Synthesis of the Low Molecular Weight Heat Shock Proteins in Plants[1]

MICHAEL A. MANSFIELD*[2] AND JOE L. KEY
Botany Department, University of Georgia, Athens, Georgia 30602

ABSTRACT

Heat shock of living tissue induces the synthesis of a unique group of proteins, the heat shock proteins. In plants, the major group of heat shock proteins has a molecular mass of 15 to 25 kilodaltons. Accumulation of these proteins to stainable levels has been reported in only a few species. To examine accumulation of the low molecular weight heat shock proteins in a broader range of species, two-dimensional electrophoresis was used to resolve total protein from the following species: soybean (*Glycine max* L. Merr., var Wayne), pea (*Pisum sativum* L., var Early Alaska), sunflower (*Helianthus annuus* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L., cv IR-36), maize (*Zea mays* L.), pearl millet (*Pennisetum americanum* L. Leeke, line 23DB), and *Panicum miliaceum* L. When identified by both silver staining and incorporation of radiolabel, a diverse array of low molecular weight heat shock proteins was synthesized in each of these species. These proteins accumulated to significant levels after three hours of heat shock but exhibited considerable heterogeneity in isoelectric point, molecular weight, stainability, and radiolabel incorporation. Although most appeared to be synthesized only during heat shock, some were detectable at low levels in control tissue. Compared to the monocots, a higher proportion of low molecular weight heat shock proteins was detectable in control tissues from dicots.

Exposure of plants to elevated temperatures induces the synthesis of a limited number of hsp[3] (1, 2, 4, 5, 10, 14, 19, 22). In animal systems, the greatest proportion of hsp synthesis is represented by the high molecular mass hsp of 68 to 110 kD, with polypeptides of 70 kD being the predominant species (see Ref. 21 for review). In *Drosophila*, it has been shown that several low mol wt hsp are synthesized in significant amounts during normal development (3, 18). The functions of the hs proteins at control temperatures may not be the same as at the hs temperature (3).

Although plants synthesize a similar set of high mol wt hs proteins, most of the translational capacity is devoted to the synthesis of the low molecular mass hsp of 15 to 25 kD based on incorporation of radioactive amino acids (12). Through two-dimensional electrophoresis and fluorography, the low mol wt hsp have been shown to be comprised of a diverse array of polypeptides in several species (6, 14, 19, 22, 29). Although this group of proteins accumulates to stainable levels in tomato (22), soybean (11), cotton (4), and *Tradescantia paludosa* (29), there are reports that the low mol wt hsp are not synthesized in substantial quantities in maize (5, 6). If the generalization that the low mol wt hsp are the major hsp in all plant species (12) is to hold true, then it would be expected that their synthesis is induced by hs and that they accumulate in all plant species. Given the complexity of the low mol wt hsp, selective expression of certain hsp during normal development may also be occurring in some plant species. The purpose of this study is to characterize more precisely the number of low mol wt hsp accumulated in a variety of crop plants using two-dimensional electrophoresis and silver staining. The protein patterns generated from hs tissue are compared to those from control tissue to identify low mol wt hsp synthesized in the absence of hs.

MATERIALS AND METHODS

Plant Material. The following species were utilized: soybean (*Glycine max*, L. Merr., var Wayne), pea (*Pisum sativum* L., var Early Alaska), sunflower (*Helianthus annuus* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L., cv IR-36), maize (*Zea mays* L.), pearl millet (*Pennisetum americanum* L. Leeke, line 23DB), and *Panicum miliaceum* L. Seeds were surface-sterilized in 10% Clorox for 10 min, rinsed thoroughly in tap water, soaked for 3 to 4 h, and then planted in moist Kimpak rolls (14). Surface sterilization was omitted for millet and *P. miliaceum* since fungal growth was not a problem. All species were germinated at 28 to 30°C in the dark except for pea which was germinated at 24°C. Seedlings were grown until their size was sufficiently large to facilitate experimental manipulation (Table I).

Incubation and Labeling Conditions. Seedlings, or portions thereof, were incubated for 3 h in 10 ml of incubation buffer (1 mM potassium phosphate, pH 6.0; 1% [w/v] sucrose; 50 µg/ml chloramphenicol) with gentle shaking. L-[4,5-³H]leucine (New England Nuclear) at a concentration of 20 µCi/ml was included in all incubations except for rice and *P. miliaceum* where the concentration was 10 µCi/ml. Whenever possible, tissues serving as endogenous reservoirs of amino acids (*i.e.* cotylendons and endosperm) were removed prior to incubation (Table I) so that labeling of the hsp with [³H]leucine would be maximized. If removal of storage tissues could not be performed without mutilating the seedlings, they were left intact until the end of the incubation period. For pearl millet and *P. miliaceum*, the entire seedling was used for protein analysis.

Incubation temperatures (Table I) were selected so as to maximize synthesis of the low mol wt hsp. Optimal temperatures for hs protein synthesis have been reported for wheat (1, 14), pea (10), sunflower (10), wheat (10), maize (5), and millet (10). For rice, 40°C was selected as the experimental temperature; a hs temperature of 40°C is consistent with its natural distribution. In the case of *P. miliaceum*, 45°C was found to be the optimal temperature for induction of synthesis of the low mol wt hsp.

Protein Extraction and Two-Dimensional Electrophoresis. Total protein was extracted in 5 ml of SDS extraction buffer as

[2] Present address: MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824.

[3] Abbreviations: hsp, heat shock protein(s); hs, heat shock; poly(A)RNA, polyadenylated RNA; IEF, isoelectric focusing.

Table I. *Conditions and Tissues Used for Incubation and Homogenization*

The experimental conditions and tissues used in each incubation are specified. The incubation protocol and protein extraction procedure are outlined in "Materials and Methods."

| Species | Age at Harvest | No. of Seedlings | Incubation Temperature | | Tissues Excised | |
			Control	hs	Prior to incubation	Prior to homogenization
	d		°C			
Soybean	2	5	28	40	Cotyledons	None
Pea	3	5	24	36	Cotyledons	None
Sunflower	3	5	28	40	None	Cotyledons
Wheat	3	5	28	40	Endosperm	None
Rice	4	20	28	40	Endosperm	None
Maize	3	5	28	40	Endosperm	Scutellum, shoot
Millet	3	15	28	46	None	None
P. miliaceum	3	20	28	45	None	None

described in Lin *et al.* (16) except that the tissue was ground in a mortar and pestle. Protein content of the final samples was determined using the assay procedure of Schaffner and Weissman (25). Amino acid incorporation was determined as described by Mans and Novelli (17). For electrophoresis, protein was precipitated from aliquots of each sample by adding 4 volumes of acetone and incubating overnight at −20°C. The precipitates were pelleted, dried briefly under vacuum, and resuspended in O'Farrell (24) sample buffer. Samples were loaded on the acidic end of the tubes gels and run for 5800 V h. The effective range of the IEF gels was pH 4 to 7. Proteins were separated on the second dimension using SDS-PAGE on 12.5% (w/v) acrylamide gels (15). Unlabeled (Sigma) and [^{14}C]formaldehyde-labeled (Bethesda Research Laboratories) protein standards were included on all gels. Proteins were visualized by silver staining (23) or fluorography (16).

For each species, equal amounts of protein were loaded on gels to be used for silver staining. The upper limit on protein that could be loaded per gel was found to be 200 µg. However, for wheat, millet, and sunflower, only 100 µg were loaded per gel. In the cases of wheat and millet, this was necessitated by the relatively low amounts of protein recovered after homogenization. Sunflower seedlings did yield sufficient protein to permit loading 200 µg, but other components in the samples interfered with resuspension in O'Farrell sample buffer and entry of protein into the IEF gel when more than 100 µg was used. To compensate for these difficulties, color development during silver staining was allowed to proceed for a longer period of time. On gels to be analyzed by fluorography, the amount of TCA-precipitable cpm loaded for each species was maximized within these protein limits.

RESULTS

The two-dimensional IEF/SDS-PAGE gel system used in this analysis resolved the low molecular mass hsp of 15 to 25 kD with isoelectric points between pH 4 and 7. Polypeptides were characterized as hsp on the basis of both increased stainability and incorporation of [^{3}H]leucine after 3 h of hs at the 'optimum' hs temperature of each species. By this technique, two classes of low molecular weight hsp were identified (Table II): polypeptides apparently induced by hs and polypeptides present in control tissue and increased after hs. In soybean (Fig. 1), a total of 27 acidic polypeptides was characterized as low mol wt hsp by these criteria (Fig. 1C). Of these 27 polypeptides, six were detectable by silver staining in control tissue (Fig. 1A), although in amounts considerably less than observed in hs tissue. This was true for the low mol wt hsp found in control tissue of all species examined here. The 22 low mol wt hsp of pea were also diverse in distri-

Table II. *Low Mol Wt hsp Synthesized by Various Plant Species*

Values represent the number of polypeptides characterized as low molecular mass hsp of 15 to 25 kD in Figures 1 through 8. Induced polypeptides are detectable only after hs. Enhanced polypeptides are detectable in control tissue but exhibit increased stainability and radiolabel incorporation after hs.

Species	No. of Induced Polypeptides	No. of Enhanced Polypeptides	Total No. of hsp
Soybean	21	6	27
Pea	13	9	22
Sunflower	19	4	23
Wheat	11	1	12
Rice	13	2	15
Maize	23	1	24
Millet	16	1	17
P. miliaceum	21	2	23

bution (Fig. 2, C and D), but at least nine were detectable in control tissue (Fig. 2A). In sunflower, a total of 23 low mol wt hsp was detected with four present in low amounts in control tissue (Fig. 3A). Compared to the monocots examined, these dicots exhibited a higher proportion of low mol wt hsp that were detectable in control tissue, with pea exhibiting the maximum (41%).

Among the monocot species examined, there was more variability in the number of low mol wt hsp detected. Wheat (Fig. 4) synthesized and accumulated 12 low mol wt hsp (Fig. 4C) with only one detectable in control tissue (Fig. 4A). A total of 15 low mol wt hsp was detected in rice seedlings (Fig. 5C), three being present in control tissue (Fig. 5A). Unexpectedly, 24 low mol wt hsp were detected in maize (Fig. 6C). Only one of these was observed in control tissue (Fig. 6A). Millet (Fig. 7) synthesized 17 low mol wt hsp in response to hs (Fig. 7C). As observed in wheat and maize, only one low mol wt hsp from millet was observed in control tissue (Fig. 7A). *P. miliaceum* (Fig. 8) produced 23 low mol wt hsp (Fig. 8C), two of which were found in control tissue (Fig. 8A).

DISCUSSION

In each of the species examined in this study, the low mol wt hsp resolved into a diverse array of polypeptides by two-dimensional electrophoresis. The number of proteins detected ranged from as few as 12 for wheat up to 27 for soybean (Table II). Within each species, the low mol wt hsp exhibited considerable heterogeneity in isoelectric point, mol wt, relative stainability, and radiolabel incorporation. Although differences in stainability
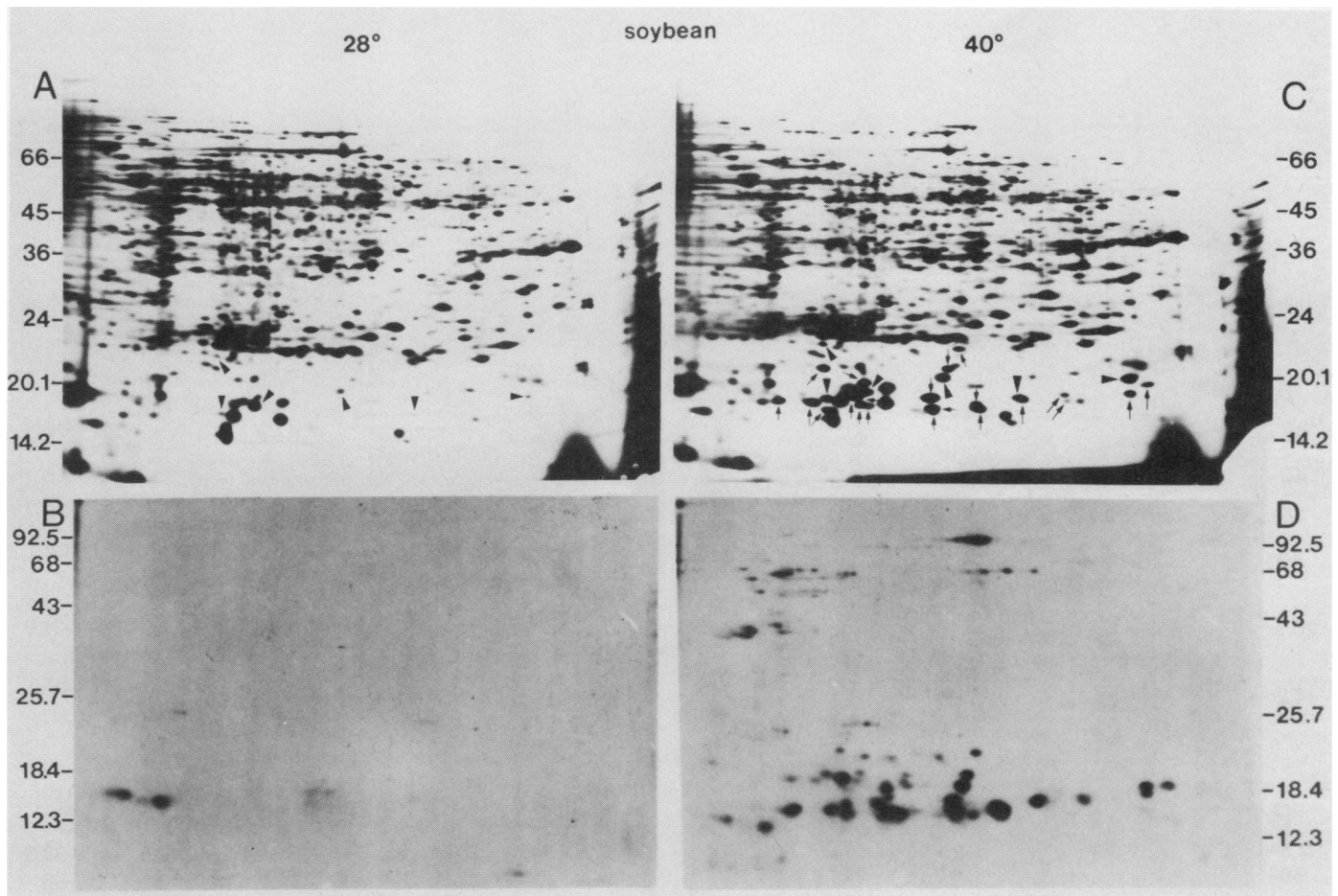
FIG. 1. Two-dimensional separation of total protein from control and hs soybean seedlings. Total protein, extracted from soybean seedlings incubated in the presence of [³H]leucine for 3 h at 28 or 40°C, was resolved by two-dimensional IEF/SDS-PAGE. Protein from control tissue (A, B) and hs tissue (C, D) was visualized by silver staining (200 μg per gel in A, C) or fluorography (1.75 × 10⁶ cpm per gel in B, D). Arrows in C indicate low molecular weight hsp induced by hs. Arrowheads in A and C indicate low molecular weight hsp also present in low amounts in control tissue. Molecular masses (kD) of protein standards are indicated.

and radiolabel incorporation could be accounted for in several ways, they most probably reflect differences in the levels to which specific polypeptides accumulate. In soybean, the diversity of these proteins can be attributed at least in part to their derivation from multigene families (8, 11, 20, 26, 27). These proteins may also be related between different species as cDNA clones for the low mol wt hsp of soybean hybridize with poly(A)RNA from pea, sunflower, millet, and maize (10).

While most of the low mol wt hsp appear to be synthesized *de novo* upon temperature shift, each of the eight species synthesized one or more of these polypeptides in non-hs tissue. Pea had the greatest percentage (41%) detectable in control tissue. Although small amounts of some hsp have been reported in control tissue for tomato (22) and maize (6), detection of the low mol wt hsp in control seedlings was unexpected. In soybean, Northern analysis of poly(A)RNA from control seedlings failed to detect the presence of any mRNA for this group of proteins (7, 13). These polypeptides may represent a subgroup of the low mol wt hsp, constitutively synthesized at low levels, with sufficient stability to permit accumulation. The presence of these proteins in control tissue suggests that they may have a physiologically important function under normal conditions. It also implies that the expression of these proteins is controlled by mechanisms independent of hs induction. Alternatively, the polypeptides detected in control tissue may represent proteins which co-migrate with the hsp in question.

For soybean, pea, and millet, there is good agreement between the staining patterns observed in this study and fluorographic patterns reported previously (10, 14). The reduced intensities of spots seen on the fluorograms presented here is not considered significant as the exposure times were kept comparatively short to permit maximum resolution of closely spaced clusters of polypeptides. Reduced exposure times also make it appear that few polypeptides were radiolabeled in control tissue. This observation can be explained by the translational switch that occurs during hs (21). Prior to hs, a large number of polypeptides is being synthesized. But within minutes of the initiation of hs, translation of control proteins is greatly reduced and synthesis of hs proteins is induced. While many proteins are synthesized in control tissue, comparatively few proteins are synthesized in hs tissue. The net effect is that the radiolabel in control samples is distributed over a much larger number of polypeptides than in hs samples, where it is primarily concentrated in the hsp. Thus, hsp were easier to visualize by fluorography than control proteins labeled for the same time interval. Prolonged exposure of control gels did indeed show that control proteins incorporated radioisotope during the incubation period (*e.g.* Fig. 4 and 7, and Refs. 10 and 14).

The observation that maize synthesized and accumulated 24 low mol wt hsp is not consistent with previous reports (2, 6). In one study (2), a single low mol wt hsp was observed. And in a second study (6), while several low mol wt hsp were detected by
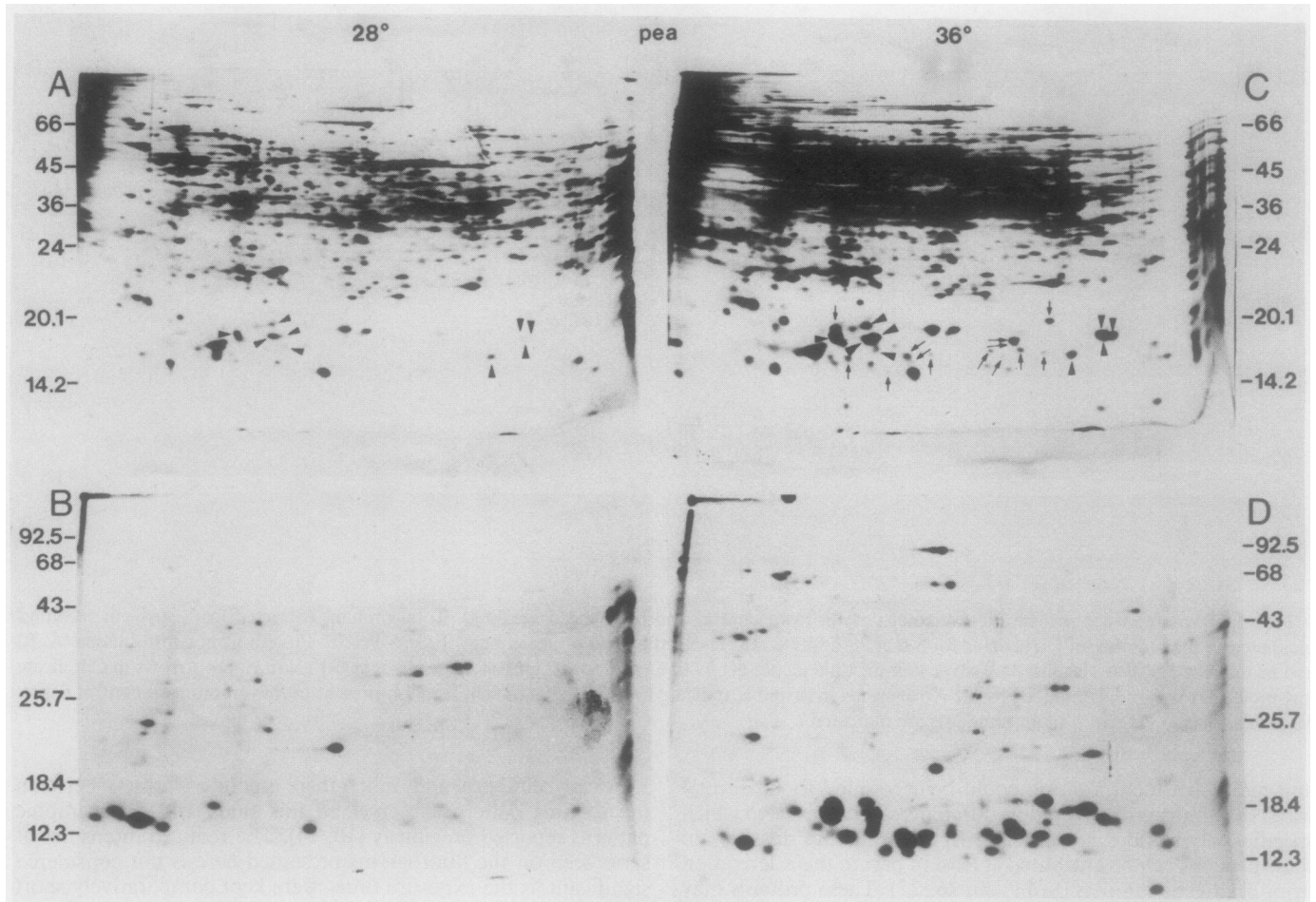
FIG. 2. Two-dimensional separation of total protein from control and hs pea seedlings. Total protein, extracted from pea seedlings incubated in the presence of [³H]leucine for 3 h at 24 or 36°C, was resolved by two-dimensional IEF/SDS-PAGE. Protein from control tissue (A, B) and hs tissue (C, D) was visualized by silver staining (200 μg per gel in A, C) or fluorography (3.5 × 10⁵ cpm per gel in B, D). Symbols are defined in the legend to Figure 1.
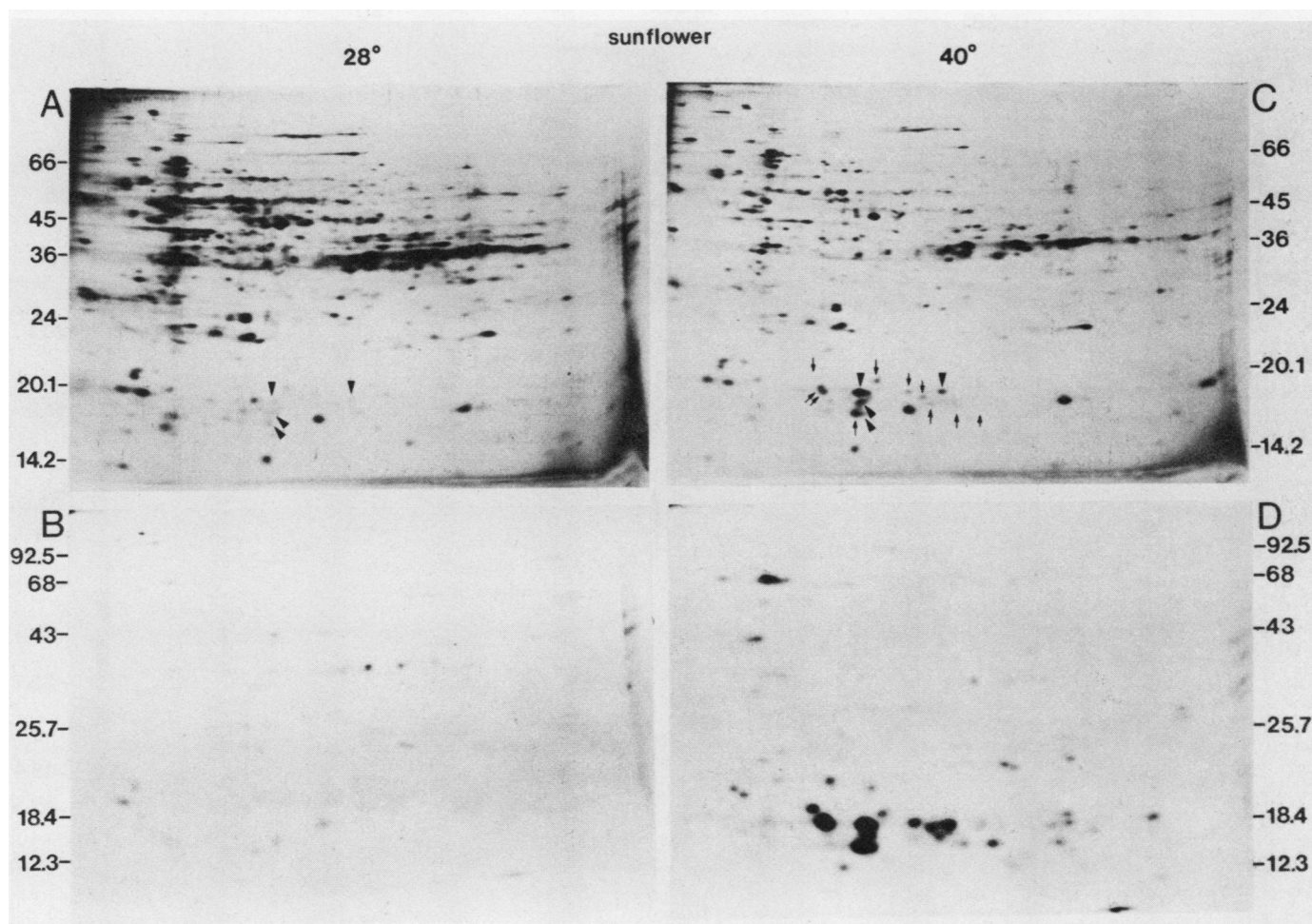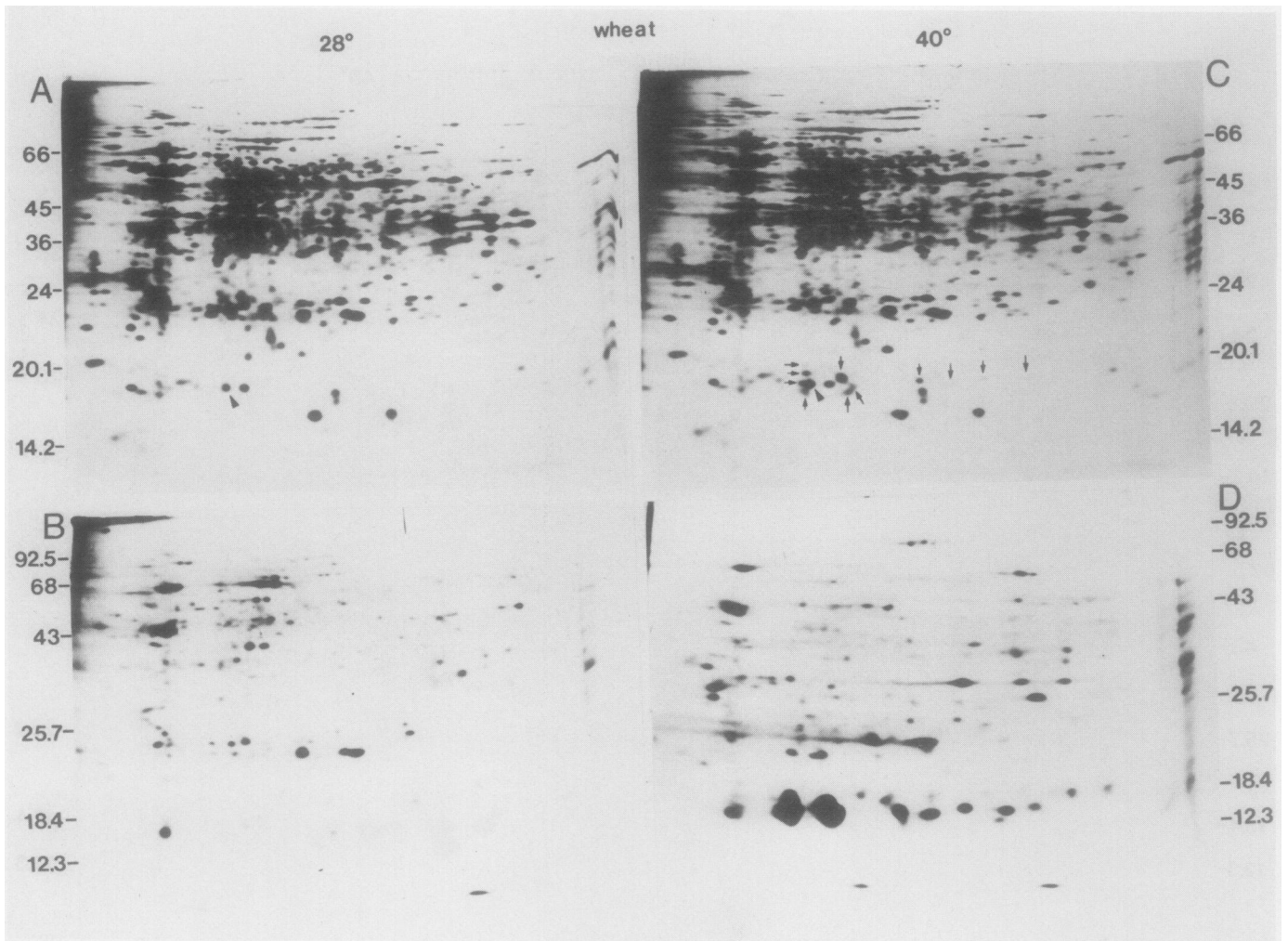
FIG. 3. Two-dimensional separation of total protein from control and hs sunflower seedlings. Total protein, extracted from sunflower seedlings incubated in the presence of [³H]leucine for 3 h at 28 or 40°C, was resolved by two-dimensional IEF/SDS-PAGE. Protein from control tissue (A, B) and hs tissue (C, D) was visualized by silver staining (100 μg per gel in A, C) or fluorography (7.5 × 10⁵ cpm per gel in B, D). Symbols are defined in the legend to Figure 1.

FIG. 4. Two-dimensional separation of total protein from control and hs wheat seedlings. Total protein, extracted from wheat seedlings incubated in the presence of [³H]leucine for 3 h at 28 or 40°C, was resolved by two-dimensional IEF/SDS-PAGE. Protein from control tissue (A, B) and hs tissue (C, D) was visualized by silver staining (100 μg per gel in A, C) or fluorography (2.6 × 10⁵ cpm per gel in B, D). Symbols are defined in the legend to Figure 1.
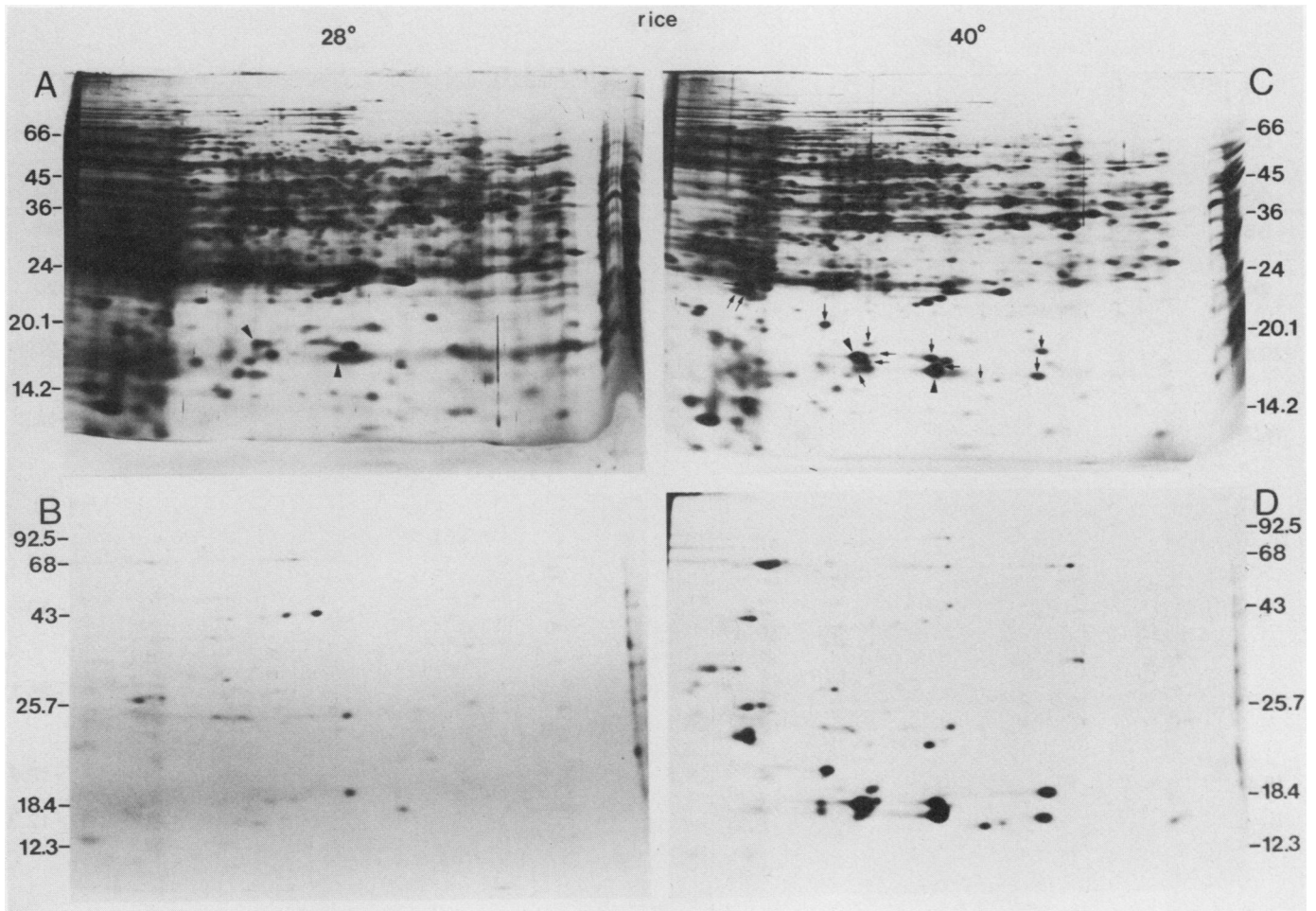
FIG. 5. Two-dimensional separation of total protein from control and hs rice seedlings. Total protein, extracted from rice seedlings incubated in the presence of [³H]leucine for 3 h at 28 or 40°C, was resolved by two-dimensional IEF/SDS-PAGE. Protein from control tissue (A, B) and hs tissue (C, D) was visualized by silver staining (200 μg per gel in A, C) or fluorography (7.5 × 10⁵ cpm per gel in B, D). Symbols are defined in the legend to Figure 1.
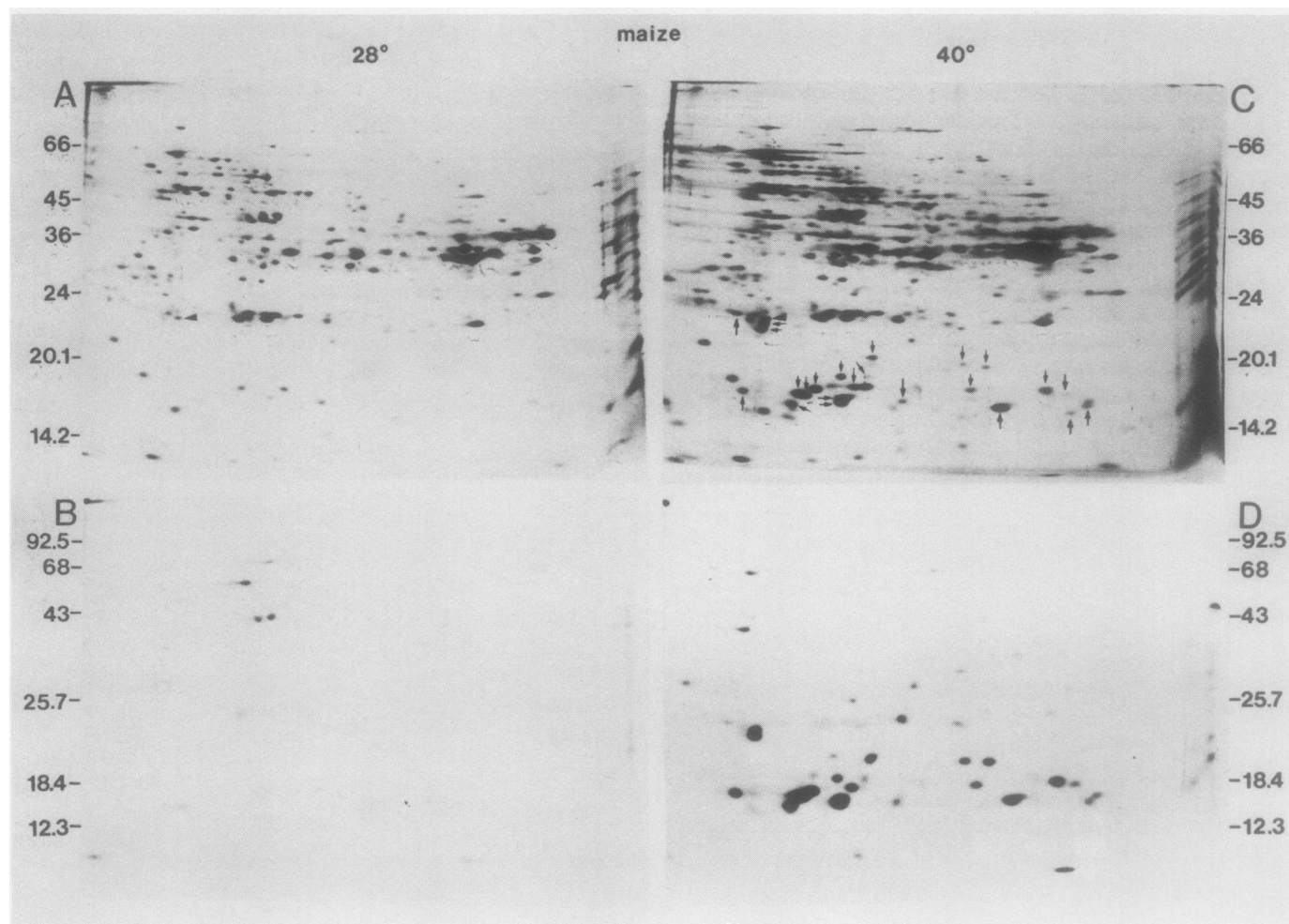
FIG. 6. Two-dimensional separation of total protein from control and hs maize seedlings. Total protein, extracted from maize seedlings incubated in the presence of [³H]leucine for 3 h at 28 or 40°C, was resolved by two-dimensional IEF/SDS-PAGE. Protein from control tissue (A, B) and hs tissue (C, D) was visualized by silver staining (200 μg per gel in A, C) or fluorography (5 × 10⁵ cpm per gel in B, D). Symbols are defined in the legend to Figure 1.
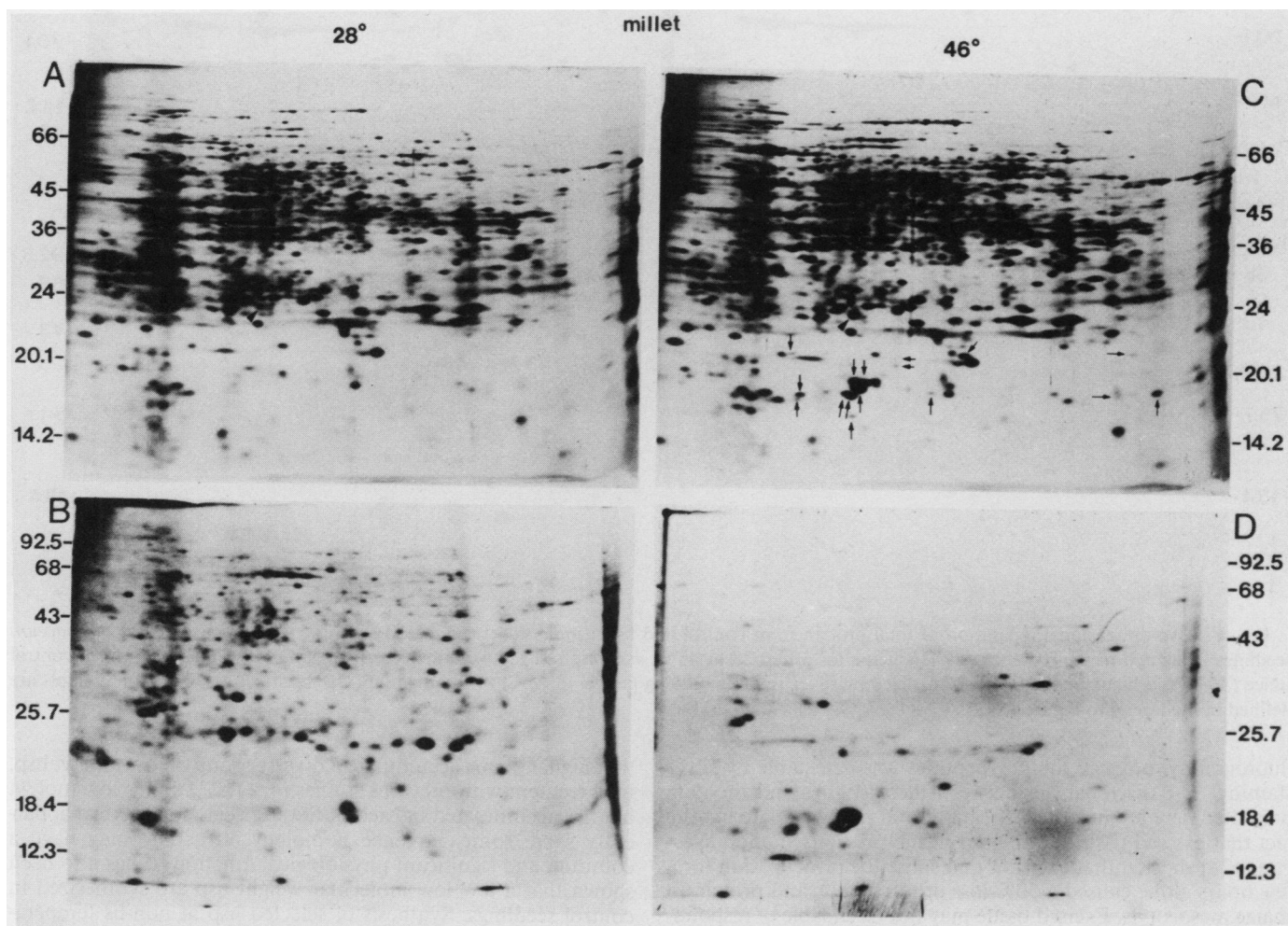
FIG. 7. Two-dimensional separation of total protein from control and hs pearl millet seedlings. Total protein, extracted from millet seedlings incubated in the presence of [³H]leucine for 3 h at 28 or 46°C, was resolved by two-dimensional IEF/SDS-PAGE. Protein from control tissue (A, B) and hs tissue (C, D) was visualized by silver staining (100 μg per gel in A, C) or fluorography (2.1 × 10⁵ cpm per gel in B, D). Symbols are defined in the legend to Figure 1.
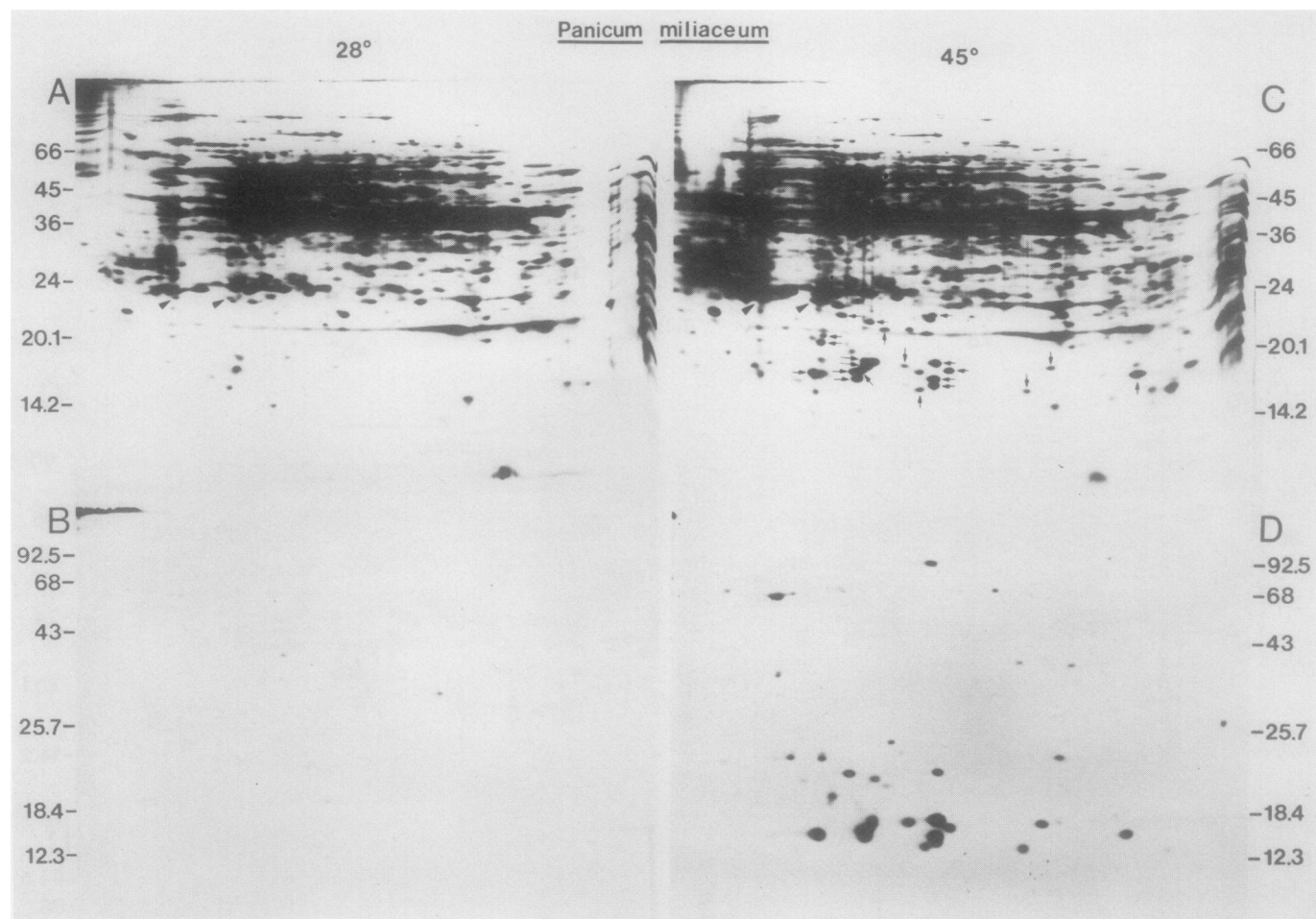
FIG. 8. Two-dimensional separation of total protein from control and hs *P. miliaceum* seedlings. Total protein, extracted from *P. miliaceum* seedlings incubated in the presence of [³H]leucine for 3 h at 28 or 45°C, was resolved by two-dimensional IEF/SDS-PAGE. Protein from control tissue (A, B) and hs tissue (C, D) was visualized by silver staining (200 μg per gel in A, C) or fluorography (10⁶ cpm per gel in B, D). Symbols are defined in the legend to Figure 1.

fluorography, none of the polypeptides was detectable by silver staining. The discrepancies between these studies and the data presented here are not entirely clear, but it may be related to the fact that excised tissues were used in the previous studies. West *et al.* (28) showed that excision and subsequent incubation for a few hours alone caused a 50% loss of total RNA and protein in maize mesocotyls. Excised tissue may not be capable of responding to hs in the same manner as intact tissue. Nevertheless, by using essentially intact seedlings, it was shown that maize synthesizes and accumulates at least 24 low mol wt hsp.

It must be noted that the techniques used may not detect all of the low mol wt proteins. Two-dimensional IEF/SDS-PAGE (24) resolves acidic proteins with isoelectric points from pH 4 to 7. Basic hsp, as reported in tomato (22) and tobacco (19), would have to be resolved on a different gel system. Furthermore, Nover and Scharf (22) have detected minor hsp after enrichment of specific cellular fractions. Although tissues containing large endogenous pools of proteins were excised from the seedlings prior to homogenization, minor hsp could still be insufficiently concentrated to permit detection. And finally, identification of a polypeptide as a hsp by both silver staining and radiolabeling requires that it contains lysine (9) and leucine, respectively. A polypeptide lacking one or both of these amino acids would not be identified as a major hsp. In fact, there were polypeptides detectable by either silver staining or radiolabeling after hs which were not characterized as hsp in this analysis.

In summary, the data presented in this report demonstrate

that higher plants accumulate a diverse group of low mol wt hsp, in agreement with previous studies (4, 11, 22). Since low mol wt hsp are accumulated in each of these species and the hsp apparently share some sequence homology (10), they may have a common and significant physiological function. It has also been shown that a few low molecular weight hsp are synthesized in control seedlings. Synthesis of selected hsp at non-hs temperatures suggests that they are involved in essential physiological functions, although their functions at normal temperatures may be different from those during hs (3).

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