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The Mechanism of Hardening on the Basis of the $\text{SH} \rightleftharpoons \text{SS}$ Hypothesis of Freezing Injury

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Abstract

It has long been known that the accumulation of sugars during hardening of young plants at low temperature (0 to 5°C) is due to a greater decrease in utilization of reserves than in rate of photosynthesis. The increase in freezing resistance is usually ascribed to this accumulation of sugars. From the point of view of the $\text{SH} \rightleftharpoons \text{SS}$ hypothesis, the reducing action of the light reactions of photosynthesis is more important than the sugar accumulation. It is probable that these light reactions show less of a decrease in rate at low temperatures than do the dark reactions of photosynthesis. As a result, the accumulated NADPH is available for protein SS reduction to SH. This would lead to exposure of free chain ends, permitting hydrolysis by proteolytic enzymes. As a result, protein molecular size would decrease and non-protein (amino acid or peptide) SH would be released. The latter increase has been found, though accumulation is in the SS form, due presumably to oxidation of the freed non-protein SH to SS. After a preliminary rise during the first 1-2 weeks of hardening, protein SH decreased steadily. Any newly formed protein must, therefore, have been essentially free of reactive SH groups, and therefore unable to form intermolecular SS bonds on freezing. Other available evidence is explainable on the basis of this concept.

Introduction

On the basis of several years' experimental results, a $\text{SH} \rightleftharpoons \text{SS}$ hypothesis of freezing injury has been proposed (Levitt, 1962). Since then, further evidence from several laboratories has supported the hypothesis (Levitt, 1966). The more recently determined changes that occur during the development of freezing resistance (Kohn and Levitt, 1966) will now be made use of in an attempt to explain the mechanism of hardening by means of the $\text{SH} \rightleftharpoons \text{SS}$ hypothesis.

I. Relation between Photosynthesis and Hardening

The hardening process leads to an increase in the freezing resistance of plants. In the case of most plants that have been investigated, both low temperature (5°C or lower) and light are necessary for the hardening to occur (Levitt, 1956). In those that have been tested, it has been shown that photosynthesis must take place during the light period and this is accompanied by a gradual accumulation of sugars. The inter-relationships between these processes can be explained as follows (Fig. 1).

When young plants are raised at normal growing temperatures (*e.g.* 25°C) nearly all of the photosynthate is used up in the production of new growth, either as the raw materials or in the energy-supplying respiratory process. A small amount accumulates as sugars or starch. When the temperature is dropped to 5°C, growth nearly stops and

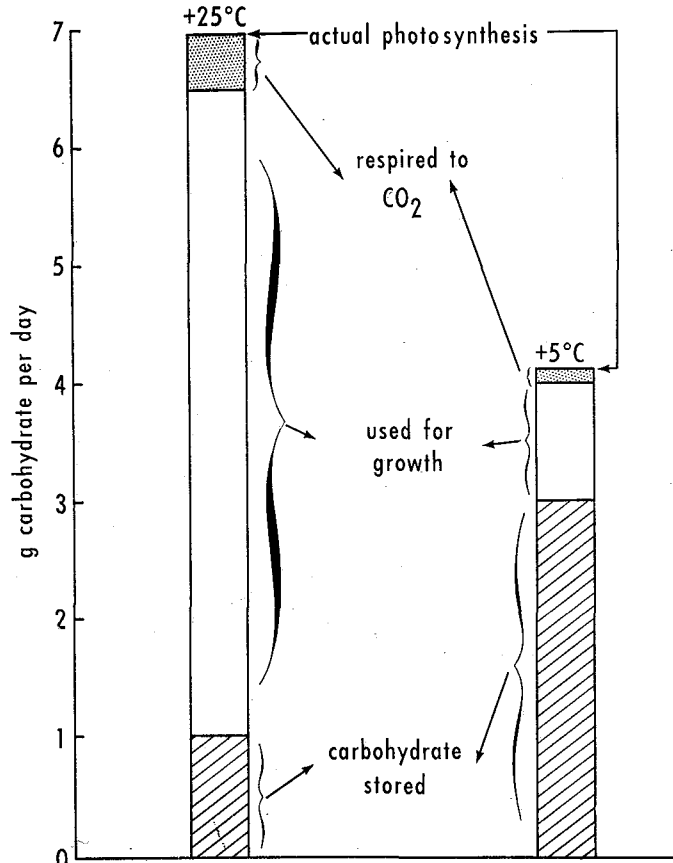


Fig. 1. Schematic representation of changes in photosynthesis, growth and carbohydrate storage due to low (hardening) temperatures. Relative photosynthesis and respiration values based on Andersson (1944)

photosynthesis continues at a decreased rate. In hardy plants, the decrease in utilization of photosynthate due to the nearly complete growth stoppage, is greater than the decrease in rate of photosynthesis. As a result, the net accumulation of photosynthate is greater than at 25°C, and the concentration of sugars in the plant increases.

All this has been known for a long time, and it has frequently been suggested that the accumulation of sugars is in itself the cause of the increased freezing resistance. But there are so many high sugar plants with no resistance, and low sugar plants with high resistance, that this explanation appears invalid. Furthermore, as shown below, the second stage of hardening can occur in the dark without any further accumulation of sugars.

From the point of view of the $SH \rightleftharpoons SS$ theory of frost resistance, another aspect of the metabolic changes at hardening temperatures is more important. Although the net accumulation of carbohydrates at 5°C is greater than at 25°C, the actual rate of photosynthesis definitely and markedly decreases. This is to be expected, since the process is measured by the rate of CO₂ assimilation to carbohydrates, consisting of a

series of ordinary dark chemical reactions which have relatively high temperature coefficients. But the "light reactions" of photosynthesis are not usually measured, and since they are more closely related to the true photochemical reactions of photosynthesis, they occur much more rapidly and they must therefore have low temperature coefficients. In other words, they must take place at nearly the same rate at 5°C as at 25°C. As direct evidence of this, photophosphorylation has been found to occur in spinach at a good rate not only at 0°C but even at -10°C (Hall, 1964), whereas oxidative phosphorylation stopped completely at -2°C. At 25°C, the ATP and NADPH produced photosynthetically are undoubtedly all or nearly all used up by the rapid CO₂ assimilation. At 5°C, due to the markedly decreased CO₂ assimilation, only a small part is used up photosynthetically, and the major part is therefore available for other metabolic processes (Fig. 2).

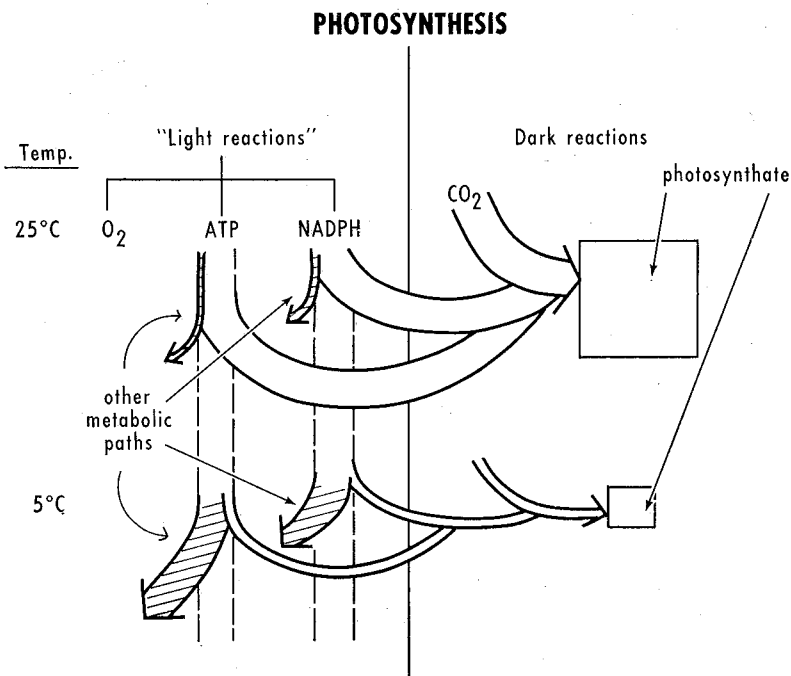


Fig. 2. Schematic representation of diversion of ATP and NADPH to non-photosynthetic pathways at 5°C

Recent measurements on hardening plants have suggested what some of these other metabolic processes may be. During the first week or two of hardening, there is a rise in protein SH (Fig. 3). Since there are known SS reduction systems capable of converting protein SS to SH, and since NADP is a cofactor (Hatch and Turner, 1960; Arrigoni, 1960; Mapson and Isherwood, 1963), it is reasonable to assume that the NADPH produced photosynthetically at 5°C and not used up by the low-temperature-retarded assimilation, is available for protein SS reduction. That the ATP produced photosynthetically may be used for non-photosynthetic processes has been indicated at least in the case of ion absorption (Weigl, 1964).

This scheme is, of course, hypothetical and must be accepted as such in a general

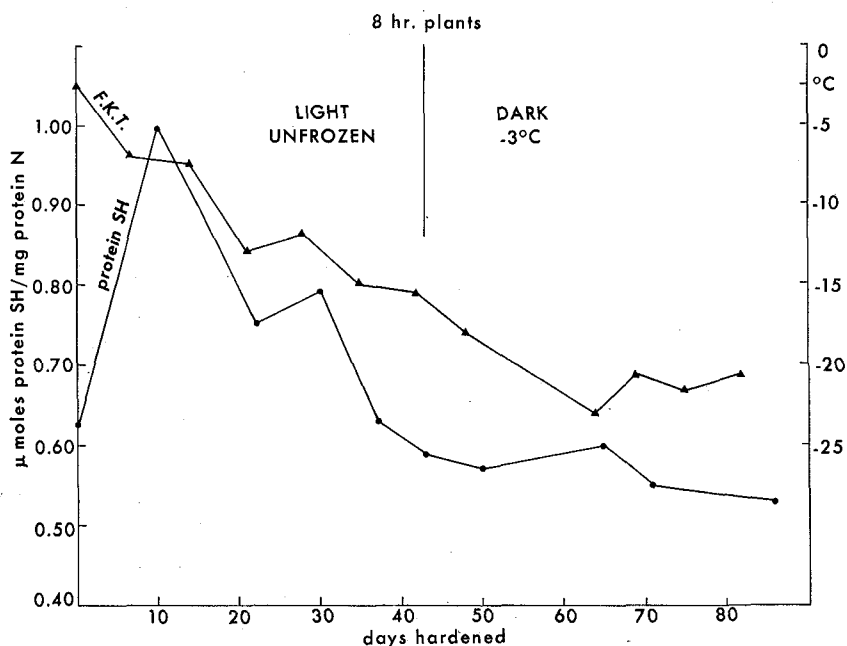


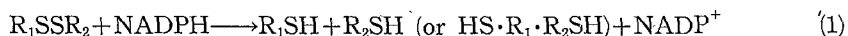
Fig. 3. Preliminary rise in protein SH during hardening, followed by a steady decrease paralleling hardiness (From Kohn and Levitt, 1966)

sense without expecting the specific reactions to be known at this time. Thus, recent results (Asahi, 1964) have shown that spinach chloroplasts do indeed reduce protein disulfides in the light, but not in darkness. NADPH was not however, able to reduce the protein in the dark. Asahi concluded from these and other results, that reduction was due to the photosynthetic electron transport system, but did not involve NADPH. This does, of course, support the general concept of hardening described above, though suggesting the replacement of NADPH by some other reducing substance formed photosynthetically.

In the case of the photosynthesizing bacterium *Chromatium*, direct evidence has been produced of this dependence of protein SH on photosynthesis (Hudock *et al.*, 1965). Triosephosphate dehydrogenase prepared from cells grown in the light had 4.2 SH groups per mole enzyme, whereas the same enzyme extracted from non-photosynthesizing cells grown on organic medium in the dark had only 2.4 SH groups per mole enzyme.

II. Proposed Changes during Development of Resistance

One of the first metabolic changes involved in hardening would then be:



According to Markus (1963), SS bonds may play one of two roles in proteins: 1) protection of the secondary and tertiary structure against distortion and 2) tying subunits together. The second type of bond could be readily broken without denaturing the protein. It would also lead to a decrease in molecular weight. The rise in SH on

hardening could therefore readily explain the marked increase in soluble proteins during hardening, which has been found by so many investigators. This may also explain what happens in SD cabbage plants, since soluble protein does increase at the same time as protein SH increases (the first 1-2 weeks of hardening...Kohn and Levitt, 1966).

Due to the reduction of these protein SS bonds, the newly freed protein ends would become accessible to proteolytic enzymes. The proteolytic enzymes themselves are SH enzymes, and therefore would be activated by the reduction. As a result, CSH or GSH and other amino acids and peptides would be removed hydrolytically (Fig. 4). The second metabolic change would therefore be:



Evidence of this second reaction is the decrease in protein SH which follows the initial rise (Fig. 3), and the accompanying increase in non-protein SH+2SS (Table 1).

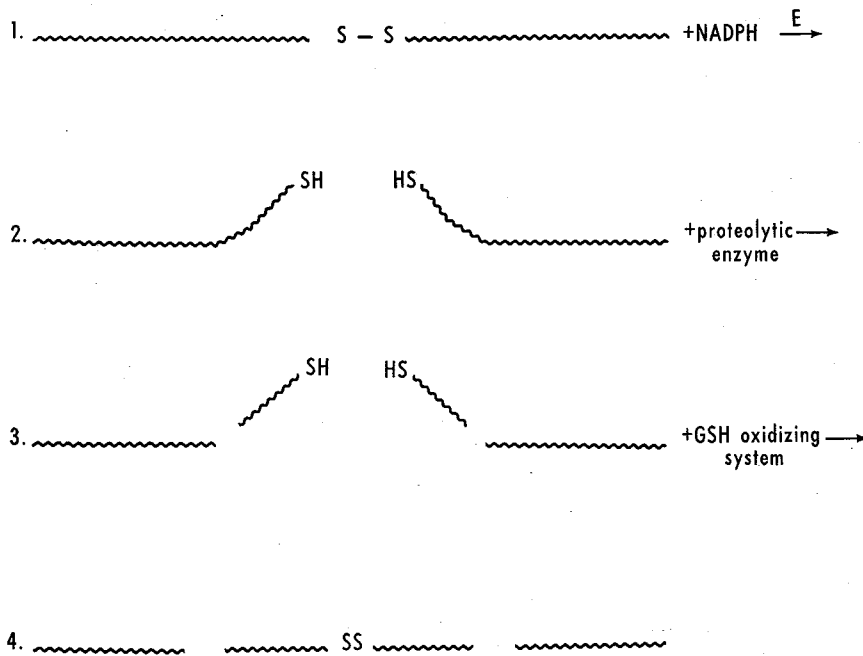


Fig. 4. Schematic interpretation of $\text{SS} \rightleftharpoons \text{SH}$ changes in proteins and non-proteins

Table 1. Increase in non-protein SH+2SS ($\mu\text{moles/gfw}$) on hardening in the light (From Kohn and Levitt, 1966)

Weeks hardened	Photoperiod (hrs)			
	8	12	18	24
0	0.84	0.58	0.67	0.51
1	1.13	1.14	0.76	0.73
2	1.34	1.01	1.13	0.70
3	1.87	1.51	1.70	1.56

Total soluble protein also decreased in the SD cabbage plants following the initial rise, and in the LD plants almost from the beginning of hardening.

Step (2) may follow so soon after step (1) that the initial rise in protein SH may be missed. This apparently happens when cabbage plants are hardened during long days. These reactions also explain the previously found large "increases" in SH on hardening, and the conclusion that these increases were artifacts. When hardy and non-hardy plants are compared, due to the higher reduction capacity of the hardy plants, the SH groups will be less readily oxidized during preparation of the sample than in the case of the non-hardy plants, and an artificially produced difference between the two will be detected. Thus, though the measured SH increase on hardening is an artifact, it is due to an amplification of a true SH increase during hardening, which occurs as a result of the increase in reduction intensity. But this true increase in the cells may not itself be detected, due to a rapid hydrolysis which splits off the SH part from the protein.

The fact that this artificial difference in SH content, between hardy and non-hardy tissues, arises due to oxidation during the first few minutes after homogenization (Schmutz, personal communication) and that the difference in oxidation rate between the hardy and non-hardy plants cannot be detected later at room temperature, is further evidence of this concept. Benson *et al.* (1949) long ago showed that the photosynthetic reducing potential is available for C-assimilation for 10 minutes after the light is turned off, but that after longer periods in the dark, photosynthetic C-reduction cannot occur.

Due to the GSH oxidizing system, previously shown to be particularly active in hardy plants (Levitt, 1962), the released GSH would be quickly oxidized (Fig. 4):



Since some amylases are SH enzymes, the reaction (1) above could also explain why the carbohydrates accumulate at 5°C as sugars rather than starches. Any starches formed would soon be hydrolysed to sugars by the activated amylase. If this amylase activation is reversible, it may not be possible to detect it in extracts due to the absence of photosynthesis. Whether or not the ATP produced photosynthetically plays any role in the above metabolic reactions, cannot be decided at this time. It may play a regulatory role in the metabolic changes. It has been shown that AMP, ADP and ATP are positive or negative effectors for many enzyme reactions. When the ratio $\frac{\text{ATP}}{\text{ADP}}$ is high, respiration rate is low, and the respiratory carriers are in the more reduced state. A high $\frac{\text{ATP}}{\text{ADP}}$ ratio may, also switch the metabolism from carbohydrate to fat accumulation in some organisms (Atkinson, 1965). In the case of hardening plants, the increase in this ratio may conceivably switch the metabolism from starch accumulation to the accumulation of sugars. If any of the reactions are endergonic (*e.g.* protein synthesis), the ATP would obviously be required.

From the point of view of the $\text{SH} \rightleftharpoons \text{SS}$ theory of frost injury, the question is how the above changes could impart freezing resistance to the hardened plant. The sugar accumulation could itself be physically protective by holding unfrozen water between the proteins, since this would help to keep the SH groups of adjacent proteins far enough

apart to prevent SS formation. This has, in fact, been shown in the case of the model system (Thiogel). But the sugar must accumulate in the cytoplasm in order to have this effect, and yet much of it accumulates in the vacuole. This may, in fact, explain why penetrating solutes such as glycerol have not proved as effective protective agents for plant cells as for animal cells. In plant cells they are excreted from the protoplasm into the vacuole where they can no longer exert their protective effect. On the other hand, external solutions containing sugars or other non-penetrating solutes may markedly protect plant cells against freezing injury. Even in this case, recent evidence (Krull, 1966) indicates that SH groups are involved, for the protective effect can be markedly altered by the addition of a small amount of a mercaptan. But this mercaptan apparently must penetrate the cell in order to exert its effect (Levitt and Hasman, 1964); therefore, intracytoplasmic and not surface SH groups are involved. It seems unlikely that sugars or other such solutes can permanently prevent SS formation. It is more likely that they merely slow down the process. But they would also decrease the amount of stress on the proteins and therefore the denaturation during thawing, since the rehydration would be smaller on account of the water held osmotically by the sugar during the freezing.

It is possible that the rise in reduction capacity due to the light reactions of photosynthesis might tend to oppose SS formation on freezing. However, it is doubtful whether this rise could be carried over to the night when photosynthesis does not occur. A lasting protection against SS formation would, however be provided by the loss of reactive SH groups from proteins. The SS formation would be expected to decrease at least according to the square of the decrease in SH content of the protein molecules. Determinations on the model system (Thiogel) have shown an even greater effect. Thus a Thiogel with 6 SH groups/100,000 M.W. is protected from SS formation by 1/20 the concentration of GSH required to protect a Thiogel with 12 SH groups/100,000 M.W. These results are particularly suggestive since the SH contents of the two Thiogels corresponds nearly exactly to the quantities found in hardened and unhardened cabbage proteins (Levitt, 1966). It is also instructive that the concentration of GSH required to protect the Thiogel SH against SS formation is much higher than the quantity of non-protein SH+2SS in cabbage, and since very little of this occurs as SH, this mechanism of protecting SH groups against SS formation is certainly not used by plant. On the contrary, the conversion of GSH to GSSG would prevent the accumulation of small-molecular weight SH substances that might be capable of triggering a chain reaction of $SH \rightleftharpoons SS$ interchange between protein molecules, leading to intermolecular SS formation.

Though the first stage of hardening must occur in the light, in order for photosynthesis to continue, the hardening can subsequently progress steadily in the dark if the temperature is lowered from 0 to -3°C . This has been called the second stage of hardening, though there is no sign of discontinuity in the progress of hardening. During this second stage of hardening, there is a dramatic net synthesis of soluble proteins, and these have little or no reactive or unmasked SH groups (Fig. 3), presumably because all of the free amino acid SH has been converted intramolecularly to SS. Such proteins would have no need for sugars or other protective agents to prevent intermolecular SS formation. The absence of unmasked SH groups would also presumably inactivate the SH enzymes leading to a low metabolic activity (the rest period) in hardy

plants. Although this second stage of hardening below 0°C may take place in the dark in cabbage (Kohn and Levitt, 1964) and in wheat (Tumanov and Trunova, 1963), recent results indicate that light is necessary in the case of conifers (Scheumann and Börtitz, 1965).

The first stage of hardening would then consist of protein reduction, partial unfolding, hydrolysis, and oxidation of the released SH units. The second stage would consist of a synthesis of soluble protein with little or no reactive SH. The transition from the first to the second stage may conceivably occur when most of the unfolded portions of the protein have been hydrolytically removed. It is obvious that the second stage changes would impart far better protection against intermolecular SS formation than the first. This is in agreement with the far greater freezing resistance—a survival of freezing at -20°C compared to about half this value at the end of the first stage. The striking parallel between the decrease in protein SH and the freezing resistance (Fig. 3) supports the above conclusion.

III. Analysis of Accumulated Evidence

It is, of course difficult to generalize for all plants on the basis of the above SH results obtained mainly with cabbage plants. However, some of the above concepts can be checked against the evidence accumulated over the years.

Necessity of photosynthesis for frost hardiness. Many investigators have shown that seedlings will not harden if photosynthesis does not occur (see Levitt, 1956). This has been proved by withholding either light or CO₂. Some hardening may occur in the dark if starch has accumulated (Dexter, 1933), but this hardening is slight. Photosynthesizing conifers are the most resistant trees, but deciduous trees may be nearly as hardy, and their hardening occurs after leaf drop, and therefore presumably in the absence of photosynthesis. But hardy cortical cells of twigs (in fall and winter) contain ample chlorophyll that colors the cytoplasm layer an intense green. In this way, the cytoplasmic proteins are sufficiently close to the chloroplasts to be directly affected by the high reduction potential. Furthermore, the chlorophyll content of the shoots has been found to increase from October to March; followed by a decrease (Smol'skaya, 1964). Consequently, the chlorophyll content is maximal when the plants are most resistant to freezing injury. One investigator has also reported a higher chlorophyll content during winter in the bark of hardier trees than in that of less hardy trees (Borzakivska, 1965). Yet, in spite of this maximum chlorophyll content in winter, photosynthetic C-assimilation declines over the winter months to zero in January, in the case of *Pinus strobus* (Shiroya *et al.*, 1966). A similar decline occurs in the case of wheat seedlings, from a maximum during the early stages of hardening, to a minimum at maximal hardening (Anderssen, 1944). All these results point to a maximum accumulation of ATP and NADPH due to high chlorophyll content and to a lack of their utilization by the essentially zero C-assimilation.

It is even possible to explain the decrease in photosynthetic C-assimilation on the basis of SH changes. Lindahl (1966) showed that tetramethylthiuram disulfide (TMTD) inhibits photosynthesis. The most plausible explanation is a reaction between TMTD

and the functional thiol groups. Similarly, Losada *et al.* (1965) completely inhibited the photoreduction of NADP by use of PCMB, which ties up the SH groups. If hardening results in the second stage disappearance of these functional SH groups, it would explain 1) the zero photosynthesis, 2) the minimal respiration rate, and 3) the dormancy, all of which are associated with the hardened state.

The internal xylem cells do not contain chlorophyll and are less frost resistant than the cortical cells. Presumably even they undergo an increase in reduction potential because they are separated from the air by the surrounding chlorophyll-containing cortical cells. It is true that non-photosynthesizing tissues not surrounded by chlorophyll-containing cells may also become fully hardy...*e.g.* buds and cambium. But these are meristematic and such tissues have high reduction intensities (Van Fleet, 1954). The non-meristematic chlorophyll-free tissues such as the mature root, are far less frost resistant than the photosynthesizing tissues. Direct evidence of the increase in reduction capacity is the accumulation of ascorbic acid on the hardening of wheat (Andrews and Roberts, 1961).

But how is this reduction capacity maintained a) at very low temperature when presumably photosynthesis ceases, b) at night? Many investigators have succeeded in measuring photosynthesis in hardy plants at temperatures below freezing. The extreme is a recent measurement in very hardy lichens below -20°C (Lange, 1965). If this has any survival value it cannot be due to the negligible accumulation of carbohydrates at these low temperatures, but could be due to the maintenance of the high reduction capacity. Presumably, at such low temperatures the oxidation process would be so much more inhibited than photosynthesis (see Fig. 3), that the reduction level would be lowered a negligible amount overnight in the dark. The oxidizing effect of respiration would be decreased still more by the usually lower night than day temperatures, and by the lower respiratory rate in hardy than in tender plants at low temperatures (see Levitt, 1956).

The Russian investigators (Tumanov and Trunova, 1963) have recently developed methods of hardening wheat plants artificially in complete darkness and therefore, of course, in the absence of photosynthesis. They do this by feeding sugars to the seedlings *via* their roots, at low temperatures and over about a two week period. But this is done only after the plants have begun to harden, and therefore presumably have already undergone the first SH changes. Furthermore they have found that not all sugars work. Only those that are metabolized by the plant give good results. It is, therefore, conceivable that the sugars may be partially oxidized at the expense of protein SS reduction.

Protein RSSR reduction to RSH. The increase in protein SH during the early hardening has been confirmed many times and in many species (Levitt, 1962). Though the measured increase is an artifact, it is probably an "amplification artifact" as indicated above, which amplifies to a measurable level the otherwise undetectable SH increase. Further evidence is the rise in frost resistance of sea urchin eggs on fertilization, which also is accompanied by a rise in SH (Asahina and Tanno, 1963).

Prevention of hardening by high NO_3 fertilization. It has long been known (Levitt, 1956), that heavy NO_3 fertilization markedly decreases the ability of plants to harden. But the chloroplast enzyme system which mediates the reduction of NO_3 involves

NADPH as a cofactor (Wessels, 1965). The reduction of the absorbed NO_3 would therefore compete for the NADPH produced photosynthetically and would therefore decrease the amount available for protein SS reduction.

Role of sugars in hardening. Many explanations have been proposed for the commonly found sugar increase accompanying the hardening of plants. None has proved satisfactory. The accumulation of sugars has long been known to inhibit the C-assimilation of photosynthesis (Hartt, 1963), whereas the accumulation of starch does not. The same relation has been found during the hardening of wheat seedlings. A higher rate of photosynthesis occurred at first during the hardening process, but as sugar accumulated, photosynthetic rate decreased though hardness increased (Anderssen, 1944). Conversion of starch to sugar could, therefore, protect by inhibiting photosynthetic C-assimilation, and releasing more of the NADPH and ATP for other metabolic pathways.

On the basis of the above SH theory of hardening, it is not the hardening process that lacks an explanation but the inability of tender plants to harden at hardening temperatures. It is, of course, known that some plants have high temperature minima for photosynthesis. Although this has been determined by measuring the C-assimilation only, it is conceivable that the enzymes or cofactors for the light reactions are also involved. Unfortunately, little information is available as to their metabolism at hardening temperatures. Direct information on these points is needed before reasonable speculation is possible. At present, it can only be suggested that tender plants are those which cannot photosynthesize actively enough at hardening temperatures to raise the reduction potential of the plant.

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