THE ACCUMULATION AND METABOLISM OF GLYCINEBETAINE BY BARLEY IN RELATION TO WATER STRESS

By

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ABSTRACT

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The objectives of this research were: (1) To test the hypothesis that in barley the betaine level of shoot organs may be an index of the stress history of the plant; this could have application in crop breeding and management; (2) To survey genetic diversity for betaine levels in Hordeum vulgare and H. spontaneum to determine if physiological-genetic studies to evaluate the purported adaptive role of betaine accumulation would be feasible; (3) To seek evidence bearing on the adaptive value of betaine accumulation.

Radiochemical tracer experiments and chemical analyses with both laboratory- and field-grown plants demonstrated that: (1) Betaine was a metabolic end-product accumulated during water-stress by young leaves as a result of in situ synthesis and translocation via the phloem; (2) Betaine levels in the uppermost leaves were significantly correlated with an integrated value, based on water potential measurements, of the seasonal plant water stress, irrespective of plant N-status; (3) During grain filling, betaine partitioned neither like total-nitrogen nor like dry matter and remained predominantly in

vegetative tissue. Although these findings support use of betaine level in the uppermost leaves as a stress index, this use cannot be recommended because: (a) The difference in betaine level between irrigated and non-irrigated crops was quite small and variable; (b) Betaine accumulation occurred no earlier than readily-detected morphological changes.

Genetic variability for betaine accumulation was found among genotypes in laboratory trials with seedlings and in field studies with mature plants. The betaine level of the uppermost leaves of non-irrigated, mature, field-grown plants was significantly correlated with the betaine level of laboratory-grown, well-watered seedlings. Apparently, in field-grown plants betaine levels were not related to values of solute potential measured when the leaves were harvested.

Seedlings fed betaine via the root showed slightly reduced leaf area; this probably explained the slowed rate of soil-water depletion and retarded wilting of betaine-treated plants during water stress. High internal levels of betaine in well-watered seedlings depressed [2-^{14}C]ethanolamine incorporation into both betaine and phosphatidyl choline, suggesting that betaine synthesis is subject to retroinhibition.

Results from autoradiographic studies at the light microscope level indicated that in stressed-rewatered seedlings, betaine was distributed equally between vacuolar and cytoplasmic compartments.

DEDICATION

Oats, peas, beans and barley grow
Oats, peas, beans, and barley grow
Will you or I or anyone know
How oats, peas, beans and barley grow?

Anonymous

To Midge and Syd Ladyman

and to my sister, Tonia

ACKNOWLEDGMENTS

To thank and express my appreciation to all the people who have helped me throughout my graduate career at M.S.U. would double the length of this dissertation. Especial thanks go to the faculty, post-docs, and graduate students of the D.O.E. Plant Research Laboratory for their advice, assistance and moral support. Particular thanks goes to Ms. Kimberly Ditz, not only for her considerable technical assistance in screening genotypes of barley for betaine levels, but also for her friendship and support. I thank my parents, and my sister Tonia, for their unflagging interest in all I do, and for their assistance and understanding, especially while on "vacation" with me in Michigan.

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TABLE OF CONTENTS

Chapter

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ABA	Abscisic acid
CCC	(2-chloroethyl)trimethyl ammonium chloride
Ci	Curie
DAP	Days after planting
GLC	Gas-liquid chromatography
G-M	Geiger-Müller
h	Hour
min	Minute
PC	Phosphatidyl choline
TLC	Thin-layer chromatography
TLE	Thin-layer electrophoresis
U.S.D.A.	United States Department of Agriculture
wt	Weight
ψ	Water potential
ψ_s	Solute potential

INTRODUCTION

Nomenclature, Properties, and Natural Occurrence of Betaine

Onium compounds have been defined as "substances that are formed by an addition reaction, in the course of which some atom increases its valence by one unit, and in doing so, increases its formal charge algebraically by one unit" (Wheeland, 1953). This broad definition includes aliphatic quaternary ammonium compounds, e.g., choline and fully N-methylated amino acids (classed as "betaines"), heterocyclic compounds that contain a quaternary nitrogen in the ring structure, e.g., thiamine and the curare alkaloids, and sulphonium compounds, e.g., S-adenosyl methionine and dimethylpropiothetin (Cantoni, 1960). Glycine betaine, or N,N,N trimethylglycine $[(CH_3)_3{}^{+}NCH_2COO^{-}]$, is structurally the simplest of the class of aliphatic compounds termed betaines and in biological literature, is generally referred to as betaine.

Betaine is highly water soluble (160 g/100 ml) and sparingly soluble in ether (Stecher, 1968). At 0.2 molar concentration, betaine has an activity coefficient (γ) close to unity (γ = 1.07); γ increases nearly 2-fold with a 10-fold increase in concentration (γ = 1.95 at 2 molar concentration) (Smith and Smith, 1940). Betaine is excluded from the hydration water of some polymers (London et al., 1967) including proteins (Wyn Jones and Pollard, 1981). Betaine is a

zwitterion with only one titratable group, the carboxyl, which has a pk_a of 1.85 at 25°C; the positive charge on the N is fixed (Wyn Jones and Storey, 1981). Hence, at physiological pH betaine has no net charge and no buffering capacity.

Betaine is widely distributed throughout the animal and plant kingdoms. Betaine has been reported in bacteria (Schieh, 1965), fungi (Küng, 1914), crustaceans (Carr, 1978), molluscs (Yasumoto et al., 1978), pisces (Carr, 1978; Bowlus and Somero, 1979), and terrestial vertebrates (Cantoni, 1960; Greenberg, 1969). In higher plants, betaine appears to be present in significant amounts (>10 μmol/g dry wt) only in certain taxa; thus it is found in vegetative tissues among at least some members of the Chenopodiaceae, Gramineae, Leguminosae, and Amaranthaceae families (Wyn Jones and Storey, 1981). Betaine has also been reported to be present in the juice of citrus fruits (family Rutaceae; Lewis, 1966).

In animals and micro-organisms considerable research has been conducted on the oxidation of choline to betaine (e.g., Mann and Quastel, 1937; Mann et al., 1938; Williams, 1952; Schieh, 1965; Tyler, 1977). The intermediate of the oxidation, betaine aldehyde, is quite labile and does not usually accumulate *in vivo* (De Ridder and Van Dam, 1973). In animals and micro-organisms choline is derived by sequential N-methylations of ethanolamine, the product of serine decarboxylation. The methylation steps involve both *de novo* synthesis of a methyl group from one-carbon precursors (Sakami and Welch, 1950) and transfer of a methyl group from a methyl donor

compound (Stekol et al., 1958). The decarboxylation and methylation steps probably occur when the bases are in the phospholipid-bound form in membranes (Kates and Marshall, 1975; Hanson and Hitz, 1982). Enzymes of the biosynthetic pathway of betaine are mainly membrane-bound (Williams, 1952; Van den Bosch, 1974), although a cytoplasmic betaine aldehyde oxidase has been implicated (Christiansen and Daniel, 1953; Rothschild and Guzman Barron, 1954).

In barley, beet and spinach plants, betaine is also synthesized via three sequential N-methylations of the decarboxylation product of serine rather than by methylation of glycine (Delwiche and Bregoff, 1958; Hanson and Nelsen, 1978; Hanson and Scott, 1980). In contrast to animal and microbial systems, in barley, sugar beet, and spinach (Hitz et al., 1981; Coughlan and Wyn Jones, 1982; Hanson and Wyse, 1982) water-soluble intermediates (perhaps phosphoryl base derivatives) appear to be involved in betaine biosynthesis. In sugar beet, phosphorylcholine is the direct precursor of the choline destined for oxidation to betaine (Hanson and Wyse, 1982). In barley, release of free choline from phosphorylcholine is indirect, via phosphatidyl choline (Hitz et al., 1981). It is not yet known whether physiological significance attaches to the difference between the two pathways.

Betaine can be metabolized by microbes and animals (Trier, 1931; Greenberg, 1969). In animals betaine can act as a donor of methyl groups (Du Vigneaud et al., 1946; Muntz, 1950; Greenberg, 1969; Tyler, 1977); betaine-homocysteine methyltransferases have been

demonstrated in the liver of a wide variety of vertebrates but not in plants or microbes (Ericson, 1960). A clinical application for the methylating capacity of betaine has been proposed in the treatment of benzene poisoning. After administration of betaine and choline to mammals that had been exposed to high levels of benzene, benzene levels in the blood were lowered as a result of benzene methylation to toluene, a less toxic hydrocarbon (Brair, 1977).

Physiological Functions of Betaine

Betaine can apparently serve several functions among animals and microbes. Betaine acts as a feeding stimulus to some crustaceans, amphibians (bullfrog), and fish (Carr, 1978; Takagi et al., 1978). In the cells of some marine invertebrates betaine is present in sufficient concentrations to be a significant osmoticum (Schoffeniels, 1976). In studies with marine invertebrates and elasmobranch fish betaine has been found to offset the perturbing effects of urea and inorganic ions, e.g., NaCl, on *in vitro* enzyme activity (Bowlus and Somero, 1979; Yancy and Somero, 1980). However, the concentration of betaine in the muscle tissue of elasmobranch fish has rarely been accurately measured; in shark, concentrations appear to be variable (11-100 mM) but under most circumstances the contribution of betaine to osmotic regulation seems unlikely to be large (Yancey and Somero, 1980). In moderately halophilic bacteria, exogenous betaine had a stabilizing effect on respiration, a membrane-associated process, in the presence of a high concentration of salt (Shkedy-Vinkler and Avi-Dor, 1975).

The functions of betaine in higher plants are not well understood. Early reports indicated that betaine had weak methyl donor activity in tobacco and barley (Scribney and Kirkwood, 1954; Byerrum et al., 1956) but these experiments were subject to artifacts arising from microbial contamination. More recent experiments have indicated that betaine is a metabolic end product in wheat and barley (Bowman and Rohringer, 1970; Ahmad and Wyn Jones, 1979).

Some halophytes contain high levels of betaine (> 100 μmol/g dry wt) which rise further in response to salinity (Storey and Wyn Jones, 1975). Betaine also accumulates in some mesophytes in response to both water and salt stress (Hanson and Nelsen, 1978; Wyn Jones and Storey, 1978). Because of the ecological link between high betaine levels and salt tolerance, an adaptive role for its presence and subsequent accumulation during stress has been proposed (Wyn Jones et al., 1977b). One possible function of betaine is that it acts as a cytoplasmic osmoticum (Wyn Jones et al., 1977a). The results from three types of experiments lend support to this hypothesis: (1) Betaine was found in low concentrations in intact vacuoles isolated from red beet roots (Wyn Jones et al., 1977a; Leigh et al., 1981); (2) In the leaves of Suaeda maritima, betaine was judged to be localized in the cytoplasm by a histochemical staining technique (Hall et al., 1978); (3) In vitro studies showed that relatively high concentrations (1 M) of betaine were not toxic--and, in the presence of salt were slightly protective--to cytoplasmic enzymes from plants as well as from animals (Pollard and Wyn Jones, 1979; Paleg et al., 1981).

Results from experiments in which exogenous betaine was fed to plants in order to study the role of betaine accumulation under stress have been inconclusive due, in part, to microbial contamination (Wyn Jones et al., 1974).

If genetic variability for betaine levels exists within or among inter-fertile species, physiological-genetic studies would be a means of determining if betaine accumulation was beneficial to plants. Correlating genotype performance of mesophytic crops under stress with a specific metabolic response has been attempted by Larqué-Saavedra and Wain (1976) and Quarrie (1980b) for abscisic acid accumulation, and by Richards (1978) and Hanson et al. (1977) for proline accumulation.

Use of Indicators of Water Stress in Plant Breeding

One aim of many breeding programs is to develop varieties that yield well under both adverse conditions such as water stress and favorable conditions. Instead of selecting for yield itself, various other criteria have been, or could potentially be, used in breeding programs to identify such stress-adapted genotypes (Moss et al., 1974). Criteria of this sort fall into two categories: Criteria based on characteristics believed to confer adaptation to stress, and criteria that are essentially stress symptoms. Characteristics with adaptive value would be selected for; those symptomatic of the stress would be selected against. The general concept of characters symptomatic of stress includes the specific cases of stress-induced phenological,

morphological, physiological and metabolic plant responses as indicators of the cumulative stress experienced by the plant. For example, one can envisage selecting against characters that indicate that the plant under drought had been unable to maintain a high internal water-status. Indeed, phenological, morphological, and physiological symptoms of plant stress have been successfully used as selection critera in this way (Hurd, 1974; O'Toole and Chang, 1978; Atsmon, 1979; Hall et al., 1979). The disadvantage with many phenological and morphological symptoms is that they are usually evident only after considerable stress has been experienced. Most physiological indicators of plant water stress (e.g. water potential, solute potential, and leaf diffusive resistance) are based on dynamic plant characteristics which at any instant in time are unlikely to represent an integrated value of the performance of the plant. Therefore, many time consuming measurements throughout the growing season must be made to gain accurate information in order to judge the overall fitness of a genotype to droughty environments.

Potential Use of Metabolic Responses to Stress in Plant Breeding

Selection procedures based on metabolic responses to stress are not currently used, mainly because plant metabolism under stress is poorly understood (Boyer and McPherson, 1975; Hanson, 1980; Quarrie, 1980a; Elmore and McMichael, 1981). The levels of two metabolites, namely proline and abscisic acid, that increase in response to water-stress have been proposed as being suitable indicators of the

fitness of a genotype to droughty environments. It was proposed, for wheat, that high levels of proline (Singh et al., 1972) but low levels of ABA (Quarrie, 1978) should be positive selection criteria. When using a metabolic, or in fact, any other indicator of plant water stress, an important consideration is that the selection criteria may be affected by environmental conditions that are unrelated to water stress; this would confound selection of any sort. For example, mineral stress in certain cases affects proline accumulation (Elmore and McMichael, 1981).

Like many physiological parameters, levels of proline and ABA are likely to be closely related only to the stress that the plant is experiencing at about the time of measurement. Because both proline and ABA are subject to rapid catabolism after relief of stress, the level of neither metabolite could reflect the cumulative internal stress experienced by a plant subjected to episodic stress (Zeevaart, 1980; Boggess et al., 1976a; Boggess et al., 1976b; Boggess and Stewart, 1976). Thus, an ideal metabolic index of cumulative stress would be one that was: (1) An accurate indication of the stress experienced prior to measurement; (2) Reproducible among gentoypes experiencing the same duration and severity of internal stress; (3) Unaffected by environmental conditions other than the specific one (viz. water availability) under investigation; (4) Readily measured.

Betaine Accumulation as an Index of Stress History

Because in wheat and barley, betaine appeared to be a metabolic end product (Bowman and Rohringer, 1970; Ahmad and Wyn Jones, 1979) which accumulated in response to water stress, Hanson and Nelsen (1978) proposed that the betaine content of a plant may reflect its internal-stress history and could thus be used as a cumulative stress index. Such an index could have applications in irrigation management as well as in plant breeding. In a plant-breeding context, selection could be practiced against high betaine levels. Note, however, that should betaine accumulation be found to be adaptive, then the advisability of breeding specifically either against or for high betaine accumulation should be re-evaluated.

The Objectives of the Research Reported in This Dissertation

This study of the metabolism, translocation, and genetic diversity of betaine accumulation in barley was undertaken in order to: (1) Test the hypothesis that the betaine concentration of certain plant organs could be used as an index of the cumulative stress experienced by the plant (Chapters I and II); (2) Determine whether a physiological-genetic study of the function of betaine accumulation under water-stress would be feasible (Chapter III); (3) Attempt to obtain evidence bearing on the postulated adaptive role of betaine accumulation by barley (Chapters IV and V).

CHAPTER I

THE TRANSLOCATION AND METABOLISM OF GLYCINEBETAINE BY BARLEY PLANTS GROWN IN CONTROLLED ENVIRONMENTS

1.1 Introduction

The betaine accumulation induced by water stress is a result of de novo synthesis from 1-C and 2-C fragments (Hanson and Nelsen, 1978; Hanson and Scott, 1980). Preliminary studies (Hanson et al., 1978) and two published reports (Ahmad and Wyn Jones, 1979; Bowman and Rohringer, 1970) indicated that betaine, once synthesized, was not further metabolized and was probably mobile within the plant. One conflicting report from Dekhuijzen and Vonk (1974) concluded that the [^{14}C]betaine synthesized in wheat from supplied [^{14}C]-(2-chloroethyl)trimethylammonium chloride (CCC) underwent degradation to glycine. However, in reaching that conclusion, the authors ignored a more likely route of ^{14}C entry to glycine, namely refixation in the light of the $^{14}CO_2$ generated from degradation of the labeled CCC.

This study of the metabolism and mobility of betaine within barley plants grown in controlled environments was the first step in determining whether the betaine concentration of either the whole shoot, or organs thereof, could be used as a cumulative index of the internal water stress experienced by the plant.

1.2 Materials and Methods

1.2.1 Plant Material and Stress Regimes

Seeds (caryopses) of spring barley (Hordeum vulgare L. cv. Proctor, CI 11806) were obtained originally from the U.S.D.A. Small Grains Collection, Beltsville, Md., and were subsequently multiplied each year in field plots at Michigan State University. Plants were grown to the three-leaf stage (17-22 days old) in plastic pots (12 cm high, 7 cm diameter) in either a soil mixture (peat:loam:sand, 1:1:2 v/v) or perlite (Chem Rock Corp., Nashville, Tenn.), in a controlled environment chamber under 16-h days (photosynthetically active radiation, 5 mW cm^{-2}; day/night temperature 22/16°C; day/night relative humidity 70/85%) (Tully et al., 1979). The plants (six per pot) were watered on alternate days with half-strength Hoagland's nutrient medium. One day before each experiment started, plants were routinely thinned to four per pot.

Mature plants (12-13 weeks old) used to investigate the translocation of betaine to the spike (Fig. 2B), were grown in 15 cm clay plots in a soil mixture (peat:loam:sand, 1:1:2 v/v) and thinned at the four-leaf stage to two per pot. They were grown under supplemental light (16-h day) in a greenhouse (day/night temperature, 22/16°C; relative humidity 70 to 80%). Plants were watered with half-strength Hoagland's solution daily.

Plants grown in perlite were osmotically stressed with a solution of polyethylene glycol (PEG) 6000 (Union Carbide Chemicals and Plastics Institute, W. Va.) in half-strength Hoagland's nutrient

medium, osmotic potential about -19 bars (Hanson et al., 1977). Plants grown in soil were stressed by withholding water for up to 3 days. In some experiments, stress was relieved by washing PEG from perlite as described by Hanson et al. (1977), or by rewatering soil. For the experiment of Figure 3 (page 25), soil-grown plants were subjected to two cycles of water withholding (both of 3 days) each followed by a 2-day period of daily watering. Control plants in this experiment were thinned to three per pot to reduce interplant competition which caused premature leaf senescence toward the end of the experiment when there were four plants per pot; the control plants were watered daily.

Shoot water potential (ψ) was measured on the second leaf blade with a pressure chamber (PMS Instrument Co., Corvallis, Or.). To reduce water loss as the measurement was being taken, the blade was wrapped in damp absorbent paper (Meiri et al., 1975; Ritchie, 1975). Leaf diffusive resistance was measured with a diffusion porometer (Model LI-65, Li-Cor Inc., Lincoln, Nb.).

1.2.2 Betaine Extraction and Determination

Whole shoots, or their component organs were freeze-dried and weighed. Tissue, having a dry weight greater than 30 mg was ground in a Thomas-Wiley intermediate mill to pass through mesh 40. Subsamples (15-20 mg) of the milled tissue, or whole organs (15-30 mg), were extracted with 5 ml of water at 100°C for 30 minutes and centrifuged to clear. The betaine in the clear supernatant was

determined either by: (1) pyrolysis-GLC after ion-exchange purification (Hitz and Hanson, 1980); or (2) separating the constituents of the supernatant by thin layer electrophoresis on pre-coated 0.1mm cellulose glass-backed plates (Cat. No. 5757; E. Merck, Darmstadt, FRG) with sodium tetraborate buffer (70 mM, pH 9.3) at 2 kV for 10 minutes. The betaine was identified on the plates by spraying with Dragendorff's reagent. Estimates of the amounts of betaine present in spots were made by comparing the size and intensity of the spots with a range of similarly prepared standards (range 5-30 μg). This semi-quantitative method was used for preliminary measurements of betaine content in meristematic tissue.

1.2.3 Labeled Compounds and Mode of Application to Plants

[6,6'(n)-^{3}H]Sucrose (15.5 Ci/mmol) and [U-^{14}C]sucrose (625 μCi/μmol) were purchased from Amersham Corp., Arlington Heights, Ill. [Methyl-^{14}C]Betaine of high specific activity was prepared from [methyl-^{14}C]choline (59 μCi/μmol; Amersham Corp.) by oxidation with acidified potassium permanganate solution, according to Lintzel and Fomin (1931). [Methyl-^{14}C]Betaine aldehyde of high specific activity was prepared from [methyl-^{14}C]choline (59 μCi/μmol) using fresh rat-liver mitochondria prepared as described by Williams (1952). (For details of synthesis, purification, and yields, see Appendix A).

For studies of metabolism using whole plants, [methyl-^{14}C]-betaine and [methyl-^{14}C]betaine aldehyde were used carrier free (i.e., at specific activity of 59 μCi/μmol). When investigating extracellular

oxidation of betaine aldehyde, 30 leaf disks (5 mm diameter, 0.2 g fresh wt) were incubated on a shaker at room temperature with [methyl-^{14}C]betaine aldehyde (0.2 µCi, 0.2 µCi/mmol) in potassium-phosphate buffer (20 mM, pH 7). Aliquots (20 µl) were taken from the incubation medium at 15-min intervals for the first hour, then at hourly intervals. At the end of 6 h the leaf disks were rinsed twice in potassium-phosphate buffer (1 ml), blotted dry and extracted in cold 0.5N formic acid (Hanson and Scott, 1980).

For translocation studies with [methyl-^{14}C]betaine, [methyl-^{14}C]choline, L-[U-^{14}C]glutamic acid (282 µCi/µmol; Amersham Corp.) and [U-^{14}C]sucrose, radiochemicals were diluted with sufficient unlabeled compound to give a total dose of 0.5 µmol/plant; specific activities ranged from 10 to 20 µCi/µmol.

For studies of both metabolism and translocation, labeled compounds were fed in a 5-µl droplet of 20 mM potassium-phosphate buffer (pH 7) to the second leaf blade (seedlings) 10 cm up from the ligule or to the flag leaf sheath (mature plants) 5 cm below the ligule through a carborundum-abraded spot of 5-6 mm diameter (Housley et al., 1977). After the droplet had been taken up, a second 5-µl droplet containing only buffer was applied. When the second droplet had been absorbed, the spot was covered with parafilm to minimize dessication of the abraded area.

Gaseous $^{14}CO_2$ was generated by mixing 20 µl $Na^{14}CO_3$ (10 µCi, 60 mCi/mmol; Amersham Corp.) with a 200-µl drop of lactic acid (43%, v/v) in a 10-ml syringe barrel with the plunger at the 10-ml mark. The total 10 ml volume of $^{14}CO_2$ was injected into the leaf-feeding

chamber. The chambers were glass tubes (1.5 cm diam x 10.5 cm) with a serum cap at one end; the top 10 cm of leaf blade was inserted into the other end which was then plugged around the leaf with cotton wool over which was stretched parafilm. The $^{14}CO_2$ was administered for 15 minutes.

1.2.4 Extraction and Separation of Labeled Metabolites

When [methyl-^{14}C]betaine was fed, extraction was by the methanol-chloroform-water (MCW) procedure described by Hanson et al. (1977). When [methyl-^{14}C]betaine aldehyde was fed, extraction was in cold 0.5 N formic acid, to minimize degradation of the [^{14}C]-betaine aldehyde (Hanson and Scott, 1980). The aqueous fraction of MCW extracts and the formic acid extracts were evaporated to dryness in a stream of N_2, redissolved in H_2O, and separated by the three-column ion-exchange procedure described by Hitz and Hanson (1980). Column fractions eluted from AG-50 and Bio-Rex 70 were further analyzed by thin-layer electrophoresis (TLE) and thin-layer chromatography (TLC). TLE systems were: on pre-coated 0.1 mm glass-backed cellulose plates (Cat. No. 5757; E. Merck) with either sodium tetraborate buffer (70 mM, pH 9.3) or formic acid (1.5 N) at 2 kV for 6-12 min; on "ITLC" plates (Gelman Instrument Co., Ann Arbor, Mi.) with formic acid (1.5 N) at 2 kV for 16 min. The TLC system was: pre-coated 0.25 mm Silica Gel-G plastic-backed plates (Brinkman Instruments, Westbury, N.Y.) developed for 90 min in methanol-acetone-conc. HCl (90:10:4 v/v).

1.2.5 Detection and Determination of Radioactivity

Labeled compounds separated by TLE and TLC were located by autoradiography using SB-5 X-ray film (Eastman-Kodak, Rochester, N.Y.); radioactive zones were eluted as described in Hanson and Tully (1979). Radioactivity in such eluates and in other soluble samples was determined by scintillation counting. Radioactivity in the insoluble fractions remaining after MCW extraction, and in freeze-dried samples of whole tissue, was also determined by scintillation counting after combustion in a Packard Tricarb sample oxidizer, model 306 (Packard Instrument Corp., Downers Grove, Ill.).

In the experiment of Table 1, in which [methyl-^{14}C]betaine was fed, plants were enclosed for 9 h in sealed 20-l glass tanks through which humidified air was passed at a flow rate of 5 l/h (Fig. 1). The enclosure was on the second day of the 3-day experimental period. After passage through the tanks, the gas streams were bubbled through traps for volatile bases (0.1 N HCl) and for CO_2 (Carbosorb II; Packard Instrument Corp.). The traps were checked for radioactivity after 9 h. A second CO_2-trap (saturated barium hydroxide) was included after the Carbosorb II; absence of any precipitate of $BaCO_3$ at the end of the experiment confirmed complete absorption of CO_2 by Carbosorb II.

1.2.6 Translocation of Labeled Compounds

These experiments were carried out under a bank of four 20-W fluorescent tubes (F20T12 Agro-Lite; Westinghouse, Pittsburgh, Pa.),

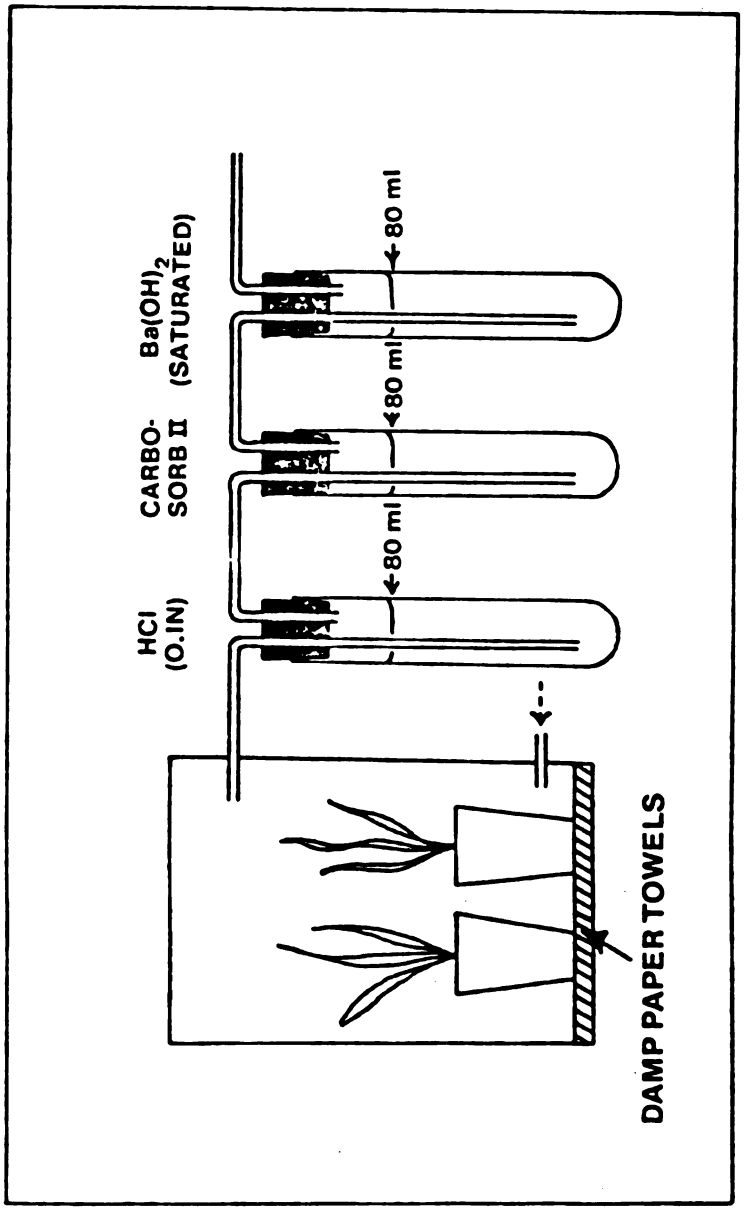


Fig. 1. Apparatus used to detect any evolution of $^{14}CO_2$ and volatile [^{14}C]bases by barley seedlings supplied with [methyl-^{14}C]betaine.

photosynthetic fluence about 9.9 mW cm^{-2} at plant level (PSI Model 65 Radiometer; Yellow Springs Instrument Co., Yellow Springs, Ohio). This relatively low light level was used to reduce further desiccation of the stressed plants during the experimental period. Both the amount of labeled compounds applied (0.5 μmol/leaf) and the abrasion method used to enhance their uptake into the leaf were based on techniques developed for studying translocation of sugars and amino acids (e.g., Housley et al., 1977; Yamaguchi and Islam, 1967). Before each experiment, diffusive resistance was measured on the fed leaf to confirm that stomata were open.

To follow translocation of labeled compounds, two Geiger-Müller (G-M) tubes were used. In studies with seedlings (Fig. 2A) a mask of aluminum sheet (thickness 0.2 mm) with a slot (20 x 5 mm) was placed on the second leaf blade centered 5 cm up from the ligule and another such mask was centered 5 cm down the sheath from the ligule (Hanson and Tully, 1979). In studies investigating the translocation of betaine to the spike (Fig. 2B), a mask similar to the ones used in seedling experiments was centered on the flag leaf sheath 1 cm above its node. Arrival of radioactivity was monitored at the masked-off 20-mm segments of both seedlings and mature plants with G-M tubes (Model D34; Dosimeter Corp., Ohio) and at the spike of the mature plant with a "pancake" G-M probe (Model HP-210; Eberline Instrument Co., Sante Fe, N.M.), the window of which covered the whole spike. The G-M tubes were connected to an Eberline radiation monitor which, in turn, was connected to a custom-made digital counter (Tully et al., 1979).

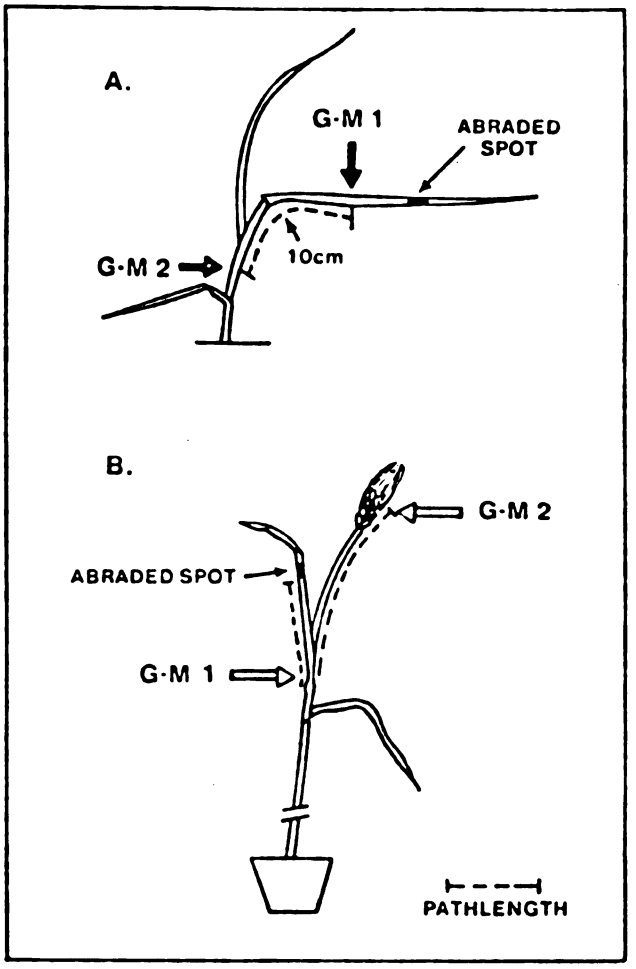


Fig. 2. Diagrams to show the translocation paths and the positions of Geiger-Müller (G-M) probes 1 and 2, for studies of the translocation of radioactively labeled compounds in seedlings (A) and mature plants (B). Arrival of ^{14}C-activity was monitored at G-M probes 1 and 2 after the radioactive compound had been administered.

At all G-M probes the front of ^{14}C-activity was taken to have arrived when the count was significantly ($p \leq 0.05$) higher than the long-term background count rate. The velocity of ^{14}C-label movement was calculated from the pathlength that separated the probes and the time the ^{14}C-front took to travel between the probes.

Leaves were heat-girdled by directing a stream of hot air (approx. 60°C) from a commercial hair dryer to a 1-cm section of sheath situated 2 cm below the ligule. The xylem remained intact because the blades remained fully turgid for at least 8 h after girdling.

For phloem exudation experiments, attached second leaves were fed with [^{14}C]substrates as above, and the second leaf sheath was then cut under water, 6 cm below the ligule, one h after the radioactive front had passed the second G-M tube. The cut end of the sheath was re-cut under water and then placed in a conical vial containing 200 µl ethylenediamine tetraacetate (EDTA) 5 mM, pH 7 (King and Zeevaart, 1974). The mouth of the vial was sealed around the sheath with parafilm to prevent evaporation of the collection medium. The EDTA was changed every 2 h. After 6 h the blade and sheath were separated, cut into 5-mm segments and washed in 1 mM unlabeled compound (betaine or betaine plus choline, respectively) for 5 minutes. The blade and sheath were then frozen in liquid nitrogen and extracted separately. Blade and sheath extracts and exudate were analyzed by TLE using glass-backed cellulose plates with sodium tetraborate buffer (70 mM, pH 9.3) at 2 kv for 9 min as described in Section 1.2.4.

1.3 Results

1.3.1 Betaine Levels During Stress and Rewatering Cycles

The betaine concentration in shoot tissue rose during the first and second stress episodes, and decreased during the rewatering periods (Fig. 3). The decline in betaine concentration upon rewatering was the consequence of the dilution by dry weight added as growth resumed, not of net degradation of betaine because the total betaine content per shoot did not decrease during the first rewatering period (Fig. 3 inset). Figure 4 confirms that the total quantity of betaine per shoot did not fall upon rewatering, and shows further that the distribution of betaine among shoot organs changed markedly upon stress relief. At the end of the stress period, most of the betaine (82% of the total, i.e., 5.0 μmol) was present in the mature leaves (1, 2, and 3). Two days after rewatering, only 2.7 μmol remained in these leaves, while the betaine contents of the expanding leaf 4, the spindle and the tillers had risen markedly (from a combined total of about 1.1 μmol to about 4.2 μmol). Analysis of shoots from well-watered plants which were 2 days younger than, but comparable in size to, the rewatered plants showed that the well-watered shoots contained only about one-fourth as much betaine; the distribution of betaine among shoot organs of well-watered plants was similar to that of stressed-rewatered plants. The stress-induced increase in betaine concentration was greatest in apices ($\leq$ 8 mm in length), for it rose about 6-fold, from about 17 μmol/g dry wt in well-watered plants to about 100 μmol/g dry wt in stressed seedlings.

Fig. 3. Betaine levels in shoots of soil-grown barley plants subjected to two cycles of stress-rewatering. Main Figure shows betaine concentration of stressed-rewatered (—●—) and well-watered control (---▲----) shoots during the 12-day experiment. Initial shoot dry weight (control and stressed plants): 148 mg. Final shoot dry weights: control plants, 620 mg; stressed plants, 330 mg. Values of shoot water potential (ψ) at end of each stress period are indicated. Inset shows betaine content of stressed-rewatered shoots on a per-shoot basis for the first 6 days only. Beyond 6 days betaine content increased further during the second period of water-withholding, and did not fall significantly upon rewatering at 9 days (not shown). The bar indicates the least significant difference (LSD) at the 5% probability level and is appropriate for comparing values vertically.

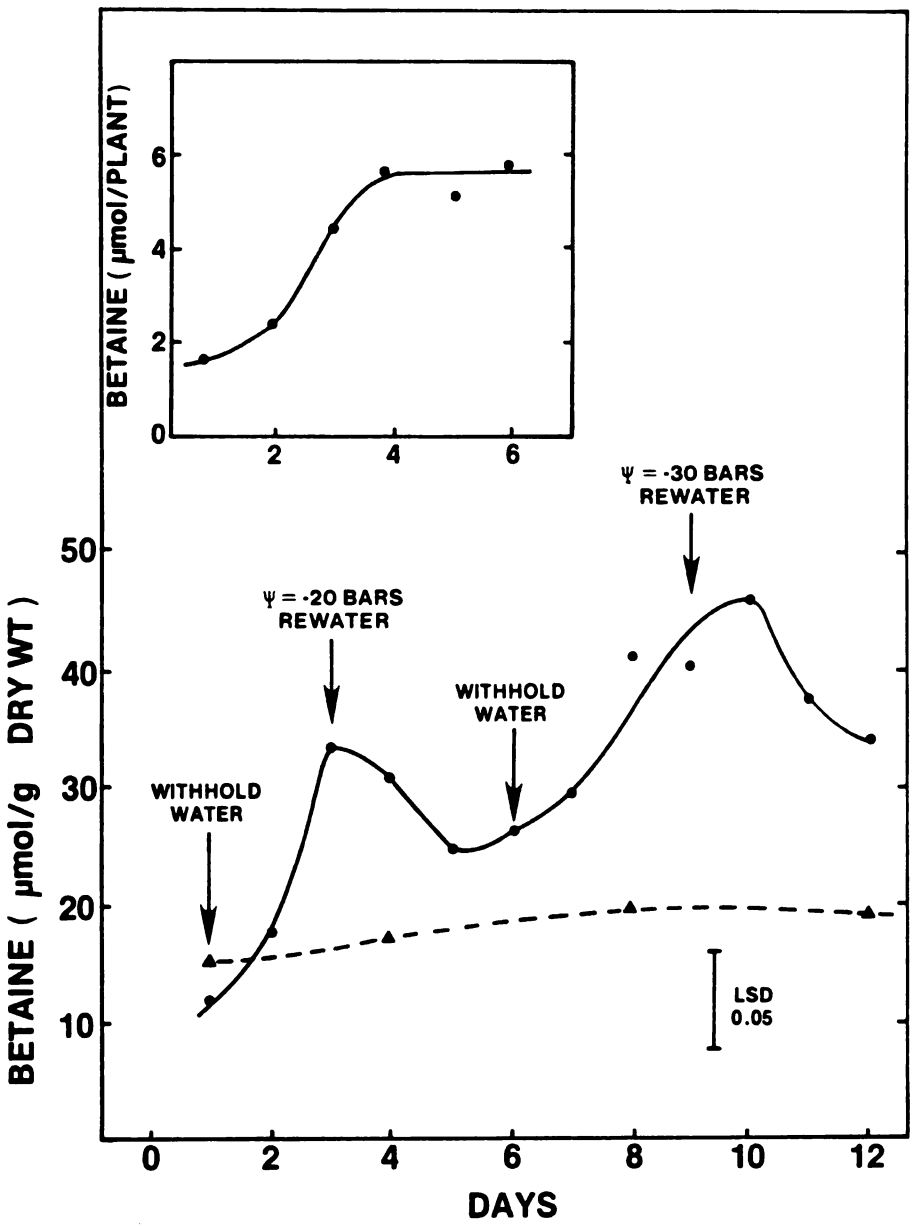
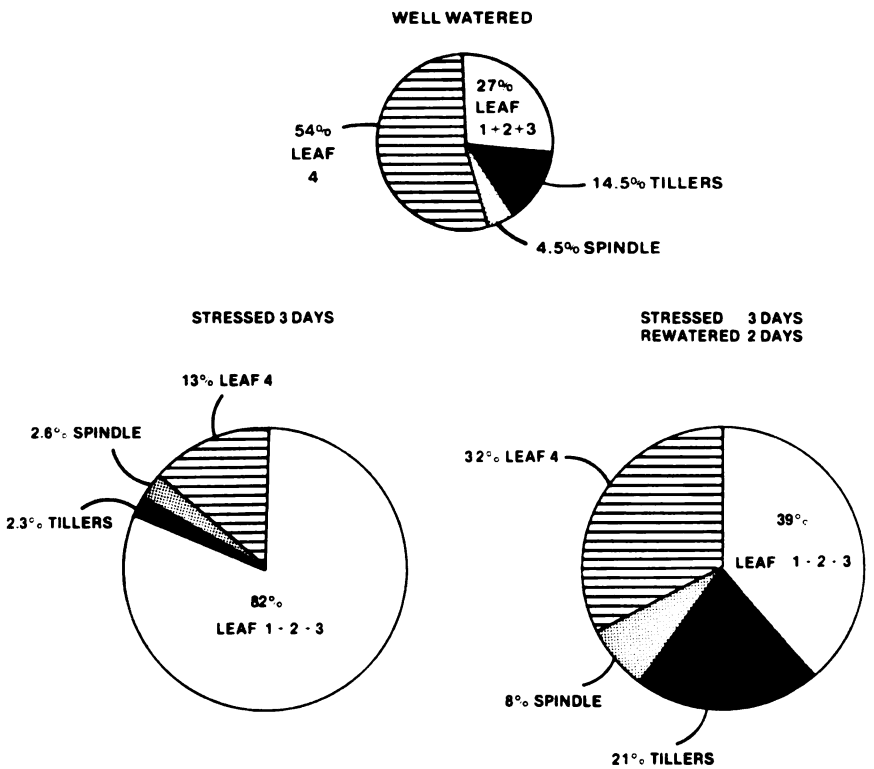

BETAINE (µmol/PLANT)
0
2
4
6
2
4
6
BETAINE (µmol/g DRY WT)
10
20
30
40
50
0
2
4
6
8
10
12
DAYS
WITHHOLD WATER
Ψ = -20 BARS REWATER
WITHHOLD WATER
Ψ = -30 BARS REWATER
LSD 0.05

Fig. 4. The distribution of betaine amongst various shoot organs in relation to water-stress history. The areas of the circles are proportional to the betaine content per plant. Sectors of the circles represent the distribution of betaine among organs; numbers in, or adjacent to, sectors give the percentage of total betaine present in each organ. The term "spindle" refers to the apical meristem plus leaf 5 and all younger leaves. Total betaine was 6.1 μmol per stressed plant, 6.9 μmol per rewatered plant and 2.6 μmol per well-watered plant. Average shoot dry weights: stressed plants, 126 mg; rewatered plants, 226 mg; well-watered plants 254 mg.


WELL WATERED
27% LEAF 1+2+3
54% LEAF 4
14.5% TILLERS
4.5% SPINDLE
STRESSED 3 DAYS
13% LEAF 4
2.6% SPINDLE
2.3% TILLERS
82% LEAF 1 - 2 - 3
STRESSED 3 DAYS
REWATERED 2 DAYS
32% LEAF 4
39% LEAF 1 - 2 - 3
8% SPINDLE
21% TILLERS

1.3.2 Fate of [Methyl-^{14}C]Betaine Applied to Mature Leaves

The results of the previous experiment indicated that upon rewatering, betaine is not retained by the mature leaves in which it accumulated. Betaine could either be transferred to the actively-growing regions of the shoot, without net destruction occurring, or it could be simultaneously degraded in mature leaves and synthesized in growing tissues upon stress relief. To decide between these alternatives, [methyl-^{14}C]betaine (8.5 nmol) was fed to the second leaf blade of plants given one of three different treatments (well-watered; continuously stressed; stressed and rewatered). Plants were then incubated for 3 days and dissected for analysis. Relative to the endogenous betaine contents of both well-watered and stressed second leaves (about 0.4 and 1.2 μmol, respectively) 8.5 nmol betaine is a tracer quantity.

The added radioactivity was recovered quantitatively from the various plant organs in all three treatments; release of $^{14}CO_2$ and ^{14}C-labeled volatile bases were negligible in all cases. The distribution of ^{14}C among the various plant organs is summarized in Table 1; the distribution pattern was consistent with the results of the previous experiment. The rewatered plants mobilized the added label extensively, and generally had larger amounts of ^{14}C in their actively growing regions than did the well-watered controls of the continuously stressed plants, which mobilized it least. In no case was the radioactivity recovered in the organs in any form other than betaine itself. There was thus no detectable metabolic

TABLE 1. Distribution of ^{14}C among organs of barley 3 days after supplying 8.5 nmol of [methyl-^{14}C]betaine (specific radioactivity 59 μCi/μmol) to the blade of the second leaf. Plants were grown in perlite; well-watered control plants were irrigated with nutrient solution daily before and after [^{14}C]betaine application. Continuously stressed plants were irrigated with polyethylene glycol 6000 (PEG) solution for 2 days before [^{14}C]betaine application and also thereafter; stressed-rewatered plants were irrigated with PEG solution for 2 days before [^{14}C]betaine application and rewatered with nutrient solution immediately following application of the [^{14}C]betaine. Mean recovery of ^{14}C was 97%, based on all three treatments (2 replicates per treatment).

Organ	Distribution (%) of ^{14}C Recovered		
	Well-watered Controls	Continuous Stress	Stress-rewater
Leaf 2, blade	61.8	97.5	38.5
Leaf 2, sheath	4.2	0.8	6.7
Leaf 1	1.3	0.06	1.6
Leaf 3	5.3	0.2	10.6
Leaf 4	14.3	0.1	16.9
Spindle[§]	2.0	0.6	2.3
Lower crown	0.6	0.6	0.3
Tillers	5.8	0.06	8.0
Roots	4.7	0.04	15.1

[§]Spindle = apical meristem + leaf 5 and all younger leaves.

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degradation of betaine over a 3-day period. The separatory system used would have permitted detection of the following possible metabolites of [methyl-^{14}C]betaine: trimethylamine, dimethylamine, monomethylamine, N,N-dimethylglycine, sarcosine, choline, betaine aldehyde, trimethylamine-N-oxide, phosphorylcholine, trigonelline, hordenine and gramine.

1.3.3 Phloem Translocation of [Methyl-^{14}C]Betaine

The patterns of endogenous betaine redistribution and [^{14}C]-betaine mobilization described above were consistent with transport of betaine in the phloem. To test this, four types of experiments were conducted with seedling plants.

In the first type, [methyl^{14}C]betaine (0.5 µmol, 10 µCi/µmol) was applied to second leaves of well-watered plants, plants stressed for 1 day only, and stressed-rewatered plants; the movement of ^{14}C to the sheath was then monitored (Fig. 5). Phloem translocation would be expected to be active not only in well-watered and rewatered plants, but also in the plants stressed for 1 day only, because Tully et al. (1979) showed that the movement of ^{14}C-photosynthate and nitrogenous compounds from the second leaf of barley seedlings did not cease until plants had been wilted for 2 days at which time leaf water potential had reached -18 bars. For all treatments, the [^{14}C]betaine front (B_1, B_2, Fig. 5) traversed the 10-cm distance between the 2 Geiger-Müller probes at about 0.25 cm/min (Fig. 5, Table 2). The count rate at each probe increased linearly over seven hours. Heat-girdling

Fig. 5. Representative data for translocation of applied [Methyl-^{14}C]betaine and [^{14}C]sucrose, fed in sequence, from blade to sheath in the second leaf of barley. Points B_1 and B_2 indicate times at which the [^{14}C]-betaine front was first detected at Geiger-Müller (G-M) probes 1 and 2, respectively; points S_1 and S_2 are when the [U-^{14}C]sucrose front was first detected. The respective rates of betaine and sucrose transport were calculated by dividing the path length (10 cm) by either (B_2-B_1) or (S_2-S_1). Broken lines depict the linear patterns of [^{14}C]betaine arrival found to continue for at least 7 h in similar experiments where [U-^{14}C]sucrose was not added. The amounts of [^{14}C]betaine and [^{14}C]-sucrose were 0.5 μmol/leaf, at specific activities of 10 μCi/μmol and 20 μCi/μmol, respectively.

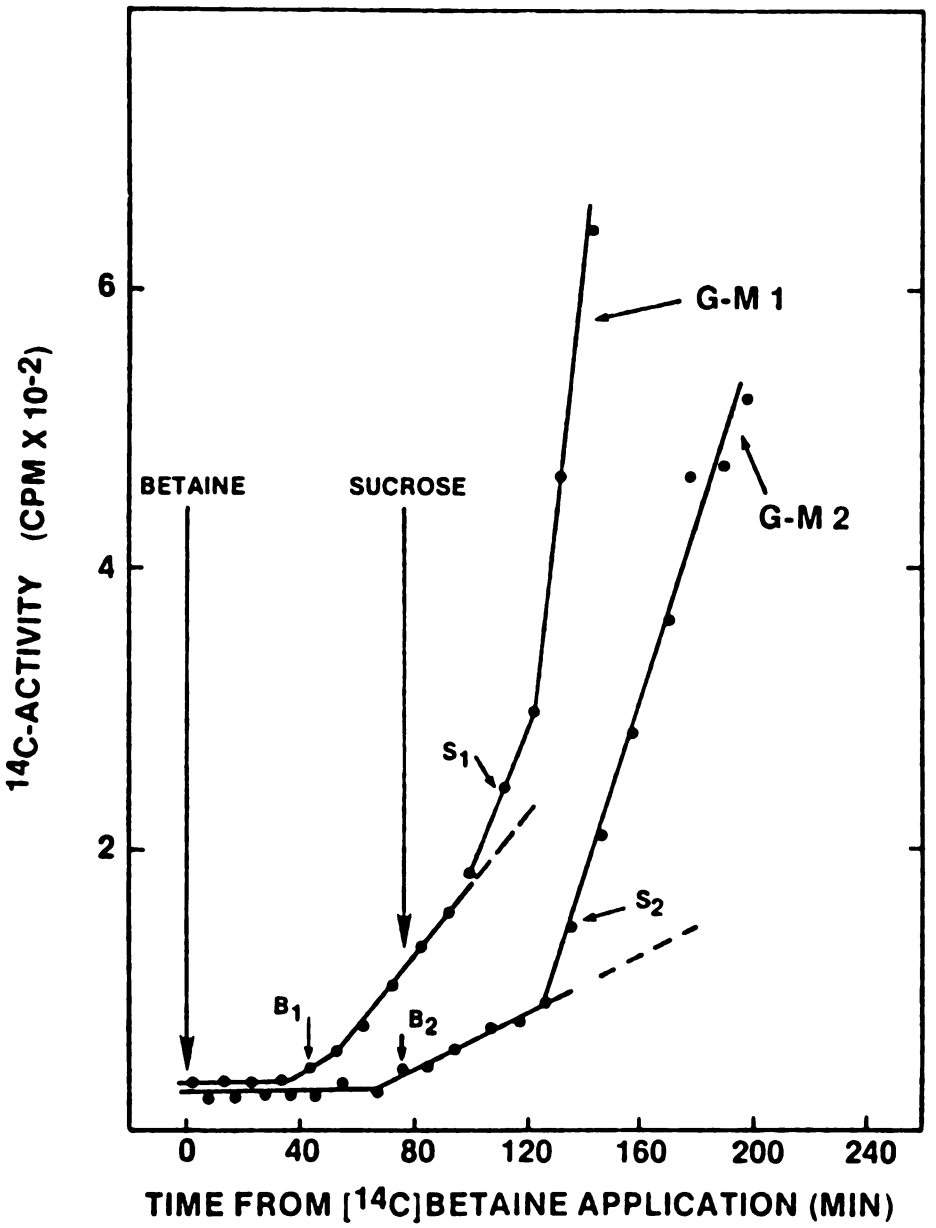

6
4
2
^{14}C-ACTIVITY (CPM X 10^{-2})
BETAINE
SUCROSE
G-M 1
G-M 2
S_1
S_2
B_1
B_2
0
40
80
120
160
200
240
TIME FROM [^{14}C]BETAINE APPLICATION (MIN)

TABLE 2. Translocation velocities for ^{14}C-labeled compounds applied to the second leaves of barley seedlings. [Methyl-^{14}C]-Betaine and [U-^{14}C]sucrose were applied to the second leaf blades, as shown in Figure 2. The values for well-watered plants are the means ± standard errors for 5 experiments. For stressed and rewatered plants, stress was imposed by polyethylene glycol 6000 in one pair of experiments, (+PEG), and by withholding water in the other ($-H_2O$).

Treatment	Methods of Stress Imposition	ψ bars	Velocity of ^{14}C transport (cm/min)	
			Betaine	Sucrose
Well-watered	--	-2	0.24±0.03	0.28±0.02
Stressed 1 day	+PEG	-10	0.19	0.22
	$-H_2O$	-9	0.27	0.25
Stressed 2 days-rewatered 1 day	+PEG	-2	0.27	0.29
	$-H_2O$	-2	0.31	0.35

the sheath completely prevented radioactivity reaching the lower probe, and caused ^{14}C to accumulate just above the girdle. [^{14}C]-Glutamate (0.5 μmol, 10 μCi/μmol), which is recognized as being phloem-mobile (Pate, 1976) was fed to a comparable heat-girdled plant. As in the case when [^{14}C]betaine was fed, radioactivity did not pass the girdle and accumulated immediately above it.

Although these data are consistent with phloem movement, the velocity is in the lower part of the typical range (see reviews by Canny, 1973, pp. 205-207; Wardlaw, 1974). To confirm that the observed velocity indeed reflected phloem transport, an "internal standard" of [U-^{14}C]sucrose was fed through the same abraded spot as soon as the [methyl-^{14}C]betaine reached the second probe. Arrival of the [^{14}C]sucrose at probes 1 and 2 was detected by sudden sharp increases in count rate (S_1, S_2 in Fig. 5). The velocities of sucrose movement between probes 1 and 2 estimated in this way were similar to those estimated for betaine (Table 2).

In the second type of translocation experiment, a mixture of [6,6'(n)-^{3}H]sucrose (0.5 μmol, 163 to 286 μCi/μmol) and [^{14}C]-betaine (0.5 μmol; 10 μCi/μmol) was applied to the second leaf blade of rewatered and well-watered plants. After a short (60-90 min) period to permit translocation, the $^{3}H/^{14}C$ ratio in the conducting path (sheath tissue) was determined. Both ^{3}H and ^{14}C activities had qualitatively similar distribution patterns down the sheath. The $^{3}H/^{14}C$ ratio on the upper part of the sheath was the same as that of the applied mixture (Table 3), although the ratio tended to become about 50% higher than that of the fed mixture in the lower

TABLE 3. Movement of applied [6,6'(n)-^{3}H]sucrose and [methyl-^{14}C]-betaine from blades to sheaths of the second leaves of soil-grown barley seedlings. A droplet of feeding solution containing both [^{3}H]sucrose (0.5 µmol) and [^{14}C]betaine (0.5 µmol) was applied to leaves of well-watered and stressed--rewatered plants. When radioactivity first arrived at a Geiger-Müller tube centered 2 cm below the ligule, a 4-cm section of sheath tissue (which included the tissue directly beneath the probe, plus that about 1 cm above and below it) was harvested. This section was cut into 5-mm pieces and freeze-dried; each piece was assayed for ^{3}H and ^{14}C after combustion.

Treatment	^{3}H/^{14}C Ratio	
	Feeding Solution	Sheath Tissue[§]
Well-watered	16.0	15.8±0.2
Stressed 2 days--rewatered 1 day	12.4	13.0±0.4
Stressed 2 days--rewatered 1 day	28.5	30.5±1.0

[§]Mean ± standard error for eight 5 mm pieces.

part of the sheath, i.e., as the leading edges of the radioactive fronts were approached (data not shown). These results are quite consistent with the transport of [^{14}C]betaine, like that of [^{3}H]-sucrose, in the phloem but indicate that applied sucrose may be loaded into the phloem more rapidly than applied betaine, at least in the first minutes after application.

In the third type of translocation experiment, [^{14}C]betaine was sought in phloem exudate. When [methyl-^{14}C]betaine (5 μCi, 10 μCi/μmol) was fed to the second leaf blade of a well-watered seedling, betaine was the only labeled compound found in either the exudate collected over 6 h, the blade or the sheath. The exudation rate was constant for 6 h (Fig. 6) which is consistent with the results of Figure 5. Transpiration reduced the volume of the exudation solution by 23% over the 6-h experiment period. From the data of Figure 6, a minimum estimate of the betaine exported from well-watered leaves is 17 pmol/h; it should be noted that this rate is only about 0.1% of the *in vivo* rate of net betaine export from the second leaf blade of stressed-rewatered seedlings (Fig. 4).

To confirm that endogenously-synthesized betaine, like exogenous betaine, could be loaded into and transported by the phloem, a fourth type of experiment was carried out, in which precursors of betaine were fed to leaf blades. [Methyl-^{14}C]Choline (0.5 μmol, 20 μCi/μmol) was fed to the second leaf blade of a well-watered seedling and the radioactive products in the exudate examined at the end of 5 h. As well as betaine, which accounted for 14% of the

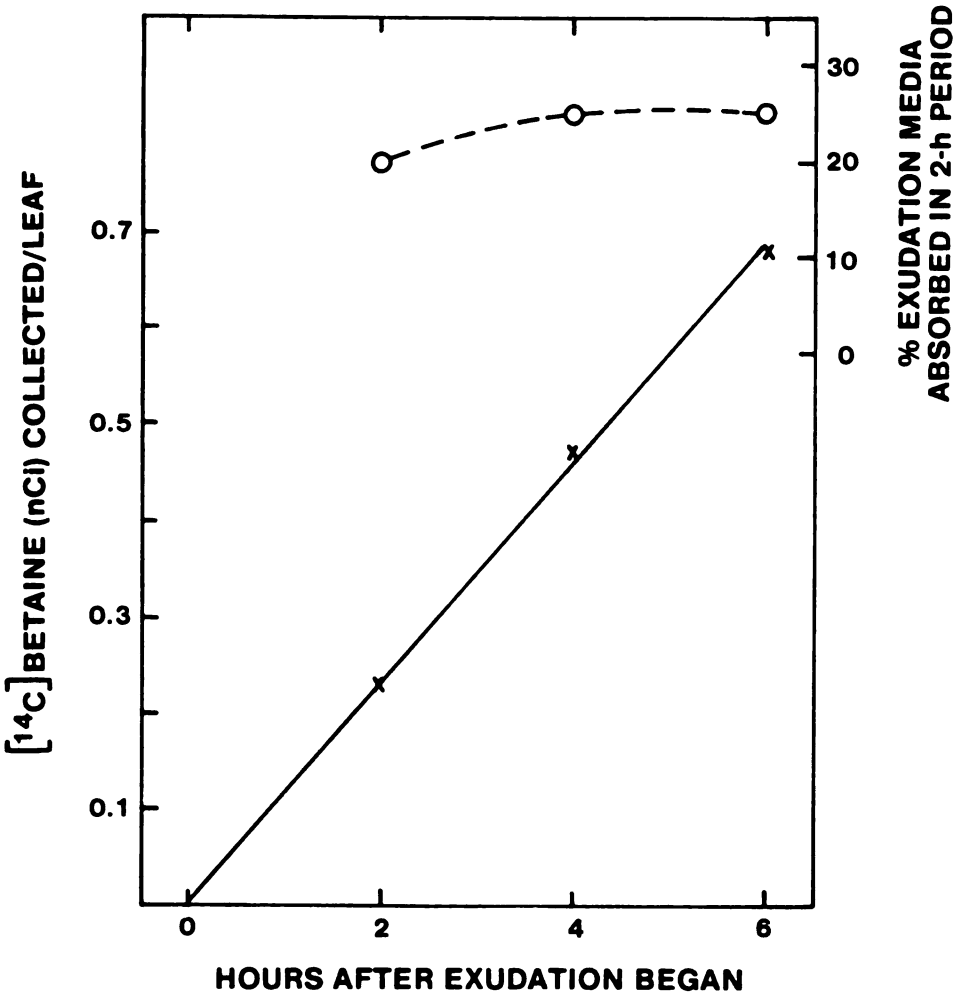


Fig. 6. Exudation of [^{14}C]betaine from the cut end of the second leaf of barley. [Methyl-^{14}C]Betaine (5 μmol; 10 μCi/μmol) was applied to an abraded spot on the second leaf blade about 2 h before exudation period began. Exudation medium was changed every 2 h. Inset graph describes the percentage of exudation media taken up by the plant during 2-h periods.

radioactivity in the exudate, choline, phosphorylcholine and an unidentified compound were labeled in the blade, sheath and exudate. The unidentified compound was not Dragendorff-positive and ran immediately behind betaine on the TLE plate. In a second precursor-feeding experiment, a tracer quantity (14 nmol) of the immediate precursor of betaine, [methyl-^{14}C]betaine aldehyde, was applied to second leaf blades of well-watered seedlings. When ^{14}C-activity was detected at the second probe (about 130 min after label application), the sheath was harvested, freeze-dried and extracted. Essentially all (>96%) of the ^{14}C was in the form of betaine.

1.3.4 Metabolism of [Methyl^{14}C]-Betaine Aldehyde by Leaf Disks

Because the preceding experiment, and those of Hanson and Scott (1980) showed that betaine aldehyde was oxidized to betaine very actively by leaf tissues, it seemed possible that betaine aldehyde was not oxidized intracellularly to betaine, but was a product of extracellular, nonspecific oxidase activity. To test this, leaf disks from the second leaf blade of a 21-day old seedling were incubated for 6 h with [methyl-^{14}C]betaine aldehyde (0.2 μCi, 0.2 μCi/mmol). Betaine aldehyde was the only ^{14}C-labeled compound found at the end of the experiment in the incubation medium, and in the buffer used to rinse the disks. Of the betaine aldehyde supplied, 44% was taken up by the leaf disks; of the ^{14}C taken up by disks, 32% was converted to betaine.

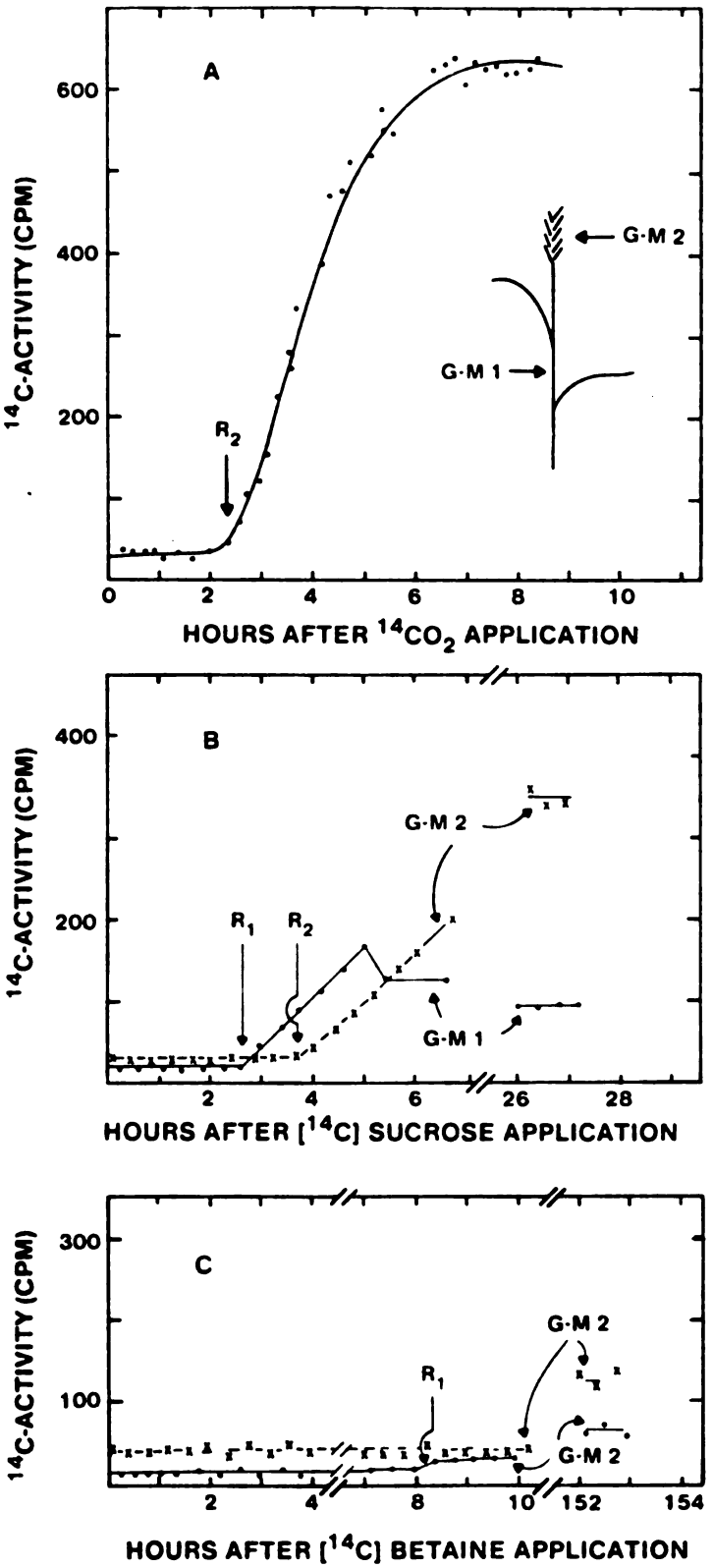
1.3.5 Translocation of ^{14}C-Labeled Compounds in Mature Plants

$^{14}CO_2$ (10 μCi), [U-^{14}C]sucrose (10 μCi, 20 μCi/μmol), and [methyl-^{14}C]betaine (10 μCi, 20 μCi/μmol) were administered to the flag leaves of well-watered plants of uniform size about 1 week after anthesis (Fig. 7). The experiment with $^{14}CO_2$ confirmed that the plants were assimilating CO_2 and transporting assimilates normally; the $^{14}CO_2$ assimilates from the flag leaf reached the spike after 2.2 h. When [U-^{14}C]sucrose was fed through an abraded area on the flag leaf sheath, the ^{14}C-activity reached the spike 3.9 h after application, and required 1.5 h to transverse the pathlength between probes 1 and 2 (Fig. 7B). Betaine was supplied to the flag leaf sheath in exactly the same manner as [^{14}C]sucrose but it was 6 days before measurable radioactivity reached the spike. The spike, flag leaf sheath, and a 4-cm segment of culm immediately below the spike were extracted in MCW and analyzed by TLE and TLC. Radioactivity was recovered only in betaine. Thus, under laboratory conditions, the spike did not appear to act as a sink for betaine in well-watered plants (Fig. 7C).

1.4 Discussion

During episodes of water stress betaine accumulated in expanding leaves of barley but was not appreciably degraded by these or any other organs before, during, or upon relief of stress. After relief of stress the betaine was exported from the expanded leaves to the young, actively growing regions of the shoot. This is similar

Fig. 7. Representative data for translocation of ^{14}C-labeled assimilates (A), [^{14}C]sucrose (B) and [^{14}C]betaine (C) from flag-leaf sheath to spike in mature barley plants. Inset in frame (A) describes position of Geiger-Müller (G-M) probes on plant. Points R_1 and R_2 indicate times at which radioactivity was first detected at G-M probes 1 and 2 respectively. When $^{14}CO_2$ was fed the output was monitored from G-M probe 2 only.


A
600
400
200
^{14}C-ACTIVITY (CPM)
G-M 2
G-M 1
R_2
0
2
4
6
8
10
HOURS AFTER $^{14}CO_2$ APPLICATION
B
400
200
^{14}C-ACTIVITY (CPM)
G-M 2
R_1
R_2
G-M 1
2
4
6
26
28
HOURS AFTER [^{14}C] SUCROSE APPLICATION
C
300
100
^{14}C-ACTIVITY (CPM)
G-M 2
R_1
G-M 2
2
4
8
10
152
154
HOURS AFTER [^{14}C] BETAINE APPLICATION

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to the behavior of another simple quaternary ammonium compound, CCC, which preferentially accumulates in the meristematic tissue of winter barley (Belzile et al., 1972). This observation raises the question: Has betaine, like many other natural and synthetic quaternary ammonium compounds, appreciable growth-regulating activity (Karanov, 1979)? This question is addressed in Chapter IV of this dissertation.

The export of betaine from older leaves most probably occurs in the phloem. In young vegetative barley plants the youngest leaves were the major sinks for both betaine and sucrose. However, although the spike readily imported supplied sucrose, it did not appear to be a dominant sink for betaine in well-watered, mature plants during grain-fill. In the mature plant the longer period of time to elapse between detection of measurable ^{14}C-activity in each G-M tube for [^{14}C]betaine as compared to [^{14}C]sucrose was probably due to [^{14}C]-betaine arriving at the spike in quantities below the threshold of detection, and not that betaine was translocated at a slower velocity than sucrose. Slow translocation of betaine to the spike is consistent with reports that wheat grains accumulate small quantities of betaine during development (Wyn Jones and Storey, 1981); the highest concentrations of betaine in wheat grains occur in the embryo and aleurone layer (Chittenden et al., 1978).

In this study the betaine concentration in the apices was higher than in any other organ of the water-stressed vegetative shoot. This observation may bear indirectly upon the subcellular compartmentation of betaine; Göring et al. (1978) suggested that

high concentrations of a metabolite in cytoplasm-rich cells, compared to its concentration in highly vacuolated cells, provides indirect evidence for cytoplasmic localization.

Two additional points emerge from the results of this study regarding the sites of synthesis and the translocation of betaine in well-watered plants. The first point: Betaine concentration in shoots of well-watered plants remained quite steady (at about 15 μmol/g dry wt) as dry weight increased about 3-fold (Fig. 3). Betaine must therefore be synthesized continuously during growth. This synthesis probably occurs at least in part in the mature leaves because mature leaves of well-watered plants can convert various ^{14}C-precursors to betaine at low rates (Hanson and Nelson, 1978; Hanson and Scott, 1980). The second point: Since the betaine content of individual mature leaves (e.g., blade of the second leaf) remains fairly constant (Hanson and Nelsen, 1978), any betaine synthesized in such leaves must be exported from them. The results contain some direct experimental support for such a continuous export of betaine from mature leaves of unstressed plants. Exogenously supplied [^{14}C]betaine, and betaine endogenously synthesized from either [^{14}C]betaine aldehyde or [^{14}C]choline, moved from the second leaves, apparently in the phloem. An element of spatial separation between sites of synthesis and accumulation is quite often shown with secondary plant products, which include nitrogenous bases like betaines (see discussions in Floss et al., 1974; McKey, 1974).

That betaine is both metabolically inert and phloem-mobile lends support to its promise as an indicator of the cumulative water

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stress experienced by a barley crop (Hanson and Nelsen, 1978). The most appropriate organs to sample for analysis in further tests of this concept appear to be the youngest leaves.

CHAPTER II

THE ACCUMULATION AND METABOLISM OF GLYCINEBETAINE IN FIELD-GROWN BARLEY

2.1 Introduction

Whereas considerable research has been conducted on the accumulation of betaine in barley plants grown in controlled environments (e.g., Hanson and Nelsen, 1978; Wyn Jones and Storey, 1978), no data are available on the accumulation of betaine in field-grown barley. Indeed, there are very few studies that include results on betaine synthesis and accumulation in any mesophytic plants in the field. Among these few studies are those on the relationship between betaine accumulation and rust infection in wheat (Bowman and Rohringer, 1969), and on the contribution of betaine to osmotic adjustment during water stress in tropical pasture crops (Ford and Wilson, 1981).

Laboratory work (Chapter I; Hanson and Nelsen, 1978; Ahmad and Wyn Jones, 1979) indicated that the betaine that accumulates during stress is not readily degraded upon stress relief. This observation led to the hypothesis that the betaine concentration in a plant may reflect its previous stress history (Hanson and Nelsen, 1978), and so serve as index of the cumulative internal stress suffered by the plant. Indices that distinguish the most adapted cultivars (in this context, those cultivars producing high yields under

stress) are much sought after as selection criteria in crop breeding programs (Fischer, 1970; Wilson, 1976). It is desirable that such indices be integrated measures or predictors of plant performance because many single parameters, for example, those that reflect water status, may differ between adapted cultivars that have adopted different strategies to cope with the same environment (Hanson and Nelsen, 1980). Numerous indices of overall adaptation to water stress have been proposed, but their practical value has generally not been adequately demonstrated (Petinov, 1965; Hall et al. 1979). In most cases measurement of proposed indices is very time consuming, e.g. measurements of average relative water content and related parameters (Dedio, 1975), transpiration productivity (Petinov, 1965) and stomatal function (Jones, 1980). Summarizing, the prospect of some plant character (measured either during or at the end of the growing season) that sums or integrates the internal stress experienced by the plant prior to measurement is a very attractive one. Hiler et al. (1972) introduced the concept of a cumulative stress index that was a "measure of the degree and duration of plant water deficit" when investigating the sensitivity of southern peas to plant water deficits. Similarly, an integrated stress index (IS) based on shoot water potential measurements, was recently applied to relate the depression of seed yield to the cumulative stress experienced in cowpea (Shouse et al., 1981).

Seedlings subjected to short-term water deficits under laboratory conditions do not necessarily respond to stress in the same way

as a mature plant growing in dryland conditions. Hence the objective of this research was to evaluate, under field conditions, the hypothesis that the betaine concentration in the shoot, or organs thereof, of barley is a reliable index of the cumulative internal stress experienced by the plant. To accept betaine concentration as such an index, the following criteria must be met: (1) Betaine concentration, at all stages of growth, must be positively and highly correlated with the cumulative stress experienced by the plant; (2) The difference in betaine concentration between water-stressed and irrigated crops should be evident before morphological symptoms of stress develop; (3) There should be no significant differences for betaine concentration between cultivars given the same degree of internal stress; (4) Differences in nitrogen (N) fertilization should not, per se, significantly alter the betaine concentration of the tissue.

The fourth consideration, that N-fertilization must not be critical, is important as soil-N fertility frequently varies across a field under normal environmental conditions and the concentrations of individual nitrogenous compounds are frequently affected by N-availability (Waller and Nowacki, 1978; Elmore and McMichael, 1981).

Thus the following questions were addressed in this study:

1. Is betaine metabolically inert in field-grown plants?
2. To what extent and in which organs does betaine accumulate?
3. At what time during the growing season does betaine accumulate?

4. Are there significant differences in betaine concentration between cultivars with the same stress history?
5. Is betaine accumulation significantly depressed by low soil-N?

2.2 Materials and Methods

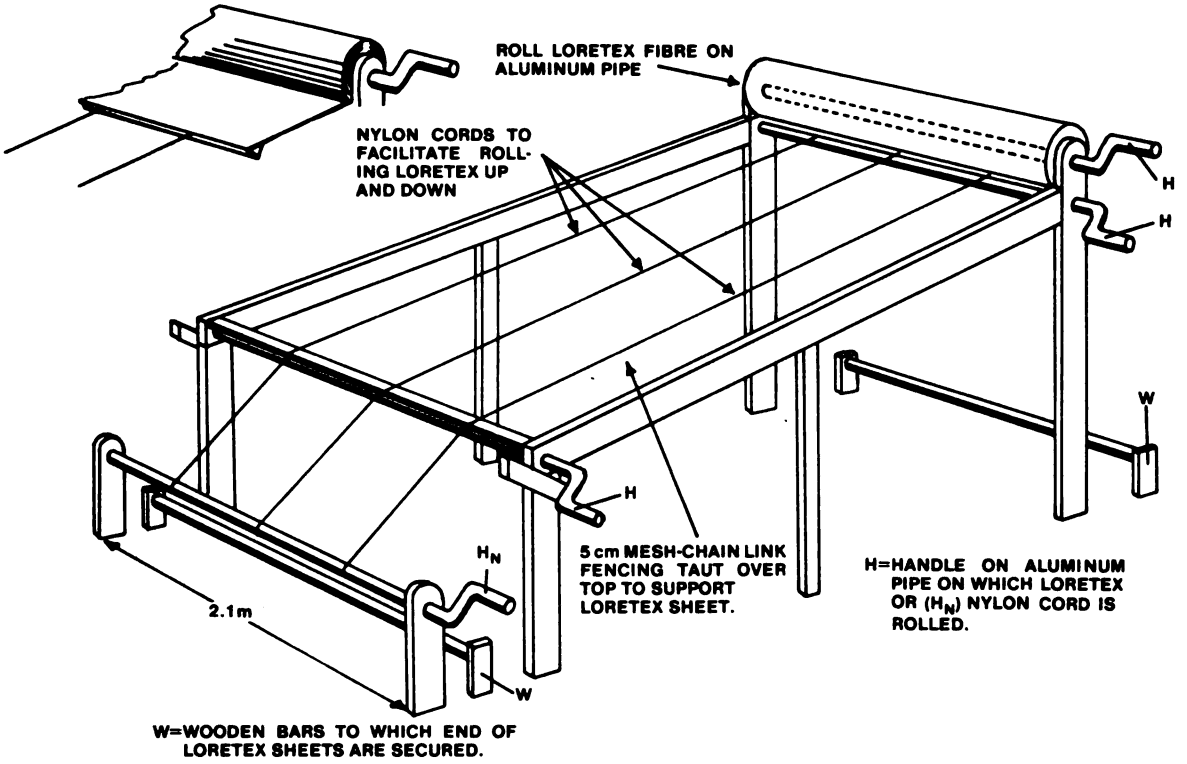
2.2.1 Plant Material

The spring barley cultivars "Proctor" CI 11806, "Bankuti Korai" CI 12972, and "Arimar" CI 13626 were used. Proctor was planted on April 13, 1979, April 17, 1980, and April 22, 1981; Bankuti Korai was planted on July 19, 1979, and Arimar on July 25, 1980. Seed was treated before planting with Vitavax-200 (2.5 g/kg; Uniroyal Chem., Naugatauk, Ct.).

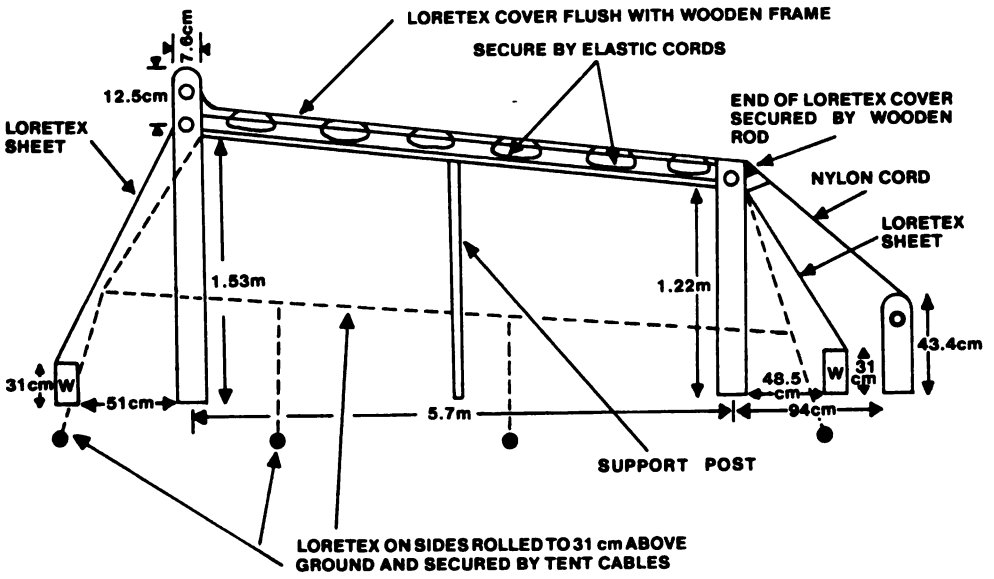
2.2.2 Field Plots

In 1979 and 1980, two plots (irrigated, I; non-irrigated, NI) and in 1980, also a third plot (water-stressed/rewatered; RW) were laid out; in 1981 two NI plots were laid out. In all three years the plots were on a Spinks sandy, mixed mesic soil (Psammentic Hapludalfs); plot size 2.1 m x 3.6 m in 1979, 2.1 x 5.7 m in 1980 and 1981. All plots were protected from rain by shelters that each comprised a permanent wood frame (average height about 1.4 m) equipped with movable sheets of clear, flexible plastic (Loretex, A. H. Hummert Seed Co., St. Louis, Mo.). The frames were covered with the Loretex sheets at night and during rain, and were uncovered at all other times. Figure 8 gives detailed specifications of the shelters; views of the shelters are shown in the photographs of Figure 9. At all

Fig. 8. Specification sketches of the manual rain-out shelters used in 1980. (A) 3-dimensional view; (B) side view. Inset of frame A describes the attachment of the nylon cord to Loretex sheet; at the free end of the sheet a wooden slat is affixed through which are drilled holes. The nylon cord is secured through each hole by a knot.


ROLL LORETEX FIBRE ON ALUMINUM PIPE
NYLON CORDS TO FACILITATE ROLLING LORETEX UP AND DOWN
H
H
W
H
H_N
5 cm MESH-CHAIN LINK FENCING TAUT OVER TOP TO SUPPORT LORETEX SHEET.
H=HANDLE ON ALUMINUM PIPE ON WHICH LORETEX OR (H_N) NYLON CORD IS ROLLED.
2.1m
W
W=WOODEN BARS TO WHICH END OF LORETEX SHEETS ARE SECURED.

A.


LORETEX COVER FLUSH WITH WOODEN FRAME
SECURE BY ELASTIC CORDS
7.6cm
12.5cm
LORETEX SHEET
END OF LORETEX COVER SECURED BY WOODEN ROD
NYLON CORD
LORETEX SHEET
1.53m
1.22m
43.4cm
31cm
W
51cm
5.7m
48.5 cm
W
31 cm
94cm
SUPPORT POST
LORETEX ON SIDES ROLLED TO 31 cm ABOVE GROUND AND SECURED BY TENT CABLES

B.

Fig. 9. Photographs of the rainout shelters in fine(A) (1980) and rainy (B) weather (1979). The side cover of the plot on the left (B) is rolled up for the photograph.

A

B

times, the sides and top of the shelters were covered by netting (mesh size = 2 cm) placed underneath the plastic to prevent crop damage by birds and mammals. On the west side of each shelter (not shown in Fig. 8) snow fence was erected as a precaution against storm damage. To ensure adequate ventilation at all times, the plastic covers were never secured nearer than 30 cm to the ground. Black plastic covered the shelters for 10 days after seeding at the second plantings of each year (Bankuti Korai and Arimar) to reduce heat injury on emergence.

Soil pH was measured before each planting and sufficient lime was routinely applied to raise soil pH to about 7.0. For the crops of Proctor (1979, 1980) and Bankuti Korai the plots beneath the shelters were divided in half, and each received a preplant fertilization of 56 kg P/ha and 52 kg K/ha. One half of each plot received 64 kg N/ha, the other half was not N-fertilized. The Arimar crop was not subdivided with respect to nitrogen fertilization and each plot received a preplant fertilization of 64 kg N/ha, 56 kg P/ha, and 52 kg K/ha. In 1981 preplant fertilization for the Proctor crop was 52 kg N/ha, 46 kg K/ha and 7 kg Mn/ha. Prior to fertilization, all plots received a preplant irrigation (at least 15 cm) to bring the soil to field capacity. Seed was planted (40 seeds/m) in 12 rows spaced 17.8 cm apart at rates calculated to give stands of 230 plants/m^2. In all plots, twin-wall drip-irrigation lines (4 mil, 10.2 cm outlet spacing; Chapin Watermatics Inc., Watertown, N.Y.) were laid between rows after emergence, and a single irrigation (about 1-2 cm water) was given. (For details of the irrigation

system, see Fig. 10.) Thereafter, N-I plots received either a single irrigation at about 5 weeks after planting (Proctor, 1979, 2 cm; Proctor, 1980, 5 cm) or no irrigation. I plots were irrigated once (1979) or twice (1980) per week, with sufficient water to replace calculated evapotranspiration losses. [Water lost from an open pan of water is closely related to evapotranspiration; when there is full plant cover evapotranspiration is about 85% of open pan evaporation (Vitosh, 1977). Evapotranspiration losses at all stages in the growing season were estimated by summing daily open pan evaporation values (cm lost/day; supplied by C. Vandenbrink, MSU, East Lansing, Mi.) between irrigation days and multiplying the total by 0.85.]

RW plots (Proctor) were irrigated at 50 and 68 (anthesis) days after planting (DAP) with 4.6 cm water; RW plots (Arimar) were given a single irrigation of 4.6 cm water at 41 DAP (anthesis). After 68 (Proctor) or 41 (Arimar) DAP, RW plots were irrigated as frequently as I plots.

Table 4 summarizes the pest control measures that were taken.

2.2.3 Sampling Times and Measurements Taken in the Field

Leaf water potential (ψ), leaf diffusive resistance (r), and leaf temperature (t), were measured weekly (1979) or twice weekly (1980) between noon and 3 p.m. E.S.T. on the blade of either the youngest mature leaf, or the flag-1 leaf. (The uppermost leaf of each mature tiller is termed the flag leaf; the leaf immediately below

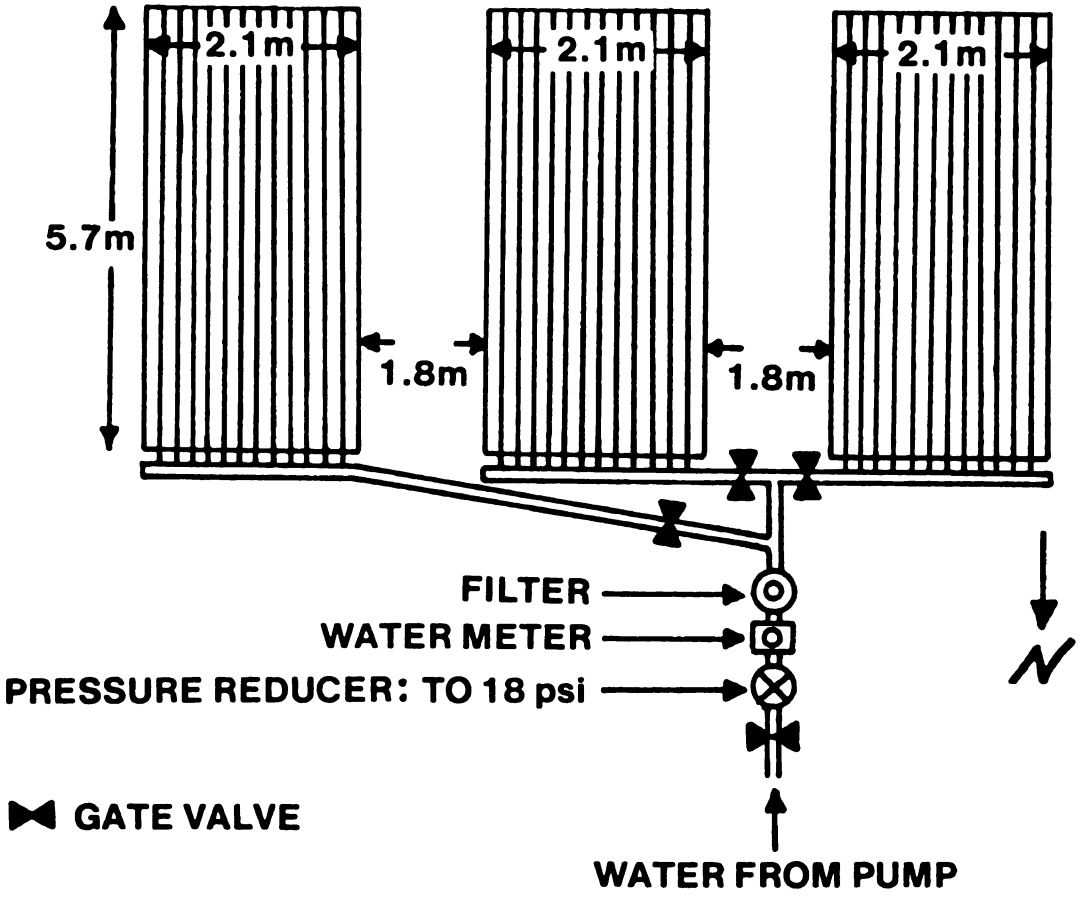


Fig. 10. Plan of twinwall trickle irrigation installation used in 1980.

TABLE 4. Approximate dates of appearance during 1979 of common pests of barley and control measures adopted.

Date of Outbreak[§]	Pest	Control Measure[†]
May 19 Jun 15 Aug 10	Aphids and/or Greenbugs	Orthene 75S (0.7 kg/ha)
May 27 Aug 15	Powdery mildew (Erysiphe graminis)	Benomyl (1.7 kg/ha)
Jun 15	Hessian fly (Mayetiola destructor)	Orthene 75S (0.7 kg/ha) [Especially directed into spindle]
Aug 30	Rust (Puccinia hordei)	Plantvax (2.3 kg/ha)

[§]These dates were approximately (±4 days) the same in 1980 and 1981.

[†]Sprayed at weekly intervals until the pest was no longer evident.

the flag leaf is referred to, in this dissertation, as the flag-1 leaf). Leaf water potential was measured with a pressure chamber (Model 1000 PMS; Instrument Co., Corvallis, Or.). Leaf diffusive resistance and leaf temperature were determined on the upper leaf surface with a Lambda Model LI-65 Autoporometer (Li-Cor Inst. Co., Lincoln, Nb.). [The Li-Cor diffusive resistance meter uses a lithium chloride coated sensor, partially enclosed by a plastic cup. When the sensor is placed on a leaf, electrical conductivity of the lithium chloride increases as water vapor, which diffuses from the leaf, is absorbed. The conductivity increases at a rate proportional to the rate of

diffusion of water vapor into the cup. The time (ΔT) taken for the increase in conductivity between 2 preselected values is recorded. At the same time, leaf temperature is measured by a bead-thermistor that is fixed between the LiCl sensor. The ΔT value is then converted to standard temperature (25°C) using standard conversion factors. The corrected value of ΔT, ΔT_{25C}, is used to calculate leaf diffusive resistance from a calibration curve of ΔT_{25C} vs. diffusive resistance (sec/cm).]

To confirm that the plants under the shelters behaved as a typical field crop, plant height and fertile tiller number/m^2 were measured during grain filling. Grain number/spike and grain weight were measured at harvest; grain yield was estimated as the product of the three yield components (Grafius, 1964).

In 1979 whole plants of Proctor were harvested at weekly intervals and divided into spindle, or spike, and remaining vegetative tissue, for determination of betaine content (and in some weeks, nitrogen content). At 70 DAP additional flag and flag-1 leaf blades were harvested and pooled for betaine and nitrogen determination. To confirm that water-stress induces betaine accumulation in Bankuti Korai the betaine in the pooled (flag plus flag-1) leaf blades was assayed at mid-grain fill.

In 1980 the two youngest mature leaf blades and the youngest organs (the spindle early in the growing season, the spike late in the growing season) were harvested at weekly intervals for betaine determination. At 71 DAP (Proctor) or 49 DAP (Arimar), when individual

spikes weighed more than 100 mg, they were subdivided; for each spike separate betaine determinations were made on the awns and remainder of the spike, which comprised the rachis, caryopses and glumes. At anthesis, anthers from Proctor were also collected for betaine determination. All samples were taken from the central 8 rows of the plots, or subplots.

2.2.4 Estimation of Cumulative Stress Experienced by Plants

The ψ of the blade of the youngest mature leaf, or flag-1 leaf, was used as the basis for calculating an index of integrated water stress (IS) as defined by Turk and Hall (1980). Measurements of ψ were made at weekly intervals in 1979, and every 3-4 days in 1980; ψ values were assumed to be representative for these periods. The integrated plant water stress (IS) for each treatment

$$IS = \int_{t_1}^{t_2} \psi \cdot dt$$

was estimated as the area above the ψ vs. time curve (Figs. 14, 16 and 17 lower frames) by a trapezoidal approximation method; "trend" lines were judged by eye. To give IS units of "bar-weeks" the area above the curve was divided by the area of 1 bar-week.

In 1979 IS was calculated for Proctor from 28 DAP (t_1) to 77 DAP (t_2). In 1980 IS was calculated from either 29 DAP (t_1 for all treatments of Proctor) or 21 DAP (t_1 for all treatments of Arimar)

to the date of maximum betaine level in the two youngest mature leaves (t_2); t_2 = 78 DAP for I and NI treatments of Proctor, t_2 = 85 DAP for RW treatments of Proctor, and t_2 = 56 DAP for all treatments of Arimar.

2.2.5 Chemical Analyses

For betaine determination, plant material [2 replicates (1979) or 5 replicates (1980) from each treatment] was transported on ice to the laboratory, frozen in liquid N_2, and freeze-dried. The dried organs, divided as described in Section 2.2.3, were weighed and ground in a Micro-Wiley mill to pass a 40-mesh screen. An accurately weighed subsample of milled material (about 20 mg) from each plant was extracted with 5 ml of H_2O at 100°C for 1 h and centrifuged to clear. Betaine in the cleared extract was determined by pyrolysis-GLC after ion-exchange purification (Hitz and Hanson, 1980). Because phosphorylcholine can, in some circumstances, appear in the betaine-containing fraction of the ion-exchange procedure, a number of selected plant extracts, from both I and NI treatments, containing 200 μg betaine were analyzed by thin layer electrophoresis [0.1 mm-thick cellulose glass-backed plates (Cat. No. 5757; E. Merck) 2 kv, 10 min, 1.5 N formic acid]. Semi-quantitative estimates of the phosphorylcholine present were made by comparing Dragendorff-reagent sprayed plates with similarly treated standard plates on which had been loaded betaine (200 μg) and phosphorylcholine (5-50 μg, respectively); 10 μg phosphorylcholine was easily discernible. As no phosphorylcholine was found in the betaine fraction by this method,

it was concluded that there was $\leq$ 4 μmol phosphorylcholine/g dry wt in NI plants and $\leq$ 2 μmol phosphorylcholine/g dry wt in I plants.

To extract grains, an additional step before ion-exchange was taken to remove soluble polysaccharides. After reducing the cleared supernatant under a stream of N_2 (at about 80°C) to a 2-ml volume, ethanol (100%, 4 ml) was added, mixed thoroughly; the mixture was then left to stand for 1 h at 4°C to precipitate polysaccharide. After centrifugation the supernatant was dried in a stream of N_2 (at about 80°C) and redissolved in 2 ml H_2O. The betaine could then be determined as described above. A 0.5-nmol "spike" of [^{14}C]betaine was added to each of 3 grain samples. At the end of the extraction and ion-exchange procedure $\geq$ 96% of applied ^{14}C was recovered; ^{14}C was found only in the form of betaine after thin layer electrophoretic analysis (Section 1.2.4).

Kjeldahl-N was determined on selected organs of Proctor [2 (1979) or 3 (1980) replicates per treatment] at selected sampling dates. Tissue was frozen, dried and ground as for betaine determination. Duplicate subsamples (50 mg) were analyzed by the method of Cataldo et al. (1974), except that NO_3^--N was reduced by a 1-h predigestion with 3% salicylic acid in H_2SO_4 (conc., 2 ml) prior to the Kjeldahl procedure to ensure total tissue-N was measured (L. Schrader, personal communication).

For Proctor (1979), NO_3^--N was determined on the vegetative shoot tissue 56, 63, and 85 DAP by the method of Cataldo et al. (1975), and α-amino-N was determined on vegetative shoot tissue 63 DAP and on

spike tissue 70 DAP, using the method of Rosen (1957). For each treatment the analysis was carried out with two replicates, each of which consisted of the pooled organs from 2 plants.

2.2.6 Radioactive Labeling Experiments

[Methyl-^{14}C]Betaine (59 μCi/μmol) was prepared by the oxidation of [methyl-^{14}C]choline as described in Appendix A. The leaf blade that was to be fed was trimmed to about 10 cm, and a 3-μl droplet of K^+-phosphate buffer (20 mM, pH 7) containing [methyl-^{14}C]-betaine (0.75 or 1 μCi) was applied to the cut end. After droplet absorption was complete (15-25 min), four 2-μl droplets of water were fed sequentially at 10-15 minute intervals; other details of this method are given by Hanson and Scott (1980).

$^{14}CO_2$ was generated from Na[^{14}C]bicarbonate (3.75 μmol; 40 mCi/mmol, Amersham Corp., Ill.) and 43% lactic acid, and administered to the trimmed leaf blade as described in Section 1.2.3. For experiments with Proctor on the translocation and metabolism of betaine (experiment 1, Table 18), [^{14}C]betaine was fed to various leaves 46-61 DAP. At 74 DAP the whole shoots were harvested and freeze-dried. In experiment 3, flag-1 leaf blades were fed 62 DAP and harvested either 74 or 85 DAP. In experiment 1, shoots (except that one used in Fig. 19) were dissected before freeze-drying; in experiment 3 (Table 19) dissection of shoots was performed after freeze-drying and subsequent to localizing the ^{14}C-activity by autoradiography on SB-5 X-ray film (Eastman-Kodak). The amount and

chemical form of the ^{14}C present in various organs was determined by extracting the organs or, in case of the culm, 20 mg subsamples in duplicate, in MCW as described in section 1.2.4. The aqueous fraction was analyzed by ion-exchange chromatography on 1.5 ml columns AG- 50 (H^+) followed by AG-1 (formate) (Atkins and Canvin, 1971). The AG-1 column was sequentially eluted with formic acid (6N, 10 ml) and then HCl (2N, 8 ml). The AG-50 column was eluted with ammonium hydroxide (4N, 8ml). [^{14}C]Betaine in the AG-50 eluate was identified by co-migration with authentic unlabeled betaine using the TLE and TLC methods described in Section 1.2.4. For experiments with Arimar (experiment 2) that investigated translocation during developing water stress in the NI plants, various leaves were fed either $^{14}CO_2$ or [^{14}C]betaine 33 or 41 DAP. Whole shoots were harvested 55 DAP and freeze-dried intact. The ^{14}C-activity was localized in the shoots by autoradiography using SB-5 X-ray film (Eastman Kodak).

2.3 Results and Discussion

2.3.1 1979 Pilot Field Experiment

The field study conducted in 1979 was a pilot project designed to guide detailed planning for the second year of field experimentation.

2.3.1.1 Physical and physiological parameters of the crops.

Early-season Proctor Crop: The values for the yield components (Table 5) and the trends in dry matter accumulation, tillering habit (Fig. 11) and leaf diffusive resistance (Fig. 12) for the Proctor

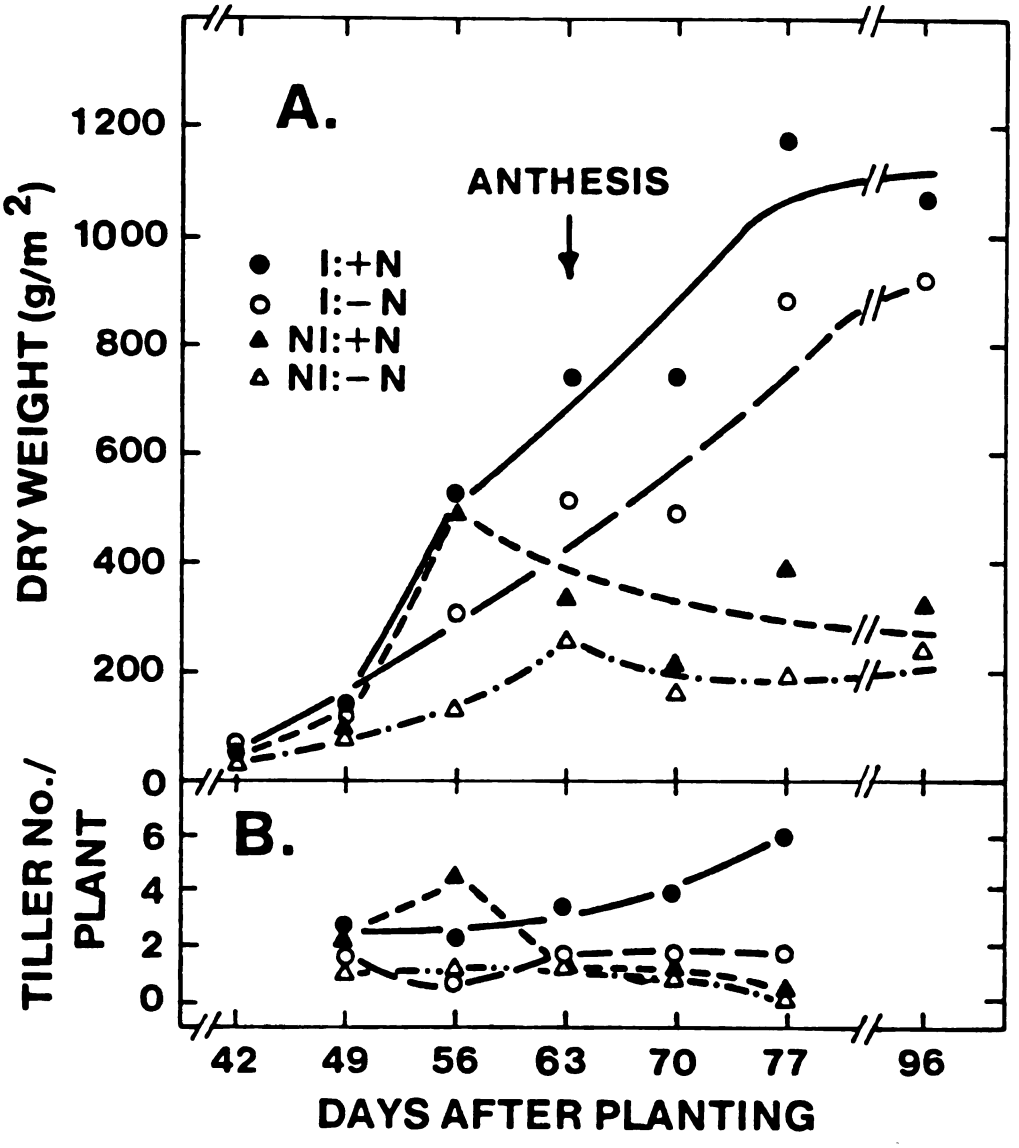


Fig. 11. Trends in above ground dry matter accumulation (A) and tillering (B) of Proctor barley (1979).

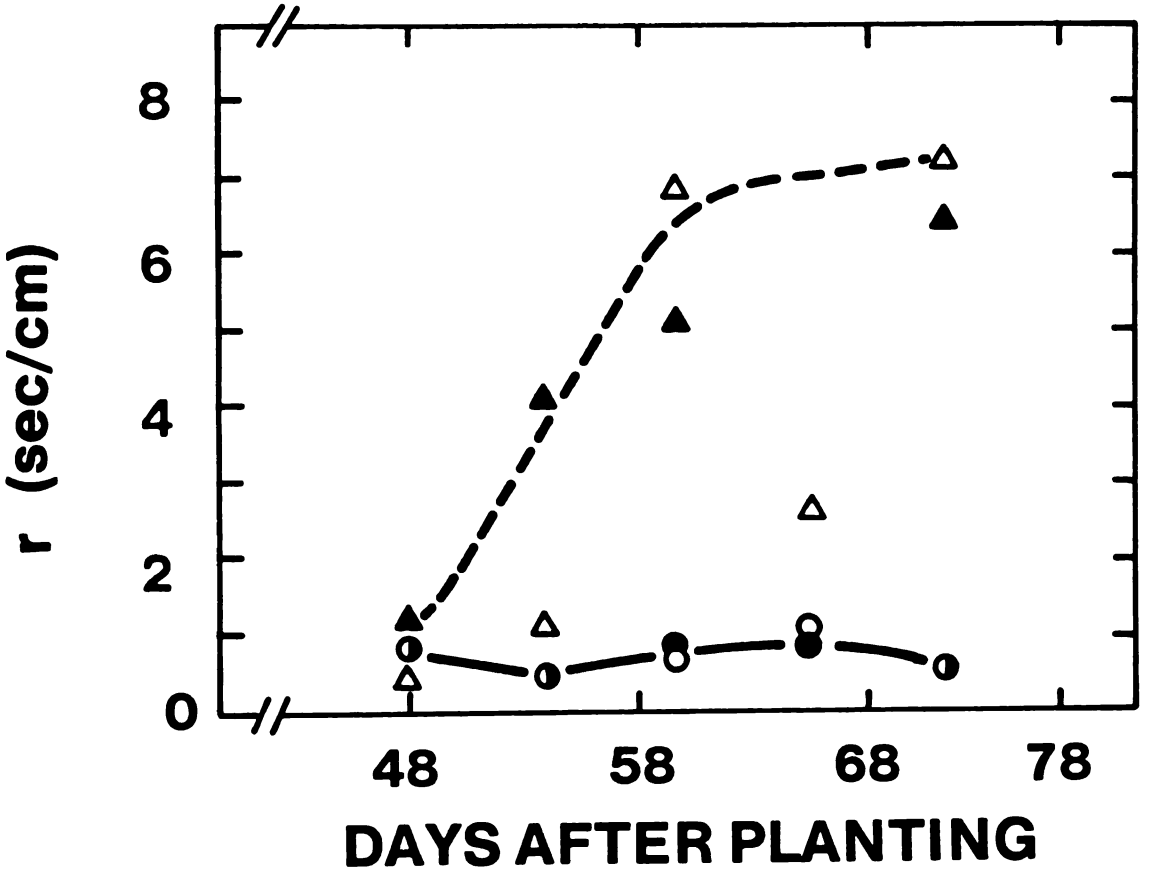


Fig. 12. The trends through the growing season of leaf diffusive resistance (r, sec/cm) of the youngest mature leaves of Proctor (1979). Symbols as in Figure 12.

TABLE 5. Yield, yield components, and selected growth characteristics of the Proctor crops in 1979.

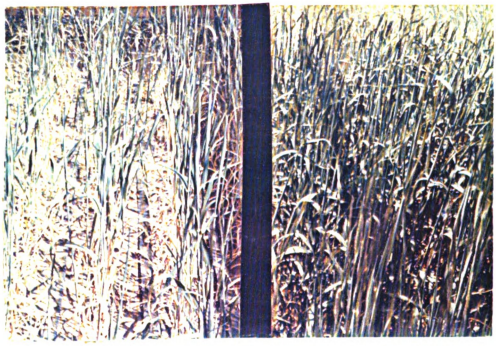
Parameter	Treatment			
	I : +N	I : -N	NI : +N	NI : -N
Mature plant ht (cm)	90	85	40	35
Days to 50% anthesis	69	69	69	69
Fertile tillers/m^2	393	264	79	75
Grain no./spike	26	25	12	16
Grain wt. (mg)	36	35	31	34
Grain yield (kg/ha)	3530	2270	290	410

crop in 1979 were all in accordance with the expected performance of a normal barley crop subjected to I and NI conditions (Briggs, 1978; Legg et al., 1979). Leaf temperature on any sampling day was not significantly different between treatments. Both tillering and dry weight increase (Fig. 11) were sensitive to water deficit (Fig. 13; Aspinall et al., 1964). As the season advanced, ψ declined steadily in the NI crop and, by 10 days before anthesis, leaf resistance was consistently higher than in the I crop. The water stress developed slowly (Fig. 15C) so that changes in growth pattern occurred that were typical for dryland crops (Fig. 11; Fig. 13). The yield of the irrigated crop (at about 3.5 tons/ha) compared favorably to the average yield for spring barley of 1979 for the State of Michigan(about 2.9 tons/ha; R. Morrison, Agricultural Extension Office, Mason, Mi.).

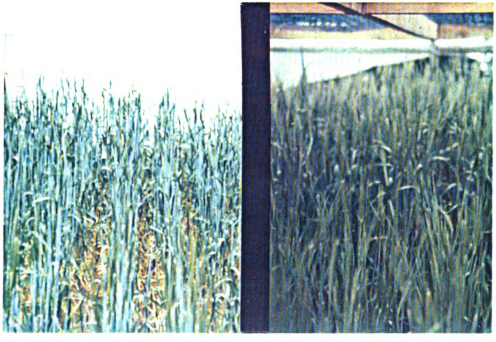
Fig. 13. Photographs of Proctor barley crops at ear emergence.

A and C: Non-irrigated crops in 1979 and 1980, respectively

B and D: Irrigated crops in 1979 and 1980, respectively.



A B



C D

The extensive reduction in tiller number/m^2, and the consequent low yield, of the NI crop was not caused by a failure in plant establishment; it was due to rodent damage to the NI plants in the later part of the growing season.

Above-ground dry matter accumulation, which probably reflected leaf area development, was lower in the N-deficient crop which suggested that the rate of soil-water depletion would be less than in the N-fertilized crop.

Late-Season Crop: The growing season in Michigan for spring barley is between March or April and June or July, but to fully exploit the summer months for field experimentation, a trial crop of barley, cv. Bankuti Korai, was planted in July. Bankuti Korai is an early maturing cultivar and thus could complete its life cycle within the remaining three months of late summer. Water potential measurements at different stages of growth were recorded (Fig. 14) to use as a guide for management of water-stress development in later summer plantings in future years.

For both early- and late-sown crops morphological differences between the plants grown under different levels of N-fertilization were evident--the N-deficient plants having typically a shorter stature, fewer tillers per plant and lighter-green colored leaves than the N-fertilized plants.

2.3.1.2 Results of chemical analyses. As ψ declined, the betaine concentration in the vegetative tissue of the NI, N-fertilized

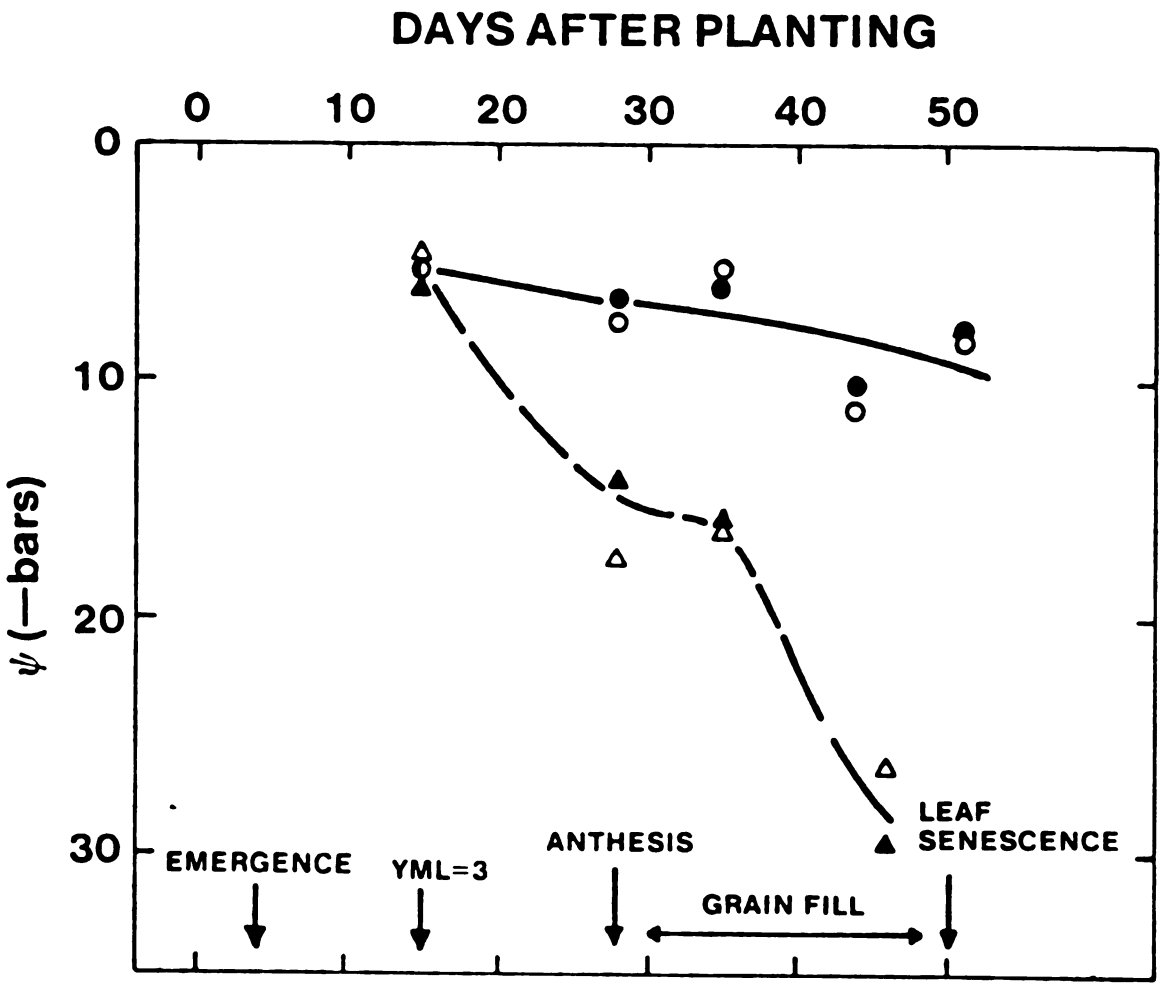


Fig. 14. Stage of growth, and trends in water potential (ψ) of the blade of the youngest mature leaf (YML), or flag-1 leaf, of Bankuti Korai planted July 19, 1979. Symbols as in Figure 11.

crop increased to a maximum of 50 μmol/g dry wt at about one week after anthesis (Fig. 15A). However, the betaine concentration in the vegetative shoot tissue of NI, N-deficient plants remained fairly constant at values similar to those of I plants which had consistently high ψ values (Fig. 15A). Such a depression of betaine accumulation by N-deficiency was not unexpected. In the converse situation when N-fertilization was applied to the halophytic grass Spartina alterniflora that grows normally in low N, high salt conditions the betaine levels increased (Cavalieri and Huang, 1981), indicating a link between soil-N status and betaine synthesis.

In the N-fertilized, N-I crop the betaine concentration of the vegetative shoot tissues appeared to decline about two weeks after anthesis. Assuming this drop in betaine level after anthesis to be a real effect, it could have been due either to degradation or to translocation to roots or spikes. The highest concentration of betaine in Proctor barley shoots was observed in the (flag plus flag-1) leaf blades (Table 6) where soil-N availability had little effect on betaine accumulation. The betaine concentration in the (flag plus flag-1) leaf blades of Bankuti Korai was also high--of similar magnitude to Proctor--under NI conditions at both levels of N-fertilization (Table 7). In contrast to whole-shoot tissue, the betaine concentration of the spike tissue rose in both N-fertilized, and N-deficient, NI plants (Fig. 15B).

Betaine, as a nitrogenous compound, did not appear to partition either like total-N or like dry matter (see respective harvest

Fig. 15. Betaine level of the whole shoot, excluding spike tissue (A) and the developing spike (B) for each irrigation and nitrogen treatment of Proctor barley (1979). The bottom frame (C) indicates trends in leaf water potential (ψ) for each irrigation and nitrogen regime. Symbols as in Fig. 11.

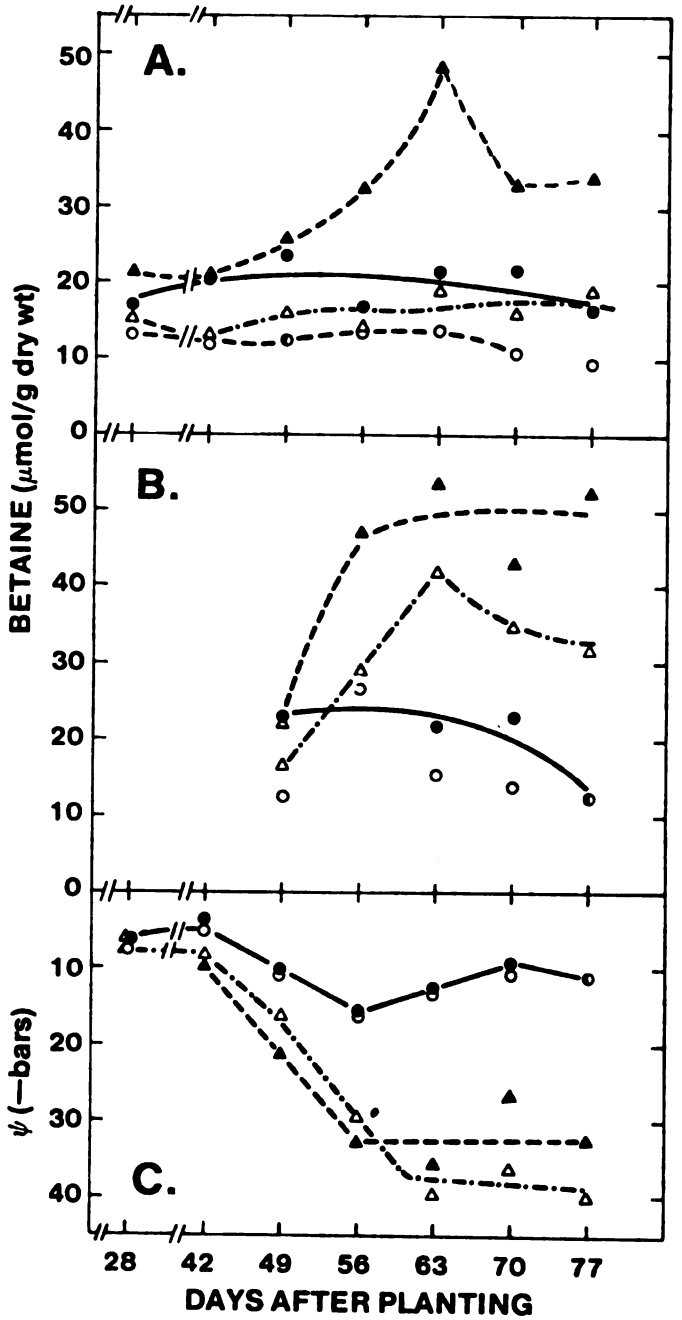

A.
B.
C.
BETAINE (μmol/g dry wt)
ψ (—bars)
50
40
30
20
10
0
10
20
30
40
28
42
49
56
63
70
77
DAYS AFTER PLANTING

TABLE 6. Betaine concentration of selected organs during the growing season for Proctor (1979).

Shoot Tissue	Betaine Concentration			
	I:+N	I:-N	NI:+N	NI:-N
	μmol/g dry wt			
Spindle (leaves 5+ younger) 48 DAP	17	19	27	27
Youngest mature leaf blades 56 DAP	41	33	115	49
(Flag + flag-1) leaf blades 70 DAP	58	34	155	101
Grain at maturity	8	6	9	8
Straw[§] at maturity	24	17	72	26

[§]Straw comprises chaff + vegetative shoot.

TABLE 7. Concentration of betaine in (flag plus flag-1) leaf blades of Bankuti Korai at 42 DAP

Treatment	Betaine (μmol/g dry wt)
I : +N	35
I : -N	40
NI : +N	173
NI : -N	100
	$LSD_{0.05}$ = 31

indices, Table 8). The distribution of betaine at maturity suggested that it remained in the leaves and stems of the maturing crop. It is interesting to note that in the straw of N-fertilized, NI plants 10% of the total-N was in betaine (Table 9B).

In agreement with other reports (e.g. Halse et al., 1969) the percentage of N in dry matter was similar in plants grown with and without N-fertilization (Table 10). There was, however, far less N in the N-deficient crop because it had less above-ground dry matter accumulation than the N-fertilized crop (Fig. 11). The slightly higher N content (as % dry weight), especially in the grain, of the NI plants as compared to I plants is commonly observed (Day et al., 1978) and can be explained by depressed photosynthate production and accumulation in NI plants.

In all treatments, NO_3^--N (Table 11) was a small proportion of the total-N in the shoot (cf Table 10). No consistent accumulation of NO_3^--N occurred in the NI plants, although NO_3^- is known to accumulate in the stems of some water stressed crops (Hanway and Englehorn, 1958).

The proportion of total-N in the form of free amino acids (α-amino-N) in vegetative and spike tissues was somewhat higher in NI plants (Table 9), but this effect of water stress was minor as might be expected from laboratory data on amino acid content of barley seedlings subjected to rapidly-induced water stress (Singh et al.,1973; Tully et al., 1979). Betaine-N (2.2% total-N) approached the contribution to total N of α-amino-N (3.8% total-N) in NI, N-fertilized plants at anthesis. This was not true in N-deficient

TABLE 8. Harvest indices of dry weight, nitrogen and betaine of Proctor barley at maturity (1979).

Treatment	HI[§]	NHI	BHI
I:+N	0.25	0.5	0.1
I:-N	0.25	0.41	0.1
NI:+N	0.1	0.22	0.1
NI:-N	0.13	0.39	0.1

[§]HI, Harvest Index = $\frac{\text{Grain Yield}}{\text{Total above-ground dry matter}}$

NHI, nitrogen harvest index = $\frac{\text{Total-N in grain}}{\text{Total-N in total above-ground dry matter}}$

BHI, betaine harvest index = $\frac{\text{Betaine in grain}}{\text{Betaine in total above-ground dry matter}}$

TABLE 9. α-Amino-N (A) and betaine-N content (B) of selected organs of Proctor (1979) at various dates after planting

A. α-Amino-N as % Total-N

DAP	Organ	α-Amino-N			
		Treatment			
		I : +N	I : -N	NI : +N	NI : -N
		% Total-N			
63	Vegetative tissue	3.0	3.4	3.8	6.2
70	Spike	14.5	15.0	16.2	14.5

B. Betaine-N as % Total-N

DAP	Organ	Betaine -N			
		Treatment			
		I : +N	I : -N	NI : +N	NI : -N
		% Total-N			
63	Shoot (excluding spike)	1.2	1.1	2.2	1.2
70	Spike	1.9	1.5	3.6	3.2
70	Flag + Flag-1 leaf blades	2.3	1.8	4.5	4.0
84	Spike	0.9	1.0	3.2	2.4
84	Main culm (excluding spike)	2.6	1.2	3.7	2.2
96	Grain	0.6	0.5	0.4	0.4
96	Straw	4.5	2.7	10.0	5.3

TABLE 10. Total-N (including NO_3^--N) content of selected organs of Proctor barley (1979) at various dates after planting.

DAP	Organ	Nitrogen Content			
		Treatment			
		I:+N	I:-N	NI:+N	NI:-N
		% dry wt.			
63	Shoot (excluding spike)	2.0	1.8	2.1	1.7
70	Spike	1.8	1.5	2.1	1.8
	Flag + flag-1 leaf blades	4.3	3.3	4.2	3.3
84	Spike	1.9	1.1	2.5	1.8
	Shoot (excluding spike)	1.0	1.7	1.3	1.2
90	Grain	2.1	1.7	3.0	2.8
	Straw	0.7	0.8	1.0	0.6

TABLE 11. NO_3^--N level in the shoot vegetative tissue of Proctor barley (1979) at various dates after planting.

DAP	NO_3^--N Level			
	Treatment			
	I:+N	I:-N	NI:+N	NI:-N
	% dry wt.			
56	0.27	0.13	0.09	0.14
63	0.09	0.10	0.15	0.09
84	0.11	0.09	0.07	0.09

plants where in fact, with the exception of the (flag plus flag-1) leaf blades, betaine-N (expressed as % total-N) in all the organs sampled tended to be lower than in N-fertilized plants. The amino acid-N content of the spike represented a much larger proportion of the total-N than did betaine (Table 9).

Comparison of data for betaine in the spike at 1 week after anthesis (70 DAP), and in the grain after harvest (96 DAP) (Table 9B) shows that the contribution of betaine to total grain-N was far lower than to total spike-N. This might be explained in one or more of the following ways: (1) Betaine was metabolized by spike tissue; (2) Betaine ceased to be translocated to the spike later in the growing season and so its relative contribution to total-N decreased as grain protein levels rose; (3) Betaine was largely localized in awn and/or rachis tissue.

In order to use betaine as a cumulative stress index there must be a strong correlation between an integrated value of the stress experienced by the plant and the betaine level of either the whole shoot or organs thereof. The whole vegetative shoot of N-deficient plants did not accumulate betaine. However, the spike and youngest leaves did accumulate betaine regardless of soil-N level; regression of betaine level in spike tissue on IS, calculated from the weekly measurements of ψ (Fig. 14; see Section 2.2.4 for definition) showed a significant correlation between the two parameters ($r = 0.74^{**}$, N-fertilized; $r = 0.79^{**}$, unfertilized). This result encouraged further testing of the hypothesis that the betaine level

of certain organs could be used as an index of the cumulative stress experienced by a crop.

2.3.2.3 Conclusions from the preliminary 1979 field study. It proved possible to simulate dryland farming conditions in Michigan using manual rain-out shelters. The shelters did not adversely affect the performance of the I or NI crops. Symptoms of N-deficiency in plants grown without N-fertilization were evident. N-deficiency severely depressed betaine accumulation in whole shoots, but only moderately lowered betaine accumulation in the spike and youngest leaves. In addition, it was unclear whether the lowered betaine levels in the unfertilized NI crop was a consequence of a higher water status, resulting from slower depletion of soil water by a thinner stand. Betaine contributed up to 4.5% to the total-N in NI (flag plus flag-1) leaf blades and up to 10% total-N in the straw at harvest. Betaine appeared to remain predominantly in vegetative tissue, and not to be massively translocated to the main sinks (grains) of the maturing plant.

The influence of N-fertilization on betaine accumulation in whole shoots was too great for the betaine concentration in this tissue to be considered further for practical use as an index of cumulative stress. The most likely organs to sample to further test the hypothesis appeared to be the spike (possibly specifically the awns) and the youngest mature leaves.

2.3.2 1980 Field Experiment

2.3.2.1 Physical and physiological parameters of the crops. Both the Proctor and Arimar barley crops developed and matured satisfactorily (Table 12). The water-stress experienced by the NI crops were less severe than in 1979 and thence leaf firing and tiller dieback were less pronounced (Fig. 13).

Leaf ψ declined in NI periods but after rewatering rose to values similar to those recorded in the I crop. Leaf diffusive resistance of the NI crop was consistently higher than that of the I crop. Leaf diffusive resistance of the RW crop increased during stress, but fell after irrigation (see Table 13 for average values of leaf resistance for each treatment).

Yields of Proctor were higher than in 1979 and this was due to an increase in tiller number/m^2; grain weight was similar in all treatments in both years. This observation agrees with the report of Gallagher et al. (1975) that the grain weight of Proctor remains stable (29-41 mg/grain) under various environmental conditions. The yield of the RW crop was low in comparison to the I crop. In the RW crop, after rewatering, there was a proliferation of small tillers which did not set grain.

In cv. Proctor, differences due to N-fertilization were discernible under all three irrigation regimes, being especially pronounced in NI and RW plots. All N-deficient plants were characteristically 3-8 cm shorter than the corresponding N-fertilized plants with fewer tillers and paler green leaves.

TABLE 12. Yield, yield components and selected growth characteristics of the Proctor crop (A) and Arimar crop (B) of 1980

A. Proctor 1980

Parameter	Treatment					
	I:+N	I:-N	NI:+N	NI:-N	RW:+N[§]	RW:-N[§]
Mature Plant Height (cm)	90	85	50	45	50	40
Days to 50% anthesis	62	62	62	62	62	62
Fertile tiller/m^2	562	376	261	206	330	196
Grain no./spike	24.2	19.8	15.6	15.6	18.4	14.4
Grain wt (mg)	36.6	31.2	29.3	31.2	36.3	31.3
Grain yield (kg/ha)	5000	2320	1200	1000	2210	890

B. Arimar

Parameter	Treatment		
	I:+N	NI:+N	RW:+N[§]
Days to 50% anthesis	40	40	40
Fertile tiller no./m^2	523	162	263
Grain no./spike	26.6	13.4	27.0
Grain wt (mg)	43.2	38.4	34.7
Grain yield (kg/ha)	5870	800	1290

[§]Small tillers that sprouted after each rewatering were not included in the observations of this table.

TABLE 13. The average leaf diffusive resistance of the youngest mature leaf blade over the growing season (1980).

Treatment	Leaf Diffusive Resistance	
	Proctor[§]	Arimar
	sec/cm	
I	7	7
NI	30	28
RW		
Stressed period	13	23
Irrigated period	5	9

[§]Since no significant differences existed between nitrogen treatments, values were pooled for Proctor.

2.3.2.2 Results from chemical analyses. The total-N (including NO_3^--N) content of the whole shoot of Proctor plants was measured at anthesis as an indicator of the N-status of the crops (Table 14). The values were lower, but comparable to those of the previous year (Table 11).

The irrigated plants had consistently higher ψ values and lower, relatively constant, betaine levels than the NI plants (Fig. 16 and 17). In Proctor, betaine accumulated in the (flag plus flag-1) leaf blades of both N-fertilized and N-unfertilized plants that had been either continuously or periodically stressed (Fig. 16). The slightly lower levels of betaine in NI, N-deficient plants than in NI, N-fertilized plants may be explained by the former maintaining, on average, a higher water status during the season.

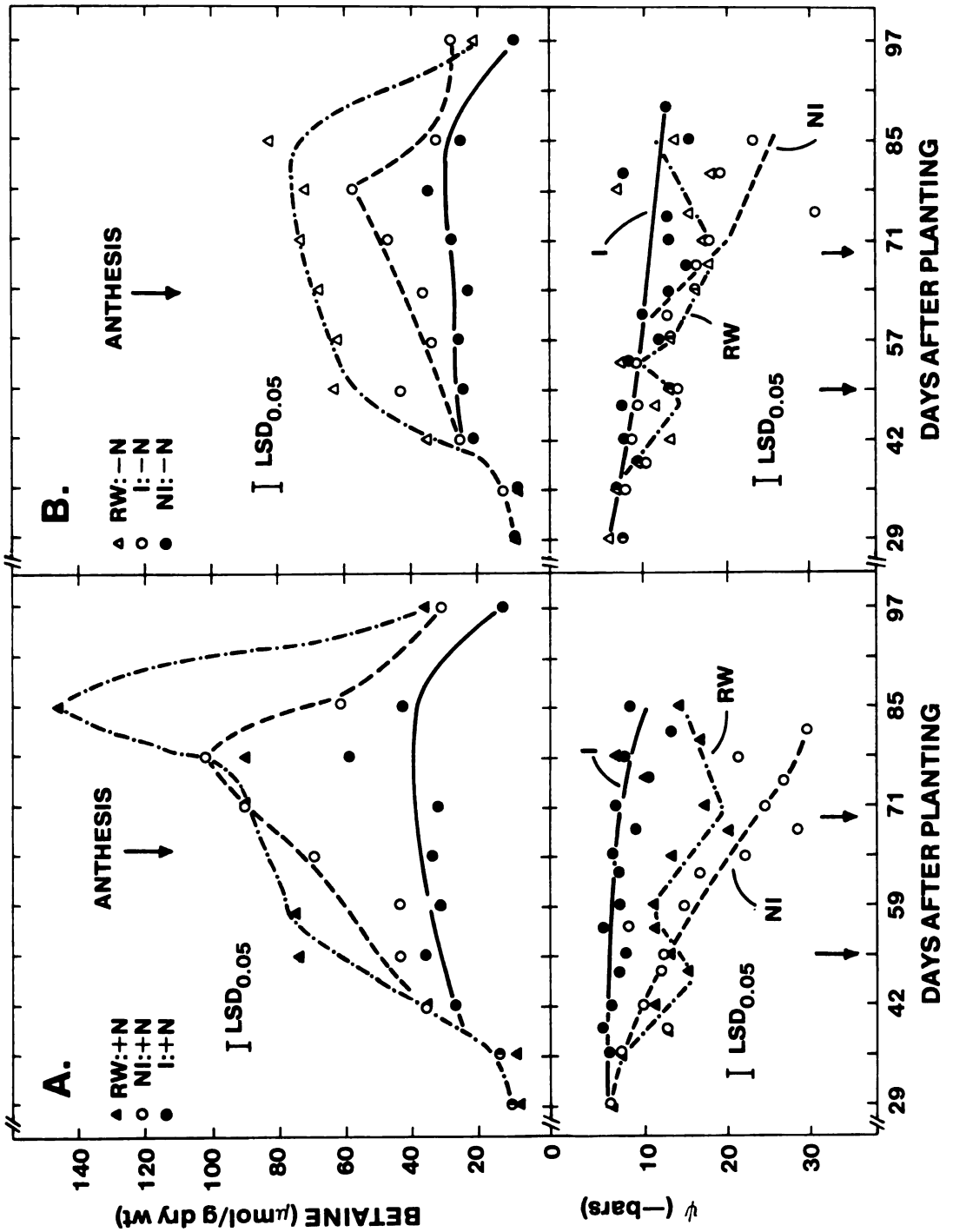
TABLE 14. Nitrogen content (as % dry weight) of the total shoot of Proctor (1980) at anthesis.

N-Treatment	Total-Nitrogen Content[§]		
	Irrigation Regime		
	I	NI	RW
	% dry wt.		
+N	1.3a	1.8b	1.7b
-N	1.2a	1.3a	1.2a

[§]Data are means of 3 replicates. Means followed by different letters were significantly different (p = 0.05) according to Duncan's Multiple Range Test.

The betaine concentration in the (flag plus flag-1) leaf blades of the N-deficient, RW plants appeared to reach a plateau, whereas that in the N-fertilized, RW plants continued to increase until about 3 weeks after flowering. The decline in betaine concentration in the (flag plus flag-1) leaf blades began about 1 week before yellowing, suggesting that betaine synthesis ceased before senescence began, and that net export of betaine from the blade, or betaine degradation, began late in the growing season. Approximate calculations in which it was assumed (a) that the spike was a sink for betaine translocation from (flag plus flag-1) leaves, and (b) that there was no *in situ* synthesis of betaine in spikes between 77-85 DAP, suggested only 20-25% of the betaine lost during this period from mature (flag plus flag-1) leaf blades was attributable to translocation to the

Fig. 16. Betaine level of the two youngest mature leaf blades of N-fertilized (A) and N-unfertilized (B) Proctor barley (1980). Lower frames indicate the trends of water potential (ψ) measured on the youngest mature leaf, or the flag-1 leaf. Trend lines are hand-drawn. Vertical arrows in lower frame indicate dates of rewatering of RW plots. Symbols for treatments in the lower frames correspond to the symbols described in the frames immediately above.


A.
B.
RW:+N
NI:+N
I:+N
RW:−N
I:−N
NI:−N
ANTHESIS
LSD0.05
BETAINE (μmol/g dry wt)
ψ (−bars)
RW
NI
I
DAYS AFTER PLANTING
0
20
40
60
80
100
120
140
10
20
30
29
42
59
71
85
97
57

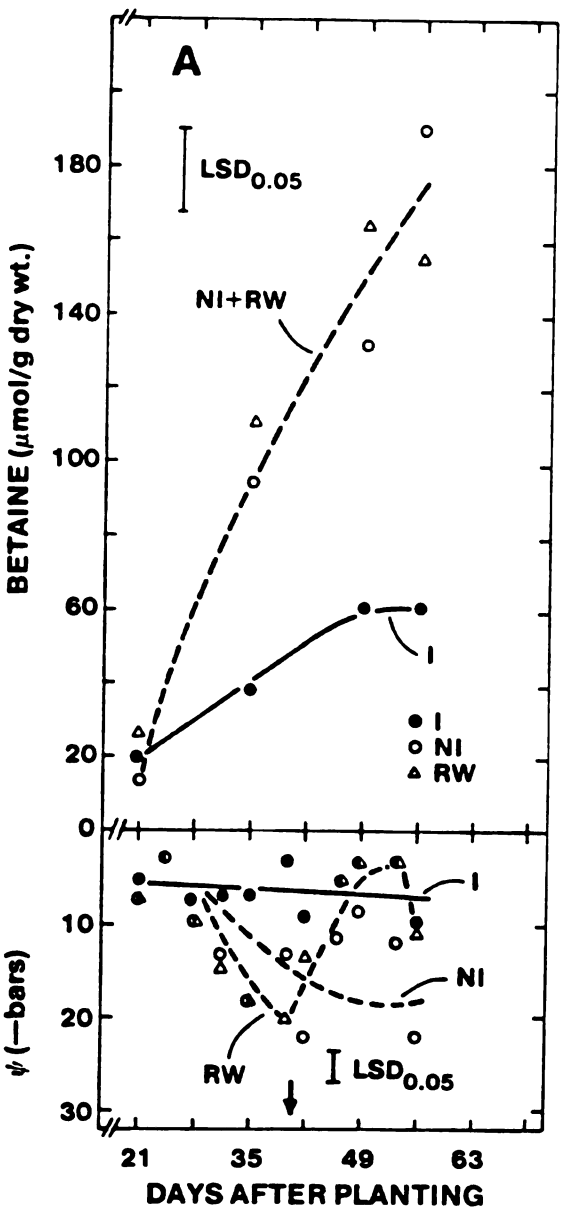


Fig. 17. Betaine concentration of the two youngest mature leaf blades of Arimar barley (1980). The lower frame indicates the trends in water potential (ψ) measured on the youngest mature leaf, or flag-1 leaf. Vertical arrows in lower frame indicate date of rewatering RW plot.

(●:I, o:NI, Δ:RW)

spike. Thus, if betaine export is to account for the loss of betaine from the upper leaves, the sink may be elsewhere than in the developing spike.

The high betaine concentration in the leaf blades of NI plants involved an active accumulation and could not be ascribed solely to a constant amount of betaine (i.e. a finite capacity, unaffected by stress, for a plant leaf blade to accumulate betaine) being present in the smaller leaves of the NI plants. This deduction was based on data (Table 15) collected from the N-fertilized plants 71 DAP. Although the NI treatment reduced the area and the weight of the flag-1 leaf blades, this blade contained about 4.9 μmol of betaine in the NI crop, and only about 2.2 μmol in the I crop.

TABLE 15. The average betaine content and dimensions of the flag-1 leaf blade of N-fertilized Proctor barley 71 DAP (1980).

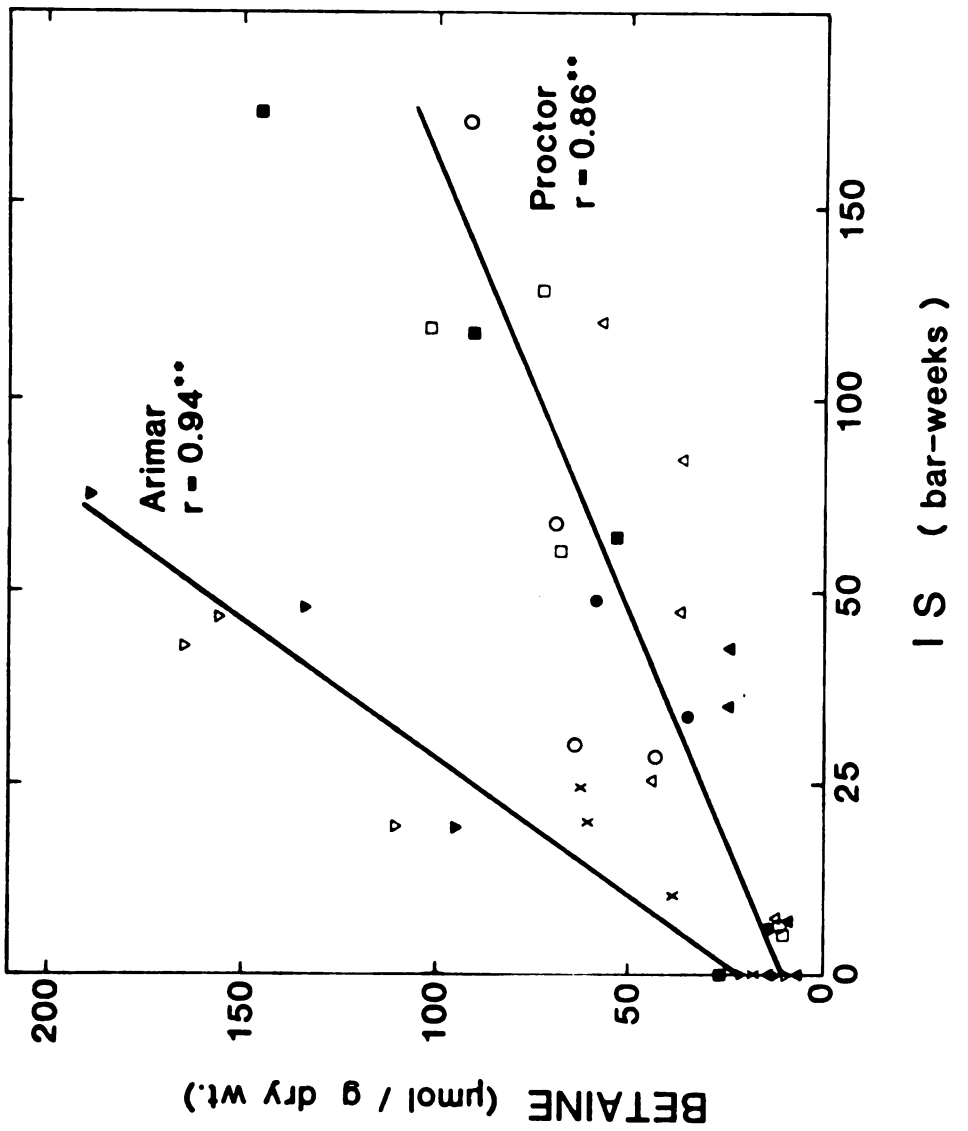
Parameter	Treatment[§]	
	I:+N	NI:-N
Leaf area (cm^2)	27.8	15.7**
Leaf weight (mg)	69.2	55.3**
Betaine level (μmol/g dry wt)	31.4	89.0**
Total betaine content/leaf (μmol)	2.2	4.9**

**Indicates significance at $p \leq 0.01$.

[§]All pairs of values were tested for significance by Student t test.

Even though the cumulative stress (IS) experienced by Arimar was less than Proctor (see Fig. 18) the concentration of betaine in the (flag plus flag-1) leaf blades of NI Arimar reached a maximum of 200 μmol/g dry wt (Fig. 17). This was twice the maximum concentration observed in Proctor (Fig. 16A) and about 3-4 times the maximum level usually found in wilted leaves of young barley plants (e.g., Section 1.3; Hanson and Nelsen, 1978; Wyn Jones and Storey, 1978). This high concentration approaches those reported in leaves of several halophytes (Storey et al., 1977). Arimar was planted after Proctor had been harvested and so some environmental parameters, e.g., daylength, potential evapotranspiration, and average daily range in temperature, differed between the respective growing seasons for those two cultivars. In laboratory experiments light was not required for betaine synthesis (Bregoff and Delwiche, 1955; Bowman and Rohringer, 1970). This being so, and because the difference in the average daylength between the two crops was only about 2 hours, daylength was unlikely to contribute to the difference in betaine accumulation, especially as the higher level of betaine was in the cultivar which experienced fewer hours of daylight. Laboratory experiments using three-week old barley seedlings indicated that growing temperatures up to 34°C did not greatly affect betaine levels when the evaporative demand was held constant (A. D. Hanson, unpublished results). From these results it could be argued that the warmer temperatures encountered by the late-season Arimar crop, as compared to the early-season Proctor crop, would be unlikely to account for the

Fig. 18. The relationship between the betaine concentration of the youngest mature leaf blades and the IS experienced by Proctor and Arimar barley crops (1980). Proctor, N-fertilized: ●, I; o, NI; ■, RW. Proctor, N-deficient: ▲, I; △, NI; □, RW. Arimar N-fertilized: x,I; ▽, NI; ▼, RW. Primary data are those of Figures 16 and 17.


BETAINE (µmol / g dry wt.)
200
150
100
50
0
0
25
50
100
150
I S (bar-weeks)
Arimar
r = 0.94**
Proctor
r = 0.86**

high betaine levels in Arimar plants. These considerations, taken with evidence for genetic variation for betaine accumulation in barley (Hanson and Nelsen, 1978; see also this dissertation, Section 3.3) suggested that the two-fold higher betaine level in Arimar as compared to Proctor was more likely to be genetic than environmental in origin.

The betaine concentration in the youngest mature leaf blades was highly correlated with IS (expressed as bar-weeks, see Section 2.2.4 for definition) experienced by the plant (Fig. 18). The regressions for Proctor and Arimar could not be pooled because of the differences between the times and lengths of growing seasons, but all the treatments, with respect to nitrogen and irrigation, for any one cultivar were combined. To confirm that pooling the data from the two N-regimes was legitimate, it was confirmed by covariance that the slopes of the two regression lines (N-fertilized/N-deficient, Proctor) could have come from the same population and that the differences in betaine concentration were mainly resulting from differences in degree of stress experienced.

In the 1979 study with Proctor there was an anomaly apparent in betaine levels in spikes and grains; whereas young spike tissues including developing grains were fairly high in betaine, the mature grains were not. Accordingly, meristematic tissues and various spike organs were analyzed in detail in both Proctor and Arimar. Although betaine levels in the unexpanded leaves and the apical meristem of young N-fertilized and N-deficient NI plants began rising (Table 16),

TABLE 16. Betaine level in the youngest organs of Proctor barley (1980).

DAP		Betaine Level					
		Treatment					
		I:+N	I:-N	NI:+N	NI:-N	RW:+N	RW:-N
		µmol/g dry wt.					
29	Spindle[§]	25	25	27	22	26	17
36	Spindle	31	22	49	32	40	31
43	Spindle	46	30	86	56	89	81
50	Developing Spike	21	15	41	24	48	55
57	Developing Spike	12	10	17	13	18	26
78	Emerged Spike:						
	Awns	28	17	41	29	34	37
	Remainder[†]	9	7	10	8	10	10
85	Emerged Spike:						
	Awns	23	16	43	44	52	36
	Remainder	7	7	12	6	13	11

[§]Spindle refers to the young unexpanded leaves.

[†]Remainder comprises the glumes, caryopses and rachis.

as the spike tissues differentiated and then matured, the betaine levels in these organs began to decline. After anthesis, betaine levels in awn tissues of NI and RW plants of both cultivars rose, but levels in the grains and other spike parts did not (Table 16; Table 17). This is consistent with the results of laboratory experiments (Section 1.3.3) which indicated that the spike was not a major sink for betaine. That the betaine concentration in the spike (excluding awns) was relatively constant (Table 16; Table 17) as dry weight increased implies either a continuous synthesis of betaine in situ or continuous translocation of betaine from other organs.

TABLE 17. Betaine level of the youngest organs of Arimar barley shoots at 3 dates after planting.

DAP		Betaine Level		
		Treatment		
		I	NI	RW
		μmol/g dry wt		
35	Young Spike	12	28	74
49	Emerged Spike			
	Awns	23	46	30
	Remainder[§]	6	7	7
56	Emerged Spike			
	Awns	24	51	38
	Remainder	14	14	18

[§]The remainder of spike consisted of the rachis, caryopses and glumes.

The contribution of the awns to total spike weight decreased from about 43% at 64 DAP to about 7% at 85 DAP. Thus the difference between the contribution of betaine to total-N in the spike at 70 DAP and the grain at maturity (Table 9) can be explained by preferential localization (on a dry wt basis) of betaine in awn tissue; the contribution of betaine to total-N fell as protein accumulated in the grain during development. Betaine has been identified in wheat anthers as a substance stimulating the growth of Fusarium graminearum (Strange et al., 1974). Anthers, which were shedding pollen, were collected from I and NI Proctor plots. Anthers from all treatments had high levels of betaine (average about 100 μmol/g dry wt) with no significant differences between treatments.

2.3.2.2 Translocation and metabolism of betaine. Laboratory experiments of a few days' duration (Chapter I) indicated that betaine was not degraded by barley seedlings and that it was transported in the phloem from older to younger organs. Radiochemical experiments in the field sought firstly to evaluate the contribution that translocation made to betaine accumulation in the (flag and flag-1) leaf blades, and secondly, to investigate the decline in betaine concentration in the flag-1 leaf blade two weeks after anthesis (see Fig. 16).

In the first experiment, [^{14}C]betaine was applied to various Proctor leaves on three dates after planting. At harvest, the only ^{14}C-labeled compound found in any organ was betaine (Table 18). The recovery of ^{14}C was low, especially at the earlier planting dates. Roots could not be harvested, but because in laboratory experiments

TABLE 18. Distribution of [methyl-^{14}C]betaine in shoots of non-irrigated Proctor barley in 1980 (Experiment 1)

Feeding Date (Days after Planting)	Leaf Fed[§]	^{14}C-Distribution Among Shoot Organs at Harvest (% of ^{14}C-Fed)[†]					
		Fed Leaf	Flag + Flag-1 Blades	Spike	Remainder of Main Culm	Tillers	Total
46	Flag-4	3	5	4	19	13	44
53	Flag-3	3	8	4	34	18	67
61	Flag-2	52	< 1	< 1	18	11	83

[§]Average leaf number per main culm was eight.

†Data are means of three replicates. Harvest date was 74 days after planting. In all organs, ≥97% of the ^{14}C was in the form of [^{14}C]betaine.

roots imported up to 15% of the applied betaine after 3 days (Section 1.3), it is probable that incomplete recovery of ^{14}C in the field reflects slow downward translocation of betaine to roots. However, loss of $^{14}CO_2$ and other volatile metabolites cannot be ruled out as explanations for the low ^{14}C-recovery.

The applied [^{14}C]betaine was extensively exported from the lower two leaves fed (flag-4 and flag-3) and in these cases the main sinks in the shoot were tillers, and leaves above those fed. In contrast, much of the fed [^{14}C]betaine was retained by the flag-2 leaf, and little ^{14}C migrated upwards (Table 18). These results confirm that applied betaine can be translocated from mature to expanding leaves, but indicate that the import of betaine into the (flag-1 plus flag) leaf blades was probably only a minor term in their total betaine accumulation.

The diminished translocation of betaine from the flag-2 leaf blade might have been caused by a general depression of translocation during the developing water-stress. Figure 19 illustrates the translocation of [^{14}C]betaine fed through the flag-2 leaf of Proctor 54 DAP (experiment 1). This autoradiograph serves to orient the reader so autoradiographs of experiment 2 are clear. Results (Fig. 20) from feeding [^{14}C]betaine and $^{14}CO_2$ to the flag-1 leaf blades of Arimar clearly demonstrate that the failure of betaine to be exported did not reflect a generalized failure of translocation. Thus, $^{14}CO_2$-assimilates and [^{14}C]betaine fed early in the season (Fig. 20A, C) were extensively translocated around the plant but, whereas

Fig. 19. Autoradiograph of a Proctor barley plant fed [^{14}C]betaine to flag-2 leaf blade 54 DAP. Harvest at 75 DAP. At harvest, before autoradiography, main culm was cut at the flag leaf node.

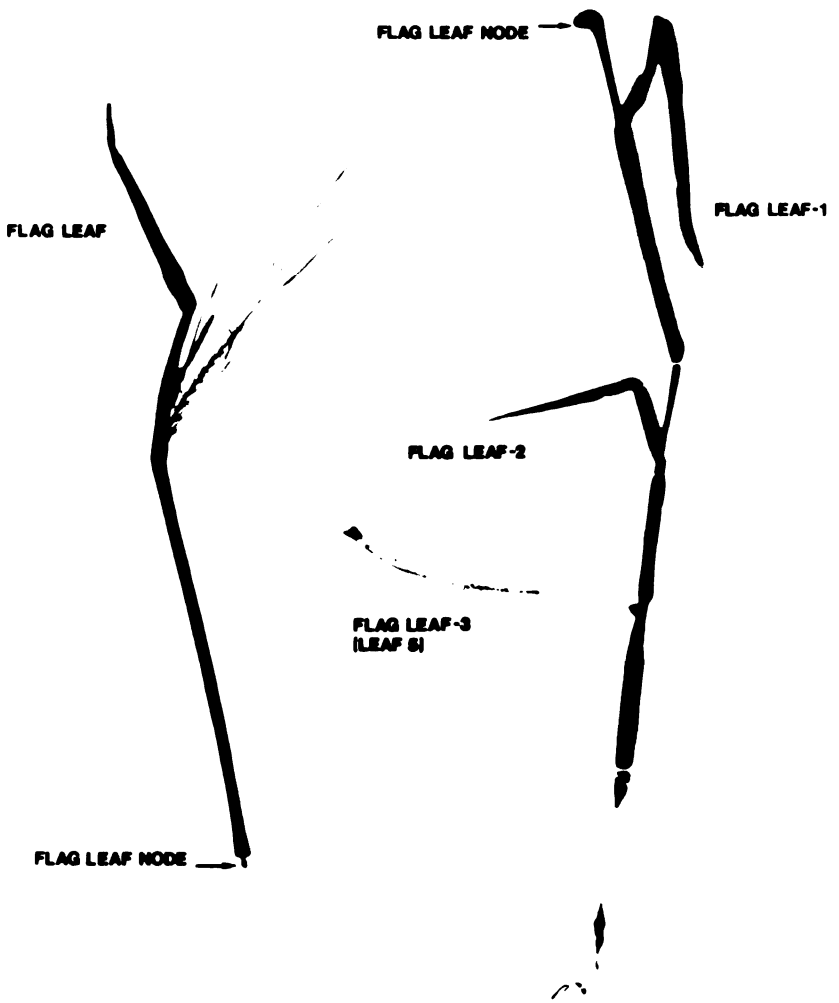
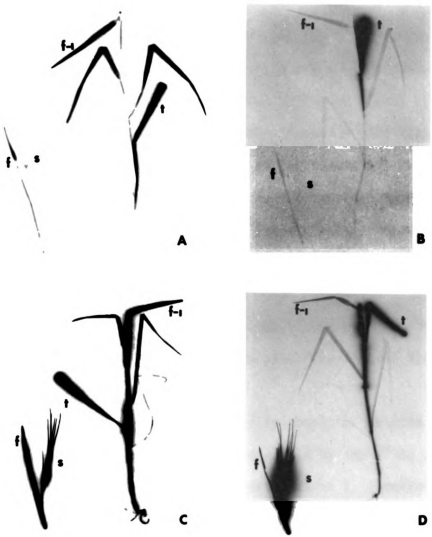

FLAG LEAF NODE
FLAG LEAF-1
FLAG LEAF
FLAG LEAF-2
FLAG LEAF-3
(LEAF 6)
FLAG LEAF NODE

Fig. 20. Autoradiographs of barley plants fed 1 μCi [^{14}C]betaine (A,B) or 50 μCi $^{14}CO_2$ (C,D) at 22 (A,C) or 41 (B,D) DAP. Harvest at 55 DAP. [Experiment 2]. At harvest the main culm was cut at the flag leaf node.

Key to letters: f = flag leaf; s = spike; f-1 = leaf immediately below flag; t = fed leaf.


f-1
f
s
A
f-1
f
s
B
f-1
f
f
s
C
f-1
f
f
s
D

$^{14}CO_2$-assimilates were exported from the flag-1 leaf later in the season (Fig. 20D), applied [^{14}C]betaine was largely retained by this leaf (Fig. 20B).

The flag-1 leaf blades of the NI plants of the third experiment (Table 19) were fed [^{14}C]betaine 62 DAP in 1981 and then were harvested after 12 days (74 DAP) and 23 days (85 DAP), respectively. Autoradiographs of the whole plant prior to extraction indicated considerably more ^{14}C-activity was in the blades of plants of the early, than in those of the late harvest. Plants of the late harvest had correspondingly more radioactivity in the sheath and culm. Little ^{14}C-activity (< 2.5%) was found in any form other than betaine in the four parts of the plant analyzed and ^{14}C-distribution resembled, in a general way, that of the endogenous betaine at the two dates (Table 19). This result, that betaine moves out of the blade and into other vegetative organs, explains, to a large extent, the drop in betaine levels observed in the (flag plus flag-1) leaf blades after anthesis (Fig. 16).

The above results indicate that synthesis of betaine <u>in situ</u> is the major source of the betaine accumulated by the (flag plus flag-1) leaf blades, and that in Proctor, betaine is exported from the flag-1 leaf blade late in the season to be relocated in the sheath of this leaf and in the culm. The implication of these results is that export, and possibly import, of betaine is regulated in some specific way so that betaine does not simply follow the translocation patterns of other nitrogen compounds or of CO_2-assimilates.

TABLE 19. Distribution of ^{14}C-activity in selected organs of non-irrigated Proctor barley (1981) after [^{14}C]betaine (0.75 μCi) was fed to the flag-1 leaf blade 62 DAP (Experiment 3).

Harvest Time (DAP)	Organ	Betaine		^{14}C[§]	
		μmol/ g dry wt	μmol/ organ	% applied	nCi/ organ
74	Flag-1 blade	93	1.8	80	602
	Flag-1 sheath	50	2.3	2	16
	Culm 2nd internode	17	0.6	0	0
85	Flag-1 blade	59	0.9	35	266
	Flag-1 Sheath	69	2.9	12	96
	Culm 2nd Internode	89	4.1	9	70
	Flag leaf sheath	83	4.7	2	12

[§] $\geq$ 97.5% ^{14}C was in the form of betaine.

2.3.2.4 Conclusions from the 1980 field study. The accumulation of betaine in the (flag plus flag-1) leaf blades of NI barley is not a shock response to rapidly imposed water deficit but occurs gradually, as ψ declines, at a time when the plants are making morphological changes in response to their water-stressed condition. Betaine accumulation apparently ceased well before the onset of senescence, judged from incipient yellowing. Within the mature plant betaine is not metabolically labile and can be translocated, probably in the phloem. Although betaine is translocated in such mature plants, it does not follow the partitioning pattern of total plant N and photosynthate, and remains primarily in the leaves and culm; some betaine does accumulate in the awns. The concentration of betaine in the grain is always low (< 10 μmol/g dry wt) which agrees with values obtained for wheat grains (Chittenden et al., 1978).

Circumstantial evidence from this work, and from the radiochemical experiments of Hitz et al. (1982), indicates that the accumulation of betaine in the (flag plus flag-1) leaf blades is predominantly due to synthesis in situ and not to translocation from other organs. Unlike betaine accumulation in the shoot as a whole, which is severely depressed by low soil-N, betaine accumulation in the two youngest mature leaves is largely unaffected by N-fertilization.

Betaine concentration of the youngest mature leaf pair was correlated ($r \approx 0.9^{**}$) with the cumulative degree of stress, expressed as bar-weeks, experienced by the plant. The criteria that were set in order to judge whether the betaine content of the (flag plus flag-1)

leaf blades could be used as an index of the cumulative stress experienced by the plant, as described in the Introduction, were largely met. However, betaine concentration could not be recommended as such an index for the following reasons: (1) The difference in betaine level between I and NI plants was comparatively small (approximately 2- to 3-fold). In addition, the levels among I and NI plants were too variable to give an accurate estimation of the cumulative stress experienced by the plants unless many samples were taken; (2) The increase in betaine concentration accompanied, but did not precede, morphological changes in response to water stress. The latter, in any practical situation, are easier to detect and to measure; (3) From studying just two cultivars of barley, genetic variability for basal and stress induced levels of betaine was implicated; this could confound interpretation of results in a breeding program.

The timing, the extent, and the site of betaine accumulation (viz. in the two leaves that contribute most to grainfill), are all consistent with betaine accumulation having an adaptive role under stressed conditions.

CHAPTER III

GENETIC VARIATION FOR BETAINE LEVELS IN CULTIVATED AND WILD BARLEY

3.1 Introduction

Halophytes are known to show osmotic adjustment when subjected to saline conditions (Flowers et al., 1977); this permits maintenance of turgor and continued water flux through the plant. Many halophytes, especially those from the families Chenopodiaceae and Gramineae, have high shoot concentrations of betaine (100-300 μmol/g dry wt) even when grown in non-saline environments; betaine levels increase further (to 300 - 1000 μmol/g dry wt) in response to salinity (Storey and Wyn Jones, 1975; Wyn Jones et al., 1977b). In such halophytes it has been suggested that betaine accumulation is an adaptive response to salinization. Betaine is believed to act as a nontoxic, or "compatible" osmoticum in the cytoplasm, balancing the accumulation of potentially toxic salts in the vacuole (Wyn Jones et al., 1977a; Hall et al., 1978; Leigh et al., 1981). Some mesophytes of the above-mentioned families, (e.g., the grasses, barley and rhodes grass) also accumulate betaine in resonse to salt stress, but generally to lower maximum levels than their halophytic relatives (Wyn Jones et al., 1977b).

Recent investigations have demonstrated that many mesophytic crops are able to lower solute potentials by accumulating solutes when

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subjected to water stress, i.e., show osmotic adjustment (Morgan, 1977; Jones and Turner, 1978; Turner and Jones, 1980; Wilson et al., 1980). Betaine has been found to accumulate in response to water stress in several mesophytes, notably the cereals barley, wheat, and rye (Hitz and Hanson, 1980), the grasses, green panic and buffel grass (Ford and Wilson, 1980) and spinach (Pan et al., 1981); In barley and wheat, betaine is apparently a metabolic end product accumulated by stressed young leaves as a result of enhanced in situ synthesis and translocation from older leaves (Bowman and Rohringer, 1970; Hitz et al., 1982; Section 1.3 and 2.3). In one cultivar of barley grown under non-irrigated conditions in the field, betaine levels in the uppermost leaves reached 200 μmol/g dry wt (Section 2.3) which approaches the concentrations observed in some halophytes (Wyn Jones et al., 1977b; Ahmad et al., 1981).

An adaptive role for betaine accumulation in water stressed cereals has been postulated essentially by extrapolation from (a) the association of betaine with ecological halotolerance (Wyn Jones et al., 1977b) and (b) from the protection afforded by betaine to bacteria growing in NaCl (Shkedy-Vinkler and Avi-Dor, 1975). There is now some evidence from field studies of barley in dry environments (Hitz et al., 1982) that is consistent with an adaptive role for betaine accumulation.

One approach to assessing the adaptive worth of a metabolic trait, such as betaine accumulation, is to perform physiological-genetic studies similar to those undertaken to evaluate the adaptive

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role of proline in barley (Hanson et al., 1977) and rapeseed (Richards and Thurling, 1979) and of abscisic acid in wheat (Quarrie, 1980b). The objective of this research was to evaluate the genetic variability for betaine accumulation in cultivated barley and its progenitor, Hordeum spontaneum, and so determine if such a physiological-genetic approach would be feasible. The investigation was limited to H. vulgare and H. spontaneum as crosses involving these two species have been highly successful with no pronounced sterility or abnormal segregation ratios (Price, 1968); among the other species of the genus Hordeum a considerable degree of incompatibility exists and interspecific crosses resulting in fertile hybrids are rare (Price, 1968).

3.2 Materials and Methods

3.2.1 Plant Material

Seed for both field and laboratory tests was obtained from the USDA Small Grains Collection (Beltsville, Md.). CI 13626 (cv. Arimar), CI 11806 (cv. Proctor), and CI 11509 (cv. Excelsior), originally from the USDA collection, were multiplied in 1979 at sites in E. Lansing, Mi. Seed for laboratory studies was used untreated. Seed was treated with Vitavax-200 (2.5 g/kg, Uniroyal, Naugatuck, Ct.) before planting in field trials.

3.2.2 Betaine Determination

All plant material was freeze-dried. Whole shoots of laboratory-grown plants were weighed and ground in a Wiley Mill to pass mesh size 40. Accurately weighed subsamples (18-21 mg) were

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extracted by boiling in 5 ml water for 1 h and standing overnight at 4°C. Flag-1 leaf blades from field-grown plants were individually weighed and extracted without grinding. Betaine was estimated using the 2-column ion exchange/gas chromatography-pyrolysis procedure described by Hitz and Hanson (1980).

3.2.3 Water Potential (ψ) and Solute Potential (ψ_s) Measurements

In laboratory trials ψ was measured on the blade of the second leaf and in field studies on the blade of either the youngest mature leaf or the flag-1 leaf, using a pressure chamber (PMS Instrument Co., Corvallis, Or.). To reduce water loss during the measurements, blades were wrapped in damp paper towels. For ψ_s measurements, duplicate or triplicate 8-mm disks were cut from the mid-blade position of leaves, wrapped in foil, frozen in liquid N_2 and then thawed. A Wescor HR-33T dewpoint micro-voltmeter was used to estimate ψ_s on the frozen-thawed disks. It should be noted that ψ_s estimated in this way is actually more accurately described as the sum of solute potential and matric potential. However, it is reasonable to assume that matric potential is small ($\leq$ -2 bars) in relation to ψ_s, and that it does not differ markedly among the cultivars tested, so that the over-estimation of ψ_s was a minor and consistent effect across cultivars.

3.2.4 Controlled Environment Tests

Growth chamber conditions for all tests were: 16-h day, PAR 5 mW cm^{-2}, 21°C, relative humidity 70%; 8-h night, 16°C, relative humidity 85%. The plants were watered on alternate days with

half-strength Hoagland's nutrient medium. The statistical design for all trials was a partially balanced (3 replicates) 7 x 7 lattice square (Cochran and Cox, 1957). The replications were conducted sequentially, not concurrently, due to limited space in the growth chamber.

A total of 147 entries, in 3 sets of 49, were screened for betaine concentration in whole shoot tissue in 3 independent trials (A, B, C). Within each trial, genotypes were assessed for betaine concentration under well-watered and water-stressed conditions in two sub-trials which were conducted concurrently. In each replicate, the 49 genotypes were planted in completely different groups of 7; the groups of 7 in irrigated and water-stressed sub-trials of each replicate were the same. This design was adopted to reduce the effects of genotypic differences in pot water-depletion and plant water status that occur as stress develops (Quarrie and Jones, 1979; Hanson and Hitz, 1982), by ensuring that each genotype had a different set of neighbors in each replicate.

Trial A comprised 28 genotypes of H. spontaneum and 20 cultivars of H. vulgare selected for their purported adaptation to diverse environmental conditions (high salt, high altitude, high temperature, dry or moist conditions). Trial B included 42 genotypes of H. spontaneum and 7 cultivars of H. vulgare chosen from diverse geographical locations. Trial C comprised 48 cultivars of H. vulgare from 32 countries. Both CI 11806 (Proctor) and CI 13626 (Arimar) were included as checks in each trial to enable inter-trial comparisons. In Trials A and C cv. Proctor was entered twice.

Unglazed clay pots (15 cm diam.) were divided into 7 sectors marked off with plastic strips. In Trials A and B, seed was stratified in darkness at 4°C on moist filter paper for one week in order to break dormancy, and transferred at room temperature for 3 days to allow germination. Sprouting seeds were then planted in a soil mixture (peat:loam:sand; 1:1:2 v/v), placing 3 seeds of one genotype per sector. In Trial C, 3 seeds per sector were planted directly into the soil mixture. In all trials, CI 11509 (Excelsior) was planted in the center of each pot; the water status of Excelsior served as a biological "benchmark" for the severity of water stress. After 8 days the seedlings were thinned to one per sector. The well-watered seedlings were harvested 16 days after planting (DAP; 4-leaf stage), at which time water-withholding from the pots of the water-stressed trial began. The water-stressed plants were harvested 22 DAP; they had been wilted for 4 to 5 days.

3.2.5 Field Experiment

The 8 cultivars with the lowest and the 8 cultivars with the highest betaine concentration after stress in laboratory Trial C (Table 20) were chosen to assess the variability for betaine accumulation among genotypes under non-irrigated conditions in the field. Growth habit and length of growing season of the cultivars were unknown and so to ensure that at least 6-10 cultivars would be developmentally similar, and hence comparable with respect to water status and to betaine level, 16 cultivars were planted. In fact, it was necessary to omit only three of the cultivars (CI 11806,

TABLE 20. Designation and origin of barley cultivars tested for betaine accumulation in non-irrigated field conditions.

CI Number[§]	Provenance	Class[†]
High-Betaine		
14936	Ethiopia	1
1114	Peru	2
6577	Afghanistan	2
3480	Syria	1
11961	Morocco	2
13626	USA	1
Low-Betaine		
10064	Ecuador	1
2318	India	1
10138	Afghanistan	1
10359	Indonesia	1
709	USSR	3
13057	USSR	3
9309	China	2

[§]Cultivars chosen were the 6 highest and 7 lowest accumulators under stress in laboratory trial C; ranges for betaine concentrations were: 6 highest, 76-83 μmol/g dry wt; 7 lowest, 51-54 μmol/g dry wt.

[†]The class designation refers to the betaine accumulation pattern in the flag-1 leaf blade under field conditions.

CI 1610, and CI 742) because they were markedly later in maturity; CI 742 remained vegetative.

Preplant fertilization was 52 kg/ha N, 46 kg/ha K and 7 kg/ha Mn in accordance with results from soil analysis. Seed was planted on April 23, 1981, under 2 rain-out shelters (2.1 m x 5.7 m; as described in Section 2.2.1) in E. Lansing, Mi., on a Spinks sandy mixed mesic soil (Psammentic-Hapludalfs).

The land under each shelter was divided into two 2.6 m blocks separated by a path. Each block was then subdivided into two sections which were also separated by a path. In each section there were 12 1-m rows, 17.8 cm apart; seed was planted at a density of 50 per meter of row. Each cultivar was entered once per block in a design in which no two cultivars were adjacent in more than one block. Two guard rows of barley were planted around the plots in each shelter. No irrigation was given after seedlings were established.

Water potential and ψ_s are known to undergo diurnal variation (Hsiao et al., 1976). Sampling all 4 blocks required about 3 h and so to ascertain whether ψ_s would vary more during the morning or early afternoon, ψ and ψ_s measurements were taken on the blades of the youngest mature leaf (5 replicates) of Proctor and Arimar 47 DAP at 9 a.m., 1 p.m. and 3:30 p.m. E.S.T. The differences between ψ_s at 1 p.m. and 3:30 p.m. were less than during the morning (Table 21); hence, samples were taken in the early afternoon. Water potential, as expected, varied throughout the day.

TABLE 21. Variation in ψ and ψ_s in the youngest mature leaf of Arimar and Proctor Barley

Time of Day	Cultivar§			
	Proctor		Arimar	
	ψ	ψ_s†	ψ	ψ_s
	bars			
9:00 a.m.	-4.6	-11.0	-8.5	-13.0
1:00 p.m.	-9.3	-12.0	-14.0	-14.2
3:30 p.m.	-6.2	-12.5	-10.1	-14.5
$LSD_{0.05}$	2.9	0.5	2.7	1.1

§5 replicates at each date.

†Triplicate samples on each leaf.

Blocks were sampled at 41, 59, and 63 DAP. Four replicates, one from each block, were taken at each sampling date; all entries in each block were harvested together. Water potential measurements were made on the youngest mature leaf blade at 41 DAP; and on the flag-1 leaf blade at 59 and 63 DAP. The same leaves were then transported on ice to the laboratory for ψ_s measurements, followed by freeze-drying for betaine assay.

3.3 Results

3.3.1 Results of Controlled Environment Tests

In all three trials, there was statistically significant variation in betaine concentration amongst genotypes both before

and after water-stress (Fig. 21); for details of genotypes included in each trial, and their average pre- and post-stress shoot betaine concentrations, see Appendix B. Insofar as environmental conditions were very similar for all cultivars and trials, a genetic component in the cultivar variance is implied (Hanson and Hitz, 1982).

Under well-watered conditions, the betaine concentrations within the cultivars Proctor and Arimar were not significantly different in all three trials (Table 22) so that it was reasonable for purposes of comparison to combine the results of all 3 well-watered trials (Fig. 21, uppermost frame). The severity of water-stress varied between trials, and there were significant differences in betaine levels within the cultivars Proctor and Arimar between trials (Table 22); the highest betaine levels were recorded in trial C, the lowest in trial A. Such differences between water-stressed trials might be anticipated as betaine accumulation in barley seedlings is proportional in a general way to the severity and duration of stress in the range -10 to -20 bars (Wyn Jones and Storey, 1978; Hanson and Nelsen, 1978; Hanson and Scott, 1980). These differences in betaine levels between trials for both Proctor and Arimar were consistent with the ψ data for the benchmark cultivar, Excelsior. In trial C, the benchmark ψ value (-27 bars) was lower than trial A (-16 bars). As direct comparison between water-stressed trials is therefore precluded, results from each trial are shown separately (Fig. 21, lower frames).

In analyzing the primary data of Figure 21, two questions were asked:

Fig. 21. Frequency distributions for betaine concentration in cultivated and wild barley genotypes entered in 3 laboratory trials (A, B, C). The uppermost frame summarizes data for the well-watered seedlings from all 3 trials. The lower 3 frames are data for water-stressed seedlings in the individual trials. Average ψ values of the benchmark plants (cv. Excelsior) in the trials are indicated. The vertical arrows show the means, and the horizontal bars indicate the $LSD_{0.05}$ for the population sampled. One $LSD_{0.05}$ value has been given in the uppermost frame as values were the same among well-watered trials.

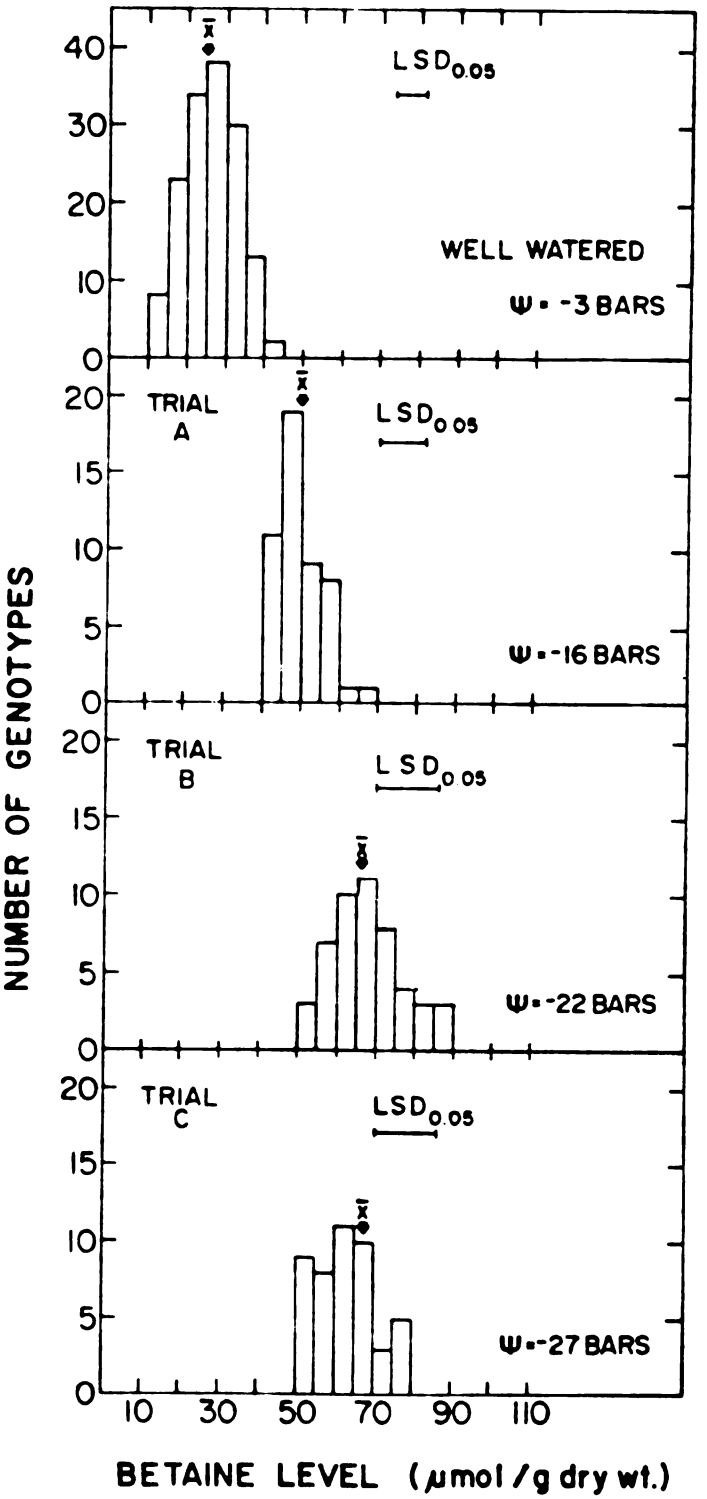

NUMBER OF GENOTYPES
BETAINE LEVEL (µmol/g dry wt.)
WELL WATERED
Ψ = -3 BARS
TRIAL A
Ψ = -16 BARS
TRIAL B
Ψ = -22 BARS
TRIAL C
Ψ = -27 BARS
LSD0.05
0
5
10
15
20
30
40
50
70
90
110

TABLE 22. Shoot betaine concentrations of Proctor and Arimar in laboratory trials A, B, and C.

Cultivar	Treatment	Shoot Betaine Concentration[§]		
		Trial		
		A	B	C
		μmol/g dry wt		
Proctor	Well-watered	11.7[a]	16.0[a]	18.0[a]
	Well-stressed	44.3[cd]	52.0[d]	52.7[d]
Arimar	Well-watered	25.7[ab]	33.3[bc]	34.3[bc]
	Water-stressed	49.7[d]	67.0[e]	83.7[f]

[§]Mean of 3 replicates. Values followed by the same letter are not significantly different ($p < 0.05$) according to Duncan's Multiple Range Test; comparisons made between and within columns.

1. Is the betaine level in water-stressed seedlings (y) related to the betaine level under well-watered conditions (x)? If so, additional screening for high- and low-accumulators might be based solely on trials with unstressed seedlings. Betaine levels in water-stressed seedlings were significantly correlated with the betaine levels of well-watered seedlings (Table 23); this relationship between the pre- and post-stress betaine levels was stronger in the two trials in which the water-stressed sub-trials reached the lowest ψ values (Table 23). The data did not permit a distinction to be drawn between the accumulation of the same amount of betaine by all genotypes and the accumulation of various amounts of betaine proportional to the basal level characteristic of the genotype

TABLE 23. The relationship between the betaine concentration in the shoots of water-stressed (y) and well-watered (x) seedlings of barley in 3 laboratory screening trials.

Trial	Regression Equation	Correlation Coefficient	Average[§] Ratio ± s_d	Average[†] Increase ± s_d
A	y = 0.38x + 41.5	0.29*	2.8 ± 0.7	29.6 ± 6.1
B	y = 1.27x + 27.95	0.72**	2.2 ± 0.3	35.6 ± 7.5
C	y = 1.10x + 35.13	0.64**	2.5 ± 0.4	37.7 ± 7.0

*,** Indicate significance at p = 0.05 and p = 0.01, respectively.

[§] Average Ratio = average value of $\left(\frac{\text{Betaine concentration after stress}}{\text{Betaine concentration before stress}}\right)$ among genotypes in individual trials.

[†] Average Increase = average value of (betaine concentration after stress - betaine concentration before stress) among genotypes in individual trials.

(Table 23). Either or both of these possibilities could underlie the positive correlation noted between betaine levels before and after stress.

2. Is the variation in betaine level normally distributed? Assuming the genotypes sampled were representative of the whole germplasm and if the distribution of betaine levels was not significantly different from normal then the data could be used as a base to predict the number of additional genotypes that must be screened in order to identify types separated by several multiples of the s_d and lying outside the range already sampled. The two- to three-fold range in betaine levels actually found among the genotypes tested is probably only barely sufficient for an evaluation of the value of betaine accumulation using a physiological-genetic approach.

The range of betaine levels ($\bar{x}$ = 25.7 µmol/g dry wt; s_d = 6.9 µmol/g dry wt) in well-watered seedlings conformed to a normal distribution (X^2 = 7.1, 4 df n.s.; skewness = -0.0013, n.s.). It follows that about one thousand additional genotypes would have to be screened in order to identify a pair with a 6-fold difference in betaine level.

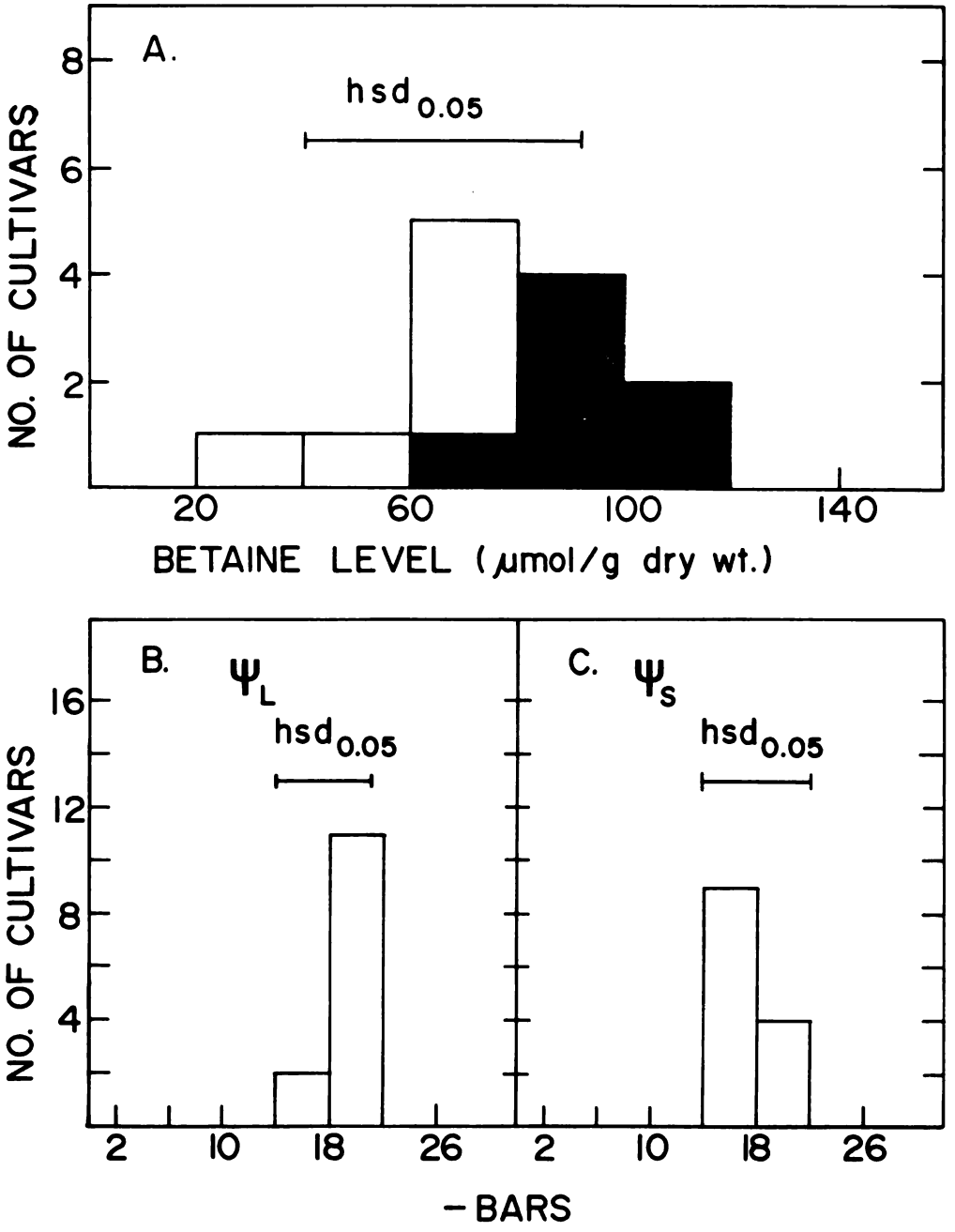
In view of the diversity for other secondary nitrogenous compounds common among species of the same genus (Jeffrey and Tso, 1964; Waller and Nowacki, 1978; Hanson et al., 1981) the narrow range of betaine concentration uncovered was somewhat unexpected as the genotypes investigated were genetically diverse and were developed for, or collected in, widely differing geographical locations. There was no clear association between betaine concentration and provenance,

or habitual area of cultivation, e.g., low betaine accumulators were represented among genotypes from both moist and arid environments. However, the relationship between overall adaptation to the environment and the expression of an individual metabolic trait is likely to be tenuous; morphological and phenological characteristics probably have a far larger influence in determining the overall adaptation of a genotype to a given environment (Passioura, 1976; Hanson and Hitz, 1982).

3.3.2 Results of Field Study

All the cultivars included in the field study were at comparable developmental stages at all three sampling dates. On the first sampling date (41 DAP) there were significant differences for ψ_s among cultivars but no differences among cultivars for betaine level. At the two later sampling dates (59 and 63 DAP) there were no significant differences among cultivars for ψ and ψ_s but there were significant differences for betaine level (see Appendix B for water status and betaine levels of cultivars at all three sampling dates). With the exception of CI 13056 all genotypes had higher betaine concentrations in stressed flag-1 leaf blades than in stressed seedling shoots. The patterns of betaine accumulation among cultivars grown in the field fell into three classes (Table 20). In Class 1 betaine continued to accumulate in the flag-1 leaf blades throughout the season. In Class 2 the betaine concentration reached a maximum at about one week after anthesis and then declined. In Class 3 there was no appreciable accumulation of betaine after 41 DAP by the flag-1 leaf blades.

Fig. 22. Frequency distributions for 13 barley cultivars: A, betaine concentration; B, ψ; C, ψ_s. Measurements were made 59 DAP on flag-1 leaf blades in non-irrigated field-grown plants. The shaded areas in A identify the six genotypes which were the highest betaine accumulators in laboratory trial C (see Table 20). Horizontal bars indicate the honestly significant differences. [The Tukey Multiple Comparison Test, in which the error rate applies on an experimentwise, rather than a per comparison basis, was chosen to determine the significance of differences observed as it keeps the probability of a type I error small and the aim of this research was to make the most conservative estimate of the variability for betaine concentrations among genotypes.]


A.
hsd 0.05
NO. OF CULTIVARS
8
6
4
2
20
60
100
140
BETAINE LEVEL (μmol/g dry wt.)
B. ψL
hsd 0.05
C. ψs
hsd 0.05
NO. OF CULTIVARS
16
12
8
4
2
10
18
26
-BARS

Insofar as water stress is probably the principal factor causing betaine accumulation in these experiments (Section 2.3.2), the variation observed among lines at the same ψ_s and ψ is likely to represent true genetic variation (Hanson and Hitz, 1982); variation in ψ_s and ψ between cultivars within sampling dates cannot account for the differences in betaine levels. The decrease in concentration of betaine in flag-1 leaf blades of cultivars of Class 2 may be due to translocation of betaine from the blade to the sheath and culm late in the grain filling period (Section 2.3.2.2).

The non-significant differences between ψ_s along with the significant differences for betaine level could, in principle, be a statistical artifact arising from differences in the precision of the estimates of these parameters. This was probably not the case, insofar as the coefficients of variability for measurements of betaine level and ψ_s were 3.4%, and 3.0%, respectively.

Data for the second sampling date (59 DAP; Fig. 22) were selected for detailed analysis because all cultivars were in mid-grain fill, none had lost betaine relative to the first sampling date, and none showed any sign of senescence in the leaves sampled for betaine. At the second sampling date, there was significant correlation ($r = 0.71^{**}$) between the betaine levels in the flag-1 leaf blades and the betaine levels in unstressed seedling shoots. The shaded areas in the frequency distribution of betaine levels in leaves of mature plants in Figure 22 (Frame A) correspond to those cultivars that had high levels of betaine as well-watered seedlings.

The lower frames of Figure 22 indicate the ψ_s and ψ values of the cultivars sampled 59 DAP.

There were no obvious differences between cultivars for performance during stress; no relationship between morphological symptoms of stress (e.g., leaf curling, firing, or wilting) and betaine accumulation was apparent.

3.4 Discussion

In this investigation, genetic variability for betaine concentration in non-stressed and water-stressed barley was evaluated in seedlings grown in pots in controlled environment chambers, and in mature plants grown in the field. Two difficulties beset pot experiments to evaluate metabolic responses of plants to water stress. First, genetic variation for rate of soil water depletion [caused, for example, by variation in root/shoot ratios (Quarrie and Jones, 1979)] can confuse interpretation of results. Second, the relatively rapid imposition of stress on seedlings may not elicit the same metabolic response as a long-term drought in a mature plant in the field. With these problems in view, the pot experiment of this study was designed to nullify or reduce the effects of unequal rates of soil water depletion between genotypes; the field experiment served to determine if the betaine levels of pot-grown seedlings were likely to be reliable predictors of betaine levels in leaf blades of mature plants subjected to water-stress in the field.

In all the laboratory trials, pre- and post-stress betaine levels were positively and significantly correlated, but this does

not exclude the possibility that there was genetic variability either for the threshold water deficit required for the onset of betaine accumulation or for the rate of accumulation. For example, a genotype with a low overall betaine accumulating potential, but one that began accelerated betaine synthesis at -10 bars, could conceivably have a higher betaine content at -16 bars than a high overall betaine-accumulator that only began betaine accumulation at a threshold ψ of -14 bars. The field results not only demonstrated cultivar differences for the maximum levels of betaine reached during prolonged stress, but also provided some evidence for variation in the onset and rate of accumulation. As well as grossly different patterns of betaine accumulation among cultivars that could be broadly categorized (Table 20), there were subtle differences in patterns of betaine accumulation within classes. For example, within Class 1, in which betaine continued to accumulate throughout the season, the rates of accumulation among culitvars differed; betaine accumulated steadily in some cultivars between the first and last sampling dates, whereas in other cultivars, the betaine that accumulated between the second and last sampling dates was much more than between the first and second dates (see Appendix B, Table B-4, for further details). The range of betaine concentration among diverse genotypes, although significant, was relatively narrow; thus betaine was never absent or at a low level. This encourages the speculation that the synthesis of betaine plays some significant metabolic role, and that betaine is not merely a functionless secondary product.

Cultivars having both high and low levels of betaine apparently developed normally and filled similar numbers of grains per plant under the simulated dry land conditions of the field experiment. These results might be construed as evidence that high levels of betaine did not improve performance (grain fill) under water stress. The problem with this interpretation is that the effect of an individual metabolic trait in any situation is likely to be small, and so may only enhance the fitness (reproductive output) of a plant by a few percent (Swain, 1977). Thus, although the advantage of a metabolic response like betaine accumulation might become apparent among genotypes of natural populations over an evolutionary time scale, in a single field experiment an advantage in grain yield might be too small to detect.

Both severity and duration of water-stress can affect betaine accumulation (Section 2.3). Although the variation in betaine levels in the field was not a function of differences in instantaneous ψ_s, variation in duration of water stress among cultivars in the field cannot be ruled out as an explanation of the betaine variation. However, there was significant variation for betaine in unstressed plants in laboratory trials, where plant water status was uniformly high. It is therefore probable that the observed variability for betaine levels in both field and laboratory represents true genetic variation in plant metabolism and <u>not</u> merely apparent variation for metabolism underlain by variation in plant water status (Quarrie and Jones, 1979; Hitz and Hanson, 1982). Wyn Jones et al. (1977a) indicated

that there was a positive correlation between ψ_s and betaine level among many species of the Gramineae. Within the range of ψ_s values measured in this study there was no indication of such a relationship; when ψ_s varied between cultivars (41 DAP) there were no significant differences for betaine level, and at the later sampling dates the converse was true, i.e., there were significant differences for betaine levels among cultivars having the same ψ_s.

In the absence of specific inhibitors of betaine synthesis, physiological-genetic experiments appear to be the most promising way of determining the adaptive significance of betaine accumulation in barley. If intensive screening of genotypes were to be undertaken, a logical preliminary step to identify potentially high and low accumulators would be to examine unstressed seedlings. Testing of chosen genotypes could then be extended to water-stressed conditions. It should be noted that although the betaine concentrations of mature leaves of field-grown plants were correlated with the betaine concentrations in well-watered seedling shoots of the same genotypes, by screening seedlings it would be impossible to identify some potentially interesting genotypes, such as CI 13056, which had ceased accumulating betaine by 41 DAP in the field.

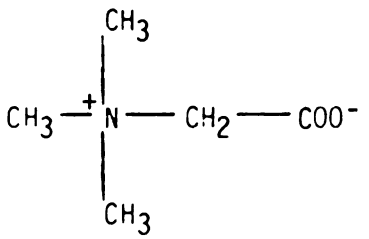
In view of the comparatively narrow range in betaine concentration observed among genotypes, generating greater variability through breeding, e.g., exploiting the possibilities of transgressive segregation (Quarrie, 1981), may be appropriate.

CHAPTER IV

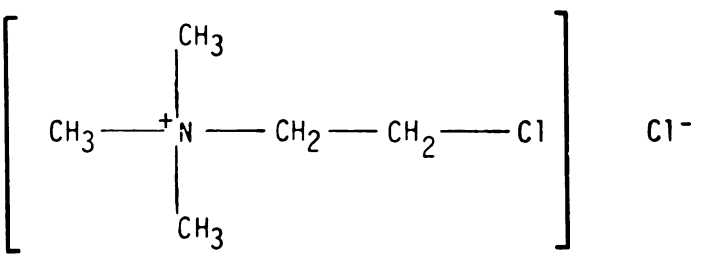
THE EFFECT OF EXOGENOUS BETAINE ON GROWTH AND BETAINE SYNTHESIS IN BARLEY SEEDLINGS

4.1 Introduction

Three hypotheses that rationalize the accumulation of betaine by stressed plants are: (1) Betaine acts as a cytoplasmic osmoticum (Wyn Jones et al., 1977a; Flowers and Hall, 1978; Hamza, 1980; Cavalieri and Huang, 1981); (2) Betaine serves as a protectant for enzymes, membranes and organelles under stress conditions (Schobert, 1969; Rafaeli-Eskhol and Avi-Dor, 1968; Paleg et al., 1980; Nash et al., 1980); (3) Betaine serves as a growth regulator (Wheeler, 1969); this hypothesis is based on the observation that many natural and synthetic quaternary ammonium compounds (QACs) are biologically active (Karanov, 1979). It has been claimed that application of (2-choloroethyl) trimethyl ammonium chloride (CCC), a QAC structurally similar to betaine (Fig. 23), can increase the drought resistance of wheat crops (El Damaty et al., 1965). Since CCC is known to reduce vegetative growth (Larter et al., 1965; Larter, 1967; Lovett and Kirby, 1971; Jung, 1978) it may be that its application tends to impose water conservation by lowering leaf area; this could account for CCC effects on crop drought-resistance. By analogy with other QACs, and in particular with CCC, betaine may thus have growth



Betaine



CCC

Fig. 23. The structures of betaine and CCC.

regulating activity. Perhaps the betaine which accumulates during stress contributes to the morphological changes (e.g., reduction in plant size) that are associated with water conservation.

The following question was asked: Would an external supply of betaine to turgid plants, resulting in high but physiological internal betaine levels have favorable effects on subsequent plant performance under water-stressed conditions? This type of approach, i.e., supplying a compound to a plant in order to investigate its role _in vivo_, is most useful when the supplied compound is not degraded and when, for reasons that are genetic or due to prior application of specific chemical inhibitors, there is a negligible amount of the compound in the treated plant at the time of feeding. Betaine and choline were reported to stimulate the germination of maize seedlings under saline conditions (Wyn Jones et al., 1974). However, the extent of microbial contamination, and thus possible breakdown of betaine, was questionable and in order to interpret results from experiments where exogenous compounds are fed, the importance of sterility, and confirmation that the fed compound is not metabolized either before or after entering the plant, is paramount.

In addition, even if sterility is confirmed and the compound in question is identified within appropriate plant organs, conclusions drawn from feeding studies will be beset by potential errors arising from subcellular compartmentation differences between the exogenous compound and the metabolite synthesized _in situ_.

Different internal levels of betaine in seedlings not only permit an approach to the question of the adaptive value of betaine

under stress but the condition may be exploited to investigate if a feedback regulation of betaine synthesis operates: Does an external supply of betaine suppress internal synthesis? The synthesis of proline, another metabolite that accumulates during water-stress is subject to feedback inhibition in irrigated barley (Boggess et al., 1976a). In wilted barley the feedback inhibition system is inactive (Boggess et al., 1976a) which may reflect a deleterious breakdown in the normal regulatory system of a healthy cell (Stewart and Hanson, 1980). Studies with non-irrigated field grown barley indicated that the termination of betaine synthesis was not due to either relief of stress nor to the onset of senescence (Hitz et al., 1982). Hence if retroinhibition of betaine is indicated, it may be proposed that betaine accumulation is not a disintegrative process associated with eventual stress-induced death but that its synthesis is related to adaptive metabolic changes in response to the stress.

Thence, the objective of this research was to: (1) Evaluate the effects of betaine on growth and performance of barley seedlings under stress; (2) Determine if feedback inhibition of betaine synthesis operates in well-watered seedlings.

4.2 Materials and Methods

4.2.1 Plant Material and Seed Sterilization Procedures

Barley seed (cv. Proctor, CI 11806) was originally from the U.S.D.A. Small Grains Collection (Beltsville, Md.). Seed used in experiments 1-3 (inclusive) was multiplied in 1977 in East Lansing, Mi. Seed used in experiments 4 and 5 and in [2-^{14}C]ethanolamine

labeling experiments was multiplied in 1978 in Mesa, Ariz. Proctor barley was chosen because,compared to other cultivars, it has a low betaine concentration under both irrigated and water-stressed conditions (Section 3.3).

Two seed sterilization procedures were used. For embryo culture (experiments 1-3): the outer husk was carefully removed and seeds were incubated 1.5 h with 0.56% Na hypochlorite (1 vol. Chlorox + 9 vol. H_2O). After rinsing with sterile distilled water, the seeds were left a further 24 h in 0.1% Na hypochlorite (1 vol Chlorox + 49 vol H_2O). At both sterilization steps, Tween-20 (0.5% v/v; Sigma Chemical Co., St. Louis, Mo.) was used as a wetting agent. The seeds were finally rinsed with sterile distilled water and the embryos dissected from endosperm.

For experiments using intact seeds (experiments 4 - 7): the husk was thoroughly scraped off the whole seed with a scalpel and the seed then washed in ethanol (70% v/v; 7 min, continuous stirring), followed by 5.26% Na hypochlorite (concentrated cholrox) plus Tween 20 (0.5% v/v; 10 min, continuous stirring). Seeds were then rinsed in 4 changes of sterile distilled water (Bailey, 1980).

4.2.2 Media

Wick's medium (Steidl, 1976) B 5 complete medium (Gamborg and Wetter, 1975), and Murashige and Skoog's (MS) medium (1962) minus their respective plant hormones were tested for their suitability for use in mature barley embryo culture. For embryo culture, agar (0.9% w/v; Bacto Difco, Detroit, Mi.) was included in media.

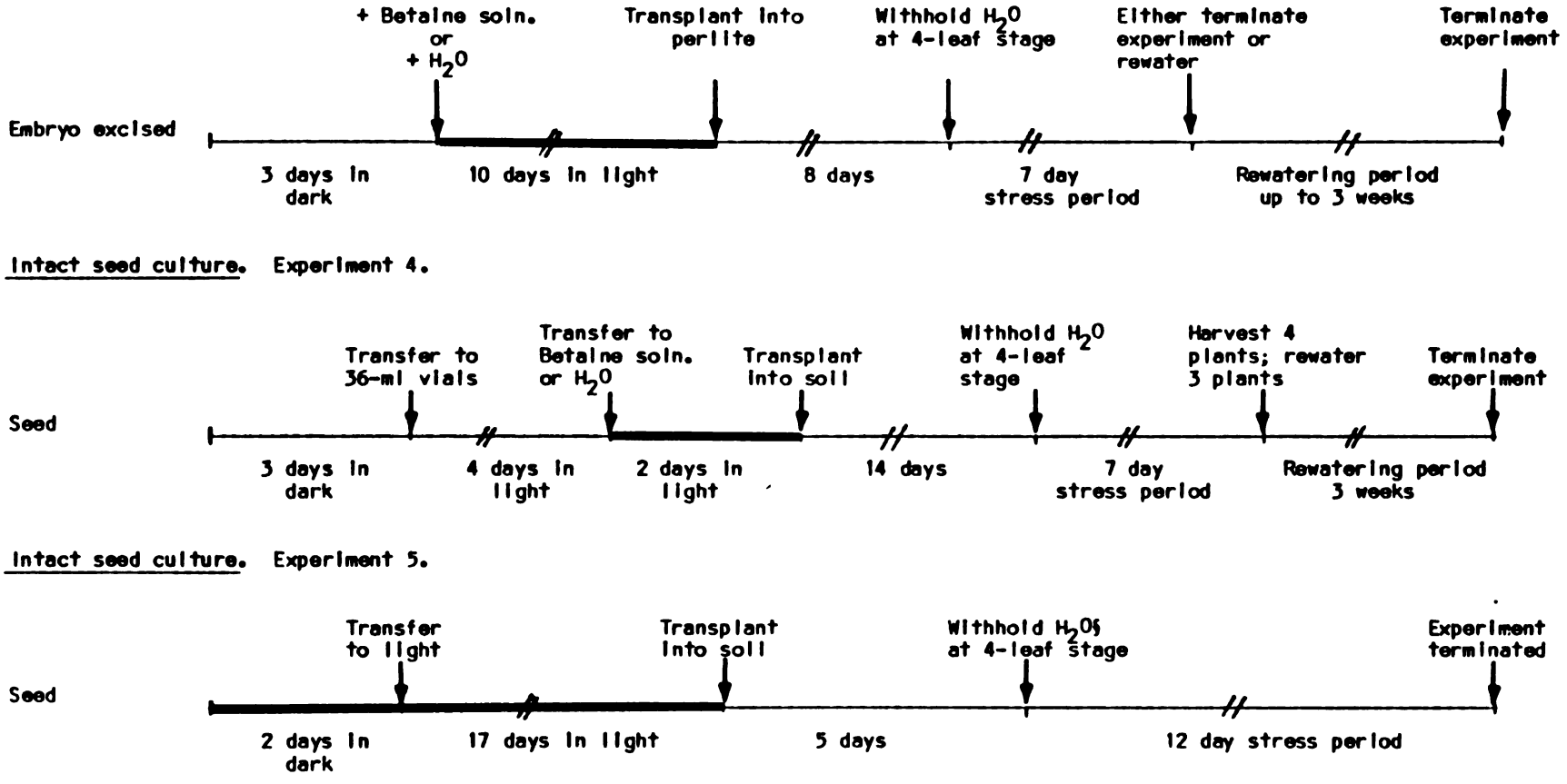
When intact seeds were used, half-strength and quarter-strength Hoagland's solution was adequate for robust seedling development (for constituents of media, see Appendix C).

Sucrose was found to be essential for vigorous seedling development from excised embryos. In the absence of sucrose only 4 out of 31 embryos grew into plantlets as compared to 27 out of 31 when sugar was included in the medium. Embryos germinated and developed on Wick's, B 5 and MS media, plus or minus betaine supplements, equally well. Presumably because the embryos were mature, media pH values higher than 5.2 did not interfere with development (Narayanaswami and Norstog, 1964).

Contamination of media was tested by incubating aliquots of liquid media (0.2 - 1 ml) or 3 mm^3-cubes of agar media on either 0.08% w/v or 0.8% w/v nutrient broth (Difco Labs, Detroit, Mi.; 1% agar) plates for 3-5 days in the dark at 25°C. More specific tests for bacterial contamination using bacto-typtone or bacto-peptone based media were made if contamination was suspected but not confirmed after incubation on nutrient broth-agar (see Appendix C for media formulations). All contaminated plantlets were discarded.

4.2.3 Plant Culture Conditions

For clarity, the chronological order of culture and stress events is outlined in Figure 24. Essentially, seedlings were exposed to betaine under sterile or semi-sterile conditions and were then transplanted into either perlite (Chem Rock Corp., Tenn.) or a soil mix (Section 1.2.1). Seedlings were irrigated alternate days until



Period in which roots were exposed to betaine; in experiment 5 as soon germinated seedlings were exposed to betaine as soon as roots contacted nutrient solution.

§ Time at which 2-^{14}Clethanolamine feeding studies were carried out.

Fig. 24. Schemes describing chronological order of culture and stress events for seedlings grown from embryo culture.

they reached the 4-leaf stage (fourth leaf not fully expanded); water stress was then imposed by withholding water for either 7 or 12 days. During stress periods, aluminum foil, fitting closely around seedling culms, covered the soil surface. At the end of the stress period some plants were rewatered for up to three weeks to determine if pretreatment with betaine was beneficial to recovery after water stress.

4.2.3.1 Embryos. Ten-ml aliquots of media were dispensed into 75-ml boiling tubes (2 cm diam.); agar was predissolved by autoclaving media for 5 min (121°C). Tubes were capped with a wad of cotton wool which was covered by aluminum foil (Fig. 25A) and autoclaved (121°C; 20 min). The embryos (1 per tube) were set gently on the agar surface. In order to reduce the amount of light to reach the roots the bottom of the tubes were wrapped with aluminum foil. After the embryos were left 3 days in the dark (25°C), either water alone (0.5 ml) or betaine (OH^-) solution (15.3 or 153 µmol betaine/0.5 ml H_2O/tube) that had been sterilized by Millipore filter (mesh size 0.22 µm; Millipore Corp. Bedford, Ma.) was added. At this time tubes were transferred to the light (16-h day; day/night temperature 22/16°C). After 10 days when plantlets were at the 2-leaf stage, they were individually transplanted into perlite (pot size, 7 cm diam. x 12 cm; 1 seedling/pot) and kept in the light as before (day/night relative humidity 70/85%). At the time of transplantation the agar media were tested for microbial contamination.

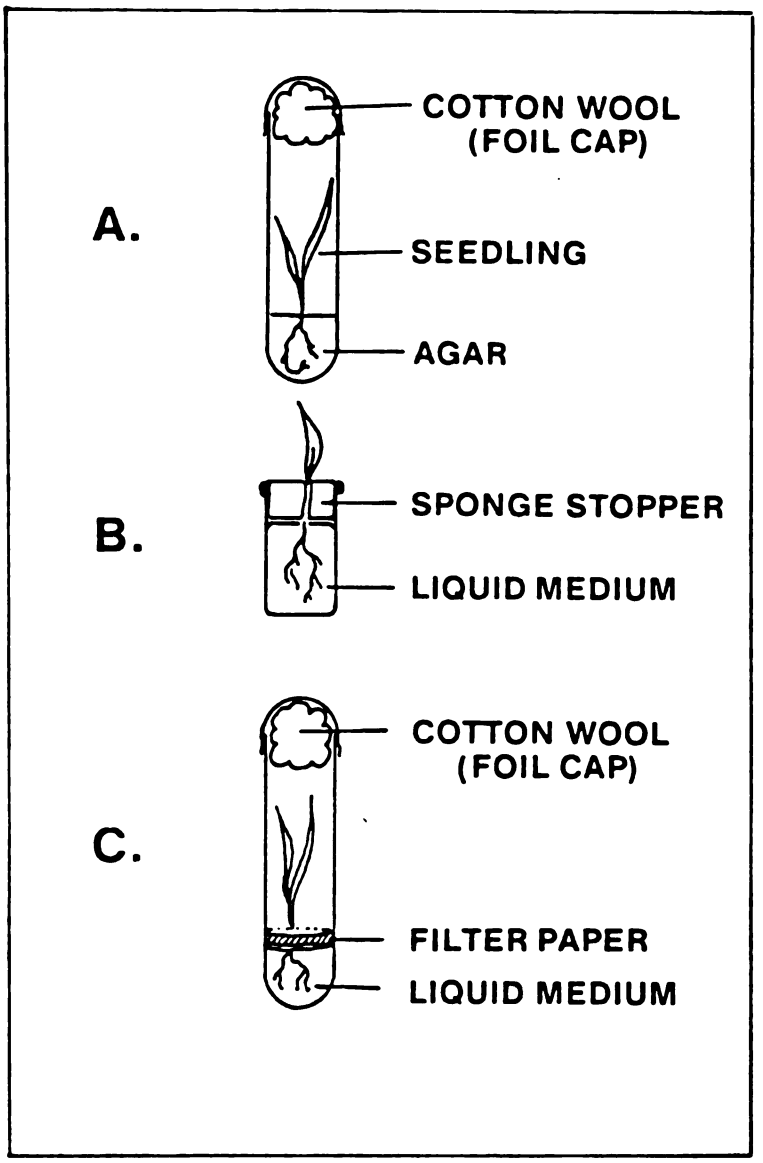


Fig. 25. Culture conditions for embryo (A) and intact seeds in semi-sterile (B) and sterile(C) conditions.

4.2.3.2 Intact seeds under semi-sterile conditions. Equipment and media were sterilized by autoclaving (121°C, 20 min). Betaine was not degraded upon autoclaving as shown by TLC and TLE analysis of sterilized media which had been supplemented with [^{14}C]-betaine (Section 1.4). Sterilized seeds were germinated on sterile filter paper for 3 days in the dark (25°C). The germinated seeds were individually placed between the 2 halves of a sponge stopper (0.5 cm thick, cut vertically to generate the 2 halves) and fitted into the top of 36-ml glass vials (Fig. 25B) into which had been measured 20 ml of quarter-strength Hoagland's solution. To prevent light reaching the roots, aluminum foil was wrapped around the vials. The seedlings were left in the growth chamber (16-h day, day/night temperature 22/16°C, relative humidity 70/85%) for 4 days. Then, still with seed secured by sponge, they were placed in 10-ml beakers which contained either water (3 ml) or betaine (OH^-) solution (27 mM, 3 ml). After 2 days, 2 to 2.5 ml of liquid had been taken up by the seedlings which were then transplanted into a soil mix (Section 1.2.1), one seedling per pot (7 cm diam. x 12 cm). Aliquots (0.3 - 1 ml) were taken from all root media and tested for microbial contamination. Seedlings were grown in the greenhouse under supplemental light (16-h day, day/night temperature 22/16°C, relative humidity 70 to 80%).

4.2.3.3 Intact seeds under sterile conditions. Half-strength Hoagland's solution (10 ml), plus either betaine (40 mM) in K^+-phosphate buffer (2 ml; pH 7, 20 mM) or K^+-phosphate buffer alone was measured into 75-ml boiling tubes (2 cm diam.). A circle of filter paper (Whatman #4; 2 cm diam.) was positioned about 0.5 cm above the

liquid surface (Fig. 25C). The intact seed was placed in a perforation (≤ 3 mm diam.) in the center of the filter paper. The tubes were closed with a wad of cotton wool and covered by aluminum foil. In order to reduce the amount of light reaching the roots, aluminum foil was wrapped around the bottom of the tubes. After 2 days in the dark at 25°C the tubes were transferred to the growth chamber (16-h day, day/night temperature 22/16°C). After 17 days the seedlings were transplanted into a soil mix (Section 1.2.1) in pots (7 cm diam. x 12 cm; 1 seedling/pot) and transferred to the greenhouse under supplemental light (16-h day, day/night temperature 22/16°C, relative humidity 70 to 80%). Before transplantation, nutrient media (1-ml aliquots) were tested for microbial contamination.

4.2.4 Incubation with [^{14}C]Compounds

In all experiments, [methyl-^{14}C]betaine (9.7 µCi/µmol; radiochemical purity > 99%; Amersham Corp., Ill.) was used to spike representative tubes of root feeding media that contained unlabeled betaine; 0.5 µCi of [^{14}C]betaine was added per tube. Uptake of betaine by seedlings was estimated from either ^{14}C-activity in the plant or ^{14}C-activity remaining in the root feeding solution, assuming: (1) There were no isotope effects; (2) Betaine was not degraded to volatile [^{14}C]products.

[2-^{14}C]Ethanolamine (44 µCi/µmol; radiochemical purity > 98.4%; Amersham Corp., Ill.) was used to investigate the effect of varying endogenous betaine levels on betaine synthesis in well-watered seedlings. The fully expanded, third-leaf blade was cut

10 cm up from the ligule. After waiting 5 min to check that guttation was not occurring from the cut end, the cut end was bevelled and a 1-μmol dose of [2-^{14}C]ethanolamine (1.2 μCi/μmol in experiment 6; 0.74 μCi/μmol in experiment 7) was fed to the underside of the cut end in a 3-μl droplet of K^+-phosphate buffer (pH 7, 20 mM). For further details of feeding procedure, see Hanson and Scott (1980).

The experiment was terminated 9 h after the radiochemical was applied by detaching the leaf blade plus the uppermost stem portion of leaf sheath from the seedling. The leaf tissue was weighed and frozen in liquid N_2.

4.2.5 Localization, Extraction and Separation of Labeled Metabolites

[Methyl-^{14}C]Betaine used as a "spike" in the root medium was localized in selected seedlings by autoradiography on SB-5 X-ray film (Eastman-Kodak, Rochester, N.Y.). The betaine in selected plants and in each root-feeding solution was confirmed to be undegraded by TLC and TLE analysis using the procedures described in Section 1.2.4.

In experiments with [2-^{14}C]ethanolamine, the frozen leaf blade, plus the uppermost 5-cm portion of leaf sheath was ground to a fine powder, boiled in 2 ml of isopropyl alcohol for 5 min, and then stored under N_2 at -20°C until further processing. Thereafter, extraction, separation, and determination of ^{14}C-labeled metabolites was essentially by the same ion-exchange/TLC-TLE procedures described by Hitz et al. (1981). The isopropanol phase was removed from the insoluble residue after centrifugation and the residue was then

further extracted by grinding with 2 ml chloroform-methanol (2:1, v/v) followed by centrifugation and removal of the solvent. This step was repeated. The chloroform-methanol extracts were combined with the isopropanol extract and partitioned once against $CaCl_2$ (1.2 ml, 20 mM) and twice against 1.2 ml aliquots from the upper phase from a chloroform:isopropanol:methanol:20 mM $CaCl_2$ (22:16:10:10, v/v) mixture. The washed chloroform-alcohol phase was diluted with 6 ml chloroform and an aqueous layer which separated out was aspirated off. The chloroform phase was dried over anhydrous Na_2SO_4 and then reduced to a small volume (about 0.3 ml) in a N_2 stream at 40 to 45°C. For isolation of water-soluble metabolites, the combined aqueous washes from the chloroform-alcohol phase were added back to the remaining isoluble residue and heated to 60°C for about 10 min, centrifuged to clear, and evaporated to dryness in a stream of N_2 at about 70°C.

The aqueous phase was separated by passage through three ion-exchange columns (1.5 ml each) arranged in sequence; AG-1 (OH^-), BioRex 70 (H^+), and AG-50 (H^+). The Ag-50 column was eluted with 6 ml NH_4OH (4N). AG-50 column eluates were reduced to dryness at about 80°C in a stream of N_2. The dried Ag-50 eluate was redissolved in water (100 µl).

[^{14}C]Betaine was separated from phosphorylcholine in the AG-50 eluate by TLE [on ITLC (Gelman Instruments), in 1.5 N formic acid at 2kv for 10 min. at 4°C].

Phospholipids in the chloroform-alcohol phase were separated by TLC on Silica Gel-G (Brinkmann) in a one-dimensional, two solvent

system. Samples equivalent to 0.25 leaf-segments were applied to TLC plates which were developed first in acetone:petroleum ether (3:1, v/v) followed by development in chloroform:methanol:glacial acetic acid:water (85:15:10:3.5, v/v).

^{14}C-labeled metabolites were located on TLC or TLE plates by autoradiography. For betaine the ^{14}C-activity in TLE zones was determined as described by Hanson and Scott (1980). ^{14}C in TLC zones was scraped into scintillation vials and shaken with 0.2 ml chloroform:methanol:ammonia (65:25:5 v/v) to which 10 ml dioxane based scintillant was then added.

4.2.6 Betaine Determination

In some plants of experiments 4 and 5 and in [^{14}C]ethanolamine-labeling experiments the betaine concentration of shoot and leaf tissue was determined by the ion exchange/pyrolysis chromatography method as described by Hitz and Hanson (1980).

In the ^{14}C-labeling experiments unlabeled blades comparable to those fed were fresh- and dry-weighed so that betaine concentration as "µmol/g fresh wt" of labeled plants could be converted to "µmol/g dry wt" [dry wt = 9.6 (±0.5)% fresh wt].

4.2.7 Statistical Analysis of Results

Where two treatments were involved, means and standard deviations of selected parameters were calculated and compared by Students "t" test. Where 3 or more treatments were compared, significance of results was tested by analysis of variance.

4.3 Results

4.3.1 Effects of Exogenous Betaine on Seedling Performance during Stress and Rewatering

Observations of the seedlings during and after water stress evaluated the effect of high internal levels of betaine on the following plant characters: (1) The time wilting first became evident; (2) The extent of leaf firing (the percentage of the total area of the fully expanded leaf blades that was fired); (3) Leaf blade area, or leaf length; (4) Tillering upon rewatering after stress.

Unstressed barley seedlings (17-26 days old) grown in controlled environments have shoot betaine levels of about 14 μmol/g dry wt; after water is withheld for 7 days levels increase up to about 54 μmol/g dry wt.

4.3.1.1 Stress experiments with seedlings from embryo culture.

Seedlings raised from excised embryos were variable in size. The uptake of betaine by these seedlings was also variable (Table 24); as far as possible comparisons were made between groups of seedlings of comparable size.

Results from experiments 1-3 that used seedlings transplanted into perlite from embryo culture are summarized in Table 24. In each experiment an equal number of untreated plants (controls) were subjected to the same culture conditions and stress periods as betaine-treated seedlings. [^{14}C]Betaine label taken up by the treated seedlings was distributed between root and shoot; in both organs

TABLE 24. Summary of results of experiments in which seedlings were grown from excised embryos. All experiments comprised a number of controls equal to the number of plants fed betaine.

Experiment	Media	Betaine Supplied	Estimated Betaine[§] Uptake		No. of Reps	Observations
		µmol/ Plant	% Supplied ± $s_{\bar{d}}$	µmol/ plant		
1	MS	15.3	93 ± 1	14	10	No differences between treatments in onset of wilt symptoms. Leaf area fired[†] (%): Treated = 31.5%; Control = 43.5% ns. No. tillers/plant[#]: Treated = 2.8; Control = < 1**
2	Wick's	15.3	83 ± 1	13	3	15.3 µmol-treated seedlings wilted about 2 days later than control and 153 µmol-treated seedlings.
		153.0	38 ± 15	58	3	Leaf area fired (%)[†]; 15.3 µmol-treated = 69[a]; 153 µmol treated = 70[a]; Control = 75.4[b].
						Leaf-3 blade area (cm^2): 15.3 µmol-treated = 11.1; 153 µmol-treated = 11.1; Control = 11.8 ns.
						No. tillers/plant[#]: 15.3 µmol-treated = 2; 153 µmol-treated = 1; Control = <1.
3	Wick's	15.3	65 ± 29	10	10	Betaine-treated plants wilted about 8 h after control plants.
		153.0	51 ± 12	78	10	Leaf(blade plus sheath)length (cm): Wick's medium, 15.3 µmol-treated = 157[a]; 153 µmol-treated = 100[b]; Control = 153[a]; B 5 medium, Treated = 124, Control = 116 ns.
	B-5	15.3	53 ± 27	8	5	No. tillers/plant[#]; Wick's, Treated = 1; Control = 0.**

[§]Dry weights of seedlings before stress was imposed were between 120 - 180 mg.

[†]Leaf area fired indicates the percentage of the total area of the fully expanded leaf blades that was fired.

[#]No. of tillers per plant after rewatering.

**Indicates significance at p = 0.01; ns indicates non-significance; values followed by a different letter for any parameter within treatments is significantly different according to Duncan's Multiple Range Test (p = 0.05).

^{14}C-activity was only in the form of betaine [recovery of ^{14}C in the six seedlings analyzed was 91.5 (± 3.5)%].

From these experiments, it was possible to conclude only that betaine was not toxic when supplied externally, and that pre-treatment with betaine tended to delay the first signs of wilting.

4.3.1.2 Stress experiments using plants from intact seed culture. In order to overcome the problems of variability for size and vigor among seedlings grown from embryo culture, intact seeds were used in two later experiments. A preliminary experiment (experiment 4) using semi-sterile conditions was completed before an experiment using sterile intact seed culture was conducted.

In experiment 4 plants (7 replicates) exposed to 80 µmol betaine (27 mM; shoot betaine concentration, 109 ± 30 µmol/g dry wt) wilted about 24 h after the untreated plants (Fig. 26). Pot weights and leaf-3 blade areas were measured at the beginning and end of the stress period. At the end of the stress period, 4 plants were randomly selected and their shoot dry weights, leaf-3 and leaf-4 blade areas were measured (Table 25). The remaining 3 plants in each treatment were rewatered for 2 weeks to observe differences in recovery.

At the end of the stress period, betaine-treated plants had a smaller leaf-4 blade area and had lost significantly less water (calculated from differences in pot weights at the beginning and at the end of the stress period; Table 25). These results are consistent, in that a smaller transpiring suface is likely to be associated with slower soil moisture depletion. Differences in pot weights should

Fig. 26. Experiment 4. The effect of betaine pretreatment on the development of wilting symptoms during water-withholding. Photograph shows that the seedlings pretreated with betaine (shoot betaine level before stress ~ 109 μmol/g dry wt) were less severely wilted than untreated controls (shoot betaine level before stress ≈ 14 μmol/g dry wt) 5 days after irrigation was stopped. Seedlings were grown from intact seeds using semi-sterile culture conditions.

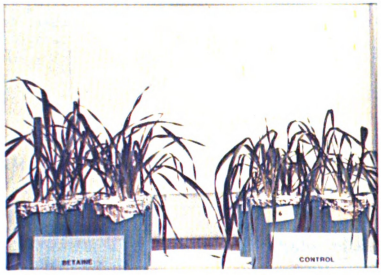

BETAINE
CONTROL

TABLE 25. Selected growth characteristics of betaine-treated and untreated barley seedlings, measured after stress in experiments 4 and 5.

Experiment	Organ	Parameter	Untreated	Betaine Treated
4	---	Total H_2O lost (g)	97.0	88.0**
	Total Shoot	Dry weight (mg)	418.0	409.0
	Leaf-3 blade	Area (cm^2)	13.0	11.2*
	Leaf-4 blade	Area (cm^2)	21.3	18.9*
	Leaf blades: 1 + 2 + 3 + 4	% fired	60.0	33.0**
5	---	Total H_2O lost (g)	115.0	110.0
	Leaf-3 blade		8.6	8.0
	Leaf-4 blade		15.6	14.1
	Leaf blades: 1 + 2 + 3 + 4	% fired	77.0	65.0**

*,** Indicates significance at p = 0.05 and p = 0.01, respectively. Unless indicated, differences between treatments were statistically non-significant.

closely reflect the quantity of water transpired as growth would be minimal during stress. No differences in recovery from stress were evident between treatments.

The experiment was repeated (experiment 5), but using sterile conditions when supplying betaine (160 μmol) to the seedling shoots, and imposing a longer stress period. As observed previously, the betaine-treated plants (shoot betaine concentration ≈ 150 μmol/g dry wt) exhibited retarded (≈ 20 h) wilting as compared to control plants. Although the total water lost (calculated from the difference in pot weights at the beginning and end of the stress period) over the 12 days were not significantly different between the two treatments, the pattern of water loss could account for the delayed signs of stress (Fig. 27). The betaine-treated plants depleted the pot water supply at a slower rate than untreated plants. The leaf blades of betaine-treated plants were smaller than those of untreated plants but the difference was not statistically significant in this experiment.

The blade of leaf-5 was fully emerged in both betaine-treated and untreated plants. However, during the stress period there was more blade expansion (growth) of leaf-5 in betaine-treated plants. This greater expansion of leaf-5 of betaine-treated plants compared to untreated plants is consistent with the former remaining turgid for a longer period of time as growth is very sensitive to loss of turgor (Hsiao et al., 1976). Stress induced leaf-firing (leaves 1, 2, 3, and 4) was significantly less in treated than in control plants (Table 25).

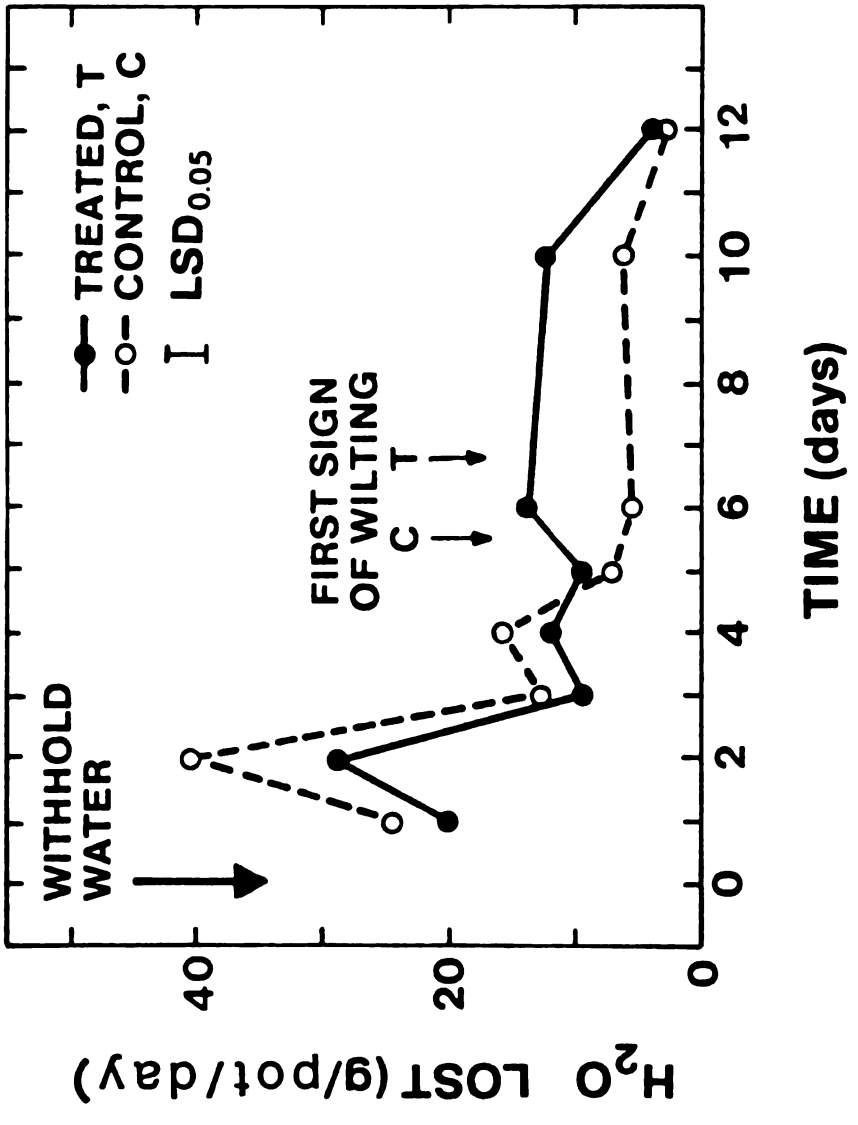


Fig. 27. Experiment 5. Representative data describing pot water loss during a 12-day period of water-withholding from barley seedlings (6 replicates) that had been pre-treated with 160 µmol betaine (●) or untreated (○). There was one seedling per pot.

One plant, selected randomly from each of experiments 4 and 5 was extracted and analyzed to confirm absence of [^{14}C]betaine metabolism. [^{14}C]Betaine was distributed between shoot and root, 4:1. No ^{14}C-label was in any form other than betaine.

To determine if betaine-treated plants may have had a smaller leaf area at the beginning of the stress period, the leaf blade areas of leaf-1 and leaf-2 were measured in a separate experiment using seedlings grown from intact seeds under sterile culture conditions. The second leaf blades of betaine-treated plants were significantly ($p = 0.05$) smaller ($\bar{x} = 5.8$ cm^2 for 15 replicates) than those of untreated plants ($\bar{x} = 6.6$ cm^2 for 11 replicates).

4.3.2 Metabolism of [2-^{14}C]Ethanolamine

The major pathway of betaine biosynthesis that is believed to operate in barley (Hitz et al., 1981) is schematically described in Figure 28. [Minor pathways that may contribute to the synthesis of betaine could involve N-methylation reactions occurring either on the free bases, on nucleotide derivatives of the bases, or on phosphatidyl derivatives of the bases (Hitz et al., 1981). These alternative pathways were not shown in Figure 28.] The point at which the supplied [^{14}C]ethanolamine is likely to join the endogenous precursors to betaine is indicated.

The incorporation of ^{14}C-label from [2-^{14}C]ethanolamine into betaine and phosphatidyl choline (PC) at various internal levels of betaine are given in Table 26. Since the pool sizes of other QAC

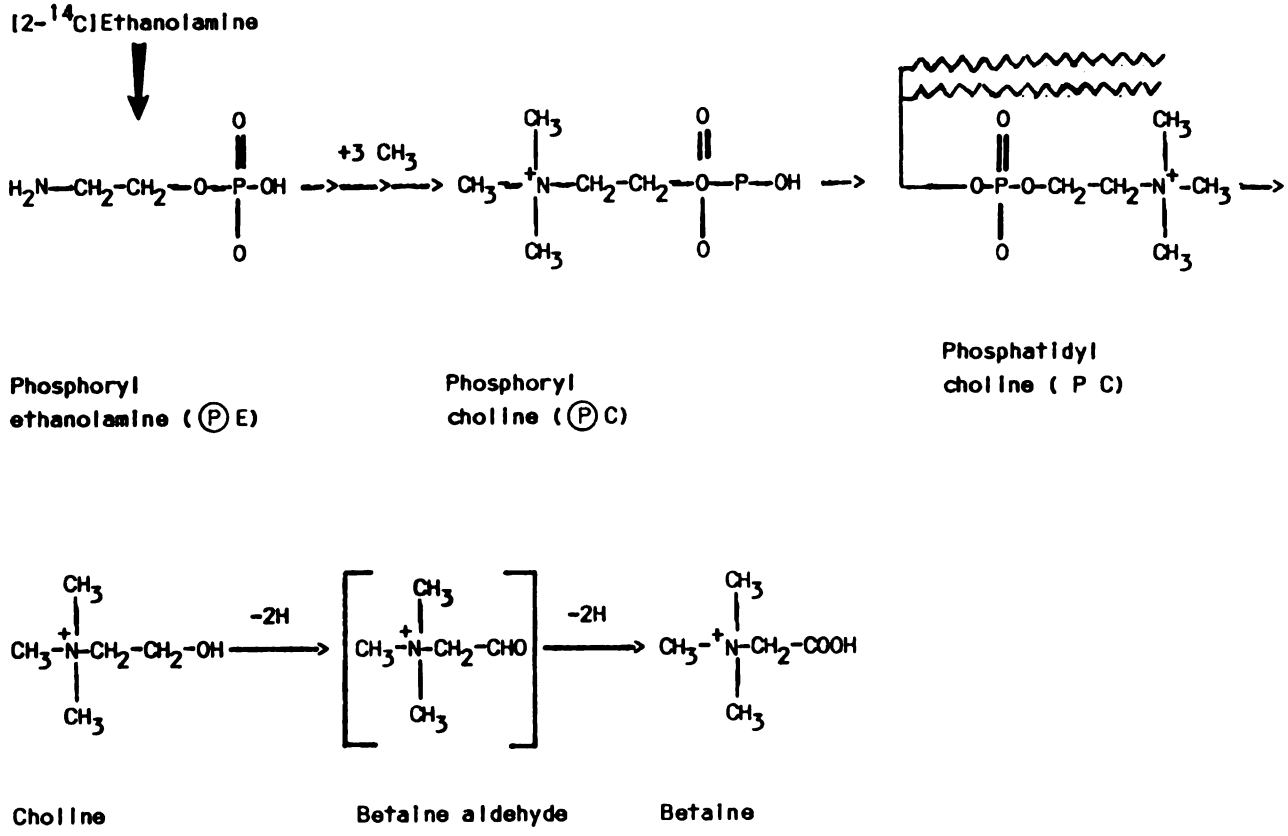


Fig. 28. A schematic representation of the major pathway believed to operate in the biosynthesis of betaine from ethanolamine in barley (after Hitz et al., 1981).

TABLE 26. Incorporation of ^{14}C-activity (nCi) into betaine and phosphatidylcholine by the second leaf blade of barley seedlings having various internal levels of betaine.

Experiment	Betaine Level	^{14}C-content[§]	
		Betaine	PC
	µmol/g dry wt	nCi/(blade plus sheath segment)	
6	15	4.5	22.0
	15	4.6	14.0
	22	4.8	16.0
	65[†]	2.3	13.0
	76[†]	2.9	14.0
	95[†]	1.9	8.3
7	8	4.1	14.5
	12	2.3	18.7
	15	3.2	10.6
	42[†]	1.8	5.8
	48[†]	1.5	6.3
	63[†]	3.1	7.4
	64[†]	2.4	6.1
	69[†]	2.3	6.2
	73[†]	0.61	3.6
	77[†]	1.9	6.1
	85[†]	2.3	3.8

[§][2-^{14}C]ethanolamine supplied = 1 µmol/leaf. Specific radioactivity (nCi/µmol): Experiment A = 1202, B = 737. Incubation = 9 h.

[†]Indicates seedlings treated with 100 µmol betaine.

intermediates between ethanolamine and betaine are small (Hitz et al., 1981), PC and betaine are the major products of the betaine biosynthesis pathway after periods of several hours. Increasing the internal levels of betaine tended to depress synthesis of both [^{14}C]PC and [^{14}C]betaine. The relationship between betaine level and [^{14}C]PC and [^{14}C]betaine synthesis are clearly shown by the scatterplots in Figure 29. As the slopes of the two regression lines in Figure 29 are similar, it would appear that the rates of synthesis of both PC and betaine were depressed to about the same extent. In view of the general growth inhibitory effect of QACs, this result could be ascribed to betaine causing a general reduction in metabolic activities, in which case the observed effects would be non-specific. However, this trivial explanation seems unlikely, as in the studies of the effect of betaine on growth, only small differences between betaine-treated and untreated seedlings were observed. Such small differences in growth cannot be used to explain the 2- to 3-fold reductions in the rates of [^{14}C]PC and [^{14}C]betaine synthesis. As there is no net synthesis of PC after feeding ethanolamine to turgid barley seedlings (Hitz et al., 1981), the lower incorporation of ^{14}C-label into PC in the presence of elevated levels of betaine probably reflects a diminished turnover of the base moiety of PC.

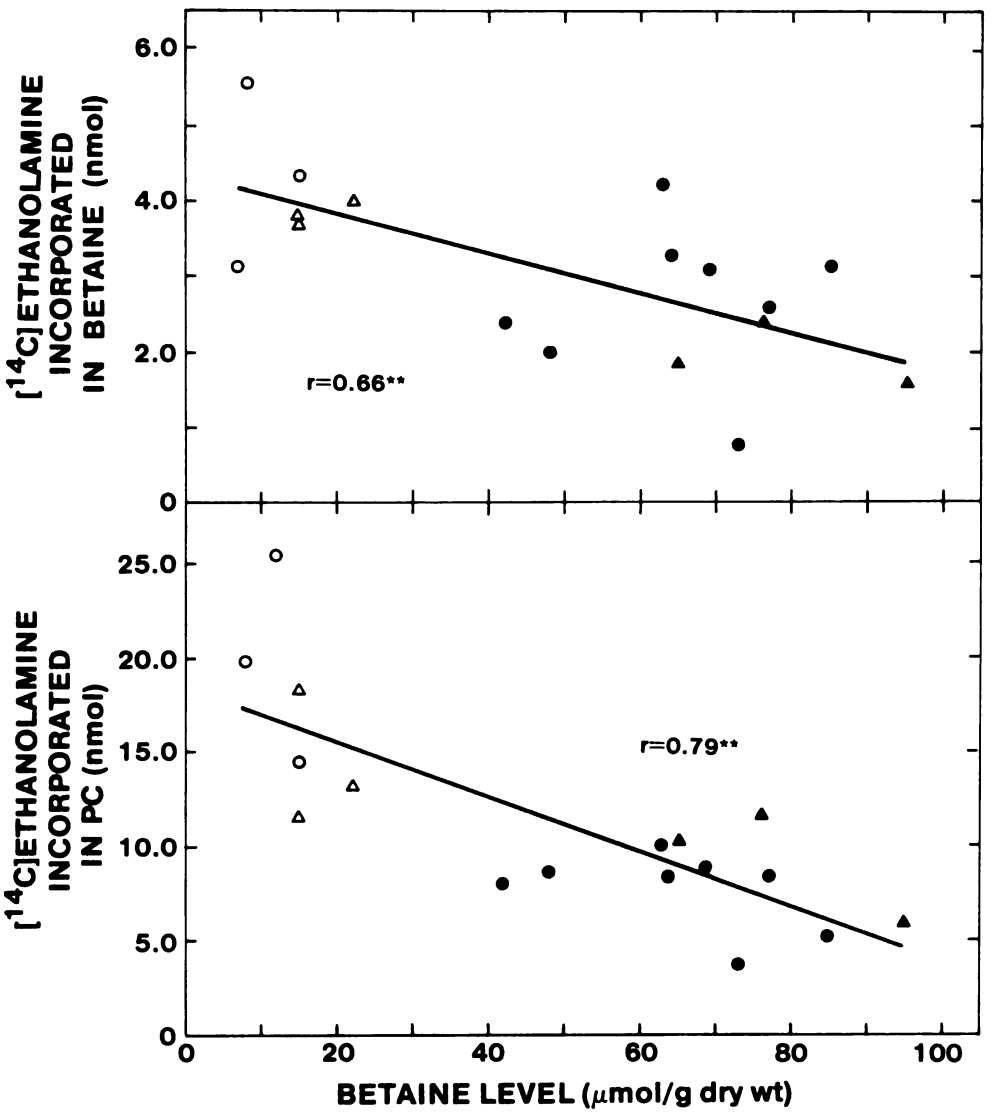
4.4 Discussion

Exogenous application of betaine led to elevated internal betaine levels, and appeared to retard development of symptoms of water stress, i.e., wilting, in barley seedlings. This phenomenon

Fig. 29. Relationship between endogenous betaine level and [^{14}C]ethanolamine (nmol/leaf) conversion to betaine and phosphatidylcholine (PC) when [2-^{14}C]ethanolamine was fed to the third leaf blade of well-watered seedlings. Treated (▲) and untreated (△) plants of experiment 6; treated (●) and untreated (o) plants of experiment 7. The relationships could be described by the linear regression equations:

Ethanolamine (nmol) incorporated into PC = -0.13 (betaine level) + 17.83; r = 0.66.**

Ethanolamine (nmol) incorporated into betaine = -.0.03 (betaine level) + 4.33; r = 0.79.**


[14C]ETHANOLAMINE INCORPORATED IN BETAINE (nmol)
6.0
4.0
2.0
0
r=0.66**
[14C]ETHANOLAMINE INCORPORATED IN PC (nmol)
25.0
20.0
15.0
10.0
5.0
0
r=0.79**
0
20
40
60
80
100
BETAINE LEVEL (μmol/g dry wt)

was apparently due to slower soil-water depletion by betaine-treated plants which, in turn, was probably a result of the latter having a slightly smaller transpiring surface. Observations by Wheeler (1969) on Phaseolus vulgaris lend some support to the notion that betaine may act as a mild growth retardant; betaine inhibited enlargement of leaf disks from P. vulgaris over a 24-h period, although there was no decrease in leaf area when betaine was supplied, via a soil drench, to the intact plant.

The relatively large contribution that betaine makes to total nitrogen efflux from the aleurone layer of wheat grains during germination led Chittenden et al. (1978) to suggest that betaine was an important source of nitrogen for the germinating seedling. This is unlikely to be correct because degradation of betaine was not observed during barley grain germination in any of the experiments reported here.

An increase in the endogenous betaine concentration of leaf blades tended to depress the incorporation of ^{14}C-label from [2-^{14}C]-ethanolamine into both PC and betaine. Accepting that this depressive effect is specific to betaine synthesis and does not apply to cellular metabolism in general, one may suggest that the regulatory steps in betaine accumulation can be located at one or more of the following stages: (1) Choline moiety turnover at the phospholipid level; (2) Choline transfer between water-soluble and lipid intermediates; (3) N-methylation. These alternatives are described schematically by the dashed or dotted lines in Figure 30.

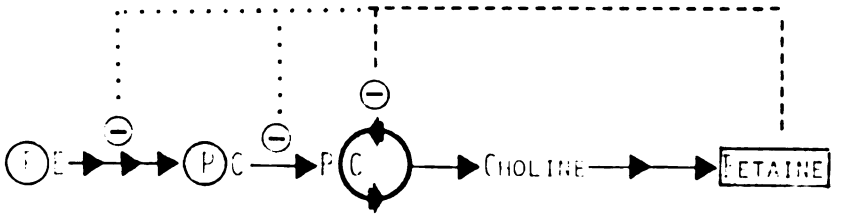


Fig. 30. A schematic diagram indicating the possible regulatory steps in betaine accumulation.

A rationalization for the regulatory step in the synthesis of betaine occurring at PC turnover (indicated by the dashed line in Fig. 30) may be formulated thus: It is known that water stress may induce membrane deterioration that could be detrimental to cellular metabolism (Gaff, 1980). Possibly one facet of deterioration in membrane structure is an increase in the turnover rate (i.e., breakdown and resynthesis) of PC in membranes. Since betaine has been indicated as having a stabilizing effect on membrane functions, (Rafaeli-Eshkol and Avi-Dor, 1968; Schobert, 1977), it can be envisaged that a mechanism for regulating PC turnover is to convert a fragment (viz. choline) that is a consequence of undesirable membrane turnover, to a protective substance (viz. betaine) that, by its very accumulation, cuts off the source of its precursor (i.e., choline from PC breakdown). This explanation has bearing on the physiological significance of betaine accumulation by water-stressed barley. Although it is not reasonable to infer from the results of experiments with well-watered seedlings that the synthesis of PC and betaine is subject to retroinhibition in stressed

plants grown in the field, results from field studies are consistent with feedback regulation of betaine synthesis in mature, stressed barley (Hitz et al., 1982).

Since betaine was administered to the seedlings early in their development, it is reasonable to believe that the exogenous betaine had joined the subcellular site of the endogenous betaine by the time that [^{14}C]ethanolamine was fed. The results thus lend support to the hypothesis that at least some betaine is located in the cytoplasm; it seems unlikely a metabolite stored in the vacuole could interfere with a cytoplasmic activity, viz. betaine synthesis. Because of the long-term nature of the experiment, it was impossible to distinguish between inhibition of enzyme activity or enzyme synthesis as explanation of the observed retroinhibition.

CHAPTER V

SUBCELLULAR LOCALIZATION OF [METHYL-^{14}C]BETAINE BY MICROAUTORADIOGRAPHY

5.1 Introduction

Betaine has been suggested to be an osmotic effector in both halophytes and mesophytic crops (Wyn Jones, 1979). Data collected in the field for water-stressed barley (cv. Arimar) permit a crude estimation of the potential contribution that betaine accumulation could make to the osmotic adjustment in the flag-1 leaf blades. The maximum difference between betaine levels in the flag-1 leaf blade of irrigated and non-irrigated barley grown in the field was 125 μmol/g dry wt, or about 44 mM on a plant-water basis. The difference in solute potential between non-irrigated and irrigated leaves was about 10 bars. Thus if the accumulated betaine were distributed throughout the cell, its contribution (about 1 bar) to the drop in solute potential would be relatively small. However, were betaine to be localized in the cytoplasm (assumed to be about 10% of cell volume) betaine accumulation could account for all the drop in solute potential. Thus, crucial to the hypothesis that betaine is a significant osmotic effector is that it be localized in the cytoplasm.

Knowledge about the subcellular distribution of enzymes and metabolites is essential to understanding many aspects of cellular

metabolism (e.g., Emes and Fowler, 1979). Three approaches to distinguish between vacuolar and cytoplasmic localization are available: (1) Isolation of intact vacuoles and determining the presence or absence of the metabolite (Heck et al., 1981; Leigh et al., 1981; Martinoia, 1980); (2) Histochemical localization of the metabolite using specific staining procedures (Jensen, 1962); (3) Microautoratiographic procedures that localize radio-labeled metabolites (Jensen, 1962; Evans and Callow, 1978).

In order to obtain evidence of the subcellular compartmentation of betaine, intact vacuoles from red beet have been isolated (Wyn Jones et al., 1977a; Leigh et al., 1981). These experiments indicated that the levels of betaine were variable, but were always higher in the cytoplasm than in the vacuole. Both nitrate (Martinoia, 1980) and proteinases (Heck et al., 1981) have been successfully identified as vacuolar constituents in barley by isolating vacuoles from turgid primary leaves. Objections to using the procedure of vacuole isolation to identify the subcellular localization of betaine in barley include: (1) The subcellular localization is of greatest interest in tissues subjected to moderate long-term stress. Vacuoles could not be isolated from such tissues without the superimposition of osmotic shock. (2) Betaine may leak from vacuoles during isolation; (3) The potential problem associated with assaying an unrepresentative sample of vacuoles due to low yields of vacuoles relative to the fresh weight of whole tissue.

Hall et al. (1978) investigated the subcellular distribution of betaine in leaf cells of Suadeda maritima by using a histochemical

procedure that was based on the fact that quaternary ammonium compounds (QAC) form colored complexes with platinum halogenates. The iodoplatinate solution that was used in their study reacts with any quaternary ammonium compound, e.g., choline (Dierichs and Inczedy-Marcsek, 1976) and thus was not specific to betaine. Although leakage of betaine from the tissues did not appear to be a problem in this histochemical study, it would appear from the aqueous and alcohol incubation procedures used that loss or displacement of betaine can not be completely ignored when interpreting the results.

Using cryofixation and freeze substitution procedures, water- and alcohol-soluble ^{14}C-labeled compounds have been successfully localized in leaves and maize kernels at the light-and electron microscope level (Fisher and Housely, 1972; Felker and Shannon, 1980).

In an intact barley plant, older leaves are known to export applied [^{14}C]betaine to younger organs (Section 1.3 and 2.3). It is reasonable to assume that the [^{14}C]betaine in the young organ will then enter the pool of endogenous betaine.

This study sought to identify the subcellular site of [methyl-^{14}C]betaine accumulation in the fully expanded younger leaves of barley after supplying [methyl-^{14}C]betaine to the second leaf blade of turgid 3-week old seedlings using microautoradiographic techniques at the light microscope level.

5.2 Materials and Methods

5.2.1 Plant Material

Barley seed, originally from the USDA Small Grains Collection (Beltsville, Md.) was multiplied in East Lansing, Mi.

5.2.2 Growing Conditions and Stress Regimes

In the experiment where [methyl-^{14}C]betaine was fed, seedlings were grown in a soil mix (sand:peat:loam, 2:1:1) in plastic pots (12 cm x 7 cm diam.) in a controlled environment chamber under 16-h days (PAR 5 mW cm^{-2}; day/night temperature 22/16°C; day/night relative humidity 70/85%). The seedlings (6/pot) were irrigated on alternate days with half-strength Hoagland's solution. One day before the experiment began [19 days after planting, (DAP)] seedlings were thinned to 4 per pot and the seedlings irrigated. [Methyl-^{14}C]Betaine was fed to the second leaf blade of a seedling 20 DAP and water was then withheld from the plant. After 3 days the plants were rewatered for 4 days. At the end of the rewatering period the potted seedlings were taken to the laboratory for cryofixation of selected organs.

In experiments using $^{14}CO_2$, plants were grown in the greenhouse under supplemental light (16-h day; day/night temperature 22/16°C; relative humidity 70 to 80%) in a soil mix [peat:loam:sand, 1:1:2; fertilized with NH_4SO_4 (0.5 g) and P_2O_5 (0.1 g)] in large, well-drained plastic pipes (1 m long x 16 cm diam.). Immediately before seeds (7/pot) were planted, sufficient half-strength Hoagland's solution was applied to the soil to completely fill the profile.

Seedlings were irrigated with half-strength Hoagland's solution alternate days until seedlings were at the 2-leaf stage. The seedlings were then thinned to 1 per pot and water was withheld. The plants matured and successfully completed grain fill using only stored water.

5.2.3 Labeled Compounds and Mode of Application

[Methyl-^{14}C]Betaine (7 μCi, 59 μCi/μmol; prepared from [methyl-^{14}C]choline as described in Appendix A) was fed in a 3 μl-droplet of K^+-phosphate buffer (20 mM, pH 7) to the cut end of the second leaf blade as described in Section 2.2.6 (Hanson and Scott, 1980). Water was withheld from the plant for 4 days. The plant was then rewatered for 2 days after which it was taken from the growth chamber to the laboratory for preparation for microautoradiography.

At 64 DAP the flag-1 leaf blade was placed in a feeding chamber (Section 1.2.3) and exposed for 15 min to $^{14}CO_2$ (1.25 μCi, ~60μCi/μmol), generated as described in Section 1.2.3. After 3 h, the leaf sheath was cut at the node of insertion, and the leaf was quickly transported on ice from the greenhouse to the laboratory for preparation for microautoradiography.

5.2.4 Preparation for Microautoradiography

The procedure used was based on methods reported by Fisher and Housely (1972) and Felker and Shannon (1980). All steps in the procedure described were conducted in a dry box through which a

continuous stream of dry N_2 was passed (relative humidity < 10%). A 1-cm^2 segment of the flag-1 leaf blade from $^{14}CO_2$-treated plants, or a 1-cm^2 segment from the first expanded leaf of the first tiller (defined by Briggs, 1978, p. 17) from the [methyl-^{14}C]betaine treated seedling, was quickly cut into small (about 1 mm^2) pieces of tissue which were plunged into liquid Freon-12 held at its freezing point with liquid N_2. The frozen specimens were rapidly transferred into vials of cold (< -40°C) propylene oxide (20 ml); propylene oxide was kept cold by placing the vials in an acetone/dry ice bath. Prior to use, the propylene oxide was thoroughly dried for $\geq$ two weeks over a molecular sieve (propylene oxide:molecular seive 5:1 v/v, 8-12 mesh beads; Davison Chemical, Baltimore, Md.). Typically only 4-8 small pieces of tissue were substituted in the same vial to ensure a high ratio of solvent to tissue. The tissue was then left for 3 weeks in a plastic box filled with $CaSO_4$ (Hammond Drierite Co., Xenia, Ohio) at -100°C for freeze substitution in propylene oxide.

At the end of three weeks the tissue was allowed to warm slowly to room temperature; tissue was kept for periods of 12 h at -49°C, -20°C, -10°C, -4°C and then in ice at room temperature for 16 h. The slow warm up procedure was a precaution against trapping air bubbles in the tissue. When the vials were at room temperature the propylene oxide used for substitution was changed for fresh, dry propylene oxide. After a further 2 h in the fresh proplyene oxide at room temperature the tissue was then slowly (10% increments of

Spurr's medium into propylene oxide at 45 min intervals) infiltrated with Spurr's resin (Appendix D; Spurr, 1969). The tissue was left for two 8-h periods in 100% Spurr resin before final polymerization overnight at 70°C.

Sections (1 μm thick) were cut with a diamond knife on an ultramicrotome. Sections were fixed to microscope slides which had been pretreated by dipping them in an aqueous solution containing 1% gelatin (w/v) and 1% potassium chromium sulphate (w/v). The sections were transferred to the slides in a drop of ethylene glycol and dried in a desiccator at room temperature.

5.2.5 Microautoradiography and Section Staining

Microautoradiographs were prepared by dipping the slides in Kodak NTB-2 (Eastman-Kodak Co., N.Y.) nuclear track emulsion after it was melted at 40°C in a water bath. Slides were stored at 4°C in light-tight boxes. After exposure of 16 days the slides were developed by dipping them sequentially in: Microdol-X developer (7 min; Eastman-Kodak), acetic acid (1%, 1 min), fixer (7 min; Eastman Kodak), H_2O (10 min). Developer and fixer were prepared according to manufacturer's instructions. Dipping and development of slides was conducted in total darkness.

Some sections were stained with toluidine blue [filtrate from toluidine blue (1 g) plus Na borate (1 g) in 100 ml H_2O] and not treated with the emulsion, for use in interpreting the pattern of exposed silver grains on the microautoradiographs.

5.3 Results and Discussion

Results from autoradiography were interpreted in terms of the hypothesis that betaine might be largely confined to the cytoplasm. Thus, the question asked was: What proportion of silver grains present over the vacuolar compartment could not be accounted for by ^{14}C-activity in the cytoplasm?

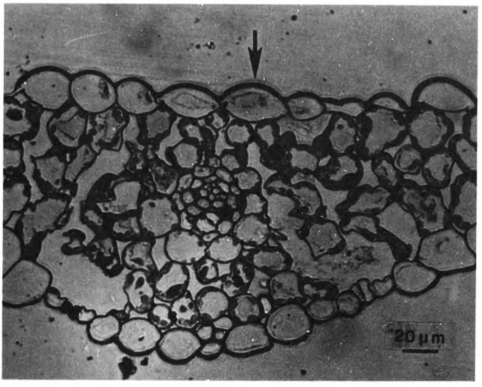
Tissue integrity was satisfactory after the substituting and embedding procedure; $\leq$ 5% ^{14}C-activity leached out of the tissue into the substituting solvent prior to embedding the tissue pieces in resin.

The cross-sectional area of cells of the flag-1 leaf blade of stressed, mature plants grown in the greenhouse was much smaller (about 150-300 μm^2) than that of cells of the seedling leaf blades (about 500-800 μm^2). As the track of a ^{14}C β-particle can be expected to be about 2-3 μm (personal communication, R. Hahn, Eastman-Kodak, N.Y.) with the thickness of emulsion used in this study, the larger cell size of seedlings made it possible to ascribe the silver grains to either vacuolar, cytoplasmic or extracellular compartments; the smaller size of the flag-1 leaf cells precluded such an analysis. The preliminary $^{14}CO_2$ feeding experiment served only to confirm that the substituting/embedding/autoradiographic procedures could be completed successfully; the stained sections indicated that a high percentage of cell volume (30-50%) was cytoplasm.

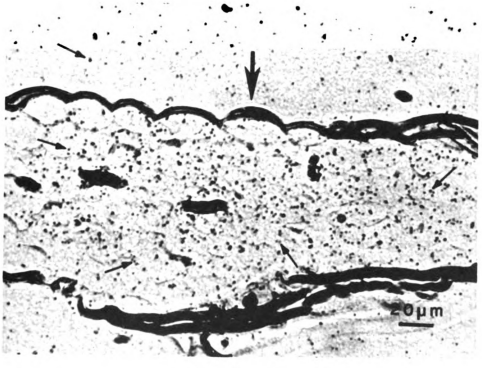
Microautoradiographs of sections (Fig. 31B) were matched with adjacent sections (Fig. 31A) that had been stained with toluidine blue;

thus the cytoplasm and extra-cellular spaces could be delineated. That part of the total vacuolar area unlikely to contain silver grains exposed by β-particles emitted from [^{14}C]betaine localized in the cytoplasm was defined by drawing a line 2-3 μm within the internal perimeter of the cytoplasm. The numbers of grains/unit area were not significantly different between the areas so defined; ^{14}C-activity attributable to extracellular spaces was significantly lower (Table 27) which indicates the silver grains over the vacuole are not artifacts of the procedure used. Thus, in stressed-rewatered seedlings, it appears that betaine is not preferentially localized in the cytoplasm. Since the seedlings were stressed and rewatered prior to freeze-substitution, the possibility must be recognized that continuously stressed tissues might show a different distribution of betaine.

Fig. 30. Photographs of transverse sections through the first leaf of the first tiller of a stressed-rewatered barley plant supplied with [^{14}C]betaine via a leaf on the main culm. (A) stained with toluidine blue. (B) Microautoradiograph of a section adjacent to that in (A). The epidermal cells on the lower leaf surface on the microautoradiograph are folded; a reference point is marked by a large vertical arrow. ^{14}C-activity was about 2 μCi/ml on a plant water basis. In the lower frame the numerous discrete black particles are the silver grains. For clarity five of such grains (one outside the section, which would be considered background, and four inside the section) are indicated by small arrows.



A



B

TABLE 27. The number of silver grains/10 μm^2 in the vacuolar and cytoplasmic compartments of mesophyll cells and in the surrounding extra-cellular spaces in the first leaf blade of a stressed-rewatered seedling tiller. The grain number described is corrected for a background of 11 grains/10 μm^2. Grains were counted on photographs of sections; magnification at the microscope level was 320 x.

Section Number	Number of Cells	Number grains/10 μm^2/compartment Extra-cellular:Vacuolar:Cytoplasmic		
1	7	0	5	14
2	18	7	12	33
3	11	8	17	18
4	7	4	21	20
Average		4.7	13.8	21.3

($LSD_{0.05}$ = 10.6)

CONCLUSION

The objectives of this study on the metabolism and accumulation of betaine by barley were: (1) To evaluate the hypothesis that the level of betaine in certain organs would reflect the stress history of the plant; (2) To investigate the feasibility of physiological-genetic studies to test the hypothesis that betaine accumulation under water stress was of adaptive value; (3) To obtain evidence bearing on the adaptive value of betaine accumulation in barley.

Studies with seedlings, grown in controlled environments, and mature plants, grown in the field, demonstrated that betaine was an inert metabolic end product that accumulated via translocation and *in situ* synthesis in the youngest vegetative organs. In seedlings, betaine was shown to be transported in the phloem. In field-grown plants, betaine accumulated in the flag and flag-1 leaves and during grain filling did not partition either like other nitrogenous compounds or like dry matter. The developing spikes of field-grown plants were not strong sinks for [^{14}C]betaine applied to flag leaves; this was in accordance with results from laboratory experiments on phloem translocation in mature plants. Betaine could represent up to 10% of the total-N in NI, N-fertilized vegetative shoot tissue at grain-maturity. Low soil-N depressed betaine accumulation in total shoot tissue but did not significantly affect betaine

accumulation in the youngest leaves. The betaine level in the youngest mature leaves was significantly correlated with an integrated value, based on ψ measurements, of the stress experienced by the plant prior to the measurement of betaine. The accumulation of betaine in field grown plants occurred slowly during the growing season at a time when the plant was making morphological changes in response to the stress; betaine accumulation is thus not a short-term shock response.

Genetic variability for betaine accumulation was found among genotypes of H. vulgare and H. spontaneum in trials with seedlings grown in controlled environments, and among cultivars of H. vulgare grown under non-irrigated conditions in the field. The range in betaine levels among seedlings grown in controlled environments was 3-4 fold under well-watered conditions, and 2-3 fold under water-stressed conditions. In the field, the difference among cultivars for maximum levels of betaine in the flag-1 leaf blades was about 4-fold; apparently betaine levels were not related to the instantaneous values of ψ_s. The extent of variability identified in this study was only barely sufficient for physiological-genetic studies, but was large enough to encourage one to believe that greater variability exists among species within the primary gene pool of cultivated barley.

The relatively limited extent of betaine accumulation by water-stressed, field-grown barley and the indications of genetic variability for its levels under well-watered and non-irrigated conditions make the betaine level of any shoot organ of barley

unsatisfactory as a practical index of the cumulative stress experienced by a crop.

Under conditions of a limited water supply pretreatment of seedlings with exogenous betaine tended to delay wilting which was a result of a reduction in the rate of soil-water depletion. This phenomenon was ascribed to betaine causing a slight reduction in leaf area.

When [2-^{14}C]ethanolamine was applied to the second leaf blade of well-watered barley seedlings, high, but physiological, internal levels of betaine tended to depress the synthesis of [^{14}C]-betaine and its precursor, [^{14}C]phosphatidyl choline. These results suggested that a feedback regulatory system in betaine synthesis operates in well-watered seedlings. This observation led to speculation on the physiological significance of betaine accumulation; possibly betaine accumulation mitigates an undesirable acceleration of PC turnover, which may be an aspect of membrane deterioration, during stress. A reduction in the rate of betaine synthesis that was consistent with retroinhibition was noted in studies with NI, field-grown barley (Hitz et al., 1981).

Results from autoradiographic studies at the light microscope level showed that betaine was distributed about equally between the cytoplasm and the vacuole of mesophyll cells of turgid barley leaves.

As betaine represents up to 4.5% of total-N in metabolically active, water-stressed leaf blades, but does not serve as a source of either re-usable nitrogen or energy, it is difficult to believe that

betaine accumulation is an incidental response to stress. The slow, but progressive, accumulation of betaine throughout a droughty growing season, the site of betaine accumulation (viz. in the youngest mature leaves which are most important to grain fill), and the effect of exogenous betaine on growth and regulation of PC turnover, are circumstantial evidence consistent with the notion that betaine accumulation has adaptive value. However, there remains the possibility that betaine accumulation is an 'evolutionary relic', in that its function is not recognized because it is no longer needed in the conditions in which barley is grown today.

APPENDICES

APPENDIX A

SYNTHESIS OF [METHYL-^{3}H]BETAINE, [METHYL-^{14}C]BETAINE, AND [METHYL-^{14}C]BETAINE ALDEHYDE

APPENDIX A

SYNTHESIS OF [METHYL-^{3}H]BETAINE, [METHYL-^{14}C]BETAINE, AND [METHYL-^{14}C]BETAINE ALDEHYDE

A.1 Introduction

The oxidation of choline to betaine proceeds via the aldehyde (Fig. A-1). [Methyl-^{14}C]Betaine was prepared chemically and [methyl-^{14}C]betaine aldehyde was biosynthesized using a rat liver mitochondrial preparation.

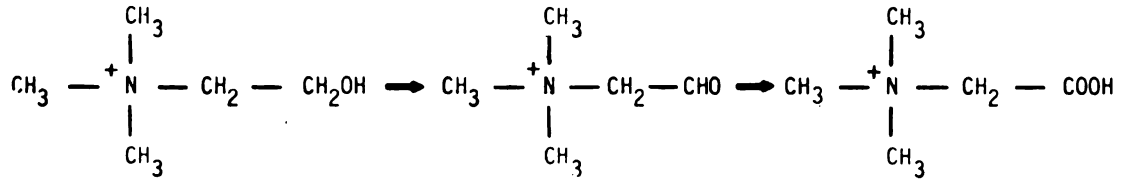


Figure A-1. Oxidation of Choline.

A.2 Materials and Methods

A.2.1 Synthesis of [Methyl-^{3}H] Betaine and [Methyl-^{14}C]-Betaine (after Lintzel and Fomin, 1931)

[Methyl-^{14}C]Choline chloride (100-200 μCi, 59 μCi/μmol) or [methyl-^{3}H]choline chloride (1 mCi, 15 mCi/mmol), $KMnO_4$ (1 mg) and conc. H_2SO_4 (2 μl) in water (200 μl) were mixed throughly and heated for 1 h in a boiling water bath. The solution was centrifuged for 5 minutes;

the supernatant was carefully removed and the pellet washed 3 times with warm water (3 x 0.5 ml). The final pellet was discarded and all the supernatants were combined and passed in sequence through 3 columns (each of 1.5 ml volume): AG-1 (OH^-), BioRex 70 (H^+), AG-50 (H^+). The AG-50 column was eluted with ammonium hydroxide (4N, 8 ml) and the eluate dried at about 80°C under a stream of N_2. The eluate was redissolved in water.

Radiochemical purity of [methyl-^{14}C]betaine (>95%) was confirmed by the TLC and TLE systems described in Section 1.2.4. The percent recovery of label in betaine was 74-90% when [methy-^{14}C] choline was the precursor and 45% when [methyl-^{3}H]choline was the precursor. Specific radioactivity has taken as that of the original [^{14}C]choline (59 μCi/μmol for ^{14}C; 15 mCi/mmol for ^{3}H).

A.2.2 Synthesis of [Methyl-^{14}C]Betaine Aldehyde

A.2.2.1 Preparation of mitochondria following a similar procedure to Williams (1952). The liver of a freshly killed male rat (Sprague-Dawley strain) was weighed, chilled, cut into small pieces and added to ice cold isotonic sucrose solution (0.25M; 50 ml). The preparation was homogenized for 3 min in a Virtis Hi-Speed "23" Homogenizer (Virtis Co., Gardiner, N.Y.). The suspension was then poured into an ice-cold Potter homogenizer with a loose fitting pestle and further gently homogenized until the liver tissue was no longer discernible. The resulting brei was centrifuged at 600 g (10 min) in a refrigerated centrifuge. The 600 g supernatant was then

centrifuged at 8500-9000 g (10 min). This 8500-9000 g supernatant was discarded and the pellet was rinsed twice in ice-cold sucrose solution (0.25 M, 2 x 20 ml), and re-suspended in 7.0 ml ice cold sucrose solution (0.25 M); the protein concentration of the final solution was estimated by Bradford's reagent (Bradford, 1976). The suspension was further centrifuged at 8500 g and the pellet resuspended in sufficient ice cold K^+-phosphate buffer (25 mM; pH 7.8) to give a concentration of 8.3 mg protein/ml. During the preparation of the mitochondria all vessels and media were maintained at approximately 2-4°C.

A.2.2.2 Oxidation of choline following a similar procedure to Kensler and Langemann (1951). The reaction mix comprised [methyl-^{14}C]choline (100 μCi, 59 μCi/μmol; Amersham Corp., Ill.), $CaCl_2$ (1 mM), cytochrome-C (1.6 x 10^{-5}M; Horse heart-type XIII acid modified; Sigma Chemical Co., Mo.) and mitochondria (2.5 mg protein) in 300 μl K^+-phosphate buffer (25 mM; pH 7.8). The preparation was sparged with O_2 and incubated for 2 h at 37°C. The reaction mix was then centrifuged and the supernatant passed sequentially through three columns (each of 5 ml volume): AG-1 (OH^-), BioRex 70 (H^+), AG-50 (H^+).

The BioRex 70 column was eluted with HCl (0.5 N, 40 ml). This eluate was evaporated to dryness overnight under an infra-red lamp and ventilated by a fan, and then redissolved in water (150 μl). Betaine aldehyde was separated from choline by TLE on Whatman #3MM paper (27 x 5 cm strips) in 1.5 N formic acid at 2 kv for 20 min.

After the band corresponding to betaine aldehyde was localized by autoradiography (1-h exposure) on SB-5 X-ray film (Eastman Kodak, N.Y.) it was cut out and the betaine aldehyde eluted from the paper with water (4 ml) overnight. The elute was dried down under a stream of N_2 (~60°C) and redissolved in water (1 ml).

Radiochemical purity (>95%) was confirmed by the TLC and TLE systems described in Section 1.2.4. The percent recovery of label in [^{14}C]betaine aldehyde was 48%.

There are negligible amounts of free betaine aldehyde in rat liver mitochondria (De Ridder and Van Dam, 1973). The choline in the mitochondrial preparation was estimated to be $\leq$ 160 nmol (Colbeau et al., 1971), thus the specific activity of the [methyl-^{14}C]betaine aldehyde was taken to be between 54 to 59 μCi/μmol.

APPENDIX B

WILD AND CULTIVATED BARLEY GENOTYPES SCREENED IN LABORATORY AND FIELD TRIALS

APPENDIX B

WILD AND CULTIVATED BARLEY GENOTYPES SCREENED IN LABORATORY AND FIELD TRIALS

For laboratory screening trials, genotypes of H. vulgare and H. spontaneum are entered by CI (H. vulgare) or PI number, or where no such numbers have been assigned, by provenance (H. spontaneum). Number and provenance designations were made by the USDA Small Grains Collection (Beltsville, Md.). The average shoot betaine level (3 replicates) of irrigated and water-stressed seedlings are given. In field trials cultivars of H. vulgare are entered by CI number. Average betaine level and ψ_s of the youngest mature leaf, or flag-1 leaf, for each cultivar at each sampling date are indicated.

TABLE B.1 Betaine level in shoots of irrigated and water-stressed seedlings of genotypes included in Laboratory Trial A.

Genotype	Betaine Level	
	Irrigated	Water-Stressed
CI Number	——— μ mol/g dry wt ———	
3625	16	46
6566	21	42
6939	19	40
7055	18	45
7243	18	51
8093	15	53
10083	21	55
10146	12	41
10149	14	45
10501	19	40
10870	15	53
10975	13	45
10979	12	47
10996	18	52
11009	15	49
11037	16	46
11806	19	49
13626	26	51
15289	12	48
15480	14	42
PI Number		
284742	19	67
284749	23	61
296912	25	54
391071	24	56
391074	22	51
391080	25	40
391081	19	45
391124	18	55
391131	19	49
391133	21	46
391136	25	47
391126	21	48
Provenance		
Mt. Meron Israel	24	57
Mt. Meron Israel	23	49
Mt. Meron Israel	29	59
Mt. Meron Israel	25	56

TABLE B-1 Continued.

Genotype	Betaine Level	
	Irrigated	Water-Stressed
	μmol/g dry wt	
Mt. Hermon Israel	25	56
Mt. Hermon Israel	17	50
Mt. Hermon Israel	19	44
Mt. Hermon Israel	19	57
Bor Mashash Israel	19	46
Bor Mashash Israel	20	49
Bor Mashash Israel	19	44
Bor Mashash Israel	22	43
Bor Mashash Israel	22	49
Wadi Qilt Israel	25	53
Wadi Qilt Israel	25	45

TABLE B.2 Betaine level in shoots of irrigated and water stressed seedlings of genotypes included in laboratory Trial B.

Genotype	Betaine Level	
	Irrigated	Water-Stressed
CI Number	µmol/g dry wt	
3455	25	67
9201	23	52
10161	25	62
10180	34	82
11806	18	51
13060	29	57
13626	34	65
PI Number		
211041	38	84
212305	35	89
220341	38	87
220523	27	71
220664	36	66
227019	36	70
235639	39	86
244776	40	74
244777	31	76
244778	40	79
245739	31	67
249983	24	62
253573	32	66
254894	39	80
268244	34	66
282572	29	59
282576	32	77
282577	34	74
282595	32	72
282592	37	67
282616	23	53
296910	28	60
361678	24	56
370759	31	74
354929	36	72
354934	27	69
354936	31	62
354938	32	75
354941	35	63
354947	25	61
354949	29	68
391000	34	66

TABLE B.2 Continued.

PI Number	Betaine Level	
	Irrigated	Water-Stressed
	μmol/g dry wt	
391001	30	72
391002	31	60
391003	27	56
391007	31	58
391009	32	62
391012	36	68
391013	33	61
391014	24	59
391015	29	60
391019	20	57

TABLE B.3 Betaine level in shoots of irrigated and water-stressed seedlings of genotypes included in laboratory Trial C.

CI Number	Betaine Level	
	Irrigated	Water-Stressed
	μmol/g dry wt	
709	25	51
742	31	84
1114	25	76
1610	36	78
1624	22	59
1641	26	65
2318	22	54
2380	33	62
3480	33	82
5199	21	56
6576	28	61
6577	34	81
6952	32	71
7341	24	73
8044	24	64
8834	30	67
9203	30	62
9309	19	51
9366	30	63
10064	18	54
10138	18	53
10152	23	63
10171	21	70
10333	21	57
10359	26	53
10371	34	60
10500	29	65
10807	20	64
10840	30	67
11197	27	67
11211	26	63
11271	25	59
11622	29	58
11719	27	70
11806	18	54
11832	26	65
11961	27	81
12083	31	66
12456	32	73
13057	23	53
13452	29	68
13626	35	84

TABLE B.3 Continued.

CI Number	Betaine Level	
	Irrigated	Water-Stressed
	µmol/g dry wt	
14935	30	66
14936	34	76
15293	19	55
366465	21	55
382211	39	69
382673	31	62

TABLE B.4 Betaine concentration and solute potential (ψ_s) of the youngest mature leaf, or flag-1 leaf, for thirteen cultivars of H. vulgare at three dates during the growing season (1981)

CI Number	Days after Planting					
	Betaine Level			ψ_s		
	41	59	63	41	59	63
	——μmol/g dry wt ——			——— (-) bars ———		
709	60	64	64	16	19	20
1114	51	86	66	16	20	18
2318	67	93	110	15	18	22
3480	72	87	96	18	19	20
6577	54	92	84	17	17	19
9309	57	68	62	14	20	19
10064	50	59	68	13	15	18
10138	53	60	116	16	18	21
10359	56	65	71	17	20	19
11961	51	76	72	17	21	19
13057	39	36	37	15	18	18
13626	70	107	62	16	20	19
14936	76	104	110	14	18	19

APPENDIX C

MEDIA USED IN STERILE EMBRYO AND INTACT SEED CULTURE

APPENDIX C

MEDIA USED IN STERILE EMBRYO AND INTACT-SEED CULTURE

TABLE C.1 B-5 medium (Gamborg and Wetter, 1975).

B 5 Major	g/l Medium[§]
$NaH_2PO_4 \cdot H_2O$	0.15
KNO_3	2.5
$(NH_4)_2 SO_4$	0.13
$MgSO_4 \cdot 7H_2O$	0.25
$CaCl_2 \cdot 2 H_2O$	0.15
Sucrose	20
B 5 Minor	**mg/l Medium**
$MnSO_4 \cdot H_2O$	100
H_3BO_3	30
$ZnSO_4 \cdot 7H_2O$	20
$Na_2MoO_4 \cdot 2H_2O$	2.5
$CuSO_4 \cdot 5 H_2O$	0.25
$CoCl_2 \cdot 6 H_2O$	0.25
KI	7.5

TABLE C.1 Continued.

Na_2EDTA	37.4
$FeSO_4 \cdot 7\ H_2O$	27.8
Thiamine HCl	10.0
Pyridoxine HCl	1.0
Nicotinic acid	1.0
Inositol	100.0

[§]Adjust complete medium to pH 6 with KOH and HCl.

TABLE C.2 Murashige and Skoog medium (Murashige and Skoog, 1962)

Major Elements	mg/l Medium [§]
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2 H_2O$	440
$MgSO_4 \cdot 7 H_2O$	370
KH_2PO_4	170
Na_2 - EDTA	37.3
$FeSO_4 \cdot 7 H_2O$	27.8
Minor Elements	
H_3BO_3	6.2
$MnSO_4 \cdot 4 H_2O$	22.3
$ZnSO_4 \cdot 4 H_2O$	8.6
KI	0.83
$Na_2MoO_4 \cdot 2 H_2O$	0.25
$CuSO_4 \cdot 5 H_2O$	0.025
$CoCl_2 \cdot 6 H_2$	0.025
Nicotinic Acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0
Myo-Inositol	100.0
	g/l medium
Sucrose	30.0

[§]Complete medium adjusted to pH 6.0 with KOH and HCl.

TABLE C.3 Modified Wick medium (Steidl, 1976).

MAJOR MINERALS	g/liter Medium[§]
Na_2SO_4	0.8
$Ca(NO_3) \cdot 4H_2O$	0.58
$MgSO_4 \cdot 7\ H_2O$[†]	0.33
KNO_3	0.08
KCl	0.005
$NaH_2PO_4 \cdot H_2O$	0.038
MICRONUTRIENTS	**mg/liter medium**
$MnSO_4$	0.45
$ZnSO_4$	0.6
H_3BO_3	0.00375
KI	0.03
Glycine	3.0
Thiamine HCl	0.1
Ca-pantothenate	2.5
$CuSO_4 \cdot 5\ H_2O$	0.025
$NaMoO_4$	0.025
$CoCl_2 \cdot 6\ H_2O$	0.25
Sequestrene (sodium ferric diethylenetriamine pentaacetate)[#]	16.0
	g/liter medium
Sucrose	20.0

[§]Adjust complete medium to pH 5.4 with KOH and HCl. [†]Dissolve separately.

[#]Sequestrene, 13% Fe in form of $NaFe^{3+}$, Ciba Geigy.

TABLE C.4. Hoagland's medium (full strength).

Minerals	mg/l Medium[§]
Major	
$Ca(NO_3)_2 \cdot 4H_2O$	590.0
Sequestrene (NaFe 13%)	77.0
KH_2PO_4	68.4
KNO_3	253.3
$MgSO_4 \cdot 7H_2O$	125.5
Minor	
$ZnSO_4 \cdot 7 H_2O$	0.1
$MnSO_4 \cdot H_2O$	0.8
$CuSO_4 \cdot 5H_2O$	4.2×10^{-5}
H_3BO_3	1.5
$MoO_3 \cdot 2H_2O$	1×10^{-5}

[§]Adjust complete medium to pH 5.2 with KOH and HCl.

TABLE C.5 Tests for bacterial contamination

Media	Constituents	g/l Media
i	Yeast extract	10
	Bacto - Peptone	20
	$MgSO_4 \cdot 7\ H_2O$	20
	Agar	10
ii	Bacto-tryptone	10
	NaCl	5
	D-glucose	10
	Agar	15
	pH 7	
iii	Bacto-tryptone	10
	Yeast extract	5
	NaCl	10
	$CaCl_2 \cdot H_2O$	0.75
	Agar	15

APPENDIX D

RESIN FORMULA USED IN MICROAUTO-RADIOGRAPHIC STUDIES

TABLE D.1. Composition of Spurr's resin used for microautoradiographic studies in Chapter I (Spurr, 1969).

Component[§]	Firm Plastic formula[†]
	— g —
NSA (Nonenyl succinic anhydride)	26.0
ERL-4206 (Vinyl cyclohexene dioxide)	10.0
DER-4206 (Diglycidyl ether of polypropylene glycol)	6.0
DMAE (Dimethylaminoethanol)	0.4

[§]The constituents must be weighed into a beaker for mixing in the order described in the table. The mix is stirred throughly for 3 min at three 30-min intervals before using. Pot life is 3 days.

[†]Resin is cured for 8 h at 70°C.

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