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DELAYED LIGHT STUDIES ON PHOTOSYNTHETIC ENERGY CONVERSION

VII. EFFECT OF THE HIGH ENERGY STATE, COUPLED TO 2,3,5,6-TETRAMETHYL *p*-PHENYLENEDIAMINE-CATALYZED CYCLIC ELECTRON FLOW, ON MILLISECOND EMISSION FROM CHLOROPLASTS AND DIGITONIN SUBCHLOROPLAST PARTICLES

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SUMMARY

The effect of 2 3,5,6-tetramethyl *p*-phenylenediamine-catalyzed cyclic electron flow on millisecond delayed light emission from chloroplasts has been compared to the effect on subchloroplast particles. Non-cyclic electron flow of both chloroplasts and subchloroplast particles was blocked with 3-(3,4-dichlorophenyl)-1,1-dimethylurea. 2,3,5,6-tetramethyl *p*-phenylenediamine-catalyzed cyclic electron flow increased the millisecond delayed emission by 2–4 times in both chloroplasts and subchloroplast particles. Uncoupling conditions which collapse only the pH gradient component of the proton motive force reduced the 2,3,5,6-tetramethyl *p*-phenylenediamine stimulation of delayed light in chloroplasts but not in particles. The 2,3,5,6-tetramethyl *p*-phenylenediamine stimulation of delayed light in particles was sensitive to uncoupling conditions which are presumed to destroy the transmembrane potential. Energy transfer inhibitors were without effect on the 2,3,5,6-tetramethyl *p*-phenylenediamine stimulation in both chloroplasts and particles.

The 2,3,5,6-tetramethyl *p*-phenylenediamine stimulation of millisecond delayed emission appears to reflect the particular form of the proton motive force; in chloroplasts it seems to be correlated with the proton concentration gradient, whereas in particles it is more closely correlated with the transmembrane potential.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CF_1, chloroplast coupling factor$_1$; Diaminodurene, 2,3,5,6-tetramethyl *p*-phenylenediamine; DCCD, dicyclohexyl carbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TTFB, 4,5,6,7-tetrabromo-trifluoromethylbenzimidazole; TES, *N*-Tris(hydroxymethyl)-2-aminoethane sulfonic acid; Tricine, *N*-Tris (hydroxymethyl)methyl glycine.

INTRODUCTION

Mayne [1] originally suggested that the high energy coupling state of photophosphorylation might modulate the quantum conversion process at Photoreaction II. A number of observations have subsequently added weight to the idea that coupling state can somehow provide energy to increase the rate of the back reaction quantum conversion as measured by delayed light emission [2–8]. Experiments on emission stimulated by sudden shifts in pH [9–11], ionic strength [12], or salt addition [10, 13, 14] have suggested that a proton motive force (pmf) across thylakoid membranes can provide energy for delayed emission from Photoreaction II. In the present study experiments have been performed with chloroplasts where the pmf is mainly in the form of a chemical concentration gradient for protons [15–18], and with subchloroplast particles where the pmf is largely in the form of a transmembrane potential [19–21]. The experimental results, interpreted in terms of Mitchell's chemiosmotic coupling theory [22–25], suggest that the rate of the back reaction of quantum conversion can be increased by both components of the pmf; pH gradient and transmembrane potential.

MATERIALS AND METHODS

Preparation of chloroplasts and subchloroplast particles

Chloroplasts were prepared from either market spinach, greenhouse-grown Good King Henry (*Chenopodium bonicus henricus*), or 1–2 week old oat seedlings (*Avena sativa* var. Garry), according to Jagendorf and Avron [26]. Leaves were homogenized in a medium containing 0.4 M sucrose, 0.05 M *N*-Tris (hydroxymethyl) methyl glycine–NaOH (Tricine–NaOH), pH 7.8 and 0.01 M NaCl. The chloroplast pellet was resuspended in the sucrose–Tricine–NaCl buffer, washed once ($6500 \times g$ for 5 min) and finally resuspended in 0.1 M sucrose, 0.005 M TES–NaOH (*N*-Tris (hydroxymethyl)-2-aminoethane sulfonic acid–NaOH), pH 7.4 and 0.02 M NaCl or in sucrose–Tricine–NaCl buffer at a final concentration of 1 mg/ml chlorophyll. Chlorophyll concentration was determined according to Arnon [27].

EDTA-treated chloroplasts were prepared in the following manner; the chloroplast pellet (prepared as above) was resuspended in 0.1 M sucrose, 0.005 M TES–NaOH, pH 7.4 and washed once ($6500 \times g$ for 5 min). The chloroplasts were then suspended in 0.001 M EDTA, pH 7.5, 10 ml per mg chlorophyll, washed once ($27\,500 \times g$ for 5 min) and finally resuspended in the sucrose–TES–NaCl medium described above.

Subchloroplast particles were prepared from spinach, using digitonin, as described by Nelson et al. [20]. The fraction sedimenting between $10\,000 \times g$ and $90\,000 \times g$ was normally used. The subchloroplast particles were stored at -70 °C at a chlorophyll concentration of 1–2 mg/ml.

Measurement of light-induced proton uptake

The 5-ml sample contained in a temperature-controlled plexiglass chamber was illuminated with red actinic light. The light source was a 650 W tungsten-iodide lamp, whose output passed through 7 cm of water and a Corning red glass filter (CS2-62). The light intensity incident on the sample was $1.0 \cdot 10^6$ ergs/cm^2 per s.

Changes in pH were detected with an Ingold (Cat. 20371) combination electrode connected to a Corning Model 12 pH meter and displayed on a Sargent SRG strip recorder. They were calibrated by the addition of a standard aliquot of HCl.

Measurement of ATP formation

Cyclic photophosphorylation mediated by 2,3,5,6-tetramethyl *p*-phenylenediamine (diaminodurene) was measured by the proton uptake method of Nishimura et al. [28]. The apparatus employed was identical to the one described above for proton uptake measurements. In calculating rates of ATP formation it was assumed that 0.9 μmole of H^+ was consumed per 1 μmole of ATP formed.

Measurement of the light-induced 515 nm absorbance change

Light-induced changes in absorbance at 515 nm were monitored with an Aminco-Chance Dual Wavelength Spectrophotometer, using 540 nm as the reference wavelength. The light source was a 650 W tungsten-iodide lamp, whose output passed through 7 cm of water, a Schott KG-1 heat filter, and a Schott RG665 red glass filter. The light intensity incident on the sample was $8 \cdot 10^5$ ergs/cm^2 per sec. The photomultiplier tube was protected from stray exciting light by a saturated $CuSO_4$ solution and a Corning blue glass filter (CS4-96). All absorbance changes reported represent the initial rapid response induced by the exciting light (on-response).

Measurement of millisecond delayed light emission

Delayed light emission from 0.8–3.2 ms after the centers of repeating flashes of white light were measured with a modified Becquerel phosphoroscope as previously described [29, 30]. To increase the sensitivity of the measurement, the RCA 7102 photomultiplier previously employed was replaced by an RCA 8852 "Quantacon" photomultiplier tube. The sample was illuminated for 30 s at 10 % of full intensity, for 30 s at full intensity, and a photograph of the oscilloscope face was made. Diaminodurene was added to a final concentration equal to 0.25 mM and a second photograph was taken after a steady state was established.

Biochemicals

Diaminodurene was a gift from Dr. N. Good; fresh solutions were prepared daily. The nigericin-like antibiotic X-464 was a gift from Dr. W. E. Scott of Hoffmann-LaRoche. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were gifts from Dr. P. Heytler of Dupont. 3-thiocyanatoindole and the dichloro analogue of 4,5,6,7-tetrabromo-trifluoromethylbenzimidazole (TTFB) were kindly supplied by Dr. A. T. Jagendorf. Dicyclohexyl carbodiimide (DCCD) and gramicidin were obtained from K and K Laboratories. Digitonin was obtained from British Drug Houses. Valinomycin was purchased from Calbiochem. All other chemicals were reagent grade.

RESULTS

Effects of diaminodurene on millisecond delayed emission from chloroplasts

Fig. 1 shows that addition of diaminodurene to DCMU-poisoned chloroplasts results in an increase in the intensity of delayed light emission, with little effect on

TABLE I

EFFECT OF UNCOUPLERS ON THE DIAMINODURENE STIMULATION OF MILLISECOND DELAYED LIGHT EMISSION FROM SPINACH CHLOROPLASTS, AND ON PROTON UPTAKE, THE 515 nm SHIFT AND ATP FORMATION

The reaction mixtures contained: 25 mM KCl, either 25 mM Tricine–NaOH, pH 7.5 (515 nm shift and delayed light measurements) or 1 mM Tricine–NaOH, pH 7.5 (proton uptake and ATP formation), 2 mM $MgCl_2$, 0.01 mM DCMU and 0.25 mM diaminodurene. The chlorophyll concentrations employed in the various assays were as follows: proton uptake (30 μg/ml), ATP formation (30 μg/ml), 515 nm shift (30 μg/ml) and delayed light emission (10 μg/ml). N.D., not determined.

Additions	Large transient on addition of diaminodurene	Delayed light intensity at 1 ms (+ diaminodurene/ − diaminodurene)	H^+ uptake (μmoles per mg chlorophyll)	515 nm shift ($\Delta A \cdot 10^{-3}$)	ATP formation (μmoles per mg chlorophyll/h)
None	+	3.89	1.05	1.04	180
NH_4Cl (10^{-2} M)	−	1.94	0.20	0.96	22
X-464 ($2.5 \cdot 10^{-7}$ M)	−	1.57	0.20	1.04	7
Valinomycin (10^{-6} M)+KCl ($2.5 \cdot 10^{-2}$ M)	+	3.33	0.90	1.12	180
Valinomycin (K^+)+NH_4Cl	−	1.37	N.D.	0.72	0
Valinomycin (K^+)+X-464	−	1.18	N.D.	0.56	2
Gramicidin (10^{-6} M)	−	1.25	0.30	0	0

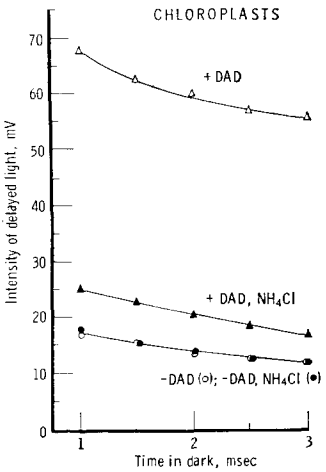

Fig. 1. Effect of NH_4Cl on the diaminodurene stimulation of millisecond delayed light emission from Good King Henry chloroplasts in the presence of DCMU. The reaction mixture (in 2 ml) contained; 50 mM NaCl, 50 mM Tricine–NaOH (pH 7.8), 0.01 mM DCMU, 0.25 mM diaminodurene and chloroplasts equivalent to 20 μg chlorophyll. NH_4Cl was present at $5 \cdot 10^{-3}$ M. DAD, diaminodurene.

millisecond dark-decay kinetics shown as previously reported [8]. On adding diaminodurene there is a transient increase in delayed light intensity of approx. 10-fold followed by a slow decline, over a 30 s period, to a steady state which is 2–4-fold higher than the level in the absence of diaminodurene. Inclusion of an uncoupler, NH_4Cl, prevented the large transient observed on the addition of diaminodurene, and Fig. 1 shows that the diaminodurene-induced increase in the steady state level was small in the presence of NH_4Cl when compared to the control situation (Fig. 1).

Table I shows the effect of several uncouplers on the diaminodurene stimulation of delayed light, as well as on light-induced H^+ uptake, the steady-state 515 nm absorption shift and on ATP formation. NH_4^+ and X-464 inhibited the diaminodurene stimulation, inhibited light-induced proton uptake and inhibited ATP formation, but had no effect on the light-induced 515 nm shift. The inclusion of valinomycin with either NH_4^+ or X-464 slightly inhibited the 515 nm shift and further inhibited the diaminodurene stimulation. Valinomycin (K^+), alone, did not affect any of the measured parameters. In contrast, gramicidin inhibited all of the measured parameters.

Effect of EDTA treatment on the diaminodurene stimulation and its reversal by DCCD

Removal of the chloroplast coupling factor (CF_1) by washing with low concentrations of EDTA (1 mM) inhibited both the diaminodurene stimulation and light-induced proton uptake in a parallel fashion. McCarty and Racker [31] and Uribe [32, 33] have shown that proton uptake in EDTA-treated chloroplasts can be restored by DCCD. We observed that addition of DCCD to EDTA-washed chloroplasts restored the diaminodurene stimulation of delayed light and proton uptake in a parallel fashion. This is shown in Table II. The larger diaminodurene effect in the EDTA-treated chloroplasts in the presence of DCCD was the result of a slight lowering of the delayed light intensity in the absence of diaminodurene.

TABLE II

EFFECT OF EDTA WASHING ON THE DIAMINODURENE STIMULATION OF MILLISECOND DELAYED LIGHT EMISSION FROM SPINACH CHLOROPLASTS AND ON PROTON UPTAKE

The reaction mixtures were similar to those described in Table I, except that KCl was replaced by NaCl.

Condition	Large transient on addition of diaminodurene	Delayed light intensity at 1 ms (+diaminodurene/ −diaminodurene)	H^+ uptake (μmoles per mg chlorophyll)
Control	+	3.69	0.77
Control + 10^{-4} M DCCD	+	3.84	0.67
EDTA-washed	−	1.41	0.08
EDTA-washed + 10^{-4} M DCCD	+	5.13	0.73

Effects of diaminodurene on millisecond delayed light emission from digitonin subchloroplast particles

Digitonin subchloroplast particles [20], are characterized by relatively ammonia-insensitive photophosphorylation and very little capacity for light-induced proton uptake. Addition of diaminodurene to DCMU-poisoned subchloroplast particles results in an increase in delayed light intensity of the same magnitude as that observed with chloroplasts (Fig. 2). The time-dependent changes in emission on the addition of diaminodurene were also similar. Addition of NH_4Cl to digitonin subchloroplast particles did not alter the diaminodurene stimulation (Fig. 2).

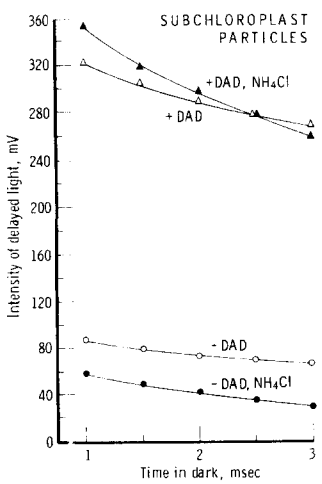

Fig. 2. Effect of NH_4Cl on the diaminodurene stimulation of millisecond delayed light emission from digitonin subchloroplast particles in the presence of DCMU. The reaction mixture (in 2 ml) contained; 50 mM NaCl, 50 mM Tricine–NaOH (pH 7.8), 0.01 mM DCMU, 0.25 mM diaminodurene and subchloroplasts equivalent to 20 μg chlorophyll. NH_4Cl was present at $4 \cdot 10^{-3}$ M.

TABLE III

EFFECT OF UNCOUPLERS ON THE DIAMINODURENE STIMULATION OF MILLISECOND DELAYED LIGHT EMISSION FROM DIGITONIN SUBCHLOROPLAST PARTICLES, AND ON PROTON UPTAKE, THE 515 nm SHIFT AND ATP FORMATION

The reaction mixtures were similar to those described in Table I, except that the chloroplasts were replaced by an equivalent amount of subchloroplast particles.

Additions	Large transient on addition of diaminodurene	Delayed light intensity at 1 ms (+diaminodurene/ −diaminodurene)	H^+ uptake (μmoles per mg chlorophyll)	515 nm shift ($\Delta A \cdot 10^{-3}$)	ATP formation (μmoles per mg chlorophyll/h
None	+	3.50	0.10	2.88	54
NH_4Cl (10^{-2} M)	+	4.57	0	2.56	33
X-464 ($2.5 \cdot 10^{-7}$ M)	+	4.30	0	2.72	33
Valinomycin (10^{-6} M) +KCl ($2.5 \cdot 10^{-2}$ M)	slow rise	2.64	0.10	1.20	42
Valinomycin (K^+)+NH_4Cl	−	1.59	0	1.44	0
Valinomycin (K^+)+X-464	−	1.00	0	0.88	0
Gramicidin (10^{-6} M)	−	1.00	0.03	0	0

TABLE IV

EFFECT OF DCCD ON THE DIAMINODURENE STIMULATION OF MILLISECOND DELAYED LIGHT EMISSION FROM OAT CHLOROPLASTS, PROTON UPTAKE AND ATP FORMATION

The reaction mixtures were similar to those described in Table I.

Condition	Large transient on addition of diaminodurene	Delayed light intensity at 1 ms (+diaminodurene/ −diaminodurene)	H^+ uptake (μmoles per mg chlorophyll)	ATP formation (μmoles per mg chlorophyll/h)
No additions	+	2.00	1.00	
+DCCD (10^{-4} M)	+	2.28	1.03	
+ADP ($5 \cdot 10^{-4}$ M), P_i($1 \cdot 10^{-3}$ M), Mg^{2+}($2 \cdot 10^{-3}$ M)	+	1.85		172
+DCCD, ADP, P_i, Mg^{2+}	+	2.00		0

An experiment similar to the one described in Table I was performed with subchloroplast particles and the results are shown in Table III. Neither NH_4^+ nor X-464 inhibited the diaminodurene stimulation or the 515 nm absorbance change, whereas they totally inhibited light-induced proton uptake and inhibited ATP formation by 40 %. The inclusion of valinomycin with either NH_4^+ or X-464 led to an inhibition of the diaminodurene stimulation, the 515 nm shift and ATP formation. Valinomycin (K^+), alone, inhibited the diaminodurene stimulation and the ATP synthesis slightly and inhibited the 515 nm shift. Gramicidin strongly inhibited the diaminodurene stimulation, the 515 nm shift and ATP formation. In other experiments we observed that the diaminodurene stimulation of millisecond emission in both chloroplasts and subchloroplast particles was inhibited by CCCP ($5 \cdot 10^{-6}$ M), FCCP ($1 \cdot 10^{-6}$ M), the dichloro analogue of TTFB ($7.5 \cdot 10^{-6}$ M) and 3-thiocyantoindole ($2 \cdot 10^{-4}$ M].

It should be noted that the amplitude of the 515 nm shift in subchloroplast particles in the presence of diaminodurene was routinely greater than that observed in chloroplasts. This result should be contrasted with the observations of Neumann et al. [21] with PMS.

Effects of coupling conditions and energy transfer inhibitors on the diaminodurene stimulation

With chloroplasts that were actively phosphorylating, the inclusion of ADP and P_i in a reaction mixture for the delayed light measurement resulted in a slight attenuation of the diaminodurene stimulation, both on the large transient and on the steady-state level (Table IV). DCCD added to normal chloroplasts acts as an energy transfer inhibitor [31]; when added alone to DCMU-poisoned chloroplasts, DCCD slightly enhanced proton uptake and the diaminodurene stimulation. In the presence of DCCD, ADP and P_i did not affect the diaminodurene stimulation. Similar effects were observed with digitonin subchloroplast particles.

DISCUSSION

The stimulation of millisecond delayed light emission observed on the addition of diaminodurene to DCMU-poisoned chloroplasts or digitonin subchloroplast particles appears to be related to the formation of the high-energy state of phosphorylation. This interpretation is based on the sensitivity of the stimulation to uncouplers and ionophorous antibiotics. Diaminodurene, in the presence of DCMU, is a mediator of cyclic electron flow [34]. The high-energy state generated by this cyclic electron transport appears to be modulating the production of delayed light by Photoreaction II, although Photoreaction II in the presence of DCMU is clearly not running the cyclic system.

According to the chemi-osmotic hypothesis of Mitchell [22–24], the high-energy state is equivalent to an electrochemical activity gradient for protons across a membrane, the pmf. The pmf has a chemical concentration term (ΔpH) and an electrical term ($\Delta\Psi$) due to charge differences across the membrane. ATP synthesis in chloroplasts appears to depend almost entirely on the chemical concentration component since the membrane potential is small [15–18, 35]. In contrast, ATP production in subchloroplast particles seems to depend on membrane potential [19–21], the pH gradient being small.

The diaminodurene stimulation of delayed light in chloroplasts seems to depend mainly on light-induced uptake of protons; uncouplers which dissipate a pH gradient, such as NH_4^+, nigericin, etc. [15], prevent the diaminodurene stimulation (Fig. 2; Table I). This hypothesis is further supported by the experiment on EDTA uncoupling and its reversal by DCCD (Table II). The addition of DCCD restores both H^+ uptake and the diaminodurene stimulation in a parallel fashion. A similar observation was made with respect to the diaminodurene-induced quenching of fluorescence in EDTA-uncoupled chloroplasts [17].

The delayed light emission of subchloroplast particles, with low proton uptake, is stimulated by diaminodurene to the same extent as chloroplasts (Fig. 2). Uncouplers such as NH_4^+ and nigericin, which dissipate ΔpH, had no effect on the diaminodurene stimulation (Table III). The combination of valinomycin with ammonia or with nigericin apparently reduces the membrane potential, as indicated by an attenuated 515 nm shift, and also inhibits the diaminodurene stimulation of delayed light. These results tend to suggest that the diaminodurene stimulation in particles is associated with the proton motive force mainly in the form of a membrane potential. Sherman [36] also observed a correlation between membrane potential and a diaminodurene stimulation of delayed light intensity with bacterial chromatophores.

Valinomycin, alone, attenuated the diaminodurene stimulation in subchloroplast particles slightly (25 %) while inhibiting the 515 nm shift to a greater extent. The larger effect on the 515 nm shift could be associated with a valinomycin-induced stimulation of H^+ uptake, thus increasing ΔpH and decreasing $\Delta\Psi$ as reported by McCarty [37] for subchloroplast particles and D-144 particles in the presence of PMS at pH 6.2. However, addition of valinomycin (K^+) to our digitonin subchloroplast particles at pH 7.5, did not enhance proton uptake (unpublished observations).

Uncouplers such as the cyanide carbonyl phenylhydrazones and gramicidin, which can dissipate both ΔpH and $\Delta\Psi$ [15, 38] largely prevent the diaminodurene stimulation in both chloroplasts and in subchloroplast particles.

The phosphorylating reagents (ADP, P_i, Mg^{2+}) had a small effect on the diaminodurene stimulation in both chloroplasts and particles (Table IV). In systems which have not been poisoned with DCMU, the inclusion of ADP, P_i, Mg^{2+} in reaction mixtures seems to have a somewhat larger effect on attenuating the emission in the presence of a non-cyclic acceptor [1, 7, 8]. Energy transfer inhibitors, alone, slightly enhance the diaminodurene stimulation and also block the small lowering effect of ADP/P_i. The diaminodurene-induced quenching of prompt fluorescence [17] responds to energy transfer inhibitors in a similar fashion (Cohen, W. S., unpublished observations). The observation that ADP/P_i have a smaller effect on the diaminodurene stimulation than uncouplers could be explained by a slower decay of the high-energy state via the coupling mechanism. This hypothesis is supported by measurements of ΔpH and proton efflux under coupling conditions [39, 40] and in the presence of various uncouplers [40–42].

Crofts et al. [6] have suggested that the pmf (ΔpH and $\Delta\Psi$) could modulate the intensity of delayed light emission in the millisecond time range. Our data suggest that in chloroplasts the pmf, mainly in the form of a proton concentration gradient, is the dominant factor whereas in digitonin subchloroplast particles the membrane potential is the regulating factor. Neumann et al. [7] have implied that the magnitude of the delayed light emission reflects the size of the pmf in chloroplasts and since

the pmf is mainly in the form of ΔpH in chloroplasts, our results are in general agreement with those of Neumann. The effects of diaminodurene on prompt fluorescence, which are also related to the high energy state, can be interpreted solely in terms of the proton concentration gradient. In chloroplasts [17], subchloroplast particles [17] and chromatophores [43], the degree of diaminodurene-induced quenching appears to be a reflection of either ΔpH or the internal pH of the thylakoid.

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