Planta 144, 153–159 (1979)

Planta

Photosynthetic Apparatus in Chilling-sensitive Plants

IV. Changes in ATP and Protein Levels in Cold and Dark Stored and Illuminated Tomato Leaves in Relation to Hill Reaction Activity

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Abstract. Changes in the levels of both ATP and protein in relation to Hill reaction activity following cold and dark storage and illumination of leaves of *Lycopersicon esculentum* Mill. were studied. Loss of Hill reaction activity observed during cold and dark storage of leaves for 3–4 days was accompanied by about 50% decrease of both ATP and protein levels while the content of chlorophyll was not affected Illumination of cold and dark stored leaves (8000 lx for 2 h) resulted in almost a complete restoration of both ATP and protein levels as well as Hill reaction activity. The latter process proceeded, however with different kinetics than the former ones. The rate of Hill reaction activity increase very rapidly from the beginning of illumination while the ATP level diminished during the first hour of illumination. In addition there was a lag in the increases in protein content. By about two hours of illumination all these processes reached the maximum values. Following illumination of leaf discs stored in the cold and dark in the presence of either cycloheximide or DCMU, both ATP and proteins levels as well as Hill reaction activity were greatly diminished. These data seem to suggest that the lack of reactivation of Hill reaction activity in the presence of these two inhibitors is due to inhibition of ATP synthesis required primarily for manganese reincorporation into the thylakoid membrane and thereby restoration of Hill reaction activity (Kaniuga, Zabek and Sochanowicz, Planta 1978 b). Contribution of cytoplasmic protein synthesis in this process appears to be of secondary importance, although the inactivation and reactivation of electron transport are accompanied by a large loss (as high as 50%) and the restoration of the initial protein content in leaves following illumination.

Key words: ATP – Chilling-sensitive plant – Cold treatment – Hill reaction – *Lycopersicon* – Protein.

Abbreviations: DCIP = 2,6-dichlorophenolindophenol; DCMU = 3-(3,4-dichlorophenyl)-1,1 dimethylurea; FFA = free fatty acid

Introduction

Detached leaves maintained in darkness at room temperature are frequently used as a standard system for the study of leaf senescence, which is accompanied by changes in several metabolic parameters as a decrease in protein and chlorophyll content as well as a decline in RNA content. All these changes become clearly observable usually after 48 h and sometimes after 24 h, and light was found to delay senescence in leaves mainly because it allowed photosynthesis. Recently it was proposed that the light exerts its effect by photoproduction of ATP (Thimann et al., 1977).

Metabolic changes induced by the cold treatment of leaves of chilling-sensitive plants seem to resemble those observed in senescence of leaves. However, in contrast to many detailed studies concerning the changes in lipid composition induced by chilling or hardening of plants, there are only few data concerning the changes in ATP (Stewart and Guinn, 1969; Jones, 1970; Wilson, 1978) and protein levels (Wright and Simon, 1973; Guinn, 1971) in leaves following the cold treatment.

In the previous papers in this series, it was shown that the cold treatment of leaves of chilling-sensitive plants results in a loss of Hill reaction activity (Kaniuga et al., 1978a) and a decline of the galactolipid content in chloroplasts (Michalski et al., 1976) accompanied by an increase in chloroplast unsaturated free fatty acids (Kaniuga and Michalski, 1978), and a decrease of loosely bound manganese content (Kaniuga et al., 1978b). All these changes were fully reversible upon illumination of the cold and dark stored leaves but not of isolated chloroplasts or leaf homogenate. This seemed to suggest that the effect of light is due to the energy supply for both chloroplast and cytoplasmic processes involved in the restoration of Hill reaction activity. The present work deals with the effect of cold and dark storage of tomato leaves and their illumination on the level of ATP and protein

0032-0935/79/0144/0153/$01.40

in leaf cells in relation to the changes in Hill reaction activity. A preliminary report of these studies has been presented (Frąckowiak-Sochanowicz et al., 1976).

Materials and Methods

Plant Material

Tomato leaves *Lycopersicon esculentum,* Mill. were obtained from plants grown in the greenhouse. Cold storage of leaves, reactivation with light, isolation of chloroplasts as well as determination of both Hill reaction activity and chlorophyll content in chloroplast preparations from fresh (F), cold and dark stored (A), and reactivated leaves (R) are described in the previous paper (Kaniuga et al., 1978a).

Determination of Leaf Protein and Chlorophyll Content

Total protein and total chlorophyll content were determined as follows: Leaf discs or fragments of leaves were ground in a Potter Elvehjem homogenizer in 80% ethanol. The homogenate was centrifuged and the precipitate was washed with 80% ethanol. The combined supernatants were made up to 10 ml with ethanol, and the chlorophyll content was determined spectrophotometrically at 665 nm.

For estimation of protein content the washed precipitate was resuspended in 5 ml of 1 *N* NaOH, incubated for 10 min at 90° C, and centrifuged. The supernatant was diluted up to 10 ml and assayed for the protein by the method of Lowry et al. (1951) using bovine serum albumin as the protein standard. The amount of the protein in leaf homogenates used in experiments presented in Fig. 3 were estimated in the same way using 96% ethanol.

Extraction and Determination of ATP Content

First, 0.3 g of tomato leaves or leaf discs were ground in a Potter Elvehjem homogenizer in 2% $HClO_4$ at 0° C. At this temperature ATP was extracted by perchloric acid for 1 h. The suspension was then neutralized to pH 7.0–7.4 with 2 M K_2CO_3 and centrifuged for removing the cell material and insoluble $KClO_4$. ATP was determined spectrophotometrically in the supernatant liquid by the enzymatic method described by Williamson and Corkey (1969). Hexokinase and glucose-6-phosphate dehydrogenase used in this measurement were obtained by the method described by McDonald (1955) and Kornberg and Horecker (1955), respectively.

Results

The Effect of Cold and Dark Storage of Leaves on the Levels of Chlorophyll and Protein

When detached leaves or leaf segments of both chilling-sensitive (Goldthwaite and Laech, 1967; Takegami, 1975) and chilling-resistant plants (Martin and Thimann, 1972; Thomas and Stoddart, 1975; Wittenbach, 1977; Choe and Thimann, 1977) are maintained for few days at room temperature in darkness, a large loss of up to 50% of the initial value of chlorophyll and protein levels is observed.

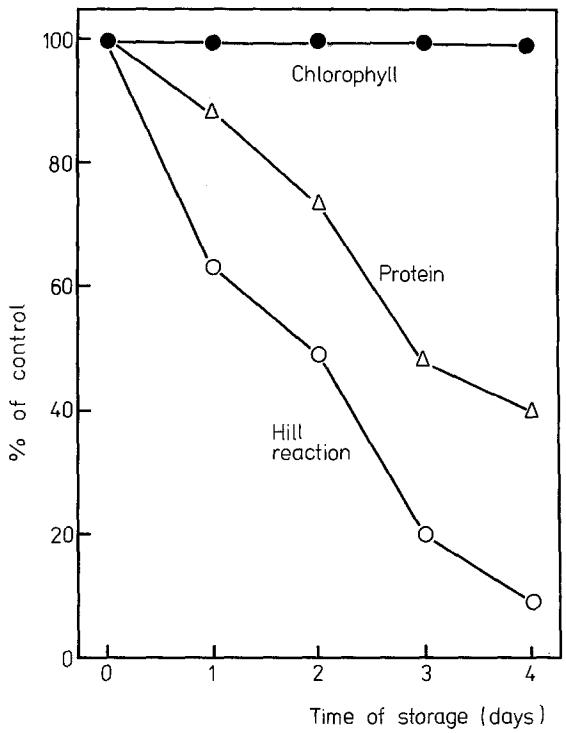

Fig. 1. Effect of the cold and dark storage of tomato leaves on Hill reaction activity and protein and chlorophyll contents. Leaves were stored at 0° C in the dark for the time indicated. Chloroplast photochemical activity and protein content in control leaves were 48.4 μmol DCIP red. mg $chl^{-1}h^{-1}$ and 19.3 μg protein mg fresh weight of $leaf^{-1}$, respectively. Chlorophyll content in control leaves: the extract from 50 mg of fresh leaves in 10 ml 80% acetone, $A_{652}=0.430$

Figure 1 shows that following cold and dark storage of detached tomato leaves only the protein content decreases very fast, in parallel to the loss of Hill reaction activity reaching 50% and 20% of the initial value, respectively, after 3 days of storage. In contrast the chlorophyll level is not affected by such a treatment.

Restoration of Hill reaction activity upon illumination of cold stored detached leaves or retention of this activity in intact plants by applying intermittent light (Kaniuga et al., 1978a) suggested that illumination of leaves could induce protein synthesis. Figure 2 indicates that restoration of Hill reaction activity upon illumination of cold and dark stored leaves is accompanied by almost the complete restoration of the level of protein synthesis. However, the increase in the level of protein synthesis was observed only in homogenate obtained after illumination of aged leaves (Fig. 3) or leaf discs (cf. Fig. 8) but not following illumination of homogenate from cold and dark stored leaves (Fig. 3). These data indicate that for

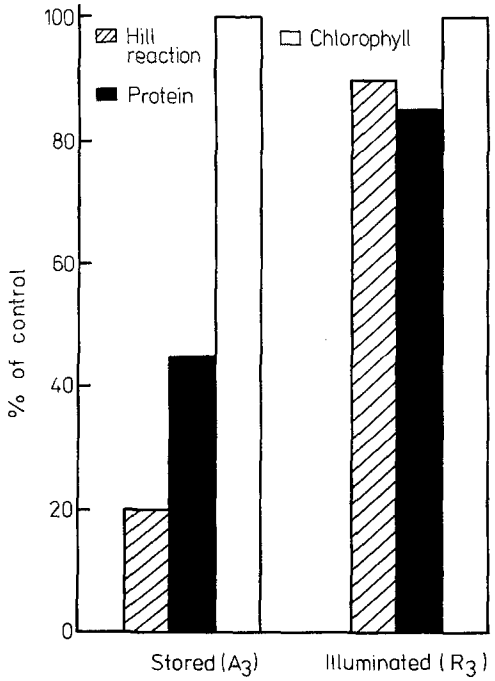

Fig. 2. Restoration by light of Hill reaction activity and the protein level in cold and dark-stored leaves. The leaves were stored for 3 days at 0° C in the dark (A_3) followed by the illumination (8000 lx) at 25° C for 2 h (reactivated, R_3). Hill reaction activity and the protein content in control leaves were 68.5 μmol DCIP red. mg $chl^{-1}h^{-1}$ and 17.1 μg protein mg fresh weight of $leaf^{-1}$, respectively. Chlorophyll content in control leaves: the extract from 50 mg of fresh leaves in 10 ml 80% acetone, $A_{652}=0.350$

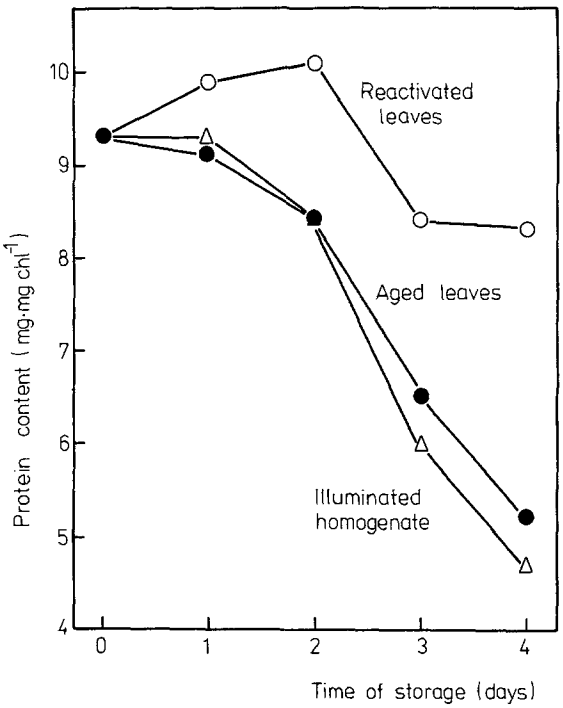

Fig. 3. Effect of illumination of leaves and leaf homogenate on protein level following the cold and dark storage of leaves. Leaves were stored (aged) in the cold (0° C) and dark for time indicated. Then leaves were either illuminated (reactivated) 8000 lx for 2 h at 25° C or directly taken up for the preparation of the homogenates using a Waring Blendor and 15 s homogenization in medium containing 0.4 M sucrose, 20 mM NaCl, 50 mM Tris-HCl (pH 7.5), 40 mM ascorbate. The homogenates filtered through cheese cloth were used for protein content determination either directly or after illumination of the homogenate obtained from aged leaves. In the latter case homogenate was placed in petri dishes and illuminated as described for leaves

restoration of a level of protein synthesis similar to that observed with reactivation of Hill reaction activity (Kaniuga et al., 1978a), an intact cell structure is required.

Changes in ATP and Protein Levels During Cold and Dark Storage and Illumination of Leaves

The changes in ATP levels in leaves of chilling-sensitive plants subjected to chilling treatment were studied in cotton (Stewart and Guinn, 1969; 1971) and bean leaves (Jones, 1970; Wilson, 1978). In all these experiments intact plants were used, while the light conditions were varied. The cold and dark storage of detached tomato leaves results in a rapid drop in the ATP level by about 50% in leaf cells, which is accompanied by a decrease (by about 80%) of Hill reaction activity (Figs. 4 and 5) following 4 days of storage. Illumination of leaves (for 2 h at 8000 lx) causes a restoration of both Hill reaction activity and the initial ATP level. It is interesting that restoration of ATP synthesis in leaves stored in the cold and dark for 3 days is easily reversible upon illumination of leaves, similarly as Hill reaction activity.

Figure 6 shows the time course of dark and light induced changes in ATP level in tomato leaves stored at 0° C and illuminated at 25° C. As can be seen there is a further rapid fall in the ATP level during the first hour of illumination while there is a restoration of both Hill reaction activity and chloroplast manganese content (Kaniuga et al., 1978b). Between the first and second hour of illumination, the ATP level rapidly increases.

When the rate of protein synthesis was examined under the same conditions (Fig. 7), it was found again that the increase of Hill reaction activity precedes protein synthesis. Following 30 min of illumination electron transport is reactivated by about two-thirds, while protein synthesis is hardly visible. This may

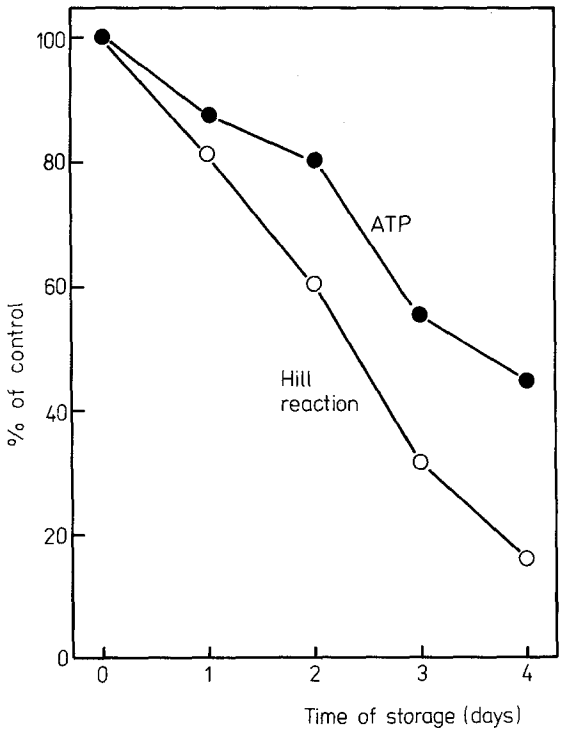

Fig. 4. Decrease of both ATP content and Hill reaction activity during cold and dark storage of leaves. ATP content and Hill reaction activity in the control were 1.67 μg fresh weight^{-1} and 54.0 μmol DCIP red. mg chl^{-1} h^{-1}, respectively

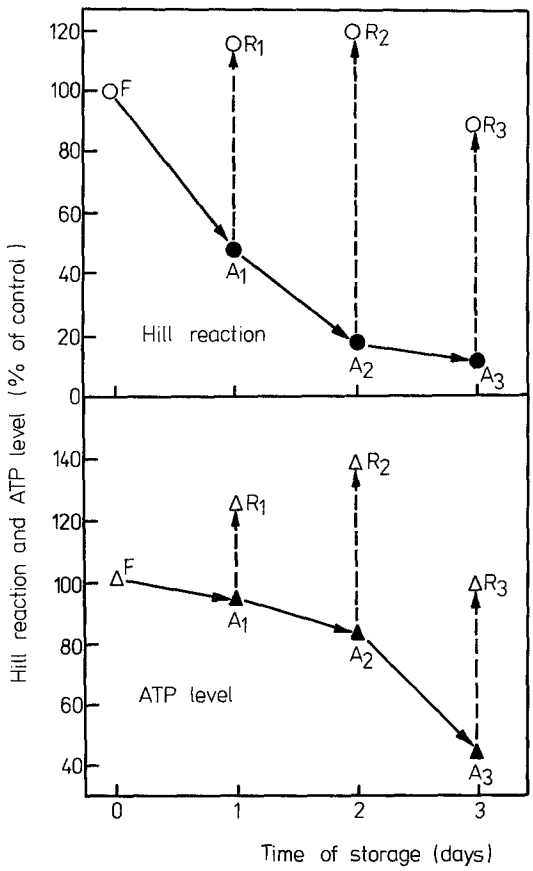

Fig. 5. Restoration of ATP synthesis and Hill reaction activity upon illumination of leaves stored in the cold and dark for 1–3 days. Leaves were stored at 0° C in dark (*A*) for the time indicated and then illuminated (*R*) with 8000 lx for 2 h. *Dashed arrows* indicate increased levels of both Hill reaction activity and ATP content upon illumination of leaves. Hill reaction activity and ATP content in control leaves were 90.0 μmol DCIP red. mg chl^{-1} h^{-1} and 1.20 μg fresh weight^{-1}, respectively

suggest that restoration of Hill reaction activity is independent of protein synthesis. The rates of both processes are very fast, and the original values are reached after about 2 h of illumination of the leaves.

The Effect of Cycloheximide and DCMU on ATP and Protein Levels in Leaf Discs

The requirement of the intact cell structure for reversibility of cold and dark induced inactivation of Hill reaction activity in leaves of chilling-sensitive plants has suggested an interaction between cytosolic and chloroplast processes. To check the relationship between Hill reaction activity and ATP synthesis as well as cytosolic protein synthesis, we used two inhibitors – DCMU for inhibition of ATP synthesis and cycloheximide – since it is known that some chloroplast proteins are synthesized in the cytoplasm (Machold and Aurich, 1972; Cashmore, 1976).

In the experiment presented in Figure 8 tomato leaf discs were incubated either with cycloheximide or DCMU in the cold and dark and then illuminated. As can be seen, the presence of cycloheximide in the cold and dark stored sample (A) results in a decline of the protein level to one-half that of the control, probably due to an enhancement of proteolysis. Upon illumination of leaf discs incubated with cycloheximide, the Hill reaction activity does not increase while the ATP level decreases by 50%. This may be due either to the consumption of ATP for some synthetic processes, e.g., synthesis of chloroplast galactolipids as reported by Michalski et al. (1976) for cold and dark stored tomato leaves following illumination or to a decrease in the ATP pool in the presence of cycloheximide (McMahon, 1975).

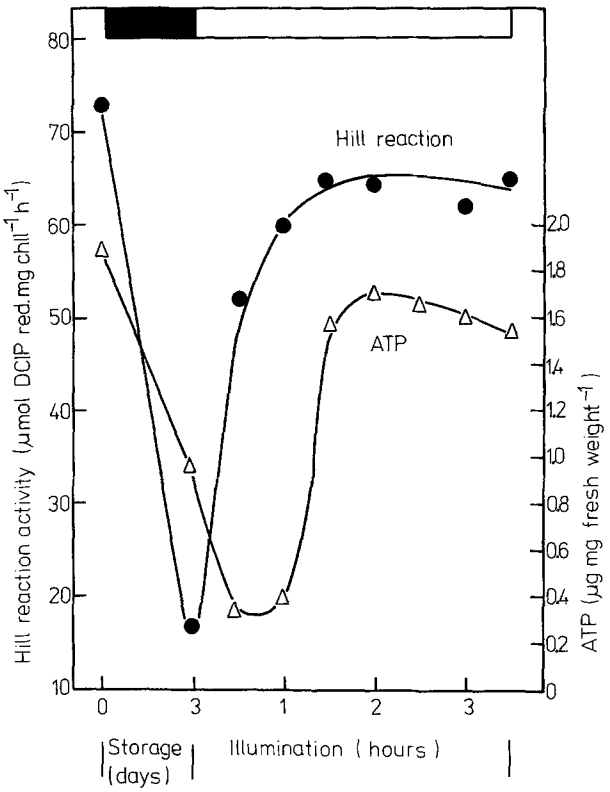

Fig. 6. Time course of light-induced restoration of ATP synthesis and Hill reaction activity in cold and dark stored leaves. Leaves were stored at 0° C in the dark for 3 days followed by illumination (8000 lx) at 25° C for the time indicated

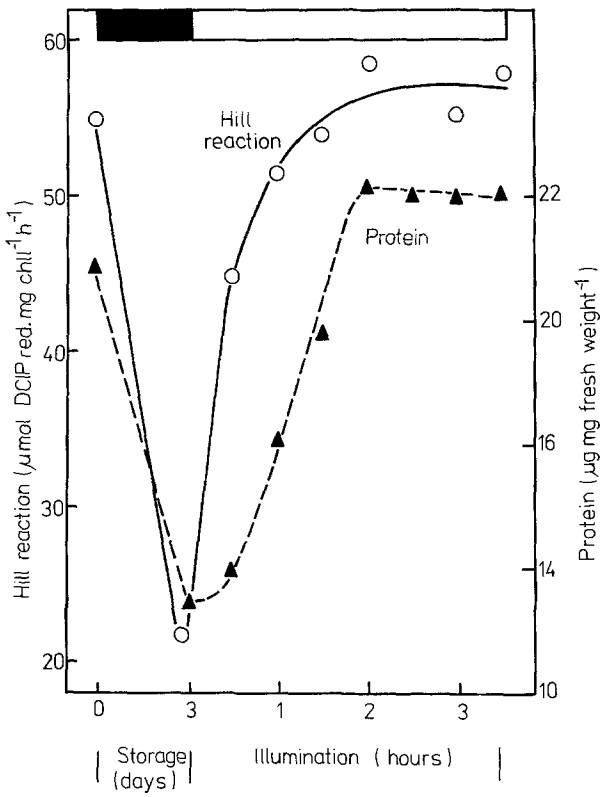

Fig. 7. Time course of light-induced restoration of protein synthesis and Hill reaction activity in cold and dark stored leaves. Conditions as in Figure 6

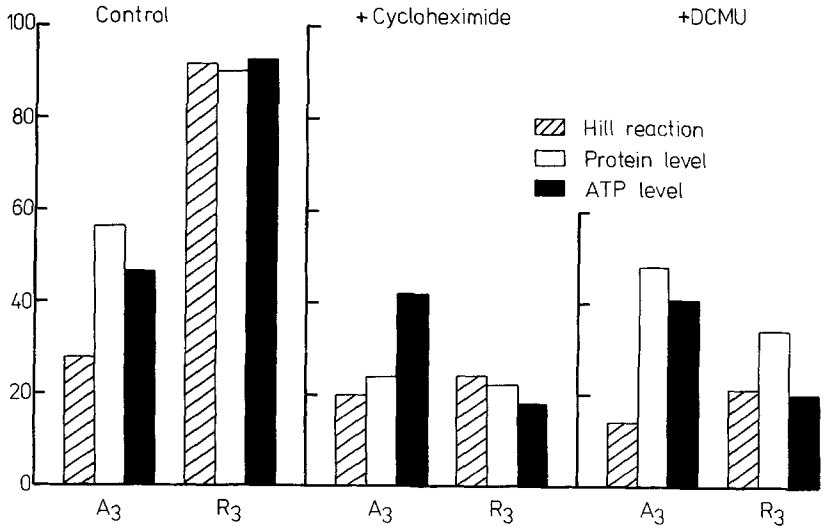

Fig. 8. Effect of cycloheximide and DCMU on protein and ATP levels and on Hill reaction activity in cold and dark stored and illuminated leaf discs. Tomato leaf discs, 0.6 cm in diameter, were punched from the leaves, main veins being excluded, with a cork borer. Discs were floated backside up in petri dishes containing distilled water, cycloheximide (10^{-5} M), or DCMU (10^{-6} M) solutions for 3 days in the dark at 0° C (symbol *A*) For reactivation, leaf discs were incubated at 25° C in white light, 8000 lx, for 2 h (symbol *R*)

In the presence of DCMU, the ATP level in dark-stored samples (A) is not affected more than, that in the control or in samples incubated with cycloheximide. Upon illumination of leaf discs incubated with DCMU, ATP content is diminished by one-half, similarly as in the presence of cycloheximide (R). In contrast, the protein level in the dark stored sample is similar to that of the control; however, upon illumination it decreases by about 25%. Thus, the inhibitory effect of both cycloheximide and DCMU on the ATP

level seems to point out the improtance of energy supply for processes involved in restoration of cold and dark inactivated Hill reaction activity.

Discussion

Detached leaves of chilling-sensitive plants that have been subjected to cold and dark storage exhibit several metabolic changes that are characteristic of senescence at room temperature, of which degradation of protein and chlorophyll is the most typical and commonly studied. It is interesting that about 50% of tomato leaf protein is degradated during 3–4 days of storage of leaves in darkness at 0° C (Figs. 1, 2 and 7). Thus the rate of protein loss is as fast as that observed at room temperature in detached leaves of several other plants of both chilling-sensitive (Goldthwaite and Laetch, 1967; Takegami, 1975) and chilling-resistant plants (Martin and Thimann, 1972; Peterson and Huffacker, 1975; Thomas and Stoddart, 1975; Choe and Thimann, 1977; Wittenbach, 1977). It appears that an accelerated proteolysis induced by excision of leaves cannot be slowed down by low temperature. Moreover, cold and dark treatment of tomato leaves results in a rapid and large (up to 50%) depletion of chloroplast manganese (Kaniuga et al., 1978b), degradation of chloroplast galactolipids (Michalski et al., 1976), increase in FFA (Kaniuga and Michalski, 1978), as well as in a large decrease in the ATP level (Figs. 4, 5, and 6). All these changes indicate the strong deleterious effect of the two main factors – detachment of leaves and darkness – and to a lesser extent that of cold.

In contrast to very fast loss of chlorophyll during senescence of leaves in the dark at room temperature, cold and dark storage of tomato leaves does not affect the chlorophyll content (Figs. 1 and 2). However, when cucumber plants were chilled for 3 days at 5° C and 85% r.h. in the light but not in the dark, one-third of the chlorophyll content was lost (Wright and Simon, 1973). Thus low temperature and darkness seem to prevent chlorophyll degradation in chilling-sensitive plants.

The response of detached leaves and intact plants to cold and dark treatment is different even when the same conditions of light or darkness are applied. Thus, the loss of Hill reaction activity following cold storage in the dark occurs more rapidly in detached leaves than in growing plants (Kaniuga et al., 1978a). While in cold and dark-stored detached tomato leaves ATP level drops (Figs. 5 and 6), chilling of bean plants in the dark at 4° C leads to an increase of ATP level (Jones, 1970; Wilson, 1978). Accumulation of ATP was more effective when hardening preceded chilling at 5° C (85% r.h.) or when water loss was prevented by maintaining a saturated (100% r.h.) atmosphere (Wilson, 1978). However, in some chilling-sensitive plants such as cotton (Stewart and Guinn, 1969, 1971) and *Episcia reptans* (Wilson, 1978), the ATP level decreased during 24 h of chilling at 5° C.

Requirement of both intact cell structure and light for restoration of cold and dark inactivated Hill reaction activity could suggest an interrelationship between processes occurring in the chloroplasts and cytosol. It is known that major lamellar protein associated with photosystem II is synthesized on cytoplasmic ribosomes (Machold and Aurich, 1972; Cashmore, 1976). Very large (up to 50%) losses of both soluble leaf protein (Figs. 1, 2, and 7) and ATP (Fig. 4, 5 and 6) and their rapid resynthesis to the initial levels following 2 h of illumination seem to indicate a contribution of both chloroplast ATP and cytosolic protein synthesis. When DCMU and cycloheximide were used to eliminate ATP and cytoplasmic protein synthesis, respectively, it was found (Fig. 8) that: (1) besides the inhibitory effect on protein synthesis cycloheximide also diminished the ATP level, as was postulated by McMahon (1975), (2) DCMU prevents an increase of both ATP and protein synthesis during illumination of cold and dark-stored leaf discs, probably due to limiting ATP synthesis. Since it is known that neither DCMU nor cold and dark treatment of leaves of chilling-sensitive plants affects cyclic electron transport, it may be concluded that production of ATP by cyclic photophosphorylation is too low to provide energy for all processes involved in the restoration of damaged noncyclic electron transport. At the beginning of illumination of leaves (Fig. 6), ATP consumption is so effective that some decrease in the ATP level is observed. Thus the primary effect of light on the restoration of damaged electron transport is to produce ATP required for incorporation of manganese into thylakoid membrane; several other processes, such as protein synthesis are of secondary importance for the reactivation of Hill reaction activity.

The authors thank Mrs. M. Froelich and Mr. M.J. Nałęcz for participation in experiments. The skillful technical assistance of Mr. W. Łasiński is acknowledged. We are grateful to the Administration of Państwowe Gospodowstwo Ogrodnicze "Mysiadło" for providing plant material.

This work was carried out under Project No 10.2.10 coordinated by the Institute of Ecology, Polish Academy of Science.

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Received 13 June; accepted 16 September 1978