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Long-Lived and Short-Lived Heat-Shock Proteins in Tobacco Mesophyll Protoplasts

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ABSTRACT

We have studied modifications in the pattern of proteins synthesized by tobacco (*Nicotiana tabacum* var Maryland) mesophyll protoplasts when they are transferred from 25°C to 40°C. The synthesis of one group of proteins is practically unaffected by the heat shock. On the other hand, the synthesis of most other 25°C proteins is greatly reduced, while specific heat-shock proteins appear: 17 stable, neutral, major proteins, which are synthesized throughout the culture period at the higher temperature and which correspond to those observed in other organisms, and two basic proteins with a short lifetime and which are synthesized only during the first 2 hours of heat shock. We suggest that these latter proteins are regulatory peptides which intervene in the inhibition of 25°C syntheses.

Considerable changes in protein synthesis are seen when *Drosophila* cells or tissues, normally cultivated at 25°C, are maintained at 37°C. Although most 25°C proteins are no longer synthesized at 37°C, six to eight 'heat-shock' proteins appear. The function of these proteins is unknown, but they constitute, nonetheless, one of the best systems for the study of the differential regulation of protein synthesis in eukaryotes. It has been shown that the cessation of synthesis of 25°C proteins is due both to the cessation of transcription of the corresponding genes (4) and to a specific block of translation of 25°C mRNA that are present at the beginning of culture at 37°C but are not degraded at this temperature (8, 16, 18). The appearance of heat-shock proteins coincides with an extremely abundant synthesis of their mRNA (4), which are specifically translated in shocked cells (16). Studies using cloned genes of heat shock and 25°C proteins open the way to the characterization of the molecular mechanisms responsible for this differential expression (20).

Similar responses concerning protein synthesis at high temperature have been described in other invertebrates (19), birds (6), mammals (1, 3, 15), and fungi (5, 9). It is only very recently that two articles have reported similar phenomena in higher plants, using monodimensional electrophoresis to characterize the proteins (2, 7). Because of the potential interest of such a system for the study of the regulation of gene expression in higher plants, it is of interest to obtain precise information on the phenomenon in these organisms.

The monodimensional pattern of proteins synthesized at 40°C is complex compared with that of heat-shock proteins of *Drosophila*. It is uncertain whether this is the result of the synthesis of a large number of induced proteins or of the superposition of induced and residual 25°C syntheses.

By using the high resolution method of O'Farrell *et al.* (13, 14) for polypeptide analysis, we have shown that certain major 25°C proteins continue to be abundantly synthesized at 40°C, whereas synthesis of others is considerably reduced. Simultaneously, major new proteins appear at 40°C, which we designate as heat-shock proteins. The characterization of these three classes of proteins has allowed us to follow the kinetics of appearance of heat-shock proteins and that of the reduction of synthesis of 25°C proteins during the heat shock. We also demonstrate the recovery of 25°C protein synthesis when the culture is continued at the higher temperature.

In addition, as protoplasts constitute a perfectly dissociated cellular suspension, we have been able to carry out pulse labeling followed by an efficient chase and thus estimate the stability of proteins under different conditions.

MATERIALS AND METHODS

Preparation and Cultivation of Protoplasts. Tobacco (*Nicotiana tabacum* var Maryland) mesophyll protoplasts were prepared and cultivated as previously described in medium WO-6 (10); in a few experiments, the medium lacked auxin or cytokinin as indicated in the text. Petri dishes, 3 cm in diameter, contained 1 ml of culture solution and 1 to 3 × 10^4 protoplasts. The frequency of division was determined as described previously (11) after orcein staining of the nuclei.

Temperature Conditions. Culture was normally at 25 ± 2°C. For the heat shock, Petri dishes were transferred to a water bath, generally at 40 ± 0.5°C, although certain experiments were carried out at other temperatures as indicated in the text.

Uptake and Incorporation of Methionine. Protoplasts were labeled with 2 μCi [^{35}S]methionine (1,300 Ci/mmol, Commissariat à l'Energie Atomique) per dish. After 15 min labeling, they were centrifuged 2 min at 1,000*g*, resuspended in 1 ml fresh medium, and pelleted 3 min at 1,000*g*. The pellet was resuspended in 1 ml 10% TCA, and the radioactivity of an aliquot measured. This amount of radioactivity is not modified if 1 mM unlabeled methionine is added to the washing medium, indicating a low adsorption of [^{35}S]methionine to the surface of the protoplasts. Consequently, this radioactivity represents the uptake. The remainder of the sample was heated 5 min at 95°C in a water bath (to degrade [^{35}S] methionine tRNA), and the pellet was recovered by centrifugation (Janetzki microcentrifuge, 10 min), resuspended, and washed twice in 5% TCA. The final pellet was resuspended in 60 μl 5% SDS, 5% β-mercaptoethanol and counted.

Conditions of Labeling of Proteins for Electrophoresis. Unless otherwise noted, protoplasts were kept for 1 h at the heat-shock temperature, then labeled 2 h with 10 μCi [^{35}S]methionine per Petri dish (1 ml culture, 1–3 × 10^4 protoplasts). In some experiments, 30-min labelings were carried out using 20 μCi [^{35}S]methionine per dish. Where the labeling was followed by a chase, protoplasts were recovered by centrifugation for 2 min at 100*g* and resuspended in fresh medium containing 1 mM unlabeled methionine.

Preparation of Samples. At the end of the labeling period, protoplasts were recovered by centrifugation (Janetzki, 2 min) and

the pellet frozen at −80°C. Soluble proteins from one pellet (one dish) were extracted for 30 min at 4°C in 100 μl 10 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$, 100 μg/ml pancreatic DNase, and 50 μg/ml pancreatic RNase. After centrifugation, the pellet was reextracted with 50 μl of the same buffer for 15 min. The two supernatants were mixed and NP 40[1] added to 2%, ampholines (LKB 3.5–10) to 2%, β-mercaptoethanol to 5%, and urea to saturation (9.5 M). In most experiments, this extract was used without further treatment, but in some cases it was further dialyzed in microchambers for 2 h at 25°C against 9.5 M urea, 2% NP 40, 5% β-mercaptoethanol. This treatment does not alter the pattern on pH 3.5 to 10 NEPHGE gels but considerably reduces the background and streaking. In addition, it is indispensable for IEF at pH 5 to 7, in order to avoid the collapse of the pH gradient, probably due to the presence of salts in the crude extracts.

Electrofocusing. Proteins were separated either by NEPHGE at pH 3.5 to 10 (14) or by IEF (13) at pH 5 to 7 in cylindrical gels (13.5 cm × 2 mm). NEPHGE gels contained 4% acrylamide, 9 M urea, 2% NP 40, and 2% pH 3.5 to 10 LKB ampholines. Twenty-μl samples were loaded at the acid end of the gels, covered with 20 μl overlay solution (8 M urea, 1% pH 3.5–10 LKB ampholines, 2% NP 40 [13]). Electrodes consisted of 10 mM H_3PO_4 and 20 mM NaOH. The voltage was increased in steps (30 min at 100 v, 45 min at 200 v, 160 min at 300 v, and 2 h at 500 v) to 2,000 vh. IEF gels contained 4% acrylamide, 9 M urea, 2% NP 40, Pharmalyte ampholines (0.5% pH 3–10, 0.5% pH 4–6.5, 1% pH 5–8). Samples were loaded as described above, using 1 M glutamic acid and 30 mM ethanolamine as electrodes. The voltage was increased in steps (30 min at 100 v, 45 min at 200 v, 16 h at 300 v, and 1 h at 1,000 v) to 6,000 vh. Gels were subsequently equilibrated in 10 ml buffer 0 (10% glycerol, 5% β-mercaptoethanol, 2.5% SDS, 62.5 mM Tris-HCl [pH 6.8]) containing 0.01% bromophenol blue for 30 min and then frozen and stored at −70°C.

Denaturing Electrophoresis. After thawing, first-dimension cylindrical gels were dialyzed a further 30 min in 10 ml fresh buffer 0 containing 0.01% bromophenol blue. The second-dimension gels were prepared in a Bio-Rad apparatus (model 220) using 1.5-mm spacers. The separating gel was 12.5% acrylamide, 0.1% SDS, 0.37 M Tris-HCl (pH 8.8), and the stacking gel was 0.125 M Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 0.1% SDS, 5% glycerol, 1% Pharmacia IEF agarose. The stacking gel was poured at about 50°C and the first-dimension gel inserted directly on top. Migration was carried out at 20 mamp/gel until the bromophenol blue reached the lower buffer (about 4–5 h). The following mol wt markers were used: lysosyme (mol wt 14,300), carbonic anhydrase (mol wt 30,000), ovalbumin (mol wt 46,000), BSA (mol wt 69,000), phosphorylase b (mol wt 92,500), myosin (mol wt 200,000) as ^{14}C methylated form (Amersham). The gels were subsequently treated as previously described (12).

RESULTS

Effect of Temperature on the Development of Protoplasts and on the Uptake and Incorporation of Methionine. Freshly isolated protoplasts were cultivated at different temperatures (Fig. 1). Because the first mitoses appear after 40 h in culture at 37°C as opposed to 46 h at 25°C, development is more rapid at 37°C than at 25°C. At 40°C, considerable perturbations of development are noted. Cell wall regeneration is greatly inhibited and only 10 to 20% of protoplasts undergo mitosis. At 42°C, 50% of protoplasts die within 6 h and no mitoses are observed. Similar results were obtained with protoplasts cultivated 2 h at 25°C, then transferred to the higher temperatures.

The uptake of methionine is not greatly affected by transfer of

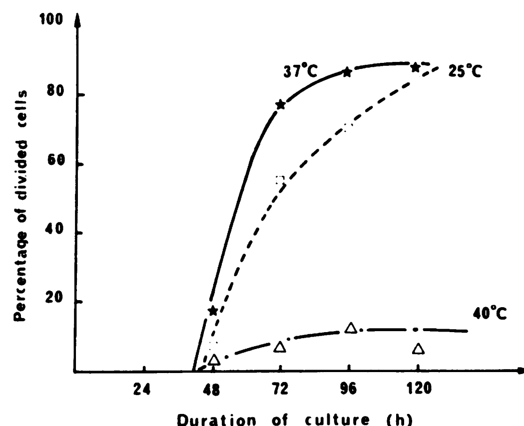


FIG. 1. Effect on mitosis. Protoplasts were cultivated immediately after isolation at 25°C (□), 37°C (★), or 40°C (△).

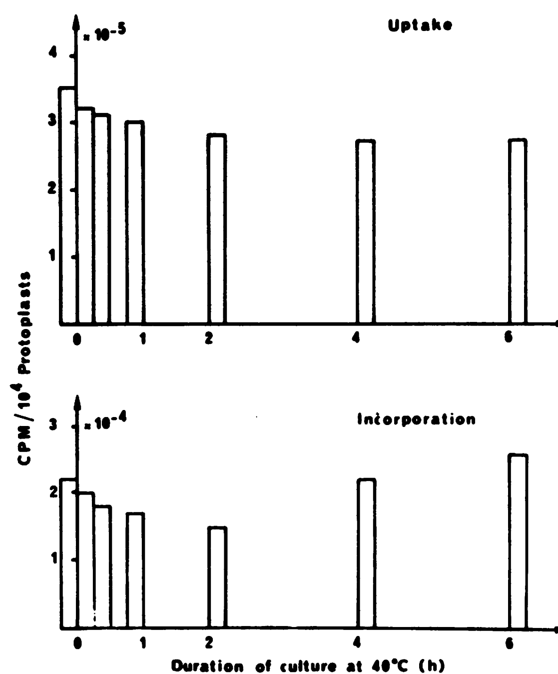


FIG. 2. Effect of transfer of protoplasts from 25°C to 40°C on the uptake and incorporation of methionine. Protoplasts were labeled for 15 min either at 25°C or at various times after transfer to 40°C. Uptake and incorporation were estimated as indicated in "Materials and Methods."

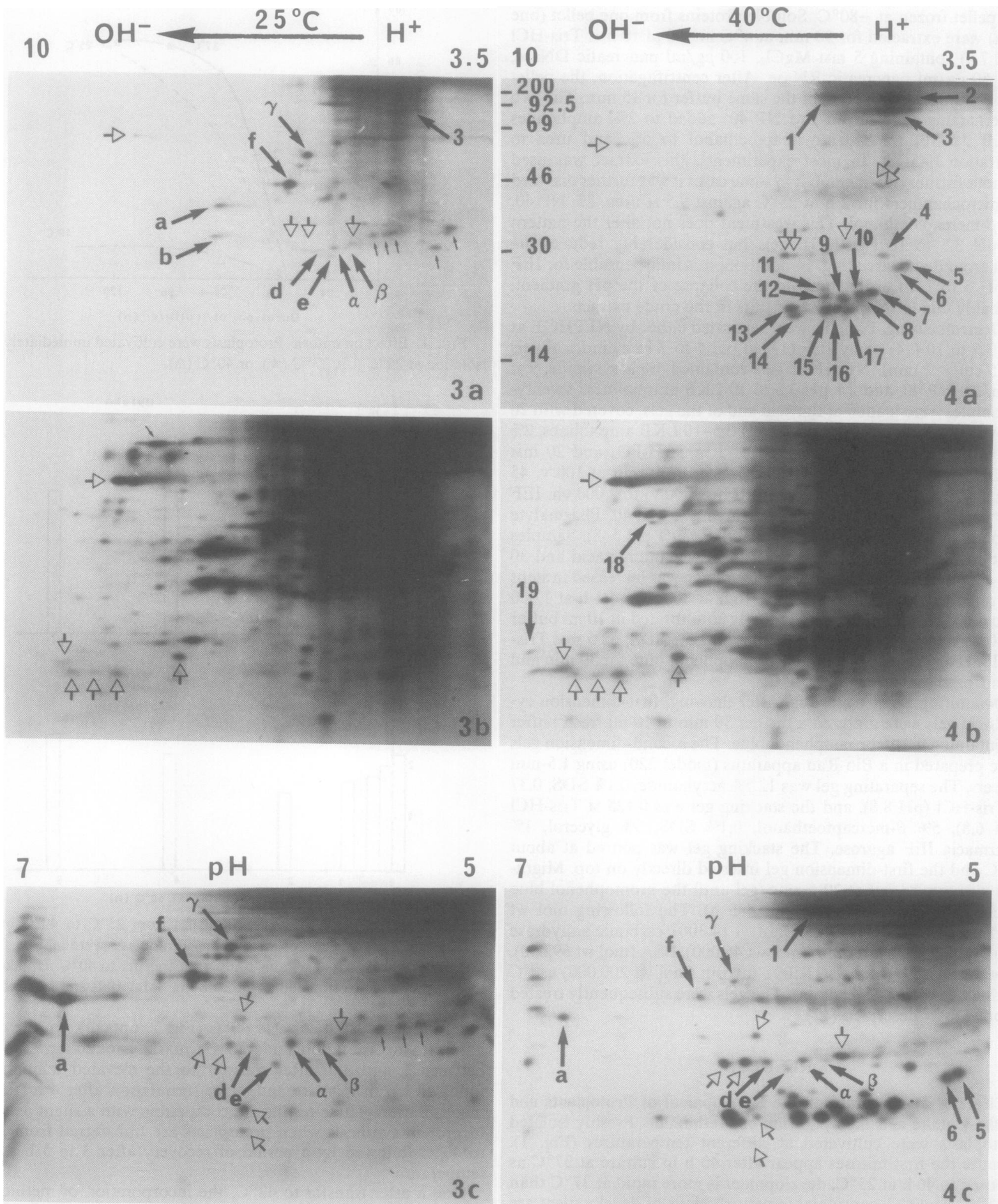
the protoplasts from 25°C to 40°C (Fig. 2, uptake). On the other hand, its incorporation undergoes a marked reduction (30–40%) between 1 and 3 h after transfer to the elevated temperature followed by an increase and slight stimulation after 4 h (Fig. 2) incorporation. These results are compatible with a slight decrease in protein synthesis when protoplasts are transferred from 25°C to 40°C followed by a period of recovery after 3 to 5 h at this temperaure.

One h after transfer to 42°C, the incorporation of methionine falls sharply. This decrease continues and, after 6 h, the incorporation corresponds to about 2 to 5% of that at 25°C, while the uptake remains more or less constant (if corrected for the number of surviving protoplasts).

Effect of Heat Shock on the Protein Pattern. In this series of experiments, protoplasts were cultivated 2 d at 25°C, transferred to 40°C for 1 h, then labeled for 2 h at this temperature. Controls consisted of protoplasts of the same age maintained constantly at 25°C and labeled for 2 h at this temperature.

To allow a comparison with earlier work (12), we show in

[1] Abbreviations: NP 40, Nonidet P 40; NEPHGE, nonequilibrium pH gradient electrophoresis; IEF, isoelectrofocusing.



FIGS. 3 and 4. Pattern of newly synthesized proteins in 2-d-old protoplasts at 25°C or after a heat shock at 40°C. Protoplasts were cultivated for 2 d at 25°C then labeled for 2 h either at 25°C (Fig. 3) or after transfer to 40°C (Fig. 4). Crude extracts were dialyzed before separation by NEPHGE (a,b) or IEF (c) followed by SDS-polyacrylamide gel electrophoresis.

Figure 3, a and c, proteins which are stimulated (Greek letters) or inhibited (Roman letters) by the presence of auxin in the culture medium. Spots γ (stimulated by auxin) and f (inhibited by auxin) correspond to proteins with a short lifetime which were not detected in the earlier work where longer labeling times were used. Three kinds of proteins can be distinguished. The first group, shown by white arrows (Fig. 4a), continues to be synthesized at 40°C at a level more or less comparable with that at 25°C (Fig. 3a). A longer exposure of these gels shows a similar result for certain minor basic proteins (Figs. 3b and 4b). As the neutral zone shows a high background of radioactivity in this separation system, we analyzed neutral proteins using IEF in the first dimension. It

Table I. *Characteristics of Tobacco Protoplast Heat-Shock Proteins*

Polypeptides	Apparent Mol Wt	Stimulation Factor	Other Characteristics
1	120,000	≥ 10	Neutral: pH_i between 5 and 7. Stable proteins synthesized throughout culture at high temperature.
2	100,000	≥ 10	
3[a]	75,000	≃ 5	
4	26,000	≥ 10–20	
5[a]	25,000	≃ 5	
6[a]	25,000	≃ 5	
7–17	20,000 to 18,000	≥ 20–50	
18	50,000	≥ 50	$pH_i > 7$. Short-lived, synthesized only at the beginning of heat shock.
19	20,000	≥ 50	

[a] These proteins are also synthesized at 25°C but at a reduced level.

is again clear that certain neutral proteins continue to be synthesized at 40°C (Figs. 3c and 4c). We have confirmed that these proteins comigrate by analyzing a mixture of 25°C and 40°C proteins. This group is constituted of both major and minor proteins, which are both neutral and basic.

The synthesis of a second group of 25°C proteins is greatly inhibited by the heat shock. They will subsequently be referred to as 'heat-sensitive' proteins. Certain representatives of this group are indicated by black arrows in Figure 3.

The third group is composed of proteins undetectable or barely detectable at 25°C which are synthesized at 40°C and will be referred to as heat-shock proteins. We have numbered 17 major heat-shock proteins. Table I shows their apparent mol wt as well as the minimal stimulation factor by comparison with the background to the corresponding region of the 25°C gel. With the exception of proteins 3, 5, and 6, which comigrate with 25°C proteins and whose radioactivity is stimulated about 5 times, the other heat-shock proteins are stimulated at least 10 times and up to 50 times the 25°C level. Labeling for 2 h at 40°C followed by an 18-h chase shows that these proteins are stable. If the 2-h labeling is carried out at 25°C and the chase at 40°C, these proteins do not appear on the autoradiographs, indicating that they are most probably not maturation (or degradation) products of 25°C proteins. A longer exposure of the gels shows that certain minor proteins, basic as well as neutral, can also be considered as heat-shock proteins. Among these, we show two basic proteins (18 and 19) which will be considered later. Pulse-chase studies show that these proteins have a short lifetime. They become undetectable after a 2-h chase.

Kinetics of Changes in the Protein Pattern after Transfer to 40°C. In this series of experiments, protoplasts were cultivated for 2 d at 25°C, transferred to 40°C, then labeled for 30 min at this temperature, either immediately after transfer or after cultivation for a certain time at 40°C. With the exception of spots f and γ which are more intense for the short labeling, patterns obtained at 25°C for a 30-min labeling show no great differences from those obtained after a 2-h labeling. Thus, 25°C gels shown in Figure 3a serve as controls. From 0 to 30 min, the synthesis of heat-sensitive proteins is unchanged, but heat-shock proteins begin to appear at a very low level (Fig. 5a). Between 30 min and 1 h, synthesis of heat-sensitive proteins is markedly inhibited, while heat-shock proteins become clearly visible. The relative level of radioactivity of proteins 1 to 17 is more or less the same as that observed in Figure 4a. On the other hand, basic proteins 18 and 19 show a high level of radioactivity. Between 1 and 1.5 h, the maximum inhibition of heat-sensitive proteins is obtained. Heat-shock proteins constitute the greater part of the major proteins. The radioactivity of proteins 18 and 19 begins to decrease at this moment. Between 1.5 and 2 h (Fig. 5d), the pattern resembles the previous pattern, except that the level of radioactivity in proteins 18 and 19 decreases considerably.

For longer times of cultivation at 40°C, with labeling between 5.5 and 6 h, heat-shock proteins 1 to 17 continue to be synthesized, while proteins 18 and 19 are no longer detectable (Fig. 5e) even after long exposure to the autoradiogram. However, we observe the recovery of the synthesis of heat-sensitive proteins at this time. As this recovery of synthesis of 25°C proteins at 40°C had not previously been described, we wished to compare it with the recovery when heat-shocked cells are returned to 25°C. In this series of experiments, protoplasts were transferred to 40°C for 2 h and were then returned to 25°C and labeled for 2-h intervals at different times. If labeling is carried out immediately after the return to 25°C, the pattern is indistinguishable from that obtained in Figure 4a, *i.e.* when the labeling is carried out at 40°C. After 2 h at 25°C (Fig. 6a), the recovery of 25°C syntheses is obvious, but heat-shock proteins remain the major proteins. After 4 h at 25°C, *i.e.* 6 h after the beginning of the heat shock (Fig. 6b), 25°C syntheses have reappeared but heat-shock proteins are still synthesized. The phenomenon of recovery of 25°C syntheses after returning to 25°C is thus not much more rapid than the phenomenon of spontaneous recovery when protoplasts are constantly maintained a 40°C. However, after 16 h at 25°C, syntheses of heat-shock proteins are no longer detectable.

Effect of Several Factors on the Heat-Shock Pattern. Different heat-shock temperatures were tested. At 35°C, heat-shock proteins are induced but the synthesis of 25°C heat-sensitive proteins is almost uninhibited. At 37°C, the synthesis of heat-sensitive proteins is clearly inhibited. The pattern at 39°C is indistinguishable from the 40°C pattern. At 42°C, there is a marked inhibition of methionine incorporation and the inhibition of 25°C heat-sensitive proteins is only partial.

We have already shown that the pattern of proteins synthesized at 25°C undergoes modifications during protoplast development (12). When protoplasts underwent a heat shock at different times after isolation, the heat-shock pattern evolved with the age of the protoplasts but only the level of proteins insensitive to the higher temperature varied (Fig. 7).

The presence of auxin and cytokinin is necessary for the mitotic development of protoplasts. We have previously shown that the radioactive labeling of a limited number of spots is auxin-dependent (12). All these proteins are heat-sensitive 25°C proteins. No heat-shock protein is affected by the presence of auxin and cytokinin.

DISCUSSION

Although protoplasts are capable of growing within a wide range of temperature, as mitotic frequency is not affected between 15°C and 38°C, above the latter temperature an abrupt transition occurs. At 40°C, the frequency of division is greatly reduced with no marked modification of the ability to synthesize proteins, while at 42°C protein synthesis is considerably reduced and 50% of the protoplasts die within 6 h. We thus chose 40°C, which is not lethal for protoplasts, as the standard heat-shock temperature. This result is in agreement with previous work on the heat shock in plants (2, 7).

Analysis by two-dimensional separation of newly synthesized

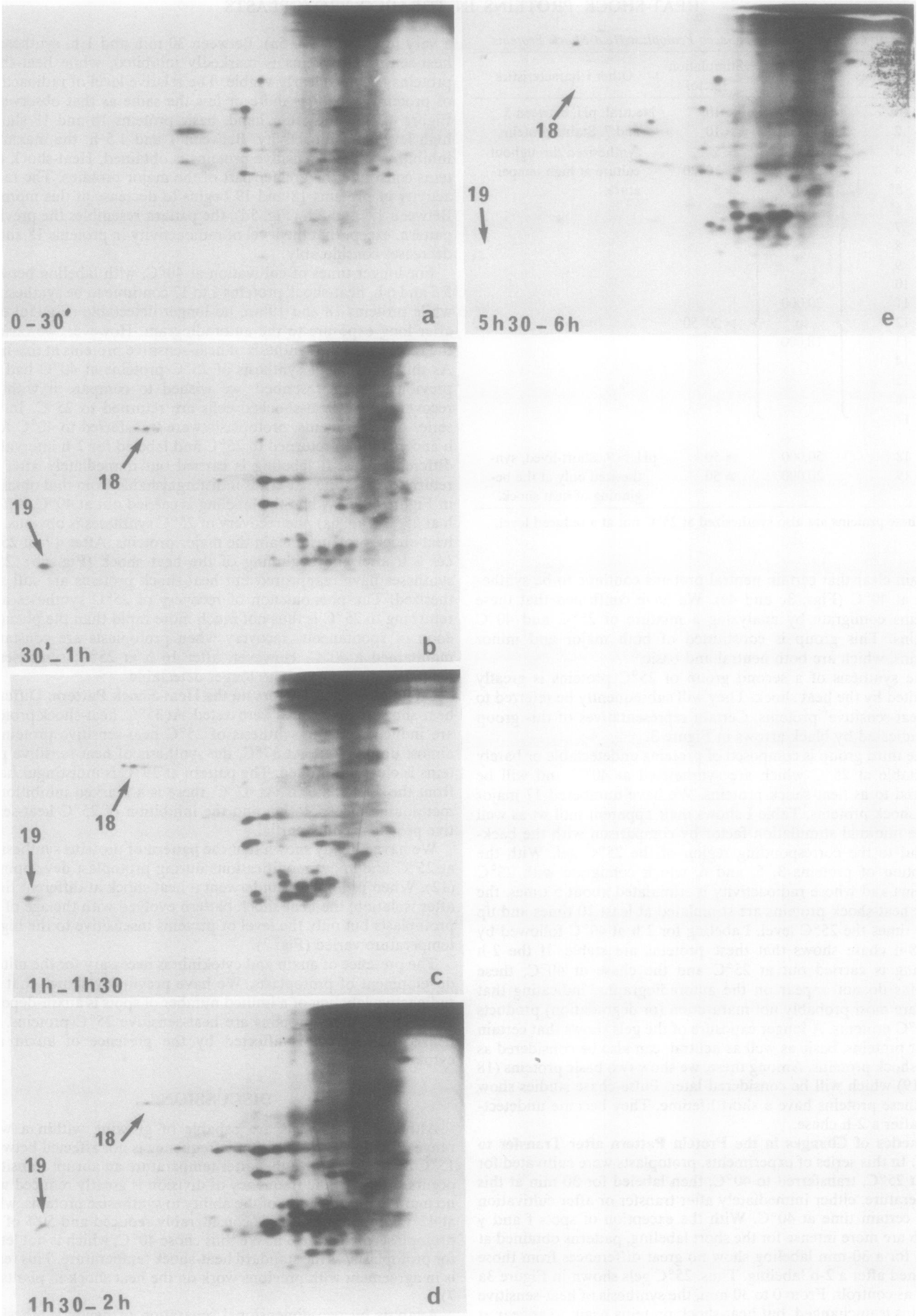


FIG. 5. Modification of the pattern of newly synthesized proteins during the heat shock. Protoplasts were labeled for 30 min at the times, after transfer to 40°C, indicated at the left of each photograph. Extracts were dialyzed and separated by NEPHGE and polyacrylamide gel electrophoresis.

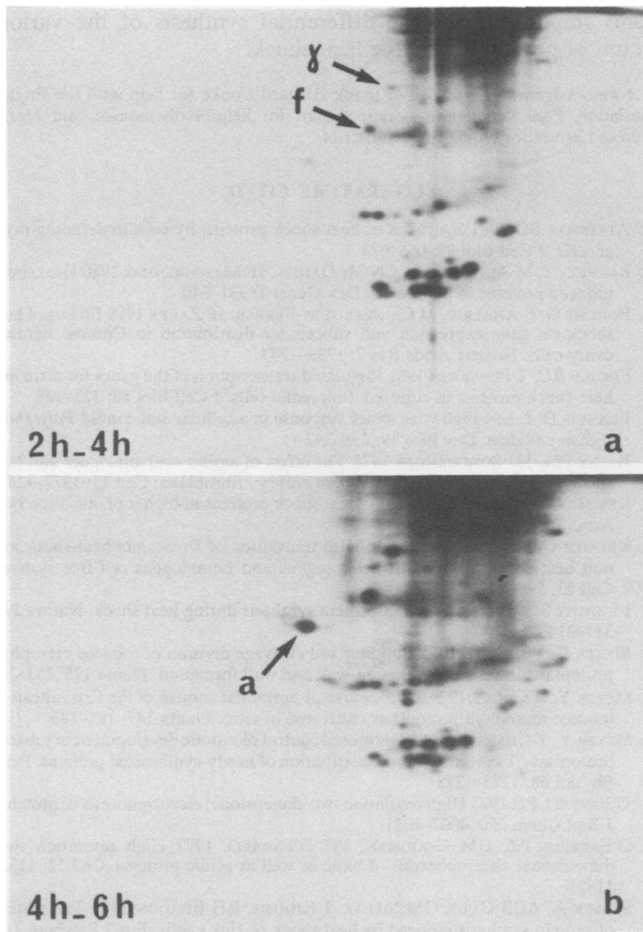


FIG. 6. Modification of the protein pattern after a 2-h heat shock and return to 25°C. Protoplasts were labeled for 2 h at the times, after return to 25°C, indicated at the left of the photographs. Dialyzed extracts were analyzed by NEPHGE and polyacrylamide gel electrophoresis.

proteins has allowed us to show that a certain number of proteins synthesized at 25°C continue to be synthesized at 40°C whereas the level of radioactivity associated with the rest of the 25°C proteins is greatly reduced after a heat shock. The complete inhibition of 25°C proteins is not common to all organisms when they are exposed to a heat shock, as in several cases all 25°C proteins continue to be synthesized (1, 9). Even in *Drosophila*, where the synthesis of all major 25°C proteins is inhibited, histone synthesis is not affected by the temperature shock (17). In their pioneering work in plants, Key *et al.* (7) presented neutral two-dimensional gels, but only to show that several bands visualized on monodimensional gels are in fact composed of several polypeptides. However, an examination of their photographs clearly shows that some 25°C proteins continue to be synthesized at 40°C in soybean seedlings as in tobacco. Barnett *et al.* (2) have also provided results supporting this conclusion by analyzing tryptic fragments of certain 25°C protein bands and their 40°C homologs. The persistence of synthesis of several 25°C proteins during the heat shock is thus probably the rule in higher plants.

A second group of proteins is composed of 25°C proteins whose synthesis is inhibited by the heat shock. It has been shown in *Drosophila* that 25°C mRNA is not degraded but that ribosomes acquire a specificity of translation of heat-shock messengers. Key *et al.* (7) have presented several results suggesting that the situation is similar in the case of soybean seedlings. The approach which we have adopted here does not allow us to determine at which level heat-sensitive 25°C syntheses are blocked. However, the fact that these proteins are synthesized normally during the first 0.5 h

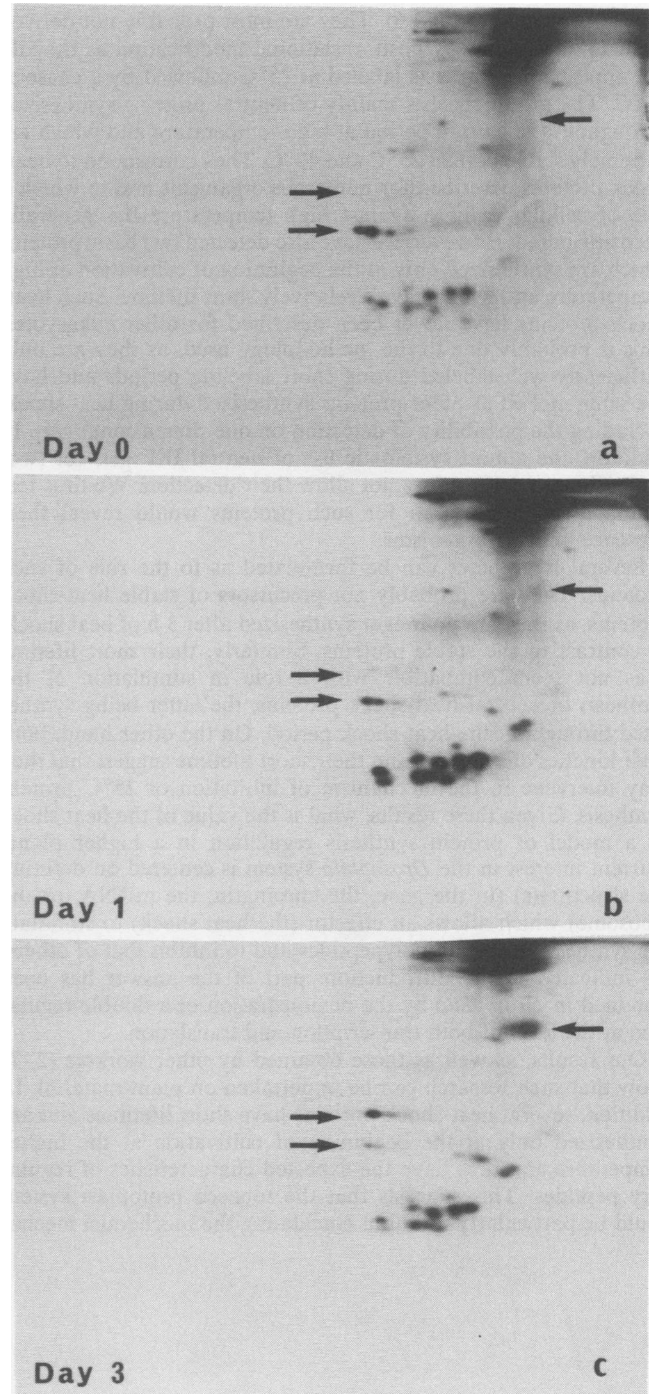


FIG. 7. Modification of the protein pattern with the age of protoplasts. Freshly isolated protoplasts (a) or after 24 h (b) or 72 h (c)—see Fig. 4a for 48 h—were kept for 1 h at 40°C, then labeled for 2 h. Undialyzed extracts were analyzed by NEPHGE and polyacrylamide gel electrophoresis.

of heat shock and again during the recovery phase at 40°C shows that this is not due to an inability *per se* of the cells to synthesize sensitive 25°C proteins at 40°C but requires a dynamic inhibition mechanism. The kinetics of this inhibition as well as the fact that the synthesis of sensitive 25°C proteins is less specifically inhibited at 42°C, at which temperature protein synthesis is limited, than at 40°C suggests that this mechanism possibly depends on synthesis of regulatory proteins.

The third group of proteins is composed of the heat-shock proteins, which are undetectable at 25°C (perhaps with the excep-

tion of proteins 3, 5, and 6). They are most probably not derived from 25°C proteins by posttranslational modification as they do not appear in protoplasts labeled at 25°C followed by a chase at 40°C. The group consists mainly of neutral proteins synthesized throughout the culture period at high temperature and which are extremely stable both at 25°C and 40°C. They correspond to heat-shock proteins described for numerous organisms and to which a role of cellular defense against high temperature has generally been attributed. However, we have also detected two basic proteins which are synthesized only at the beginning of cultivation at high temperature and which have a relatively short lifetime. Such heat-shock proteins have never been described for other eukaryotes. This is probably due to the methodology used, as they are only sufficiently well labeled during short labeling periods and have the same mol wt as other proteins synthesized during heat shock, excluding the possibility of detection on one-dimensional gels. In addition, the almost systematic use of neutral IEF gels for two-dimensional analysis does not allow their detection. We thus feel that a systematic search for such proteins would reveal their presence in other organisms.

Several hypotheses can be formulated as to the role of such proteins. They are probably not precursors of stable heat-shock proteins, as they are no longer synthesized after 3 h of heat shock, in contrast to the stable proteins. Similarly, their short lifetime does not seem compatible with a role in stimulation of the synthesis of neutral heat-shock proteins, the latter being synthesized throughout the heat-shock period. On the other hand, both their kinetics of synthesis and their short lifetime suggest that they may intervene in the mechanism of inhibition of 25°C protein synthesis. Given these results, what is the value of the heat shock as a model of protein synthesis regulation in a higher plant? Current interest in the *Drosophila* system is centered on defining the structure(s) (in the gene, the chromatin, the mRNA, or the ribosome) which allows an effector (the heat shock) to stimulate the synthesis of certain polypeptides and to inhibit that of others. As indicated in the introduction, part of the answer has been obtained in *Drosophila* by the demonstration of a double regulation at the level of both transcription and translation.

Our results, as well as those obtained by other workers (2, 7) show that such research can be undertaken on plant material. In addition, several heat-shock proteins have short lifetimes and are synthesized only at the beginning of cultivation at the higher temperaure and thus have the expected characteristics of regulatory peptides. This suggests that the tobacco protoplast system could be particularly useful in elucidating the biochemicl mechanisms responsible for the differential synthesis of the various groups of protein during the heat shock.

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