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Stabilization of Thylakoid Membranes by Spermine during Stress-induced Senescence of Barley Leaf Discs[1]

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ABSTRACT

The effect of spermine on photochemical activity and polypeptide composition of chloroplasts from barley leaf discs during senescence in the dark was studied. Chloroplast membranes did not show photosystem II activity after spermine treatment when water was the electron donor, but in the presence of diphenylcarbazide, this activity was observed. The diphenylcarbazide-stimulated photoreduction of dichloroindophenol was 3-fold greater in leaf discs incubated for 72 hours in spermine than in water. Photosystem I activity was reduced by about 90% within the first 24 hours in the spermine-treated samples. This reduction, however, was not due to a decrease in the photosynthetic unit size. A preferential loss of polypeptides other than those associated with photosystem II was observed during senescence of the leaf discs in water, but this loss was reduced by spermine. Spermine treatment also prevented the appearance of several additional chlorophyll proteins found in the controls during senescence. The results have been interpreted on the basis of the interaction of spermine with thylakoid membranes resulting in stabilization of membrane function during senescence.

The role of polyamines in plant cell metabolism is not fully understood, although their significance in biochemical processes such as protein synthesis (6, 11, 13) and RNA degradation (19) has been recognized. Polyamines have been shown to inhibit senescence in oat leaf protoplasts (1) and in whole leaves of a number of plant species (18), and to stabilize bacterial spheroplasts against lysis (29). In the preceding study (12) we found that of the polyamines tested spermine was the most effective in reducing Chl destruction and maintaining the thylakoid system within the chloroplasts of barley leaf discs during stress-induced senescence. It was suggested that the cationic polyamines exert their influence through interaction with the negatively charged loci on the membranes. We extended the investigation to examine the photoreductive activities and thylakoid membrane polypeptide composition of barley leaf discs incubated in spermine. The results, in general, provide further evidence that polyamines stabilize the chloroplast membranes, thereby preventing the loss of Chl during senescence.

MATERIALS AND METHODS

Preparation of Chloroplasts. The procedures used for the growth of plants, incubation of the leaf discs, and the isolation of chloroplasts were described in the preceding paper (12). The leaf discs were incubated in the dark in deionized H_2O or 0.1 mM spermine for 24, 48, or 72 h before analysis.

Photoreduction of DPIP[3]. The photoreduction of DPIP was measured by the decrease in *A* at 600 nm following irradiation with saturating red light. The reaction mixture had a final volume of 3.0 ml and contained 10 mM phosphate buffer (pH 7.4), 0.1 mM $MgCl_2$, 0.1 mM DPIP, and chloroplasts (2–5 mg Chl/ml). DPC was used at a concentration of 0.5 mM and DCMU at 5 μM.

PSI Light Saturation Measurements. PSI activity was assayed polarographically in the presence of DCMU using the TMPD-ascorbate couple and methyl viologen as previously described (12). Saturating red light at an energy of 1.5×10^6 ergs/cm^2·s was provided by a 500-w projection lamp fitted with a Corning 2-62 red filter. A water reservoir and a condensing lens focused the light onto the sample cuvette. A series of neutral density filters (Balzers) were used to vary the light intensity.

Sample Preparation for Electrophoresis. The chloroplast preparation was washed twice with 50 mM Hepes buffer (pH 7.6), 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and sedimented by centrifugation at 3,500*g* for 2 min. The pellet was then washed twice with a high salt buffer (0.5 M NaCl, 50 mM Hepes [pH 7.6], 2 mM ascorbate, 1 mM $MgCl_2$) to remove adhering soluble proteins such as ribulose bisphosphate carboxylase (20), and twice again in a magnesium-deficient buffer (50 mM Hepes [pH 7.6], 2 mM ascorbate, 1 mM EDTA, and 0.5 mM K_2HPO_4) to wash off the chloroplast-coupling factor (16). The intrinsic membrane proteins were solubilized by suspending the membrane pellet in 62.5 mM Tris-HCl (pH 6.7), 1% mercaptoethanol, 0.02% bromophenol blue, 10% glycerol, and 0.1% SDS for 30 min at 5 C prior to electrophoresis (SDS to Chl ratio was 10:1 [w/w]).

Electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out at 7 C using the discontinuous buffer system of Laemmli (22). Gels were prepared in glass tubes (0.6 × 12 cm) and consisted of a 9% running gel and a 3% stacking gel. The gels were prerun for 30 min at 1 mamp/gel prior to the application of the sample and were run at 1 mamp/gel until the sample reached the interface between stacking and running gels at which time the current was increased to 2 mamp/gel for the remainder of the run (about 8 h). After the run, the gels were scanned at 670 nm in a Beckman model 25 spectrophotometer to record the position of the Chl containing bands. The gels were then stained with Coomassie Brilliant Blue R (17) and scanned at 595 nm.

Chl and Protein Measurements. Chl was measured by the method of MacKinney (23). Chloroplast membrane protein was determined by the method of Bradford (10).

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[3] Abbreviations: DPIP: 2,6-dichloroindophenol; DCP, 1,5-diphenyl carbazide; TMPD: N,N,N'N'-tetramethyl-*p*-phenylenediamine; CPI: P-700 chlorophyll *a*-protein complex; CPII: light-harvesting chlorophyll *a*/*b*-protein complex; FC: free-running chlorophyll; Kd: kilodalton.

RESULTS

Chl to Protein Ratio. In both water control and spermine-treated leaf discs, there was a loss of Chl relative to thylakoid protein during senescence. The loss of Chl was much reduced with the spermine treatment (12) and the change of the Chl to protein ratio with time was only one-half that of the control (Fig. 1). Thus, the spermine treatment resulted in an increased stabilization of this ratio.

Photochemical Reduction of DPIP. Chloroplasts isolated from leaf discs treated with spermine for 24 h showed no photochemical reduction of DPIP with water as the electron donor while the control samples showed a steady decrease to about 5% of their original activity after 72 h on a leaf disc basis (Table I). With DPC as an artificial donor to PSII, however, the activity of the spermine-treated samples could be restored to about 50% of that of the controls after 24 h, and this level of activity remained constant throughout the remaining 48 h of the experiment. After 72 h the activity of the spermine-treated samples using DPC was about 3-fold greater than the controls on a leaf disc basis. On a Chl basis using DPC, 40% of the control activity was restored in the spermine-treated samples and again this level of activity was stable from the 24-h to the 72-h sampling period (Table I). On a thylakoid membrane protein basis, the DPC-stimulated photoreduction of DPIP increased significantly in control leaf discs during the 72 h of senescence, while this activity for the spermine-treated samples increased only slightly (Table I).

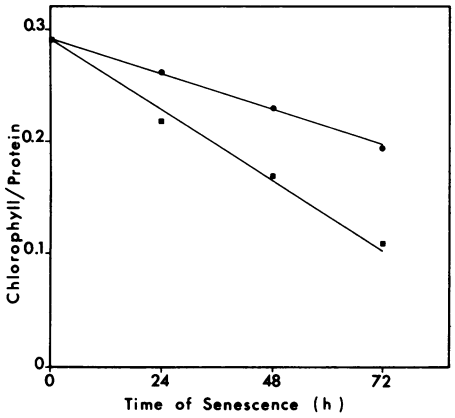

FIG. 1. Change in the Chl to thylakoid membrane protein ratio (μg/μg) of chloroplasts from barley leaf discs during senescence in 0.1 mM spermine (●) or water (■).

Table I. *Photochemical Reduction of DPIP by Chloroplasts from Barley Leaf Discs Incubated in 0.1 mM Spermine or Water*

Time	−DPC		+DPC[a]	
	Water	Spermine	Water	Spermine
h				
	μmol DPIP/leaf disc·h			
0	915		915	
24	374	0	426	251
48	97	0	147	233
72	46	0	70	228
	μmol DPIP/mg Chl·h			
0	312		312	
24	216	0	246	94
48	138	0	210	92
72	138	0	210	90
	μmol DPIP/mg protein·h			
0	106		106	
24	93	0	106	36
48	82	0	124	40
72	132	0	194	46

[a] Corrected for DCMU-insensitive photoreduction.

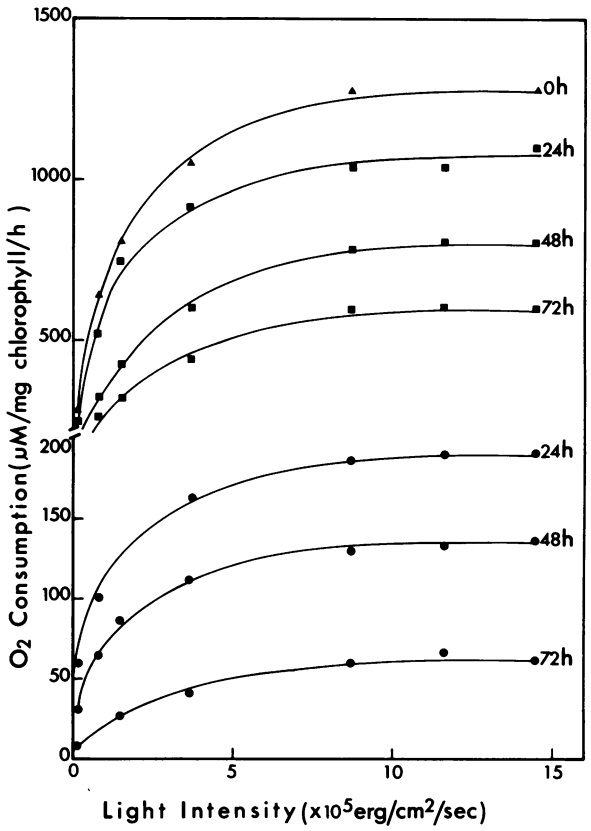

FIG. 2. PSI activity of isolated chloroplasts from barley leaf discs during senescence in 0.1 mM spermine (●) or water (■). (▲): Time 0, unincubated sample. PSI activity was measured by O_2 evolution from a 1-ml reaction chamber containing 3 mM sodium isoascorbate, 0.2 mM TMPD, 0.1 mM methyl viologen, 1 to 5 mg Chl, and 15 μM DCMU in 50 mM Hepes buffer (pH 7.6) containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$.

Light Saturation of PSI. The PSI activity measured under saturating light conditions decreased by only 15% after 24 h of incubation of the leaf discs in water, whereas it decreased by 85% after 24 h in spermine (Fig. 2). The relative reductions in the maximum PSI activity over the remaining 48 h of senescence were similar in both water and spermine-treated tissues. Throughout the study, the PSI activities of the water and spermine-treated samples showed identical light saturation characteristics, although there were considerable differences in the PSI efficiency.

Chl Proteins. Polyacrylamide gel scans indicating the positions of the Chl proteins are shown in Figure 3. The three characteristic Chl bands corresponding to the P-700 Chl *a*-protein complex (CPI) (14), the light-harvesting Chl *a*/*b*-protein complex (CPII) (30), and free-running Chl (FC) were obtained from samples before incubation. The apparent mol wt of these bands were 85 Kd, 27 Kd, and less than 13 Kd, respectively. True mol wt could only be determined if the samples were heated with SDS prior to electrophoresis, in which case the SDS entirely displaces the Chl and the mol wt of these proteins change considerably (8, 31). Throughout the 72 h of senescence, the scans of the spermine-treated samples changed very little apart from a decrease in the amount of CPI with respect to CPII in the first 24 h. In the controls, however, a major change characterized by the appearance of three additional Chl bands was apparent within the first 24 h. At 48 h, there was very little FC, the amount of CPI was approximately equal to that of CPII, and only one of the additional

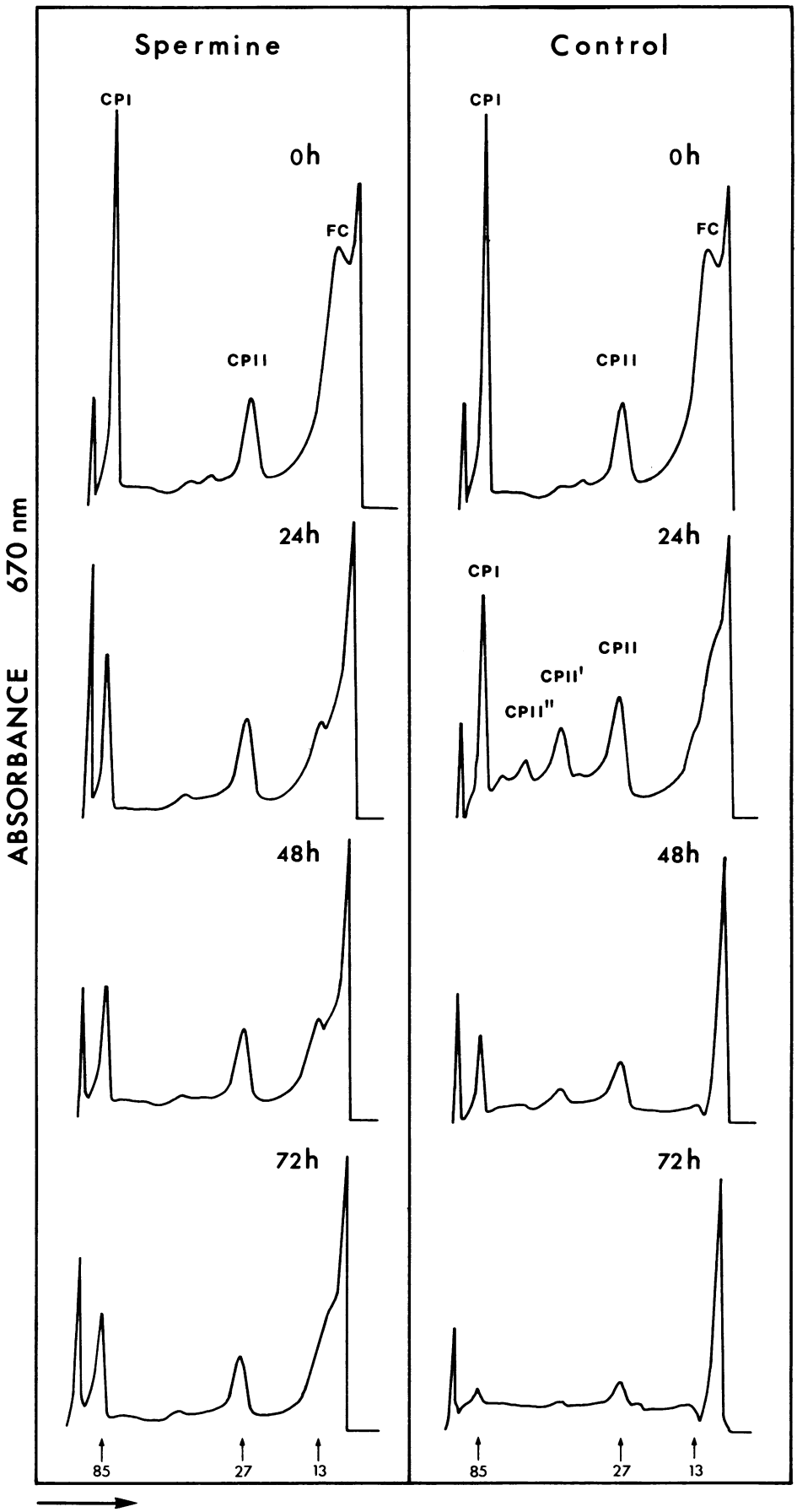

FIG. 3. SDS-polyacrylamide gel electrophoretic scans of Chl proteins extracted from chloroplast membranes of barley leaf discs incubated in the dark in 0.1 mM spermine or water. Samples equivalent to 150 μg of protein were applied to each gel. Gels were scanned at 670 nm immediately after the termination of electrophoresis.

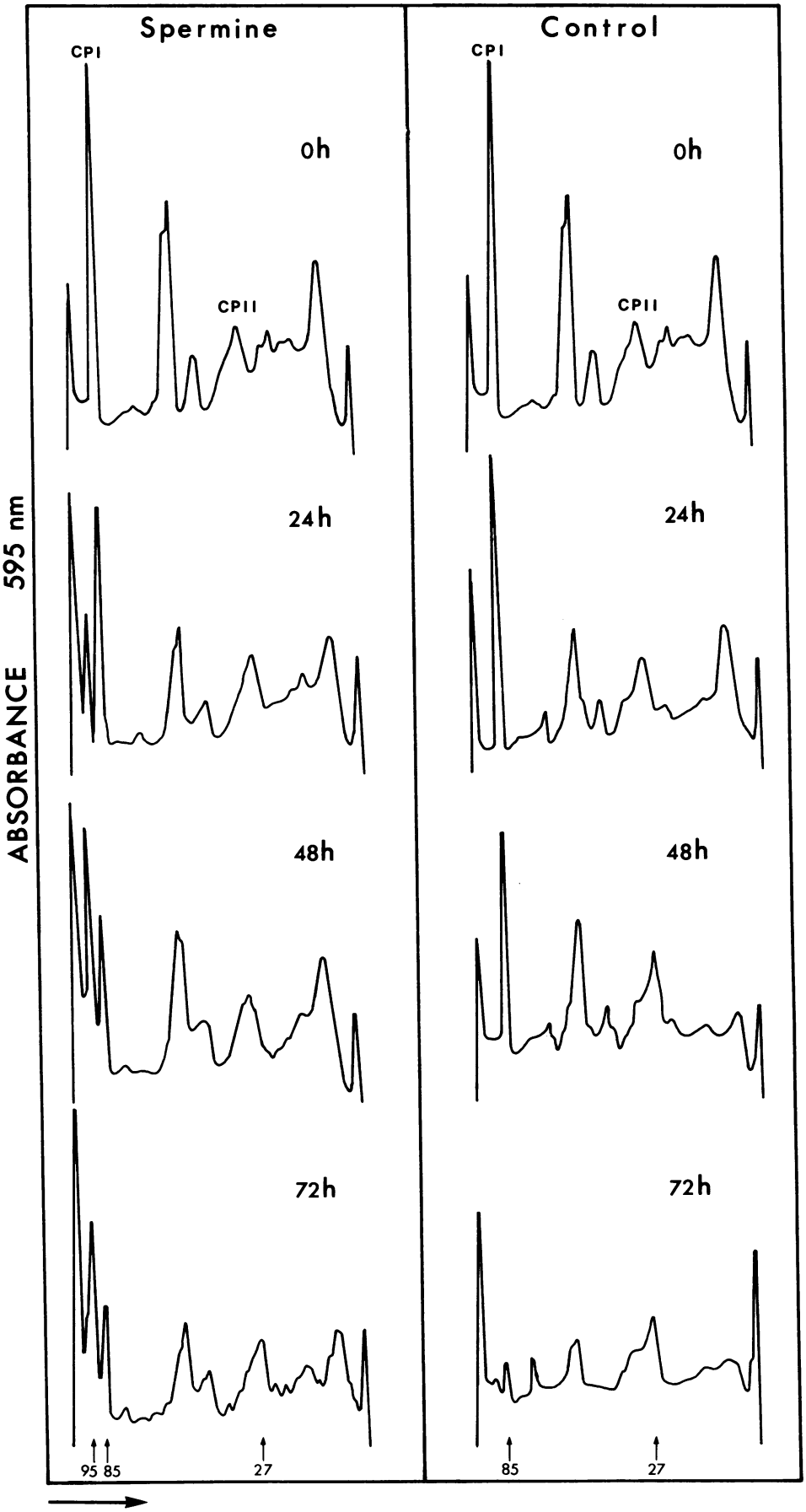

FIG. 4. SDS-polyacrylamide gel electrophoretic scans of chloroplast membrane proteins of barley leaf discs incubated in the dark in 0.1 mM spermine or water. Samples equivalent to 100 μg of protein were applied to each gel. Gels were scanned at 595 nm after staining.

Chl bands remained. After 72 h, most of the Chl still in the gels was associated with CPII.

Polypeptide Profiles. The polypeptide profiles of the thylakoid membranes are shown in Figure 4. Within 24 h of the spermine treatment, a new high mol wt band of major intensity appeared. This band had an apparent mol wt of about 95 Kd, and it was a major thylakoid polypeptide in these samples after 48 h of senescence. This high mol wt polypeptide, however, was never seen in the control samples. The membrane protein profiles of the control samples showed a continuous decrease in the intensity of the 85 Kd CPI band from 24 to 72 h while the band corresponding to CPII increased relative to the other membrane polypeptides. However, in the spermine-treated samples there was little change in the relative amounts of the membrane proteins with the exception of CPI and the newly formed high mol wt band (Fig. 4).

DISCUSSION

Polyamines have been reported to protect Chl and maintain the thylakoid membrane system in chloroplasts of barley leaf discs during stress-induced senescence (12). To elucidate further the mechanism of this action, we have characterized the effect of spermine on changes in thylakoid membrane polypeptides and photoreductive activities during senescence. We have shown that despite the stabilization of membrane structure, Chl content, and polypeptide pattern in spermine-treated tissues, a loss of photochemical activities occurs. The losses of membrane constituents such as Chl and intrinsic thylakoid protein which are associated with senescence were retarded *in situ* by spermine. The membranes were thus less accessible to the hydrolytic activities associated with senescence.

The restoration of PSII activity with DPC in spermine-treated leaf discs (Table I) indicated that the PSII trap remained intact but the oxidizing side of PSII was inhibited. This is supported by the fact that CPII was maintained as a major polypeptide in membranes of spermine-treated tissue (Figs. 3 and 4).

In the preceding study (12) electron microscopic analysis revealed that incubation of the leaf discs in spermine caused a separation of grana stacks into a series of single thylakoids. Similar effects have been noted by other authors when chloroplast membranes were washed with low ionic strength buffer lacking Mg^{2+} (26, 27). The unstacking of grana in Mg^{2+}-deficient buffer has been shown to cause a dramatic loss in PSII activity (9). Two possible mechanisms for the effect of spermine on PSII can be suggested. First, spermine as a polycation may compete with Mg^{2+} at the site of Hill activity. Second, spermine may bind directly to the polyanionic membranes causing a net repulsion of thylakoids within grana stacks. In addition to the unstacking of the grana membrane, conformational changes could have been sufficient to cause the loss of the Hill activity of PSII.

The activity of PSI was reduced by about 80% within the first 24 h of spermine treatment (Fig. 2). This loss of PSI activity, like that of PSII, might have been due to a competition with Mg^{2+} or to induced membrane conformational changes. Since Mg^{2+} is not known to have a major effect on PSI (9), it seems more likely that the decrease in activity due to the spermine treatment was a result of membrane conformational changes.

The data in Figure 2 show that although the over-all efficiency of the photochemical activities varied with treatment or time of senescence, the light intensity required to saturate the activity was identical in all cases. This indicates that the photosynthetic unit size did not change (7, 15, 21). Since Chl was lost during senescence, but the photosynthetic unit size apparently remained unchanged, one may conclude that complete photosynthetic units were being destroyed.

An increase in PSII activity on a thylakoid protein basis for both control and spermine-treated tissues during senescence was found (Table I). This could be explained by a preferential loss of membrane proteins other than those associated with PSII. This is probable since proteinase activities increase during senescence (25) and the PSII reaction center is thought to be on the internal side of the thylakoid membrane (3, 4, 28). This possibility is supported by the results in Figure 4 which show increased stability of CPII with respect to other membrane polypeptides, especially in control samples. In both the increase in PSII activity on a thylakoid protein basis during senescence (Table I) and the preferential loss of membrane polypeptides other than those associated with PSII (Fig. 4), the spermine-treated tissue showed a smaller change, providing additional evidence of the stabilizing influence of spermine on thylakoid membranes.

The appearance of a series of Chl-protein bands in the polypeptide profiles of control samples but not in the spermine-treated tissue is puzzling. It is similar to the finding by Aro and Valanne (5) that spinach membranes contained only the two bands characteristic of CPI and CPII when prepared in the presence of Mg^{2+}, but had additional Chl-protein bands in the absence of Mg^{2+}. In our experiments spermine appears to be acting like Mg^{2+} in preventing the appearance of the additional Chl-protein bands (Fig. 3).

Anderson *et al.* (2) and Markwell *et al.* (24) have recently characterized additional complexes from higher plants using similar electrophoretic procedures. The bands CPII′ and CPII″ (Fig. 3) are probably similar to $LHCP^2$ and $LHCP^1$ of Anderson *et al.* (2) and AB-1 and AB-2 of Markwell *et al.* (24), which represent a dimer and trimer, respectively, of CPII. Since the additional Chl-protein bands seen at 24 h in control samples (Fig. 3) were not seen before incubation, their appearance may, in this instance, be related to chloroplast degradation. After the 48 h of senescence of the control samples, most of the Chl in the membrane which was not bound to CPI or CPII was lost as indicated by the reduction of the FC band (Fig. 3). The similarity in appearance of Chl-protein bands from 0 to 72 h of senescence in spermine solution provides still further evidence for the stabilization of these membranes by spermine.

The recent literature regarding the biological significance of polyamines has been primarily restricted to effects on regulation of nucleic acid metabolism and protein synthesis (6, 11, 13). In this paper, we provide further evidence that spermine interacts directly with the internal membranes of the chloroplasts. The evidence suggests that the polyamine interacts with the membranes so that they become more stable to degradation during senescence. This is consistent with the finding that the spermine-treated leaf discs remained green long after the water control discs had blanched (12), and indeed, after 72 h of spermine treatment, the DPC-stimulated photoreduction of DPIP was 3-fold greater than the controls on a leaf disc basis (Table I). Further consideration of the biological role of polyamines must now take into account the possibility that these polycations can directly affect membrane processes in higher plants.

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