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Preparation and Properties of Chloroplasts Depleted of Chloroplast Coupling Factor 1 by Sodium Bromide Treatment[1]

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AYALA KAMIENIETZKY AND NATHAN NELSON
Department of Biology, Technicon-Israel Institute of Technology, Haifa, Israel

ABSTRACT

Chloroplasts were treated with 2 M sodium bromide. The resulting particles lost their ATPase activity and chloroplast coupling factor 1 subunits were detected in the supernatant by means of gel electrophoresis and specific antibodies. The chloroplast coupling factor 1 depleted particles show high rates of Hill reaction with pH optimum shifted toward lower pH. The sodium bromide treatment also abolished the light-induced proton uptake. In the presence of N-methylphenazonium methosulfate light-induced proton release, insensitive to uncouplers, was observed. Addition of dicyclohexylcarbodiimide reversed the light-induced pH changes to the normal proton uptake and increased the pH optimum of the Hill reaction.

The resolution of the energy-transducing system in chloroplasts met more difficulties than in mitochondria. In the latter, particles fully depleted of ATPase (F_1)[2] and active in electron transport are available (19). Chloroplast particles depleted of CF_1 were prepared by EDTA or silicotungstate treatment (10, 11). The EDTA-treated particles can be reconstituted by addition of CF_1; however, the degree of the reconstitution depends on the amount of CF_1 retained on the particles (H. Nelson and N. Nelson, unpublished observations; 10, 20). The electron transport properties of these particles were not damaged and they responded like uncoupled chloroplast (2, 8, 11). In silicotungstate-treated particles which are highly depleted of CF_1, the electron transport is badly damaged (10).

The need for highly resolved chloroplast particles (16) prompted us to look for a treatment which will deplete all of the CF_1 while the electron transport remains intact. It is the purpose of this communication to describe the preparation of NaBr-treated chloroplasts which are fully depleted of CF_1 while their electron transport remains intact.

MATERIALS AND METHODS

Tricine, digitonin, ATP, ADP, and BSA were obtained from Sigma. Acrylamide, methylenbisacrylamide, and SDS were obtained from Bio-Rad. Tricine, Tricine-maleate, and Tricine-MES buffers were prepared by adjusting the pH with NaOH.

Photophosphorylation (1), O_2 evolution (10), proton uptake (17), and ATPase activity (15) were performed by published procedures. [γ-^{32}P]ATP was prepared (15) and NADP photoreduction was measured as previously described (13). Gel electrophoresis in the presence of SDS was performed as described by Weber and Osborn (22). The gels were fixed, stained, and destained as previously described (14).

Preparation of Chloroplasts. About 60 g of lettuce (*Lactuca sativa* var. romaine) leaves were homogenized in a Waring Blendor at low speed for 5 to 8 sec in 200 ml of medium containing 0.4 M sucrose, 10 mM NaCl, 10 mM Tricine (pH 8), 20 mM sodium ascorbate, and 0.5 mg/ml of BSA (Sigma fraction V). The homogenate was filtered through gauze and centrifuged, with SS-34 rotor in RC2-B Sorval centrifuge, until it reached 3000 rpm. The precipitate was discarded, and the supernatant was centrifuged at 1500*g* for 7 min. The pellet was suspended in 10 mM Tricine (pH 8) and centrifuged at 20,000*g* for 5 min. The pellet was dispersed by glass-Teflon homogenizer in 5 ml of medium containing 0.4 M sucrose, 10 mM NaCl, 10 mM Tricine (pH 8), and 10 mg/ml of BSA.

Preparation of Sodium Bromide-treated Chloroplasts. Chloroplasts suspended in the above medium but without BSA were incubated with 2 M NaBr at 0 C for 30 min. The NaBr was added as 5 M solution. An equal volume of H_2O was added, and the suspension was centrifuged at 35,000*g* for 15 min. The pellet was suspended in medium containing 0.4 M sucrose, 0.01 M NaCl, 0.01 M Tricine, (pH 8), and 10 mg/ml of BSA to give a Chl concentration of about 1 mg/ml.

RESULTS

Chloroplasts treated with NaBr lost photophosphorylation and heat-activated Ca^{2+} ATPase activity (Fig. 1). Photophosphorylation was more sensitive than the ATPase to NaBr treatment. At 0.4 M NaBr about 60% of the Ca^{2+}-ATPase activity was retained while the photophosphorylation was almost fully inhibited. However, treatment with 2 M NaBr completely inhibited both reactions. Treatment of purified CF_1 with 2 M NaBr abolished its Ca^{2+}-ATPase activity. The supernatant of 2 M NaBr-treated chloroplasts was analyzed on SDS gel electrophoresis. Figure 2 shows that it contains bands in the positions of α, β, and γ bands of CF_1. These bands were so prominent that it appears that NaBr treatment removed preferentially the CF_1 or its above mentioned subunits. Further identification of CF_1-released subunits was carried out using their specific antibodies (14). The supernatant obtained after NaBr treatment produced on Ouchterlony plates precipi-

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[2] Abbreviations: CF_1: chloroplast coupling factor 1; F_1: mitochondrial coupling factor 1; SDS: sodium dodecyl sulfate; DCCD: dicyclohexylcarbodiimide; PMS: N-methylphenazonium methosulfate; FCCP: carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

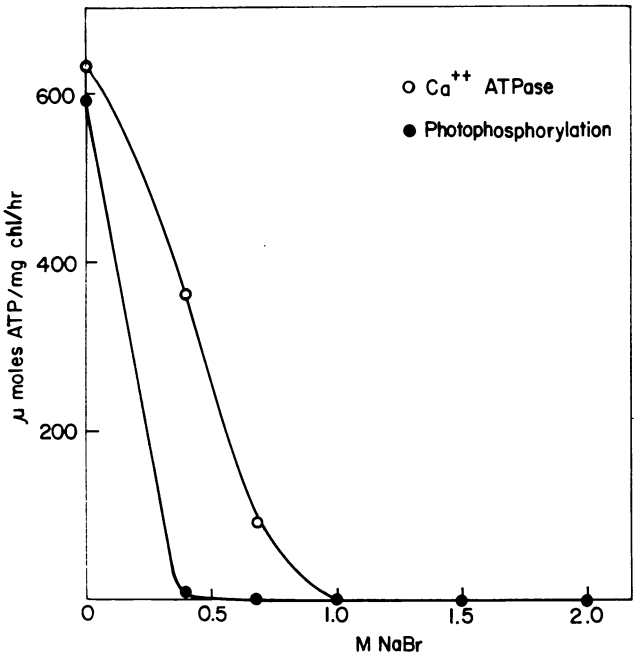

FIG. 1. Effect of sodium bromide treatment on photophosphorylation and Ca^{2+}-ATPase activities in chloroplasts. The reaction mixture for photophosphorylation contained the following in a final volume of 2 ml: 33 μmoles of Tricine (pH 8), 33 μmoles of NaCl, 13 μmoles of $MgCl_2$, 6.6 μmoles of sodium Pi (pH 8), 2 μmoles of ADP, 0.06 μmole of PMS, about 10^6 cpm of ^{32}Pi, and chloroplasts or NaBr-treated chloroplasts equivalent to 12 μg Chl. After 1 min of illumination by white light (2.3×10^5 ergs per cm^2 per sec) the reaction was stopped with 0.2 ml of 30% trichloroacetic acid, centrifuged, and the supernatant assayed for incorporation of radioactivity. The reaction mixture for Ca^{2+}-ATPase contained the following in a final volume of 1 ml: 30 μmoles of Tricine (pH 8), 4 μmoles of [γ-^{32}P]ATP, 8 μmoles of $CaCl_2$, and heat-activated chloroplasts or NaBr-treated chloroplasts equivalent to 9 μg Chl. After 10 min at 37 C, 0.1 ml of 30% trichloroacetic acid was added, and after centrifugation the liberation of ^{32}Pi was determined in the supernatant. Chloroplasts or NaBr-treated chloroplasts were heat activated at 64 C for 4 min in 0.9 ml of solution containing the following: 2.5 μmoles of Tricine (pH 8), 10 μmoles of ATP, 2.5 μmoles of DTT, 10 μmoles of sucrose, 0.3% digitonin, and chloroplasts equivalent to 300 μg of Chl.

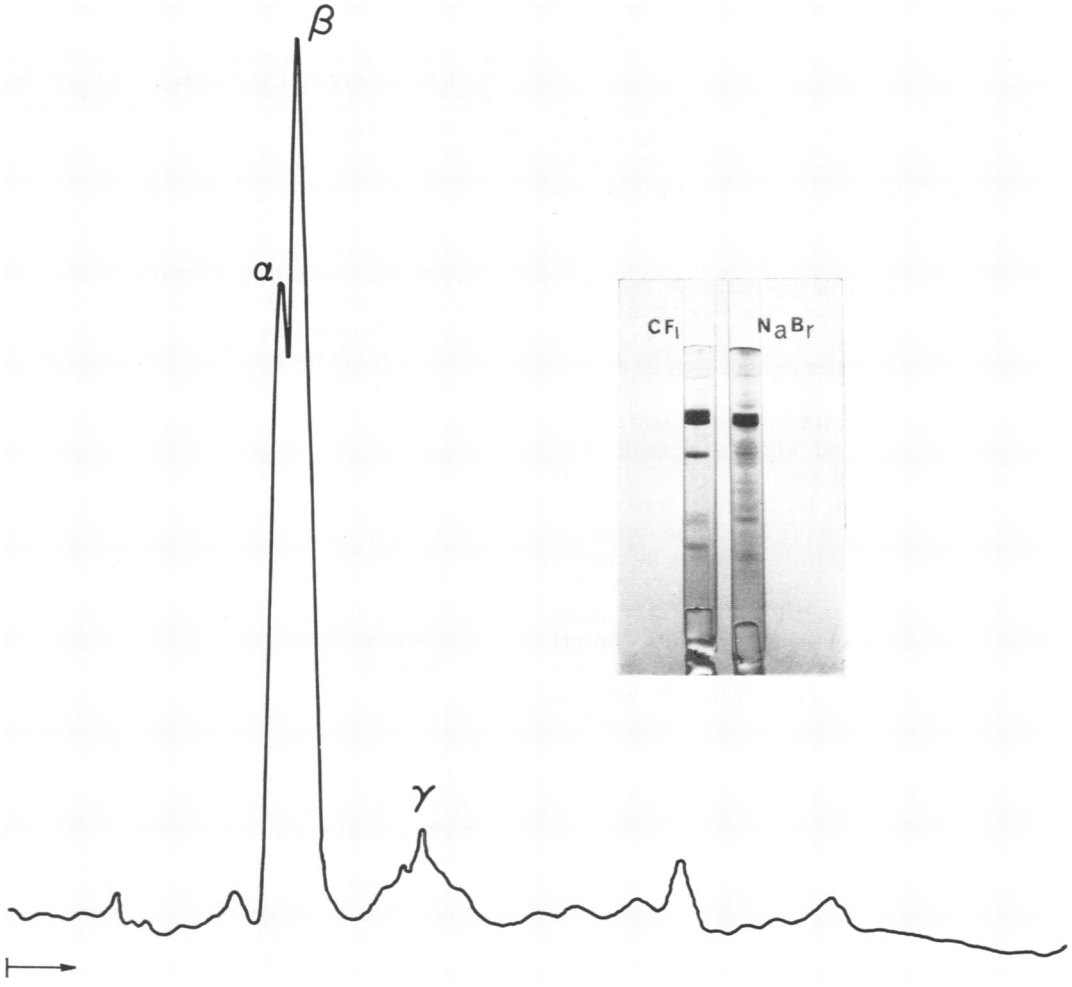

FIG. 2. SDS gel electrophoresis pattern of purified CF_1 and of the supernatant after sodium bromide treatment of chloroplasts. To 2 ml of the supernatant obtained after sodium bromide treatment of chloroplasts, 0.2 ml of 30% trichloroacetic acid was added. After centrifugation the pellet was mixed with 1 ml of acetone and centrifuged. The pellet was dissolved in 0.2 ml solution containing 10 μmoles tris, 2% SDS, 2% mercaptoethanol, and about 10% sucrose. Purified CF_1 (0.6 mg) was dissolved in 1 ml of the same solution. Fifty microliter samples were applied to the gels and run for 4.5 hr at constant current of 7 mamp. per tube.

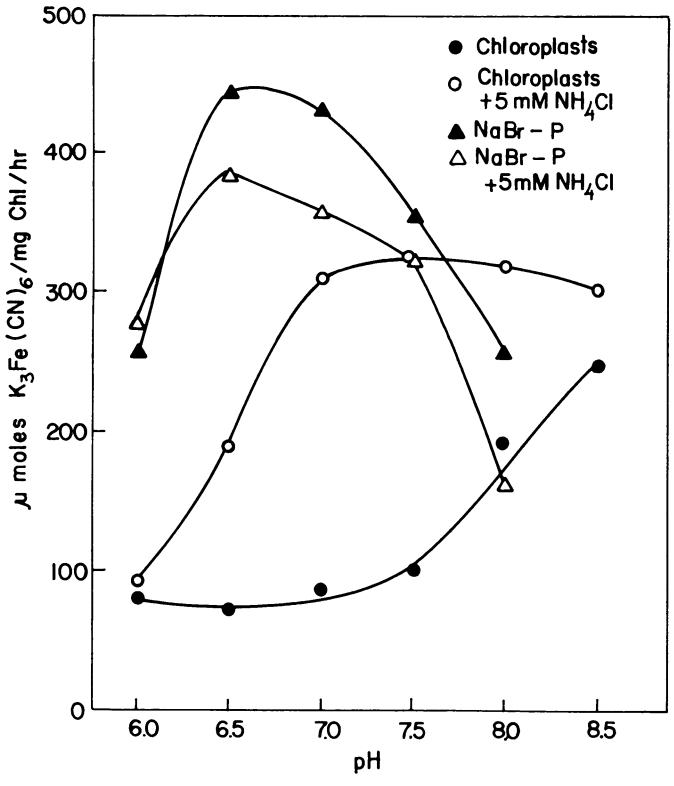

FIG. 3. Effect of sodium bromide treatment on the Hill reaction with ferricyanide. The reaction mixture contained the following in a final volume of 4 ml: 100 μmoles of Tricine-MES at the specified pH, 200 μmoles of NaCl, 10 μmoles of ferricyanide, and chloroplasts equivalent to 63 μg of Chl or 2 M NaBr-treated chloroplasts (NaBr-P) equivalent to 88 μg of Chl. The mixture was illuminated by white light (1.5×10^6 ergs per cm^2 per sec) and the O_2 evolution was determined with a Clark type electrode. The temperature was kept at 23 C by a thermostat and a water jacket.

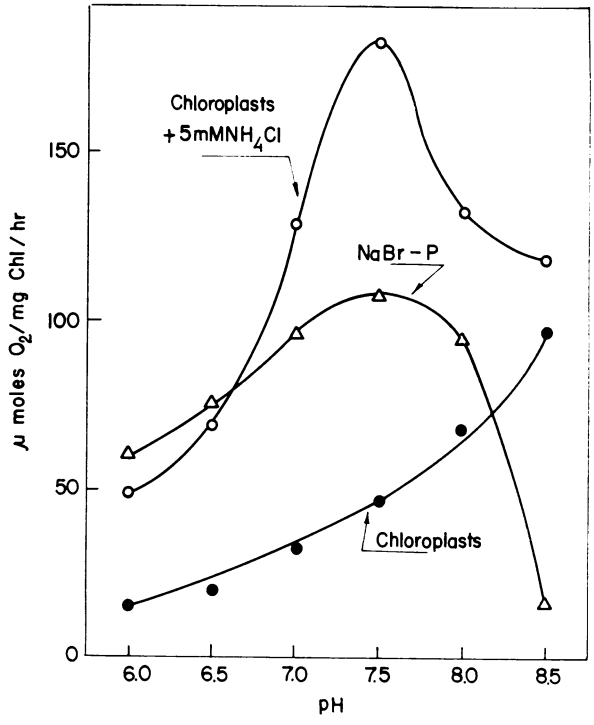

FIG. 4. Effect of sodium bromide treatment on the Hill reaction with methyl viologen. Experimental conditions were as described in Fig. 3 except the ferricyanide was omitted and 1 μmole of methyl viologen was added.

tation lines with the specific antibodies against each of the five CF_1 subunits.

The electron transport of the particles was measured and high specific activities were obtained with ferricyanide (Fig. 3) or methyl viologen (Fig. 4) as electron acceptors. The pH optimum for the Hill reaction with ferricyanide was shifted to pH 6.5 and with methyl viologen to pH 7.5. These values are similar to the pH optimum for electron transport in chloroplasts in the presence of uncouplers such as NH_4Cl. NADP photoreduction was actually accelerated by the NaBr treatment (Table I). Here, too, the pH optimum was shifted to pH 7 and addition of ferredoxin NADP-reductase did not alter the rate of NADP photoreduction. The reduction was absolutely dependent upon addition of ferredoxin to the reaction mixture and plastocyanin addition had no effect.

When DCCD was included in the reaction mixture, the NaBr particles behaved exactly like untreated chloroplasts. The pH optimum for the Hill reaction was shifted back to pH 8.5, and the electron transport was accelerated by uncouplers. When light-induced pH changes were measured with NaBr particles, a light-induced proton release was observed that was reversed in the dark (Fig. 5). The amount of the released protons was about half of the light-induced proton uptake in control chloroplasts. This proton release was insensitive to uncouplers and was completely dependent on PMS. Addition of DCCD reversed the proton movement to the original light-induced proton uptake which was sensitive to uncouplers.

The pH optimum for the light-induced proton release was at pH 7.5 (Fig. 6), whereas the pH optimum for proton uptake in control chloroplasts was about pH 6 (17).

The reversal of the light-induced pH change by DCCD in the NaBr particles was time-dependent. Incubation for about 15 min at room temperature was required for 5 μM DCCD to reverse the pH effect. Increasing concentrations of DCCD shortened the lag period (Fig. 7). Table II summarizes the effect of FCCP on light-induced pH changes and on the Hill reaction catalyzed by chloroplasts and sodium bromide-treated chloroplasts. FCCP abolished the proton uptake by chloroplasts and accelerated the Hill reaction with ferricyanide and methyl viologen. In NaBr particles FCCP inhibited both Hill reaction and light-induced proton release with the former more sensitive than the latter. The light-induced proton release

Table I. *Effect of Sodium Bromide Treatment on NADP Photoreduction*

The reaction mixture in final volume of 1 ml contained the following: 20 μmoles of Tricine-MES at the specified pH, 0.5 μmole of NADP, 60 μmoles of NaCl, 0.002 μmole of ferredoxin, and chloroplasts or 2 M NaBr-treated chloroplasts equivalent to 9 μg of Chl. The absorbance of the reaction mixture was determined at 340 nm before and after illumination for 2 min.

Particles	pH	NADP Photoreduction
		μmoles NADP/mg Chl·hr
Chloroplast	6	57
Chloroplast	7	131
Chloroplast	8	252
NaBr particles	6	120
NaBr particles	7	439
NaBr particles	8	285

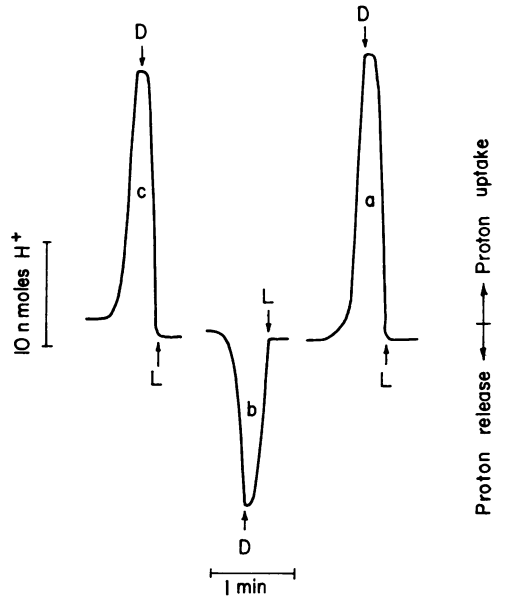

FIG. 5. Light-induced proton release by sodium bromide-treated chloroplasts. The reaction mixture contained the following in a final volume of 4 ml: 400 μmoles of NaCl, 0.5 μmole of Tricine, 0.06 μmole of PMS, and chloroplasts or NaBr particles equivalent to 30 μg Chl. The illumination and temperature control were as described in the legend of Fig. 3. The initial pH was 6.5. *a:* Chloroplasts; *b* sodium bromide particles; *c* NaBr particles; and 25 μM DCCD.

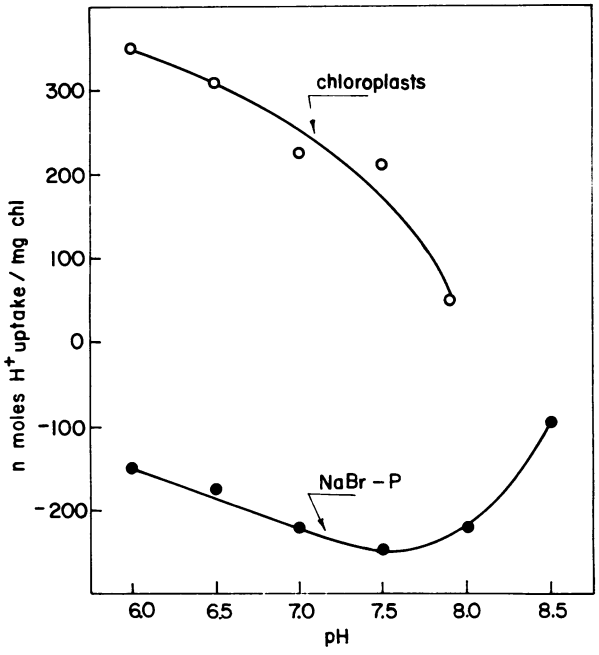

FIG. 6. Effect of pH on the light-induced proton release by sodium bromide particles. Experimental conditions were as described in Fig. 5. Chloroplasts equivalent to 50 μg of Chl or NaBr particles equivalent to 30 μg of Chl were added.

by NaBr particles was sensitive to DCMU (Table III). However, the proton release cannot be explained by reduction of PMS in the light and reoxidation in the dark, because in some experiments the amount of protons released was severalfold higher than the amount of PMS added.

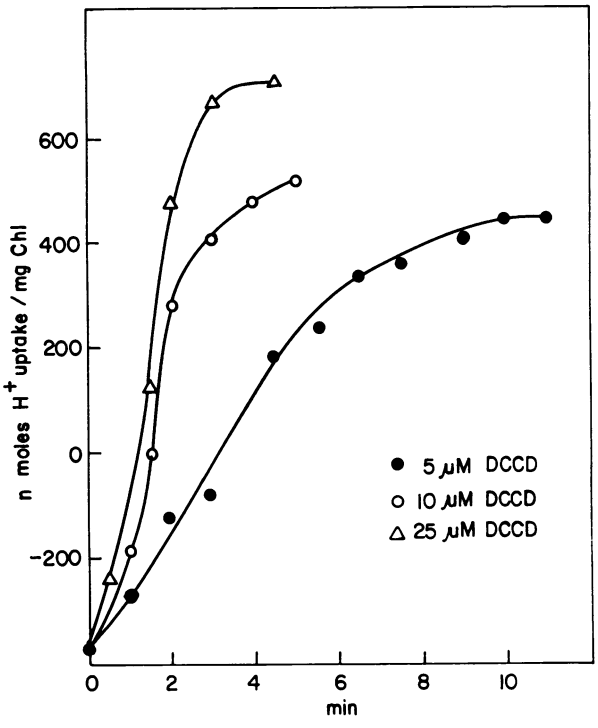

FIG. 7. Effect of DCCD on light-induced pH changes by sodium bromide particles. Experimental conditions were as described in Fig. 5. The initial pH was 6.5 and 2 M NaBr particles equivalent to 30 μg of Chl were added.

Table II. *Effect of FCCP on Hill Reaction and Light-induced Proton Release by Sodium Bromide Particles*

Proton uptake and proton release were measured as described in Fig. 6. Oxygen uptake with methyl viologen as electron acceptor was measured as described in Fig. 4. The reaction was carried out at pH 7. Chloroplasts equivalent to 40 μg of Chl and NaBr particles equivalent to 28 μg of Chl were used. A red filter (Corning No. 2403) was placed in the illumination light path and the light intensity at the level of the reaction mixture was 7.5 × 10^5 ergs per $cm^2 \cdot sec$.

Particles	FCCP	PMS Proton Uptake or Release	Methyl Viologen Oxygen Uptake
	μM	*nmoles H^+/mg Chl*	*μmoles O_2/mg Chl·hr*
Chloroplast	0	272	80
Chloroplast	2.5	130	214
Chloroplast	5.0	66	160
Chloroplast	7.5	0	133
Chloroplast	12.5	0	96
NaBr particles	0	−211	271
NaBr particles	2.5	−167	116
NaBr particles	5.0	−156	69
NaBr particles	7.5	−139	62
NaBr particles	12.5	−120	54

DISCUSSION

NaBr-treated chloroplasts lost all of their ATPase activity, which suggests that the particles are depleted of active CF_1. The SDS gels of the supernatant after the NaBr treatment showed bands in the positions of CF_1 subunits. The particles behave like uncoupled chloroplasts. The fact that this uncoupled state can be fully reversed by DCCD suggests that

Table III. *Effect of DCMU on Hill Reaction and Proton Release by Sodium Bromide Particles*

Experimental conditions were as described in Table II except that oxygen uptake with chloroplasts was carried out in the presence of 5 mM NH_4Cl and chloroplast equivalent to 32 μg of Chl and NaBr particles equivalent to 30 μg of Chl were added.

Particles	DCMU	PMS Proton Uptake or Release	Methyl Viologen Oxygen Uptake
	μM	*nmoles H^+/mg Chl*	*μmoles O_2/mg Chl·hr*
Chloroplast	0	310	171
Chloroplast	0.05	310	65
Chloroplast	0.1	305	40
Chloroplast	0.2	315	28
NaBr particles	0	−146	210
NaBr particles	0.05	−132	84
NaBr particles	0.1	−73	33
NaBr particles	0.2	−48	15

the uncoupling was a result of removal of CF_1 as observed with EDTA-treated chloroplasts (12). The only difference is that in EDTA particles a considerable amount of CF_1 remains attached to the membrane (10, 20). Addition of DCCD failed to induced any photophosphorylation in NaBr-treated chloroplasts. Since the uncoupled state was reversed by similar treatment, it might suggest that the particles were fully depleted of active CF_1.

Tzagoloff *et al.* (21) found that NaBr treatment depleted F_1 from submitochondrial particles. This is another remarkable similarity between mitochondrial F_1 and chloroplasts CF_1 (16).

The NaBr particles reduced methyl viologen and NADP at high rates. The fact that only addition of ferredoxin was required for NADP reduction suggests that not only were both photosystems not damaged but that sufficient ferredoxin NADP-reductase was left (13).

The pH optimum for Hill reaction with NaBr particles corresponds to the "membrane" pH optimum in isolated chloroplasts as calculated by Bamberger *et al.* (3).

NaBr particles show light-induced proton release in the presence of PMS. A similar kind of proton movement was found by Douglas and Packer (6) in submitochondrial particles and at low pH after harsh treatment. We also observed that, in the presence of PMS, addition of FCCP or NH_4Cl at high concentrations caused light-induced proton release.

The O_2 evolution of the NaBr particle was very sensitive to Triton X-100, FCCP, and tetraphenylboron. Tetraphenylboron and FCCP at high concentrations diminished the light-induced proton release but lower concentrations were required to inhibit the O_2 evolution. Both reactions were similarly sensitive to DCMU.

The fact that FCCP, at concentrations which strongly inhibited the O_2 evolution, failed to prevent the light-induced proton release might suggest that we are dealing with cyclic electron transport probably within photosystem II. The sensitivity to DCMU might support this suggestion. Pyocyanin failed to mediate this reaction probably because of its lower redox potential. The finding that there is proton release in NaBr particles under red light and under conditions when O_2 evolution is blocked by FCCP or tetraphenylboron ruled out the possibility that the pH change is caused by reduction of PMS in the light and reoxidation in the dark. Under these conditions photosystem I can provide a limited amount of electrons and thus can only give an effect which is two orders of magnitude lower than the observed one. It was frequently suggested that specific binding sites for protons play a role in the over-all light-induced proton movements in chloroplasts (4, 5, 9, 7). If the NaBr particles are permeable to protons and probably to other monovalent ions the light-induced proton release might be caused by sodium and H^+ exchange on specific sites within the membrane. In the light, Na will exchange bound protons which will result in pH decrease, while in the dark the reverse of this reaction takes place and the pH is rising again. Alternatively, light-induced conformational changes in the membrane may expose bound protons and release them to the aqueous medium. In the dark the conformation is reversed and the protonated functional groups are buried in the lipid phase of the membrane. This might also explain the light-induced changes in buffer capacity of chloroplasts observed by Polya and Jagendorf (18).

The NaBr particles might prove useful as a tool not only for the study of the energy transfer machinery but also to get better understanding of the interaction of protons with functional groups within the membrane.

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