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PHOTOSYNTHETIC APPARATUS IN CHILLING-SENSITIVE PLANTS

VII. COMPARISON OF THE EFFECT OF GALACTOLIPASE TREATMENT OF CHLOROPLASTS AND COLD-DARK STORAGE OF LEAVES ON PHOTOSYNTHETIC ELECTRON FLOW *

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Summary

1. Both galactolipase treatment of tomato chloroplasts and the cold and dark storage of leaves induce a large degradation of chloroplast monogalactosyl diacylglycerol and digalactosyl diacylglycerol as well as an accumulation of free fatty acids accompanied by the inhibition of Hill reaction activity with water as electron donor. All these changes are reversed upon illumination of the leaves.

2. Inhibition of diphenylcarbazide (DPC) → dichlorophenolindophenol (DCIP) activity by free fatty acids released following galactolipase treatment of chloroplasts isolated from either fresh or cold and dark-stored and illuminated leaves is almost completely reversed by either bovine serum albumin or Mn^{2+}, while that in chloroplasts from the cold and dark-stored leaves is reversed by bovine serum albumin and Mn^{2+} only up to about 60 and 25%, respectively.

3. Fatty acids released during the treatment of chloroplasts with galactolipase affect the electron transport mainly in the same site as exogenous

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Abbreviations and symbols: PS I (II), Photosystem I (II); Hepes, 2-4-(2-hydroxyethylpiperazine)-2-ethanesulphonic acid; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, 1,5-diphenylcarbazide; Chl, chlorophyll; cold and dark-stored and illuminated leaves have been illuminated at 25°C (8000 lux) for 2 h following cold and dark storage for 3 days; cold and dark-stored leaves have been stored at 0°C in the dark for 3 days.

unsaturated fatty acids do, while those released due to endogenous galactolipase activity appear to affect also in the region damaged by either Tris washing of chloroplasts or the cold and dark treatment of leaves.

4. The loss of manganese from chloroplasts (Kaniuga, Z., Ząbek, J. and Sochanowicz, B. (1978) Planta 144, 49—56) seems to be the main reason of cold and dark-induced inactivation of Hill reaction activity in chloroplasts of chilling-sensitive plants, while both the degradation of galactolipids and the accumulation of fatty acids are of secondary importance.

Introduction

Inhibition of the Hill reaction of Photosystem II due to the treatment of chloroplasts with galactolipase has been observed in spinach chloroplasts [1—3]. This inhibition has been largely prevented by the presence of high concentration of defatted bovine serum albumin. It has been postulated that if precautions are taken to prevent the binding of fatty acids to chloroplasts, large amounts of galactolipids might be removed without any marked effect on the electron flow [1,3].

In a previous paper of this series [4] it has been shown that the cold and dark storage of detached tomato leaves results in a release of endogenous polyunsaturated fatty acids from chloroplast membranes, accompanied by a decrease of Hill reaction activity. Inhibition of the electron flow in chloroplast from fresh and cold and dark-stored and illuminated leaves is reversed by bovine serum albumin [4]. In contrast, in chloroplasts isolated from cold and dark-stored leaves the addition of bovine serum albumin to the reaction mixture is not sufficient to restore Hill reaction activity [4], probably due to the loss of about 40—50% of total Mn content [5].

In the present paper the effects of both, cold and dark storage of leaves and the galactolipase treatment of isolated chloroplasts on electron flow were compared in order to elucidate whether the Hill reaction activity is more affected by the loss of manganese or by a degradation of galactolipids and a release of fatty acids. Preliminary results of these studies have been reported [6].

Materials and Methods

Plant material. Leaves of tomato (*Lycopersicon esculentum* Mill. var. Eurocross and Revermoon) were harvested from the plant grown under greenhouse conditions used for commercial purpose. Leaves of bean (*Phaseolus vulgaris* L. var. Piekny Jaś) were harvested usually between the 12th and 16th day after planting the bean seeds. Conditions of both seed germination and growing of the bean plant have been described in detail in the previous paper [7].

Cold and dark storage of leaves, reactivation with light, isolation of chloroplasts and determination of photochemical activities. Procedures for all these treatments are described in the previous paper [7]. Photosystem I activity of isolated chloroplasts was determined by photoreduction of $NADP^+$ at 340 nm,

following 30 s illumination (20 000 lux) of the 3-ml reaction mixture, containing: 0.4 M sucrose, 15 mM NaCl, 3 mM $MgCl_2$, 20 mM Hepes buffer (pH 7.0), 0.5 mM ascorbate, 50 μM DCIP, 200 μM $NADP^+$, saturating amount of spinach ferredoxin (10 μM), 10 μM DCMU and chloroplast suspension corresponding to about 50 μg of chlorophyll.

Extraction of chloroplast lipids. Chloroplast suspension (about 5 mg of chlorophyll) was heated with 10 ml isopropanol at 40° C for 3—5 min and extracted according to the classical method of Folch et al. [8] as modified by Sastry and Kates [9].

Separation and determination of fatty acid content. Free fatty acids were estimated in lipid extracts of untreated and galactolipase-treated chloroplasts. Procedures for both separation and determination of free fatty acids content with Rhodamine 6G have been described previously [4].

Determination of glycolipids. Individual lipids were obtained directly by thin-layer chromatography of total lipid extracts [10]. Acetone or acetone/acetic acid/water (100 : 2 : 1, v/v) was effective for separation of monogalactosyl diacylglycerol, digalactosyl diacylglycerol and sulpholipid [11] from phospholipids and neutral lipids. These solvents were applied following prior thin-layer chromatography procedure using chloroform/methanol/water (65 : 25 : 4, v/v) for preliminary removal of pigments and neutral lipids. Glycolipids were detected by spraying the plates with either 10% phenol in H_2SO_4 (1 : 1, v/v) or with 1% α-naphthol in H_2SO_4 (1 : 1, v/v) following heating.

For quantitative analysis galactolipids and sulpholipid were identified in iodine vapour and estimated by determination of galactose in the eluate following elution of spots from the chromatograms [12]. Factors 4.3 and 2.6 were used to convert galactose to monogalactosyl- and digalactosyl-diacylglycerol, respectively [13]. Amounts of lipids were converted to mg of chlorophyll, using the following calculated molecular weights: monogalactosyl diacylglycerol = 787, digalactosyl diacylglycerol = 947, sulpholipid = 837.

The fraction of total chloroplast phospholipids was separated by thin-layer chromatography, and following elution of the identified spots and ashing of phospholipids, phosphorus was determined by the method Fiske and Subba-Row [14]. Phosphatidylcholine and phosphatidylcholine-freed rabbit brain phospholipids were separated from the Folch brain lipid extract [8] by thin-layer chromatography with chloroform/methanol/water (65 : 25 : 4, v/v). Individual phospholipids were detected on the plates by spraying with either Rhodamine 6G or molybdate [15].

Galactolipase preparation

Bean leaf galactolipase. The protein fraction containing galactolipase was obtained from fresh bean leaves following their storage at 4° C in the dark for 24—48 h to remove starch. All procedures for partial purification of the enzyme were performed according to Anderson et al. [1]. The ammonium sulphate fraction (so-called galactolipase) was applied for experiments. The activity of galactolipase was assayed by determination of fatty acids released from chloroplast fragment (equivalent to 50 μg of chlorophyll) obtained by disruption of chloroplasts in isotonic solution in a Potter-Elvejhem homogenizer with a Teflon pestle [16], and the activity was approximately 1.0

(1.08—1.20) μmol fatty acid released/min per mg protein at 25° C and pH 7.0. The specificity of this enzyme was also estimated for other substrates: monogalactosyl diacylglycerol, phosphatidylcholine, glycerylpalmitate, glycerylstearate and phosphatidylcholine-freed rabbit brain phospholipids. Lipids dispersed in Triton X-100 (0.1% final concentration) were used as substrates. The activity was observed only for monogalactosyl diacylglycerol (100%) and phosphatidyl choline (15%) of that measured with monogalactosyl diacylglycerol. Galactolipase was free of proteolytic activity.

Tomato leaf galactolipase. The galactolipase containing crude extract from the chloroplast fraction of tomato leaves was obtained as described elsewhere for bean galactolipase [1]. The chloroplast fraction was obtained following the centrifugation at 3500 × *g* of the filtered leaf homogenate. Thus, it was a mixture of class I and II chloroplasts as well as of chloroplast fragments. The activity of this enzyme with chloroplast fragments as substrate, was assayed by the same method as bean galactolipase [16]. The specificity of this enzyme was tested with the same substrates as for bean galactolipase and the activity with monogalactosyl diacylglycerol, phosphatidylcholine and glycerylstearate of 100, 18 and 3%, respectively, was found.

Galactolipase treatment of chloroplasts

In all experiments class II chloroplasts from fresh, cold and dark-stored, and cold and dark-stored and illuminated tomato leaves with very low endogenous galactolipase activity (see legend to Fig. 1) were used. Chloroplasts were incubated with the indicated amount of bean galactolipase in a reaction mixture containing the same components as described for photochemical activity determination, except electron donors and acceptors. Incubation was performed at 25° C in the dark at pH 7.0 and then both fatty acid released and galactolipid contents were estimated as described above.

Other methods

Chlorophylls *a* and *b* were assayed by the method of Arnon [17]. Protein content was estimated by the method of Lowry et al. [18]; manganese was determined as described previously [5].

Reagents

Hepes buffer and spinach ferredoxin were purchased from Sigma. Crystallized, defatted bovine serum albumin and Triton X-100 were obtained from BDH Chemicals. DPC was purchased from Eastman and recrystallized from a water/methanol mixture. Glycerylpalmitate and glycerylstearate were from Analabs. The other substrates of galactolipases were separated from fresh lipid extracts (see above). Rhodamine 6G was obtained from Hopkin-Williams. The other chemicals, of analytical grade, were from P.O.Ch. Poland, except $NADP^+$ which was from Reanal, Hungary.

Results

Changes in chloroplast galactolipid content following cold and dark storage of leaves and their illumination

As is shown in Table I the storage of young tomato leaves at 0° C in the dark

TABLE I

EFFECTS OF DEVELOPMENT, STORAGE AND ILLUMINATION OF TOMATO LEAVES ON THE EXTENT OF CHANGES IN CHLOROPLAST GLYCOLIPID CONTENT AND HILL REACTION ACTIVITY

Values of the representative experiment as well as the extremal values found in the indicated number of experiments (in parentheses) performed are included. MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol.

Source of chloroplasts	Hill reaction activity (μmol DCIP reduced/h per mg chl.)	Glycolipids (μmol per mg chlorophyll)			MGDG/DGDG ratio
		MGDG	DGDG	Sulpholipid	
Young leaves					
Fresh	116	7.20 (7.05—8.45) (6)	2.40 (2.30—2.70) (6)	0.70 (0.65—0.75) (3)	3.00
Stored	5	4.70 (4.60—5.50) (5)	1.70 (1.70—1.85) (5)	0.65 (0.64—0.55) (3)	2.75
Stored and illuminated	85	6.10 (6.00—6.35) (5)	2.00 (1.90—2.15) (4)	0.65 (0.60—0.65) (3)	3.05
Expanded leaves					
Fresh	36	8.70 (8.50—9.05) (3)	2.70 (2.60—2.70) (3)	—	3.20
Stored	28	8.10 (7.90—8.40) (3)	2.60 (2.60—2.70) (3)	—	3.10
Stored and illuminated	44	10.20 (9.40—11.30) (3)	2.80 (2.70—2.80) (3)	—	3.65

results in a marked decrease (35% of control) of the chloroplast galactolipid content, whereas following storage of expanded leaves the content of chloroplast galactolipids is reduced by only 5% of control. Upon illumination of cold and dark-stored leaves the level of galactolipids increased in chloroplasts from young and expanded leaves to about 85—90% and 115—120%, respectively. The monogalactosyl/digalactosyl diacylglycerol ratios indicate that monogalactosyl diacylglycerol is somewhat more rapidly degradated and resynthetized following cold and dark storage and illumination of leaves, respectively. Sulpholipid content in chloroplasts from young leaves accounts for only about 8% of the sum of galactolipids. The changes in sulpholipid content (equal to about 0.05 μmol free fatty acid per mg chlorophyll) are too small to be regarded as an important source of fatty acids released during cold and dark treatment of leaves. The data of Table I indicate also that loss of galactolipid content is accompanied by an inhibition of Hill reaction activity especially in chloroplasts from young leaves. Illumination of the cold and dark-stored leaves restores both galactolipid content and Hill reaction activity. All these changes are much smaller in chloroplasts from expanded leaves.

Effect of galactolipase treatment of chloroplasts on galactolipid content and photochemical activities

Tomato chloroplasts like spinach chloroplasts [9,19] exhibit very low endogenous lipolytic activity (about 0.02 μmol fatty acid released/min per mg chlorophyll). This feature makes tomato chloroplasts suitable for studies

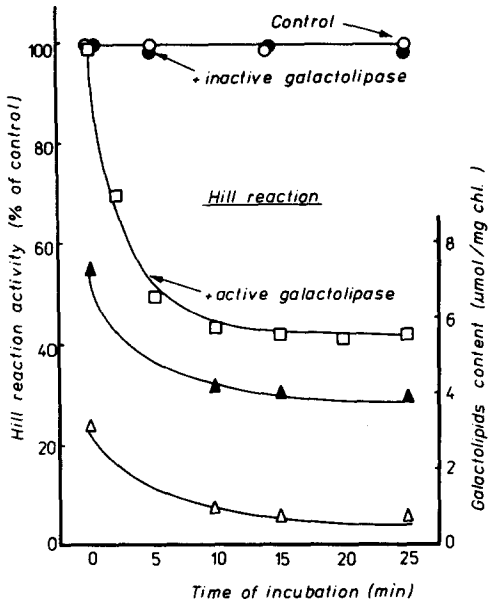

Fig. 1. Changes in galactolipid content and Hill reaction activity, following treatment of chloroplasts from fresh tomato leaves with bean galactolipase. Hill reaction activity in the presence of galactolipase was measured following the incubation of chloroplasts (50 μg chlorophyll) with the enzyme (2.5 μg protein per μg chlorophyll) for the time indicated and under conditions described in Methods. Then DCIP (50 μM) was added and its reduction was measured at 620 nm following the illumination of chloroplast suspension for 30 s. Incubation of class II chloroplasts at 25°C for 25 min in the absence of enzyme resulted in a nonsignificant decrease in galactolipid content. The activity of endogenous lipolytic enzymes of class II chloroplasts was approximately 0.5 μmol fatty acid released during 25 min of incubation per mg chlorophyll. Inactive bean galactolipase was obtained by heating at 80°C for 5 min. The activity of exogenous bean galactolipase was equal to 1.08 μmol fatty acid liberated/min per mg protein, as measured with chloroplast fragments as substrate (see Methods). Hill reaction activity of the control was 65 μmol DCIP reduced/h per mg chlorophyll. Chloroplast glycolipids were estimated after incubation with galactolipase under the same conditions as these applied for the photochemical reaction, but the amount of chloroplasts equivalent to 5 mg chlorophyll was used. ▲, monogalactosyl diacylglycerol; △, digalactosyl diacylglycerol.

of degradation of endogenous chloroplast galactolipids by exogenous galactolipase. Incubation of chloroplast preparation from fresh tomato leaves with galactolipase from bean leaves (Fig. 1) results in a decrease of endogenous chloroplast galactolipids to about 50 and 60% of monogalactosyl diacylglycerol and digalactosyl diacylglycerol, respectively, accompanied by a decrease of Hill reaction activity being especially pronounced during the first 5 min of the incubation. Since galactolipase treatment under these conditions does not result in a release of Mn from thylakoid membranes (data not presented), the observed changes in Hill reaction activity seem to be mainly due to the inhibitory effect of released fatty acids.

Fig. 2 shows the effect of galactolipase treatment of chloroplasts isolated from fresh, cold and dark-stored, and stored and illuminated leaves on the activity of electron transport dependent on PS II and PS I. For measurement of PS II activity DPC was applied as electron donor since it was found that the cold and dark storage of leaves results in the inactivation of Hill reaction activity measured with water as electron donor [7]. It is evident that following

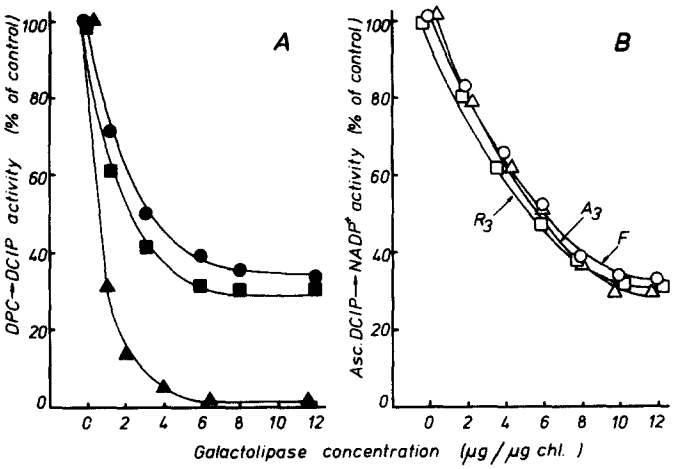

Fig. 2. Effect of bean galactolipase treatment of tomato chloroplasts on the activities of PS I and PS II. Chloroplasts were incubated with the indicated amount of enzyme at 25°C for 5 min, as described in Methods. After incubation the electron donors (300 µM DPC or ascorbate with 50 µM DCIP) and acceptors (DCIP or ferredoxin plus $NADP^+$) were added and then photoreduction of acceptors following the illumination of chloroplast suspension was determined. (A) Photosystem II activity of chloroplasts from fresh (●), cold and dark-stored (▲), and stored and illuminated (■) leaves was 120, 117 and 116 µmol DCIP reduced/h per mg chlorophyll, respectively as studied in the presence of DPC. (B) For Photosystem I activity-(ascorbate DCIP → $NADP^+$) the corresponding values were 65, 59 and 63 µmol $NADP^+$ reduced/h per mg chlorophyll, respectively, for fresh (○, F), cold and dark-stored (△, A_3), and cold and dark-stored and illuminated (□, R_3) leaves.

incubation of chloroplasts isolated from all three kinds of leaves with increasing amount of galactolipase a decline of both the PS II and PS I activity occurs. The PS II activity of chloroplasts isolated from leaves stored at 0° C in the dark (Fig. 2A) appeared to be the most sensitive to galactolipase treatment. The complete loss of this activity in chloroplasts of the stored leaves is observed in the presence of 5 µg galactolipase per µg chlorophyll, whereas in chloroplasts from fresh as well as from stored and illuminated leaves about 30% of the control activity is still found in the presence of 10—12 µg galactolipase per µg chlorophyll. Moreover, the rate of inactivation of PS II activity in chloroplasts from stored leaves at low galactolipase concentration (4 µg per µg chlorophyll) is much faster than in chloroplasts from both fresh as well as from stored and illuminated leaves. Since it is known that in chloroplasts from stored leaves the free fatty acid content is increased [4] both free fatty acids accumulated during the storage of leaves and those released during the treatment with exogenous galactolipase seem to be responsible for the extent and the rate of inactivation of PS II activity in these chloroplasts.

The sensitivity of the PS I activity to galactolipase treatment (Fig. 2B) is similar for all three kinds of chloroplast preparations. Incubation of chloroplasts with 10—12 µg galactolipase per µg chlorophyll results in a decline of activity by about 70% in all chloroplast preparations studied. It is interesting that the rate of inactivation of PS I activity is somewhat slower than that of the PS II activity, especially at low concentrations of galactolipase. A higher sensitivity of PS II activity than that of PS I to the galactolipase treatment seems to be due to the inhibitory action of free fatty acids on Hill reaction

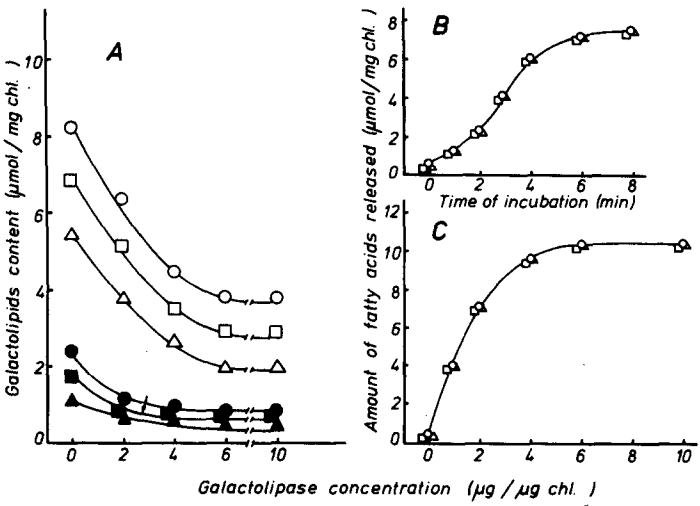

Fig. 3. Degradation of galactolipids (A) and release of fatty acids (B and C) following treatment of tomato chloroplasts with bean galactolipase. Conditions of chloroplast treatment with bean galactolipase and measurement of photochemical activity were the same as described in Methods and in the legend to Fig. 2, respectively. Fatty acid release was estimated following addition of 2 μg bean galactolipase per μg chlorophyll. ○ and ●, fresh leaves; □ and ■, cold and dark-stored leaves; △ and ▲, cold and dark-stored and illuminated leaves. Open symbols in (A) are for monogalactosyl diacylglycerol and black symbols digalactosyl diacylglycerol.

activity. PS I activity is known to be relatively resistant to the inhibition by free fatty acids [20,21]. Moreover, in heptan- or galactolipase-treated chloroplasts it can be unspecifically restored by addition of galactolipids [22].

Fig. 3 presents the changes in both galactolipid and fatty acid contents of galactolipase treated chloroplasts from fresh, cold and dark-stored, as well as stored and illuminated leaves. It is striking that in the presence of galactolipase the rates of both galactolipid (Fig. 3A) and the fatty acids release (Fig. 3B and C) are similar in chloroplasts of all three kinds of leaves studied.

Endogenous lipolytic activity of tomato leaf chloroplast fraction

Since the process of galactolipid degradation can be exerted by both temperature and darkness one might consider whether it results from an enhanced activity of the endogenous galactolipase or whether it is caused by inhibition of the light-dependent galactolipid synthesis in the darkness while the hydrolytic activity is being preserved. It was found that galactolipolytic activity of chloroplasts is not affected by the cold and dark storage of leaves. When measured at 25°C it ranges from 2.9 to 3.1 nmol fatty acid released/min per mg protein with chloroplast fragments used as substrate despite the treatment of leaves.

Although the activity of endogenous galactolipase in chloroplasts from tomato leaves is greatly reduced at about 4°C (to 0.15 μmol fatty acid released/min per mg protein) the storage of tomato leaves for 3 days results in an increase of the level of free fatty acids up to 5—6 μmol per mg chlorophyll (cf. Table IV) accompanied by a decrease of the total galactolipid content by about 3 μmol per mg chlorophyll (Table I). Illumination of leaves stimulates

the disappearance of free fatty acids (cf. Table IV) which can be reutilized for both synthesis of galactolipids (cf. Table I) and photoperoxidation [4]. Thus, it appears that during the cold and dark treatment of leaves the equilibrium between hydrolysis and synthesis of galactolipids in chloroplasts is greatly influenced primarily due to the lack of light which is known to be required for the lipid synthesis [23].

Effect of bovine serum albumin on electron transport in galactolipase-treated chloroplasts

Since inhibition of Hill reaction activity following the cold and dark treatment of leaves of chilling-sensitive plants is due to both an accumulation of free fatty acids [4] and depletion of chloroplast manganese [5], we have tried to separate effects of Mn depletion from those of lipid degradation and the inhibitory effect of free fatty acids on PS II activity. Thus, DPC was applied as electron donor for these studies since it donates electrons beyond the site affected by the cold and dark treatment of leaves [7]. Data shown in Table II indicate that bovine serum albumin added to the galactolipase-treated chloroplasts from either fresh or stored and illuminated leaves results in the complete restoration of DPC → DCIP activity, while it is restored only up to 60% in galactolipase-treated chloroplasts from cold and dark-stored leaves. This appears to be due to a more drastic damage of thylakoid membrane resulting from the action of both endogenous and exogenous galactolipases. In accordance with the observation made by Anderson et al. [1] bovine serum albumin is more efficient in protecting the electron transport against the inhibitory effect of free fatty acids when added before the treatment of chloroplasts with galactolipase since proteins of thylakoid membrane can also bind fatty acids.

Comparing the sensitivity of PS I and PS II activities of chloroplasts from fresh leaves to galactolipase treatment it is evident that the former one is much less affected by the galactolipase treatment than the latter one (Table II). Moreover, as concluded from the lack of restoration of PS I activity on the addition of albumin either prior or after galactolipase treatment, the inhibition by about 30% of PS I activity following the galactolipase treatment appears to be due to the degradation of membrane galactolipids and depletion of plastocyanin and not to accumulation of free fatty acids.

An incomplete restoration of Hill reaction activity by albumin in chloroplasts from the cold and dark-stored leaves (Table II) was probably due to a very drastic damage of thylakoid membrane induced by a large excess of galactolipase (10 μg per μg chlorophyll). Therefore, in experiments presented in Table III two levels of galactolipase were applied in order to control the hydrolysis of membrane galactolipids. DPC was used as the electron donor to avoid a deleterious effect of cold and dark treatment of leaves on electron transport at the site occupied by Mn. Following the incubation of chloroplasts with 5 μg galactolipase per μg chlorophyll DPC → DCIP activity is only 15% of control. The addition of as much as 800 μg albumin per μg chlorophyll restores this activity only to about 60% of control. However, when the chloroplasts were treated with 1 μg galactolipase per μg chlorophyll Hill reaction activity was slightly higher (about 35% of control) and only 400 μg albumin

TABLE II

EFFECT OF BOVINE SERUM ALBUMIN LIGHT-DEPENDENT PS I AND PS II ACTIVITIES OF GALACTOLIPASE-TREATED CHLOROPLASTS FROM FRESH, COLD AND DARK-STORED, AND STORED AND ILLUMINATED TOMATO LEAVES

Chloroplasts were incubated with galactolipase at 25°C for 5 min as described in the legend to Fig. 2. The activity of both Photosystems is expressed in μmol acceptor reduced/h per mg chlorophyll.

Conditions (components and sequence of additions)	Reaction measured						
	Photosystem II						Photosystem I Ascorbate DCIP → $NADP^+$
	H_2O → DCIP			DPC → DCIP			
	Fresh	Cold and dark-stored	Cold and dark-stored and illuminated	Fresh	Cold and dark-stored	Cold and dark-stored and illuminated	Fresh
None	58	14	48	114	110	118	65
+ enzyme *	17	2	13	41	14	40	38
+ enzyme + BSA **	48	7	37	97	59	96	40
+ BSA ** + enzyme	53	9	44	103	66	105	43
+ BSA **	55	14	46	108	106	110	63

* 10 μg galactolipase per μg chlorophyll was added.

** 400 μg bovine serum albumin per μg chlorophyll was added.

TABLE III

REVERSAL BY BOVINE SERUM ALBUMIN OF THE INHIBITION OF DPC → DCIP ACTIVITY IN GALACTOLIPASE-TREATED CHLOROPLASTS ISOLATED FROM THE COLD AND DARK-STORED TOMATO LEAVES

PS II activity was measured with 300 μM DPC as electron donor.

Conditions (components and sequence of addition)	DPC → DCIP activity (μmol DCIP reduced/h per mg chl.)
Control	
(untreated chloroplasts)	110.0
Galactolipase-treated chloroplasts	
+ 1 μg enzyme per μg chl.	38.0
+ 1 μg enzyme per μg chl.	
+ 400 μg BSA per μg chl. *	93.5
+ 400 μg BSA per μg chl. **	
+ 1 μg enzyme per μg chl.	105.5
+ 5 μg enzyme per μg chl.	17.0
+ 5 μg enzyme per μg chl.	
+ 800 μg BSA per μg chl. *	60.5
+ 800 μg BSA per μg chl. **	
+ 5 μg enzyme per μg chl.	72.0

* Chloroplasts were incubated at 25°C for 5 min with the indicated amount of bean galactolipase and then bovine serum albumin was added.

** Bovine serum albumin was included into reaction medium 5 min before the addition of enzyme.

per μg chlorophyll was sufficient to almost completely restore activity. It should be pointed out that treatment of chloroplasts with 5 μg instead of 1 μg galactolipase per μg chlorophyll increases the level of free fatty acids released only about 2-fold (cf. Fig. 3C). Since in all these experiments bovine serum albumin was added to the chloroplasts suspension (either prior or together with the enzyme) in an amount sufficient to bind all fatty acids present, it may be concluded that the extent of restoration of DPC → DCIP activity depends not only upon the removal of released fatty acids by bovine serum albumin but also upon the degree of damage of thylakoid membranes. In chloroplasts from the cold and dark-stored leaves in which a fraction of chloroplast galactolipids has been hydrolyzed during the cold and dark storage, the exogenous galactolipase augmented the effect of the endogenous one on the thylakoid membranes. Thus, removal of free fatty acids by albumin is not sufficient to restore a damaged electron flow in chloroplasts from the cold and dark-stored leaves.

Effect of Mn^{2+} on electron flow in galactolipase-treated chloroplasts

Siegenthaler [24] has recently demonstrated that the inhibition of Hill reaction activity induced by exogenous unsaturated fatty acids is reversed by exogenous Mn^{2+} which forms a shunt by-passing the fatty acid block.

As can be seen in Table IV in chloroplasts from fresh and stored and illuminated leaves the presence of 1.0—1.5 μmol free fatty acids per mg chlorophyll does not practically affect the DPC → DCIP activity. In chloroplasts from cold and dark-stored leaves, which contain about 6 μmol free fatty acids per mg

TABLE IV

EFFECT OF Mn^{2+} ON LIGHT-DEPENDENT PHOTOSYSTEM II ACTIVITY OF GALACTOLIPASE-TREATED CHLOROPLASTS FROM FRESH, COLD AND DARK-STORED AND STORED AND ILLUMINATED TOMATO LEAVES

Incubation of chloroplasts with galactolipase (10 μg per μg chlorophyll) was performed at 25°C for 5 min as described in legend to Fig. 2. The photochemical activity is expressed in μmol acceptor reduced/h per mg chlorophyll. 5 mM $MnCl_2$ was present in the reaction medium. The content of free fatty acids was estimated as described in Methods.

Source of chloroplasts	Conditions	Free fatty acid content (μmol per mg chl.)		Photosystem II activity			
				$H_2O \rightarrow DCIP$	%	DPC → DCIP	%
Experiment A							
Fresh leaves	Control	1.23		76.0	100	91.0	100
	+ enzyme	9.48	(Δ = 8.25)	21.0	27	32.0	35
	+ enzyme + Mn^{2+}			77.8	102	97.7	107
	+ Mn^{2+}			88.9	117	106.0	116
Stored leaves	Control	5.75		12.6	100	78.0	100
	+ enzyme	14.00	(Δ = 8.25)	2.3	18	3.6	5
	+ enzyme + Mn^{2+}			10.0	80	24.0	30
	+ Mn^{2+}			16.0	127	93.5	120
Stored and illuminated leaves	Control	1.43		66.2	100	72.5	100
	+ enzyme	9.75	(Δ = 8.32)	23.1	35	31.0	42
	+ enzyme + Mn^{2+}			77.0	116	98.5	135
	+ Mn^{2+}			80.5	122	100.5	140
Experiment B							
Fresh leaves	Control	1.00		79.0	100	109.0	100
	+ enzyme	9.93	(Δ = 8.93)	26.3	33	35.0	35
	+ enzyme + Mn^{2+}			90.0	100	102.0	93
	+ Mn^{2+}			90.0	110	118.0	115
Stored leaves	Control	5.64		8.6	100	89.0	100
	+ enzyme	13.94	(Δ = 8.34)	1.5	17	7.0	8
	+ enzyme + Mn^{2+}			6.0	70	18.0	20
	+ Mn^{2+}			12.0	140	87.0	98
Stored and illuminated leaves	Control	1.50		56.5	100	94.0	100
	+ enzyme	10.00	(Δ = 8.50)	21.0	37	29.0	30
	+ enzyme + Mn^{2+}			50.0	88	90.0	96
	+ Mn^{2+}			54.0	96	93.0	100

chlorophyll, DPC → DCIP activity is inhibited only by 15—20% of controls. However, when the level of free fatty acids increases to about 8 μmol per mg chlorophyll following galactolipase treatment of chloroplasts both H_2O → DCIP and DPC → DCIP activities are inhibited by 70%. The highest inhibition of DPC → DCIP activity amounting to 90% was observed in galactolipase-treated chloroplasts isolated from cold and dark-stored leaves in which the level of fatty acids increased up to 14 μmol per mg chlorophyll.

In galactolipase-treated chloroplasts from both fresh and stored and illuminated leaves containing an elevated level of fatty acids, exogenous Mn^{2+} stimulated both DPC → DCIP and H_2O → DCIP activities up to the control level. Thus, Mn^{2+} added to these chloroplasts was able to establish a shunt by-passing the fatty acid block, similarly to that postulated in the presence of exogenous unsaturated fatty acids [24]. However, in galactolipase-treated chloroplasts isolated from cold and dark-stored leaves the Hill reaction activity stimulated by Mn^{2+} is equal only to about 25% of control, probably due to damage of the site or carrier donating electrons to exogenous Mn^{2+}. As shown previously [7] there is a common site in electron transport chain of chloroplasts of chilling-sensitive plants affected by either Tris or cold and dark treatment of leaves.

Discussion

In the mechanism of inactivation of Hill reaction activity induced by cold and dark treatment of leaves of chilling-sensitive plants at least two factors are involved: (1) a depletion of chloroplast manganese [5,25], and (i) an enzymic hydrolysis of chloroplast membrane lipids [4]. In the latter case two different mechanisms could be expected for the inhibition of photochemical activities: (i) an inhibitory effect of free fatty acids [3,21,27—29], and (ii) a large damage of the membrane structure due to the hydrolysis of galactolipids [2,29].

Cold and dark storage of tomato leaves results in a degradation of total galactolipids by about 40%. Chloroplast monogalactosyl diacylglycerol is more readily affected than digalactosyl diacylglycerol by both cold and dark treatment of leaves (Table I) as well as by exogenous galactolipase treatment (Fig. 1). The diminished content of galactolipids by about 3 μmol per mg chlorophyll corresponds to an increase of free fatty acid level (by about 5—6 μmol per mg chlorophyll) which induces a low inhibition of DPC → DCIP activity (Table IV). On the other hand, induced by endogenous free fatty acids nearly complete inhibition of PS II activity in spinach chloroplasts was observed at concentrations of about 1.0 [28,29], 1.5 [21] or 2.0—3.0 [3] μmol free fatty acid per mg chlorophyll. Exogenous fatty acids, however, are not as effective as linolenic acid released from the thylakoid membrane by lipase [28]. Moreover, the inhibitory effect caused by exogenous fatty acids on PS II activity is more readily reversed by the addition of bovine serum albumin than that caused by endogenous linolenic acid [3,4,30].

Different effects of lipolytic enzymes on PS I and PS II activities in spinach chloroplasts were found by Hirayama and Matsui [2] who observed that potato lipolytic enzyme rapidly hydrolyzed monogalactosyl diacylglycerol and preferentially inhibited PS I activity while phospholipase A_3 decom-

posed predominantly phosphatidylglycerol and caused a strong inhibition of PS II activity. Also in tomato chloroplasts hydrolysis of monogalactosyl diacylglycerol predominates over that of digalactosyl diacylglycerol following bean galactolipase treatment (Table I and Fig. 1). PS II was found to be more sensitive than PS I, especially at low enzyme concentrations (Fig. 2). According to Shaw et al. [3] inhibition of electron flow in spinach chloroplasts by galactolipase treatment is mainly due to free fatty acids released enzymatically since the inhibition is prevented by serum albumin even under conditions where as much as 50—60% of the acyl lipids of chloroplast membrane are hydrolyzed. The same was observed for PS II activity in tomato chloroplasts isolated from fresh, and stored and illuminated leaves, but not in chloroplasts from cold and dark-stored leaves (Tables II and III). Similarly, PS I activity could not be restored by albumin added either prior or after galactolipase treatment of chloroplasts (Table II). This seems to indicate that the release of plastocyanin due to damage of the thylakoid membrane following treatment of chloroplasts with galactolipase may result in a PS I inactivation which is not reversed in the presence of albumin. It was reported [22] that the reactivation of PS I activity in either heptan- or galactolipase-treated spinach chloroplasts depends on the addition of plastocyanin.

The cold and dark-induced inactivation of PS II electron flow appears to be a common result of manganese depletion from chloroplasts [5] as well as of hydrolysis of galactolipids providing inhibitory free fatty acids. Postulated sites of the action of those two factors are depicted in Fig. 4. A very low inhibition of DPC → DCIP activity (10—15% of control) observed in the presence of 5—6 μmol free fatty acid accumulated during the cold and dark storage of leaves may result from either an unspecific binding of fatty acids which are slowly released during 3 days storage of leaves at 0° C or from their specific binding to the membrane at the site before donation of electrons by DPC. The latter possibility is, however, difficult to demonstrate in these chloroplasts due to the loss of Mn [5] resulting in the inhibition of H_2O → DCIP activity.

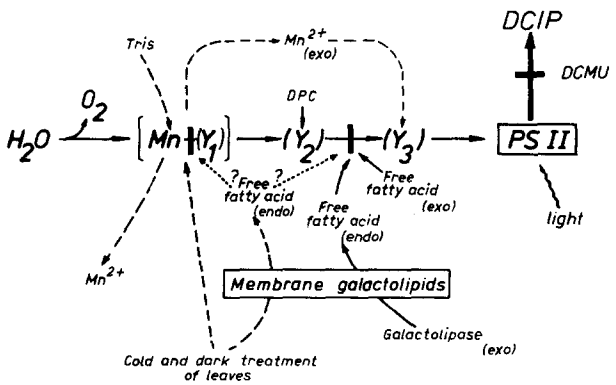

Fig. 4. Postulated sites of inhibition of Photosystem II electron flow in chloroplasts resulting from the cold and dark storage of tomato leaves and/or from the galactolipase treatment of isolated chloroplasts (see text for details). Note that in chloroplasts from cold and dark-stored leaves or in Tris-washed chloroplasts external Mn^{2+} does not form a shunt by-passing fatty acid block due to damage of the site occupied by the endogenous Mn.

As in the presence of exogenous linolenic acid in spinach chloroplasts [24] the elevation of free fatty acid levels up to 14 μmol/mg chlorophyll due to the treatment of chloroplasts with bean galactolipase results in 90% inhibition of DPC → DCIP activity (Table IV). The site of action of both exogenous and endogenous free fatty acids accumulated during treatment of chloroplasts with galactolipase, may be located on the oxidizing site of Photosystem II, i.e. between the hypothetical carriers Y_2 (which may be reduced by DPC) and Y_3 [24]. 70% inhibition of DPC → DCIP activity in galactolipase-treated chloroplasts due to accumulation of about 10 μmol fatty acid per mg chlorophyll is restored completely by the addition of Mn^{2+} which, as proposed by Siegenthaler [24], forms a shunt by-passing the fatty acid block. The restoration of DPC → DCIP activity is, however, possible only in chloroplasts from fresh and stored and illuminated leaves. Depletion of chloroplast manganese by either cold and dark treatment of leaves [5,25] or Tris-washing of chloroplasts [5,31] abolishes the ability of chloroplasts to use exogenous Mn^{2+} as an electron donor [24,26] and to form a by-pass in the presence of free fatty acids (Table IV).

Although both depletion of Mn and hydrolysis of membrane lipids proceed simultaneously it appears that the main reason of PS II inactivation following cold and dark treatment of tomato leaves is the loss of chloroplast Mn content [5]. It is interesting to note that neither aging of isolated chloroplasts at 0°C for a few days [5] nor digestion of chloroplasts with the exogenous galactolipase decreases the chloroplast Mn content.

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