Heat Shock Response of the Chloroplast Genome in *Vigna sinensis*

(S. Krishnasamy†, R. Mannan Mannan, M. Krishnan‡, and A. Gnanam†)

From the Department of Plant Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai 625021, India

The light-dependent protein synthesis occurring in chloroplasts isolated from the leaves of 7-day-old *Vigna sinensis* responded to heat shock with the induction of a set of four heat shock proteins (HSPs). The synthesis of these four HSPs at the elevated temperature was transcriptionally regulated, and all of them were found to be thylakoid membrane-bound. The synthesis of these chloroplast-coded HSPs was also observed in etioplasts during protein synthesis at the elevated temperatures with the exogenously added ATP. The in vitro induction of these plastid-coded HSPs was observed only in the leaves subjected to gradual increase in temperature but not in leaves subjected to rapid heat shock. Further, the in vitro synthesis of these plastid-coded HSPs was seen only in chloroplasts isolated from the control and from leaves subjected to gradual increase in temperature and not in chloroplasts isolated from leaves subjected to rapid heat shock. Taken together these observations suggest that in *V. sinensis*, the plastid genome contains a definite heat shock response and that in vitro expression of this response corresponds to the gradual rise in temperature normally occurring under field conditions.

A wide range of organisms from bacteria to higher plants and animals, including cells in culture, respond to elevated temperatures by reducing the synthesis of most of the normal proteins and by the induction of a set of new proteins called HSPs1 (1). The apparent evolutionary conservation of this heat shock response across the biological world is an indication of a fundamental role (2). There is a growing body of evidence to suggest that HSPs play an important role in the induction of thermotolerance (3, 4). The acquisition of thermotolerance appears to depend not only on the synthesis of HSPs but also on their selective intracellular localization (3, 5).

Chloroplast and mitochondria are semiautonomous intracellular organelles having their own genomes and an independent protein synthetic machinery, and they are thought to have been derived from prokaryotic organisms (9). It is logical, therefore, to expect that in the eukaryotic organisms, besides the nuclear coded HSPs, there may be certain HSPs coded by the organelle genome and synthesized by their own protein synthetic machinery. Contrary to this expectation, the HSPs associated with the mitochondrial fraction in heat-shock soybean seedlings are not mitochondrial in origin (3) although there is a recent report that a 60-kDa HSP is coded for by the mitochondrial genome of maize and *Brassica campestris* (10). In the case of chloroplast, Kloppstech et al. (8) reported that the 22-kDa HSP which is localized in the chloroplast in heat-shocked pea leaves and *Chlamydomonas* cell is nuclear coded and there seems to be no HSP coded by the chloroplast genome (8). Subsequently, Vierling et al. (11) demonstrated that in three plant species, soybean, pea, and corn, certain nuclear-encoded HSPs are transported into chloroplasts and their in vivo synthesis can be inhibited by cycloheximide and not by chloramphenicol. Recently, Kloppstech et al. (12) observed the induction of synthesis of a HSP with an apparent molecular weight of 70,000 in the nucleated cells of *Arabidopsis thaliana* subjected to heat shock. Although this report indicated that the site of synthesis of this HSP is the chloroplast, as the chloramphenicol inhibits protein synthesis of the chloroplasts of unicellular algae more readily than that of their mitochondria (13).

Our results on the studies of the heat shock response of the plastids obtained from *Vigna sinensis* seedlings indicate that the plastid genome in this species does have a heat shock response and the induction of this response depends on the mode of heat shock treatment (rapid or gradual) given to leaves.

**EXPERIMENTAL PROCEDURES**

*Plant Material—Seeds of *V. sinensis* and *Sorghum vulgare* were allowed to germinate in moist vermiculite for 7 days at 25 °C in a controlled growth chamber provided with illumination (45 μE/m²/s). A 12-h light/dark cycle was followed. Etiolated seedlings were raised in the same manner but under continuous darkness in a light-proof growth chamber.*

*Isolation of Chloroplast and Etioplast—Intact chloroplasts were isolated from 7-day-old seedlings as in Ref. 14. The etioplast isolation was carried out as described in Ref. 15. The subfractionation of the plastids was done following the method of Poincelet and Day (16) as modified by Mendiola-Morgenthaler et al. (17).*

*Protein Synthesis by Leaf Segments—About 500 mg of leaves were cut into 1-mm² segments and transferred to a 25-ml conical flask containing 3 ml of sterile water and kept in a thermostated water bath shaker at a specified temperature. Illumination was provided at a photon flux density of 37.2 μE/m²/s. For gradual heat shock, the temperature was increased at the rate of 3 °C/h. After incubating the leaf segments at a specified temperature for 30 min, 50 μCi of [4C]-Chlorella protein hydrolysate (26 μCi/m mole of C) was added. After 90 min of incubation the leaf segments were homogenized and chlo-
roplasts were isolated. Total leaf homogenate as well as the chloroplas
t fraction were precipitated with 10% trichloroacetic acid at 4 °C.
The precipitate was washed with cold diethyl ether three times,
dissolved in a small volume of 2% SDS solution and subsequently
used for SDS-PAGE.

Protein Synthesis by Chloroplasts and Etioplasts—For light-
dependent protein synthesis by isolated chloroplasts, the reaction
mixture in a total volume of 200 μl contained chloroplasts equivalent
to 20–30 μg of Chl, 50 mM HEPES-KOH, pH 8.3, 350 mM sorbitol, 1
mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, and 2 mM 2-mercaptoethanol.
After incubating the chloroplast suspension at a specified temperature
for 5 min, 5 μCi of [¹⁴C]-Chlorella protein hydrolysate (20 mCi/m mole of
C) was added. Energy source for protein synthesis was provided
through illumination at a photon flux density of 50 μE/m²/s. For
etoplasts ATP at the concentration of 3 mM served as the energy
source. The time course of incorporation of [¹⁴C]-amino acids into
trichloroacetic acid precipitable material was determined by the
method of Bollum (18) with modification (19).

Polyacrylamide Gel Electrophoresis and Fluorography—SDS-PAGE
was carried out as described by Laemmli (20) with modifications. The
gel contained a linear 9–18% acrylamide concentration gradient with
5–10% glycerol co-gradient. The stacking gel contained
0.1% glycerol co-gradient. Electrophoresis was carried out at 20 °C for 12 h at 17 mA with
initial 1 h at 5 mA. Fluorography was carried out at −90 °C as
described earlier (21) with screen type x-ray film.

RESULTS

Effect of Incubation Temperature on Protein Synthesis by
Isolated Chloroplasts—Intact chloroplasts of V. sinensis med-
ated protein synthesis in a light-dependent reaction, inde-
pendent of the addition of ATP. No protein synthesis was
seen in the dark. The incubation temperature had a significant
influence on the pattern of protein synthesis. At 25 °C protein
synthesis measured in terms of [¹⁴C]-amino acids incorporated
into trichloroacetic acid insoluble fraction was linear for only
20 min. Total protein synthesis proceeded at 40 °C for about
20 min and was 25% less than that of the chloroplasts incu-
bated at 25 °C.

Fig. 1a shows the fluorographic profile of the proteins synthesized at various temperatures. The general profile of the polypeptides synthesized was not altered very much by the change in incubation temperature. The synthesis of the large subunit of ribulose-1,5-bisphosphate carboxylase, subunits of the coupling factor, and of the 32-kDa polypeptide associated with photosystem II occurred normally at elevated

temperatures as it was at 25 °C. However, at 35 °C and above,
t here was a distinct increase in the synthesis and/or accumu-
lolation of four polypeptides in the molecular size of 85, 70,
60, and 23 kDa. All four polypeptides could be observed, at
least as faint bands, even at 25 °C.

This heat shock response of the plastid genome detectable in V. sinensis was also observed in S. vulgare (Fig. 1b) except that the lower molecular weight (23 kDa) HSP was not detectable. Further, while the heat shock response could be observed at 35 °C, in V. sinensis, a definite heat shock re-
response with respect to the synthesis of all three high molecular
weight proteins could be seen in S. vulgare only at 40 °C.

The synthesis of HSPs at 40 °C in the isolated chloroplasts were completely inhibited by chloramphenicol but not by
cycloheximide. The transcriptional inhibitors like actinomy-
cin-D and rifamycin were also inhibitory to the HSP synthesis
(Fig. 2) suggesting that the synthesis of these four HSPs by
the isolated chloroplast was regulated at the transcriptional
level.

In order to establish the localization of these HSPs, chloroplas
t incubated with [¹⁴C]-amino acids at 25 and 40 °C were
fractionated into stroma and thylakoids and their fluorog-
graphic profiles were compared (Fig. 3). All four HSPs seen in
the intact chloroplasts were detectable only in the thylakoid
membrane and not in the stromal fraction. Another interest-
ing observation is the complete absence of 85- and 60-kDa
HSPs in the thylakoid membranes of the chloroplasts labeled
at 25 °C, while the 70 and 23 kDa could be detected as a faint
band. In contrast in the fluorographic profile of the total
chloroplast proteins, all four heat-induced polypeptides could
be detected as a faint band at 25 °C. From these two observa-
tions, it can be concluded that the 60- and 85-kDa polypep-
tides associated with the thylakoid membranes are newly
synthesized only at the elevated temperatures, while the 70
and 23 kDa are synthesized in small amounts even at 25 °C.

FIG. 2. Fluorographic profile of proteins synthesized by iso-
lated intact chloroplasts of V. sinensis incubated in the pres-
ence of various inhibitors. Chloroplasts were labeled with [¹⁴C]-
Chlorella protein hydrolysate as described in the legend of Fig. 1.
After the labeling was over, the samples were processed and subjected
to SDS-PAGE as described under "Experimental Procedures." 150
μg of protein was loaded in all the lanes. Lane A, chloroplasts
incubated at 25 °C; lane B, chloroplasts incubated at 40 °C; lane C,
chloroplasts incubated at 40 °C with chloramphenicol (150 μg/ml);
lane D, chloroplasts incubated at 40 °C with cycloheximide (5 μg/ml);
lane E, chloroplasts incubated at 40 °C with actinomycin-D (50 μg/
ml); lane F, chloroplasts incubated at 40 °C with rifamycin (100 μg/
ml); lane G, chloroplasts incubated at 40 °C with ethidium bromide
(150 μg/ml).
Therefore, 60- and 85-kDa HSPs associated with the thylakoid membranes could not be equivalent to the polypeptides of similar molecular mass detectable as faint bands in the fluorographic profile of total proteins from the 25°C incubated sample.

**Effect of Incubation Temperature on Protein Synthesis by Etioplasts**—The etioplast preparations obtained from 7-day-old dark grown V. sinensis seedlings were found to be active in protein synthesis. The energy source, however, was exogenous ATP. The optimum rate of protein synthesis with the etioplasts was obtained at the ATP concentration of 3 mM. As in the case of protein synthesis occurring in the isolated chloroplasts, both the rate and linearity of protein synthesis occurring in the etioplasts were found to decline at the elevated temperatures. Thus, the ATP-dependent protein synthesis mediated by the etioplasts incubated at 25°C was linear for about 50 min, while the etioplast preparation incubated at 40°C showed a linearity only for about 25 min. The total protein synthesis occurring during the period of 20 min in the etioplasts incubated at 40°C was found to be 20–30% lesser when compared to the protein synthesis occurring for the same duration in the etioplast incubated at 25°C.

Fig. 4 shows the fluorographic profile of the polypeptides synthesized by the etioplast preparations incubated at different temperatures ranging from 25 to 40°C. As in the case of chloroplasts, in the etioplast preparations incubated at elevated temperatures, there was an induction of synthesis of a set of HSPs (85, 70, and 60 kDa). Although the HSPs detectable in the etioplasts incubated at the elevated temperature are comparable to the HSPs observed in the chloroplasts, there was a significant difference in the temperature range for the induction of HSPs between chloroplasts and etioplasts. In the case of chloroplasts, the synthesis of the three high molecular mass HSPs (85, 70, and 60 kDa) could be observed at the incubation temperature of 35°C itself while the synthesis of three HSPs of similar molecular weight in the etioplast was observed only at 40°C. Further, the low molecular mass HSP (23 kDa) detectable in the chloroplast in the temperature range of 35–40°C was not seen in the etioplasts in the temperature range tested.

**Effect of Heat Shock on Protein Synthesis by Vigna Leaf**

Fig. 5 illustrates the heat shock response of the young green leaves of V. sinensis subjected to rapid heat shock. At temperatures higher than 35°C, most of the normally occurring polypeptides were not synthesized and only a set of HSPs (96, 80, 75, 22, 20, and 15 kDa) made their appearance in the fluorographic profile of the total leaf homogenate. The 75-kDa protein is synthesized in considerable amounts even at 25°C suggesting its constitutive expression.

The fluorographic profile of chloroplasts isolated from leaves heat shocked at 35°C was closely comparable to that of chloroplasts isolated from leaves at 25°C (Fig. 5b). But at heat shock temperatures higher than 35°C, there was a marked decrease in the amount of radioactivity associated with the chloroplast fraction, and consequently the number of polypeptides detectable in the fluorographic profile decreased. At the incubation temperature of 40°C, the synthesis of most of the high molecular weight polypeptides associated with the chloroplast fraction was decreased and only a few low molecular weight polypeptides appeared as distinct bands. At the incubation temperature of the leaves of 42°C, the chloroplast fraction showed the presence of only five low molecular mass polypeptides (22, 20, 19, 17, and 15 kDa) of which one polypeptide (20 kDa) appeared only as a faint band in the fluorograph. None of the high molecular weight polypeptides could be seen in the fluorograph. A 22-kDa HSP associated with the chloroplast in pea and *Chlamydomonas* has earlier been shown to be a nuclear-coded HSP, synthesized in the cytoplasm and localized in the chloroplast (8). These chloroplast-associated low molecular weight HSPs are...
not synthesized in vivo in leaves infiltrated with cycloheximide but could be readily observed in leaves infiltrated with chloramphenicol (data not shown). It is thus possible that the 22 kDa and other lower molecular mass HSPs detectable in chloroplasts obtained from heat-shocked leaves could be of nuclear origin. The interesting result from the analysis of the fluorographic profile of the chloroplasts isolated from leaves subjected to rapid heat shock is the absence of the high molecular mass HSPs detectable either in the total leaf homogenate (96, 80, and 75 kDa) or in the chloroplasts heat shocked in vitro (85, 70, and 60 kDa).

In the total leaf homogenate of the Vigna leaves subjected to a gradual heat shock treatment, in addition to high molecular mass HSPs (96, 80, and 75 kDa) detectable in the rapid heat-shocked leaves, a 60-kDa HSP was detected as a distinct band in the fluorographic profile (Fig. 6). Furthermore, two new HSPs in the molecular size of 85 and 70 kDa were also detectable as a faint band. These three HSPs (85, 70, and 60 kDa) detectable in the total leaf homogenate of the heat-shocked leaves are comparable to the HSPs detectable in the chloroplasts subjected to heat shock in vitro. In the fluorographic profile of the chloroplast preparation obtained from these leaves subjected to gradual heat shock, these three HSPs (85, 70, and 60 kDa) appeared as distinct bands comparable to the chloroplasts heat shocked in vitro. From the results it appears that the chloroplast-coded HSPs are expressed in vivo only when the leaves are subjected to gradual heat shock while rapid heat shock seems to suppress this expression.

There was a subtle difference in the status of chloroplast protein synthesis between the leaves subjected to rapid heat shock treatment for 30 min and those subjected to heat shock treatment for only 5 min. In the leaves subjected to 30 min of heat shock treatment, the chloroplast protein synthesis including HSPs was strongly suppressed and the synthesis of chloroplast-coded proteins such as ribulose 1-5 bisphosphate carboxylase could not be detected as well (Fig. 6, lane F).

However, in leaves subjected to rapid heat shock treatment for 5 min only, the synthesis of this particular protein is comparable to that in the control leaves incubated at 25 °C while the synthesis of HSPs was totally suppressed (Fig. 6, lane G).

In Vitro Protein Synthesis in the Chloroplasts Isolated from the Preheat-shocked Leaves—In order to see whether the rapid heat shock treatment given to leaves damages the chloroplast protein synthesis in a general way or suppresses specifically the expression of chloroplast-coded HSPs, in vitro protein synthesis at 40 °C was performed with the chloroplast preparations obtained from the leaves subjected to rapid and gradual heat shock treatments. In chloroplast preparations from leaves subjected to rapid heat shock either for 2 h or for 15 min, the synthesis of all three high molecular weight HSPs was not detectable, while the synthesis of chloroplast proteins could be seen at least as faint or diffuse bands. However, in chloroplast preparations from leaves subjected to gradual heat shock treatment all three high molecular weight polypeptides (HSPs) were synthesized as in the case of chloroplasts isolated from control leaves (Fig. 7).

**DISCUSSION**

Beginning with the report of the occurrence of HSPs in soybean seedlings (22), there has been a growing interest in the study of HSPs in higher plants. Attempts have been made to see whether there are any HSPs coded by the organelle...
that the genes for HSPs are retained only in the chloroplast plast-coded HSPs could be detected only when the leaves were preincubated at 40°C for 2 h and the labeling was done at 40°C. Lane D, chloroplasts were isolated from leaves preincubated at 40°C for 15 min and the labeling was done at 40°C. Lane E, chloroplasts were isolated from leaves subjected to gradual heat shock treatment. The leaves were originally kept at 25°C and the incubation temperature was gradually raised from 25°C to 40°C; then the chloroplasts were isolated, and the labeling was done at 40°C.

It has been reported that in maize and *B. campestris*, a 60-kDa HSP is encoded within the mitochondria (10). But to date there is no information as to whether the chloroplast genome of higher plants can code for any HSPs. In the case of pea leaves and *Chlamydomonas* cells subjected to rapid heat shock, it has been shown that all of the *in vivo* labeled HSPs can also be detected in the pattern of *in vitro* translation of poly(A)* RNAs. This observation has been taken as evidence to suggest that during the course of evolution, the HSP genes originally present in the progenitors of the chloroplast have either been lost or transferred from the prokaryotic genome to the nuclear genome (8). However, the results of the present investigation suggest that at least in *V. sinensis* and *S. vulgare* the chloroplast genome apparently retained the genes for HSPs.

The interesting observation of this investigation concerns the conditions required for the expression of chloroplast genes for HSPs. In *V. sinensis*, the heat shock response of the chloroplast genome with the induction of synthesis of a set of HSPs is readily seen in the isolated chloroplast subjected to heat shock. However, the *in vitro* expression of these chloroplast-coded HSPs could be detected only when the leaves were subjected to a gradual rather than a rapid heat shock treatment. This specific treatment required for the *in vivo* expression of the chloroplast coded HSPs might have been the reason for the inability of Kloppstech *et al.* (8) to detect these organelle-specific HSPs in pea leaves and *Chlamydomonas* cells subjected to rapid heat shock. However, the possibility that the genes for HSPs are retained only in the chloroplast genome of tropical plants like *Vigna* and *Sorghum* while lost in temperate plants like pea, cannot be ruled out at this moment, but is of interest in understanding chloroplast evolution.

The expression of chloroplast-coded HSPs *in vivo* with gradual heat shock and not with rapid heat shock treatment may reflect the temperature changes occurring under field conditions. Plants growing under field conditions do not experience abrupt temperature shifts. Instead, they experience daily gradual fluctuations in temperature that are often greater than 10°C and can be as much as 20°C on sunny days. Thus, temperature-dependent regulations involved in chloroplast-coded HSPs have adapted to the conditions prevailing in the natural environment.

The explanation for the absence of *in vivo* expression of chloroplast-coded HSPs with rapid heat shock treatment is not immediately clear. This is analogous to the effect of rapid heat shock treatment on the *in vivo* synthesis of the chloroplast-coded large subunit of ribulose-1,5-bisphosphate carboxylase in soybean cell suspension cultures. A 40°C heat shock treatment for 2 h decreases the *in vivo* synthesis of this polypeptide by 80%, and this decrease is not a result of decrease in the level of mRNA for this polypeptide (23). A suggested explanation for this observation is that by reducing the metabolite or energy supply to the chloroplast or by inactivating the chloroplast translational machinery, heat shock could depress total chloroplast protein synthesis. It is possible that unlike during rapid heat shock, gradual heat shock enables the metabolite or energy supply between the chloroplast and cytoplasm to get adjusted in such a way so that chloroplast protein synthesis is not significantly affected. The results of the *in vitro* heat shock experiments with chloroplasts isolated from rapid heat-shocked leaves reported here indicates, that the rapid heat shock treatment besides reducing the *in vivo* chloroplast protein synthesis in general, also seems to have some inhibitory effect on the transcription of mRNAs for chloroplast-coded HSPs, since in chloroplasts isolated from the leaves subjected to rapid heat shock, *in vitro* synthesis of HSPs could not be observed.

Furthermore, the observation that in leaves subjected to rapid heat shock treatment for a shorter duration, the *in vivo* expression of chloroplast-coded HSPs was suppressed while the synthesis of ribulose-1,5-bisphosphate carboxylase was not significantly affected also lends support to the suggestion that rapid heat shock treatment *in vivo* seems to have some inhibitory effect on the transcription of mRNAs for chloroplast-coded HSPs.

The observation that high temperature decreases the quantum yield for CO₂ fixation (24) has been taken as evidence to suggest that one of the first components of the photosynthetic apparatus to be damaged by heat shock is the thylakoid membrane. Under these conditions the localization of all four chloroplast-coded HSPs in the thylakoid membranes assumes a functional significance in offering thermal tolerance to activities bound to thylakoid membranes.

**Acknowledgments**—We are indebted to Professor Andre T. Jagentorff, Cornell University, Professor Joseph S. Kahn, North Carolina State University, and Professor J. Jayaraman, Madurai Kamaraj University, for critically reading the manuscript and Mary Andrews for neatly typing the manuscript.

**REFERENCES**

1. Schlesinger, M. J., Aliperti, G. & Keely, P. M. (1982) *Trends Biochem. Sci.* **7**, 222-225.
2. Tanguay, R. M. (1983) *Can. J. Biochem. Cell. Biol.* **61**, 387-394.
3. Lin, C. Y., Roberts, J. K. & Key, J. L. (1984) *Plant Physiol.* **74**, 152-160.
4. Loomis, W. F. & Wheeler, S. A. (1982) *Dev. Biol.* **90**, 412-418.

**FIG. 7.** Fluorographic profile of the polypeptides synthesized by isolated chloroplasts from leaves subjected to rapid or gradual heat shock treatment. Lane A, chloroplasts were isolated from leaves incubated at 25°C and labeling was done at 25°C. Lane B, chloroplasts were isolated from leaves incubated at 25°C and the labeling was done at 40°C. Lane C, chloroplasts were isolated from leaves preincubated at 40°C for 2 h and the labeling was done at 40°C. Lane D, chloroplasts were isolated from leaves preincubated at 40°C for 15 min and the labeling was done at 40°C. Lane E, chloroplasts were isolated from leaves subjected to gradual heat shock treatment. The leaves were originally kept at 25°C and the incubation temperature was gradually raised from 25°C to 40°C; then the chloroplasts were isolated, and the labeling was done at 40°C.
Heat Shock Proteins of Chloroplast

5. Vincent, M. & Tanguay, R. M. (1982) *J. Mol. Biol.* 162, 365–378
6. Velazquez, J. M., DiDomenico, B. J. & Lindquist, S. (1986) *Cell* 20, 679–689
7. Vincent, M. & Tanguay, R. M. (1979) *Nature* 281, 501–503
8. Kloppsteich, K., Meyer, G., Schuster, G. & Ohad, I. (1985) *EMBO J.* 4, 1901–1909
9. Yamamori, T., Ito, K., Nakamura, Y. & Yura, T. (1978) *J. Bacteriol.* 134, 1133–1140
10. Sinha, R. M. & Turben, T. (1986) *J. Biol. Chem.* 260, 15382–15385
11. Vierling, E., Mishkind, M. L., Schmidt, G. W. & Key, J. L. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 362–365
12. Kloppsteich, K., Ohad, I. & Schweiger, H. (1986) *Eur. J. Cell Biol.* 42, 239–245
13. Ohad, I. & Drews, G. (1982) in *Photosynthesis* (Govindjee, ed) pp. 89–140, Academic Press, New York
14. Fish, L. E. & Jagendorf, A. T. (1982) *Plant Physiol.* 70, 1107–1114
15. Daniell, H., Ramanujam, P., Kirshnan, M., Gnanam, A. & Rebeiz, C. A. (1983) *Biochem. Biophys. Res. Commun.* 111, 740–749
16. Poincelot, P. R. & Day, P. R. (1974) *Plant Physiol.* 54, 780–783
17. Mendiola-Morgenthaler, L. R. & Morgenthaler, J.-J. (1974) *FEBS Lett.* 49, 152–155
18. Bollum, F. G. (1960) *J. Biol. Chem.* 235, 2399–2403
19. Mans, R. J. & Novelli, G. D. (1961) *Arch. Biochem. Biophys.* 94, 48–53
20. Laemmli, U. K. (1970) *Nature* 227, 680–685
21. Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* 56, 335–341
22. Key, J. L., Lin, C. Y. & Chen, Y. M. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 3526–3530
23. Vierling, E. & Key, J. L. (1985) *Plant Physiol.* 78, 155–162
24. Björkman, O. (1975) *Carnegie. Inst. Wash. Year Book* 74, 748–751