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The Stacking of Chloroplast Thylakoids

Quantitative Analysis of the Balance of Forces between Thylakoid Membranes of Chloroplasts, and the Role of Divalent Cations

M. J. SCULLEY,[1] J. T. DUNIEC, S. W. THORNE,[2]
W. S. CHOW,[3] AND N. K. BOARDMAN

CSIRO, Division of Plant Industry, P.O. Box 1600, Canberra, Australian Capital Territory, 2601, Australia

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An analysis is made of the van der Waals dispersion attractive forces and electrostatic repulsive forces between the grana thylakoid membranes of chloroplasts. These forces are determined for negatively charged surfaces with a pK_a value of 4.7 for a bulk pH of 7.0 with a range of mono- and divalent cation concentrations and intermembrane spacing in the range 10 to 80 Å. For equilibrium under dark conditions, it is concluded that either there is extensive electrostatic binding of divalent cations (Mg^{2+}) to the negatively charged membrane groups (phospholipid, sulfolipid, and protein carboxyl), or a redistribution of these groups between stacked and unstacked regions must be invoked.

A unique feature of the higher plant chloroplast is the differentiation of the internal membranes (thylakoids) of the chloroplast into stacked (grana) and unstacked regions. If isolated chloroplasts are resuspended in appropriate media of low ionic strength, the close contact between thylakoids in the grana regions is lost, but the grana regions may be reconstituted by addition of cations to the medium (1). Divalent cations are more effective than monovalent cations in the reconstitution. When dark equilibrated chloroplasts are irradiated with light, there is an uptake of protons by the thylakoids from the medium, accompanied by an efflux of Mg^{2+} ions from the thylakoids (2). From these and other studies, it is concluded that Mg^{2+} ions play an important role in stacking of thylakoid membranes. Cations also regulate the distribution of quanta of radiation between the two photosystems to give a balanced input of quanta between the reaction centers of photosystem I and photosystem II (3–5). Several authors have suggested that the close appression of thylakoids in the grana is necessary for the regulation of excitation energy distribution but the evidence is not conclusive (5).

In dark-adapted chloroplasts, grana thylakoid stacks are in equilibrium with a balance between attractive and repulsive forces between adjacent thylakoid membranes. The attractive forces between lipoprotein membranes are van der Waals dispersion forces and possibly osmotic forces, while the repulsive forces are electrostatic in nature due to the excess negative surface charge on the thylakoids. At very short range (<25 Å) further repulsive forces are operative between lipoprotein membranes due to structural or hydration effects (ion hydration shells). Osmotic effects are not likely to be of significance, because of the ion effects observed with low salt chloroplasts.

In an earlier investigation (6), the effects of monovalent and divalent cations on the electrostatic forces between charged lipid membranes were analyzed quantitatively.

[1] Department of Applied Mathematics, Institute of Advanced Studies, Research School of Physical Sciences, Australian National University, P.O. Box 4, Canberra, A.C.T., 2600, Australia.

[2] To whom all correspondence and requests for reprints should be addressed.

[3] Present address: Department of Botany, Imperial College, London SW 7, United Kingdom.

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In particular, an explanation was provided for the experimental observations of Gross and Prasher (7) and Boardman and Thorne (8) with ion-free chloroplasts. Low concentrations of monovalent ions caused an unstacking of the grana thylakoids, while restacking occurred on addition of divalent cations. Although it is clear that divalent cations stabilize grana thylakoids under dark conditions, the mechanism of stacking was not explored in detail, since only electrostatic repulsive forces were considered as a function of the following parameters; pK_a of membrane surface groups, pH, concentration of monovalent and divalent cations in the medium, with an assumed noninteraction with surface anions and distance between membranes. The presence of balancing attractive forces between the membranes was assumed under "high salt" conditions.

In this paper, an analysis is made of the van der Waals dispersive attraction and the electrostatic repulsion between lipoprotein membranes, under conditions that are relevant to the chloroplast thylakoid membranes. We conclude that for equilibrium of grana thylakoid stacks in the dark, a considerable fraction of the negatively charged surface sites of the lipoprotein membrane must be charge-neutralized by adsorption or electrostatic binding with divalent cations (Mg^{2+}). Alternatively, a redistribution of the charge between stacked and nonstacked regions must occur.

CALCULATIONS

The van der Waals Force

Charge fluctuations occur at every point in a material body due to thermal agitation or because of natural uncertainties in the positions and momenta of atomic nuclei. It is the interaction of the electric charges and currents during these fluctuations that constitute the van der Waals force.

The first rigorous analysis of the interaction between two macroscopic bodies was derived by Dzyaloshinskii, *et al.* (9). Using quantum field theory they obtained an expression for the force between two semi-infinite bodies of uniform isotropic substances separated by a thin planar slab. Israelachvili (10) has written a thorough review on van der Waals forces in biological systems.

Parsegian and Ninham (11) extended this theory to the case which is of relevance to granal membranes, i.e., the interaction between two thin planar slabs immersed in an aqueous medium.

Neglecting retardation effects due to the finite propagation speed of the electromagnetic wave their expression (Eq. [8] of Ref. (11)) can be written, with x a variable (Eq. [3] of Ref. (11)), as

$$G(d_a,d_m,T) = \frac{kT}{4\pi d_a^2} \times \sum_{n=0}^{\infty}{}' \int_0^{\infty} x\{\ln(1 - \Delta^2(d_m)e^{-2d_a x})\}\,dx \quad [1]$$

$$\Delta(d_m) = \frac{[\epsilon_a(i\xi_n) - \epsilon_m(i\xi_n)][1 + e^{-2d_m x}]}{\left|\epsilon_a(i\xi_n) + \epsilon_m(i\xi_n) + \frac{(\epsilon_a(i\xi_n) - \epsilon_m(i\xi_n))^2}{(\epsilon_a(i\xi_n) + \epsilon_m(i\xi_n))} e^{-2d_m x}\right|}, \quad [2]$$

where $G(d_a,d_m,T)$ is the van der Waals free energy of interactions between two planar membranes of thickness, d_m, separated by an aqueous layer of thickness d_a at temperature T; k is Boltzmann's constant and ϵ_m and ϵ_a are the dielectric permittivities of the membranes and the aqueous region, respectively.

The sum in Eq. [1] is taken over the discrete angular frequencies

$$\xi_n = \frac{[2\pi kT]}{\hbar} n, \quad n = 0, 1, 2 \ldots, \quad [3]$$

where $2\pi\hbar$ is Planck's constant, and for the $n = 0$ term only a multiplier of 1/2 is included. These frequencies arise out of the mathematical analysis and are not to be confused with photon frequencies. Equation [1] states that the van der Waals interaction is due to the difference in material polarizabilities as measured by the function Δ at each frequency ξ_n.

Expanding the logarithm in Eq. [1] and retaining only leading terms we have

$$\ln(1 - \Delta^2(d_m)e^{-2d_a x}) \approx -\left[\frac{\epsilon_a(i\xi_n) - \epsilon_m(i\xi_n)}{\epsilon_a(i\xi_n) + \epsilon_m(i\xi_n)}\right]^2 \times [e^{-2d_a x} + e^{-(2d_a+4d_m)x} + 2e^{-2(d_a+d_m)x}] \quad [4]$$

Substituting this expression in Eq. [1], we have for the energy per unit area of the interacting layers the approximate expression

$$G(d_a,d_m,T) = \frac{A}{12\pi}\left|\frac{1}{d_a^2} - \frac{2}{(d_a + d_m)^2} + \frac{1}{(d_a + 2d_m)^2}\right|, \quad [5]$$

where A, the effective Hamaker constant, is given by

$$A = \frac{3kT}{2}\sum_{n=0}^{\infty}{}' \left|\frac{\epsilon_a(i\xi_n) - \epsilon_m(i\xi_n)}{\epsilon_a(i\xi_n) + \epsilon_m(i\xi_n)}\right|^2. \quad [6]$$

Differentiating Eq. [5] with respect to d_a, we obtain for the effective force per unit area between the interacting plates

$$F_a = \frac{A}{6\pi}\left|\frac{1}{d_a^3} - \frac{2}{(d_a + d_m)^3} + \frac{1}{(d_a + 2d_m)^3}\right|. \quad [7]$$

In order to calculate the van der Waals force between two thylakoid membranes we require a compound model for the dielectric permittivities ϵ_m and ϵ_a.

In principle these functions give complete information concerning the strength and location of the energy absorption spectrum for all frequencies from zero to X-ray frequencies. Such information is not completely available, however it has been shown (12) that on the imaginary frequency axis the functions can be adequately represented by

$$\epsilon(i\xi) = \frac{\sum C_{rot}}{(1 + \xi/\xi_{rot})} + \frac{\sum C_j}{1 + (\xi/\xi_j)^2} + 1. \quad [8]$$

The first term describes simple Debye rotational relaxation and the second Lorentz harmonic oscillation dispersion. The constants C_{rot} and C_j give the strength of absorption at the frequencies ξ_{rot} and ξ_j and can be obtained by fitting Eq. [8] to the dielectric data.

This has been carried out by LeNeveu *et al.* (13) who obtained for the aqueous dielectric permittivity

$$\epsilon_a(i\xi) = \frac{74.8}{[1 + \xi/6.5 \times 10^{-5}]} + \frac{1.464}{[1 + (\xi/0.021)^2]} + \frac{0.737}{[1 + (\xi/0.067)^2]} + \frac{0.153}{[1 + (\xi/0.092)^2]} + \frac{0.014}{[1 + (\xi/.2)^2]} + \frac{0.075}{[1 + (\xi/.42)^2]} + \frac{0.78}{[1 + (\xi/12.7)^2]} + 1 \quad [9]$$

where ξ is measured in electron volts.

Measurements show that thylakoid membranes consist of about 50% proteins and 50% lipids, (14). The mean dielectric permittivity of such a membrane will consist of a sum of the permittivities of each component. To a first approximation we consider the proteins to be cylinders spanning the membrane. The dielectric permittivity of the whole membrane is given by

$$\epsilon_m = \nu_p\epsilon_p + (1 - \nu_p)\epsilon_h \quad [10]$$

where ν_p is the volume fraction of proteins in the membrane (15).

The dielectric permittivity of the lipid component can be accurately described by that for hydrocarbon. This is known to be essentially constant from zero to optical frequencies with a value of approximately $\epsilon_h = 2$. The complicated and incompletely known ultraviolet spectrum can be summarized for the near ultraviolet by the first ionization energy of 10.4 (eV) giving (12)

$$\epsilon_h = 1 + 1/[1 + (\xi/10.4)^2]. \quad [11]$$

The permittivity value for protein is larger than that for lipid but has a similar frequency dependence. Following Eq. [11] we will assume a single ionization energy of 10 eV giving

$$\epsilon_p = 1 + C_1/[1 + (\xi/10)^2] \quad [12]$$

where C_1 is a constant determined by the

strength of the permittivity. The static dielectric permittivity for protein is given by Pethig (16) as $\epsilon_p \approx 2-3$. Substituting $\xi = 0$ in Eq. [12], this gives a range of C_1 from 1–2.

Values of ϵ_h and ϵ_p determined by analysis from Eqs. [11] and [12] were substituted in Eq. [10] to give the mean value of membrane permittivity ϵ_m by taking the volume fraction ν_p in the range 0.4 to 0.6. From Eqs. [3] and [9] values of the aqueous permittivity were determined and used to evaluate upper and lower limits of the Hamaker constant A from Eq. [6]. Finally the van der Waals attractive force F_a was determined from Eq. [7] by substitution of Hamaker constant values for a range of aqueous spaces d_a from 10 to 80 Å taking the compound membrane thickness as 50 Å. The limits of these attractive forces as a function of d_a are shown in Fig. 1 as derived from computer analysis based on the procedures outlined.

In essence the attractive force follows an inverse cube law at large separations, and rises more steeply at closer distances. The force increases with the proportion of protein in the membrane and with increasing values of the dielectric permittivity of the protein. The van der Waals force increases by about a factor of 3.5 when the protein parameters are taken from the minimum to maximum values.

The Hamaker constant also ranges from about 3.5×10^{-14} to 1.22×10^{-13} erg which is comparable with experimental values (17), but is larger than those of Nir and Andersen (18).

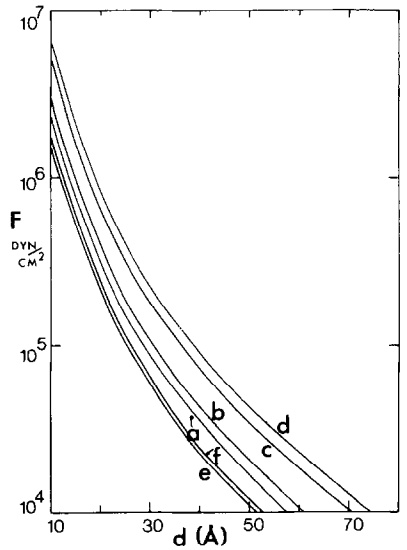

FIG. 1. Variation of van der Waals attractive force with membrane separation. (a) Protein of dielectric constant 2.5 comprising 40% of membrane. (b) Protein of dielectric constant 2.5 comprising 50% of membrane. (c) Protein of dielectric constant 3.0 comprising 50% of membrane. (d) Protein of dielectric constant 3.0 comprising 60% of membrane. (e) Protein of dielectric constant 2.1 comprising 20% of membrane. (f) Protein of dielectric constant 2.1 comprising 60% of membrane.

The Electrostatic Force

The electrostatic repulsive force between the two membranes depends on their separation, the density of electric charge on the surfaces of the membrane, and the concentration of screening ions in the aqueous region separating the membranes.

Let n_i^0 be the bulk of ions in the solution and ν_i their valence numbers with appropriate signs. Choose the x-axis of a spatial coordinate system perpendicularly to the membrane surfaces and let $x = \pm a$ be the positions of the membranes. The Poisson–Boltzmann equation:

$$\frac{d^2\psi}{dx^2} = \frac{-4\pi e}{\epsilon} \sum_i \nu_i n_i^0 \exp\left(\frac{-e\nu_i\psi(x)}{kT}\right) \quad [13]$$

describes the electrostatic potential ψ at any point between the membranes (19). Here ϵ is the dielectric constant of the solution, k the Boltzmann's constant, T the temperature, and e the electron charge. The following boundary conditions

$$\left.\frac{d\psi}{dx}\right|_{x=\pm a} = \frac{4\pi}{\epsilon}\sigma \quad [14]$$

relate the potential to the surface charge density $-\sigma$.

The surface charge density is related to the dissociation reaction constant κ and the number of surface anionic groups per unit area Γ, and is given by

$$\sigma = \frac{-e\kappa\Gamma}{\kappa + n_{H^+}^0 \exp(-e\psi s/kT)} \quad [15]$$

where $n_{H^+}^0$ is the bulk hydrogen ion concentration, and ψs is the surface potential (20). Equations [13] and [15] with boundary

conditions (Eq. [14]) are solved to give the force between the membranes

$$F_r = kT \sum_i n_i^0(C + 1) \quad [16]$$

and the corresponding membrane separation

$$d = 2 \int_{\psi_m}^{\psi_s} \frac{d\psi}{\{(8\pi kT/\epsilon) \sum_i n_i^0 | \exp(-\nu_i e\psi/kT) + C |\}^{1/2}} \quad [17]$$

where ψ_m is the potential at the midpoint between the membranes and C is an integration constant.

The computational procedure described previously (6) was used to calculate the force–distance curves for a range of the parameters; surface dissociation $pK_a = 4.7$ (21) and a bulk pH of 7.0. Representative results are shown in Fig. 2 for 1 electron charge per 1000 Å^2 or per 2000 Å^2 and cation concentrations as detailed in the legend. It can be seen that the slope of the curves increases with increasing ionic strength, and that at constant ionic strength, the force increases with increasing charge density.

As mentioned earlier in the paper, thylakoid membranes contain 50% protein and 50% lipid (14). The proteins of the thylakoid membrane, such as the chlorophyll–protein complexes and the cytochromes of the photosynthetic electron transport chain, are viewed as globular particles embedded in or attached to a lipid bilayer. The glycolipids, monogalactosyl diglyceride and digalactosyl diglyceride, account for 80% of the lipids of grana thylakoids, with the phospholipids and sulfolipids making up most of the remaining 20% (22). The glycolipids have neutral hydrophilic head groups, while the phospholipids and sulfolipids each carry a single negatively charged head at neutral pH. It is characteristic of thylakoids that most of the phospholipids are singly charged, rather than being zwitterionic. The pK_a values of the charged groups on the phospholipids and sulfolipids are in the region of 2.0 in an open solution. The negatively charged groups due to protein at the surface of the membrane at neutral pH will be due mainly to the side chain carboxyl groups of aspartic acid ($pK_a = 3.8$) and glutamic acid ($pK_a = 4.25$). Positively charged groups will be contributed by the side chain basic groups of lysine and arginine, which are protonated at neutral pH. The imidazole side chain of histidine with a pK_a of 6.0 will be mainly charged at pH 7.0. Chain-terminating amino acids may also make a small contribution to the net charge on the membrane surface. A phospholipid in a bilayer occupies an area of at least 60 Å^2 (23). Thus, in the lipid area of the grana thylakoids the charge density is about 1 per 300 Å^2 or in the total surface area of the thylakoid about 1 per 600 Å^2, if we assume little net contribution from the proteins at neutral pH.

A comparison of the attractive forces of Fig. 1 and the repulsive forces of Fig. 2 clearly indicates that the negative charge density of 1 per 600 Å^2 is far too high to permit any form of proximity balance to be achieved, since the repulsive forces always greatly exceed the attractive forces for the ranges being considered. It would seem that the charge density would need to be less than 1 per 2500 Å^2 or at least 80% of the available 1 per 600 Å^2 would need to be charge-neutralized by cations by direct electrostatic binding or adsorption at the surface of cations that exist in the stroma of the intact chloroplast. Stromal cation concentrations have been estimated (24, 25). The divalent cation concentration Mg^{2+} is in the range 1 to 5 mM while the monovalent cation concentration K^+ and Na^+ is in the range 5 to 20 mM. Divalent ion concentrations of 1 and 5 mM were used for the calculation of electrostatic repulsive force in Fig. 2, and monovalent ion concentrations ranged from 5 to 100 mM. These ion concentrations are appropriate to most experimental conditions used with isolated chloroplasts.

The Balance of Forces

Measurement of the repulsive forces between lipid bilayers has shown that at

membrane distances of less than about 30 Å a strong repulsive force due to the structuring of water molecules near the membrane surfaces comes into play. This force has been shown to obey the empirical formula (13, 17).

$$F = 10^{11} e^{-da/1.93} \text{ dyn/cm}^2. \quad [18]$$

Further experimental evidence for the existence of hydrostructural forces has also been obtained (26).

In Fig. 3 this force has been plotted, along with the electrostatic forces for the ionic conditions appropriate to chloroplasts but charge densities of 1 per 2000 Å^2, 3000 Å^2, and 5800 Å^2, curves c–a, together with the maximum and minimum van der Waals curves from Fig. 1.

It has been shown (27) that *in vivo* the thylakoid membrane spacing is 20 to 40 Å. In Fig. 3 it can be seen that if curve d is taken for the van der Waals force, then curve a for the electrostatic force will allow approach of the membranes into this range. The hydrostructural force provides a barrier which limits the membrane approach to about 27 Å.

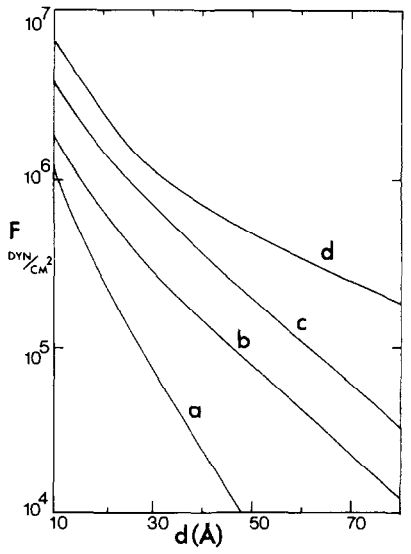

FIG. 2. Variation of electrostatic repulsive force with membrane separation. $pK_a = 4.7$, pH = 7.0 (a) 1 charge/2000 Å^2 $[C^{2+}] = 5 \times 10^{-3}$ M, $[C^+] = 1 \times 10^{-1}$ M. (b) 1 charge/2000 Å^2 $[C^{2+}] = 5 \times 10^{-3}$ M, $[C^+] = 2 \times 10^{-2}$ M. (c) 1 charge/1000 Å^2 $[C^{2+}] = 5 \times 10^{-3}$ M, $[C^+] = 2 \times 10^{-2}$ M. (d) 1 charge/1000 Å^2 $[C^{2+}] = 1 \times 10^{-3}$ M, $[C^+] = 5 \times 10^{-3}$ M.

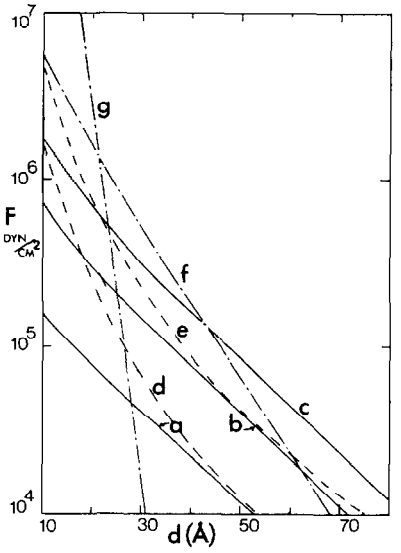

FIG. 3. Comparison of forces involved in membrane equilibrium. (a) Electrostatic force as in Fig. 2d except that charge density = 1/5800 Å^2. $[C^{2+}] = 1 \times 10^{-3}$ M, $[C^+] = 5 \times 10^{-3}$ M. (b) As in Fig. 3a except that charge density = 1/3000 Å^2. (c) As in Fig. 3a except that charge density = 1/2000 Å^2. (d) Minimum van der Waals force from Fig. 1e. (e) Maximum van der Waals force from Fig. 1d. (f) Electrostatic force. 1 charge/700 Å^2, $[C^+] = 1 \times 10^{-1}$ M. (g) Hydrostructural force from Eq. [18].

Curve c for a charge density of 1 per 2000 Å^2 would not allow equilibrium thylakoid stacking, however, because the electrostatic force is greater than the van der Waals force for all distances in the range of interest.

It must be concluded from these curves that the maximum charge density on the membrane surface which will allow membrane stacking will range from 1 per 5800 Å^2 up to 1 per 3000 Å^2 depending on the parameters chosen for the van der Waals force with a stacking equilibrium gap in the region of 30 Å. These values correspond to those measured by Itoh (28).

It can be seen that for the membranes to approach a stacked equilibrium in the dark, the majority of the charged lipid head groups must be electrically neutralized, and this can be achieved either by the electrostatic binding of the divalent Mg^{2+} cations

or by lateral redistribution between stacked and nonstacked regions of membrane.

The first conclusion agrees with that of McLaughlin (29) who showed that measurements of membrane surface potential as a function of divalent ion concentration could be explained by a degree of binding at the surface. In experiments (30) with single layers of methylphosphatidic acid (MPA)[4] at pH 8.0 and 20°C it was shown that considerable binding of divalent cations takes place up to the ratio limit of $(MPA)/Ca^{2+} = 2$. In the case of our analysis for thylakoid membranes under dark conditions a binding of divalent cations to 80 or 90% of the surface groups is required to permit membrane approach and close spaced equilibrium. The second conclusion agrees with that of Barber (31) who considered a lateral redistribution of charges.

Curve f in Fig. 3 shows the electrostatic repulsion force for membranes with a charge density of 1 per 700 Å^2 and an assumed high monovalent cation concentration of 100 mM. As can be seen, the slope of the electrostatic force is now steeper than that of the van der Waals force, thus a balance between them rather than between electrostatic and hydrostructural forces can be obtained. This expanded equilibrium possibility serves to explain the results (7) where is was observed that the addition of large concentrations of monovalent cations could cause a restacking of thylakoid membranes, although in a swollen state.

CONCLUSIONS

Our analysis has compared the relative magnitudes of van der Waals attractive forces and a combination of electrostatic and short-range hydration structural repulsive forces as a function of membrane spacing for a range of parameters representative of the grana thylakoid membranes of chloroplasts and their ionic environment. We conclude that the thylakoid membranes under dark conditions may only attain the stacked condition when the surface charge density on the appressed membranes is less than 1 charge per 3000 Å^2. This contrasts with measured values of 1 charge per 640 Å^2 (32), and a more recent value of 1 charge per 1500–2000 Å^2 (33), but corresponds with the value obtained by Itoh (28) for illuminated chloroplasts.

[4] Abbreviation used: MPA, methylphosphatidic acid.

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