Plant Physiol. (1987) 84, 872–878
0032-0889/87/84/0872/07/$01.00/0

# Induction of Freezing Tolerance in Spinach Is Associated with the Synthesis of Cold Acclimation Induced Proteins[1]

Received for publication September 29, 1986 and in revised form March 13, 1987

CHARLES L. GUY* AND DALE HASKELL
*Ornamental Horticulture Department, Institute of Food and Agricultural Sciences, The University of Florida, Gainesville, Florida 32611*

## ABSTRACT

**Spinach (*Spinacia oleracea* L. cv Bloomsdale) seedlings cultured *in vitro* were used to study changes in protein synthesis during cold acclimation. Seedlings grown for 3 weeks postsowing on an inorganic-nutrient-agar medium were able to increase their freezing tolerance when grown at 5°C. During cold acclimation at 5°C and deacclimation at 25°C, the kinetics of freezing tolerance induction and loss were similar to that of soil-grown plants. Freezing tolerance increased after 1 day of cold acclimation and reached a maximum within 7 days. Upon deacclimation at 25°C, freezing tolerance declined within 1 day and was largely lost by the 7th day. Leaf proteins of intact plants grown at 5 and 25°C were *in vivo* radiolabeled, without wounding or injury, to high specific activities with [$^{35}$S]methionine. Leaf proteins were radiolabeled at 0, 1, 2, 3, 4, 7, and 14 days of cold acclimation and at 1, 3, and 7 days of deacclimation. Up to 500 labeled proteins were separated by two-dimensional gel electrophoresis and visualized by fluorography. A rapid and stable change in the protein synthesis pattern was observed when seedlings were transferred to the low temperature environment. Cold-acclimated leaves contained 22 polypeptides not found in nonacclimated leaves. Exposure to 5°C induced the synthesis of three high molecular weight cold acclimation proteins (CAPs) ($M_r$ of about 160,000, 117,000, and 85,000) and greatly increased the synthesis of a fourth high molecular weight protein ($M_r$ 79,000). These proteins were synthesized during day 1 and throughout the 14 day exposure to 5°C. During deacclimation, the synthesis of CAPs 160, 117, and 85 was greatly reduced by the first day of exposure to 25°C. However, CAP 79 was synthesized throughout the 7 day deacclimation treatment. Thus, the induction at low temperature and termination at warm temperature of the synthesis of CAPs 160, 117, and 85 was highly correlated with the induction and loss of freezing tolerance. Cold acclimation did not result in a general posttranslational modification of leaf proteins. Most of the observed changes in the two-dimensional gel patterns could be attributed to the *de novo* synthesis of proteins induced by low temperature. In spinach leaf tissue, heat shock altered the pattern of protein synthesis and induced the synthesis of several heat shock proteins (HSPs). One polypeptide synthesized in cold-acclimated leaves had a molecular weight and net charge ($M_r$ 79,000, pI 4.8) similar to that of a HSP ($M_r$ 83,000, pI 4.8). However, heat shock did not increase the freezing tolerance, and cold acclimation did not increase heat tolerance over that of nonacclimated plants, but heat-shocked leaf tissue was more tolerant to high temperatures than nonacclimated or cold-acclimated leaf tissue. When protein extracts from heat-shocked and cold-acclimated leaves were mixed and separated in the same two-dimensional gel, the CAP and HSP were shown to be two separate polypeptides with slightly different isoelectric points and molecular weights. The results suggested that a single temperature stress protein was not responsible for both freezing and heat tolerance.**

In a great number of plant species exposure to low-nonfreezing temperatures induces a greater tolerance to freezing (17). This process whereby plants adjust to low temperature and/or freezing tolerance is increased is termed cold acclimation. In spinach, induction of greater freezing tolerance is rapid, beginning within 1 d of exposure to low temperature (9). During prolonged cold acclimation, spinach will reach a maximum hardiness in 2 to 3 weeks, but the major portion of this increase in hardiness occurs during the first 4 d (9, 11).

It has been suggested that the development of freezing tolerance during cold acclimation results from altered gene expression (26). This concept is supported by much indirect evidence (5, 10, 16, 20, 21), but only recently has direct evidence to support an altered gene expression hypothesis been reported. In spinach, a qualitative alteration in the translatable mRNA content of leaf tissue was observed during cold acclimation (11). This induction of freezing tolerance upon exposure to low temperature was found to be correlated with the appearance of two mRNAs that encode high mol wt polypeptides. The two mRNAs were present by the second day of low temperature exposure when freezing tolerance was increasing and they continued to be expressed during a 16 d low temperature treatment. The inducibility and continuous expression of the two mRNAs suggested a possible function in the freezing tolerance of spinach.

We report here data that indicate the pattern of *in vivo* protein synthesis is changed during cold acclimation. Additionally, the synthesis of three high mol wt proteins was correlated with the induction and loss of freezing tolerance in spinach during cold acclimation and deacclimation.

## MATERIALS AND METHODS

**Plant Materials.** A commercially available cultivar of spinach (*Spinacia oleracea* L. cv Bloomsdale) was used for all experiments. Plants were grown from seed germinated on a culture medium *in vitro* or in a soil mix as described previously (13). The plants were grown to the true leaf stage (3 weeks postsowing) for all experiments reported in this communication.

**Cold Acclimation, Deacclimation, and Heat Shock Treatment.** Plants germinated and grown in a growth chamber at 25°C with a 12 h photoperiod were transferred to cold-acclimating conditions consisting of a constant 5°C and a 12 h photoperiod. Illumination of the plants exposed to 5°C was provided by a combination of cool-white fluorescent lamps (60% input wat-

[1] Supported by a USDA competitive research grant 85-CRCR-1-1649 and the Institute of Food and Agricultural Sciences, The University of Florida. Florida Agricultural Experiment Station Journal Series, No. 7583. This research was made possible by the Nancy F. Winston Foundation for Advanced Studies.

tage) and incandescent bulbs (40% input wattage) with a total average irradiance (400–700 nm) over the growing area at plant height of 480 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The transition from light to dark was abrupt. Plants were cold acclimated for 1, 2, 3, 4, 7, and 14 d. CA[2] plants were deacclimated following transfer from 5°C conditions to 25°C with a 12 h photoperiod for 1, 3, and 7 d.

Seedlings grown at 25°C were subjected to a 3 h HS treatment by transfer to an incubator at 45°C.

**Cold and Heat Tolerance.** Freezing tolerance ($LT_{50}$) was determined by previously described methods (13). Heat tolerance was determined by exposing seedlings to high temperatures. Individual seedlings were wrapped in tissue paper and inserted into a 2.2 × 22 cm test tube. The tissue paper was saturated with water and the tube stoppered. Seedlings were subjected to a single temperature ranging from 45 to 60°C for 30 min in a circulating water bath. Heat-treated seedlings were returned to 25°C and incubated overnight before the leaf tissue was visually evaluated and the electrolyte leakage determined (13).

**Protein Radiolabeling and Extraction.** Leaf proteins of *in vitro* grown seedlings were radiolabeled using the procedures developed specifically for the study of spinach cold acclimation (13). Only axenic plants were used for the radiolabeling studies. Plants were radiolabeled at 25°C, NA; at 5°C, CA; and at 45°C, HS. Plants subjected to cold acclimation were radiolabeled at 1, 2, 3, 4, 7, and 14 d of exposure to low temperature. CA seedlings returned to 25°C were radiolabeled at 1, 3, and 7 d of deacclimation. Leaf proteins were radiolabeled following the application of 10 $\mu$Ci of L-[$^{35}$S]methionine (1205 Ci/mmol Amersham) in 10 $\mu$l of sterile distilled water containing 0.01% v/v Triton X-100 to the underside of the leaf. The [$^{35}$S]methionine solution was applied at the beginning of the light period and uptake and incorporation proceeded for 24 h at either 5 or 25°C with the normal 12 h light and dark diurnal cycle. Heat-shocked plants were radiolabeled for 2 h at 25°C and then transferred to 45°C for 3 h.

Uptake was terminated by rinsing the underside of the leaf tissue with distilled water to remove label remaining on the surface. Incorporation of label into protein was stopped by homogenization of the tissue (100 mg fresh weight) and extraction of the protein in 1 ml of 80% v/v distilled phenol buffered with 120 mM Tris-HCl (pH 6.8), 50 mM EDTA, 100 mM KCl, 1 mM PMSF, 2% v/v 2-mercaptoethanol, 2% w/v SDS (22, 24). The phenol extract was vortexed with 1 ml of the above buffer and then centrifuged at 1,000*g* for 1 min. The aqueous phase was removed and the phenol phase washed twice with a 1 ml volume of buffer. Cell debris was removed from the phenol phase by two centrifugations at 15,000*g* for 10 min. Proteins were precipitated from the phenol by the addition of five volumes of −20°C 100 mM ammonium acetate, 1% v/v 2-mercaptoethanol in absolute methanol and cooling for 2 h at −80°C. Proteins were pelleted by centrifugation at 15,000*g* for 5 min and the supernatant discarded. The protein was washed twice with 1 ml of the ammonium acetate precipitation solution and twice with acetone containing 1% v/v 2-mercaptoethanol, briefly air dried, and dissolved in 100 $\mu$l O'Farrell lysis buffer (18). The ampholyte composition of the lysis buffer was modified to contain two parts pH 5 to 7, two parts pH 4 to 6, and one part pH 3 to 10 to accommodate the acidic nature of the leaf proteins. Protein extracts were stored at −80°C.

**Gel Electrophoresis.** Radiolabeled proteins were separated by a modification of the standard O'Farrell two-dimensional gel electrophoresis technique (18). Equal amounts of TCA insoluble radioactivity (usually 1 or 2 X $10^6$ cpm and containing less than 67 $\mu$g protein) were loaded onto the 1.5 mm diameter IEF gels. The IEF gels were 3.5% acrylamide and electrode buffer concentrations were 50 mM NaOH and 25 mM $H_3PO_4$. Electrophoresis was at 800 V for 15 h at 22°C (8). During the later stages of this work the catholyte and anolyte solutions and the electrical polarity were reversed. This caused the proteins to focus from the acidic end of the gel to the basic end, the reverse of the standard O'Farrell method. This change produced better overall separations of leaf proteins. Following isoelectric focusing the IEF gels were equilibrated twice for 15 min in 5 ml of O'Farrell equilibration buffer (18) and then subjected to electrophoresis on 10% acrylamide gels containing 0.1% w/v SDS. Separated proteins were visualized following fluorography at −80°C on preflashed Kodak X-AR no screen film. Nonradiolabeled proteins were extracted and separated on one-dimensional SDS gels (13).

## RESULTS AND DISCUSSION

When subjected to low-nonfreezing temperatures, spinach cold acclimated and developed a greater tolerance to intercellular freezing stress (9–11). Even very young spinach seedlings cultured *in vitro* have shown the capacity to increase freezing tolerance when exposed to low temperatures that promote cold acclimation (13). In this study, plants cultured *in vitro* showed the same hardiness potential and freezing-tolerance-induction kinetics as soil-grown plants (Table I). Furthermore, when CA plants were subjected to deacclimation by return to 25°C growth conditions, the rate of hardiness decline was nearly the same for both *in vitro* cultured and soil-grown plants. Photoperiod was never altered in these studies and was not a factor in the induction or loss of hardiness as it is for many woody species (26). Thus, in spinach, freezing tolerance appeared to be an inducible characteristic regulated by exposure to low temperature.

Alterations in the protein content of plant tissues have long been associated with cold acclimation and the cold hardy state. This includes both quantitative increases in protein content and alterations in electrophoretic profiles. Briggs and Siminovitch (2)

Table I. *Comparison of the Induction and Loss of Freezing Tolerance during Cold Acclimation and Deacclimation of True Leaves from Plants Grown on Soil or in Vitro on a Culture Medium*

Seedlings were grown for 3 weeks postsowing at 25°C with a 12 h photoperiod and were designated as NA. The cotyledons were removed after the first true leaves had emerged. Uniform seedlings were CA at 5°C with a 12 h photoperiod. Seedlings kept at 25°C were subject to freezing along with CA seedlings to ensure changes in hardiness were not the result of developmental processes. Values are the mean ± SD for the $LT_{50}$s.

| Treatment | Days | Growth Medium | |
|---|---|---|---|
| | | Soil | Agar |
| | | °C | |
| Nonacclimated | | −5 ± 1 | −4 ± 1 |
| Cold acclimated | | 5 | |
| | 1 | −6 ± 1 | −8 ± 2 |
| | 2 | −7 ± 1 | −7 ± 2 |
| | 3 | −8 ± 1 | −9 ± 1 |
| | 4 | −9 ± 1 | −11 ± 1 |
| | 7 | −9 ± 2 | −9 ± 2 |
| | 14 | −11 ± 2 | −10 ± 2 |
| Deacclimated | | 25 | |
| | 1 | −8 ± 2 | −8 ± 1 |
| | 3 | −7 ± 2 | −6 ± 1 |
| | 7 | −6 ± 2 | −5 ± 1 |

[2] Abbreviations: CA, cold acclimated; CAP, cold acclimation protein; HS, heat shock; HSP, heat shock protein; IEF, isoelectric focusing; LS, large subunit of Rubisco; $LT_{50}$, temperature required to kill 50% of the tissue; NA, nonacclimated; PMSF, phenylmethylsulfonyl fluoride; Rubisco, ribulose bisphosphate carboxylase oxygenase; SS, small subunit of Rubisco.

observed an accumulation of water-soluble protein during the winter season when cold hardiness was maximal. Many others have recorded similar increases in the protein content of CA plants (reviewed in Ref. 17). In spinach, cold acclimation did not lead to higher protein levels (Table II). A slight decline in the total protein content was observed during cold acclimation which was accelerated in the early stages of deacclimation. However, after 7 d of deacclimation, total protein content reached a concentration almost twice that of preacclimation levels. Incorporation of [$^{35}$S]methionine into protein was reduced during the first 4 d of low temperature exposure, but approached NA levels by the 7th d (Table II). Deacclimated plants had the highest rate of incorporation of label into protein. Taken together, these observations were consistent with previous findings that incorporation of [$^{35}$S]methionine into protein is increased at low temperature during the course of cold acclimation (11). It is unclear whether this resulted from increased uptake or changes in rates of synthesis and turnover of proteins or from altered methionine pools. The findings did suggest that proteins were rapidly degraded during the early stages of deacclimation. In the later stages of deacclimation, protein synthesis appeared to be accelerated and resulted in elevated protein content. This pattern of protein synthesis and degradation would have resulted in a rapid turnover of the proteins present in CA tissue during deacclimation. Thus, proteins synthesized at low temperature, that function to increase freezing tolerance, would quickly disappear along with freezing tolerance.

Table II. *Protein Content and Incorporation of [$^{35}$S]Methionine in Protein of Nonacclimated, Cold-acclimated, and Deacclimated Spinach Seedlings*

The data in this table are from a representative experiment.

| Treatment | Days | Protein | Incorporation | |
|---|---|---|---|---|
| | | *μg/ mg fresh wt* | *μCi/ mg fresh wt* | *cpm × 10$^{-4}$/ μg protein* |
| Nonacclimated | | 12.7 | 0.21 | 4.97 |
| Cold acclimated | | | 5°C | |
| | 1 | 11.5 | 0.14 | 3.01 |
| | 2 | 10.5 | 0.14 | 3.02 |
| | 4 | 11.9 | 0.17 | 3.19 |
| | 7 | 10.4 | 0.17 | 4.71 |
| | 14 | 10.1 | 0.20 | 3.02 |
| Deacclimated | | | 25°C | |
| | 1 | 8.9 | 0.21 | 5.90 |
| | 3 | 7.6 | 0.35 | 10.04 |
| | 7 | 22.7 | 0.63 | 6.18 |

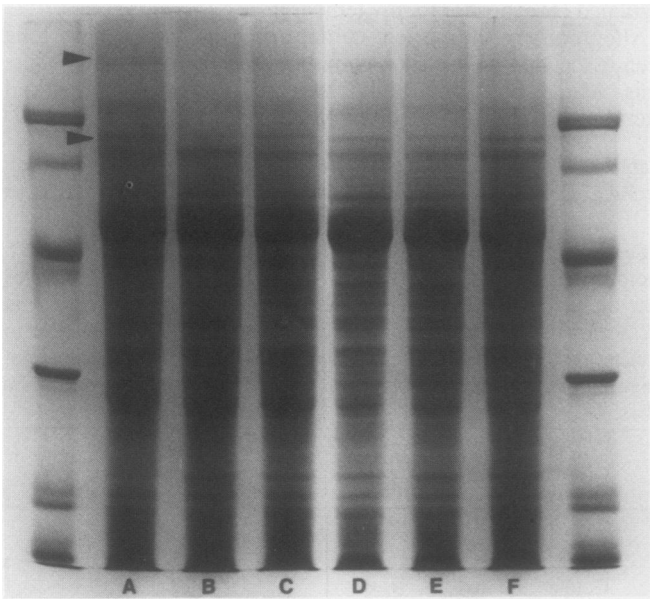


FIG. 1. SDS-PAGE of leaf proteins at different stages of cold acclimation. Proteins were solubilized in SDS buffer; 120 mM Tris-HCl (pH 6.8), 20 mM DTT, 20% v/v glycerol, and 2% w/v SDS. Aliquots of the protein extract (50 μg protein) were electrophoresed on a 10% acrylamide gel. Proteins were stained with Coomassie blue. Lanes are plants CA for A, 14 d; B, 7 d; C, 4 d; D, 2 d; E, 1 d; F, NA. Cold acclimation was at 5°C. Protein standards $M_r$ x 10$^{-3}$; phosphorylase *b* 92, BSA 66, ovalbumin 45, carbonic anhydrase 31, soybean trypsin inhibitor 22, lysozyme 14. Arrows indicate position of protein bands present only in extracts from CA leaves.

Changes in protein electrophoretic patterns characteristic of CA tissues have been demonstrated for a number of species (3, 7, 16, 27). When spinach leaf proteins from NA and CA tissue were separated on SDS gels and stained with Coomassie blue, banding patterns were very similar (Fig. 1). Comparison of the profiles of NA and CA proteins failed to reveal any major shifts in protein composition resulting from low temperature exposure. This fact and the incorporation data (Table II) suggested that the overall effect of low temperature on protein content was subtle or involved alterations only in low abundance polypeptides that were obscured by the low resolving power of the technique used in this experiment. However, two very faint bands with mol wt of about 160,000 and 85,000 (indicated by arrows in Fig. 1) were present in leaf tissue CA for 14 d that were not observed in NA tissue. The bands were not observed at earlier stages of cold acclimation and indicated that an extended period of cold acclimation was necessary for the proteins to accumulate to detectable levels.

Leaf proteins of NA, CA, and deacclimated plants were *in vivo* radiolabeled without injury at either 5 or 25°C. Separation of *in vivo* radiolabeled leaf proteins by two-dimensional gel electrophoresis greatly increased the resolution (up to 500 proteins were observed) and revealed the synthesis of several putative CAPs. These proteins were synthesized at 5°C, but not at 25°C (Figs. 2–4). The changes in protein synthesis, induced by low temperature, were rapid and stable. The synthesis of four high mol wt CAPs (160, 117, 85, 79) was detected at the earliest labeling interval possible, the first 24 h of cold acclimation, and throughout the 14 d cold acclimation treatment (Fig. 2). In some experiments, CAP 117 was resolved into two proteins (Fig. 5). When CA plants were returned to 25°C growth conditions, the synthesis of three CAPs (160, 117, and 85) was almost halted at the earliest labeling interval (the 24 h immediately following the transfer from low to warm temperature) (Fig. 3). However, synthesis of CAP 79 continued throughout the 7 d deacclimation treatment.

Other changes in the protein synthesis patterns were observed in lower mol wt proteins (19,000–48,000), but they were not consistent from experiment to experiment (Fig. 5). Additional work is needed to verify and characterize these changes. We did not find significant quantities of polypeptides that were present only in CA tissues in the mol wt range of 10,000 to 20,000 that might correspond to the small cryoprotective protein isolated from spinach (25). Also, nine lower mol wt polypeptides found in nonacclimated leaves appeared not to be synthesized in leaves exposed to 5°C. This observation was consistent with earlier findings using *in vitro* translation assays (11).

The finding that a rapid and stable change in the protein synthesis occurred in spinach leaves upon exposure to low temperature was consistent with the hypothesis put forth by Weiser (26) that cold acclimation, leading to greater freezing tolerance, involved altered gene expression. Changes in the translatable mRNA content of spinach leaf tissue exposed to low temperature have been reported and were supported by the data presented in this communication. Guy *et al.* (11) found that exposure to 5°C

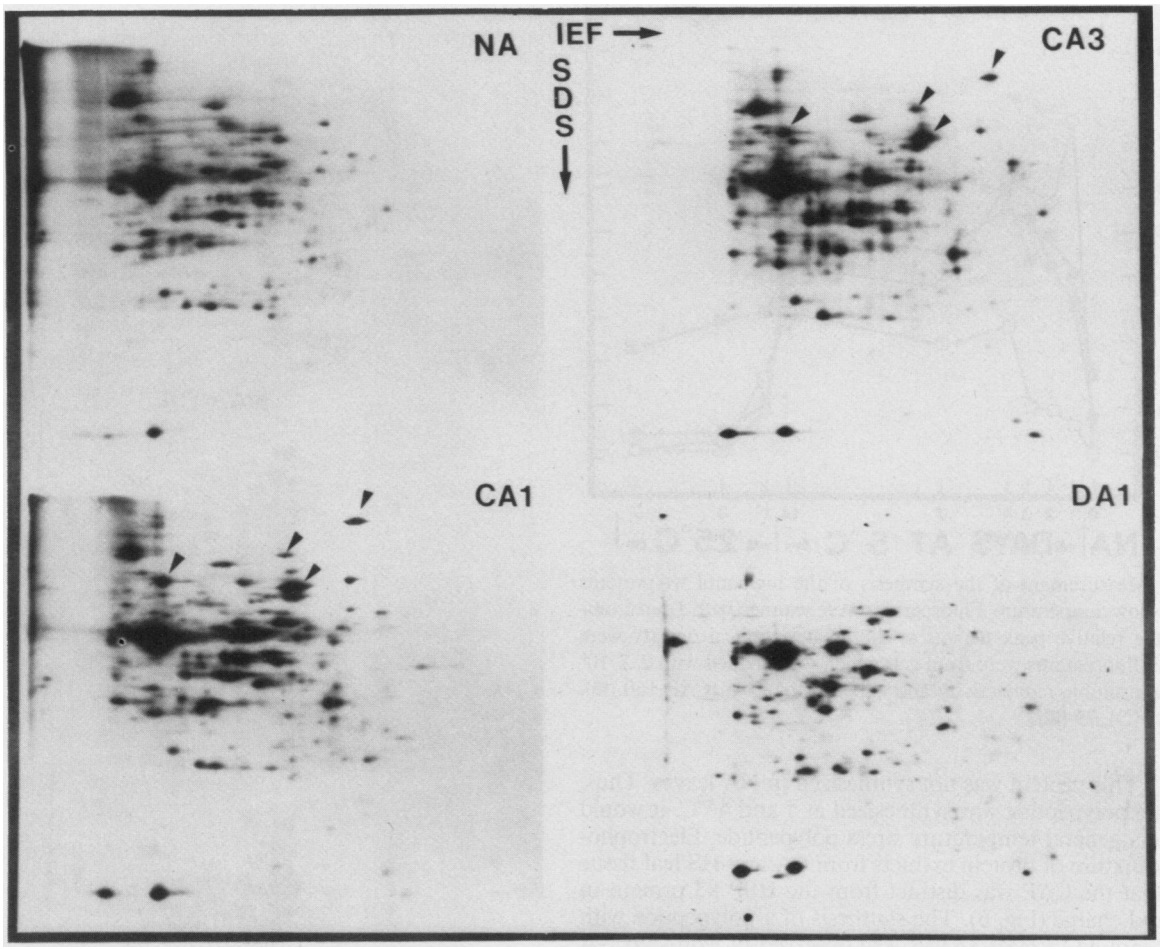


FIG. 2. Fluorograms of *in vivo* radiolabeled leaf proteins separated by two-dimensional gel electrophoresis. Nonacclimated, NA; cold-acclimated, CA; and deacclimated, DA. Numbers indicate the days of treatment. Arrows point out the four high mol wt proteins synthesized at 5°C. All gels were loaded with aliquots equal to 1 X $10^6$ cpm of TCA insoluble radioactivity and exposed for 72 h at −80°C.

resulted in the rapid appearance and stable expression of two high mol wt mRNAs that were correlated with the induction of freezing tolerance. Two of the CAPs (Figs. 2, 5) could possibly be the *in vivo* translation products of the two high mol wt mRNAs detected in the previous work. Additional evidence that supported an altered gene hypothesis in cold acclimation was the high correlation that existed between the synthesis of the CAPs and the induction and loss of freezing tolerance (Table I; Fig. 3). Freezing tolerance was rapidly induced and lost during cold acclimation and deacclimation at precisely the same time the CAPs synthesis was induced and halted. This correlated response suggested that synthesis of CAPs was required for the induction and maintenance of freezing tolerance at low temperature.

The locations on the gels and the intensity of the label incorporation suggested the high mol wt CAPs were newly synthesized and not the result of a posttranslational modification of proteins already present in NA tissue. Experiments, where proteins from NA and CA tissues were mixed and run on the same two-dimensional gel did not reveal large scale net charge or mol wt differences which would indicate a general posttranslational modification of housekeeping proteins (Fig. 4). Growth of rye for long periods at low temperature was shown to cause a change in the net charge and conformation of the large subunit of Rubisco (14). We saw no evidence of a change in net charge of spinach leaf proteins (including the large subunit of Rubisco) resulting from exposure to low temperature. The absence of a change in the net charge of the large subunit and other leaf proteins in spinach in contrast to rye could have resulted from species differences or from different durations of low temperature treatment (4 *versus* 90 d).

The effect of HS on plant protein synthesis has been well documented (1, 6, 15). Since altered protein synthesis at low temperature is another type of temperature response, it was important to compare protein synthesis during cold acclimation and HS. Unlike HS, cold acclimation did not shut down synthesis of housekeeping proteins in favor of CAPs (Figs. 2, 4, 5). Protein synthesis was reduced at 5°C, not through a form of translational control, but as a result of the general effect that reducing temperature has on protein synthetic activity.

In these experiments, a number of non-HSPs were labeled in the heat-shocked tissue. The incorporation of label into non-HSPs (the large and small subunits of Rubisco) occurred during the 2 h labeling period prior to the HS treatment. The kinetics of label uptake had indicated low incorporation of label into protein would occur in 2 h at 25°C (13). Apparently, under subsequent HS conditions the small amount of incorporation at 25°C was significant relative to the incorporation at 45°C and should not be construed as synthesis at the higher temperature.

Major differences in the patterns of protein synthesis of CA leaves and heat-shocked leaves were observed (Fig. 5). The response of spinach to cold acclimation and HS was characterized by the synthesis of polypeptides unique to each treatment. Based on mol wt and charge, one polypeptide synthesized during HS (HSP 83) also appeared to be synthesized during cold acclimation

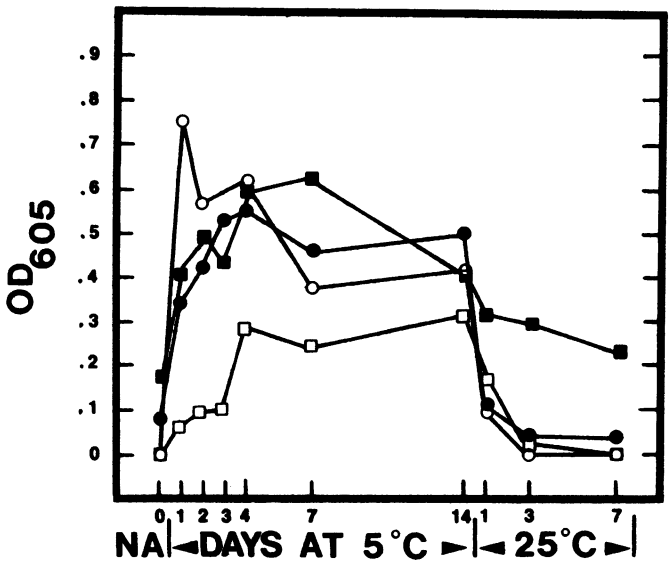


FIG. 3. Measurement of the synthesis of the high mol wt proteins induced by low temperature. Fluorograms were scanned with a densitometer and the relative peak heights at the greatest optical density were plotted. All fluorograms were from gels which were loaded with 2 X $10^6$ cpm TCA insoluble radioactivity and exposed for 24 h. CAP 160 (●), 117 (□), 85 (○), 79 (■).

(CAP 79). This protein was not synthesized in NA leaves. Thus, if the same polypeptide was synthesized at 5 and 45°C, it would constitute a general temperature stress polypeptide. Electrophoresis of a mixture of protein extracts from CA and HS leaf tissue verified that the CAP was distinct from the HSP 83 protein in mol wt and charge (Fig. 6). The synthesis of a polypeptide with a similar mol wt to HSP 83 was also observed in wounded NA tissue (11). Had it not been possible to radiolabel the leaf proteins without injury, it is unlikely that we would have observed that both CA and heat-shocked leaves synthesized a protein with a mol wt near 80,000, while NA leaves did not. It has been clearly demonstrated that excision or wounding dramatically altered the rate and the qualitative pattern of protein synthesis in root tips (23). Both of these effects would have significantly complicated our analyses.

Cross-protection experiments, where CA and heat-shocked seedlings were subjected to freezing and heat stress, provided additional evidence to support the conclusion that the CAP 79 was a different protein from the HSP 83. If the CAP and the HSP were the same protein, then stress tolerance might be induced for both temperature extremes by either cold acclimation or HS alone. Evidence for such a cross-protection has been reported for plants and fungi. Cold acclimation of *Solanum commersonii* increased the tolerance of the leaf tissue to high temperature (19). Conversely, when germinating conidiospores of *Neurospora crassa* were given a HS immediately prior to freezing, survival was increased 4- to 5-fold over nonheat-shocked conidiospores (12). Spinach plants subjected to HS and then frozen, showed no increased freezing tolerance over that of the control plants (Table III). The HS treatment did, however, confer a small increase in heat tolerance. Cold-acclimated plants were more freezing tolerant, but were no more heat tolerant than NA plants that were not given a HS treatment. In these experiments, no cross-protection was observed that could be linked to the synthesis of the 83,000 $M_r$ protein at either high or low temperature. Our results contrast with the findings of Palta *et al.* (19) that cold acclimation could induce greater thermotolerance. Furthermore, our findings largely support the conclusions of Chen *et al.* (4) that cold acclimation did not induce a greater

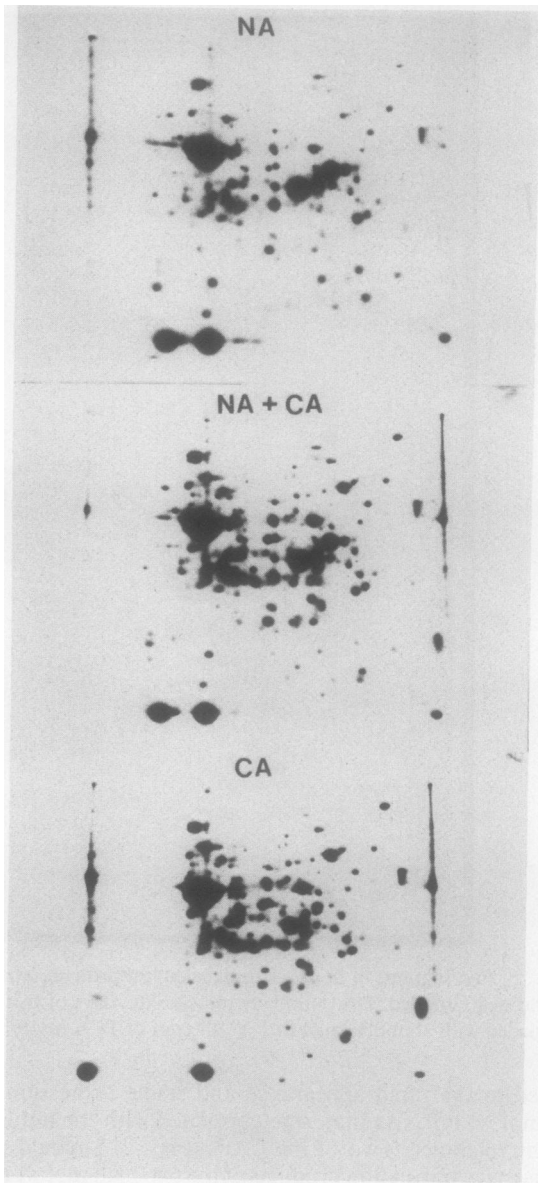


FIG. 4. Fluorograms of *in vivo* radiolabeled leaf proteins. Nonacclimated tissue, NA; nonacclimated and cold acclimated tissue protein extracts mixed together (5 X $10^5$ cpm of TCA insoluble radioactivity each), NA + CA; and cold-acclimated tissue, CA. The fluorograms for NA and CA were from separations that had 1 X $10^6$ cpms loaded on to the first dimension. Exposure time was 72 h at −80°C.

tolerance to high temperature stress and that heat acclimation did not increase freezing tolerance.

In summary, we have shown that exposure of spinach to a temperature of 5°C alters the relative pattern of protein synthesis. A few proteins present in NA leaves were not detected in cold-acclimated tissues, while approximately 20 proteins appeared to be synthesized in CA leaves that were not in NA leaves. The synthesis of three high mol wt polypeptides during cold acclimation was highly correlated with the induction of freezing tolerance. The response of spinach to temperature extremes caused by HS and low temperature was different. Cold acclimation did not induce heat tolerance and HS did not induce freezing tolerance. The function and subcellular location of the CAPs remain unknown. The synthesis of the CAPs at 5°C may result from the expression of genes involved in adjustment of tissue metabolism to low, nonfreezing temperatures or play a direct

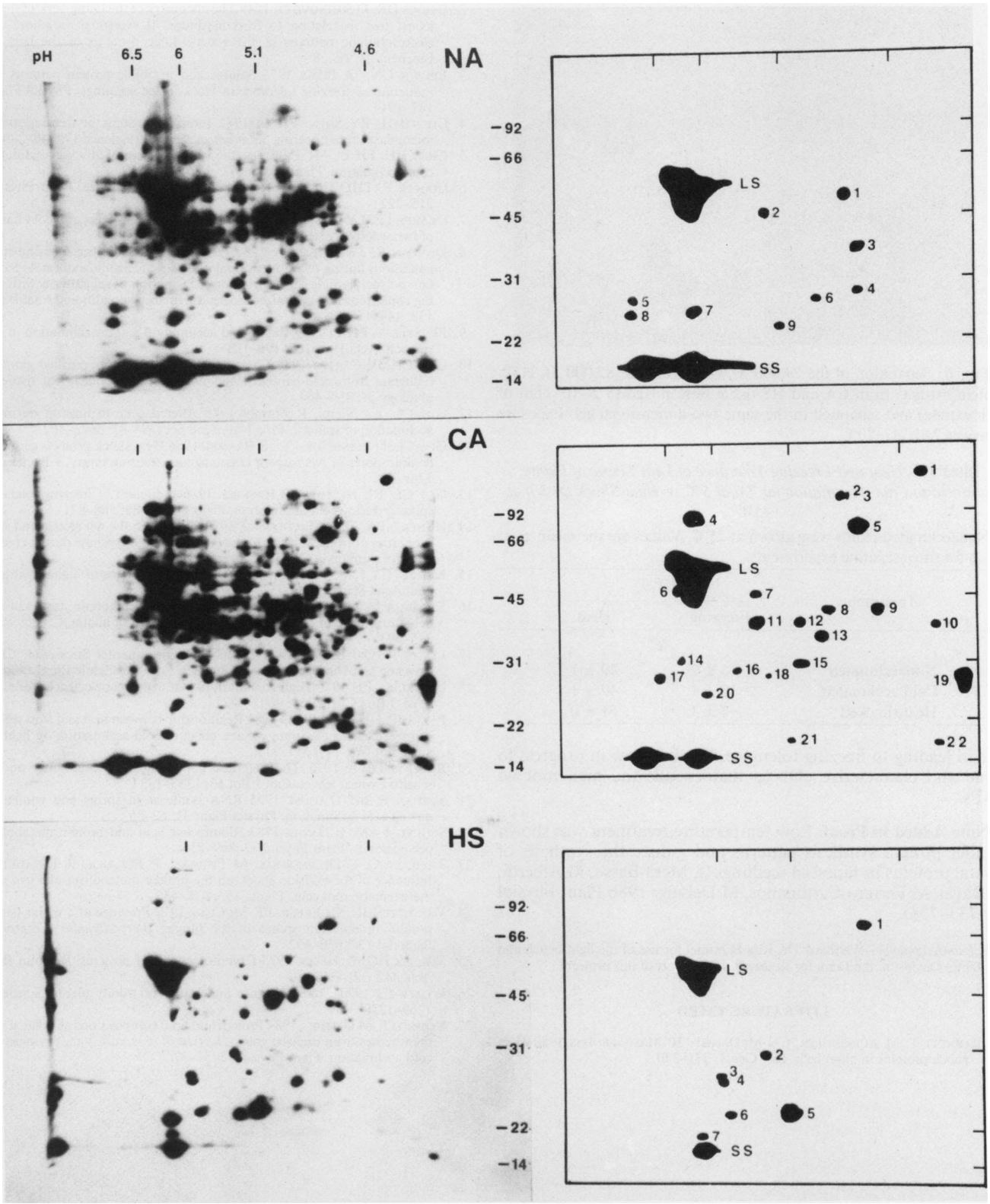


FIG. 5. Comparison of spinach leaf protein synthesis of NA, CA, and HS plants. Gels for NA and CA tissue proteins were loaded with 2 X $10^6$ cpm TCA insoluble radioactivity. The gel for HS tissue proteins was loaded with 7 X $10^5$ cpm TCA insoluble radioactivity. Exposure time for HS was 72 h and for NA and CA 24 h at −80°C. Polypeptides unique to each temperature treatment are indicated by the numbered spots. The position of protein standards is indicated. IEF dimension pIs: phycocyanin 4.6, β-lactoglobulin 5.1, bovine carbonic anhydrase 6.0, and human carbonic anhydrase 6.5; SDS dimension $M_r$ X $10^{-3}$: phosphorylase *b* 92, BSA 66, ovalbumin 45, carbonic anhydrase 31, soybean trypsin inhibitor 22, lysozyme 14.

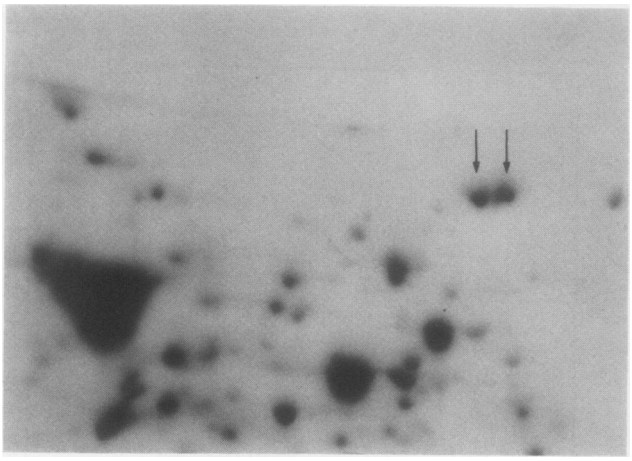

FIG. 6. Separation of the 79,000 $M_r$ CAP from the 83,000 $M_r$ HSP. Protein extracts from CA and HS tissue were mixed (5 X $10^5$ cpm of each extract) and separated in the same two-dimensional gel. Exposure time was 24 h at −80°C.

Table III. *Heat and Freezing Tolerance of Leaf Tissue of Plants Subjected to Cold Acclimation for 7 d at 5°C or Heat Shock for 3 h at 45°C*

Nonacclimated plants were grown at 25°C. Values are the mean ± SD $LT_{50}$s for three separate experiments.

| Treatment | $LT_{50}$ | |
|---|---|---|
| | Freezing | Heat |
| | °C | |
| Nonacclimated | −5 ± 2 | 49 ± 1 |
| Cold acclimated | −10 ± 1 | 49 ± 1 |
| Heat shocked | −4 ± 1 | 51 ± 0 |

role in leading to freezing tolerance. Work is now in progress to clone and characterize cDNAs that encode the high mol wt CAPs.

**Note Added in Proof.** Low temperature treatment was shown to alter protein synthesis patterns and induce the synthesis of several proteins in rapeseed seedlings (L Mesa-Basso, M Alberdi, M Rayal, M Ferrero-Cardinanos, M Delseny 1986 Plant Physiol 82: 733–738).

*Acknowledgments*—We thank Dr. Rita Hummel for use of the light bench and Dr. James Davidson, the Dean for Research, for support of this project.

## LITERATURE CITED

1. BARNETT T, M ALTSCHULER, CN MCDANIEL, JP MASCARENHAS 1980 Heat shock proteins in plant cells. Dev Gen 1: 331–340
2. BRIGGS DR, D SIMINOVITCH 1949 The chemistry of the living bark of the black locust tree in relation to frost hardiness. II. Seasonal variations in the electrophoretic patterns of the water-soluble proteins of the bark. Arch Biochem 23: 18–28
3. BROWN GN, JA BIXBY 1975 Soluble and insoluble protein patterns during induction of freezing tolerance in black locust seedlings. Physiol Plant 34: 187–191
4. CHEN HH, ZY SHEN, PH LI 1982 Interrelationships of freezing and heat tolerance in tuber bearing *Solanum* species. HortScience 17: 248–249
5. CHEN HH, PH LI, ML BRENNER 1983 Involvement of abscisic acid in potato cold acclimation. Plant Physiol 71: 362–365
6. COOPER P, THD HO 1983 Heat shock proteins in maize. Plant Physiol 71: 215–222
7. CRAKER LE, LV GUSTA, CJ WEISER 1969 Soluble proteins and cold hardiness of two woody species. Can J Plant Sci 49: 279–286
8. DUNCAN R, JWB HERSHEY 1984 Evaluation of isoelectric focusing running conditions during two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis: variation of gel patterns with changing conditions and optimized isoelectric focusing conditions. Anal Biochem 138: 144–155
9. FENNELL A, PH LI 1985 Rapid cold acclimation and deacclimation in winter spinach. Acta Hort 168: 179–183
10. GUY CL, JV CARTER 1984 Characterization of partially purified glutathione reductase from cold-hardened and nonhardened spinach leaf tissue. Cryobiology 21: 454–464
11. Guy CL, KJ Niemi, R Brambl 1985 Altered gene expression during cold acclimation of spinach. Proc Natl Acad Sci USA 82: 3673–3677
12. GUY CL, N PLESOFSKY-VIG, R BRAMBL 1986 Heat shock protects germinating conidiospores of *Neurospora crassa* against freezing injury. J Bacteriol 167: 124–129
13. GUY CL, RL HUMMEL, D HASKELL 1986 Induction of freezing tolerance in spinach during cold acclimation. Plant Physiol 84: 868–871
14. HUNER NPA, FDH MACDOWALL 1979 Changes in the net charge and subunit properties of ribulose bisphosphate carboxylase-oxygenase during cold hardening of puma rye. Can J Biochem 57: 155–164
15. KEY JL, CY LIN, YM CHEN 1981 Heat shock proteins of higher plants. Proc Natl Acad Sci USA 78: 3526–3530
16. KRASNUK M, GA JUNG, FH WITHAM 1976 Electrophoretic studies of several dehydrogenases in relation to the cold tolerance of alfalfa. Cryobiology 13: 375–393
17. LEVITT J 1980 Responses of Plants to Environmental Stresses. I. Chilling, Freezing and High Temperature Stresses, Ed 2. Academic Press, Orlando
18. O'FARRELL PH 1975 High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007–4021
19. PALTA JP, HH CHEN PH LI 1981 Relationship between heat and frost resistance of tuber-bearing *Solanum* species: effect of cold acclimation on heat resistance. Bot Gaz 142: 311–315
20. ROBERTS DWA 1975 The invertase complement of cold-hardy and cold-sensitive wheat leaves. Can J Bot 53: 1333–1337
21. SARHAN F, MJ D'AOUST 1975 RNA synthesis in spring and winter wheat during cold acclimation. Physiol Plant 35: 62–65
22. SCHUSTER AM, E DAVIES 1983 Ribonucleic acid and protein metabolism in pea epicotyls. Plant Physiol 73: 809–816
23. THEILLET C, F DELPEYROUX, M FISZMAN, P REIGNER, R ESNAULT 1982 Influence of the excision shock on the protein metabolism of *Vicia faba* L. meristematic root cells. Planta 155: 478–485
24. VAN ETTEN JL, SN FREER, BK MCCUNE 1979 Presence of a major (storage?) protein in dormant spores of the fungus *Botryodiplodia theobromae*. J Bacteriol 138: 650–652
25. VOLGER HG, U HEBER 1975 Cryoprotective leaf proteins. Biochim Biophys Acta 412: 335–349
26. WEISER CJ 1970 Cold resistance and injury in woody plants. Science 169: 1269–1278
27. YOSHIDA S, M UEMURA 1984 Protein and lipid compositions of isolated plasma membranes from orchard grass (*Dactylis glomerata* L.) and changes during cold acclimation. Plant Physiol 75: 31–37