of proline accumulated as segments exposed to light prior to stressing accumulated more proline than segments exposed to darkness during a similar period. However, other explanations are possible and increasing the level of stress also induces higher amounts of proline. Segments in light throughout (i.e. L-L) appeared sensitive to even -5 bars stress, the rate of accumulation increasing up to -27 bars. The effect of the higher stress levels, therefore demonstrates that the provision of precursors is not the only limitation on proline accumulation. The reasons for the decreased proline levels in segments stressed in darkness after exposure to 48 hours continuous light, are not apparent.

Proline accumulation was dependant on the duration of the light period and appeared to be independant of the time at which the light treatment was given for up to at least 12 hours after the imposition of stress in darkness (Fig 2.7). A similar increase in proline accumulation during stress as a result of transfer has been reported in

diatoms (Schobert, 1977a) and Chlorella (Greenway and Setter, 1979).

# SECTION I WATER STRESS

## 3. Stress stimulated proline accumulation

### - Irradiance effect

# 3 Stress stimulated proline accumulation - irradiance effect

## 3.1 Introduction

Results presented so far show that the continued presence of light is required for the enhancement of stress-stimulated proline accumulation. Light may conceivably induce this effect via photosynthesis (quantitative) or through a phytochrome response (qualitative). The experiments described below were conducted to further elucidate this response.

The experiments described in this section were performed to determine whether there were quantitative relationships in the effect of light on proline accumulation. Qualitative aspects are considered in the following section.

## 3.2 Proline accumulation at different irradiances

It is well established that increasing the energy (i.e. irradiance) incident upon turgid leaves leads to increased photosynthesis in a linear fashion until saturating levels of light are reached. Increase in irradiance beyond this point does not result in an increase in photosynthetic rate, and very high irradiances can lead to a decrease in photosynthesis due to chloroplast photooxidation and a consequent loss of efficiency.

In this experiment, the response of proline accumulation to three different irradiances was compared with accumulation in the dark. The irradiances selected were 300 $\mu E\ m^{-2}s^{-1}$, i.e. the irradiance at which the plants had been growing, and two below this level at 150 and 75 $\mu E\ m^{-2}s^{-1}$.

### 3.2.1 Methods

Barley plants were grown in sand in a 16 hour photoperiod at 20°C at an irradiance of 300 μE $m^{-2}s^{-1}$ for 14 days, at the end of which the first leaves were excised and cut into segments 1.5 cm in length. Ten segments per petri dish were floated on 10 ml of -15 bars PEG solution or water at the desired irradiance or in darkness for 24 hours. The harvested tissue was frozen in liquid nitrogen, freeze dried and weighed and then analysed for proline, total ethanol soluble carbohydrates and chlorophyll content as described in Materials and Methods Section 2.8.

### 3.2.2 Results

#### 3.2.2.1 Proline content

Segments floated on -15 bars PEG accumulated significantly more proline than those floated on water (Fig 3.1 and Table 3.1Bi). Exposing segments to a 75 μE $m^{-2}s^{-1}$ irradiance resulted in the accumulation of significantly more proline than incubation in darkness (ca. 6 mg $g^{-1}$ dry wt) but incubation at higher irradiances caused no significant further increase. Segments floated on water for 24 hours at various irradiances also showed a significantly higher accumulation of proline than segments floated in the dark, accumulating to similar amounts at the three different irradiances (Fig 3.1 and Table 3.1 Bii).

#### 3.2.2.2 Carbohydrate content

Exposure to light resulted in an increase in carbohydrate content in both turgid and stressed tissue when compared to darkness. Stress also caused a higher carbohydrate concentration in the light though

Fig 3.1

Effect of different irradiances on proline accumulation in barley leaf segments floated on PEG solution or water.

Barley plants were grown in sand for 14 days in a 16 hour photoperiod and floated on PEG (-15 bar) or water in the dark or light of different irradiances for 24 hours.

Each point is the mean of 4 replicates.

■——■ Stressed

□——□ Turgid

Table 3.1

A. F values of results in Fig 3.1

| Source of variation | Variance ratio (VR) |
|---|---|
| Stress | 132.47** |
| Irradiance | 6.76** |
| Stress x Irradiance | 2.86 |

B. Table of means of results in Fig 3.1

(i) Stress

| | |
|---|---|
| Turgid | 3.66 |
| Stress | 14.71 |
| LSD | 1.98 |

(ii) Irradiance ( $\mu E\ m^{-2}s^{-1}$)

| | |
|---|---|
| 300 | 10.90 |
| 150 | 10.50 |
| 75 | 9.83 |
| Dark | 5.50 |
| LSD | 2.81 |

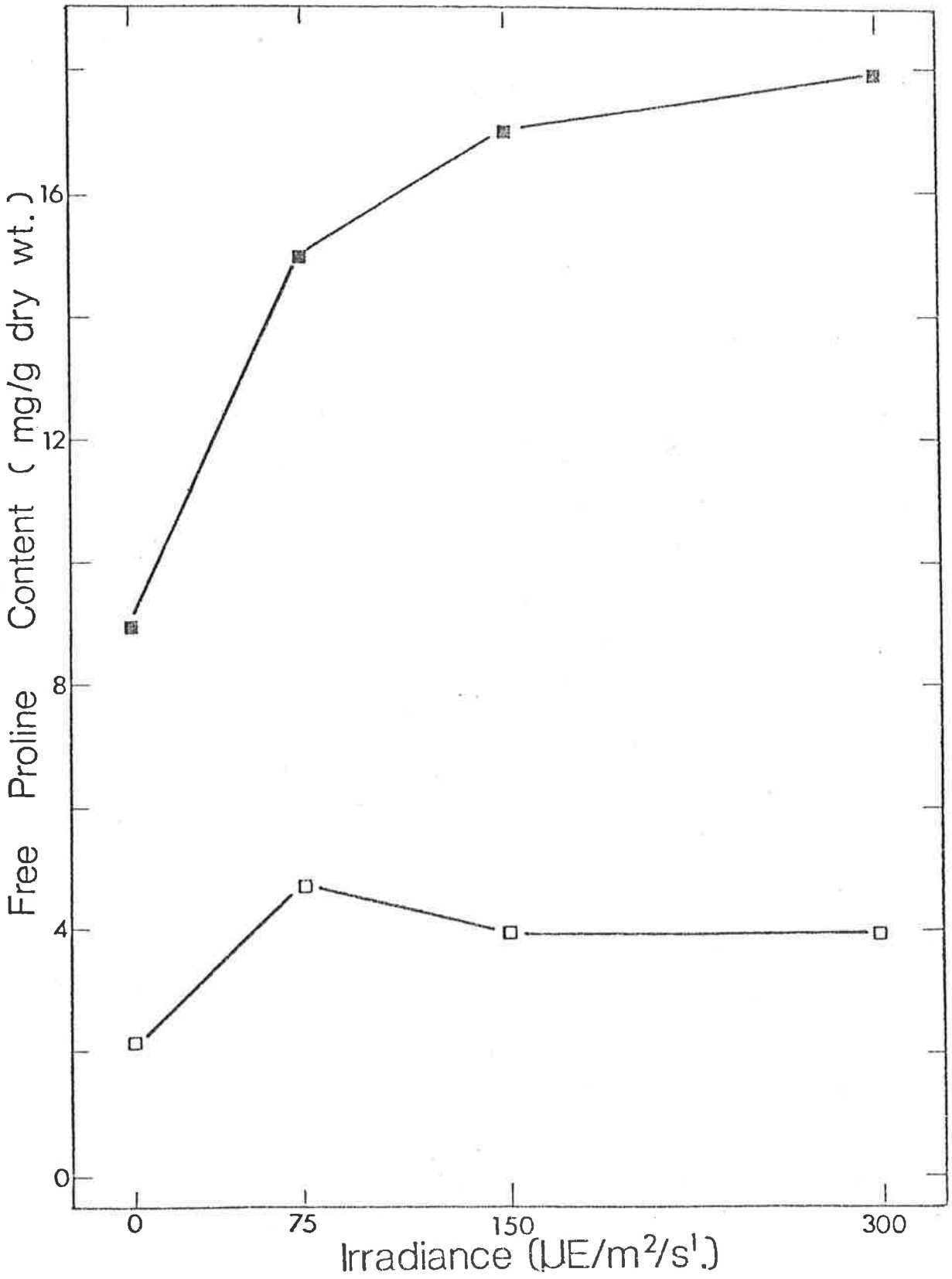

Free Proline Content ( mg/g dry wt. )
16
12
8
4
0
0
75
150
300
Irradiance (μE/m²/s¹.)

Table 3.2

Total soluble carbohydrate content in stressed tissue at different irradiances.

A. Total soluble carbohydrate content (mg $g^{-1}$ DW)

| Irradiance | Turgid | Stress | Mean | |
|---|---|---|---|---|
| 300 µE | 84.3 | 111.8 | 98.1 | LSD |
| 150 µE | 116.6 | 123.4 | 120.0 | 23.5 |
| 75 µE | 97.6 | 126.0 | 111.8 | |
| Dark | 72.4 | 83.9 | 78.2 | |
| Mean | 92.7 | 111.3 | 102.0 | |
| LSD | 16.6 | | | |

B. F Table for results in Table 3.2A

| Source of variation | VR |
|---|---|
| Treatment | 5.32* |
| Irradance | 5.17** |
| Treatment x Irradiance | 0.47 |

not in the dark (Table 3.2A and B); but there was no interaction between the effects of light and stress. From the table of means, it is clear that generally there is a difference between dark and light treatment.

3.2.2.3 Chlorophyll content

Unlike both proline and carbohydrate content, the total chlorophyll content decreased in stressed tissue in comparison with turgid tissue (Table 3.3A). It is apparent from the table that the chlorophyll content of leaf segments incubated at 300 μE $m^{-2}s^{-1}$ was significantly lower than that in any of the other treatments.

## 3.3 Low irradiance, length of period of darkness during $P_1$ and proline accumulation

In the previous experiment there was no significant difference in proline accumulation between segments stressed at 75 and those at 150 or 300 μE $m^{-2}s^{-1}$, in tissue previously exposed to a 16 hour photoperiod. It was conceivable that this lack of response to increasing irradiance was due to the exposure to light during the previous photoperiod (i.e. a carry over effect) and this experiment was designed therefore, both to measure accumulation at irradiances as low as 15 μE $m^{-2}s^{-1}$ and to examine the effect of conditions during period 1 ($P_1$) on proline accumulation.

### 3.3.1 Methods

Plants were grown till they were 11 days old as described in Section 1.2.1. On the 12th day, some plants were transferred to continuous darkness (4 hours after the beginning of the photoperiod)

Table 3.3

Irradiance and stress effect on chlorophyll content

A. Total chlorophyll (mg $g^{-1}$ DW)

| Irradiance | Turgid | Stress | Mean | |
|---|---|---|---|---|
| 300 µE | 10.58 | 8.03 | 9.31 | LSD |
| 150 µE | 12.50 | 13.05 | 12.78 | 1.64 |
| 75 µE | 13.22 | 11.80 | 12.51 | |
| Dark | 14.65 | 12.55 | 13.60 | |
| Mean | 12.74 | 11.36 | 12.05 | |
| LSD 1.16 | | | | |

B. F values for results in Table 3.3A

| Source of variation | VR |
|---|---|
| Treatment | 6.07* |
| Irradiance | 11.28** |
| Treatmnt. x Irradiance | 1.49 |

while the remaining plants were transferred to continuous light or darkness on the 13th day. On the 15th day, the first leaves of all the plants were excised, segmented and floated on -15 bar PEG in the dark or at different irradiances (15, 70 and 265 μE $m^{-2}s^{-1}$) (Fig 3.2, experimental design for experiment 3.3). The segments were then rinsed, dried, frozen in liquid nitrogen and freeze dried. The freeze dried samples were used for the estimation of proline content (Materials and Methods, Section 2.8). Analysis of variance was performed to test for significance.

### 3.3.2 Results

An increase in irradiance increased proline accumulation. This stimulation was evident even at an irradiance of 15 μE $m^{-2}s^{-1}$ (Fig 3.3). The successive increases in proline content between darkness 15 and 70 μE $m^{-2}s^{-1}$ were significant, but irradiance greater than 70 μE $m^{-2}s^{-1}$ did not significantly further increase the proline level (Table 3.4 Bii). Prestress conditions ($P_1$) also significantly influenced the level of proline. Tissue exposed to 48 hours continuous light during $P_1$ accumulated the greatest amount of proline while tissue in darkness during $P_1$ accumulated significantly lower amounts of proline (Table 3.4 Bi), being the least in tissue exposed to 72 hours of continuous darkness during this period. The prestress exposure to light and the irradiance during stress did not interact in their effects, however, and appearedto act in a strictly additive manner.

## 3.4 Irradiance and proline accumulation

The previous experiment demonstrated a considerable stimulation of proline accumulation at the comparatively low irradiance of 15 μE $m^{-2}s^{-1}$. It may well be that stimulation occurs at all irradiances greater than

Fig 3.2

EXPERIMENTAL DESIGN FOR EXPERIMENT 3.3

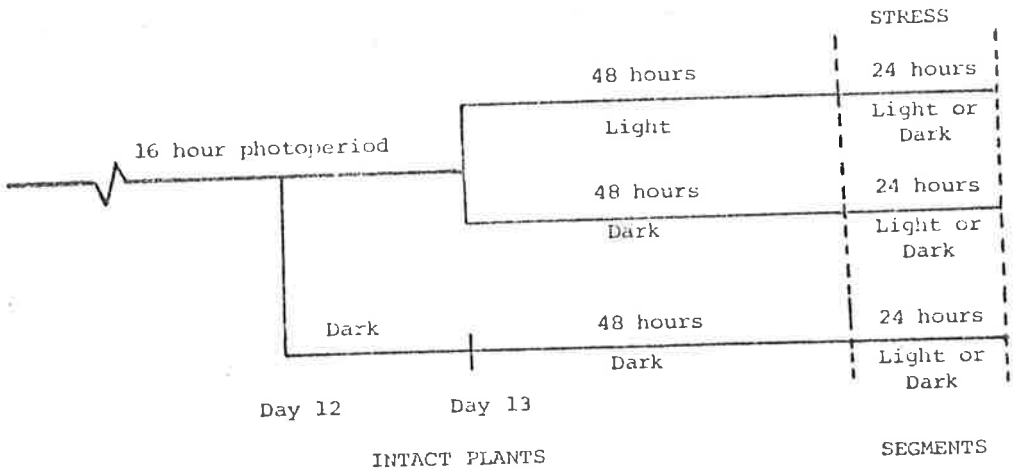


Fig 3.3

Light/dark treatment prior to stress and its effect on proline accumulation at different irradiances in tissue floated on PEG (-15 bar).

Plants were grown for 11 or 12 days in a 16 hour photoperiod and were then transferred to darkness for 2 or 3 days or left in 48 hours continuous light. The first leaves were segmented floated in darkness or light at 15, 70 or 210 $\mu E\ m^{-2}s^{-1}$ for 24 hours. Each point is the mean of 4 replicates.

Prestress treatment ($P_1$)

○——○ 2 days light

□——□ 2 days darkness

△——△ 3 days darkness

Table 3.4

A. F table for results in Fig 3.3

| Source of variation | VR |
|---|---|
| $P_1$ Treatment | 46.42** |
| Irradiation | 46.13** |
| Treatmnt x Irrad | 0.56 |

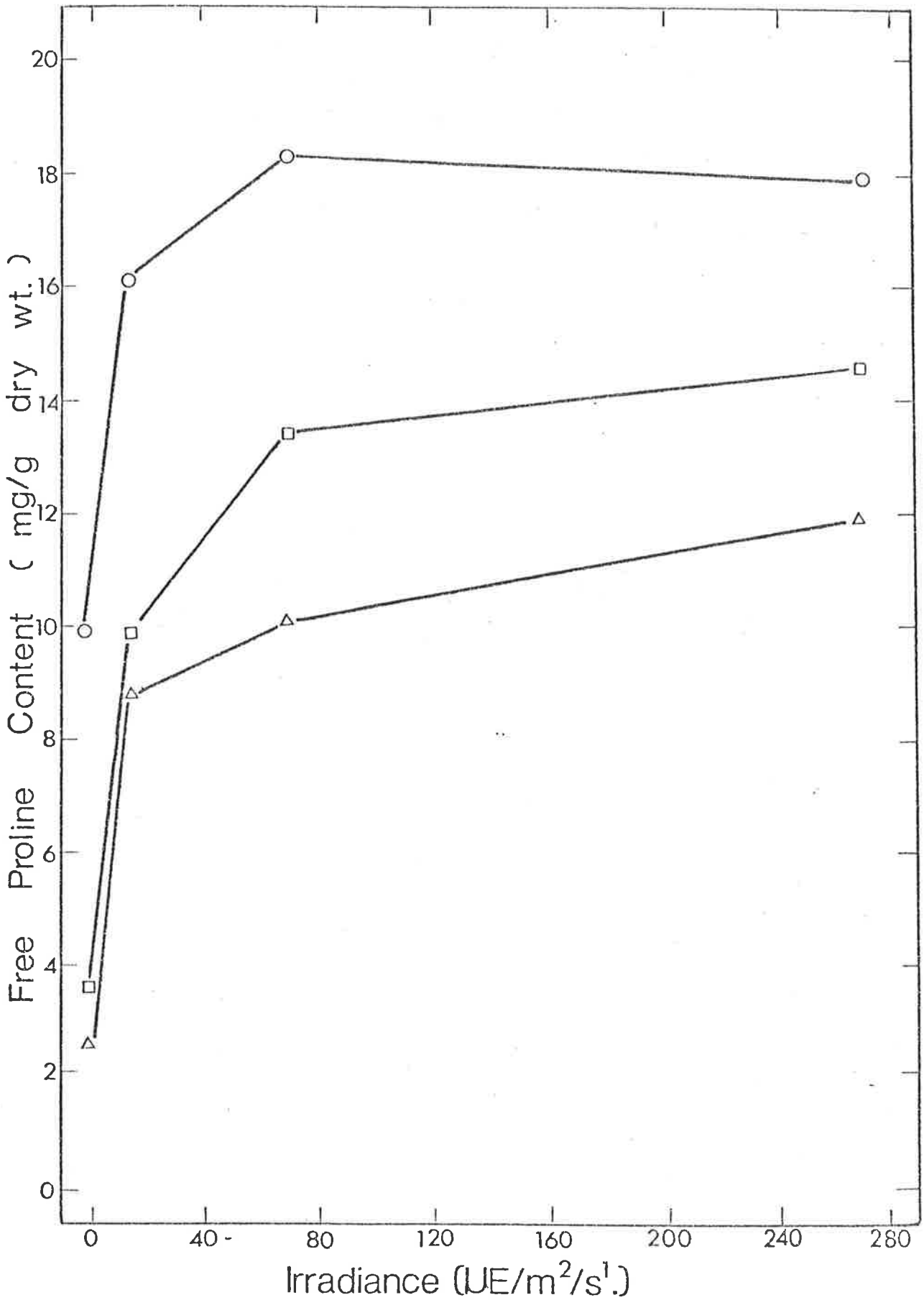
B. Table of Means for results in Fig 3.3

(i) $P_1$ Treatment

| | |
|---|---|
| 2 days light | 15.59 |
| 2 days dark | 10.40 |
| 3 days dark | 8.37 |
| LSD | 1.60 |

(ii) Irradiance

| | |
|---|---|
| Dark | 5.35 |
| 15 | 11.62 |
| 70 | 13.99 |
| 270 | 14.85 |
| LSD | 1.80 |


Free Proline Content ( mg/g dry wt. )
20
18
16
14
12
10
8
6
4
2
0
0
40
80
120
160
200
240
280
Irradiance (μE/m²/s¹.)

the light compensation point ( 6 μE $m^{-2}s^{-1}$), the determination of which is detailed in Appendix 1. This experiment was therefore designed to study this response to both lower irradiances (around light compensation point and below) and to much higher irradiances; one objective being to determine the threshold irradiance (if any) below which accumulation was not stimulated.

### 3.4.1 Methods

Plants were grown for 12 days in a 16 hour photoperiod at 20°C and were then transferred to 48 hours continuous light or darkness, after which the first leaves were excised, segmented and floated on -15 bar PEG solution for 24 hours at different irradiances - high (20, 70, 140, 260, 520 and 880 μE $m^{-2}s^{-1}$), and low (0.1, 1.0 and 10 μE $m^{-2}s^{-1}$) or in the dark. Since it was not feasible to provide the various irradiance in one experiment, two experiments were conducted, one covering irradiance of 20 and above and the other at irradiances below 10 μE $m^{-2}s^{-1}$. The light source was a bank of flourescent tubes supplemented with eight incandescent bulbs except for the 520 and 880 μE $m^{-2}s^{-1}$ irradiances where a 'Metalarc' mercury lamp served as the light source. The samples were harvested and analysed for proline after freeze drying and weighing as detailed in Section 2.8 of Materials and Methods.

### 3.4.2 Results

Expt 1. Effect of irradiance between 20 and 880 μE $m^{-2}s^{-1}$ on proline accumulation

Proline accumulation was again stimulated by exposure to even the comparatively low irradiance (20 μE $m^{-2}s^{-1}$) compared with dark

treatment in either $P_1$ treatments. This response to light appeared to be saturated at this irradiance (20 μE $m^{-2}s^{-1}$) in tissue previously exposed to light (in $P_1$) and at 70 μE $m^{-2}s^{-1}$ in those segments which had been in darkness in $P_1$. There was no further increase in proline content at irradiances upto 880 μE $m^{-2}s^{-1}$ in either treatment and there may even have been a slight decrease at the highest irradiance in D-L treatment (Fig 3.4).

Expt 2. Very low irradiance and proline accumulation.

An irradiance of 0.1 μE $m^{-2}s^{-1}$ did not stimulate proline accumulation above that in darkness (Fig 3.4 and Table 3.5 Bii) but an increase in irradiance to 1 μE $m^{-2}s^{-1}$ resulted in a significant enhancement of proline accumulation in both the L-L and D-L treatments, and this increased still further at 10 μE $m^{-2}s^{-1}$ (Table 3.5 Bii).

## 3.5 Rate of proline accumulation

The amount of proline accumulated in stressed plants in 24 hours did not change significantly with an increase in irradiance above 20 or above 70 μE $m^{-2}s^{-1}$ in the L-L and D-L treatments respectively. However, the rate of photosynthesis in turgid plants is reported to increase linearly over this range (Leopold and Kriedemann 1975). This discrepancy between proline accumulation and photosynthesis may be more apparent than real, since there may be a maximum amount of proline that can be stored, and the different irradiances, over 24 hours, may all have produced the maximum. Alternatively, of course, the irradiance at which this reaction is saturated may be very low and increasing the irradiance beyond this saturation point, does not induce higher accumulation.

Fig 3.4

Effect of irradiances (high and very low) on proline accumulation in stressed barley leaf segments.

Twelve day old barley plants were transferred to 48 hours continuous light or dark treatment. First leaves were then segmented and floated on -15 bar PEG for 24 hours at different irradiances. Each point is the mean of 4 replicates.

N.B.: Results of Experiment 1 and 2 are represented in the same figure. Irradiance values are plotted on a log scale.

$P_1$ treatment

O——O Light - Light

□——□ Dark - Light

● Light - Dark

■ Dark - Dark

Table 3.5

A. F table for results in Fig 3.4

| Source of variation | (Expt 1) VR | (Expt 2) VR |
|---|---|---|
| $P_1$ | 106.34** | 90.70** |
| Irradiance | 18.49** | 26.77** |
| $P_1$ x Irradiance | 2.45* | 0.32 |

B. Table of means for Expt 2

(i) $P_1$

| | |
|---|---|
| Light | 11.21 |
| Dark | 5.45 |
| LSD | 1.25 |

(ii) Irradiance

| | |
|---|---|
| Dark | 5.92 |
| 0.1 | 5.49 |
| 1.0 | 10.08 |
| 10.0 | 11.87 |
| LSD | 1.76 |

Free Proline Content (mg/g dry wt.)
0
4
8
12
16
20
24
Log Irradiance (μE m⁻²s⁻¹)
0·1
1
10
20
70
140
260
520
880

To distinguish between these hypotheses, the following experiment was conducted.

### 3.5.1 Methods

Barley plants were grown until they were 12 days old as described in Section 3.3.1. The first leaves were then excised, segmented and floated on -15 bar PEG solution in the dark or light of different irradiances (2, 15, 75 and 240 μE $m^{-2}s^{-1}$). Single samples of segments at each irradiance were harvested at two hour intervals over a 28 hour period and preserved for the estimation of proline as described in Materials and Methods, Section 2.8. A polynomial equation was fitted to the data to relate proline content to time at each irradiance and these were tested to ascertain whether they differed significantly using the F test.

### 3.5.2 Results

The stimulation of proline accumulation by irradiances of 15 μE $m^{-2}s^{-1}$ or higher was apparent within 4 to 8 hours in both L-L and D-L treatments (Fig 3.5A and B) - also note the difference in the ordinate scale). With the L-L curves (Fig 3.5) it is obvious that irradiances of 15, 75 and 240 μE $m^{-2}s^{-1}$ show very similar increases with time. Although statistical analysis showed the positions of the various points and the slopes to be significantly different when all the irradiances (including dark) were compared, the figure clearly shows that the significant difference is mainly between very low irradiances (or dark) and the higher irradiances. Similarly in D-L samples, the difference between 75 and 240 μE $m^{-2}s^{-1}$ is not significant while

Fig 3.5

Rate of proline accumulation at different irradiances (fitted)

Barley plants were grown at 20°C in a 16 hour photoperiod in sand for 12 days and then transferred to 48 hours continuous light or continuous darkness. The first leaves were segmented and floated on -15 bar PEG at different irradiances.

○ 240 μE $m^{-2}s^{-1}$

△ 75 μE $m^{-2}s^{-1}$

□ 15 μE $m^{-2}s^{-1}$

▽ 2 μE $m^{-2}s^{-1}$

● Dark

A. Prestress treatment in light. B. Prestress treatment in darkness

Table 3.6

A. F values for results in Fig 3.5A

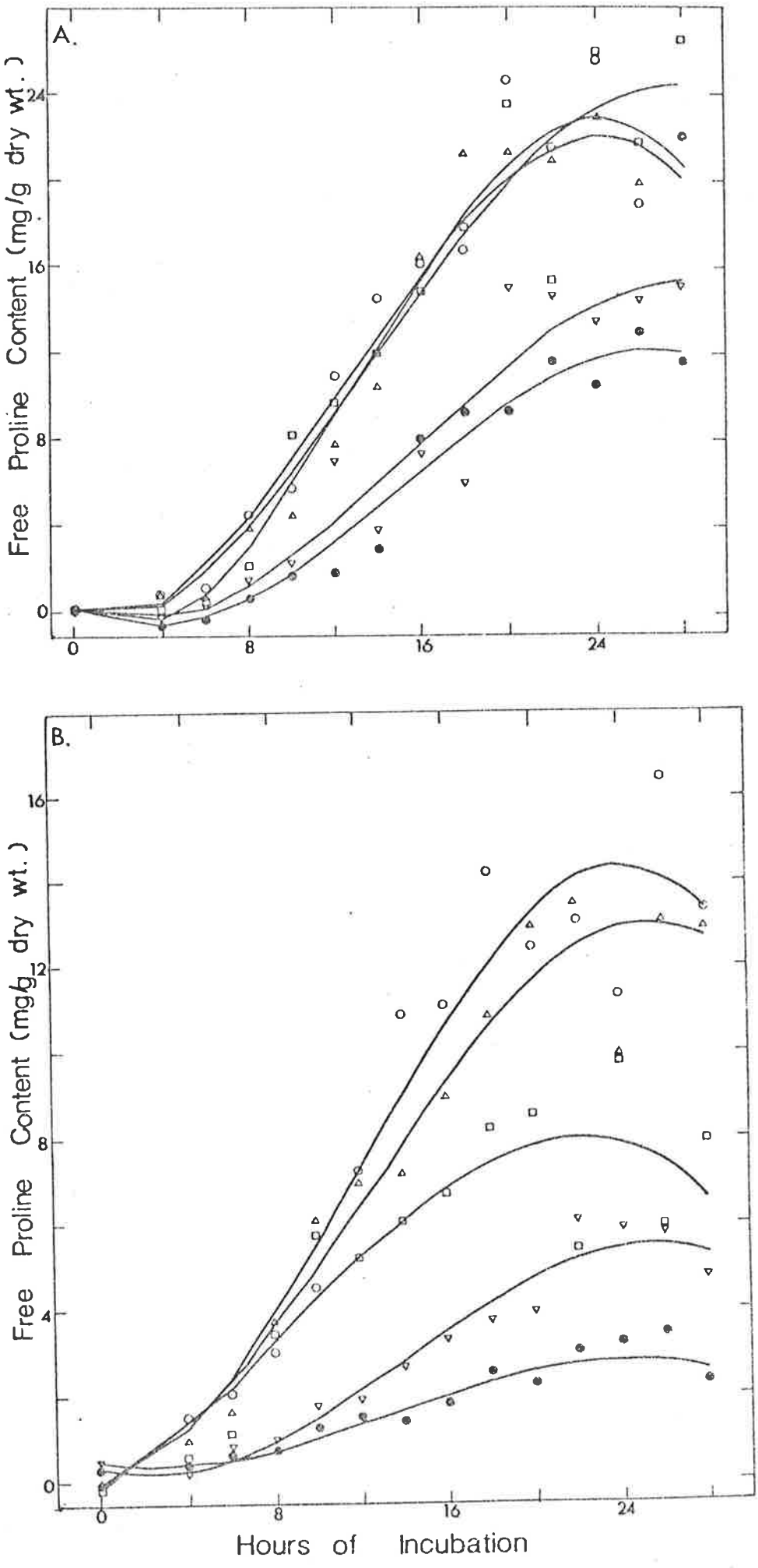
| Source of variation | VR |
|---|---|
| Positions difference | 26.45* |
| Regression (slope) diff. | 3.46* |

B. F values for results in Fig 3.5 B

| Source of variation | VR |
|---|---|
| Position difference | 74.90** |
| Regression difference | 8.70* |

Significance * $P \leq 0.05$

** $P \leq 0.01$


A.
B.
Free Proline Content (mg/g dry wt.)
Hours of Incubation
0
8
16
24
4
12

when compared with those below 75 μE $m^{-2}s^{-1}$, the curves are different. Thus, the hypothesis that similarity in total proline accumulated at these various irradiances could be due to sampling after 24 hours by which time proline accumulation may have reached a steady state level in all irradiances, is eliminated. Thus, in L-L it appears that the reaction is saturated at 15 μE $m^{-2}s^{-1}$. In D-L treatment, however, light at 15 μE $m^{-2}s^{-1}$ induced significantly less proline accumulation than 75 μE $m^{-2}s^{-1}$ or higher irradiance, after any length of time of imposition of stress.

## 3.6 Discussion

Although light enhancement of stress stimulated proline accumulation was saturated at different irradiances in tissue exposed to light or darkness during the prestress period, there was a significant stimulation at even 1 μE $m^{-2}s^{-1}$ in both treatments (Table 3.5 B ii). Thus the threshold irradiance for stimulation of proline accumulation is extremely low (below the light compensation point). Subsequent experiments have also shown clearly that the apparent saturation of the response at such low irradiance is not an artefact of the sampling time but is real (Fig 3.5A and B), as accumulation rates in all the irradiances showing similar final levels of proline were also similar.

Since there is some stimulation of proline accumulation even below the light compensation point (i.e. when there is no net increase in dry weight) it would appear that the influence of light is through

the provision of assimilatory power (ATP and NAD(P)H). Of course it is possible that although net carbohydrates may not increase, the newly fixed $CO_2$ may enter directly into proline. The saturation level of this light response is low especially in tissue high in carbohydrates. Thus, the carbohydrates present in the system seemingly play a more indirect function of both regulating the extent of proline accumulation as well as the irradiance level necessary to saturate the response. Similar correlations between the carbohydrate status of the tissue and proline accumulation have been reported in stressed turnip leaves (Stewart *et al*, 1966).

SECTION I WATER STRESS

4. Qualitative effect of light

# 4. Qualitative effect of light

## 4.1 Introduction

Enhanced accumulation of proline during stress appears to be responsive to irradiation as low as 10 μE $m^{-2}s^{-1}$. Conceivably such a response may be attributable to either photosynthesis or to a phytochrome controlled response.

In this chapter, the hypothesis that light induced enhanced accumulation of proline through a phytochrome reaction was tested. The phytochrome activation may be due either to a low or to a high energy response. If the response is mediated through enhanced enzyme synthesis, then it may show induction/reversion occurring in a short period of time or may require the continued presence of light (Schopfer, 1972). However, the earlier chapter showed that the response was not induced by exposure to a short duration of light. Therefore, the alternative possibility that the enzyme requires continued presence of far red (FR) light was studied. In addition to providing continuous far red light, the effect of continuous red light was also studied.

### 4.1.1 Methods

Barley plants were grown in sand at 20°C in a 16 hour photoperiod until they were 12 days old. They were then exposed to 48 hours continuous light or continuous darkness, commencing 4 hours after the beginning of the photoperiod, at the end of which, the first leaves were excised and cut into 1.5 cm segments and floated

on 10 ml of -15 bar PEG solution in petri dishes for 24 hours. The petri dishes were placed in the dark, white (incandescent and fluorescent lights in the growth cabinets with a spectral energy distribution as shown in Fig 4.1), red (red-fluorescent tubes - wavelength of 660 nm), or far-red (FR filter of black plexiglass, wavelength of 750 nm) light of equal energy. [A spectral radiometer (ISCO) was used to measure the wavelengths used.] The distance between the light source and the tissue was adjusted so that the total energy incident in the different treatments was similar. A LI-COR quantum sensor was used to measure the irradiance under red light which was adjusted to 10 $\mu E\ m^{-2}s^{-1}$. The total energy in the FR region was measured with a Kipp radiometer photovoltmeter equipped with a R/FR filter; and the distance adjusted so that the total energy under R and FR using the respective filters was similar. The segments were left under the various light treatments or darkness for 24 hours. They were then harvested, rinsed and dried, frozen in liquid nitrogen and freeze dried. The tissue proline content was estimated after the dry weights had been taken. The data was analysed statistically using a conventional analysis of variance test.

### 4.1.2 Results

Continuous red light induced greater accumulation of proline than the dark treatment in both L-L (non-starved) and D-L (starved) treated tissue, but this stimulation was not as great as that caused by white light (Fig 4.2). Continuous far-red light showed no significant enhancement of proline accumulation above that in the dark treatment (Table 4.1C). Statistical

Fig 4.1

Spectral energy distribution in the growth cabinet as measured with an 'Isco' spectroradiometer.

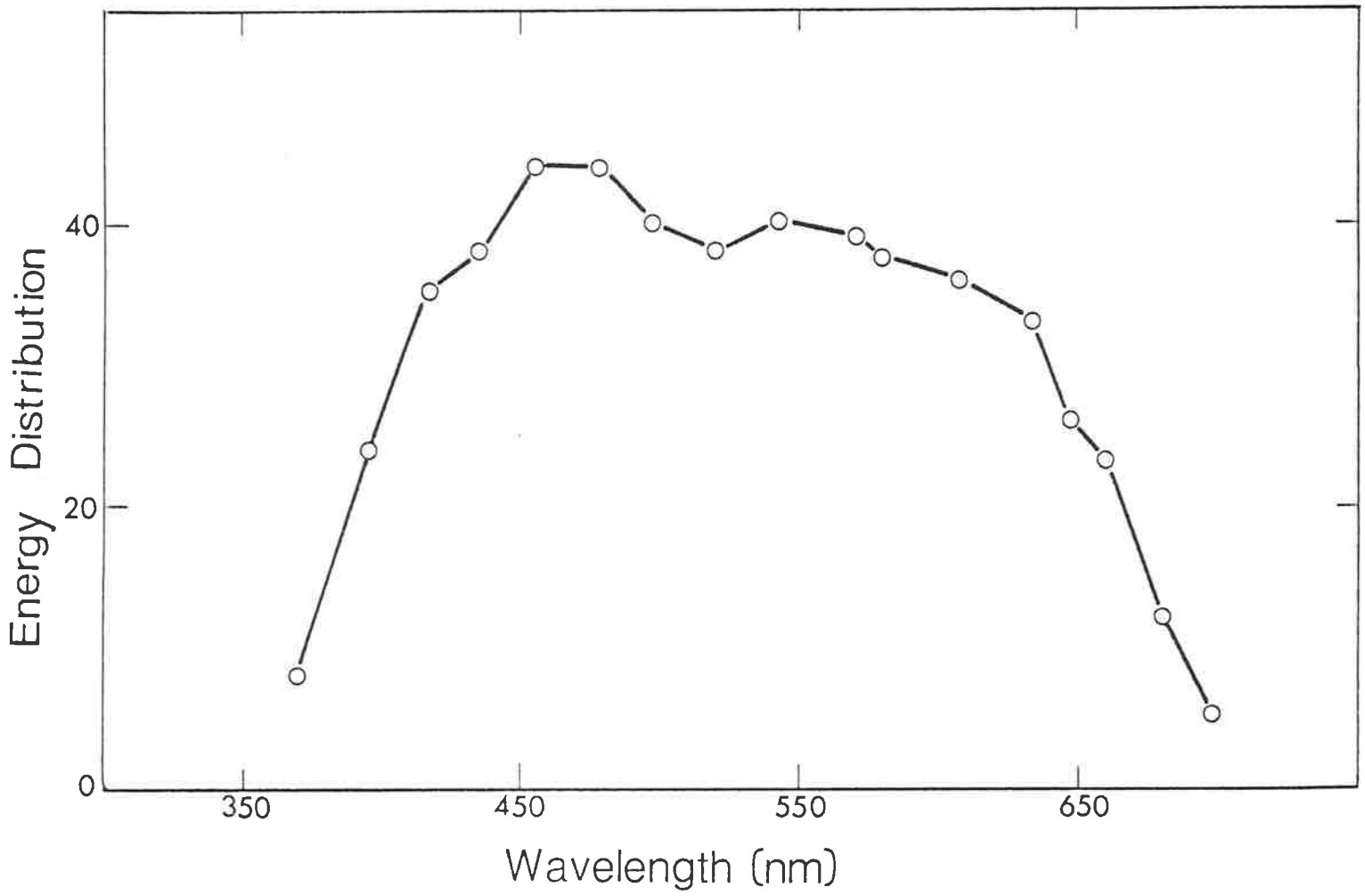

Energy Distribution
40
20
0
350
450
550
650
Wavelength (nm)

Fig 4.2

Effect of continuous red and far red light on proline accumulation

Twelve day old plants grown in a 16 hour photoperiod at 20°C were transferred to 48 hours continuous light or continuous darkness. The first leaves were then cut into segments and floated on PEG (-15 bars) in red, or far red light, whitelight or in the dark for 24 hours.

Each value is the mean of 4 replicates.

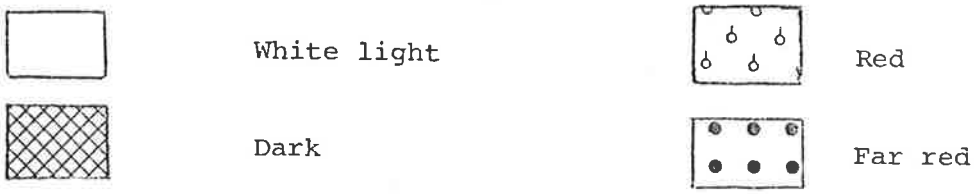



Table 4.1

A. F table for results in Fig 4.2

| | VR |
|---|---|
| Light | 145.710** |
| Wavelength | 52.314** |
| Light x wavelength | 1.600 |

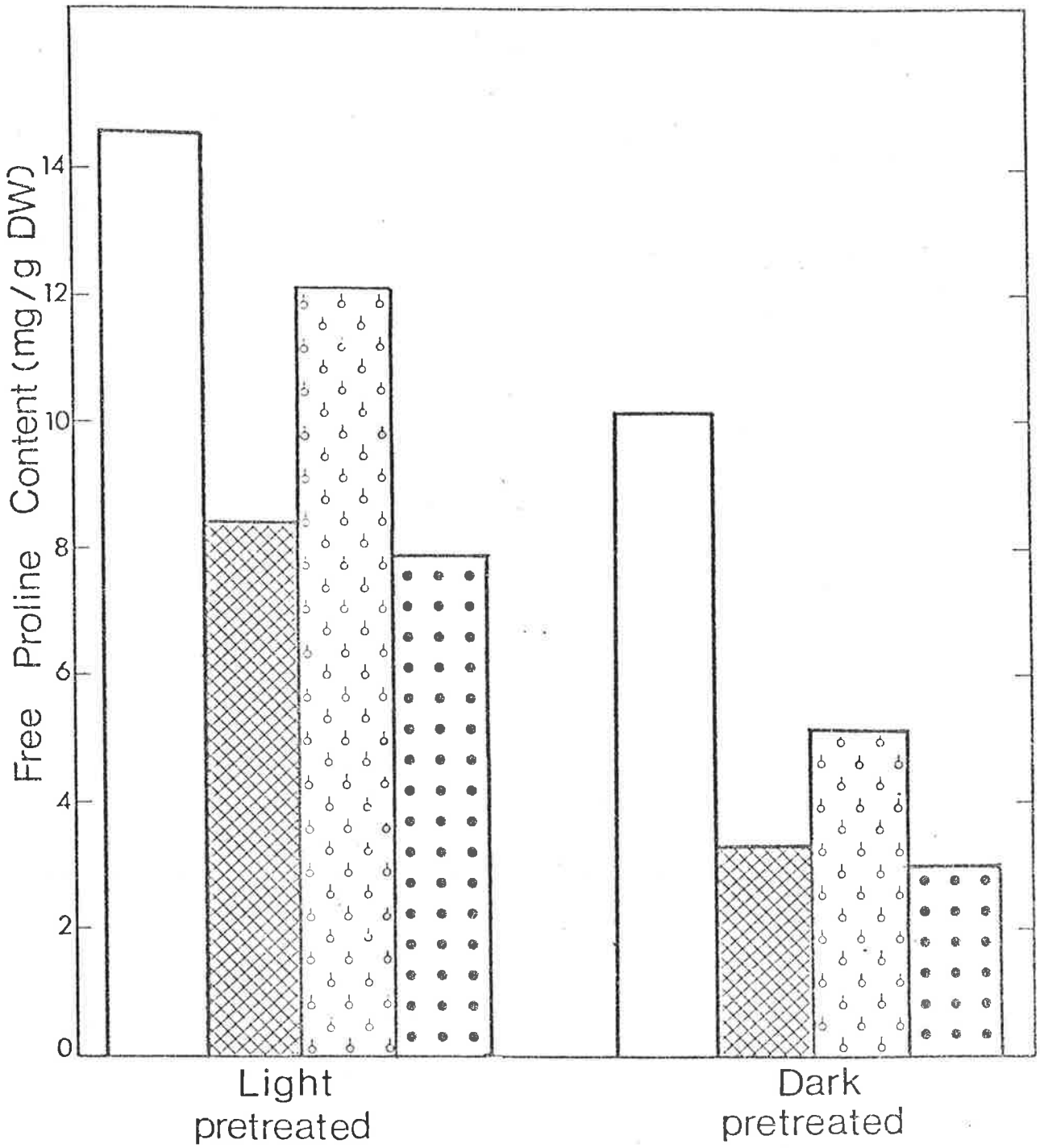
Table of means for results in Fig 4.2

B. Light effect

| | |
|---|---|
| Light | 10.74 |
| Dark | 5.43 |
| LSD | 0.91 |

C. Wavelength effect

| | |
|---|---|
| White light | 12.37 |
| Dark | 5.88 |
| Red | 8.60 |
| Far red | 5.47 |
| LSD | 1.28 |


Free Proline Content (mg / g DW)
0
2
4
6
8
10
12
14
Light pretreated
Dark pretreated

analysis showed the light effect (red, FR, white or dark) to be highly significant, and so too the difference between D-L and L-L treatment, while the interaction of the two was not significant (Table 4.1A). All treatments showed greater accumulation of proline in L-L (non-starved) treatment than D-L (starved) treatment which is similar to what has been observed in the earlier experiments (Table 4.1B).

The results clearly demonstrate that continued FR light is unable to enhance proline accumulation.

## 4.2 Relative effectiveness of different wavelengths

Since the response is not a short term response nor one induced by continuous FR (750 nm) light, but is enhanced by red light, the reaction may be a High Irradiance Response showing peak activity at the violet-blue region or at 700 nm in the FR region. Also, information on the effectiveness of various wavelengths in the visible region of the spectrum on proline accumulation is lacking. This experiment was designed to examine the enhancement of proline accumulation at different wavelengths.

### 4.2.1 Methods

Twelve day old plants growing in sand at 20°C in a 16 hour photoperiod were transferred to 48 hour continuous light or continuous darkness, 4 hours after the commencement of the photoperiod. The first leaves were then harvested, cut into 1.5 cm segments and floated on -15 bar PEG solution in continuous light of different wavelengths or continued darkness or white light for

24 hours. This was done to obtain an action spectrum and to study the relative response of the tissue to the same light flux at 20° ± 0.5°C with respect to proline accumulation. Light of different wavelengths was obtained with the Ceres Spectrograph (CSIRO Phytotron, Canberra). The light source for this was a 2500-W air cooled xenon arc lamp, housed in a Zeiss xerosol III projection unit. The beam emerging from the aperture slit, which was about 2.5 cm, was projected by a front-aluminized concave mirror onto a Bausch and Lomb diffraction grating, with a ruled area of 206 by 206 mm and 1200 grooves per millimeter blazed at 410 nm, mounted face down on the ceiling. From the grating, the beam was projected to the floor of a dark room below. Linear dispersion at the floor level was approximately 3.0 mm $nm^{-1}$. The flux density measured with a LI-COR quantum sensor was greater than 25 $\mu E\ m^{-2}s^{-1}$ between 420 and 660 nm and fell sharply to 10 $\mu E\ m^{-2}s^{-1}$ and at 360 and 705 nm (Fig 4.3). In order to obtain a uniform irradiance of $\simeq$12 $\mu E\ m^{-2}s^{-1}$ at almost all wavelengths, neutral nylon mesh were placed over the petri dishes. The petri dishes which were used in this experiment were approximately 4.5 cm (diameter) and were placed as close together as possible, so that each petri dish spanned approximately 15 nm of the beam. With each set, one petri dish was placed in a growth cabinet lit with fluorescent and incandescent light, and the irradiance adjusted to 12 $\mu E\ m^{-2}s^{-1}$, and another petri dish was placed in continuous darkness.

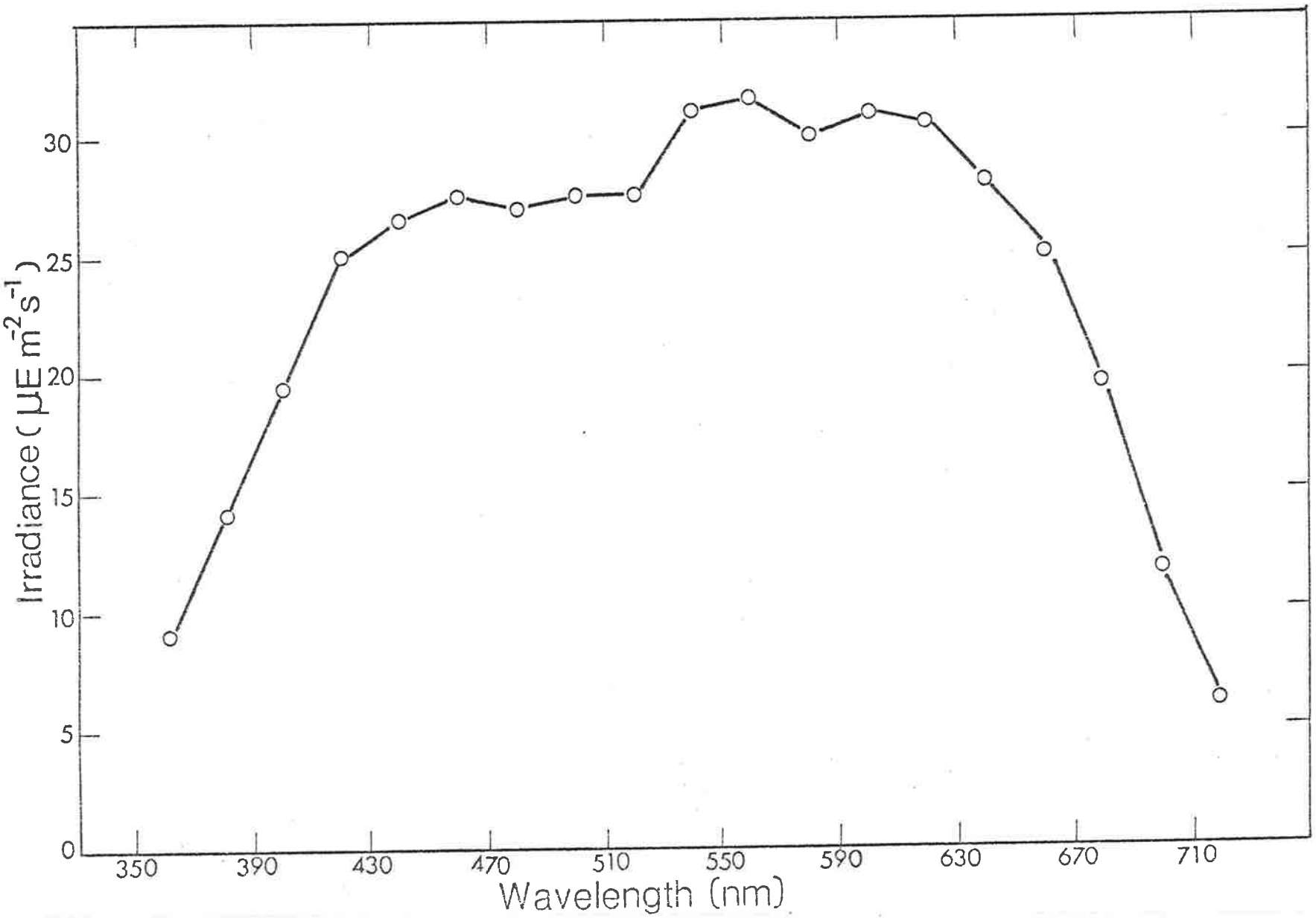
A relative action spectrum for proline accumulation was plotted, which was calculated in the following way :

$$\% \text{ change at } nm_x = \frac{\text{Proline at } nm_x - \text{Proline in dark}}{\text{Proline in light} - \text{Proline in dark}} \times 100$$

where proline is expressed in mg $g^{-1}$ dry wt.

Fig 4.3

Flux density at different wavelengths in the spectrograph. The various wavelengths were established using interference filters.


Irradiance ( μE m-2 s-1 )
0
5
10
15
20
25
30
350
390
430
470
510
550
590
630
670
710
Wavelength (nm)

Plants were sown a day apart, so that replicates, although harvested on consecutive days, were of the same age. The treatment received by plants prior to harvesting consisted of 12 days growth in a 16 hour photoperiod at an irradiance of 300 μE $m^{-2}s^{-1}$ and temperatures of 20°C, followed by 48 hours continuous light or continuous darkness. The first leaves were then harvested, cut into 1.5 cm segments and floated on PEG solution or water in petri dishes, which were then placed under the sepctrograph at an irradiance of 12 μE $m^{-2}s^{-1}$ or transferred to continuous light or continuous darkness. After 24 hours, the segemnts were harvested, rinsed in water, dried between filter papers and frozen in liquid nitrogen. When all the samples had been harvested, they were freeze dried and analysed for free proline content, after recording their dry weights. In addition, one set of petri dishes were exposed to 25 μE $m^{-2}s^{-1}$, but the comparisons are only relevant for wavelengths between 420 and 660 nm which were all adjusted to 25 μE $m^{-2}s^{-1}$.

It was only possible to use green tissue in these experiments as proline does not accumulate in stressed dark grown seedlings of barley (Singh et al, 1973b).

### 4.2.2 Results

At 12 μE $m^{-2}s^{-1}$, the continued presence of white light was most effective in enhancing proline accumulation in both L-L (non-starved) and D-L (starved) stressed tissue (Fig 4.4 A,B) none of the wavelengths showing 100% effectiveness. If relative change >70% is considered, however, it is clear that in L-L segments the maximum change occurs in the red region (600-690 nm) with two narrow peaks in the blue and violet regions. In D-L

Fig 4.4

Proline accumulation in tissue exposed to various wavelengths, relative to accumulation in white light over darkness.

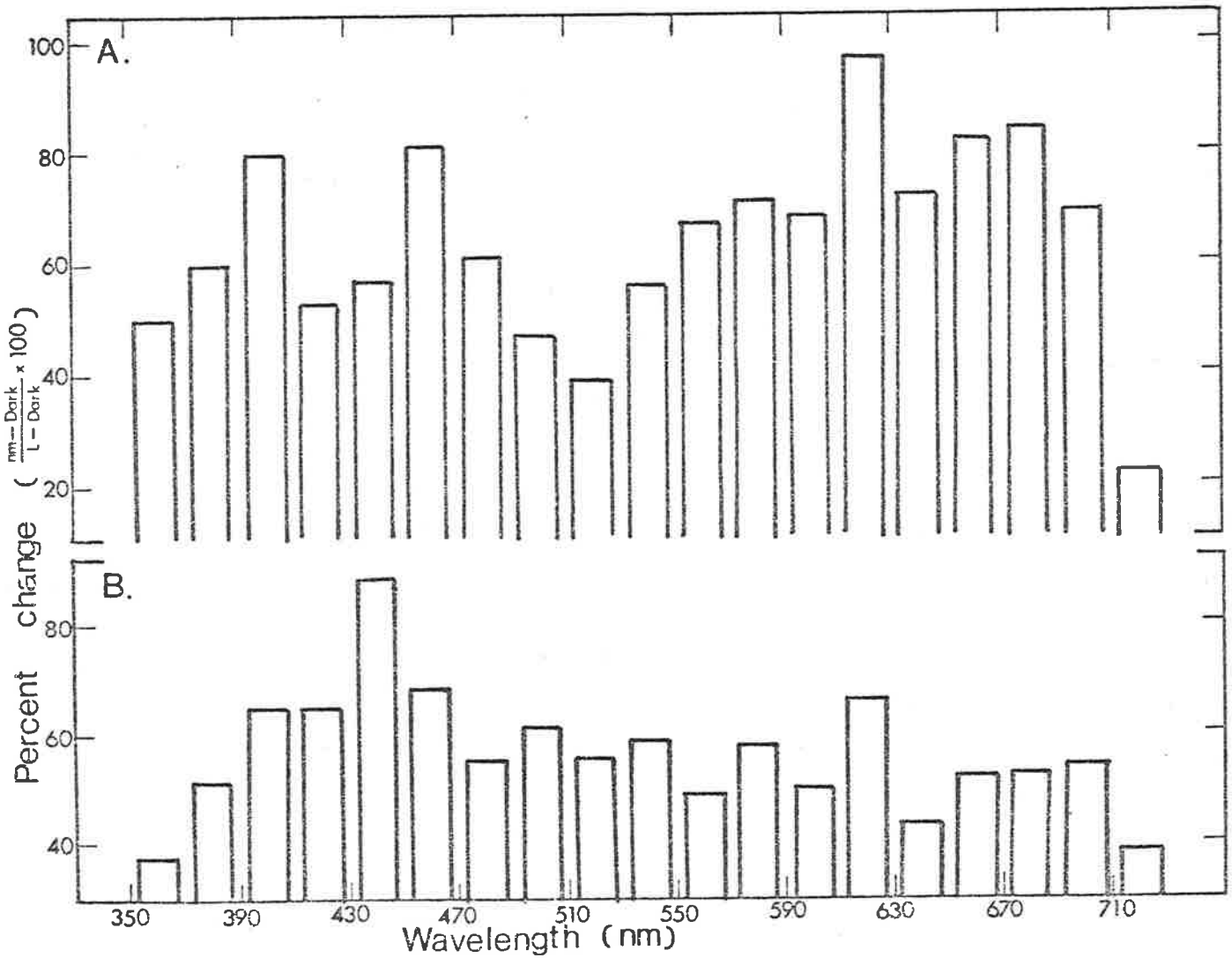
A. Tissue exposed to 48 hours continuous light prior to stressing L-L (non-starved). Each value is the mean of 2 replicates.

B. Tissue exposed to 48 hours continuous darkness prior to stressing D-L (starved). Each value is the mean of 3 replicates.

Table 4.2

Proline content (mg $g^{-1}$ dry wt) of segments incubated in dark or light

| Treatment | Dark | 12 $\mu Em^{-2}s^{-1}$ | 25 $\mu Em^{-2}s^{-1}$ |
|---|---|---|---|
| Non-starved stressed | 8.90 | 17.47 | 19.96 |
| Starved stressed | 1.97 | 10.82 | 12.52 |
| Turgid non-starved | 1.70 | 3.21 | - |
| Turgid starved | 0.92 | 0.50 | - |


A.
B.
100
80
60
40
20
80
60
40
Percent change ( nm − Dark / L − Dark × 100 )
350
390
430
470
510
550
590
630
670
710
Wavelength (nm)

on the other hand, there appears to be only the blue region showing activity greater than 70%.

When segments were exposed to higher irradiances (25 μE $m^{-2}s^{-1}$), however, the effectiveness increased greatly, particularly in the blue region. It is possible that the response may have increased in the red region beyond 660 nm, if the irradiance could have been maintained at 25 μE $m^{-2}s^{-1}$ (Fig 4.5 A,B).

This higher irradiance appears to saturate the response at most wavelengths, as relative effectiveness was greater than 70% at most wavelengths.

In the L-L turgid samples proline accumulated to the same concentration as in white light in both the blue region (450-510 nm) and in the red region (630, 690 and 710 nm) (Fig 4.6). In the turgid D-L samples, however, where light appeared to reduce the amount of proline in the tissue compared to darkness, the proline levels were lower than the light treatment at all wavelengths except at 490 ± 10 nm and are not presented. Results from turgid samples must be interpreted with caution due to the magnification inherent when data are presented as a percentage relative change. The actual changes in proline concentration were small (Table 4.2), an increase of 1.5 mg $g^{-1}$ dry wt of proline constituting a relative change of 100%.

## 4.3 Discussion

Light may influence proline accumulation via a phytochrome response or photosynthetic reaction. In the earlier chapter (3) the lack of enhancement by a short (5 or 30 min) duration of light at high or low irradiances was evident. When tissue was transferred to

Fig 4.5

Proline accumulation in tissue exposed to various wavelengths, relative to accumulation in white light over darkness at an irradiance of 25 $\mu Em^{-2}s^{-1}$.

A. Tissue exposed to 48 hours continuous light in the pre-stress period (non-starved)* missing

B. Tissue exposed to 48 hours continuous darkness in the prestress period (starved).

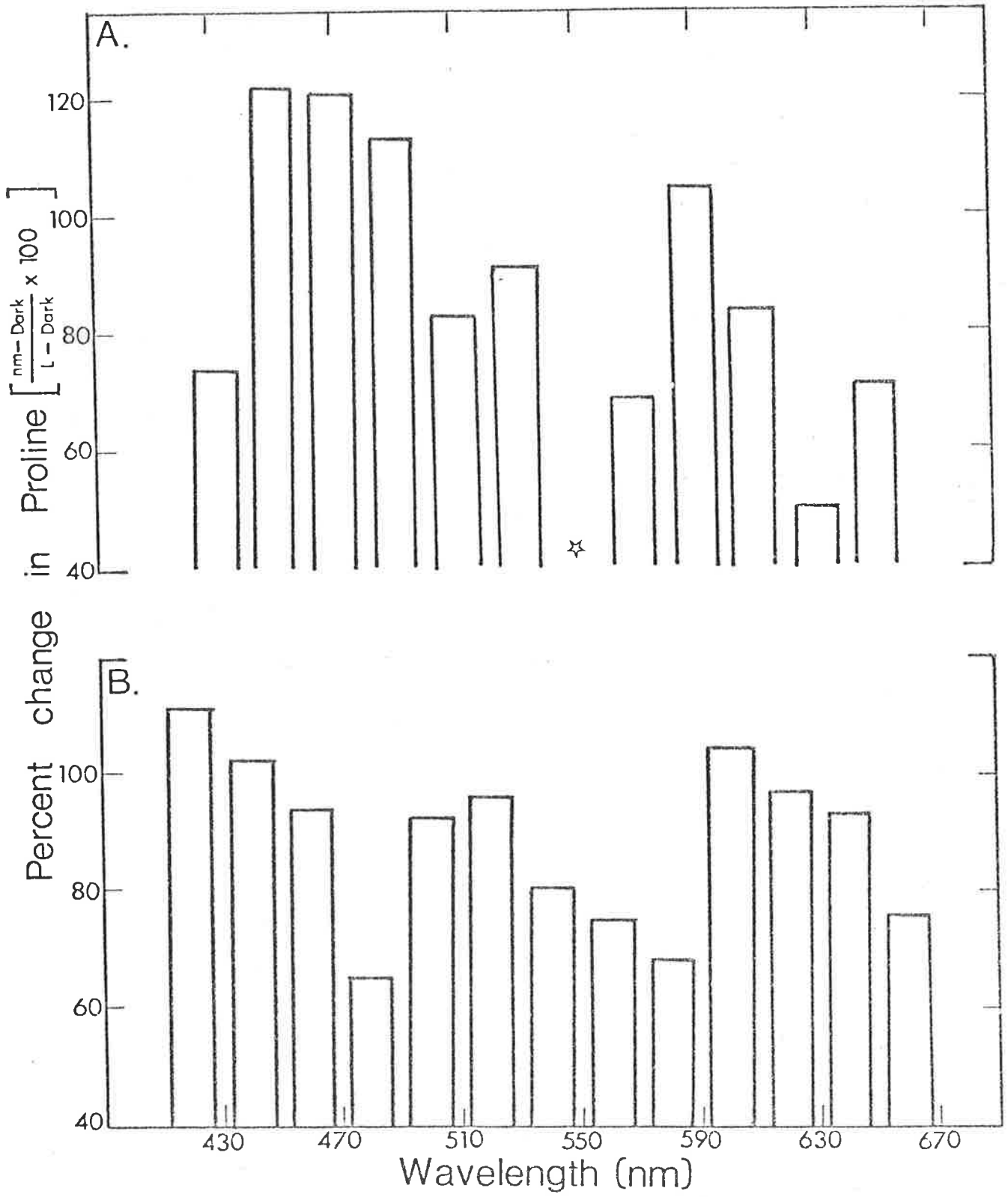
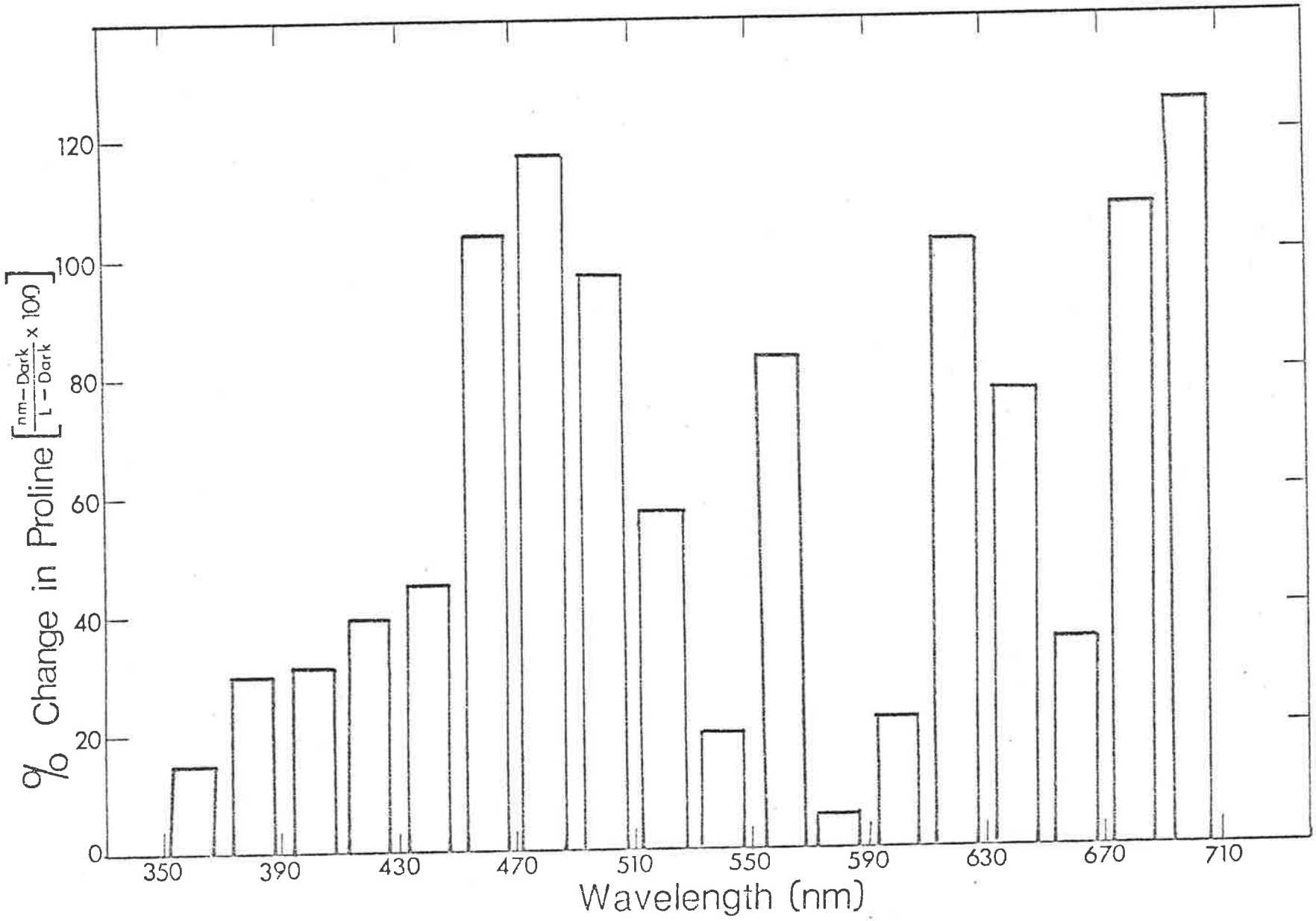

A.
120
100
80
60
40
Proline [ nm − Dark / L − Dark x 100 ]
B.
Percent change in
100
80
60
40
430
470
510
550
590
630
670
Wavelength (nm)

Fig 4.6

Proline accumulation in turgid tissue exposed to various wavelengths relative to accumulation in white light over darkness at an irradiance of 12 $\mu E\ m^{-2}s^{-1}$.

Tissue was exposed to 48 hours continuous light during the prestress period.


% Change in Proline [(nm−Dark)/(L−Dark) x 100]
0
20
40
60
80
100
120
350
390
430
470
510
550
590
630
670
710
Wavelength (nm)

darkness after 12 hours light it was unable to maintain a rate of accumulation equal to that in the light and continued light was required to obtain maximum accumulation. This is in contrast to other phytochrome mediated responses where a brief exposure to the appropriate wavelength entrains consequent changes in the dark. The responses observed are thus consistent with the known features of phytochrome mediated mechanisms. They are consistent, however, with a photosynthetic response. The peaks of maximum proline accumulation lie in the blue and red region of the spectrum, similar to the absorption peaks of chlorophyll A and B (Leopold and Kriedmann, 1975). In addition, proline accumulation varied with the irradiance being saturated at a low irradiance of 20 and 70 $\mu E\ m^{-2}s^{-1}$ in L-L and D-L samples respectively. Low energy phytochrome responses are saturated at irradiances below this level ( 1 $\mu E\ m^{-2}s^{-1}$) (Schopfer, 1977).

Thus, the evidence obtained suggests that proline accumulation in light is not mediated by phytochrome but, rather is dependant on photosynthesis.

# SECTION I WATER STRESS

## 5. Photosynthesis and proline accumulation

# 5. Photosynthesis and proline accumulation

## 5.1 Introduction

Results described in the earlier section have indicated clearly that the enhancement of proline accumulation by light is not a low-energy phytochrome response. Two possibilities remain, the response may be dependant on photosynthesis or on a high energy photoreaction. Since proline does not accumulate in etiolated or dark grown tissue (due probably to limitation of precusors, Singh et al, 1973b), it is difficult to unequivocally eliminate the latter possibility directly. The alternate hypothesis that enhanced proline accumulation is linked to photosynthesis is tested in this chapter.

Two groups of processes are involved in photosynthesis : the first group consists of those processes concerned in carbon dioxide ($CO_2$) transfer from the bulk atmosphere above the plant surface to the carboxylation centres in the chloroplasts of photosynthesizing tissues. This is dependant on the relationship between $CO_2$ flux rate, the $CO_2$ concentration gradients as the driving force of the $CO_2$ flux rate, and the resistance to $CO_2$ transport along the pathway. The second group consists of the biochemical processes of photosynthesis (i.e. photochemical reactions and dark reactions) rather than $CO_2$ transport. Reports of an inhibition of net photosynthesis by water stress are numerous, for example El-Sharkawy and Hesketh, 1964; Boyer, 1970; Redshaw and Meidner, 1972; Beardsell et al, 1973; to mention but a few. Both stomatal (Brix, 1962; Willis and Balasubramanian, 1968; Moldau, 1972) and nonstomatal effects of mild (Boyer and Bowen, 1970;

Keck and Boyer, 1974) and severe (Nir and Poljakoff-Mayber, 1967; Fry, 1970) desiccation have been implicated.

In the experiments reported here, although stomatal resistance has not been measured, some indication of the contribution of stomatal closure to the effect of stress on photosynthesis can be obtained from measurements of net photosynthesis and oxygen evolution. Net photosynthesis includes both net $CO_2$ transfer and the biochemical activity, while $O_2$ evolution only measures the biochemical process. Therefore by studying the effects of water stress on these two processes, some idea of the contribution of stomatal resistance to inhibition of photosynthetic activity can be obtained.

## 5.2 $Co_2$ flux studies

Experiments described in this section deal with the overall photosynthetic rates and include biophysical alteration and biochemical changes which affect the gas exchange rate of the tissue under water stress conditions.

### 5.2.1 Methods

Pregerminated barley seedlings were grown in sand at 20°C and a 16 hour photoperiod, until they were 12 days old. The plants were then transferred to 48 hours continuous light or continuous darkness, at the end of which the first leaves were excised and cut into 1.5 cm segments. The $CO_2$ flux with these segments was then measured with a Beckman 215 infra red gas analyser (IRGA) equipped with a differential mode for estimation of change in $CO_2$ concentration. The net $CO_2$ exchange was calculated by the following formula :

$$F = \frac{\Delta C.J}{A}$$

where $F$ = flux density of $CO_2$ ($cm^3\ cm^{-2}s^{-1}$)

$J$ = air flow rate through the assimilation chamber ($cm^3\ s^{-1}$)

$A$ = leaf area (the area of one surface ($cm^2$))

$\Delta C$ = the difference in $CO_2$ concentration of the air stream between the 'reference' and 'sample' air, measured at the same temperature and pressure ($cm^3\ cm^{-3}$).

This formula is used to derive the $CO_2$ exchange in volumetric units of $cm^3\ CO_2.cm^{-2}$ leaf area.unit $time^{-1}$.

However, to express the $CO_2$ exchange rate in terms of mass flux of $CO_2$, the temperature and pressure of the laboratory must be taken into account -

$$F = \frac{\Delta C}{A} \times \frac{J.44}{22414} \times \frac{273}{T} \times \frac{P}{1013} \quad (g\ cm^{-2}s^{-1})$$

where T and P are the temperature and pressure of the room (from Sestak et al, 1971).

Measurement of $CO_2$ flux in segments

Compressed air containing 320 to 340 ml $CO_2$ per litre was bubbled through water in a conical flask, maintained at 20°C in a waterbath (hydrater) and then through a test tube to condense excess water. It was necessary to humidify the air in this manner or the concentration of the PEG solution in the assimilation chamber increased with time. The air stream emerging from the test tube was divided and the flow rate of each stream was monitored. One stream served as the 'reference' gas $CO_2$ concentration, while the

other was connected to the assimilation chamber via the 'entry' port. The air leaving the chamber at the exit port (which was at the opposite end of the chamber to that of the entry port, thus ensuring movement of air over the leaf tissue) was connected to the IRGA after passing through a flow meter, and served as the 'sample' air (Fig 5.1A). The flow rates of the 'reference' air and 'sample' air were adjusted to equality with the help of pinch cocks connected to the flow meters; the excess 'sample' air was liberated into the room. Output from the IRGA was monitored with a chart recorder.

The assimilation chamber (Fig 5.1B) consisted of a metal chamber (outer dimension 10.3 x 5.3 x 4 cm) which contained two separate chambers - the upper (10.0 x 5 x 1.5 cm) containing an air entry and exit ports on opposite walls 'the true assimilation chamber', and the lower (10 x 5 x 2.5 cm) containing a reticulating metal tube, entering and leaving from adjacent ports. Water of the desired temperature was circulated through this tube and helped maintain a constant temperature within the chamber. To further control the temperature, a large clear glass petri dish containing water was placed between the light source and the chamber. The sample temperature was monitored with a copper-constantan thermo-couple (connected to a chart recorder) placed in the solution on which the segments were floated. The distance between the light source, a 100W incandescent bulb, and the segments was adjusted to obtain the desired irradiance. This system was found to be satisfactory for measuring the net $CO_2$ exchange of up to 20 segments. When turgid tissue was studied, the segments were floated on water, while stressed segments were floated on PEG (-15 bars) solutions.

Fig 5.1

A. Diagrammatic representation of the apparatus for measurement of $CO_2$ flux in segments

A Compressed air cylinder

B Conical flask in water bath at 20°C (hydrater)

C Tube to condense water vapour

D Flow meter

E Assimilation chamber

F Reference air

G Sample air

H IRGA

I Chart recorder

B. Assimilation chamber details

Inner chamber volume 10 x 5 x 1.5 $cm^3$

Outer chamber volume 10.3 x 5.3 x 4 $cm^3$

Lower chamber volume 10 x 5 x 2.5 $cm^3$

A = Inlet for air from cylinder

B = Outlet connected to IRGA

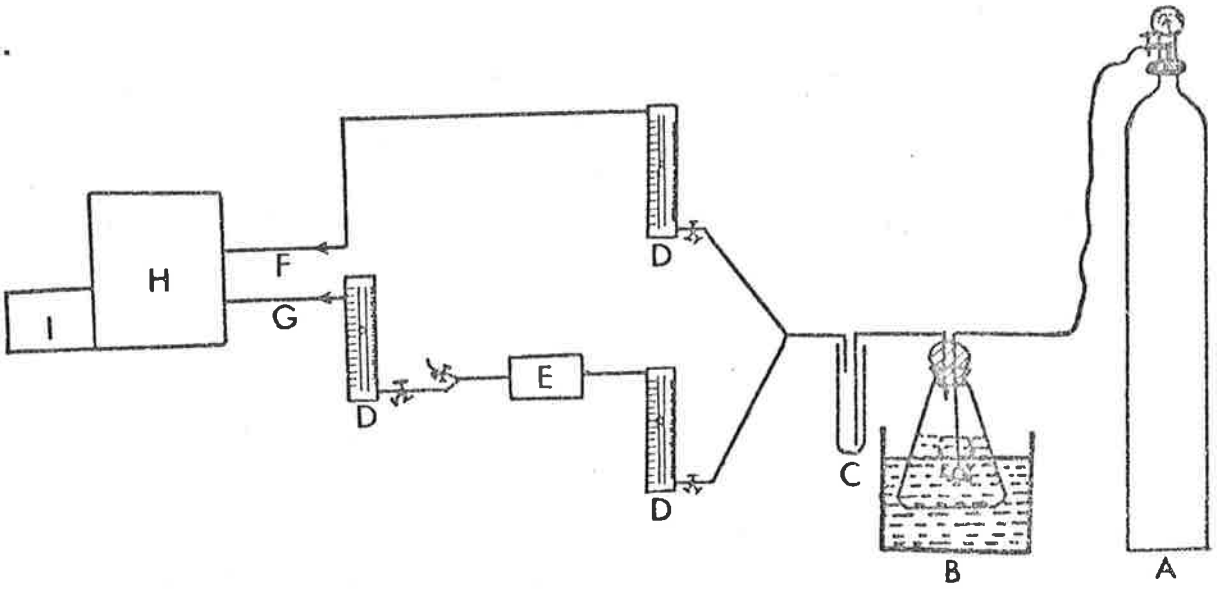
TC = Copper-Constantan thermocouple

= Reticulated tube for conducting water

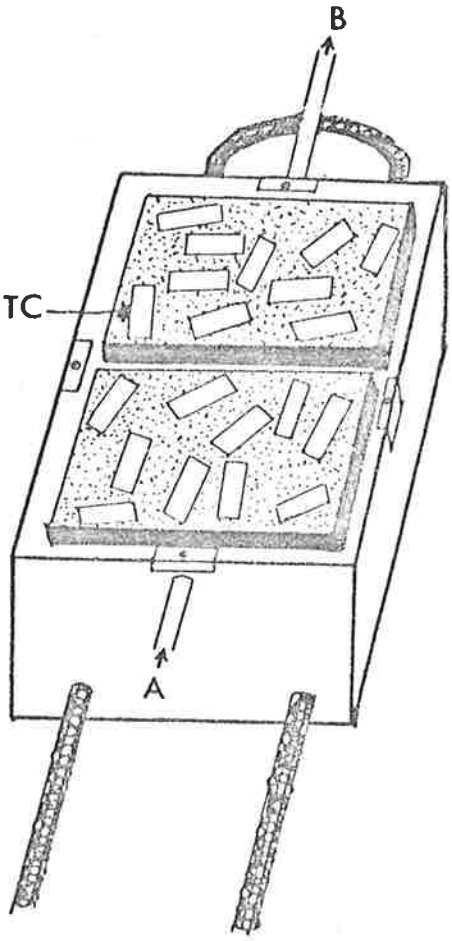
= Floating segments

= Solution on which segments are floated

A.




B.



Segments were floated on 5 ml of the desired solution which was placed in two plastic weighing dishes (45 x 4.5 x 0.7 cm) which fitted into the chamber.

### 5.2.2 Results

#### 5.2.2.1 Exchange in turgid segments

The photosynthetic rate for turgid tissue (i.e. floated on water) showed little variation over a 24 hour period in both L-L and D-L segments (Fig 5.2). Rates showed some variation on different sampling dates, possibly due to changes in the microclimate during the growth of the plants.

Segments of L-L treatment had consistently lower rates of net photosynthesis than D-L segments (the rate varying between 65% to 85% that of D-L segments) (Table 5.1 and Fig 5.3 B,C). Rates in DL segments varied between 75 to 100 µg $CO_2$ $hr^{-1}cm^{-2}$ while in L-L they varied between 50 and 75 µg $CO_2hr^{-1}cm^{-2}$.

Dark respiration, which is equivalent to the $CO_2$ efflux rate in darkness, was slightly higher in L-L segments than in D-L segments, the difference becoming prominent when expressed as a percentage of net exchange in light (Table 5.3). It is worth noting the difference in the post-illumination burst (a measure of photorespiration) in the treatments (Fig 5.3 A,B,C) with DL showing the lowest level. Segments originating from plants grown in a 16 hour photoperiod (10 hours after commencement of the photoperiod) showed similar rates of net exchange as D-L treated tissue, while dark efflux rate expressed as a percentage of net exchange was similar to that of L-L segments (Table 5.2).

Fig 5.2

Carbon dioxide flux of segments of barley floated on water for 24 hours; measured with a infra red gas analyser (Beckman Model 215)

Barley plants were grown at 20°C in a 16 hour photoperiod until they were 12 days old, when they were transferred to 48 hours continuous light or continuous darkness. The first leaves were then excised, cut into 1.5 cm segments and floated on water. They were transferred to the assimilation chamber and the $CO_2$ exchange in the presence of light monitored on the IRGA.

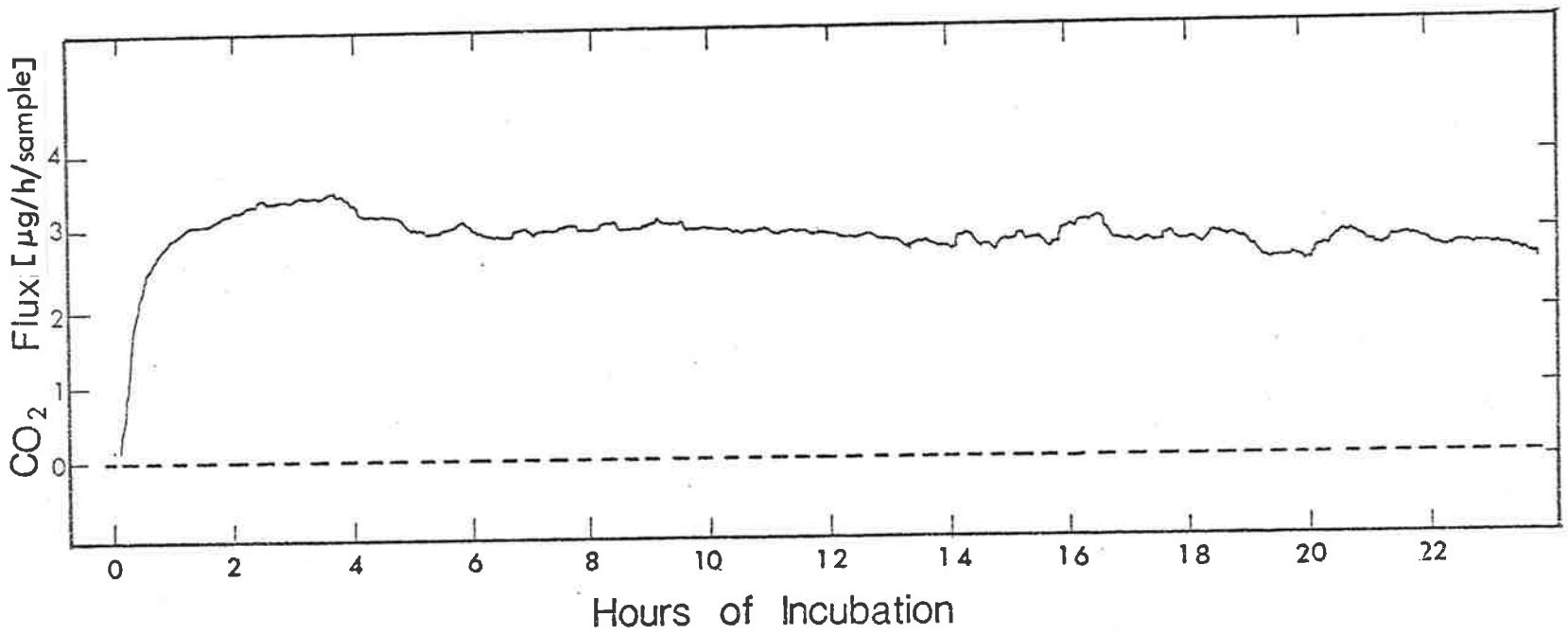

$CO_2$ Flux [µg/h/sample]
0
1
2
3
4
0
2
4
6
8
10
12
14
16
18
20
22
Hours of Incubation

Fig 5.3

Carbon dioxide exchange of barley segments floated on water; measured with an infra red gas analyser

Barley plants were grown in a 16 hour photoperiod at 20°C until they were 12 days old. They were then transferred to continuous light or continuous darkness for 48 hours, after which the first leaves were excised and cut into 1.5 cm segments and floated on water in the assimilation chamber. Carbon dioxide exchange of 20 segments for each treatment was monitored.

A. Exchange by segments from 16 hour photoperiod

B. Exchange by segments from 2 days continuous darkness

C. Exchange by segments from 2 days continuous light

Commencement of ↑ Light

↑ Dark

Table 5.1

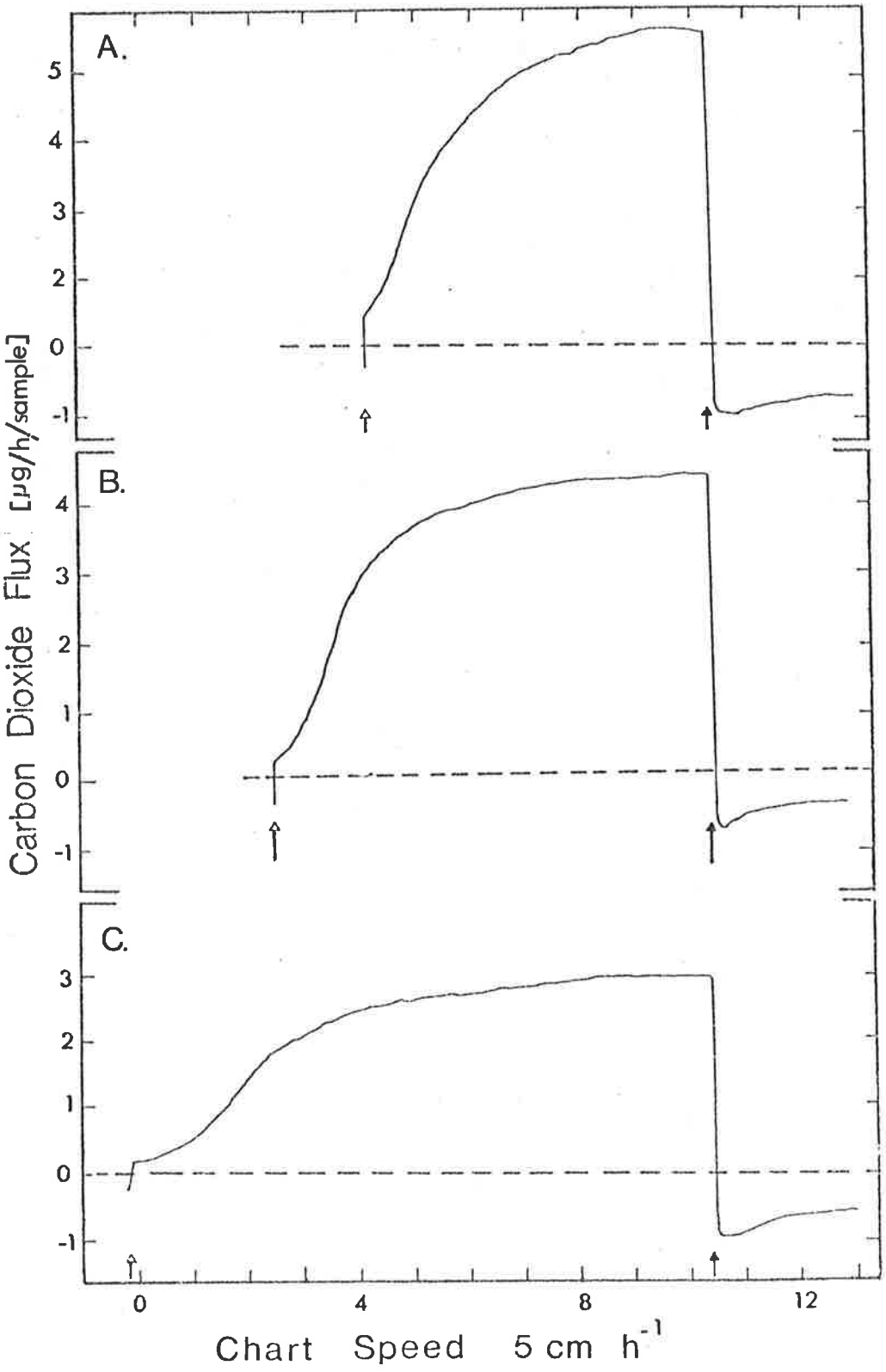
Net $CO_2$ exchange rate

| Treatment | Net $CO_2$ exchange ($\mu g\ CO_2 hr^{-1} cm^{-2}$) | |
|---|---|---|
| | Expt 1 | Expt 2 |
| L-L turgid | 73. 8 | 52.8 |
| D-L turgid | 87. 8 | 76.4 |
| 16 hr photoperiod turgid | - | 78.2 |

Table 5.2

Dark respiration rate

| | $Co_2$ efflux ($\mu g\ CO_2 hr^{-1} cm^{-2}$) | % of net exchange |
|---|---|---|
| L-L turgid | 9.9 | 18.7 |
| D-L turgid | 7.9 | 10.3 |
| 16 hr turgid | 14.1 | 18.0 |


A.
B.
C.
Carbon Dioxide Flux [µg/h/sample]
5
4
3
2
0
-1
4
3
2
1
0
-1
3
2
1
0
-1
0
4
8
12
Chart Speed 5 cm h-1

5.2.2.2 Exchange in stressed segments

Net $CO_2$ exchange was monitored for 24 hours from the time the segments were floated on -15 bar PEG (Fig 5.2 and 5.4). Stressing the segments induced a rapid decline in $CO_2$ influx within the first 4 hours and a more gradual decline thereafter (Table 5.3), till after 12 hours, the $CO_2$ exchange rate was about 5% of that of the initial peak; and remained constant during the following 12 hours. It is interesting that even after 24 hours stress, there was some uptake of $CO_2$,although this was small.

## 5.3 Studies on non-stomatal photosynthetic activity

The previous section has clearly shown that net $CO_2$ influx in the light is dramatically reduced within a few hours of the imposition of stress. It is possible that the biochemical processes of photosynthesis may be inhibited by water stress, which would lead to reduced utilisation of $CO_2$ and a consequential decrease in $CO_2$ influx. The experiments described in this section were conducted to study the effect of water stress on the photochemical processes of $CO_2$ fixation. Photosynthesis was measured on leaf slices as oxygen evolution with a polarographic electrode.

### 5.3.1 Methods

Barley plants were grown in sand at 20°C in a 16 hour photoperiod for 12 days. They were then transferred to continuous light or continuous darkness for 48 hours, after which the first leaves were excised, cut into 1.5 cm segments and floated on PEG (-15 bars) or water.

Fig 5.4

Carbon dioxide flux of stressed segments of barley measured with an infra red gas analyser.

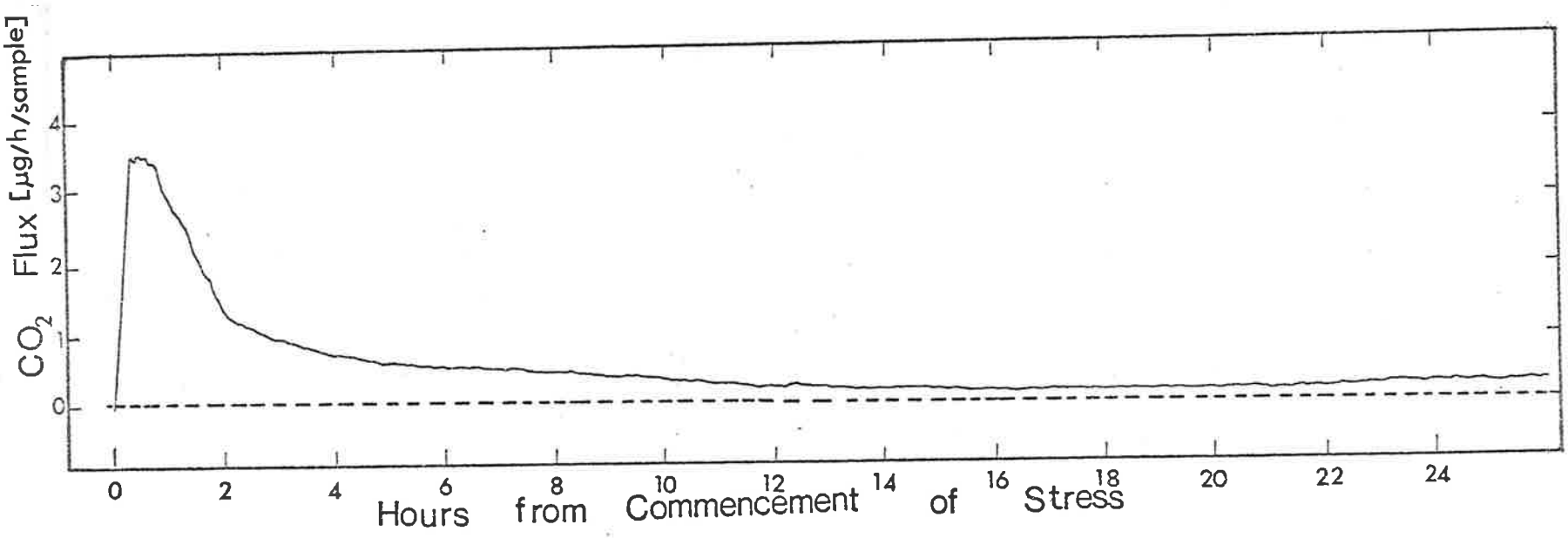
Barley plants were grown at 20°C in a 16 hour photoperiod for 12 days and were then transferred to continuous darkness for 48 hours before the first leaves were excised and cut into 1.5 cm segments. The $CO_2$ exchange in light of these segments was monitored with an infra red gas analyser. Segments were floated on -15 bar PEG solution.

Table 5.3

Net $CO_2$ exchange ($\mu g\ CO_2 h^{-1} cm^{-2}$)

| Time in hr | 1 | 2 | 4 | 8 | 12 | 22 |
|---|---|---|---|---|---|---|
| Turgid | 68.3 | 71.5 | 74.7 | 69.4 | 63.0 | 59.8 |
| Stress | 82.8 | 42.0(49) | 18.7(76) | 10.5(87) | 5.6(93) | 4.2(95) |

Figures in paranthesis indicate % inhibition over 0 hr photosynthesis rate


$CO_2$ Flux [µg/h/sample]
0
1
2
3
4
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours from Commencement of Stress

Measurement of oxygen evolution

Oxygen evolution was measured polarographically, using a Clark-type electrode on leaf slices. The techniques was initially developed for study of ionic relations of leaf cells (Osmond, 1964; Rains, 1968) while more recently, Luttge et al, (1971); Jones and Osmond (1973); Pitman et al (1975) and Ishii et al (1977) have used such a system for studying photosynthesis and respiration. This method has the following advantages :

a) It is possible to eliminate the effect of stomatal diffusion resistance on $CO_2$ absorption, because it is considered that $CO_2$ is absorbed mainly through the cut surface of the leaf slice.

b) It is possible to measure the rate of photosynthesis and respiration of a small part of each organ.

c) It is easy to detect the effect of stress treatment or inhibitor treatment on the rate of photosynthesis and respiration.

Preparation of leaf tissue

Ten segments were taken from the petri dish, blotted dry and placed on a clean glass sheet and sliced with a razor blade by hand. A finely minced preparation was obtained (sliced 0.6 to 1 mm). Oxygen evolution was measured polarographically in a sealed lucite vessel (with a circulating bath, at 20°C) of 3.0 ml volume using a Clark electrode (Yellow Springs Instrument Company, Cleveland, Ohio) connected to a 1 mvolt recorder (Rikadenki, Japan). The vessel was dried with a suction pump, and 3 ml of MES buffer (pH 6.5) was added, and bubbled with nitrogen gas to reduce

the oxygen content. To this, the prepared sample was quickly added, and last of all, 20 µl of 5% $NaHCO_3$ ( 4 mM) was added. The cup was quickly sealed, and the reading obtained in the dark gave respiration rate while that obtained when the light (from a projection lamp i.e. at saturating irradiance) was switched on, gave apparent photosynthetic oxygen evolution rate.

The zero was adjusted by using sodium dithionite to remove all the oxygen from water and the 100% line was set by using a well-aerated solution, assuming air oxygen concentration of 240 µM. When the oxygen evolution rate was steady, the vessel was emptied with a suction tube and washed several times with water before being used again. 'Respiration' was measured as oxygen uptake in the dark; 'photosynthesis' was the difference in oxygen exchange rate in the dark and light.

### 5.3.2 Results

The photosynthesis to respiration ratio showed a decline with stress to 80% of that of the control (Table 5.4 and Fig 5.5). Although absolute rates of both photosynthesis and respiration have been tabulated, it is unwise to compare absolute values because of differences resulting from preparation and handling errors, such problems are overcome when comparing relative rates within the same preparations. Both the ratio of oxygen evolution:oxygen absorption and photosynthesis:respiration, show a decline with stress to about 60% to 70% when comparing the former ratios, and about 80% to 85% in the latter case. The interesting point is that neither ratio changes dramatically between 7 and 24 hour stress, the change being less than that between turgid and 7 hour stress samples in both cases.

Fig 5.5

Effect of stress on oxygen exchange in barley leaf slices measured with a polarographic oxygen electrode.

Barley plants were grown in sand at 20°C and in a 16 hour photoperiod until they were 12 days old. They were given 48 hours dark treatment prior to excision of the first leaves which were segmented into 1.5 cm and floated on water on PEG until required. All values are expressed as percentage of exchange in control.

O Percentage change in ratio of photosynthesis to respiration over that of turgid samples

Δ Percentage change in ratio of $O_2$ evolution to $O_2$ absorption over that of turgid samples

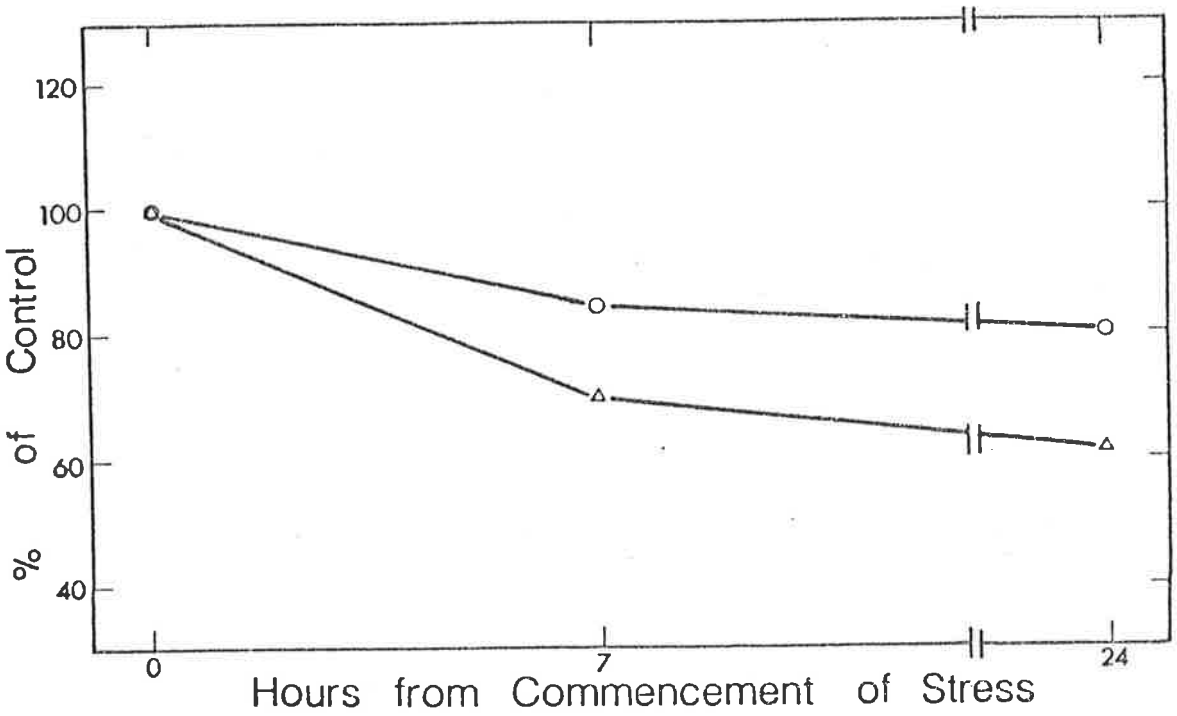
Table 5.4

Oxygen exchange rates (in the light and dark) of stressed and turgid tissu

| Treatment | n moles $O_2$ $cm^{-2} h^{-1}$ 'Photosynthesis' (P) | 'Respiration' (R) | Ratio P/R | Ratio of $O_2$ evoln/ $O_2$ absorpn. |
|---|---|---|---|---|
| Turgid | 347.8 | 169.2 | 2.06 | 1.06 |
| 7 hr stress | 401.0 | 230.4 | 1.74 | 0.74 |
| 24 hr stress | 251.5 | 152.3 | 1.65 | 0.65 |

'Photosynthesis' = Total $O_2$ efflux (net efflux-influx)

'Respiration' = $O_2$ influx


120
100
80
60
40
% of Control
0
7
24
Hours from Commencement of Stress

It is clear from the results of this section and the earlier one, that a decline in photosynthetic activity can occount for only up to 60% - 80% inhibition of photosynthesis even after 24 hours, while observed net $CO_2$ exchange rates declined to 5% that of turgid tissues. It can be concluded that both stomatal closure and photochemical inhibition occur and cause reduced photosynthesis rates in stressed tissue. It is noteworthy that it was extremely difficult to obtain photosynthesis rates using the oxygen electrode in LL samples perhaps partly attributable to the very high respiration rates recorded ( 560 nmoles $cm^{-2}hr^{-1}$).

## 5.4 Carbon dioxide concentration and proline accumulation

### 5.4.1 Effect of enhanced $CO_2$ concentration on proline accumulation

Since $CO_2$ exchange is reduced during stress perhaps through increased stomatal resistance, it was hoped that by increasing external $CO_2$ concentration, it may be possible to enhance $CO_2$ fixation and thereby influence proline accumulation. It was envisaged that if proline accumulation involved competing with $CO_2$ fixation for NAD(P)H and ATP, then an increase in $CO_2$ concentration should reduce the NAD(P)H available for proline synthesis, resulting in a reduction in proline content. On the other hand, if carbon dioxide fixation was providing the carbon precursors required for proline synthesis, then an increase in $CO_2$ concentration should induce higher proline accumulation.

5.4.1.1 Methods

Plants were grown in a 16 hour photoperiod in sand at 20°C for 12 days following which they were transferred to 48 hours continuous light or continuous darkness. The first leaves were then excised, cut into 1.5 cm segments and floated on -15 bar PEG in 140 ml glass conical flasks. The flasks were fitted with one holed rubber stoppers, through which passed a glass tube connected to a rubber tubing with a pinch cock attached to it (Materials and Methods Fig 1). After the segments had been transferred to the flask, the $CO_2$ concentration within the flask was increased by injecting varying amounts of $CO_2$ gas into the flasks and then sealing them. The volume of $CO_2$ required was calculated in the following manner - when final $CO_2$ concentrations required within the flask were 0.06% and 0.15%, pure $CO_2$ was diluted to a 1% $CO_2$ concentration by adding 10 ml of $CO_2$ to a litre flask. From this flask, 4 ml and 14 ml $CO_2$-enriched air was removed with a Hamilton syringe and introduced into the flasks to obtain a final concentration of 0.06% and 0.15%. For a final concentration of 0.53%, 0.6 ml pure $CO_2$ was directly added to the flask. There were 4 replicates for each $CO_2$ concentration. The results were analysed for statistical significance with a conventional analysis of variance test.

5.4.1.2 Results

Increasing the $CO_2$ concentration within the flasks had no significant effect on proline accumulation in either starved (D-L) (tissue exposed to 48 hours continuous darkness) or non-starved (L-L) (exposed to 48 hours continuous light) stressed tissue (Fig 5.6). The experiment was repeated and gave very similar results.

Fig 5.6

Effect of $CO_2$ concentration on proline accumulation in barley segments floated on -15 bar PEG.

Barley plants were grown for 12 days in sand in a 16 hour photoperiod and were then transferred to 48 hours continuous light or continuous darkness. The first leaves were excised and 1.5 cm segments were floated on -15 bar PEG in different $CO_2$ concentrations for 24 hours.

Each value is the mean of 4 replicates.

- o L-L (non-starved)
- □ D-L (starved)

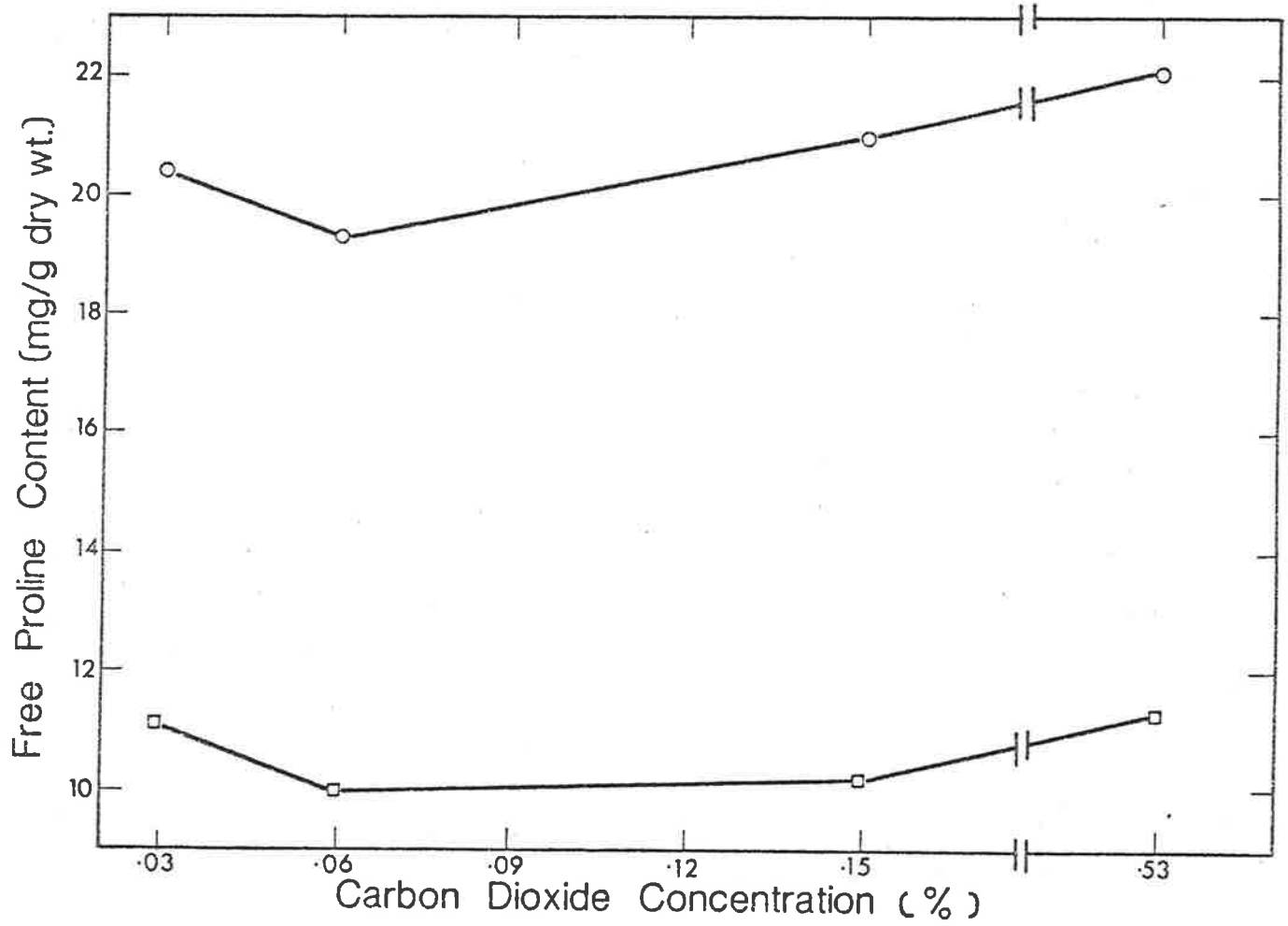
Table 5.5

F Table for results in Fig 5.6

| Source of variation | VR |
|---|---|
| $CO_2$ | 1.392 |
| Light | 188.241** |
| Light x $CO_2$ | 0.359 |

Significance level * $P \leq 0.05$

** $P \leq 0.01$


Free Proline Content (mg/g dry wt.)
22
20
18
16
14
12
10
·03
·06
·09
·12
·15
·53
Carbon Dioxide Concentration (%)

### 5.4.2 Effect of $CO_2$ free air on proline accumulation

The lack of any effect of increased $CO_2$ concentration in the ambient atmosphere on proline accumulation could be explained by any of the following -

a) Proline synthesis is either not competing with carbon fixation for NADPH or ATP; or if it is, these energy rich compounds are in excess and therefore increasing $CO_2$ fixation by providing higher $CO_2$ concentration does not affect proline levels.
b) If carbon fixation products are being utilized in synthesis of proline, they are not limiting at 0.03% $CO_2$.

It is possible that $CO_2$ fixation is not required for proline accumulation. This experiment was therefore designed to study the effect of a $CO_2$ free atmosphere on proline accumulation.

#### 5.4.2.1 Methods

Barley plants were grown on sand at 20°C in a 16 hour photoperiod for 12 days when they were transferred to 48 hours continuous light or continuous darkness. The first leaves were then harvested, cut into 1.5 cm segments and floated on -15 bar PEG solution or water in the presence or absence of $CO_2$ in the ambient atmosphere. Carbon dioxide ($CO_2$) free air was obtained by passing air from an air pump through a soda lime tower (filled with self-indicating soda lime granules 'carbsorb' consisting of a 1:1 mixture of 4-10 mesh and 14-22 mesh soda lime granules. The air was tested by bubbling through a solution of calcium hydroxide ($Ca(OH)_2$). No turbidity was found, indicating that the air was $CO_2$ free. On the other hand, when air from the pump was passed through the $Ca(OH)_2$ solution prior to passing through

the soda lime tower, massive turbidity was formed. The $CO_2$ scrubbed air was then bubbled through water and distributed to sixteen 125 ml one armed conical flasks, via a manifold. The flow rate into each flask was equalized with the help of pinch cocks fitted at the junction of the tubing and the flask (Fig 5.7). Flasks exposed to $CO_2$ treatment were arranged in a similar manner except that the soda lime tower was omitted. In addition, stressed segments were also tested for proline accumulation in the presence or absence of $CO_2$ at different irradiances. The irradiances were provided during the stress treatment, and different levels obtained by shading the flasks with 'neutral' nylon netting.

5.4.2.2 Results

Stress induced a highly significant increase in proline content in both light and dark pretreated tissue compared to turgid segments (Fig 5.8 and Table 5.5). Carbon dioxide treatment on the other hand mainly affected proline accumulation in D-L stressed segments. In L-L segments, the presence of $CO_2$ did not influence proline accumulation (standard errors have been provided in the figure to facilitate comparisons). It is noteworthy that the increase in proline content with stress was very large in comparison with the effect of $CO_2$ deprivation.

Reducing the irradiance to 185 or 30 $\mu E\ m^{-2}s^{-1}$ did not affect the response of stressed segments to the presence or absence of $CO_2$ (Table 5.6B). D-L segments at all irradiances showed inhibition of proline accumulation when stressed in the absence of $CO_2$ while L-L segments showed accumulation of proline under both conditions (+ or - $CO_2$).

Fig 5.7

Diagrammatic representation of the method of providing carbon dioxide free air.

A - Air pump

B - Soda lime tower filled with 'carbsorb' granules

C - Hydrater

D - Manifold connected to one armed conical flasks (containing segments floating on water or PEG solution).

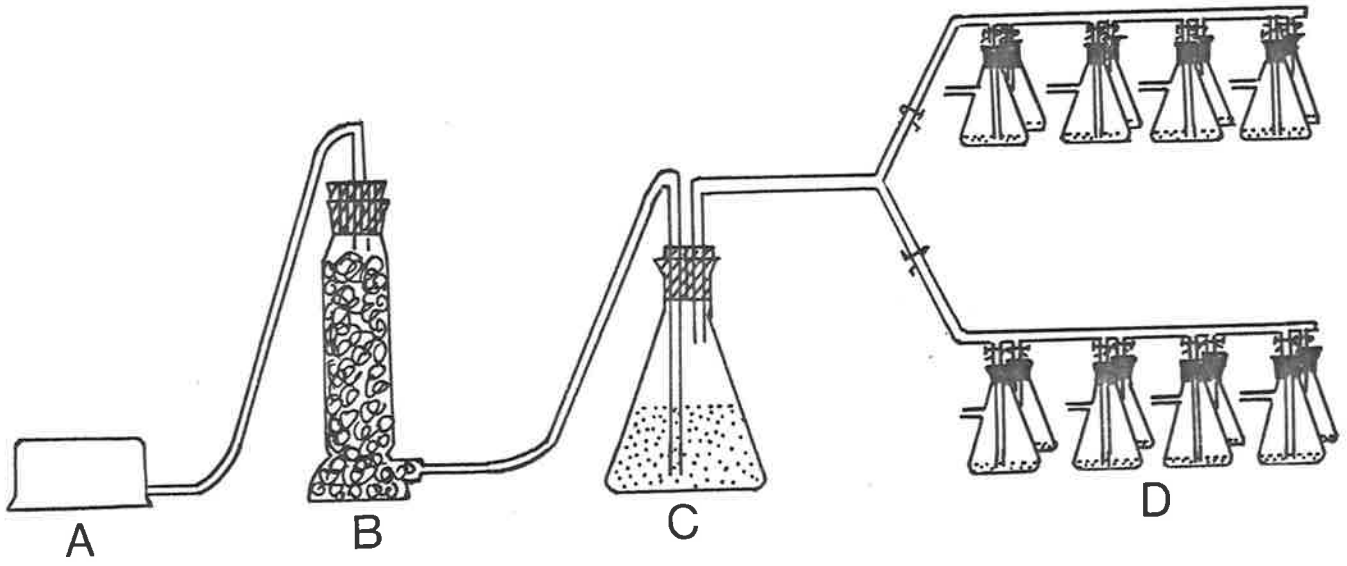
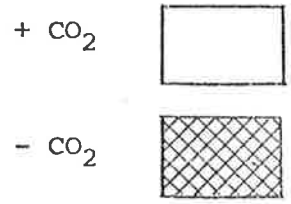

A
B
C
D

Fig 5.8

Effect of $CO_2$ on proline accumulation.

Barley plants were grown on sand in a 16 hour photoperiod until they were 12 days old. They were then transferred to 48 hours continuous light or continuous darkness, after which the first leaves were harvested, segmented and floated on water or PEG for 24 hours.




Turgid - floated on water for 24 hours

Stress - floated on -15 bars PEG for 24 hours

Table 5.6A

F Table for results in Fig 5.8

| Source of variation | V.R. |
|---|---|
| Light | 120.69** |
| $CO_2$ | 11.33** |
| Treat | 557.00** |
| Light x $CO_2$ | 1.13 |
| Light x Treat | 97.35** |
| $CO_2$ x Treat | 3.08 |
| Light x $CO_2$ x Treat | 2.49 |

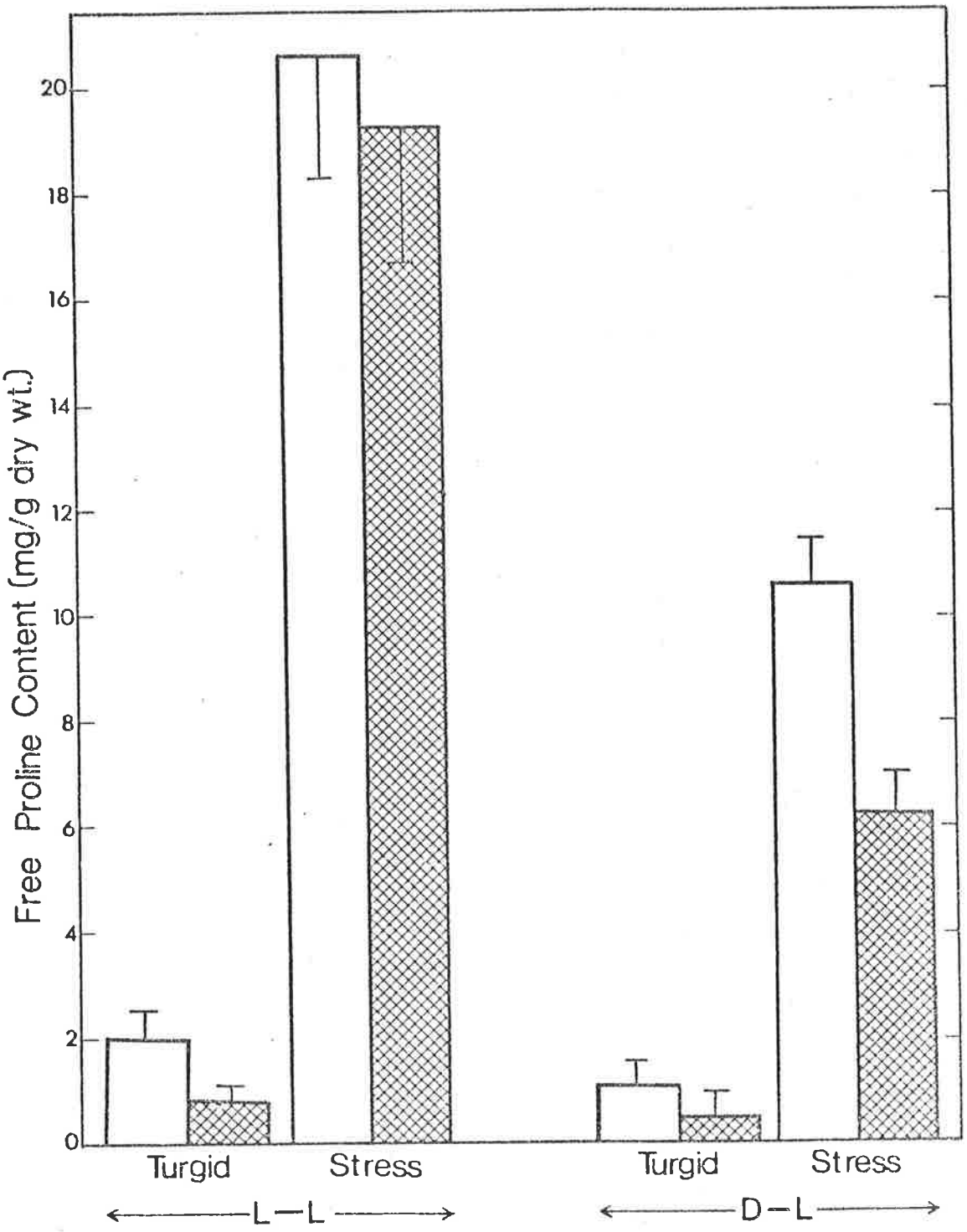

Free Proline Content (mg/g dry wt.)
0
2
4
6
8
10
12
14
16
18
20
Turgid
Stress
Turgid
Stress
L—L
D—L

Table 5.6B

Effect of $CO_2$ on proline accumulation in segments stressed at different irradiances

| | % change over $+CO_2$ treatment | | | |
|---|---|---|---|---|
| Treatment | L-L | | D-L | |
| | $+CO_2$ | $-CO_2$ | $+CO_2$ | $-CO_2$ |
| 290 µE $m^{-2}s^{-1}$ | 100 | 106 | 100 | 69 |
| 185 µE " | 100 | 110 | 100 | 61 |
| 30 µE " | 100 | 103 | 100 | 61 |

It is evident from the results presented so far, that the presence of $CO_2$ during stress is necessary for proline accumulation in D-L segments but not in L-L segments; yet both D-L and L-L segments show an enhancement of proline accumulation during stress in the presence of light. even at low irradiances (10 $\mu E\ m^{-2}s^{-1}$). In order to test this relationship between proline accumulation and photosynthesis, studies on the effect of photosynthetic inhibitors on proline accumulation were conducted.

## 5.5 Effect of inhibitors - electron transport inhibitors

Two types of inhibitors were studied - inhibitors of electron transport (DCMU, CMU) and uncouplers of photosynthesis ($NH_4Cl$, CCCP), and the effect of these substances on proline accumulation in tissue stressed on PEG was examined. In this section, the experiments performed using electron transport inhibitors are described first.

### 5.5.1 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) treatment and proline accumulation

CMU is known to be a specific inhibitor of photosynthesis. This inhibitor acts on the reducing side of photosystem II, more specifically - the unknown electron acceptor Q for photosystem II becomes almost totally reduced, suggesting that it is no longer capable of passing its electrons to photosystem I (Good and Izawa, 1973) (Fig 5.9).

Fig 5.9

Model for the mechanism of electron transport and photophosphorylation in illuminated chloroplast lamellae (thylakoids).

Q - unknown primary electron acceptor for photosynthesis II

PQ - plastoquinares

Cyt f - cytochrome f

PC - plastocyanin

P 700 - reaction centre chlorophyll of photosystem I

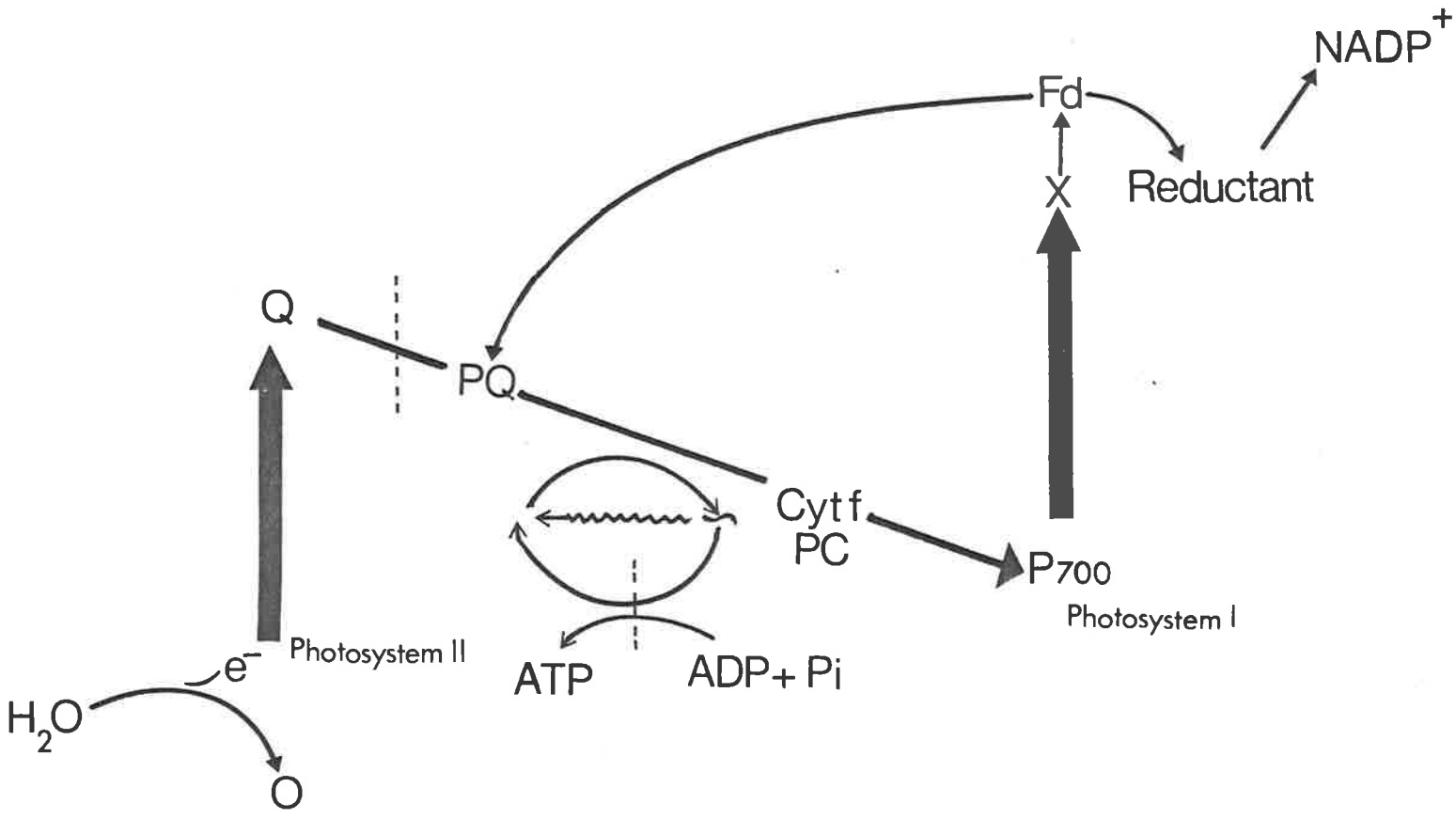
X - unknown primary electron acceptor for photosystem I

Fd - ferredoxin

┆ inhibition

<∿∿∿ uncoupling

(Good and Izawa, 1973)


NADP+
Fd
X
Reductant
Q
PQ
Cyt f
PC
P700
Photosystem I
Photosystem II
e−
H2O
O
ATP
ADP+ Pi

5.5.1.1 Methods

The plants were grown until they were 12 days old at 20°C in a 16 hour photoperiod. They were then transferred to 48 hours continuous light or continuous darkness. The first leaves were excised, cut into 1.5 cm segments which were floated on the inhibitor (concentrations used were $10^{-5}$, $10^{-4}$, $5x10^{-4}$M and saturated aqueous solutions) or water for 3 hours in large petri dishes. Tissue from plants exposed to 48 hours light was floated on inhibitor solution in light while that from plants in darkness was floated on inhibitor in the dark. The segments were transferred to -15 bar PEG solutions and left in continuous light or darkness for 24 hours, after which they were rinsed quickly in water, dried on filter paper and frozen in liquid nitrogen. Proline content was estimated after the segments had been freeze-dried and weighed.

5.5.1.2 Results

Increasing CMU concentrations to greater than $10^{-4}$M led to an inhibition of proline accumulation in all treatments except D-D (Fig 5.10). Lower concentrations had no inhibitory effect (Table 5.8) and at $10^{-4}$M may have even stimulated proline accumulation. Saturated CMU solutions inhibited proline accumulation most.

5.5.2 3-(3',4'-Dichlorophenyl)-1,1-dimethylurea (DCMU) effect on proline accumulation

The decrease in proline accumulation following CMU treatment in segments maintained entirely in the dark during stress, was unexpected and indicated that CMU was probably inhibiting proline

Fig 5.10

Effect of CMU on proline accumulation

Twelve day old barley plants grown at 20°C in a 16 hour photoperiod were transferred to 48 hours continuous light or continuous darkness prior to excision and segmentation of the first leaves. Segments were floated on CMU solution for 2 hours and then on PEG (-15 bars) in light or darkness for 24 hours.

Each value is the mean of 4 replicates.

○ L-L

● L-D

□ D-L

■ D-D

Table 5.7

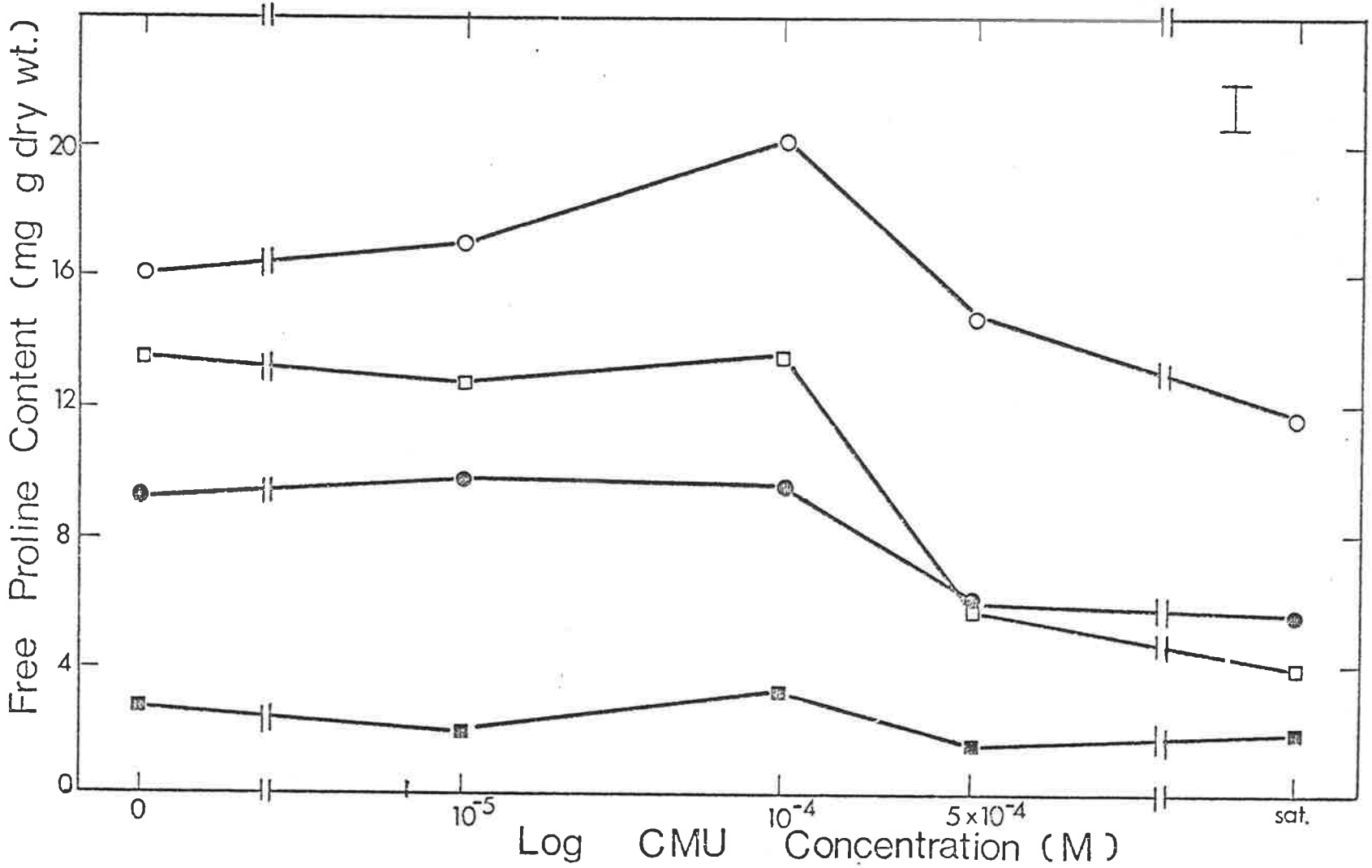
F table for results in Fig 5.10

| Source of variation | F value |
|---|---|
| $L_1$ | 587.89** |
| $L_2$ | 1012.87** |
| Treat (CMU) | 86.31** |
| $L_1.L_2$ | 0.51 |
| $L_1$.Treat | 2.61 |
| $L_2$.Treat | 20.78** |
| $L_1.L_2$.Treat | 13.47** |

Table 5.8

Proline accumulation in various treatments expressed as a % of accumulation in untreated stressed segments

| Inhibitor conc | 0 | $10^{-5}$ | $10^{-4}$ | $5x10^{-4}$ | saturated |
|---|---|---|---|---|---|
| LL | 100 | 106 | 126 | 92 | 73 |
| LD | 100 | 104 | 103 | 63 | 60 |
| DL | 100 | 94 | 100 | 41 | 29 |
| DD | 100 | 69 | 116 | 52 | 62 |


Free Proline Content (mg g dry wt.)
20
16
12
8
4
0
0
10⁻⁵
10⁻⁴
5x10⁻⁴
sat.
Log CMU Concentration (M)

accumulation through a process independant of photosynthesis. One possible cause for this inhibition could be impurities present in CMU and it was decided to study the response of accumulation to DCMU, which was available as a purer compound.

5.5.2.1 Methods

Barley plants were grown in sand at 20°C for 12 days in a 16 hour photoperiod; and treatment given in an identical manner to that described in 5.4.1.1 except that segments were floated on DCMU solutions instead of CMU solutions. The concentrations of DCMU studied were $10^{-5}$, $10^{-4}$, $2x10^{-4}$M and saturated aqueous solutions.

5.5.2.2 Results

Only concentrations of DCMU greater than $10^{-4}$M were effective in inhibiting proline accumulation in most treatments (Fig 5.11). Both $10^{-5}$ and $10^{-4}$M enhanced accumulation of proline in all light and dark treatments (Table 5.10). Maximum inhibition occurred in segments treated with saturated solution of DCMU while amongst the various light/dark treatments, D-L treatment appeared to be the most sensitive to the inhibitor. It is interesting that relative inhibition of proline accumulation in D-L was much greater than that in L-L suggesting that D-L may be more dependent on photosynthesis for proline accumulation.

5.5.3 Effect of inhibitors on gas exchange

Both CMU and DCMU appeared to inhibit proline accumulation in L-D and D-D segments, in spite of the segments being stressed in the dark. Since it is possible that these inhibitors may be

Fig 5.11

Effect of DCMU on proline accumulation.

Twelve day old barley plants grown for a 16 hour photoperiod at 20°C were transferred to 48 hours continuous light or continuous darkness prior to excision and segmentation of the first leaves. The segments were floated in DCMU of various concentrations for 2 hours before they were floated on PEG in light or darkness for 24 hours.

Each value is the mean of 4 replicates.

○ L-L

● L-D

□ D-L

■ D-D

Table 5.9

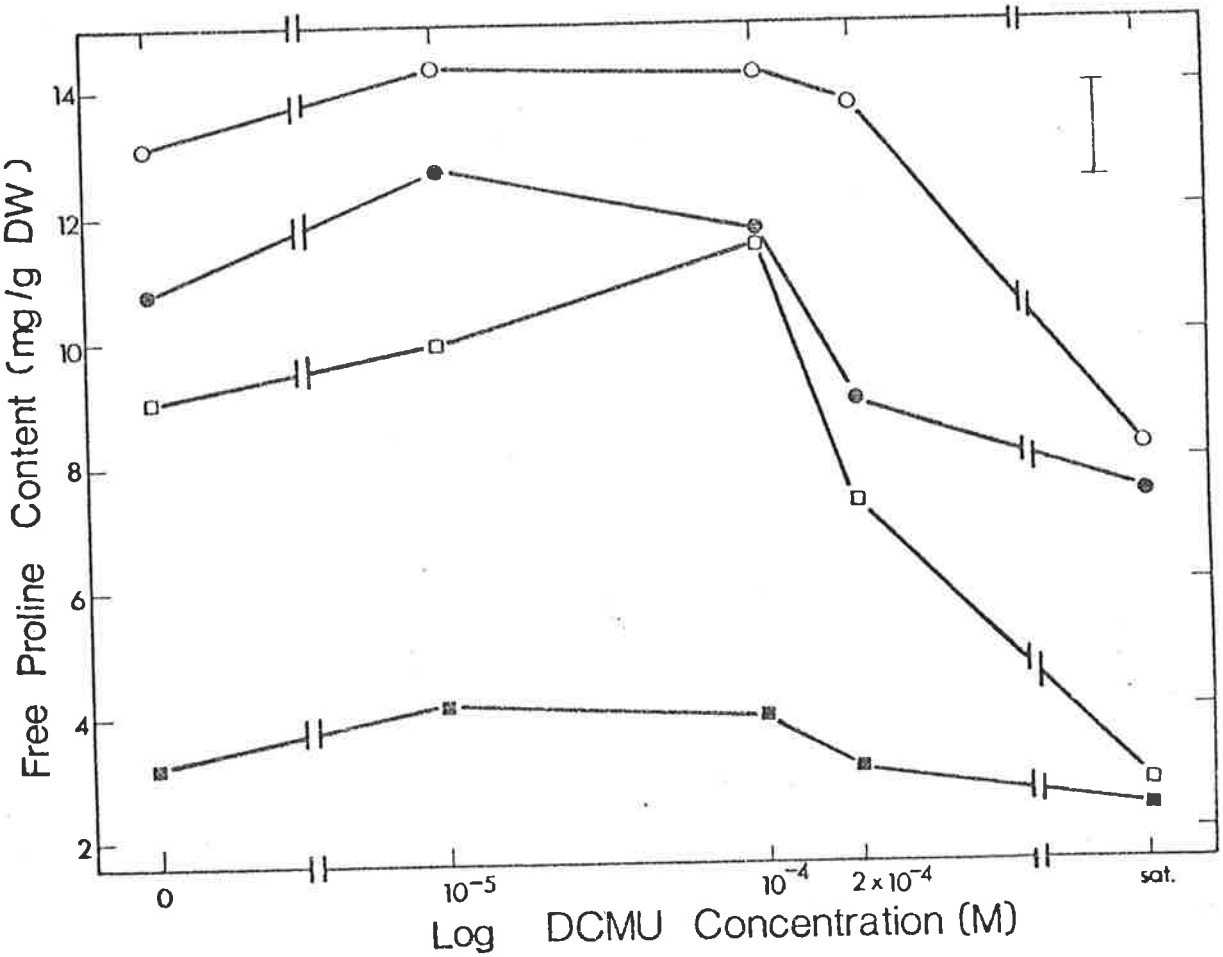
F values for results in Fig 5.11

| Source of variation | VR |
|---|---|
| $L_1$ | 592.16** |
| $L_2$ | 227.64** |
| Treat | 62.55** |
| $L_1.L_2$ | 25.27** |
| $L_1$.Treat | 1.04 |
| $L_2$.Treat | 10.87** |
| $L_1.L_2$.Treat | 6.22 |

Table 5.10

Proline accumulation in the presence of DCMU as a % of accumulation in untreated stressed tissue

| Inhibitor conc | 0 | $10^{-5}$ | $10^{-4}$ | $2x10^{-4}$ | saturated |
|---|---|---|---|---|---|
| LL | 100 | 110 | 108 | 105 | 63 |
| LD | 100 | 118 | 109 | 84 | 69 |
| DL | 100 | 109 | 127 | 81 | 30 |
| DD | 100 | 131 | 123 | 96 | 75 |


Free Proline Content (mg/g DW)
14
12
10
8
6
4
2
0
$10^{-5}$
$10^{-4}$
$2 \times 10^{-4}$
sat.
Log DCMU Concentration (M)

influencing proline accumulation by affecting some metabolic reaction linked to proline accumulation, it was decided to study the effect of these inhibitors on rates of gas exchange, i.e. $O_2$ evolution (net 'photosynthesis') and $O_2$ absorption ('respiration'). Total photosynthesis was the difference in $O_2$ exchange rate in the dark and light.

5.5.3.1 Methods

Plants were grown for 12 days in sand at 20°C in a 16 hour photoperiod and were treated with different inhibitor concentrations (after 2 days continuous light or darkness) as described in Section 5.4.1.1. The $O_2$ evolution absorption of these segments was studied using a Clark's polarographic electrode described in Section 5.2. In addition, the $CO_2$ exchange was monitored with an infra red gas analyser, the detailed methods of which are described in Section 5.1.1.

5.5.3.2 Results

A. Oxygen electrode results

Results show clearly that the ratio of photosynthesis to respiration was greatly reduced at $10^{-4}$M and higher concentrations while $10^{-5}$M seemed to stimulate photosynthesis. The reason for this is not clear (Fig 5.12 and Table 5.11). Oxygen absorption was not inhibited by the presence of DCMU, but was enhanced at all concentrations of inhibitor particularly at $10^{-4}$ and $2x10^{-4}$M DCMU. The reason for this too is not known. This enhanced respiration rate, however, cannot be responsible for the decline in proline accumulation during dark treatment, as if this were so, the inhibition should appear at all concentrations of DCMU. Once again, readings for D-L

Fig 5.12

Effect of DCMU on oxygen exchange in barley leaf sections measured with a polarographic oxygen electrode.

Barley plants were grown in sand at 20°C in a 16 hour photoperiod, until they were 12 days old. They were given 48 hours dark treatment prior to excision of the first leaves which was then segmented into 1.5 cm pieces and floated on water or DCMU solutions of various concentrations for 3 hours.

O Percent change in ratio of photosynthesis to respiration over that in untreated turgid sample.

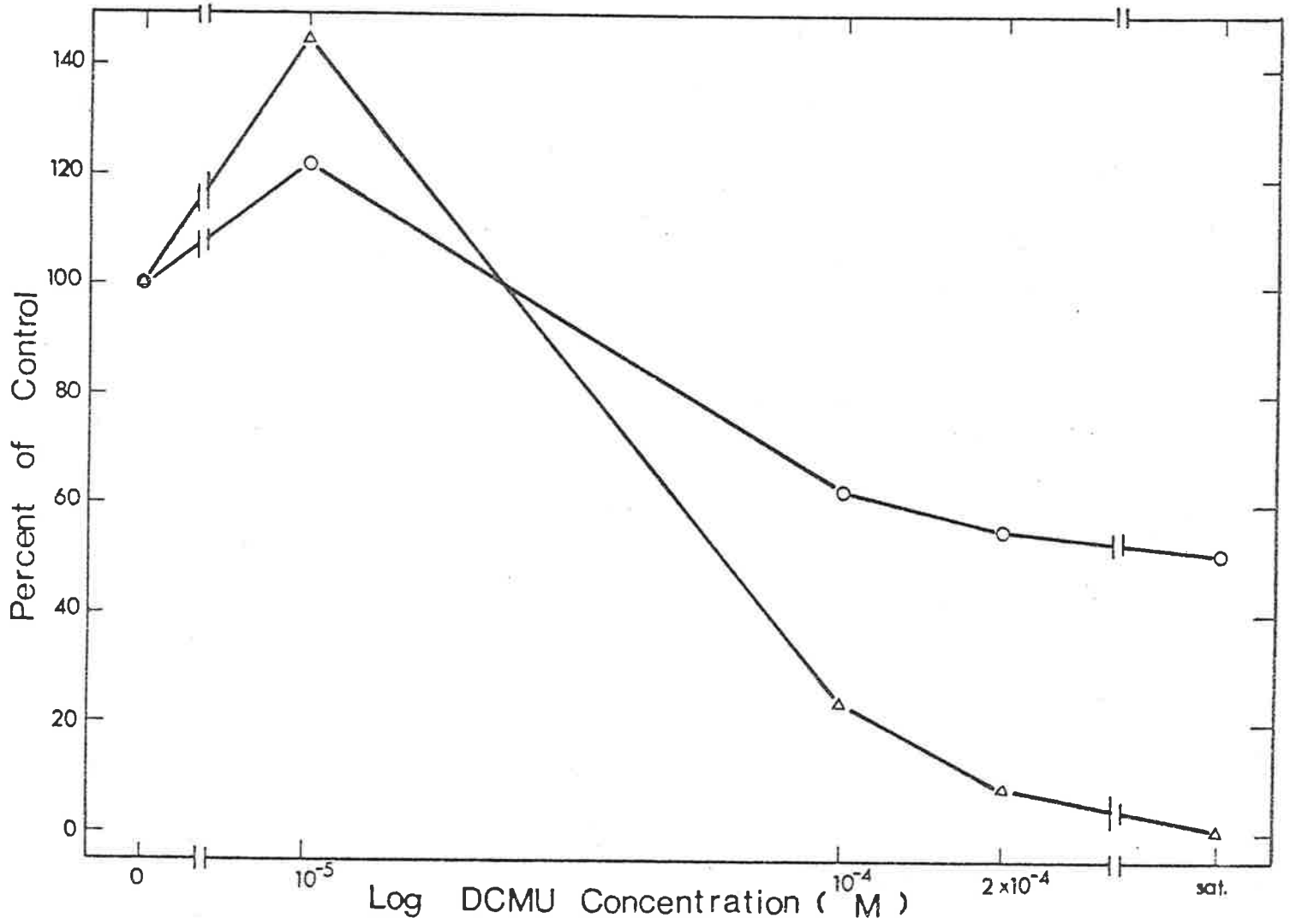
Δ Percent change in ratio of oxygen evolution (net photosynthesis) to oxygen absorption (repiration) over that of turgid untreated samples.

Table 5.11

Oxygen exchange rates in light and darkness

| Treatment | nmoles oxygen $cm^{-2}$ $hr^{-1}$ | | | P/R | Net $O_2$ evoln/ $O_2$absor |
|---|---|---|---|---|---|
| | Total photosynth. " $O_2$ evolution (P) | Net photosynth. $O_2$ evolution | Respiration $O_2$ absorption (R) | | |
| Turgid | 332.2 | 163.0 | 169.2 | 1.96 | 0.96 |
| $10^{-5}$M DCMU | 435.8 | 254.0 | 181.8 | 2.40 | 1.40 |
| $10^{-4}$M " | 362.8 | 66.1 | 296.7 | 1.22 | 0.22 |
| $2x10^{-4}$M " | 283.6 | 19.1 | 264.5 | 1.07 | 0.07 |
| sat. " | 173.1 | 0 | 173.1 | 1.00 | 0.00 |

Total 'Photosynthesis' = net photosynthesis - (respiration)


Percent of Control
140
120
100
80
60
40
20
0
0
$10^{-5}$
$10^{-4}$
$2 \times 10^{-4}$
sat.
Log DCMU Concentration ( M )

samples only were recorded, due to L-L samples showing very high respiration rates.

B. Net $CO_2$ exchange

In light, the $CO_2$ exchange (measured with the IRGA) remained the same as that of segments in the dark, when segments had been floated on a saturated solution of DCMU for 3 hours prior to studying $CO_2$ flux. This would imply that in light this tissue was not taking up any $CO_2$, but that respiration was unaffected by inhibitor treatment (Table 5.12).

### 5.5.4 Incubation on inhibitor in the dark prior to stress treatment

Though unlikely, the observed effect of inhibitors on proline accumulation in segments stressed in the dark may be due to enhanced respiration rates induced by DCMU treatment (Table 5.11). Another factor which may influence accumulation of proline is the incubation condition (viz in light or darkness) since it is possible that segments incubated on water in light (control) can continue to assimilate $CO_2$ during the incubation period while those on inhibitor do not. In this experiment, therefore, all incubations were conducted in the dark for 3 hours prior to stressing on PEG solutions in the light.

#### 5.5.4.1 Methods

Plant culture until 12 days of age was similar to that described in Section 5.4.1.1.1. Plants were transferred to continuous light or continuous darkness for 48 hours after which the first leaves were excised and segmented. The segments were floated on

Table 5.12

Carbon dioxide exchange in DCMU treated segments

| Treatment | Net $CO_2$ flux ($\mu g\ CO_2\ h^{-1} cm^{-2}$) | |
|---|---|---|
| | Light | Dark |
| L-L DCMU (sat) treated | 5.32 | 5.32 |
| L-L Turgid | 50.99 | 8.27 |

DCMU solutions of various concentrations or water for 3 hours in the dark, following which the segments were floated on -15 bar PEG for 24 hours, in continuous light or continuous darkness. The segments were then harvested and preserved and analysed for proline as detailed in Section 2.8 of the Materials and Methods.

5.5.4.2 Results

When segments were floated on DCMU in the dark prior to stressing, both L-L and D-L treatment showed a highly significant decline in free proline content (Fig 5.13), both accumulating proline to similar levels as the corresponding dark treatments when treated with saturated DCMU solutions. Although L-D treated segments showed a significant decline when treated with saturated DCMU solution, the decline was less than that of light stressed tissue (e.g. L-L showed a difference of 10 mg $g^{-1}$ dry wt with saturated inhibitor treatment, while L-D showed a corresponding decrease of 1.7 mg $g^{-1}$ dry wt in proline content) Table 5.14 and Fig 5.15 . D-D treatment on the other hand showed a decrease in proline content when treated with DCMU, which was not significant.

Thus the effect of DCMU on proline accumulation by segments stressed in light is dramatic, while in segments stressed in darkness the inhibition, though small, is still present. One possible cause for this could be the effect of DCMU on oxygen absorption (Table 5.11). One interesting point apparent upon comparing the results of the two experiments (Fig 5.11 and 5.13) is that the final amount of proline accumulated when segments are treated with saturated DCMU in either the stressed L-D or D-D treatments, in both experiments is very similar (ca 2.2 mg $g^{-1}$ dry wt in D-D and 7.0 mg $g^{-1}$ dry wt in L-D

Fig 5.13

Effect of different concentrations of DCMU on proline accumulation in stressed barley leaf segments.

Each value is the mean of 4 replicates.

○ L-L

□ D-L

● L-D

■ D-D

Table 5.13

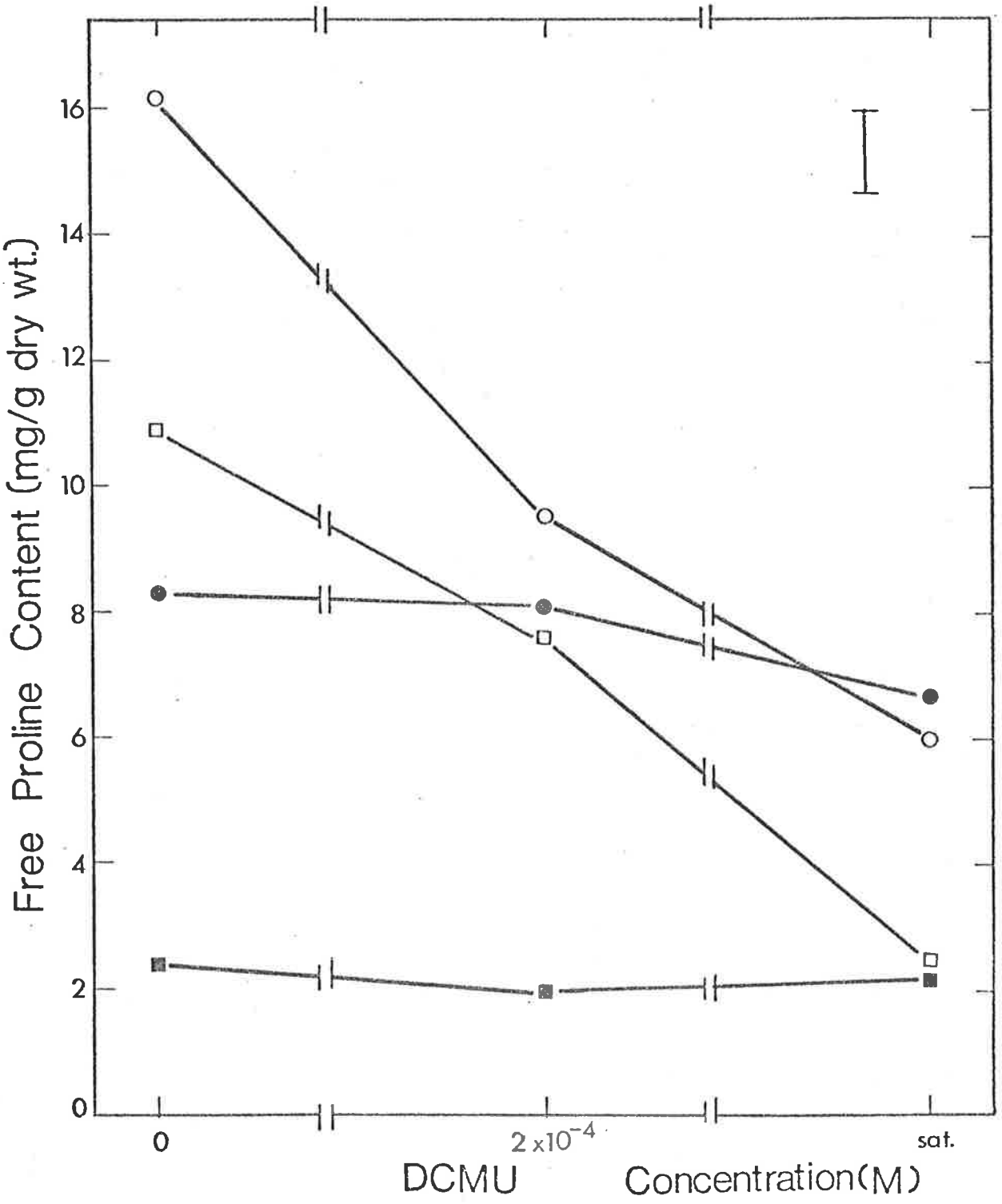
F table for results in Fig 5.13

| Source of variation | VR |
|---|---|
| $P_1$ | 275.54** |
| $P_2$ | 195.71** |
| Treat | 115.59** |
| $P_1 x P_2$ | 12.74** |
| $P_1$xTreat | 3.61 |
| $P_2$xTreat | 79.62** |
| $P_1 x P_2$xTreat | 4.55* |

Table 5.14

Proline accumulation in the presence of DCMU (provided in the dark) as a percentage of accumulation in untreated stressed tissue

| DCMU conc | 0 | $2x10^{-4}$M | saturated |
|---|---|---|---|
| L-L | 100 | 59 | 37 |
| L-D | 100 | 98 | 81 |
| D-L | 100 | 70 | 23 |
| D-D | 100 | 81 | 92 |


Free Proline Content (mg/g dry wt.)
16
14
12
10
8
6
4
2
0
0
2 x10⁻⁴
sat.
DCMU Concentration(M)

treatment). In contrast, the level of proline accumulation in stressed tissue not supplied with inhibitor was different in two experiments. This may perhaps imply that there is a distinct basal level of proline accumulation during stress, which is not affected by inhibitor treatment.

Results presented so far, show that inhibitors which impede electron flow or photosystem II activity also significantly inhibit proline accumulation. A characteristic of electron flow inhibitors is that both electron flow and the associated ATP formation are depressed to a similar extent (Good and Izawa, 1973). Very high concentrations of DCMU ($>10^{-4}$) are also reported to produce a secondary effect, an inhibition of cyclic phosphorylation probably through uncoupling (Good and Izawa, 1973). Since a significant inhibition of proline accumulation occurs only at concentrations greater than $10^{-4}$M DCMU (Fig 5.11 and 5.13) it is possible that proline accumulation is only inhibited when cyclic phosphorylation is impeded. Alternatively, if the contribution of photosynthesis (photosynthates and/or assimilatory power) to proline accumulation is small, then a significant decrease in proline level would occur only when photosynthesis is severely inhibited (Table 5.11).

The next experiment was designed to study the contribution of photophosphorylation to proline accumulation using uncouplers of phosphorylation.

## 5.6 Effect of uncouplers

Electron transport may be dissociated from ATP formation. This dissociation frees the electron transport in such a manner that it may proceed appreciably faster than when coupled to photophosphorylation. ATP formation ceases and the dissociation of electron transport from phosphorylation is known as uncoupling (Good and Izawa, 1973).

### 5.6.1 Effect of carbonylcyanide m-chloro phenylhydrazone (CCCP) on proline accumulation

This compound is a powerful inhibitor of oxidative phosphorylation (effective at $10^{-6}$M) and also photophosphorylation (though at a higher concentration than that required to inhibit oxidative phosphorylation Heytler, 1963). (Isolated mitochondrial phosphorylation was inhibited 100% at $7.6 \times 10^{-6}$M while isolated chloroplast phosphorylation was inhibited only 55 to 60% at a similar concentration of CCCP (Heytler, 1963).)

It was hoped that studies of the effect of this uncoupler on proline accumulation would provide some data on the contribution of phosphorylation to proline accumulation.

#### 5.6.1.1 Methods

Plant growth to an age of 12 days was exactly as described in Section 5.1.1.1. After 48 hours continuous light or darkness, the first leaves were excised and segmented (1.5 cm). These segments were either floated on aqueous CCCP solution ($10^{-5}$M or saturated CCCP) or water for 3 hours under light conditions prevalent during $P_1$; some segments were infiltrated with a saturated solution of CCCP for 2 minutes with

the help of a water pump. The segments were then transferred to -15 bar PEG solutions and left in continuous light or dark conditions, for 24 hours . Segments were harvested, rinsed, preserved and analysed for proline as detailed in Section 2.8 of Materials and Methods.

5.6.1.2 Results

A. Floating segments on inhibitor solution inhibited accumulation in segments in darkness during $P_1$ treatment (i.e. D-L and D-D), while segments exposed to 48 hours light during $P_1$ showed no inhibition of proline accumulation (Fig 5.14, Table 5.15).

B. Infiltrating segments with inhibitor solution on the other hand, had a marked effect in all treatments (Table 5.13). Maximum inhibition occurred in segments stressed in darkness while segments in light during $P_2$ also showed some inhibition (Fig. 5.14 and Table 5.17).

One possible reason for the lack of inhibition when segments were floated on CCCP in light could be the sensitivity of CCCP to light resulting in its rapid breakdown (Wiskich, pers comm).

Since inhibition is the least in L-L treated segments, and oxidative phosphorylation is more sensitive to CCCP than photophosphorylation (Heytler, 1963) it could be interpreted to mean that proline accumulation is more dependant on oxidative phosphorylation in segments in the dark during $P_1$ or stressed in darkness; while in L-L segments either contribution of total phosphorylation is small or that of oxidative phosphorylation is insignificant.

Fig 5.14

Effect of uncoupler CCCP on proline accumulation

Barley plants were grown in sand at 20°C in a 16 hour photoperiod until they were 12 days old. The plants were transferred to 48 hours continuous light or continuous darkness at the end of which the first leaves were excised and segmented (1.5 cm long). The segments were either floated on water or CCCP solutions ($10^{-5}$M and saturated) for 3 hours or infiltrated with saturated CCCP for 2 minutes. Segments were then transferred to -15 bars PEG solution and left for 24 hours in continuous light or continuous darkness.

Each value is the mean of 4 replicates.

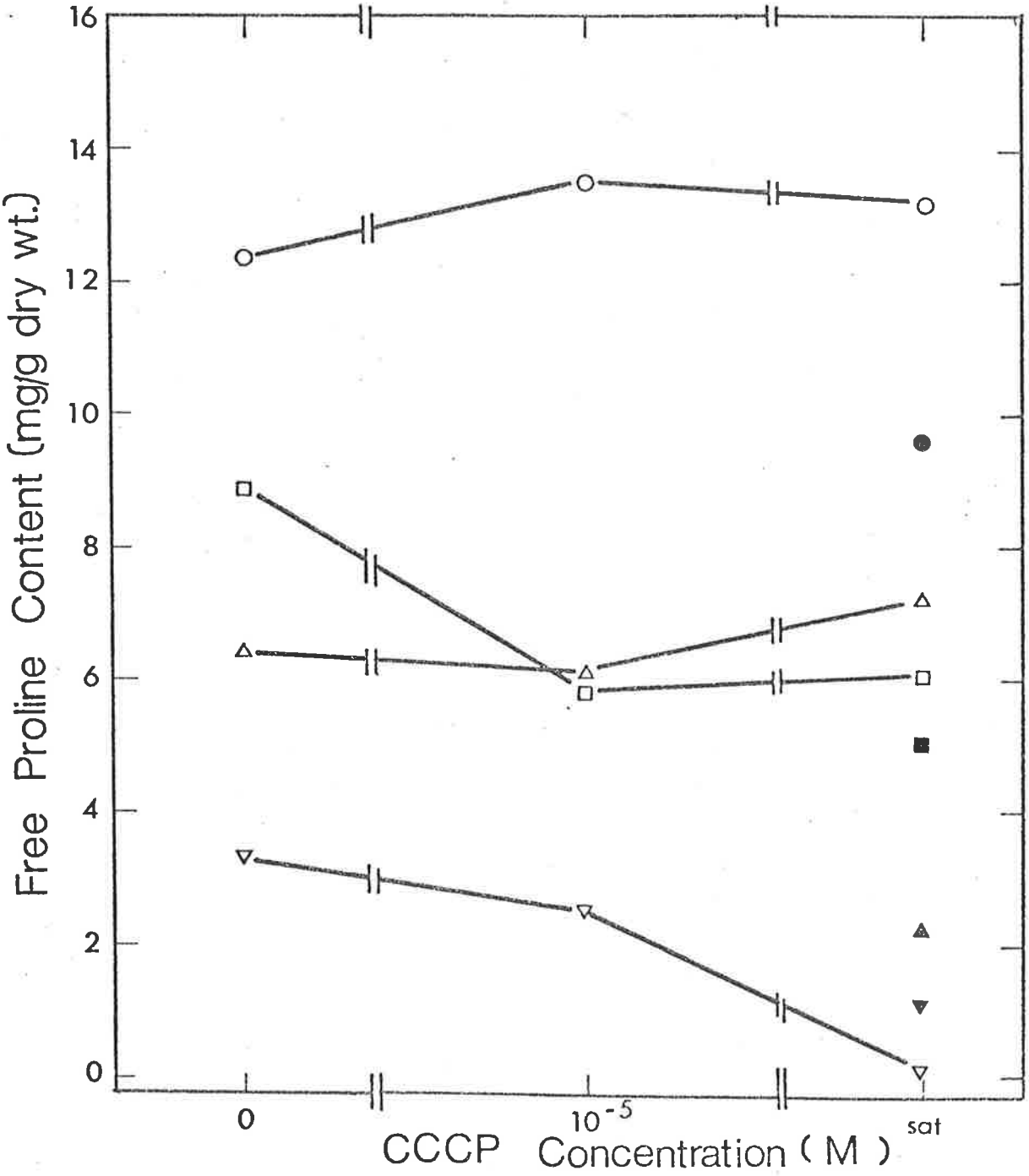
| Floated on inhibitor | | Infiltrated with inhibitor | |
|---|---|---|---|
| ○ | L-L treatment | ● | L-L treatment |
| □ | D-L " | ■ | D-L " |
| △ | L-D " | ▲ | L-D " |
| ▽ | D-D " | ▼ | D-D " |

Table 5.15

Proline accumulation in the presence of CCCP as a % of accumulation in untreated tissue

| Inhibitor Concentration | 0 | $10^{-5}$M | saturated | Infiltrated saturated |
|---|---|---|---|---|
| Treatment | | | | |
| L-L | 100 | 107 | 107 | 78 |
| L-D | 100 | 96 | 113 | 37 |
| D-L | 100 | 65 | 69 | 58 |
| D-D | 100 | 75 | - | 36 |

- not detectable


Free Proline Content (mg/g dry wt.)
16
14
12
10
8
6
4
2
0
0
10-5
sat
CCCP Concentration ( M )

### 5.6.2 Ammonium chloride and proline accumulation

Ammonium chloride is an uncoupler. However, it differs from CCCP in that it does not inhibit oxidative phosphorylation (Good and Izawa, 1973). Since CCCP inhibits proline accumulation to some extent, it was hoped that by studying the effect of ammonium chloride on proline accumulation, it would be possible to derive the contribution of photophosphorylation to the phenomenon.

#### 5.6.2.1 Methods

Plant culture and treatments were identical to those described in Section 5.5.1.1 except that segments were floated on different concentrations of ammonium chloride ($10^{-3}$, $10^{-2}$ or $10^{-1}$M $NH_4Cl$) or water for 3 hours prior to stressing on PEG (-15 bar) solution for 24 hours in continuous light or darkness. Segments were harvested and preserved for analysis as explained in Materials and Methods in Section 2.8.

#### 5.6.2.2. Results

A stimulation of proline accumulation by the presence of $10^{-3}$M ammonium chloride was observed (Fig 5.15 and Table5.17) in all treatments except D-D which showed a marked decrease. Increasing ammonium chloride concentration, however, led to a decrease in proline accumulation particularly in treatments where stress was imposed in the dark (D-D and L-D). Tissue stressed in the light showed less inhibition by ammonium chloride at all concentrations.

A likely cause for this lack of inhibition by ammonium chloride

Fig 5.15

Effect of different concentrations of $NH_4Cl$ on proline accumulation in first leaf segments of barley floated on -15 bars PEG solution. Each value is the mean of 4 replicates

○ L-L

□ D-L

△ L-D

▽ D-D

Table 5.16

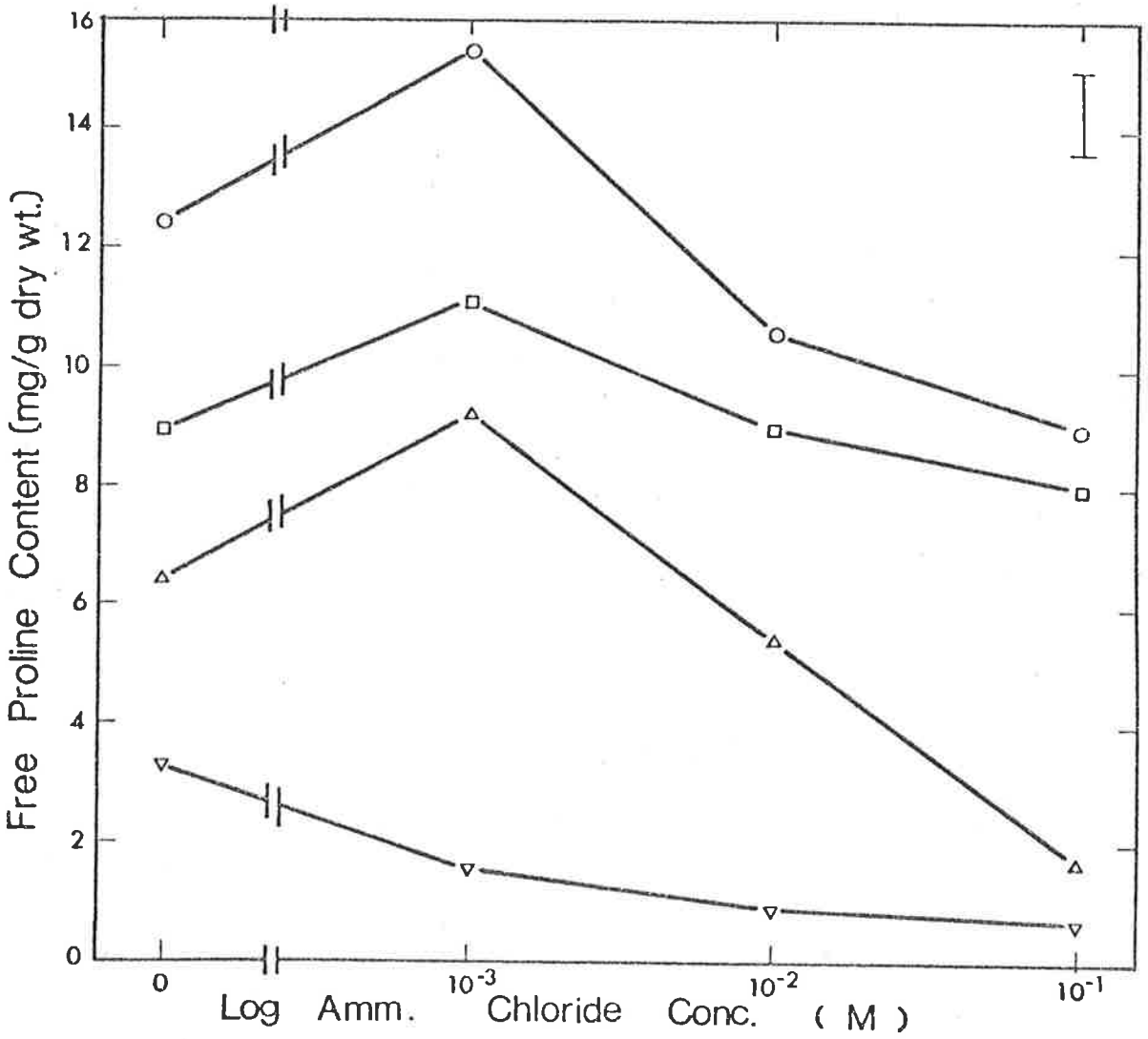
F table for results in Fig 5.15

| Source of variation | F value |
|---|---|
| $P_1$ | 195.56** |
| $P_2$ | 844.04** |
| Treat | 63.57** |
| $P_1.P_2$ | 9.28** |
| $P_1$.Treat | 17.70* |
| $P_2$.Treat | 3.31 |
| $P_1.P_2$.Treat | 4.10* |

Table 5.17

Proline accumulation in the presence of NH4Cl as a percentage of accumulation in untreated stressed tissue

| Inhibitor conc | 0 | $10^{-3}$ | $10^{-2}$ | $10^{-1}$ |
|---|---|---|---|---|
| L-L | 100 | 123 | 86 | 73 |
| L-D | 100 | 143 | 84 | 27 |
| D-L | 100 | 125 | 101 | 90 |
| D-D | 100 | 49 | 27 | 22 |


Free Proline Content (mg/g dry wt.)
16
14
12
10
8
6
4
2
0
0
$10^{-3}$
$10^{-2}$
$10^{-1}$
Log Amm. Chloride Conc. ( M )

in light treated samples could be the alkaline pH requirement for effectivity (Wiskich pers. comm.). When pH was increased from 5 to 10, the concentration of ammonium chloride necessary to uncouple isolated chloroplast photophosphorylation decreased from 1.0M to 0.05M. Also it is possible that the continued presence of ammonium ions is necessary for uncoupling chloroplasts (Wiskich, pers. comm.).

### 5.6.3 Proline accumulation and ammonium chloride treatment under varying conditions

Lack of inhibition of proline accumulation by ammonium chloride (when segments were floated on the solution for 3 hours) in segments exposed to light during stress may well have been due to the problem mentioned above. In this next section therefore, experiments were designed to test the effect of ammonium chloride at a higher pH (8.0) and also at a higher concentration. In addition, the continued presence of ammonium chloride (over the 24 hour stress period) was also studied.

#### 5.6.3.1 Methods

A 0.1M potassium phosphate buffer at pH 8.0 was prepared. To this buffer, an equivalent quantity of ammonium chloride and PEG was added to result in a 0.1M ammonium chloride solution in a -15 bar PEG solution. Controls consisted of segments floated on -15 bar PEG solution prepared in the above buffer. Also 0.1M and 1M ammonium chloride solutions were prepared on -15 bar PEG solutions at pH 7.1. In addition, the response of segments to ammonium vapours was also studied, by floating segments on -15 bar PEG solution in a petri dish, which also had a concentrated $NH_4OH$ solution in a small petri dish floating in it.

Plants were grown for 12 days and given 48 hours continuous light/dark treatment as detailed in Section 5.1.1.1. The first leaves were then excised, cut into 1.5 cm segments and floated on the various solutions mentioned. Each treatment consisted of 4 replicates. After 24 hours, the segments were harvested, preserved and analysed for proline as detailed in Materials and Methods (Section 2.8).

5.6.3.2 Results

It was found that tissue on ammonium chloride at pH 8.0, when stressed tended to get oxidized (bleached) particularly D-L segments which were completely bleached, while L-L segments appeared green except for the ends which were bleached. Segments stressed in the presence of ammonia vapours were also completely bleached (Table 5.18).

Samples on PEG (-15 bars) at pH 8.0 showed an enhancement in L-L, while D-L showed a decrease in proline accumulation compared with accumulation on PEG (-15 bars) at pH 7.1 (Table 5.18).

The continued presence of 0.1M ammonium chloride inhibited proline accumulation in all treatments. 1M ammonium chloride appeared to be an extremely severe treatment, completely inhibiting proline accumulation resulting in levels present in turgid tissue. This is probably expected as 1M solutions of any compound can induce toxic and damaging (and also osmotic) effects on plant metabolism.

The continued presence of 0.1M ammonium chloride was effective in inhibiting proline accumulation, but did so in the dark treated tissue as well. The $CO_2$ flux was studied in order to see the effect

Table 5.18

Effect of pH and ammonium chloride on proline accumulation (mg $g^{-1}$ dry wt) in barley segments. Each value is the mean of 4 replicates.

| Treatment | L-L | L-D | D-L | D-D |
|---|---|---|---|---|
| PEG only (pH 7.1) | 17.21 (100) | 6.60 (100) | 10.22 (100) | 2.21 (100) |
| PEG+0.1M $NH_4Cl$ (pH 7.1) | 14.46 (84) | 2.47 (37) | 5.83 (57) | 1.02 (47) |
| PEG+1M $NH_4Cl$ (pH 7.1) | 0.53 (3) | - | 0.26 (3) | - |
| PEG only (pH 8.0) | 20.96 (122) | - | 6.33 (62) | - |
| PEG+0.1M $NH_4Cl$ (pH 8.0) | 10.41? (61) | - | ?? | - |
| PEG + $NH_3$ vapours | ?? | - | ?? | - |

? = Treatments cuasing bleaching of ends of segments

?? = Treatments causing bleaching of the entire segments

- = Treatment not performed

Figures in parantheses are the proline content: expressed as a percentage of that in segments floated on -15 bars PEG solution at pH 7.1.

of this inhibitor on respiration and photosynthesis.

The results showed that segments floated on 0.1M ammonium chloride solution for 3 hours showed no inhibition of $CO_2$ influx (apparent photosynthesis) while $CO_2$ efflux (respiration) was drammatically increased ($\simeq$ two-fold) (Fig 5.16, Table 5.19). This may be a possible cause for the inhibition by ammonium chloride of proline accumulation. It may be that enhanced respiration implies less precursors available for proline synthesis, resulting in lowered proline content, particularly under limiting conditions where reserves are limiting (i.e. D-D, L-D treatment). One possible cause for enhanced respiration may be active chloride uptake by the leaf segments which were floated on ammonium chloride solution (Luttge et al, 1971 and Rains, 1968).

## 5.7 Discussion

Gas exchange. The $CO_2$ flux of turgid segments after exposure to 48 hours continuous light was less than that of turgid tissue exposed to continuous darkness for a similar period. The idea that photosynthesis may be under negative feedback control from its products has been discussed for some years (Neales and Incoll, 1968). Milford and Pearman (1975) have compared photosynthesis rates of sugar beet leaves exposed to 48 hours continuous light or continuous darkness and also concluded that the leaves of light pretreated plants containing a large concentration of carbohydrates photosynthesized more slowly than leaves of dark pretreated plants containing much less carbohydrates (Fig 5.16). Pitman et al (1975) studied diurnal trends and also showed a decrease in photosynthesis rate in barley leaf slices at midday when assimilate concentration was higher. Thus, the lower $CO_2$

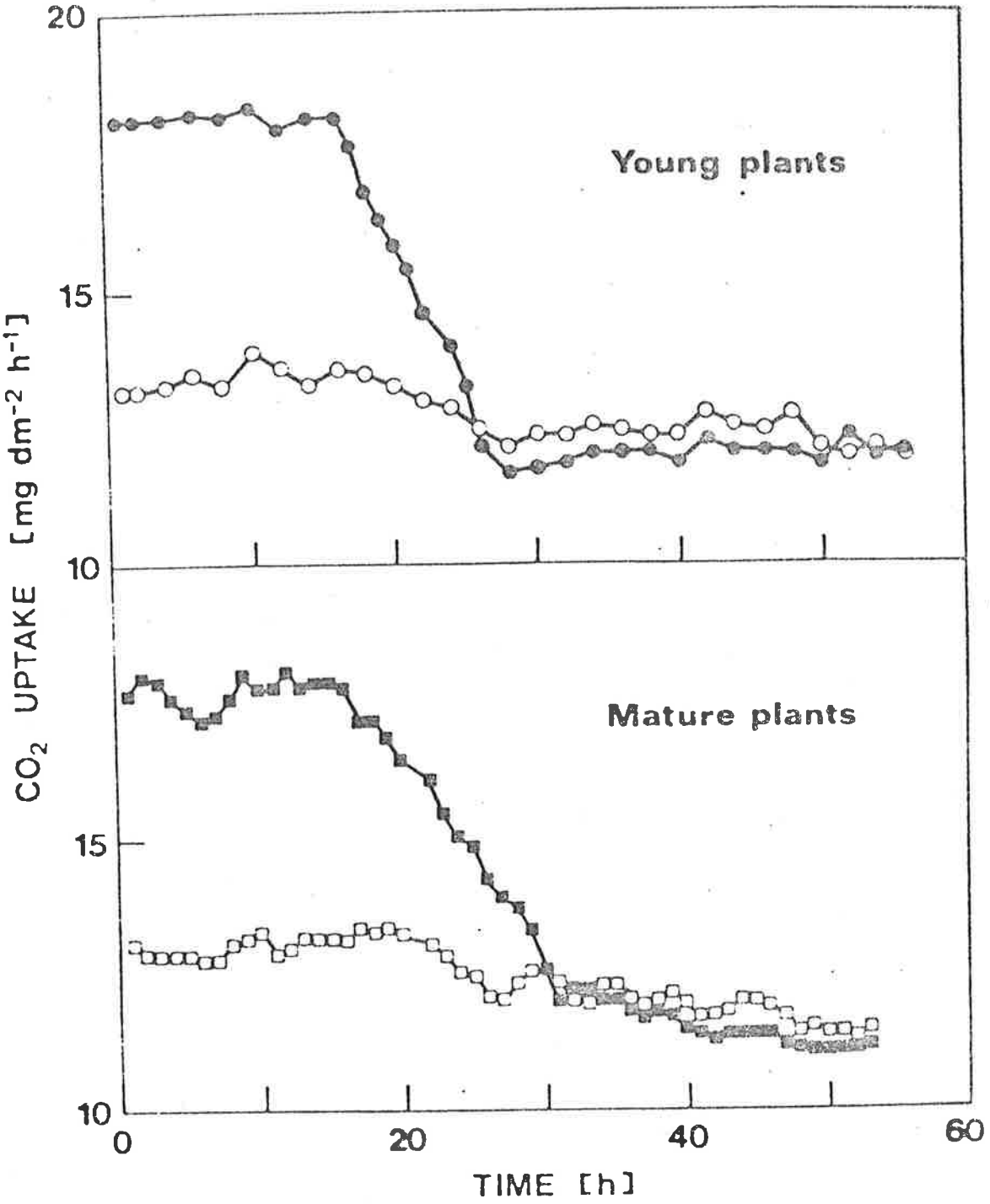
Table 5.19

Carbon dioxide exchange of inhibitor treated samples

| | | $CO_2$ flux in light 'Photosynthesis' | $CO_2$ flux in dark 'Respiration' | $CO_2$ efflux as % of $CO_2$ influx |
|---|---|---|---|---|
| | L-L | 52.81 | 9.985 | 18.5 |
| .1M $NH_4Cl$ | L-L | 56.39 | 17.73 | 31.4 |

Fig 5.16

Changes in photosynthetic rates of leaves of light-pretreated (O, □ ) and dark-pretreated (●, ■ ) sugar beet in continuous light. (Adapted from Milford and Pearman, 1975).


20
15
10
Young plants
$CO_2$ UPTAKE [mg dm$^{-2}$ h$^{-1}$]
15
10
Mature plants
0
20
40
60
TIME [h]

uptake observed in light pretreated turgid tissue (L-L) compared to dark pretreated trugid tissue (D-L) could be due to assimilate control over photosynthesis. In addition, in the present experimental system, such feedback inhibition may have been accentuated by the use of leaf segments, since translocation in the phloem has been eliminated.

Stressing the tissue induced a rapid decline in the $CO_2$ flux rate. Using leaf segment systems, it was shown that the decline occurs approximately 1 hour after the commencement of stress. A similar rapid decrease in $CO_2$ uptake during water stress has been reported for sunflower leaves (Boyer, 1973) and wheat leaves (Lawlor, 1976a,b). This decline in photosynthesic rate may be due to stomatal or mesophyll resistances, and/or inhibited chloroplast activity. Results obtained with the infra red gas analyser and the oxygen electrode would support the hypothesis that the decrease in $CO_2$ exchange in light during stress is a consequence of a decrease in chloroplast activity (20% to 40% inhibition after 7 hours stress) as well as increased resistance to $CO_2$ exchange, since total inhibition of net $CO_2$ exchange during stress was 95% of that of turgid samples. Studying oxygen evolution only, Jones and Osmond (1973) reported a similar effect of -17 bars osmotic stress on cotton leaf slices.

The lack of stimulation of proline accumulation when the $CO_2$ concentration was increased could be due to either an absence of an increase in $CO_2$ fixation at enhanced $CO_2$ levels or a lack of effect of enhanced photosynthesis on proline accumulation. Since photosynthesis rates were not measured, it is difficult to eliminate either possibility. However, it is interesting to note that photosynthesis in stressed wheat leaves (-18 bars) failed to respond to enhanced $CO_2$ concentration (Lawlor, 1976).

While increased $CO_2$ concentration above ambient appeared to not affect proline accumulation, depleting the $CO_2$ from the air induced a significant reduction (40%) in proline accumulation in dark pretreated segments. In L-L segments on the other hand, $-CO_2$ treatment had no effect. One possible explanation is that $CO_2$ assimilation during the prestress period in L-L treatment provided sufficient carbon compounds (utilised for proline synthesis) or influenced proline accumulation indirectly (e.g. carbohydrates which have been reported to affect proline accumulation either by stimulating synthesis of proline or inhibiting proline oxidation (Stewart, 1972, 1978).. In the D-L treatment, on the other hand, it is possible that they were depleted in reserves and that $CO_2$ assimilation products were consequently important for proline accumulation. Yet both dark and light pretreated tissue show a marked enhancement of proline accumulation when subjected to stress in light rather than in the dark. This could be explained in light pretreated tissue, where carbohydrate reserves are not limiting, only the photochemical energy rich products are required for proline accumulation while in the dark pretreated tissue, where the reserves are depleted, both carbon compounds and assimilatory power are essential.

It is also possible that $-CO_2$ treatment does not inhibit proline accumulation in light pretreated segments because of intercellular reassimilation of respired $CO_2$, especially since $CO_2$ loss to the atmosphere would be limited by increased stomatal and mesophyll resistances during stress. Indeed, the respiration rates in L-L were higher than in D-L, suggesting that more $CO_2$ would be available for refixation. Also, since proline accumulation of stressed tissue within a 125 ml flask (Appendix II) over a 24 hour period, it would appear that a basal low $CO_2$ fixation rate is necessary and sufficient for proline accumulation.

It has also been suggested that $-CO_2$ treatment inhibits monocyclic photophosphorylation and electron flow but does not affect cyclic photophosphorylation, so long as there is a reductant present (Simonis and Urbach, 1973). Therefore, it could be postulated that the lack of inhibition by $-CO_2$ treatment in L-L segments is due to cyclic phosphorylation being sufficient to support proline accumulation in this treatment. That this is not true is clear from the results obtained with inhibitors. Both CMU and DCMU inhibit proline accumulation significantly although cyclic phosphorylation is known to be insensitive to these compounds. Similarly in Chlorella, Greenway and Setter (1979) have reported an inhibition of proline accumulation in cells stressed in the light in the presence of DCMU. Also, if cyclic photophosphorylation was adequate to enhance proline accumulation in L-L segments, then exposing segments to 700 nm wavelength should have enhanced proline accumulation to levels induced by white light. However, this was not observed (Section 4.2). It is interesting that the effect of uncouplers and inhibitors of photosynthesis was more profound in dark pretreated tissue (D-L) than light pretreated ones. This again suggests the relative contribution of photosynthesis towards proline accumulation in light.

Thus, it can be concluded that both carbon dioxide fixation products and assimilatory power are important and responsible for enhanced accumulation of proline by segments stressed in light, particularly if the tissue is low in reserves at the commencement of stress.

## SECTION I WATER STRESS

### 6. Studies with radioactive carbon compounds

# 6. Studies with Radioactive Carbon Compounds

## 6.1 Introduction

Results presented so far show that photosynthesis during stress influences proline accumulation in both starved (D-L) and non-starved (L-L) tissue, but the manner in which this occurs is not clear. Both the carbohydrates and the energy produced during photosynthesis have been held responsible for enhancing proline accumulation during stress (Stewart et al, 1966; Stewart, 1972, 1978; Hanson and Tully, 1979). Interpretations of the manner in which carbohydrates influence proline accumulation include a direct role in the supply of carbon skeletons for proline synthesis (Stewart et al, 1966) and an indirect effect on proline metabolism (Stewart, 1972, 1978). The following experiments were performed to study the manner in which light stimulated proline accumulation in the excised barley leaf and to explore the role of current photosynthesis on proline accumulation.

## 6.2 Total ethanol soluble carbohydrate content and stress

This experiment was designed to study the changes occurring in the total ethanol soluble carbohydrate content during stress, at different irradiances.

### 6.2.1 Methods

Twelve day old barley plants growing in sand at 20°C in a 16 hour photoperiod were transferred to 48 hours continuous light or continuous darkness. The first leaves were then excised, segmented and floated on -15 bar PEG solution or water in either darkness or light at 30 or

300 μE $m^{-2}s^{-1}$. The segments were harvested and frozen after 20 and 26 hours and the carbohydrate content of the freeze dried sample was analysed as described in Materials and Methods Section 2.8. An analysis of variance was performed on the data to test for significance.

### 6.2.2 Results

Tissue exposed to 48 hours light during the prestress period showed significantly higher carbohydrate content than segments in continuous darkness during the prestress period in both stress and turgid treatments at all irradiances (Fig 6.1 and Table 6.1, 6.2a,c,f) in both harvests. Segments floated in darkness contained a similar concentration of carbohydrates in stress and turgid tissue at both harvests and this was significantly lower than that in illuminated tissue (Table 6.2b,d,e). Illuminated segments had significantly higher carbohydrate concentrations after 26 hours of incubation. It is interesting that, whereas the carbohydrate content of illuminated and stressed segments changed little between the 20 and 26 hour samples, the illuminated turgid segments markedly increased in carbohydrate content (Fig 6.1). The total carbohydrate content of the D-L stressed segments was also similar to that of the L-D stressed segments. Thus, all stressed samples at 30 and 300 μE $m^{-2}s^{-1}$ showed similar carbohydrate content while samples in the dark showed significantly lower concentrations particularly in starved segments.

## 6.3 Effect of an exogenous supply of carbon compounds on proline accumulation

It is evident from previous results (Section 6.2) that segments stressed in light show a higher carbohydrate content than ones in

Fig 6.1

Total soluble carbohydrate content in barley segments floated on water or PEG (-15 bars) at different irradiances.

Twelve day old barley plants were transferred to 48 hours continuous light or darkness. The first leaves were then segmented and floated on water or -15 bar PEG solution at 250 μE $m^{-2}s^{-1}$, 30 μE $m^{-2}s^{-1}$ or in the dark. The carbohydrate content was estimated in the freeze dried samples.
Each value is the mean of 4 replicates.

○ turgid)
Light during $P_1$ (prestress period)
● stress)

□ turgid)
Darkness during $P_1$ (prestress period)
■ stress)

Fig A. After a 20 hour incubation period

B. After a 26 hour incubation period

Table 6.1

F Table of results in Fig 6.1

| Source of variation | VR (20 hr) | VR (26 hr) |
|---|---|---|
| Prestress treatment ($P_1$) | 43.12 ** | 31.79 ** |
| Irradiance (I) | 19.73 ** | 46.13 ** |
| Stress (S) | 0.32 | 6.94 * |
| $P_1$ x I | 1.05 | 1.09 |
| $P_1$ x S | 5.33 * | 1.22 |
| I x S | 1.09 | 4.93 * |
| $P_1$ x I x S | 0.55 | 0.98 |

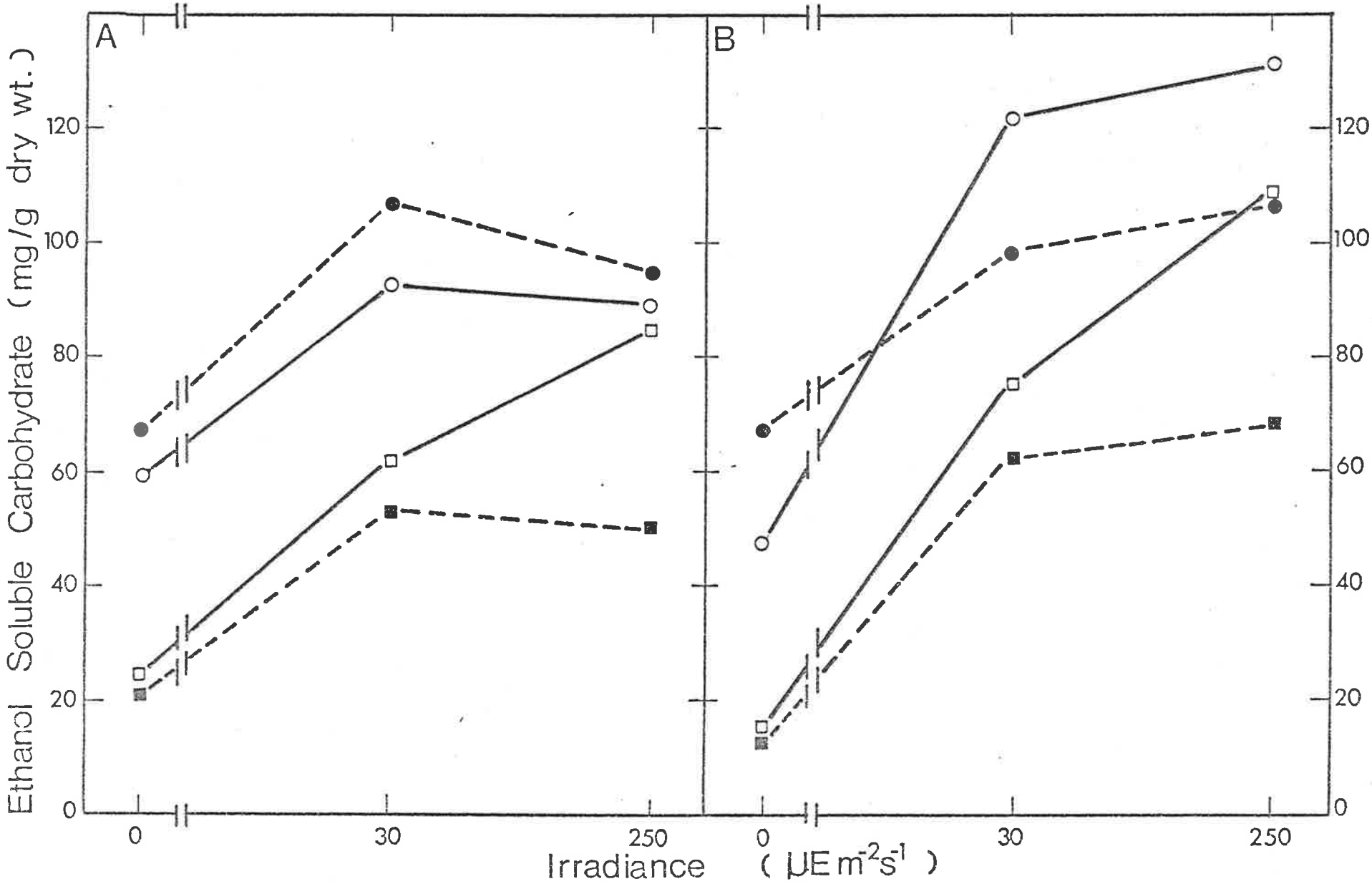

A
B
Ethanol Soluble Carbohydrate ( mg/g dry wt. )
0
20
40
60
80
100
120
0
30
250
Irradiance ( μE m-2s-1 )

Table 6.2 Table of Means of results in Fig 6.1

20 hour sample

a) $P_1$ x Stress

| Treatment | Stress | Turgid |
|---|---|---|
| $P_1$ | | |
| Light | 89.6 | 80.1 |
| Dark | 41.4 | 57.0 |
| LSD | 13.0 | |

b)

| Irradiance | |
|---|---|
| 300 | 79.5 |
| 30 | 78.6 |
| Dark | 42.9 |
| LSD | 11.2 |

c)

| $P_1$ | |
|---|---|
| Light | 84.9 |
| Dark | 49.2 |
| LSD | 9.2 |

26 hour sample

d) Irradiance x Stress

| Treatment | Stress | Turgid |
|---|---|---|
| Irrad | | |
| 300 | 87.0 | 127.3 |
| 30 | 80.0 | 98.2 |
| Dark | 39.8 | 31.4 |
| LSD | 22.3 | |

e)

| Irradiance | |
|---|---|
| 300 | 107.2 |
| 30 | 89.1 |
| Dark | 35.6 |
| LSD | 15.7 |

f)

| $P_1$ | |
|---|---|
| Light | 95.1 |
| Dark | 59.4 |
| LSD | 12.9 |

g)

| Stress Treatment | |
|---|---|
| Stress | 68.9 |
| Turgid | 85.6 |
| LSD | 12.9 |

darkness especially in starved (held in darkness for 2 days prior to stressing) samples. Light enhances proline accumulation in response to stress in both starved and non-starved (held in light for 2 days prior to stressing) segments. Light could increase the supply of either the necessary carbon skeletons and/or the reduced nitrogen for proline synthesis. The aim of this experiment was therefore to study proline accumulation in samples provided with carbon and nitrogen compounds. Compounds containing both carbon and nitrogen (amino acids) or only carbon were provided to the tissue either prior to stressing or continuously throughout the stress period.

### 6.3.1 Methods

Twelve day old barley plants growing in a 16 hour photoperiod at 20°C were transferred to 48 hours continuous light or continuous darkness. The first leaves were then excised and placed in a beaker containing the desired solution and allowed to take up the solution through the leaf base, which was re-cut after placing them in the beakers. The leaves were harvested after 3 hours and cut into 1.5 cm segments and floated on -15 bar PEG solution for 24 hours. In some cases (dark pretreated samples only) where the continuous presence of the metabolite was being studied, the segments were floated on a -15 bar PEG solution made up in the metabolite solution instead of water. The various metabolites tested were glutamate (0.5 mg $ml^{-1}$), glutamine (0.5 mg $ml^{-1}$), α-oxoglutarate (0.5 mg $ml^{-1}$), sucrose (0.1M) glucose (0.1M) and 3-phosphoglyceric acid (0.5 mg $ml^{-1}$). A student's T-test was performed on the data to test for significance.

### 6.3.2 Results

Samples prestressed in light

The proline concentration in tissue stressed in the dark was lower than that in tissue stressed in the light (Table 6.3), despite the presence of the metabolites. Glutamate and 3-phosphoglycerate (3PGA) increased the proline content of such segments, but glutamine had no effect. In samples stressed in the light 3PGA alone increased proline content while both glutamate and glutamine were ineffective. This response is difficult to interpret as 3PGA, an energy rich compound, is utilized in glycolysis to yield ATP, in addition to carbon, and the stimulation due to this compound may suggest that proline accumulation was enhanced by the provision of energy. But proline accumulation is saturated at an irradiance well below that supplied in this experiment and the energy from photosynthesis should not have been limiting.

Samples prestressed in darkness

As with the previous response, the proline concentration in samples stressed in darkness was always lower than that in samples in the light (Table 6.4). Only the carbohydrates (glucose and sucrose) increased proline content significantly in dark stressed samples. In samples stressed in the light, all the metabolites except oxoglutarate tended to reduce proline accumulation, particularly where they were provided continuously (Table 6.4).

These results support earlier reports (Stewart et al., 1966; Singh et al., 1973b; Greenway and Setter, 1979) that dark treated tissue show enhanced accumulation of proline during stress when supplied with carbohydrates. In this case, however, exogenous glutamate increased

Table 6.3

Influence of exogenous metabolites on proline accumulation in segments prestressed in light

| | Proline content (mg $g^{-1}$ dry wt) | |
|---|---|---|
| Treatment | L-L | L-D |
| Untreated | 16.2 | 7.0 |
| Glutamate (@ 0.5 mg $ml^{-1}$) | 16.4(101) | *9.3(134) |
| Glutamine (@ 0.5 mg $ml^{-1}$) | 16.5(102) | 7.1(101) |
| 3 PGA (@ 1 mg $ml^{-1}$) | *18.2(112) | *8.6(123) |

* Significantly different from untreated samples at $P \leq 0.05$

Figures in parenthesis are the proline content expressed as a percentage of that in the untreated samples.

Each value is the mean of 3 replicates.

No adjustment for differences in pH was made.

Table 6.4

Influence of exogenous metabolites on proline accumulation in segments prestressed in the dark

| | Proline content (mg $g^{-1}$ dry wt) | | | |
|---|---|---|---|---|
| | D - L | | D - D | |
| Treatment | (3 hours) | (continuous) | (3 hours) | (continuous) |
| Untreated | 6.6 | 6.6 | 1.2 | 1.2 |
| Glutamate @ 0.5 mg $ml^{-1}$ | 5.2(80) | *3.4(52) | 1.2(100) | 1.0(83) |
| Glutamine @ 0.5 mg $ml^{-1}$ | 6.1(92) | *3.4(52) | 1.1(94) | 1.4(125) |
| α-oxoglutarate @ 0.5 mg $ml^{-1}$ | 7.2(109) | - | 0.8(71) | - |
| Sucrose (.1M) | 5.7(86) | 5.6(84) | *1.8(156) | *3.4(294) |
| Glucose (.1M) | - | - | *2.2(195) | - |

* Significantly different from untreated samples at 5% level of probability.

Figures in parenthesis are the proline content expressed as a per cent of that in untreated samples.

Each value is the mean of 3 replicates.

proline accumulation only in light prestressed samples (i.e. samples fairly high in carbohydrates). This contrasts with the reports of Singh et al (1973b) who demonstrated increased accumulation of proline in etiolated barley segments in response to glutamate alone or with sucrose.

## 6.4 Studies with radioactive carbon dioxide

The contribution of current photosynthates to stress stimulated proline accumulation is as yet unknown. Products of photosynthesis (PGA and carbohydrates) and glutamate were the only exogenous compounds which significantly enhanced proline accumulation and it is conceivable that a major proportion of the photosynthate fixed during stress serves as precursor for proline synthesis. This was examined in the following experiment.

### 6.4.1 Methods

Twelve day old barley plants grown in a 16 hour photoperiod at 20°C were transferred to 48 hours continuous darkness. The first leaves were then excised, segmented and floated on PEG (-15 bar) solution in 250 ml conical flasks for 24 hours. At the same time, 10 µl $NaH^{14}CO_3$ (1 µCi) was introduced in the flask (as described in Materials and Methods) and $^{14}CO_2$ liberated with excess 50% lactic acid. The flask was sealed and left in the light at 20°C for 24 hours. Proline accumulates to the same extent in a closed atmosphere as in an open system, so estimation of the contribution of current photosynthesis to proline accumulation during the 24 hour period of this experiment should not be limited by $CO_2$ availability (see Appendix II). Turgid segments were floated on water and were exposed to 10 µCi of $^{14}CO_2$ for 2 hours (10 µCi was used in

order that sufficient radioactivity was still present in the segments following the 22 hours of incubation in unlabelled air at normal rates of photosynthesis and respiration). They were then transferred to petri dishes maintained at 20°C in continuous light for the remaining period (22 hours). The harvested segments were frozen in liquid nitrogen, freeze dried and weighed. The ethanol soluble extract and residue were obtained as described in Materials and Methods (Section 2.8.6) and the radioactivity estimated. Also counts in various amino acids and glucose, fructose and sucrose were estimated and the total content measured (as detailed in Materials and Methods, Section 2.8.6) and expressed on a dry weight basis. There were 4 replicates in each treatment and a conventional analysis of variance was performed on the data to test for significant differences. (N.B. An arc sin transformation on the data calculated as a percent of total radioactivity was performed, but did not yield significantly different results from those achieved with untransformed data).

### 6.4.2 Results

Incorporation and distribution of $^{14}C$

The total radioactivity assimilated by each sample was obtained by adding the counts in the ethanol insoluble fraction (estimated by the ACS gel method - see Materials and Methods, Section 2.8.6)to that in the soluble extract. The efficiencies of the various ACS systems used were comparable but the toluene based scintillant systems were only 75% as efficient as the ACS systems. Due corrections have been made to the data from these toluene systems. The efficiency of the liquid scintillation counter was 85%. The counts have been expressed as a precentage of the total radioactivity in the system to allow for exposure to varying

radioactivities (1 μCi and 10 μCi) in the two systems. While both the stress and turgid treatments resulted in similar proportions of the total radioactivity being found in the ethanol insoluble residue and the ethanol soluble extract (Table 6.5), the percentage of counts in the sum total of amino acids and total sugars were significantly different ( 10% in turgid L-L and D-L segments and 35% in the stressed samples). This suggests that in turgid samples 68% of the total radioactivity of the ethanol soluble extract was in some other soluble carbon compound(s), while the corresponding percentage in stressed samples was only 39% (Table 6.5).

The radioactivity in sugars was greater in D-L samples (both turgid and stressed) than in the corresponding L-L samples, but radioactivity in amino acids was greater in the L-L samples especially with turgid tissue (Table 6.5).

Free amino acid concentration and radioactivity

The total free amino acid concentration of stressed tissue was significantly greater than that of turgid segments (Table 6.6). In stressed tissues, the total amino acid content of L-L and D-L samples was not significantly different, but in turgid samples the L-L samples contained a significantly higher concentration of amino acid than did the D-L samples.

A comparison of individual amino acids concentrations between turgid and stressed samples revealed, with the exception of glycine, a generally lower content of most amino acids in the turgid samples (Fig 6.2). When D-L and L-L samples were compared, the D-L stressed samples had a very similar amino acid content to the L-L stressed samples, apart from the proline content. Turgid D-L samples, on the other hand had a significantly lower concentration of almost all amino acids when compared with the L-L turgid tissue (Fig 6.2 A,B).

Table 6.5

Incorporation and distribution of $^{14}C$

| | Total radio-activity cpm x $10^{-6}$ | Percentage of total radioactivity | | | |
|---|---|---|---|---|---|
| | | Alcohol insoluble residue | Alcohol soluble extract | Total amino acids | Sugars (Glu+Fru+Suc) |
| Turgid | | | | | |
| L-L | 8.745 | 21.70 | 78.30 | 6.97 | 3.12 |
| D-L | 8.735 | 21.64 | 78.36 | 3.08 | 7.42 |
| V.R. | 0.00 | 0.00 | 0.00 | 44.03* | 6.81* |
| Stress | | | | | |
| L-L | 0.883 | 25.29 | 74.71 | 14.44 | 18.00 |
| D-L | 0.924 | 22.49 | 77.51 | 13.79 | 27.30 |
| V.R. | 0.18 | 5.22 | 5.67 | 0.12 | 5.67 |

Significance level * $P \leq 0.05$

** $P \leq 0.01$

Fig 6.2

Amino acid content and relative distribution of counts in amino acids (expressed as a percentage of the total $^{14}C$ in the sample) in turgid and stressed barley segments.

Twelve day old barley plants were transferred to 48 hours continued darkness or light after which the first leaves were harvested and segmented. All the segments were floated on PEG (-15 bar) or water in light and exposed to $^{14}CO_2$ (released from $NaH^{14}CO_3$ by lactic acid). Segments on water were exposed to $^{14}CO_2$ for only 2 hours while stressed segments were exposed for 24 hours.
Each value is the mean of 4 replicates.

—— L-L treatment

--- D-L treatment

A. Free amino acid content (mg $g^{-1}$ dry wt) in turgid segments

B. Free amino acid content (mg $g^{-1}$ dry wt) in stressed segments

C. Percentage of total $^{14}C$ in individual amino acids in turgid samples

D. Percentage of total $^{14}C$ in individual amino acids in stressed samples.

Significance level * $P \leq 0.05$

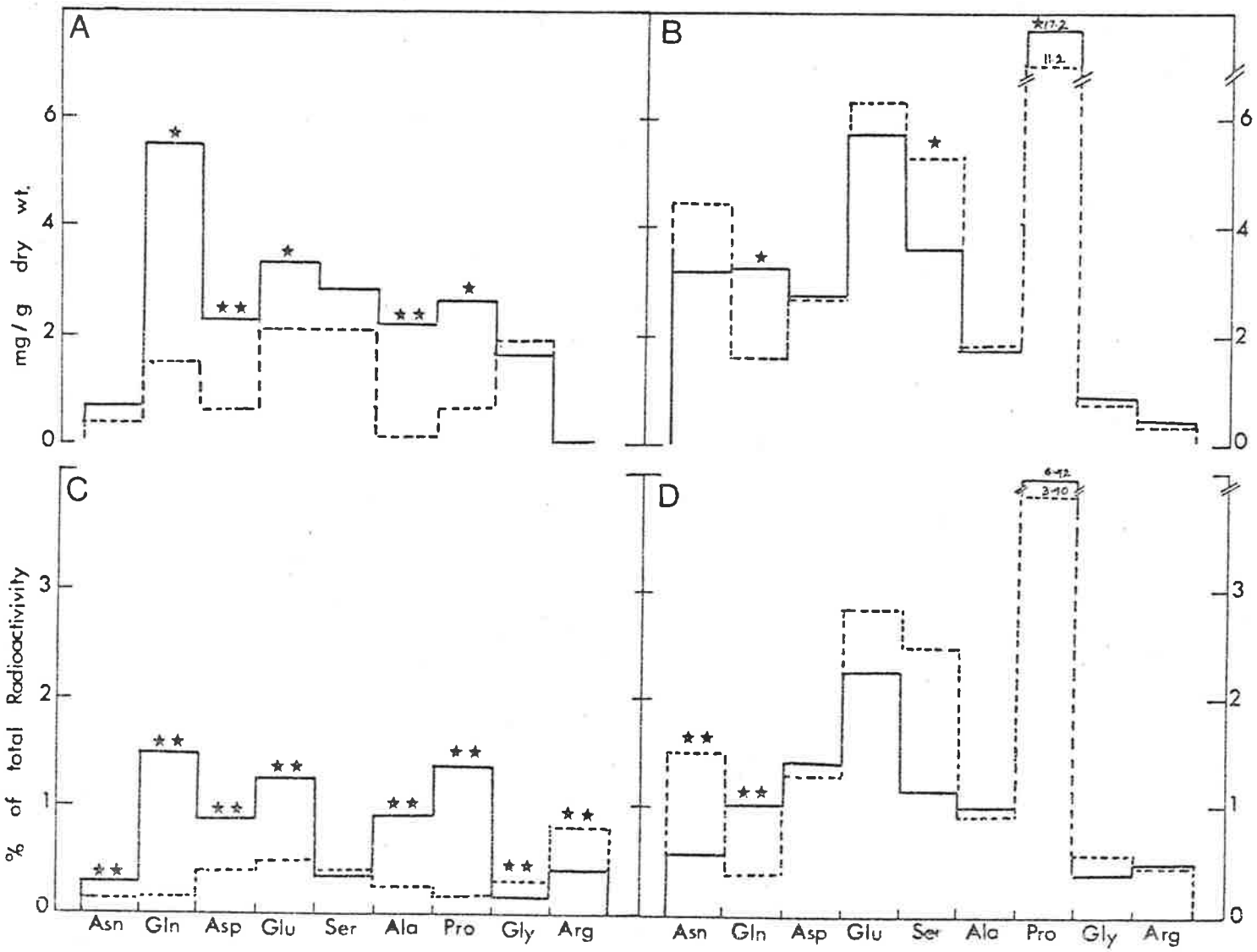
** $P \leq 0.01$

Table 6.6

Total amino acid content (mg $g^{-1}$ dry wt) in stressed and turgid samples

| Treatment | Turgid | Stress |
|---|---|---|
| L-L | $21.2^{a}$ | $39.2^{c}$ |
| D-L | $8.6^{b}$ | $36.4^{c}$ |

N.B. : Figures with similar letters (a,b or c) are not significantly different from each other.


A
B
C
D
mg / g dry wt.
% of total Radioactivity
0
2
4
6
1
3
Asn
Gln
Asp
Glu
Ser
Ala
Pro
Gly
Arg

As the amount of radioactivity introduced into the two systems differed (turgid 10 μCi, stress 1 μCi), the radioactivity present in the various amino acids is expressed a a percentage of the total radioactivity in the sample (Fig 6.2C). The turgid L-L samples had significantly higher percentage radioactivity than D-L samples in all amino acids except glycine and arginine (Fig 6.2C). In L-L turgid samples, the radioactivity in proline constituted about 20% of the total amino acid radioactivity, as against 5% in D-L turgid samples.

Stressed samples showed a significantly higher percentage of the total radioactivity in proline and glutamine and less in asparagine in L-L samples compared to D-L samples (Fig 6.2 C). $^{14}C$ in proline accounted for 44% and 29% of the total amino acid radioactivity in L-L and D-L samples respectively.

Total sugar content and radioactivity

Total alcohol soluble carbohydrates (estimated by the anthrone method - see Materials and Methods Section 2.8) were significantly different between turgid and stressed samples (Table 6.7). Stressed samples showed significantly less concentration probably because stressed segments were incubated under limiting $CO_2$ conditions (in closed 250 ml flasks).

Variation in individual sugar content between samples was high. Although D-L turgid segments showed a higher content of glucose, fructose and sucrose than L-L segments, the differences were not significant at the 5% level of probability (Table 6.8A). In stressed segments also, the D-L treatment showed higher levels of all 3 sugars, but only the fructose content was significantly higher than that in the L-L treatment

Table 6.7

Total alcohol soluble carbohydrate content (mg $g^{-1}$ dry wt) in turgid and stressed samples

| | Turgid | Stress |
|---|---|---|
| L-L | $136.0^{a}$ | $65.8^{b}$ |
| D-L | $165.6^{a}$ | $43.9^{c}$ |

N.B. Figures with similar letters (a, b or c) are not significantly different from each other.

Table 6.8

Sugar concentration (mg $g^{-1}$ dry wt) and radioactivity

A. Sugar concentration (mg $g^{-1}$ dry wt)

| | Turgid | | | Stress | | |
|---|---|---|---|---|---|---|
| | Glucose | Fructose | Sucrose | Glucose | Fructose | Sucrose |
| L-L | 26.2 | 31.1 | 43.5 | 11.7 | 12.3 | 4.7 |
| D-L | 42.9 | 45.7 | 54.5 | 15.9 | 19.8 | 5.8 |
| V.R. | 1.79 | 4.99 | 1.27 | 4.23 | 14.05* | 1.94 |

B. Radioactivity in sugars as a percentage of total $^{14}C$ in the sample

| | Turgid | | | Stress | | |
|---|---|---|---|---|---|---|
| | Glucose | Fructose | Sucrose | Glucose | Fructose | Sucrose |
| L-L | 0.92 | 0.66 | 1.50 | 5.18 | 2.48 | 10.30 |
| D-L | 1.90 | 1.35 | 4.15 | 6.97 | 3.86 | 16.50 |
| V.R. | 2.85 | 3.68 | 6.17* | 1.18 | 3.60 | 8.13* |

Significance level * $P \leq 0.05$

(Table 6.8A). When the sugar contents of stressed and turgid samples were compared, the turgid samples (both L-L and D-L ) had significantly higher contents than the stress treatment in every case.

Stressed samples showed a higher percentage of the total $^{14}C$ in sugars compared with turgid samples (Table 6.8B). This could arise from greater synthesis or less rapid utilization of sugars during stress. However, since the concentration of sugars was much higher in turgid samples compared to stressed ones (Table 6.8A) higher radioactivity due to greater synthesis would seem unlikely. Comparison of radioactivity in sugars between L-L and D-L samples showed a generally higher percentage in the D-L samples particularly in sucrose (Table 6.8B). Here, however, since these samples also contained greater concentrations of these sugars (Table 6.8A) greater synthesis of sugars may have occurred.

Estimation of the contribution of carbon assimilated during stress towards proline accumulation

The total radioactivity present in proline, the total amount of proline accumulated, the $CO_2$ concentration in the atmosphere and the radioactivity liberated in the system are known for this experiment. Accordingly the percentage of accumulated proline molecules which are labelled with radioactive carbon can be calculated, given the assumption that only one carbon atom per molecule of proline is labelled.

Total $^{14}CO_2$ liberated = 1 μCi ≡ $2.22 \times 10^6$ dpm

Dpm x Efficiency = cpm

Efficiency of the instrument ≡ 86.75%

and assuming efficiency of liberation of $^{14}CO_2$ from $NaH^{14}CO_3$ by lactic acid to be 100%,

then the total cpm released into the flask

$= 2.22 \times 10^6 \times 0.8675$

$= \underline{1.926 \times 10^6}$ cpm

The volume of the flask = 260 ml -10 ml (volume of stopper)

∴ Nett volume = 250 ml

In 250 ml of air, volume of $CO_2 = 250 \times \frac{0.032}{100} = 0.08$ ml

We know that 22.4 l $CO_2$ ≡ 1 mole $CO_2$ ≡ $6.023 \times 10^{23}$ mols of $CO_2$

∴ 0.08 ml $CO_2$ contains ? mols of $CO_2$

$$\equiv \frac{0.08 \times 6.023 \times 10^{23}}{22.3 \times 10^3}$$

$$= \frac{48.184 \times 10^{18}}{22.4}$$

$= \underline{\underline{2.151 \times 10^{18}}}$ mols of $CO_2$

As the quantity of $CO_2$ released by $NaH^{14}CO_3$ was negligible,

[as specific radioactivity was 57.0 mci/mmol,

∴ 1 μCi ≃ 0.0175 μmol ≃ $1 \times 10^{16}$ mols of $CO_2$, which is about 0.5% of the $CO_2$ in the flask], the molecules of $CO_2$ corresponding to 1 cpm can be calculated in the following way, as we know that $2.151 \times 10^{18}$ mols contain $1.926 \times 10^6$ cpm,

∴ 1 cpm ≡ $\frac{2.151 \times 10^{18}}{1.926 \times 10^6} = \underline{\underline{1.115 \times 10^{12}}}$ mols of $CO_2$

L-L stressed samples

Amount of proline per sample = 466 µg ≡ 4.049 µmoles

i.e. $2.4385 \times 10^{18}$ mols of proline

The total label present in this proline was 59469 cpm

which would correspond to $59469 \times 1.115 \times 10^{12}$ mols of $CO_2$

$= 5.8119 \times 10^{16}$ mols of $CO_2$

If there is one labelled carbon per proline molecule, then total molecules of proline that are labelled ≡ $5.8119 \times 10^{16}$

$$\therefore \text{ \% of proline labelled} = \frac{5.8119 \times 10^{16} \times 100}{2.4385 \times 10^{18}} \equiv \underline{\underline{2.72\%}}$$

If only the increase in proline content which is attributable to light is considered (i.e. LL-LD), then the total increase in number of proline molecules due to light is

466 - 200 µg = 266 µg ≡ 2.313 µmoles

$= 1.393 \times 10^{18}$ mols of proline

And we know that $5.8119 \times 10^{16}$ mols of proline are labelled

$$\therefore \text{ \% label in proline} = \frac{5.8119 \times 10^{16}}{1.393 \times 10^{18}} = \underline{\underline{4.76\%}}$$

A similar calculation can be performed for the D-L treatment.

Proline content per sample is 229 µg = 1.991 µmole = $1.994 \times 10^{18}$ mols of proline

Total radioactivity in proline is 37738 ≡ $4.2077 \times 10^{16}$ mols of proline

$$\therefore \text{ \% of proline labelled} = \frac{4.2077 \times 10^{16}}{1.994 \times 10^{18}} = \underline{\underline{3.51\%}}$$

Light stimulated proline content = 163 µg ≡ 1.417 µmoles

$= 8.537 \times 10^{17}$ mols of proline

Total label present is 37738 cpm ≡ $4.2077 \times 10^{16}$ mols of proline

$$\therefore \text{ \% proline labelled} = \frac{4.2077 \times 10^{16}}{8.537 \times 10^{17}} = \underline{\underline{4.93\%}}$$

Thus, from the above calculations, the contribution of current assimilate during stress to proline synthesis appears small (< 5%). It must be kept in mind that although 1 μCi of radioactivity was liberated in the flask, only 50% of it could be accounted for in total counts in the sample. But even if we assume that only 50% of the radioactivity is assimilated, the contribution of current $CO_2$ assimilation would be approximately 10% only. Also of interest is the similarity in contribution of $CO_2$ fixation to proline in both L-L and D-L treatments.

## 6.5 $^{14}CO_2$ fixation at a low irradiance and 7 hours after the commencement of stress

It would appear, therefore, that the contribution of carbon fixed by photosynthesis during the stress period into proline accumulation is small within the period of the experiment. This however, is 4 to 6% of the total $^{14}C$ fixed during the stress period (Fig. 6.2) which is considerable considering the various other carbon compounds e.g. glycine betaine and triglycerides (Storey et al., 1977 and Douglas and Paleg, 1981) which have been reported to accumulate during stress. In conclusion, therefore, although only a small proportion of proline synthesized during stress contains carbon from current photosynthesis, a large proportion of the carbon fixed ends up in proline. In order to further test this conclusion, the carbon dioxide fixation and labelling pattern at a lower irradiance was also examined. In addition, radioactive carbon fixation 7 hours after the imposition of stress was studied. This latter treatment was included to check whether the pattern of $CO_2$ fixation and diversion of carbon to proline differed between the period immediately following stress imposition and later, when the rate of P/S would have declined significantly.

### 6.5.1 Irradiance and carbon dioxide fixation

#### 6.5.1.1 Methods

Twelve day old barley plants growing in a 16 hour photoperiod at 20°C were transferred to 48 hours continuous light or darkness. The first leaves were excised, segmented and floated on -15 bar PEG solution in 125 ml conical flasks at an irradiance of 300 or 30 μE $m^{-2}s^{-1}$. Radioactive $NaH^{14}CO_3$ was also introduced in the flasks (@ 1 μCi per flask) and the $^{14}CO_2$ liberated with 50% lactic acid. Segments were harvested, preserved and the various fractions analysed as described in Section 6.4.1.

#### 6.5.1.2 Results

Incorporation and Distribution of $^{14}C$

Segments incubated at 30 μE $m^{-2}s^{-1}$ assimilated significantly less radioactive carbon dioxide than segments exposed to 300 μE $m^{-2}s^{-1}$ in both the D-L and L-L treatments (Table 6.9). Despite this difference, the distribution of counts between the ethanol soluble and insoluble fractions were similar at the two irradiances in both the L-L and D-L treatments. However, L-L segments incorporated a greater proportion of the total radioactivity into the ethanol insoluble residue than did the D-L samples. While the proportion of radioactivity present in amino acids did not vary greatly between treatments, that in the sugars did. The D-L segments at 30 μE $m^{-2}s^{-1}$ showed the highest percentage of radioactivity in sugars while L-L at 300 μE $m^{-2}s^{-1}$ showed the least (Table 6.9).

Comparison of these results with those from the previous experiment (D-L and L-L stressed samples at 300 μE $m^{-2}s^{-1}$) (Table 6.5) show that there is a greater incorporation of total radioactivity in the present experiment, although the same amount (1 μCi) of radioactivity was introduced. Small differences may be attributed to random differences

Table 6.9

Total radioactivity and its distribution in the various fractions at different irradiances

Barley plants were grown for 12 days in a 16 hour photoperiod at 20°C prior to transferring them to 48 hours of continuous light or continuous darkness. The first leaves were then excised, segmented and floated on -15 bar PEG solution in a $^{14}CO_2$ labelled atmosphere and illuminated at 30 or 300 μE $m^{-2}s^{-1}$ for 24 hours. Radioactive $CO_2$ was introduced at the commencement of the stress period.

| Treatment | Total Radioactivity cpm x $10^{-6}$ | Percentage of total radioactivity: Alcohol insoluble residue | Alcohol soluble extract | Total amino acids | Sugars (Glu+Fru+Suc) |
|---|---|---|---|---|---|
| L-L 300 | 1.431 | 23.80 | 76.20 | 14.53 | 9.96 |
| L-L 30 | 1.172 | 24.96 | 75.04 | 12.98 | 21.32 |
| D-L 300 | 1.372 | 18.38 | 81.62 | 14.90 | 26.61 |
| D-L 30 | 1.148 | 17.84 | 82.16 | 14.30 | 36.00 |
| V.R. | | | | | |
| Irrad | 42.78** | 0.05 | 0.05 | 1.06 | 58.32** |
| L/D | 1.26 | 22.08** | 22.08** | 0.65 | 133.03** |
| IrradxL/D | 0.20 | 0.40 | 0.40 | 0.20 | 0.52 |

Significance level * $P \leq 0.05$

** $P \leq 0.01$

in the tissue used but a further possible cause for this discrepancy could be the difference in the volume of the incubation flask (250 ml in the previous experiment versus 125 ml in the present experiment). This would have led to the specific radioactivity of $CO_2$ in the present experiment being twice that in the previous. On comparing the distribution of radioactivity, it is apparent that the percentage of the radioactivity in the amino acid fraction was unchanged. L-L samples at 300 μE $m^{-2}s^{-1}$ showed less percentage of total radioactivity in sugars while D-L showed a higher percentage of the total radioactivity present in the soluble extract in the present experiment compared to the relative distribution in the previous one.

Proline content and radioactivity in proline

The free proline content of the L-L 300 and 30 μE $m^{-2}s^{-1}$ samples were not significantly different (Table 6.10) but total radioactivity was. Samples incubated at 300 μE $m^{-2}s^{-1}$ showed significantly higher radioactivity than those at 30 μE $m^{-2}s^{-1}$; even when expressed as a percentage of the total $^{14}C$ in the sample (Table 6.10). The radioactivity in proline in 300 μE $m^{-2}s^{-1}$ samples accounted for 42.5% of the total $^{14}C$ in the amino acid fraction as against 28% in 30 μE $m^{-2}s^{-1}$ samples.

$^{14}C$ in proline in D-L samples on the other hand was greater at 30 than at 300 μE $m^{-2}s^{-1}$ despite lower free proline content at 30 μE $m^{-2}s^{-1}$ (Table 6.10).

The total content and radioactivity in the other amino acids and sugars are presented in Appendix III (Tables 1,2,3 and 4). Despite similar proline concentrations in L-L segments stressed under 300 or 30 μE $m^{-2}s^{-1}$ (Table 6.10) the radioactivity differed significantly. This, apart from confirming that the contribution of currently assimilated carbon to proline accumulation is small, also suggests that this contribution does not determine the rate of accumulation.

Table 6.10

Effect of irradiance on free proline content and radioactivity in proline

| | Free proline content (mg $g^{-1}$ dry wt) | Radioactivity cpm x $10^{-3}$ | Percentage of Total $^{14}C$ | Percentage of Total amino acid $^{14}C$ |
|---|---|---|---|---|
| L-L | | | | |
| 300 | 18.92 | 87.431 | 6.11 | 42.5 |
| 30 | 18.81 | 42.192 | 3.60 | 27.9 |
| D-L | | | | |
| 300 | 12.74 | 34.986 | 2.55 | 17.2 |
| 30 | 10.64 | 40.869 | 3.56 | 25.0 |

### 6.5.2 Carbon dioxide fixation and duration of stress

#### 6.5.2.1 Methods

The plant growth and prestress treatments were identical to those described in 6.5.1.1. The segments were floated on -15 bar PEG solution in light in 125 ml flasks. At zero or seven hours after the imposition of stress, radioactive carbon dioxide was liberated into the flask from $NaH^{14}CO_3$ by excess 50% lactic acid (@ 1 μCi $flask^{-1}$). Segments were left in the flask until harvested. Twenty four hours after the commencement of the stress period the segments were harvested, frozen in liquid nitrogen and freeze dried. The various fractions were estimated and radioactivity was determined as detailed in Materials and Methods Section 2.8.6. Since experiments 6.5.2 and 6.5.1 were conducted on the same set of plants, the data for the zero time samples shown here are the same as those in 6.5.1.

#### 6.5.2.2 Results

##### Incorporation and distribution of $^{14}C$

The total radioactivity fixed by the various samples was not significantly different, even though some tissue was exposed to radioactive $CO_2$ 7 hours after the commencement of stress and some immediately upon stress being imposed (Table 6.11). Although total $^{14}C$ incorporated was similar, it does not necessarily mean that the rates of photosynthesis were similar in the 2 samples, as samples exposed to $^{14}CO_2$ at time zero may have assimilated a major proportion of the radioactivity within an hour while those exposed to $^{14}CO_2$ 7 hours after the imposition of stress may have taken longer to assimilate the same amount. However, it does confirm earlier results (Section 5.1) that carbon fixation continues in stressed samples, albeit at a reduced rate.

Table 6.11

Incorporation and distribution of radioactivity as a percentage of total radioactivity in sample

| Treatment | Total cpm x $10^{-6}$ | Ethanol insoluble residue | Ethanol soluble extract | Total amino acids | Total sugars (Glu + Fu + Suc) |
|---|---|---|---|---|---|
| L-L 0 | 1.431 | 23.80 | 76.20 | 14.53 | 9.96 |
| L-L 7 | 1.400 | 17.57 | 82.43 | 16.54 | 19.44 |
| D-L 0 | 1.372 | 18.38 | 81.62 | 14.90 | 26.61 |
| D-L 7 | 1.379 | 13.35 | 86.65 | 15.60 | 37.52 |
| V.R. | | | | | |
| Time(T) | 0.14 | 20.04** | 23.04* | 1.34 | 108.08** |
| LL/DL(L) | 1.55 | 16.88* | 16.88* | 0.05 | 313.77** |
| T x L | 0.34 | 0.26 | 0.26 | 0.31 | 0.54 |
| LSD | - | - | - | - | - |

Of the radioactivity assimilated, a greater proportion was found in the ethanol insoluble residue and less radioactivity in the soluble extract in the 0 hour L-L segments than in any of the other treatments. As observed in the previous 2 experiments (6.4 and 6.5.1) the percentage of total radioactivity found in the free amino acids was similar in all treatments, but the proportion in the sugar fraction differed considerably. Here too, tissue prestressed in darkness (D-L) showed a significantly greater proportion of fixed radioactivity in the sugar fraction than those prestressed in light. Moreover, samples exposed to $^{14}CO_2$ 7 hours after the commencement of stress had significantly more label in sugars. Compared to the '0' hour samples (Table 6.11). Thus, samples containing higher radioactivity in sugars contained less radioactivity in the ethanol insoluble residue. One possible explanation for this is that L-L tissue which has a high carbohydrate content may also have a rapid turnover of these sugars, resulting in a reduced level of radioactivity in sugars. D-L treated tissue on the other hand, is initially low in sugar content, accumulates sugars and consequently contains greater radioactivity. However, in the D-L treatment, sugars are metabolised (albeit at a slower rate than L-L) as samples exposed to $^{14}CO_2$ for 24 hours showed less radioactivity remaining in sugars than those exposed to $^{14}CO_2$ for 17 hours. This was also the case in L-L treatments. Since the 17 hour samples which showed higher radioactivity in sugars also showed lower radioactivity in the insoluble residue, it is possible that with time the radioactive sugars were converted into alcohol insoluble polysaccharides.

Amino acids - radioactivity

Proline

The radioactivity present in proline (when expressed as a percentage of the total $^{14}C$ in the sample) was very similar between 0 and 7 hours in both L-L and D-L samples (Table 6.12). A significantly less amount of $^{14}C$ entered into proline in D-L samples compared with that in L-L samples. The significance of the similar percentage of label in proline in the samples exposed to $^{14}CO_2$ after 0 and 7 hours of stress can be interpreted as being due to any of the following -

i) Incorporation of radioactivity into proline (by enhanced synthesis) commences 7 hours after stress is imposed or later (i.e. a lag phase of 7 hours or more is present. Results in Figs. 3.5 and 3.6 showed significant proline accumulation occurred 6-8 hours after the commencement of stress treatment.)

ii) Incorporation and equilibration of $CO_2$ or photosynthates occurs rapidly at any time

iii) Since the radioactivity in the insoluble residue is significantly greater in the '0' time sample than in the 7 hour sample, it is possible that some of the radioactive proline formed in the segments fed at 0 hours is incorporated into proteins (bound proline) and hence does not contribute to the free proline radioactivity

iv) The contribution of current photosynthates to proline is relatively insignificant so that even if the radioactivity in proline from this source is reduced, it has no significant effect.

Any or all of the above explanations may be valid but one conclusion from the data is that free proline synthesis from photo-

Table 6.12

Radioactivity in proline in samples exposed to $^{14}CO_2$ after 0 or 7 hours of stress

| Treatment | Radioactivity cpm x $10^{-3}$ | Percentage of Total $^{14}C$ | Percentage of Total amino acid $^{14}C$ |
|---|---|---|---|
| L-L | | | |
| 0 | 87.42 | 6.11 | 42.5 |
| 7 | 86.81 | 6.20 | 37.5 |
| D-L | | | |
| 0 | 35.00 | 2.55 | 17.2 |
| 7 | 31.99 | 2.32 | 14.9 |

synthates synthesized during stress forms a very small proportion of the proline synthesized.

Radioactivity in other amino acids and sugars are given in Appendix III - Tables 5 and 6.

It can be concluded that the proline accumulated is not derived exclusively from carbon fixed during stress. This is in agreement with the earlier calculation where the contribution of current $^{14}CO_2$ fixation to proline synthesis was calculated to be small (< 10%). However, since $^{14}CO_2$ was not provided continuously but assumed to be present in the flask during the entire stress period (albeit in decreasing concentration) it is possible that the refixation of respired $CO_2$ may have provided precursors for proline synthesis.

## 6.6 Estimation of NAD(P)H production in light in stressed segments

Differences in proline accumulation between the various treatments have been shown to be due to factors in addition to differences in the contribution of carbon assimilated during stress which is directed to proline synthesis. However, it is possible that current photosynthesis provides the energy rich compounds (NAD(P)H) required for proline synthesis from glutamate. Since the irradiance, leaf area and amount of proline accumulated is known, it should be possible to calculate the reduced nucleotides produced and compare this figure with the amount required for the proline synthesis which occurs.

If the incident light on the segments is 20 μE $m^{-2}s^{-1}$ for 24 hours then total light over 24 hours will be -

$= 20 \times 24 \times 60 \times 60$

$= 20 \times 86400$ μE $m^{-2}$ $day^{-1}$

We know that 1 μE = $6.023 \times 10^{17}$ quanta

∴ Total energy incident $m^{-2}$ $day^{-1}$ is $20 \times 86400 \times 6.023 \times 10^{17}$ quanta

As the total area of 10 segments is 10 $cm^2$

total incident energy on segments = $\frac{20 \times 86400 \times 6.023 \times 10^{17} \times 10}{100 \times 100}$

$= 6.023 \times 10^{20} \times 1.728$ quanta $day^{-1}$

If 80% of the incident energy is absorbed

then absorbed energy = $6.023 \times 10^{20} \times 1.728 \times 0.8$ quanta $day^{-1}$

With maximum trapping efficiency, the reduction of

1 NAD(P) to NAD(P)H requires 4 quanta

∴ Maximum NAD(P)H that can be produced from the energy absorbed is

$= \frac{6.023 \times 10^{20} \times 1.728 \times 0.8}{4}$

$= 6.023 \times 10^{20} \times 0.3456$

Thus, the maximum number of NAD(P)H molecules that can be produced at 20 μE irradiance over a 24 hour period on 10 segments of barley leaf is

$6.023 \times 10^{19} \times 3.456 = 2.082 \times 10^{20}$

It is known that proline synthesis from glutamate requires 2NAD(P)H

Glutamate $\xrightarrow{(NAD(P)H}$ Pyroline-5-carboxylate

Pyroline-5-carboxylate $\xrightarrow{(NAD(P)H}$ Proline

In the non-starved light treatments (L-L), the increase in proline content in light in stressed segments was 266 μg sample$^{-1}$

$$\equiv \frac{266}{115} = 2.313 \text{ μmoles}$$

To synthesize 1 μmole proline will require 2 μmoles of NAD(P)H

$\therefore$ 2.313 μmoles proline will need 4.626 μmoles of NAD(P)H

$= \underline{4.626 \times 6.023 \times 10^{17}}$ mols of NAD(P)H

$= \underline{\underline{2.786 \times 10^{18}}}$ mols of NAD(P)H

Thus, at 20 μE irradiance, the system is apparently capable of synthesizing (NAD(P)H at a rate 75 times greater than that required for enhanced proline synthesis.

However, the following assumptions have been made in reaching this conclusion -

i) Maximum absorption of total incident energy (80%)

ii) Maximum efficiency of energy trapping (4 quanta per NAD(P)H)

iii) Glutamate to proline requires 2NAD(P)H.

Although the NAD(P)H produced is theoretically much greater than that required for proline synthesis a major proportion of this may well be used for other reactions including the synthesis of other compounds. However, even if the energy-trapping efficiency is reduced by stress to 70% of that in turgid samples (Section 5.2), the total energy produced will still exceed that necessary for proline synthesis by fifty-fold.

Thus even making generous allowance for these factors, the calculation supports the hypothesis that even the energy produced at an irradiance of 20 μE $m^{-2}s^{-1}$ is sufficient to support the enhanced proline synthesis encountered, in addition to providing the carbon skeletons for the proline molecule itself.

## 6.7 Metabolism of glutamate

From the above calculations, it would seem that light even at the (20 $\mu Em^{-2}$ $s^{-1}$), can provide abundant energy for light-stimulated proline accumulation. This energy may come directly from reductants produced during photosynthesis or may result from oxidation of newly fixed carbohydrates. If the stimulation of proline synthesis by light is due to this energy being available, then it is implied that proline accumulation is limited by energy supply in the dark. The major source of energy for synthesis in dark incubated segments would come from the oxidation of carbohydrates and it is possible to estimate the energy requirement for the proline synthesized (in light incubated segments) and the potential energy available from the oxidation of carbohydrates. Additional proline synthesized in the light in 24 hours (LL-LD) $\simeq$ 10 mg $g^{-1}$ dry wt $\equiv$ 0.09 mmoles $g^{-1}$ dry wt. Complete oxidation to $CO_2$ and $H_2O$ yields 6 NADH + 2NAD(P)H + 2$FADH_2$ + 2ATP per molecule of glucose while partial oxidation to α-oxoglutarate yields 2NAD(P)H + 2ATP. If partial oxidation is assumed to occur, with the carbon skeleton remaining for proline synthesis, then for every molecule of proline synthesized from α-oxoglutarate, (requiring 3NAD(P)H), 1.5 molecules are needed to satisfy the energy requirement. The additional 0.09 mmoles of proline synthesized in light would require 0.135 mmoles of glucose. In L-D segements there was 0.33 mmoles $g^{-1}$ dry wt glucose equivalent of soluble carbohydrate present even after 24 hours stress in darkness. This would be sufficient to support additional proline synthesis to the rate found in light for more than 2 days. This suggests that, unless carbohydrates in dark incubated segments were not available for oxidation, energy availability alone did not limit proline accumulation in the dark, where the leaves were pretreated in the dark.

A similar calculation can be performed for samples pretreated in the dark (D-L and D-D samples) where the additional proline synthesized in the light in 24 hours (DL-DD) $\simeq$ 8 mg $g^{-1}$ dry wt $\equiv$ 0.07 mmoles $g^{-1}$ dry wt. This would require 0.10 mmoles of glucose. However, the glucose equivalent of soluble carbohydrates present after 24 hours stress in darkness (D-D) was only 0.08 mmoles $g^{-1}$ dry wt. This would not be sufficient to support the additional proline synthesis rate found in light stressed samples. Thus in dark pretreated segments, the energy available for proline synthesis is limiting.

To confirm whether light is required for the synthesis of proline from glutamate, in both light and dark prestressed samples the following experiment using labelled glutamate was performed. The treatments studied were turgid and stressed samples given light and dark pretreatments. Samples were incubated at 250 or 30 µE $m^{-2}s^{-1}$ or in the dark.

### 6.7.1 Methods

Twelve day old barley plants growing in a 16 hour photoperiod at 20°C were transferred to 48 hours continuous light or darkness. The first leaves were excised and floated on -15 bar PEG solution or water for 20 hours in the dark or light of 30 or 250 µE $m^{-2}s^{-1}$. The leaves were then taken out of the PEG solution and placed in a small vial containing 0.5 ml radioactive L-U-$^{14}$C glutamic acid (specific radioactivity of the glutamate solution was 270 mCi $mmol^{-1}$ and concentration was 250 µCi/5 ml). The bases of the leaves were cut under the solution to facilitate uptake. The leaves were allowed to take up the solution for 1 hour in the light or darkness. (To ensure that leaves were capable of taking up solutions through their base, a trial run using eosin dye was performed which confirmed that despite being wilted and placed in the dark,

leaves still took up some solution albeit less than that by turgid leaves). The leaves were then replaced on PEG solution or water and left for a further 5 hours before they were rinsed in water, surface dried and frozen in liquid nitrogen. The samples were then freeze dried, weighed and extracted in boiling 80% ethanol (see Materials and Methods Section 2.8.2). Counts in the alcohol insoluble residue and the soluble extract were estimated as detailed in Materials and Methods Section 2.8 .

Two hundred µl of the 1 ml concentrated extract was spotted on paper and the amino acids separated using descending paper chromatography in a phenol ammonia solvent system (Smith, 1969). To 500 g phenol, 125 ml water was added. One ml ammonia per 200 ml of the above solution was added just prior to use. The papers were placed in the tanks and run for 16 hours. The papers were removed, left to dry for 1 week in a fume cupboard and the markers (run on the margin of the paper) cut and sprayed with ninhydrin solution. The amino acids were identified by their Rf and the corresponding positions on the sample strips were marked, cut and counted in a liquid scintillation counter using a toluene-PPO-POPOP cocktail (3 g PPO and 0.3 g POPOP per litre of toluene).

### 6.7.2 Results

The uptake of $^{14}C$ glutamate showed considerable variation especially between turgid and stressed samples. To overcome this variation, counts in the various amino acids have been expressed as a percentage of the total counts in the ethanol soluble extract.

Distribution of radioactivity between soluble extract and insoluble residue

Samples prestressed in light (non-starved)

All samples showed the soluble extract to contain 90% or more of the total radioactivity except for stressed samples incubated at 30 µE $m^{-2}s^{-1}$

which also took up significantly less radioactivity (Table 6.13A). The distribution of radioactivity was similar in both turgid and stressed samples as well as between dark and light incubated samples (Table 6.13A).

Samples prestressed in darkness (starved)

Stressed samples (prestressed in the dark) showed no significant difference in relative amount of radioactivity in the soluble extract and the insoluble residue between samples stressed at 30, 250 or darkness (Table 6.13B). Turgid samples incubated in light, however, showed a significantly greater proportion of total radioactivity in the insoluble residue compared with turgid samples incubated in the dark and all stressed samples. A comparison of the distribution of radioactivity between light and dark prestressed samples demonstrated there was a similar distribution pattern in most samples with the exception of the D-L turgid samples which contained a higher proportion of counts in the insoluble residue.

Total amino acids

Non-starved samples

The total radioactivity present in the various amino acids accounted for 40 to 70% of the total counts in the soluble extract with the maximum percentage being present in samples stressed in the dark (Table 6.14).

Starved samples

The total free amino acid fraction constituted very similar percent of total radioactivity in both stressed and turgid samples (Table 6.14). Once again the dark stressed samples contained a higher proportion of

Table 6.13

Radioactivity in the ethanol soluble extract and the insoluble residue in L-U-$^{14}$C glutamate fed samples

| | Soluble extract | Insoluble residue |
|---|---|---|
| **A. Pretreated in light (nonstarved)** | | |
| Treatment | | |
| 250 μE $m^{-2}s^{-1}$ | | |
| Turgid | 873943 (92.3) | 72354 (7.7) |
| Stress | 51310 (93.7) | 3463 (6.3) |
| 30 μE $m^{-2}s^{-1}$ | | |
| Turgid | 248355 (92.6) | 19902 (7.4) |
| Stress | 17073 (84.4) | 3144 (15.6) |
| Dark | | |
| Turgid | 193765 (93.2) | 14217 (6.8) |
| Stress | 49308 (91.3) | 4718 (8.7) |
| **B. Pretreated in darkness (starved)** | | |
| 250 μE $m^{-2}s^{-1}$ | | |
| Turgid | 321870 (81.4) | 73738 (18.6) |
| Stress | 26400 (92.6) | 2095 (7.4) |
| 30 μE $m^{-2}s^{-1}$ | | |
| Turgid | 271653 (87.5) | 38887 (12.5) |
| Stress | 29140 (90.7) | 2982 (9.3) |
| Dark | | |
| Turgid | 137815 (95.6) | 6319 (4.4) |
| Stress | 44225 (93.7) | 2949 (6.3) |

Figures in parenthesis are the radioactivity expressed as a percentage of the total radioactivity in the sample

Table 6.14

Radioactivity in total amino acids as a percentage of total soluble extract radioactivity in $^{14}C$ glutamate fed samples

| Prestress treatment | Light | Dark |
|---|---|---|
| Treatment | | |
| 250 µE $m^{-2}s^{-1}$ | | |
| Turgid | 45.3 (41.8) | 54.3 (44.2) |
| Stress | 57.8 (54.2) | 51.8 (48.0) |
| 30 µE $m^{-2}s^{-1}$ | | |
| Turgid | 54.8 (50.8) | 57.3 (50.1) |
| Stress | 50.3 (42.5) | 56.6 (51.3) |
| Dark | | |
| Turgid | 48.5 (45.2) | 41.5 (39.7) |
| Stress | 71.0 (64.8) | 63.6 (59.6) |

Figures in parenthesis are the amino acid counts expressed as a percentage of the total radioactivity (soluble + insoluble) in the sample

radioactivity in this fraction.

As the radioactivity in total free amino acids only has been estimated it is possible that the radioactivity is in the bound amino acids. However, the radioactivity in the insoluble residue (containing proteins) was low (Table 6.13) which must mean that the proportion of radioactivity in bound amino acids is low in both starved and non-starved samples.

Proline

Non-starved samples

The radioactivity in proline, when expressed as a percentage of the total radioacticity in the soluble fraction, was similar in all stressed samples and varied between 4 and 9% (Fig 6.3A). The apparently low radioactivity present in the proline of stressed samples incubated at 30 $\mu E\ m^{-2}s^{-1}$ was probably related to the exceptionally low counts in the alcohol soluble fraction of this treatment (Table 6.13A).

In turgid samples, proline synthesis from $^{14}C$ glutamate was not significantly different between 30 and 250 $\mu E\ m^{-2}s^{-1}$ samples, but was lower in the dark (Fig 6.3). Stress did not enhance the synthesis of proline from $^{14}C$-glutamate in these non-starved samples except where the samples were stressed in darkness.

Starved samples

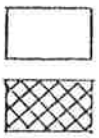
Proline synthesis at 30 and 250 $\mu E\ m^{-2}s^{-1}$ was again similar in both stressed and turgid samples (Fig 6.3B) while synthesis in the dark was also significantly reduced (Table 6.15).

Unlike non-starved samples, in the starved samples the stress treatment did not enhance proline synthesis in darkness (Fig 6.3A,B). Synthesis in light appeared similar in both the starved and non-starved segments. These relationships were unchanged when the radioactivity in

Fig 6.3

Radioactivity in proline as a percentage of the radioactivity in the soluble extract in L-U-$^{14}$C glutamate fed samples

Twelve day old barley plants were transferred to 48 hours continuous light or darkness following which the first leaf was excised and floated on water or PEG (-15 bars) for 20 hours under light of 30 or 250 μE $m^{-2}s^{-1}$ or in darkness. The leaves were allowed to take up $^{14}$C glutamate solution for 1 hour and then replaced on PEG solution or water and left for a further 5 hours before they were harvested.



Turgid

Stressed

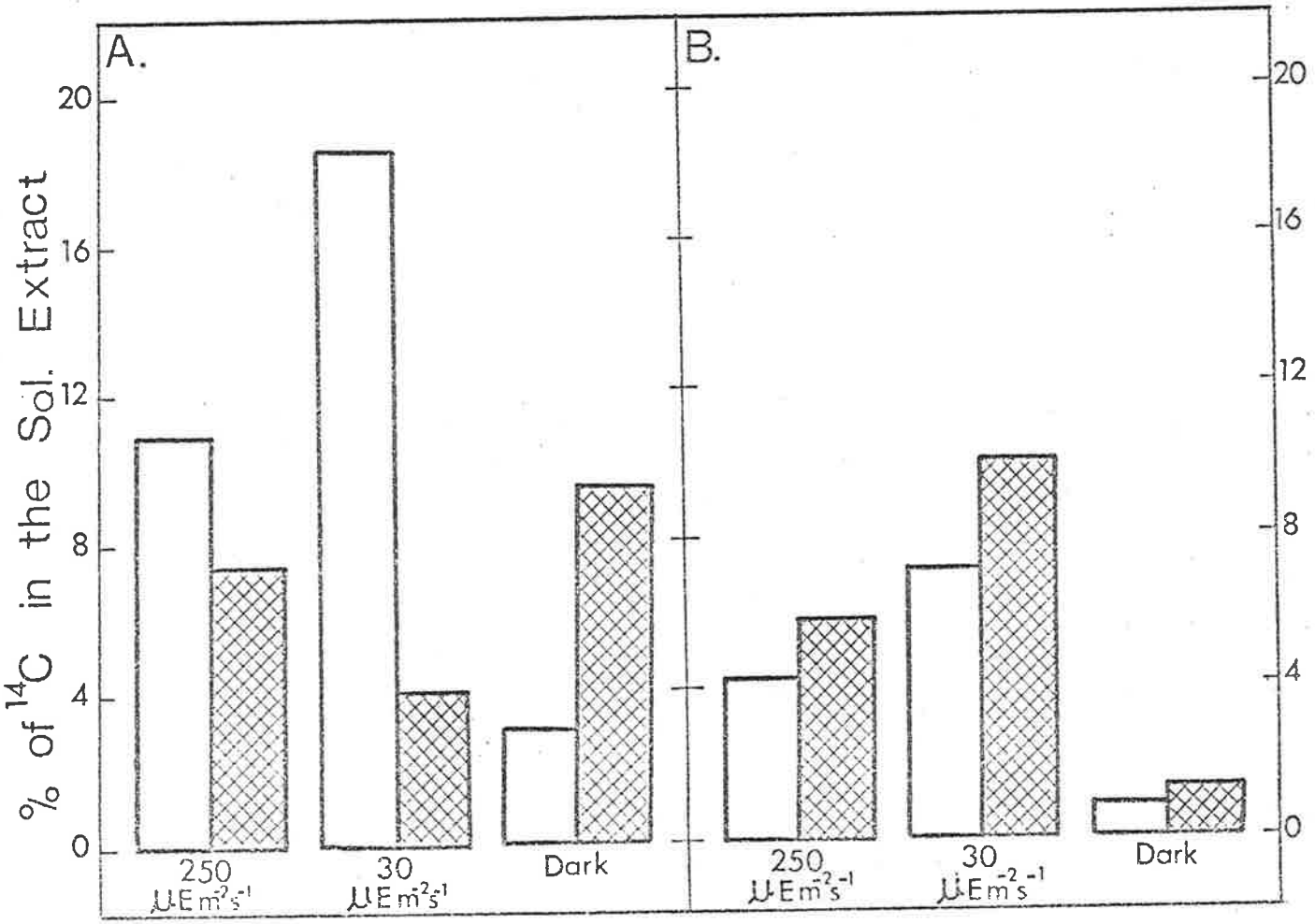
A. Prestressed in light (non-starved)

B. Prestressed in darkness (starved)

Table 6.15

F Table for results in Fig 6.3

| Prestress treatment | Light | Dark |
|---|---|---|
| Source of variation | | |
| Stress (S) | 2.52 | 1.40 |
| Irradiance (I) | 1.39 | 9.69* |
| Interaction (S x I) | 6.28* | 0.25 |


A.
B.
% of $^{14}C$ in the Sol. Extract
0
4
8
12
16
20
250 μEm⁻²s⁻¹
30 μEm⁻²s⁻¹
Dark
250 μEm⁻²s⁻¹
30 μEm⁻²s⁻¹
Dark

proline was expressed as a percentage of the total radioactivity (soluble + insoluble).

Other amino acids

Non-starved samples

The total counts found in glutamate were significantly greater in samples incubated in the dark (in both turgid and stressed samples) than in any illuminated sample (Fig 6.4a,b,c and Table 6.16A) i.e. the conversion of glutamate to other amino acids was reduced in dark treated samples.

Stress induced significantly higher amounts of labelled aspartate in both dark and illuminated samples (Fig 6.4a,b,c and Table 6.16A).

Starved samples

$^{14}C$ in the glutamine fraction (containing alanine and arginine as well as glutamine) was significantly greater in the light than in the dark, but the opposite was true for the serine fraction (serine + glycine + asparagine). The radioactivity remaining in glutamate was high in stressed samples in the dark compared to illuminated samples (Fig 6.4 d,e,f).

Stressed samples showed significantly higher label in $\gamma$ aminobutyrate and lower label in glutamine compared to turgid samples (Fig 6.4 d,e,f and Table 6.16B). Net synthesis of proline from labelled glutamate in light pretreated tissue, thus appears to proceed at a similar rate whether the tissue is illuminated or not during stress. In tissue maintained in the dark during the prestress period, however, proline synthesis is significantly reduced if stressed in the dark and the radioactivity appears to remain in glutamate. Isotope trapping cannot account for this difference as turgid samples had significantly smaller proline pools, yet incorporated a greater proportion of the label in proline.

Fig 6.4

Radioactivity in the individual amino acids as a percentage of the radioactivity in the soluble extract

Twelve day old barley plants were transferred to 48 hours continuous light or continuous darkness. The first leaves were excised and floated on -15 bar PEG solution or water for 20 hours at different irradiances. They were then fed $^{14}C$ glutamic acid and incubated for a further 5 hours before they were harvested.

..... Turgid samples

_____ Stress samples

abc - Prestressed in light def - Prestressed in darkness

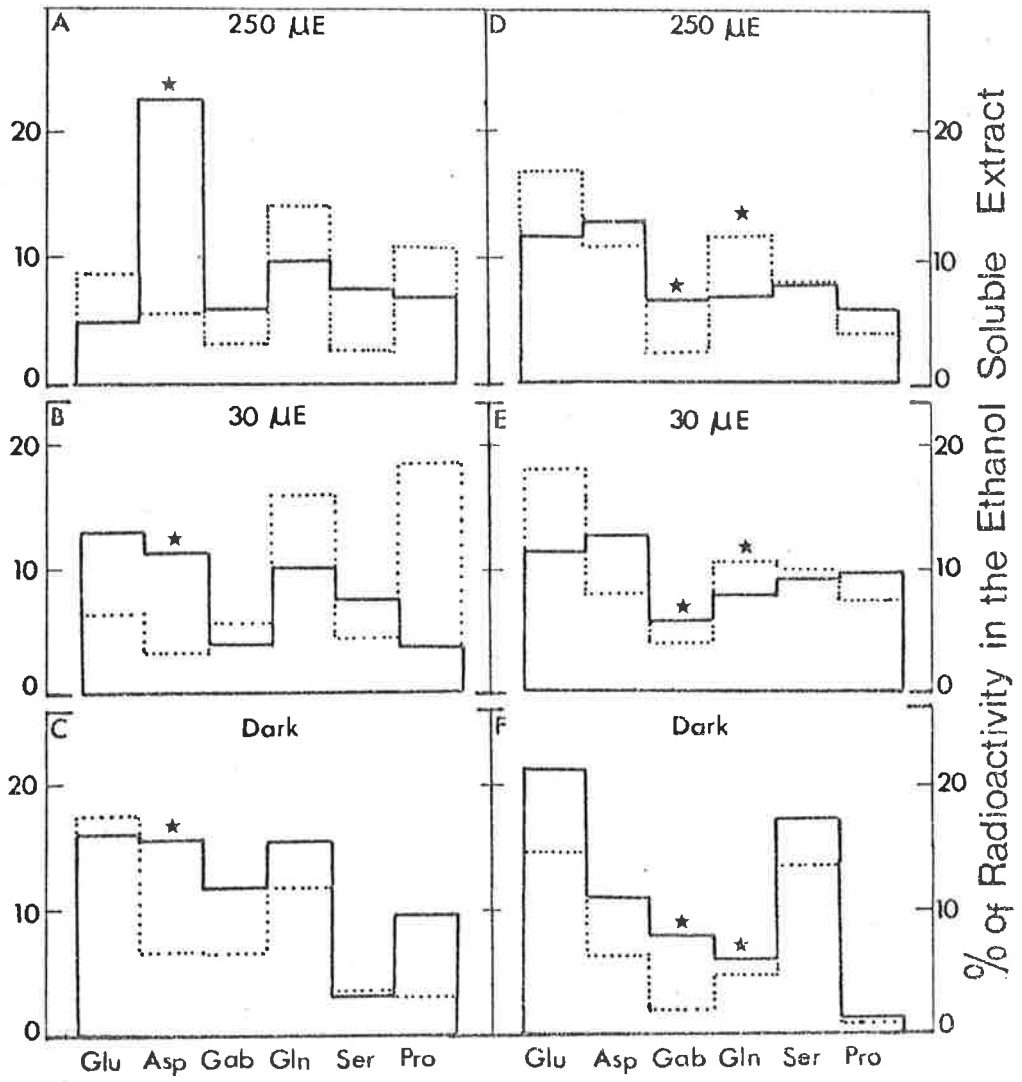
N.B : Glu = glutamine + alanine + arginine

Ser = serine + asparagine + glycine

Table 6.16

F Table for results in Fig 6.4

| Source of variation | Glut | Asp | Gab | Gln | Ser | Prol |
|---|---|---|---|---|---|---|
| | A. Prestressed in light | | | | | |
| Stress | 0.08 | 34.69* | 2.35 | 0.54 | 3.24 | 2.52 |
| Irrad | 13.48* | 4.17 | 4.44 | 0.10 | 1.40 | 1.39 |
| S x I | 3.88 | 2.18 | 2.33 | 0.87 | 1.13 | 6.28* |
| | B. Prestressed in darkness | | | | | |
| Stress | 0.15 | 2.75 | 5.56* | 6.29* | 0.37 | 1.40 |
| Irrad | 0.28 | 0.66 | 0.01 | 8.75* | 8.16* | 9.69* |
| S x I | 1.05 | 0.18 | 0.31 | 4.06 | 0.75 | 2.25 |


A
250 µE
D
250 µE
B
30 µE
E
30 µE
C
Dark
F
Dark
20
10
0
Glu Asp Gab Gln Ser Pro
Glu Asp Gab Gln Ser Pro
% of Radioactivity in the Ethanol Soluble Extract

From these results it appears unlikely that enhanced synthesis of proline from glutamate is responsible for the greater accumulation of proline in L-L compared with L-D samples during stress treatment. In starved segments, however, light may enhance proline accumulation by increasing the rate of synthesis of proline from glutamate. Results of this experiment have shown that in dark treated tissue depleted of carbohydrates (D-D) an extremely low percentage of the total radioactivity was accumulated as proline.

Thus, this experiment further confirms that the synthesis of proline from exogenous glutamate is similar in segments illuminated at 30 or 250 $\mu E\ m^{-2}s^{-1}$ in both starved and non-starved segments. In addition, it clearly shows that in stressed segments, proline synthesis is reduced in starved tissue (low in carbohydrates) incubated in the dark but not in non-starved ones (high in carbohydrates).

## 6.8 Oxidation of proline

Tissue exposed to light before the period of stress accumulated radioactivity from $^{14}C$ glutamate in proline at a similar rate whether exposed to darkness or light during stress (L-D and L-L). Earlier experiments had shown that dark-stressed tissue accumulated only about half the amount of proline accumulated in light-stressed tissue, however. This discrepancy could be due to a difference in the rate of proline oxidation between the two treatments. In order to evaluate the role of proline oxidation in proline accumulation, the following experiment was performed.

6.8.1 Methods

The plant culture and treatments were identical to those described in Section 6.7.1, except that 0.5 ml L-U-$^{14}$C proline (specific radioactivity 285 mCi mmol$^{-1}$ supplied as 50 µCi ml$^{-1}$) solution was fed to the leaves instead of L-U-$^{14}$C glutamate. All the other procedures (viz harvesting, extraction and estimation of radioactivity) were exactly as has been explained in Section 6.7.1

6.8.2 Results

Incorporation of radioactivity into the alcohol soluble extract and the insoluble residue.

Non-starved samples

Again, there was a much higher uptake of radioactivity by turgid samples than by stressed samples (Table 6.17A). The percentage of the total radioactivity present as insoluble residue was high (compared to samples fed $^{14}$C-glutamate) throughout apart from samples stressed at 250 µE m$^{-2}$s$^{-1}$.

Starved samples

Turgid samples again took up more label than stressed samples (Table 6.17B). Here the turgid samples incorporated most of the radioactivity into the insoluble residue in the illuminated segments. The stressed samples (both illuminated and dark) and the dark turgid samples incorporated a much lower percentage of total radioactivity into the insoluble residue (Table 6.17B).

Table 6.17

Radioactivity in the ethanol soluble extract and the insoluble residue in L-U-$^{14}$C proline fed samples.

A. Prestressed in light (non-starved)

| | Soluble extract | Insoluble extract |
|---|---|---|
| Treatment | | |
| 250 µE $m^{-2}s^{-1}$ | | |
| Turgid | 255410 (86.4) | 40139 (13.6) |
| Stress | 62490 (95.3) | 3058 (4.7) |
| 30 µE $m^{-2}s^{-1}$ | | |
| Turgid | 343455 (87.0) | 51520 (13.0) |
| Stress | 30975 (83.9) | 5954 (16.1) |
| Dark | | |
| Turgid | 120625 (79.5) | 31066 (20.5) |
| Stress | 37060 (83.1) | 7526 (16.9) |

B. Prestressed in darkness (starved)

| | Soluble extract | Insoluble residue |
|---|---|---|
| Treatment | | |
| 250 µE $m^{-2}s^{-1}$ | | |
| Turgid | 127840 (27.1) | 344650 (72.9) |
| Stress | 58860 (89.2) | 7101 (10.8) |
| 30 µE $m^{-2}s^{-1}$ | | |
| Turgid | 133000 (17.9) | 608148 (82.1) |
| Stress | 38580 (90.1) | 4240 (9.9) |
| Dark | | |
| Turgid | 258553 (80.3) | 63238 (19.7) |
| Stress | 53380 (85.0) | 9407 (15.0) |

Figures in parenthesis are the radioactivity expressed as a percentage of the total radioactivity in the sample

Total amino acids

Non-starved

Label from $^{14}C$ proline, unlike that from glutamate was recovered as a high percentage in the total amino acid fraction (Table 6.18) in both turgid and stressed samples. When the radioactivity in amino acids was expressed as a percentage of the total radioactivity present in the sample, the proportion was still high (< 70%) in all treatments (Table 6.18), meaning that a higher percentage remained as free amino acids.

Starved

Again total amino acids constituted the major proportion of the total soluble extract radioactivity (Table 6.18) in both stressed and turgid samples. When the amino acid radioactivity was expressed as a percentage of the total radioactivity (soluble + insoluble), however, the illuminated turgid samples showed a reduced percentage of total counts in the amino acids (< 20%, Table 6.18) due to the high radioactivity of the insoluble residue. These results imply that in turgid samples, $^{14}C$ from proline is rapidly incorporated into proteins and other compounds present in the insoluble residue. This was not the case in any of the stressed samples or the dark turgid samples (Table 6.18).

Proline

Non-starved samples

Samples pretreated in light retained a high percentage of the total radioactivity in proline in both turgid and stressed samples in light and darkness (Fig 6.5A) suggesting a low rate of oxidation.

Starved samples

Dark pretreated samples retained a high proportion of the applied radioactivity in proline only in tissue illuminated during stress. All tissue maintained turgid (both in darkness and in light) and stressed

Table 6.18

Radioactivity in total amino acids as a percentage of total radio - activity in the alcohol soluble extract in $^{14}C$ proline fed samples

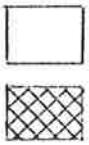
| Prestress treatment | Light | Dark |
|---|---|---|
| Treatment | | |
| 250 µE $m^{-2}s^{-1}$ | | |
| Turgid | 83.3 (72.0) | 65.9 (17.9) |
| Stress | 84.0 (80.1) | 92.3 (82.3) |
| 30 µE $m^{-2}s^{-1}$ | | |
| Turgid | 100.3 (87.3) | 86.3 (15.5) |
| Stress | 85.4 (71.7) | 90.5 (81.5) |
| Dark | | |
| Turgid | 94.0 (74.7) | 87.8 (70.5) |
| Stress | 92.6 (77.0) | 70.8 (60.2) |

Figures in parenthesis are the amino acid counts expressed as a percentage of the total radioactivity (soluble + insoluble) in the sample

Fig 6.5

Radioactivity in proline as a percentage of the radioactivity in alcohol soluble extract in L-U$^{14}$C proline fed samples

Twelve day old barley plants were transferred to 48 hours continuous light or darkness following which the first leaves were excised and floated on water or PEG (-15 bars) for 20 hours under light of 250 or 30 $\mu E\ m^{-2}s^{-1}$ or in darkness. The leaves were allowed to take up $^{14}$C-proline solution and left for a further 5 hours before they were harvested.



Turgid

Stressed

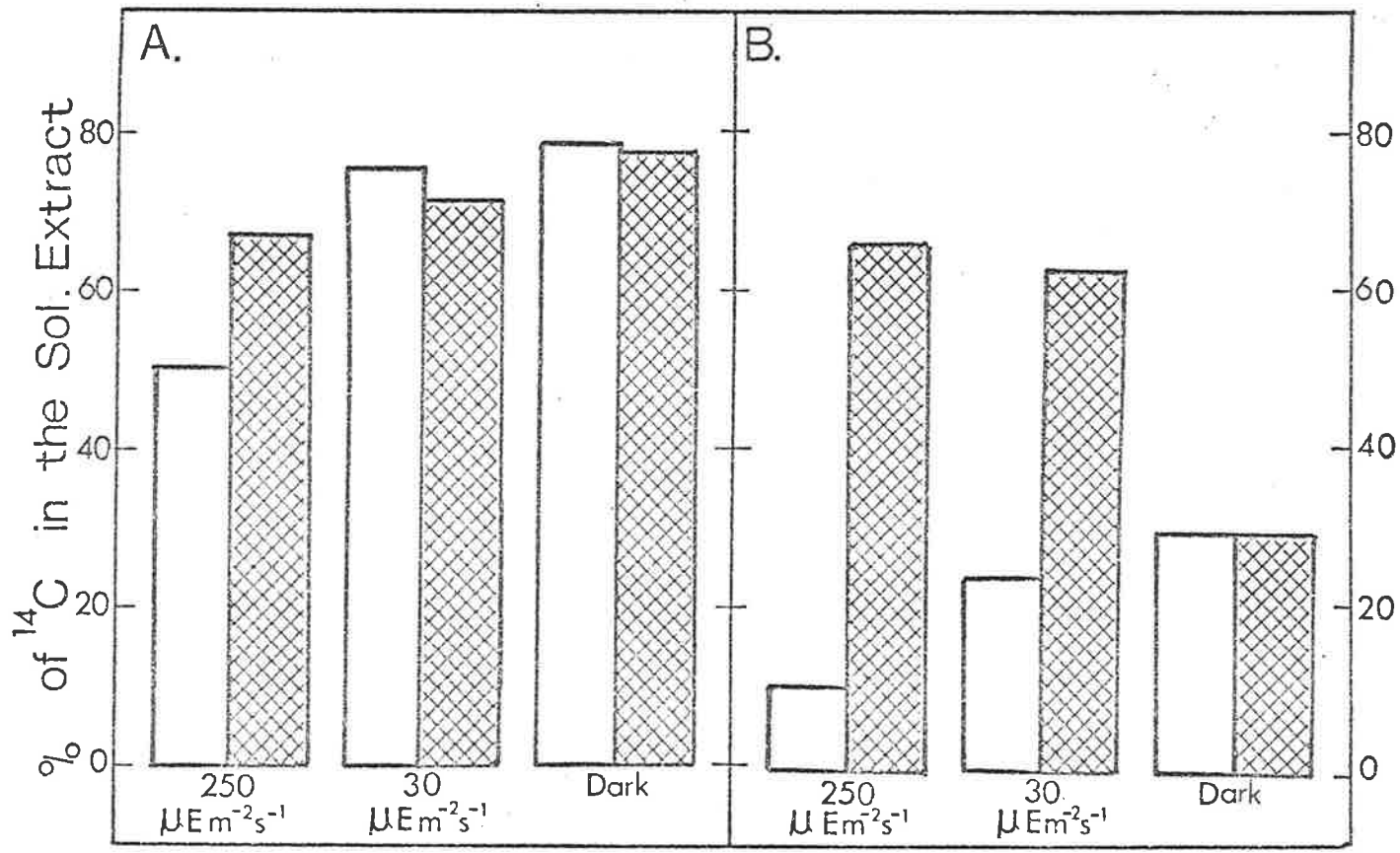
A. Prestressed in light (non-starved)

B. Prestressed in darkness (starved)

Table 6.19

F values for results in Fig 6.5

| Prestress treatment | Light | Dark |
|---|---|---|
| Source of variation | | |
| Stress (S) | 0.36 | 12.01* |
| Irradiance (I) | 3.48 | 0.52 |
| Interaction (SxI) | 1.11 | 5.03 |


A.
B.
% of $^{14}C$ in the Sol. Extract
0
20
40
60
80
250 μEm$^{-2}$s$^{-1}$
30 μEm$^{-2}$s$^{-1}$
Dark

tissue incubated in the dark showed very low radioactivity remaining in proline (Fig 6.5B and Table 6.19) with turgid samples at 250 μE $m^{-2}s^{-1}$ the least radioactivity. It is interesting that proline oxidation in the dark in both stressed and turgid tissue was apparently identical. Thus, it seems that with starved tissue, light promotes proline utilization in turgid segments but inhibits this process in stress tissue (Fig 6.5).

When the radioactivity in proline was expressed as a percentage of the total radioactivity in the tissue (Table 6.20) the difference between turgid and stressed samples was magnified, because of the considerable amount of radioactivity present in the insoluble residue of the illuminated turgid samples.

Other amino acids

Non-starved samples

The distribution of radioactivity in the various amino acids in stressed samples was similar between illuminated and dark treatments (Fig 6.6A). In turgid samples, the dark treated samples contained lower radioactivity in glutamate and glutamine than illuminated ones. Turgid samples illuminated at 250 μE $m^{-2}s^{-1}$ showed a less percentage of total $^{14}C$ in proline but greater percentage in γ-amino butyrate and aspartate when compared with 20 μE $m^{-2}s^{-1}$ and dark treated samples.

When turgid and stressed samples are compared, the radioactivity in glutamate and glutamine was significantly higher in turgid samples in all three treatments ( Fig 6.6A and Table 6.12A).

Starved samples

Stress treatment in tissue incubated in the dark induced conversion of a greater percentage of the labelled proline to glutamate compared to that converted to light (Fig 6.6 and Table 6.21B). Turgid samples did

Table 6.20

Radioactivity in proline as a percentage of the total radioactivity (soluble + insoluble) in the sample in $^{14}C$ proline fed samples

| Prestress treatment | Light | Dark |
|---|---|---|
| 250 µE $m^{-2}s^{-1}$ | | |
| Turgid | 42.1 | 2.2 |
| Stress | 64.0 | 59.8 |
| 30 µE $m^{-2}s^{-1}$ | | |
| Turgid | 62.0 | 4.2 |
| Stress | 59.3 | 59.7 |
| Dark | | |
| Turgid | 60.8 | 23.6 |
| Stress | 64.3 | 25.0 |

Fig 6.6

Distribution of radioactivity in the various amino acids when expressed as a percentage of the total radioactivity in the ethanol soluble extract, in samples fed $^{14}C$ proline.

..... Turgid

——— Stress

A. Prestressed in light

B. Prestressed in darkness

Table 6.21

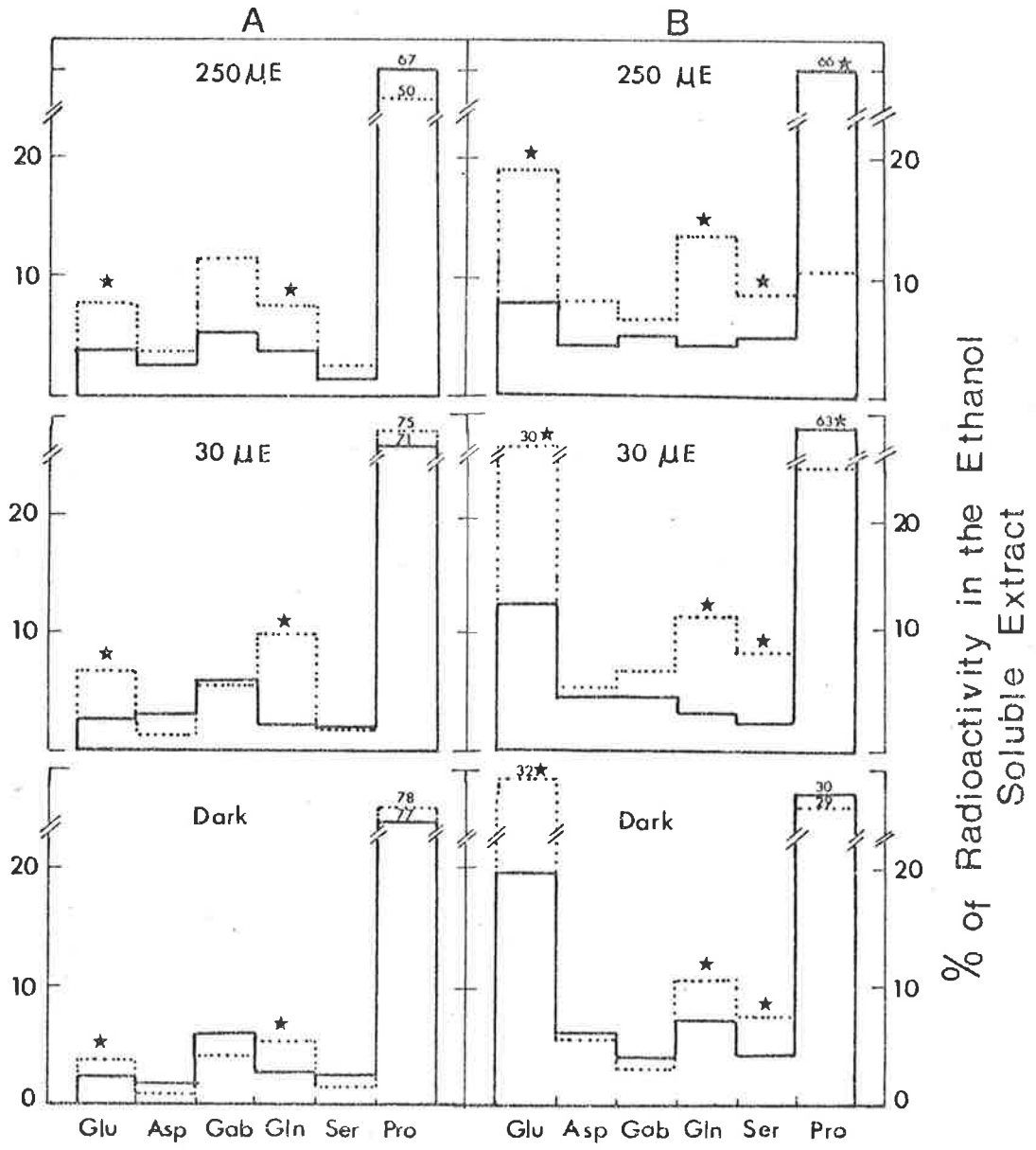
F table of results in Fig 6.6

| A Source of variation | GLUT | ASP | GAB | GLN | SER | PROL |
|---|---|---|---|---|---|---|
| Stress (S) | 45.39** | 1.67 | 0.29 | 136.77** | 0.08 | 0.36 |
| Irradiance(I) | 10.74* | 9.46 | 0.77 | 8.99* | 0.14 | 3.48 |
| Interaction (SxI) | 2.76 | 5.32* | 1.11 | 15.11** | 1.75 | 1.11 |
| B Source of variation | | | | | | |
| Stress (S) | 40.52** | 5.49* | 0.69 | 96.31** | 35.55** | 12.01* |
| Irradiance (I) | 8.76* | 1.97 | 0.90 | 1.84 | 1.04 | 0.52 |
| Interaction (SxI) | 2.18 | 2.77 | 0.85 | 6.37* | 1.40 | 5.03* |

A - Prestressed in light

B - Prestressed in darkness

Significance level * $P \leq 0.05$


A
B
250 µE
30 µE
Dark
67
50
75
71
78
77
66
30
63
32
30
29
20
10
0
Glu
Asp
Gab
Gln
Ser
Pro
% of Radioactivity in the Ethanol Soluble Extract

not show any significant differences between illuminated and dark treated samples. When stressed and turgid samples were compared, the percentage of radioactivity in glutamate, glutamine and serine was significantly greater in the turgid treatments (Table 6.21B).

Thus, one can conclude that the oxidation of proline in samples initially high in carbohydrates (i.e. non-starved samples ) (Fig 6.1) is relatively slow in stressed and turgid samples whether they are incubated in light or darkness (with the possible exception of turgid tissue at 250 $\mu E\ m^{-2}s^{-1}$, Fig 6.6). In samples pretreated in the dark and hence low in initial carbohydrate content, both illumination and stress seem to be essential to inhibit the oxidation of proline. Apart from the requirement for carbohydrate, stress seems to be equally important since illuminated, turgid, starved samples despite having a higher carbohydrate content, showed very high rates of utilisation compared to otherwise similar stressed samples (Fig 6.1). Of course, turgid samples have a smaller free proline pool and consequently will exhibit a more rapid oxidation of supplied radioactive proline. However, in turgid non-starved (L-L) samples where the proline pool is small, oxidation is slow. Therefore, higher utilization of proline observed in D-L turgid samples is unlikely to be due solely to the smaller proline pool.

## 6.9 Discussion

Photosynthesis may act in several ways in influencing proline accumulation in stressed tissue. Although it does not provide the bulk of carbon atoms for *de novo* proline synthesis (<5%) in starved (D-L) and non-starved (L-L) leaves, it is likely to provide the energy and carbohydrates which regulate proline accumulation. The provision of carbohydrates exogenously enhanced accumulation to a certain extent in

starved tissue in the dark, although it was not as effective as light (Table 6.4). The provision of light but not $CO_2$ (i.e. in a $CO_2$ free air) to starved segments reduced proline accumulation compared to segments incubated in normal air (Section 5.3) suggesting that carbon fixation is required for maximum proline accumulation, particularly if the tissue is depleted of carbohydrates. In non-starved segments (i.e. high in carbohydrates), $CO_2$ free air did not inhibit proline accumulation but transfer to the dark did suggesting that light was able to promote proline accumulation and may have been acting in some way in addition to carbon fixation.

Illuminated segments at 30 or 250 $\mu E\ m^{-2}s^{-1}$ had similar rates of net proline synthesis and of oxidation in starved and non-starved segments. However, the prestress environment greatly influenced these rates in stressed segments compared with turgid segments.

Starved samples

Tracer studies clearly show that light significantly enhanced proline synthesis in both turgid and stressed tissue. A similar enhancement of proline synthesis by light has been reported in turgid tobacco leaves (Mizusaki et al, 1964) and stressed barley leaves (Hanson and Tully, 1979).

Illuminated turgid samples showed rapid oxidation of proline to glutamate and glutamine (alanine + arginine + glutamine) fraction or incorporation into the insoluble fraction. Rapid incorporation of proline into the insoluble residue has been reported previously in turgid barley leaves (Boggess et al, 1976; Stewart et al, 1977 and Hanson and Tully, 1979).

Thus, stress treatment may have induced proline accumulation, in illuminated leaf segments due more to an inhibition of

oxidation in these segments than to enhanced synthesis. When compared with dark treated samples, illumination may have induced proline accumulation due to both reduced oxidation and enhanced synthesis. Of course, there may be other factors not immediately apparent, inducing higher proline accumulation in illuminated stressed segments.

Non-starved samples

In contrast to the starved samples, the reason for greater proline accumulation in non-starved samples stressed in light is less apparent. Stressed samples show no difference in either synthesis or oxidation rates between illuminated and dark tissue. This could result from the presence of high initial carbohydrate contents. Turgid tissue on the other hand showed greater synthesis of proline in the light compared with segments incubated in the dark although oxidation rates were similar. This could account for the greater accumulation of proline in illuminated turgid tissue compared with dark treated tissue. Both Mizusaki et al (1964) and Wang (1969) have reported very slow oxidation of proline in turgid tobacco and corn leaves.

The lack of a difference in either synthesis or oxidation rates between illuminated and dark tissue in stressed samples is puzzling. However since illuminated tissue accumulates $\simeq 2$ times as much proline as dark tissue when stressed, it must imply that it is not the difference in proline synthesis or oxidation rates that are responsible for greater accumulation of proline in light, but possibly that glutamate supply or some other step leading to glutamate is limiting in dark stressed samples. However, there may be other explanations; for example, the exogenous labelled compounds supplied may enter a different compartment to that of the endogenous compounds. However, assuming that

the labelled compounds introduced enter the same metabolic pool in the various treatments, it can be concluded that proline synthesis is enhanced by light and/or stress (segments left in the dark show reduced synthesis of proline in all instances except for non-starved stressed segments (L-D)). Loss of radioactivity in proline is rapid in turgid samples depleted of reserves (mainly incorporated into the insoluble residue) and also in starved samples incubated in the dark. Stress treatment seems to prevent this incorporation of proline into the insoluble residue which therefore remains as proline. In non-starved samples the oxidation of proline is inhibited in all samples (both dark and illuminated, stressed and turgid samples).

## SECTION II LOW TEMPERATURE STRESS

### 7. Proline accumulation at low temperatures

## Section 2 Low temperature stress

### 7. Proline accumulation at low temperature

#### 7.1 Introduction

Proline has been shown to accumulate at low temperature in a number of species including wheat (Trione et al, 1966; Jones and Weinberger, 1970 and Stefl et al, 1978), Barley (Chu et al, 1974, 1978) radish (Chu et al, 1974, 1978), rape (Kacperska-Palacz et al, 1977; Chu et al, 1978 and Sosinska and Maleszewski, 1978) and apples (Benko, 1968), etc. Although reports on accumulation of proline in response to lowered temperatures are plentiful, there have been relatively few (Chu et al, 1974, 1978; Sosinka and Maleszewski, 1978) on the physiological and biochemical changes which characterise this response.Of special interest is the apparent absolute requirement of light for proline accumulation in barley plants reported by Chu et al, 1978. The following experiments were designed to confirm that light is essential for proline accumulation in the cold and to test if conditions prevalent during the prestress period could influence proline accumulation during the stress period.

#### 7.2 Intact plants

##### 7.2.1 Effect of continuous light or darkness on proline accumulation in intact barley plants

###### 7.2.1.1 Methods

Barley plants were grown in sand for 10 days at 20°C in a 16 hour photoperiod and irradiance of 250 $\mu Em^{-2}s^{-1}$ before being transferred

to 48 hours continuous light or darkness. The plants were then transferred to 5°C or left at 20°C for 3 days in continuous light or darkness (the cold room was illuminated with a bank of fluorescent lights and irradiance adjusted to approximately 250 $\mu E\ m^{-2}s^{-1}$). The first leaves were then harvested, frozen in liquid nitrogen, freeze dried and weighed. Samples were also harvested at the end of the prestress period (48 hours). The weighed samples were then analysed for free proline content as detailed in Materials and Methods Section 2.8.

7.2.1.2 Results

At the end of 48 hours continuous darkness at 20°C, the free proline content of the plants was greater than that of plants exposed to continuous light (compare Fig 7.1A and B, 0 time sample). Further growth in the light at 20°C during the following 3 days produced plants with a low free proline content (<0.5 mg $g^{-1}$ dry wt), whereas transfer of the plants to darkness at 20°C gave higher levels of proline. Plants transferred from darkness to light for 3 days also showed a decrease in proline concentration (Fig 7.1 A,B) .

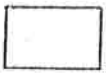
Low temperature (5°C) for 3 days in darkness did not change the proline content of plants and prestress levels were maintained during the stress treatment irrespective of exposure to light or darkness during the prestress treatment. Plants in light during the low temperature treatment, however, showed a significant accumulation of proline when they had been prestressed in light (Fig 7.1A). In contrast, plants exposed to darkness for 48 hours before being moved to a 5°C light environment accumulated no proline within the 3 days.

Fig 7.1

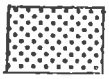
Effect of low temperature on proline accumulation in barley plants exposed to continuous light or darkness.
10 day old barley plants were exposed to 48 hours continuous light or continuous darkness prior to transferring one half of the plants to 5°C for 3 days. Plants were either exposed to continuous light or darkness during this 3 day period.
Each point is the mean of 4 replicates

A. Prestressed in light

B. Prestressed in darkness



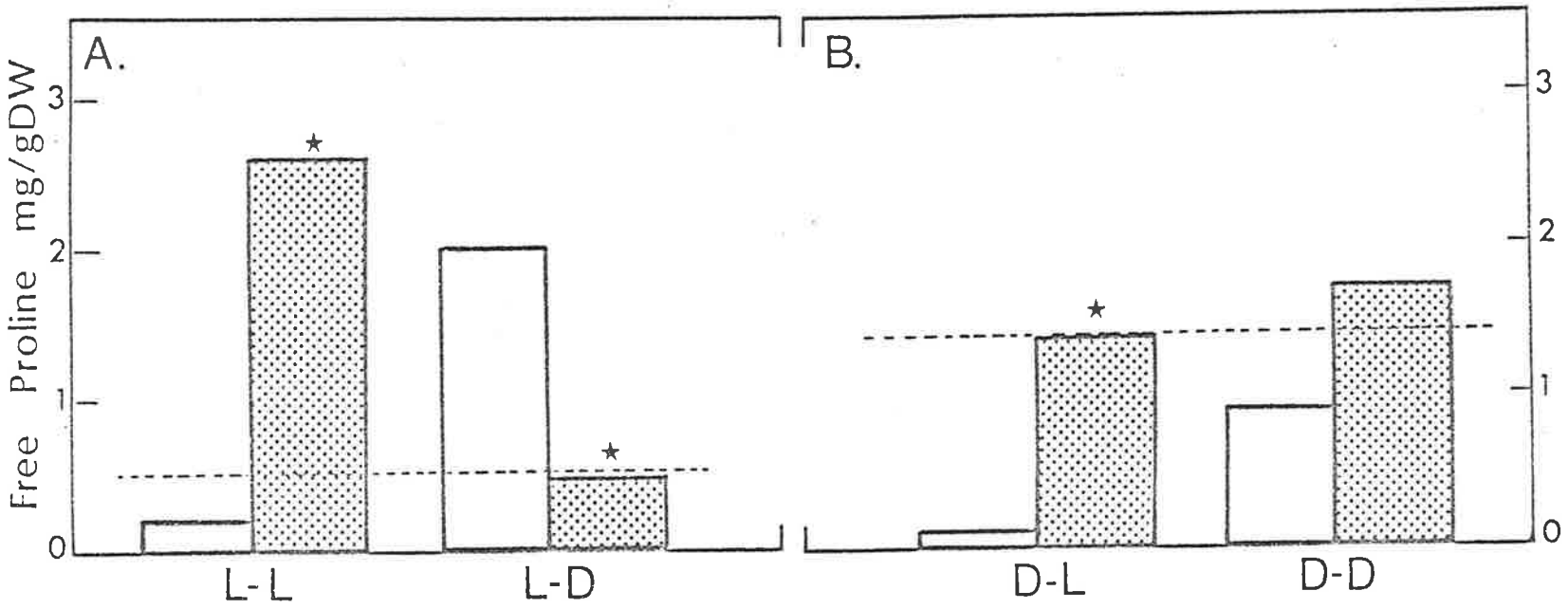
Incubated at 20°C for 3 days



Incubated at 5°C for 3 days

----- 0 day, 20°C samples

Significantly different from 20°C sample


A.
B.
Free Proline mg/gDW
0
1
2
3
L-L
L-D
D-L
D-D

Thus, proline accumulation at 5°C required light both immediately before and during exposure to the low temperature.

### 7.2.2 Effect of low temperature over a prolonged period on proline accumulation

From the results obtained in the previous experiment, light during low temperature treatment appears to have no effect on proline accumulation in dark prestressed plants during 3 days of cold treatment. Whether this was due to a delay in accumulation (i.e. a greater lag phase) or to a total lack of accumulation in plants prestressed in darkness was not clear. To further elucidate this point, plants were exposed to low temperatures (5°C) for a longer duration.

#### 7.2.2.1 Methods

Growth and treatment of plants were identical to those in the previous experiment except that plants were sampled over a longer period of time (6 days). Proline was estimated in the preserved samples as detailed in Materials and Methods Section 2.8.

#### 7.2.2.2 Results

Plants maintained in continuous darkness at 5°C showed no significant change in proline content over the entire 6 day sampling period whether the prestress period had been in light or in darkness (Fig 7.2). Plants in light during exposure to low temperature showed a significant accumulation of proline; which was affected by the prestress treatment. Plants prestressed in light showed a rapid increase in proline concentration until day 4, after which the proline content did not change. In plants prestressed in darkness, however, the free proline content was not significantly different from either the dark stressed

Fig 7.2

Proline accumulation in intact barley plants in the cold under different conditions of light/dark treatment during and prior to cold stress.

Barley plants were grown till they were 10 days old in a 16 hour photoperiod at 20°C in sand. They were then transferred to 48 hours continuous light or darkness before exposure to 5°C in continuous light or darkness.

Each value is the mean of 4 replicates

| | Prestress ($P_1$) | Stress ($P_2$) |
|---|---|---|
| ○ | Light | Light |
| ● | Dark | Light |
| □ | Light | Dark |
| ■ | Dark | Dark |

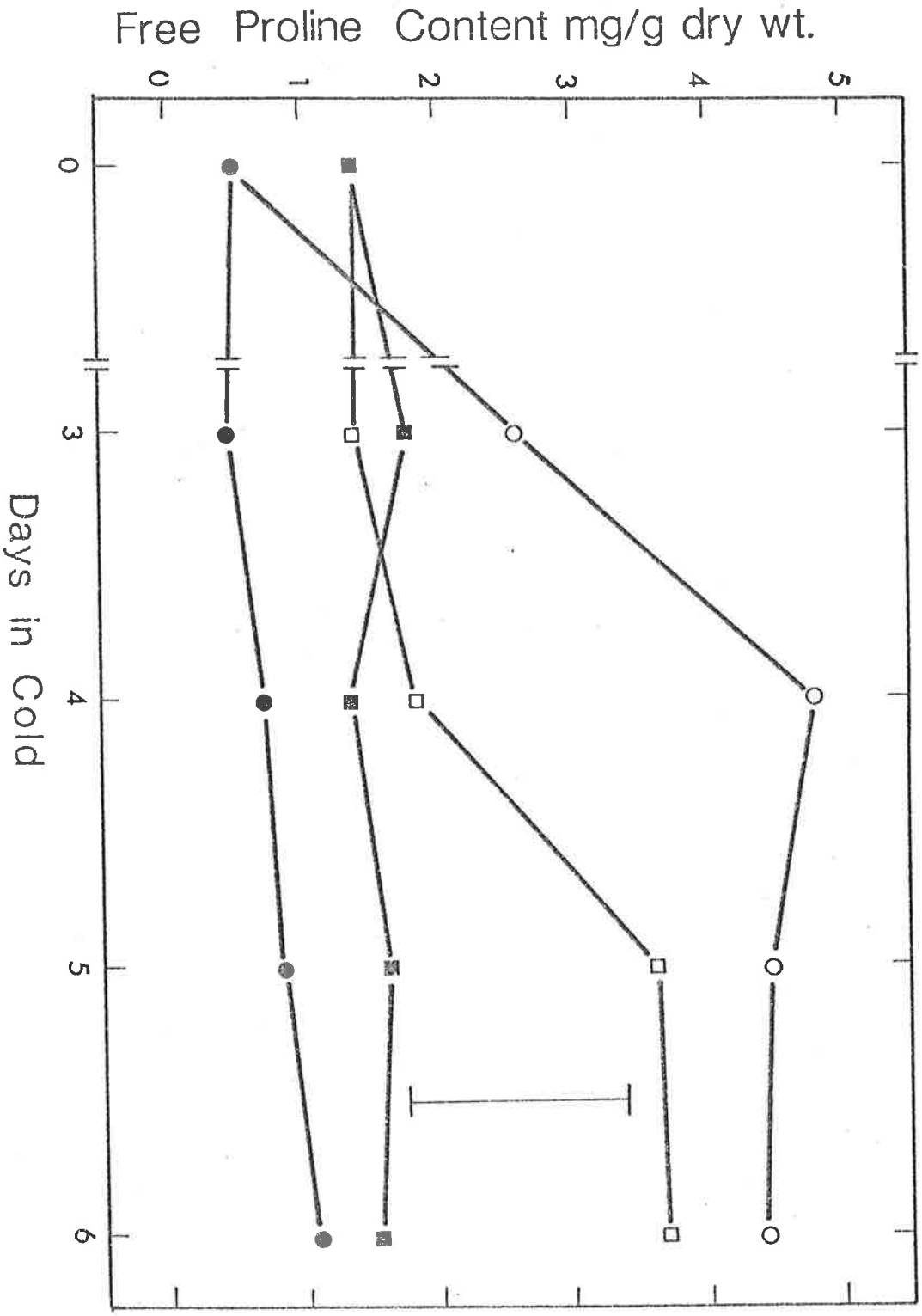
Table 7.1

F values for results in Fig 7.2

| Source of variation | Variance ratio (VR) |
|---|---|
| Prestress ($P_1$) | 8.36** |
| Stress ($P_2$) | 414.41** |
| Duration | 23.82** |
| $P_1$ x $P_2$ | 110.00** |
| $P_1$ x Duration | 6.89** |
| $P_2$ x Duration | 17.07** |
| $°_1$ x $P_2$ x Duration | 6.14** |

Significance level * probability $\leq 0.05$

** probability $\leq 0.01$


Free Proline Content mg/g dry wt.
0
1
2
3
4
5
Days in Cold
0
3
4
5
6

(D-D) or day 0 samples until day 4 after which the free proline content increased significantly. Thus, both conditions during prestress and during the stress treatment significantly influenced the rate and extent of proline accumulation (Table 7.1).

It can be concluded that a prestress period in darkness delays, but does not prevent or inhibit, the subsequent accumulation of proline in light at 5°C. Exposure to light during the period at low temperature is a requirement for accumulation, however.

## 7.3 Excised leaf segments

### 7.3.1 Introduction

Although the problems of using intact plants in water stress studies (such as reduced $\Psi$ and enhanced transpiration in light) were not present at 5°C, experiments on excised leag segments were conducted to facilitate comparisons with the previous water status results. Moreover, manipulation of the atmosphere (e.g. $CO_2$), light conditions and treatment with inhibitors were greatly facilitated by the use of an excised segment system. Excised leaf segments also were not subject to possible changes in water status through reduced root conductivity at 5°C.

### 7.3.2 Effect of light and darkness on proline accumulation at 5°C

#### 7.3.2.1 Methods

Barley plants were grown in sand at 20°C in a 16 hour photoperiod until they were 10 days old. The plants were then exposed to 48 hours continuous light or darkness at 20°C. The first leaves were then excised, segmented and floated on water (10 segments per petri dish).

The petri dishes were incubated at 5°C either in continuous light or darkness. They were harvested after 48 hours and every 24 hours thereafter until day 6. The harvested segments were dried between filter papers (Whatman No. 1), frozen in liquid nitrogen and freeze dried. The samples were analysed for proline after their dry weights had been recorded. There were 4 replicates of each treatment.

### 7.3.2.2. Results

#### A. Dry weight

Segments incubated in the dark at 5°C showed a significant decrease in dry weight within 2 days of cold treatment in both light and dark prestressed samples (Fig 7.3) after which the dry weight did not change significantly. Illuminated tissue (irrespective of the prestress treatment) showed a significant increase in dry weight over the entire period, but particularly during the first 3 days. The dry weight of illuminated samples prestressed in light was always greater than those prestressed in darkness up to day 6 (Fig 7.3). Statistical analysis showed that all the three treatments i.e. prestress, stress and duration of stress, had a significant effect on dry weight of the tissue (Table 7.2). Evidently the segments continued to photosynthesise at an appreciable rate at 5°C.

#### B. Proline accumulation

Illuminated segments showed a significant accumulation of proline (in both light and dark prestressed (D-L) samples), accumulating the most in light pretreated samples. It is interesting that proline

Fig 7.3

Dry weight of 10 segments at 5°C under different conditions of illumination both during and prior to stress.

Barley plants were grown in a 16 hour photoperiod at 20°C for 10 days and then transferred to 48 hours continuous light or darkness. The first leaves were then segmented and floated on water at 5°C in the light or dark for varying lengths of time.

Each value is the mean of 4 replicates.

| | Prestress (48 h) | Stress (5°C) |
|---|---|---|
| ○ | Light | Light |
| ● | Light | Dark |
| □ | Dark | Light |
| ■ | Dark | Dark |

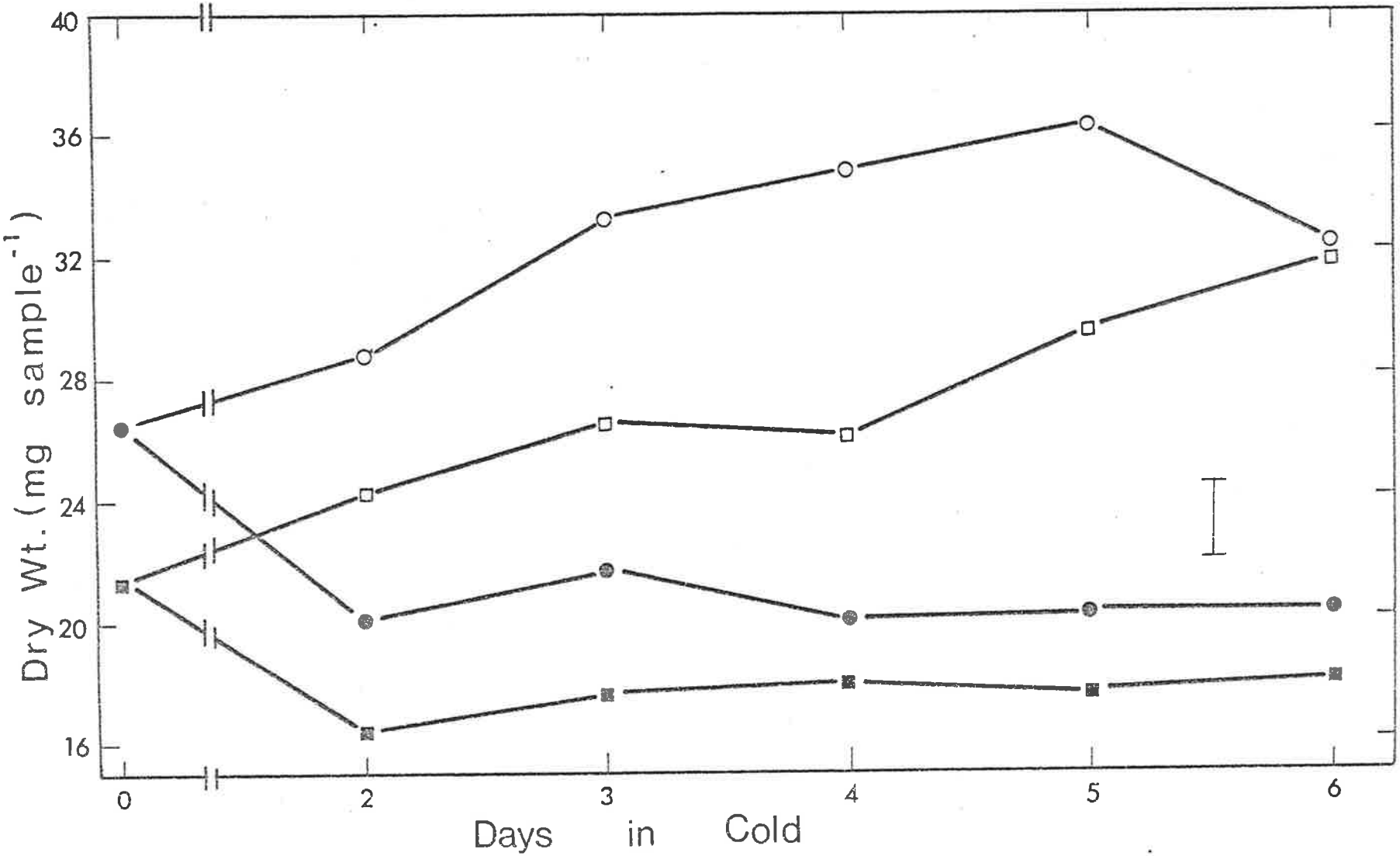
Table 7.2

F table for results in Fig 7.3 (DW)

| Source of variation | Variance ratio |
|---|---|
| Period 1 ($P_1$) | $163.34^{**}$ |
| Period 2 ($P_2$) | $775.35^{**}$ |
| Duration | $9.13^{**}$ |
| $P_1 \times P_2$ | $9.29^{**}$ |
| $P_1 \times$ Duration | $3.36^{*}$ |
| $P_2 \times$ Duration | $37.04^{**}$ |
| $P_1 \times P_2 \times$ Duration | $3.49^{*}$ |

Significance level * $P<0.05$

** $P<0.01$


40
36
32
28
24
20
16
Dry Wt. (mg sample$^{-1}$)
0
2
3
4
5
6
Days in Cold

concentration appeared to reach a maximum at 5°C in the L-L samples and may have reached a maximum in D-L samples at 5 days (Fig 7.4). Segments incubated in the dark showed no significant accumulation of proline over the entire duration of the experiment (Fig 7.4). All 3 treatments ($P_1$, $P_2$ and duration) significantly influenced proline accumulation (Table 7.3). As in the intact plants, then, light during exposure to 5°C is essential for proline accumulation, although here lack of light in the period before low temperature did not delay accumulation to the same extent.

### 7.3.3 Effect of transfer of segments between light and darkness on proline accumulation

#### 7.3.3.1 Methods

The growth of barley plants until 10 days was identical to that described in Section 7.2.1. All the plants were exposed to 48 hours continuous light during the prestress treatment. The leaves were then excised, segmented and floated on water at 5°C in either light or darkness for 72 hours, samples were interchanged between the two environments at 24 and 48 hours. All the segments were harvested after 72 hours, frozen in liquid nitrogen, freeze dried and weighed. The samples were analysed for proline as detailed in Materials and Methods Section 2.8.

#### 7.3.3.2 Results

Segments exposed to light for the entire 72 hour period accumulated the most proline and samples in darkness throughout this period the least (Fig 7.5 A,B and Fig 7.6). Those transferred between light and darkness accumulated varying amounts of proline, depending

Fig 7.4

Proline accumulation at 5°C under different conditions of illumination, both during and prior to stress.
Barley plants growing in 16 hour photoperiod at 20°C for 10 days were transferred to 48 hours continuous light or darkness. The first leaves were then segmented and floated on water at 5°C in the light or darkness for varying lengths of time. Each point is the mean of 4 replicates.

| | Prestress | Stress |
|---|---|---|
| ○ | Light | Light |
| ● | Light | Dark |
| □ | Dark | Light |
| ■ | Dark | Dark |

Table 7.3

F table for results in Fig 7.4

| Source of variation | Variance ratio |
|---|---|
| Period 1 ($P_1$) | 65.14** |
| Period 2 ($P_2$) | 555.05** |
| Duration | 71.03** |
| $P_1$ x $P_2$ | 60.73** |
| $P_1$ x Duration | 11.59** |
| $P_2$ x Duration | 52.16** |
| $P_1$ x $P_2$ x Duration | 5.08** |

Significance level * $P \leq 0.05$

** $P \leq 0.01$

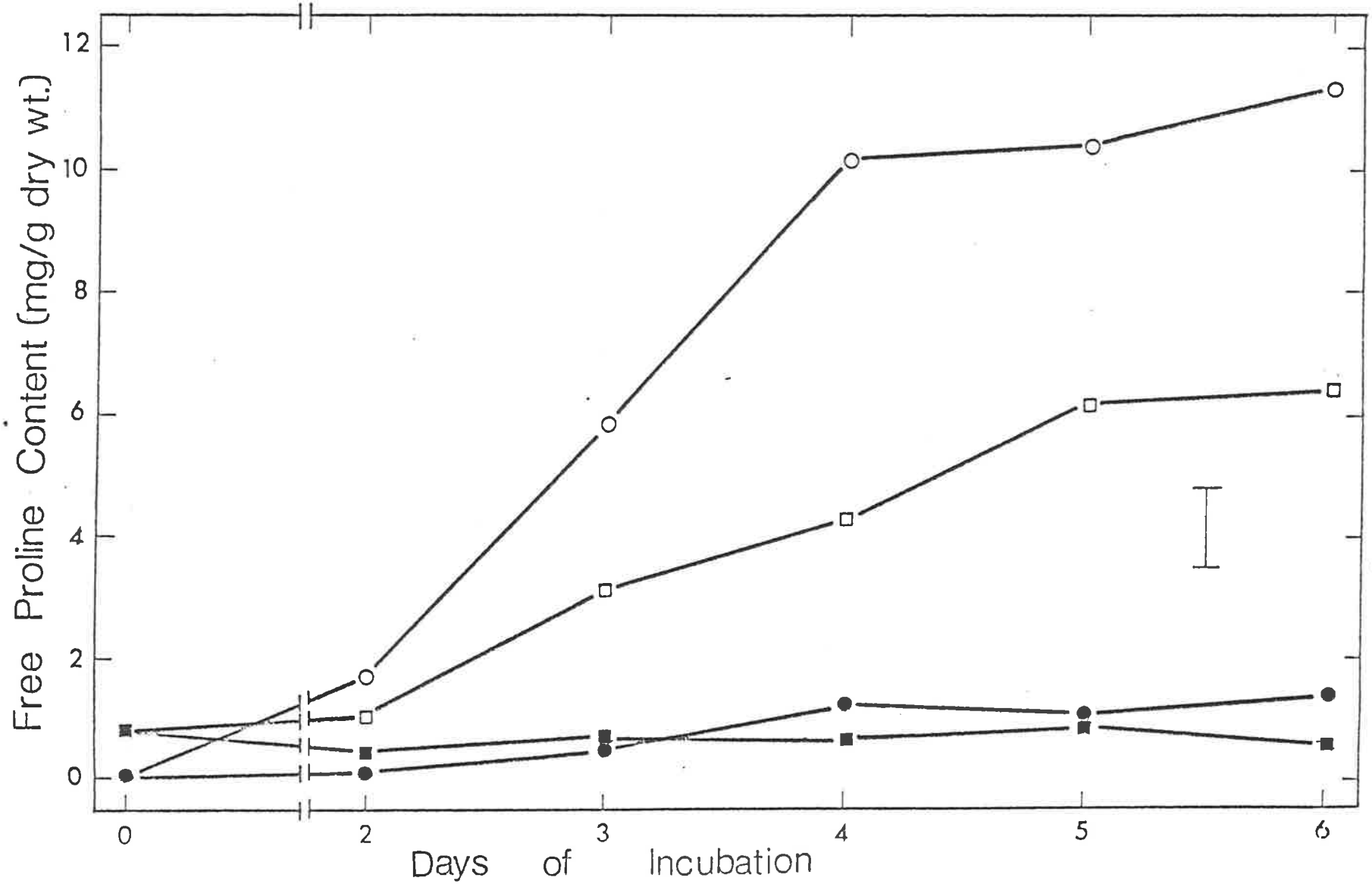

Free Proline Content (mg/g dry wt.)
12
10
8
6
4
2
0
0
2
3
4
5
6
Days of Incubation

Fig 7.5

Effect of transfer between light and darkness on proline accumulation in barley segments at 5°C.

Each point is the mean of 4 replicates.

O Light treatment

● Dark treatment

A. Transfer of segments from light to darkness

B. Transfer of segments from dark to light

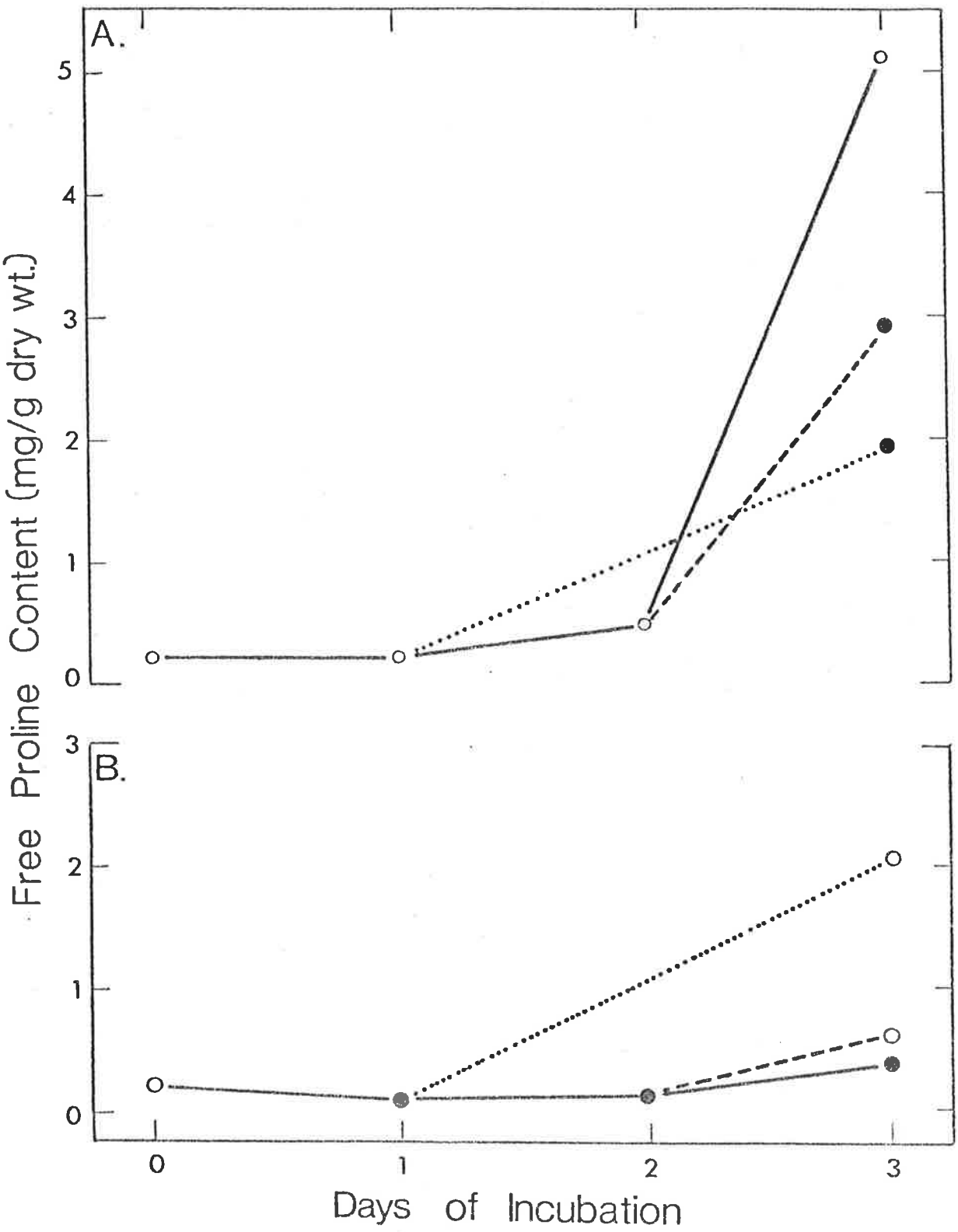

A.
B.
Free Proline Content (mg/g dry wt.)
Days of Incubation
0
1
2
3
4
5

Fig 7.6

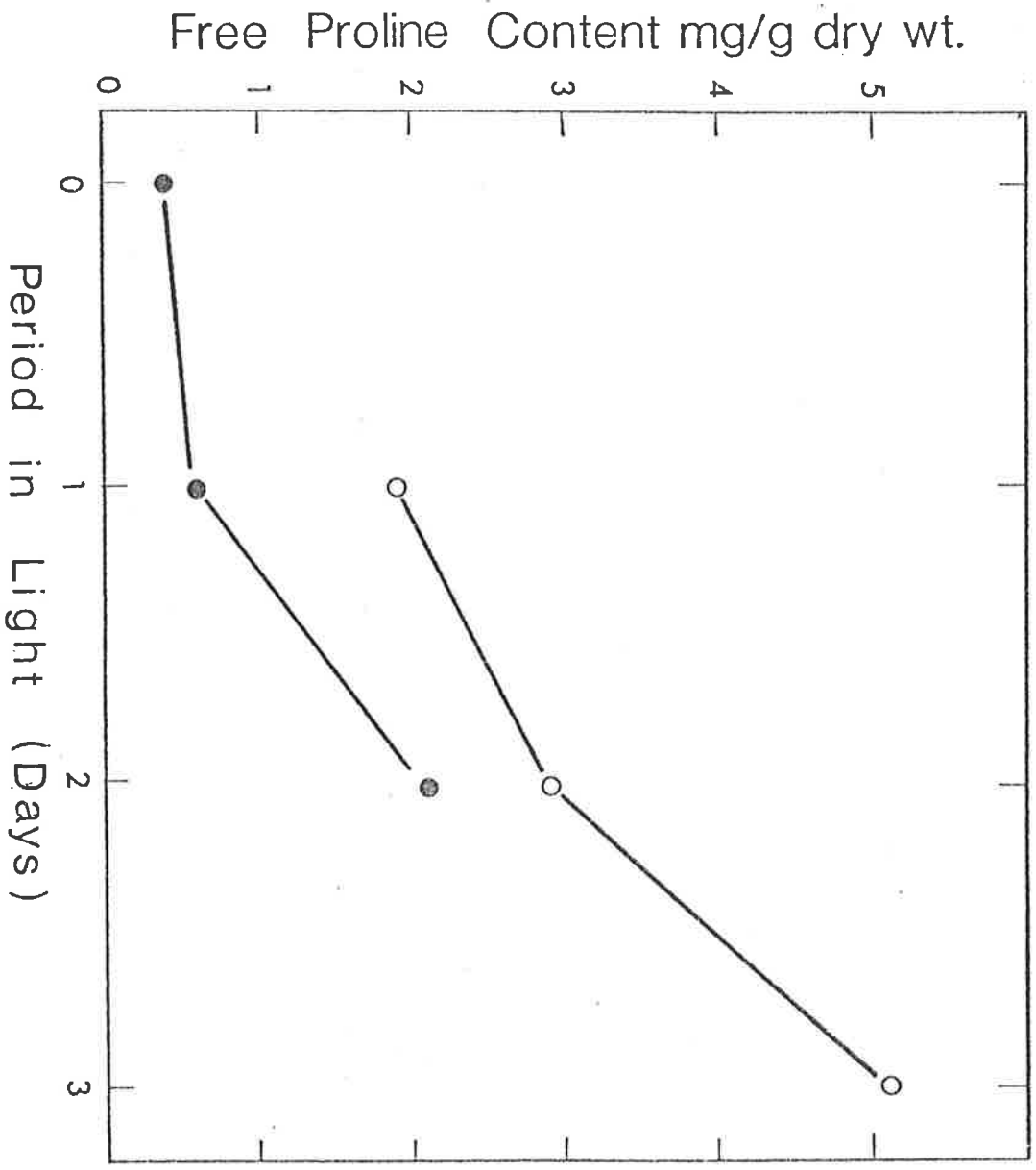
Proline accumulation in segments exposed to light for varying lengths of time during incubation at 5°C.

The data points are the same as those in Fig 7.5.

Sequence of light/dark treatment at 5°C

○ Light, Dark

● Dark, Light


Free Proline Content mg/g dry wt.
0
1
2
3
4
5
Period in Light (Days)
0
1
2
3

on the duration and sequence of the light and dark treatment. However, one salient feature is that proline accumulated in darkness in samples exposed to light for the first 24 hours of stress alone (Fig 7.5A and Fig 7.6).

### 7.3.4 Effect of irradiance on proline accumulation at 5°C

Since light is required for proline accumulation at low temperature, the response to irradiance was examined.

#### 7.3.4.1 Methods

Plant growth and prestress treatment were identical to those described in Section 7.3.3.1. After excision, the tissue was floated on water at 5°C for 3 days (experiment A) or 5 days (experiment B) and exposed to different irradiances (experiment A 1, 30, 70, 140 and 220 μE $m^{-2}s^{-1}$; experiment B 15, 35, 70, 140 and 230 μE $m^{-2}s^{-1}$) or left in darkness. The segments were then harvested and preserved for the estimation of proline.

#### 7.3.4.2 Results

Experiment A

Samples prestressed in light showed a significant stimulation of proline accumulation at 30 μE $m^{-2}s^{-1}$ and above when compared with segments at 5°C in darkness. Segments prestressed in darkness, however, showed a significant enhancement of accumulation only at an irradiance of 140 μE $m^{-2}s^{-1}$ or greater (Fig 7.7 and Table 7.4). An irradiance of 1 μE $m^{-2}s^{-1}$ failed to enhance the accumulation of

Fig 7.7

Effect of irradiance on proline accumulation at 5°C in tissue exposed to light or darkness, 48 hours prior to stressing.

Ten day old barley plants were transferred to 48 hours continuous light or darkness and then excised and segmented. The segments were floated on distilled water at 5°C for 72 hours under different irradiances.

Each point is the mean of 4 replicates.

○ L - L ● L - D

□ D - L ■ D - D

N.B. The irradiances are plotted on a log scale.

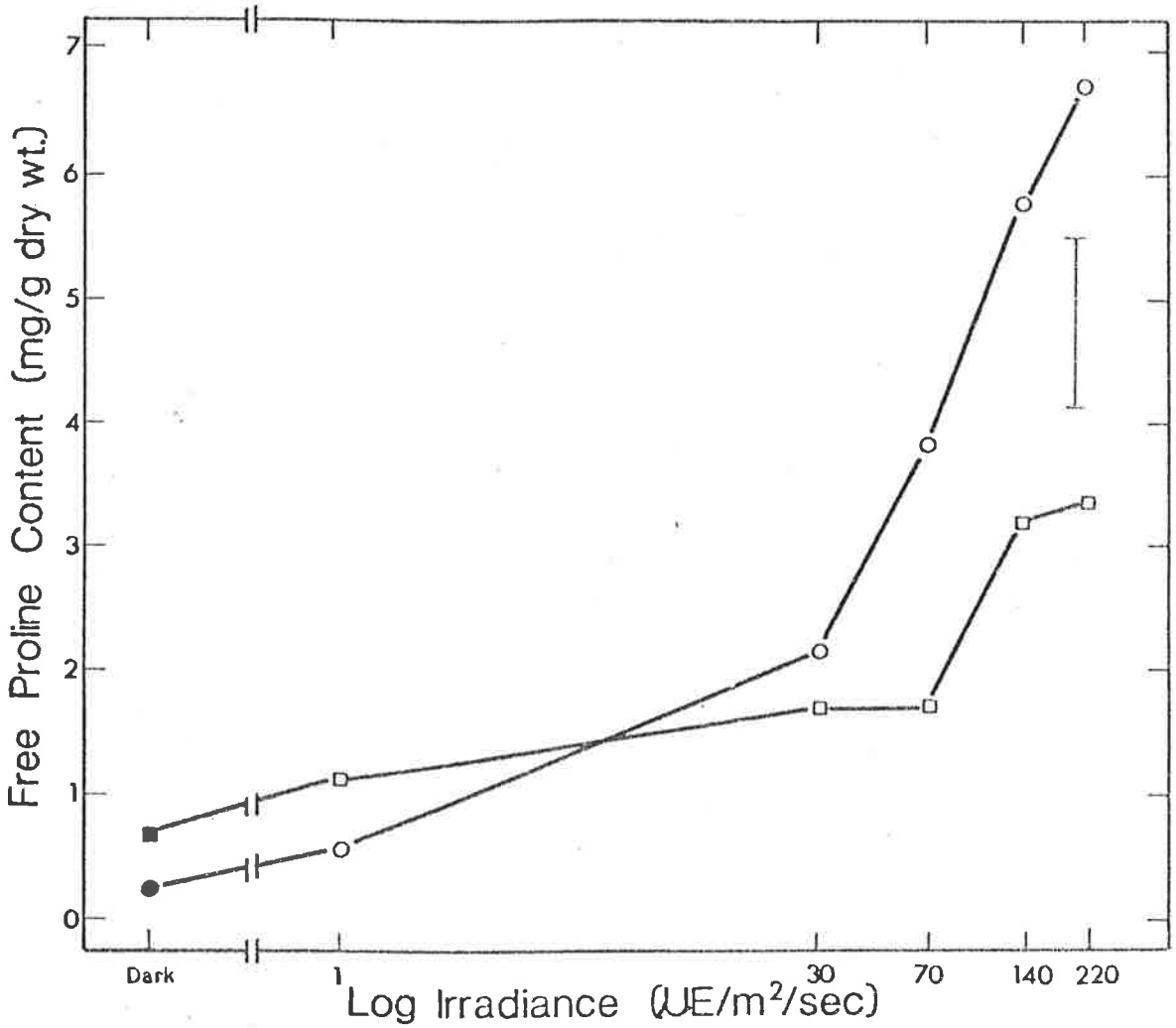
Table 7.4

F table for results in Fig 7.7 (LL/DL irradiance)

| Source of variation | Variance ratio |
|---|---|
| Prestress ($P_1$) | $20.72^{**}$ |
| Irradiance (I) | $30.44^{**}$ |
| $P_1$ x I | $5.98^{**}$ |

Significance level * $P \leq 0.05$

** $P \leq 0.01$


Free Proline Content (mg/g dry wt.)
0
1
2
3
4
5
6
7
Dark
1
30
70
140
220
Log Irradiance (μE/m²/sec)

proline in either treatment (but did so with water stress Fig 3.3). Segments prestressed in light accumulated significantly more proline than those prestressed in darkness when incubated at 5°C in an irradiance of 70 μE $m^{-2}s^{-1}$ or greater.

Experiment B

The effect of irradiance on proline accumulation was much more marked following 5 days incubation at 5°C than following 3 days (Fig 7.8). This was particularly true at the lowest irradiance used, 15 μE $m^{-2}s^{-1}$. The statistical analysis table (Table 7.5) clearly demonstrates this effect of duration on proline accumulation.

## 7.4 Discussion

Proline accumulation at 5°C was stimulated by light in both intact plants and excised leaf segments. In excised segments, a low irradiance of 15 μE $m^{-2}s^{-1}$ was effective in significantly enhancing proline accumulation . It is also clear that there was a carry over effect of light in samples transferred to the dark, i.e. if light was present for some duration during incubation at 5°C, subsequent dark treatment at 5°C did not enhance proline accumulation, but neither did it inhibit the synthesis of light stimulated proline. It is interesting that, unlike water stress, the presence of light during the pretreatment (at 20°C) had no effect on subsequent proline accumulation in the dark at 5°C. One possible explanation for this observation could be that the carbohydrate content after 3 days dark treatment at 5°C is reduced below the threshold necessary for proline to accumulate. This may not occur in water stress if the carbohydrate threshold for proline accumulation is lower than that

Fig 7.8

Irradiance on proline accumulation during 3 and 5 days of low temperature stress.

Ten day old barley plants growing in a 16 hour photoperiod at 20°C were exposed to 48 hours continuous light prior to segmentation of the first leaves. The segments were incubated on water at 5°C in the dark or light at various irradiances for 3 or 5 days.

Each point is the mean of 4 replicates.

● 3 days in the cold

★ 5 days in the cold

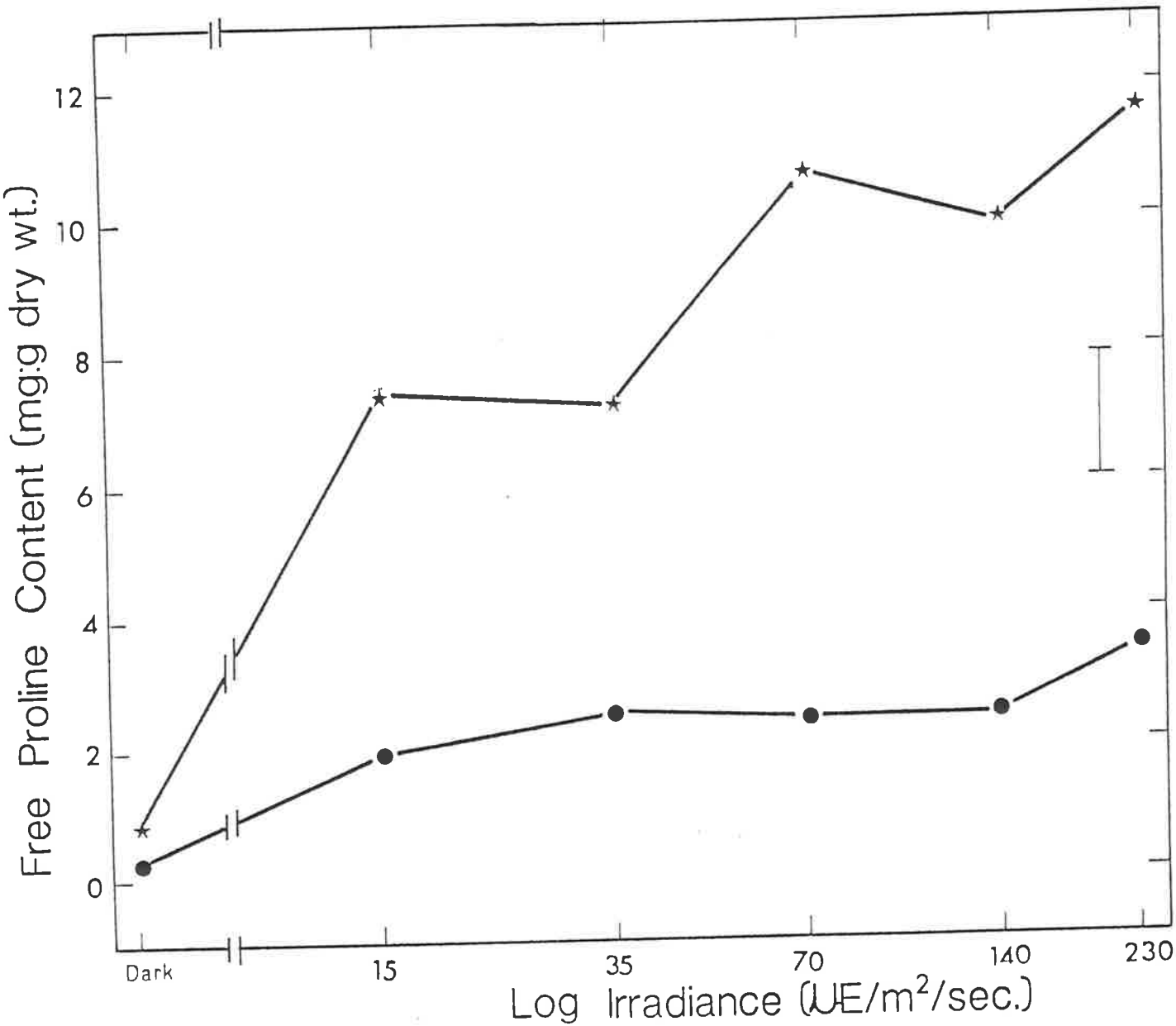
Table 7.5

F table for results in Fig 7.8

| Source of variation | Variance ratio |
|---|---|
| Duration | 242.70** |
| Irradiance | 29.43** |
| Duration x Irradiance | 10.71** |

Significance level * $P \leq 0.05$

** $P \leq 0.01$


Free Proline Content (mg:g dry wt.)
12
10
8
6
4
2
0
Dark
15
35
70
140
230
Log Irradiance (μE/m²/sec.)

necessary for proline accumulation during cold stress. Providing light for even 1 day may increase carbohydrate content above this threshold, resulting in proline accumulation. Of course, there may be other factors e.g. nitrogen metabolism which may require light and in turn influence proline accumulation.

Also in contrast to water stress, proline accumulation was the least in light prestressed samples, stressed in the dark (L-D). These samples showed even lower proline content than dark stressed samples which were also prestressed in the dark (D-D). The reason for this is that the tissue at low temperature in the dark appears to show no significant change in proline content with time, thus maintaining the prestress level, and this was greater in the dark pretreated samples. Since proline levels do not change significantly over the 6 day period, it must mean that the metabolism of proline is very slow. In fact, in considering the sample dry weights, it appears that general metabolism was negligible in the dark at 5°C. Chu et al (1978) also concluded that there was reduced metabolism of proline in the dark at 5°C and that light probably induced greater synthesis.

## SECTION II LOW TEMPERATURE STRESS

### 8. Photosynthesis and proline accumulation at 5°C

# 8. Photosynthesis and proline accumulation at 5°C

## 8.1 Introduction

The previous chapter has shown that light is essential for proline accumulation at 5°C. Light may have a qualitative (phytochrome mediated) or a quantitative (photosynthetic) effect on proline accumulation. That the response is not a low energy phytochrome response is evident from the results obtained. The accumulation of proline was proportional to both the duration of light as well as the irradiance level. In their report on proline accumulation in response to cold in barley, Chu et al (1978) presumed that the rate of photosynthesis at 5°C was probably not significant and hence the supply of photosynthetically derived carbohydrate precursors was unlikely to be critical to the light dependance of accumulation. The following experiments were performed to test the above hypothesis and to further elucidate the manner in which light might influence proline accumulation.

## 8.2 Carbon dioxide exchange in barley segments incubated at 5°C

### 8.2.1 Methods

Barley plants were grown in sand at 20°C in a 16 hour photoperiod for 10 days. The plants were then transferred to 48 hour continuous light or continuous darkness. The first leaves were then excised, segmented and floated on water at 5°C. The $CO_2$ exchange was measured with an infra red gas analyser (IRGA) as detailed in chapter 5.1. All conditions were identical to those of the earlier experiment except the temperature of the assimilation chamber which was maintained

at 5°C by pumping cold water from a water bath maintained at 5°C. The temperature within the chamber was monitored continuously with a copper-constantan thermocouple placed against the underside of the leaf. The carbon dioxide exchange of dark pretreated samples alone was monitored continuously from the commencement of incubation at 5°C. This treatment was chosen in preference to the light pretreated samples as earlier results (Fig 5.3) has shown that $CO_2$ exchange was greater in dark pretreated than light pretreated samples, and response to cold would be easier to detect.

Also, it was not possible to perform replicates of the net carbon exchange, mainly due to the time required for each measurement.

### 8.2.2 Results

Leaf segments showed a steady rate of photosynthesis for the first 12 to 13 hours (Fig 8.1A). The $CO_2$ exchange then dropped rapidly such that within the following 12 hours, the $CO_2$ exchange rate was 55% of the initial photosynthesis rate. The rate of decline in the $CO_2$ exchange became slower in the next 24 hours after which $CO_2$ exchange remained steady up to 4 days of cold treatment (Fig 8.1B). Thus, cold stress induced a significant decline in the photosynthesis rate ( 50% in D-L and 30% in L-L). The segments pretreated in light and darkness showed a similar photosynthetic response with time, although D-L samples showed significantly higher exchange rates than the L-L samples. Low temperatures also induced a 50% decline in the $CO_2$ efflux rate in the dark (Fig 8.1B). In this case, the $CO_2$ efflux in light pretreated samples (L-L) was always greater than in the dark pretreated tissue (D-L).

Thus, the assumption of a very low, non-significant photosynthesis rate in tissue in the cold is wrong, as significant $CO_2$ exchange is present even after 4 days cold treatment.

Fig 8.1 A

Continuous monitoring of net $CO_2$ exchange of barley leaf segments from the beginning of the incubation period at 5°C in tissue exposed to 48 hours continuous darkness during the prestress treatment (D - L samples).

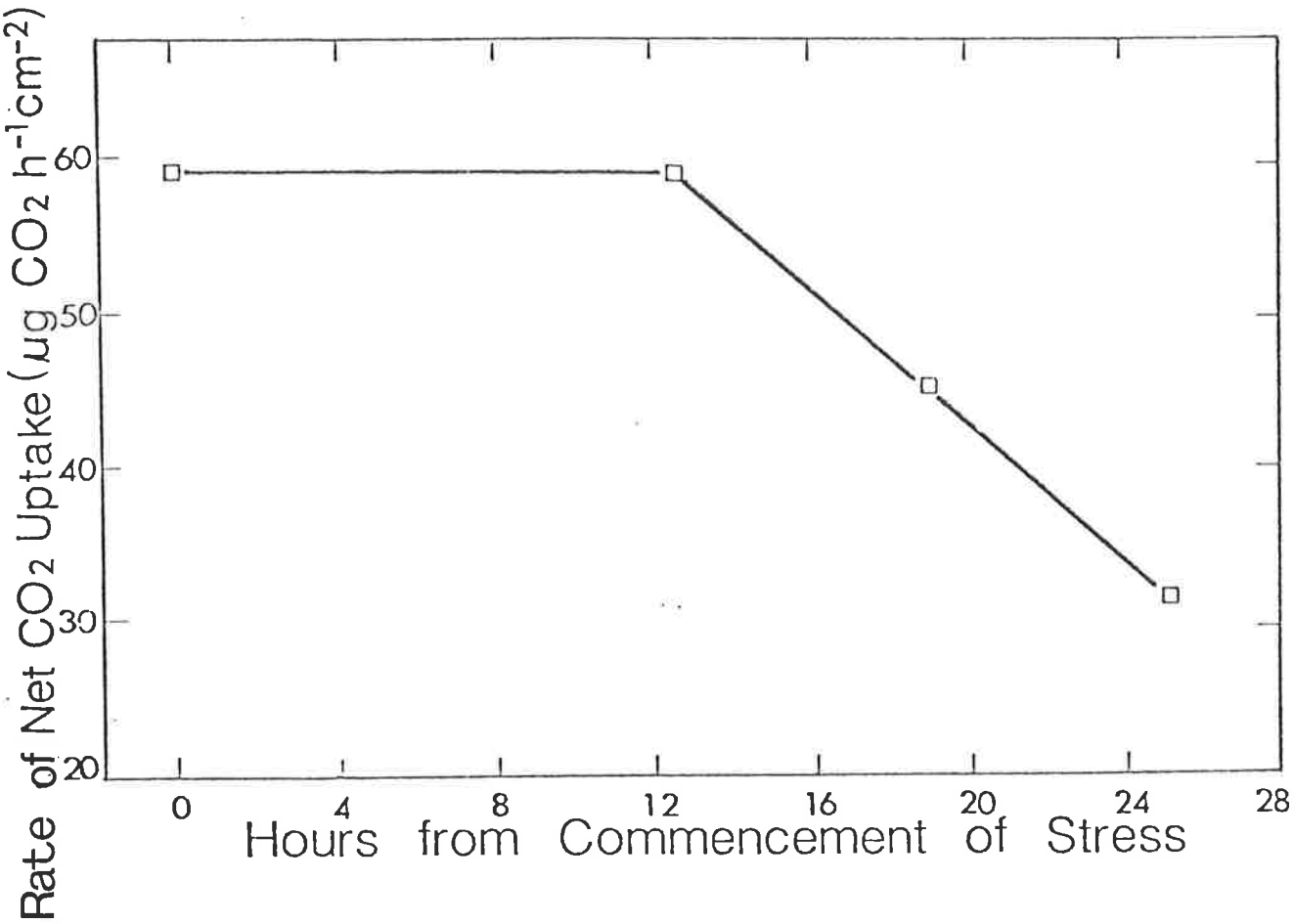

Rate of Net $CO_2$ Uptake ($\mu g\ CO_2\ h^{-1}cm^{-2}$)
60
50
40
30
20
0
4
8
12
16
20
24
28
Hours from Commencement of Stress

Fig 8.1 B

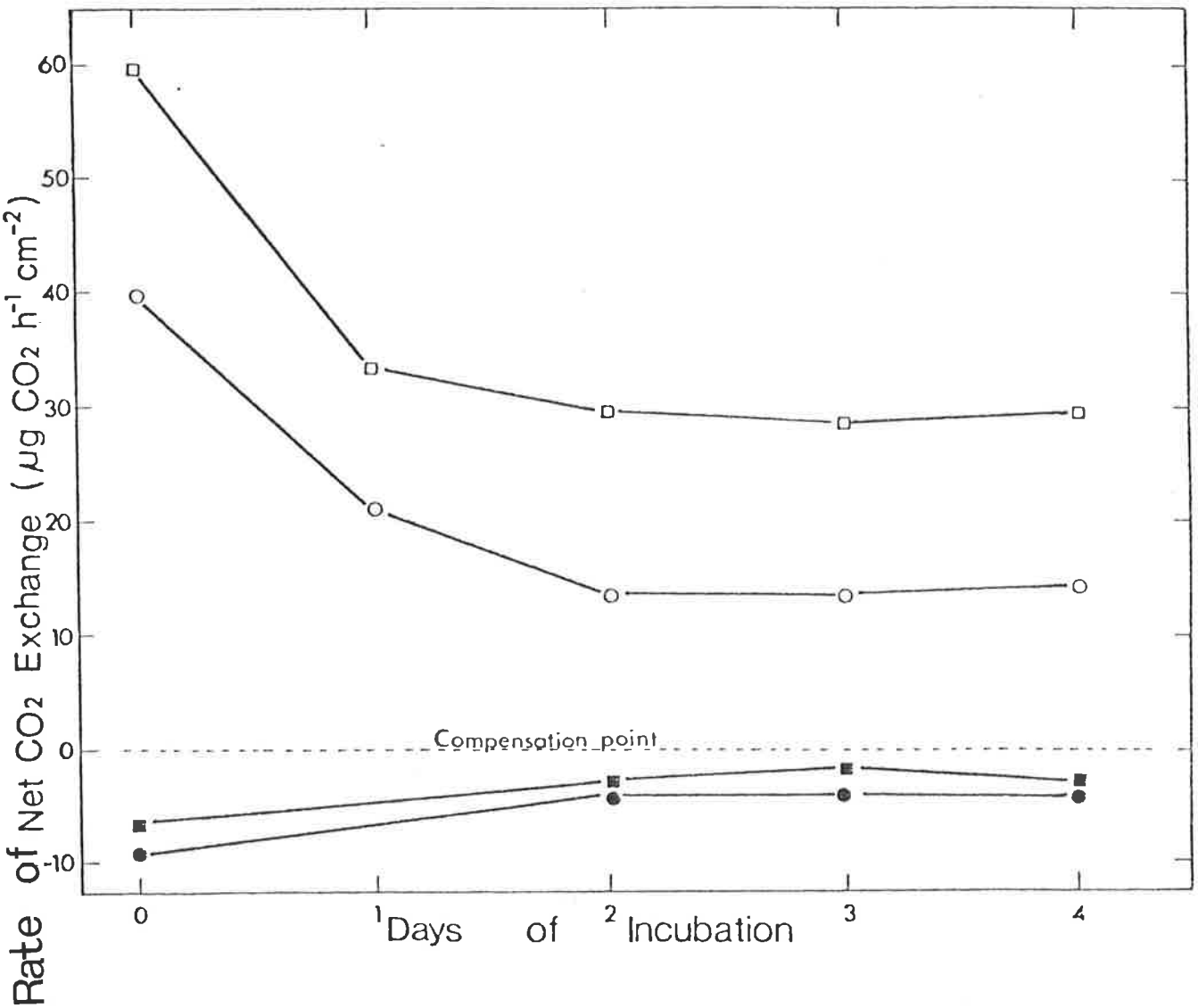
Effect of incubation at 5°C on the net $CO_2$ exchange in barley segments.

Prestressed in light

- ○ $CO_2$ exchange in light
- ● $CO_2$ exchange in dark

Prestressed in darkness

- □ $CO_2$ exchange in light
- ■ $CO_2$ exchange in dark


Rate of Net CO2 Exchange (µg CO2 h-1 cm-2)
60
50
40
30
20
10
0
-10
Compensation point
0
1
2
3
4
Days of Incubation

## 8.3 Effect of photosynthetic inhibitors on proline accumulation

Although photosynthesis does occur during exposure to low temperatures, it is possible that it is not essential for proline accumulation. In order to establish the relationship between photosynthesis and proline accumulation at 5°C, the following experiments using inhibitors of photosynthesis were conducted.

### 8.3.1 Effect of electron transport inhibitors on proline accumulation at 5°C

#### 8.3.1.1 Methods

Ten day old plants were exposed to 48 hours continuous light and the first leaves were then excised, segmented and floated on saturated CMU or DCMU solutions for 3 hours. The segments were then transferred to 72 hours continuous light or darkness (DCMU treatment only) and floated on water at 5°C. The segments were harvested after 72 hours blotted dry on filter papers, frozen and freeze dried. The proline content was estimated as detailed in Materials and Methods 2.8.

#### 8.3.1.2 Results

CMU significantly inhibited proline accumulation in the light at 5°C (Fig 8.2). DCMU treatment also significantly inhibited proline accumulation in segments exposed to light during cold treatment. In segments in the dark, however, DCMU did not have a significant effect on proline accumulation (Fig 8.2).

### 8.3.2 Effect of ammonium chloride on proline accumulation

#### 8.3.2.1 Methods

Ten day old plants were exposed to 48 hours continuous light and the first leaves were then excised, segmented and floated on ammonium

Fig 8.2

Effect of photosynthesis inhibitors on proline accumulation in segments incubated at 5°C for 3 days.

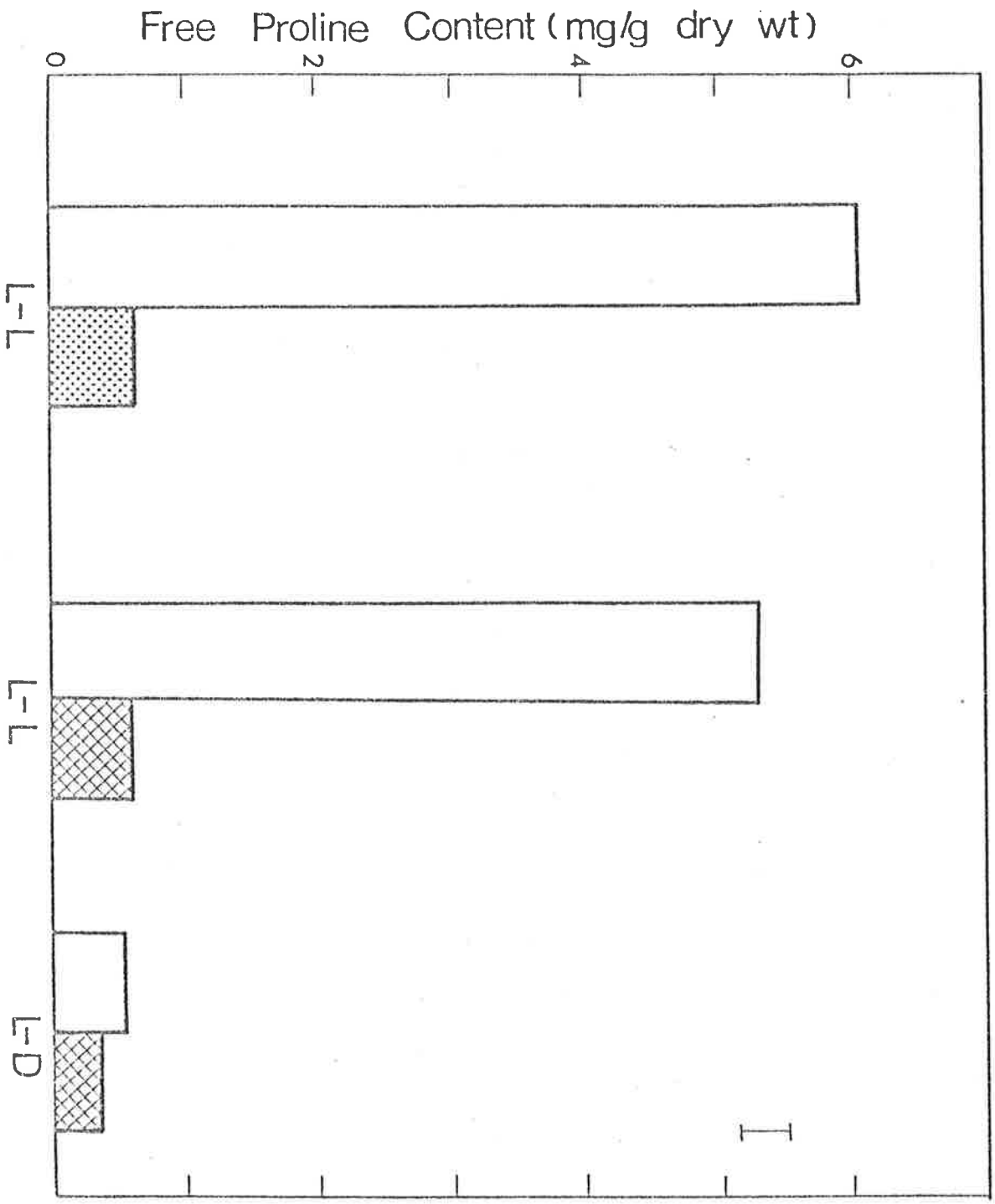
Ten day old barley plants were transferred to 48 hours continuous light. The first leaves were then harvested and segmented. The segments were floated on the inhibitor for 3 hours and then transferred to water and incubated at 5°C for 3 days in light or darkness.

Each value is the mean of 4 replicates.

Untreated

CMU treated

DCMU treated


Free Proline Content (mg/g dry wt)
0
2
4
6
L-L
L-L
L-D

chloride solutions of various concentrations at 5°C for 72 hours. The segments were floated in continuous light or darkness throughout the 3 day incubation period. [The continued presence of ammonium chloride is essential for inhibition - see Section 5.5.]

The segments were harvested, preserved and analysed for proline content.

8.3.2.2 Results

0.01M Ammonium chloride had no significant effect on proline accumulation, while concentrations of 0.1M and greater, significantly inhibited accumulation ( 80% inhibition) in samples stressed in the light. Samples stressed in the dark, however, showed no effect of the uncoupler (Fig 8.3). Both inhibitors and uncouplers inhibit proline accumulation in the light significantly, suggesting a profound involvement of photosynthesis in proline accumulation at 5°C.

8.3.3 Effect of inhibition of photosynthesis after varying duration of stress

It appears that photosynthesis is necessary for proline accumulation but the relative contribution of photosynthesis during the 3 days of stress is not known. The following experiment was designed to test the effect of inhibition of photosynthesis during the 3 day stress period.

8.3.3.1 Methods

Ten day growth and prestress treatment was identical to that described in Section 8.3.1.1. Inhibitor treatment was given by replacing the incubation solution (water) with ammonium chloride solution (0.01M). Samples were exposed to light and were treated with the uncoupler for

Fig 8.3

Effect of ammonium chloride on proline accumulation in barley segments at 5°C.

Ten day old barley plants were transferred to 48 hours continuous light prior to excision and segmentation of the first leaf. The segments were floated in light or darkness for 72 hours in the presence of various concentrations of ammonium chloride.

Each point is the mean of 4 replicates.

○ L - L (Stressed in light)

● L - D (Stressed in darkness)

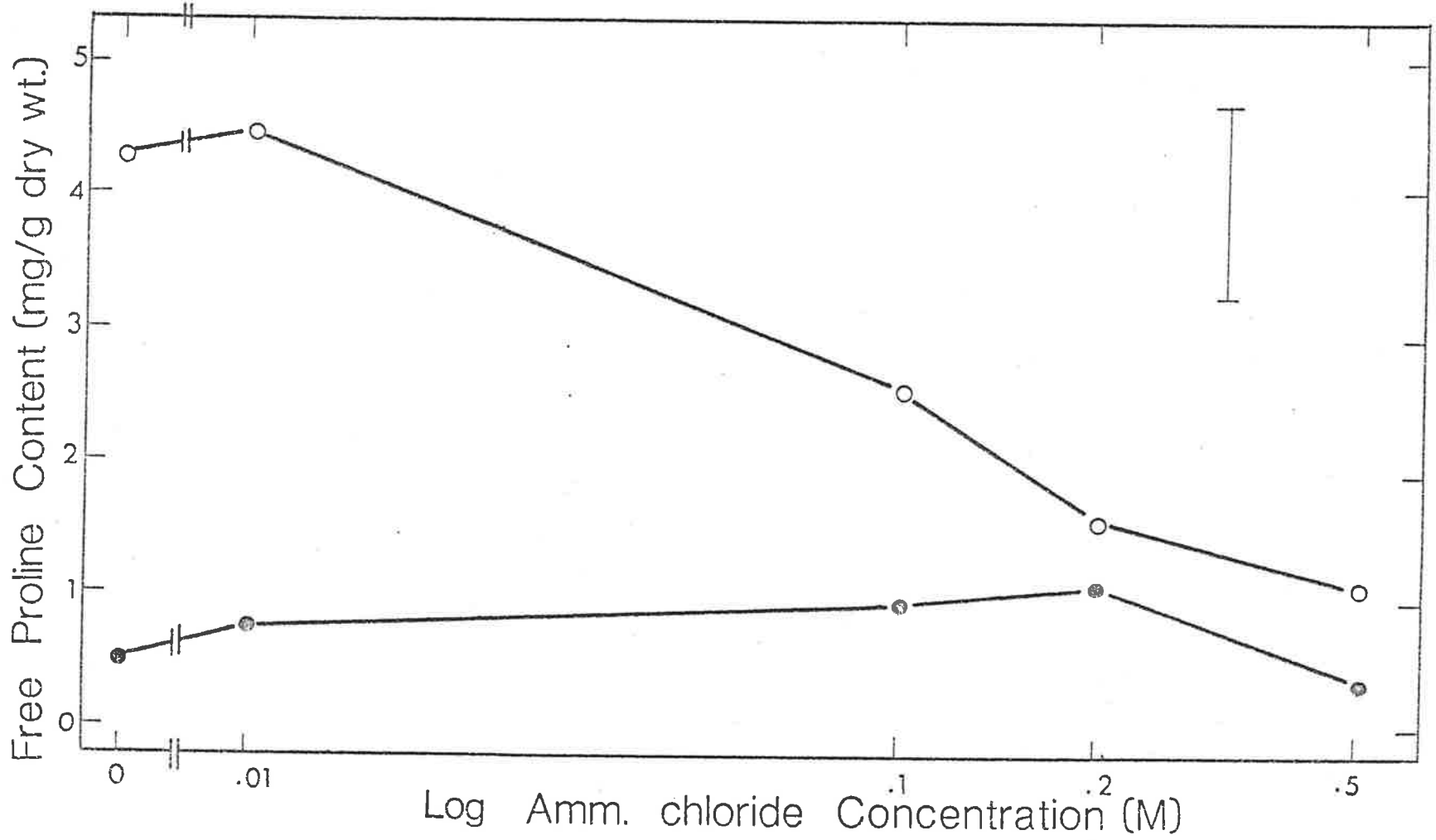
Table 8.1

F values for results in Fig 8.3

| Source of variation | Variance ratio |
|---|---|
| Light/Dark (L) | 47.93** |
| Inhibitor (I) | 5.58** |
| L x I | 5.69** |

Significance level * $P<0.05$

** $P<0.01$


Free Proline Content (mg/g dry wt.)
0
1
2
3
4
5
0
.01
.1
.2
.5
Log Amm. chloride Concentration (M)

all 3 days, or the latter 2 days or the last day only. All samples were harvested after 72 hours of stress treatment and preserved for the analysis of proline.

8.3.3.2 Results

Significant inhibition of proline accumulation by inhibitor treatment was evident after 1, 2 or 3 days of its presence in both light (L-L) and dark (D-L) prestressed samples (Fig 8.4). It is noteworthy that the decrease in proline content caused by 24 hours of ammonium chloride treatment, compared with appropriate control samples, was very similar in both L-L and D-L ( 2 mg per g dry wt).

## 8.4 Effect of closed systems on proline accumulation

The above results demonstrate that photosynthesis during cold stress is essential for maximum proline accumulation. Photosynthesis may influence the proline content through the supply of currently fixed $CO_2$ or provide the necessary energy (ATP and NADPH) for proline accumulation. In the following experiment, an attempt has been made to distinguish between these two possibilities by studying proline accumulation under limiting $CO_2$ conditions (closed flask).

### 8.4.1 Methods

After 10 days growth in a 16 hour photoperiod at 20°C, plants were transferred to 48 hours continuous light. The first leaves were then excised, segmented and floated on water at 5°C in continuous light. The segments were incubated in petri dishes or 125 ml stoppered flasks

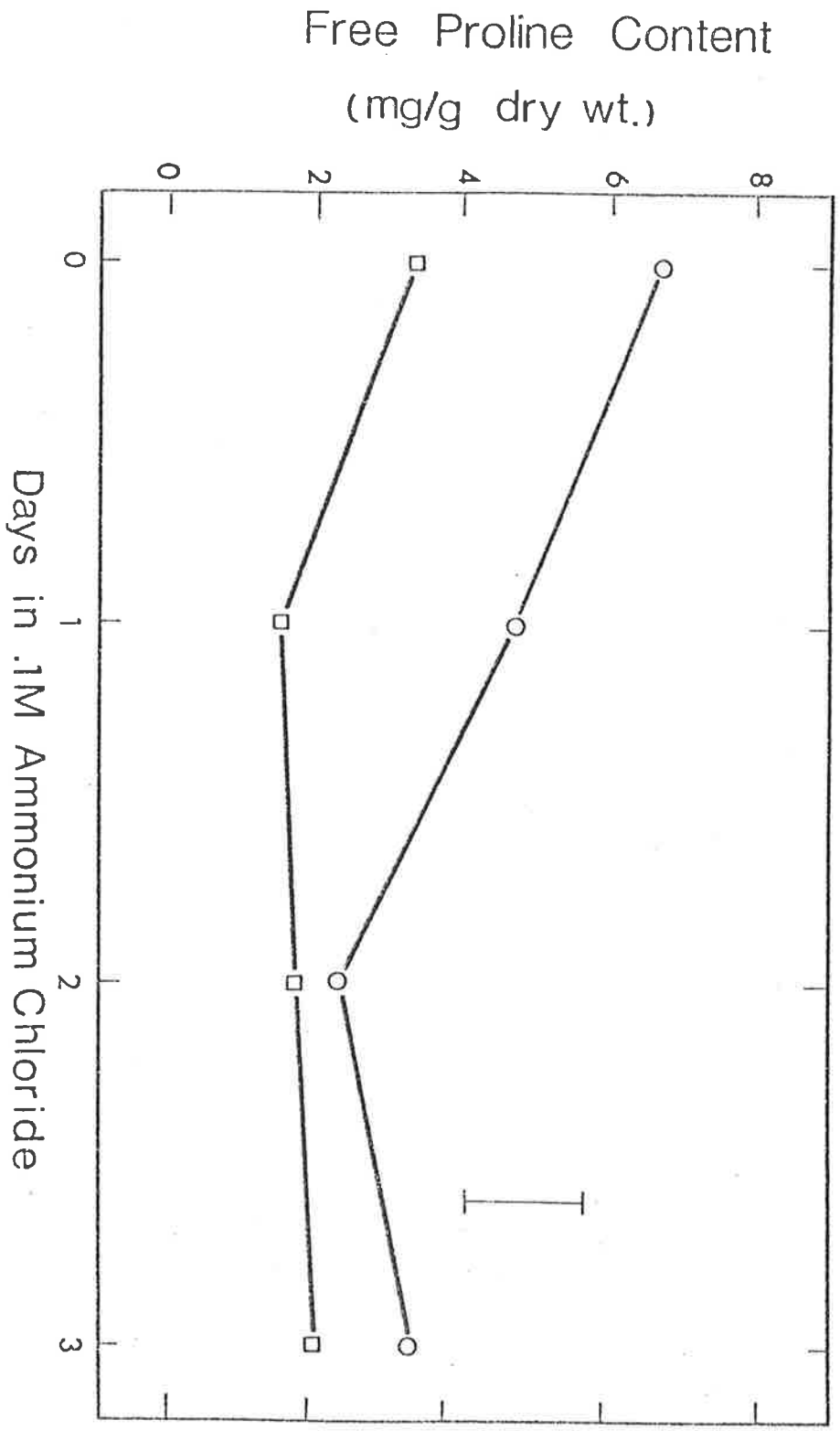
Fig 8.4

Inhibition of proline accumulation by ammonium chloride supplied during 3, 2 or 1 day of incubation at 5°C.

Each point is the mean of 3 replicates.

○ Prestressed in light

□ Prestressed in darkness


Free Proline Content
(mg/g dry wt.)
0
2
4
6
8
0
1
2
3
Days in .1M Ammonium Chloride

for the entire incubation period (i.e. 3 days) or transferred to 125 ml flasks after 1 or 2 days of incubation in petri dishes. All the samples were harvested after 72 hours of cold treatment. The samples were frozen in liquid nitrogen, freeze dried, weighed and then analysed for the total free proline content.

### 8.4.2 Results

#### 8.4.2.1 Dry weight

The dry weight of the sample was directly proportional to the duration of the incubation in petri dishes (i.e. open system). Segments incubated in flasks throughout showed the lowest dry weights while those in petri dishes the highest (Fig 8.5A).

#### 8.4.2.2 Proline content

Segments incubated in flasks over the entire 3 day period showed significantly lower proline content than any of the other treatments (Fig 8.5B).

These results suggest that the refixation of photorespired $CO_2$ alone is not sufficient for proline accumulation over the 3 day incubation period. In addition to light, free air flow for at least 1 day is essential for proline accumulation.

Fig 8.5

Effect of incubation in stoppered flasks for varying periods during stress.

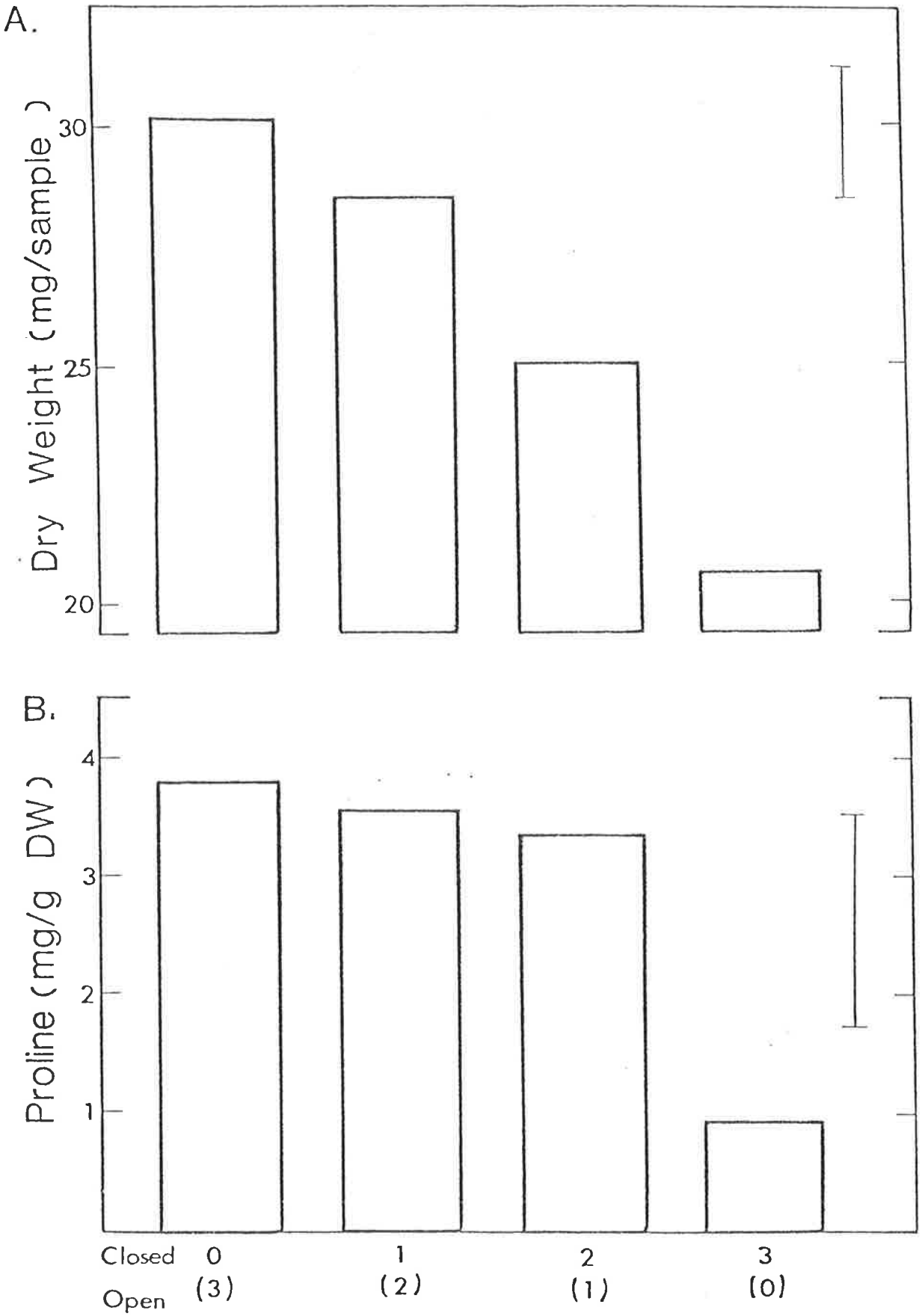
Ten day old barley plants were exposed to 48 hours continuous light. The first leaves were then excised, segmented and incubated in petri-dishes or sealed flasks for 3 days or transferred from the petri dish to the flask after 1 and 2 days of commencement of stress.

Each value is the mean of 4 replicates.

A. Dry weight per 10 segments

B. Proline content

N.B. Figures in paranthesis refer to number of days in petri dish i.e. open system.


A.
Dry Weight (mg/sample)
30
25
20
B.
Proline (mg/g DW)
4
3
2
1
Closed
Open
0
(3)
1
(2)
2
(1)
3
(0)
Days of Incubation

## 8.5 Carbon dioxide concentration and proline accumulation

Since proline accumulation appears to be quantitatively dependant on photosynthesis, the effect of altered $CO_2$ concentration on proline accumulation was investigated. Enhancing $CO_2$ concentration between day 3 and day 5 had no effect on proline accumulation in both L-L and D-L samples at 5°C in light (results not presented). In the following experiment, therefore, the $CO_2$ concentration was altered over the entire incubation period.

### 8.5.1 Methods

Growth of plants until they were 10 days old was under similar conditions as detailed in Section 8.4.1. All plants were prestressed in 48 hours continuous light. The first leaves were then excised, segmented and floated on water at 5°C in petri dishes in light. The $CO_2$ concentration was enhanced by placing the petri dishes (partially open) within a 100 litre perspex box fitted with a fan (to circulate the air). Only one concentration of $CO_2$ was tested, the $CO_2$ within the chamber being increased 16-fold by adding 480 ml in pure $CO_2$ directly into the chamber (since normal air contains 320 $Ul^{-1}$, the volume of $CO_2$ within the chamber would be 320 x 100 = 32 ml). The petri dishes were left in the chamber for 3 or 5 days. After removing the petri dishes on the third day, however, the chamber was resealed and the $CO_2$ concentration increased as described above. In addition to enhanced $CO_2$, the effect of $CO_2$ free air was also studied. To obtain $CO_2$ free air, a small dish containing self-indicating soda-lime was floated alongside the segments in the petri dish. The petri dish was sealed with vacuum grease and left in the light at 5°C

Fig 8.6

Effect of carbon dioxide concentration on proline accumulation in barley segment after 3 and 5 days at 5°C.

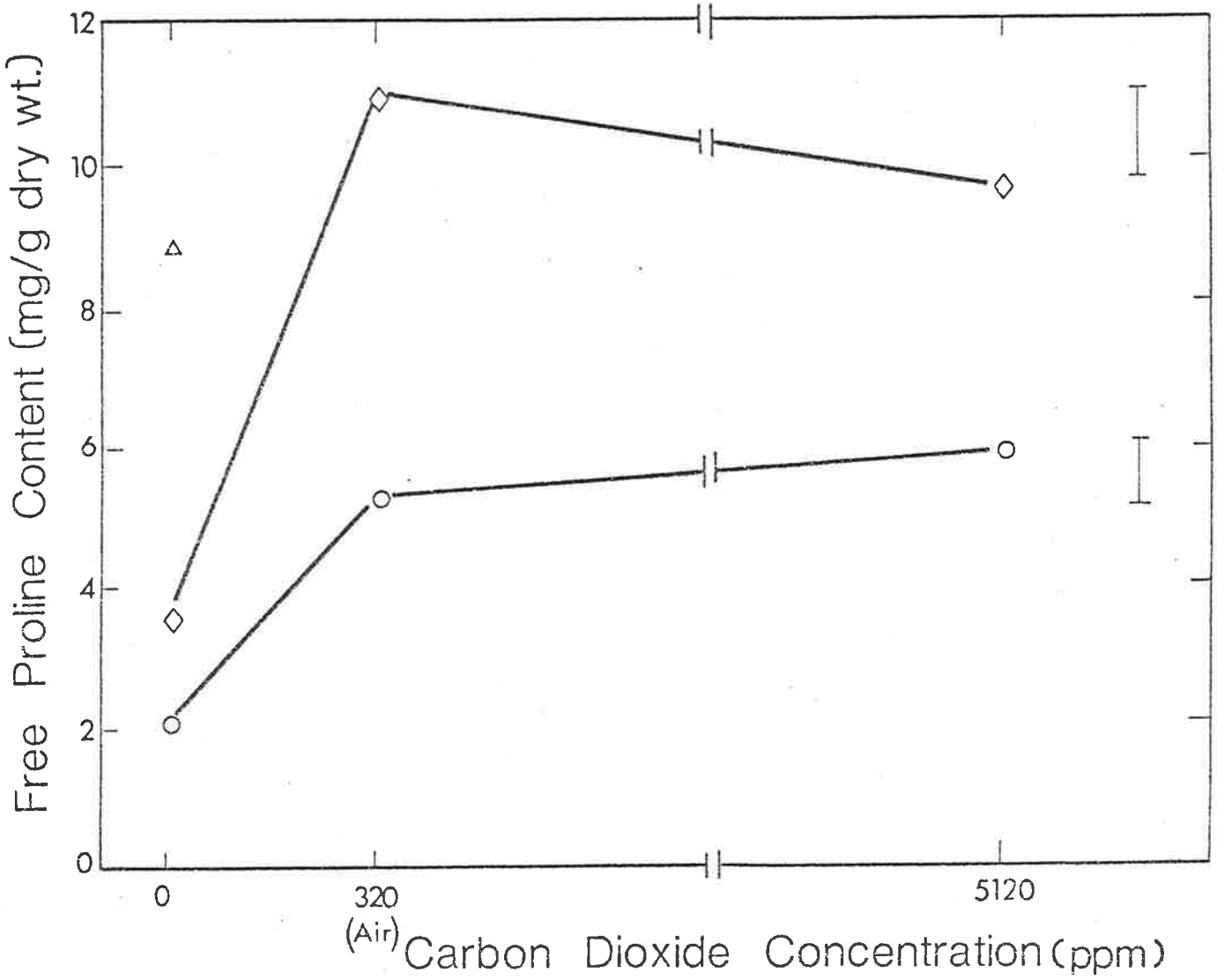
Ten day old barley plants were exposed to 48 hours continuous light and then excised, segmented and floated on water at 5°C. The $CO_2$ concentration was monitored as detailed in the text. Segments were harvested after 3 or 5 days.

Each point is the mean of 4 replicates

○ 3 days at 5°C

◇ 5 days at 5°C

△ Transferred to $CO_2$ free conditions after 3 days, harvested at 5 days


Free Proline Content (mg/g dry wt.)
12
10
8
6
4
2
0
0
320
(Air)
5120
Carbon Dioxide Concentration (ppm)

for 3 or 5 days. An extra treatment was included where the $CO_2$ absorbent (sodalime dish) was introduced after 3 days of cold treatment.

### 8.5.2 Results

Increasing $CO_2$ concentration over the entire incubation period had no significant effect on proline accumulation (Fig 8.6 ). The effect of $CO_2$ free air, however, was highly significant after 3 and 5 days of stress. The proline accumulated in segments exposed to $CO_2$ free air was reduced to <40% of that in normal air in both incubation periods. However, it is noteworthy that proline did accumulate to some extent in samples deprived of $CO_2$. Also segments continued to accumulate proline when deprived of $CO_2$ at 3 days (Fig 8.6 ).

## 8.6 Carbon compounds and proline accumulation

Free access to air at least for one day appears essential for proline accumulation, while photosynthesis is required for a longer period (2 days). Photosynthesis may provide precursors required for proline synthesis or the compounds which are essential to induce accumulation. On the other hand, photosynthesis may be required for the provision of energy rich nucleotides and ATP. The following experiment was conducted to explore the former hypothesis. Carbon compounds were supplied to test if it was possible (a) to induce proline accumulation in segments stressed in darkness and (b) to influence proline accumulation in light stressed samples.

8.6.1 Methods

Ten day old barley plants growing in a 16 hour photoperiod at 20°C were given 48 hours continuous light treatment. The first leaves were then excised, segmented and floated on sucrose (0.1M), glutamine (0.5 mg $ml^{-1}$) solutions or water at 5°C in the light or darkness for 3 days. Sucrose was used because it is one of the major stable products of photosynthesis while glutamine is known to be the mobile form of reduced nitrogen in barley (Cocking and Yemm, 1961; Oji and Izawa, 1972). In addition, Schobert (1977) was able to obtain a transient enhancement in proline levels in stressed diatoms with this latter compound. [Preliminary experiments in light stressed segments showed that the provision of glutamate at 0.5 mg $ml^{-1}$ over the entire stress period did not influence proline levels while glutamine enhanced them.] At the end of 3 days, the segments were harvested, frozen and proline content estimated.

8.6.2 Results

Both glutamine and sucrose were effective in enhancing proline levels in samples stressed in the dark. Sucrose increased proline levels approximately seven-fold while glutamine induced are approximately two-fold increases (Fig 8.7).

In samples stressed in the light, only glutamine had a significant enhancing effect on proline accumulation (Fig 8.7). the enhancement of proline accumulation in samples stressed in the dark by sucrose is to 70% of dark stressed samples by sucrose is to 70% of light stressed samples compared to 10% in untreated tissue.

Fig 8.7

The effect of the continued presence of precursors on proline accumulation at 5°C.

Ten day old barley plants were transferred to 48 hours continuous light and then the first leaves were harvested, segmented and floated on water or precursor solution at 5°C. Segments were incubated in the light or darkness for 72 hours.

Each value is the mean of 4 replicates.

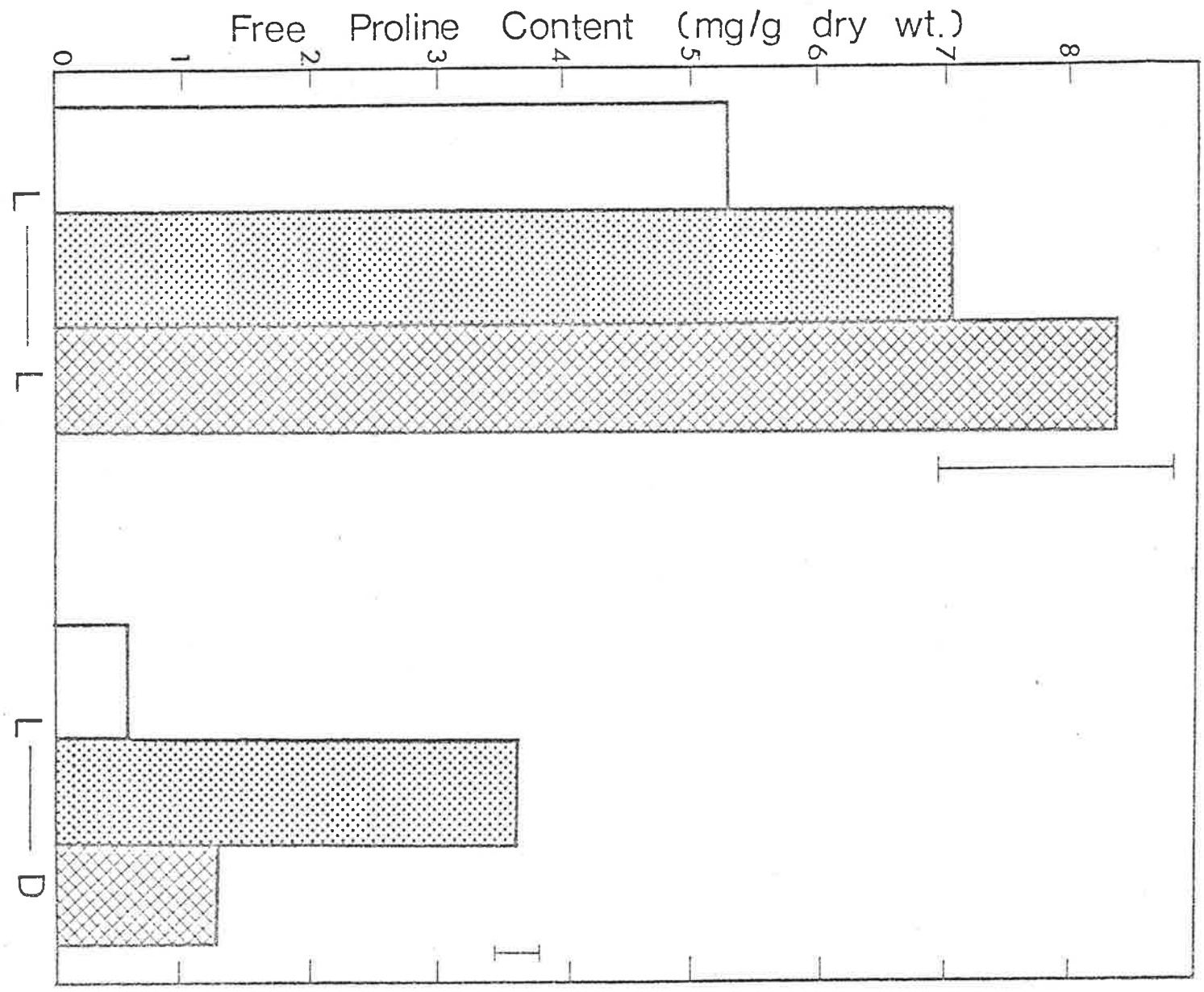
Incubated on water

Incubated on sucrose (0.1M)

Incubated on glutamine (1 mg $ml^{-1}$)

L - L Prestressed in light and stressed in light

L - D Prestressed in light and stressed in darkness


Free Proline Content (mg/g dry wt.)
0
1
2
3
4
5
6
7
8
L
L
L
D

## 8.7 Effect of carbon compounds in the presence of DCMU

It is possible to conclude from the previous experiment that photosynthesis is providing carbohydrates which are essential for proline accumulation. If this is the case, then it should be possible to ameliorate the inhibition by DCMU of proline accumulation at 5°C by the provision of these compounds. The following experiment was designed to test this.

### 8.7.1 Methods

Ten day old barley plants were transferred to 48 hours continuous light and then the first leaves were excised, segmented and floated on DCMU solution for 3 hours. The segments were then transferred to 5°C and were floated on water, sucrose (0.1M) or glutamine (0.5 mg $ml^{-1}$) for 3 days in light. [Glutamine was included as there was considerable enhancement of proline accumulation particularly in light stressed samples in the presence of this compound.] In addition, one set of petri dishes were incubated in the dark at 5°C. After 3 days, the segments were all harvested, frozen, freeze dried and analysed for proline content.

### 8.7.2 Results

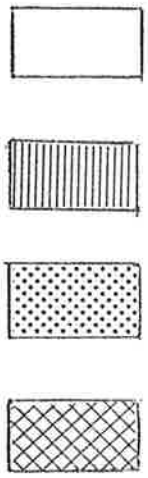
DCMU treatment significantly inhibited proline levels to 10% of that of untreated samples. The addition of sucrose to DCMU treated segments, however, significantly enhanced proline accumulation to 85% of that of untreated samples (Fig 8.8). Glutamine on the other hand, had no effect on DCMU inhibition of proline accumulation.

Fig 8.8

Effect of precursors on alleviation of DCMU inhibition of proline accumulation at 5°C.

First leaves of 12 day old barley plants were segmented and incubated at 5°C in light after exposure to 48 hours continuous light. Segments were treated with DCMU and floated on water or solutions of sucrose or glutamine for 72 hours.

Each value is the mean of 4 replicates.



Untreated

DCMU treated

DCMU + Sucrose (0.1M)

DCMU + glutamine (1 mg $ml^{-1}$)

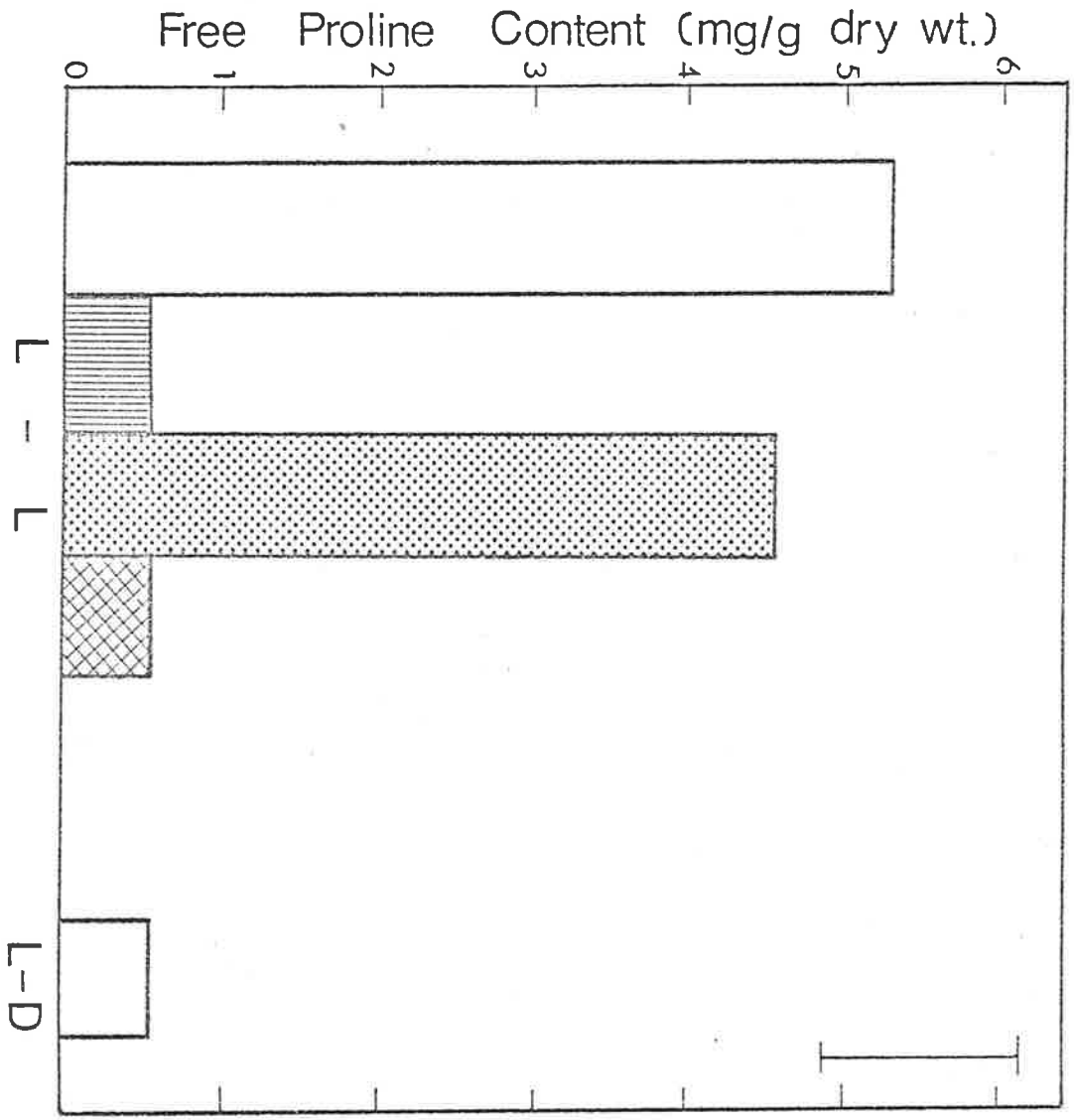
L - L Prestress (48 h) and stress (72 h) in light

L - D Prestressed in light, stressed in darkness.

F value for treatments in Fig 8.8 59.16**

Significance level * $P \leq 0.05$

** $P \leq 0.01$


Free Proline Content (mg/g dry wt.)
0
1
2
3
4
5
6
L - L
L-D

Thus, one can conclude that proline accumulation is increased by photosynthesis probably through the provision of carbohydrates.

## 8.8 Discussion

A requirement for photosynthesis for proline accumulation is evident. The assumption that photosynthesis at low temperatures is not significant and therefore could not be providing the precursors required (Chu et al, 1978) is incorrect. Cold temperatures appear not to affect photosynthesis for the first 12 hours after which the decline is rapid. The inhibition of photosynthesis at low temperatures may be due to reduced photochemical activity, e.g. tomato (Sochanowicz and Kanuiga, 1979), cucumber (Kaniuga et al, 1978) or to stomatal aperture changes (Phaseolus vulgaris (Wilson, 1979)). Such a cold-induced reduction in photosynthesis of intact sorghum plants has also been reported (Bagnall,1979).

The effect of photosynthesis on proline accumulation over the 3 day period appears cumulative, since inhibition of photosynthesis for just one day is able to reduce proline accumulation significantly (Fig 8.4). Refixation of respired $CO_2$ over the entire 3 day period is insufficient to sustain proline accumulation yet accumulation is not affected when transferred to $CO_2$ limiting conditions after 24 hours in an open system (Fig 8.5). Similarly proline levels are not affected by transfer to $CO_2$ free air after 3 days of incubation in petri dishes with free access to $CO_2$ (Fig 8.6B).

All the results obtained so far appear to support the hypothesis that photosynthesis provides carbohydrates or some other product which is essential for proline accumulation. Although Chu et al

(1978) failed to observe a stimulation of proline accumulation by glutamate or sucrose, results obtained here have shown that precursors can significantly enhance proline accumulation. The reason for the lack of response in their experiment may have been the shorter duration (only $2^1/2$ hours) of exposure and to the precursors and also shorter duration of exposure to cold (only 48 hours). That photosynthesis provides carbohydrates or its products which influence proline content was further confirmed with inhibitor studies where the inhibition of proline accumulation by DCMU treatment was alleviated by the provision of sucrose. Glutamine, however, was not effective. One possible explanation for this could be that in addition to supplying carbon skeletons for proline synthesis which glutamine can fulfil, carbohydrates may influence proline metabolism in some other way not accomplished by glutamine. However, one cannot conclude that the role of light in proline accumulation is confined to the provision of carbohydrates as transfer of segments to darkness after 2 days of light at 5°C induced significantly less proline accumulation than when they were left in the light for 3 days (Fig 7.5); yet segments transferred to flasks (limited $CO_2$) for the latter 2 days of stress showed no significant reduction in proline levels in spite of a significant decline in the dry weight (Fig 8.5 A,B). Moreover, the difference in proline content between segments incubated with DCMU and sucrose in the light and segments incubated with sucrose alone in the dark, supports the hypothesis that carbohydrates and light together are required for maximum accumulation of proline (Fig 8.7 and 8.8). Of course, there may be other explanations for these results, for instance, reduced uptake of sucrose in dark stressed samples or an increase in the carbohdyrate at the expense of other carbon compounds in segments incubated in the enclosed system in light.

## SECTION II LOW TEMPERATURE STRESS

### 9. Studies with radioactive carbon

## 9. Studies with Radioactive Carbon

### 9.1 Introduction

Previous results have shown a requirement for photosynthesis for proline accumulation at 5°C. Photosynthesis provides the necessary carbohydrates and energy which may be required directly for proline synthesis or may indirectly regulate accumulation. When photosynthesis was inhibited for 3 days at 5°C, proline accumulation was also inhibited. However, when inhibited on the last 2 days alone, the inhibition of proline accumulation was just as great. On the other hand, enclosing the segments in flasks for the last 2 days at 5°C did not reduce proline accumulation, yet when they were enclosed in flasks for all 3 days, proline accumulation was significantly reduced. It is conceivable that the contrasting results obtained can occur in either of the following ways :

A) Photosynthesis during the first day of stress provides compounds which facilitate proline accumulation but do not serve as carbon precursors for proline synthesis. The carbon for proline synthesis originates exclusively from carbon fixed subsequently (in enclosed flasks, it is conceivable that reassimilated $CO_2$ may serve as the precursor for proline accumulation since atmospheric $CO_2$ present in the 125 ml flask $\simeq$ 88 µg. This will be exhausted within 1 hour at photosynthesis rates of 140 µg $CO_2$ $h^{-1}$ per sample (10 segments) (see Section 8.2)).

B) Photosynthesis during the entire stress period provides carbon for proline synthesis while the necessary energy for proline synthesis is supplied by photosynthesis subsequent to the first day (hence inhibition of photosynthesis on the last 2 days was as effective in

reducing proline accumulation as when photosynthesis was inhibited on all 3 days of stress).

The following experiment was designed to (A) test the relative contribution of carbon fixed on different days of stress (0,2,4) at 5°C towards proline accumulation 24 hours after feeding labelled $CO_2$; and to (B) trace the contribution of carbon fixed on day 0 and day 2 to proline synthesis up to the fifth day at 5°C. Approach (A) would test the relative contribution of carbon fixation occurring on different days at 5°C towards the proline accumulated within 24 hours. If there is an increased incorporation of carbon fixed on the later days of stress into proline, this would support hypothesis (A).

In approach (B) the ultimate contribution of carbon fixed on day 0 and day 2 of stress towards proline accumulation is traced. If there is significant radioactivity present in proline at the end of 5 days it must mean that carbon fixed on day 0 also serves as a carbon precursor for proline synthesis at 5°C

## 9.2 Verification of methods

$^{14}CO_2$ can be supplied to leaf discs most conveniently in a closed system, e.g. a conical flask, but it was necessary to ascertain that such an incubation system did not affect proline accumulation adversely. Previous experiments (see Fig 8.5B) had shown that incubation in a closed 125 ml flask for up to 48 hours on day 2 and day 3 of exposure to low temperature did not influence proline accumulation but here $^{14}CO_2$ was to be supplied for 2 hours on the first day. Accordingly leaf segments, prepared as before, were incubated for 1-4 hours on day 1 in a closed flask (at other times floated on petri dishes) and

compared with discs floated on petri dishes with free aeration. There was no difference in proline accumulation between any of the treatments after 72 hours at 5°C (mean proline content 5.0 - 4.0 mg $g^{-1}$dry wt) and this method was adopted.

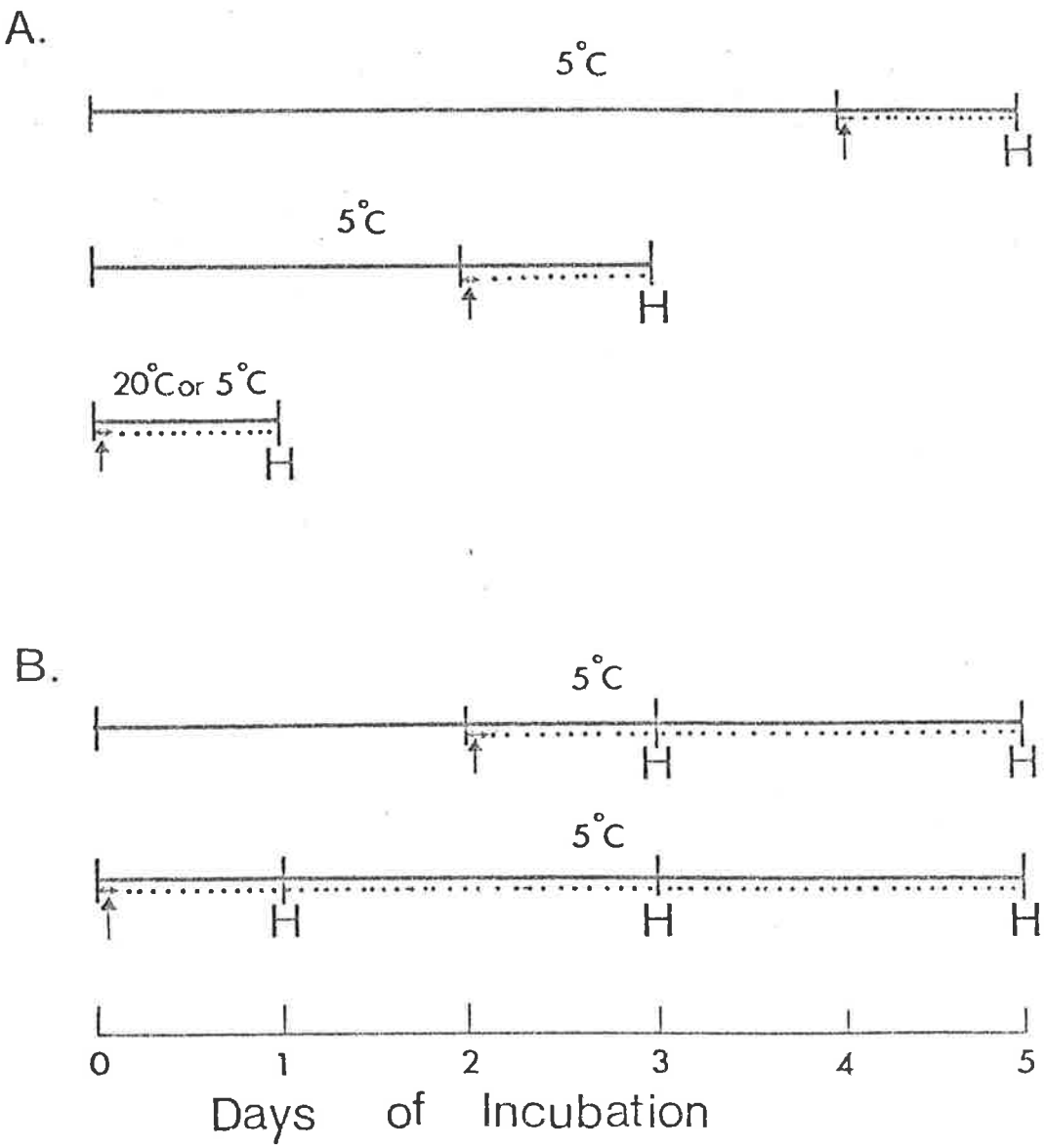
## 9.3 $^{14}CO_2$ Studies

### 9.3.1 Experimental design

Basically, two treatments were involved, in the first (A) $^{14}CO_2$ was fed for a short period (2 hours) after various periods of incubation at 5°C and the segments were harvested after a fixed period of incubation (2 + 22 hours) and in the second (B) $^{14}CO_2$ was fed immediately upon exposing the segments to 5°C and 2 days later, and the segments were harvested at intervals thereafter. The first sampling following $^{14}CO_2$ feeding on day 1 and day 3 was the same for both A and B. In addition, there was one treatment in which the segments were exposed to $^{14}CO_2$ for 2 hours at 20°C and transferred to petri dishes which were incubated in light at 20°C for a further 22 hours (Fig 9.1 experimental design for experiment9.3).

### 9.3.2 Methods

Barley plants were grown for 10 days in a 16 hour photoperiod at 20°C in a sand culture. The plants were then transferred for 48 hours to continuous light prior to excision and segmentation of the first leaves. The segments were floated on water at 5°C or 20°C in light for various lengths of time (Fig 9.1 ).


A.
5°C
H
5°C
H
20°C or 5°C
H
B.
5°C
H
H
5°C
H
H
H
0
1
2
3
4
5
Days of Incubation

Exposure to $^{14}CO_2$

Segments were placed in 125 ml conical flasks which were then sealed with rubber stoppers. A glass tube was passed through a hole in the stopper and connected to a small vial floating on water in the flask. At the other end, the tube was connected to rubber tubing to which was attached a pinch cock (see page 70). Before sealing the flask with the stopper, 10 micro curies of $NaH^{14}CO_3$ was placed in the vial which was gently lowered into the flask, which was then sealed. The $^{14}CO_2$ was liberated by introducing excess 50% lactic acid through the glass tubing. The pinch cock was tightened and the flasks left in light for 2 hours for $^{14}CO_2$ assimulation. At the end of 2 hours, the flasks were opened and the segments were removed and floated on water in petri dishes. Segments were harvested at various times (Fig 9.1 experimental design for experiment 9.3), frozen in liquid nitrogen, freeze dried and weighed.

Counts in the soluble extract, insoluble residue, amino acids and sugars were estimated (see Materials and Methods, Section 2.8).

There were 3 replicates for each treatment and a conventional one way analysis of variance was performed to test for significant differences between treatments.

9.3.3 Results

Results of experiment A will be reported first.

A. Effect of duration of stress on the fixation and distribution of assimilated $^{14}CO_2$

Total radioactivity in various fractions

In this experiment, $^{14}CO_2$ was supplied at the beginning of exposure

to 20°C or 5°C, after 48 hours or after 96 hours at 5°C and the radioactivity fixed was assessed after 24 hours incubation in each case. The total radioactivity (soluble + insoluble) fixed during the 2 hours was not significantly less than that fixed by segments supplied with $^{14}CO_2$ at 20°C except in samples exposed to $^{14}CO_2$ after 96 hours of cold treatment (Fig 9.2a).

The number of counts in the ethanol soluble fraction was significantly different only between day 1 and day 5 samples at 5°C (Fig 9.2b). Counts in the insoluble residue (Fig 9.2c) and amino acid fraction (Fig 9.2e) were significantly higher in 20°C incubated samples than in those incubated at 5°C. Radioactivity in sugars, however, was greater in the 5°C day 1 samples than in samples incubated at 20°C (Fig 9.2d).

Within 24 hours of $^{14}CO_2$ feeding and incubation at 20°C more than 20% of the $^{14}CO_2$ fixed had been converted to ethanol insoluble compounds while segments incubated and fed with $^{14}CO_2$ at 5°C converted only 7% - 13% of the fixed $^{14}CO_2$ to such compounds (Fig 9.2C).

## Amino acids

### Proline

Proline content increased significantly during prolonged exposure to 5°C although only traces of proline were present in samples incubated at 5°C for 1 day. Samples incubated at 20°C for 1 day on the other hand, showed significantly greater proline content than 5°C samples incubated for similar period (Fig 9.3a).

The radioactivity appearing in proline was significantly greater in samples incubated at 20°C than in 5°C treated samples (Fig 9.3b).

Fig 9.2

Distribution of radioactivity in various fractions after incubation with $^{14}CO_2$ during different days of stress.

Ten day old barley plants growing in a 16 hour photoperiod at 20°C were transferred to 48 hours continuous light prior to excision and segmentation of the first leaves. Segments were floated on water in light at 5°C and also exposed to $^{14}CO_2$ for 2 hours in flasks on day 0, 2 or day 4 of cold treatment. The segments were then transferred to petridishes at 5°C and harvested after 22 hours.

Each point is the mean of 3 replicates.

 20°C  5°C

a Total counts present

b Ethanol soluble counts

c Counts in ethanol insoluble residue

d Total glucose + fructose + sucrose

e Total amino acids

N.B. Figures in paranthesis are the counts expressed as a percent of total counts in the sample.

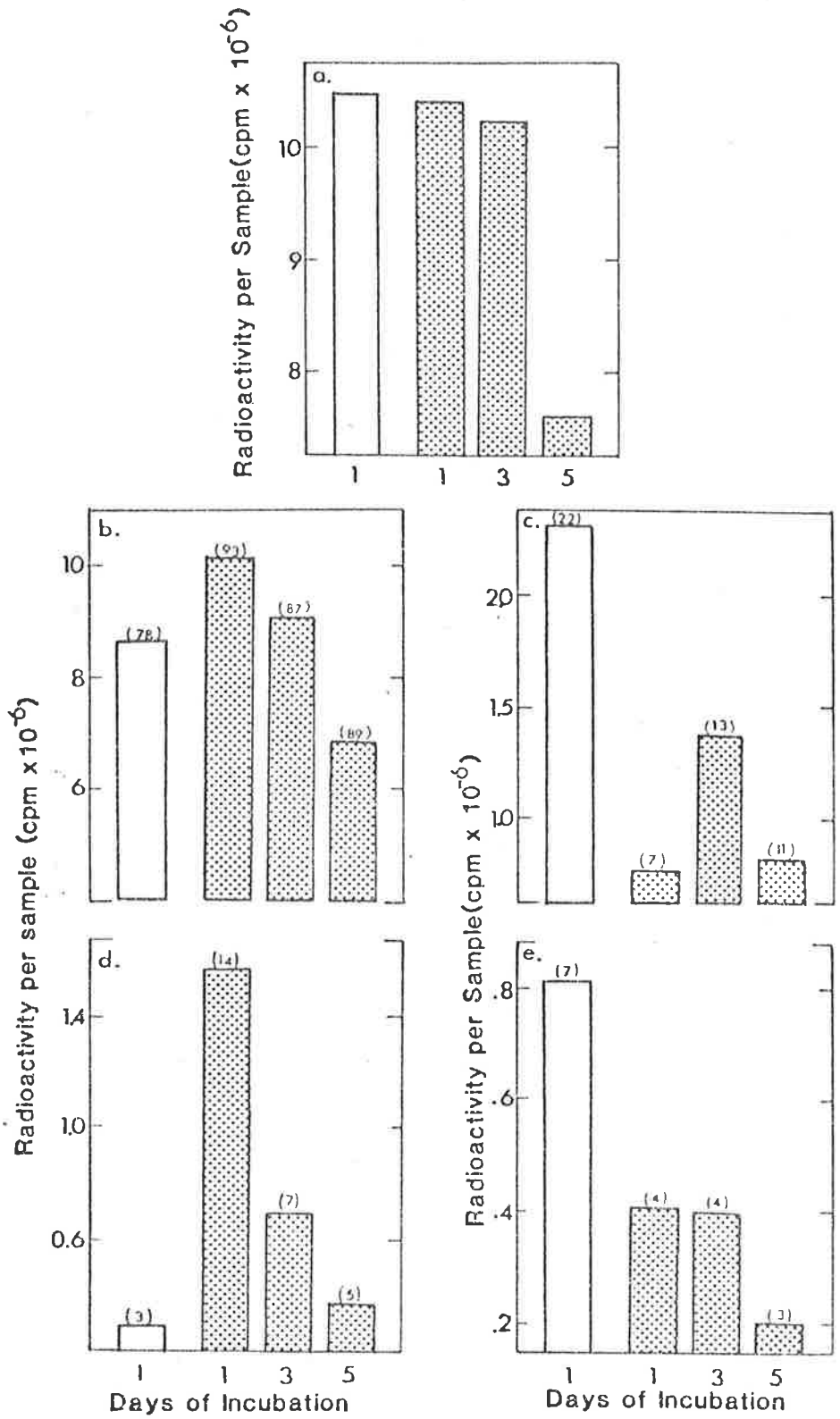
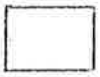

a.
Radioactivity per Sample(cpm x 10⁻⁶)
10
9
8
1
1
3
5
b.
(78)
(92)
(87)
(69)
c.
(22)
(7)
(13)
(11)
d.
(3)
(14)
(7)
(5)
e.
(7)
(4)
(4)
(3)
Radioactivity per sample (cpm x10⁻⁶)
Radioactivity per Sample(cpm x 10⁻⁶)
Days of Incubation
Days of Incubation

Fig 9.3

Content and radioactivity of proline

Plant growth and treatment as described in Fig 9.2

 20°C  5°C

a. Proline content (mg $g^{-1}$ dry wt)

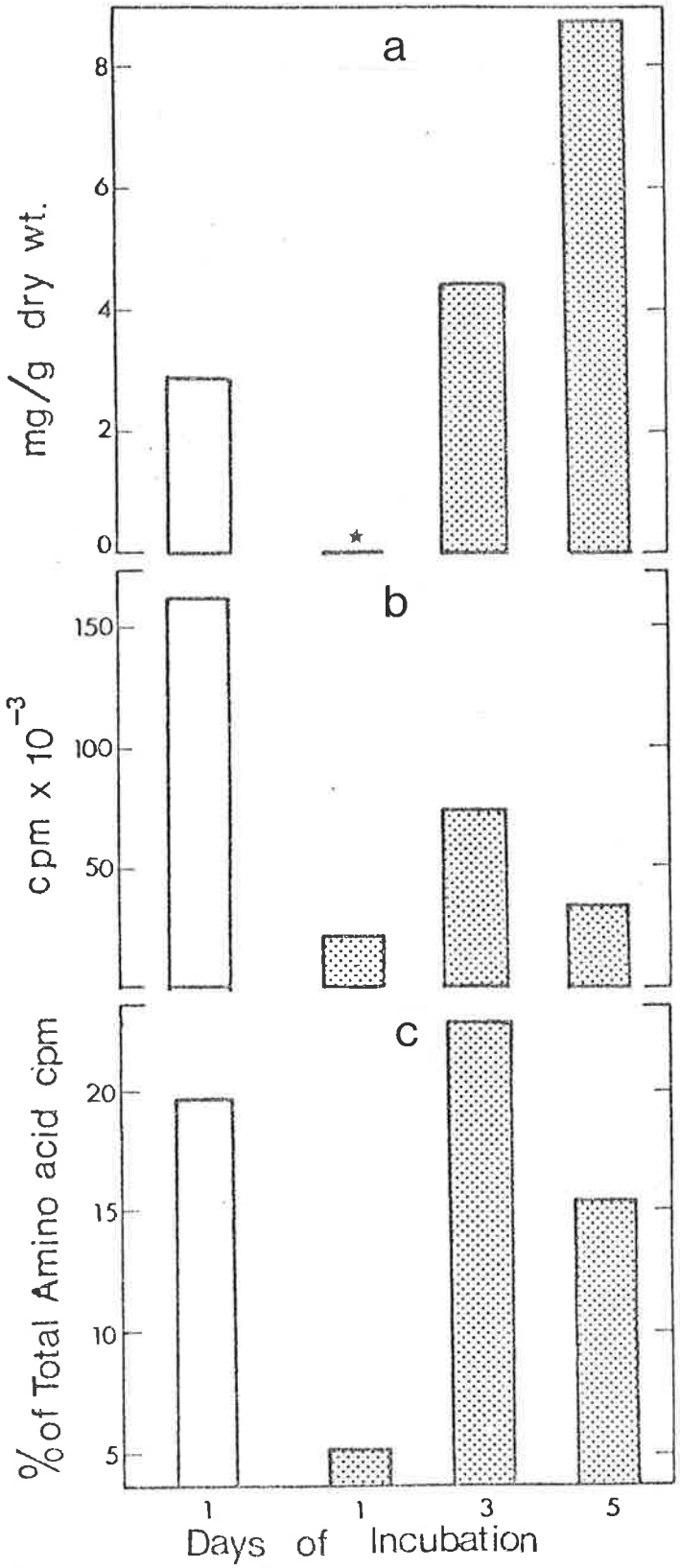
b. Total counts in proline (cpm x $10^{-3}$)

c. Precent of radioactivity of total amino acid radioactivity

* Traces

Table 9.1 Specific radioactivity in proline after incubation with $^{14}CO_2$ during different days of stress

| Temperature | 20°C | 5°C | | |
|---|---|---|---|---|
| Incubation period (days) | 1 | 1 | 3 | 5 |
| Specific Radioactivity (cpm $\mu g^{-1}$) | 2106 | 82900 | 512 | 98 |


a
b
c
mg/g dry wt.
cpm x 10⁻³
% of Total Amino acid cpm
0
2
4
6
8
50
100
150
5
10
15
20
1
1
3
5
Days of Incubation

When the radioactivity was expressed as a percentage of the counts in the amino acid fraction, however, only the 1 day 5°C samples showed significantly lower counts (5% of amino acid fraction). In the other 3 treatments, proline constituted 15% to 23% of the radioactivity in the total amino acids. Calculations of specific radioactivity (cpm $\mu g^{-1}$ proline) showed extremely high specific activity in the 1 day 5°C treatment suggesting that although proline accumulation was small, $^{14}C$ incorporation into proline was high (Table 9.1). In the 3 day and 5 day incubations, the specific radioactivity of proline was low probably due to the large amounts of proline present resulting in isotope dilution.

Other amino acids

Asparagine and serine were the only 2 amino acids which were found in significantly greater concentration in the 20°C treated samples than in any of the 5°C samples (Table 9.2A). Glutamate, aspartate and alanine concentrations were significantly reduced initially but after 5 days in the cold, the concentration had returned to the level in the 20°C sample (Table 9.2A). Glutamine and glycine content did not show any significant changes between any of the treatments while arginine was present in traces in all the treatments. Radioactivity in each of the amino acids except glycine, was at the highest level at 20°C (Table 9.2B). Of these asparagine, glutamate and alanine showed statistically significantly more counts at 20°C than in any 5°C sample. On examining the $^{14}C$ incorporation into various amino acids, it is evident that most of the amino acids showed a tendency to incorporate less $^{14}C$ (Table 9.2B) with duration of cold treatment, particularly glutamine which showed almost $100 \times 10^{-3}$ cpm less on day 5 compared with day 1 samples at 5°C.

Table 9.2 Amino acid changes accompanying incubation at low temperatures

Table 9.2A

Amino acid content (mg $g^{-1}$ dry wt)

| Incubation temperature | 20°C | 5°C | | | Variance ratio |
|---|---|---|---|---|---|
| Incubation period (days) | 1 | 1 | 3 | 5 | |
| Asparagine | 2.78 | 0.41 | 0.85 | 0.52 | 5.01* |
| Glutamine | 5.78 | 7.61 | 6.52 | 5.11 | 0.57 |
| Aspartate | 2.14 | 0.88 | 0.90 | 2.48 | 4.47* |
| Glutamate | 3.05 | 1.48 | 2.46 | 3.03 | 5.17* |
| Serine | 3.14 | 1.15 | 1.73 | 1.30 | 8.94** |
| Alanine | 2.04 | 0.82 | 0.86 | 1.38 | 2.38 |
| Proline | 2.92 | - | 4.44 | 8.74 | 50.40** |
| Glycine | 1.55 | 1.74 | 1.74 | 0.94 | 3.54 |
| Total | 22.80 | 13.58 | 19.50 | 23.49 | |
| (Total - Proline) | 19.88 | 13.58 | 15.06 | 14.75 | |

Significance level *P ⩽ 0.05

**P ⩽ 0.01

- Traces

Table 9.2B

Radioactivity in amino acids (cpm x $10^{-3}$)

| Incubation temperature | 20°C | 5°C | | | Variance ratios |
|---|---|---|---|---|---|
| Incubation period (days) | 1 | 1 | 3 | 5 | |
| Arginine | 44.92 | 26.88 | 34.38 | 20.27 | 2.03 |
| Asparagine | 33.04 | 14.08 | 11.68 | 5.73 | 8.47** |
| Glutamine | 134.75 | 115.82 | 32.51 | 19.00 | 10.54** |
| Aspartate | 85.44 | 74.54 | 35.13 | 32.89 | 3.62 |
| Glutamate | 147.71 | 63.72 | 70.33 | 40.98 | 9.99** |
| Serine | 38.25 | 32.43 | 24.13 | 21.54 | 1.49 |
| Alanine | 105.07 | 41.47 | 22.76 | 15.02 | 13.80** |
| Proline | 163.53 | 20.73 | 74.04 | 35.51 | 11.66** |
| Glycine | 18.59 | 19.54 | 27.92 | 14.43 | 1.06 |

Level of significance *P≤ 0.05

**P≤ 0.01

Sugars

The sucrose content was much higher after 1 day incubation at 5°C than in any other treatment (Table 9.3A). Apparently sucrose content rose initially when the temperature was lowered but thereafter fell to the level in segments maintained at 20°C. Glucose content exhibited a similar trend, although the change in concentration was not as great and did not reach statistical significance (except in the comparison between 1 and 5 day samples). The fructose content was greatest in the 20°C incubated sample and appeared to decline progressively upon incubation at 5°C (Table 9.3A).

The changes in sucrose concentration were accompanied by identical changes in the radioactivity in sucrose, with the 5°C, 1 day sample containing far more radioactivity than any other treatment (Table 9.3B). Although, radioactivity in glucose and fructose tended to be the highest in the 3 day incubated samples, it was not statistically significantly different from that in any of the other treatments (Table 9.3B).

Both sucrose content and total radioactivity in sucrose were always greater than the content or radioactivity of glucose and fructose in all treatments, radioactivity in sucrose constituting 50 to 85% of the total radioactivity found in sugar.

Summarizing the results obtained so far, it is evident that total fixation of $^{14}CO_2$ is significantly reduced only following 4 days of cold treatment. Segments at 20°C showed more counts in the insoluble residue and the amino acid fraction than similar tissue at 5°C. Of the various compounds estimated, the extremely high radioactivity and relatively high concentration of sucrose in the 24 hour 5°C treated samples is notable, here sucrose accounted for 13% of total radioactivity

Table 9.3

Changes in sugars accompanying incubation at low temperature

(A) Sugar content ( mg $g^{-1}$ dry wt)

| Incubation temperature | 20°C | 5°C | | | Variance ratio |
|---|---|---|---|---|---|
| Incubation period (days) | 1 | 1 | 3 | 5 | |
| Glucose | 23.72 | 28.81 | 20.75 | 19.59 | 2.82 |
| Fructose | 26.72 | 22.92 | 17.64 | 14.82 | 2.96 |
| Sucrose | 35.84 | 90.91 | 39.14 | 34.49 | 7.43** |

(B) Radioactivity in sugars (cpm x $10^{-3}$)

| Incubation temperature | | | | | Variance |
|---|---|---|---|---|---|
| Incubation period (days) | 1 | 1 | 3 | 5 | ratio |
| Glucose | 143.08 | 126.72 | 203.40 | 100.77 | 0.64 |
| Fructose | 57.63 | 66.57 | 133.73 | 49.10 | 2.81 |
| Sucrose | 133.72 | 1375.40 | 379.12 | 222.00 | 4.96* |

Significance level * $P \leq 0.05$

** $P \leq 0.01$

in the sample. The radioactivity accumulated in proline at 5°C was less than at 20°C, despite the concomitant accumulation of proline. This evidence does not support the proposition that proline synthesis from current photosynthesis is enhanced by low temperatures; or that proline is not synthesized from carbon fixed during the first day of stress but is synthesized from carbon fixed subsequently.

B. Metabolism of labelled compounds during incubation at 5°C for 5 days

The alternate hypothesis for proline accumulation at 5°C - hypothesis (B) that carbon for proline synthesis is derived from photosynthesis occurring over the entire stress period was tested in this experiment. Barley segments were exposed to $^{14}CO_2$ for 2 hours immediately upon transfer to 5°C and 2 days after incubation at 5°C. The segments were transferred to petri dishes and left in air at 5°C. Segments were harvested after 1, 3 and 5 days of incubation at 5°C and counts in the various fractions were estimated as detailed in the Materials and Methods section.

Total radioactivity in various fractions

Total radioactivity present in the samples decreased significantly between 1 and 3 days of exposure to 5°C (Fig 9.4a). The counts in the ethanol-soluble extract fell significantly with time (Fig 9.4b) but those in the insoluble residue did not change significantly (Fig 9.4c).

Two components of the soluble fraction, total sugars (glucose + fructose + sucrose) and amino acids both showed a fall in radioactivity with time, but only the fall in soluble sugars was statistically significant (Fig 9.4d,e).

Fig 9.4

Change in total incorporation of radioactivity and that in various fractions during incubation at 5°C.

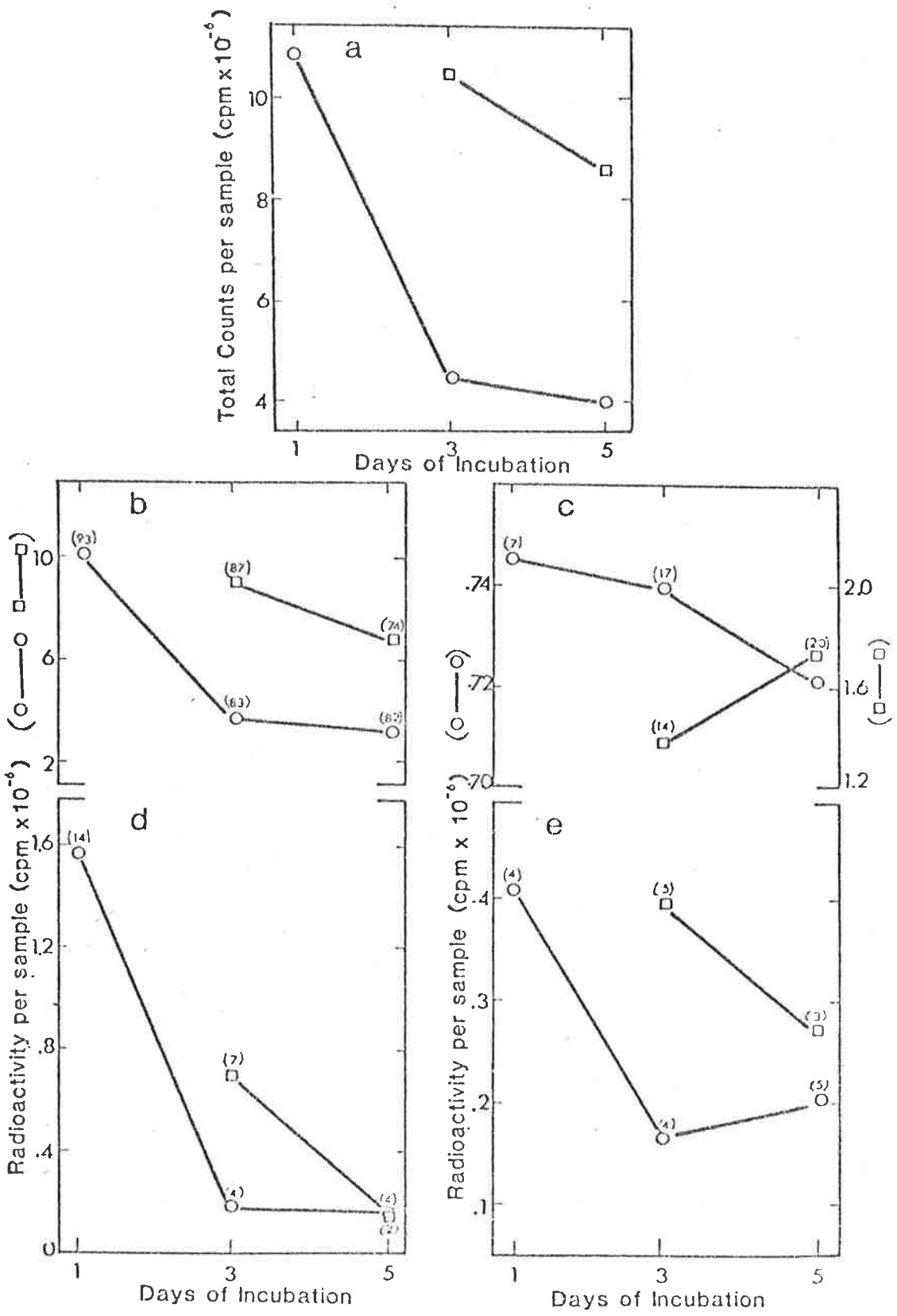
Ten day old barley plants growing in a 16 hour photoperiod at 20°C were transferred to 48 hours continuous light prior to excision and segmentation of the first leaves. Segments were floated on water in light at 5°C and also exposed to $^{14}CO_2$ for 2 hours in flasks. The segments were then transferred to petridishes at 5°C and harvested 24, 72 and 120 hours after commencement of cold treatment. Each point is the mean of 3 replicates.

a. Total radioactivity in the sample
b. Total radioactivity in the ethanol soluble fraction
c. Total radioactivity in the ethanol insoluble residue
d. Total radioacticity in sugars (glucose + fructose + sucrose)
e. Total radioactivity in total amino acids

I ○ samples exposed to $^{14}CO_2$ on day 0

II □ samples exposed to $^{14}CO_2$ on day 2.

Figures in parenthesis are the radioactivity expressed as a precent of total radioactivity in the samples


a
Total Counts per sample (cpm x 10⁻⁶)
Days of Incubation
b
c
d
e
Radioactivity per sample (cpm x 10⁻⁶)
Days of Incubation

When the radioactivity in the various fractions was expressed as a percentage of the total radioactivity in the sample (to study relative distribution amongst the different fractions), the counts in sugars alone were significantly reduced in day 3 and day 5 samples compared with day 1 samples (Fig 9.4d). The sugar fraction in the day 1 sample constituted 14% of the total radioactivity, while after 3 and 5 days incubation at 5°C, it fell to 4%.

Samples exposed to $^{14}CO_2$ on day 2 and harvested on day 3 and day 5 showed a similar change in radioactivity as did samples exposed to $^{14}CO_2$ on day 0 and harvested on day 3 to day 5 (Fig 9.4). The radioactivity was not significantly altered in any fraction.

Thus the total radioactivity in the leaf segments is reduced with time during incubation at 5°C primarily between dey 1 and day 3. The decrease occurs in the ethanol-soluble extract alone and is partly due to the decrease in the sugar fraction. There are two ways by which the radioactivity can be reduced - through transformation to insoluble compounds or through loss to the atmosphere by respiration. As there was no significant increase in radioactivity in the insoluble fraction during the cold treatment, the loss of radioactivity must have resulted from respiration. This loss was most apparent between 1 and 3 days and subsequently there was no loss of counts. This may signify a decline in respiration with prolonged exposure to low temperature.

## Amino acids

### Proline

The proline content of the tissue increased significantly with the duration of cold treatment as before (Fig 9.5a). The radioactivity in proline also increased with time (Fig 9.5b), and when the counts were expressed as a percentage of total counts in all amino acids, this increase with time was emphasised with counts in proline constituting up to 60% of the total amino acid radioactivity (Fig 9.5c). Although proline did not account for a large proportion of the total radioactivity of the sample, this proportion also increased with time, 2% and 3% of the total radioactivity in the sample on day 3 and day 5 respectively as against 0.3% of total sample radioactivity on day 1.

The increase in proline content between day 3 and day 5 in proline samples exposed to $^{14}CO_2$ on day 2 (II) was similar to that of 3 and 5 day samples exposed to $^{14}CO_2$ on day 1 (I) (Fig 9.5a). The total radioactivity in proline on the other hand, increased significantly (two-fold) between day 3 and day 5 (Fig 9.5b). When the counts in proline on day 5 were expressed as a percentage of the total radioactivity in the amino acid fraction, however, it constituted 44% in sample II as against 60% in sample I (Fig 9.5c). When expressed as a percentage of the total radioactivity in the sample, proline increased from 0.7% on day 3 to 1.7% on day 5 in sample II. The specific radioactivity calculations showed little difference in counts per µg between any of the day 3 and day 5 samples irrespective of the day on which they were exposed to $^{14}CO_2$ (Table 9.4) while day 1 samples showed very high counts in proline which was present in trace amounts only.

### Other amino acids

Glutamic acid was the only amino acid which increased significantly after 3 and 5 days of cold treatment (Table 9.5). None of the

Fig 9.5

Change in proline content and radioactivity with length of duration of cold stress.

Plant culture and treatments are as detailed in Fig 9.4

A. Free proline content

B. Radioactivity in proline (cpm x $10^{-3}$)

C. cpm as % of total radioactivity in all amino acids

Figures in parenthesis arethe counts in proline expressed as a percent of the total radioactivity in the sample.

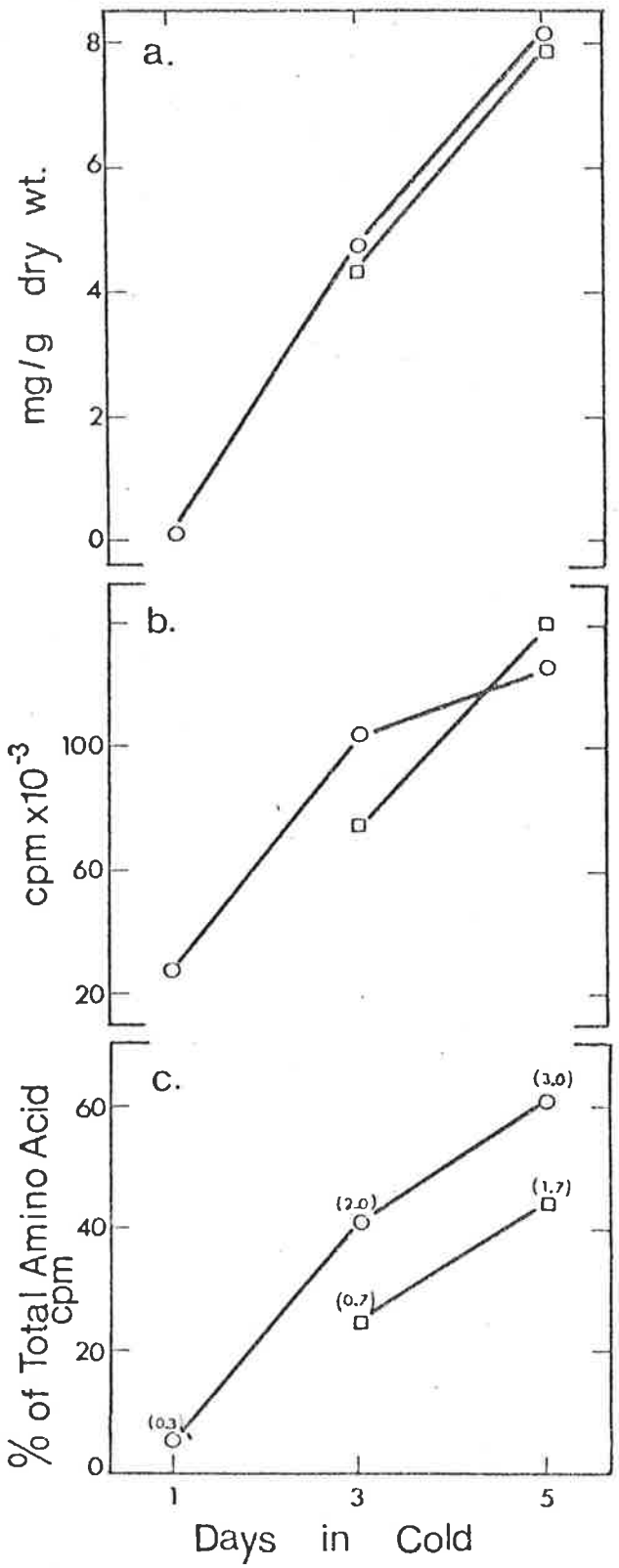
I ○ samples exposed to $^{14}CO_2$ on day 0

II □ samples exposed to $^{14}CO_2$ on day 2

Table 9.4

Specific radioactivity of proline (cpm $\mu g^{-1}$)

| Incubation period (days) | 1 | 3 | 5 |
|---|---|---|---|
| Sample I | 82900 | 537 | 368 |
| Sample II | | 512 | 416 |


a.
mg/g dry wt.
8
6
4
2
0
b.
cpm x10-3
100
60
20
c.
% of Total Amino Acid cpm
60
40
20
0
(3.0)
(2.0)
(1.7)
(0.7)
(0.3)
1
3
5
Days in Cold

apparent changes in concentration in the other amino acids were statistically significant (Table 9.5). (Amino acid content of sample II was identical to sample I day 3 and day 5 samples and therefore have not been presented.) In fact, when the total amino acid content (excluding proline) was calculated, there was no significant change with time (Table 9.5). In general, the radioactivity in all the amino acids except proline (and alanine in sample II) decreased significantly with time (Fig 9.6). As described earlier, proline showed a significant increase in radioactivity over the entire stress period. In Sample I, the decrease in radioactivity between day 3 and day 5 appeared less than between day 1 and day 3. This may have been partly due to isotope dilution since in sample II where the radioactivity was higher than sample I on day 3, the decline between day 3 and day 5 was as rapid as that between day 1 and day 3 of sample I (Fig 9.6). Thus, it can be concluded that the amino acid pools (excluding proline) turned over rapidly at 5°C since the content did not change significantly although the radioactivity showed a rapid decline with time (Fig 9.6).

Sugars

The sucrose content of the leaf samples fell considerably with prolonged exposure to low temperature. There was a rapid decrease from day 1 to day 3 but thereafter the decline was more gradual (Table 9.6). The changes in glucose and fructose content were less and not statistically significant. In sample II as well, none of the sugars changed significantly with time (tested with a T-test) (Table 9.6). The sucrose content expressed as a percentage of total sugars (glucose + fructose + sucrose) decreased from 86% to 50% from day 1 to day 5 in sample I and remained at 50% in day 2 samples.

Fig 9.6

Change in radioactivity in amino acids with time at 5°C

Plant culture and treatments are described in Fig 9.4

ARG - Arginine

ASN - Asparagine

GLN - Glutamine

GLU - Glutamate

ASP - Aspartate

SER - Serine

ALA - Alanine

GLY - Glycine

I ○ Fed $^{14}CO_2$ on day 0

II □ Fed $^{14}CO_2$ on day 2

Table 9.5

Change in amino acid content (mg $g^{-1}$ dry wt) with time at 5°C

| Incubation period (days) | 1 | 3 | 5 | Variance ratio |
|---|---|---|---|---|
| Asparagine | 0.41 | 0.85 | 0.25 | 1.77 |
| Glutamine | 7.61 | 6.82 | 3.73 | 1.53 |
| Aspartate | 0.88 | 1.78 | 1.43 | 0.39 |
| Glutamate | 1.48 | 2.64 | 3.40 | 4.61* |
| Serine | 1.15 | 0.83 | 0.78 | 0.48 |
| Alanine | 0.82 | 1.06 | 1.26 | 0.25 |
| Proline | - | 4.75 | 8.14 | 25.48** |
| Glycine | 1.24 | 0.59 | 1.59 | 0.64 |
| Total | 13.58 | 19.31 | 20.58 | |
| (Total Proline) | 13.58 | 14.56 | 12.44 | |

Significance level * $P \leq 0.05$ ** $P \leq 0.01$

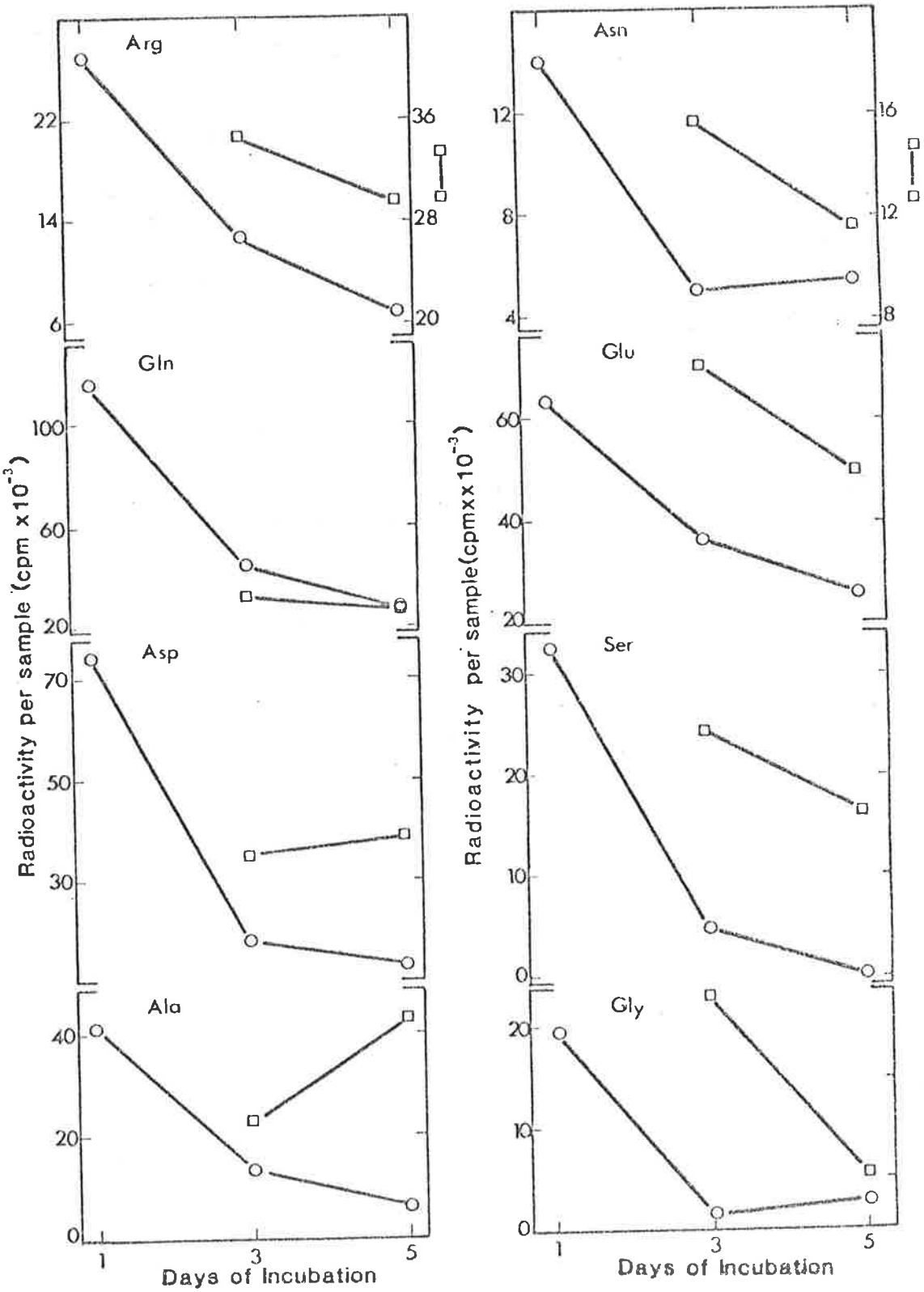

Arg
Gln
Asp
Ala
Asn
Glu
Ser
Gly
Radioactivity per sample (cpm x10⁻³)
Radioactivity per sample(cpmxx10⁻³)
Days of Incubation
Days of Incubation

Table 9.6

Changes in sugar content (mg $g^{-1}$ dry wt) with time at 5°C

| | | | | | Variance |
|---|---|---|---|---|---|
| Incubation period (days) | | 1 | 3 | 5 | ratio |
| I | Glucose | 28.81 | 24.13 | 30.86 | 0.45 |
| | Fructose | 22.92 | 18.15 | 18.39 | 0.96 |
| | Sucrose | 90.91 | 53.46 | 45.77 | 4.17* |
| II | Glucose | | 20.75 | 24.48 | 1.076 |
| | Fructose | | 17.64 | 18.67 | 3.118 |
| | Sucrose | | 39.14 | 40.58 | 3.542 |

Significance level * $P \leq 0.05$

** $P \leq 0.01$

The radioactivity in the sugar fraction declined rapidly between day 1 and day 3 after which there was no further decline in sample I. The radioactivity in sucrose decreased significantly from $1.4 \times 10^{-6}$ counts per minute to less than $0.2 \times 10^{-6}$ counts per minute between day 1 and day 3, but the decreases in both glucose and fructose radioactivity over the same period were not significant. In sample II on the other hand, the decrease in counts in glucose, fructose and sucrose were similar (Fig 9.7A).

Calculation of specific radioactivity showed counts in sucrose were higher on day 1 (I) not solely as a result of greater content but also due to greater labelling of sucrose (Fig 9.7B). Once again sample I showed very little difference in specific radioactivity between day 3 and day 5, while sample II showed a significant decrease. The reason for this could be isotope dilution in sample I or extremely rapid turnover rates resulting in rapid depletion of radioactivity.

From the results described so far, it is clear that both the radioactivity and the amount of proline accumulated increased significantly with the duration of the cold treatment in samples exposed to $^{14}CO_2$ on both day 0 and day 2. Other amino acids declined in radioactivity but their concentration did not vary with time. Sucrose concentration and radioactivity also declined during this period and it is difficult to rule out either class of compounds as a source for proline synthesis.

## 9.4 Total anthrone sugars

Sucrose is one of the major soluble carbohydrates and it was apparent that the sucrose content decreased with continuing stress (Table 9.6). However, the effects of duration of 5°C treatment on

Fig 9.7

Changes in radioactivity and specific radioactivity with time at 5°C in the various sugars.

Plant culture and treatments were as described in Fig 9.4

Glu - Glucose

Fru - Fructose

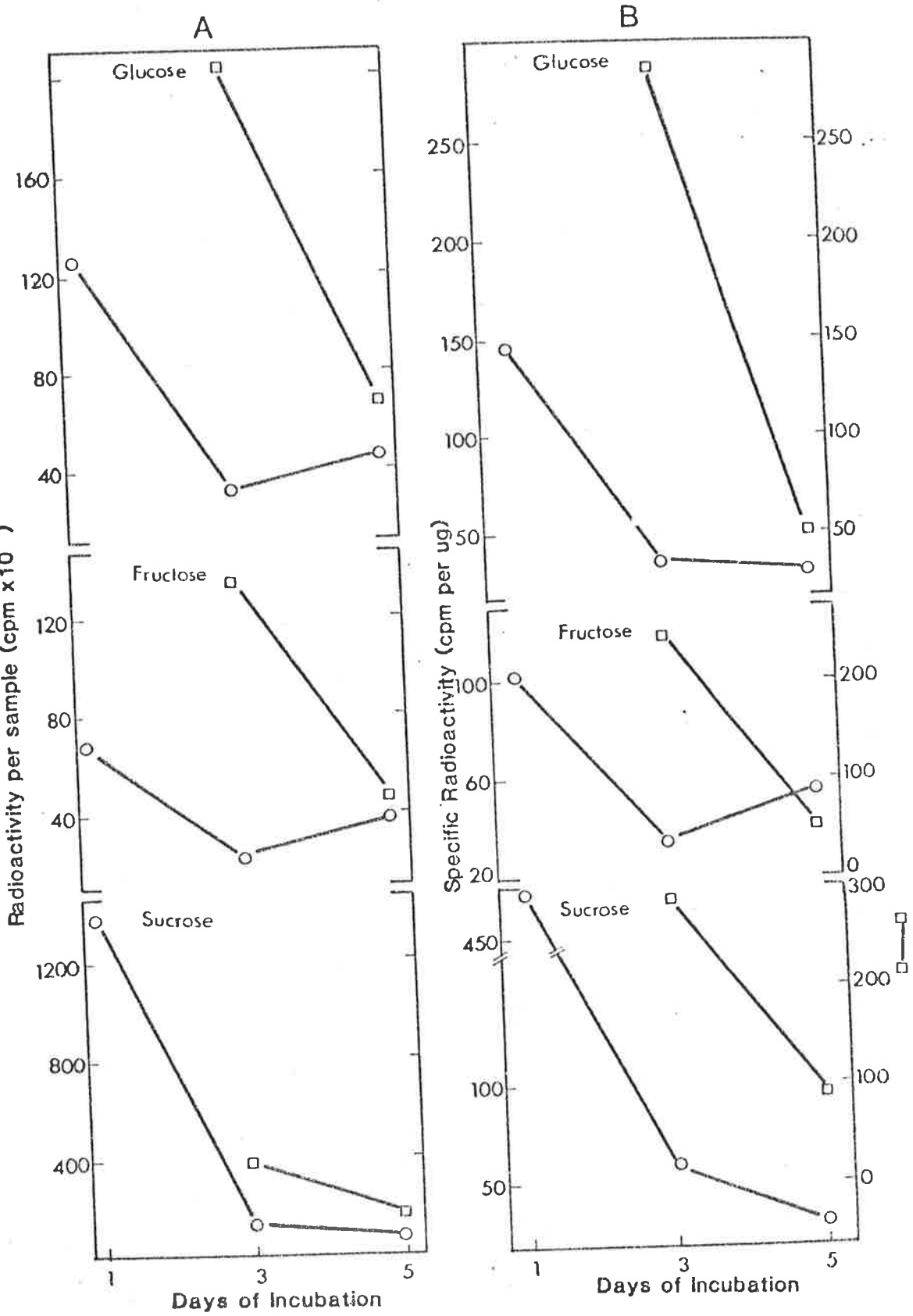
Suc - Sucrose

I Exposed to $^{14}CO_2$ on day 0

II Exposed to $^{14}CO_2$ on day 2

A. Radioactivity per sample (cmp x $10^{-3}$)

B. Specific radioactivity (cpm per µg)


A
B
Glucose
Fructose
Sucrose
Radioactivity per sample (cpm x10$^{-3}$)
Specific Radioactivity (cpm per ug)
Days of Incubation
Days of Incubation

the total soluble carbohydrates were not known and have been examined in this section.

The total anthrone sugars increased significantly over the entire stress period, accumulating to levels up to three-fold greater than 20°C samples (Fig 9.8). Contents in both experiments (A and B) were very similar in the same duration samples (Table 9.7 and Fig 9.8).

## 9.5 Discussion

Proline accumulation is dependent on photosynthesis over the entire incubation period since the inhibition of photosynthesis even for 1 day resulted in a significant inhibition of proline accumulation (Fig 8.4). Also, the transfer of segments from light to darkness in the third day of the cold treatment was able to reduce proline accumulation (Fig 7.5) compared to segments incubated in light for all 3 days. However, in the dark or in inhibitor treated samples in the light when incubated at 5°C the continued supply of carbohydrate was able to induce significant proline accumulation (Fig 8.7 ) which suggests that photosynthesis may be providing carbohydrates essential for proline accumulation. The incubation of segments in closed systems for up to2 days during a 3 day incubation period did not reduce proline content implying that although the net carbon content of the tissue was not increased, the re-assimilation of respired $CO_2$ could have occurred providing the necessary photosynthates and energy for proline accumulation. Under water stress conditions, carbohydrates have been implicated in the induction of proline accumulation either by providing the carbon skeletons essential for proline synthesis (Stewart et al, 1966) or by inhibiting the oxidation and stimulating the synthesis of proline (Stewart, 1972, 1978).

Fig 9.8

Effect of duration of incubation on total ethanol soluble carbohydrate content in barley leaf segments.

Ten day old barley plants were transferred to 48 hours continuous light prior to excision, segementation and incubation in light at 5° or 20°C for varying periods of time.

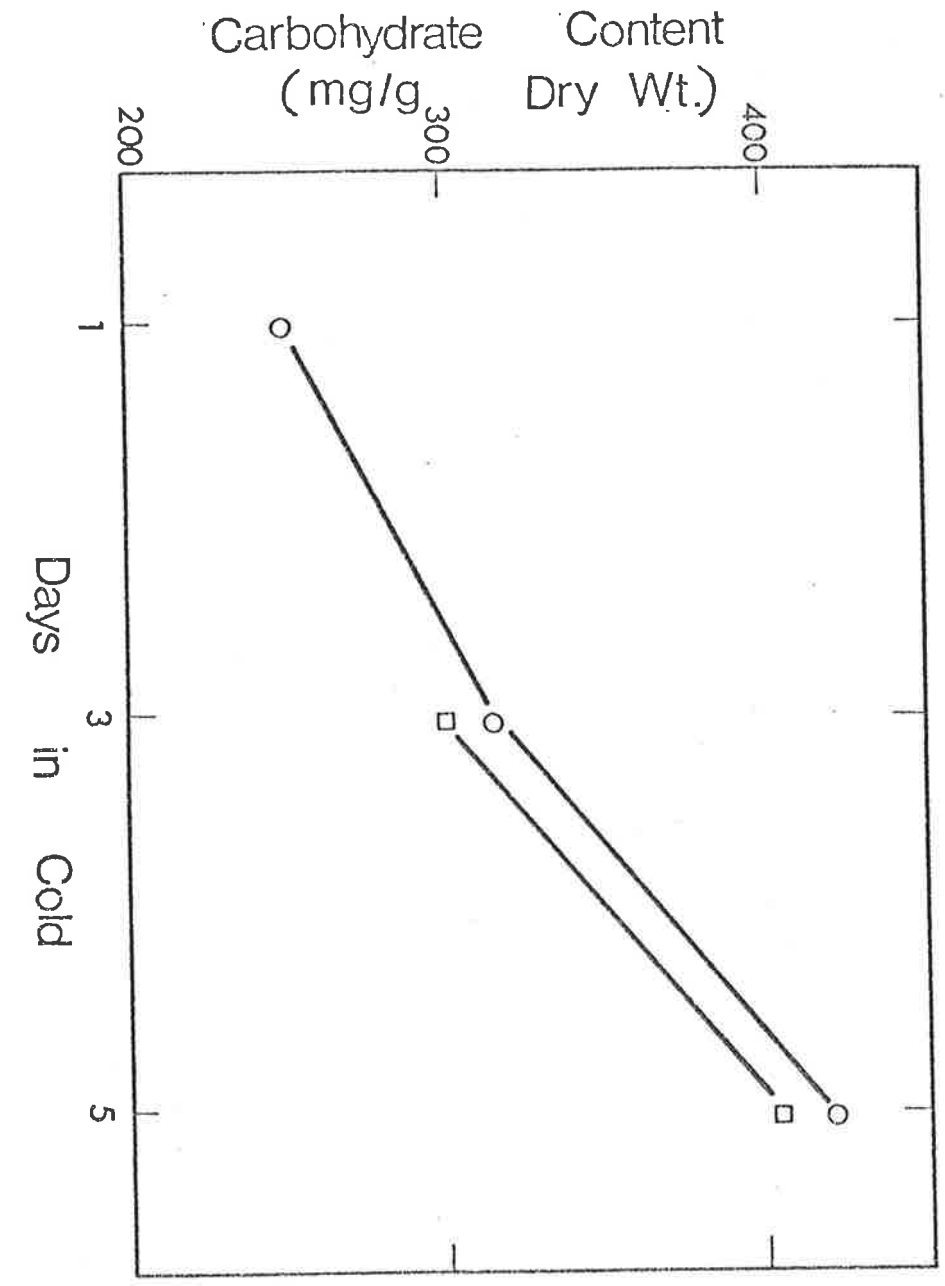
Each point is the mean of 3 replicates

○ Exposed to $^{14}CO_2$ on day 0

□ Exposed to $^{14}CO_2$ on day 2

Table 9.7 Soluble carbohydrate content (mg $g^{-1}$ dry wt) after incubation with $^{14}CO_2$ during different days of stress

| Incubation temperature | 20°C | 5°C | | |
|---|---|---|---|---|
| Incubation period (days) | 1 | 1 | 3 | 5 |
| Carbohydrate content (mg $g^{-1}$ dry wt) | 136 | 248 | 300 | 424 |


Carbohydrate Content
(mg/g Dry Wt.)
200
300
400
1
3
5
Days in Cold

Proline accumulation may occur due to increased partitioning of carbon fixed during the stress period towards proline synthesis or may result from reduced oxidation of proline and continued gradual synthesis over the entire incubation period. In rape plants exposed to low temperatures for 2 days, Sosinska et al (1977) concluded that proline accumulated as a consequence of an increased incorporation of the assimilated carbon into proline and less into carbohydrates (sugars and starch) compared with 25°C controls. Results obtained here showed that this was not true for barley plants at 5°C. Comparisons of samples treated for 1, 3 or 5 days at 5°C with controls (1 day at 20°C) showed no increase in the incorporation of carbon into proline at 5°C. In fact, 20°C samples showed significantly higher radioactivity in proline than any of the 5°C samples (Fig 9.3). This difference in incorporation of radioactivity into proline at 5°C was present even where similar amounts of total radioactive carbon were incorporated in both 20° and 5°C samples. Thus, enhanced proline synthesis from current photosynthates during low temperature treatment is not the cause of an increase in proline content. From results obtained in experiment B it would appear that carbon for proline accumulation may originate from carbon compounds fixed on day 0 as well as day 2 as tissue exposed to $^{14}CO_2$ on either of these days showed considerable accumulation of radioactivity in proline despite a significant reduction in the total radioactivity with time (Fig 9.5) as well as isotope dilution of existing radioactivity. Thus it appears that proline accumulates not because of increased synthesis but due to a continuation of synthesis coupled with a reduction in utilization during cold treatment.

Since the accumulation of radioactivity into proline is not immediate upon transfer to cold temperatures any of the following compounds could serve as the intermediate for proline synthesis - proteins, amino acids or carbohydrates. A decrease in total protein content (either due to reduced synthesis or increased degradation) would result in an increase in amino acid concentration (Table 9.5) which could then be transformed into proline; similarly carbohydrates can provide the carbon skeletons essential for proline synthesis.

According to Kudrev (1968) proline accumulates during water stress as a consequence of the breakdown of proteins resulting in the release of glutamate and other amino acids which then served as precursors for proline. Results obtained here would suggest that this was unlikely to be the case at low temperatures as the radioactivity in the insoluble residue did not change significantly over the 5 day period (Fig 9.4) yet that in proline increased significantly. Stefl et al (1978) proposed that free proline accumulated in cold treated wheat plants as a surplus after the synthesis of proline rich proteins had occurred within the first six hours after transfer to cold temperatures. The authors, however, failed to explain how an increase in free proline available for incorporation into proteins occurred in cold treated plants. Results of Experiment A, however, clearly showed significant incorporation of radioactivity in the insoluble residue in samples exposed to $^{14}CO_2$ 4 days after incubation at 5°C. Of course, it is possible that proteins were being synthesized during the 5 days at 5°C but not ones rich in proline.

Sosinska and Maleszewski (1978) suggested alanine to be the source of carbon for proline synthesis. When $^{14}C$ alanine was fed to cold

treated rape plants, proline was found to contain radioactivity unlike 25°C controls which showed no radioactivity in proline. Results obtained here showed a rapid decline in radioactivity in alanine with time from 40,000 cpm on day 1 to 6000 cpm on day 5 while that in proline increased by 1u0,000 cpm during a similar period (Figs 9.5 and 9.6) in segments exposed to $^{14}CO_2$ on day 0 (I). Samples exposed to $^{14}CO_2$ on day 2, on the other hand behaved differently. These segments showed a two-fold increase in radioactivity in alanine and proline between day 3 and day 5 samples. This suggests that alanine may not be the carbon precursor for proline synthesis.

Since most of the amino acids as well as sugars showed a decline in radioactivity over the 5 day incubation period while radioactivity in proline increased, it is difficult to identify the carbon precursor for proline synthesis. Although neither glucose, fructose nor sucrose content showed a good correlation with proline accumulation, the total alcohol soluble carbohydrates (data in Figs 9.3, 9.5 and 9.8 and Table 9.7) were highly correlated with proline content (correlation coefficient 0.94) suggesting that they were important for proline accumulation although the nature of the relationship is not evident.

# V GENERAL DISCUSSION

# V GENERAL DISCUSSION

## 1. Introduction

Proline accumulates in barley plants in response to various environmental stresses, including water, low temperature and salt stress (Singh et al 1973a, Chu et al 1974). Light is able to increase this accumulation of proline in water stressed tissue, independent of changes in tissue water status (Appendix II), while in tissue exposed to low temperatures, there appears to be an absolute requirement for light for proline accumulation (Fig. 7.4). This response to light could conceivably be mediated through a phytochrome response or be due to photosynthesis. The present results show that neither exposure to a brief period of light (Fig. 2.4) nor illumination with a very low irradiance [<1 $\mu E\ m^{-2}s^{-1}$ during water stress (Fig. 3.4) and <15 $\mu E\ m^{-2}s^{-1}$ during cold (Fig. 7.7)] increased proline accumulation, thus rendering it unlikely that proline accumulation is controlled by a low energy phytochrome response. The wavelengths of maximum effectiveness for proline accumulation appeared in the blue and red regions similar to those for photosynthesis, while far red light did not increase proline accumulation above accumulation in the dark (Figs. 4.2 and 4.4). These data are consistent with the hypothesis that light acts on proline accumulation through photosynthesis and that a phytochrome response is not involved. This view is further strengthened by the results of the experiments with photosynthetic inhibitors in which proline accumulation in response to water or low temperature stress was inhibited when these inhibitors were supplied (Figs. 5.13, 5.15, 8.2 and 8.3). Other workers have also shown a link between photosynthesis and proline accumulation during stress. Singh et al (1973b) were able to invoke some proline accumulation in water

stressed, etiolated barley leaf segments by the supply of carbon compounds (both sucrose and/or glutamate). Moreover in rape plants exposed to low temperatures, Sosinska et al (1977) reported an increased transfer of carbon fixed from alanine to proline during stress. Hanson and Tully (1979) reported a stimulation of the synthesis of proline from glutamate in stressed barley plants which were illuminated.

2. The Role of Light

Such experiments do not clarify the role of photosynthesis in proline accumulation, as photosynthesis can influence proline accumulation by the provision of -

a) Fixed carbon

b) Trapped energy, or

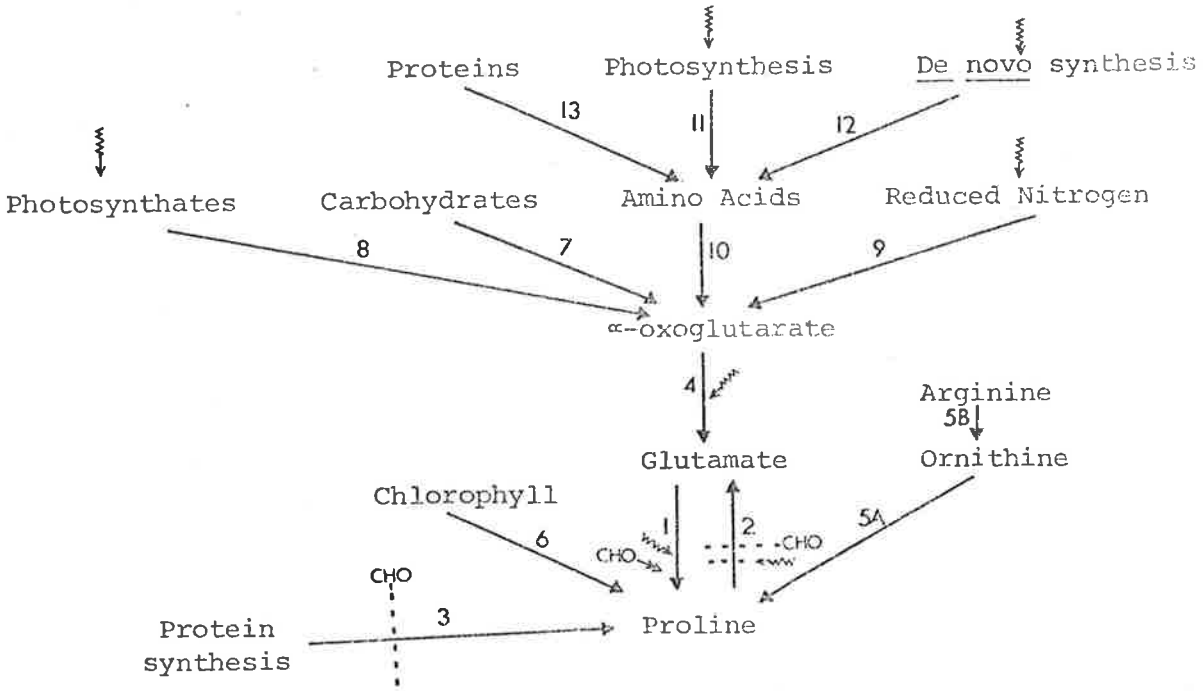
c) Both carbon and energy.

a) Fixed carbon

Since carbon dioxide exchange continues in stressed tissue, albeit at a reduced rate [>90% reduction in PEG stressed tissue (Fig. 5.3) and >50% reduction in tissue exposed to low temperatures (Fig. 8.2)] it is possible that concurrent photosynthesis may be providing all of the carbon required for enhanced proline accumulation in illuminated tissue (Fig. 1 reactions 1 and 7). Evidence against this hypothesis for both water stress and low temperature stress includes -

i) Experiments in which segments incubated on PEG in enclosed flasks throughout the stress period did not show inhibited proline accumulation despite $CO_2$ becoming limiting for photosynthesis within less than an hour (Appendix II and Fig. 6.2B). During exposure to $5^{o}C$, enclosure of the segments in flasks (limited $CO_2$) after 1 day in air did not inhibit

Fig 1 THE INFLUENCE OF LIGHT ON PROLINE METABOLISM




| | |
|---|---|
| CHO | carbohydrates |
| ⇝ | Light energy (NAD(P)H, ATP) |
| - - - | Inhibition |
| ↠ | Stimulation |

proline accumulation which increased by ≈ 3 mg $g^{-1}$ dry weight during this period (Fig. 8.5B).

ii) Similarly, the incubation of segments in $CO_2$-free air did not inhibit proline accumulation in light pretreated segments (Fig. 5.8) nor did it affect the increase in proline accumulation (≈ 6 mg $g^{-1}$ dry weight) in cold treated segments which were transferred to this treatment following 3 days of stress in an open system (Fig. 8.6). Enclosure in $CO_2$-free air did however inhibit proline accumulation in dark pretreated and cold stressed samples possibly because here photosynthesis even from recycled $CO_2$ was inhibited resulting in an inhibition of energy production as well as carbon fixation (Fig. 8.6 and Fig. 5.8).

iii) The provision of carbohydrates to samples stressed in the dark induced some proline accumulation, but this did not equal the accumulation occurring in samples incubated in light even without exogenous carbohydrates (Table 6.4 and Fig. 8.7).

iv) The transfer of PEG stressed segments from darkness to light induced a rapid increase in the rate of proline (Fig. 2.7B,D) accumulation despite net carbon exchange having dropped to <10% of that in turgid tissue (Fig. 5.3). Similarly, the transfer from light to darkness decreased the rate of proline accumulation (Fig. 2.7A,C). During exposure to low temperatures too, the transfer of segments to darkness following incubation in light for 1 day induced less proline accumulation than occurred in segments which were in light throughout but enclosed in limiting $CO_2$ conditions (Fig. 7.5A and 8.5B).

v) The incubation of segments at an irradiance of <6 μE $m^{-2}s^{-1}$ (i.e. below light compensation, when no net increase in $CO_2$ occurs) increased proline accumulation above that taking place in segments floated on PEG in the dark (Fig. 3.4).

vi) $^{14}CO_2$ studies showed that <10% of the proline accumulated in light was labelled with carbon fixed during stress (see page 210). Results obtained in samples exposed to 5°C also showed that the conversion of $^{14}CO_2$ fixed in light, into proline was similar over the entire stress period (5 days) as that in samples incubated at 20°C (Fig. 9.3).

The results of these various experiments indicate strongly that although illumination may result in some currently fixed carbon being channelled to the accumulating proline, this forms by no means a large proportion of the carbon required for proline synthesis and certainly does not account for the light response of proline accumulation.

b) Trapped Energy

Calculations of the potential energy (NAD(P)H) that could be generated at an irradiance of ≃ 20 μE $m^{-2}s^{-1}$ showed that despite a trapping efficiency of 75% of that in turgid tissue, the NAD(P)H produced was sufficient to support the enhanced synthesis of proline in light (Fig. 1 - reaction 1)(Section 6.6). That photosynthetic energy is essential for enhanced proline accumulation is concluded from results with inhibitors of photosynthesis (Figs. 5.13, 5.15 and 8.2, 8.3), where the inhibition of photosynthesis for just 24 hours reduced proline accumulation significantly.

Also, since exogenous $CO_2$ is not essential, yet photosynthesis is, it can be inferred that trapped energy is required for proline accumulation in light (as the two reactions which constitute photosynthesis are $CO_2$ fixation and the light reaction responsible for energy trapping).

c) Both carbon fixation and trapped energy

Although, as has been seen previously (a) current photosynthesis during stress does not exclusively or even largely furnish the carbon

skeletons required for proline synthesis, it appears that during exposure to low temperatures, there is a prerequisite carbohydrate concentration which must be attained in order that proline may accumulate. During exposure to PEG stress, however, this does not seem to be the case. This is concluded from experiments in which -

i) Light pretreated samples incubated at 5°C in light failed to accumulate proline when enclosed in flasks on all 3 days of the stress period (Fig. 8.6). Yet, similar tissue accumulated proline rapidly when incubated on PEG solution in such flasks over a similar period at low temperature, following 1 day incubation in an open system (Fig. 8.6).

ii) Enclosure of light pretreated segments in $CO_2$-free air throughout the cold stress period inhibited proline accumulation, but enclosure between days 3 and 5 was without effect (Fig. 8.6). During water stress on the other hand, light pretreated samples showed no inhibition of proline accumulation due to incubation in $CO_2$-free air (Fig. 5.8).

iii) Proline failed to accumulate in cold treated segments at irradiances below 15 $\mu E\ m^{-2}s^{-1}$ (Fig. 7.7) i.e. above the light compensation point but at negligible rates of net $CO_2$ fixation.

Thus, both water and cold stress induced proline accumulation through photosynthesis, mainly from the provision of energy in the case of water stressed samples, and from the provision both of energy and of fixed carbon for samples in cold treatment.

Besides photosynthesis, photorespiration also occurs in light. However, the possibility that light influences proline accumulation through photorespiration seems unlikely as neither $CO_2$-free air (Fig. 5.8) nor incubation at irradiances below the light compensation point (Fig. 3.5) affected proline accumulation. Both these treatments would have enhanced relative photorespiration (i.e. ratio of photorespiration to photosynthesis).

Also, increasing the $CO_2$ concentration, which should inhibit or reduce photorespiration, failed to have any effect on proline accumulation (Figs. 5.8 and 8.6).

3. Synthesis of Proline

Since photosynthesis influences proline accumulation, and there are a number of ways in which light can influence carbon metabolism culminating in proline accumulation (see Fig. 1), the effect of light on proline synthesis and metabolism (i.e. Reactions 1, 2 and 3) was explored. The immediate precursors of proline are glutamate and ornithine (Fig. 1 reactions 1 and 5A). In water-stressed barley tissue, however, Boggess and Stewart (1976) showed that the primary precursor for proline synthesis is glutamate, with synthesis from ornithine and arginine (Fig. 1 - 5B) occurring subsequent to substantial proline accumulation. During exposure to low temperatures, however, the contribution of ornithine and arginine to proline accumulation has not been assessed.

Illumination was able to stimulate the synthesis of proline from $^{14}C$ glutamate supplied exogenously (Fig. 6.3). This stimulation in light occurred in both stressed and turgid samples. A similar stimulation of proline synthesis in light has been reported in turgid tobacco leaves (Mizusaki et al 1964) and stressed barley leaves (Hanson and Tully 1979).

4. Oxidation of Proline

In turgid tissue, proline is oxidized to glutamate (Fig. 1 - 2) or utilized for protein synthesis (Fig. 1 -3), the tissue thus maintaining a constant concentration of free proline. This mechanism is suspended during stress, resulting in proline accumulation (Stewart 1973, Boggess et al 1976 and Iwai et al 1979). In illuminated barley tissue proline utilization is significantly reduced during stress (Fig. 6.5), and

utilization also seems to be inhibited by low temperature (5°C). This latter conclusion arises from the results of experiments performed with radioactive $CO_2$, in which the radioactivity in proline alone increased linearly with time at 5°C, while the radioactivity in other amino acids and sugars fell (Fig. 9.5b). Although experiments using labelled proline during exposure to low temperatures have not been performed in this work, an inhibition of $^{14}C$ proline oxidation at 5°C has been reported in illuminated barley tissue (Chu 1974). Thus, during water stress (PEG), light influences both proline synthesis and proline utilization.

5. Control by Precursor Availability

Proline can only accumulate to high concentration if sufficient pools of precursors (both carbon and nitrogen) exist in the tissues. Tully et al (1979) suggest that an increase in proline is a consequence of the breakdown of translocation of (carbon and nitrogen) organic compounds out of the leaf in stressed intact barley plants. However, the experiments reported here showed an enhancement of proline accumulation in light during stress in excised segments, while turgid tissues which were also excised ailed to show massive proline accumulation (Fig. 2.2). This must imply that proline accumulation is not only a consequence of severed translocation of organic compounds out of the tissue, but also the result of an increased stimulation of proline synthesis (and, possibly, decreased utilization) in response to stress. One mechanism for this could be an increased channelling (partitioning) of organic compounds to proline accumulation over dark and/or turgid treatments (Fig. 2.2) this must also necessitate a greater availability and/or more efficient utilization of the precursors for proline. In addition to influencing proline metabolism (Fig. 1, reactions 1 and 2) light can influence other processes interrelated with proline synthesis (Fig. 1, reactions 4, 7, 8, 9, 11, 12, 13). For example, the

supply of carbon can be partially oxidized to yield α-oxoglutarate (Fig. 1 - 7, 8), which can then be aminated either from glutamine or from concurrently reduced nitrogen 9, to produce glutamate. A stimulation of proline accumulation was obtained when water- and cold-stressed tissue were supplied with glutamine (Table 6.4 and Fig. 8.7), which would support the hypothesis that other amino acids can influence proline accumulation.

An increase in the amino acid concentration may occur as a result of a reduction in the total protein content (either due to reduced synthesis or increased degradation of protein) reaction 13, or may arise from de novo synthesis of amino acids (reaction 11 and 12 respectively). The amount of labelled carbon fixed into the ethanol-insoluble residue including protein, in stressed tissue was less than that in turgid tissue - in both types of stress (Table 6.5 and Fig. 9.2). Another suggested source of proline is chlorophyll. Breyhan et al (1959) suggest proline as the precursor for chlorophyll synthesis; and Tyankova (1969) postulated that the degradation of chlorophyll during stress yields proline. However, the correlation between leaf chlorophyll content and proline concentration was poor in leaves subjected to water stress (Table 1.4) and chlorophyll degradation was not observed at 5°C (Chu 1974). It is conceivable that light energy may also influence the availability of stored reserves (carbon and nitrogen) for the synthesis of amino acids. The provision of 3PGA to segments incubated in the dark induced proline accumulation during water stress (Table 6.3) supporting the notion that photosynthesis (energy) influences proline accumulation.

6. Indirect Effect of Carbohydrate - Regulation of Proline Metabolism

Since proline accumulates to significantly greater concentrations in tissue pretreated in light (L-D) rather than darkness (D-D), prior to

incubation on PEG in the dark, as proposed previously, the carbohydrate status may be able to regulate proline metabolism independent of illumination. The tissue which was low in carbohydrates (D-D) showed very low rates of synthesis of proline and rapid rates of loss of radioactivity from $^{14}C$ proline. The L-D samples on the other hand, although stressed in darkness showed both an enhanced synthesis rate, as well as an inhibited rate of utilization of proline during stress (Figs. 6.3 and 6.5).

Thus, although proline accumulation during stress is greatly stimulated by the presence of light (which influences both proline metabolism as well as the provision of precursors for proline synthesis) the carbohydrate status of the tissue may also be able to influence proline accumulation. A similar role of carbohydrates, in which proline utilization is inhibited, has been shown in turgid maize tissue (Oaks et al 1970), and stressed bean leaves (Stewart 1973). In barley tissue, Stewart (1978) proposed the stimulation of synthesis of proline from glutamate as the role for carbohydrates.

## 7. Conclusion

In can be concluded, therefore, from the results discussed here, that during water stress, light provides the energy, trapped during photosynthesis, which influences proline metabolism directly and also provides the precursors essential for proline accumulation, independent of a net gain in carbon. In the cold the requirement for light for proline accumulation is for carbon as well as energy trapped during photosynthesis. Thus, there appears to be no single unique role for light in proline accumulation, but various responses contribute.

# VI APPENDICES

Appendix I

Measurement of light compensation point

The light compensation point is defined as that low irradiance at which net photosynthesis is equal to zero (Sestak, et al, 1971).

1. Intact Plants

The net carbon dioxide ($CO_2$) exchange of whole plants in a pot was measured by placing the pot in a 35 litre plastic assimilation chamber irradiated from above by a 400 watt metal arc lamp so that the irradiance at the canopy level was about 450 $\mu E\ m^{-2}\ s^{-1}$. Air exchange within the chamber was provided by a compressed air supply at 6-18 litres $min^{-1}$ via an oil filter, a 50 litre hydrating and buffer drum, through a flow meter to the chamber and then to waste. Air was circulated in the chamber by a small fan at a downward velocity over the canopy of about 0.5 m $s^{-1}$. Air temperature in the chamber was maintained as close to 20°C as possible by a small heat exchanger with water constantly circulated from a water bath. A stream of air from the entry port and one from the exit port were each dried and compared for $CO_2$ concentration in a Beckman 215 infra red gas analyser (IRGA) at flow rates of 0.5 litres $min^{-1}$. Air temperature in the chamber was monitored continuously with a copper constantan thermocouple (Fig 1).

Before placing the pot in the chamber, the chamber zero was obtained by circulating the air through the chamber and adjusting the differential output on the chart recorder to read zero. The pot was then placed in the chamber and left to equilibrate for one hour in the light, before readings were taken. The irradiance was then reduced by using neutral nylon mesh, until the output recorded zero indicating that next $CO_2$ exchange was zero. The irradiance at this point was measured and was called the light compensation point.

The light compensation point for intact barley plants was 162 $\mu E\ m^{-2}\ s^{-1}$ at the canopy level when the intensity was 70 $\mu E\ m^{-2}\ s^{-1}$ at pot level.

2. Excised leaf segments

The net $CO_2$ exchange of segments was also measured with a Beckman Model 215 infra red gas analyser. The general methods were similar to those with intact plants, however, some alterations were necessary because of the small size of the sample. The sample assimilation chamber was a small metal container fitted with a clear glass cover (see Section 4.1.1 of Results and Discussion) in which the segments were floated on distilled water in 2 smaller containers which fitted inside the assimilation chamber. The temperature of the chamber was maintained at 20°C by circulating water at this temperature through the lower half of the chamber which was especially designed for this purpose. Also a large petri dish containing water was placed between the light source and the chamber to minimise heating by the light. The air source from a compressed air cylinder was bubbled through water (to minimise loss of water by the sample) and then entered the chamber. The flow rate of air into the chamber was adjusted to 0.7 $l.min^{-1}$, using a flow meter. Some of the air from the exit port was passed through a flow meter at the rate of 0.5 $l\ min^{-1}$ (the remaining air being vented) and was connected to the IRGA and served as the 'sample air'. The 'reference air' consisted of a subsample of the air (after hydration) before it entered the chamber. This 'reference air' flow rate was also adjusted to 0.5 $l.min^{-1}$ and was connected to the IRGA.

The light source was a 100 watt incandescent bulb and the irradiance was altered by varying the distance between the light and the chamber. Neutral nylon mesh was also used when very low irradiances were required.

Measurement of carbon dioxide exchange in intact plants

IRGA - Infra Reg Gas Analyses

A - Flow meter

B - Assimilation chamber containing pot with plants

C - Chart recorder

≷ - Light

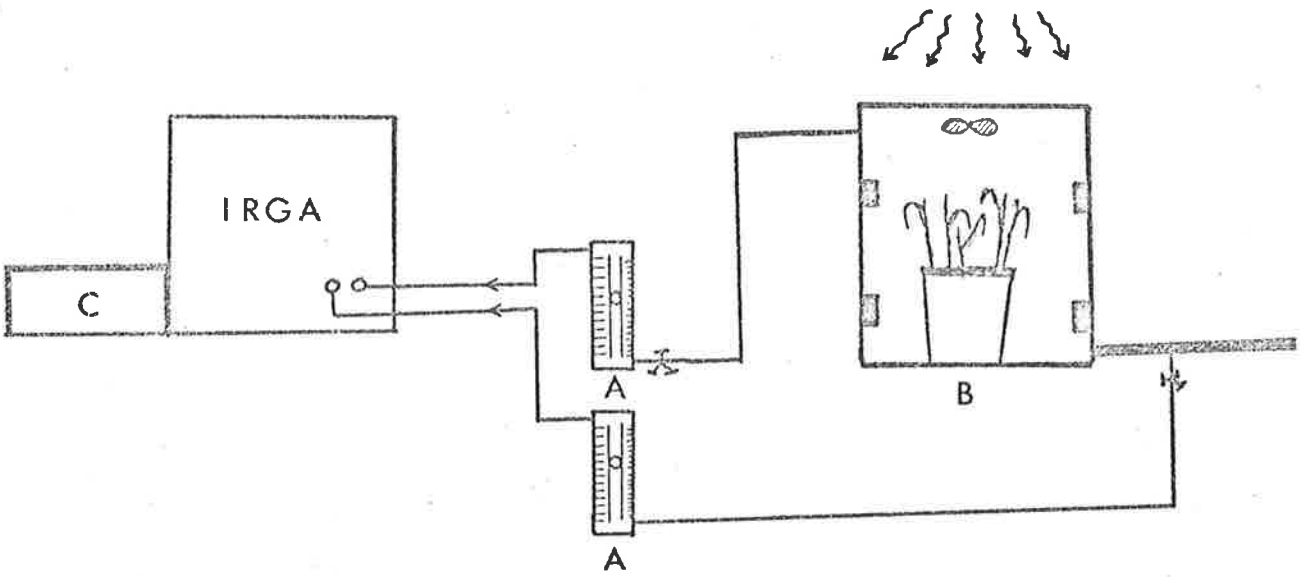
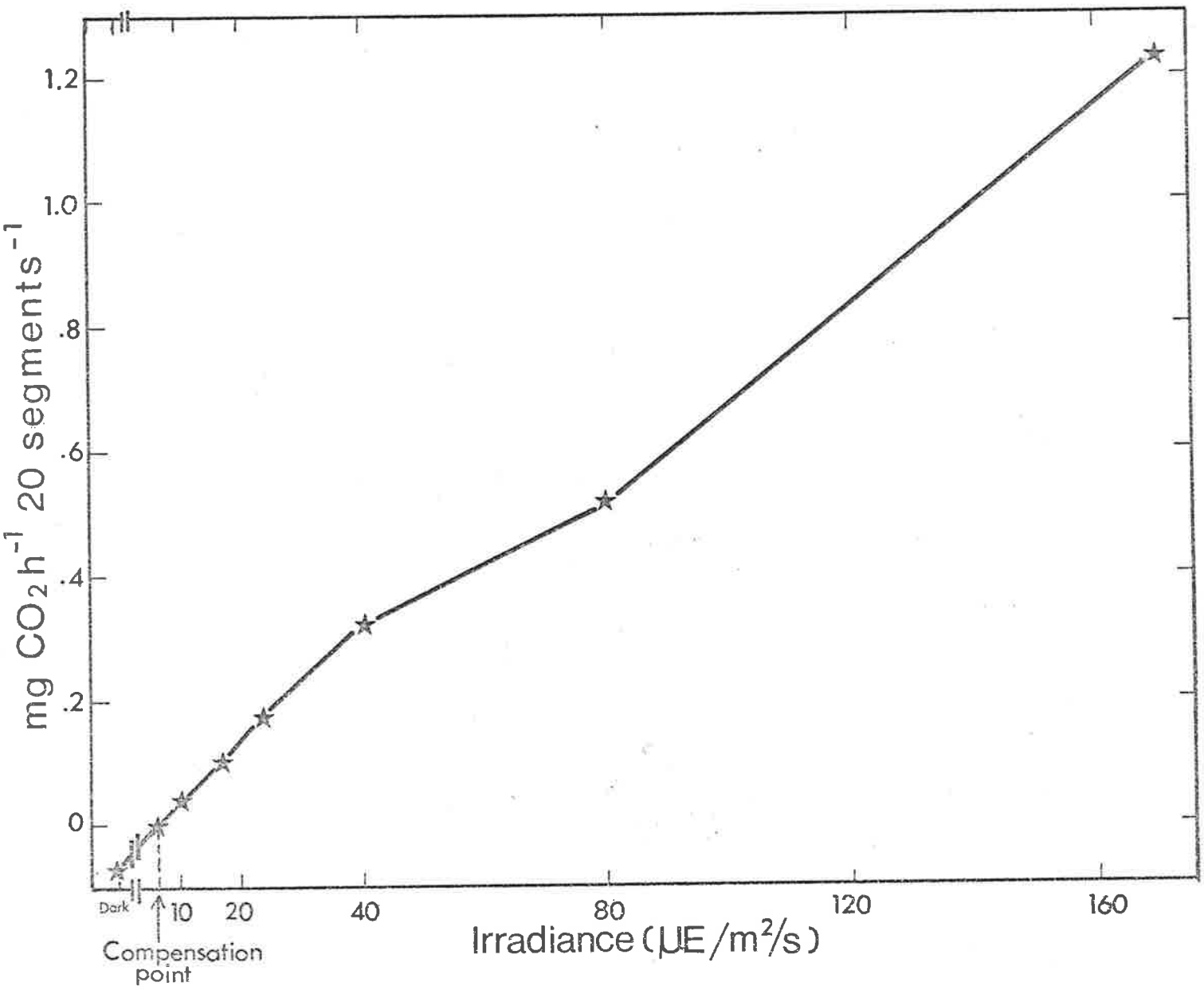

IRGA
C
A
A
B

Fig 2

Effect of irradiance on net carbon dioxide exchange in barley leaf segments


1.2
1.0
.8
.6
.4
.2
0
mg $CO_2 h^{-1}$ 20 segments$^{-1}$
Dark
10
20
40
80
120
160
Irradiance ($\mu E/m^2/s$)
Compensation point

Reducing the irradiance resulted in a linear decrease in $CO_2$ exchange until at the compensation point, no exchange occurred. This was at an irradiance of 6 μE $m^{-2}$ $s^{-1}$ (Fig 2).

Conclusions

The light compensation point for intact plants is much higher than that for leaf segments. This is to be expected since, with intact plants, the presence of leaves, stem and roots causes a lower ratio of photosynthetic to non-photosynthetic area. Respiration rate, on the other hand, would be relatively higher resulting in light compensation point occurring at a higher irradiance. Lamina segments would have a photosynthetic to non-photosynthetic ratio of almost unity, and also have relatively lower respiration rates.

## Appendix II

## Excised segment systems

Changes in water potential accompanying differences in irradiance with water-stressed intact plants complicate the analysis of the effect of light (Results and Discussion -Chapter 1). It was therefore necessary to design a system which could overcome this problem. The use of excised leaf segments (1.5 cm long), floated on water (for control or turgid treatments) or on PEG of desired water potentials (for stress treatments), was tested as an alternative system. The experiments described here were performed to characterize the system and establish its suitability for water stress studies.

### 1. Effect of light or darkness on water potential of segments floated on -15 bar PEG solution

In this experiment, the effect of light on the water potential of segments floated on PEG was studied. Only the two extreme treatments were considered, i.e. tissue in light for 72 hours (periods 1 and 2) or in darkness for 72 hours (periods 1 and 2). The duration of period 2 (when stress was applied) was reduced to 24 hours compared to the 72 hours for intact plants in order to minimise the effects of senescence following upon leaf excision which could complicate the interpretation of the results (McKee, 1951; Ranjan and Laloraya, 1960).

Barley plants were grown in a 16 hour photoperiod for 12 days at 20°C and an irradiance of 300 μE $m^{-2}s^{-1}$. They were then exposed to 48 hours continuous light or continuous darkness, at the end of which the first leaves were excised, cut into 1.5 cm segments and floated in a large petri dish (15 cm) containing water. Sample of ten segments per treatment were selected at random from these pools

and floated on 10 ml of -15 bar PEG in 9 cm petri dishes for 24 hours in either light or darkness. The two treatments thus consisted of tissue exposed to light during both period 1 and period 2 (L-L), and tissue maintained in darkness during period 1 and period 2 (D-D) (Fig 1). At the end of the treatment, the segments were quickly rinsed in water (to remove adhering PEG) dried on filter paper and enclosed in glass vials. The tissue water potential was then measured using a spanner thermocouple psychrometer (as described in Materials and Methods Section 2.6.1). Five replicates of each treatment were taken; and a student's T-test was used to test for significance.

The water potential of both treatments was lower than that of the PEG solution (-15 bar) and the water potentials of the tissue in light or in darkness were not significantly different (-17.8 and -19.1 bars respectively).

It can be concluded that the effect of light on water potential is eliminated in this system. Neither the presence of PEG solution or the washing are likely to have influenced this result as the water potential of the leaf tissue was lower than that of the PEG solution used for incubation.

2. Proline accumulation in segments with either the upper epidermis or the lower epidermis exposed to light

As the stomatal resistance of the upper and lower epidermis differed (Section 3.3.1), it was considered important to establish whether this difference (which would also influence photosynthetic rate) influenced proline accumulation in the excised segment system where only one surface was available for gas exchange and the other was in contact with the treatment solution. As the effect of exposing

Fig 1

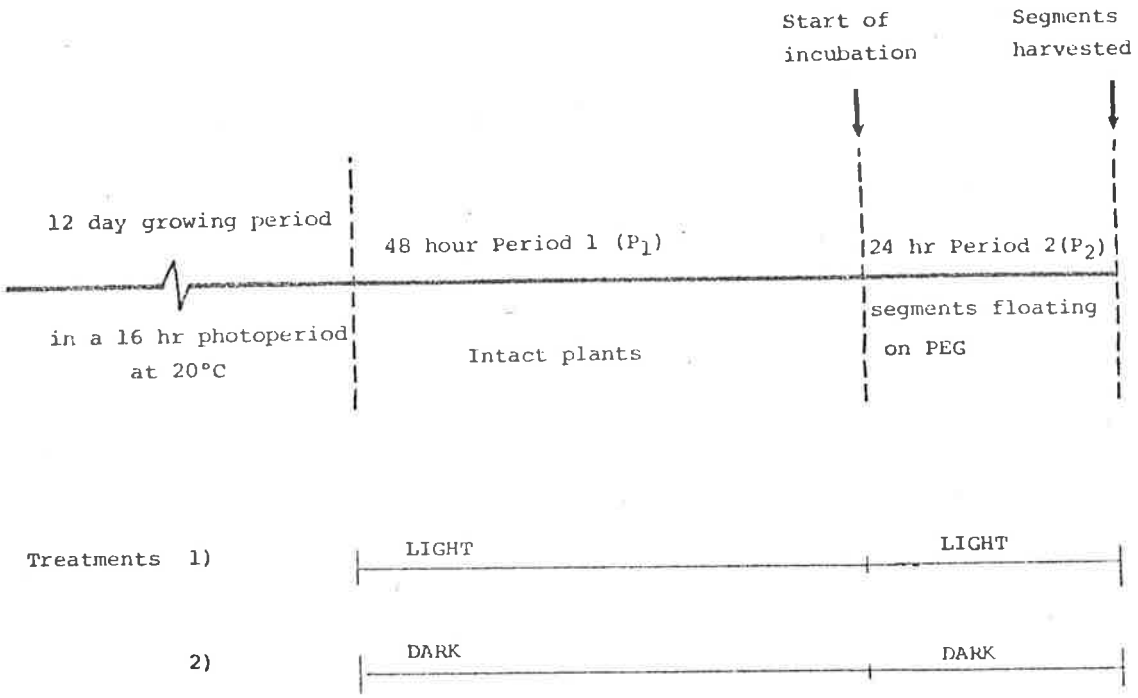



Table 1 Effect of exposing upper epidermis or lower epidermis of segments to light, on proline accumulation

| | Proline (mg $g^{-1}$ dry wt) | |
|---|---|---|
| Treatment | L-L | D-L |
| Upper epidermis | 19.8 | 14.8 |
| Lower epidermis | 20.4 | 15.3 |
| Effect of treatment | n.s. | n.s. |

different surfaces to light was being studied, only light treatments were considered.

Plants were treated as described in Appendix II.1, until they were 12 days old. The period 1 treatment consisted of 48 hours continuous light or darkness given to intact plants, while in the period 2 treatment, all the segments were exposed to light (Fig 4). The segments were floated on PEG (-15 bar) with either the upper or lower epidermis exposed to light during period 2; at the end of which they were harvested, rinsed in water, dried and frozen in liquid nitrogen. The tissue was lyophilized, weighed and analysed for proline (Materials and Methods 2.8.1). Four replicates of each treatment were taken; and significance was tested using the student T-test.

It is evident from the results that the proline accumulated by segments with their upper epidermis exposed was not significantly different from that of segments with their lower epidermis exposed to light during the stress treatment (Table 2). It can be concluded that floating segments on PEG with either surface exposed to light has no significant effect on the proline content.

3. Proline accumulation in stressed segments floated on PEG solution in closed systems

It was necessary to incubate segments in a closed system during the course of experimentation to measure gas exchange, feed with $^{14}CO_2$, etc. and a preliminary experiment was undertaken to test that proline accumulation was not limited by the volume of air for gas exchange in such a system.

The conditions of growth were identical to Appendix II.1. Plants were given 48 hours light or darkness during period 1, then

the first leaves were excised and the segments floated on -15 bar PEG in the light for 24 hours (Fig 2). Segments were floated in petri dishes, sealed petri dishes (sealed by applying vacuum grease along the rim of the lower dish) or in 125 ml conical flasks sealed with rubber stoppers. Four replicates of each treatment were taken At the end of the period 2, the segments were harvested, and treated in a similar manner as in Appendix II.1. A student's T-test was performed on the results, to test for significant differences between treatments.

Proline accumulated to a similar level in all three systems in both L-L and D-L segments (Table 2). It is clear from the results that the volume of air in a sealed petri dish or 125 ml flask did not limit proline accumulation within 24 hours.

The system for imposing water stress on excised segments, by floating them on PEG solution, appears to be appropriate for detailed experiments on water stress. It is evident that the effect of light on the water potential of tissue, seen in intact plants, has been eliminated. In addition, it has been demonstrated that leaf segments can be floated with either surface exposed to light, and that experiments can be performed in an enclosed atmosphere without affecting proline accumulation.

Fig 2

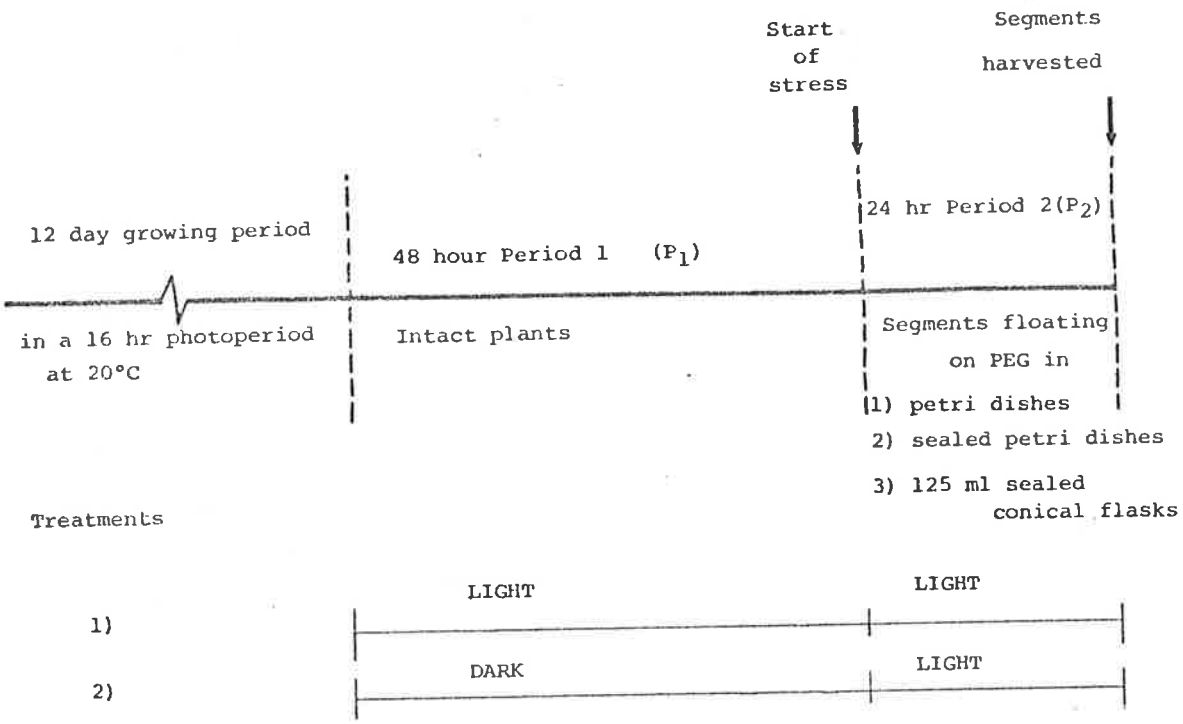



Table 2 Effect of closed systems on proline accumulation in barley leaf segments

| Treatment | Proline (mg $g^{-1}$ dry wt) L - L | D - L |
|---|---|---|
| Petri dish | 17.0 | 11.6 |
| Sealed petri dish | 17.4 | 13.1 |
| Sealed flask | 17.3 | 12.4 |
| Effect of treatment | n.s. | n.s. |

## APPENDIX III

Table 1

Effect of irradiance on amino acid content in D-L and L-L samples (mg $g^{-1}$ dry wt)

| | ARG | ASN | GLN | ASP | GLU | SER | ALA | PRO | GLY | TOTAL |
|---|---|---|---|---|---|---|---|---|---|---|
| L-L | | | | | | | | | | |
| 300 | 0.26 | 4.68 | 4.20 | 2.21 | 4.74 | 5.04 | 0.92 | 18.92 | 0.38 | 38.96 |
| 30 | 0.22 | 3.36 | 5.77 | 2.74 | 3.74 | 7.27 | 1.55 | 18.81 | 0.62 | 43.96 |
| D-L | | | | | | | | | | |
| 300 | 0.02 | 5.59 | 1.88 | 1.49 | 5.31 | 4.22 | 1.02 | 12.74 | 0.34 | 33.92 |
| 30 | 0.49 | 7.31 | 2.05 | 2.08 | 4.32 | 5.73 | 1.42 | 10.64 | 0.37 | 34.40 |
| V.R. | | | | | | | | | | |
| Irradiance (I) | 4.65 | 0.04 | 1.41 | 2.61 | 5.35* | 15.36** | 6.72* | 1.80 | 2.13 | 4.38 |
| L-L/D-L(L) | 0.02 | 6.34 | 17.03** | 3.95 | 1.80 | 6.19* | 0.01 | 76.09** | 2.34 | 31.09** |
| Interaction (LxI) | 6.33 | 2.46 | 0.93 | 0.01 | 0.00 | 0.56 | 0.36 | 1.48 | 1.35 | 2.98 |
| L.S.D. (LxI) | 0.14 | | | | | | | | | |

Significance level * $P \leq 0.05$

** $P \leq 0.01$

## APPENDIX III

Table 2

Effect of irradiance on radioactivity in amino acids when expressed as a percentage of the total $^{14}C$ in the sample

| | ARG | ASN | GLN | ASP | GLU | SER | ALA | PRO | GLY | TOTAL $^{14}C$ ($cpmx10^{-6}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| L-L | | | | | | | | | | |
| 300 | 0.64 | 1.02 | 1.02 | 1.26 | 1.71 | 1.65 | 0.87 | 6.11 | 0.20 | 1.431 |
| 30 | 0.84 | 0.94 | 1.37 | 1.31 | 1.13 | 2.48 | 0.81 | 3.60 | 0.51 | 1.172 |
| D-L | | | | | | | | | | |
| 300 | 0.67 | 2.87 | 1.08 | 0.93 | 2.00 | 3.59 | 0.72 | 2.55 | 0.50 | 1.372 |
| 30 | 0.83 | 2.33 | 0.56 | 1.22 | 1.95 | 2.55 | 1.04 | 3.56 | 0.32 | 1.148 |
| V.R. | | | | | | | | | | |
| Irradiance(I) | 16.80** | 2.79 | 0.05 | 1.43 | 1.30 | 0.06 | 0.67 | 6.36 | 1.32 | 42.79** |
| L-L/D-L(L) | 0.07 | 78.44** | 0.98 | 2.17 | 4.12 | 5.86 | 0.07 | 36.35** | 1.23 | 1.26 |
| Interaction (LxI) | 0.26 | 1.59 | 1.29 | 0.71 | 0.96 | 5.03 | 1.48 | 34.63** | 20.55** | 0.21 |
| L.S.D. Interaction | | | | | | | | 1.17 | 0.21 | |

Significance level * $P \leq 0.05$

** $P \leq 0.01$

# APPENDIX III

Table 3

Effect of irradiance on sugar content in D-L and L-L samples (mg $g^{-1}$ dry wt)

| Treatment | Glucose | Fructose | Sucrose |
|---|---|---|---|
| L-L | | | |
| 300 | 10.13 | 9.00 | 11.75 |
| 30 | 10.85 | 10.57 | 17.77 |
| D-L | | | |
| 300 | 12.99 | 9.17 | 9.31 |
| 30 | 14.28 | 11.21 | 10.88 |
| V.R. | | | |
| Irradiance(I) | 0.38 | 1.75 | 3.23 |
| L-L/D-L (L) | 3.76 | 0.08 | 4.89 |
| Interaction IxL) | 0.03 | 0.03 | 1.11 |

Table 4

Effect of irradiance on radioactivity in sugars when expressed as a percentage of the total $^{14}C$ incorporated in sample

| Treatment | Glucose | Fructose | Sucrose |
|---|---|---|---|
| L-L | | | |
| 300 | 3.2 | 2.7 | 4.1 |
| 30 | 5.7 | 3.7 | 11.9 |
| D-L | | | |
| 300 | 8.9 | 9.4 | 8.8 |
| 30 | 9.6 | 14.8 | 11.6 |
| V.R. | | | |
| Irradiance(I) | 0.30 | 18.86** | 9.08* |
| L-L/D-L(L) | 2.79 | 146.23** | 1.06 |
| Interaction(IxL) | 0.10 | 8.65 | 1.44 |

Significance level * $P \leq 0.05$
** $P \leq 0.01$

## APPENDIX III

Table 5

Radioactivity in amino acids as a percentage of the total counts in the sample in 0 and 7 hour fed samples

| | ARG | ASN | GLN | ASP | GLU | SER | ALA | PRO | GLY |
|---|---|---|---|---|---|---|---|---|---|
| L-L | | | | | | | | | |
| 0 | 0.64 | 1.02 | 1.02 | 1.26 | 1.71 | 1.65 | 0.87 | 6.11 | 0.20 |
| 7 | 0.94 | 0.94 | 1.49 | 1.42 | 1.98 | 2.58 | 0.73 | 6.20 | 0.28 |
| D-L | | | | | | | | | |
| 0 | 0.67 | 2.87 | 1.08 | 0.93 | 2.00 | 3.59 | 0.72 | 2.55 | 0.50 |
| 7 | 1.07 | 3.44 | 0.63 | 1.03 | 2.27 | 3.87 | 0.67 | 2.32 | 0.33 |
| V.R. | | | | | | | | | |
| Time(E) | 34.56** | 0.66 | 0.00 | 0.53 | 0.81 | 6.67 | 0.38 | 0.20 | 1.55 |
| L-L/D-L (L) | 1.98 | 51.87** | 1.01 | 3.96 | 0.90 | 46.70** | 0.46 | 510.12** | 24.18** |
| Interaction (TxL) | 0.72 | 1.16 | 1.41 | 0.02 | 0.00 | 1.9 | 0.06 | 0.92 | 12.79** |
| L.S.D | | | | | | | | | 0.14 |

Significance level * $P \leq 0.05$
**$P \leq 0.01$

# APPENDIX III

Table 6

Radioactivity in sugars as a percentage of the total counts in the samples exposed to $^{14}CO_2$ after 0 or 7 hours after imposition of stress

| Treatment | | Glucose | Fructose | Sucrose |
|---|---|---|---|---|
| L-L | 0 | 3.16 | 2.67 | 4.13 |
| | 7 | 5.13 | 4.18 | 10.12 |
| D-L | 0 | 8.91 | 9.44 | 8.25 |
| | 7 | 14.10 | 12.91 | 10.36 |
| V.R. | | | | |
| Time (T) | | 7.73* | 16.30** | 11.61** |
| L-L/D-L (L) | | 32.70** | 158.35** | 3.37 |
| Interaction (TxL) | | 1.56 | 2.52 | 2.67 |

Significance level * $P \leq 0.05$

** $P \leq 0.01$

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