

INFLUENCE OF OSMOTIC STRESS, ETHANOL, AND A SUBSTITUTED PYRIDAZINONE,
BAS 13-338, ON THE GROWTH AND LIPID COMPOSITION OF TWO CHLORELLA SPECIES

by

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(ABSTRACT)

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Chlorella vulgaris and Chlorella pyrenoidosa were compared relative to their abilities to grow at osmotic potentials of -0.1, -0.5, -1.0, -1.5, -2.0 MPa, [polyethylene glycol (PEG)-induced], and for osmotically-induced changes in lipid composition. C. vulgaris growth was inhibited as osmotic potentials decreased, while C. pyrenoidosa growth was moderately inhibited at -2.0 MPa. C. vulgaris produced increasing concentrations of triglycerides and sterol esters and decreasing levels of polar lipids and sterols as osmotic concentrations increased. Polar lipids, triglycerides, and sterols declined in C. pyrenoidosa while steryl esters remained constant. Ratios of free sterols to polar lipids were 10-fold greater in C. pyrenoidosa and were unaffected by reduced osmotic potentials. In C. vulgaris the sterol to polar lipid ratio declined.

Decreasing osmotic potentials in a continuous culture of C. vulgaris, lowered cell lipid concentration, and had no effect on chlorophyll concentrations. The greatest decrease occurred as the osmotic potential decreased from -0.1 to -0.5 MPa. Decreasing osmotic potential caused the phospholipid concentrations to decline.

Saturation of triglycerides and free fatty acids increased and decreased, respectively, while polar lipids remained fairly constant. However, the sterol to phospholipid ratio increased as the osmotic potential was lowered.

BAS 13-338 (4-chloro-5-(dimethylamino)-2-phenyl-5-3(2H)pyridazinone) had no effect on C. vulgaris resistance to osmotic stress, but caused growth inhibition as concentrations increased. However, BAS 13-338 was effective in decreasing growth inhibition of C. vulgaris grown in inhibitory levels of ethanol. BAS 13-338 had differing effects on the lipid composition of C. vulgaris when grown in PEG at an osmotic potential of -1.5 MPa compared to -0.1 MPa with 0.33% ethanol. The greatest effects were observed in the ethanol treatments where the qualitative composition of precursor sterols increased as the level of BAS 13-338 increased.

This investigation confirmed the important role of lipids in responding to environmental stress through observations of lipid responses to osmotic stress and by manipulation of lipid concentrations using BAS 13-338. Resistance to ethanol inhibition but not osmotic inhibition was achieved in the investigation.

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Chapter I

Introduction

Worldwide, limited water resources, arid environments, seasonal and even diurnal fluctuations in precipitation or insolation result in periods of plant water stress. Additionally, increased urban usage of water resources and depletion of aquifers is intensifying demands to limit the access of the agricultural sector to current water supplies. Even as world food demands are increasing, the decreased availability of water may increase crop areas subjected to water stress. Since water stress limits plant productivity, an understanding of water stress resistance in plants is imperative.

Drought-tolerant species continue growth and productivity under conditions of lowered cell water potential. Several mechanisms of tolerance to water stress have been proposed. Accumulation of osmoprotectants and inorganic ions and membrane stabilization and function under stress conditions may play a vital role in drought tolerance. Several researchers have correlated changes in the lipid composition of membranes as well as total lipids to water stress exposure. Thus, a careful documentation of water-stress resistance afforded by lipid alterations and the controlled chemical or genetic manipulation of lipid composition of plants may afford the opportunity to increase plant tolerance to water stress.

The purpose of the present study was to observe the impact of

water stress on the lipids of two Chlorella species grown under defined environmental conditions. Growth rates of these species were monitored as an indication of tolerance at each of five osmotic potentials. It was hoped that stress resistance would correlate with differences in lipid accumulation or disappearance. Additionally Chlorella vulgaris was grown under continuous culture conditions to allow daily monitoring and sampling of algae grown at constant density and concentrations of mineral nutrition minimizing interference with other culture influences. The final goal of this study was to determine whether or not lipid alterations observed in response to stress were merely non-adaptive responses rather than significant adaptations which resulted in stress tolerance. Algae were treated with BAS 13-338, a compound shown to influence lipid unsaturation. It was hoped that BAS 13-338 treatment would result in beneficial alterations in lipid composition, and that beneficial alterations could be observed as increased growth rates of treated algae under conditions of water stress.

CHAPTER II

LITERATURE REVIEW

A. Water Stress: A Regional and Global Issue

Stress has been defined as an external factor or force applied to a plant which causes a response or strain in the plant (Levitt 1972). In particular, water stress is a condition in which the water potential of a plant's environment has decreased relative to the potential of free water. A water-stressed plant will experience a strain to the degree that the environmental stress lowers the plant water potential below the potential of free water. The degree of water stress is expressed in units of pressure, MPa, since a force is acting on an area. More negative pressure values indicate greater stress exerted by the environment. However, in common usage water stress is often used to refer to the water potential of the plant rather than the environment. Whether a stress or a strain, the growth of water stressed plants is reduced when compared to growth of non-stressed plants.

Worldwide, 46.6% of the total land area of 1.33×10^{10} hectares has been classified as arid or semiarid. Irrigated land is estimated to cover 2.3×10^9 hectares (Epstein 1980). Although arid and semiarid cannot be equated with plant water stress per se, these figures help to illustrate the global dimensions of potential plant water stress.

At the national level, examination of selected regional cases adequately illustrates the increasing crisis of water availability. Currently municipalities and industries are demanding increasing proportions of available water resources. Legislation has been proposed which would restrict agricultural uses of water. Also essentially non-renewable water resources have been depleted to such an extent in some regions that agriculture has been adversely affected.

Currently, in the state of Texas water is obtained from underground aquifers or from collected surface water. Roughly two-thirds of the state is underlain by one of 23 aquifers. In 1980 groundwater supplied 8.1×10^{11} liters of water or approximately 61% of the total water needs of Texas. This level of use combined with insufficient groundwater recharge resulted in a net deficit or overdraft of 4.1×10^{11} liters in 1980. While the total surface water storage capacity of Texas is 2.4×10^{11} liters, only 8.1×10^{11} liters are dependable on a yearly basis. In 1980, Texas agriculture used 8.3×10^{11} litres, municipalities used 2.1×10^{11} liters, and manufacturing used 1.1×10^{11} of water. A comparison of these rates of water use has resulted in pressure on the agricultural sector to give up a substantial portion of its current water share. If the non-agricultural users of water are successful in their demands, an increasing reversion to dryland farming will result (Kelton 1985).

While blessed with an ample supply of water in excess of total water demands, the state of Virginia does not possess across the board water surpluses. Although irrigated agriculture in Virginia has not

increased over the past three decades and is expected to remain stable, the increasing urbanization of the state is expected to increase the frequency and seriousness of regional water shortages. For this reason the Virginia State Water Plan Advisory Committee to the State Water Control Board proposed the Virginia Water Withdrawal Act. This bill would establish a system to regulate all withdrawals of water in excess of 1.1×10^4 liters of water per month. A permit would be required for all uses of surface and ground water in excess of a 1.1×10^4 liter threshold, an exception being agricultural irrigators using water from a pond fed by diffused surface water and holding less than 2.25×10^4 liters of water. Another amendment to the present Virginia Code would rescind the exemption of agricultural users of water from regulation by the State Water Control Board in sensitive groundwater areas (primarily south of the James River and the Eastern Shore). The threshold for regulation would be lowered from 1.89×10^5 to 3.78×10^4 liters of water per day. Thus, these proposed bills, while not currently denying the rights of Virginia agriculture to water, will balance water rights of agriculture against the needs of other water users in the future (Virginia Cooperative Extension, October, 1985).

A final case demonstrating the declining water resources available to agriculture is the Ogallala aquifer. The Ogallala aquifer, encompassing 5.8×10^5 km², underlies parts of six states; Nebraska, Colorado, Kansas, New Mexico, Oklahoma, and Texas. Currently 20% of all land irrigated in the United States depends on the Ogallala aquifer. In 1980, 6.1×10^4 km² of land in this region were irrigated

with 1.6×10^{12} liters of water. At the same time recharge to the aquifer is very small. The region is faced with the ultimate exhaustion of its water resource. Major irrigated crops in the region and typical productivity under irrigated and non-irrigated management are as follows:

<u>crop</u>	<u>irrigated yield</u>	<u>non-irrigated yield</u> (bu./acre)
wheat	47	23
corn	118	42
milo	80	31
cotton	529 pounds fiber	340 pounds fiber

(Banks 1980).

The greater energy required for pumping groundwater from greater depths and a 60% increase in energy costs since 1977 or the lowered yields using dryland farming techniques will all decrease farming profitability. Compounding this serious situation has been a 20% decline in crop prices over the past two years (Federal Reserve Bank of Chicago 1986). Experts now predict abandonment of fields in the near future.

In other areas of the country with overall sufficiency of water, the potential exists for seasonal patterns of drought. Excellent examples of seasonal drought have been the spring rainfall patterns in the Southeastern United States during both 1985 and 1986 and the Midwest in 1987. In addition to the above considerations which affect water availability, plants in all areas periodically undergo internal water stress even if the soil is maintained near field capacity. During a diurnal cycle incident solar radiation can lead to dramatic

shifts in water potential and thus, daily periods of water stress.

In conclusion, competition for limited water resources, legislation affecting water use, and depletion of present sources of water will increase the need for methods to maintain productivity and profitability as water resources available to the agricultural sector decline. Inevitably as water resources decline the probability of conditions leading to water stress in plants will increase. The great demands and finite supplies of agricultural water resources will possibly be the most important driving force for furthering our knowledge of plant water stress. Understanding plant responses to water stress and investigations into ameliorating stress are increasingly important objectives.

B. Comparison of Salinity Stress With Water Stress

The primary focus of many previous water stress investigations has been the role of solutes and salinity in plant stress. Researchers have often lumped together investigations of salinity and water stress as overlapping components of osmotic stress. Le Rudulier et al. (1984) stated: "Salinity is another form of water-related stress responsible for major crop losses worldwide...". Yancey et al. (1982) state "organisms facing water stress possess a solute adaptation strategy that has a minimal requirement for genetic change and a high degree of flexibility in allowing the organism to conform to wide ranges of external salinity".

However, several investigations have shown that the primary strain

in plants subjected to salinity is directly the result of ion toxicity.

Salinity stress has been shown to be a tripartite process having osmotic, nutritional, and toxic effects (Leopold and Willing 1984). Soybean leaf discs were pretreated by floatation in isoosmotic solutions of either sorbitol or NaCl. After pre-treated discs were transferred to water, a ten-fold increase in leakiness of the sodium chloride pre-treated discs was observed. Increased leakiness was interpreted as a toxic response to sodium chloride. Similarly, experiments in which leaf discs were pretreated with various monovalent and divalent salts indicated that the monovalent salts induced greater leakiness than the divalent salts. Thus, salinity stress is dependent on the ionic species involved. During the adaptation of barley, Hordeum vulgare L., seedlings to extremely high levels of NaCl, the alteration in chloroplast membrane lipids was found to result from the high concentration of the monovalent ions rather than the increase in water potential (Muller and Santarius 1978). The stress stability of thylakoid membranes was compared by measuring photophosphorylation of two drought tolerant plants Cratogeomys plantagineum and Ceterach officinarum with the drought-sensitive plant spinach, Spinacia oleracea L. (Schwab and Heber 1984). Drastic lowering of the water potential with solutes such as sorbitol in thylakoid suspensions from these plants did not cause irreversible membrane damage. However, using a chaotropic salt such as NaCl to lower the water potential caused irreversible membrane damage. The toxic effect was strongly ion dependent. The chaotropicity of various ions differed according to the

ion position in the lyotropic series of Hofmeister. Because the studies indicated that sensitivity to stress was comparable in thylakoid membranes from tolerant and sensitive species, stress tolerance of a plant was not attributed to specific membrane structures which would cause increased membrane stability (Schwab and Heber 1984).

Further evidence for specific ion toxicity was supplied by experiments in which the ratio of K^+ and Na^+ supplied to two inbred sugarbeet, Beta vulgaris L., lines was varied (Stuiver et al. 1981). Depending on the inbred line Na^+ either stimulated or inhibited growth of sugarbeets as the Na^+ to K^+ ratio increased.

Although evidence presented above indicates that salt perturbation rather than induced osmotic stress plays a major factor in plant stress responses, other investigators have shown parallel plant responses are induced by salinity and water stress (Lerner et al. 1984). Studies using a NaCl-resistant line of tobacco Nicotiana tabacum L. cells indicated incremental additions of NaCl resulted in a stepwise accumulation of proline in the cultured cells. Exposing this same line of cells to mannitol-induced osmotic stress resulted in a parallel accumulation of proline with added increments of mannitol. Additionally, wild-type tobacco cells, which were sensitive to NaCl were also sensitive to mannitol-induced water stress and were unable to accumulate higher concentrations of proline with successive increasing increments of stress.

Observations of plant responses to salinity and interpretations of the value of these adaptations vary quite widely. A unified scheme of

water stress responses based on the striking convergent evolution in the properties of organic osmotic solute systems in bacteria, plants, and animals was proposed (Yancey et al. 1982). All of these life forms except the halobacteria were observed to make use of polyhydric alcohols, free amino acids, and combinations of urea and methylamines. These organic osmolytes are compatible with macromolecular structure and function at both high and low osmolyte concentrations. Thus, use of this system has a high degree of flexibility in allowing the organism to adapt to a wide range of external salinities with a minimal requirement for genetic change (Yancey et al. 1982). Other researchers have argued that salt absorption is the most widely used adaptation to salinity stress (Epstein 1980). The use of ions from the external medium to adapt osmotically is thought to be energetically cost effective compared with the production of organic solutes. This can be especially effective if the ions are sequestered in vacuoles minimizing contact with sensitive portions of the plant cell. Research with Chlorella autotrophica has shown that this alga continues to photosynthesize over a range of external salinity from 1% to 600% relative to the salinity of seawater. Proline is synthesized but large quantities of Na^+ , K^+ , and Cl^- are accumulated to provide over 50% of the osmotic adjustment (Ahmad and Hellebust 1984).

In conclusion, it appears that inherent differences in water and salinity stress have been poorly defined. Although patterns of plant adjustment may appear to be similar in both salinity and water stress, the actual plant stresses and strains encountered can be considerably

different. For this reason, the study of water stress should be considered discrete from, although overlapping with, the study of salinity stress.

C. Plant Stress Research Methodology

A procedural foundation useful in evaluating information generated by past and future water-stress research approaches was proposed based on a review of plant-stress research techniques (Hanson and Hitz 1982). The following research approaches to determine the adaptive significance of metabolic responses to water deficits were highlighted:

- 1.) Researchers postulate an adaptive response for one genotype based on theoretical calculations and observational data on the one genotype. Data collected are used to decide if the response is timed correctly, in the correct direction, and to a sufficient extent.
- 2.) Researchers intervene in the metabolism of one genotype to block or reinforce metabolic responses to stress and then evaluate the overall performance of the treated plant in response to stress.
- 3.) Researchers make comparisons among species, races, or cultivars from contrasting environments searching for consistent patterns of stress responses.
- 4.) Researchers make comparisons of metabolic responses between "drought resistant" and "drought susceptible" cultivars.
- 5.) Researchers use genetics to develop populations or lines of a plant that differ in extent of expression of a metabolic trait and then

test the lines in the field for overall performance.

Hanson and Hitz (1982) emphasized that research approaches must determine if metabolic alterations have resulted from stress-induced lesions at vulnerable sites in metabolism or if the alterations have resulted from adaptive changes reflecting ordered operation of metabolic regulatory mechanisms. Another possibility is that metabolic alterations observed are incidental and accompany stress but are neutral in their effect at alleviating stress (Hanson 1980). An experiment in which the phospholipid content and composition of 13 cultivars of wheat, Triticum aestivum L. differing in frost hardiness were compared illustrates the principle of incidental metabolic alterations well. The phospholipid content of leaves at the end of the hardening period was related to acquired hardiness. This relationship was described by a regression equation (Horvath et al. 1980). Although the researchers noted hardening caused increased phospholipid concentrations and phospholipid increases were correlated with increased survival, no attention was drawn to the fact that the distribution of survival of unhardened plants was the same as the distribution of hardened plants. Undoubtedly factors aside from phospholipid content were related to cold survival. Thus, a mathematically described relationship suggested a causal relationship between the two variables when, in fact, none probably existed.

Finally, the design principles coming into play when a water deficit develops over a period of days and weeks appear very different from principles governing plant responses to sudden changes in water

stress (diurnal variations). Short-term stress experiments fail to elicit full expression of metabolic potentials important in the field (Hanson and Hitz 1982, Hasegawa et al. 1984). For example, investigations of 10 barley cultivars subjected abruptly to identical degrees of internal water stress indicated that the proline-accumulating ability of the barley was positively correlated with the grain yield under conditions of limited water (Singh et al. 1972). In a reevaluation of the work Hanson et al. (1977) attempted to determine if proline accumulation was a plant-adaptive mechanism to withstand drought or a response to a lesion. Although proline accumulation had been postulated to act as a desiccation protectant or the source of nitrogen and reducing power during plant recovery from stress, none of these speculations were supported by unequivocal data. The results with barley, 'Proctor' and 'Excelsior' indicated that stringent selection for high proline accumulation following abrupt imposition of severe osmotic stress to the root would most likely result in the selection of cultivars susceptible to environmental water stress rather than selection of cultivars resistant to chronic drought (Hanson et al. 1977).

Methodologies in plant water-stress research must take into account the various whole plant water-stress avoidance responses which aid survival. Plants such as the desert ephemerals actually escape water stress by completing their life cycles during short time periods of high water availability in environments classified as arid or semi-arid (Levitt 1972). Other drought-avoiders prevent conditions of

strain during water stress by carefully restricting transpiration through such techniques as opening stomates only at night or during the early morning hours, having thickened cuticles, or reducing the amount of leaf area (Hasegawa et al. 1984, Levitt 1972). Drought avoiders can also use a strategy of maintaining high rates of water uptake using adaptations such as increasing the root to shoot ratio (Levitt 1972).

In contrast to being drought-avoidant, some plants are classified as drought tolerant by virtue of their growth under conditions of low cell water potential (Hasegawa et al. 1984). Study of the drought tolerant plants should be of the greatest use agriculturally, since this group tends to maintain productivity with lowered water availability.

Water-stress adaptations can occur at four plant organizational levels: phenological, morphological, physiological, or metabolic (Hanson and Hitz 1982). Water-stress research has shown that adaptations at any given level may influence plant responses at levels both above and below the level of adaptation. Of the above plant responses to water stress, probably the least is understood about the cellular and metabolic levels (Hanson and Hitz 1982). For this reason, several investigators have used in vitro cell culture systems as approaches to studying water-stress adaptations (Hasegawa et al. 1984, Lerner et al. 1984). The following have been listed as advantages of the in vitro cell culture techniques (Hasegawa et al. 1984):

- 1.) All responses but those operating at the cellular level are eliminated.

- 2.) Rigorous control of cell environment and nutrition is allowed.
- 3.) Ontogenetically uniform cells are obtained with reproducible patterns of growth. In whole plant studies, a large proportion of cells are beyond the active growth phase while exposed to stress.
- 4.) Changes in water status can be achieved rapidly and uniformly.
- 5.) Water relations parameters can be obtained for a cell population aliquot. Cell lines varying only in stress resistance can be isolated and compared.

In conclusion, the research approaches outlined above should yield information at both the whole-plant and cellular levels which can be used to delineate causal relationships from correlative events. Adaptations at the cellular and metabolic levels have been the least understood because of the whole plant responses which can mask metabolic responses to stress. For this reason, cell culture experiments should continue to provide information of great importance.

D. Stress Influences on Plant Lipid Composition

Hypotheses have been proposed and many experiments have been carried out in an attempt to explain the functions of various lipid classes in regulating membrane fluidity. Model vesicle systems and micelles have been developed to study lipid effects on permeability of membranes. The role of individual lipid substituents and the ratios of these substituents have been shown to alter permeabilities of these model systems which are relatively simple compared with complex cellular membranes. Therefore, it is important to observe changes in

natural systems that mirror or substantiate effects observed in the model systems. Numerous investigations of diverse environmental stresses have in fact shown that plant lipid composition is very sensitive to an assortment of environmental factors. A short review of some of these stresses and their effects on lipid composition serves to illustrate the divergent paths mediating the plant stress responses.

Temperature Stress

Studies of plant responses to temperature demonstrate that altering lipid composition is not the sole adaptive response. In work with pea, Pisum sativum L., lower growth temperatures resulted in an increase in the lipid content of the thylakoid membranes, which was named environmental enrichment. The increase in thylakoid lipid content decreased the rates of whole chain electron transport. To verify that this decrease resulted solely as a response to lipid content thylakoid membranes were artificially supplemented with extra lipid. These membranes also demonstrated a decrease in electron transport rates. The reductions in rates of electron transport were attributed to a greater separation of photosynthetic complexes in the lipid milieu which increased the effective distance of the diffusion path of inter-system mobile electron carriers (Chapman et al. 1982). Thus, adjustments to the environment can take place at the ultrastructural level, without changes in the lipid composition.

Numerous studies have also demonstrated that changes in temperature are paralleled by changes in individual lipid substituents

or in ratios of several lipid substituents. Chilling injury has been proposed to be induced by the formation of a lipid gel phase in membranes. When injury occurs the membranes become leaky to small electrolytes and ion gradients across the membranes that are essential for the maintenance of physiological activities of cells are diminished. Some investigations demonstrating that lipid phase transitions occur at physiological temperatures have focused on one particular phospholipid substituent: phosphatidyl glycerol. Analysis of dipalmitoylphosphatidyl glycerol present in mung bean (Vigna radiata L. 'Berken'), wheat, 'Falcon', and pea, 'Massey Gem', indicated a correlation existed between the concentration of this substituent and transition exotherm temperatures (Raison and Wright 1983). The concentration of dipalmitoylphosphatidyl glycerol and transition temperature, respectively, for each species were: mungbean - 1.7%, 10 C; pea - 0.3%, 0 C; and wheat - 0.0%, -3 C. These results indicated that the transition exotherm could be influenced by small amounts of high melting point lipids and involved only a small proportion of the membrane lipids. In addition to the importance of the head group substituents, the composition of the acyl chains also influence chilling resistance. The lipid groups phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), monogalactosyldiglyceride (MGDG), digalactosyldiglyceride (DGDG), and sulphoquinovosyl diglyceride (SQDG) in fluorescence polarization studies of aqueous dispersions of the separated lipids. A clear difference was noted in the phase behavior

of PG from chilling sensitive versus chilling-resistant plants (Murata and Yamaya 1984). No changes were observed with other lipid classes. This indicated to the authors that only PG could induce thermotropic phase transition at room temperature in the cellular membranes. In comparing the chilling-sensitive species [sweet potato (*Ipomoea batatas* Lam.), sponge cucumber (*Luffa cylindrica* Roem.), squash (*Cucurbita moschata* Durch.)] with chilling-resistant species [spinach (*Spinacia oleracea* L., cluster amaryllis (*Lycoris radiata* Herbert), lettuce (*Lactuca sativa* L.)], researchers noted that the percentage of 16:0, 16:1(3 trans), and 18:0 fatty acids in the PG lipid class was 67 to 82% in sensitive plants and only 54% in the chilling-resistant plants. Thus, lipid molecular species having two molecules of either 16:0, 18:0, or 16:1 (3-trans), or their combinations underwent thermotropic phase transition from liquid crystalline to gel state at room temperature or above. Molecular species having even one cis-unsaturated bond underwent phase transition below room temperature.

Studies of the polar and acyl composition of phospholipids of *Pinus pinea* seeds germinated at either 4 C or 28 C indicated that low temperature seedlings had an increased phospholipid content primarily because of an increase in PC. The double bond index of chilled seedlings was almost twice as great as seedlings germinated at 28 C. The largest changes in the double bond index were evidenced in phosphatidylethanolamine and phosphatidylinositol. Chilling resistance appeared to be mediated by both head group and acyl chain adjustments (Palacios-Alaiz et al. 1982). Plants of *Atriplex lentiformis* had more

saturated leaf lipids when grown at 43 C day / 30 C night as compared to 23 C day / 18 C night temperatures (Pearcy 1978). In MGD6 the presence of hexadecatrienoic acid occurred at low but not at high temperatures. Observed changes in other lipid classes also involved decreases in the proportion of 18:3 and increases in the concentration of more highly saturated fatty acids as the temperature increased. Increased lipid saturation was correlated with greater thermostability of the photosynthetic apparatus at high growth temperatures.

Some researchers have proposed that temperature-dependent changes in lipid composition are actually manifestations of changes in oxygen solubility. Sycamore (Acer pseudoplatanus L.) cambial cells were grown in liquid culture and oxygen concentrations of the media were varied from 12.5 to 305 μ M. While no significant differences were observed in the distribution of radioactivity between oleate and linoleate with different temperatures, the level of oxygen concentration did affect the fatty acid pattern of the total lipids. At oxygen concentrations below 60 μ M, the molar proportion of oleate increased dramatically while linoleate decreased. These results suggested temperature played only an indirect role in controlling the degree of unsaturation (Rebeille et al. 1980).

Plant sterol composition is also sensitive to temperature influences. Wheat, 'Monon', exhibited a decrease in the shoot levels of sitosterol, stigmasterol, and campesterol as the temperature changed from 10 C to 1 C (Davis and Finker 1972). No significant change occurred in shoot concentrations of cholesterol. Roots responded by

initially reducing sterol levels and recovering to higher sterol concentrations than the initial starting conditions. Crown tissues exhibited an intermediate response to cold temperatures. Studies of plant survival at freezing temperatures indicate sterol and phospholipid metabolism are affected by the hardening process. An increase in phospholipids began immediately after exposing the seedlings of winter rape (Brassica napus L. var. olifera 'Gorczański') to reduced temperatures (Sikorska and Farkas 1982). Free sterol concentrations declined at the end of the hardening period. An inverse relationship between the sterol / phospholipid ratio and the temperature at which one-half of the seedlings died indicated that adaptation involved sterol-phospholipid interactions. The observed alterations in sterol to phospholipid ratios possibly facilitated water efflux and reduced the possibility of intracellular freezing.

Nutrient and Salt Stress

Investigations of the role of essential and non-essential mineral elements on membrane lipids have relied heavily on comparing plants adapted to high-stress environments with plants from "normal" environments. Alterations in plant lipid composition have been attributed to ionic species, ratios of salts, and salts in the presence of nutrient-rich or nutrient-poor conditions. Investigations with specific elements in the growth medium indicate that stresses caused by salinity are ion specific and not only the result of osmotic stress. Also, membrane lipid alterations in response to nutrient and salinity

stress are diverse, occur in all the major lipid classes, and often involve interactions of several classes of lipids simultaneously.

Nutrient levels in culture can affect the concentration of the lipid constituents. Batch culture growth of Chlorella vulgaris at different nitrogen levels influenced the metabolism of lipids. Low nitrogen concentrations (.001 - .003% KNO_3) resulted in increased production of neutral lipids and fatty acids with a low degree of unsaturation. At higher nitrogen levels (>.003% KNO_3) the algae synthesized predominantly polyunsaturated fatty acids. As a percentage of dry weight, total lipids increased from 22.6% at 0.1% KNO_3 to 57.9% at 0.0003% KNO_3 . The ratio of polar lipids to neutral lipids increased from 0.2 to 2.0 as the nitrogen concentration increased from 0.0003% to 0.1% KNO_3 . With increasing nitrogen concentrations, the decline in neutral lipids was greater than the concomitant increase in polar lipids resulting in an overall decrease in the total lipid content (Piorreck et al. 1984).

Phospholipid metabolism was shown to depend strongly on the Ca^{+2} concentrations of the growth medium (Kuiper and Kuiper 1978). The phospholipid concentrations in plants declined as the Ca^{+2} concentration increased in the root environment. Since the amount of Ca^{+2} uptake and translocation is greatly reduced by salinity in many species, some observed salinity effects in other investigations may relate to plant Ca^{+2} nutrition.

Reports of the synthesis of novel lipids in response to salinity are rare. In general, the lipids of resistant and susceptible species

are similar. The lipids of the halotolerant algae Dunaliella parva and Dunaliella tertiiecta grown at 500 mM NaCl were analyzed (Evans et al. 1982). The algal lipid composition of the two halotolerant species was similar to the composition of other non-halophilic photosynthetic species except for the presence of the unique polar lipid diacylglycerol-O-N,N,N-trimethylhomoserine. However, it would be difficult to attribute salinity tolerance to the presence of this single polar lipid without further studies.

Biochemical and ultrastructural changes associated with salinity stress were correlated in chloroplasts isolated from alfalfa (Medicago sativa L. cv. 'Gages') seedlings. Chlorophyll, carotenoid, and polar lipid concentrations were reduced by nearly half in the presence of KCl. Concomitantly, the proportion of 18:2 in the acyl lipids increased. Salt-treated seedling chloroplasts were smaller and had swollen granal thylakoids (Harzallah-Skhiri et al. 1980).

Increasing the growth medium concentration of NaCl from 0 to 150 mM in the growth medium resulted in a decreased lipid concentration of both the leaves and roots of sunflowers (Helianthus annuus L. 'Airelle'). The linolenic acid concentration of NaCl-treated plants decreased in the shoots, while linoleic acid concentration increased in the roots (Gharsalli and Cherif 1979). Observations of the desaturation of both acetate and oleate precursors indicated the rate of desaturation of treated plants at 150 mM NaCl was only half the rate of untreated plants (Ellouze et al. 1982). Decreasing the unsaturation of membrane fatty acids was proposed to reduce the membrane permea-

bility to Na^+ while affecting membrane permeability to K^+ less (Ellouze et al. 1982).

Rootstocks of Rangpur lime (Citrus reticulata var. austera), Kharna khatta (Citrus karna), and Etrog citron (Citrus medica) differed in their ability to exclude Cl^- (Douglas and Walker 1983). Salt-induced changes in the ratios of the 'more planar' (campesterol and cholesterol) to 'less planar' (sitosterol and stigmasterol) sterols correlated well with the Cl^- -exclusion capacity. The qualitative and quantitative differences in sterol composition of roots were proposed to reflect the relative abilities of higher plants to regulate membrane permeability to ions and tolerate or adapt to a saline environment. In examining the activation energy of the ATPase for the same three citrus rootstocks differing in Cl^- -exclusion abilities, an inverse relationship was noted between the activation energy of the ATPase and the phospholipid to free sterol ratio of the plasma membrane preparations in the presence and absence of salt treatment (Douglas and Walker 1984). The investigators suggested that changes in membrane fluidity, particularly those induced by free sterols, had the potential to influence active as well as passive ion transport processes and possibly played a significant role in the Cl^- -exclusion mechanism.

Plantago species differing in salt sensitivity, exhibited varying patterns of lipid accumulation when exposed to increasing concentrations of NaCl . Concentrations of phospholipids, galactolipids, sulfolipids, sterols, and sterol esters declined in the salt-sensitive Plantago media as the NaCl concentration increased (Erdei et al.

1980). In two tolerant species of Plantago, the concentrations of lipids were maintained or even increased until grown at the highest experimental levels of NaCl. These tolerant plants exhibited an increase in the sterols and sterol esters and a decrease or no change in the free sterol concentrations. In salt-tolerant plants, phospholipid synthesis appeared stimulated by a minor salt stress. Within the sterols, the relative level of sitosterol decreased with increasing salinity, while cholesterol increased which indicated cholesterol was more effective in regulating ionic permeability of root cells. Thus, differences in tolerance seemed to result from quantitative changes in lipids, and the quantitative changes indicated that the degree of regulation of ionic permeability was determined by the lipid-containing plant cell membranes (Erdei et al. 1980).

Investigations of the growth of six species of Plantago in low-salt regimes indicated that, as with high salt concentrations, lipid adjustment occurred. When Plantago major ssp. major and Plantago maritima were exposed to conditions of low salinity, total lipids declined, sterol esters showed a pronounced decrease, and the total fatty acids were elevated. Concurrently, low salinity resulted in an increase in the degree of saturation of fatty acids. Sterol esters remained unaffected. Plantago cornatus, normally adapted to saline but nutrient poor conditions, showed a high degree of control of lipid composition under fluctuating nutritional regimes. When this species was transferred to low salt conditions, the concentration of free sterols increased matching an increase in the total fatty acids. This was

indicative of a highly regulated membrane permeability. With other Plantago species, lower levels of galactolipids were induced by low salt conditions. This was proposed as a mechanism reducing leakage of ions back to low salt solutions (Kuiper and Kuiper 1978).

Sugarbeet, 'Monohill', growth at concentrations of 1 to 100 mM Na_2SO_4 was reduced at 100 mM. On a total lipid basis, the content of the phospholipids, sulfolipids, and sterols plus sterol esters of the shoots was unaffected at the concentrations of Na_2SO_4 tested. Glycolipids in the shoots, but not in the roots, declined as Na_2SO_4 concentrations were increased. Although the growth of the sugarbeet shoots did not correlate directly with the glycolipid content, the authors stated that the increase in salt tolerance might be related to the glycolipid fraction (Stuiver et al. 1984).

An extensive comparison was made of Na^+ and K^+ effects on two sugarbeet lines which differed in their relative uptake of the two ionic species. The inbred sugarbeet line 'ADA' maintained a high K^+/Na^+ ratio while inbred line 'FDA' had a relatively low ratio. Increasing the sodium concentration in the growth medium from 0 to 150 mM NaCl or varying the ratio of K^+/Na^+ from 5 meq of Na^+ alone to 5 meq of K^+ alone indicated that the ratio of K^+/Na^+ caused the greater changes in the lipid composition of the sugarbeet lines. The direction of changes in the roots and shoots of plants were often opposite. Phospholipids and sulfolipid concentrations declined in roots and increased in shoots as the K^+ in solution was replaced with Na^+ . The interactions between the inbred lines and alterations in the K^+ to Na^+

ratio or alterations in Na^+ concentrations in solution indicated that the physiology of the lines differed in response to the internal concentrations of Na^+ and K^+ (Stuiver et al. 1978).

In conclusion, great variations in plant response to salinity and nutrition are possible. Some variation in plant responses are caused by sensitivity to particular ions while others are due to inherent plant differences. Plant adjustments to salinity stress appear universal. The question is whether or not the responses are adaptive. Experiments in which various lipid class concentrations are manipulated before exposure to stress may help elucidate more clearly the precise interactions between lipid adaptation and stress resistance.

Water Stress

Various hypotheses have been proposed as to how water stress mediates changes in plants. Changes in the cellular distribution of lipids have been observed and possibly reflect altered roles of lipids in stress. The cellular organization and ultrastructure of maize (Zea mays L.) subjected to water stress was correlated with both abscisic acid levels and measurements of water potentials. With increasing levels of water stress, cytoplasmic vesicles appeared in the bundle sheath and mesophyll cells. After 7 days of water stress the water potential dropped to -1.85 MPa. Osmiophilic granules formed around the edge of the chloroplasts (Giles et al. 1974).

Farkas et al. (1982) also proposed that water-stress-induced accumulation of abscisic acid altered the lipid composition. Water-

stressed and abscisic-acid-treated leaves of corn, bean (Phaseolus vulgaris L., and Capsicum vulgaris exhibited no changes in fatty acid composition of isolated phosphatidylcholine. In water stressed leaves a reduction in the formation of free sterols and an increase in the formation of phosphatidylcholine were noted in experiments in which labeled mevalonic acid and glycerol incorporation were monitored. The ratio of the formation of free sterols to phosphatidylcholine declined during a 4-day period of water stress. Abscisic-acid-treated leaves exhibited a similar change in the sterol to phosphatidylcholine ratio as the concentration of abscisic acid in the incubation medium increased. The reduction of sterol to phosphatidylcholine was proposed to result in more fluid membranes. This long-term effect is different from the direct interaction abscisic acid has with the membrane upon initial exposure (Farkas et al. 1982).

In experiments monitoring the effects of short-term water stress on lipid composition, 28-day-old corn, 'Style Pak', plants were subjected to a polyethylene glycol-induced osmotic stress of -1.5 MPa for a period of 48 hours. The stress resulted in increased levels of triglycerides in the stem and leaf tissues, and increased the stem steryl ester concentration. Stem and leaf triglyceride concentrations increased, with linolenic acid being the primary fatty acid substituent increasing in leaves. As a whole, the plant phospholipid composition seemed to be relatively resistant to rapid environmental perturbation. No new lipid constituents were found to be produced in response to water stress (Douglas and Paleg 1981).

Further experimental evidence has indicated that alterations in cellular lipid composition are induced during short term exposure to water stress. In Spirodela, grown for 15 hours on medium containing polyethylene glycol, the concentrations of protein, lipids, chlorophyll, and carotenoids in the fronds were reduced. The reduction was less for phospholipids and sulfolipids than for glycolipids where the greatest reduction was in MGDG. In all lipid classes examined, linoleic acid increased, while linolenic acid decreased except in the phosphatidyl glycerol class. A decrease in the total fatty acid concentration (expressed on a per gram dry weight basis) was noted with the onset of stress. Neutral lipids increased during stress treatments from 8.7% to 20%. MGDG, DGDG, PC, and PE decreased with stress. PG, SL, and PI remained stable. Trans- γ -hexadecanoate concentration decreased with increased levels of stress (Lechevallier 1977).

Experiments dealing with longer-term exposure of plants to water stress have noted different changes from the short-term experiments. Extended stress of wheat and barley caused an increase in the total phospholipid concentration of entire leaves (Chetal et al. 1980). Increases of phosphatidylcholine and decreases of phosphatidylglycerol were greatest in the drought-susceptible cultivars of both crops. Since phosphatidylinositol and phosphatidylcholine are components of the non-photosynthetic membranes, observed increases in phosphatidylcholine appeared to indicate that during water stress non-photosynthetic organelles such as the golgi, mitochondria, and endoplasmic reticula were increasing. Thus, studies of the

concentrations of chloroplastic lipids were initiated (Chetal et al. 1981, 1983). The glycolipid concentration of chloroplasts decreased in both wheat and barley when plants were stressed. The decrease in glycolipids was most apparent in stress-sensitive cultivars. Also the greatest reduction of phospholipids occurred in cultivars of wheat and barley requiring greater quantities of water. The chloroplast content of phosphatidylcholine increased, while the content of phosphatidylglycerol decreased. The decline in chloroplastic phospholipid concentration was attributed to the destruction of membranes. After being released from water stress, stress-resistant varieties were better able than stress sensitive varieties to restore phospholipid concentrations to non-stressed levels (Chetal et al. 1983). Unfortunately, in the preceding long term studies, no measurements were made of the actual degree of water stress experienced by the cultivars of wheat and barley. Stress resistance was designated on the basis of water requirements for the cultivars.

Oat (*Avena sativa* L.) plants subjected to consecutive treatments of water stress for periods of 18 to 24 hours with intervening 24-hour periods of recovery were assayed for fresh weight, dry weight, and lipid composition (Liljenberg and Kates 1982). The degree of water stress was expressed as the ratio of root fresh weight to dry weight rather than as actual water potentials of the plant material. Leaves of stressed plants were close to wilting. While stress treatment resulted in a decrease in membrane acyl lipids, the production of sterols did not change. The result was an increased ratio of

sterols / phospholipids plus glycolipids. A persistent increase in the fatty acid saturation of all acyl lipids in both the microsomal and plasmalemma fraction resulted from stress, even 10 days after the stress was removed.

To study the effects of water-deficit stress on sterol metabolism, oats, 'Seget', were grown using sand culture and exposed to alternating periods of stress and rewatering (Liljenberg et al. 1985). At the end of the fourth stress period, the fresh weight to dry weight ratio declined 50% relative to the unstressed control plant ratio. This stress resulted in a significant increase in free sterol concentrations without altering the sterol composition. These results contrast with the work of previous researchers who observed slight reductions (Farkas et al. 1982) or no changes in the concentration of free sterols (Grunwald 1978). Liljenberg et al. (1985) proposed that several stress induction periods or a certain stress threshold value would cause sterol accumulation in membranes. Additionally, the acyl lipids of oat root cells decreased sharply in response to water deficit stress resulting in an increase in the overall sterol to acyl lipid ratios. Finally, the noted increase in saturation in the acyl lipids of the whole root as well as in the membrane vesicle fractions could be expected to result in changed physical behavior and permeability properties of plasma membranes in the root cells after stress in direction leading to increased water retention.

Experiments with 25-day-old wheat, 'Sonalika', seedlings subjected to polyethylene glycol-induced water stress resulted in increased

concentrations of sterols and an increase in the proportion of cholesterol (Biswas et al. 1983). As the water potential decreased from 0 to -1.5 MPa, the total sterol concentration increased from 0.48 to 3.46 mg per gram dry weight, and cholesterol in particular increased from 8.47% to 12.14%.

Water stress affected the lipid composition differently in three cultivars of cotton (Gossypium hirsutum L.) differing in drought sensitivity (Pham Thi et al. 1982). In 'Moco' and 'Mocosinho', drought resistant cultivars, the levels of both the phospholipids and galactolipids increased with mild drought but diminished with severe drought. In the drought-sensitive cultivar, 'Reba', the concentrations of phospholipids and galactolipids decreased with increasing levels of dehydration. The researchers proposed that the ability to maintain lipid and fatty acid composition was related to stress tolerance possibly by maintaining adaptation of the cellular compartments.

Further studies were initiated to study lipid composition of chloroplasts of stressed cotton chloroplasts (Ferrari-Illiou et al. 1984). Stress induced greater reductions in the galactolipid than the phospholipid fraction. Also the degree of unsaturation of fatty acids decreased due to a reduction in the percentage of linolenic acid.

Studies of the incorporation of radioactively labelled acetate with cotton were initiated to determine how water stress affected the rates of lipid metabolism (Pham Thi et al. 1985). During cotton water stress, incorporation of acetate into phosphatidylcholine and galactolipids was markedly decreased, while the incorporation into

neutral lipids increased. Water deficits also inhibited fatty acid desaturation resulting in a sharp decline in linoleic and linolenic acid biosynthesis. Researchers proposed that the greater stability of the membrane system of 'Mocosinho', a drought-resistant cotton cultivar, resulted from less pronounced variations in lipid metabolism under water stress than the drought-sensitive cultivar, 'Reba'. A decline in galactolipids resulted from an increased degradation rather than the inhibition of synthesis of galactolipids.

Few studies have used algae to examine the role of water stress on lipid composition. In one study however, polyethylene glycol-6000 was used as an osmoticum in the growth medium. Chlorella vulgaris cells were exposed to a stress of -0.7 MPa, but no conclusions as to the effects on lipid composition on this species were made (Prasad and Khan 1981).

Since plants are often exposed to several stresses during growth, reports indicating stress cross-protection through lipid adaptation are to be expected. In studies of frost tolerance in wheat, 'Jubilejnana', changes in the phospholipid content and composition were investigated (Huiteña et al. 1982). Non-stressed plants were adjusted daily to a 40% soil water holding capacity while stressed plants were allowed to deplete soil moisture to a level of 10% soil water holding capacity. The phospholipid concentration of drought-stressed plants increased. This change was similar to changes in plants exposed to frost hardening temperatures. The PC to PE ratio remained at 4.5 in nonstressed plants. However, in stressed plants the ratio increased from 4.5 at 9

days after germination to 8.0 at 25 days after germination. Such a change again paralleled changes induced by hardening temperatures. No changes were observed in the fatty acid composition of total leaf lipids of wheat grown under stressed conditions over a 15-day period relative to lipid composition of non-stressed plants.

Cross protection against drought was also provided by far-red light through effects on the lipid composition of cotton, 'Bou', (Quedraogo et al. 1984). In roots, far-red treatment caused an increased saturation of fatty acids. During water stress a general decrease in the concentration of fatty acids with a concomitant increase in saturation of fatty acids was observed. Without far-red pretreatment, water stress caused a marked decrease in fatty acids of cotton buds. However, water stress had no effect on the fatty acid concentration of cotton buds pretreated with far-red light. Sufficient saturation had occurred because of far-red exposure.

Miscellaneous Effects

Previous sections of this review indicate that studies of water-stress effects on plant lipid composition must take into account other environmental factors which can be altered as a consequence of, or in conjunction with, water stress.

For example, it is possible to culture algae heterotrophically. The sterols of Euglena gracilis Z. were shown to be qualitatively and quantitatively different when grown in the light as opposed to darkness. In the light, Euglena contained mainly free Δ^7 -sterols with

a methyl or ethyl substituent at the C-24. By contrast, dark-grown Euglena produced small amounts of free sterols and mainly sterol esters with a Δ^5 -double bond and with or without an ethyl substituent at C-24. The authors suggested that this indicated the free Δ^7 -sterols were associated with the chloroplast membranes (Anding et al. 1971).

When Chlorella ellipsoidea and Chlorella emersonii were grown either autotrophically, photoheterotrophically, or heterotrophically, lipid composition reflected growth habit (Wright et al. 1980). When grown heterotrophically, the percentage of sterol in C. ellipsoidea dropped 15-fold relative to autotrophic growth, while heterotrophic growth of C. emersonii resulted in a 60% reduction. Photoheterotrophic growth resulted in a 66% and 33% decline in sterol concentration of C. ellipsoidea and C. emersonii, respectively, compared to the autotrophic sterol composition. The percentages of the individual sterols changed less than 5% in response to the different growth habits. Heterotrophic growth resulted in a 50% decline in linolenic acid, with corresponding increases in oleic and linoleic acids. While the total saturated to unsaturated ratio declined 35% in C. ellipsoidea, no decline occurred in this ratio with C. emersonii.

The control of sterol synthesis also appears to be regulated by internal controls responding to light/dark transitions. Synchronous cultures of the green alga, Chlamydomonas reinhardtii 137+, synthesized sterol continuously with the highest rates of synthesis confined to the light period. With the onset of the dark period, sterol synthesis dropped an order of magnitude. Upon reillumination, sterol synthesis

increased 30-fold (Janero and Barnett 1982).

E. Chemical Ameliorants of Water Stress

The review of the literature as presented thus far has given indications that changes in the composition of lipids in response to environmental stresses do occur. However, the criteria set forth by Hanson and Hitz (1982) in regard to the research methodologies have not been used extensively. As a result many researchers have only been able to correlate changes in lipid composition with water stress rather than prove causation. One approach that may contribute to a better understanding of the role of lipids in plant stress responses is the work of Christensen and St. John (1984). In this study the researchers chemically manipulated the level of unsaturation of plant fatty acids and demonstrated a causal relationship between fatty acid unsaturation and plant chilling resistance (St. John 1982). Compounds inhibiting desaturation or inhibiting sterol synthesis in plants may have potential to aid in studying stress phenomena in plants. Currently, considerable interest has developed in the efficacy of several anti-gibberellins (Shanahan and Nielsen 1987). Two experimental anti-gibberellins, BAS 106..W and BAS 110..W, were applied to corn seed and the plants were grown under high or low levels of irrigation. The chemical treatments reduced plant water stress during silking and early grain fill, particularly under the low irrigation treatment. Under low irrigation, grain yields increased an average of 13% for the 2 years of the study while under high irrigation the chemicals reduced the yield

8%. Measurements indicated that reductions in water loss occurred as a result of a decline in the leaf area index. Again the question arises as to whether anti-gibberellins also influence lipid composition, possibly affecting stress resistance at the membrane level.

Application of mepiquat chloride to cotton, 'Paymaster 303', in Texas resulted in reduced plant growth and increased water potentials (0.2 to 0.45 MPa) and transpiration rates (increases of 13 to 28%) with no decrease in lint yields. The treatment did not appear to increase the extraction of soil moisture by plants (Wendt et al. 1984).

Two compounds, BAS 13-338 (4-chloro-5-(dimethylamino)-2-phenyl-3(2H)pyridazinone) and CCC (chlorocholine chloride) may be good candidates for investigations into water stress resistance, since both are reported to alter lipid composition and also affect plant resistance to water stress (St. John 1984, El Damaty et al. 1965, Goodin et al. 1966).

BAS 13-338

BAS 13-338 belongs to a herbicidal class of compounds referred to as substituted pyridazinones. These compounds have multifunctional modes of action depending on the substitution of the basic ring structure. Biochemical modes of action have been determined to include: inhibition of PSII in electron transport, interference in the accumulation of chloroplast pigments (chlorophylls and carotenoids), and inhibition of the desaturation of linoleic to linolenic acid in galactolipids and phospholipids (St. John 1982).

Unlike other herbicides in this class, BAS 13-338 has relatively little effect on chloroplast pigment accumulation. Pigment accumulation of 4-day-old shoots of wheat chloroplasts was unaffected by treatment with BAS 13-338 (Hilton et al. 1971). Slight herbicidal interference with chloroplast pigment development was reported in in vivo absorption spectral studies of barley, 'Dayton CI 9517', and mustard (Brassica juncea L. Coss. 'Southern Giant Curled') (Hilton et al. 1969). Also BAS 13-338 was a less potent inhibitor of photosynthetic electron transport than other substituted pyridazinones. Ferricyanide reduction by the Hill reaction in isolated chloroplasts was inhibited 50% at $1.4 \times 10^{-4}M$. Electron transport was inhibited at the same point of action as the phenylurea and triazine herbicides. In wheat shoots, BAS 13-338 was the most effective of the substituted pyridazinones in altering the ratios of linoleic to linolenic acid (18:2 / 18:3) of the galactolipids (St. John 1982). In untreated wheat plants, the 18:2 / 18:3 ratio was 0.32 for both MGDG and DGDG, while the ratio for plants treated with 0.1mM BAS 13-338 was 3.82 for MGDG and 3.32 for DGDG. Although the saturation ratios of the galactolipids were changed dramatically, only a slight shift was noted in the ratio of saturated to unsaturated fatty acids. These results have confirmed that the site of action of BAS 13-338 is the Δ^6 -desaturase. Differential responses in saturation levels by different species and tissues suggest that control of linolenic acid biosynthesis may vary or the action of the herbicide may be affected by some cellular constituent. Labelling of linolenic acid in plants incubated with

[^{14}C]acetate was severely inhibited in cucumber (Cucumis sativus L.), maize, and rye grass (Lolium perenne L.), while labeling was unaffected in pea and spinach leaves. In general, C-3 monocotyledonous plants were more sensitive than dicotyledons. In contrast to this activity, total labelling of acyl lipids of algae incubated with [^{14}C]acetate was severely inhibited by 10^{-4}M BAS 13-338 in Azolla mexicana, Azolla fuliculoides, Chlorella vulgaris, Chlorella pyrenoides, and Anabaena variabilis (Murphy et al. 1985).

Use of the substituted pyridazinone BAS 13-338 has recently provided the opportunity to bypass comparative studies of the role of membrane lipids and their component fatty acids in plant responses to temperature. Prior approaches compared chilling-resistant and chilling-sensitive plant species and demonstrated that chilling-resistant species contained more unsaturated fatty acids, particularly linolenic acid. With the development of BAS 13-338, it is possible to manipulate the content of linolenic acid of plant membranes in a given species and study plant responses to temperature. In studies with cotton decreased levels of linolenic acid in membranes of root tips correlated with increased sensitivity to chilling (St. John 1984). Field experiments with wheat, barley, and rye (Secale cereale L.) indicated that BAS 13-338 treated plants had reduced levels of survival and tillering after the overwintering period. The reduced survival correlated with reduced levels of linolenic acid.

BAS 13-338 also seems to provide some protection against drought stress (St John 1984). Pre-emergence soil applications of BAS 13-338 to

corn, 'Silver Queen', at a rates of 5.6 to 11.0 kg/ha resulted in significantly less leaf rolling under water stress compared to untreated corn. Treated plants transpired at a rate of 0.59 g H₂O / cm² leaf area / 24 hr while untreated plants transpired at a rate of 0.79 g H₂O / cm² leaf area / 24 hr. Similar results were noted in sorghum and cotton plants treated with BAS 13-338. No differences in diffusive resistance, leaf water potential, or internal water content were noted between treated and untreated plants. In soybeans (Glycine max L. Merr.) treated with 15 ppm BAS 13-338, the cuticular waxes were 40% greater than controls. It was suggested that water economy was partially altered by leaf cuticular wax which reduced cuticular transpiration.

Chlorocholine Chloride (CCC)

In 1960 a new group of growth retardant chemicals, the onium compounds, was reported. As a class, these compounds with a quaternary ammonium structure exhibited good growth inhibition of wheat and cotton but substantially less effect on oats (Avena sativa L.), rye, and barley and virtually no activity on rice (Oryza sativa L.) and soybean.

The most active of these compounds was CCC. By examination of various homologues of CCC, researchers determined that biologically active molecules possessed a quaternary ammonium or phosphonium atom or a tertiary sulfonium atom. In the case of ammonium compounds with moderate or greater activity, 6 or 8 carbon atoms and 16 to 18 hydrogen atoms were required (Sauter 1984).

Plant growth retardants are chemicals that slow cell division and cell elongation in shoot tissues and regulate height physiologically without formative effects. CCC has a high solubility in water, persists in soils for 3 to 4 weeks, and is effective as both a soil drench and as a foliar spray (Cathey 1964). CCC was found to be effective on selected species with no obvious correlation between taxonomic classification and plant response. Even different cultivars of the same species varied greatly in their responsiveness to applications of CCC. Of 55 species tested, 44 were reported to be sensitive to soil drenches of CCC including wheat. However, bentgrass (Agrostis sp.), red fescue (Festuca rubra L.), barley, and Kentucky bluegrass (Poa pratensis L.) were essentially unresponsive to applications of CCC.

CCC enhanced growth, photosynthesis, respiration, and nitrite and nitrate assimilation of Chlorella fusca at concentrations between $5 \times 10^{-3}M$ to $5 \times 10^{-2}M$. Chlorophyll a and b concentration also increased on a dry weight basis in treated cells (Ahmed and Abdullah 1980). In experiments in which cells of Chlorella sorokiniana were grown in synchronous culture, concentrations of CCC between $10^{-3}M$ and $10^{-2}M$ were ineffective at promoting or inhibiting cell growth. This was interpreted as indicating CCC had no regulatory effects on growth of Chlorella sorokiniana (Chen and Lin 1981). CCC slightly stimulated growth and altered protein, lipid, carbohydrate, and nucleic acid content of Chlorella pyrenoidosa at a concentration range of $10^{-4}M$ to $10^{-2}M$ (Czerpak 1979).

In addition to cell division effects, CCC and other choline derivatives are capable of influencing the lipid composition of plants. CCC at $10^{-3}M$ caused a significant increase in the concentration of triacylglycerols of Chlorella vulgaris. Also, CCC at concentrations of $10^{-3}M$ to $10^{-4}M$ tended to decrease total sterols (Orcutt et al. 1984a, 1984b).

CCC has been implicated in increasing plant tolerance to various stress conditions including: soil salinity, drought, attacks by insects, and plant diseases (Ganashan et al. 1975). Numerous investigations have been carried out to determine the effect of CCC on the water use efficiency of treated plants. CCC did not influence the transpiration coefficient of wheat, 'Opal', (El Damaty et al. 1965). However, treated plants under conditions of drought used the water more efficiently for the production of kernels as determined by the harvest index. CCC did not affect the total dry matter production or shoot/root ratios in water-stressed plants in which stress was regulated by controlling moisture level of soil or frequency of irrigation.

In comparison with control plants, water uptake of barley treated with $10^{-4}M$ to $10^{-3}M$ CCC and grown in nutrient solution was reduced 60 to 80%. When plants were decapitated and xylem exudate collected, a similar inhibition in water uptake was found. This treatment decreased the dry weight of roots (Gohlke et al. 1962).

Experiments with barley, 'Blanco', in which CCC-treated soil was dried to predetermined weights and then rewatered, indicated that CCC

did not provide a consistent improvement in water use ratios of treated plants (Goodin et al. 1966). CCC treatment did result in a significant reduction in water used per unit of seed yield. Thus, CCC appeared to offer advantages for increased grain production with any given amount of water.

CCC-treated chrysanthemum (Chrysanthemum morifolium Hensl.) plants exposed to high temperatures after growth at low light intensity were less susceptible to wilting than untreated plants (Halevy and Kessler 1963). In experiments with bean, 'Brittle Wax', treated with CCC indicated that treated plants were resistant to water stress. Water was withheld from treated and untreated plants after the full expansion of the third true leaf. Untreated plants were fully desiccated after 30 days. Treated plants maintained turgor for 30 days and did not lose leaves until the 42nd day after withholding water. Treated plants were found to deplete soil moisture more. (Halevy and Kessler 1963).

Interactions of CCC and water stress have been reported too. Experiments with spring wheat, 'Phoebus', demonstrated that CCC had little effect on the yields from irrigated plots, while yield was increased from unirrigated plots by 670 kg/ha. The investigators theorized that CCC increased yield by increasing root mass and enabling more culms to survive dry periods when shoot numbers were declining (Humphries et al. 1967). Three foliar applications of CCC to wheat plants had no effect on dry matter production, grain yield, or water requirement of plants watered regularly or exposed to short one-day wilting cycles. CCC-treated plants exposed to two drought cycles of 5

to 6 days had a pronounced increase in dry weight and grain production relative to untreated plants. No consistent effects were seen on transpiration, water requirement, or stomatal opening. The increasing ability of treated plants to withstand drought was related to the chemical's ability to delay senescence in stressed tissues (Plaut and Halevy 1966).

The keeping quality of plants after flowering was greatly increased by treatment with CCC as treated plants did not wilt as fast as control plants (Lindstrom and Tolbert 1960). In experiments with sunflower (Helianthus annuus L.) CCC applied as soil drenches resulted in increased stomatal frequency while decreasing stomatal size. Diffusive stomatal resistance increased in treated plants (Lovett and Campbell 1973).

Thus, numerous claims have been made concerning the stress resistance conferred by treatment with either BAS 13-338 and CCC. Using these compounds in a model system in which lipid composition was also monitored could help elucidate the relationship between lipid composition and water stress resistance. Additionally, by using a cellular system rather than a whole-plant system, the interference caused by whole plant responses to the environment can be minimized possibly allowing direct observation of responses of cell membranes to stress and chemical treatment.

LITERATURE CITED

- Ahmad, I. & Hellebust, J. A. 1984. Osmoregulation in the extremely euryhaline marine microalga Chlorella autotrophica. - Plant Physiol. 74: 1010-1015.
- Ahmed, J. & Abdullah, Z. 1980. Preliminary investigations on the effect of (2-chloroethyl) trimethyl ammonium chloride (CCC) on the metabolism of Chlorella. - Pak. J. Bot. 12: 81-89.
- Anding, C., Brandt, R. & Ourisson, G. 1971. Sterol biosynthesis in Euqlena gracilis Z.: Sterol precursors in light-grown and dark-grown Euqlena gracilis Z. - Eur. J. Biochem. 24: 259-263.
- Banks, H. O. 1980. Six-state high plains Ogallala aquifer area regional study. - In Western Water Resources: Coming Problems and the Policy Alternatives (Symposium by Federal Reserve Bank of Kansas City), pp. 49-66. Westview Press Inc. Boulder, Colorado. ISBN 0-86531-036-X.
- Biswas, A. K., Sarkar, U. & Chaudhury, M. A. 1983. Water-stress induced changes in total steroids and cholesterol contents in wheat seedlings. - Sci. Cult. 49: 109-110.
- Cathey, H. M. 1964. The physiology of growth retarding chemicals. - Annu. Rev. Plant Physiol. 15: 271-302.
- Chapman, D. J., Millner, P. A., Ford, R. C. & Barber, J. 1982. Lipid content of chloroplast thylakoids and regulation of photosynthetic electron transport. - In Biochemistry and Metabolism of Plant Lipids (J. F. G. M. Wintermans and P. J. C. Kuiper eds.), pp.363-368. Elsevier, New York, ISBN 0-444-80457-9.
- Chen, P. C. & Lin, H. F. 1981. Effects of growth retardants and inhibitor on the growth of green alga Chlorella sorokiniana. - Hsueh Poa 17: 241-247.
- Chetal, S., Wagle, D. & Nainwatee, H. 1980. Phospholipid changes in wheat and barley leaves under water stress. - Phytochemistry 19: 1393-1395.
- Chetal, S., Wagle, D. S. & Nainwatee, H.S. 1981. Glycolipid changes in wheat and barley chloroplasts under water stress. - Plant Sci. Lett. 20: 225-230.

- Chetal, S., Wagle, D. S. & Nainwatee, H. S. 1983. Phospholipid changes in wheat and barley chloroplasts under water stress. - *Plant Sci. Lett.* 29: 273-278.
- Christensen, M. N. & St. John, J. B. 1984. Chemical modification of plant responses to temperature extremes. - In *Bioregulators Chemistry and Uses* (R. L. Ory and F. R. Rittig eds), ACS Symposium Series 257, pp. 235-243. American Chemical Society, Washington D.C. ISBN 0-8412-0853-0.
- Czerpak, R. 1979. Effect of some natural and artificial growth regulators on the chemical composition of autotrophic microorganisms of the genera Anabaena, Chlorella, Scenedesmus. - *Bull. Acad. Pol. Sci., Sci. Biol.* 27: 591-603.
- Davis, D. L. & Finker, V. C. 1972. Influence of temperature on sterol biosynthesis in Triticum aestivum. - *Plant Physiol.* 52: 324-326.
- Douglas, T. J. & Paleg, L. G. 1981. Lipid composition of Zea mays seedlings and water stress-induced changes. - *J. Exp. Bot.* 32: 499-508.
- Douglas, T. J. & Walker, R. R. 1983. 4-Desaethylsterol composition of citrus rootstocks of different salt exclusion capacity. - *Physiol. Plant.* 58: 69-74.
- Douglas, T. J. & Walker, R. R. 1984. Phospholipids, free sterols and adenosine triphosphatase of plasma-membrane enriched preparations from roots of citrus genotypes differing in chloride exclusion ability. - *Physiol. Plant.* 62: 51-58.
- El Damaty, A. H., Kuhn, K. & Linser, H. 1965. Water relations of wheat plants under the influence of (2-chloroethyl)-trimethylammonium chloride (CCC). - *Physiol. Plant.* 18: 650-657.
- Ellouze, M., Gharsalli, M. & Cheriff, A. 1982. Effect of sodium chloride on the biosynthesis of unsaturated fatty acids of sunflower plants (*Helianthus annuus*). - In *Biochemistry and Metabolism of Plant Lipids* (J. F. G. M. Wintermans and P. J. C. Kuiper eds.), pp. 419-422. Elsevier, New York, ISBN 0-444-80457-9.
- Epstein, E. 1980. Responses of plants to saline environments. - In *Genetic Engineering of Osmoregulation: Impact on Plant Productivity for Food, Chemicals, and Energy* (D.W. Raines, R.C. Valentine and A. Hollander, eds), pp. 7-21. Plenum Press, New York.

- Erdei, L., Stuiver, C. E. E., & Kuiper, P. J. C. 1980. The effect of salinity on lipid composition and on activity of Ca^{+2} - and Mg^{+2} -stimulated ATPases in salt-sensitive and salt-tolerant Plantago species. - *Physiol. Plant.* 49: 315-319.
- Evans, R. W., Kates, M., Ginzburg, M. & Ginzburg, B. 1982. Lipid composition of halotolerant algae, Dunaliella parva Lerche and Dunaliella tertiolecta. - *Biochim. Biophys. Acta* 712: 186-195.
- Farkas, T., Nemezc, G., & Singh, B. 1982. Abscisic acid induced changes in physical state and lipid composition of plant membranes. - In *Biochemistry and Metabolism of Plant Lipids* (J.F.G.M. Wintermans and P. J. C. Kuipers eds.), pp. 471-474. Elsevier, New York, ISBN 0-444-80457-9.
- Federal Reserve Bank of Chicago. 1986. Selected agricultural economic indicators. - In *Agricultural Letter* # 1681. P.4
- Ferrari-Iliou, R., Thi, A. & Da Silva, J. V. 1984. Effect of water stress on the lipid and fatty acid composition of cotton Gossypium hirsutum cultivar Reba chloroplasts. - *Physiol. Plant.* 62: 219-224.
- Ganashan, P. & Whittington, W. J. 1975. Effects of chloromequat chloride on related tall and dwarf rice varieties. - *Ann. Appl. Biol.* 81: 219-225.
- Gharsalli, M. & Cherif, A. 1979. Action du chlorure de sodium sur la croissance et la teneur en lipides de plants de Tournesol (Helianthus annuus L.). - *Physiol. Veg.* 17: 215-229.
- Giles, K. L., Beardsell, M. F. & Cohen, D. 1974. Cellular and ultrastructural changes in mesophyll and bundle sheath cells of maize in response to water stress. - *Plant Physiol.* 54: 208-212.
- Gohlke, A. F. & Tolbert, N. E. 1962. Effect of 2-chloroethyltrimethylammonium chloride on phosphate absorption and translocation. - *Plant Physiol. Suppl.* 37: xii.
- Goodin, J. R., McKell, C. M. & Webb, F. L. 1966. Influence of CCC on water use and growth characteristics of barley. - *Agron. J.* 58: 453-454.
- Grunwald, C. 1978. Function of sterols. - *Phil. Trans. R. Soc. Lond. B.* 284: 541-558.

- Halevy, A. H. & Kessler, B. 1963. Increased tolerance of bean plants to soil drought by means of growth-retarding substances. - *Nature* 197: 310-311.
- Hanson, A. D. 1980. Interpreting the metabolic responses of plants to water stress. - *Hortscience* 15: 623-629.
- Hanson, A. D. & Hitz, W. D. 1982. Metabolic responses of mesophytes to plant water deficits. - *Annu. Rev. Plant Physiol.* 33: 163-203.
- Hanson, A. D., Nelsen, C. E. & Everson, E. H. 1977. Evaluation of free proline accumulation as an index of drought resistance using two contrasting barley cultivars. - *Crop Sci.* 17: 720-726.
- Harzallah-Skhiri, T., Guillot-Salomon, T. & Sigmol, M. 1980. Lipid changes in plastids isolated from alfalfa seedlings grown under salt-stress. - In *Biogenesis and Function of Plant Lipids* (P. Mazliak, P. Beneviniste, C. Costes, R. Douce, eds), pp. 99-102. Elsevier, New York, ISBN 0-444-80273-8.
- Hasegawa, P. M., Bressan, R., Handa, S. & Handa, A. K. 1984. Cellular mechanisms of tolerance to water stress. - *Hortscience*. 19: 371-377.
- Hilton, J. L., Scharen, A. L., St John, J. B., Moreland, D. E. & Norris, K.H. 1969. Modes of action of pyridazinone herbicides. - *Weed Sci.* 17: 541-547.
- Hilton, J. L., St John, J. B., Christensen, M. N. & Norris, K. H. 1971. Interactions of lipoidal materials and a pyridazinone inhibitor of chloroplast development. - *Plant Physiol.* 48: 171-177.
- Horvath, I., Vigh, L., Belea, A. & Farkas, T. 1980. Hardiness dependent accumulation of phospholipids in leaves of wheat cultivars. - *Physiol. Plant.* 49: 117-120.
- Huiteima, H., Woltjes, J., Vigh, L. & Van Hasselt, P. 1982. Drought induced frost resistance in wheat correlates with changes in phospholipids. - In *Biochemistry and Metabolism of Plant Lipids* (J. F. G. M. Wintermans and P. J. C. Kuiper eds), pp. 433-436. Elsevier, New York, ISBN 0-444-80457-9.
- Humphries, E. C., Melbank, P. J. & Williams, E. D. 1967. Interaction of CCC and water deficit on wheat yield. - *Nature* 215: 782.
- Janero, D. R. & Barnett, R. 1982. Sterol synthesis in Chlamydomonas reinhardtii 137⁺ cell-cycle variations. - *Biochim. Biophys. Acta* 710: 242-247.

- Kelton, E. 1985. When the well runs dry. - Texas Monthly 13: 170-172, 206, 224.
- Kuiper, D. & Kuiper, P. J. C. 1978. Lipid composition of the roots of Plantago species: responses to alteration of the level of mineral nutrition and ecological significance. - Physiol. Plant. 44: 81-86.
- Lechevallier, D. 1977. Effets du polyéthylène glycol sur les lipides et les lipochromes des colonies de Spirodèle. - Physiol. Veg. 15: 387-402.
- Leopold, A. C. & Willing, R. P. 1984. Evidence for toxicity effects of salt on membranes. - In Salinity Tolerance of Plants (R. C. Staples and G. H. Toenniessen eds), pp. 67-76. John Wiley and Sons, New York, ISBN 0-471-89674-8.
- Lerner, H., Poljakoff-Mayber, A., Reinhold, L. & Hodges, T. K. 1984. The Mechanism of Osmotic Accomodation to Water and Saline Stress in Plant Cells; and the Role of Hormones in Modulating This Response. United States - Israel Binational Agriculture Research and Development Fund Final Report Project# 1-33-79.
- Le Rudulier, D., Strom, A. R., Dandekar, A. M., Smith, L. T. & Valentine, R. C. 1984. Molecular biology of osmoregulation. - Science 224: 1064-1068.
- Levitt, J. 1972. Responses of Plants to Environmental Stresses. pp. 353-378. Academic Press. New York.
- Liljenberg, C., Karunen, P. & Ekman, R. 1985. Changes in steryl lipids of oat root cells as a function of water-deficit stress. - Physiol. Plant. 63: 253-257.
- Liljenberg, C. & Kates, M. 1982. Effect of water stress on lipid composition of oat seedling root cell membranes. - In Biochemistry and Metabolism of Plant Lipids (J. F. G. M. Wintermans and P. J. C. Kuiper eds), pp. 441-444. Elsevier, New York, ISBN 0-444-80457-9.
- Lindstrom, R. S. & Tolbert, N.E. 1960. (2-chloroethyl)-trimethyl-ammonium chloride and related compounds as plant growth substances IV. Effect on chrysanthemums and poinsettias. - Mich. State Univ. Agric. Exp. Stn. Quart. Bull. 42: 917-928.
- Lovett, J. V. & Campbell, D. A. 1973. Effects of CCC and moisture stress on sunflower. - Exp. Agric. 9: 329-336.
- Muller, M. & Santarius, K. A. 1978. Changes in chloroplast membrane lipids during adaptation of barley to extreme salinity. - Plant Physiol. 62: 326-329.

- Murata, N. & Yamaya, J. 1984. Temperature-dependent phase behavior of phosphatidyl glycerols from chilling-sensitive and chilling resistant plants. - *Plant Physiol.* 74: 1016-1024.
- Murphy, D. J., Harwood, J. L., Lee, K. A., Roberto, F., Stumpf, P. K., & St. John, J. B. 1985. Differential responses of a range of photosynthetic tissues to a substituted pyridazinone, SANDOZ 9785. Specific effects on fatty acid desaturation. - *Phytochemistry* 24: 1923-1929.
- Orcutt, D. M., Davis, G. A. & Moore, L. D. 1984 a. Influence of choline derivatives on the sterol composition of Chlorella vulgaris grown in continuous culture. - *Can. J. Bot.* 62: 78-80.
- Orcutt, D. M., Davis, G. A., Moore, L. D. 1984 b. The effects of choline derivatives on the growth and lipid content of continuously cultured Chlorella vulgaris. - *Can. J. Bot.* 62: 81-85.
- Quedraogo, M. A., Tremolieres, A. & Hubac, C. 1984. Change in fatty acid composition during water stress in cotton plants. Relation with drought resistance induced by far red light. - *Int. J. Plant Physiol.* 114: 239-245.
- Palacios-Alaiz, E., Velez, J. & Alsasua, M. T. 1982. Temperature effect on phospholipids and fatty acid content of germinating seeds. - In *Biochemistry and Metabolism of Plant Lipids* (J. F. G. M. Wintermans and P. J. C. Kuiper eds), pp.445-450. Elsevier, New York, ISBN 0-444-80457-9.
- Pearcy, R. W. 1978. Effect of growth temperature on the fatty acid composition of leaf lipids in Atriplex lentiformis (Torr.) Wats. - *Plant Physiol.* 61: 484-486.
- Pham Thi, A. T., Flood, C., Da Silva, J. V. 1982. Effects of water stress on lipid and fatty acid composition of cotton leaves. - In *Biochemistry and Metabolism of Plant Lipids* (J. F. G. M. Wintermans and P. J. C. Kuiper eds), pp. 451-454. Elsevier, New York. ISBN 0-444-80457-9.
- Pham Thi, A. T., Borrel-Flood, C., Da Silva, J. V., Justin, A. M. & Mazliak, P. 1985. Effects of water stress on lipid metabolism in cotton leaves (Gossypium hirsutum). - *Phytochemistry* 24: 723-727.
- Piorreck, M., Baasch, K. & Pohl, P. 1984. Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. - *Phytochemistry* 23: 207-216.

- Plaut, Z. & Halevy, A. H. 1966. Regeneration after wilting, growth and yield of wheat plants, as affected by two growth-retarding compounds. - *Physiol. Plant.* 19: 1064-1072.
- Prasad, R. & Khan, A. H. 1981. Water stress effects on the lipid metabolism of Chlorella. - *Proc. Nat. Acad. Sci. India* 51: 51-52.
- Raison, J. K. & Wright, L. C. 1983. Thermal phase transitions in the polar lipids of plant membranes. Their induction by desaturated phospholipids and their possible relation to chilling injury. - *Biochim. Biophys. Acta* 731: 69-78.
- Rebeille, F., Bligny, F. & Douce, R. 1980. Role de l'oxygene et de la temperature sur la composition en acides gras des cellules isolees d'erable (Acer pseudoplatanus L.). - *Biochim. Biophys. Acta* 620: 1-9.
- Sauter, H. 1984. Chemical aspects of some bioregulators. - In *Bioregulators Chemistry and Uses* (R. L. Ory & F. R. Rittig eds), ACS Symposium Series 257, pp.9-21. American Chemical Society, Washington, D.C., ISBN 0-8412-0853-0.
- Schwab, K. B. & Heber, U. 1984. Thylakoid membrane stability in drought-tolerant and drought-sensitive plants. - *Planta* 161: 37-45.
- Shanahan, J. F. & Nielsen, D. C. 1987. Influence of growth retardants (anti-gibberellins) on corn vegetative growth, water use, and grain yield under different levels of water stress. - *Agron. J.* 79: 103-109.
- Sikorska, E. & Farkas, T. 1982. Sterols and frost hardening of winter rape. - *Physiol. Plant.* 56: 349-352.
- Singh, T. N., Aspinall, D. & Paleg, L. G. 1972. Proline accumulation and varietal adaptability to drought in barley: a potential measure of drought resistance. - *Nature New Biol.* 236: 188-190.
- St. John, J. B. 1982. Effects of herbicides on the lipid composition of plant membranes. - In *Biochemical Responses Induced by Herbicides* (D. E. Moreland, J. B. St. John, and F. D. Hess eds), pp. 97-109. American Chemical Society, Washington D.C. ISBN 0-8412-0699-6.
- St. John, J. 1984. Inhibition of carotenoid and lipid biosynthesis by herbicides. - In *Biochemical and Physiological Mechanisms of Herbicide Action* (S. O. Duke ed), pp. 58-71. USDA/ARS, Stoneville, Mississippi.

- Stuiver, C., DeKok, L., Santens, J. & Kuiper, P. 1984. The effect of Na_2SO_4 on the lipid composition of sugar beet plants. - *Int. J. Plant Physiol.* 114: 187-191.
- Stuiver, C., Kuiper, P. J. C. & Marschner, H. 1978. Lipids from bean, barley, and sugar beet in relation to salt resistance. - *Physiol. Plant.* 42: 124-128.
- Stuiver, C., Kuiper, P. J. C., Marschner, H. & Kylin, A. 1981. Effects of salinity and replacement of K^+ by Na^+ on lipid composition in sugar beet inbred lines. - *Physiol. Plant.* 52: 77-82.
- Wendt, C. W., Isbell, V. R., Stuart, B. L. & Abernathy, J. R. 1984. Effects of 1,1-dimethylpiperidinium chloride on growth and water relations of cotton in a semiarid environment. - In *Bioregulators Chemistry and Uses* (R. L. Ory and F. R. Rittig eds), ACS Symposium Series 257, pp.205-213. American Chemical Society, Washington D.C. ISBN 0-8412-0853-0.
- Wright, D. C., Berg, L. R. & Patterson, G. W. 1980. Effect of cultural conditions on the sterols and fatty acids of green algae. - *Phytochemistry* 19:783-785.
- Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. & Somero, G. N. 1982. Living with water stress: evolution of osmolyte systems. - *Science* 217: 1214-1222.

Chapter III

OSMOTIC-INDUCED CHANGES IN THE GROWTH AND LIPID COMPOSITION OF CHLORELLA VULGARIS AND CHLORELLA PYRENOIDOSA GROWN IN BATCH CULTURE

Abstract

The growth and lipid composition of two algal species, Chlorella vulgaris and Chlorella pyrenoidosa, subjected to polyethylene glycol-induced water stress were compared. The logarithmic growth rate of C. vulgaris declined with each incremental reduction in water potential. C. pyrenoidosa growth was stimulated at -0.5 MPa, not significantly affected at -1.0 or -1.5 MPa and declined significantly at -2.0 MPa.

Algal lipid composition varied as the water potential declined. In C. vulgaris, the free sterol concentration decreased, while steryl esters increased as the water potential was reduced. The concentration of free sterols of C. pyrenoidosa declined, while the steryl esters remained constant as water potential declined. The concentrations of C. vulgaris polar lipids declined, triglycerides increased, and free fatty acids remained constant with decreases in the water potential. The concentration of C. pyrenoidosa polar lipids, triglycerides, and free fatty acids all declined with decreases in water potential.

Introduction

Several recent reviews have outlined the role of lipids in plant adjustments to several environmental stresses (Harwood 1983, Kuiper 1985). Until recently, however, little attention was given to the possible role of lipids in plant adaptation to water stress. Possibly this oversight was a consequence of equating water stress with salinity stress (Yancey et al 1982, Le Rudulier et al 1984). Although these two stresses can be considered to be facets of a common stress, several investigations have established the primary strain in plants subjected to salinity stress results from ion toxicity (Leopold and Willing 1984, Muller and Santarius 1978).

Investigations of cell membrane stability under conditions of moisture stress have correlated drought tolerance with cell membrane stability in a number of crop species (Blum and Ebercon 1981). Since membrane stability is a function of the physical properties of the lipid substituents (Kuiper 1985), recent investigations have focused on lipid changes in response to natural and artificially-induced water stress (Lechavallier 1977, Douglas and Paleg 1981, Pham Thi et al. 1982, Biswas et al. 1983, and Liljenberg et al. 1985). These studies have clearly shown that lipid adjustment differs in drought-resistant and drought-tolerant species (Chetal et al. 1980, 1983, Pham Thi et al. 1982).

A difficulty in working with cellular level adaptations in whole plant systems is that plants make structural and functional level adjustments which aid in drought resistance and which affect the

process of adjustment at the cellular level. For this reason, in vitro cell culture techniques have many advantages in studying stress responses (Hasegawa et al. 1984). In vitro cell culture allows all responses but those operating at the cellular level to be eliminated, rigorous control of the cell environment, and the use of ontogenetically uniform cells with reproducible patterns of growth. Also, the manner in which a plant is subjected to stress can influence responses to the stress. Principles involved in water stress responses are believed to differ when a plant is suddenly subjected to a water stress rather than slowly developing water stress over days or weeks (Hanson and Hitz 1982, Hasegawa et al. 1984, Thompson 1986).

As a consequence of the foregoing observations, research was initiated with two unicellular algal species to determine how differing levels of water stress affected their respective growth rates and lipid composition when grown under defined environmental conditions. To simulate water stress, PEG was added to the growth medium to serve as a non-penetrating osmoticum. PEG appears to impose a stress similar to that experienced during desiccation (Bressan et al. 1981). In this manner it was hoped that growth changes and analysis of whole cell lipid patterns of the two species would reflect true adaptations to stress, rather than alterations resulting from stress-induced lesions at vulnerable sites in cellular metabolism (Hanson 1980).

Abbreviations: FFA, free fatty acid; FS, free sterol; GC, gas-liquid chromatography; PEG, polyethylene glycol; PFA, polar lipid fatty acid; PL, polar lipid; SE, steryl ester; TG, triglyceride; TFA, triglyceride

fatty acid; TLC, thin-layer chromatography.

Materials and Methods

Plant Material.--Chlorella vulgaris Beyerinck (Pratt strain) was obtained from Carolina Biological Co., Burlington, North Carolina. Chlorella pyrenoidosa Chick (UTEX 251) was obtained from the Culture Collection of Algae at the University of Texas at Austin. Axenic cultures were maintained under continuous illumination on 1% agar slants of Knop's medium (Vela & Guerra 1966). In preparation of Knop's medium, $\text{CoCl}_2(2\text{H}_2\text{O})$ was substituted for $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and the initial pH was adjusted to 6.5. An inoculation medium was prepared by growth of algae in modified Knop's medium aerated with 1% CO_2 and illuminated continuously at a photon flux density of $25 \mu\text{mol m}^{-2}\text{s}^{-1}$. Three ml of log phase inoculum were added to 150 ml of Knop's medium osmotically adjusted to the treatment levels of -0.1 (control), -0.5, -1.0, -1.5, -2.0 MPa by addition of PEG 4000 (MW 3350) (Sigma Chemical Company, St. Louis, Missouri). This solution was partitioned to provide 40 ml of inoculated medium in each of three pyrex test tubes (25 X 200mm). Osmotic potentials were verified by freezing point depression using a Fiske 'OS' Osmometer (Fiske Assoc., Uxbridge, Massachusetts). Each test tube was fitted with a foam rubber stopper through which a glass aeration tube (2.0 mm o.d.) was inserted the full tube length. Non-absorbent cotton plugs were inserted into each glass tube to filter the 1% CO_2 and air mixture which was bubbled through each tube culture at a

rate of 5 ml/ min. Cells were incubated at 27 C by suspending the culture tubes in a styrofoam collar along the sides of a 56.8 l aquarium. Two 40-watt, cool-white fluorescent tubes (Philips Lighting Corporation, Bloomfield, New Jersey) provided continuous illumination with a photon flux density of $25 \mu\text{mol m}^{-2}\text{s}^{-1}$.

Growth Determination-- The technique of optical density determination as described by Sorokin (1973), which allows linearity over wide ranges of population densities, was used to monitor and analyze growth of batch cultures. Transmittance at 678 nm of the axenically maintained cultures was measured with a Spectronic 20 spectrophotometer. The growth rate was determined for the exponential growth phase. Growth data were expressed as \log_2 growth for plotting and analysis of data. Growth data presented are the means of four experiments with three replications per treatment.

Lipid Isolation-- Late exponential phase algal cultures were harvested for lipid analysis (60% transmittance of a 1:4 dilution). A procedure described by Nichols (1963) was modified as follows to extract lipids. The cells from the three replications of each treatment were combined to obtain sufficient material for lipid analysis. Cells were concentrated by centrifugation at 3000 X g. To remove PEG, the concentrated cells were washed twice with an isotonic NaCl solution and reconcentrated by centrifugation. A 1 ml aliquot of cell solution, from the total 10 ml of cells in an isotonic NaCl solution, was removed

for dry weight determination. The aliquot of cells was washed and centrifuged twice with distilled water and dried at 70 C. The remainder of the cell solution was re-centrifuged, and the supernatant was decanted. Cells were then suspended in 5 ml of isopropanol to deactivate hydrolytic enzymes (Harborne, 1984). An additional 5 ml of isopropanol was added to the screwcap vials containing the cells and the vials were flushed with N₂ gas. Cells were concentrated by centrifugation and the isopropanol layer was removed. An additional extraction with 10 ml of isopropanol was performed. The cellular debris was extracted under N₂ for 12 hours on a rotary shaker at 100 rpm with 40 ml of chloroform:isopropanol (1:1 v/v) in a stoppered 125 ml erlenmyer flask. The isopropanol and chloroform-isopropanol extracts were combined, filtered through Whatman #50 paper and concentrated by rotary evaporation at 45 C. The extracted lipids were Folch washed (Folch et al 1957). The washed lipids were transferred to tared tubes to determine lipid weight. Internal standards of 100 ug methylpentadecanoate, 40 ug cholesterol, and 30 ug cholesterol oleate (Sigma Chemical, St. Louis, Missouri) were added to the lipid extracts to aid in quantification.

Lipid characterization and quantitation--Lipid classes were separated on Kieselgel 60 G (EM Science, Elmsford, New York) TLC plates (0.5 mm thickness) using a solvent system of n-hexane:diethylether:acetic acid (85:15:1 v/v/v) (Ginger and Fairbairn 1966). Lipid bands corresponding to PL, TG, FFA, FS, and SE were visualized with 0.2% 2',7'-dichloro-

fluorescein in 95% ethanol under long-wave ultraviolet light, removed from TLC plates, and processed further. The PL, FFA, and TG were analyzed as described (Orcutt et al. 1978) with the exception that samples were not separated by degree of unsaturation. PL and TG were quantified on the basis of the fatty acids liberated by hydrolysis and were expressed as PFA and TFA. GC analyses were performed on a Bendix 2500 gas chromatograph (Bendix Process Instrument Division, Ronceverte, NV) equipped with a flame ionization detector. Fatty acids were separated isothermally at 190 C on a 2 m column packed with 10% DEGS on 80-100 Chromasorb WAW. Quantification was based on the internal standard methylpentadecanoate. Identification of the fatty acids was by comparison of GLC retention times with those of known standards.

Trimethylsilyl ether derivatives of sterols and hydrolyzed steryl esters were prepared and quantified as described previously (Bradford et al. 1982). Silylated sterols were separated isothermally at 275 C on a (2m X 2mm i.d.) coiled glass column packed with 3% SE-30 on 80/100 Gas Chrom Q. Sterols were identified by comparing GLC relative retention times with known standards and published data (Patterson 1971, Orcutt et al. 1984). Retention times and peak areas were determined using a Hewlet Packard 3392A Integrator. Quantification of steryl esters is expressed in terms of hydrolyzed free steryl concentrations.

Identification of sterol constituents involved combined capillary column gas chromatography and mass spectroscopy of acetate derivatives of the sterols run under the conditions previously described (Orcutt et

al. 1984). Based on molecular weights, comparisons with previously published relative retention times, and previously published mass spectra of sterols, the major constituents were identified (Patterson 1971).

Results

Growth data

The growth responses of C. vulgaris and C. pyrenoidosa to stress differed substantially (Fig. 3-1 & 3-2, Table 3-1). With increasing levels of stress an increasing period of adjustment was required before the algae resumed exponential growth. Thus, measurements of optical density changes began at different time intervals for the different treatments. Data presented in Fig. 3-1 & 3-2 are representative data of one experiment (three replications), while the data of Table 3-1 presents the accumulated data from four experiments. In Table 3-1 the growth of C. vulgaris and C. pyrenoidosa were each set equal to 100 and all other growth rates for each species are compared to this rate.

Although C. vulgaris possessed a higher growth rate under nonstressed conditions, this species was the more sensitive to water stress. With each increase in water stress, a decrease in growth rate occurred (Fig. 3-1, Table 3-1). In contrast C. pyrenoidosa growth was stimulated at osmotic potentials of -0.5 and -1.0 MPa. Even at -1.5 MPa, the growth rate was not significantly different from the growth of controls. Only at -2.0 MPa was there a significant decrease in cell division.

Lipid Analysis

The fatty acid composition of the PL, FFA, and TG fractions of both C. vulgaris and C. pyrenoidosa were not significantly altered by increased levels of osmoticum (data not shown). PFA concentrations at -0.1 MPa were higher in C. vulgaris than C. pyrenoidosa (0.21 vs 0.15 mg PFA/mg lipid). In both species concentrations of PFA declined significantly with increased osmotic concentration. As the osmotic potential decreased from -0.1 to -2.0 MPa, concentrations of PFA declined 58% in C. vulgaris while decreasing 85% in C. pyrenoidosa (Fig. 3-3, 3-4). The TFA content of C. pyrenoidosa and C. vulgaris were differentially affected by the treatments. The TFA concentration increased 40-fold in C. vulgaris but declined 63% in C. pyrenoidosa as osmotic potentials decreased from -0.1 to -2.0 MPa (Fig. 3-3, 3-4).

The concentrations of FFA remained at a low constant level in C. vulgaris but decreased approximately 70% in C. pyrenoidosa (Fig. 3-3, 3-4).

The sterol composition of the two algal species differed quite significantly qualitatively and quantitatively. The free sterol composition of C. vulgaris was a complex mixture of sterols and included: ergost-7,22-dien-3 β -ol, ergost-8(14)-en-3 β -ol, ergost-8(9)-en-3 β -ol, 24-methylenepollinastanol, ergost-7-en-3 β -ol, 4-methylergosta-8(9),14-dien-3 β -ol, 24-methylpollinastanol, stigmasta-7,22-dien-3 β -ol, stigmasta-8(9)-en-3 β -ol, and stigmast-7-en-3 β -ol (Fig. 3-5A). The three major sterols identified from C. pyrenoidosa included

ergost-7-en-3 β -ol, stigmasta-7,22-dien-3 β -ol, and stigmast-7-en-3 β -ol (Fig. 3-5B). Because of the complexity of the separation pattern in C. vulgaris, it was not possible to quantitate the individual sterol constituents separately using conventional GLC columns available. Therefore total sterol concentrations are reported. However, identification of the sterols was based on GC-MS separation using a capillary column.

At -0.1 MPa, the concentration of FS in C. pyrenoidosa was 6.5 times greater than that observed in C. vulgaris (26 vs 4.0 ug sterol /ug lipid) (Fig. 3-6, 3-7). Although the concentration of free sterols declined significantly in C. pyrenoidosa as osmotic potential decreased, the percentage composition of the individual sterol substituents remained constant (Fig. 3-7, Table 3-2). With decreasing osmotic potential, FS concentrations also decreased significantly in C. vulgaris (Fig. 3-6). At -2.0 MPa the sterol concentrations had declined 77% in C. pyrenoidosa and 88% in C. vulgaris relative to concentrations of the control. In C. pyrenoidosa, the concentration of SE remained at a low constant level with decreasing osmotic potential (Fig. 3-7), while in C. vulgaris the SE concentration exhibited an inverse relationship with FS concentrations and increased 350% as the water potential decreased from -0.1 to -2.0 MPa (Fig. 3-6). At -2.0 MPa, the concentration of SE nearly matched the concentration of FS of the control.

The two species of algae also differed dramatically in their ratios of FS to PFA (Table 3-3). Under low stress conditions this

ratio was 10-times greater in the water-stress resistant C. pyrenoidosa than in C. vulgaris. With increasing concentrations of osmoticum, this ratio increased 50% in C. pyrenoidosa. In contrast, this ratio actually declined in C. vulgaris (Table 3-3). Thus, at the highest osmotic level, the ratio of FS to PFA in C. pyrenoidosa increased to a 50-fold greater value than in C. vulgaris.

Discussion

The data presented indicate that the two species of Chlorella differed substantially in growth maintenance and lipid responses during osmotic. The growth data indicate that, C. pyrenoidosa at all osmotic levels and C. vulgaris at -1.5 and -2.0 MPa underwent an initial adjustment when transferred from the inoculation medium to the growth tubes. The period of adjustment increased as the osmotic potential, to which the algae were transferred, decreased. After acclimation, the algae entered an exponential growth phase at all of the osmotic levels tested. Although the rates of growth relative to unstressed algae declined with decreasing water potential, the exponential growth indicated a degree of adaptation had occurred.

Growth data indicated that C. pyrenoidosa was considerably more tolerant to osmotic stress than C. vulgaris. Since Chlorella are unicellular, these differences in tolerance can be considered to originate within the cell. Previously observed differences in stress have been proposed to result from either significant qualitative or quantitative differences in lipid composition (Chetal et al. 1980,

1983, Douglas & Paleg 1981, Lechevallier 1977).

The comparison of these two species of Chlorella differing in osmotic sensitivity provided a good test system. Although on the basis of sterol composition and other biochemical evidence, the genus Chlorella is suggested to represent a combination of morphologically similar taxa of polyphyletic origin (Kessler 1982), lipid composition of the two species was very similar. An examination of the fatty acids present in the FFA, TG, and PL classes and an examination of the polar lipids revealed all the constituents were common to both species. The difference in the sterol composition of C. vulgaris and C. pyrenoidosa seemed to result largely because of an accumulation of sterol precursors in C. vulgaris. Six major groups of Chlorella have been distinguished on the basis of qualitative and quantitative differences in sterol composition (Holden and Patterson 1982). Both C. pyrenoidosa and C. vulgaris are members of the Group II Chlorella which are characterized by the absence of a double bond in all sterols at the C-5 position.

Of great interest in determining the manner of adaptation to stress is the fact that C. pyrenoidosa, the stress resistant species, contained similar lipids to those isolated from C. vulgaris. From this observation one might infer that the osmotic susceptibility of C. vulgaris was not the result of qualitative differences in lipids. However, C. vulgaris possessed sterol precursors not identified in C. pyrenoidosa. If osmotic resistance is based on sterol composition, this may indicate that C. vulgaris possessed the necessary sterols

required for resistance but possibly was not able to incorporate them into membranes in the most advantageous ratio to provide for resistance. However reports with other species have indicated sterol synthesis is responsive to environmental conditions (Grunwald 1978, Sikorska and Farkas 1982). C. pyrenoidosa did not appear to modulate osmotic resistance through regulation of the relative proportions of the three sterols which this species possessed. Since water-stress adaptation appeared not to depend on qualitative factors of lipid composition, the role of quantitative changes in lipid composition would appear to be of great importance.

The pattern of FS and PL decreases and SE and TG increases exhibited by the sensitive alga, C. vulgaris, are similar to those observed in yeast (Saccharomyces cerevisiae) during late exponential growth phase. At stage, yeast accumulate large amounts of SE and TG (Taylor and Parks 1978, 1979). In yeast a rapid and efficient interconversion of sterols and steryl esters occurs. Also in yeast, triglyceride metabolism appeared to be coupled to phospholipid synthesis. However, in the current experiment all treatments were harvested at uniform culture densities; so it appears unlikely that the above observations could directly explain the accumulations of sterols and steryl esters. However, the capability of converting membrane-active components (FS and PL) to storage forms of these components (SE and TG) can be an adaptive mechanism which also confers dehydration resistance as well as acting as a response to the depletion of nutrients. Such an adaptation would allow storage of compounds for use

under more favorable conditions. This extensive conversion of active lipid components to storage forms may be triggered at different stress thresholds in different organisms. Previously Liljenberg and Kates (1985) noted that a certain number of stress cycles were required to initiate lipid responses in oat (Avena sativa L.) roots. Only at the lowest water potential treatment tested, was growth of C. pyrenoidosa strongly inhibited. Possibly with the application of further stress a similar lipid conversion pattern would be noted.

Another possible explanation of what is occurring in C. vulgaris is suggested by the work of Piorreck et al. (1984). Investigations of lipid composition of two green algae, Chlorella vulgaris and Scenedesmus obliquus, under different nitrogen regimes in batch culture indicated that the lipid metabolism of green algae was influenced by the nitrogen concentration of the growth medium. At low nitrogen concentrations, algae have a tendency to synthesize neutral lipids and fatty acids with a low degree of unsaturation. At higher culture nitrogen concentrations, green algae predominantly synthesized polar lipids containing polyunsaturated fatty acids. Depletion of nitrogen during growth of the algae during osmotic adjustment might explain some of the observed changes in C. vulgaris but would fail to answer why the same mechanism was not operative in C. pyrenoidosa.

Observations of water stress induced changes in lipid composition have varied depending on the plant source and length of stress adaptation period. Investigations of sterols of oat root cells indicated that both FS and SE increased with stress unlike the

observations of the current investigation (Liljenberg et al. 1985). In another study, the content of acyl lipids including TG, PL, and SE were found to decrease. Decreases in the degree of unsaturation of the fatty acids with increased water stress have been noted in the galactolipid fractions (Ferrari-Illiou et al. 1984, Pham Thi 1984, Liljenberg and Kates 1985). The results of the current study did not reveal saturation changes in the lipid classes analyzed. Thus, it would appear modification of saturation was not required for adaptation. Some changes in saturation might have been observed had the various PL constituents been analyzed individually rather than as a single class.

Previously the low concentration of glycolipids under water stress has been proposed to result from a degradation process rather than an inhibition of synthesis. Observation, in actively growing algae, of declining concentrations of PL with increasing stress could be explained by the concept of degradation (Pham Thi et al. 1985). If increased stress resulted in enhanced rates of degradation, stable but lower rates of various polar lipid constituents could result with each increase in water stress.

In both C. vulgaris and C. pyrenoidosa, the levels of the polar lipids declined. This in itself did not seem to be indicative of osmotic resistance. Rather the greatest difference in lipid composition was the ratio of the sterol to phospholipid concentrations. Resistance to other environmental stresses has been correlated with this ratio (Sikorska and Farkas, 1982). In oat roots subjected to

periodic water stress, the ratio of sterols to phospholipids also increased as a response to stress (Liljenberg et al. 1985). Unlike the observations in oat seedling roots, FS concentrations were not increased by stress but actually declined. In C. vulgaris the rate of FS decline was more rapid than the decline in PFA, so the result was a decline in the FS to PFA ratio. The rate of decline of FS in C. pyrenoidosa was less rapid than the PFA decline and this resulted in an actual increase in the FS:PFA ratio. C. pyrenoidosa may have been able to maintain higher rates of growth under water stress as a result of increasing the FS to PFA ratio during stress. If maintaining or increasing such a ratio is advantageous, why didn't C. vulgaris follow the same adaptive pathway? Free sterols in C. vulgaris were not degraded but appear to have been converted to SE, a storage form. Could it be that the enzyme(s) which control SE synthesis in C. vulgaris and C. pyrenoidosa differ in sensitivity to water stress and when SE synthesis begins, free sterols are removed from membranes causing the destabilization of membranes leading to formation of other storage components such as TG? Although CP could maintain growth rates with increasing levels of stress, such an adaptation might not confer an overall advantage in terms of survival in stressful environments.

This study has supported previous work indicating that the ratio of free sterol to polar lipids may be an important determinant of adaptation to water stress. These results from actively growing unicellular organisms were not affected by modifications at the tissue or organ level.

Literature Cited

- Biswas, A. K., Sarkar, U. & Chaudhury, M. A. 1983. Water-stress induced changes in total steroids and cholesterol contents in wheat seedlings. - *Sci. Cult.* 49: 109-110.
- Blum, A. & Ebercon, A. 1981. Cell membrane stability as a measure of drought and heat tolerance in wheat. - *Crop Sci.* 21: 43-47.
- Bradford, B., Moore, L. D. & Orcutt, D. M. 1982. The free sterol, steryl ester, and steryl glycoside content of tomato cultivars resistant and susceptible to Phytophthora infestans. - *Can. J. Bot.* 60: 1469-1473.
- Bressan, R. A., Hasegawa, P. M. & Handa, A. K. 1981. Resistance of cultured higher plant cells to polyethylene glycol-induced water stress. - *Plant Sci. Lett.* 21: 23-30.
- Chetal, S., Wagle, D. S. & Nainawatee, H. S. 1980. Phospholipid changes in wheat and barley leaves under water stress. - *Phytochemistry* 19: 1393-1395.
- Chetal, S., Wagle, D. S. & Nainawatee, H. S. 1983. Phospholipid changes in wheat and barley chloroplast under water stress. - *Plant Sci. Lett.* 29: 273-278.
- Douglas, T. J. & Paleg, L. G. 1981. Lipid composition of Zea mays seedlings and water stress-induced changes. - *J. Exp. Bot.* 32: 499-508.

- Ferrari-Illiuo, R., Pham Thi, A. T. & da Silva, J. V. 1984. Effect of water stress on the lipid and fatty acid composition of cotton (Gossypium hirsutum) chloroplasts. - *Physiol. Plant.* 62: 219-224.
- Folch, J., M. Lees, and G.H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. - *J. Biol. Chem.* 226:497-509.
- Ginger C. & Fairbairn D. 1966. Lipid metabolism in helminth parasites. 1. The lipids of Hymenolepis diminuta (cestoda). - *J. Parasitol.* 52: 1086-1096.
- Grunwald, C. 1978. Function of sterols. - *Phil. Trans. R. Soc. Lond. B.* 284: 541-558.
- Hanson, A. D. 1980. Interpreting the metabolic responses of plants to water stress. - *Hortscience* 15: 623-629.
- Hanson, A. D. & Hitz, W. D. 1982. Metabolic responses of mesophytes to plant water deficits. - *Annu. Rev. Plant Physiol.* 33: 163-203.
- Harborne, J. B. 1984. *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis.* pp. 153-160. Chapman and Hall, New York, ISBN 0-412-25550-2.
- Harwood, J. L. 1983. Adaptive changes in lipids of higher-plant membranes. - *Biochem. Soc. Trans.* 11: 343-346.
- Hasegawa, P. M., Bressan, R., Handa, S. & Handa, A. K. 1984. Cellular mechanisms of tolerance to water stress. - *Hortscience* 19: 371-377.
- Holden, M. J. & Patterson, G. W. 1982. Taxonomic implication of sterol composition in the genus Chlorella. - *Lipids* 17: 215-219.

- Kessler, E. 1982. Chemotaxonomy in the chlorococcales. - In Progress in Phycological Research, (F. E. Round and D. J. Chapman eds), Vol. 1, pp. 111-135. Elsevier, New York, ISBN 0-444-80396-7.
- Kuiper, P. J. C. 1985. Environmental changes and lipid metabolism of higher plants. - *Physiol. Plant.* 64: 118-122.
- Lechevallier, D. 1977. Effets du polyethylene glycol sur les lipides et les lipochromes des colonies de *Spirodele*. - *Physiol. Veg.* 15: 387-402.
- Leopold, A. C. & Willing, R. P. 1984. Evidence for toxicity effects of salt on membranes. - In *Salinity Tolerance of Plants* (R. C. Staples and G. H. Toenniessen, eds), pp.67-76. John Wiley and Sons, New York, ISBN 0-471-89674-8.
- Le Rudulier, D., Strom, A. R., Dandekar, A. M. & Smith, L. T. 1984. Molecular biology of osmoregulation. - *Science* 224: 1064-1068.
- Liljenberg, C., Karunen, P. & Ekman, R. 1985. Changes in steryl lipids of oat root cells as a function of water-deficit stress. - *Physiol. Plant.* 63: 253-257.
- Liljenberg, C. & Kates, M. 1985. Changes in lipid composition of oat root membranes as a function of water-deficit stress. - *Can. J. Biochem. Cell Biol.* 63: 77-84.
- Muller, M. & Santarius, K. A. 1978. Changes in chloroplast membrane lipids during adaptation of barley to extreme salinity. - *Plant Physiol.* 62: 326-329.

- Nichols, B.W. 1963. Separation of the lipids of photosynthetic tissues: improvements in analysis by thin-layer chromatography. - *Biochim. Biophys. Acta.* 70:417-422.
- Orcutt, D. M., Davis, G. A. & L. D. Moore. 1984. Influence of choline derivatives on the sterol composition of Chlorella vulgaris grown in continuous culture. - *Can. J. Bot.* 62: 78-80.
- Orcutt, D. M., Fox, J. A., & Jake, C. A. 1978. The sterol, fatty acid, and hydrocarbon composition of Globodera solanacearum. - *J. Nematology* 10: 264-269.
- Patterson, G. W. 1971. Relation between structure and retention time of sterols in gas chromatography. - *Anal. Chem.* 43: 1165-1170.
- Pham Thi, A. T. 1984. Action de la secheresse sur les lipides polaires des feuilles de Cotonnier (Gossypium hirsutum L.). - *Bull. Soc. Bot. Fr.,* 131, Actual. Bot. 1: 89-97.
- Pham Thi, A. T., Flood, C. & Da Silva, J. V. 1982. Effects of water stress on lipid and fatty acid composition of cotton leaves. - In *Biochemistry and Metabolism of Plant Lipids* (J. F. G. M. Wintermans and P. J. C. Kuiper, eds), pp. 451-454. Elsevier, New York, ISBN 0-444-80457-9.
- Pham Thi, A. T., Borrel-Flood, C., Da Silva, J. V., Justin, A. M. & Mazliak, P. 1985. Effects of water stress on lipid metabolism in cotton leaves. - *Phytochemistry* 24: 723-727.

- Piorreck, M. K., Baasch, K. & Pohl, P. 1984. Formation of biomass, total protein, chlorophylls, lipids and fatty acids in green and blue-green algae during one growth phase. - *Phytochemistry* 23: 217-223.
- Sikorska, E. & Farkas, T. 1982. Sterols and frost hardening of winter rape. - *Physiol. Plant.* 56: 349-352.
- Sorokin, C. 1973. Dry weight, packed cell volume, and optical density. - In *Handbook of Phycological Methods Culture Methods and Growth Measurements* (J. R. Stein, ed.), pp. 331-343. Cambridge University Press, New York. ISBN 0-521-20049-0.
- Taylor, F. R. & Parks, L. W. 1978. Metabolic interconversion of free sterols and steryl esters in *Saccharomyces cerevisiae*. - *J. Bacteriol.* 136: 531-537.
- Taylor, F. R. & Parks, L. W. 1979. Triacylglycerol metabolism in *Saccharomyces cerevisiae* relation to phospholipid synthesis. - *Biochim. Biophys. Acta* 575: 204-214.
- Thompson Jr., G. A. 1986. Metabolism and control of lipid structure modification. - *Can. J. Biochem. Cell Biol.* 64: 66-69.
- Vela, G. R. & Guerra, C. N. 1966. On the nature of mixed cultures of *Chlorella pyrenoidosa* TX71105 and various bacteria. - *J. Gen. Microbiol.* 42: 123-131.
- Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. & Somero, G. N. 1982. Living with water stress: evolution of osmolyte systems. - *Science* 217: 1214-1222.

Table 3-1. Relative growth rates of logarithmic phase Chlorella vulgaris and Chlorella pyrenoidosa at five osmotic potentials.

Osmotic potential (MPa)	Species	
	<u>C. vulgaris</u>	<u>C. pyrenoidosa</u>
-0.1	100	100
-0.5	86 ± 7.0	106 ± 0.4
-1.0	81 ± 7.0	104 ± 4.0
-1.5	73 ± 3.0	90 ± 9.0
-2.0	61 ± 3.0	67 ± 3.0

Data entries are the means of four experiments of three replications per treatment ± standard deviation.

Table 3-2. Percentage composition of free sterols of Chlorella pyrenoidosa grown in batch culture at five water potentials.

Osmotic potential (MPa)	Sterol constituent		
	1	2	3
-0.1	22.8 ± 2.9	71.8 ± 4.6	5.1 ± 2.8
-0.5	25.8 ± 5.2	66.7 ± 7.8	7.5 ± 3.1
-1.0	23.9 ± 3.0	67.2 ± 6.3	8.8 ± 3.9
-1.5	28.2 ± 3.6	65.5 ± 6.0	8.4 ± 1.4
-2.0	27.7 ± 1.9	67.4 ± 2.6	3.2 ± 2.8

Data represent the averages of four replications ± standard deviation.

Sterol constituents are: 1, ergost-7-en-3 β -ol; 2, stigmasterol-7,22-dien-3 β -ol; 3, stigmasterol-7-en-3 β -ol.

Table 3-3. Ratios of ug free sterol / mg polar lipid of Chlorella vulgaris and Chlorella pyrenoidosa grown at five water potentials.

Osmotic potential (MPa)	Species	
	<u>C. vulgaris</u>	<u>C. pyrenoidosa</u>
-0.1	42 ± 57	178 ± 15
-0.5	13 ± 5	207 ± 62
-1.0	28 ± 28	162 ± 54
-1.5	5 ± 3	212 ± 55
-2.0	5 ± 5	281 ± 46

Data represent the averages of four replications ± standard deviation.

Figure 3-1. Growth of logarithmic phase Chlorella vulgaris at five osmotic potentials; Symbols, ○ - -0.1 MPa, $r^2=0.98$; △ - -0.5 MPa, $r^2=0.99$; □ - -1.0 MPa, $r^2=0.99$; ▽ - -1.5 MPa, $r^2=0.99$; ◇ - -2.0MPa, $r^2=0.99$.

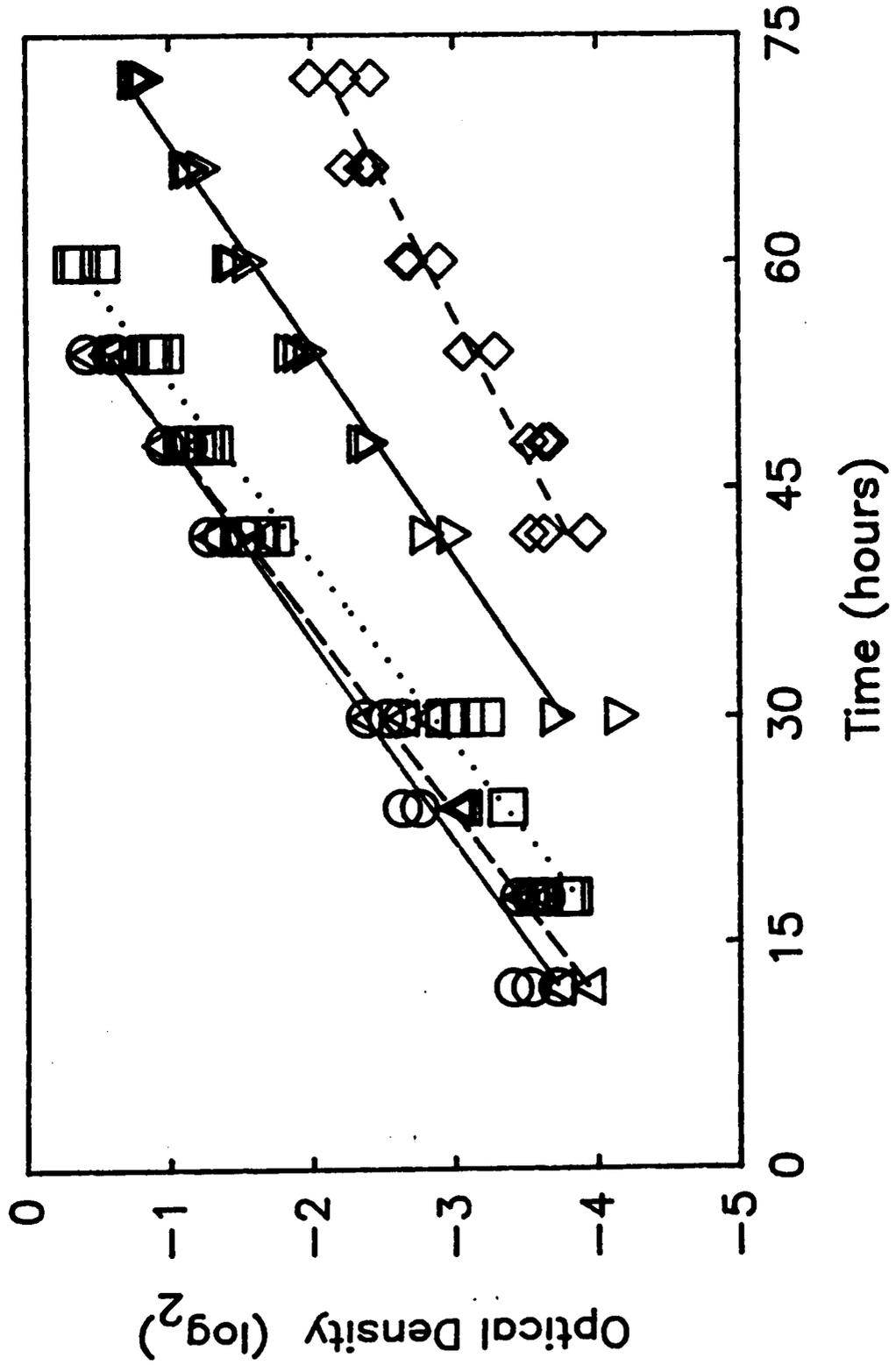


Figure 3-2. Growth of logarithmic phase Chlorella pyrenoidosa at five osmotic potentials; Symbols, ○ - -0.1 MPa, $r^2=0.99$; △ - -0.5 MPa, $r^2=0.99$; □ - -1.0 MPa, $r^2=0.99$; ▽ - -1.5 MPa, $r^2=0.99$
◇ - -2.0MPa, $r^2=0.98$

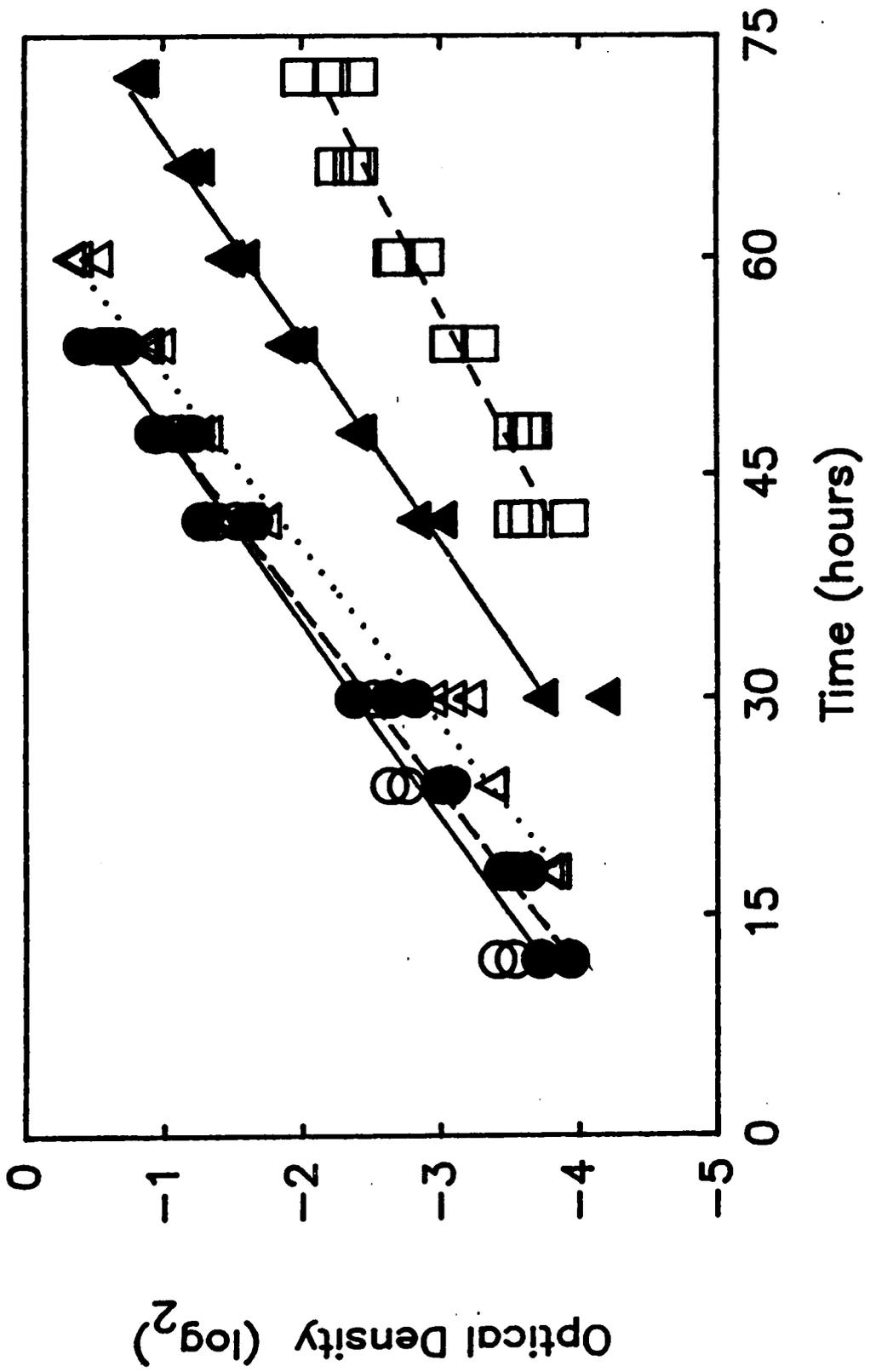


Figure 3-3. Osmotic-induced alterations in the concentrations of PFA, TFA, and FFA in Chlorella vulgaris; Symbols, ○ - polar lipid fatty acid, $r^2=0.46^*$; △ - triglyceride fatty acid, $r^2=0.65^{**}$; □ - free fatty acid, $r^2=0.22$ n.s.

n. s. = not significant

* = significant at 0.05 level of probability

** = significant at 0.001 level of probability

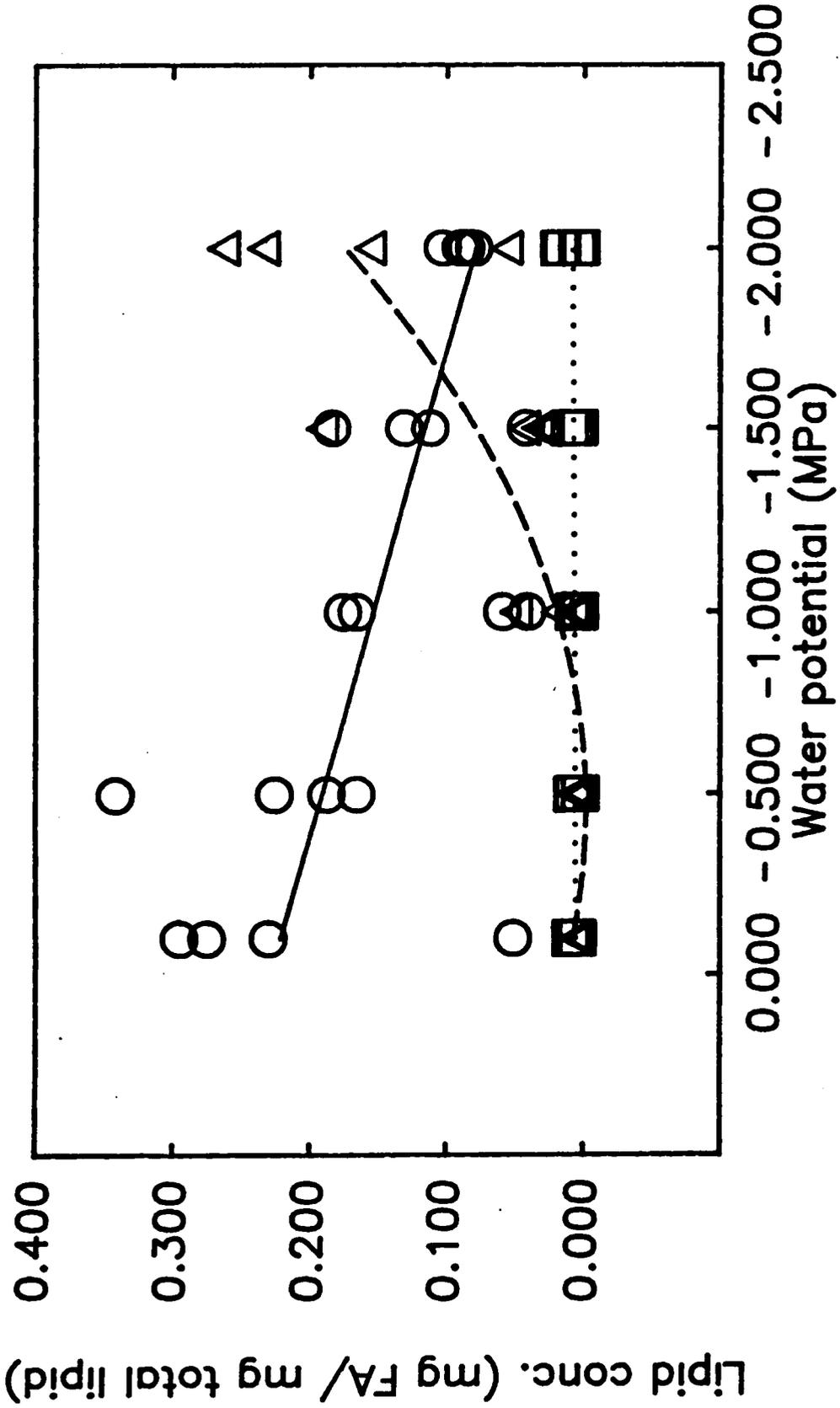


Figure 3-4. Osmotic-induced alterations in the concentrations of PFA, TFA, and FFA in Chlorella pyrenoidosa; Symbols, ○ - polar lipid fatty acid, $r^2=0.67^{**}$; Δ - triglyceride fatty acid, $r^2=0.20$ n.s.; □ - free fatty acid, $r^2=0.63^*$

n. s. = not significant

* = significant at the 0.01 level of probability

** = significant at the 0.001 level of probability

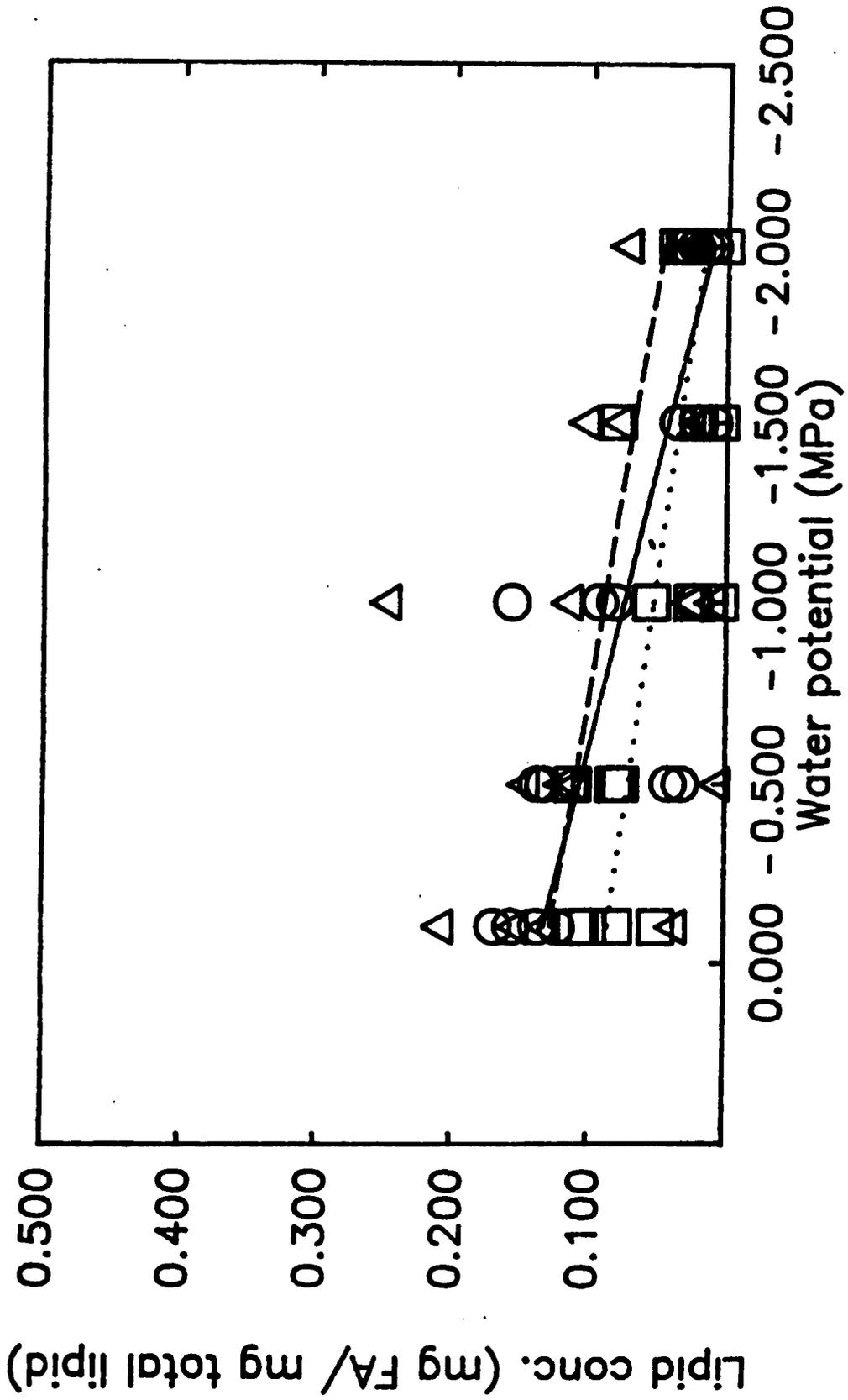


Figure 3-5. Total ion chromatogram of total sterol acetates isolated from: A. Chlorella vulgaris, B. Chlorella pyrenoidosa.

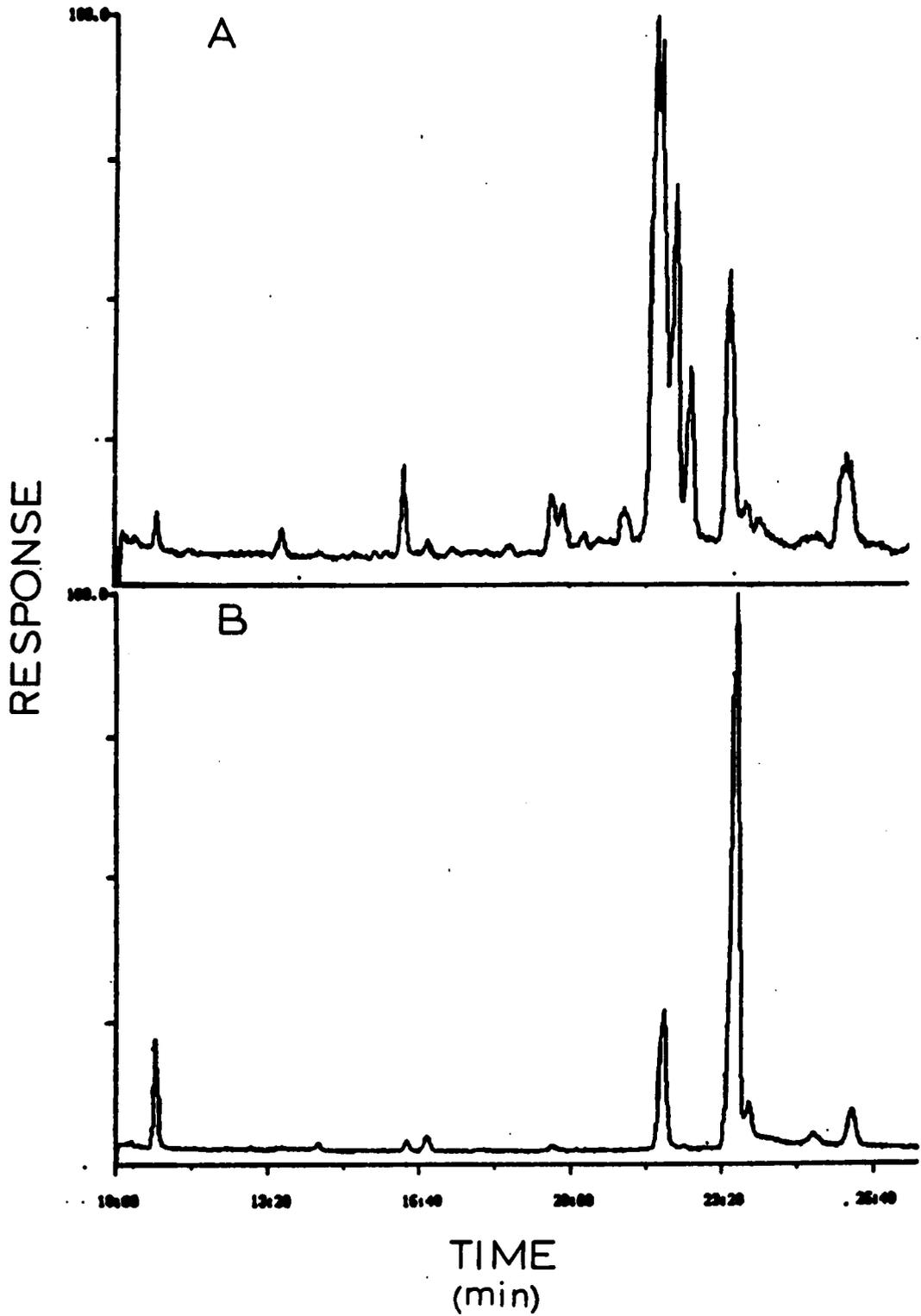


Figure 3-6. Osmotic-induced alterations in the concentrations of FS and SE of Chlorella vulgaris; Symbols- ○ - free sterols, $r^2=0.64^{**}$; Δ - steryl esters, $r^2=.54^*$

* = significant at the 0.01 level of probability

** = significant at the 0.001 level of probability

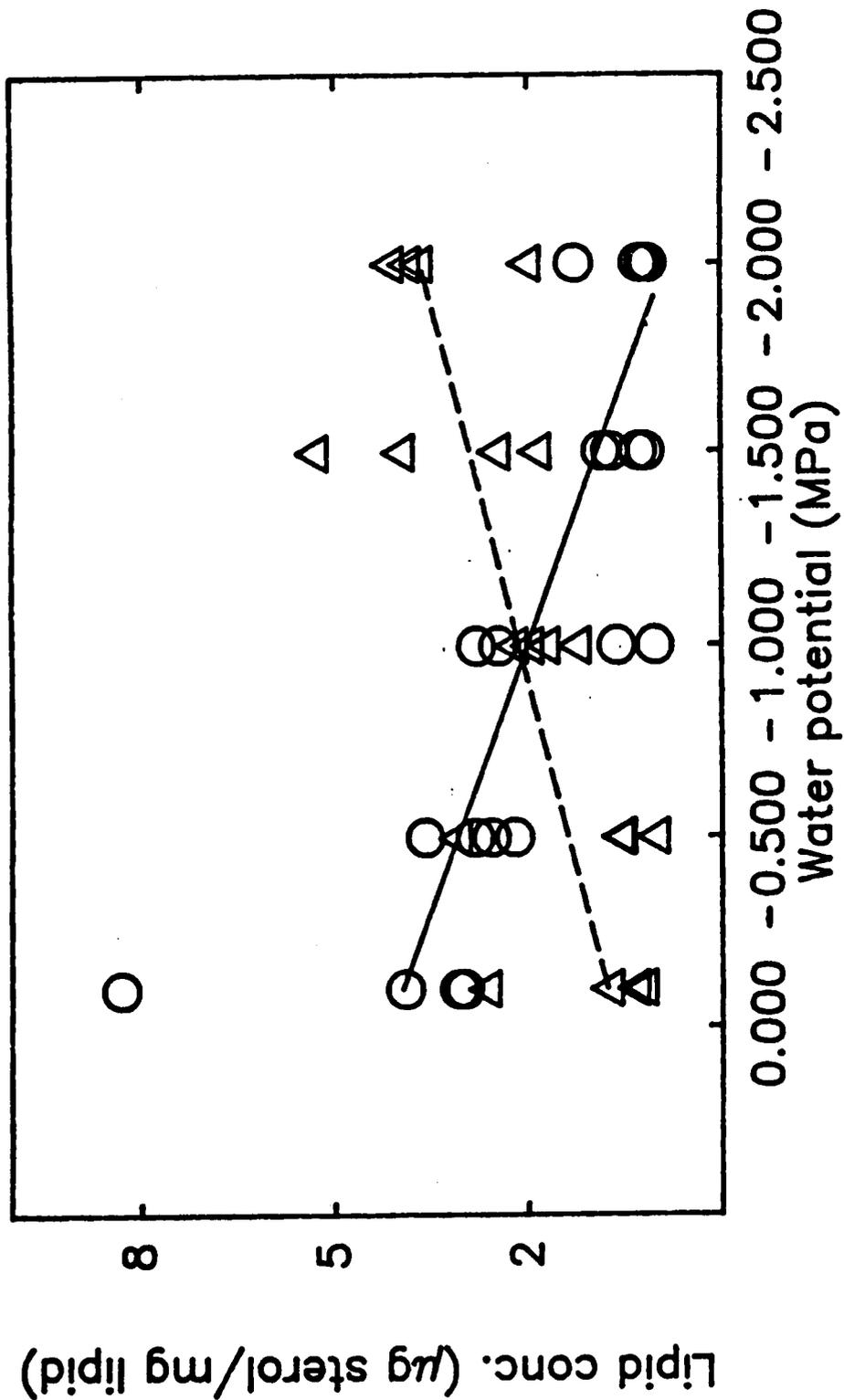
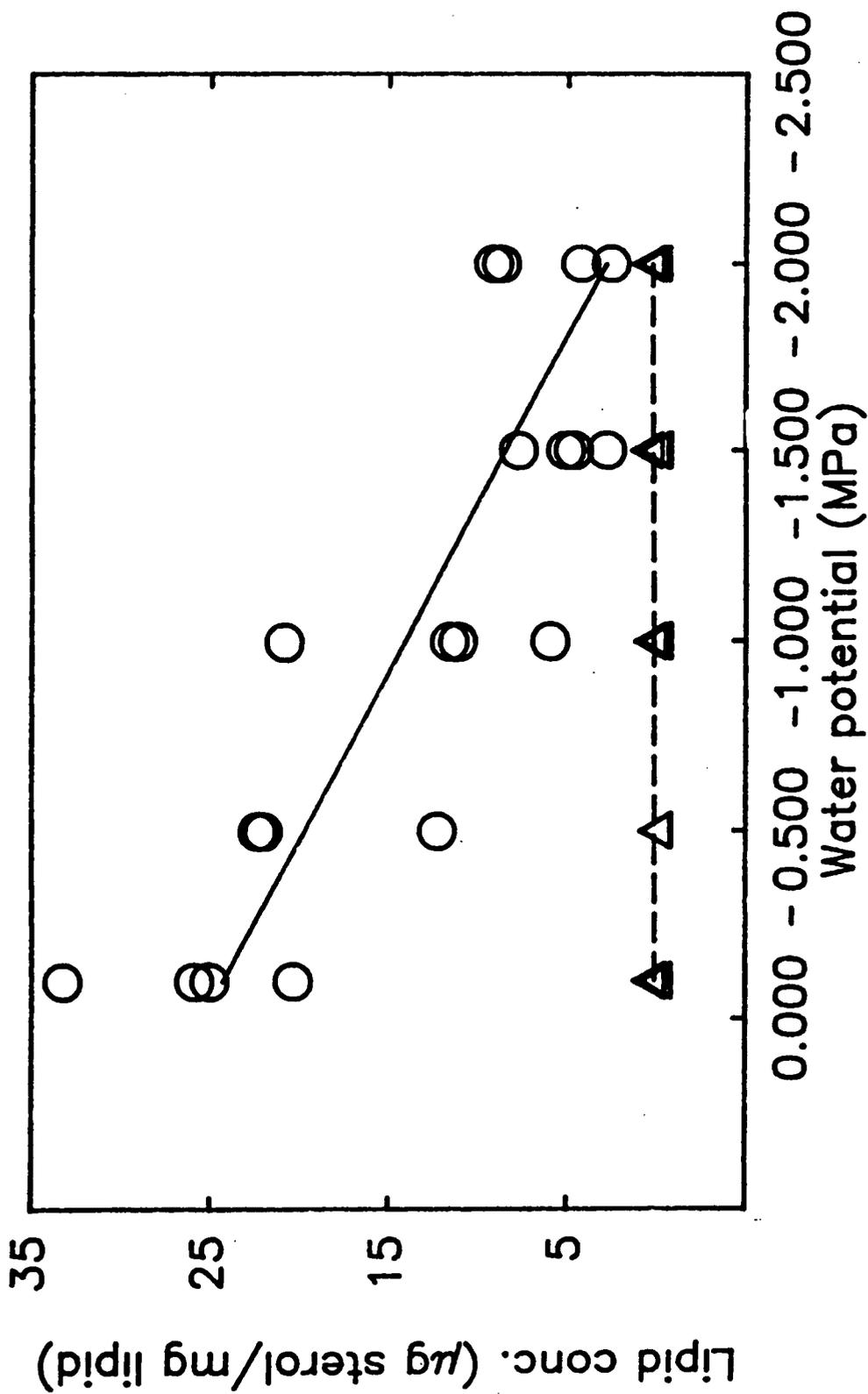


Figure 3-7. Osmotic-induced alterations in the concentrations of FS and SE of Chlorella pyrenoidosa; Symbols- ○ - free sterols, $r^2=0.80^{***}$; Δ - steryl esters, $r^2=0.23$ n. s.

n. s. = not significant

*** = significant at the 0.0001 level of probability



Chapter IV

OSMOTIC-INDUCED CHANGES IN GROWTH, PHOTOSYNTHESIS, AND LIPID COMPOSITION OF CHLORELLA VULGARIS GROWN IN CONTINUOUS CULTURE

Abstract

Growth, lipid composition, and photosynthetic rates were determined for Chlorella vulgaris grown in nutrient solution containing polyethylene glycol at osmotic potentials of -0.1, -0.5, -1.0, and -1.5 MPa. Total lipid and CO₂ fixation decreased as osmotic potential increased. Chlorophyll concentration did not change but cell dry weight increased by about 36% at -1.0 MPa compared to the control (-0.1 MPa). Storage lipids (triglycerides) increased while free fatty acids and polar lipid fatty acids declined as osmotic potentials decreased. Free sterols and sterol esters remained unchanged. The ratio of linolenic to linoleic acids increased in the polar lipid fatty acids, remained unchanged in the triglyceride fatty acids, and decreased in the free fatty acid lipid classes as the osmotic potential declined. Unsaturated fatty acids increased in the triglyceride, decreased in the free fatty acid and remained constant in the polar lipid fatty acid classes. Phosphatidic acid, phosphatidyl choline, and phosphatidyl glycerol decreased while phosphatidyl inositol plus phosphatidyl ethanolamine remained unchanged as the osmotic potential decreased. The results suggest a metabolism is initiated which leads to the production of neutral lipids of high energy and low water affinity.

This may aid C. vulgaris in stress survival and in resumption of growth once the stress is removed. The use of the continuous culture system may also allow for more defined studies leading to the illucidation of cause and effect relationships relative to specific environmental stress effects on lipids.

Abbreviations: FFA, free fatty acid; FS, free sterol; GC, gas liquid chromatography; HPTLC, high performance thin layer chromatography; PA, phosphotidic acid; PC, phosphatidyl choline; PE, phosphatidylethanolamine; PEG, polyethylene glycol; PG, phosphatidyl glycerol; PI, phosphatidylinositol; PFA, polar lipid fatty acid; PPL, phospholipid; SE, steryl ester; TFA, triglyceride fatty acid.

Introduction

Previous research has shown that exposure of plants to water stress resulted in lipid changes that correlated with adaptation to drought stress (Lechevallier 1977, Chetal et al. 1980, 1983, Liljenberg and Kates 1985). Some of the difficulties in illucidating the effects of drought stress on the lipid composition of plants can be attributed to the influence on lipid composition of environmental changes (light, temperature, nutrition) (Harwood 1983), ontogenetic variables such as age, genetic differences, and the level of complexity of a plant system (multicellular as opposed to single celled organisms) (Hasegawa et al. 1984). Although the use of single-celled organisms can reduce many of these variables, frequently, such studies are conducted in "batch" culture where nutrient levels, pH, and culture density and age are

important variables to consider. Thus the utilization of single-celled organisms in conjunction with continuous culture systems to study osmotic stress effects on plant lipids can eliminate most of the previously mentioned variables. This approach was recently used to study the effects of chlorocholine chloride, choline chloride, and phosphoryl choline chloride on the lipid composition of Chlorella vulgaris (Orcutt et al. 1984).

The objectives of the current study were to determine the effects of polyethylene glycol-induced osmotic stress on the lipid composition of Chlorella vulgaris grown in continuous culture. The lipid components studied were the polar lipids (PL), phospholipids (PPL), free fatty acids (FFA), triglycerides (TG), free sterols (FS), and steryl esters (SE).

Material and Methods

Culture conditions-- Chlorella vulgaris Beyerinck (Pratt strain) was obtained from Carolina Biological Co., Burlington, North Carolina. Axenic cultures were maintained under continuous illumination on 1% agar slants of Knop's medium as described previously (Goedhart and Orcutt 1988a). Algal cells from a slant were inoculated directly into a Bethesda Research Laboratories Airlift Fermenter (Life Technologies Inc., Gaithersburg, MD.) and the culture allowed to reach a density of 65% transmittance (1:4 dilution) measured at 678 nm. The culture was then operated as a chemostat using a peristaltic pump in which the rate of media influx was 16% greater than the efflux which allowed for daily sampling from the culture vessel. A filtered air/carbon dioxide

mixture (1000 ppm CO₂) was bubbled through the 2-l culture vessel at a rate of 850 ml/min, providing a carbon source and culture circulation necessary for growth. Light intensity, pH, and temperature were maintained at 25 $\mu\text{mol m}^{-2}\text{s}^{-1}$, 7.6, and 29 C respectively.

CO₂ analysis-- Carbon dioxide concentrations were determined daily for the experimental period using an Analytical Development Corporation (ADC) 225-MK3 Infrared Gas Analyzer (Hoddesdon, Hertfordshire, England). The CO₂ analyzer was calibrated using a 6001-01 gas calibration cylinder (Licor, Lincoln, Nebraska). The net CO₂ uptake was calculated as follows: $([\text{influent CO}_2 \text{ (1000 ppm)}] - [\text{effluent CO}_2]) \times (\text{gas flow rate } (.85 \text{ l}\cdot\text{min}^{-1})) \times (2 \text{ l}^{-1} \text{ growth medium}) \times 1 \text{ growth medium}\cdot\text{ug chl}^{-1}) = \text{mg CO}_2 \text{ fixed min}^{-1} \text{ ug chl}^{-1}$.

Osmotic regulation and sampling-- Osmotic adjustment was attained by addition of polyethylene glycol (PEG) 4000 (Mazei Chemicals, Gurnee, Illinois) to create water potentials of -0.1 MPa (Knop's solution only), -0.5, -1.0, and -1.5 MPa. These osmotic potentials were introduced sequentially in the order indicated. As cell density declined with decreasing osmotic potential, the culture density was maintained by decreasing the dilution rate. Three or four samples for lipid analysis were collected from the culture system at each osmotic level.

Lipid analysis-- The analysis of PL, TG, FFA, FS, and SE extracted from algal cells was conducted as previously described (Goedhart and Orcutt 1988a). Individual phospholipids were separated and quantified using high performance thin layer chromatography (HPTLC). HPTLC plates, 10 x

10 cm, precoated with Kieselgel 60 G (EM Science, Elmsford, New York) were washed in methanol prior to use to remove binders which interfered with visualization. Plates were activated at 100 C for 1 h and five 10-ug lipid samples were applied to each plate using the Camag Linomat III (Camag Scientific Inc, Wrightsville Beach, NC) sample applicator. Phospholipid standards containing PA, PC, PI, PE, PS, PG, and DG DG were also applied to each plate. One dimensional chromatography using a solvent system of acetone:benzene:water (91:30:8 v/v/v) was used to separate the lipids. The solvent front was allowed to run 6.0 cm above the point of lipid application. HPTLC plates were removed from the chamber, air dried for 5 min, and sprayed with 10% CuSO₄ in 8% H₃PO₄ until translucent (Touchstone et al. 1980). Plates were air dried an additional 5 min, heated at 150 C for 15 min, and cooled for 0.5 h before scanning with a thin layer densitometer (Camag TLC Scanner II). Peak integration was determined with a Spectra Physics 4270 integrator (Spectra Physics, San Jose, CA).

Results

Culture data-- The osmotic potential of the continuous culture was adjusted sequentially from -0.1 to -1.5 MPa using PEG. The adjustments to -0.5, -1.0, and -1.5 MPa were made at 4, 18, and 34 days, respectively, into the experiment (Fig. 4-1). Each time the osmotic potential was changed, the dilution rate was reduced in an effort to maintain an optical density near 65% transmittance. Cells for lipid,

chlorophyll, and dry weight determinations were collected from the culture after osmotic adjustment and density stabilized (Table 4-1).

The greatest reduction (38%) in CO₂ uptake and growth occurred when the osmotic potential was adjusted from -0.1 to -0.5 MPa on day 4 (Fig. 4-1 and Table 4-1). Although the growth rate of the culture was much reduced, the effect on chlorophyll concentration and cell dry weight was minimal (Table 4-1). Reductions in osmotic potential below -0.5 MPa had only a limited effect on growth and CO₂ fixation as was true for -1.5 MPa (data missing). However, lipid content of the cells decreased substantially at -1.5 MPa.

Lipid data-- Table 4-2 summarizes the effects of osmotic-induced changes in the polar lipid fatty acids (PFA), triglyceride fatty acids (TFA), free fatty acids (TFA), free fatty acids (FFA), free sterols (FS), and steryl esters (SE) of C. vulgaris grown in continuous culture. PFA and FFA were 56 and 95% percent lower, respectively, than the control (-0.1 MPa) at an osmotic potential of -1.5 MPa. PFA decreased sequentially as the osmotic potential declined, however this was not true of FFA. FFA increased as osmotic potential declined and was 3.8 times higher at -1.5 MPa compared to the control. FS and SE remained relatively unchanged.

Tables 4-3 through 4-5 summarize the relative percentages of n-tetradecanoic (14:0), n-hexadecanoic (16:0), n-hexadecenoic (16:1), n-hexadecadienoic (16:2), n-octadecanoic (18:0), n-octadecenoic (18:1), n-octadecadienoic (18:2), and n-octadecatrienoic (18:3) acids identified in the PFA, FFA, and TFA fractions of C. vulgaris subjected

to decreasing osmotic potentials. The relative percentage of PFA changed very little as the osmotic potential decreased with the exception that 18:0 declined slightly and 18:3 increased (Table 4-3). However, in the TFA lipid class, the relative percentage of all unsaturated fatty acids increased, while the saturated fatty acids decreased as the osmotic potential was reduced (Table 4-4). The opposite was true of the FFA lipid class (Table 4-5). The double bond index (DBI, Table 4-6) also reflects these relationships. The ratio of 18:3/18:2 did not change for the PFA or TFA class while decreasing in the FFA class as the osmotic potential decreased.

Table 4-7 summarizes the effects of osmotic-induced changes on the phospholipids (PPL) of *C. vulgaris*. Total PPL, phosphatidic acid (PA), phosphatidyl inositol plus phosphatidyl ethanolamine (PI + PE), and phosphatidyl glycerol decreased by 31, 68, 45, and 31 percent, respectively, as the osmotic potential was lowered. Phosphatidyl choline (PC) remained relatively constant. The FS to PPL ratio initially decreased below the control level and then increased as the osmotic potential increased.

Discussion

Water-deficit stress can cause reduction in photosynthesis, chlorophyll concentration, respiration, protein, and RNA concentration (Levitt 1972). Sullivan and Eastin (1974) found that endogenous oxygen evolution by cell free homogenates of sorghum leaves was reduced 15 to 49% by decreasing the osmotic potential from -0.54 to -1.14 MPa and was

further reduced by 58 to 92% by decreasing the osmotic potential to -3.1 MPa. Reductions in photosynthesis may result from changes in membrane stability as reflected in changes in lipid composition. Water-deficit stress reportedly causes a decrease in polar lipids and glycolipids of cotton (Gossypium hirsutum L.) (Ferrari-Iliou et al. 1984) wheat (Triticum aestivum L.) and barley (Hordeum vulgare L.) chloroplasts (Chetal et al. 1981, 1983). In cotton, chloroplast stability was related to the degree of stress to which the plants had been subjected; the greater the stress, the less stable the chloroplasts were. In the present study, photosynthesis was reduced by 41% at 0.5 MPa and 56% at -1.0 MPa (Table 4-1) which is in close agreement with observations of photosynthesis of sorghum chloroplasts stressed at -1.14 MPa (Sullivan and Eastin 1974). The decrease in photosynthetic rate in C. vulgaris does not appear to be related to chlorophyll concentration since it remained relatively unchanged with stress (Tab 4-1). Work with oxygen solubility in PEG-4000 solutions indicate that the solubility drops as the PEG concentration increases and that the dimensionless mass transport of oxygen decreases very rapidly between the osmotic potentials of -0.1 and -0.5 MPa (Mexal et al. 1975). Although CO₂ solubility may not mirror O₂ solubility, under the concentrations of CO₂ used (1000 ppm), the mass transport of CO₂ to algal cells may be a limiting factor in preventing algal growth as the water potential decreases from -0.1 to -0.5 MPa.

Thompson (1986) proposed that one of the most rapid and beneficial responses to stress is the control of saturation levels of cellular

lipids and further suggested that alterations of sterol or phospholipid composition could not occur quickly enough for rapid changes in environmental conditions. Changes in the degree of lipid unsaturation as well as alterations in sterols and other lipids have been reported. In Spirodela, water stress resulted in a decrease in the ratio of linolenic to linoleic acid in phospholipids and other acyl lipids (Lechevallier 1977). In cotton, [1-¹⁴C]acetate was incorporated into saturated fatty acids rather than unsaturated fatty acids associated with the phospholipids and glycolipids (Pham Thi et al. 1985). The decrease in unsaturated fatty acid production was primarily a result of decreased linolenic acid. Also, the phospholipid concentration decreased in the leaves. In the present study, the polar lipid fatty acids decreased as the osmotic potential decreased but the ratio of linolenic to linoleic acid increased (Table 4-6). The only decreases in unsaturated fatty acids occurred in the FFA fraction (Table 4-6) where all unsaturated fatty acids declined as did the ratio of linolenic to linoleic acid.

Triglycerides, free sterols and steryl esters were reported to increase in corn tissue stressed with PEG at an osmotic potential of -1.5 MPa (Douglas and Paleg 1981). Oat seedlings exposed to four periods of water-deficit stress alternating with rewatering periods exhibited a 60% increase in steryl esters and methyl sterols (Liljenberg et al. 1985). In the current study with C. vulgaris, TFA increased with stress while PFA declined. The FS and SE concentrations changed little when exposed to stress in the continuous culture (Table

4-2). In the batch culture of C. vulgaris free sterols decreased and the steryl esters increased in an inverse manner as the osmotic potential was reduced (Goedhart and Orcutt 1988a). Such differences may reflect the more controlled conditions of the continuous culture compared to the batch culture system.

In general, all the phospholipids in C. vulgaris decreased as the osmotic concentration increased except for PC which was relatively stable (Table 4-7). An increase in PC concentrations has been observed in wheat and barley leaves subjected to drought-stress (Chetal et al. 1980) while PG decreased and PI remained unchanged. The concentration of PC was found to decline in drought-sensitive cultivars of cotton subjected to drought stress (Pham Thi et al. 1985). Liljenberg and Kates (1985) suggested that increases in the ratio of sterols to membrane acyl lipids might be beneficial in water stress because a more rigid, less fluid, membrane would lower the permeability to water and to other small molecules. Thus, the observed increases in the FS/PPL ratios in C. vulgaris at -1.0 and -1.5 MPa (Table 4-7) may contribute to the survival of the organisms at higher osmotic concentrations. In conclusion, the increase in triglycerides and reduction in PPL and FFA suggest interconversions among lipids which allow for the sequestering of lipids into neutral forms such as triglycerides when lipid synthesis and growth are limited (Taylor and Parks 1984) by stress. These lipid storage forms can later be used for growth upon release from stress. In addition, the hydrophobic character of triglycerides prevents interaction with the H-bonds of water. Limited water resources can be

made available as protective hydration shells around proteins (Douglas and Paleg 1981). The lack of interconversion of free sterols and sterol esters in the continuous culture may reflect the importance of controlled conditions of the continuous culture system. This may aid in elucidating those lipid factors susceptible to osmotic stress as opposed to those alterations which are induced by other cultural variables such as nutrient levels, pH, and stage of growth.

Literature Cited

- Chetal, S., Wagle, D. S. & Nainawatee, H. S. 1980. Phospholipid changes in wheat and barley leaves under water stress. -
Phytochemistry 19: 1393-1395.
- Chetal, Wagle, D. S. & Nainawatee, H. S. 1983. Phospholipid changes in wheat and barley chloroplast under water stress. -
Plant Sci. Lett. 29: 273-278.
- Douglas, T. J. & Paleg, L. G. 1981. Lipid composition of Zea mays seedlings and water stress-induced changes. -
J. Exp. Bot. 32: 499-508.
- Goedhart, C. L. & Orcutt, D. M. 1988a. Osmotic-induced changes in the growth and lipid composition of Chlorella vulgaris and Chlorella pyrenoidosa grown in batch culture. -
Physiol. Plant. (submitted)
- Harwood, J. L. 1983. Adaptive changes in lipids of higher-plant membranes. - Biochem. Soc. Trans. 11: 343-346.
- Hasegawa, P. M., Bressan, R., Handa, S. & Handa, A. K. 1984. Cellular mechanisms of tolerance to water stress. -
Hortscience 19: 371-377.
- Lechevallier, D. 1977. Effets du polyethylene glycol sur les lipides et les lipochromes des colonies de Spirodele. -
Physiol. Veg. 15: 387-402.
- Levitt, J. 1972. Responses of Plants to Environmental Stresses.
Academic Press. New York. 697 p.

- Liljenberg, C., Karunen, P. & Ekman, R. 1985. Changes in steryl lipids of oat root cells as a function of water-deficit stress. - *Physiol. Plant.* 63: 253-257.
- Liljenberg, C. & Kates, M. 1985. Changes in lipid composition of oat root membranes as a function of water-deficit stress. - *Can. J. Biochem. Cell Biol.* 63: 77-84.
- Mexal, J., Fisher, J. T., Osteryoung, J. & Reid, C. P. P. 1975. Oxygen availability in polyethylene glycol solutions and its implications in plant-water relations. - *Plant Physiol.* 55: 20-24.
- Orcutt, D. M., Davis, G. A. & Moore, L. D. 1984. The effects of choline derivatives on the growth and lipid content of continuously cultured Chlorella vulgaris. - *Can. J. Bot.* 62: 81-85.
- Pham Thi, A. T., Borrel-Flood, C., Da Silva, J. V., Justin, A. M. & Mazliak, P. 1985. Effects of water stress on lipid metabolism in cotton leaves. - *Phytochemistry* 24: 723-727.
- Sullivan, C. Y. & Eastin, J. D. 1974. Plant physiological responses to water stress. - *Agric. Meteor.* 14: 113-127.
- Taylor, F. R. & Parks, L. W. 1978. Metabolic interconversions of free sterols and steryl esters in Saccharomyces cerevisiae. - *J. Bacteriol.* 136: 531-537.
- Thompson Jr., G. A. 1986. Metabolism and control of lipid structure modification. - *Can. J. Biochem. Cell Biol.* 64: 66-69.
- Touchstone, J. C., Chen, J. C. & Beaver, K. M. 1980. Improved separation of phospholipids in thin layer chromatography. - *Lipids* 15: 61-62.

Table 4-1. Osmotic-induced changes in the lipid weight, chlorophyll concentration, and CO₂ fixation rates of Chlorella vulgaris grown in continuous culture.

Osmotic potential (MPa)	Dry weight mg/ml medium	Lipid wt/dry wt mg/mg	Chl conc. ug/ml medium	CO ₂ fixation rate ug CO ₂ ·ug chl ⁻¹ ·min ⁻¹
-0.1	.24 ± .01	.23 ± .0	12.7 ± 0.3	7.8
-0.5	.25 ± .01	.29 ± .03	13.3 ± 0.4	4.6
-1.0	.38 ± .06	.29 ± .11	13.6 ± 1.4	3.4
-1.5	*	.15 ± .03	*	*

* Missing data

Figure 4-1. Growth, CO₂ uptake, and dilution rate of
Chlorella vulgaris grown in continuous culture.

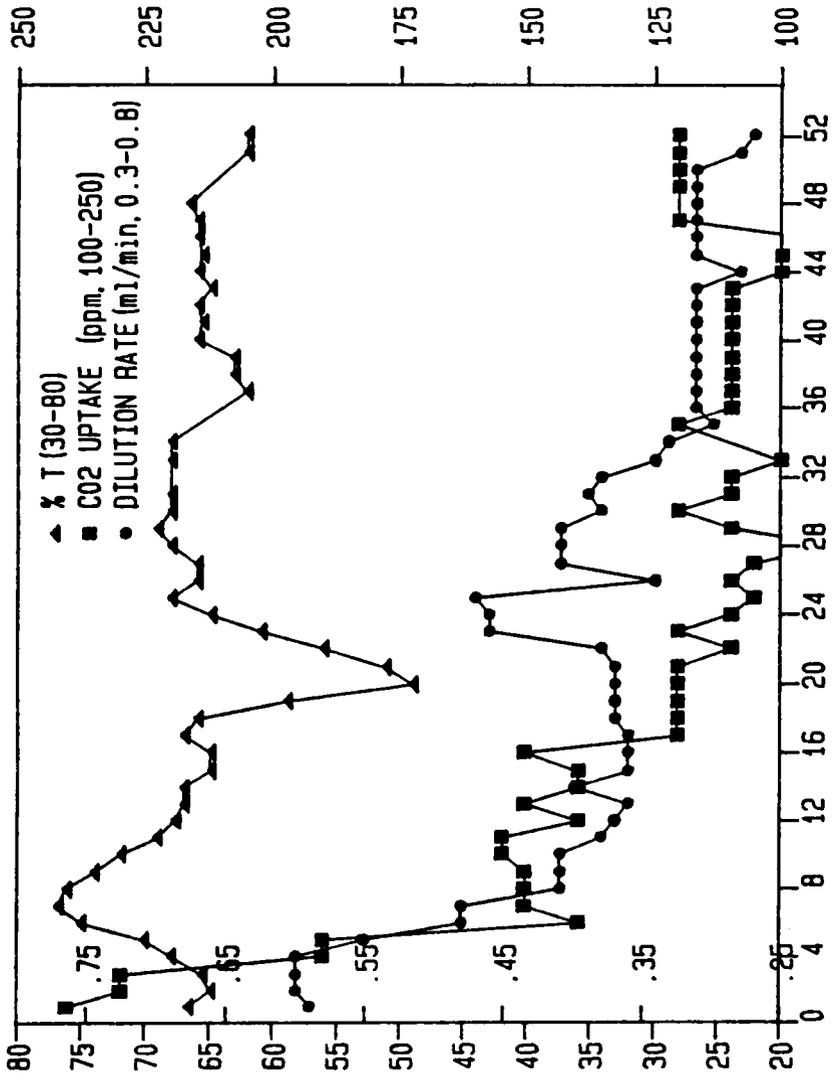


Table 4-2. Osmotic-induced changes in the lipid composition of Chlorella vulgaris grown in continuous culture.

Osmotic Treatment	Lipid concentration (ug / mg total lipid)					
	PFA	TFA	FFA	FS	SE	FS/PPL
-0.1 MPa	338 ± 8.6	2.54 ± .04	42.7 ± 4.2	8.2 ± 0.5	3.9 ± .7	.024
-0.5 MPa	254 ± 8.5	3.65 ± .68	8.6 ± 0.4	3.5 ± 0.7	2.8 ± .3	.014
-1.0 MPa	185 ± 59	7.42 ± 2.6	22.6 ± 13.5	10.7 ± 0.6	4.2 ± 1.7	.058
-1.5 MPa	147 ± 12	9.64 ± .61	2.0 ± 0.4	9.3 ± 5.6	3.7 ± 1.0	.063

Data entries are the average of four replicates ± standard deviation.

Abbreviations- FFA, free fatty acid; FS, free sterol; PFA, polar lipid fatty acid; TFA, triglyceride fatty acid; SE, steryl ester.

Table 4-3. Osmotic-induced changes in the fatty acid composition of the polar lipid fraction of *Chlorella vulgaris* grown in continuous culture.

Treatment	Fatty acid constituent (%)							
	14:0	16:0	16:1	16:2	18:0	18:1	18:2	18:3
-0.1 MPa	0.6 ± 0.2	15.4 ± 0.3	1.7 ± 0.1	12.9 ± 0.5	10.2 ± 0.4	1.2 ± 0.1	22.4 ± 0.6	35.6 ± 0.7
-0.5 MPa	1.3 ± 0.4	14.4 ± 0.6	2.1 ± 0.1	10.9 ± 0.6	8.3 ± 0.4	1.8 ± 0.4	21.4 ± 0.3	39.9 ± 1.5
-1.0 MPa	0.9 ± 0.5	12.5 ± 0.9	0.7 ± 0.3	10.2 ± 0.3	8.2 ± 0.5	2.0 ± 0.4	19.5 ± 0.9	46.2 ± 0.6
-1.5 MPa	1.1 ± 0.2	15.9 ± 0.2	2.0 ± 0.2	10.7 ± 0.1	6.0 ± 0.1	1.1 ± 0.0	23.6 ± 0.5	39.6 ± 0.4

Data entries are an average of four replicates, two from each of two separate experiments ± standard deviation.

Table 4-4. Osmotic-induced changes in the fatty acid composition of the triglyceride fraction of Chlorella vulgaris grown in continuous culture.

Treatment	Fatty acid constituent (%)							
	14:0	16:0	16:1	16:2	18:0	18:1	18:2	18:3
-0.1 MPa	7.8 ± 1.9	42.0 ± 7.2	—	2.7 ± 0.4	36.5 ± 6.2	6.0 ± 8.4	3.6 ± 5.0	1.4 ± 2.0
-0.5 MPa	4.1 ± 1.2	34.4 ± 3.5	—	2.5 ± 0.8	28.3 ± 1.7	16.6 ± 2.5	10.1 ± 2.8	3.7 ± 1.0
-1.0 MPa	3.2 ± 1.0	45.2 ± 9.0	—	2.1 ± 0.5	24.1 ± 7.0	13.1 ± 3.4	14.5 ± 3.7	6.2 ± 1.9
-1.5 MPa	2.0 ± 0.3	38.1 ± 1.0	—	3.0 ± 0.2	12.6 ± 0.7	11.1 ± 0.4	23.3 ± 0.6	10.0 ± 0.4

Data entries are an average of four replicates, two each from each of two separate experiments ± standard deviation.

Table 4-5. Osmotic-induced changes in the fatty acid composition of the free fatty acid fraction of *Chlorella vulgaris*

grown in continuous culture.

Treatment	Fatty acid constituent (%)							
	14:0	16:0	16:1	16:2	18:0	18:1	18:2	18:3
-0.1 MPa	1.3 ± 0.4	18.4 ± 0.9	0.9 ± 0.2	6.2 ± 0.3	6.1 ± 0.5	2.4 ± 0.2	19.5 ± 1.4	45.2 ± 0.7
-0.5 MPa	5.2 ± 0.9	43.3 ± 5.1	—	—	8.9 ± 2.6	11.8 ± 2.3	15.6 ± 3.2	15.2 ± 1.8
-1.0 MPa	1.6 ± 0.8	54.8 ± 25	1.4 ± 1.4	0.3 ± 0.3	7.0 ± 4.2	2.6 ± 2.6	15.3 ± 12.5	17.1 ± 15
-1.5 MPa	9.4 ± 5.6	51.4 ± 5.9	0.4 ± 0.6	1.2 ± 1.2	31.6 ± 5.8	4.9 ± 8.6	1.0 ± 1.7	0.3 ± 0.4

Data entries are an average of four replicates, two from each of two separate experiments ± standard deviation.

Table 4-6. Osmotic-induced changes of the ratio of 18:2 to 18:3 and the DBI of three lipid classes of Chlorella vulgaris grown in continuous culture.

Osmotic Treatments	18:3/18:2			DBI		
	PFA	TFA	FFA	PFA	TFA	FFA
-0.1 MPa	1.59	0.39	2.32	1.80	0.22	1.90
-0.5 MPa	1.87	0.37	0.98	1.87	0.53	0.90
-1.0 MPa	2.38	0.43	1.12	2.00	0.65	0.87
-1.5 MPa	1.68	0.43	0.30	1.91	0.94	0.10

Abbreviations- DBI = Double bond index; FFA, free fatty acid; PFA, polar lipid fatty acid; TFA, triglyceride fatty acid.

Table 4-7. Osmotic-induced changes in the concentrations of phospholipids of *Chlorella vulgaris* grown in continuous culture.

Treatment	Phospholipid concentration (mg / 10 mg total lipid)					
	PA	PC	PI + PE	PG	Total PPL	FS/PPL
-0.1 MPa	.47 ± .02	1.01 ± .12	.22 ± .04	.76 ± .07	2.46 ± .19	0.033
-0.5 MPa	.42 ± .13	.79 ± .09	.20 ± .07	.72 ± .15	2.13 ± .30	0.016
-1.0 MPa	.31 ± .11	1.08 ± .68	.13 ± .06	.10 ± .10	1.50 ± .57	0.071
-1.5 MPa	.15 ± .03	.92 ± .68	.12 ± .06	.50 ± .20	1.70 ± .71	0.055

Data entries are an average of four replicates ± standard deviation.

Abbreviations- FS, free sterol; PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; PI, phosphatidyl inositol; PPL, phospholipid.

Chapter V

INFLUENCE OF A PYRIDAZINONE HERBICIDE ON ETHANOL AND POLYETHYLENE GLYCOL-INDUCED CHANGES IN GROWTH AND LIPID COMPOSITION OF CHLORELLA VULGARIS

Abstract

At a water potential of -1.5MPa, stepwise increases in the concentration of BAS 13-338 (4-chloro-5-(dimethylamino)-2-phenyl-3(2H)pyridazinone) in the growth medium resulted in incremental decreases in growth of Chlorella vulgaris. Growth of C. vulgaris in Knop's solution containing 0.33% ethanol was inhibited 65% relative to control. Addition of BAS 13-338 at concentrations of $4 \times 10^{-8}M$ to $3.2 \times 10^{-6}M$ in 0.33% ethanol solutions partially reversed ethanol inhibition, doubling growth rates from 35% to 75% of control.

Ethanol treatment at -0.1 MPa resulted in phospholipid and free sterol reductions of 11% and 48% respectively, while triglyceride fatty acids increased 40-fold. The free sterol to phospholipid ratio also declined 48%. BAS 13-338 at $4 \times 10^{-8}M$ reversed the ethanol-induced lipid alterations. At -1.5 MPa increasing the concentration of BAS 13-338 to $8 \times 10^{-8}M$ resulted in reduced levels of phospholipids and free sterols by 30% and 40%, respectively.

Fatty acid profiles of polar lipids, triglycerides, and free fatty acid fractions of C. vulgaris treated with ethanol and BAS 13-338 at -0.1 MPa and treated with BAS 13-338 at -1.5 MPa resulted in 20 and 35%

increases in the 18:2/18:3 ratio of the polar lipid and triglyceride fractions, respectively. This increase in saturation reversed the decrease in saturation caused by ethanol treatment alone.

Polar lipids of C. vulgaris were affected by both ethanol and BAS 13-338. At -0.1 MPa and 0.33% ethanol the concentrations of phosphatidyl choline and phosphatidyl glycerol declined, while the combined concentration of phosphatidyl inositol and phosphatidyl ethanolamine increased. Addition of BAS 13-338 to ethanol treatments resulted in a complete reversal of ethanol's phosphatidyl choline inhibition and resulted in a decrease in phosphatidic acid. At -1.5 MPa with 0.33% ethanol, BAS 13-338 caused decreases in concentrations of digalactosyldiglyceride and phosphatidyl glycerol, and the combined concentrations of phosphatidyl inositol and phosphatidyl ethanolamine.

BAS 13-338 treatment at -0.1 MPa of algal cells grown in 0.33% ethanol resulted in large increases in the concentrations of 4 - methylcholest-8(14)-en-3 β -ol, ergost-8(14)-en-3 β -ol, and 4 - methylergosta-8(9),14-dien-3 β -ol. BAS 13-338 treatment did not increase the concentration of ergost-7-en-3 β -ol which was inhibited by 0.33% ethanol treatment.

The changes observed seem to indicate that adaptation to ethanol toxicity by lipid alteration is poorly regulated in Chlorella vulgaris and that the addition of BAS 13-338 stimulates adaptive lipid formation.

Abbreviations - DBI, double bond index; DGDG, digalactosyldiacylglycerol; FFA, free fatty acid; FS, free sterol;

HPTLC, high performance thin layer chromatography; GC, gas chromatography; MPa, megapascal; PEG, polyethylene glycol; PFA, polar lipid fatty acid; PL, polar lipid; PPL, phospholipid; TG, triglyceride; TGFA, triglyceride fatty acid; TLC, thin layer chromatography; SE, steryl ester.

Introduction

Ethanol is produced in various herbaceous and woody plants in response to environmental stresses such as SO_2 , ozone, freezing, and water deficit (Kimmerer & Kozlowski 1982). Ethanol synthesis can continue in the plant even after the stress has been removed, as has been observed with plant exposure to SO_2 . Unlike ethanol production in response to anaerobiosis, ethanol accumulation in the above stresses appears independent of O_2 availability. Under environmental conditions of flooding or plant encasement in ice, accumulation of ethanol occurs (Andrews 1977). Reduced cold hardiness and survival of winter cereals encased in ice can be explained in part as a result of ethanol damage. However, under conditions of subfreezing temperatures and restricted O_2 availability, plant adaptation to prevent ethanol damage to membranes and eventual loss of cellular integrity would seem unlikely (Andrews and Pomeroy 1977).

Ethanol has generally shown stimulatory activity in interactions with phytochrome of light-requiring seeds with the exception of Rumex crispus L. (Taylorson, 1984). Demonstration that ethanol effects could be prevented or overcome by shifting seed germination temperature

regimes suggests that ethanol causes membrane perturbation preventing P_{4r} from acting (Taylorson 1984).

Despite these reports of ethanol accumulation and activity, few studies have investigated plant responses to ethanol. Most reports of ethanol toxicity and tolerance concern animal systems. Ethanol appears to have activity as an anesthetic. Because anesthetic properties of many drugs correlate with their lipid solubilities, anesthetics are thought to intercalate in the hydrophobic membrane regions, partially disrupting membrane function (Chin and Goldstein 1976).

Alterations in the lipid composition of various tissues and classes of lipids in response to chronic exposure to ethanol have been observed in several studies. An increased proportion of saturated fatty acids occurred in brain and heart phospholipids of mice exposed to ethanol (Littleton et al. 1980). Higher cholesterol to phospholipid molar ratios have also been observed in tissues of rats and mice (Rovinski and Hosein 1983, Goldstein et al. 1980). The cardiolipin fraction of mitochondrial membranes of chronic alcoholic rats increased in unsaturation relative to non-exposed rats (Waring et al. 1981). Moderate depression by alcohol in rates of sterol synthesis from both acetate and mevalonate occurred in parallel with reduction of HMG-CoA reductase and cholesterol 7 hydroxylase activity in livers of ethanol fed rats (Klurfeld et al. 1979).

The above observations have led researchers to propose that alcohol tolerance results from changes in membrane lipid composition.

Increased saturation causes increased membrane rigidity which leads to a reduction in membrane binding of alcohol and will allow membranes to have a normal fluidity in the presence of moderate concentrations of ethanol (Rottenberg et al. 1981). Support for such conclusions have been supplied by electron paramagnetic resonance studies (Chin and Goldstein 1976, Goldstein et al. 1980). However, conflicting results have caused some researchers to question the direct link between functional, compositional, and fluidity changes of membranes as described by Rottenberg et al. (1981) and Gordon (1984).

Several recent reviews have indicated a common response of plants to stress involves the alteration of lipid composition (Harwood 1983, Kuiper 1985). As with other stresses, water stress appears to affect phospholipid concentrations (Chetal et al. 1981, 1983), levels and degree of unsaturation of glycolipids (Ferrari-Iliou et al. 1984), and sterol to phospholipid ratios (Liljenberg and Kates 1982). Such alterations may merely reflect a change in enzymatic activity of lipid metabolism without appreciable significance for the physiology of the plant or alternatively such alteration may have adaptive value (Kuiper 1985).

Hanson and Hitz (1982) have outlined five approaches to plant stress research to determine whether metabolic alterations result from stress-induced lesions at vulnerable sites in metabolism or if the alterations result from adaptive changes reflecting ordered operation of metabolic regulatory mechanisms. One such approach was to intervene in the plant's metabolism and genetically or chemically alter

a pathway involving the compounds proposed to be involved in the adaptation process.

BAS 13-338 (4-chloro-5-(dimethylamino)-2-phenyl-3(2H)pyridazinone) is a member of the herbicidal substituted pyridazinones. This herbicide class has multiple modes of action depending on the substitution of the basic ring structure. Biochemical activity of BAS 13-338 differs from other substituted pyridazinones in that the compound is relatively ineffective in preventing chloroplast pigment accumulation, and is a poor inhibitor of photosynthetic electron transport relative to the other substituted pyridazinones (St. John 1982). However, in wheat (Triticum aestivum L.) shoots, BAS 13-338 was the most effective of the substituted pyridazinones in altering the ratios of linoleic to linolenic acid of phospholipids and galactolipids (St. John 1982, 1984). Differential responses in saturation levels of different plant species suggests that control of linoleic acid biosynthesis may vary or that the action of the herbicide may be affected by some cellular constituent.

BAS 13-338 seems to provide protection against drought stress (St. John 1984). Pre-emergence soil applications to corn at rates of 5.6 to 11.0 kg/ha resulted in a 25% reduction in transpiration rates. This reduction in transpiration was not the result of differences in diffusive resistance, leaf water potential, or internal leaf water content. In soybeans treated with 15 ppm BAS 13-338, the amount of cuticular waxes was 40% greater than that of untreated plants. This suggested that water economy was partially altered by leaf cuticular

wax (St. John 1984).

Studies of lipid composition were initiated with Chlorella vulgaris to determine whether or not this compound could provide tolerance to water stress or ethanol toxicity through alterations of specific membrane lipids. Use of BAS 13-338, a chemical which affects lipid metabolism would appear to answer the concerns of Kuiper (1985) in determining whether alterations are truly adaptive or merely reflections of alteration in enzymatic activity without adaptive significance.

Materials and methods

Plant material. Chlorella vulgaris Beyerinck (Pratt strain) was obtained from Carolina Biological Co., Burlington, North Carolina. Axenic cultures were maintained under continuous illumination on 1% agar slants of Knop's medium (Vela & Guerra 1966) with the exception that $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$ was substituted for $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and the initial pH was adjusted to 6.5. An inoculation medium was prepared by growing algae in modified Knop's medium aerated with 1% CO_2 and illuminated continuously at a photon flux density of $25 \mu\text{mol m}^{-2}\text{s}^{-1}$. Three ml of log phase inoculum were added to 150 ml of the growth medium. The growth media used in the first experiment consisted of Knop's medium containing: no added compounds, 0.33% ethanol, or 0.33% ethanol and BAS 13-338 (96% technical material, BASF Wyandotte Corporation, Fairfield, New Jersey) at concentrations of $4.0 \times 10^{-3}\text{M}$, $1.6 \times 10^{-4}\text{M}$, or $3.2 \times 10^{-4}\text{M}$. In a second experiment the growth medium consisted of Knop's

medium with PEG 4000 (MW 3350) to lower the water potential to -1.5 MPa, and concentrations of BAS 13-338 at $4.0 \times 10^{-6}M$, $2.0 \times 10^{-6}M$, $4.0 \times 10^{-7}M$, or $8.0 \times 10^{-8}M$. The 150 ml volume of algal cells was divided into 40-ml portions among each of three sterilized pyrex test tubes (25 X 200mm). Each test tube was fitted with a foam rubber stopper through which a glass aeration tube (2.0 mm o.d.) was inserted the full tube length. Non-absorbent cotton plugs were inserted into each glass tube to filter the 1% CO₂ and air mixture which was bubbled through each tube culture at a rate of 5 ml/ min. Cells were incubated at 27 C by inserting the culture tubes along the edges of a rectangular piece of styrofoam which floated in a 56.8-l aquarium. Two 40-watt, cool-white, fluorescent tubes (Philips Lighting Corporation, Bloomfield, New Jersey) provided continuous illumination with a photon flux density of $25 \mu\text{mol m}^{-2}\text{s}^{-1}$. The BAS 13-338 solutions were filter sterilized.

Growth determination-- Growth was determined by optical density measurements using a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 678 nm (Sorokin 1973). The growth rate was determined for the exponential growth phase and only this linear growth is plotted in the figures. Growth data were expressed as log₂ growth for plotting and analysis of data. Growth data presented are the means of four experimental runs with three replications per treatment.

Lipid Isolation-- Late exponential phase algal cultures were harvested

for lipid analysis (60% transmittance of a 1:4 dilution).

A procedure described by Nichols (1963) was modified as follows to extract lipids. Cells from the three replicate samples of each treatment were concentrated by centrifugation at 3000 X g followed by two successive washings with an isotonic NaCl solution and subsequent re-centrifugation. The cells were resuspended in 10 ml of NaCl solution and 1 ml was removed for dry-weight determination. This aliquot of cells for dry weight determination was washed with distilled water, centrifuged, and dried at 70 C. The remaining 9 ml of the cell solution for lipid extraction was re-centrifuged and the supernatant was decanted. Cells were then suspended in 5 ml of isopropanol to deactivate hydrolytic enzymes (Harborne, 1984). An additional 5 ml of isopropanol was added, the vials containing the cells were flushed with N₂ gas, and this material was stored for further extraction. Cells were concentrated by centrifugation and the isopropanol layer was removed. An additional 5-min extraction with 10 ml of isopropanol was performed. The remaining cellular debris was extracted under N₂ for 12 h on a rotary shaker at 100 rpm with 40 ml of chloroform:isopropanol (1:1 v/v) in a stoppered 125 ml erlenmeyer flask. The isopropanol and chloroform-isopropanol extracts were combined, filtered through Whatmann #50 paper and concentrated by rotary evaporation at 45 C. The extract was subjected to a Folch wash (Folch et al. 1957) and the lipid fraction transferred to tared tubes to determine lipid weight. Internal standards of 100 ug methylpentadecanoate, 40 ug cholesterol, and 30 ug cholesterol oleate (Sigma Chemical, St. Louis, Missouri) were

added to the lipid extracts before the Folch wash to aid in quantification.

Lipid characterization and quantitation-- Lipid classes were separated on Kieselgel 60 G (EM Science, Elmsford, New York) TLC plates (0.5 mm thickness) using a solvent system of n-hexane:diethylether:acetic acid (85:15:1 v/v/v) (Ginger and Fairbairn 1966). Lipid bands corresponding to PL, TG, FFA, FS, and SE were visualized with 0.2% 2',7'-dichlorofluorescein in 95% ethanol under long wave ultraviolet light, removed from TLC plates and processed further. The PL, FFA, and TG were analyzed as described (Orcutt et al. 1978) with the exception that samples were not separated by degree of unsaturation. TG were quantified on the basis of the fatty acids liberated by hydrolysis and were expressed as TFA. GC analyses were performed on a Bendix 2500 gas chromatograph (Bendix Process Instrument Division, Ronceverte, WV) equipped with a flame ionization detector. Fatty acids were separated isothermally at 190 C on a 4 mm i.d. X 2 m column packed with 10% DEGS on 80-100 Chromasorb NAW. Quantitation of FFA and PL was based on the internal standard C-15. Identification of the fatty acids was by comparison of GLC retention times with those of known standards.

Additionally concentrations of polar lipid constituents (phospholipids and DGDS) were quantified using HPTLC. HPTLC plates, 10 X 10 cm precoated with Silica Gel 60 (Merk # 5631), were washed in methanol prior to use to remove binders which interfered with visualization. Plates were then activated at 100 C for 1 h. Five

samples (5 ug) and three standards, containing PA, PC, PI, PE, PS, PG, and DGDG, were applied to each plate using the Camag Linomat III (Camag Scientific Inc., Wrightsville Beach, NC). Lipids were separated using one-dimensional chromatography and a solvent system of acetone:benzene:water (91:30:8 v/v/v). Samples were applied 0.5 cm from the edge of the plate and the solvent front was allowed to run 6.0 cm in a Camag HPTLC Linear Development Chamber (Camag Scientific Inc, Wrightsville Beach, North Carolina). Plates removed from the chamber were air dried for 5 min and sprayed with 10% CuSO₄ in 8% H₃PO₄ until translucent (Touchstone et al. 1980). Plates were air dried an additional 5 min, heated at 150 C for 15 min and cooled for 0.5 h before being scanned by the thin layer densitometer (Camag TLC Scanner II). Peak integration was determined with a Spectra Physics 4270 integrator (Spectra Physics, San Jose, CA).

Trimethylsilyl ether derivatives of sterols were prepared and quantified as described previously (Bradford et al. 1982). Silylated sterols were separated isothermally at 275 C on a 2-m by 2-mm (i.d.) coiled glass column packed with 3% SE-30 on 80/100 Gas Chrom Q. Retention times and peak areas were determined using a Hewlett Packard 3392A Integrator (Hewlett Packard, Avondale, PA).

Tentative identification of sterols was made by comparison of retention times relative to cholesterol with published data (Patterson 1971, Orcutt et al. 1984) and by electronic ionization mass spectra. A Finnigan model 4510 gas chromatograph-mass spectrometer fitted with an on-column injector (J&W model 200-1020) and a 30 m by 0.32 mm (i.d.)

fused silica capillary column coated with a 0.1 μm bonded methyl silicone phase was used for the mass spectral analysis. The column was held at 220 C for 1 min, then temperature programmed at a rate of 2 C / min to 255 C. Helium was used as the carrier gas at a pressure of 15 PSI. Electron ionization spectra were obtained at 70 eV with a source block temperature of 150 C.

Results

Growth data

Ethanol (0.33%) did not immediately effect the growth of C. vulgaris. Growth for the first 24 h. was at a rate similar to growth in the absence of ethanol. However beyond this time, ethanol (0.33%) reduced the growth rate of Chlorella vulgaris by 65% when grown at an osmotic potential of -0.1 MPa (Fig. 5-1a and Table 5-1). BAS 13-338, at a concentration of $4 \times 10^{-8}\text{M}$, alleviated the inhibitory effect, restoring growth to 75% of control. Higher concentrations of the herbicide also alleviated the inhibitory effects of ethanol but to a lesser extent as concentration increased. BAS 13-338 did not alleviate a PEG-induced osmotic stress (-1.5 MPa) as indicated by a incremental reductions in the growth rate of C. vulgaris with increasing concentration of the herbicide (Fig. 5-1b and Table 5-1). At the highest concentration of BAS 13-338 growth was reduced by 22% compared to the stressed control growth.

Lipid data

At -0.1 MPa and 0.33% ethanol, the concentrations of PPL declined

11%, FS declined 48%, FFA remained unchanged, TFA increased 40-fold and the ratio of FS/PPL declined 50% (Table 5-2). As increasing amounts of BAS 13-338 were added, lipid changes caused by ethanol treatment were reversed. BAS 13-338 treatments at 1.6×10^{-4} M and higher resulted in lipid concentrations similar to those of algae grown without ethanol (Table 5-2).

At a water potential of -1.5 MPa, increasing concentrations of BAS 13-338 generally caused reduced concentrations of all the major lipid classes analyzed (Table 5-2). At the highest concentration of BAS 13-338 (8.0×10^{-3} M), PPL, TFA, FFA, FS, and FS/PPL were reduced by 48.9, 35.0, 40.0, and 37.6%, respectively.

The major fatty acids identified from the PL, TG, and FFA classes were 16:0 (palmitic), 16:3 (hexadecatrienoic), 18:0 (stearic), 18:1 (oleic), 18:2 (linoleic), and 18:3 (linolenic). Figure 5-2 is a gas liquid chromatogram of the fatty acid profiles of the three lipid classes isolated from *C. vulgaris*. Table 5-3 summarizes the influence of BAS 13-338 on ethanol and PEG-induced stress on the three lipid classes, as reflected in changes in the 18:2/18:3 ratios and the DBI. The PL class was affected least by the treatments employed with very little change occurring in the ratios of 18:2/18:3 or DBI in any of the treatments. The ratio of 18:2/18:3 in the TG class declined more than 3.5-fold in the ethanol treatment. As BAS 13-338 concentrations in the ethanol treatments increased, the 18:2/18:3 ratios increased in the TG fraction. The pattern observed for the TG fraction was also observed for the FFA fraction except that the ratio of 18:2/18:3 did not drop in

the ethanol treatment and surpassed the control ratio as BAS 13-338 increased.

The TFA of the PEG plus BAS 13-338 treatments behaved similarly to the ethanol plus BAS 13-338 treatments with respect to the ratios of 18:2/18:3 and the DBI. However, in the former treatments the relative amounts of 18:2 and the degree of unsaturation were usually higher. The ratio of 18:2/18:3 in the FFA fraction increased and then declined as the concentration of BAS 13-338 increased. Also, the relative amount of 18:2 in the FFA fraction was higher in the PEG plus BAS 13-338 treatments than the ethanol plus BAS 13-338. Generally, the changes in DBI observed in the experiments were inversely related to the changes in 18:2/18:3, since the DBI is a measure of the degree of unsaturation of fatty acids.

Table 5-4 summarizes the effects of BAS 13-338 on the phospholipid composition of *C. vulgaris* cells exposed to ethanol and PEG-induced stress. Ethanol at 0.33% caused a reduction in concentration of PC and PG by 40 and 45%, respectively. Concentrations of PI plus PE increased by 25% while no change occurred in the concentrations of PA and DGDG (Table 5-4). With the addition of BAS 13-338, a reversal of PC inhibition was observed with a subsequent increase of 35% at the highest concentration of BAS 13-338 compared to the control concentration. PA continued to decline with increasing concentrations of BAS 13-338 while PI plus PE, PG, and DGDG concentrations changed little compared to the 0.33% ethanol treatment.

PEG and combinations of PEG and BAS 13-338 resulted in no

detectable levels of PA in any of the treatments, nor did PC concentrations change in any of the treatments. However, the concentrations of PI plus PE, PG, and DGDG decreased with increasing concentrations of BAS 13-338. At the highest level of BAS 13-338 ($8.0 \times 10^{-4}M$), PI plus PE, PG, and DGDG decreased by 38, 46, and 23% respectively.

Table 5-5 summarizes the relative retention times and the major m/e ions greater than 250 mass units of the acetate derivatives of sterols isolated from ethanol and BAS 13-338 treated C. vulgaris cells. Comparison of these data with previously reported information (Patterson 1971, Doyle et al. 1972, and Chan et al. 1974) allowed the tentative identification of ten major sterol constituents: ergost-7,22-dien-3 β -ol (1), ergost-8(14)-en-3 β -ol (2), 4-methylcholest-8(14)-en-3 β -ol (3), 24-methylenepollinastanol (4), ergost-7-en-3 β -ol (5), 4-methylergosta-8(9),14-dien-3 β -ol (6), 24-methylpollinastanol (7), stigmasta-7,22-dien-3 β -ol (8), stigmast-8(9)-en-3 β -ol (9), and stigmast-7-en-3 β -ol (10). These sterols are drawn in figure 5-3.

Figure 5-2 depicts graphically the changes in the various sterol components of C. vulgaris cells grown in solution with ethanol and increasing concentrations of ethanol plus BAS 13-338. Treatment of algae with 0.33% ethanol resulted in substantial decreases in ergost-7-en-3 β -ol (5), stigmasta-7,22-dien-3 β -ol (8), and 24-methylpollinastanol (7) as well as smaller reductions in ergost-7,22-dien-3 β -ol (1), and ergost-8(14)-en-3 β -ol (2). Addition of BAS 13-338 at all concentrations stimulated the production of two previously absent

sterols, 4-methylcholest-8(14)-en-3 β -ol (3) and stigmast-8(9)-en-3 β -ol (9). The relative concentrations of ergost-8(14)en-3 β -ol (2), 4-methylergosta-8(9),14-dien-3 β -ol (6), and stigmasta-7,22-dien-3 β -ol (8) also increased with BAS 13-338 treatments. Additionally, Table 5-4 also provides data showing BAS 13-338 reestablishes the sterol-to-phospholipid ratio of ethanol-treated algae to that found in untreated algae. Treatment of algae, under water stress (-1.5 MPa), with BASF 13-338 did not result in alterations in the patterns of accumulated sterols.

Discussion

Previously, BAS 13-338 has been reported to modulate resistance to environmental stresses. Treated cotton (Gossypium hirsutum L.) plants were more susceptible to chilling temperatures. Treated wheat, rye (Secale cereale L.), and barley (Hordeum vulgare L.) were shown to be less winter hardy (St. John and Christensen 1976, St. John et al. 1979). In the present study, ethanol-induced reduction in the growth of C. vulgaris was reversed by all concentrations of BAS 13-338 tested. However, BAS 13-338 was unable to stimulate C. vulgaris growth rates reduced by osmotic stress. Growth rates declined as BAS 13-338 concentrations were increased, thus providing little evidence of acquired water stress resistance. This is in contrast to previous studies in which BAS 13-338 was shown to provide water stress resistance in corn (Zea mays L.) and soybean (St. John 1984). This may indicate that stress resistance conferred by BAS 13-338 operates most

effectively at the whole plant level, such as reducing transpiration, while not modulating stress resistance at a cellular level. Additionally, the inhibitory effect of higher concentrations of BAS 13-338 under both stress regimes may point to the multifunctional activity of the compound. Although BAS 13-338 is considered a weak inhibitor of photosystem II-dependent electron transport, previous reports have indicated that 1.4×10^{-9} M BAS 13-338 will inhibit ferricyanide reduction 50% by the Hill reaction in isolated chloroplasts (St. John 1982). Such inhibition of electron transport might negate the benefits which lipid modifications might have on growth rates under water stress conditions.

Exposure of C. vulgaris to ethanol resulted in concentration changes in the observed lipid classes. The large decrease in FS concentration along with a small decrease in PPL resulted in a 50% decrease in the FS/PFA ratio (Table 5-2). The decline in FS concentration may be similar to those observed in animal systems in which alcohol was shown to depress the activity of HMG-CoA reductase (Klurfeld et al. 1979). The large decrease in the FS/PFA ratio runs counter to observations in which cholesterol to phospholipid ratios increased in animals chronically exposed to ethanol (Rovinski and Hosein 1983, Goldstein et al. 1980). Possibly the difference in the FS to PFA ratio response could be explained in terms of tolerance. Animal systems investigated are tolerant to alcohol and this tolerance is attributed to lipid adaptation. Observations of growth inhibition by ethanol in C. vulgaris clearly demonstrate that this alga is not

tolerant to ethanol, possibly the result of the decreasing FS/PFA ratio. Only when the ratio was increased above control levels by BAS 13-338 treatment did the rate of algal growth approach that of control conditions. A higher FS/PPL ratio is proposed to lead to membranes of normal fluidity in the presence of ethanol (Klurfeld et al. 1979). Thus as BAS 13-338 induced slightly greater than control concentrations of FS to PPL, tolerance to ethanol occurred.

The decline of PPL in cells exposed to ethanol was matched in magnitude by an increase in TFA. Such coupling between PPL and TFA has been observed both in C. vulgaris exposed to water stress (Goedhart and Orcutt 1988a) and also in Saccharomyces cerevisiae approaching stationary phases of growth (Taylor and Parks 1978). Thus it appears that under ethanol stress extra lipid is stored in a non-membrane form.

Under conditions of osmotic stress, the treatments of BAS 13-338 tested resulted in decreases in the the concentrations of the various lipid classes. PPL and FS concentrations declined in response to increasing BAS 13-338 concentration but the FS/PFA ratio declined erratically. Previously a reduction in the FS/PC ratio was observed in response to a 4-day period of water stress (Farkas et al. 1982), although an increase in the ratio of sterols / phospholipids plus glycolipids was reported in oats subjected to consecutive treatments of water stress (Liljenberg and Kates 1982). The observed decrease in the FS/PFA ratio as a result of BAS 13-338 treatment could result in a more permeable membranes while osmotically stressed cells require decreased permeability.

Additionally, previous research has shown that resistance to ethanol or to water stress involved decreasing membrane fluidity by increasing lipid saturation (Lechavallier 1977, Liljenberg and Kates 1982, and Littleton et al. 1980). BAS 13-338 was reported to alter the ratio of linoleic to linolenic acid in favor of linoleic acid (St. John 1982, 1984). The reversal by BAS 13-338 of ethanol induced decreases in the 18:2/18:3 ratios and increases in the DBI of all lipid classes indicated that BAS 13-338 was highly active in C. vulgaris. Treatment of osmotically stressed algae also resulted in an increased 18:2/18:3 ratio in the TFA fraction, while simultaneously decreasing the ratio in the FFA fraction. The general trend of increased saturation agreed with previous reports of BAS 13-338 activity. The observed activity of BAS 13-338 contrasted to a study in which the ratio of 18:2/18:3 of the total fatty acids of C. vulgaris was reported to be unaffected (Murphy et al. 1985). However, the increase in 18:2/18:3 in this study is consistent with reports of inhibition of desaturase activity in other species (Murphy et al. 1985) and may be one of several cellular factors resulting in protection from stress, especially ethanol toxicity.

Of interest is the fact that the degree of saturation of TGFA fraction was influenced to a greater degree than the PFA fraction in both the osmotic stress and ethanol treatments. Increased saturation of the PFA fraction would appear to play a more important role in membrane permeability, since TG are considered to have mainly a storage function. Since PA is considered to be a precursor of the TG fraction, this would indicate that TG may be more readily desaturated.

It should be noted that the saturation of a single class of phospholipids can be very significant, since previous studies have demonstrated that the level of saturation of an apparently minor phospholipid substituent, such as dipalmitoylphosphatidyl glycerol, can dramatically influence membrane fluidity (Murata and Yamaya 1984). The degree of saturation of the individual phospholipid classes were not monitored in this study.

In the treatment of algal cells with ethanol only, decreases in the concentration of PC and PG were noted as well as an increase in the PI plus PE fraction. Addition of increasing concentrations of BAS 13-338 to ethanol treatments caused a continuing decline in the concentration of PA but an increase in PC and PG compared to ethanol only. PA is considered to be the central metabolite in the pathway of polar lipid synthesis and may be utilized to produce diacylglycerol or CDP-diacylglycerol from which PC and PE or PI, PG, or PS are formed, respectively (Moore 1982). One possible explanation for a decrease in PA could be a depletion induced by the increase in PC concentrations stimulated by BAS 13-338 plus ethanol treated algae. Chetal et al. (1983) observed that alterations in PL composition differed among cultivars varying in sensitivity to water stress. The greatest PL decreases occurred in sensitive species. Chetal et al. (1983) noted increases in PC, decreases in PG and no changes in PI which probably resulted from the destruction of chloroplast membranes.

A comparison of the the general concentrations of the phospholipids of C. vulgaris grown under at -0.1 and -1.5 MPa, even though side by

side growth was not attempted in this investigation, shows that the concentrations of several substituents changed with stress. PA was no longer found in water stressed cells and the concentration of the PI plus PE fraction increased as the concentration of PG declined. No significant differences in the concentration of PC were noted.

From previous reports (Doyle et al. 1972, Chan et al. 1974), one of the possible pathways of sterol synthesis in C. vulgaris resulting in chondrillastanol formation is the following: 24-methylpollinastanol → 24-methylenepollinastanol → stigmast-8(9)-en-3B-ol → stigmast-7-en-3B-ol → stigmasta-7,22-dien-3B-ol (chondrillastanol). Treatment of C. vulgaris with either 0.33% ethanol or BAS 13-338 appears to effect sterol synthesis since the concentrations of stigmast-7-en-3B-ol and stigmasta-7,22-dien-3B-ol decline dramatically when ethanol is added, while stigmast-8(9)-en-3B-ol, stigmast-7-en-3B-ol, and stigmasta-7,22-dien-3B-ol increase with the addition of BAS 13-338. Concomitantly the concentrations of 24-methylpollinastanol (7) and 24-methylene-pollinastanol (4) decrease. BAS 13-338 treatment also results in large increases in 4-methylcholest-8(14)-en-3B-ol and 4-methylergosta-8(9),14-dien-3B-ol and in ergost-8(14)-en-3B-ol. These results indicate an accumulation of precursors in the sterol synthesis pathway. Possibly these compounds are also incorporated into membranes and provide increased resistance to stress. However at the same time, the BAS 13-338 treatment also doubles the sterol to polar lipid ratio of ethanol treated algae. This consequence of treatment will certainly play a major role in determining membrane fluidity. Such alterations

in sterol composition may not necessarily result from a direct effect of BAS 13-338 on sterol biosynthesis but could be indirectly influenced through effects on 18:3 synthesis and thus the activity of biosynthetic enzymes. The observations of lipid alteration in response to BAS 13-338 treatment in PEG-induced algal stress did not parallel responses of algae stressed by ethanol. Perhaps this reflects the higher concentrations used in the ethanol-stress study. Also ethanol could have solubilized the BAS 13-338 more effectively than PEG thus leading to differences in activity.

Previously Grunwald (1968) demonstrated that different sterol constituents varied in their ability to reduce methanol induced cellular permeability. The rates of betacyanin efflux from beet (Beta vulgaris L.) root cylinders placed in 10% solutions of methanol were measured. The order of sterols in their ability to reduce leakage was cholesterol > campesterol > 8-sitosterol, stigmasterol. Additionally, ergosterol was found to increase the permeability of beet root cylinders. These results were interpreted to indicate that only sterols with a planar configuration similar to that of cholesterol were physiologically active in membrane stabilization. The charge distribution of the sterols was also important as evidenced by ergosterol's ability to increase rather than decrease permeability. These results would support the importance of individual sterol substituents in stress resistance.

That BAS 13-338 appears to influence the metabolism of several classes of lipids, is in agreement with the observations of Murphy et

al. (1985). Because of the diversity of lipid classes affected stress adaptation could not be attributed to a single lipid class. The effects of BAS 13-338 on sterol accumulation patterns have not been previously reported. The utilization of plant species with less complex sterol synthesis pathways might prove useful in further studies of the effects of BAS 13-338. Nonetheless BAS 13-338 still provided useful information in studying stress responses in C. vulgaris.

Literature Cited

- Andrews, C. J. 1977. Accumulation of ethanol in ice-encased winter cereals. - *Crop Sci.* 17: 157-161.
- Andrews, C. J. & Pomeroy, M. K. 1977. Mitochondrial activity and ethanol accumulation in ice-encased winter cereal seedlings. - *Plant Physiol.* 59: 1174-1178.
- Bradford, B., Moore, L. D., & Orcutt, D. M. 1982. The free sterol, steryl ester, and steryl glycoside content of tomato cultivars resistant and susceptible to Phytophthora infestans. - *Can. J. Bot.* 60: 1469-1473.
- Chan, J. T., Patterson, G. W., Dutky, S. R. & Cohen, C. F. 1974. Inhibition of sterol biosynthesis in Chlorella sorokiniana by triparanol. - *Plant Physiol.* 53: 244-249.
- Chetal, S., Wagle, D. S. & Nainwatee, H. S. 1981. Glycolipid changes in wheat and barley chloroplasts under water stress. - *Phytochemistry* 19: 1393-1395.
- Chetal, S., Wagle, D. S. & Nainwatee, H. S. 1983. Phospholipid changes in wheat and barley chloroplasts under water stress. - *Plant Sci. Lett.* 29: 273-278.
- Chin, J. H. & Goldstein, D. B. 1976. Drug tolerance in biomembranes: a spin label study of the effects of ethanol. - *Science* 196: 684-685.

- Doyle, P. J., Patterson, G. W., Dutky, S. R. & Thompson, M. J. 1972. Triparanol inhibition of sterol biosynthesis in Chlorella emersonii. - *Phytochemistry* 11: 1951-1960.
- Farkas, T., Nemezc, G. & Singh, B. 1982. Abscisic acid induced changes in physical state and lipid composition of plant membranes. - In *Biochemistry and Metabolism of Plant Lipids* (J. F. G. M. Wintermans and P. J. C. Kuipers eds.), pp. 471-474. Elsevier, New York, ISBN 0-444-80457-9.
- Ferrari-Illiuo, R., Pham Thi, A. T. & Da Silva, J. V. 1984. Effects of water stress on the lipid and fatty acid composition of cotton (Gossypium hirsutum) chloroplasts. - *Physiol. Plant.* 62: 219-224.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. - *J. Biol. Chem.* 226: 497-509.
- Ginger, C. & Fairbairn, D. 1966. Lipid Metabolism in helminth parasites. 1. the lipids of Hymenolepis diminuta (cestoda). - *J. Parasitol.* 52: 1086-1096.
- Goedhart, C. L. & Orcutt, D. M. 1988a. Osmotic-induced changes in the growth and lipid composition of Chlorella vulgaris and Chlorella pyrenoidosa grown in batch culture. - *Physiol. Plant.* (submitted).
- Goldstein, D. B., Chin, J. H., McComb, J. A., & Parsons, L. M. 1980. Chronic effects of alcohols on mouse biomembranes. - In *Advances in Experimental Medicine and Biology* Vol. 126. (H. Begleiter, ed.). Plenum Press, New York, ISBN 0-306-040349-8.

- Gordon, E. R. 1984. Alcohol-induced tolerance in mitochondrial membranes. - *Science* 223: 193-194.
- Grunwald, C. 1968. Effects of sterols on the permeability of alcohol-treated red beet tissue. *Plant Physiol.* 43: 484-488.
- Hanson, A. D., & Hitz, W. D. 1982. Metabolic responses of mesophytes to plant water deficits. - *Annu. Rev. Plant Physiol.* 33: 163-203.
- Harborne, J. B. 1984. *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis.* pp. 153-160. Chapman and Hall, New York, ISBN 0-412-25550-2.
- Harwood, J. L. 1983. Adaptive changes in lipids of higher-plant membranes. - *Biochem. Soc. Trans.* 11: 343-346.
- Kimmerer, T. W. & Kozlowski, T. T. 1982. Ethylene, ethane, acetaldehyde, and ethanol production by plants under stress. - *Plant Physiol.* 69: 840-847.
- Klurfeld, D. M., Tepper, S. A., Mueller, M. A. & Kritchevsky, D. 1979. Influences of alcohol from different sources on cholesterol metabolism in rats. - In *Developments in Nutrition and Metabolism Vol. 1 Metabolic Effects of Alcohol* (P. Avogadro, C. R. Sirtori, E. Tremoli eds)
- Kuiper, P. J. C. 1985. Environmental changes and lipid metabolism of higher plants. - *Physiol. Plant.* 64: 118-122.
- Littleton, J. M., Grieve, S. J., Griffiths, P. J. & John, G. R. 1980. Ethanol-induced alteration in membrane phospholipid composition: possible relationship to development of cellular tolerance to ethanol. - *Adv. Exp. Med. Biol.* 126: 7-19.

- Lechevallier, D. 1977. Effets du polyethylene glycol sur les lipides et les lipochromes des colonies de Spirodele. - *Physiol. Veg.* 15: 387-402.
- Liljenberg, C. & Kates, M. 1982. Effect of water stress on lipid composition of oat seedling root cell membranes. - In *Biochemistry and Metabolism of Plant Lipids* (J. F. G. M. Wintermans and P. J. C. Kuiper eds), pp. 441-444. Elsevier, New York, ISBN 0-444-80457-9.
- Moore Jr., T. S. 1982. Phospholipid synthesis. - *Annu. Rev. Plant Physiol.* 33: 235-259.
- Murphy, D. J., Harwood, J. L., Lee, K. A., Roberto, F., Stumpf, P. K. & St. John, J. B. 1985. Differential responses of a range of photosynthetic tissues to a substituted pyridazinone, Sandoz 9785. Specific effects on fatty acid desaturation. - *Phytochemistry* 24: 1923-1929.
- Muruta, N. & Yamaya, J. 1984. Temperature-dependent phase behavior of phosphatidylglycerols from chilling-sensitive and chilling-resistant plants. - *Plant Physiol.* 74: 1016-1024.
- Nichols, B. W. 1963. Separation of the lipids of photosynthetic tissues: improvements in analysis by thin-layer chromatography. - *Biochim. Biophys. Acta* 70:417-422.
- Orcutt, D. M., Davis, G. A. & Moore, L. D. 1984. Influence of choline derivatives on the sterol composition of Chlorella vulgaris grown in continuous culture. - *Can. J. Bot.* 62: 78-80.

- Orcutt, D. M., Fox, J. A. & Jake, C. A. 1978. The sterol, fatty acid, hydrocarbon composition of Globodera solanacearum. - J. Nematology 10: 264-269.
- Patterson, G. W. 1971. Relation between structure and retention time of sterols in gas chromatography. - Anal. Chem. 43: 1165-1170.
- Rottenberg, H., Waring, A., & Rubin, E. 1981. Tolerance and cross-tolerance in chronic alcoholics - reduced membrane binding of ethanol and other drugs. - Science 213: 583-584.
- Rovinski, B. & Hosein, E. A. 1983. Adaptive changes in lipid composition of rat liver plasma membrane during postnatal development following maternal ethanol ingestion. - Biochim. Biophys. Acta 735: 407-417.
- Sorokin, C. 1973. Dry weight, packed cell volume, and optical density. - In Handbook of Phycological Methods Culture Methods and Growth Measurements (J. R. Stein ed.), pp 331-343. Cambridge University Press, New York. ISBN 0-521-200049-0.
- St. John, J. 1982. Effects of herbicides on the lipid composition of plant membranes. - In Biochemical Responses Induced by Herbicides (D. E. Moreland, J. B. St. John, and F. D. Hess eds), ACS Symposium Series 181, pp. 97-109. American Chemical Society, Washington, D. C., ISBN 0-8412-0699-6.
- St. John, J. 1984. Inhibition of carotenoid and lipid biosynthesis by herbicides. - In Biochemical and Physiological Mechanisms of Herbicide Action (S. D. Duke ed), pp. 58-71. USDA/ARS, Stoneville, Mississippi.

- St. John, J. B. & Christiansen, M. N. 1976. Inhibition of linolenic acid synthesis and modification of chilling resistance in cotton seedlings. - *Plant Physiol.* 57: 257-259.
- St. John, J. B., Christiansen, M. N., Ashworth, E. N. & Gentner, W. A. 1979. Effect of BASF 13-338, a substituted pyridazinone, on linolenic acid levels and winterhardiness of cereals. - *Crop Sci.* 19: 65-69.
- Taylor, F. R. & Parks, L. W. 1978. Metabolic interconversion of free sterols and steryl esters in *Saccharomyces cerevisiae*. - *J. Bacteriol.* 136: 531-537.
- Taylorson, R. B. 1984. Prevention of action of far-red-absorbing phytochrome in *Rumex crispus* L. seeds by ethanol. - *Plant Physiol.* 74: 223-226.
- Touchstone, J. C., Chen, J. C. & Beaver, K. M. 1980. Improved separation of phospholipids in thin layer chromatography. - *Lipids* 15: 61-62.
- Vela, G. R. & Guerra, C. N. 1966. On the nature of mixed cultures of *Chlorella pyrenoidosa* TX71105 and various bacteria. - *J. Gen. Microbiol.* 42: 123-131.
- Waring, A. J., Rottenberg H., Ohnishi T. & Rubin, E. 1981. Membranes and phospholipids of liver mitochondria from chronic alcoholic rats are resistant to membrane disordering by alcohol. - *Proc. Natl. Acad. Sci. USA* 78: 2582-2586.

Figure 5-1. Ethanol and osmotic stress-induced changes in rate of increase of \log_2 of optical density of batch cultures of Chlorella vulgaris as influenced by the pyridazinone herbicide BAS 13-338 ; a. water stress, b. ethanol

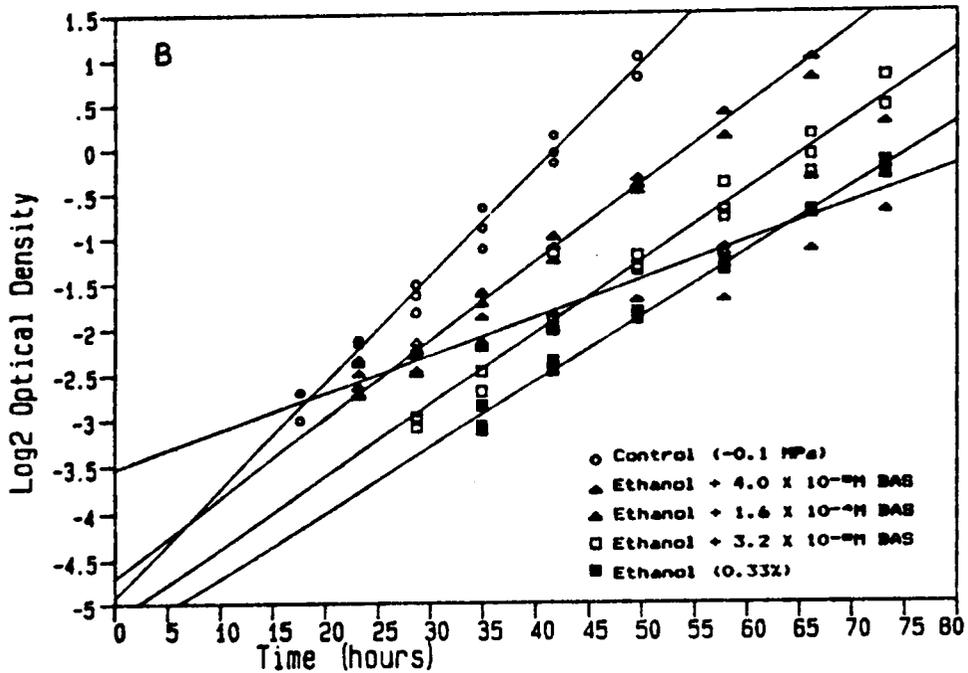
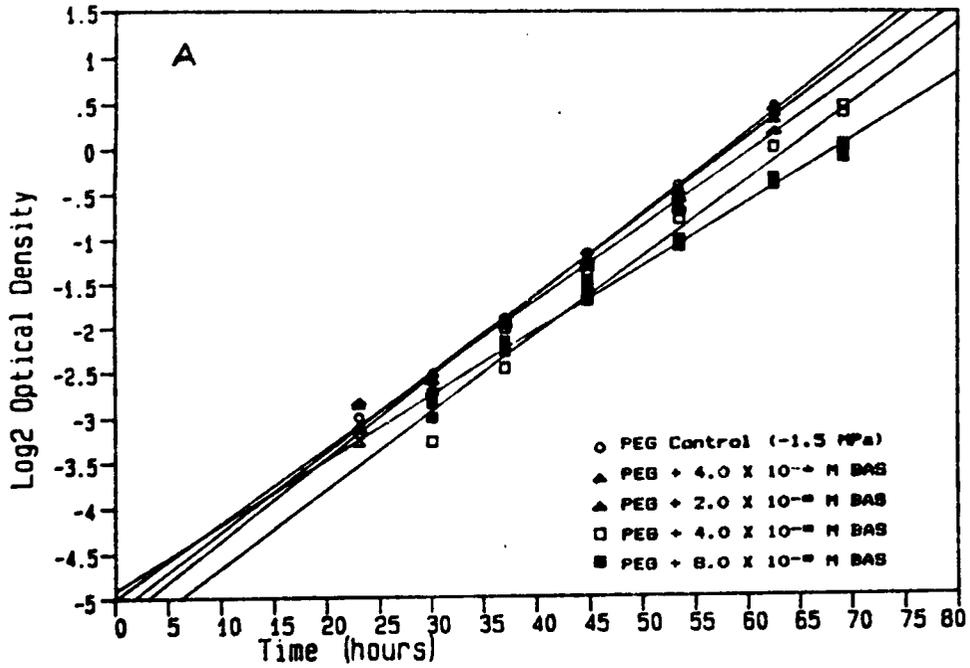


Table 5-1. Ethanol and osmotic-induced changes in the growth rate of *Chlorella vulgaris* as influenced by the pyridazinone herbicide BAS 13-339.

BAS 13-339	
Treatments	Relative growth rate (%)
Control (-0.1 MPa)	100
ETOH (0.33%)	35 ± 11
ETOH + 4.0 X 10 ⁻⁴ M	75 ± 7
ETOH + 1.6 X 10 ⁻⁴ M	73 ± 7
ETOH + 3.2 X 10 ⁻⁴ M	60 ± 3
Control (-1.5 MPa)	100
PEG + 4.0 X 10 ⁻⁴ M	97 ± 1
PEG + 2.0 X 10 ⁻⁴ M	92 ± 1
PEG + 4.0 X 10 ⁻⁴ M	85 ± 2
PEG + 8.0 X 10 ⁻⁴ M	78 ± 2

Data entries are the means of four experiments of three replications per treatment ± standard deviation. Abbreviations - ETOH, ethanol; PEG, polyethyleneglycol. Treatments involving ethanol were at 0.33% and an osmotic potential of -0.1 MPa. All osmotic stress treatments (PEG) were at an osmotic potential of -1.5 MPa.

Table 5-2. Ethanol and osmotic-induced changes in lipid composition of *Chlorella vulgaris* as influenced by the pyridazinone herbicide BAS 13-338.

BAS 13-338 Treatments	Lipid concentration ($\mu\text{g} / \text{mg}$ total lipid)				
	PFA	TFA	FFA	FS	FS/PFA
Control (-0.1 MPa)	834	4.3 \pm 0.9	8.7 \pm 7.3	8.2 \pm 0.5	.0098
ETOH (0.33%)	746	174 \pm 150	6.7 \pm 1.9	3.5 \pm 0.7	.0047
ETOH + 4.0 X 10 ⁻⁴ M	850	22 \pm 16	10.9 \pm 2.5	10.7 \pm 0.6	.0126
ETOH + 1.6 X 10 ⁻⁴ M	888	9.0 \pm 1.5	9.0 \pm 1.5	9.3 \pm 5.6	.0119
ETOH + 3.2 X 10 ⁻⁴ M	918	8.8 \pm 3.6	8.8 \pm 3.7	11.6 \pm 1.6	.0126
Control (-1.5 MPa)	474	17.4 \pm 14.9	13.7 \pm 8.0	4.5 \pm 0.6	.0117
PEG + 4.0 X 10 ⁻⁴ M	450	27.9 \pm 17.9	10.4 \pm 3.9	4.7 \pm 0.3	.0104
PEG + 2.0 X 10 ⁻⁴ M	438	9.5 \pm 8.1	8.7 \pm 3.2	3.8 \pm 1.1	.0087
PEG + 4.0 X 10 ⁻⁴ M	396	9.4 \pm 10.9	8.9 \pm 2.8	5.2 \pm 0.5	.0131
PEG + 8.0 X 10 ⁻⁴ M	368	8.9 \pm 2.8	8.9 \pm 2.8	2.7 \pm 0.2	.0073

Experiments were replicated in time and data entries are the average of four replicates \pm standard deviation. Abbreviations- ETOH, ethanol; FFA, free fatty acid; FS, free sterol; PEG, polyethylene glycol; PFA, polar lipid fatty acid. Treatments involving ETOH were at 0.33% and an osmotic potential of -0.1 MPa. All osmotic stress treatments (PEG) were at an osmotic potential of -1.5 MPa.

Figure 5-2. Total ion chromatogram of total sterol acetates isolated from Chlorella vulgaris grown at -0.1 MPa in (A) nutrient solution, (B) 0.33% ethanol, (C) 0.33% ethanol + $4.0 \times 10^{-4}\text{M}$ BAS 13-338, (D) 0.33% ethanol + $1.6 \times 10^{-4}\text{M}$ BAS 13-338, and (E) 0.33% ETOH + $3.2 \times 10^{-4}\text{M}$ BAS 13-338. (See table 4-5 for peak identification key). (a) = missing, (h) = hidden.

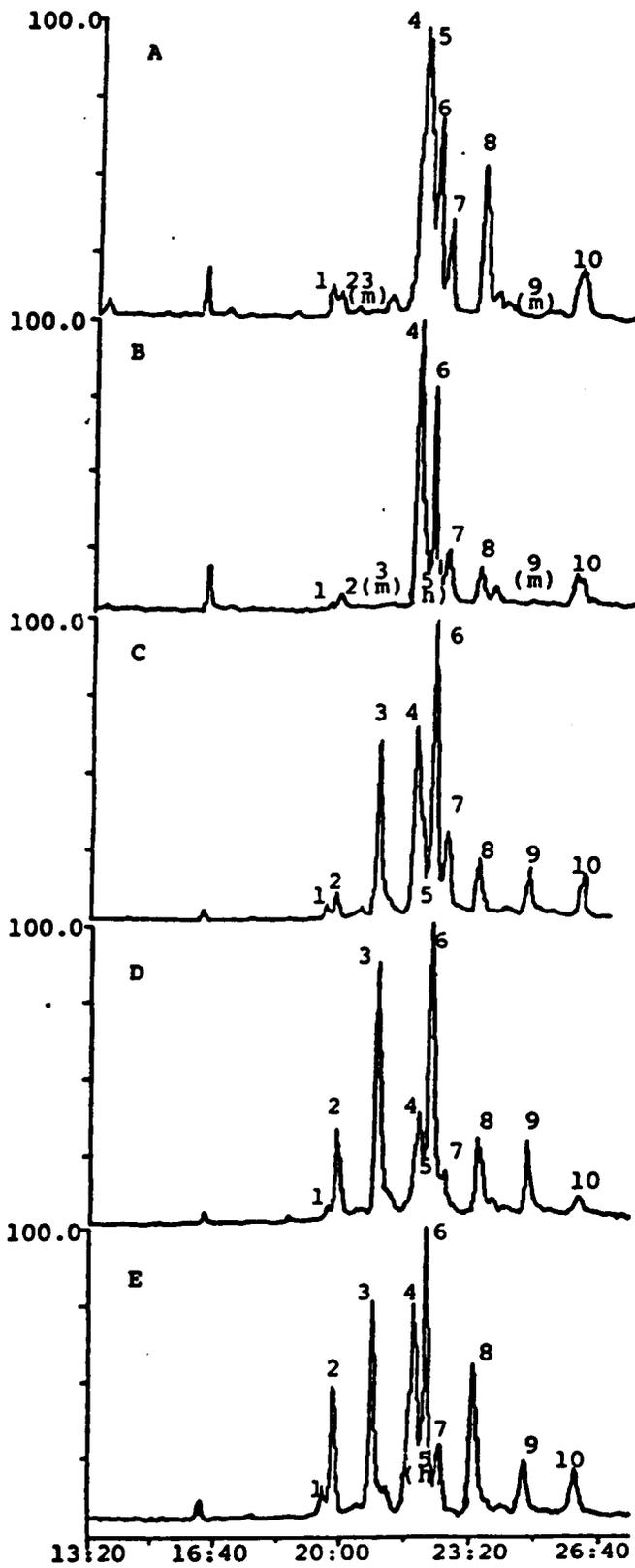


Table 5-3. Ethanol and osmotic-induced effects on the ratio of 18:2 to 18:3 and the DBI of three lipid classes of *Chlorella vulgaris*.

BAS 13-338 Treatments	18:2/18:3			DBI		
	PFA	TGF	FFA	PFA	TGF	FFA
Control (-0.1 MPa)	0.79	3.84	0.89	1.74	0.63	0.92
ETOH (0.33%)	0.60	1.03	0.89	1.70	1.70	0.97
ETOH + $4.0 \times 10^{-4}M$	0.75	1.21	1.09	1.69	1.14	0.92
ETOH + $1.6 \times 10^{-4}M$	0.72	1.34	1.25	1.51	0.87	0.36
ETOH + $3.2 \times 10^{-4}M$	0.70	1.66	1.10	1.66	0.77	0.38
Control (-1.5 MPa)	0.81	1.89	2.21	1.96	1.22	0.54
PEG + $4.0 \times 10^{-4}M$	0.82	1.37	2.60	1.95	1.41	0.49
PEG + $2.0 \times 10^{-4}M$	0.97	2.06	3.06	2.10	1.36	0.61
PEG + $4.0 \times 10^{-4}M$	0.92	2.56	1.83	1.98	1.25	0.68
PEG + $8.0 \times 10^{-4}M$	0.90	2.40	1.14	1.98	1.19	0.60

Abbreviations- DBI, double bond index; FFA, free fatty acid; PFA, polar lipid fatty acid; TGF, triglyceride fatty acid.

Table 5-4. Ethanol and osmotic-induced changes in phospholipid and digalactosyl diglyceride composition of *Chlorella vulgaris* as influenced by the pyridazinone herbicide BAS 13-338.

BAS 13-338 Treatment	Polar lipid concentration (mg / 5mg total lipid)					
	PA	PC	PI + PE	PG	DGOG	PPL
Control (-0.1 MPa)	0.65 ± .11	1.10 ± .09	1.68 ± .14	0.74 ± .09	0.76 ± .05	4.17 ± .83
ETOH (0.33%)	0.53 ± .10	0.67 ± .03	2.11 ± .05	0.42 ± .05	0.77 ± .03	3.73 ± .75
ETOH + 4.0 X 10 ⁻⁴ M	0.49 ± .12	0.90 ± .20	2.35 ± .15	0.51 ± .07	0.76 ± .01	4.25 ± .85
ETOH + 1.6 X 10 ⁻⁴ M	0.45 ± .12	1.17 ± .16	2.34 ± .17	0.48 ± .10	0.70 ± .09	4.44 ± .89
ETOH + 3.2 X 10 ⁻⁴ M	0.39 ± .10	1.48 ± .22	2.26 ± .44	0.46 ± .24	0.70 ± .12	4.59 ± .92
Control (-1.5 MPa)	—	1.02 ± .06	0.94 ± .13	0.41 ± .09	0.69 ± .02	2.37 ± .47
PEG + 4.0 X 10 ⁻⁴ M	—	1.07 ± .09	0.84 ± .12	0.34 ± .06	0.68 ± .04	2.93 ± .59
PEG + 2.0 X 10 ⁻⁴ M	—	1.04 ± .12	0.84 ± .11	0.31 ± .12	0.64 ± .06	2.83 ± .57
PEG + 4.0 X 10 ⁻⁴ M	—	1.01 ± .25	0.74 ± .05	0.23 ± .11	0.57 ± .04	2.55 ± .51
PEG + 8.0 X 10 ⁻⁴ M	—	1.04 ± .15	0.58 ± .05	0.22 ± .12	0.53 ± .06	2.37 ± .47

Experiments were replicated in time and data entries are the average of four replicates ± standard deviation. Abbreviations- ETOH, ethanol; DGOG, digalactosyldiglyceride; PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PEG, polyethylene glycol; PG, phosphatidyl glycerol; PI, phosphatidyl inositol. Treatments involving ETOH were at 0.33% and an osmotic potential of -0.1 MPa. All osmotic stress treatments (PEG) were at an osmotic potential of -1.5 MPa.

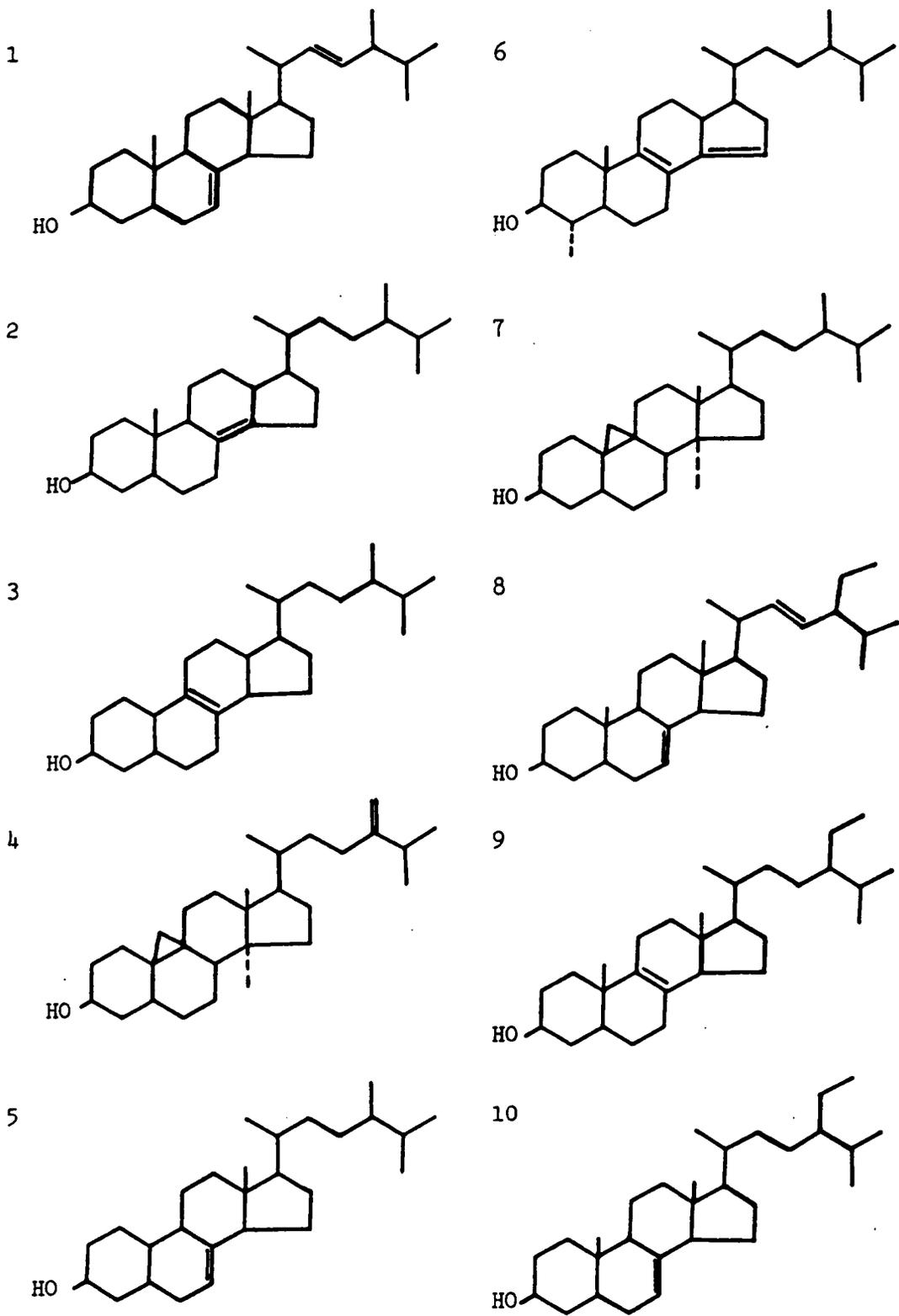
Table 5-5. Mass spectral and gas-liquid chromatographic data of acetate derivatives of sterols isolated from Chlorella vulgaris grown in ethanol plus BAS 13-338.

Peak Number	Sterol	Ions above m/e 250								Mol.	
		a	b	c	d	e	f	g	h	wt.	RRT
1	Ergost-7,22-dien-3 β -ol	255	288	313	342	355	398		425	440	1.20
2	Ergost-8(14)-en-3 β -ol	255		315		367			427	442	1.21
3	4-methylcholest-8(14)-en-3 β -ol	255		315		367			427	442	1.28
4	24-methylenepollinastanol	269		329		379	394		439	454	1.34
5	ergost-7-en-3 β -ol	255	288	315				408	427	442	1.35
6	4-methylergosta-8(9),14-dien-3 β -ol	269				379	394	411	439	454	1.37
7	24-methylpollinastanol	269				381	396	413	441	456	1.39
8	Stigmasta-7,22-dien-3 β -ol	255		313	342				439	454	1.44
9	Stigmast-8(9)-en-3 β -ol	255		315		381			441	456	1.52
10	Stigmast-7-en-3 β -ol	255	281	315					441	456	1.60

Abbreviations- RRT, Relative retention time (relative to cholesterol).

Figure 5-3. Structures of sterols isolated from Chlorella vulgaris:

- (1) ergost-7,22-dien-3 β -ol, (2) ergost-8(14)-en-3 β -ol, (3) 4-methylcholest-8(14)-en-3 β -ol, (4) 24-methylenepollinastanol, (5) ergost-7-en-3 β -ol, (6) 4-methylergosta-8(9),14-dien-3 β -ol, (7) methylpollinastanol, (8) stigmasta-7,22-dien-3 β -ol, (9) stigeast-8(9)-en-3 β -ol, (10) stigmast-7-en-3 β -ol.



Chapter VI

SUMMARY

The initial objective of this project was to look at the effects of water stress on the lipid composition of two algal species which could be cultured under defined environmental conditions. Previous studies had indicated that alterations in lipid composition were likely to occur in response to a long-term water stress. The Chlorella species sampled for lipids were considered to be adapted to stress because they maintained growth during stress conditions, although rates were slower than control growth. The two algal species selected differed in their growth response to water stress. Chlorella pyrenoidosa was able to maintain control growth rates until the water stress level declined to -2.0 MPa. Chlorella vulgaris growth rates declined with each incremental increase in water stress. Thus these two species represented an ideal opportunity to examine differences in lipid adaptation of species differing in degree of stress tolerance.

Changes in lipid composition were correlated with the degree of water stress in both species. Chlorella vulgaris was unable to maintain control ratios of sterol to phospholipid, while Chlorella pyrenoidosa maintained a 10-fold greater sterol to phospholipid ratio. Chlorella vulgaris appeared to have a mechanism which allowed for the interconversion of steryl esters and free sterols as well as an inverse relationship between polar lipid concentrations and triglycerides.

Although growth rates were not maintained in this species, an adaptation which allowed interconversion of active lipids with storage lipids might represent a successful drought avoidance strategy. Growth could be suspended until favorable conditions returned and storage lipids could then be converted to membrane components.

The continuous culture of Chlorella vulgaris provided different results from those observed in batch culture. Continuous culture allowed active growth to occur at constant nutrient concentrations. The concentration of CO₂ was only one tenth that used in the batch culture system and this could have altered some of the lipid relationships. This study did confirm previous results which indicated that lipids of plants growing under water stress conditions were more saturated. However the triglyceride and free fatty acid pools of lipids seem to be most affected.

The final objective of this project was to validate that the lipid alterations observed actually resulted in stress adaptation rather than merely reflecting a stress-induced lesion at a vulnerable site in the metabolism of Chlorella vulgaris. Research was initiated to treat the less stress-tolerant alga, Chlorella vulgaris, with a compound which had previously been proven to have the potential to alter the lipid composition. The compound BAS 13-338 was applied to algae at two osmotic potentials. At high osmotic potentials, the chemical was readily solubilized by the PEG in solution. Application of BAS 13-338, at the concentrations investigated, appeared not to increase the cellular resistance to water stress as measured by cellular growth

rates. Rather, with increases in the concentration of BASF 13-338, the rate of growth decreased.

Treatment of cells at -0.1 MPa with BASF required the dissolution of the compound in ethanol resulting in a final culture concentration of 0.33% ethanol. This concentration of ethanol alone was sufficient to decrease the algal growth rate by 65%. However application of BASF 13-338 at $4.0 \times 10^{-8}M$ effectively reversed the growth inhibitory effect of ethanol. An examination of the lipid profiles of treated algae revealed that alterations in the lipid composition resulted from algal growth in ethanol. These effects were counteracted by the application of BAS 13-338.

Thus, although no noticeable benefit was derived by the application of this compound in the presence of water stressed cells, and despite previous reports indicating that BAS 13-338 was ineffective in altering the lipid composition of Chlorella vulgaris, the results of this study indicated that the relative proportions and total amounts of lipids were altered by BASF 13-338. BAS 13-338 was shown to be partially effective in overcoming the effects of ethanol toxicity.

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