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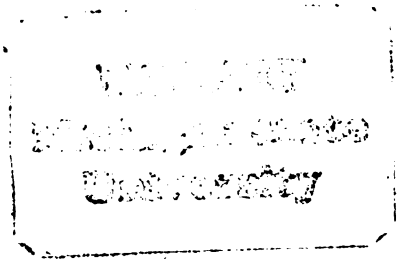

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CHLOROPLAST MEMBRANE ADAPTATION AND REPAIR
IN RESPONSE TO ENVIRONMENTAL STRESS
IN MAIZE SEEDLINGS (ZEA MAYS L.)

presented by

Bruce Raymond Runk

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Major professor
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CHLOROPLAST MEMBRANE ADAPTATION AND REPAIR IN RESPONSE TO ENVIRONMENTAL STRESS IN MAIZE SEEDLINGS (*ZEA MAYS* L.)

By

Bruce Raymond Runk

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ABSTRACT

CHLOROPLAST MEMBRANE ADAPTATION AND REPAIR
IN RESPONSE TO ENVIRONMENTAL STRESS
IN MAIZE SEEDLINGS (ZEA MAYS L.)

By

Bruce Raymond Runk

Recovery of photosystem II activity in maize (Zea mays L.) seedlings after damage by a heat treatment at 48 C for 5 minutes was studied using photosystem II-dependent electron transport assays (in vitro) and fluorescence induction transient measurements (in vivo). Requirements for recovery from heat stress were examined by testing different pre- and post- heat stress conditions. A heat treatment at 39 C for 3 hours--prior to heat stress at 49 C for 5 minutes--(preadaptation) permitted recovery to occur. Incubation in the light at 21 C following heat stress was required for recovery. Incubation in the dark at 21 C following heat stress did not allow recovery to occur but did hasten recovery during subsequent incubation in the light. 35S-methionine incorporation studies showed an inhibition of protein synthesis in heat-stressed plants which had not been preadapted at 39 C but no inhibition of protein synthesis in heat-stressed plants which had been preadapted at 39 C.

To my Lord and Savior, Jesus Christ,
The Chief Gardener

"But blessed is the man who trusts in the Lord,
whose confidence is in Him.
He will be like a tree planted by the water
that sends out its roots by the stream.
It does not fear when heat comes;
its leaves are always green.
It has no worries in a year of drought
and never fails to bear fruit."

Jeremiah 17:7,8

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TABLE OF CONTENTS

	page
LIST OF TABLES	v
LIST OF FIGURES	vi
LIST OF ABBREVIATIONS	vii
LITERATURE REVIEW	1
INTRODUCTION	13
MATERIALS AND METHODS	14
Plant material	14
Treatment of plants	14
Thylakoid isolation	15
Activity assays	17
In vivo fluorescence assay	19
Radioactive labelling	20
Gel electrophoresis	21
RESULTS	23
Experimental design	23
Damage vs heat stress temperature	24
Recovery vs preadaptation conditions	24
Conditions required for recovery	28
Recovery kinetics vs length of dark incubation	37
Radioactive labelling	40
DISCUSSION	47
BIBLIOGRAPHY	56

LIST OF TABLES

page

1. Conditions required for recovery 31

LIST OF FIGURES

		page
1.	Experimental design.	25
2.	Determination of heat stress conditions.	27
3.	Determination of preadaptation temperature . . .	29
4.	Determination of preadaptation time.	30
5.	Comparison of fluorescence transients.	33
6.	DCMU effect on <u>in vivo</u> fluorescence transient. .	36
7.	Visual observation of recovery and photodestruction	38
8.	Recovery kinetics for various dark incubation times.	41
9.	Polypeptide pattern and 35S-methionine incorporation during preadaptation	42
10.	Polypeptide patterns and 35S-methionine incorporation during dark and light incubations.	44
11.	Model of recovery process.	55

LIST OF ABBREVIATIONS

chl	chlorophyll
ATP	adenosine 5'-triphosphate
NADP	nicotinamide adenosine 5'-diphosphate
Tris	tris(hydroxymethyl)aminomethane
Tricine	N-tris(hydroxymethyl)methylglycine
DCPIP	2,6-dichlorophenolindophenol
DPC	1,5-diphenylcarbazide
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
BQ	benzoquinone
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
MeV	methylviologen
DQH_2	reduced duroquinone (2,3,5,6-tetramethyl-1,4-benzenediol)
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
kd	kilodalton
E	einstein
P	preadaptation stage
H	heat stress stage
D	dark incubation stage
L	light incubation stage

LITERATURE REVIEW

Higher plants adapt to the damaging effects of environmental stress in a number of ways. These adaptations cover a wide range of possibilities, including modifications at the organismal, tissual, cellular, subcellular, and molecular levels. Adaptations to environmental stress can be classified further into those that provide protection against damage and those that merely provide a mechanism for repair of damaged components. An adaptation that provides protection is best suited to a situation in which a certain environmental condition is constantly encountered during the life of the plant. On the other hand, an adaptation that provides a repair mechanism is best suited to a situation in which the plant encounters an environmental condition only on occasion and not as a regular, everyday occurrence.

At the organismal level, plants can respond to environmental stress through avoidance. Plants avoid stress by limiting their habitat to exclude regions where a certain form of environmental stress regularly occurs or by limiting their growth cycle to a time of year in which those conditions are unlikely to occur (1, 2). Repair at the organismal level can be seen in the production of new

plants through a variety of natural propagative methods.

At the tissual level, plants can respond to environmental stress through anatomical or structural modification. Anatomical modification is easily seen in the C_4 plants which have an altered anatomy that allows them to survive carbon dioxide deficiency by concentrating carbon dioxide in the cells in which it is to be assimilated (3). Cactus is a good example of a plant which has a modified structure that allows it to survive adverse environmental conditions. By drastically reducing its leaf area, the cactus is able to retain water during drought (2). Another form of anatomical modification is the stomate. Stomatal closure permits the plant to limit water loss, while still taking in the carbon dioxide necessary for growth. Stomatal closure is a complex process which is regulated by a variety of environmental conditions. Temperature, water availability, carbon dioxide levels, and light all regulate the closing and opening of stomates either directly or indirectly through a feedback loop (4). The repair mechanism at the tissual level involves the production of new shoots or leaves or the production of scar tissue.

Plants can adapt to environmental stress at the cellular or subcellular levels through compartmentation of the cell into organelles and modification of organelles to make them resistant to damage from stress. Compartmentation allows the plant, for example, to prevent toxic substances from interfering with important metabolic

activities of the cell. Toxic substances could arise from damage of the cell by environmental stress or from uptake of substances from the environment. Organelles can be modified themselves to provide protection from environmental stress. For instance, photosystems I and II are spatially separated in the chloroplast, allowing for the regulation of excitation energy distribution between the two photosystems (5). Repair of damage at the cellular or subcellular levels involves production of new cells and organelles.

Plants can also respond to environmental stress at the molecular level. It is this level of response that has been studied with respect to adaptation to heat stress. Studies have shown several changes in the molecular make-up of the photosynthetic apparatus in plants that are accustomed to high temperatures. Studies of excitation energy transfer from chlorophyll b to chlorophyll a, have shown that several desert plants have a resistance to dissociation of the light-harvesting chlorophyll a/b-protein complex from photosystem II when they are acclimated to high temperature conditions (6-8). Other studies have shown a change in lipid composition in high temperature acclimated plants. The fluidity of chloroplast membrane lipids was shown to decrease in Nerium oleander plants upon acclimation to higher temperatures (9). In a similar study, chilling-resistant plants were shown to have higher amounts of palmitic and trans delta 3-hexadecanoic acids

attached to phosphatidyl glycerols than their susceptible counterparts (10).

Changes in protein patterns have also been seen in response to high temperature stress. Altered gene expression has been seen to result in adaptation in a number of cases (11). Thermophilic blue-green algae, Phormidium laminosum, are known to be resistant to heat stress up to significantly higher temperatures than can be survived by most plants (12); however, the isolated photosystem II particles still show lower optimum temperature than that found in vivo.

Plants can also adapt to environmental stress at the molecular level through the development of repair mechanisms involving the production of new lipids or proteins. This type of response has been seen during cold stress (13). Although this type of adaptation has not been studied in relation to heat stress, it is likely to occur in a number plants, since heat stress is normally an occasional happening, especially in temperate climates.

Adaptation to environmental stress can also be categorized according to the component of the plant in which the particular adaptative mechanism occurs. Several environmental conditions can have a direct inhibitory effect on the photosynthetic apparatus when they reach non-optimal levels. These include: light, cold, and heat (14). For this reason, adaptation of the photosynthetic apparatus to non-optimal environmental conditions is a topic of

particular interest. The photosynthetic apparatus of higher plant chloroplasts can be classified into various functional complexes: the water-splitting complex, photosystem II, the cytochrome b_6-f complex, photosystem I, and coupling factor (CF_1 and CF_0) (15). The water-splitting complex donates electrons to photosystem II; photosystem II and the cytochrome b_6-f complex interact through the plastoquinone pool; the cytochrome b_6-f complex and photosystem I interact through plastocyanin, a soluble protein; and photosystem I donates its electrons to NADP through ferredoxin and ferredoxin-NADP reductase, both soluble proteins. Artificial electron donors and acceptors exist which can be used to determine the site of damage to components of the photosynthetic electron transport system. In addition, photosystem II can be probed through evaluation of the transient which occurs in photosystem II fluorescence as dark adapted plants or chloroplasts are subjected to light (16-18). These methods are helpful in defining the sites of action of numerous inhibitors of the photosynthetic apparatus in addition to the sites that are inhibited by different forms of environmental stress. From numerous studies using these methods, it has become well established that photosystem II is usually the first component to undergo damage by environmental stress (8).

Intense light is thought to inhibit photosystem II activity by causing more energy to be transferred to the reaction center than can be orderly dissipated (19). This

results in destruction of several components of photosystem II, possibly including a component on the oxidizing side of photosystem II or the quinone-binding secondary stable electron acceptor of photosystem II (B-binding protein) on the reducing side of photosystem II, which has been shown also to bind herbicides (20). Inhibition by intense light (photoinhibition) can be seen most readily in shade adapted plants which have developed large photosynthetic antennae systems. Photosystem I is less sensitive to inhibition by intense light because it does not include the rate limiting step of photosynthesis, namely the transfer of electrons within the B-binding protein. Photoinhibition can be intensified by extremes in other environmental conditions. Plants grown in full sun, when they are subjected to a low carbon dioxide environment, are photoinhibited by the same light intensity as shade adapted plants (21). The lack of carbon dioxide is thought to prevent the normal levels of energy dissipation, since carbon dioxide assimilation is low. Reduced electron flow can cause the build-up of the high energy state and dissipation of absorbed light energy through photodestruction. Carbon dioxide is assimilated (Calvin Cycle) by use of the reduced NADP generated by the photosynthetic apparatus and the ATP generated through photophosphorylation (19).

Cold stress can also directly affect the photosynthetic apparatus. It has been reported that incubation in the dark at 4 C caused a loss in

water-splitting activity (13). Recovery from this damage could be achieved by transferring the plants into the light at room temperature. Further examination showed a change in the free fatty acid composition in cold damaged plants (22). The total levels of free fatty acid were increased as well as the percentage of unsaturated free fatty acids upon cold stress. The free fatty acid levels went back to normal upon recovery in the light at room temperature. Exogenous linolenic acid was shown to inhibit water-splitting activity in plants at room temperature. It was concluded that cold damage was caused in part by the release of inhibitory fatty acids. Manganese levels in the cold-treated plants were also examined (23). It was found that the chloroplasts in cold damaged plants had lost 40-50% of the total amount of manganese. This was accompanied by an almost complete loss of Hill reaction activity. Restoration of Hill reaction activity upon illumination was accompanied by an increase in manganese content. Both Hill reaction activity and manganese content increased rapidly from the beginning of illumination and reached a maximum after 2 hours. ATP, chlorophyll, and total leaf protein levels were examined during cold stress and subsequent illumination (24). The ATP level was decreased by 50% during cold stress and was diminished further during the first hour of illumination before reaching a maximum level after 2 hours of illumination. The chlorophyll level was not affected by cold stress or illumination. The total

leaf protein level was decreased by 50% during cold stress and reached a maximum after 2 hours of illumination but experienced a lag at the beginning of the illumination period. The inhibitors cycloheximide and DCMU prevented restoration of Hill activity, the ATP level, and the total leaf protein level. It was concluded that ATP was necessary for the insertion of manganese into the water-splitting enzyme with cytoplasmic protein synthesis playing only a secondary role in the recovery process.

It has been concluded from several studies that other possible primary sites of action of cold stress can be dismissed. Proton efflux rates from the thylakoids were shown to be similar in both chilling sensitive and chilling resistant plants subjected to low temperatures indicating that the permeability properties of both types of membranes were the same (25). Loss of chlorophyll was shown to be a secondary effect of cold stress in tomato (26). Low temperature stress was further shown to be increased by stressing plants under light (27). Studies of fluorescence transients showed damage to both the water-oxidizing side and the reducing side of photosystem II during cold stress (28). Damage at the water-oxidizing side of photosystem II could be observed by a loss of variable fluorescence. Damage at the reducing side of photosystem II could be observed by an increase of fluorescence to maximum levels.

The photosynthetic apparatus is also sensitive to high temperature stress. As the temperature is increased,

different components of the photosynthetic apparatus are damaged by heat stress. The most sensitive of these components is the light-harvesting chlorophyll a/b-protein complex. Examination of chlorophyll fluorescence during heat treatment showed a functional dissociation of the light-harvesting chlorophyll a/b-protein complex from photosystem II both *in vivo* and *in vitro* (29, 30). At slightly higher temperatures an actual physical dissociation of the light-harvesting chlorophyll a/b-protein complex from photosystem II was shown by freeze fracture particle analysis (31). This is in contradiction to the light-harvesting chlorophyll a/b-protein complex in high temperature acclimated plants (6-8).

More severe temperature stress causes damage to other components in the thylakoid membrane. As the temperature is increased to higher levels, the water-splitting activity of the chloroplast is inhibited. DCPIP activity, an indicator of photosystem II activity, was shown to be inhibited in isolated spinach chloroplasts at high temperatures (32). Photosystem II activity of isolated thylakoids from heat-stressed intact ivy leaves was also seen to be inhibited (33). Yamashita and Butler (34) found that p-phenylenediamine and hydroquinone, which donate electrons to photosystem II, could be used *in vitro* to restore the ability of heat-treated chloroplasts to reduce NADP. This activity was inhibited by DCMU, which inhibits photosystem II electron transport at the secondary stable

quinone acceptor (herbicide-binding site (20)). These results defined the site of photosystem II damage more specifically as being the water-splitting site. Differential scanning calorimetry has also been used to show that the site of heat damage in photosystem II is the water-splitting site (35). Endothermic transitions were found that corresponded to release of manganese from the membrane, the loss of oxygen evolution with water as a donor, and a decrease in the redox potential of the hydroquinone-reducible cytochrome b-559. The first two transitions were irreversible after heating to 49 C or exposure to trypsin. This suggested that protein(s) were involved in these transitions. Studies on membranes inhibited by Tris, NH_2OH, or heat (all inhibitors of water-splitting activity) showed a loss of manganese from the chloroplasts upon these treatments (36).

Another site of heat stress damage becomes evident at temperatures higher than those that damage photosystem II. Electron transport studies utilizing methylviologen as the electron acceptor in the presence of DCMU and reduced DCPIP have shown inhibition of photosystem I activity at temperatures above 50 C (33). In general, photosystem I is much less susceptible to damage by stress than is photosystem II.

The mechanism of damage to the water-splitting activity by heat stress has been a topic of much interest. Several studies have shown changes in lipid composition

during heat treatment. Electron spin resonance spectra have shown a change in fluidity of chloroplast membranes upon heating (37). Analysis of lipid composition and release of free fatty acids during heat stress have shown no significant changes in the percentage of unsaturated fatty acids and no increase in levels of free fatty acids (38). This does not exclude the possibility of local changes within the membrane or changes in lipid-lipid interaction. A similar study showed reduction of free sterols upon heat stress even though no significant changes could be observed in the desaturation levels of the fatty acids (39). It was suggested that membrane fluidity is under a more precise control than simple changes in desaturation levels.

The possibility of changes in membrane proteins as a result of heat stress has also been investigated. Volger and Santarius (40) saw the release of several chloroplast membrane proteins, even though the total protein levels did not change. Loss of proteins of molecular weights 33 and 37 kilodaltons followed the pattern of loss of water-splitting activity. Similar studies have been made using Tris-treated chloroplasts (41) and a low fluorescent mutant of Scenedesmus (42). The Tris-treated chloroplasts were seen to release three polypeptides of molecular weights 33, 24, and 18 kilodaltons. The mutant studies showed a shift in apparent molecular weight of one protein from 34 to 36 kilodaltons upon heating. This protein has been associated

with photosystem II in Scenedesmus.

Environmental stress in the form of light, cold, or heat has a direct effect on the photosynthetic apparatus and, in particular, on photosystem II. It is therefore likely that adaptation to environmental stress would involve a modification of the thylakoid membrane or the development of repair mechanisms to deal with the damage inflicted on the thylakoid membrane by environmental stress.

INTRODUCTION

Maize (Zea mays L.) plants in the field undergo severe heat stress on a hot, dry summer day. Stomatal closure, designed to conserve moisture under dry conditions, prevents evaporation from the leaf surface which normally lowers the leaf temperature. The leaves may curl as a result of water deficiency under these conditions. Although leaf curling lessens absorption of sunlight, and thus heating of the leaves, the leaf temperature can still reach a level which, in in vitro studies, is known to cause damage to the photosynthetic apparatus (34). The plants in the field however, continue to grow with little indication of permanent damage. It therefore seems reasonable to assume that some repair or damage prevention process has been developed in the field-grown plants. The present study was carried out to determine the pre- and post- heat stress conditions required for recovery from heat-stress damage in maize and to examine the mechanism involved in the recovery process.

MATERIALS AND METHODS

Plant material

Maize (Zea mays L.) seedlings, variety FR 9 Cms X FR 37 from Illinois Foundation Seeds, Inc., Champaign, Illinois, were grown in coarse vermiculite to the three leaf stage (9 days from sowing) in individual 2 inch pots under continuous light from fluorescent and incandescent lamps of 200 $uEm^{-2}sec^{-1}$ at 27 C. Plants were watered daily with distilled water and weekly with dilute Hoagland's solution.

Treatment of plants

Plants were preadapted in the dark at various temperatures and for various lengths of time in a Precision Scientific Model 805 incubator from General Electric Company, Louisville, Kentucky. Dehydration was prevented by watering the plants thoroughly immediately before preadaptation and by maintaining high humidity in the incubator.

Plants were heat stressed at various temperatures for various lengths of time in a 50 cm X 35 cm X 10 cm insulated plastic tray filled with water. The water temperature was maintained at +/- 0.1 C by a Thermomix II mixer/thermostat from Bronwill Scientific Division of Will

Corporation, Rochester, New York. The temperature was determined by a Digi-Sense Model 8522-10 digital thermistor from Cole Parmer Instrument Company, Chicago, Illinois. Plants were submerged upside down to the bottom of the first leaf by placing the plants in a metal plant rack. The root balls were protected by inserting foam rubber pads around the stems of the plants.

Subsequent to the heat stress treatment, seedlings were allowed to recover in either light or dark. Light incubation following heat stress was conducted at 50 uEm^{-2} sec^{-1} for a variable length of time under a shading canopy set up in the same growth chamber as used for growing the seedlings. Light incubation was often preceded by a period of dark incubation of variable length of time. Dark incubation was conducted in a dark cabinet at room temperature.

Thylakoid isolation

Leaf samples were taken for several combinations of preadaptation, heat stress, dark incubation, and light incubation conditions. Samples consisted of 3-4 plants. Only the second and third leaves were used. Samples were ground in 30 ml of grinding buffer (0.015 M Tricine, pH 7.8, 0.010 M NaCl, 0.4 M sorbitol) for 5-7 seconds at top speed on a Commercial Blendor from Waring Products Division of Dynamics Corporation of America, New Hartford, Connecticut using a chilled Penner attachment from Eberbach Corporation, Ann Arbor, Michigan. The suspensions were

filtered through 4 layers and then 12 layers of cheesecloth and centrifuged at 3,000 g for five minutes in a Sorvall RC-5B Refrigerated Superspeed Centrifuge from DuPont Instruments, Wilmington, Delaware using an SS34 rotor. The pelleted chloroplasts were resuspended in 20 ml of wash buffer (0.010 M Tricine, pH 7.8, 0.010 M NaCl, 0.005 M $MgCl_2$) using a fine camel hair brush. The resuspended samples were centrifuged at 3,000 g for 5 minutes. The pelleted thylakoid membranes were resuspended in 1 ml of resuspension buffer (0.015 M Tricine, pH 7.8, 0.010 M NaCl, 0.005 M $MgCl_2$, 0.1 M sorbitol) using a fine camel hair brush. The resuspended samples were then homogenized in a hand homogenizer. Aliquots were taken for each sample for use in chlorophyll determinations. Chlorophyll determinations followed the method of MacKinney (43). Dilutions (1:100) in 80% acetone were measured for absorbance at 720 nm, 663 nm, and 645 nm in a 100-40 spectrophotometer from Hitachi Scientific Instruments-Nissei Sangyo America, Ltd., Mountain View, California. Total chlorophyll concentration was determined for each sample using the simultaneous equations:

$$A_{663} = 82.04\ c_a + 9.27\ c_b$$

$$A_{645} = 16.75\ c_a + 45.6\ c_b,$$

where A_{663} and A_{645} are the measured absorption values for the samples at 663 nm and 645 nm respectively and c_a and c_b are the concentrations in mg/ml of chlorophyll a and chlorophyll b respectively. These equations were

rearranged to give:

$c = 0.007533\ A_{663} + 0.02029\ A_{645}$,

where c is the total chlorophyll concentration in mg/ml. Adjusting for background absorbance and the dilution factor gave a final equation for total chlorophyll concentration in mg/ml:

$c = (\text{dilution factor})\ (0.007533(A_{663} - A_{720}) + 0.02029(A_{645} - A_{720}))$,

where A_{720} is the measured absorption value of the sample at 720 nm. The total chlorophyll concentrations were used to determine the amount of sample to use in activity assays. Samples were isolated and stored on ice and were used within 2 hours of isolation.

Activity assays

Photosystem II activity was generally measured using the artificial electron acceptor DCPIP (water to DCPIP) (44, 45). Aliquots of samples containing 10 ug of chlorophyll were added to 2 ml of DCPIP assay buffer (0.050 M K_2HPO_4, pH 6.8, 0.010 M NaCl, 0.005 M $MgCl_2$, 0.1 M sorbitol, 1 mM NH_4Cl, 10^{-7} M gramicidin, 0.032 mM DCPIP) and assayed in an Hitachi 100-60 spectrophotometer for 30-60 seconds against a DCPIP assay buffer blank. The samples were excited by broad red light (a Corning 2-64 filter and a Corning 1-75 heat filter from Corning Glass, Corning, New York) at saturation intensity, and the absorption change of the DCPIP was monitored at 580 nm using a X-Y Recorder Model VQ-065A from Soltec, Sun Valley, California. The

photomultiplier tube was protected from the excitation beam through the use of a Corning 4-96 blue filter. Base lines were obtained both before and after the assay by monitoring at 580 nm with no excitation light. Addition of DPC to the assay was used to measure photosystem II activity not requiring water-splitting activity (DPC to DCPIP) according to the method of Vernon and Shaw (46). DPC was added at a concentration of 20 uM, and activity was measured as in the normal water to DCPIP assay. This assay was checked for DPC donation to photosystem I by making a final addition of DCMU (1 uM) and assaying as before. Rates in the presence of DCMU were subtracted from those in its absence to obtain photosystem II-dependent values. DCPIP activity was calculated from the slopes of the assay lines using the equation:

DCPIP activity = (# squares/min) X (60 min/hr) X (O.D./square) X (1 umole reduced/18 O.D.) X (1/# mg chl), where O.D. is the optical density of the sample at 580 nm. The "1 umole reduced/18 O.D." term is determined from the extinction coefficient for reduced DCPIP at 580 nm.

Photosystem II activity was also measured using BQ as an artificial electron acceptor (water to BQ). Aliquots of samples containing 39 ug of chlorophyll were added to 1.3 ml of BQ assay buffer (0.025 M Tricine, pH 7.8, 0.010 M NaCl, 0.005 M $MgCl_2$, 0.1 M sorbitol, 0.001 M NH_4Cl, 10^{-7} M gramicidin) in a Clark oxygen electrode from Yellow Spring Instrument Corporation, Yellow Spring, Ohio. 0.25 mM BQ

and 1 uM DBMIB were then added. Oxygen evolution rates were monitored with a water-jacketed Clark type electrode and a Model 53 Oxygen Monitor, also from Yellow Spring Instrument Corporation, and recorded on a strip chart recorder from Hewlett-Packard Company, Palo Alto, California, while the samples were illuminated with saturating light. The sample was maintained at 20 C by a circulating water bath.

Photosystem I activity was measured using the artificial electron acceptor MeV (DQH_2 to MeV). Aliquots of samples containing 39 ug chlorophyll were added to 1.3 ml of MeV assay buffer (0.025 M Tricine, pH 7.8, 0.010 M NaCl, 0.005 M $MgCl_2$, 0.1 M sorbitol, 0.001 M NH_4Cl, 10^{-7} M gramicidin, 0.1 mM MeV) in a Clark oxygen electrode at 20 C. 10^{-6} M DCMU, 0.5 mM DQH_2, and 0.01 mg/ml superoxide dismutase (Sigma Chemical Company, St. Louis, Missouri) were then added. Oxygen uptake rates were monitored and recorded as before, while the samples were illuminated with saturating light.

In vivo fluorescence assay

Transients of in vivo fluorescence were taken on dark adapted plants (5 minutes dark) using red excitation light from a Model SF-10 Plant Productivity Fluorometer from Richard Brancker Research, Ltd., Ottawa, Canada and were recorded on a Model 206 oscilloscope from Nicolet Instrument Corporation, Madison, Wisconsin. Transients represent a collection of 4000 data points taken one every

2 msec. The transients are therefore 8 second transients. Transients were interpreted as described by Govindjee and Papageorgiou (16), Lavorel and Etienne (17), and Satoh (18). The characteristic stages O (original level), I (intermediary peak), D (dip), P (peak), and S (quasi steady-state) are defined in Figure 5.

Radioactive labelling

Plants were labelled *in vivo* with 35S-methionine obtained from Amersham Corporation, Arlington Heights, Illinois and allowed to incorporate the radioactive methionine while undergoing preadaptation or light or dark incubation according to the method of Leto *et al.* (47). Control plants were simultaneously labelled. 100 uCuries of 35S-methionine with a specific activity of 1450 Curies/mmole were added to 0.5 ml of a carrier solution (2-3 drops of Tween 80 from Sigma Chemical Company in 10 ml doubly distilled water) for application to a single plant using only the second leaf. The radioactive solution was applied with a fine camel hair brush, allowing the solution to evaporate between each application until all of the 0.5 ml of solution were applied. Incorporation was allowed to proceed for 3 hours under the appropriate conditions. After the incorporation period the labelled leaf was ground with a chilled pestle in a chilled mortar containing 2 ml of grinding buffer and a small amount of sterile sand. The suspension was filtered through a single layer of Miracloth (Chicopee Mills, Inc., Milltown, New Jersey) and centrifuged

at top speed for 2 minutes in a Model 235A micro-centrifuge from Fischer Scientific Company, Pittsburgh, Pennsylvania. The pellet was washed twice in wash buffer and then resuspended in resuspension buffer. Samples were assayed for chlorophyll concentration and then refrigerated for later examination by SDS-PAGE.

Gel electrophoresis

Radioactive samples were subjected to protein separation via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 20 ml volume slab gels with a gradient of 10 to 17.5% polyacrylamide (48, 49). Gradients were poured using an 2120 Varioplex II pump from LKB-Produkter AB, Bromma, Sweden. Electrophoresis grade reagents were obtained from Bio-rad Laboratories, Richmond, California. The light component for the gradient consisted of 10 ml of 10% acrylamide, 0.267% bisacrylamide, 0.9% sucrose, 0.375 M Tris, pH 8.8, 0.1% SDS, 0.08% ammonium persulfate, and 0.05% TEMED. The dense component for the gradient consisted of 10 ml of 17.5% acrylamide, 0.467% bisacrylamide, 9.3% sucrose, 0.375 M Tris, pH 8.8, 0.1% SDS, 0.08% ammonium persulfate, and 0.04% TEMED. The stacking gel consisted of 5.4% acrylamide, 0.14% bisacrylamide, 0.125 M Tris, pH 6.8, 0.1% SDS, 0.23% ammonium persulfate, and 0.11% TEMED. Some gels contained 4 M urea to allow more thorough analysis of the labelling patterns. In these gels the ammonium persulfate concentration was lowered to 0.06%, 0.06%, and 0.07% in the

light component, the dense component, and the stacking gel respectively. The sample pellets were resuspended in sample buffer (10% glycerol, 5% beta-mercaptoethanol, 2% SDS, 62.5 mM Tris, pH 6.8, and 0.001% bromophenol blue). Samples were run against a molecular weight standards mixture containing bovine serum albumin (MW 68 kd) from Polysciences, Inc., Warrington, Pennsylvania, ovalbumin (MW 45 kd) from Polysciences, Inc., human erythrocyte carbonic anhydrase b (MW 29 kd) from Sigma Chemical Company, and horse heart cytochrome c (MW 12 kd) from Sigma Chemical Company. The gels were run at 20 mAmps constant current using an LKB 2103 power supply for 6 hours with a running buffer containing 0.025 M Tris, pH 8.3, 0.192 M glycine, and 0.1% SDS. The gels were stained in staining solution (0.1% Coomassie Blue, 50% methanol, 7% acetic acid in water) for 45 minutes. The gels were destained over night in destaining solution (20% methanol, 7% acetic acid, 3% glycerol in water). The gels were dried for 45 minutes in a dual temperature slab gel dryer from Hoefer Scientific Instruments, San Fransisco, California. Autoradiograms were performed by incubating the dried gels in the dark at -80 C with Kodak XAR-5 film (Kodak Corporation, Rochester, New York) for 3-7 days according to the method of Fairbanks et al. (50). The film was processed in an automatic film processor from Machlett Corporation, Chicago, Illinois.

RESULTS

Experimental design

The experimental protocol included four stages: preadaptation, heat stress, dark incubation, and light incubation. These will be designated P, H, D, and L, respectively. The preadaptation stage was always carried out in the dark; however, the temperature and duration of preadaptation were varied. Control plants were incubated at 21 C for the same amount of time as the preadapted samples. Control plants will be designated by P(-). The heat stress stage was always carried out in the dark; however, the temperature and duration of heat stress varied. Control plants were incubated at 21 C for the same amount of time as the heat-stressed samples. Control plants will be designated by H(-). The dark incubation stage was always carried out in the dark; however, the duration of dark incubation varied. The conditions of the dark incubation will be stated within parentheses following the D notation: D(time). If no dark incubation stage was used on a particular sample, the designation D(-) will be used. The light incubation stage was always carried out in the light; however, the duration of light incubation varied. The conditions of the light incubation will be stated

within parentheses following the L notation: L(time). If no light incubation stage was used on a particular sample, the designation L(-) will be used (Figure 1).

Damage vs heat stress temperature

Photosystem II activity was measured by the DCPIP assays and the BQ assay and photosystem I activity was measured by the MeV assay for various degrees of heat stress damage (Figure 2). The water to DCPIP and water to BQ assays showed the same loss of photosystem II activity with increasing heat stress temperature. The DPC to DCPIP assay showed that there was an approximate 50% loss of photosystem II activity that could not be recovered by the artificial donor, DPC. The remainder of the photosystem II activity decline was apparently due to damage to the water-oxidizing side of photosystem II. The DQH_2 to MeV assay showed no loss of photosystem I activity over the range of temperatures which was used. A heat stress time of 5 minutes was chosen for convenience. On the basis of these data, a working heat stress state of H(48 C, 5 min) was chosen. This will be written H(+) in the following sections. These conditions gave significant loss of both overall photosystem II activity and water-splitting activity.

Recovery vs preadaptation conditions

Following heat stress and a 6 hour light incubation, the degree of recovery was measured as a function of preadaptation temperature using the water to DCPIP assay at

Figure 1. Experimental design. The experimental protocol included four stages:

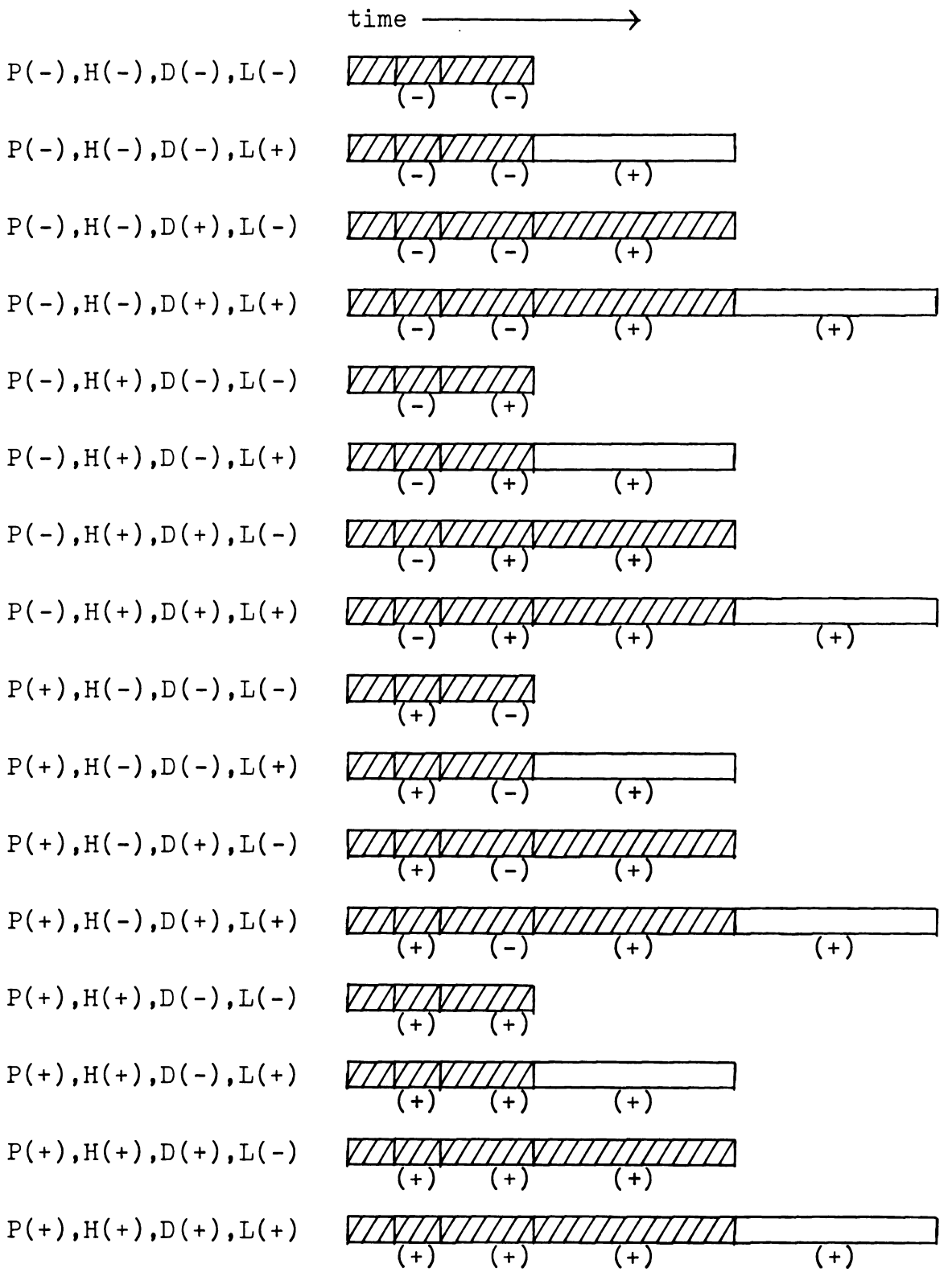
1) preadaptation--(P)--an exposure to elevated temperatures (above growth levels) preceding severe heat stress, designed to initiate the plant's mechanisms for recovery from severe heat stress without itself causing significant damage;

2) heat stress--(H)--a severe heat stress, designed to significantly damage the oxidizing side of photosystem II without causing damage to other components of the photosynthetic system;

3) dark incubation--(D)--an incubation in the dark following severe heat stress, designed to allow recovery of potential for photosystem II activity;

4) light incubation--(L)--an incubation in the light following dark incubation, designed to allow recovery of actual photosystem II activity.

Stages P and H have corresponding controls which are kept at a temperature of 21 C. Stages D and L have corresponding controls which are kept at an incubation time of 0 hours. The experimental design shows the possible combinations of the four stages and bar graphs representing each of these combinations ((+) = working conditions, (-) = control conditions, ▨ = dark, ▭ = light). Bar graph lengths do not represent actual lengths of time for each stage.


time
P(-),H(-),D(-),L(-)
P(-),H(-),D(-),L(+)
P(-),H(-),D(+),L(-)
P(-),H(-),D(+),L(+)
P(-),H(+),D(-),L(-)
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P(+),H(+),D(+),L(+)

Figure 1

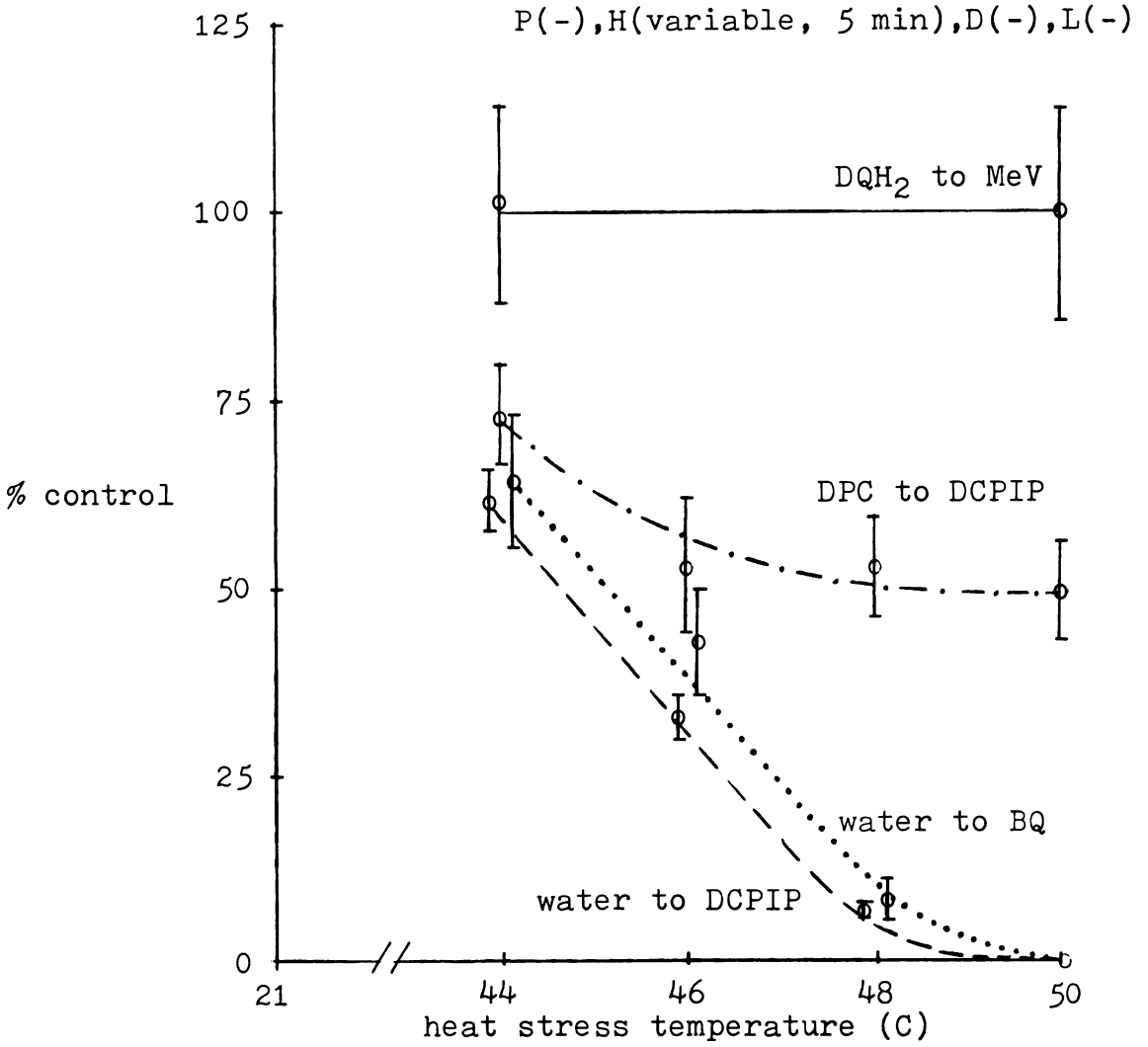


Figure 2. Determination of heat stress conditions. Unpreadapted plants were heat stressed for 5 minutes at various temperatures: P(-),H(variable, 5 min),D(-),L(-). Water to DCPIP (— —), DPC to DCPIP (—·—), water to BQ (·····), and DQH_2 to MeV (——) assays were performed on the samples after isolation of thylakoid membranes. Values are presented as a percentage of the control activity: P(-), H(-),D(-),L(-). The average control activity was 146 umoles DCPIP reduced/mg chl/hr. Data points represent an average from three samples taken on three separate days. The error bars show the standard deviation for each data point.

three different temperatures (Figure 3). At a lower preadaptation temperature (37 C), the plants were not damaged by the preadaptation but were unable to recover from the subsequent heat stress. At a higher preadaptation temperature (43 C), the plants were damaged by the preadaptation itself, but had some ability to recover. At a medium preadaptation temperature (39 C), the ability to recover was suitable for study. A working preadaptation temperature of 39 C was chosen on the basis of these data.

Following heat stress and a 6 hour light incubation the degree of recovery was determined for different preadaptation times by measuring water to DCPIP activity. The samples had undergone preadaptation at the same temperature (39 C) for various lengths of time (Figure 4). The ability to recover increased with longer preadaptation times. For convenience, a working preadaptation time of 3 hours was chosen. The working preadaptation conditions P(39 C, 3 hrs) will be written P(+) in the following sections.

Conditions required for recovery

Using the working conditions established above: P(39 C, 3 hrs),H(48 C, 5 min), various permutations of the possible conditions for each stage of the process were examined to determine the requirements for recovery. Recovery was evaluated by the water to DCPIP assay (Table 1). Photosynthetic water oxidation appeared to have been

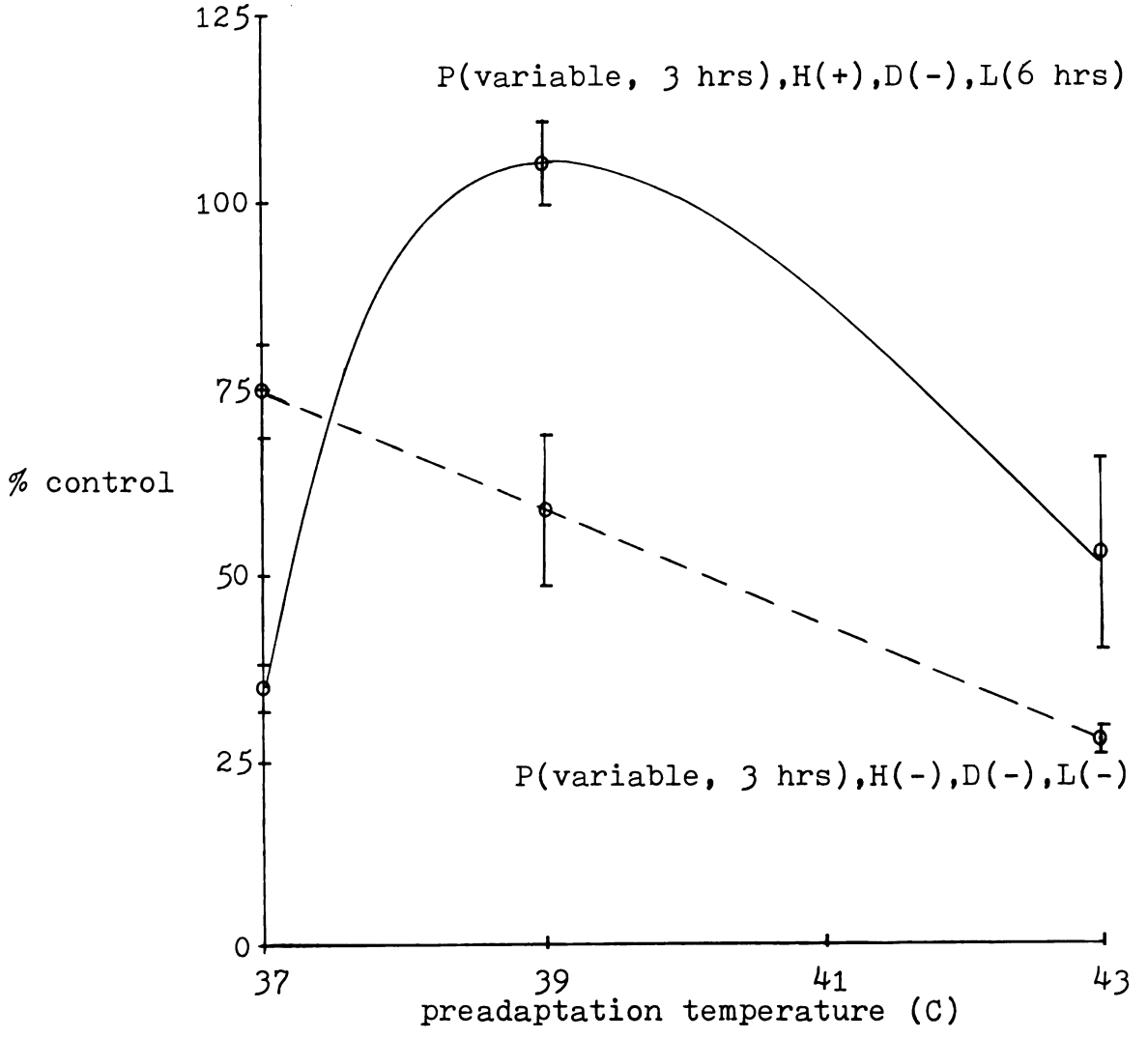


Figure 3. Determination of preadaptation temperature. Plants were preadapted for 3 hours at various temperatures, heat stressed at 48 C for 5 minutes, and incubated in the light for 6 hours: P(variable, 3 hrs),H(+),D(-),L(6 hrs) (——). Other plants were preadapted for 3 hours at the same temperatures but were not heat stressed or incubated in the light: P(variable, 3 hrs),H(-),D(-),L(-) (— —). Water to DCPIP assays were performed on the samples after isolation of thylakoid membranes. Values are presented as a percentage of the control activity: P(-),H(-),D(-),L(-). The average control activity was 166 umoles DCPIP reduced/mg chl/hr. Data points represent an average from three samples taken on three separate days. The error bars show the standard deviation for each data point.

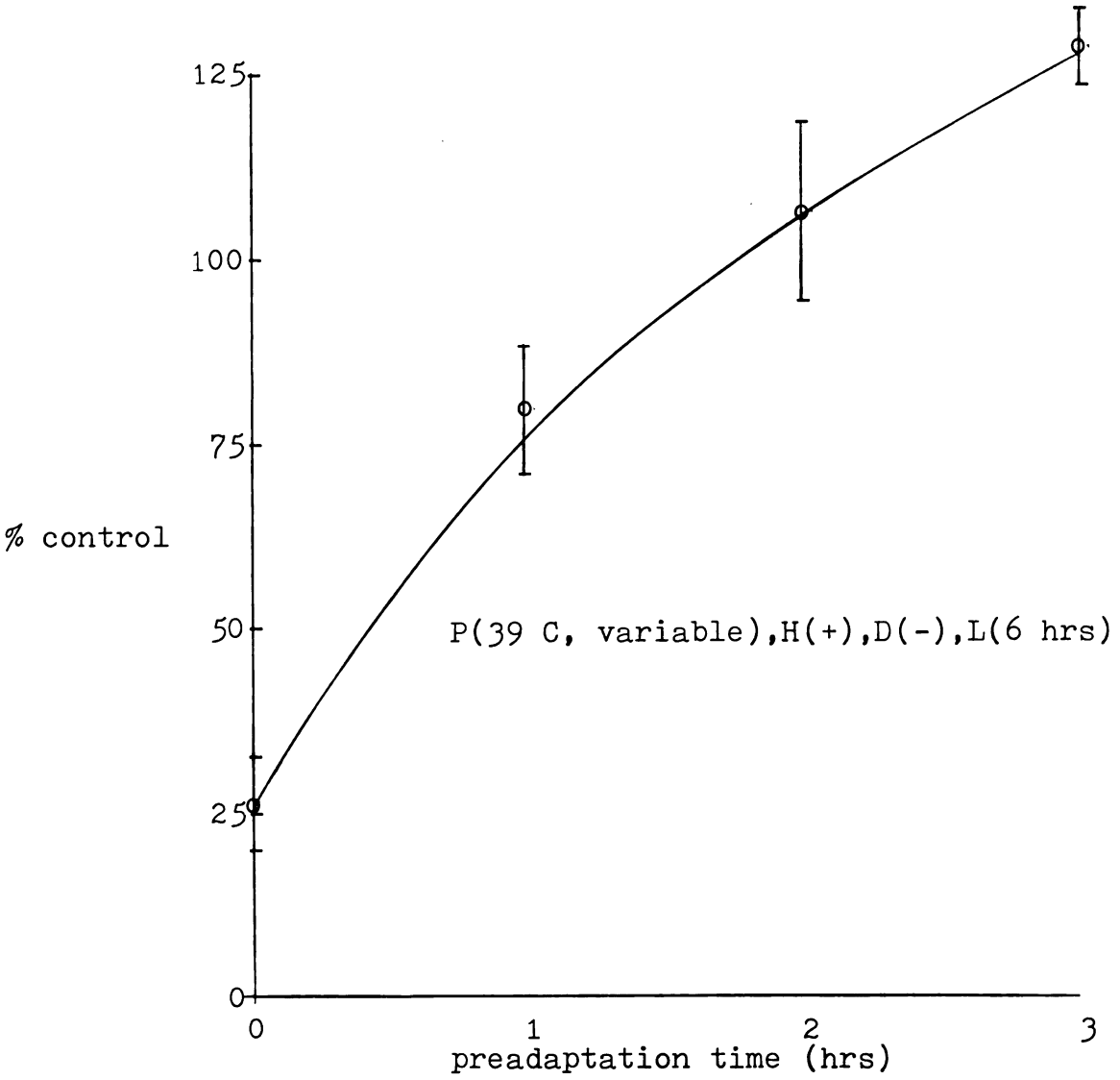


Figure 4. Determination of preadaptation time. Plants were preadapted at 39 C for various amounts of time, heat stressed at 48 C for 5 minutes, and incubated in the light for 6 hours: P(39 C, variable),H(+),D(-),L(6 hrs). Water to DCPIP assays were performed on the samples after isolation of thylakoid membranes. Values are presented as a percentage of the control activity: P(-),H(-),D(-),L(-). The average control activity was 123 umoles DCPIP reduced/mg chl/hr. Data points represent an average from three samples taken on three separate days. The error bars show the standard deviation for each data point.

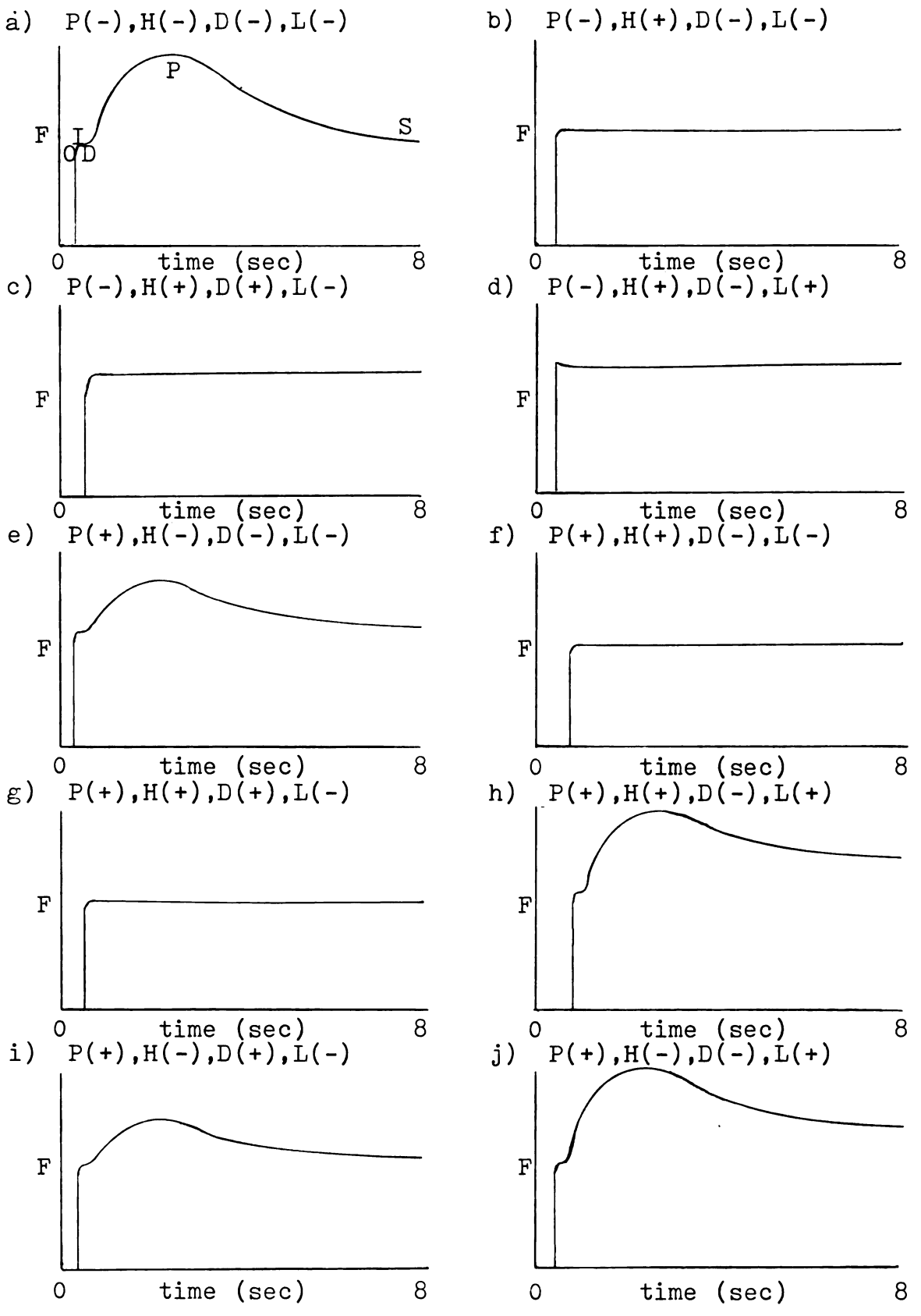
Table 1. Conditions required for recovery. Plants were processed through various permutations of the working conditions and control conditions to determine what conditions were necessary for recovery. The working conditions were 39 C and 3 hours for preadaptation and 48 C and 5 minutes for heat stress. Light and dark incubations were carried out for various amounts of time. Water to DCPIP assays were performed on the samples after isolation of thylakoid membranes. Values are presented as a percentage of the control activity: P(-),H(-),D(-),L(-). The average control activity was 132 umoles DCPIP reduced/mg chl/hr. Data represent an average from three samples taken on three separate days. Data values are followed by the standard deviation.

sample	conditions	% control (water to DCPIP)
1	P(-),H(-),D(-),L(-)	100 +/- 9
2	P(-),H(+),D(-),L(-)	9 +/- 2
3	P(+),H(+),D(-),L(-)	16 +/- 6
4	P(-),H(+),D(24 hrs),L(-)	6 +/- 3
5	P(-),H(+),D(-),L(24 hrs)	2 +/- 2
6	P(-),H(+),D(21 hrs),L(3 hrs)	2 +/- 2
7	P(+),H(+),D(24 hrs),L(-)	14 +/- 5
8	P(+),H(+),D(-),L(24 hrs)	81 +/- 10

damaged by the heat stress (48 C, 5 min) as studied by measuring electron transport activity from water to DCPIP (Table 1). A slight protection appeared to occur under preadaptation conditions regardless of a dark incubation recovery period (see samples 3 and 7). There was some ambiguity in these results because of the large standard deviation; however, some recovery may have occurred during the isolation of the chloroplasts as a result of dim room light (see Figure 7). Importantly, 80% of the control activity was recovered by incubation in the light of the preadapted, heat-stressed sample (see sample 8 in Table 1). This later result can be interpreted as requiring both preadaptation and light incubation for the recovery process to occur. Recovery did not occur in heat-stressed samples that were either not preadapted or not incubated in the light during the recovery period.

The requirements for recovery were also studied with the use of in vivo fluorescence transients (Figure 5). The characteristic in vivo chlorophyll fluorescence induction curves (Kautsky effect (16-18)) of dark adapted higher plants and algal cells have been utilized as a reflection of the photochemical and physiological state of the photosynthetic apparatus (see Figure 5a for a definition of the stages of the fluorescence transient). Changes in the "P" level have usually been considered to be associated with the oxidation state of Q through changes in electron transport activity from photosystem II to photosystem I.

Figure 5. Comparison of fluorescence transients. Plants were processed through various permutations of the working conditions and control conditions to determine what conditions were necessary for recovery. The working conditions were 39 C and 3 hours for preadaptation and 48 C and 5 minutes for heat stress. Light and dark incubations were carried out for 24 hours. Fluorescence transients were taken in vivo. a) control: P(-),H(-),D(-),L(-). b) unpreadapted and heat stressed: P(-),H(+),D(-),L(-). c) unpreadapted, heat stressed, and incubated in the dark: P(-),H(+),D(+),L(-). d) unpreadapted, heat stressed, and incubated in the light: P(-),H(+),D(-),L(+). e) preadapted control: P(+),H(-),D(-),L(-). f) preadapted and heat stressed: P(+),H(+),D(-),L(-). g) preadapted, heat stressed, and incubated in the dark: P(+),H(+),D(+),L(-). h) preadapted, heat stressed, and incubated in the light: P(+),H(+),D(-),L(+). i) preadapted control, incubated in the dark: P(+),H(-),D(+),L(-). j) preadapted control, incubated in the light: P(+),H(-),D(-),L(+). (F = fluorescence in relative units, O = original level, I = intermediary peak, D = dip, P = peak, S = quasi steady-state).


a) P(-),H(-),D(-),L(-)
P
S
F
I
O
D
0
time (sec)
8
b) P(-),H(+),D(-),L(-)
F
0
time (sec)
8
c) P(-),H(+),D(+),L(-)
F
0
time (sec)
8
d) P(-),H(+),D(-),L(+)
F
0
time (sec)
8
e) P(+),H(-),D(-),L(-)
F
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f) P(+),H(+),D(-),L(-)
F
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time (sec)
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g) P(+),H(+),D(+),L(-)
F
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time (sec)
8
h) P(+),H(+),D(-),L(+)
F
0
time (sec)
8
i) P(+),H(-),D(+),L(-)
F
0
time (sec)
8
j) P(+),H(-),D(-),L(+)
F
0
time (sec)
8

Figure 5

Loss of the "P" peak may correspond to loss of activity on the oxidizing side of photosystem II either at the water-splitting complex or at other electron donor complexes interacting with the reaction center of photosystem II, P_{680} (46). Figure 5b showed a dramatic loss in the "variable" component of fluorescence upon heat stress (48 C, 5 min), which indicated that the oxidizing side of photosystem II was affected. The heat-stressed plants were incubated in the dark at room temperature for 24 hours; the variable component of fluorescence was partially restored after this treatment (Figure 5c). An explanation of this result may be that the donor side of photosystem II had partially recovered. The transient did not return to a full amplitude of fluorescence, such as can be demonstrated by measurements of control leaves in the presence of DCMU (Figure 6). The earlier results, shown in Table 1, indicated that the recovery was not due to electron flow from water; the water-splitting complex was still "inactivated". In another experiment, the heat-stressed plants were incubated in the light at 21 C for 24 hours. Complete absence of the variable component (Figure 5d) suggested photodegradation of the photosynthetic apparatus (compare to Figures 5b and 5c, see also Table 1). This was also apparent by visual examination of the native plants under similar conditions (Figure 7). Preadaptation had previously been shown to be beneficial for recovery (Figures 2 and 3). A fluorescence transient was obtained

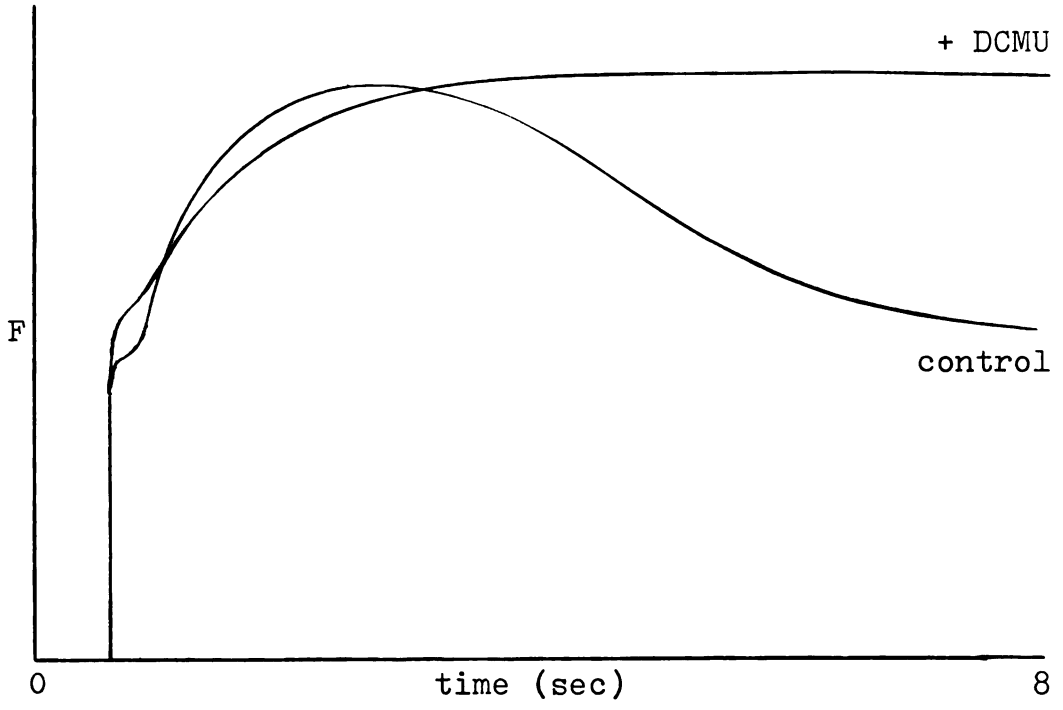

Figure 6. DCMU effect on <u>in vivo</u> fluorescence transient. Fluorescence transients were taken <u>in vivo</u> of control plants: P(-),H(-),D(-),L(-) (control) and of control plants which had had their leaves submerged for 2 minutes in 5 uM DCMU (+ DCMU). (F = fluorescence in relative units).

from plants which had been preadapted before heat stress and then incubated in the dark (Figure 5g). The results indicated that damage on the oxidizing side of photosystem II had taken place, since the variable component of fluorescence had been decreased. In contrast to the plants maintained in the dark, the fluorescence transient of plants which had been preadapted, heat stressed, and then allowed to recover in the light for 24 hours reverted to the control transient (see Figure 5h and compare to Figure 5g). This later result indicated almost complete recovery of the fluorescence transient of the stressed plant to a transient resembling that of the original control (Figure 5a). Visual in vivo examination of the plants was made under certain conditions (Figure 7). It was obvious that plants which had not undergone the preadaptation condition were drastically damaged when allowed to recover in the light (photodamage) (Figure 7a). This was confirmed by comparison with Figure 7b of plants which had been preadapted and then allowed to recover in the light. Full recovery following preadaptation conditions required light in the presence or absence of heat stress (see Figure 5h and Figure 5j).

Recovery kinetics vs length of dark incubation

Plants were preadapted at 39 C for 3 hours and then heat stressed at a slightly lower temperature than in the earlier experiments to follow initial recovery rates for various dark incubation times (46 C, 5 min). The degree of

Figure 7. Visual observation of recovery and photodestruction. a) Plants were heat stressed at 48 C for 5 minutes with no preadaptation and incubated in the light for 24 hours before making visual observations. b) Plants were preadapted at 39 C for 3 hours, heat stressed at 48 C for 5 minutes, and incubated in the light for 24 hours before making visual observations.

a) P(-),H(+),D(-),L(+)

b) P(+),H(+),D(-),L(+)

recovery was determined by measuring water to DCPIP rates. The results indicated an increase in recovery rate with longer periods of dark incubation (Figure 8). The initial rate of recovery was increased to the same degree by both the 6 hour dark incubation and the 24 hour dark incubation; however, the rate decreased at an earlier time after a 6 hour dark incubation. The time course after a 6 hour dark incubation generally showed a plateau region which was not present in the other time courses; however, the curve was drawn to give a smooth increase.

Radioactive labelling

Plants labelled with 35S-methionine in the dark for 3 hours at 39 C (preadaptation temperature) were compared to plants labelled with 35S-methionine in the dark at 21 C (normal growth temperature) to check for changes in the protein synthesis pattern (Figure 9). No detectable differences were observed. The SDS-PAGE, 10-17.5% polyacrylamide, polypeptide pattern of unpreadapted, heat-stressed plants was compared to that of control plants (Figure 10, lanes B and C). Again, no detectable differences were observed. Plants that had been preadapted at 39 C for 3 hours and heat stressed at 48 C for 5 minutes were then labelled with 35S-methionine either in the light or dark (Figure 10, lanes G and K). These samples were compared with samples that were not preadapted but were still heat stressed and labelled with 35S-methionine either in the light or the dark (Figure 10, lanes E and I).

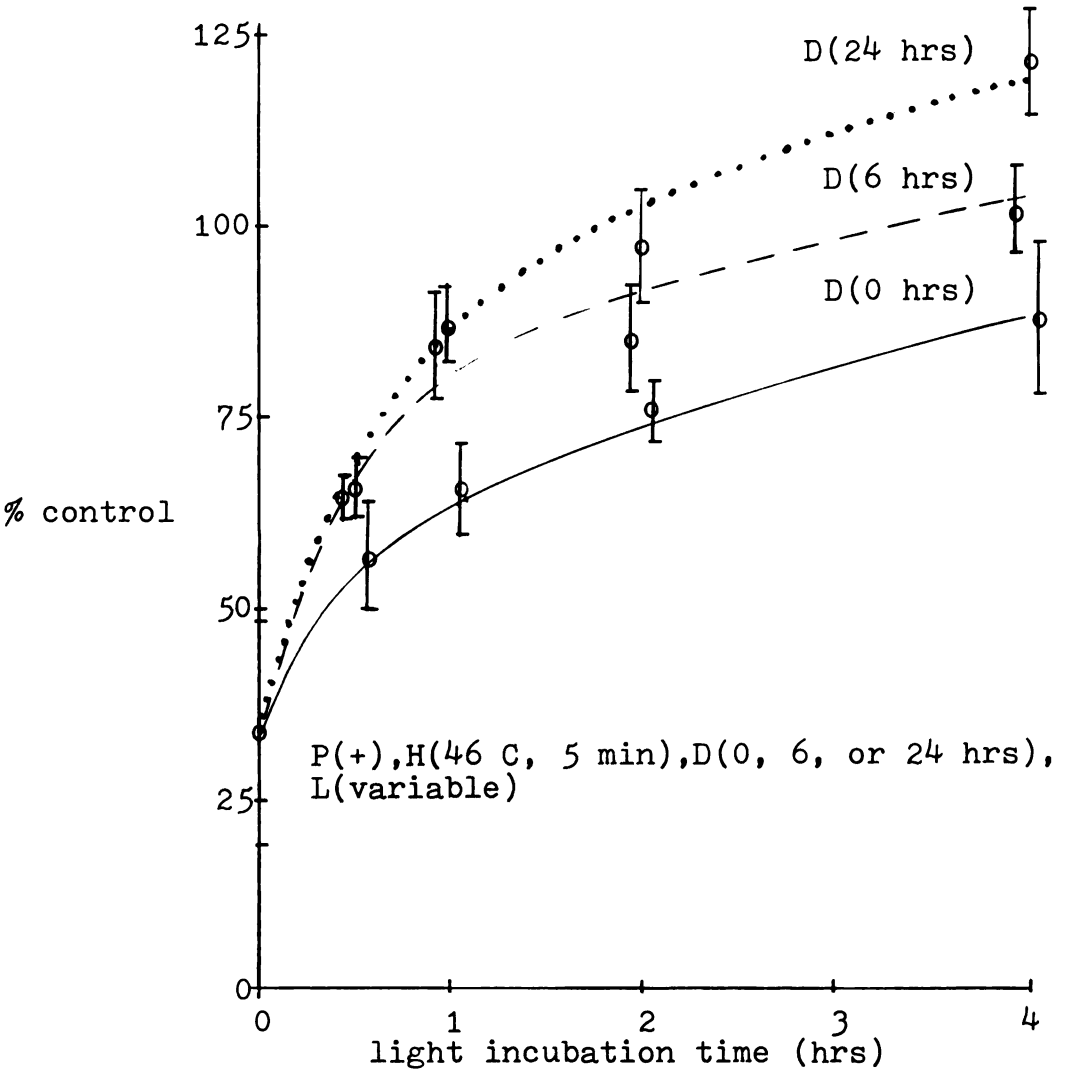

Figure 8. Recovery kinetics for various dark incubation times. Plants were preadapted at 39 C for 3 hours, heat stressed at 46 C for 5 minutes, and incubated in the dark for either 0 (——), 6 (— —), or 24 hrs (• • •), and incubated in the light for various amounts of time to produce three time courses showing the kinetics of recovery: P(+),H(46 C, 5 min),D(0, 6, or 24 hrs),L(variable). Water to DCPIP assays were performed on the samples after isolation of thylakoid membranes. Values are presented as a percentage of the control activity: P(-),H(-),D(-),L(-). The average control activities were 130, 129, and 135 umoles DCPIP reduced/mg chl/hr for the 0 hour, 6 hour, and 24 hour dark incubations respectively. Data points represent an average from three samples taken on three separate days. The error bars show the standard deviation for each data point.

Figure 9. Polypeptide pattern and 35S-methionine incorporation during preadaptation. 35S-methionine was incorporated into plants *in vivo* during preadaptation at 39 C for 3 hours in the dark and during incubation at 21 C for 3 hours in the dark. Aliquots containing 15 ug of chlorophyll were loaded from each sample into separate wells for SDS-PAGE. Lane A shows molecular weight standards: bovine serum albumin (MW 68 kd), ovalbumin (MW 45 kd), human erythrocyte carbonic anhydrase b (MW 29 kd), and horse heart cytochrome c (MW 12 kd). Lane B shows the polypeptide pattern for control plants. Lane C shows the polypeptide pattern for preadapted plants. Lane D shows the *in vivo* incorporation of 35S-methionine during dark incubation at 21 C for 3 hours. Lane E shows the *in vivo* incorporation of 35S-methionine during dark incubation at 39 C for 3 hours. The autoradiogram was developed for 7 days.

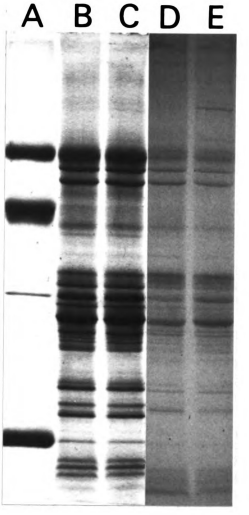

Figure 9

Figure 10. Polypeptide patterns and 35S-methionine incorporation during dark and light incubations. Thylakoid membranes were isolated from control and heat-stressed plants and aliquots containing 15 ug of chlorophyll were loaded into separate wells for SDS-PAGE. Lane A shows molecular weight standards (see Figure 9). Lane B shows the polypeptide pattern for control plants: P(-),H(-),D(-), L(-). Lane C shows the polypeptide pattern for heat-stressed plants: P(-),H(+),D(-),L(-). 35S-methionine was incorporated in vivo into plants during dark and light incubation periods for 3 hours. Lane D shows the incorporation pattern for unpreadapted control plants during dark incubation: P(-),H(-),D(3 hrs),L(-). Lane E shows the incorporation pattern for unpreadapted heat-stressed plants during dark incubation: P(-),H(+),D(3 hrs),L(-). Lane F shows the incorporation pattern for preadapted control plants during dark incubation: P(+), H(-),D(3 hrs),L(-). Lane G shows the incorporation pattern for preadapted heat-stressed plants during dark incubation: P(+),H(+),D(3 hrs),L(-). Lane H shows the incorporation pattern for unpreadapted control plants during light incubation: P(-),H(-),D(-),L(3 hrs). Lane I shows the incorporation pattern for unpreadapted heat-stressed plants during light incubation: P(-),H(+),D(-),L(3 hrs). Lane J shows the incorporation pattern for preadapted control plants during light incubation: P(+),H(-),D(-),L(3 hrs). Lane K shows the incorporation pattern for preadapted heat-stressed plants during light incubation: P(+),H(+),D(-), L(3 hrs). Arrows indicate proteins which incorporated greater amounts of 35S-methionine compared to control (lane K). Autoradiograms were developed for 7 days.

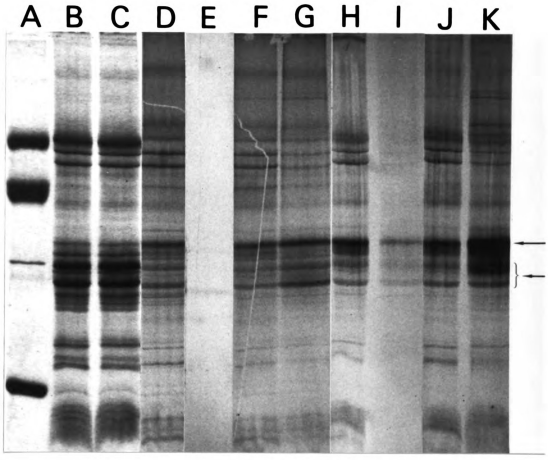

Figure 10

Both preadapted and unpreadapted plants that were not heat stressed were labelled in a similar manner to provide controls (Figure 10, lanes D, F, H, and J). The unpreadapted, heat-stressed samples showed an almost complete loss of chloroplast protein synthesis both in dark and light incubation studies (Figure 10, lanes E and I). The preadapted, heat-stressed samples showed normal levels of protein synthesis both in the light and the dark incubation studies (Figure 10, lanes G and K), but with increased synthesis of several polypeptides, especially in the sample which was labelled in the light (Figure 10, lane K). A band in the 32 kilodalton region as well as bands in the 25-27 kilodalton region appeared to have been synthesized at higher levels than normal. A 32 kilodalton protein responded to SDS-PAGE in the presence of urea in a fashion similar to that of the herbicide-binding protein of the same molecular weight (data not shown); it migrated to an apparent molecular weight of 30 kilodaltons (17). The polypeptides in the 25-27 kilodalton size class are the light-harvesting chlorophyll a/b-protein complex.

DISCUSSION

It has already been shown that heat stress causes damage to the photosynthetic apparatus by dissociating the light-harvesting chlorophyll a/b-protein complex from the photosystem II complex (29-31) and by inactivating the water-splitting enzyme, which requires manganese for its activity (32-38). Further analysis showed that this damage involves changes in the lipid composition of the photosynthetic membrane (38, 39) and release of membrane proteins (40). These findings would suggest that repair of the damaged photosynthetic membrane would require synthesis of new protein or re-processing (assembly). It has been shown, however, that damage to the photosynthetic apparatus is essentially irreversible, with recovery occurring over a period of weeks or months (8, 33, 40). An additional factor is therefore required in order for recovery to occur, since protein synthesis would be expected to repair the damage in a much shorter time span.

In addition to causing damage to the photosynthetic apparatus, heat stress also causes changes in the protein synthesis patterns of plant cells. It has been shown that at a temperature of 40 C, many higher plants start producing greater amounts of a few select proteins known as

heat shock proteins. This has been seen in tobacco (51), soybean (52), and maize (53). At even higher temperatures (45 C), general protein synthesis is inhibited. If the increase to higher temperatures is gradual or in stages, the inhibition of protein synthesis occurs at a higher temperature (49 C) than if the increase is sudden (52). A gradual increase to high temperatures or a pretreatment at a medium temperature thus provides a protection of the protein synthesis machinery against damage by the high temperature stress. The irreversibility of the damage to the photosynthetic apparatus caused by heat stress may possibly be explained by the lack of preadaptation conditions (see results in Figure 3 and Figure 4). Bauer and Senser (33) did, in fact, find that ivy plants recovered four times faster than control plants when they were hardened by a severe heat stress 20 days prior to the experimental heat stress. Even this was not consistent with the kinetics expected from a repair involving only protein synthesis, since the hastened recovery still took several days to one week to occur.

In a similar case involving the loss of water-splitting activity upon heat treatment of a temperature sensitive mutant of Chlorella pyrenoidosa, Lavintman et al. (54) found that recovery of activity required synthesis of new protein and that the recovery would not occur in the absence of light. They concluded that light was necessary for either the synthesis or assembly of the new protein.

In a series of studies using Tris-washed chloroplasts, Yamashita and Tomita (55-57) showed that the loss of water-splitting activity in Tris-washed chloroplasts could be reversed by a dark incubation with DCPIP followed by a light incubation with manganese. These studies showed that the requirement of light during the reactivation could involve a light-dependent insertion of manganese. In comparing reactivation of Tris-washed chloroplasts to heat-treated chloroplasts, they found that heat-treated chloroplasts could not be reactivated by an incubation in the light with manganese (58). This would be expected if recovery from heat stress involves protein synthesis in addition to manganese insertion; however, the results with Tris-washed chloroplasts can be interpreted to indicate the possibility that the light requirement during repair of the water-splitting enzyme involves assembly of the enzyme-manganese complex rather than protein synthesis.

In the present study, maize seedlings were pretreated at elevated temperatures, as compared to the growth conditions, but at levels which were not themselves damaging (see Figure 5, compare a and e). This allowed us to study the kinetics of recovery from heat stress and correlate the changes with the kinetics of a repair mechanism(s) involving protein synthesis and assembly. The light requirements during recovery were also studied in order to determine whether light is required for protein synthesis or protein assembly.

Photosynthetic activity in maize was shown to be damaged in stages in a manner similar to that found in other higher plants. Photosystem II was shown (see Figure 2 and Table 1) to be the most susceptible component to damage by heat stress. A component on the oxidizing side of photosystem II was shown to be preferentially damaged at slightly higher temperatures (46 to 50 C in maize), as indicated by reversal of damage by the electron donor, DPC (see Figure 2). Photosystem I was shown to be much more resistant to heat stress, with no damage occurring below 50 C in maize (see results in Figure 2).

Unpreadapted plants were unable to recover from heat stress and, in fact, suffered further damage when exposed to the same light that caused recovery in preadapted plants (see Table 1, Figure 5, and Figure 7). Preadaptation did not protect plants from the actual damage caused by heat stress but did provide protection for a mechanism involved in recovery from heat stress (52, 59, 60). This mechanism could involve protein synthesis by the chloroplasts, perhaps to replace proteins that were inactivated by the heat stress. This would be in agreement with the findings of Altschuler *et al.* (52), who have seen a protection of ribosomes from damage by severe heat stress if the samples were brought up to the high temperature gradually or were subjected to a mild heat stress prior to the more severe heat stress. This would also be in agreement with the *in vivo* 35S-methionine incorporation experiments which showed

an absence of protein synthesis in unpreadapted plants after heat stress (Figure 10, lanes E and I). The protection offered by preadaptation increases with increase in temperature and time of the preadaptation, but the damaging effects of heat stress start competing with the protective effects at higher temperatures (see Figure 3). It appears that the protection would reach a maximum level some time at or after 3 hours of preadaptation.

An incubation in the light after heat stress was shown to be necessary for recovery (see Table 1 and Figure 5). The light was not necessary for protein synthesis (see Figure 10) and was therefore required for some other aspect of the recovery mechanism. This can be seen from the ^{35}S-methionine incorporation studies which showed protein synthesis occurring in the dark as well as in the light in recovering plants (see Figure 10, lanes G and K). In addition, the kinetics of recovery showed a higher initial rate of recovery in plants that were dark incubated prior to the light incubation (see Figure 8). This would indicate a recovery of potential activity in the dark, possibly by protein synthesis. The activation of the plants would then depend on a light dependent activation of new protein, possibly the insertion of manganese into a protein involved in the oxidizing side of photosystem II. This would be in agreement with the results of Yamashita and Tomita (56, 57), which indicated that reactivation of photosystem II activity in Tris-washed heat-damaged chloroplasts in vitro

could be accomplished by incorporation of endogenous manganese in the light. If plants were given a 6 hour dark incubation prior to light incubation, the kinetics of recovery indicated a possible state in which some of the protein necessary for recovery had been synthesized in the dark but not enough for a full recovery (Figure 8). A transition zone appeared in this case which would represent the transition from the kinetics encountered with a pool of synthesized protein and the kinetics encoutered with no such protein pool. This can be interpreted to indicate a faster rate for the activation step than for the protein synthesis step.

Preadaptation did not appear to cause any changes in the protein synthesis pattern of the plants (see Figure 9). Protection could therefore not be attributed to synthesis of heat shock proteins responsible for the protection mechanism, although this proposal could not yet be completely excluded (52). The incorporation patterns after heat stress and during recovery, however, did show significant changes in protein synthesis. Unpreadapted plants lost the ability to synthesize new proteins after heat stress (Figure 10, lanes E and I). Recovery patterns in the light or dark incubations showed full protein synthesis activity with possibly the increased production of several particular proteins in the 32 kd and 25-27 kd size class (Figure 10, lanes G and K). The increased synthesis of a protein in the 32 kd range could indicate a

need to recover activity of the quinone-binding secondary stable electron acceptor of photosystem II (B-binding, herbicide-binding protein). The increased synthesis of a protein in the 25-27 kd range could indicate a need to recover activity of the light-harvesting chlorophyll a/b-protein complex. The photosynthetic parameters studied showed that heat stress damaged the oxidizing side of photosystem II (see Figure 2); however, no increase was seen in the synthesis of proteins usually associated with the oxidizing side of photosystem II (61). The incorporation patterns would not necessarily be sensitive enough to pick up changes in protein synthesis for these proteins. The data nevertheless supported the concept of a protection of the protein synthesis machinery by preadaptation, a synthesis of new protein needed for recovery during both the dark and the light incubations, and the activation of those proteins during the light incubation.

Damage of the oxidizing side of photosystem II by heat stress has previously been considered an irreversible process (8, 33, 40); however, a procedure has been devised in this study which allowed for repair of the damage to photosystem II *in vivo*. This procedure involved preadaptation of the plants by a mild heat stress which, in itself, did not cause significant damage but did allow protection of the machinery necessary for recovery (52, 59, 60). Recovery appeared to require protein synthesis and light. It is concluded that heat stress causes

inactivation of several photosystem II proteins, that preadaptation protects the protein synthesis machinery from damage during severe heat stress, that dark or light incubation allows synthesis of new proteins, and that light incubation may be involved in the activation of the newly synthesized proteins (Figure 11). This process could explain the ability of maize and other heat resistant crops to survive the gradual climb to potentially damaging temperatures during extreme heat waves.

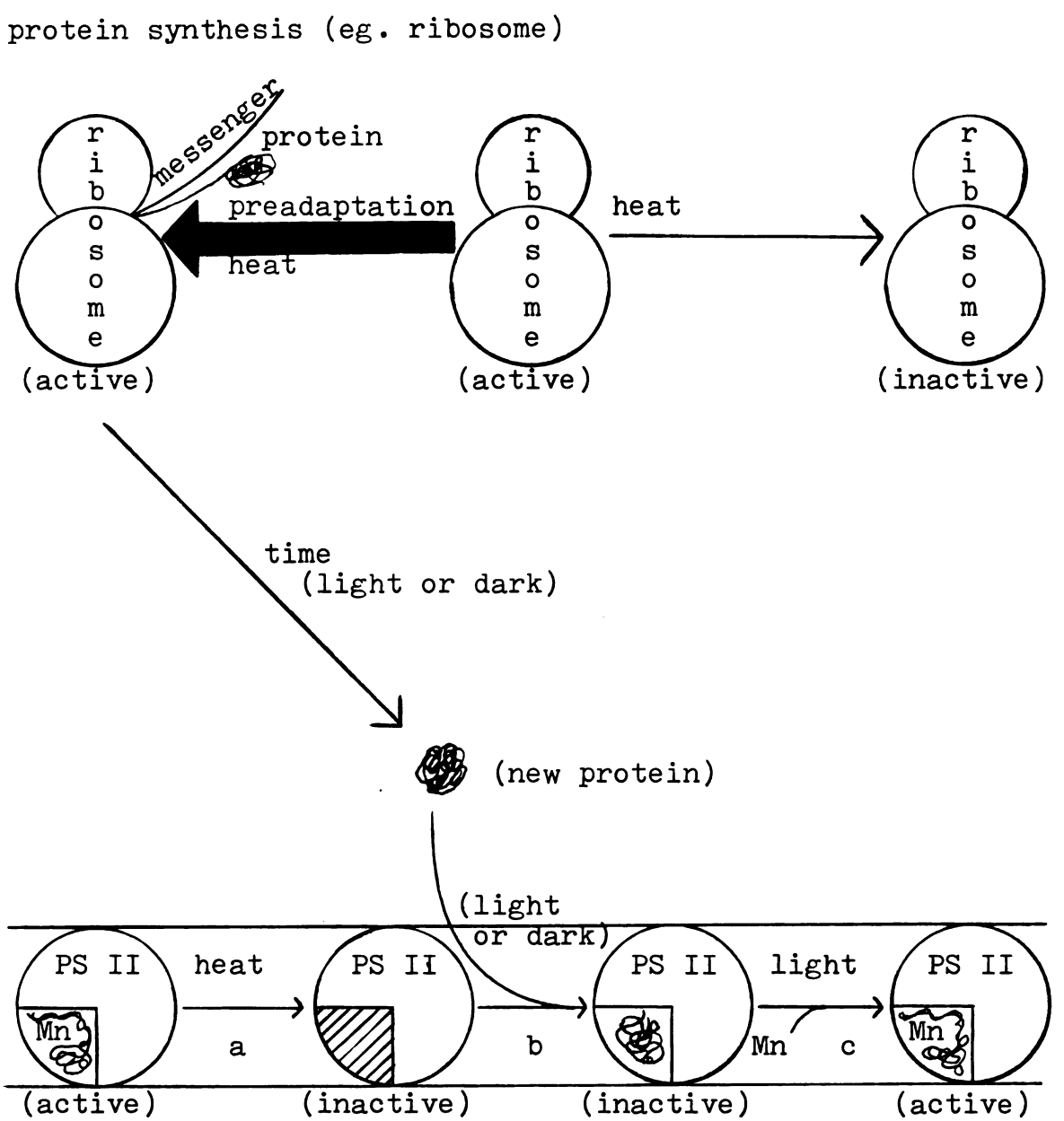

Figure 11. Model of recovery process. The response of unpreadapted and preadapted maize seedlings to heat stress can be shown by a model indicating the effects of conditions on protein synthesis and processing. The ribosome is assumed to be undamaged by heat stress if preadaptation conditions are followed (bold arrow). Without preadaptation, the ribosome is assumed to be damaged by heat stress. (a = loss of protein(s) from photosystem II, indicated by crosshatching, b = insertion of protein into photosystem II, c = activation and manganese incorporation into photosystem II, PS II = photosystem II).

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