Biochimica et Biophysica Acta 810 (1985) 200–208
Elsevier

BBA 41876

Senescence-related changes in photosynthetic electron transport are not due to alterations in thylakoid fluidity

Donald G. McRae *, James A. Chambers and John E. Thompson **

Department of Biology, University of Waterloo, Waterloo, Ontario, N2L 3G1 (Canada)

(Received May 31st, 1985)
(Revised manuscript received July 23rd, 1985)

Key words: Thylakoid; Electron transport; Senescence; Lipid fluidity

During senescence of primary bean leaves, non-cyclic electron transport declines to a greater degree than the activities of Photosystem I and Photosystem II, and intersystem electron transport becomes rate-limiting. Paramagnetic and fluorescent lipid-soluble probes have been used to measure rotational motion and lateral motion in thylakoid membranes to determine if this decrease in intersystem electron transport reflects changes in lipid fluidity that could impede the translational motion of electron carriers between the two photosystems. Probes that partition deep in the bilayer as well as those that remain anchored at the membrane surface revealed no change in rotational motion with advancing age. Translational motion was measured by determining the excimer-to-monomer ratio for pyrene partitioned into the bilayer, and this ratio also showed no change with advancing senescence, nor were there changes in phase properties or fatty-acid composition of the membranes. During in vitro aging of chloroplasts, there was also a dramatic decrease in non-cyclic electron transport that was not accompanied by changes in membrane fluidity. Thus the decline in intersystem electron transport during senescence cannot be attributed to decreased translational diffusion of mobile electron carriers. Moreover, the mechanism of thylakoid membrane deterioration during senescence appears to differ from that operative in plasma and microsomal membranes for which large decreases in lipid fluidity with advancing age are well documented.

Introduction

Loss of photosynthetic competence during senescence is attributable, at least in part, to impairment of photosynthetic electron transport in the thylakoid membranes [1]. Electron transport through Photosystems I and II decreases in senescing bean leaves, but of particular interest is the fact that the rate of non-cyclic electron transport declines to a greater degree than the activities of either of the photosystems [2]. This has prompted the proposal [2] that it is largely an impairment of electron flow between the two photosystems that limits the availability of photosynthetic reducing power with advancing senescence.

Electron transport in the mitochondrial inner membrane is thought to be achieved by energetically favourable collisions among mobile electron carriers [3] and thus influenced by membrane lipid fluidity. In thylakoid membranes, the photosystems mediating photosynthetic electron transport are spatially separated. Photosystem II is localized predominantly in the appressed granal membranes, and Photosystem I in the non-appressed

* Present address: Department of Botany, University of British Columbia, Vancouver, B.C., Canada, V6T 2B1.
** To whom correspondence should be sent.

Abbreviations: 18NP, 3-(octadecylaminomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl; I(1,14), 16-doxylstearic acid.

granal and stromal membranes [4]. Accordingly, non-cyclic photosynthetic electron transport is thought to be achieved by lateral diffusion of mobile electron carriers, specifically plastoquinone and plastocyanin [4,5]. It has been suggested [4] that the rate at which plastoquinine diffuses between Photosystem II and cytochrome *f* may limit the rate of photosynthetic electron transport. Thylakoid membranes contain high levels of unsaturated fatty acids and no sterols, and hence are normally quite fluid [6]. However, it is reasonable to expect that the rate of diffusion of mobile electron carriers would be sensitive to changes in lipid fluidity. Indeed, when the fluidity of thylakoid membranes is reduced in vitro by incorporation of cholesterol hemisuccinate into the bilayer, the rate of non-cyclic electron transport declines [7].

A senescence progresses, there are substantive changes in the lipid composition of plasma and microsomal membranes that result in large decreases in bulk lipid fluidity [8–11]. In the present study, we have examined the possibility that comparable changes in the molecular organization of thylakoid membrane lipids contribute to the decline in non-cyclic electron transport during natural senescence. Parallel measurements of electron transport, thylakoid membrane fluidity, phase properties and fatty acid composition have been made for chloroplasts isolated from senescing primary leaves of bean at various stages of senescence and for chloroplasts isolated from non-senescent tissue and aged in vitro.

Materials and Methods

Plant material and membrane isolation. Bean seedlings (*Phaseolus vulgaris* L. cv Kinghorn) were grown for 35 days under greenhouse conditions. Chloroplasts and thylakoid membranes were isolated as previously described [12] from primary leaves (10 g fresh weight) harvested 9, 14, 21, 28 and 35 days after planting. Isolated chloroplasts and thylakoids were resuspended in wash buffer (0.3 M sorbitol/50 mM Tricine, pH 8.0) at a concentration of 2 mg chlorophyll/ml.

Measurements of non-cyclic electron transport. Measurements of photosynthetic electron transport were carried out essentially as described by Jenkins and Woolhouse [2]. Rates of electron transport were measured by monitoring the changes in O_2 activity for a 2-min period using a Yellow Springs Instrument oxygen electrode. Illumination from a 150 watt light bulb was passed through a water container (3 cm diameter) to provide a light intensity of 100 W/m^2 on the reaction vessel. Rates of oxygen consumption or production in the dark were subtracted from corresponding rates measured in the light.

The basic reaction mixture used for all assays of electron transport contained sorbitol (0.1 M)/Tricine buffer (50 mM) (pH 8.0)/$MgCl_2$ (5 mM)/ADP (1 mM)/K_2HPO_4 (1 mM). For measurements of Photosystem I, the reaction mixture contained in addition dichloroindophenol (80 μM)/sodium ascorbate (1 mM)/methyl viologen (0.5 mM)/dichlorophenylmethylurea (0.1 mM). For measurements of Photosystem II, the reaction mixture also contained $K_3Fe(CN)_6$ (5 mM)/α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine (Trifluralin) (50 μM)/*p*-phenylenediaminedihydrochloride (0.5 mM), and for total non-cyclic electron transport, methyl viologen (0.5 mM) was added to the basic reaction mixture.

Electron-spin resonance. Stock solutions of two spin-labelled lipid probes, 3-(octadecylaminomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl (18NP) and 16-doxyl stearic acid [I(1,14)] dissolved in absolute ethanol at a concentration of 2 mM were stored at −20°C. In order to minimize the reduction of spin labels partitioned into thylakoids, the membranes were first suspended in wash buffer containing 10 mM hydroxylamine hydrochloride and kept on ice for 5 min. At the end of this treatment, the suspension was centrifuged at 4000 × *g* for 5 min and the membranes resuspended in wash buffer at a concentration of 2 mg chlorophyll/ml. Incorporation of spin label into the thylakoid membranes was accomplished by drying 20 μl aliquots of stock spin label solution onto the sides of a 2 ml test tube with nitrogen and adding 200 μl of thylakoid membrane suspension into the same test tube. The tube was vortexed vigorously for 5 min, and the spin-labeled thylakoid membranes were then placed in a 100 μl capillary tube, which was sealed at one end with capillary tube sealent (Miniseal) and placed in the cavity of a Varian E-12 ESR spectrometer. Spectra were recorded at 24°C, and rotational correlation times

(τ_c) were calculated as described by Legge et al. [13]. (There was no change in the value of τ_c when twice the normal concentration of spin probe was used (i.e., 40 μl rather than 20 μl of stock solution) indicating that perturbation effects attributable to the probes themselves were minimal.)

Fluorescence depolarization. Stock solutions of *cis*-parinaric acid, *trans*-parinaric acid (each 4 mM in absolute ethanol) and diphenylhexatriene (2 mM in tetrahydrofuran) were stored under nitrogen at −20°C. For thylakoid labelling, aliquots of these stock solutions were diluted 1000-fold in wash buffer, and equal volumes of fluorescent probe solution and thylakoid suspension (40 μg chlorophyll/ml in wash buffer) were mixed to give a final probe concentration of 2 μM for *cis*- and *trans*-parinaric acid and 1 μM for diphenylhexatriene. Immediately upon addition of the probe solution to the thylakoid membrane suspension, the mixture was vortexed for 10–15 s and then equilibrated in the dark for 20 min before depolarization measurements were made.

Steady-state fluorescence depolarization (P) values were determined at 24°C using an SLM 8000 spectrofluorometer as described by Ford and Barber [6]. Values of P were corrected for intrinsic fluorescence and light scattering by subtracting the background fluorescence intensity from membrane suspension that had not been labelled with fluorescent probe. For diphenylhexatriene, an excitation wavelength of 360 nm and an emission cut-off filter of 418 nm were used. For *cis*- and *trans*-parinaric acid, excitations of 325 nm and 320 nm, respectively, were used, and emitted fluorescence was passed through a 370 nm cut-off filter. The emitted fluorescence was passed through an additional band filter (transmitting from 400–450 nm) to eliminate chlorophyll fluorescence.

Pyrene fluorescence. Translational motion in thylakoid membranes was measured using the fluorescence probe pyrene as described by Kleinfeld et al. [14]. Stock solutions of 10 mM pyrene dissolved in acetone were stored under nitrogen at 2°C. Aliquots of the stock solution were diluted to 10 μM with wash buffer, and thylakoid membrane suspensions (1.2 μg chlorophyll/ml in wash buffer) were mixed with 10 μM pyrene on an equal volume basis. After stirring, the suspension was equilibrated in the dark at 24°C for 1 h prior to recording the fluorescence emission spectrum from 350–600 nm. Excitation was at 338 nm, and spectra were recorded at 24°C using an emission slit of 2 nm and an excitation slit of 8 nm.

Lipid extraction and analysis. Thylakoid membrane lipids were extracted according to the method of Nichols [15] and methylated for analysis of total fatty acids as described by Morrison and Smith [16]. Heptadecanoic acid (0.4 mg/20 mg lipid) was added as an internal standard. Derivatization for measurements of free fatty acids was accomplished using diazomethane [17]. After methylation, samples of both total and free fatty acid methyl esters in hexane were passed through a small column (3 × 0.5 cm) containing activated charcoal and celite 545 (2:1, w/w) to remove contaminating chlorophyll [18]. Aliquots of the fatty methyl esters were analyzed using a Perkin-Elmer Sigma-3B gas chromatograph equipped with flame ionization detectors and a 1.8 m high glass column (2 mm i.d.) packed with 10% diethylene glycol succinate on supelcoport, 80/100 mesh. The instrument was operated isothermally at 200°C with helium as the carrier gas (25 ml/min).

Wide-angle X-ray diffraction. For X-ray diffraction studies, isolated chloroplasts were washed three times by resuspension in 50 mM Tricine buffer (pH 8.0) and centrifugation at 4000 × g for 5 min in order to remove the envelope membranes and sorbitol. Diffraction patterns were recorded from the resulting thylakoid membranes using CuK_α radiation as previously described [19].

In vitro aging. Isolated thylakoid membranes suspended in wash buffer at 2 mg chlorophyll/ml were aged for 7 h in darkness at 2° or 24°C, and under illumination (100 W/m^2) at 24°C.

Assay procedures. Malondialdehyde was assayed using thiobarbituric acid [20], and chlorophyll was determined by the procedure of Arnon [21].

Results

Photosynthetic electron transport. Total noncyclic electron transport activity as well as the activities of the individual photosystems proved to be highest on day 9 when the leaves were still young. From day 9 onwards, chlorophyll levels decrease as senescence of the leaves progresses [22]. The activities of Photosystem I and Photosys-

tem II both declined steadily with advancing sensecence, although the degree of inhibition was greater for Photosystem II than for Photosystem I (Table I). However, total non-cyclic electron transport, which involves both photosystems, was inhibited to a greater degree than the activities of either photosystem. This was particularly evident during the early stages of senescence. For example, by day 21 non-cyclic electron transport had decreased by 50%, whereas Photosystems I and II showed inhibitions of only 15% and 25% respectively (Table I). By day 35, non-cyclic electron transport had decreased by approx. 90%, and Photosystems I and II by 57% and 74%, respectively. These observations can be interpreted as indicating the intersystem electron transport becomes rate-limiting with advancing senescence.

Rotational motion. Two spin-labeled ESR probes, I (1,14) and 18NP, which probe the interior and headgroup regions, respectively, of the membrane bilayer, were used to monitor lipid motion. Thylakoid membranes labelled with these probes gave rise to a three-line ESR spectrum at 24° from which a rotational correlation time (τ_c) could be calculated [13]. Values of τ_c for membranes labelled with either of the spin-labeled probes did not change significantly with advancing senescence (Fig. 1). However, rotational correlation times obtained using I(1,14) were consistently

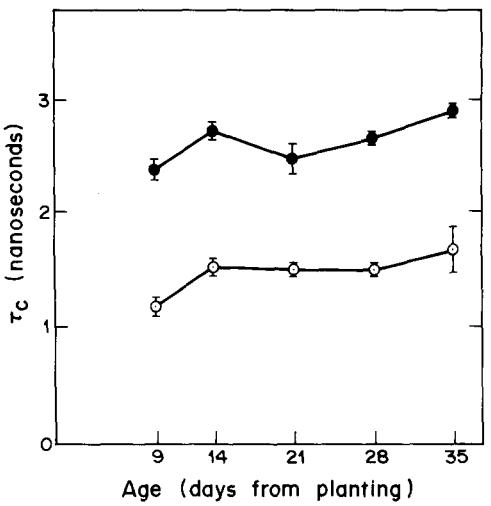

Fig. 1. Changes during senescence in the rotational correlation time (τ_c) for thylakoid membranes labelled with I(1,14) [○] or 18NP [●]. Standard errors of the means for three separate experiments are indicated.

lower than those obtained with 18NP indicating that the interior of the thylakoid membrane is more fluid than the headgroup region.

Three fluorescent probes, diphenylhexatriene, *cis-* and *trans*-parinaric acid, were also used to monitor changes in the lipid fluidity of thylakoid membranes with advancing senescence. No change in P, the degree of polarization, was noted for membranes labelled with any of these probes (Table II) indicating that by this criterion as well the lipid fluidity of thylakoid membranes remains essentially constant during senescence. Chlorophyll absorbs in the spectral range that overlaps the emission spectra of the probes used in this study. To determine the extent to which there was energy transfer from the probes to chlorophyll, the emission intensity of chlorophyll fluorescence was measured in the presence and absence of each of the probes under the same experimental conditions as those used for polarization measurements. As noted previously [23] no increase in chlorophyll emission was observed for diphenylhexatriene (Fig. 2A), and for *cis-* and *trans*-parinaric acid the changes were barely perceptible (Fig. 2B and C).

Translational motion. Translational motion in the thylakoid bilayer was measured using the fluorescence probe pyrene [14]. The emission spectrum

TABLE I

CHANGES IN PHOTOSYNTHETIC ELECTRON TRANSPORT OF CHLOROPLASTS ISOLATED FROM PRIMARY BEAN LEAVES AT VARIOUS STAGES OF SENESCENCE

Values are reported as percentage inhibition relative to activities on day 9. Standard errors of the means for three separate experiments are indicated. Rates for day 9 are 298, 308 and 204 μmol 1/2 O_2 per mg Chl per h for Photosystem I, Photosystem II and non-cyclic electron transport, respectively.

Age (days from planting) of primary leaves used for chloroplast isolation	Percentage inhibition		
	Photosystem I	Photosystem II	Non-cyclic electron transport
9	0	0	0
14	8±11	6±8	25±5
21	15± 7	25±5	50±2
28	49± 3	63±2	78±3
35	57± 3	74±2	89±1

TABLE II

SENESCENCE-RELATED CHANGES IN MOTIONAL PARAMETERS FOR THYLAKOID MEMBRANES LABELLED WITH FLUORESCENT PROBES

Polarization values (P) were obtained after labelling with diphenylhexatriene, *cis*-parinaric acid or *trans*-parinaric acid. Excimer-to-monomer ratios were obtained after labelling with pyrene. Standard errors of the means for three separate experiments are indicated.

Age (days from planting) of primary bean leaves used for thylakoid isolation	P			
	diphenyl hexatriene	*cis*-parinaric acid	*trans*-parinaric acid	excimer-to-monomer ratio
9	0.252 ± 0.002	0.356 ± 0.006	0.397 ± 0.003	0.77 ± 0.02
14	0.252 ± 0.003	0.345 ± 0.003	0.389 ± 0.004	0.75 ± 0.02
21	0.248 ± 0.001	0.361 ± 0.002	0.391 ± 0.002	0.74 ± 0.03
28	0.256 ± 0.007	0.372 ± 0.006	0.401 ± 0.003	0.71 ± 0.02
35	0.259 ± 0.003	0.365 ± 0.003	0.399 ± 0.001	0.73 ± 0.03

for pyrene incorporated into bilayers features a multicomponent peak corresponding to the monomer and a broad peak deriving from the excimer

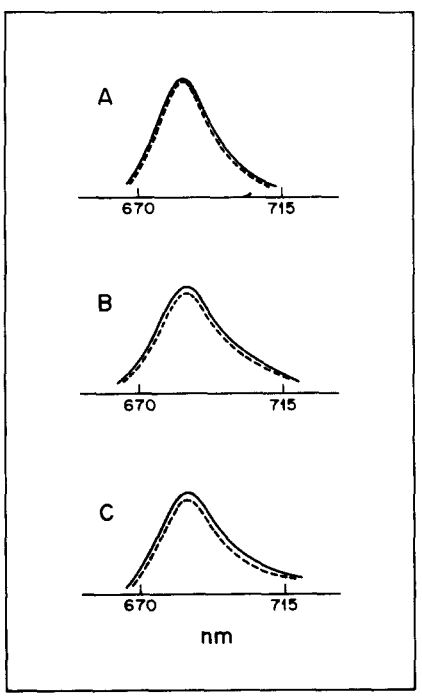

Fig. 2. Emission spectra of chlorophyll in thylakoid membranes with (solid line) and without (dashed line) fluorescent probe. (A) Diphenylhexatriene; (B) *cis*-parinaric acid; (C) *trans*-parinaric acid. Thylakoids were isolated from the primary leaves of 14-day-old plants. Spectra were recorded at 24°C with an emission slit of 2 nm and an excitation slit of 8 nm. Excitation wavelengths were 360 nm for (A), 325 nm for (B) and 320 nm for (C).

[14]. Provided the concentration of pyrene in the membrane is kept constant, the ratio of excimer to monomer peak heights provides a relative measure of translational motion in the bilayer [14]. This was achieved in the present study by maintaining a constant ratio of pyrene to chlorophyll, since in separate experiments it had been established that there is no change in the chlorophyll to fatty-acid ratio of these membranes with advancing senescence. The excimer to monomer ratio for thylakoids labelled with pyrene remained essentially unchanged as senescence progressed (Table II) indicating that there is no significant change in translational motion within the bilayer. Emission spectra for pyrene partitioned into thylakoid membranes proved to be virtually identical to those reported previously for pyrene in lymphoma plasma membranes [14] and featured a multicomponent monomer peak with maxima at 377, 384 and 394 nm and a broad excimer peak at 463 nm. Corresponding emission maxima reported for pyrene in lymphoma plasma membranes are 373, 385, 394 and 465 nm [14]. Thus there is no distortion of the emission maxima in thylakoids. As well, the dependence of the excimer-to-monomer ratio on pyrene concentration proved to be linear (as has been reported for lymphoma plasma membrane [14]) indicating that excimer formation in thylakoid membranes is diffusion-limited.

Phase change and lipid composition. Wide-angle X-ray diffraction studies of thylakoid membranes isolated from leaves at various stages of senescence indicated that there are no liquid-crystalline to gel

TABLE III

SENESCENCE-RELATED CHANGES IN THE LEVELS OF FREE AND ESTERIFIED FATTY ACIDS IN THYLAKOID MEMBRANES

Standard errors of the means for three separate experiments are indicated.

Age (days from planting) of primary bean leaves used for thylakoid isolation	Fatty acids (mg per mg total lipid)	
	esterified	free
9	0.268 ± 0.022	0.012 ± 0.001
14	0.274 ± 0.012	0.011 ± 0.001
21	0.243 ± 0.012	0.013 ± 0.002
28	0.255 ± 0.002	0.016 ± 0.003
35	0.241 ± 0.002	0.017 ± 0.003

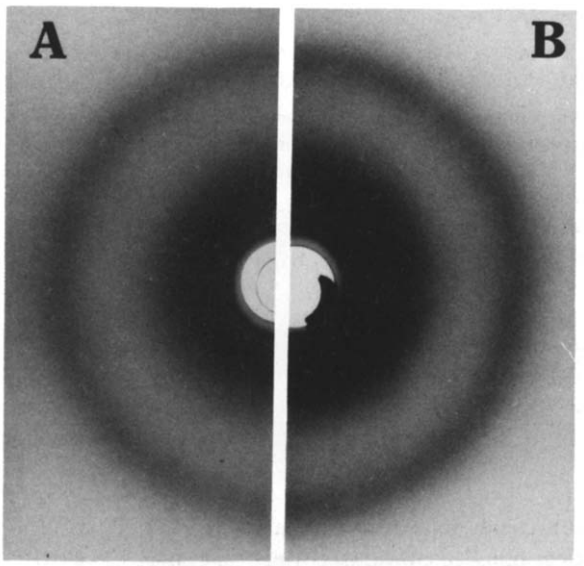

Fig. 3. Wide-angle X-ray diffraction patterns recorded at 24°C for thylakoid membranes isolated from primary leaves. (A) Thylakoid membranes from 14-day-old bean leaves; (B) thylakoid membranes from 35-day-old bean leaves. The patterns feature a broad diffuse reflection (outermost band) centered at a Bragg spacing of 0.46 nm that derives from liquid-crystalline lipid.

phase separations in these membranes with advancing age. Diffraction patterns recorded at 24°C from 14 and 35-day-old thylakoids featured only the broad diffuse X-ray reflection centered at a Bragg spacing of 0.46 nm and reflecting liquid-crystalline lipid (Fig. 3). In fact, the bilayers of young and senescent membranes both remained exclusively liquid-crystalline at temperatures as low as −20°C.

Free fatty acids are known to inhibit electron transport in chloroplasts [24] and to cause structural perturbations in lipid bilayers [25]. Thus any accumulation of free fatty acids in senescing thylakoid membranes could contribute to the decline in electron transport. However, levels of free fatty acids per mg total thylakoid lipid did not change with advancing senescence (Table III). Likewise, there was no significant change during senescence in the levels of esterified fatty acids per mg total lipid (Table III). Measurements of individual fatty acids indicated that there was also little change in fatty acid composition of the thylakoid membranes with advancing senescence (Table IV).

In vitro aging. When isolated thylakoid membranes from chloroplasts of young 14-day-old bean

TABLE IV

SENESCENCE-RELATED CHANGES IN THE FATTY-ACID COMPOSITION OF THYLAKOID MEMBRANES

Standard errors of the means for three separate experiments are shown.

Age (days from planting) of primary leaves used for thylakoid isolation	Percentage (w/w) relative to total fatty acid					
	14:0	16:0	18:0	18:1	18:2	18:3
0	0.06 ± 0.01	10.1 ± 0.4	1.8 ± 0.1	1.0 ± 0.1	3.1 ± 0.2	83.4 ± 0.5
14	0.15 ± 0.08	10.3 ± 0.5	1.9 ± 0.1	0.9 ± 0.1	3.0 ± 0.1	84.1 ± 0.8
21	0.6 ± 0.1	8.1 ± 0.2	1.8 ± 0.1	0.8 ± 0.1	2.8 ± 0.3	86.5 ± 0.8
28	1.6 ± 0.3	8.7 ± 0.2	2.0 ± 0.1	1.3 ± 0.3	2.9 ± 0.1	83.3 ± 0.9
35	2.9 ± 0.2	11.3 ± 0.5	3.0 ± 0.3	2.5 ± 0.3	3.8 ± 0.2	76.1 ± 1.4

TABLE V

EFFECTS OF IN VITRO AGING ON SELECTED FUNCTIONAL AND STRUCTURAL PARAMETERS OF THYLAKOID MEMBRANES ISOLATED FROM 14-DAY-OLD PRIMARY LEAVES

Measurements for control membranes were made immediately upon isolation. In vitro aging was for 7 h. Values for electron transport are calculated relative to the control (153 μmol 1/2 O_2 per mg Chl per h). Membranes for fluorescence polarization were labelled with diphenylhexatriene. Standard errors of the means for three separate experiments are indicated.

Treatments	Non-cyclic electron transport (% inhibition)	Malondialdehyde (nmol/ml)	Fluorescence polarization (P)
Control	0	41 ± 5	0.257 ± 0.008
Aged, 2°C (dark)	67 ± 3	44 ± 4	0.260 ± 0.009
Aged, 24°C (dark)	80 ± 3	39 ± 6	0.256 ± 0.011
Aged, 24°C (light)	100	45 ± 2	0.252 ± 0.005

leaves were aged in vitro at 24°C under illumination for 7 h, non-cyclic electron-transport activity was completely inhibited (Table V). During in vitro aging for the same duration in darkness, non-cyclic electron transport decreased by 80% at 24°C and 67% at 2°C (Table V). No liquid-crystalline-to-gel phase transitions were detectable in the aged membranes by wide-angle X-ray diffraction at temperatures as low as −20°C, and measurements of polarization (P) after labelling with diphenyl-hexatriene indicated that there was no change in membrane fluidity as a result of in vitro aging (Table V). Nor was there any increase in lipid peroxidation. Levels of malondialdehyde a product of lipid peroxidaton, proved to be virtually identical for control membranes and those aged under conditions of darkness or light (Table V).

Induction of fluidity changes in thylakoid membranes. To confirm that fluidity changes in thylakoid membranes can be detected by measurements of rotational motion, primary leaves of 14-day-old bean plants were floated on 20 mM $NaHSO_3$ (pH 3.5), for 120 min under fluorescent lights (4.3 W/m^2), and then on distilled water for an additional 24 h in the dark. This treatment is known to induce lipid peroxidation in chloroplasts [26]. As a result of this treatment, polarization values of thylakoids labelled with diphenylhexatriene rose from 0.249 ± 0.001 for control leaves to 0.321 ± 0.007 for treated leaves, and levels of linolenic acid declined from $83.2 \pm 1.01\%$ to $65.7 \pm 1.62\%$ following treatment.

Discussion

Photosynthetic electron transport declines dramatically with advancing senescence. However, of particular significance is the finding that non-cyclic electron transport declines to a greater degree than the independent activities of either Photosystem I or Photosystem II. This was first reported for senescing primary leaves of bean (*Phaseolus vulgaris*) by Jenkins and Woolhouse [2] and suggests that as senescence progresses electron transport between the donor of Photosystem II and the acceptor of Photosystem I limits the availability of photosynthetic reducing power. Jenkins and Woolhouse [2,27] have also demonstrated that there is no change in phosphorylation control of electron transport as these leaves senesce.

The reactions of photosynthetic electron transport are thought to be dependent upon the fluidity of thylakoid membranes inasmuch as the ability of electron carriers to interact is determined by their relative translational mobilities [7,28]. Indeed, Yamamoto et al. [28] were able to induce a decline in the electron transport activity of pea thylakoids by decreasing lipid fluidity. A similar proposal for the dependence of mitochondrial electron transport on membrane lipid fluidity has been advanced by Hackenbrock [3]. Changes in lipid fluidity are also known to cause vertical displacement of membrane proteins [29,30]. Thus, in addition to affecting the translational diffusion of mobile electron carriers, alterations in thylakoid fluidity could alter the function of protein complexes by inducing vertical displacement.

It is well documented for a variety of senescing plant tissues that plasma membranes and microsomal membranes sustain a decrease in bulk lipid fluidity with advancing age. The evidence for this has some mainly from experiments in which changes in fluidity have been inferred from measurements of the rotational motion of paramagnetic and fluorescent probes partitioned into the membrane bilayer [8–11]. There is also evidence for the formation of increasing proportions of gel phase lipid in senescing microsomal and plasma membranes from plant tissues [31,32]. In addition, Ford and Barber [6] have reported that during in vitro aging of pea thylakoid membranes there is a decrease in lipid fluidity detectable by fluorescence depolarization after labelling with diphenylhexatriene.

In the present study, we have examined the prospect that the decline in non-cyclic photosynthetic electron transport with advancing senescence is attributable, at least in part, to a decrease in the bilayer fluidity of thylakoid membranes. Fluidity as reflected by rotational motion was measured using a variety of paramagnetic and fluorescent compounds that probe both deep in the bilayer and near the membrane surface, and in all cases no significant change in lipid fluidity was detectable with advancing senescence. In addition, experiments using the fluorescent probe pyrene, in which fluidity is reflected by translational motion within the plane of the bilayer, indicated no change with advancing senescence.

The fluorescent probe, *trans*-parinaric acid, partitions preferentially into gel phase lipid, and thus can be used to detect phase transitions in lipid bilayers. Accordingly, the fact that there was no change with advancing senescence in the polarization value for thylakoids labelled with this probe can be interpreted as indicating that the membranes had not undergone a liquid-crystalline-to-gel phase transition. This was confirmed by wide-angle X-ray diffraction, which revealed that 35-day-old senescent thylakoids were exclusively liquid-crystalline at temperatures as low as $-20°C$. When thylakoids are isolated from senescent leaves by a different procedure that less effectively excludes chloroplasts having lost their structural integrity, small amounts of gel phase lipid are detectable by X-ray diffraction [19]. This can presumably be attributed to the presence in such preparations of chloroplasts at a very advanced stage of senescence that have lost morphological integrity. Such chloroplasts would have altered sedimentation properties and would largely be excluded by the isolation procedure used in the present study. In any case, it is clear from the present study that the lipid bilayers of thylakoid membranes showing extensive loss of photosynthetic electron-transport activity are exclusively liquid-crystalline.

Photosynthetic electron transport also declined when thylakoids isolated from young leaves were aged in vitro under continuous illumination or in darkness. There was no change in lipid fluidity of the thylakoid membranes during the 7 h in vitro aging period. These results are in contrast to those reported by Ford and Barber [6] who found an increase in polarization for pea thylakoid membranes that had been aged in vitro and labelled with diphenylhexatriene. As well, there have been previous reports of enhanced malondialdehyde production reflecting lipid peroxidation during illumination of isolated thylakoids [33], yet in the present study no increase in malondialdehyde was observed during the 7 h in vitro aging period in light or darkness. These observations collectively suggest that thylakoid membranes from bean may be uncharacteristically resistant to lipid peroxidation and ensuing changes in lipid fluidity. Thylakoid membranes are known to produce increased levels of O_2^- during both natural senescence and in vitro aging [12]. Moreover, O_2^- has been shown capable of inducing membrane lipid rigidification [34], and may well be the causative agent underlying the decrease in lipid fluidity of pea thylakoid membranes incurred during in vitro aging. It is conceivable that thylakoids from bean have a higher radical scavenging capability than those from pea, and are thus more immune to lipid peroxidation and ensuing bilayer rigidification during in vitro aging.

It has recently been reported that there is a decline in the functional concentration of the cytochrome b-f_6 complex with advancing leaf senescence [35,36]. Moreover, Holloway et al. [36] have deduced from measurements of partial photochemical reactions and concentrations of electron carriers that in senescing barley leaves the rate-limiting step is the transfer of electrons from

plastohydroquinone to the cytochrome b-f_6 complex. However, they also note that the temporal correlation between loss of electron transport activity and the decline in cytochrome f is non-linear and suggest that factors in addition to the limiting concentration of the cytochrome b-f_6 complex render this partial reaction rate-limiting.

The in vitro experiments with pea thylakoids in which increased polarization of diphenyhexatriene was observed as the membranes aged [6] suggest that changes in thylakoid membrane fluidity might also contribute to the decline in non-cyclic electron transport with advancing senescence. However, it seems clear from the present study that, at least for bean leaf thylakoids, this is not the case. This contention is supported by the fact that there were no notable changes in fatty acid composition of the membranes with advancing senescence. In particular, the level of linolenic acid, which is highly unsaturated and would tend to maintain the membrane in a highly fluid state [37], remained characteristically high during senescence. It is also apparent that the mechanism of senescence for thylakoid membranes differs from that for plasma and microsomal membranes of plant tissue, for these latter membranes do sustain pronounced decreases in bulk lipid fluidity with advancing age [8–11].

Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada. D.G.M. is the recipient of an NSERC Postgraduate Scholarship.

References

1 Thomas, H. and Stoddart, J.L. (1980) Annu. Rev. Plant Physiol. 31, 83–111
2 Jenkins, G.I. and Woolhouse, H.W. (1981) J. Exp. Bot. 32, 989–997
3 Hackenbrock, C.R. (1981) Trends Biochem. Sci. 6, 151–154
4 Anderson, J.M. (1981) FEBS Lett. 124, 1–10
5 Takano, M., Takahashi, M.-A. and Asada, K. (1982) Arch. Biochem. Biophys. 218, 369–375
6 Ford, R.C., Barber, J. (1980) Photobiochem. Photobiophys. 1, 263–270
7 Ford, R.C. and Barber, J. (1983) Planta 158, 35–41
8 Borochov, A., Halevy, A.H. and Shinitzky, M. (1976) Nature (London) 263, 158–159
9 McKersie, B.D., Lepock, J.R., Kruuv, J. and Thompson, J.E. (1978) Biochim. Biophys. Acta 508, 197–212
10 Thompson, J.E., Mayak, S., Shinitzky, M. and Halevy, A.H. (1982) Plant Physiol. 69, 859–863
11 Legge, R.L., Thompson, J.E., Murr, P.D. and Tsujita, J. (1982) J. Exp. Bot. 33, 303–312
12 McRae, D.G. and Thompson, J.E. (1983) Planta 158, 185–193
13 Legge, R.L., Thompson, J.E., Baker, J.F. and Lieberman, M. (1982) Plant Cell Physiol. 23, 161–169
14 Kleinfeld, A.M., Dragsten, P., Klausner, R.D., Pjura, W.J. and Matayoshi, E.D. (1981) Biochim. Biophys. Acta 649, 471–480
15 Nichols, B.W. (1964) in New Biochemical Separations (James, A., Morris, L.S., eds.), pp. 321–337, D. van Nostrand, Toronto
16 Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 5, 600–608
17 Schlenk, H. and Gellerman, J.L. (1960) Anal. Chem. 32, 1412–1414
18 Khor, H.T. (1979) J. Chrom. 179, 225–226
19 McKersie, B. and Thompson, J.E. (1978) Plant Physiol. 61, 639–643
20 Buege, J.A. and Aust, S.D. (1978) Methods Enzymol. 52, 302–310
21 Arnon, D.I. (1949) Plant Physiol. 24, 1–15
22 Eze, J.M.O., Dumbroff, E.B. and Thompson, J.E. (1981) Physiol. Plant. 51, 418–422
23 Ford, R. and Barber, J. (1980) Photobiochem. Photobiophys. 1, 263–270
24 Golbeck, J.H., Martin, I.F. and Fowler, C.F. (1980) Plant Physiol. 65, 707–713
25 Klausner, R.D., Kleinfeld, A.M. Hoover, R.L. and Karnovsky, M.J. (1980) J. Biol. Chem. 255, 1286–1295
26 Peiser, G.D., Lizada, M.C. and Yang, S.F. (1982) Plant Physiol. 70, 994–998
27 Jenkins, G.I. and Woolhouse, H.W. (1981) J. Exp. Bot. 32, 467–478
28 Yamamoto, Y., Ford, R.C. and Barber, J. (1981) Plant Physiol. 67, 1069–1072
29 Borochov, H. and Shinitzky, M. (1976) Proc. Natl. Acad. Sci. USA 73, 4526–4530
30 Armond, P.A. and Staehelin, A. (1979) Proc. Natl. Acad. Sci. USA 76, 1901–1905
31 McKersie, B.D. and Thompson, J.E. (1979) Biochim. Biophys. Acta 550, 48–58
32 Lees, G.L. and Thompson, J.E. (1980) Physiol. Plant. 49, 215–221
33 Takahama, U. and Nishimura, M. (1976) Plant Cell Physiol. 17, 111–118
34 Mayak, S., Legge, R.L. and Thompson, J.E. (1983) Phytochem. 22, 1375–1380
35 Ben-David, H., Nelson, H. and Gepstein, S. (1983) Plant Physiol. 73, 507–510
36 Holloway, P.J., Maclean, D.J. and Scott, K.J. (1983) Plant Physiol. 72, 795–801
37 Bishop, D.G., Kenrick, J.R., Bayston, J.H., Macpherson, A.S., Johns, S.R. and Willing, R.I. (1979) in Low-Temperatures Stress in Crop Plants (Lyons, J.M., Grahma, D. and Raison, J.K., eds.), pp. 375–389