THE PERFORMANCE AND CAPABILITIES OF

TERRESTRIAL ORGANISMS IN EXTREME AND

UNUSUAL GASEOUS AND LIQUID ENVIRONMENTS

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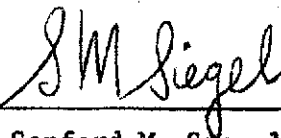
UNUSUAL GASEOUS AND LIQUID ENVIRONMENTS

Halo- and Cryotolerance of the
Alga *Dunaliella*

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THE CRYO- AND HALOBIOLOGY OF *DUNALIELLA*

Part I

A Dissertation submitted to the Graduate Division of the University of Hawaii in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Botanical Sciences

By

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INTRODUCTION

The accounts of physiological research on the active metabolism and behavior of autotrophic eukaryotes at sub-zero temperatures are relatively meagre. Possibly, this is because so few organisms remain unfrozen at temperatures of 0°C to -15°C. This report will deal with the alga *Dunaliella*, and its remarkable cryo- and halo-tolerance.

The genus *Dunaliella*, composed of green unicellular algae, is in the family Polybelpharidaceae (phylum Chlorophyta) (24). The organisms are motile, with two equal, unipolar flagella. They possess a thin, elastic cell envelope which follows changes in body outline. Reproduction occurs by longitudinal fission or by fusion of two motile, haploid cells to form a zygote (136). This genus resembles *Chlamydomonas*, but differs in the nature of the cell envelope. *Chlamydomonas* has not only a periplast but also a cell wall. The movement of most species of *Dunaliella* is a constant, linear motion. The algae rotate only slowly, and when quiescent, continue to vibrate. *Dunaliella* is of commerical importance as a source of vitamin A (105, 107, 108, 109).

Dunaliella have been recorded in oceans, salt marshes, inland salt springs, lagoons and saline pools all over the world (24, 105, 170). Some specimens have been found at depths of 315 meters in the Dead Sea (44).

The similarities and differences in salt and cold tolerance mechanisms of *Dunaliella salina* and *Dunaliella tertiolecta* will be under focus in this investigation.

THE BIOLOGY OF *DUNALIELLA TERTIOLECTA*

AND *DUNALIELLA SALINA*

D. salina and *D. tertiolecta* differ from each other in morphology. Although both species are ovoid or ellipsoid, mature *D. salina* cells usually have at least twice the volume of *D. tertiolecta* cells. The periplast of *D. salina* is less firm than that of *D. tertiolecta*. Both species have companulate chromatophores, *D. tertiolecta*'s being more rugose. The pyrenoid of *D. salina* is sub-basal and *D. tertiolecta*'s is central. *D. salina* contains a large number of refractive granules arranged in a girdle just above the chromatophore. The granules of *D. tertiolecta* are distributed evenly throughout the cell. *D. salina* has a haploid number of chromosomes of about 10, while the count for *D. tertiolecta* has not been recorded (24, 134).

Light optima for photosynthesis of both species have been reported. *D. salina* is positively phototaxic (17). Reports of optimal light intensities for growth range from 600 lux (116) to 10,000 lux (50, 170). *D. salina*'s carotene composition (63) and accumulation with higher salinities is documented (37, 38, 39, 40, 41, 42).

After high illumination, *D. tertiolecta* needs a short period in the dark to develop maximum photosynthetic rate (64). Oxygen evolution is higher with NH_3 than NO_3 as a nitrogen source (133). Grant (55) gave evidence for the existence of two independent systems of nitrate reduction, one within and the other outside

the chloroplast. The enzyme system which fixes CO_2 is likely to be the rate limiting step in photosynthesis. Glycerol is the major soluble photosynthate (31). Photopigment extractions and concentrations have been reported (34, 101, 138, 159, 163).

Redox enzymes of *D. salina* such as cytochrome oxidase, peroxidase, catalase, reductase-ascorbate oxidase and polyphenol oxidase have been studied. Its polyphenol oxidase is specific for triphenols (122). For *D. tertiolecta*, nitrate reductase (56), glucose 6-phosphate dehydrogenase, pyrophosphatase, ATP-ase (75), hexokinase (89), ribulose di-phosphate carboxylase and aldolase (64, 133) have been studied.

The general mechanism of salt tolerance for *D. salina* and *D. tertiolecta* is reported. *D. salina* is a "coper", whose cytoplasmic salt concentration is at least as high as the ambient medium. *D. tertiolecta* is an "avoider" and actively keeps salt out of the cytoplasm.

D. salina is an inhabitant of ultra-haline, neutral-alkaline waters (104, 171). Blooms in such places as the Krymsk Oblast occur in summer, which coincides with a decrease in Ca^{2+} and an increase in Mg^{2+} and SO_4^{2-} (103). Increasing NaCl concentration is increased from 2 to 4 M; from 4 to 5 M it decreases (119). Increasing NaCl results in disturbance of nucleic acid and protein metabolism. RNA per cell decreases and protein accumulates

in large amounts (122). Marre and Servattaz (102) reported high
conductivity and osmotic pressure of the cell sap of *D. salina* grown
in 3.9 M NaCl in comparison to those grown in 1.5 M NaCl. Based on
[24]Na experiments, they also concluded that the cell membrane is
freely permeable to sodium.

Trezzi (161) looked at the ultrastructural changes in *D. salina*
following large increases and decreases in osmolarity of the culture
medium.

"When the tonicity of the nutrient medium is suddenly lowered
the whole cell and single cytoplasmic structure appear equally
swollen and the state of dispersion of the stroma of cellular
organelles is increased. A conspicuous amount of water pene-
trates in every part of the cell. The mitochondria show a
uniform type of swelling, while in the inner space of the
nuclear envelope wide sacs appear upon hypotonic shock. The
outer layer of the double membrance of the nucleus may be
more permeable to water than the inner one. In chloroplasts
the flattened sacs formed by paired lamellae having blind
ends in the peristromium swell up giving rise to conspicuous
vesicles. The permeability of water of the lamellae is
greater than that of the external plastid boundary. Sudden
and strong hypertonic stress causes great loss of water from
every part of the cell. Shrinking can be seen in all cyto-
plasmic organelles together with great electron density of

their stroma. The two layers of the nuclear membrane and
the lamellar pairs forming the succuli of the chloroplasts
stick closely together causing a nearly total disappearance
of the inner space. The formation of large vesicles after
sudden hypotonic stress is due to a localized osmotic gra-
dient. In both cases the conspicuous structural variations
are reversible and the alga returns to the normal state in
a short time. During the short time span necessary for the
reestablishment of a new osmotic equilibrium and of a normal
state of the plasmic structures, the cells suffer from a lose
of motility and from an altered metabolism. A specific re-
sistance of plasma proteins to denaturation may be involved."
Concommitant with high internal salt concentration is the depressed
freezing point of the cytoplasm. The organism remains motile at tem-
peratures as low as -15°C (102). Low temperatures within the range
-15°C to +11°C depress the growth of *D. salina* and in the course of
eight days cause death of the cells. Some cells tolerated a tempo-
rary fall of the temperature to -30°C (170).

D. tertiolecta is a euryhaline organism growing in salinities
ranging from 3.75 to 150% (75, 114). The first detailed study of
the culture of *D. tertiolecta* was reported by MacLachlan (114). He
established its comparatively high sodium requirement as 10 mM.
Calcium and magnesium are inhibitory at high concentrations, but
the inhibition can be prevented if the Ca^{2+}/Mg^{2+} ratio was maintained

at 4. Jokela (75) showed that the internal concentration of both Na^+ and K^+ increase as the salinity of the medium increases. Nevertheless the internal Na concentration stayed considerably lower than that in the culture medium at high salinities. For example, when the NaCl concentration of the medium is 15%, the intracellular concentration is 4.35%. The cell membrane is therefore not freely permeable to sodium. The cell membrane is characterized by a high phosphatidic acid content and high concentrations of C-16 saturated fatty acids. It is also characterized by its divalent ion dependent stability, its relatively high hydrophobic amino acid content and the presence of a Na-K activated ATP-ase.

Both the soluble enzymes (as exemplified by glucose 6-phosphate dehydrogenase) and particulate enzymes (pyrophosphatase and ATP-ase) of *D. tertiolecta* were found to be severely inhibited by high Na and K concentrations (above 6%) (75).

Increasing the concentration of osmotic substances causes a decline in photosynthetic oxygen evolution, then oxygen consumption, accompanied by CO_2 evolution. Both show distinct maxima at 2.8 M NaCl. Fermentation probably occurs at higher osmotic pressures as indicated by respiratory quotients (165). Glycerol production is considered to be a protective mechanism for osmoregulation in *D. tertiolecta*. With increasing concentrations of NaCl, photosynthetic incorporation of ^{14}C declines, but the percentage incorporated into glycerol increases to a distinct maximum at 2.8 M NaCl (31, 47, 165).

When transferred from a medium of 15% NaCl to 4% NaCl, 15% of the total cell glycerol is released instantaneously from the cells (75).

The upper temperature limit for growth of D. *tertiolecta* increases with NaCl concentration. Greatest growth rates occur at 0.5 to 0.75 M NaCl at 33°C (46).

REVIEW OF THE LITERATURE ON HALOPHILISM

The term halophyte literally means salt plant, and is usually used for plants that grow in the presence of high concentrations of sodium salts. A halophyte can be halophilic, salt resistant, halo-tolerant or haloavoidant. There is confusion in the literature as to the proper usages of these terms. According to Levitt (97) the following explanation would probably be proper. A halophilic plant is one which grows best at high salinities. On the other hand, a salt resistant plant can withstand high salinities, but grows best at lower salinities. There are two major mechanisms utilized by halotolerance and haloavoidance. The first, halotolerance, is the same as ion accumulation. The second, haloavoidance, can be accom-plished in any of three ways; salt can be excluded passively, ex-truded actively, or can be diluted.

The salinity extremes that organisms can survive in or thrive in are very varied. Some examples are given. Certain bacteria, such as *Halobacterium salinarium* can grow in saturated NaCl (29). Selected strains of *Penicillium notatum* grow on saturated calcium acetate (143). The fungus *Scrophulariopsis parvula* grows in media with saturated NaCl. The yeast *Debaryomyces hansenii* shows respiratory activity in 24% NaCl, which is 10% of normal.

Species of algae have been reported from brine lakes, salterns, salt springs and pools with saline contents 2 to 17 times that found

in the ocean. The algae are largely Chlorophyceae, although there occur species of Myxophyceae, Bacillariophyceae and Euglenophyceae. *Dunaliella,* reported from numerous brine waters, is probably the most common of the green algae. *Chlamydomonas* has been found in the saline lakes of the Crimea. Both *Dunaliella* and *Stephanoptera* form light green areas on solid salt crusts (157). Elazari-Volcani (44) examined mud sampels taken from the Dead Sea bed. These contained green and blue green algae and diatoms. Most of the green forms resembled *Dunaliella,* with lesser amounts of *Scenedesmus bijugatus, Pediastrum simplex* and *Ulothrix.* The blue greens resembled *Aphanocapsa littoralis, Aphanothece halophytica* or an *Oscillatoria.* Of the diatoms, forms of *Melosira, Navicula, Pinnularia, Gomphonema, Cymbella* and *Synedra* occurred. Each liter of Dead Sea water contains approximately: 143 g. magnesium chloride, 87 g. sodium chloride, 37 g. calcium chloride, 11.5 g. potassium chloride and 5 g. magnesium bromide.

Species of *Enteromorpha* occur in the Great Salt Lake as well as from fresh waters. Fluctuating amounts of salinity produce specimens that have characteristics of several species in a single frond (157). The blue green algae *Sprirulina subsala, Phormidium tenue* and others grow and multiply in 3 M NaCl (65, 84).

A few examples of halophilism of higher plants are given. Seedlings of *Atriplex vesicaria* were established in 1 M NaCl (16). At

100-200 mEq/liter of Na^+, K^+ or Mg^{2+} Cl *Atriplex nummularia* grows

optimally. There is still good growth at 300 mEq/liter (57).

Halogeton glomeratus survives at 1.4 molal NaCl (137). The optimal

level of NaCl for growth of the mangrove *Avicennia marina* is 1.5%,

one half the concentration of sea water (30). Succulent halophytes

such as *Sueda depressa, Salicornia europaea* and *Spergulia marina*

germinate and develop in 5% NaCl (162). Leaves of *Nitraria schoberi*

(the most tolerant among woody plants) may contain 14% of their dry

matter in the form of NaCl, 57% as total salts (158).

Mechanisms of Osmotic Regulation

The following are mechanisms of osmotic regulation utilized by

halophilic plants and microorganisms:

A. Development of cell membranes highly permeable to ions.

A membrane which is highly permeable to sodium enables the cell

to adjust rapidly to high osmotic pressure changes. This requires

adaptation of a cell's enzyme system, nucleic acids, ribosomes, etc.,

to function at high as well as low ionic concentrations. This mech-

anism is suggested as being responsible for the osmotic regulation

of *D. salina* (102). Another species of the halophilic organism,

D. parva, is freely permeable to sucrose, inulin, starch and even

polyvinylpyrollidone with a molecular weight of 20,000 to 40,000

(53). It is impermeable however to dextran (mol. wt. 80,000). The

mycelia of a salt tolerant strain of *Penicillium notatum* accumulate

high intracellular concentrations of copper when grown on metallic

copper surfaces (154). Kylin and Gee (88) showed that a halophilic

mangrove (*Avicennia nitrida*) possesses a Na extrusion pump, but it is

inactivated at high concentrations of NaCl (0.2 to 0.4 M). Similar

inactivation also occurs with *Atriplex subcordata*, *Suaeda maratima*

and *Astes tripolium* (168).

B.   Avoidance, including active Na extrusion.

Many plant halophiles, including *Spartina townsendii*, *Limonium

latifolium* (3) and the mangroves (6) posses salt glands which extrude

NaCl against a concentration gradient (144). In the case of *Tamarix

aphylla*, the glands show no apparent selectivity between Na^+ and K^+

ions (14). Even Rb was taken up from the nutrient solution and

secreted by the glands (160). Single celled organisms may use

vacuoles to maintain a steady state low concentration of NaCl. The

non-photosynthetic flagellate *Choanogaster plattneri* is found in

water of 20% salinity. The cells have a complex vacuolar system,

producing smaller accessory vacuoles that empty outside the cells and

that decrease in activity when the salinity of the medium is lowered.

The vacuoles are said by Pochman (136) to contain a higher concentra-

tion of salt than the medium and serve to excrete salt. The contrac-

tile vacuoles of *Chlamydomonas moewusii* have an entirely different

function, the elimination of water. A mutant strain lacking contrac-

tile vacuoles survives and grows only if the osmotic pressure of the medium exceeds 1.5 atm (59).

For algae, sodium pumps have been described since Scott and Hayward (146) described a sodium excretion mechanism in *Ulva lactuca*. The mechanism of this active transport was envisioned as involving a cation sensitive ATP-ase system as demonstrated for erythrocytes (36, 137). As far as completing the comparison, Eppley in 1962 (45) stated that "an experimental approach might be facilitated if an algal equivalent of the erythrocyte ghost were available." Jokela, in 1969 (75) isolated naturally wall free, ghost forming membranes of *D. tertiolecta* and detected Na-K ATP-ase activity.

C.   Development of an active exchange-transport system.

Halobacteria avoid excessively high sodium content of the cell by an active inward transport of potassium. K^+ concentration is much higher than in the medium, and in some cases the value found intracellularly was close to the limit of solubility for KCl (29). Adaptation of the cell's enzyme structure is necessary for functioning at high ionic strength. Many of the enzymes are reversibly inactivated in the absence of high concentrations of neutral salts (91).

D.   Utilization of inert neutral compounds as osmotic agents.

The maximum proline concentration that *E. coli* can concentrate internally has been shown to be dependent on the osmolarity of the suspension medium; the labeled amino acid pool in *E. coli* has been shown to be proportionally released when the cells are washed with

low osmotic strength solutions (20). Oxalate synthesis in *Atriplex*
spp. has been shown to be related to the external osmotic pressure
(132). Polyalcohol production of yeast is influenced by the presence
of high salt concentrations (131). Glycerol production by *D. tertio-
lecta* in response to increasing osmotic pressures has been mentioned.
E.   Salt dilution.

Rhizophora mucronata does not excrete salt, yet its growing
leaves retain a constant concentration (510 to 560 mEq/liter) though
receiving 17 mEq Cl/day (6). During growth, sufficient water is
absorbed to prevent increase in concentration. This dilution of
cell sap during growth has been found in some moderately salt tol-
erant halophytes (97). Many other plants avoid increase in concentra-
tion by increasing succulence. Cells, especially parenchyma cells
enlarge, avoiding excessive salt concentrations (139).

High Salinities and Cellular Adaptations

The adaptations of some cellular constituents to high salt con-
centrations are related.
A.   Cell envelopes.

Halobacteria do not have clearly separable wall and membrane
structures (142). The term cell envelope is used to designate struc-
tures enveloping the cytoplasm. The cell envelope requires a high
salt concentration for its maintenance; at low concentrations it loses

its rigidity and collapses. Chemicals which do not possess a strong net charge in aqueous solutions do not protect the structure of the envelope (123). The hypothesis of Abram and Gibbons (1) is that the cell envelopes of the halobacteria "are held together rather loosely by hydrogen bonds, Coulomb forces or 'salt' linkages, and in the presence of high concentrations of NaCl the electrostatic forces are screened so that the bonds hold the organism together in a rod shape." The cell envelopes of the halobacteria have very few or no higher fatty acid residues, and in this way differ pronouncedly from other bacteria (27, 91). The bulk of the lipid is an analogue of diphosphatidyl glycerophophate (147). The proteins of the cell envelope of halobacteria are very acidic (21).

It is of interest that in the cell membrane of *D. tertiolecta* long chain fatty acids are absent, at least when the organism is cultured at high salinities. There is also a high phosphatidyl glycerol content and a predominantly acidic amino acid composition (75).

B. Nucleic acids.

According to Kessler (83) high osmotic pressures induce changes in copolyribonucleotides synthesized from equimolar concentrations of ADP, GDP, CDP and UDP. He suggests that osmotic stress is coded into a changed composition of polyribonucleotides which may lead to adaptive reactions by serving as primers for RNA dependent RNA polymerase. Siegel (152) has demonstrated the role of nucleotides, especially IMP, in the recovery of *Penicillium* from extreme salt effects.

C.   Ribosomes.

The ribosomes of *H. cutirubrum* are stable only in solutions

containing KCl close to saturation (4M) and Mg^{2+} in the concentration

range 0.1-0.4 M.   The KCl requirement is quite specific, and cannot

be replaced by iso-osmolar NaCl (12).   With regard to higher plants,

it has been shown that the ribosomes of salt sensitive citrus species

are less stable than those of more resistant species, but were able to

acquire an increase in stability (83).

D.   Proteins and enzymes.

In a culture of callus tissues from cabbage leaves, it was found

that salts slow down amination and amidation, as well as the transfer

of sulfides to S-containing amino acids (148).   Shevyokova (148)

suggests that an increase in derivatives of S-amino acids may be one

reason for the unfavorable effect of salts.

Baxter and Gibbons (11) made the important discovery that the

enzymes of extremely halophilic bacteria are adapted to function at

the very high salt concentrations found within the cells.   Since then

about twenty different enzymes have been reported, and all data fit

the general contention that the enzymes of extremely halophilic bacte-

ria are extremely halotolerant, and in most cases even strikingly

halophilic (11, 29, 91).   These enzymes are irreversibly inactivated

in the absence of high concentrations of salts.   For example, *Halo-*

bacterium salinarum excretes a protease which is possible to chro-

motograph only in the presence of 3.4 M NaCl (127). Another specific property of their proteins was the absence of half-cysteine residues, in opposition to the non-halophilic *E. coli* which has 0.6 moles half-cysteine per 100 moles of amino acid. Stroganov (158) suggests that the bacteria adapt to high salinity by binding of the mineral element (158). Haloavoidant organisms and non-halophiles usually have enzymes that are salt sensitive, as is the case with *D. tertiolecta* (75).

The capabilities of some enzymes under stress are enormous. *In vitro* horseradish peroxidase activity is present in 10 M lithium chloride. Considering the coordination number of the ions, a 10 M LiCl solution can have little or no uncoordinated water (153).

HALOTOLERANCE – EXPERIMENTAL INVESTIGATIONS

Growth of *D. salina* was reported by Marre (102) to occur in saturated NaCl solutions. McLachlan (114) demonstrated *D. tertiolecta* growth in 2.5 M NaCl, the highest concentration tested. This investigation corroborates these results. It was also found that *D. tertiolecta* growth occurs in saturated NaCl, though greatly attenuated ($^1/_4$ the rate of growth in 2.5 M NaCl).

All cultures were grown in Hutner's Marine *Chlamydomonas* medium, with variation only in the major chloride (69) (See Table 1). The pH was adjusted to 7.2 with KOH. This glass electrode measurement proved to be optimum for increase in cell number for *D. salina* cells grown in 300 g/l NaCl (See Table 2). It must be kept in mind that such pH measurements are inaccurate, due to dilution of water by the concentrated salts. The light intensity of all experiments was a continuous 3000 lux.

At room temperature the effect of various electrolytes and glycerol on *Dunaliella* growth are shown in Table 3. NaCl, KCl, $MgCl_2$, $CaCl_2$ and glycerol were added to the medium in concentrations yielding an osmolarity equivalent to $55^0/_{00}$ NaCl.

Table 1. Hutner's marine chlamydomonas
medium (69), pH adjusted to 7.2 with KOH.

Metal chloride	Xg/liter
EDTA	0.50 g
$K_2HPO_4 \cdot 3H_2O$	0.26 g
$MgSO_4$	1.22 g
Glycine	2.50 g
K-acetate	2.00 g
H_3BO_3	244.00 mg
$ZnCl_2$	62.00 mg
$Ca(NO_3)_2 \cdot 4H_2O$	147.00 mg
$MnSO_4 \cdot H_2O$	31.00 mg
$Na_2MoO_4 \cdot 2H_2O$	25.00 mg
$FeCl_3 \cdot 6H_2O$	14.00 mg
$CoCl_2 \cdot 6H_2O$	1.00 mg

Table 2. Effect of initial pH of Hutner's marine chlamydomonas medium on growth of *D. salina*. Cultures initially contain 250 ml of 5×10^4 cells/ml.

Glass electrode pH	% increase in cell number after 3 weeks
5.0	all dead
6.0	300
6.8	1000
7.7	1300

Table 3. Change in cell number of 250 ml cultures in Hutner's supplemented with chlorides and glycerol of osmolarities comparable to $55^0/_{00}$ NaCl. Incubation period is three weeks. The initial cell concentration (X) is 5×10^4 cells/ml.

	D. salina	*D. tertiolecta*
NaCl$_2$	14X	31X
MgCl$_2$	0X	5X
KCl	0X	0X
CoCl$_2$	0X	0X
Glycerol	0X	1X

The highest growth rates occur in NaCl. Slower division in $MgCl_2$ and glycerol was evident. All measurements were done with visible microscopy and a Levy hemocytometer. Lowering the temperature extends survival time for $CaCl_2$ cultures. (Figure 1). This fact will be elaborated upon in the next section.

To ascertain if diffusion of the major cation through the plamsa membrane is necessary for osmoregulation, survival of *D. salina* in choline$^+$ chloride$^-$ was determined. One ml aliquots of a late log culture of *D. salina* grown in 150 g/l NaCl Hutner's were transferred to either of two solutions. The first solution contained two ml of the identical medium. The second contained two ml of 35 g/l NaCl Hutner's supplemented with 27.6 g choline$^+$ chloride$^-$ / 100 ml to make it isotonic. After 10 minutes, there was a 38% drop in viability in the choline chloride solution.
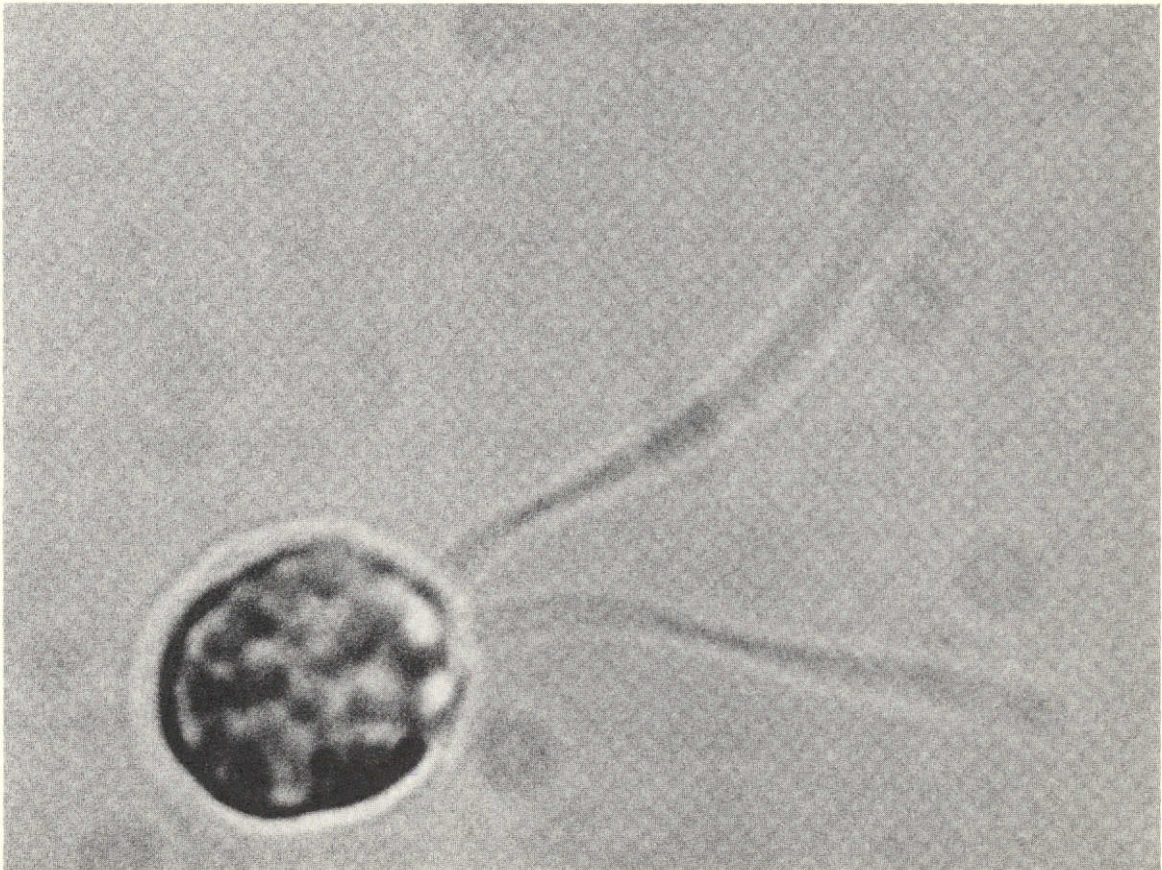
Figure 1. *Dunaliella salina* in saturated CaCl₂.

Optical resolution. Cell diameter lengthwise is 10 μm.

Osmotic Regulation

The conclusion of Marre (102) that *Dunaliella salina* plasma membranes are freely permeable to sodium is based almost entirely on his ^{24}Na experiments. Our studies have shown that his interpretations of the data were almost completely fallacious.

Marre suspended aliquots of algae in the original media indicated below and dialyzed them for ten hours against 20 volumes of the final medium containing ^{24}NaHCO$_3$.

NaCl in medium		Na^{24}CPM / cc	
original	final	medium	cell sap
2.50 M	7 M	165,000	81,000
1.25 M	7 M	163,000	112,000

The original medium did not contain the isotope. This was introduced with a specific activity as high as practical without altering the Na$^+$ ion concentration of the medium. Before monitoring radioactivity of the algae, Marre centrifuged the cells for 20,000 g for 20 minutes and decanted the medium. He somehow assumed that the intercellular volume of the packed cells was less than 3% of the total volume. No basis for this assumption is given.

A follow-up experiment involved exclusive use of iso-osmotic solutions.

NaCl conc. of the medium	Na^{24} cpm / cc after 10 hours in the medium containing the isotope		30 minutes after transport of cells from med. contain-ing Na^{24} to isotope free med.
	In medium	In cell	In cell
2.50 M	129,000	71,000	360
3.75 M	129,000	94,000	150

On the basis of these two experiments, along with the observations of Trezzi (161) that the cells shrink in hypertonic solutions and then regain their original shape — that all the subsequent literature on the passive Na^+ intracellular accumulation is based.

Assuming Marre's interpretation of his data was correct, we proceeded to look for halophilic or halotolerant enzymes in *D. salina.* This search seemed to be called for since there has never been reported a halophilic or halotolerant enzyme isolated from an alga. Both glucose-6-phosphate dehydrogenase and catalase had attenuated activities with increasing NaCl concentrations. Increasing NaCl concentration from $6^0/_{00}$ to 100 $^0/_{00}$ resulted in a 90% decrease in glucose-6-phosphate dehydrogenase activity. At $250^0/_{00}$ NaCl there was another tenfold loss in activity.

To make precise estimates of osmotically active substances in
D. salina, the following determinations for cell volume, sodium,
potassium and glycerol were performed.

Determination of Intracellular Volume of *D. salina* Cells
Using a Bluedextran Indicator Method

Cells were harvested at the end of their log phase of growth at
$25°$ in $150^0/_{00}$ NaCl Hutner's and resuspended in identical sterile
solution. Two hematocrit tubes were filled with aliquots of the
suspension and centrifuged at 2,500 g for 10 minutes. The ratio of
packed cell volume to total volume of liquid was then measured.

Four ml aliquots of the algal suspension were centrifuged under
the same conditions as the hematocrit tubes. The supernatants were
decanted, the sides of the centrifuge tubes carefully dried, and then
the packed cells were resuspended in a Blue Dextran solution of known
concentration and Optical Density at 580 mm. The cells were then
recentrifuged under the previous conditions (2,500 g for 10 minutes).
The supernatant was decanted, millipore filtered, and then the Optical
Density determined. By comparing standard Blue Dextran dilution plots
to the decrease in O. D. of the dye solution caused by dilution of the
dye by intercellular space liquid, the volume of the intercellular
space was determined. The intercellular volume was 32% of the packed
volume, ten times Marre's figure.

Assay of Glycerol Using Glycerol Dehydrogenase

D. salina cultures in late log phase were centrifuged at 2,500 for 10 minutes, and then the supernatant analyzed for glycerol.

1. To each tube containing from 0 to .4 µ moles of standard glycerol solution, or similar amount of sample, or both, the following solutions were added:

2. 1.9 ml of Sorensen's glycine II buffer, pH 9.5

3. 0.2 ml of glycerol dehydrogenase (Worthington, from Enter-obacter aerogenes) (2mg/3 ml), prepared immediately before use.

4. Distilled water to bring volume up to 3 ml.

5. 0.1 ml of DPN solution (6.6 mg/ml)

Optical density at 340 mu with appropriate standards recorded for twenty minutes.

To determine intracellular glycerol concentrations, packed cells were washed twice with 100 volumes of fresh medium at 4°, then freeze-thawed and centrifuged. The supernatant was analyzed in the manner indicated above. Results are shown in Table 4.

Table 4. Intra and extracellular glycerol, Na and K in late log
D. salina culture grown in 150 g/1 NaCl Hutner's medium. Values are
means of three determinations.

Compound	Intracellular		Extracellular	
	Molarity	Osmolarity[a]	Molarity	Osmolarity[a]
Glycerol	1.640 ± 0.260	1.64	0.004	0.004
Na	0.534 ± 0.237	1.07	2.560	5.120
K	0.084 ± 0.034	<u>0.16</u>	0.044	<u>0.088</u>
		2.87		5.212

[a] Osmolarities include anion and assume (without justification)
a 1 : 1 anion-cation ratio, in a completely free liquid cell.

Determination of Intracellular Sodium and Potassium Content

Triplicate samples of packed cells were digested in 2 ml of 17:3 nitric-perchloric acid and heated until solutions were clear. Solutions were then diluted with deionized water and analyzed with flame atomic absorption apparatus. The absorbancy of sodium at 589 nm was measured and the concentration determined by interpolating in the standard curve of Na. Potassium absorbance was measured at 768 nm. Results are summarized in Table 4.

Apparently glycerol is the principal osmotically active intracellular substance in *D. salina*. Sodium ion extrusion and potassium ion accumulation is occurring. In light of these results investigations into glycerol-salt interaction with *D. salina* enzyme are planned for the near future.

The effect of salinity on mercury toxicity was also investigated. Mid log cultures of both Dunaliella species were grown at $35^0/_{00}$ or $150^0/_{00}$ NaCl and exposed to various concentrations of $HgCl_2$ for two hours. The per cent of motile cells before and after treatment was counted using light microscopy and a hemocytometer. Results are plotted in Figure 3. Interesting to note is that Hg tolerance as reflected by ID_{50}'s is roughly doubled at higher concentrations of NaCl.

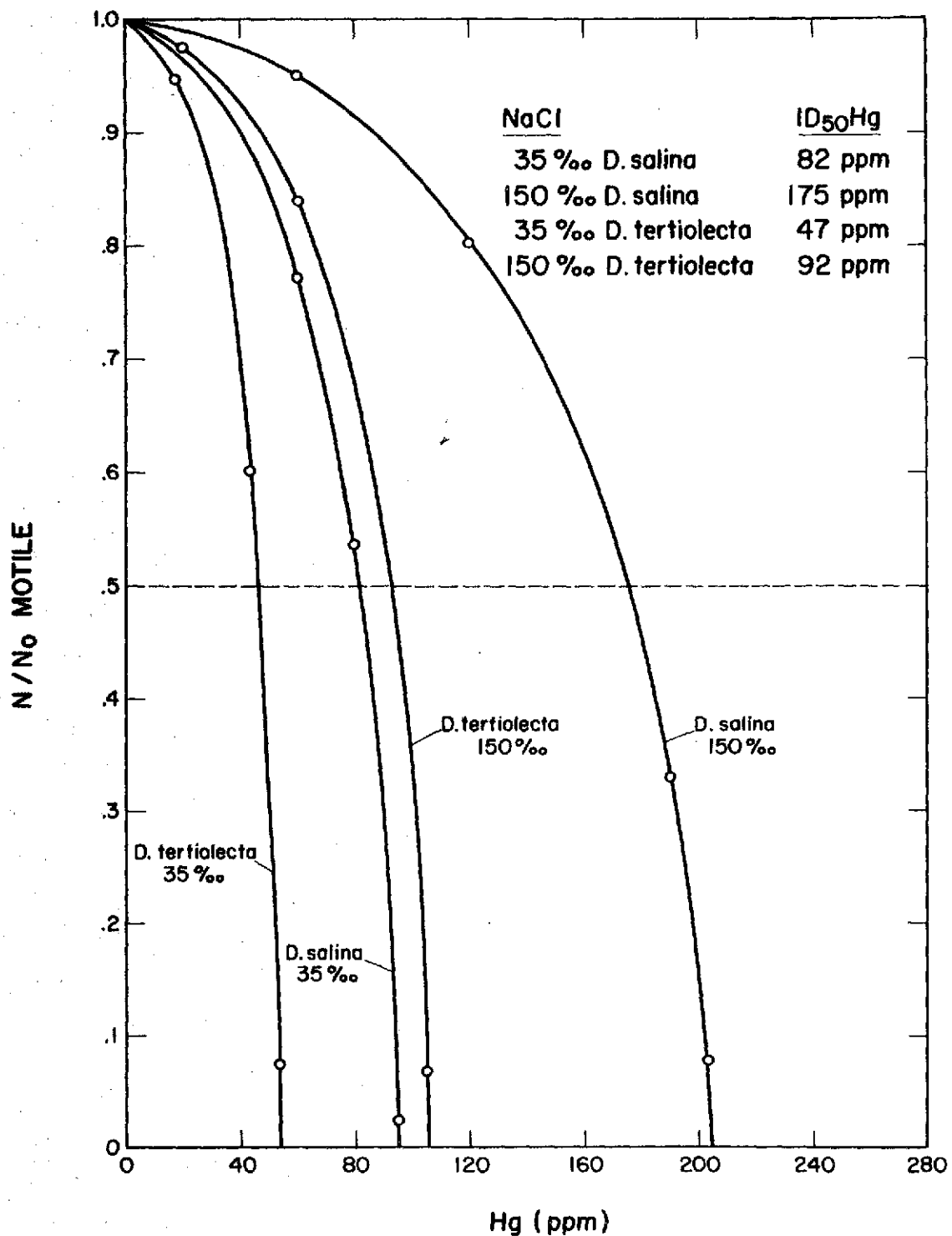| NaCl		$ID_{50}Hg$
35 ‰	D. salina	82 ppm
150 ‰	D. salina	175 ppm
35 ‰	D. tertiolecta	47 ppm
150 ‰	D. tertiolecta	92 ppm

Figure 3. Response of *Dunaliella* to $HgCl_2$ in different concentrations of NaCl. Each determination represents a count of the number of motile cells per 1000 cells before treatment and after two hours of incubation with prescribed mercury levels.

REVIEW OF THE LITERATURE OF CRYOTOLERANCE

There is an apparent contradiction between the use of very low temperatures to store cells and tissues for long periods of time and the finding that living cells are usually destroyed by sub-zero temperatures (48). With the exception of nuclear processes and combining of free radicals (140), the rates of all physical, chemical, and therefore biological processes are temperature dependent (110). When processes depend solely on the mean velocity of molecular motion, the decrease in rate with decrease in temperature is proportional to the fractional change in absolute temperature, and is therefore small over ten or twenty degree intervals. Diffusion is such a process. But for reactions requiring collision of molecules having energies over some threshold value, the effect of temperature on reaction rate can be tremendous. Thousands of enzymatically catalyzed reactions are occurring in living cells and most occur in the correct temporal and spatial sequence to be effective. Since the temperature coefficients (Q_{10}'s) and activation energies of these reactions are not identical, a decrease in temperature is likely to upset the balance (74).

Lowering the body temperature 10°C or 20°C is lethal to most homoiothermic animals and profoundly alters the metabolic rates of all poikilothermic organisms. These alterations are reversible in most poikilotherms as long as no water phase change is involved. Examples

of organisms that can withstand low temperature is given.

Bacteria

Psychrophiles are bacteria capable of growth at 0°C, but subject to enhanced rates at higher temperatures (153). The implication that there is an actual preference at or near 0°C is incorrect (72). It has been suggested that they should be called psychrotrophic, and that the term psychrophilic be reserved for organisms that grow optimally below 30°C (43, 156). Psychrophiles are ubiquitous, found not only in soils and oceans, but also in dairy products, meats and fruits.

Mostly, these bacteria are gram-negative, non-spore forming rods, including the following genera: *Serratia* (5, 34, 156), *Flavobacterium, Archromobacter, Pseudomonas* (156), *Salmonella, Proteus, Bacillus* (52), and *Lactobacillus*, Cocci, namely *Streptococcus* (32), *Micrococcus* (52, 117), and *Rhodococcus* have been reported. Strictly anaerobic psychrophiles seem to be rare in most habitats, although sulfate reducers occur in ocean deposits (159) and some spore forming anaerobes such as *Clostridium* will grow well at 6°C (13). Bacteriophage with growth temperature maxima only slightly above that of the psychrophilic host have been reported (130). ZoBell (173) has been able to culture 76 out of 88 kinds of marine bacteria at sub-zero temperatures. This is significant since over 80% of the ocean floor is 1700 m or more deep with temperatures $\leq$ 3°C (7).

Protozoa

Wolfson (165) was able to supercool suspensions of *Paramecium* to -14°C without completely stopping ciliary action. Viable cells of *Paramecium* recovered and multiplied after exposure to liquid nitrogen temperatures (155). Some amoebae survive supercooling to -5°C. (25). There do not seem to be any reports of growth or multiplication of protozoa in sub-zero temperatures.

Fungi

There exists a *Penicillium* mutant capable of growth in ordinary broth or solid media saturated with acetates or chlorides of Na, K, Mg, Ca or Sr that will grow at constant 4°C or with 16 hours per 24 hours at -30°C just as well as at constant 25°C (150). Vital activity of this same organism was found in liquid ammonia-glycerol media at -40°C (49). Cells of the yeast *Saccharomyces cerevisiae* survive in prolonged suspension supercooled to -16°C (108), and are capable of surviving liquid N_2 temperatures if slow cooled and fast warmed (70, 100, 111). Isolated mitochondria of the same organism can be preserved intact at -190°C without addition of cryoprotective agents (8). The freeze-thaw response of *Neurospora crassa* is very similar to *S. cerevisiae* (9). The list of psychrophilic fungi include *Candida, Rhodotorula, Aureobasidium, Geotrichum* and *Phoma* (161). Lichens such as *Cladonia, Lobaria,* and *Umbilicaria* are capable of withstanding temperatures well below 0°C (66).

Algae

The plants of perpetual snow and ice are aften related to plank-
tonic algae of lakes and streams (157). The vegetation is largely
made up of algae, although moss protonema, fungi and bacteria also
occur. The algal forms generally grow close to the surface. Many
forms multiply and grow in the melted snow and are therefore living
at temperatures no lower than the freezing point. Oxygen is always
ample.

Cryophytes are frequently classified on the basis of color. The
green color of the snow of European and Arctic regions is associated
usually with limestone. Species of *Chlamydomonas*, *Ankistrodesmus*
and *Mestaenium* are common (157). *Euglena* also occur (82). Red snow
is common all over the world. The algae responsible for the color
are species of *Chlamydomonas*, *Scotiella* (28), *Chionaster*, *Raphidonema*
Gleocapsa and some diatoms (90). Yellow or yellow-green snow is
caused by *Protoderma*, *Scotiella* and *Chlorosphaera* (98, 157).

Active life for these forms poses two problems, the ability to
survive freezing during extremely cold nights and the capacity to
carry on metabolic reactions required for growth and assimilation when
the day temperature is in the neighborhood of 0°C. The first problem
is common to many plants and is usually defined as that of frost
resistance. One mechanism of defense against low temperatures can be
a high osmotic pressure of the cell sap, correlated with a lowering

of the freezing point. This is the case with *Dunaliella salina.*
However, high internal osmotic values do not always result in cold
tolerance; for example in *Griffithsia,* an intertidal red alga, the
higher osmotic pressure of the apical cells is associated with a
higher sensitivity to cold damage (15). Drying of intertidal algae
is sometimes related to freeze tolerance. For example, respiration
in *Fucus* has been measured at -15°C. *Fucus* is a prominent Arctic
intertidal alga (77).

Just as an aside, at least 23 strains of green algae (*Chlorella,*
Ankistrodesmus, Coccomyxa, Scenedesmus, Euglena) and blue green
algae have been frozen in the laboratory to liquid nitrogen tem-
peratures and remained viable (23, 67, 70).

Higher Plants

Aside from conifers, winter evergreen species include *Pterido-*
phytes, and the angiosperms *Asplenium, Dryopteris, Polypodium,*
Rhododendron, Arctostaphylos and *Lauris* (153). These forms maintain
some degree of metabolic activity at sub-zero temperatures.

Higher Invertebrates

Survival of intertidal mollusks of temperatures down to -5°C have
been recorded (74). Sea urchin eggs can survive in the -5°C to -15°C
range (4). The intrinsic nontoxicity of glycerol as a cryoprotectant
is supported by many observations of high concentrations of this com-
pound found in insects. The most dramatic example is of an Alaskan

insect which elaborates glycerol to a whole body concentration of 2.5 M

and is uninjured by months of freezing at −40°C, at which temperature

concentrations in excess of 6 M will be produced (10, 128).

Vertebrates

In fish and turtles, core temperatures of −1°C have been found

(35, 144). Human erythrocytes are routinely stored at liquid nitrogen

temperatures. Bare skinned mammals such as swine tolerate −50°C (153).

Recently mouse embryos were frozen to −269°C. When reimplanted after

thawing they developed into normal mice (165).

Effects of Cold and Frost on Cells

The effects of cold and frost on different cell organelles
including membranes (33, 49, 62, 87, 96, 109, 112, 116, 125, 135),
chromosomes (95, 96, 98, 126), chloroplasts (60, 61, 62), mitochondria
(8, 60, 87), ribosomes (73), vacuoles (61, 79) and lysosomes (135)
are well documented. This is also the case with chemical constituents
such as proteins (19, 23, 34, 48, 58, 60, 61, 62, 73, 78, 81, 92, 93,
95, 96, 98, 115, 126), lipoproteins (61), lipids (2, 84, 85, 87, 96),
sugars (60, 61, 79, 113) and nucleic acids (84, 96, 98, 126). Rather
than discussing each constituent individually, general theories of
cryodamage, tolerance and avoidance will be discussed.

Plants, as poikilotherms, are unable to develop low temperature
avoidance. They are either tolerant (hardy) or else they show damage.
A number of theories of frost injury and tolerance were compiled by
Levitt (94). They will be summarized.

A. Armchair theories

Perhaps the oldest is the "caloric theory" which proclaimed that
frost resistance results from the release by the plant of enough heat
to prevent freezing. This theory was disposed long ago since it
imples avoidance of both frost and low temperature injury, whereas
frost resistance is actually tolerance.

The second oldest is the "rupture theory". According to this
theory, cellular expansion caused by ice formation resulted in cell

rupture. However, later investigations showed that frozen tissues actually contract.

B. Frost precipitation theory

Gorke (54) in 1906 found that freezing plant juice precipitated the proteins and that this precipitation was prevented by adding sugar. He therefore explained frost injury as a precipitation of the protoplasmic proteins caused by the concentration of the cell salts that occurs on freezing, and frost resistance as a result of the protection of the proteins by the sugars that accumulate on hardening. Gorke's protective effect by sugars was corroborated using grains but not in most observations. Furthermore, there are many plants that cannot become resistant in spite of high sugar content, so his explanation of frost resistance is inadequate. Finally, it has been possible to increase frost resistance by inducing the uptake of salts (141).

C. Iljin's mechanical stress theory (71)

Iljin was struck by the pronounced cell collapse that occurs on extracellular ice formation and concluded that the protoplasm of such cells must be subjected to stresses and strains. He determined that injury occurred only during the thawing, mainly because of his success in preventing injury by simply thawing in strongly plasmolyzing solutions. The factors associated with hardiness can be explained logically by Iljin's theory. The smaller the cell size, the greater the specific surface and therefore, the less the strain developed due

to any one degree of cell contraction. The greater the sugar
concentration, the smaller the water loss and therefore the less the
cell contraction and the stress at any one freezing temperature. An
increase in bound water produced by penetrating cryoprotective agents
would have the same effect. However, the theory gave no explanation
for the greater injury caused by intracellular than by extracellular
freezing, nor for the greater resistance of hardy protoplasm to
stresses and strains. Nor could it explain the injury to animal cells
without stiff cellulose walls.

D.   Intracellular freezing theory

Intracellular freezing is consistently fatal at temperatures
that fail to injure when freezing is extracellular. Thus, rapid
cooling to -30°C results in 99.9% killing of yeast cells; slow cooling
to the same temperature results in 50% kill (108). Such evidence has
frequently led to the suggestion that all frost resistance is due to
the avoidance of intracellular freezing. Scarth (143) was the first
to suggest that the higher permeability of hardy cells to water would
favor this avoidance, and to later produce evidence that hardy cells
do actually show an avoidance of intracellular freezing. For example,
sea urchin eggs are more resistant to rapid freezing injury when
fertilized than when unfertilized, and this is correlated with four
times greater membrane permeability when fertilized. Chambers and
Hale (25) first showed that cell membranes are essentially impermeable
barriers to ice seedings, except at temperatures below -40°C.

E.   The sulfhydryl-disulfide theory

According to this theory of Levitt's (94) frost injury is the
result of unfolding and therefore the denaturation of the protoplasmic
proteins. This results from the mutual approach of the protein mol-
ecules (due to their dehydration during freezing) until they are
close enough for the formation of intermolecular SS bonds. Levitt
admits "although direct evidence in its favor is admittedly still
lacking, there is much to recommend it as a working hypothesis." The
hypothesis is supported by the following facts: 1) increase in pro-
tein SS is found when freezing does not result in injury; 2) the
attenuated effect of freezing if done under low O_2 tensions (150);
3) general apparent increases in protein SH on hardening; 4) greater
injury during intracellular freezing may result from the compression
of the dehydrated molecules between expanding ice loci. This would
bring SH and SS groups closer together; 5) the protective effect of
glycerol in the case of animal cells can be explainable by the binding
of the glycerol molecules (by hydrogen bridges) to the SH groups of
the protein molecules (replacing removed water molecules), thus keeping
them too far apart for interaction; 6) the increase in GSH oxidizing
ability of the cell that occurs during hardening, which would tend to
eliminate GSH — a substance capable of triggering SH-SS interchange
reactions in protein; 7) enzymes are frequently prepared from tissues
killed by freezing and since their activity is retained, unlike other

proteins in the dead protoplasm, they cannot have been denatured. But

the enzymes so prepared are soluble ones which would not be held in

the protoplasmic framework, and therefore would remain too far apart

for SH-SS interchange.

CRYOTOLERANCE - EXPERIMENTAL INVESTIGATIONS

The premise of necessary Antarctic Dry Valley sterility as promulgated by Horowitz and Cameron in 1972 (68) is contradicted by the motility capabilities of *D. salina* at -15°C. Studies reported by this author concerning uptake of radioactively labelled organic compounds by "greenhouse sludge" in a Don Juan Pond simulator were reported in University of Hawaii Botanical Science Paper Number 31. It is a fact that there is metabolism of autotrophic eukaryotes at sub-zero temperatures. The high internal osmolarity and concommitant extensive freezing point depression capabilities (3 M glycerol depresses freezing point to -9°) were another reason for it being a likely candidate for being a cryophile.

Azenic cultures of *D. salina* and *D. tertiolecta* were grown at room temperature in Hutner's medium supplemented with 2.5 molar sodium chloride and adjusted to pH 7.2. A constant light intensity of 3000 lux candles was maintained. At late log phase with approximately 5×10^7 cells/ml, aliquots of both cultures were equilibrated to 25, 6, -1 and -8 degrees respectively under identical light intensities. After two hours, each culture was innoculated with a sterile, equimolar solution containing 40 μCi/ml ^{14}C labelled sodium carbonate. After an additional 36 hours, aliquots were withdrawn from each culture, millipore filtered (.22 M) and washed. Cells plus filters were placed in scintillation cocktail. One day was allowed

for bleaching of cocktails, and then radioactivity was read using a scintillation counter. Appropriate standards were used for calculation disintegrations per minute. As indicated by Figure 2, CO_2 reduction is appreciable below zero degrees for both species. Although extrapolations to the baseline as drawn do not reach below minus ten degrees, further experimentation is necessary to determine the actual lower limit of carbon dioxide reduction.

In an attempt to isolate labeled photosynthates, produced at -6°C, high specific activity 1 mCi/ 1.6 mg $NaHCO_3$ was utilized. A few low molecular weight organic compounds have been isolated, but not presently positively identified. They are very convincing evidence that more than non-specific absorption is occurring at -6°C.

For low temperature studies, both species of *Dunaliella* provide excellent visual indicators of their osmotic state and viability. These indicators are cell shape and motility. We have observed that approximately nine times out of ten, structural integrity and motility of individual cells occur together. The value of viability determinations for low temperatures is not mitigated if observations are made after cultures are returned to room temperature, being that no phase change of the salty medium is involved. The high correlation of structure and function which can be observed using light microscopy provides an efficient, yet non-complex system for evaluating the effect of any sort of parameter on the low temperature viability
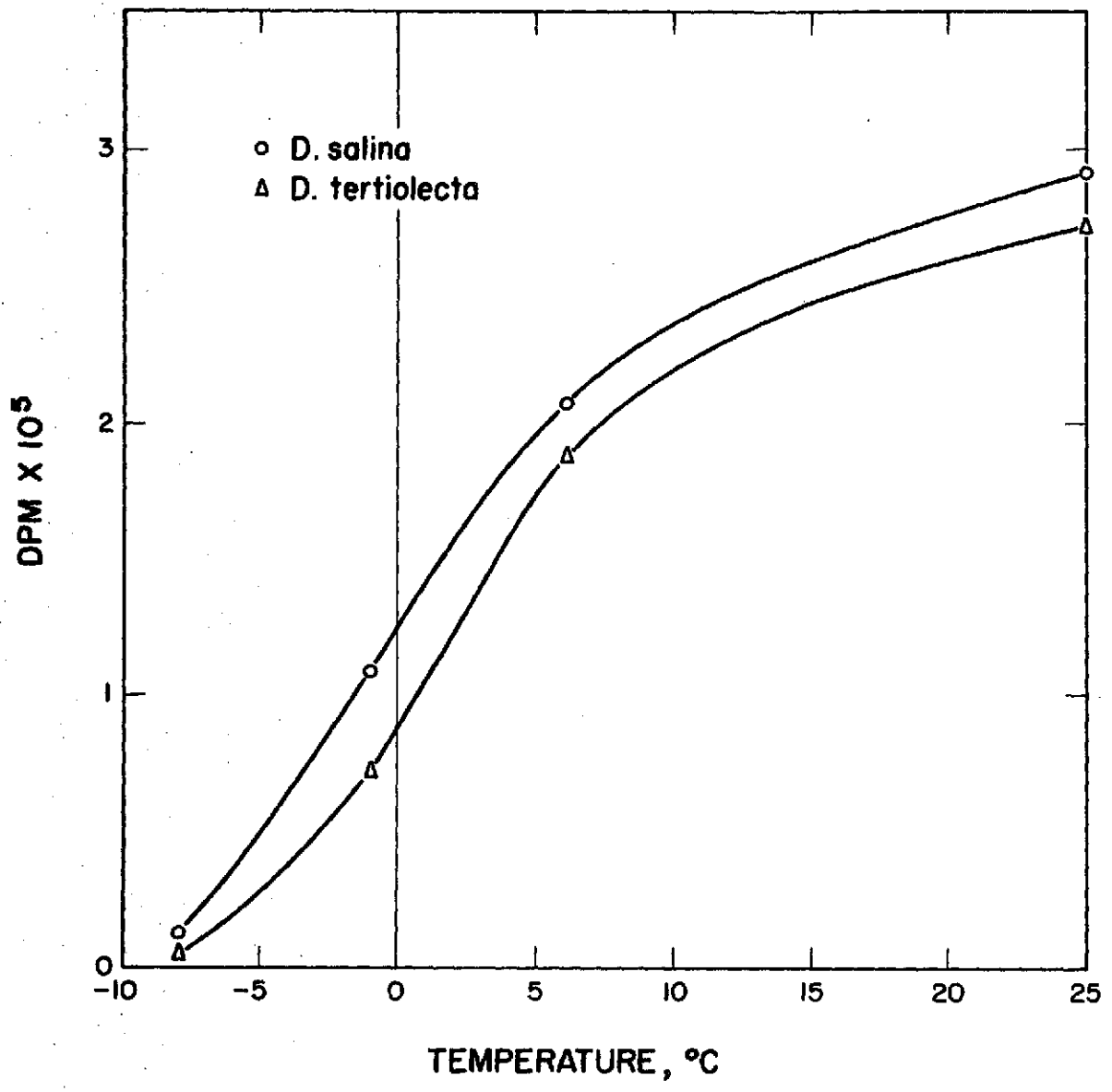
Figure 2. $^{14}CO_2$ reduction by *Dunaliella* in 150°/₀₀ NaCl at temperatures approaching -10°C.

of *Dunaliella.*

Dunaliella cells in mid-log phase growing in 2.5 M NaCl Hutner's divided into 12 aliquots per species and each of the below treatments were performed in triplicate for three weeks:

25° constant 3000 lux illumination

25° dark

-6° constant 3000 lux illumination

-6° dark

The following table indicates cell number before and after three week treatment.

		Viable cells/.1mm³		
		Pretreatment	Post-treatment	% change
D. salina				
	25° light	63	182.00 ± 28.00	288.8
	25° dark	63	4.00 ± 3.00	6.6
	-6° light	63	.55 ± 0.58	0.9
	-6° dark	63	26.60 ± 30.00	42.2
D. tertiolecta				
	25° light	31	63.00 ± 30.00	180.0
	25° dark	31	11.00 ± 6.00	31.4
	-6° light	31	13.00 ± 2.00	41.9
	-6° dark	31	6.50 ± 4.30	20.3

The increased low temperature survival in the dark for both species is most interesting. It was observed by R.T. Wilce (166) and other investigators that the intensity of light available to many Arctic deep water benthic marine algae is almost negligible (1-30 candle power) for most of the year, yet they managed to survive. He goes so far as to hypothesize facultative heterotrophy.

Viewing the Dunaliella low temperature survival findings, perhaps low light intensities, though they are not conducive to active metabolism (i.e., photosynthesis), do favor survival (preservation) before spring thaw.

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