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EFFECT OF COLD-STORAGE OF BEAN LEAVES ON PHOTOSYNTHETIC REACTIONS OF ISOLATED CHLOROPLASTS. INABILITY TO DONATE ELECTRONS TO PHOTOSYSTEM II AND RELATION TO MANGANESE CONTENT*

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SUMMARY

1. Chloroplasts from bean leaves which have been aged in darkness at 0 °C do not carry out Hill reactions. They reduce NADP with reduced 2,6-dichlorophenolindophenol (DCIP) in a reaction that is insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). They reduce NADP with hydroquinone, *p*-phenylenediamine or benzidine, and reduce DCIP with hydroxylamine, 1,4-diphenylsemicarbazide, 1,5-diphenylcarbohydrazide or manganous ion in DCMU-sensitive reactions.

2. Chloroplasts from aged leaves have low fluorescence yields at high light intensity even in the presence of DCMU.

3. When chloroplasts from aged leaves are extracted with water they lose the ability to use manganous ion but retain the ability to use other donors for reduction of DCIP.

4. Plastid manganese decreases on aging leaves and increases on restoration of photosynthetic activity by illumination of leaves.

5. It is concluded that chloroplasts from aged leaves have a defect in photosynthetic electron transport which makes them incapable of reducing the photooxidant generated by Photosystem II. The relation of this defect to the loss of plastid manganese and the site of electron donation by exogenous manganese are discussed.

INTRODUCTION

Bean leaves lose the ability to photosynthesize when they are stored in darkness at 0 °C[1]. Chloroplasts from these aged leaves do not carry out Hill reactions with ferricyanide, 2,3′,6-trichlorophenolindophenol (TCIP) or NADP[1]. However, they reduce NADP if ascorbate or ascorbate and 2,6-dichlorophenolindophenol (DCIP) ar provided[1]. When aged bean leaves are placed in the light at 20 °C, photosynthetic activity is restored, and chloroplasts active in the Hill reaction can again be isolated[1]. These results indicate that plastids from aged leaves are unable to carry out at least

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TCIP, 2,3′,6-trichlorophenolindophenol.

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one reaction between the oxidation of water and the site of entry of electrons from reduced DCIP. The present investigation was carried out to determine whether chloroplasts from aged bean leaves are deficient in ability to reduce the oxidant generated by Photosystem II.

MATERIALS AND METHODS

Plant materials

Phaseolus vulgaris, variety Black Valentine, was grown in perlite (or Vermiculite) and water by incubation in darkness for 4 days at 22 °C followed by an additional 6 tot 7 days with alternating periods of 8 h of light (25 °C) and 16 h of darkness (22 °C)[1]. Leaves were picked on the 10th or 11th days from planting (fresh leaves). They were then placed on ice and incubated in darkness for various periods of time, usually 2 days, unless indicated otherwise (aged leaves). Aged leaves were placed in light (500 ft-candles), for 2 h unless stated otherwise (reactivated leaves).

Preparation of chloroplasts

Unwashed ("intact") chloroplasts and "osmotically shocked" ("broken") chloroplasts were prepared from fresh, aged, or reactivated leaves as previously described[1]. The leaves were ground with a solution containing 0.4 M sucrose, 0.05 M Tris, 0.01 M NaCl, 0.01 M EDTA, pH 8.0. Chloroplasts were resuspended in a solution in which 0.05 M phosphate, pH 7.0 was substituted for Tris. EDTA was omitted from solutions when manganous ion was used as an electron donor. Chlorophyll content was determined spectrophotometrically[2].

Measurement of Hill reactions, photoreductions and fluorescence yield

The complete reaction micture for the DCIP Hill reaction contained 1200 μmoles sucrose, 150 μmoles (pH 7.0) phosphate, 30 μmoles EDTA, 30 μmoles NaCl, 0.1 μmole DCIP, and "intact" chloroplasts containing 25 μg chlorophyll (unless stated otherwise); final volume 3.0 ml. The complete reaction mixture for the NADP Hill reaction contained 400 μmoles sucrose, 50 μmoles phosphate (pH 7.0), 10 μmoles EDTA, 10 μmoles NaCl, 0.5 μmole NADP, 40 μg ferredoxin (Sigma Chemical Co., St. Louis, Mo.), and "broken" chloroplasts containing 25 μg of chlorophyll (unless otherwise stated); final volume 1.0 ml. EDTA was omitted when manganous ion was used as electron donor. The rates of photoreduction of NADP were 60–80 % of rates obtained with saturating amounts of ferredoxin. Other conditions (*e.g.* pH) might also have been suboptimal. Reduction of DCIP was measured spectrophotometrically at 600 nm for a 30-s illumination. Reduction of NADP was measured at 340 nm for 3 consecutive 2-min intervals. Correction was made for any reduction that occurred in darkness. Photoreduction with added donors was carried out in the same way, with exceptions as noted. All reactions were carried out in air at about 20 °C.

Fluorescence yield was measured as described by Epel and Levine[3]. Details, including intensities of measuring and actinic beams are described by them. "Intact" chloroplasts (final concentration, 8 μg chlorophyll/ml) were suspended in a solution containing 0.4 M sucrose, 0.05 M phosphate, 0.01 M NaCl, 0.01 M EDTA, pH 7.8.

Determination of manganese

Unwashed chloroplasts were prepared and DCIP Hill reaction activity was

measured. The remaining chloroplasts were collected by centrifugation for 15 min at 20000 × *g*, the pellet suspended in a small volume of water, and stored at −30 °C. Samples which contained 2.0–6.0 μmoles of chlorophyll were extracted with a mixture of nitric and perchloric acids[4]. These extracts were aspirated directly into the flame of a Jarrell Ash Atomic Absorption spectrophotometer, and absorbance compared with that of standard manganese solutions containing perchloric and nitric acids. Absorbance was proportional to manganese content of standard solutions in the range used (0.1–2.0 μg manganese/ml).

RESULTS

Leaves lose chloroplast photosynthetic activity rapidly upon aging and rapidly regain this activity when leaves are placed in the light (Table I) (*cf.* Margulies and Jagendorf[1]). Leaves that have been aged 2 days at 0 °C yield chloroplasts with less than 5 % of either DCIP or NADP Hill reaction activity of chloroplasts from fresh leaves. However, Photosystem I activity is not lost, since when chloroplasts from aged leaves are supplied with DCIP and ascorbate they photoreduce NADP (Table I). This reaction is not inhibited by 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Addition of DCIP and ascorbate makes no difference in the initial rate of photoreduction of $NADP^+$ obtained with chloroplasts from fresh leaves.

The fluorescence yield of plastids from aged leaves is lower than the fluorescence yield of plastids from fresh leaves at high light intensity only (Table II). Although DCMU increases the fluorescence yield of plastids from fresh leaves at low light intensities, it has almost no effect on plastids from aged leaves.

Hydroxylamine, 1,5-diphenylcarbohydrazide, 1,4-diphenylsemicarbazide, and manganous ion permit chloroplasts from aged leaves to photoreduce DCIP (Table III). Photoreduction with each of these donors is inhibited by 10 μM DCMU.

Hydroquinone, *p*-phenylenediamine and benzidine, each with ascorbate, permit

TABLE I

EFFECT OF TREATMENT OF LEAVES ON PHOTOSYNTHETIC ACTIVITIES OF PLASTIDS

Dispersion is the average deviation from the mean for the number of experiments which are given in parentheses. Rates of NADP reduction are for a 2-min illumination period. Where indicated, 0.03 μmole DCIP and 0.33 μmole sodium ascorbate, pH 7.0 were added per ml final volume.

Treatment of leaves	*Photoreduction*		
	moles DCIP reduced/ mg chlorophyll per h	*moles NADP reduced/ mg chlorophyll per h*	
		No addition	*DCIP + ascorbate*
None	163 ± 20 (4)	33	—
Aged 0.5 day	—	10	—
Aged 1.0 day	42 ± 14 (2)	5.2	27
Aged 1.5 days	—	1.5	22
Aged 2.0 days	5 ± 1 (4)	1.5	23
Aged 2.5 days	—	1.4	32
Aged 2.0 days + 15 min light	109 ± 30 (3)	—	—
Aged 2.0 days + 120 min light	159 ± 28 (3)	—	—

TABLE II

RELATIVE FLUORESCENCE YIELDS OF PLASTIDS FROM FRESH LEAVES AND FROM AGED LEAVES

DCMU	*Illumination sequence*	*Fluorescence yield (relative values)*	
		Plastids from fresh leaves	*Plastids from aged leaves*
None	Measuring beam on	17	19
	Actinic light on	40	21
	Actinic light off	18	19
10 μM	Measuring beam on	27	21
	Actinic light on	40	25
	Actinic light off	32	25

TABLE III

ABILITY OF VARIOUS SUBSTANCES TO ACT AS ELECTRON DONORS FOR THE LIGHT-DEPENDENT REDUCTION OF DCIP BY CHLOROPLASTS FROM AGED LEAVES

Where indicated, reaction mixtures of 3.0 ml contained: 75 μmoles hydroxylamine (pH 7.0), 1.5 μmoles 1,5-diphenylcarbohydrazide, 1.5 μmoles 1,4-diphenylsemicarbazide, 0.3 μmole $MnCl_2$, 0.03 μmole DCMU. Dispersion is average deviation for the number of experiments indicated in parentheses.

Additions	*μmoles DCIP reduced/mg chlorophyll per h*			
	Hydroxylamine	*1,5-Diphenyl-carbohydrazide*	*1,4-Diphenyl-semicarbazide*	*$MnCl_2$*
None	3 ± 1 (8)	4 ± 2 (3)	4 ± 2 (3)	0 ± 1 (2)
Donor	37 ± 5 (8)	47 ± 5 (3)	50 ± 2 (3)	15 ± 0 (2)
Donor + DCMU	2 ± 1 (2)	9 (1)	5 (1)	0 ± 0 (2)

TABLE IV

ABILITY OF VARIOUS SUBSTANCES TO ACT AS ELECTRON DONORS FOR LIGHT-DEPENDENT REDUCTION OF NADP BY CHLOROPLASTS FROM AGED LEAVES

Where indicated, reaction mixtures of 1.0 ml contained: 0.33 μmole sodium ascorbate (pH 7.0), 0.2 μmole hydroquinone, 0.033 μmole *p*-phenylenediamine, 0.033 μmole benzidine, 3.0 μmoles semicarbazide, 0.01 μmole DCMU. Dispersion is average deviation for the number of experiments indicated in parentheses. Absolute rates of NADP reduction in the presence of DCIP and ascorbate are quite variable but are comparable to the values given in Table I.

Additions	*Rate of NADP reduction* *Percent of rate of reduction with ascorbate and DCIP*			
	Hydroquinone	*p-Phenylene-diamine*	*Benzidine*	*Semicarbazide*
None	2 ± 3 (4)	4 ± 2 (3)	4 ± 2 (4)	3 ± 1 (2)
Ascorbate + donor	53 ± 16 (4)	27 ± 4 (3)	29 ± 6 (3)	9 ± 0 (2)
Ascorbate	8 ± 1 (4)	11 ± 3 (3)	8 ± 1 (3)	10 ± 1 (2)
Donor	1 ± 1 (4)	2 ± 0 (2)	11 ± 7 (3)	3 ± 1 (2)
Ascorbate + donor + DCMU	3 ± 0 (2)	7 ± 1 (2)	5 ± 2 (2)	—

chloroplasts to photoreduce NADP (Table IV). With each of these donors photoreduction is inhibited by 10 μM DCMU. Ascorbate alone permits some photoreduction of NADP as does benzidine alone. Neither hydroquinone nor *p*-phenylenediamine alone serve as donors for NADP photoreduction. The sum of the photoreduction obtained with ascorbate and hydroquinone, ascorbate and *p*-phenylenediamine, or ascorbate and benzidine is less than the photoreduction with ascorbate and donor together. Although semicarbazide serves as donor for photoreduction of ferricyanide[5] or DCIP[6] with Tris-washed chloroplasts of spinach, chloroplasts from aged bean leaves or Tris-washed plastids from fresh bean leaves do not photoreduce DCIP with semicarbazide or NADP with semicarbazide and ascorbate.

Although manganous ion serves as a donor for DCIP reduction with "intact" chloroplasts from aged leaves, it does not serve as donor with "broken" chloroplasts from aged leaves (Table V). In contrast, 1,4-diphenylsemicarbazide or 1,5-diphenylcarbohydrazide serve as donors with either "intact" or "broken" chloroplasts (Table V). Addition of chloroplast extract to broken chloroplasts from aged leaves does not restore the ability of manganous ion to serve as donor. The rate of DCIP reduction

TABLE V

EFFECT OF BREAKING CHLOROPLASTS FROM AGED LEAVES ON THE ABILITY OF $MnCl_2$, 1,4-DIPHENYLSEMICARBAZIDE OR 1,5-DIPHENYLCARBOHYDRAZIDE TO SERVE AS DONORS FOR THE REDUCTION OF DCIP

The concentrations of donors are the same as in Table III. Each 3.0 ml of reaction mixture contained "intact" chloroplasts with 43 μg of chlorophyll or "broken" chloroplasts with 47 μg of chlorophyll.

Additions	*μmoles DCIP reduced/mg chlorophyll per h*	
	"Intact" plastids	*"Broken" plastids*
None	0 (<2)	3
$MnCl_2$	11	3
1,4-Diphenylsemicarbazide	60	76
1,5-Diphenylcarbohydrazide	59	70
None	0 (<2)	3
$MnCl_2$	13	0 (<2)

TABLE VI

RELATION BETWEEN HILL REACTION ACTIVITY OF CHLOROPLASTS AND THEIR MANGANESE CONTENT

Treatment of leaves	*Mole Mn/ mole chlorophyll*	*Moles chlorophyll/ mole Mn*	*Hill reaction (μmoles DCIP reduced/mg chlorophyll per h)*	*(Mn/chlorophyll) Hill activity $\times 10^3$*
Fresh	0.0097	100	96	0.10
Aged	0.0016	630	3	0.53
Reactivated	0.0058	170	63	0.092
Fresh	0.0076	130	110	0.069
Aged	0.0016	630	1	1.6
Reactivated	0.0053	190	75	0.071

with manganous ion is considerably lower than the rates with 1,4-diphenylcarbazide and 1,5-diphenylcarbohydrazide. However, manganous ion catalyzes reoxidation of reduced DCIP in darkness in the presence of chloroplasts and air. It is estimated that real rates of photoreduction may be double those actually observed.

Chloroplasts from fresh bean leaves, unlike chloroplasts from spinach[7] lose Hill reaction activity rapidly when stored in dilute solutions of Tris. For example, plastids stored in 0.05 M phosphate, pH 7.0 do not lose DCIP Hill activity in 0.5 h, while plastids stored in 0.05 M Tris or 0.8 M Tris, both pH 8.0, lose 90 % and 99 % of their Hill reaction activity, respectively, in 0.5 h. Since washing chloroplasts with Tris removes manganese[7,8], and restoration of phytosynthetic activity to both manganese deficient cells and aged bean leaves requires light, the possibility that aging bean leaves produces chloroplasts deficient in manganese was suggested and found to be correct. Manganese content of chloroplasts from aged leaves is lower than the manganese content of plastids from fresh leaves (Table VI). In addition, reactivation of aged leaves produces an increase in the manganese content of the chloroplasts such that the ratio of manganese content of chloroplasts to their Hill reaction activity is restored to the same ratio found in chloroplasts from fresh leaves (Table VI).

DISCUSSION

DCMU blocks photosynthetic electron transport between oxidation of reduced Q and the sites of reduction and oxidation of DCIP[9,10]. Donor-dependent, DCMU-sensitive photoreductions of DCIP, NADP, *etc.* by chloroplasts that have been treated so that they will not carry out Hill reactions with DCIP, NADP, *etc.* have been reported[9]. These reactions are interpreted to mean that the treatments produce a defect in electron transport so that reduction of the oxidant generated by the action of light on Photosystem II can no longer take place. As expected, these defects in electron transport also decrease ability to maintain a high level of reduced Q, as judged be decreased fluorescence yield at high light intensities[9,11]. Since chloroplasts from aged bean leaves show donor-dependent DCMU-sensitive reductions of DCIP and NADP it is concluded that these chloroplasts have a defect which no longer permits them to reduce the oxidant generated by Photosystem II. The decreased fluorescence yield at high light intensity might likewise result from inability of plastids to reduce the oxidant produced by Photosytem II, but might also result from other modifications of Photosystem II, since in some cases electron transport but not fluorescence yield are restored by addition of suitable electron donors[11].

Manganese is a component necessary for photosynthetic electron transport[4,12] and can act as a donor for DCMU-sensitive photoreductions[7,13] (Table V). The relationship between these two biological activities is not known[9]. The ability of manganese to act as donor for photoreduction of DCIP with chloroplasts from aged leaves is destroyed by extraction of these chloroplasts with water while photoreduction of DCIP with other donors is not affected (Table V). Thus, the site of entry of manganese into the photosynthetic electron transport pathway may be different from, and more distant from Photosystem II than the site(s) of entry for the other donors, as has been suggested from work with Tris-washed lettuce chloroplasts[13].

Donor-dependent, DCMU-sensitive photoreductions of acceptors such as DCIP, ferricyanide, or NADP are observed with some mutants[3], chloroplasts from chloride-

deficient cells[14], chloroplasts that have been washed with Tris[5,8] or hydroxylamine[8] or chloroplasts treated with heat[8,11], ultraviolet light[11], ammonia or methylamine[14]. Like aging of bean leaves, development of dependence on exogenous donor for photoreductions by chloroplasts treated with heat or chloroplasts extracted with Tris or hydroxylamine is correlated with loss of manganese[8,9]. However, all the treatments which produce loss of manganese and dependence on donors for DCMU-sensitive photoreductions may not produce identical effects on electron transport. For example: by extraction with hydroxylamine or Tris one can obtain loss of Hill reaction activity or dependence on donor for photoreduction with high or low fluorescence yield, depending on extent of extraction[8]; by extraction with Tris one can obtain high fluorescence yield (in the presence of DCMU) with retention of about 30 % of plastid manganese[8], while with chloroplasts from aged bean leaves one can have low fluorescence yield (also in the presence of DCMU) with retention of about the same amount of manganese, 30 % (Table VI). The fluorescence properties of chloroplasts from aged bean leaves most nearly resemble the properties of chloroplasts heated at 50 °C and chloroplasts treated with ultraviolet light, since these chloroplasts all show low fluorescence yields at high light intensity, either in the presence or absence of DCMU[5].

The manganese measured (Table VI) is probably bound manganese. First, chloroplasts are sedimented twice from solutions which contain EDTA (see Methods). Second, the ratio of manganese to chlorophyll of chloroplasts from fresh leaves is comparable to that of chloroplasts, from a number of species of higher plants, which have been treated to remove free manganese[15].

The form in which manganese is released on aging of leaves is not known nor is its location. Possibly it is released from chloroplast membranes and stored in the chloroplast stroma. This manganese would probably not be recovered in the isolated chloroplasts, since much stroma material is lost during preparation of chloroplasts[16]. Alternately, the manganese which is released might be transferred to some other part of the cell, to other cells, or to the outside of the leaf. Similarly, the source of the manganese which becomes bound to the chloroplast during reactivation is not known. The only external source of manganese is the water on which leaves are floated during reactivation[1].

After 120 min of illumination, restoration of Hill reaction activity and manganese chloroplast content was incomplete although the ratio of manganese to Hill activity was restored to normal (Table VI). This suggests that restoration of Hill activity is directly related to restoration of chloroplast manganese. Since half-maximal restoration of Hill activity can occur in 15 min or less (Table I) (*cf.* Margulies and Jagendorf)[1], it seems probable that restoration of manganese content will likewise prove rapid, and that unlike some manganese deficient algae[15,17], no period for uptake of manganese will be required.

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