ACTIVE PLANT GROWTH AT FREEZING TEMPERATURES*

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Abstract. As a foundation for speculations in exobiology, we are attempting to understand plant responses to extreme environments on Earth. We have emphasized active plant growth at low temperatures and in response to ultraviolet light. We have studied the winter environment in the mountains near Logan, Utah and have found several plants that grow under the snow. We have measured chlorophyll synthesis, carbohydrate levels, and ion balances in these plants and established field experiments with hardy and nonhardy varieties of wheat. In the laboratory we have studied characteristics of three enzymes in two wheat varieties, finding a number of interesting differences in response to ultraviolet and low-temperature treatments. We have also examined cell ultrastructures of three grass species subjected to a range of temperatures. Chloroplasts were most affected at low temperatures, but other organelles were also influenced. Studies of ion balances substantiate the suggestion from ultrastructure work that membranes may exhibit the primary responses to low temperatures. Cytokinins are also implicated in the cold response. We are presently emphasizing the investigation of membranes.

1. Introduction

Having no extraterrestrial life to study, the exobiologist must be content with speculations based upon terrestrial life forms. Since known solar extraterrestrial environments differ markedly from those on Earth, it seems logical to study Earth organisms that survive and/or grow under environmental conditions differing as much as possible from the Earth norm. In our research we have emphasized active growth of higher plants under temperatures at or below the freezing point of water, as well as response of higher plants to relatively high intensities of ultraviolet light. This paper emphasizes the low temperatures studies.

Our research has followed two approaches: (1) We have studied plants in the field, looking for examples of growth at low temperatures. We have studied mountain areas in the vicinity of Logan, Utah, as well as some alpine snow banks in the Colorado Rockies. (2) Having found examples of low temperature plant growth (either in the literature or by our own field searches), we have initiated laboratory studies to discern how these species can grow actively at temperatures that prohibit the growth of most plants. We have studied enzymes in plants that grow at low temperatures, cell ultrastructural responses to low temperatures, carbohydrate levels during growth under snow, changes in ion flux, chlorophyll synthesis, and we are now initiating a study of hormone effects and membrane characteristics.

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2. Field Studies: Environment

Our field studies have been carried out primarily at three locations: (1) We established plots of wheat (hardy and nonhardy) on a university farm south of Logan, Utah during the winter of 1968–69. (2) We established field plots near the Beaver Mountain Ski Area at about 2135 m elevation. Snow was removed for observation of the plants at intervals during the winter. (3) Similar studies were laid out in Franklin Basin in Logan Canyon. We have constructed a tunnel there that allows us to measure penetration of light through the snow and obtain samples of plants without removing the snow or disturbing the soil (Figure 1).

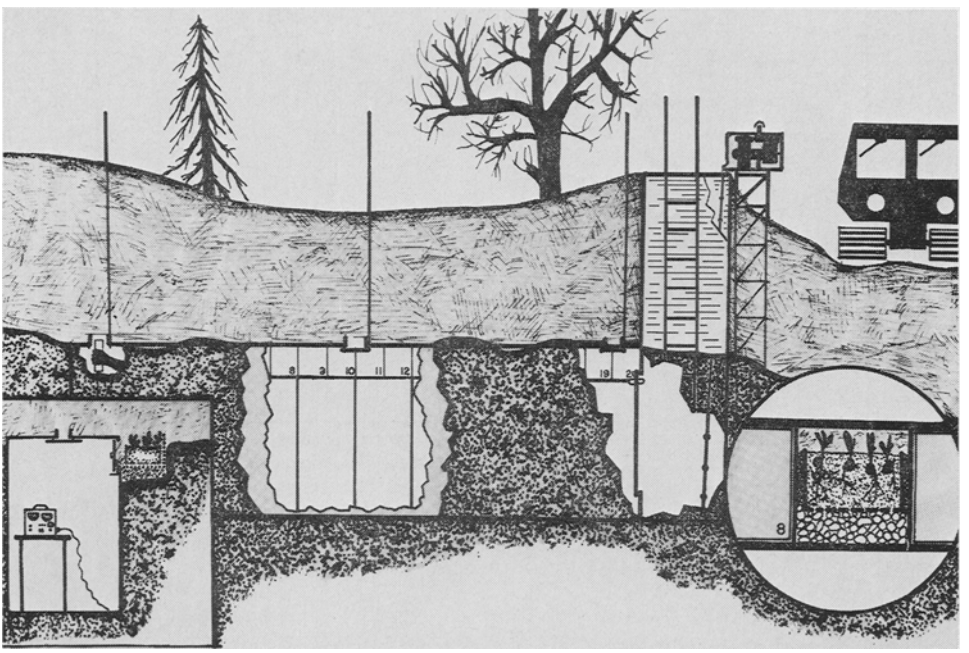

Fig. 1. The snow tunnel in Franklin Basin, east of Logan, Utah. The tunnel is entered through a tube of corrugated metal (right). Next to this is a generator under a protective covering. There are three ports in the ceiling of the tunnel through which light penetration of the snow can be measured. Depth of the snow at the ports is indicated by the vertical measuring sticks. Insert at lower left is a cross section of the tunnel, showing the balcony arrangement to the upper right, where metal boxes containing soil and plants can be removed at intervals during the winter for observation. Insert at lower right is a detail of one of these boxes. The boxes are placed on gravel so that they will not be frozen into place.

In our field studies we have measured the environment by placing thermocouples at various depths in the soil, snow, and air. We have attempted in various ways to measure light penetration of the snow and have carried out a few experimental procedures in the field, such as covering the surface of the ground with a thin layer of

black plastic or with a layer of insulating material, or attempting to heat the ground with electrical heaters (this so far not being very successful because of the huge heat capacity of the soil and snow).

Figure 2 summarizes temperature conditions as measured in one study. If the snow is late in coming, fall frosts may freeze the upper few centimeters of soil and drop the temperature to a few degrees below freezing. Soil at greater depths remains warm, however, and when the snow falls this heat is slowly transferred by conduction to the

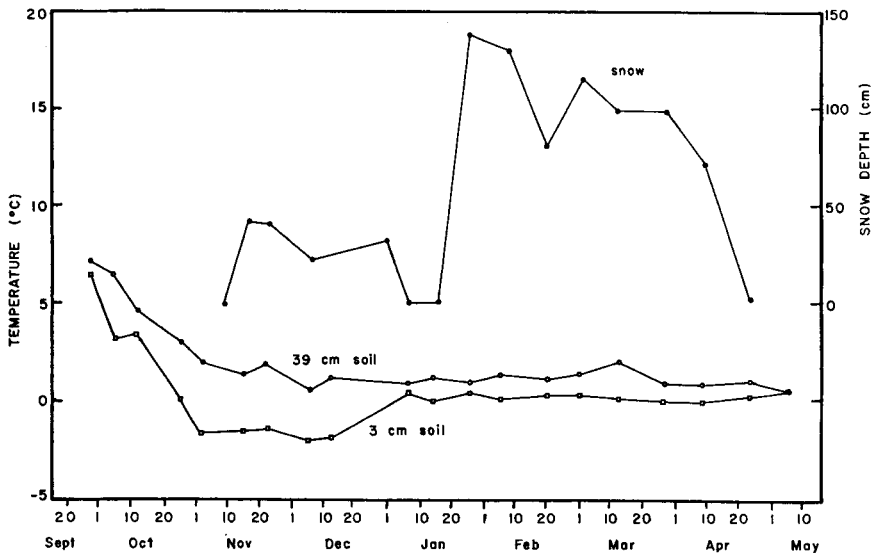

Fig. 2. Some representative temperatures along with snow depths at the Beaver Mountain study area during the winter of 1968–69. (Measurements of S. L. Kimball.)

soil surface so that the soil temperature rises to 0 °C at the point where it contacts the snow, being slightly higher below. It is conceivable that the snow could reach temperatures well below freezing, but in our measurements this has not occurred. The lower layers of snow and the upper layers of soil stay close to the freezing point during the entire winter until the snow melts off in the spring. Upper snow layers do sometimes drop below freezing, warming again on clear days.

Light penetrates the snow to depths of 2 m, as detected by the eye, although our instrumentation has so far not been sensitive enough to accurately measure these low light levels. Figure 3 shows results of spectral measurements through shallower depths of snow. In general, the blue and the green wavelengths penetrate best (as would be expected from the color of glacier ice, etc.), but this can be complicated by dirt and other impurities in the snow, so that under some conditions even red wavelengths may penetrate best.

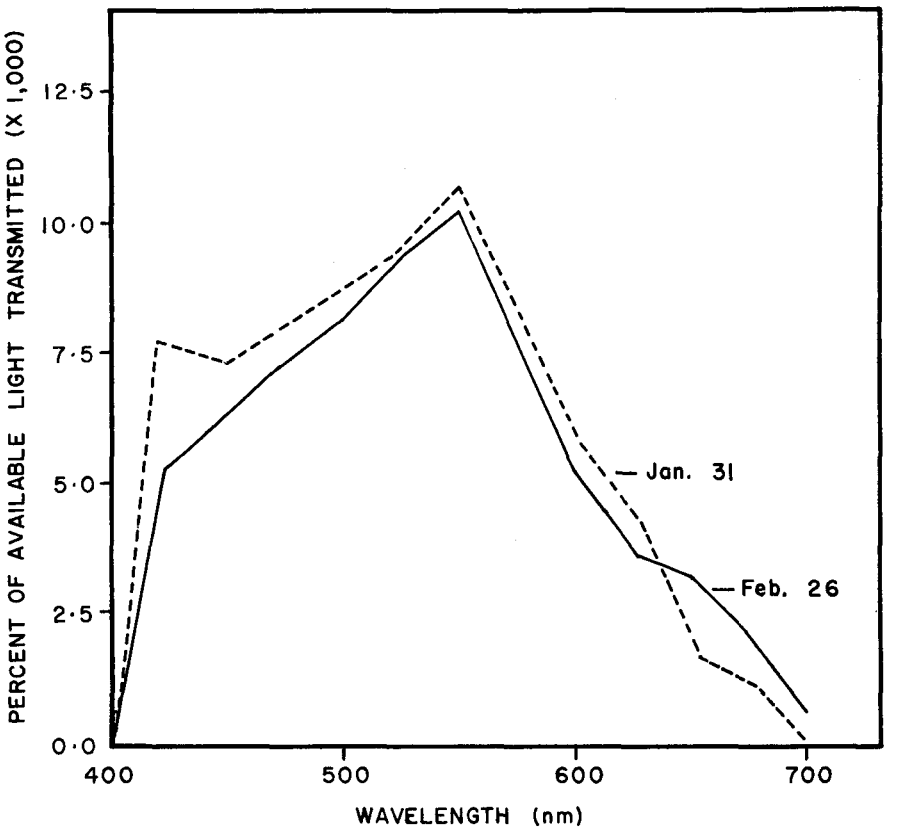

Fig. 3. Spectral distribution of light penetrating 69 cm of snow. Data are averages of 3 measurements and were obtained with an ISCO Model SR Spectroradiometer. (Measurements of B. Bennett.)

3. Field Studies: Plant Growth

A study of Brevor (winter-hardy) and Lemhi (nonhardy) wheat varieties (*Triticum aestivum* L.) during the winter months indicated that both could grow and develop during near-freezing temperature regimes (Kimball and Salisbury, 1971). Increases in numbers of roots, leaves, and tillers were considered as direct evidence for growth at the crown level. Each variety's ability to progress during low temperatures was proportional to its degree of seedling establishment. The Brevor variety reduced its activity somewhat more than the nonhardy Lemhi during the winter, but in early spring the Brevor was able to continue its modest activity, whereas the Lemhi frequently died back to ground level or completely succumbed.

Studies at the mountain research sites have revealed several species that grow slowly during the winter months. When the snow melts these flourish for a few weeks and then go into dormacy until the snow falls in autumn. These species are referred to as *spring ephemerals.* Most mountain species show no growth during the winter months and grow only slowly during the first few weeks after the snow has melted.

They reach their maximum growth in late spring and summer, completely overwhelming the spring ephemerals.

The spring ephemerals fall roughly into three broad classes, according to their behavior under the snow. The first group grows from underground storage organs through the few centimeters of soil between these organs and the surface, stopping their growth at the soil-snow interface. Representatives of this group that we have discovered include *Brodiaea douglasii*, *Lithophragma glabra*, *Orogenia linearifolia*, and *Erythronium grandiflorum* (in some of our observations). *Orogenia* may form complete flowers in the soil, these opening after the snow has melted and elongation of the shoot has raised them above the soil surface. *Erythronium* may also form complete flowers in the soil. The second category of species grows from underground storage organs

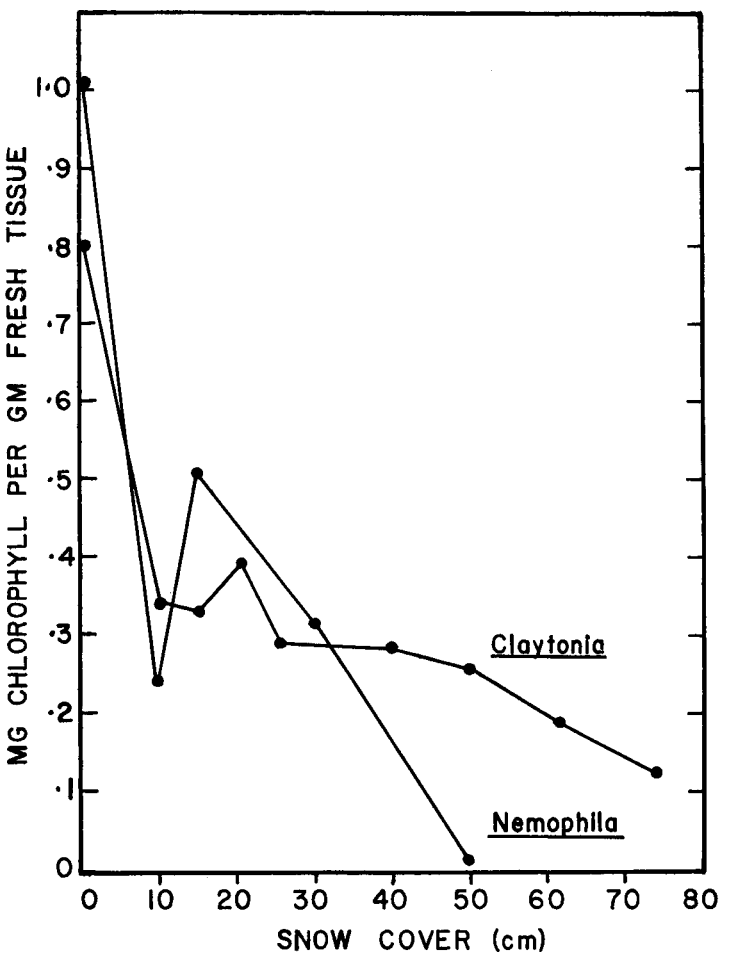

Fig. 4. Chlorophyll contents of two plant species as a function of snow depth. The 'out of line' point for *Nemophila* (10 cm) occurred in an area of relatively heavy shade. Chlorophyll contents were measured spectrophotometrically, using techniques outlined by Comar and Zscheile (1942). (Measurements by S. L. Kimball.)

through the soil and then into the snow. In our experience these species all form flowers in the snow. Our examples include *Claytonia lanceolata*, *Ranunculus jovis*, *Erythronium grandiflorum* (in some cases), and *Ranunculus adoneus* (in alpine snow banks–not our plots at lower elevations). The different species may exhibit different behaviors. *Claytonia* for example forms complete flowers with pollen, ovules, etc., but these flowers remain enclosed in two bracts that open only after the snow has melted. The *Ranunculus adoneus* flower, however, actually opens in the snow. The third category germinates from seed under the snow. We know of only one example: *Nemophila breviflora.*

In angiosperms (flowering plants) synthesis of chlorophyll from protochlorophyll depends upon light. We have often observed plants containing chlorophyll under considerable depths of snow. The question arises as to whether these have formed their chlorophyll in response to the light penetrating the snow, or if they constitute exceptions to the general rule applying to most angiosperms. Figure 4 shows results of a study in which chlorophyll contents of selected samples are plotted as a function of the depth of snow above the samples. As the snow depth decreases, chlorophyll contents increase. Samples were all collected at the same time but at different distances from the edge of the snowbank. The one point that does not fit the curve was collected

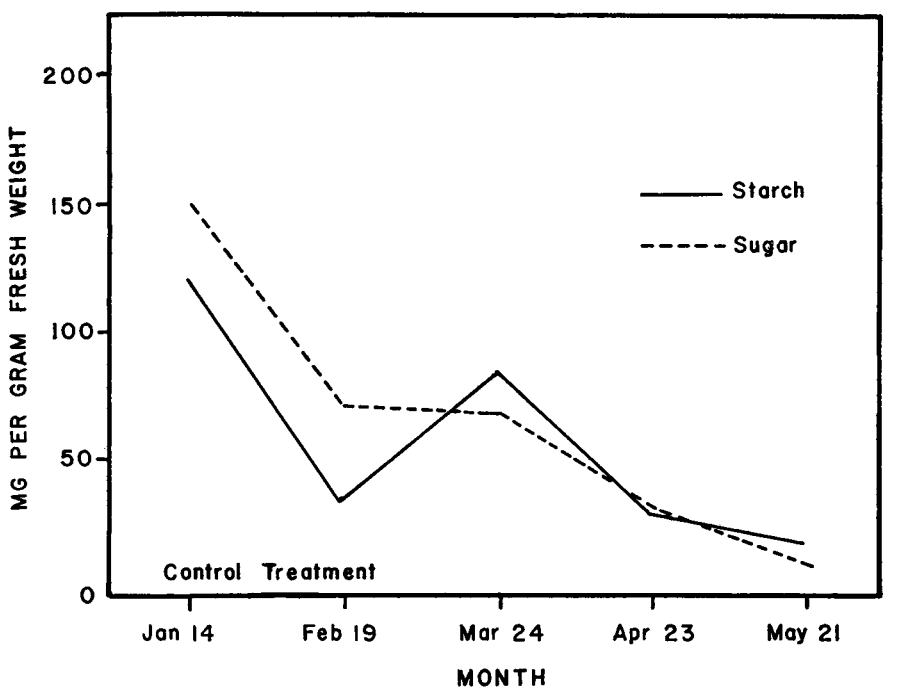

Fig. 5. Carbohydrate contents of *Claytonia lanceolata* as a function of time during the season. The increase of starch in March was replicated in several other sets of data. Carbohydrates were measured by the methods of McCready, Guggolz, Silviera, and Owens (McCready *et al.*, 1950 and Yemm and Willis, 1954). (Measurements of B. Bennett.)

in the shade of a tree. The implications are that chlorophyll development in this species (*Claytonia*) is dependent upon the light that penetrates the snow. Plants under black plastic did not contain chlorophyll except for *Nemophila* seedlings. Chlorophyll could have been present in their cotyledons before germination.

Carbohydrates were measured in Claytonia at monthly intervals during the winter of 1969–70. Results are shown in Figure 5. It is apparent that levels begin high, dropping during the winter as might be expected if growth under the snow is dependent upon carbohydrates stored in the corms. The increase in March is interesting if it proves to be real. It could imply photosynthesis under the decreasing depths of snow, or perhaps conversion of protein or fat to carbohydrate.

So far our attempts at field experimentation have been disappointing. Covering the surface with black plastic seemed to have little effect on total shoot elongation, although plants were colorless (except for seedlings of *Nemophila*). The most serious problem was that plants could not penetrate the plastic and were forced to grow horizontally beneath it. We intend to repeat this by providing a porous medium for the plants to grow into (e.g., perlite), and covering this with opaque material. Layers of insulation did cause slight increases in temperature (fractions of a degree), but no effect upon plant growth could be observed. The problem again is the vast heat sink provided by the more than 2 m of snow.

4. Laboratory Studies: Heat Transfer

For several years we studied heat transfer between the plant and its environment (Salisbury and Spomer, 1964; Drake *et al.*, 1970; Drake and Salisbury, 1972). The work is not directly related to the topic being discussed here, but it was found, for example, that plants growing at high elevations in the alpine tundra often have leaf temperatures as much as 20 °C above the ambient air temperatures (due to solar radiation), while plants at lower elevations may have leaf temperatures much closer to ambient air temperatures.

5. Laboratory Studies: Enzymes

Enzyme studies were carried out by Manfred Weidner. Ten-day-old seedlings (grown at 20 °C under continuous fluorescent light, 2000 ft-c) of a Canadian (Federation) and a Mexican (Pitic 62) selection of spring wheat show no differences in the activation energies (E_a) and the range of temperature stability for 1,5-ribulose diphosphate-carboxylase, pyruvatekinase, and nitratereductase that could be related to their different frost resistances. Figure 6 shows data for one variety. E_a values and temperature stabilities vary considerably for the three enzymes. RuDP-carboxylase is an 'inefficient' but highly stable enzyme: $E_a = 24000$ to 28000 cal, stable between 2.5 °C and 40 °C. E_a values for pyruvatekinase are in the average range: 11500 to 15500 cal. Nitratereductase has rather low activation energies ($E_a = 9000$ to 10000 cal) but only a narrow range of temperature stability. High-temperature denaturation occurs

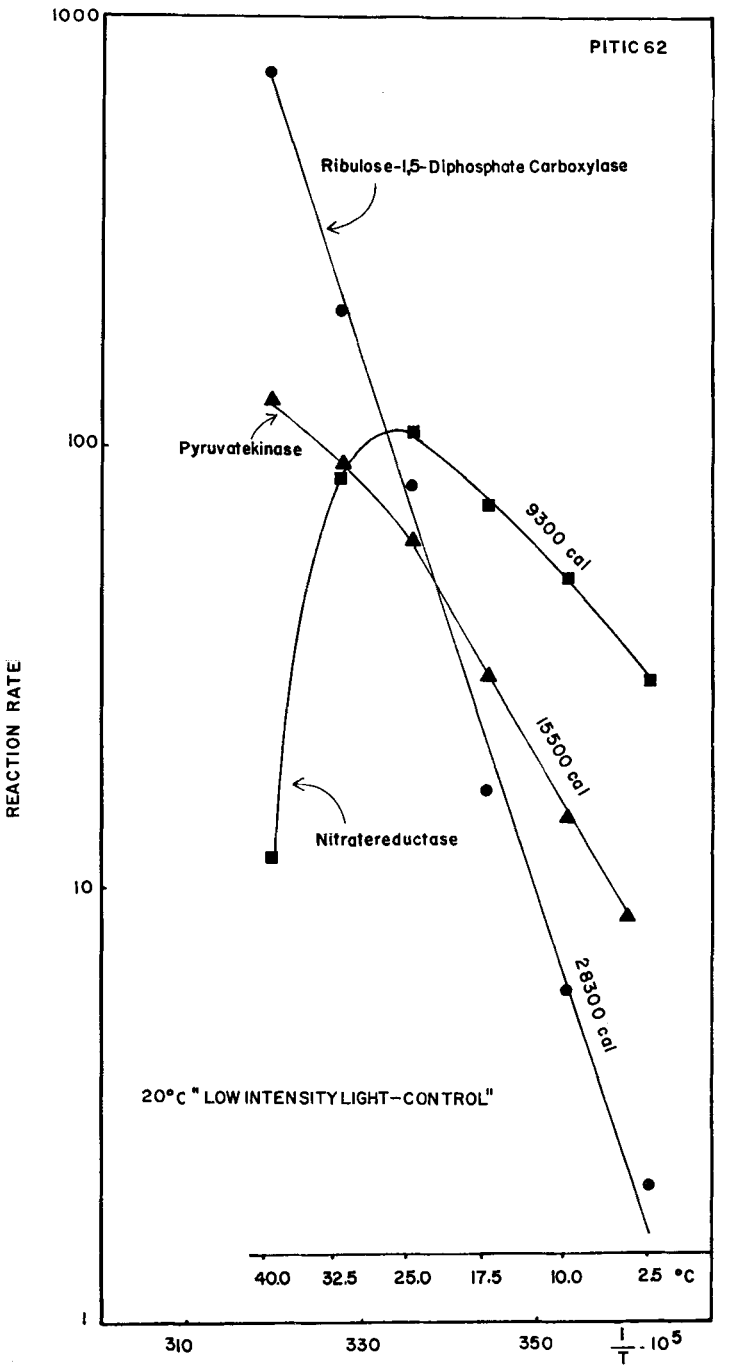

Fig. 6. Arrhenius curves for three enzymes. Note the stability of ribulose-1,5-diphosphate-carboxylase over the entire range of temperatures and the high temperature denaturation of nitratereductase, beginning at 25°C. (Measurements of M. Weidner.)

above 20–25 °C. These differences in E_a and temperature stability cause the relative enzyme activities to shift drastically at extreme temperatures. Beyond certain limits, the regulatory mechanisms may no longer succeed in keeping cell metabolism in balance. Resulting metabolic disorders could cause multiple damage to the organisms, but cold-adapted plants must avoid this.

We simulate solar radiation beyond the atmosphere with a 6 KW Osram xenon arc (intensity 1.5 cal cm^{-2} min^{-1}). Corning 9-54 filters exclude shortwave UV that otherwise produces ozone. Longwave UV is transmitted in this treatment, symbolized: V+UV. Window glass excludes UV totally in control experiments (treatment symbolized: V). V+UV-irradiated leaves have pale and brown spots and eventually die.

V+UV has no effect on E_a and temperature stability of RuDP-carboxylase. In the case of pyruvatekinase, E_a seems to be a somewhat more labile parameter, at least

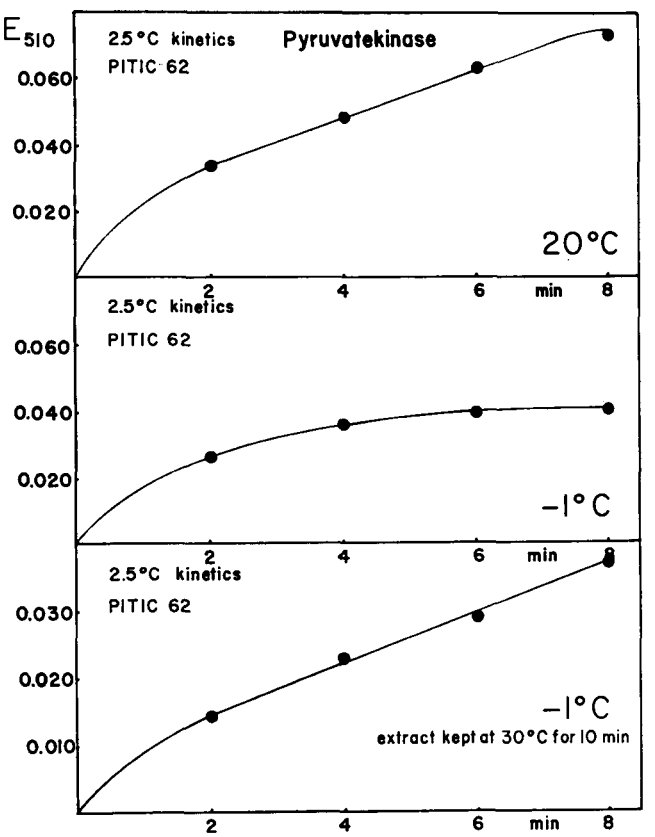

Fig. 7. Kinetics for the reaction controlled by pyruvatekinase as a function of temperatures at which the plants were grown, and at which the measurements were made. Pyruvatekinase from plants grown at −1 °C but measured at 2.5 °C shows decreasing activity with time (virtually no reaction after 6 to 8 min). The same enzyme shows a normal kinetics after the extract has been held at 30 °C for 10 min. The low temperature denaturation is thus readily reversed. (Measurements of M. Weidner.)

in Pitic 62, where both V + UV and V effect a slight decrease in the activation energy, V + UV being more efficient. The temperature stability is unchanged. The E_a for nitratereductase is significantly reduced (to 7000 cal) by V + UV in comparison to V, but only in Federation, not in Pitic 62.

In plants grown at −1 °C under low intensity light for 48 h, activation energies and ranges of enzyme stability of RuDP-carboxylase and nitratereductase are not different from 20 °C control plants. Pyruvatekinase Arrhenius curves, however, show an inflection point at about 10 °C, indicating low temperature denaturation of this enzyme in both wheat varieties. In order to obtain further information about this effect, pyruvatekinase kinetics were obtained at 2.5 °C and 15 °C for plants exposed to both 20 °C and −1 °C. The kinetics at 15 °C are identical (linear) for both plants, but kinetics at 2.5 °C show abnormalities. In cold treated plants, the reaction rate decreases rapidly, approaching 0 after 8 min. Control kinetics begin the same way but assume linearity after approximately 2 min. An extract from −1 °C plants exhibits kinetics at 2.5 °C that are identical to kinetics for control plants (20 °C) – providing the extract is held for 10 min at 30 °C (Figure 7). This indicates that the 'broken' Arrhenius curve does not consist of two sections as it might appear. Rather, an inactivation of pyruvatekinase occurs over the entire temperature range, and this is reversed immediately during the assay at temperatures above 10 °C.

Km data for RuDP-carboxylase have been determined for HCO_3^- (at 17.5°C). Plants grown at 20 °C, 5 °C (48 h), and −1 °C (48 h) were investigated. Linear Lineweaver-Burk plots were obtained for both varieties at 20 °C (Km-values about 3×10^{-3} M). At 5 °C in Pitic 62 the curve was unaffected, but in Federation the substrate capability of the enzyme was drastically reduced, only high substrate concentrations yielding reaction rates close to expectation. The Lineweaver-Burk curve is bent sharply upward. At −1 °C Pitic 62 also shows this breakdown of HCO_3^--binding capacity. In Federation the effect was enhanced at −1 °C: no CO_2 fixation occurred within the whole range of HCO_3^--concentrations used in these experiments. Perfectly normal reactions took place, however, when 10 times higher substrate concentrations were applied. Pyruvatekinase activity per seedling increased under V + UV to the same level as in controls, but a −1 °C treatment markedly reduced the enzyme. RuDP-carboxylase activity is considerably diminished by both V + UV and −1 °C. Enzyme activity values are not valid for nitratereductase, because the amount of NO_3^- in the culture medium cannot be kept constant (due to bacterial infestation), and nitrate levels strongly influence enzyme levels.

6. Laboratory Studies: Cell Ultrastructure

Except for the enzyme work, the most extensive laboratory studies so far involved an ultra-structural study of 3 species of grasses carried out by Steven Kimball. Species were chosen for their varying degrees of cold hardiness. *Secale cereale* (rye) is highly cold resistant, recovering completely from exposures to 0° (but under our nonhardened conditions not from exposures to −5°); *Cynodon dactylon* (Bermuda grass) is killed

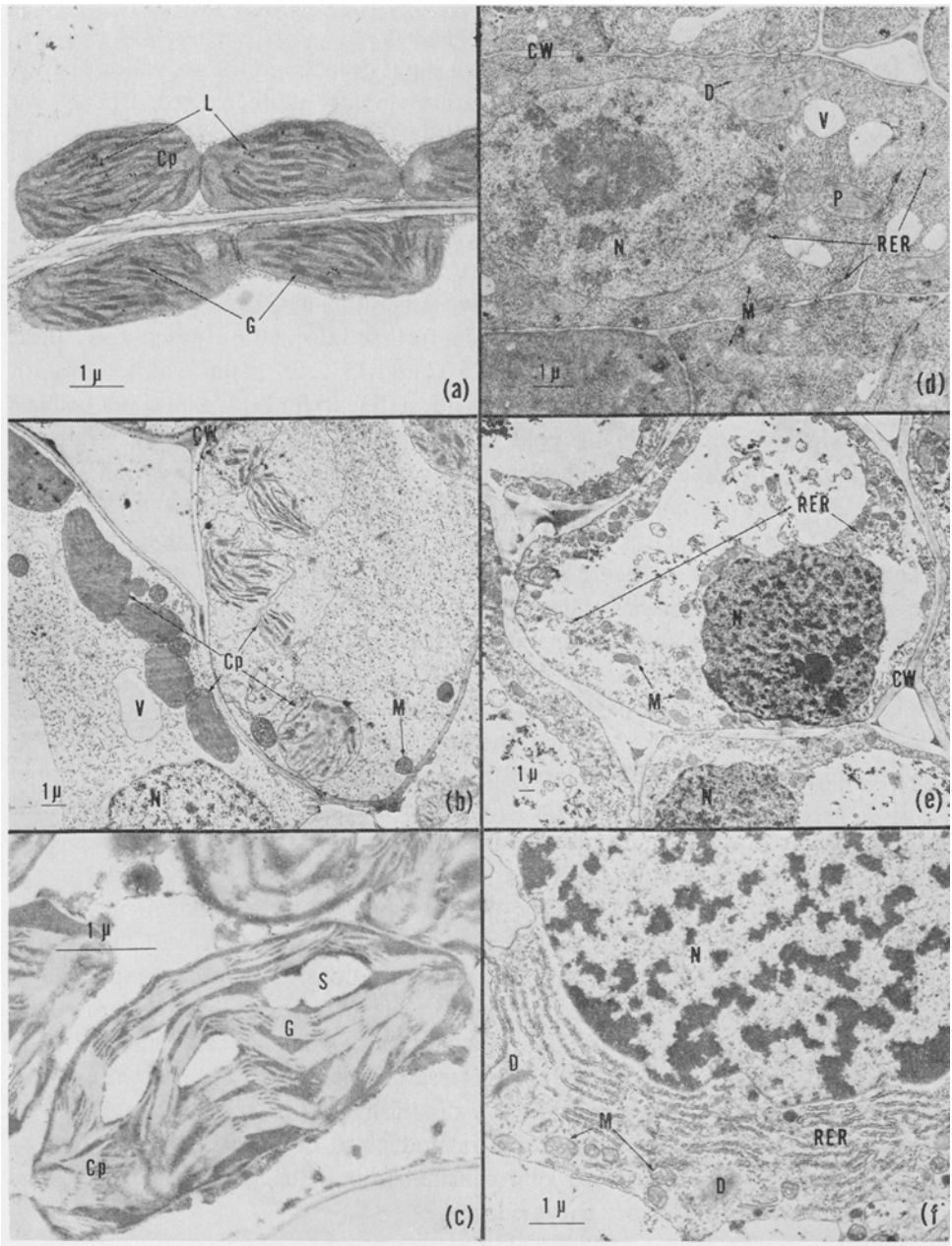

Fig. 8. Some representative electron micrographs of plants used in the low temperature study. Magnifications are indicated by horizontal lines (1 μ) on the photographs. (a) Leaf tissue of *Secale cereale*, grown at 25 °C. These are control plants. (b) *Secale cereale* leaf tissue grown at 0 °C. Note swelling of the chloroplasts in the cell on the right, but fairly normal appearance in the cell on the left. (c) *Secale cereale* leaf tissue grown at − 5 °C. Note negative staining of chloroplast internal lamella. (d) *Paspalum notatum* crown tissue grown at 25 °C. These cells do not contain chloroplasts but represent the 'typical' nonphotosynthesizing plant cell. (e) *Secale cereale* crown tissue grown at 0°C. Note vacuolar debris. It appears that the vacuolar membrane might have ruptured, allowing cytoplasmic organelles to drift into the vacuole. (f) *Secale cereale* root tissue grown at 0 °C Note rough endoplasmic reticulum laying parallel to the nuclear membrane. Key: Cp = chloroplast; L = lipid (droplet); G = granum; CW = cell wall; M = mitochondrian; V = vacuole; S = starch; RER = rough endoplasmic reticulum; D = dictyosome; P = plastid (immature); N = nucleus. (Photographs by S. Kimball.)

to an extent of about 10% of the plants in a population by exposure to 0 °C; and *Paspalum notatum* (Bahia grass) is almost completely killed (90%) by exposures to these temperatures. In the ultrastructure study, species were exposed to temperatures of −5°, 0°, 10° and 25 °C for 3 days (originally having been germinated at 25 °C for a period long enough to insure 2 mature leaves or 3 to 5 roots). After treatment, samples of leaf, crown, and root tissue were collected and prepared for examination with the electron microscope. Samples were collected from at least 2 plants on two or more occasions for each temperature treatment, and a minimum of 40 electron photomicrographs were made of typical cells from each treatment. The controls at 25 °C were repeated for each treatment and each replication. Treatments were always compared to their respective control counterparts. Some representative electron micrographs are shown in Figure 8.

Chloroplasts proved to be the most easily affected organelles in all three species, with plastids from the hardy species (rye) showing less clustering but considerably more individual swelling at colder temperatures than either of the less hardy species (Bermuda and Bahia grass). The internal lamella of all chloroplasts became increasingly disoriented as treatment temperatures were lowered. At −5 °C, the plastid lamella of Bermuda grass were greatly twisted or folded, but in Bahia grass they were somewhat less distorted but negative in contrast (i.e., membranes appeared as white instead of the typically black lines). The progressive degrees of disorientation may indicate a declining metabolism within the chloroplasts. The reverse staining was even more apparent in rye than it was in the mature chloroplasts of Bahai grass, but Bermuda grass exhibited the negative contrast only in a few young plastids found in the crown tissue. This effect could be due to dehydration of the membranes by freezing or to changes in the fatty acid composition in the membranes.

All three species showed increased rough endoplasmic reticulum (RER) at lower temperatures. The RER became oriented parallel to either the plasma membrane or the nuclear membrane as it increased. Such changes indicate an increased potential for protein synthesis – something that is known to occur as plants harden.

Mitochondria and ribosomes did not change significantly with temperatures in any of the species, but dictyosomes decreased in all three species as temperature was reduced. Cellular debris within the vacuoles of all three species increased with reduced temperatures, the temperate species again showing greater response than the tropical species.

7. Laboratory Studies: Membrane Phenomena

It has been difficult in our work to distinguish between direct cold temperature endurance mechanisms and dormancy. Probably the membrane is involved in direct endurance mechanisms, as has been noted previously (Mazur, 1969). Effects on ion balance between the cell and its environment provide one way to study any protective change in the membrane. It is possible that the overall response of the plant to low temperatures at this level is part of a general syndrome characterizing plant reponses to a wide variety of stressors. Analogies with other biological systems might lead one

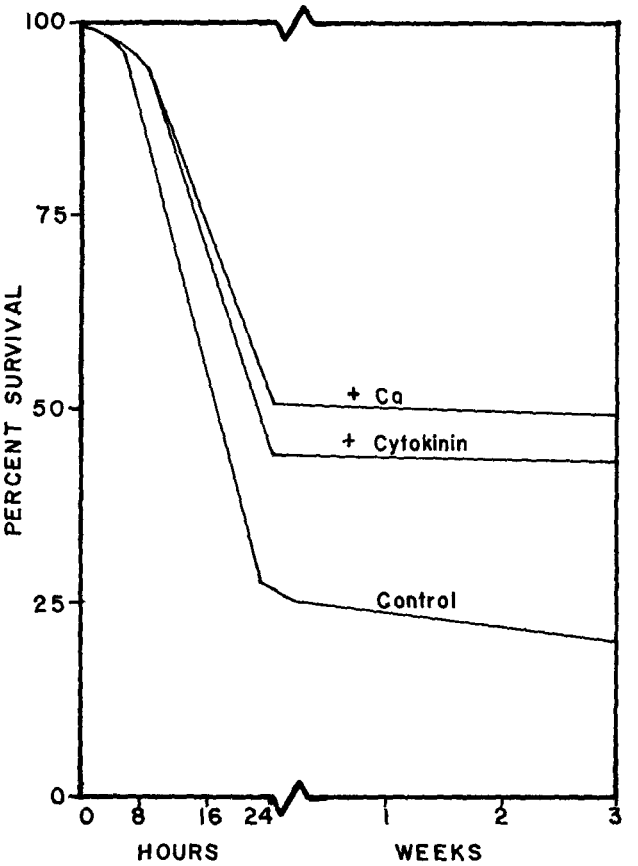

Fig. 9. Survival of *Axonopus affinis* plants subjected to -5 °C following treatment with calcium or kinetin (6-Furfurylaminopurine) solutions. (Experiment of P. Rosen.)

to believe that this system is influenced by hormones, and indeed plant cytokinins have been implicated (Kuraishi *et al.*, 1966; Lang, 1967).

Figure 9 shows that kinetin (6-Furfurylaminopurine) applied to the soil solution in concentrations of 2×10^{-5} molar protects plants from cold (mortality measured by visual examination for leaf color, green or brown; experimental plant, *Axonopus affinis*, had only one small leaf so that there were few borderline cases). Although the well-known cytokinin protection of senescing chloroplasts (through protein synthesis – Osborne, 1962) could be involved here, our results indicate that it is not. Kinetin-treated plants showed increased lipid synthesis, but this was not correlated with increased resistance to stress.

Responses to excess calcium salts applied to the soil (instead of kinetin) are also shown in Figure 9. (Mortalities are not comparable to cytokinin treatments due to the greater suitability of this method of application for calcium.) The idea that this protective action of calcium involves a direct membrane reaction is supported by experimental work in a number of converging areas, including the antagonisms of

monovalent and divalent cations occurring at the membrane (Nieman and Willis, 1971), the *in vitro* binding of lipo-protein films by calcium for greater structural integrity (Hanahan, 1960), and the effects of cation flux on metabolism as related to the chemiosmotic theory of energy production (Packer *et al.*, 1970).

Formation of edaphic ecotypes with different calcium uptake characteristics has been widely studied as a prime example of evolutionary processes over short distances

TABLE I

Contents of divalent cations in *Claytonia lanceolata*, measured after nitric acid digestion of tissue, by atomic absorption spectrophotometry

	Per cent Ca^{++}			Per cent Mg^{++}		
	Mountain		Valley	Mountain		Valley
	immature	mature	mature	immature	mature	mature
TIP	15	35	11	23	23	5
LEAF	6	4	6	13	35	25
STEM	34	46	48	48	28	32
CORM	45	15	35	16	14	38

in short periods of time. This is thought to depend directly on changing configurations of the proteins with which calcium binds for transport (Jeffries *et al.*, 1969). The data of Table I show that calcium accumulates in the tips of plants in the mountain environment (in which meristematic cells are actively dividing at 0 °C) as compared to magnesium ion and to plants in the milder valley environment.

8. Future Studies

We are continuing our field studies. We now have the instrumentation to measure extremely low light intensities, so we hope to obtain better data this winter relating to light penetration of the snow. We also hope to continue the experimental field approach, using thicker insulation, etc.

The electron microscope study needs to be extended, particularly by collecting samples at various times after specimens have been placed in the cold, and at various times after they have been returned to more suitable growing conditions. We need to know something about the development of the symptoms described above and about recovery from these symptoms.

We feel that the work so far indicates that an understanding of membrane structure may be crucial to understanding active growth of plants at low temperatures. The membrane is the interface between the cell (or organelle) and its environment. It could be a crucial region for stress response. Thus we intend to intensify our laboratory efforts, emphasizing membranes. We want to study not only their ultrastructure, but also their composition, permeability, etc. Isolated mitochondria and chloroplasts will be used.

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