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Cold Acclimation of *Hedera helix*

EVIDENCE FOR A TWO PHASE PROCESS

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ABSTRACT

The light-enhanced production and accumulation of sugars is only one step in the process of cold acclimation in *Hedera helix* L. var. Thorndale (English ivy). Applications of 2,4-dinitrophenol to plants with different portions exposed to light and dark indicated that the mere presence or accumulation of the light-generated promoters did not invoke an increase in hardiness. Kinetics of cold acclimation during alternating periods of light and dark also indicate that the light stimulation of cold acclimation is only a partial component of the total process. Incubation on 50 mM solutions of sucrose can replace the light requirement. A second phase which can proceed in the dark is thought to result in the production of proteins which, due to an altered composition or configuration, have a greater capacity to bind sugars. This is evidenced by the fact that protein from cold acclimated tissue exhibited a higher sugar-binding capacity than protein from nonacclimated tissue. Furthermore, the two phases can proceed independently of each other, but only upon complementation of the products of the two phases is an increase in cold hardiness manifested.

Many environmental factors influence the cold acclimation process, and of these, light appears to be as fundamental as low temperature in inducing maximal hardiness of many species (2, 7). Previously, light has been shown to greatly enhance the rate and degree of cold acclimation in *Hedera helix*, but it is not essential for the cold acclimation process (14). The light stimulation results in the production of translocatable promoters of hardiness and is not involved in the promotion of some predisposing condition (13). Attempts to characterize the light-generated promoters through fixation of $^{14}CO_2$ in an illuminated donor portion and subsequent translocation of the ^{14}C-labeled products to a darkened receptor portion indicated that the translocatable promoter was sucrose (13). Furthermore, sucrose is the most abundant carbohydrate accumulating during cold acclimation, and hardiness of leaves of *H. helix* can only be significantly increased by sucrose solutions and not by equimolar solutions of glucose, galactose, or mannitol (13, 15).

Although the light stimulus results in an accumulation of photosynthates in *Hedera* and in other species (9, 16, 17), it is doubtful that cold acclimation is an accumulation of photosynthates *per se*. Increases in sugar content during cold acclimation are well documented (7). And, although Heber (4) concludes that the protective influence of sugars is due to their capacity to retain or substitute water via hydrogen bonding in structures sensitive to dehydration, demonstration of a causal relationship is lacking.

MATERIALS AND METHODS

Method of Cold Acclimation. Unless otherwise stated, the standard procedure for artificial cold acclimation of *Hedera helix* L. var. Thorndale (English ivy) was as previously described (11). Plants, grown from cuttings in a greenhouse for 3 to 6 months, were placed at 5 C for a period of 6 weeks. During this time, light was provided by a combination of cool-white fluorescent lamps supplemented with incandescent lamps. An intensity of 600 ft-c at plant level was maintained during an 8-hr photoperiod.

In some instances, sucrose was substituted for the light requirement. This was accomplished by severing the vines from the root system and trimming them to eight leaves per vine. The vines were then placed in a growth chamber at 26 C in water in the dark for 3 days to reduce endogenous levels of sugars and starch reserves. The stems were then placed in the appropriate solutions and maintained in a dark growth chamber at 3.5 C. Where noted, this procedure was slightly modified by using cuttings consisting of one leaf and one internode.

Method of Freezing and Thawing. Samples to be frozen were first wrapped in aluminum foil and then placed in insulated boxes. The samples were then placed in a freezer at −6 C. After the temperature inside the insulated boxes reached −6 C, one box remained at this temperature for 2 hr and the remaining boxes were transferred to a freezer maintained at −12 C. The process continued with freezers maintained at 6 C increments. The temperature fall resulting from this procedure was approximately 3.5 C per hour. Air temperatures inside the insulated boxes were recorded at 2.5-min intervals. Following the 2-hr period at the desired temperature, the insulated boxes containing the frozen samples were removed to a 5 C cooler and allowed to thaw. Tissue survival was determined the following day.

Method of Testing Survival. The killing temperatures of tissue samples following freezing were determined by the refined triphenyl tetrazolium chloride test (12). All killing points are the means of three replications.

Kinetics of Cold Acclimation. Studies of the kinetics of cold acclimation following alternating periods of dark will indicate whether cold acclimation is solely a one step accumulation phenomenon. Proportional increases in the amount of light should invoke proportional increases in hardiness if cold acclimation is only the accumulation of cryo-protective substances produced in the light. However, this has not always been effectively proven (9). In attempting to show such a response, the usual procedure has been to vary the light intensity or duration of light, but with the latter approach difficulties arise due to photoperiodic effects. These difficulties can be overcome by

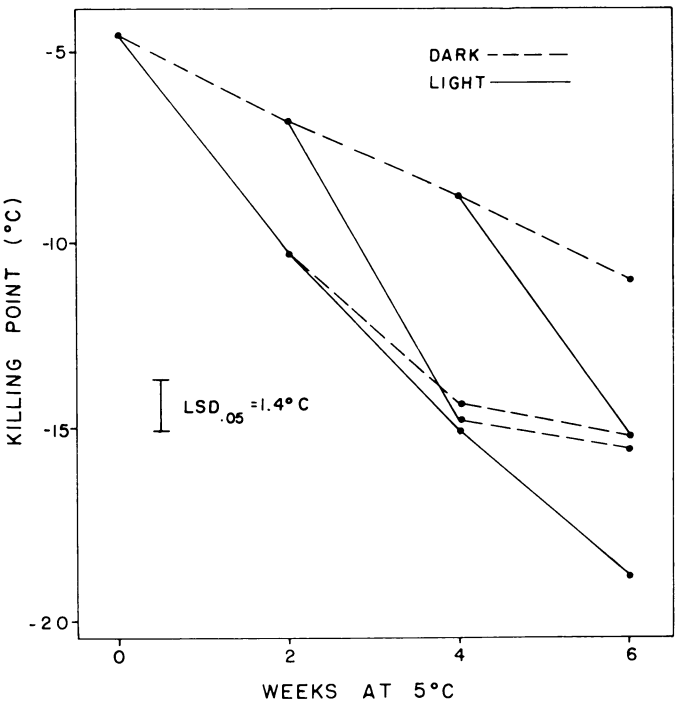

FIG. 1. Cold acclimation of leaves of intact plants following various periods of light and dark at 5 C.

maintaining the light intensity and photoperiod while manipulating the number of days the plants are exposed to the light regime. Groups of plants were placed in either the light (8-hr photoperiod, 600 ft-c) or in the dark at 5 C for a period of 6 weeks. Other groups of plants were exposed to an 8-hr photoperiod for only 2 weeks, either in the initial, mid, or terminal 2-week period of the 6-week cold acclimation period. Hardiness was determined after each 2-week period.

A similar experiment was conducted in which individual cuttings consisting of one leaf and one internode were used instead of intact plants, and the light period was replaced by incubation in 50 mM solutions of sucrose in the dark.

Translocation of Hardiness Promoters. Upper portions of intact plants were covered with aluminum foil to exclude light; the illuminated portion was considered the donor of the light-generated promoters and the darkened portion the receptor (13). The plants were then exposed to an 8-hr photoperiod at 5 C to induce cold hardiness. Foliar applications of 1.0 mM DNP[1] + wetting agent were made to various portions of the plants after 0, 2, and 4 weeks under the above conditions. Treatments were as follows (base-light top-dark): −DNP/−DNP, −DNP/+DNP, +DNP/−DNP, and +DNP/+DNP. Hardiness of leaves from the individual portions was determined after 6 weeks.

The influence of DNP on translocation and accumulation of photosynthates produced in the illuminated donor portions was determined in plants fed $^{14}CO_2$. Upper portions of plants were covered with aluminum foil, and the plants were placed under the standard acclimation conditions for a period of 2 weeks. The upper darkened portions were then immersed in 1.0 mM DNP + wetting agent solutions for 60 sec and then recovered with aluminum foil. The following day the plants were exposed to $^{14}CO_2$ (supplied by $Ba^{14}CO_3$; 5.0 mc/mM) for 6 hr at 5 C in a sealed Plexiglas chamber. Upon termination of the feeding period, three plants from the control group and three plants from the DNP treatment were immediately frozen and retained for radioassay. The remaining plants were returned to the 5 C chamber for continuation of the acclimation process, and further samples were frozen and retained for radioassay after an additional 1, 5, and 10 days of cold acclimation.

Samples were separated into leaves and stems from both the illuminated and darkened portions of individual plants. Leaves were lyophilized, ground in a mortar and pestle, and 100-mg samples of the fine powder were placed into scintillation vials to which 2.5 ml of 80% ethanol were added. After 15 hr, the extracts were exhaustively extracted with petroleum ether (density 0.60–0.70) until they were clear. The extracts were then taken to dryness under reduced pressure and re-extracted with 2 ml of absolute ethanol for 2 hr. Scintillation fluid, 2,5-*bis*-2-(5-*tert*-butylbenzoxazoly)-thiophene in toluene (4 g/liter), was added, and the vials were vigorously mixed. Within 30 min the sediment settled to the bottom of the vial, and counting was initiated in a liquid scintillation spectrometer.

Measurement of Protein-Sugar Binding. To detect protein-sugar binding a procedure using gel filtration analogous to dialysis equilibrium was utilized (5). Acetone powders of leaves were prepared for plants exposed to artificial cold acclimation conditions for 0, 2, 4, and 6 weeks. The acetone powders were then extracted in 0.01 M tris-HCl buffer, pH 7.2, and centrifuged at 20,000*g*. The resultant pellet was resuspended in buffer and recentrifuged twice. The washed 20,000*g* pellet (resuspended in buffer) and the combined supernatants, respectively referred to as the particulate and soluble fractions, were then subjected to ammonium sulfate fractionation. Protein precipitating with 75% concentrations of ammonium sulfate was dialyzed against 0.01 M tris-HCl, pH 7.2, for 72 hr. Following dialysis the samples were lyophilized. Samples (10 mg) of the lyophilized residues were dissolved in 0.5 ml of phosphate buffer (0.05 M KH_2PO_4, pH 5.00) containing either ^{14}C-glucose (6.5 μM, 4.88 mc/mM) or ^{14}C-sucrose (6.6 μM, 5.0 mc/mM) and incubated at 2 C for 1 hr. A 0.4-ml aliquot of this solution was then placed on a 5.0- × 1000-mm column of Sephadex G-25 (coarse) which was equilibrated with the same ^{14}C-sugar solution used to dissolve the protein. The column was eluted at a flow rate of 12 ml/hr with the same ^{14}C-sugar-phosphate buffer, and 0.4-ml fractions were collected at 2 C. After elution, 0.3-ml fractions were pipetted into aluminum planchets and counted in a thin window counter. Three milliliters of distilled H_2O were added to the remainder of each fraction and the A_{280} values were determined.

RESULTS

Kinetics of Cold Acclimation. Light greatly enhanced the rate of cold acclimation (Fig. 1), but at no sampling period was the amount of hardiness attained by plants receiving only 2 weeks of light directly proportional to the hardiness of control plants receiving 6 weeks of light. That is, after 4 weeks the increase in hardiness of plants receiving only 2 weeks of light was not 50% of the increase exhibited by plants receiving 4 weeks of light; nor was the expected proportion of 33% exhibited after 6 weeks. For clarity of discussion the various portions of the treatments will be referred to as L and D, where L and D refer to a 2-week period of light and dark, respectively. Plants that received a period of dark following a period of light (treatment L-D), exhibited a rate of cold acclimation during the dark period which was greater than the rate of cold acclimation during the corresponding dark period of the dark control (treatment D-D). In treatment D-L, the rate of cold acclimation during the light period was greater than the rate exhibited during the corresponding light period in the light control (treatment L-L). This was also true when the light period of the D-D-L treatment was

[1] Abbreviation: DNP: 2,4-dinitrophenol.

compared to the corresponding light period of the light control (treatment L-L-L). Thus, when a dark period follows a light period there appears to be a carryover effect of the previous light period allowing for a faster rate of cold acclimation during the dark period. When a light period follows a dark period there is a faster rate of cold acclimation during the light period in comparison to the appropriate control, indicating a more efficient utilization of the light-generated promoters.

If cold acclimation was solely the accumulation of the light-generated promoters, hardiness attained during the dark periods should have been equal and independent of the previous light regime. Also, hardiness should have been proportional to the amount of light received, but at no sampling period was this evident.

When incubation on sucrose solutions was substituted for the light periods, similar results were obtained (Fig. 2). In this case, cuttings consisting of one leaf and one internode were prestarved at 26 C for 3 days and then placed in vials containing either 50 mM sucrose or water at 3.5 C for 6 weeks. As in the case of the experiment with various light periods, there was an accelerated rate of cold acclimation in the presence of sucrose if this period was preceded by a period at low temperatures in the absence of sucrose. Similarly, there was a carryover effect of the previous sucrose treatment into the following incubation period at low temperatures without sucrose which was manifested as an accelerated rate of cold acclimation.

Separation of the Two Phases. To further substantiate that the cold acclimation process is more than an accumulation of sucrose, cuttings consisting of eight leaves were incubated in various concentrations of sucrose during exposure to low temperatures in the dark. The stems were recut and sucrose solutions changed at 3-day intervals during the 6-week period. Small increases in hardiness were elicited by increasing the concentration from 1 mM to 25 mM (Fig. 3). The largest increase in hardiness was elicited by increasing the sucrose concentration from 25 mM to 50 mM. However, further increases up to 250 mM failed to significantly increase the hardiness. Cuttings incubated in concentrations higher than 250 mM had plasmolyzed areas around the veins and were not considered to give reliable viability measurements, although when care was taken to sample nonplasmolyzed areas for viability their hardiness was no different from tissues incubated in sucrose concentrations of 50 mM to 250 mM. While the data do not completely eliminate the idea that increasing sugar concentrations increase cold hardiness, the concentration range where this is manifested is considerably below that normally encountered in acclimated tissue (8% fresh weight) (15).

An attempt was made to separate the sugar accumulation phase from the second phase. If the two phases proceed independently of each other this might be demonstrated by incubating the cuttings at low temperature on water and on progressive days changing them to sucrose solutions. If the presence of sucrose was a prerequisite for the second phase, then the cold hardiness should be proportional to the time exposed to the sucrose and independent of the total time at low temperatures. Alternatively, the second phase may be only a partial component of the cold acclimation process which can proceed in the absence of sucrose but is only manifested as an increase in hardiness when sucrose is present. In this case, the cold hardiness determined at the end of the total period should be the same regardless of when the sucrose is added.

Cuttings which were incubated in sucrose for the terminal 48 hr of the 21-day period were equal in hardiness to those incubated in sucrose for 4-, 7-, 14-, or the entire 21-day period (Fig. 4). An appreciable amount of hardiness was also elicited by incubating the cuttings on sucrose for only the final 24 hr of the 21-day period. If cuttings which had no previous low temperature treatment were incubated on sucrose solutions for 24 or 48 hr, no appreciable increase in hardiness could be detected. Furthermore, if cuttings were incubated continuously on sucrose, the increase in hardiness was linear throughout the 21-day period. Clearly, the process of cold acclimation is composed of two phases which can proceed independently of each other. The time lag of 24 to 48 hr for maximal hardiness to be manifested could be due in part to the fact that eight-leaf cuttings were used in this experiment and only the basal portions of the stems were immersed in the sucrose solutions. Thus, a considerable amount of the time could be attributed to uptake and translocation to the leaf, to say nothing about reaching the appropriate intracellular site.

A second way in which the presence of two phases could be demonstrated would be by the selective inhibition of one of the phases. Previously, it has been shown that the light stimulus results in the production of a translocatable promoter of cold hardiness, most likely sucrose (13). By darkening a portion of the plant, the light-induced production of sucrose is confined to

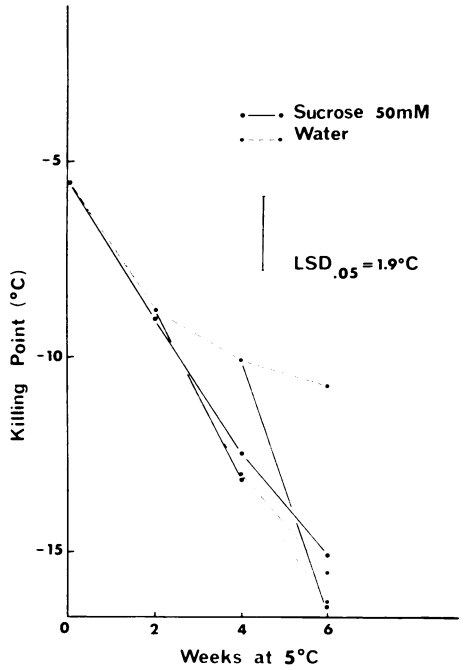

FIG. 2. Cold acclimation of excised leaves incubated in water and 50 mM sucrose in the dark at 5 C.

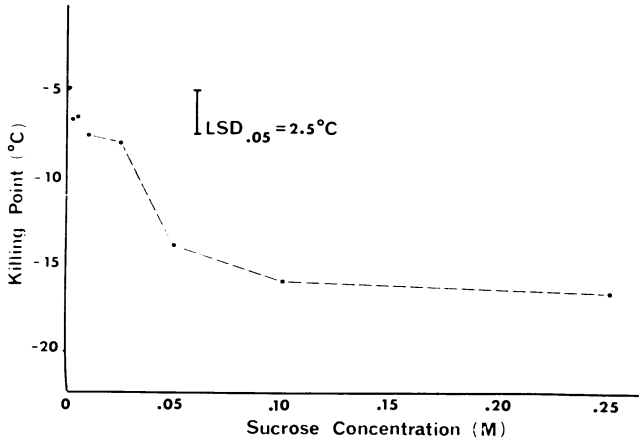

FIG. 3. Influence of sucrose concentration on cold acclimation of cuttings in the dark at 5 C.

the illuminated portion of the plant, and translocation and accumulation of sucrose in the darkened portion can be studied separately.

The cold hardiness of darkened receptor portions treated with DNP was significantly less than untreated receptor portions or the illuminated donor portions. In plants not treated with DNP, hardiness of both portions was equal. Furthermore, applications of DNP to illuminated portions of the plants failed to significantly affect hardiness in either the donor or receptor portions of the plants. The failure of DNP to interfere with photosynthate production in the illuminated donor portions is probably due to light inactivation (6, 8). The decrease in hardiness in the presence of DNP indicated that sugars synthesized in the illuminated donor portion and translocated to the darkened receptor were unable to elicit an increase in hardiness. If cold acclimation was just the accumulation of sucrose, DNP should have had no effect on hardiness in the receptor.

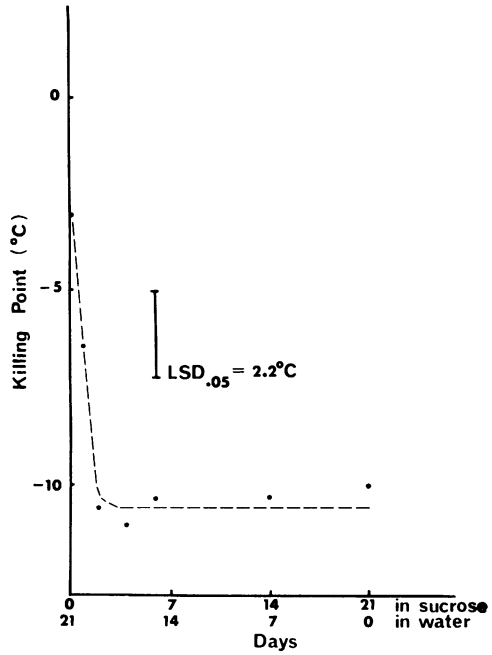

FIG. 4. Influence of time of incubation on sucrose solutions on cold acclimation. All cuttings were at 5 C in the dark for 21 days. Incubation in 50 mM sucrose solutions was for the terminal 1, 2, 4, 7, 14, or 21 days and was preceded by incubation on water. Hardiness was determined after 21 days.

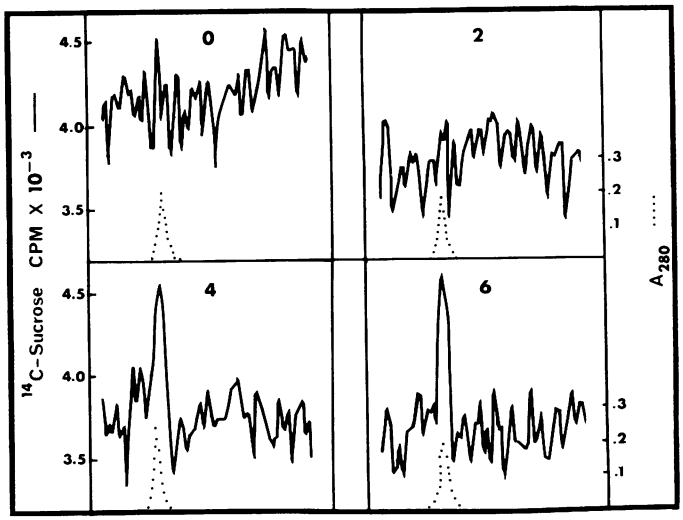

FIG. 5. Binding of ^{14}C-sucrose to particulate protein fraction isolated from plants following 0, 2, 4, or 6 weeks exposure to cold acclimation conditions.

Whereas the similarities in hardiness of DNP-treated and untreated donors would indicate that DNP was not interfering with the light stimulation of cold acclimation, the decreased hardiness in the darkened receptors could have been due to impairment of translocation by DNP. However, this was not shown when the amount of $^{14}CO_2$ fixed and translocated to the receptors was measured following DNP treatment. Initially, there appeared to be a stimulation of $^{14}CO_2$ fixation in the donor portions of plants whose receptor portions were treated with DNP. Immediately after the 6-hr exposure to $^{14}CO_2$, the amount of ^{14}C present in the darkened receptor portions was negligible regardless of the treatment. After 5 days the percentage of the total ^{14}C in the darkened receptor portions was similar for both DNP-treated (46%) and untreated (49%) plants. After 10 days there was only a slight decrease in the percentage of the total ^{14}C in the darkened receptors of DNP-treated plants (41%) as compared to untreated plants (49%).

Protein-Sugar Binding. Heber (4) has indicated that the protective influence of sugars may be due to their ability to retain or substitute water of hydration of proteins via hydrogen bonding. If this is the case, perhaps the second phase of cold acclimation resides in the sugar-binding capacity of cellular proteins. An attempt to demonstrate this was made by using Sephadex gel filtration in a manner analogous to dialysis equilibrium. If there is binding between the protein and sugar, the amount of ^{14}C-sugar will increase above the equilibrium value at a point corresponding in position to the protein and will decrease below the equilibrium value after the protein peak.

While the capacity of the particulate protein fraction of nonacclimated tissue to bind ^{14}C-sucrose was slight, the ^{14}C-sucrose binding capacity of this fraction was greatly increased in plants exposed to 4 and 6 weeks of cold acclimation conditions. In Figure 5, a representative example of these elution profiles, note the increase in ^{14}C-sucrose content above the equilibrium value in those fractions containing protein from acclimated tissue. A similar increase in the capacity of the soluble protein fraction to bind ^{14}C-sucrose was also observed. With ^{14}C-glucose, there was no binding by soluble and particulate protein fractions from nonacclimated tissue, and it increased only slightly during cold acclimation. Thus, sucrose, the predominant sugar accumulating during cold acclimation and the most effective sugar in increasing cold hardiness when added in artificial studies, exhibited a higher affinity for the protein preparations than did glucose. Furthermore, the capacity of the protein fractions to bind ^{14}C-sucrose increased during cold acclimation.

DISCUSSION

The results support the concept that cold acclimation is a two phase process involving (a) a light-stimulated production of a translocatable promoter, most likely sucrose; and (*b*) a dark reaction requiring low temperatures enabling the plant to respond to the presence of sucrose. Furthermore, the two phases can proceed independently of each other but only upon complementation of the products of the two phases is an increase in hardiness manifested. It is speculated that the second phase may be an alteration in the protein complement of the cell enabling frost-sensitive proteins to bind the sugar. Thus, during the second phase the protein assumes a new configuration or composition which provides sites which bind with the hydroxyl groups of sucrose. This binding or complementation results in stabilization of the protein and hence is manifested as an increase in hardiness.

Re-examination of Figure 1 in view of the proposed concept

will demonstrate the existence of two individual phases and their independence. Consider the D portion of the L-D treatment: the increased rate of cold acclimation during D is the result of an accumulation of sucrose from the previous L portion of the treatment. Sucrose accumulated during the L portion because the rate-limiting step was the second phase; but when the plants were placed in the dark and the second phase continued, the previously accumulated sugars were used for complementation. Thus, treatments L-L and L-D resulted in the same degree of hardiness after 4 weeks because the rate of the second phase was identical, and the amount of sucrose produced during the 2-week light period was sufficient for maximal complementation.

Considering the L portion of the D-L treatment, the existence of two independent phases is also shown. During the initial D period of the D-L treatment, the second phase proceeded but was not expressed as an increase in hardiness due to the lack of sucrose required for complementation (prestarved plants were used). However, when the plants were exposed to light, the sucrose produced was used more efficiently due to the progress of the second phase during the previous dark period. Thus, after 4 weeks, treatments L-L, L-D, and D-L all exhibit the same degree of hardiness due to the rate of the second phase being equal in all cases, even though more sucrose is produced in the L-L treatment, and the rates during each period were different.

The L portion of the D-D-L treatment is also demonstrative of the more efficient utilization of the photosynthates when preceded by a dark period. Since the rate of cold acclimation during this period does not greatly exceed that of the L portion of the D-L-D treatment, it appears that the amount of photosynthates produced during a 2-week light period is not sufficient to complement all of the products of the second phase that have been produced during the 6-week period. This is also substantiated by the fact that the terminal dark periods of L-D-D and D-L-D had a rate of cold acclimation similar to the dark control (D-D-D). Thus, after 6 weeks, hardiness was lower in treatments L-D-D, D-L-D, and D-D-L than in treatment L-L-L because the amount of photosynthates produced was rate limiting in the former treatments. The increase in hardiness over the dark control elicited by exposure to only 2 weeks of light was 60% of the increase in hardiness resulting from 6 weeks exposure to light. Since in plants receiving only a 2-week light period, the light reaction is limiting after 6 weeks; the 60% increase in hardiness is directly proportional to the duration of the light period. Therefore, an additional week of light should have elicited the same increase in cold acclimation over the D-D-D treatment as the L-L-L treatment. The 2:1 ratio (weeks of dark period–weeks of light period) is compatible with the results received after 4 weeks of cold acclimation. That is, 4 weeks of the dark period was complemented to the maximal extent by 2 weeks of light. If this ratio were based on an hourly basis, it would mean that two periods of dark, *i.e.*, 48 hr would be complemented by one period of light, *i.e.*, 8 hr (since the photoperiod was only 8 hr). Therefore, on an hourly basis, the ratio is 6:1, and during a 24-hr cycle a 4-hr photoperiod (under these experimental conditions) would be sufficient for light saturation to occur. It is important to note that previously a 4-hr photoperiod was shown to be as effective as an 8- or 16-hr photoperiod in promoting maximum cold hardiness (14).

In the experiment where sucrose was substituted for the light periods (Fig. 2) results identical to those described above were obtained. There is little doubt that the light requirement can be replaced by sucrose. Furthermore, Figure 3 demonstrates that maximal cold hardiness is elicited by low concentrations of sucrose. Previously (14), it was concluded that only a small portion of the total photosynthates produced during cold acclimation are actually used for the cold acclimation process. Recognition of the fact that photosynthate production and sugar accumulation is only one phase and not the rate-limiting phase of cold acclimation may explain a number of inconsistencies in the literature concerning the relationship of sugar content to cold hardiness. Many workers, most notably Weiser (18), have dismissed sugars from the cold acclimation process on the basis of either poor correlation of sugar content with cold hardiness or failure to increase cold hardiness by increasing the sugar content. Since sugar accumulation is only one phase and not the rate-limiting phase of cold acclimation, one would not expect to show a perfect correlation between hardiness and the total sugar content. Furthermore, for the same reasons, addition of sugars to increase hardiness will be effective only if the endogenous levels are reduced to limiting quantities, such as by prestarvation.

The existence of two independent phases of cold acclimation is also demonstrated in Figure 4. Under normal conditions or when incubated on sucrose solutions, plants or cuttings will exhibit a linear increase in hardiness during the first 4 weeks. This experiment demonstrates that this linearity is determined by the second phase of cold acclimation. Furthermore, within 48 hr of exposure to sucrose, the hardiness of the plants preconditioned at low temperatures can be increased to the same level as plants maintained continuously on sucrose. If the two phases were not independent, this would not be possible. Furthermore, the concept that the products of the two phases must complement each other for an increase in cold hardiness to be manifested is evident. The ability of the sucrose to rapidly increase the hardiness of tissue preconditioned at low temperatures in the dark would indicate that the second phase involves some alteration in frost-sensitive sites in the cell that allows the sugars to be bound or positioned in some way to confer stability on the frost-sensitive sites. Without doubt, these frost-sensitive sites are the cellular membranes or membrane systems (4), and sugars have been shown to confer stability on such membrane systems as chloroplasts and mitochondria. Furthermore, it has been shown that sugars are able to form hydrogen-bonded complexes with proteins (3). It is possible that the increased sugar binding capacity of the particulate protein fractions from acclimated plants may represent the second phase of cold acclimation. Thus, an accumulation of sugars in a cell would not confer cold hardiness unless there was a concomitant change in the protein's capacity to bind the sugars and effectively stabilize the proteins to endure the rigors of freezing.

Whether the increased ability to bind sugars is the result of synthesis *de novo* or simply a configurational change or the insertion of amino acids which favor binding is now under investigation. It is interesting to note that polar amino acids of proteins were shown to increase dramatically during cold acclimation (10). Furthermore, it was suggested that these changes may have been the result of unmasking of the sites. Giles and McKay (3) have suggested that in protein-carbohydrate hydrogen bonding, the carbohydrate is the proton donor. Thus, an enrichment in the dicarboxylic amino acids, glutamic acid and aspartic acid, could provide an increase in sites available for hydrogen bonding. Brandts (1) has shown that hydrophobic bonds are extremely labile at low temperatures, and their disruption leads to enzyme denaturation and the formation of membrane-bound vesicles in the case of membrane proteins. While hydrophobic regions are usually restricted to the interior of the protein molecule with polar groups on the exterior, stabilization of the polar groups on the exterior by sugars could confer stability to the interior hydrophobic regions.

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