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Hyphothesis

ELECTRICAL DIFFUSE LAYERS AND THEIR INFLUENCE ON PHOTOSYNTHETIC PROCESSES

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1. Introduction

There are several observations in the literature concerning chlorophyll fluorescence, thylakoid stacking and electron-transport which may be accounted for by changes in the nature of the diffuse electrical layer at the surface of the thylakoid membrane. In this paper we present the basic concepts of diffuse electrical layers associated with charged surfaces and then we use these to give explanations to the above mentioned phenomena.

2. Theory

Consider a charged membrane surface in a solution containing diffusible ions. The distribution of ions in the solution at equilibrium will be such that the electrochemical-potential ($\bar{\mu}$) of an ion (i) at any point is the same. For example:

$$\bar{\mu}_i = \bar{\mu}_{ia}$$

where $\bar{\mu}_i$ is the electrochemical-potential of species i at a point near the membrane and $\bar{\mu}_{ia}$ is the electrochemical-potential of the same species at an infinite distance from the membrane.

Assuming that $\bar{\mu}$ is completely described by chemical and electrical thermodynamic quantities and neglecting pressure terms and activity coefficients, the distribution of the ith ion at any point x cm from the membrane surface is described by the Boltzmann equation:

$$C_i = C_{ia} \exp\left(-ZF\psi/RT\right) \qquad (1)$$

where C_i is the concentration at a point x cm from the membrane surface, C_{ia} is the concentration in the bulk solution (at an infinite distance from the membrane surface), ψ is the electrical potential difference at point x relative to the bulk solution (where $\psi = 0$) and the other terms have their usual meanings.

Assuming the membrane surface to be infinitely flat and bearing a uniformly spread charge density of q μC/cm^2, the electrical potential difference at any point x, relative to the bulk solution is given by the Gauss equation:

$$\frac{d\psi}{dx} = -\frac{4\pi}{\epsilon} q \qquad (2)$$

where ϵ is the permittivity of water, = 78.4 ϵ_0 (where ϵ_0 is the permittivity of a vacuum).

For a membrane suspended in aqueous solution at equilibrium, q is balanced by an equal diffuse charge density of opposite polarity in the layer of solution adjacent to the membrane surface, thus:

$$q = \int_{x=0}^{x=a} -\rho \, dx \qquad (3)$$

where ρ is the space charge density of ions in solution in a plane parallel to the membrane surface at x cm. ρ is also given by eq. (4).

$$\rho = \sum_i Z_i F C_i \qquad (4)$$

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

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where C_i is concentration of ith ion in the plane.

Combination of eq (2) and eq. (3) leads to the Poisson equation:

$$\frac{d^2\psi}{dx^2} = -\frac{4\pi}{\epsilon}\rho \tag{5}$$

The Poisson equation can be combined with eq. (1) and eq. (4) to yield the Poisson-Boltzmann expression:

$$\frac{d^2\psi}{dx^2} = -\frac{4\pi}{\epsilon}\sum_i Z_iFC_{ia}\exp\left(\frac{-Z_iF\psi}{RT}\right) \tag{6}$$

Equation (6) can be integrated to yield

$$\frac{d\psi}{dx} = \pm 2\left(\frac{2\pi RT}{\epsilon}\right)^{1/2}\left|\sum_i C_{ia}\left(\exp\left(\frac{-Z_iF\psi}{RT}\right)-1\right)\right|^{1/2} \tag{7}$$

The field strength ($d\psi/dx$) can be related at any point with the potential difference at that point (ψ) relative to the bulk solution ($x = a$). Since the Gauss equation also relates the field strength at the membrane surface ($x = 0$) to the surface charge density on the membrane q, combinations of eq. (2) and eq. (7) yields

$$q = \pm\left|\frac{RT\epsilon}{2\pi}\sum_i C_{ia}\left(\exp\left(\frac{-Z_iF\psi_0}{RT}\right)-1\right)\right|^{1/2} \tag{8}$$

When the membrane is suspended in a medium containing Z–Z electrolytes such as KCl or $MgSO_4$ eq. (8) reduces to

$$q = 2A\,(C_a)^{1/2}\sinh\left(\frac{ZF\psi_0}{2RT}\right) \tag{9}$$

where $A = \left(\frac{RT\epsilon}{2\pi}\right)^{1/2}$

Numerical substitution for 25°C gives

$$q = 11.74\,(C_a)^{1/2}\sinh\frac{Z\psi_0}{51.7} \tag{10}$$

where q is in $\mu C/cm^2$, C_a in mol/l and ψ_0 in mV.

The value of ψ decays to zero with increasing distance x perpendicular to the membrane plane. The value of ψ at any point x can be found by integrating eq. (7). For a Z–Z electrolyte the integration gives

$$\kappa x = \ln\left(\tanh\left(\frac{ZF\psi_0}{4RT}\right)\right) - \ln\left(\tanh\left(\frac{ZF\psi}{4RT}\right)\right) \tag{11}$$

where $\kappa = \frac{8\pi Z^2F^2C_a}{RT\epsilon}$

For explaining the photosynthetic phenomena mentioned in the introduction it is necessary to consider the effect of bathing the thylakoid membranes in solutions of mixed electrolytes (i.e., Z^+/Z^- and Z^{2+}/Z^{2-}).

Defining C'_a and C''_a as the bulk concentrations of monovalent and divalent salts, respectively, then application of eq. (8) yields, after rearrangement, the following quadratic:

$$2C''_a\cosh^2\left(\frac{F\psi_0}{RT}\right) + C'_a\cosh\left(\frac{F\psi_0}{RT}\right) - \left(2C''_a + C'_a + \frac{q}{2A}\right) = 0 \tag{12}$$

Similarly substitution into eq. (7) yields

$$\frac{d\psi}{dx} = \pm\left(\frac{8\pi RT}{\epsilon}\right)^{1/2}\left(4C'_a\sinh^2\left(\frac{F\psi}{2RT}\right) + \left(4C''_a\sinh^2\left(\frac{F\psi}{RT}\right)\right)\right)^{1/2} \tag{13}$$

Before considering actual experimental data and the application of eq. (12) and eq. (13) it is worth commenting on the assumptions and validity of these expressions.

(a) The membrane is assumed to be infinitely flat with a fixed negative charge 'smeared-out' uniformly over the surface. In practice the membrane can be considered infinitely flat if the radius of curvature of the surface is 30-times the perpendicular distance of the ion from the centre of the membrane. Since the radius of a single thylakoid is approximately 2500 Å for the major surface area, whereas the electrical double-layer can be normally considered to have little influence on ions at a distance greater than 100–200 Å from the surface, this assumption appears to be reasonably valid. More serious is the fact that it is very unlikely that the charge is either smeared-out or homogeneous on the membrane surface.

(b) It is assumed in all the integration steps that the dielectric constant term, ϵ, is independent of ψ. Grahame [1] has shown that this assumption is valid for a field strength ($d\psi/dx$) not exceeding 10^6 V/cm. In our analyses the field strength never exceeds this value.

(c) In the Gouy-Chapman treatment of the double-layer, ions in solution are considered to be point charges which can approach the membrane to any distance including $x = 0$. In practice however, the closest plane of approach will be defined by the radius of the hydrated ion, which for small metal cations is of the order of 2–4 Å. This error may be accounted for by dividing the double-layer into two regions, (i) the compact double-layer between the charged surface ($x = 0$) and the plane of closest approach of the ion ($x = x_2$) and (ii) the diffuse double-layer which extends out from the plane of closest approach. Thus the potential ψ_0 ascribed to the point $x = 0$ in eq. (10), eq. (11) and eq. (12) should be redefined as the potential at the plane of closest approach, ψ_2. However, when the surface charge density q is small (e.g., 2.5 $\mu C/cm^2$ as used in the analysis below) the difference between ψ_0 and ψ_2 may be small and has for convenience been neglected.

(d) Ion-binding and changes in the activity coefficients relative to the values of the bulk ions have not been allowed for. Although such effects almost certainly will occur they are difficult to quantify and introduce into the classical approach.

3. Experimental observations

3.1. *Fluorescence changes*

When isolated broken chloroplasts treated with DCMU are illuminated with bright light in a suspending medium containing essentially no cations, the chlorophyll fluorescence yield is at a maximum. Under these conditions the photosystem-two (PS2) traps are closed. Addition of a low concentration of monovalent cations initially decreases the fluorescence but on increasing the monovalent cation composition above 5–10 mM the fluorescence yield rises to reach a maximum at about 40–60 mM. This pattern of monovalent cation induced changes in fluorescence yield is independent of fluctuations in the redox-state of the PS2 trap (kept closed by DCMU) and was first discovered by Gross and Hess [2]. The original data of Gross and Hess is shown in fig.1(A) and fig.1(B) and has been confirmed by Vandermeulen and Govindjee [3] and Barber and Mills [4]. The fluorescence rise from the low level can also be induced by divalent cations but at much lower concentrations than required with monovalent cations as shown in fig.1(C) taken from the original paper of Gross and Hess [2]. As shown later by Vandermeulen and Govindjee [3] and by Mills and Barber [5] the actual levels of divalent cations required to bring about the rise is dependent on the level of monovalent cations present in the medium. Under no conditions do divalent cations

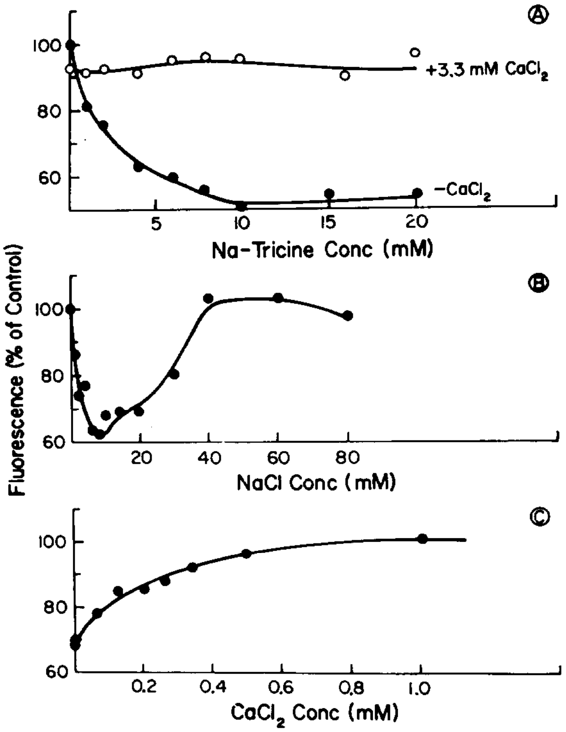


Fig.1. Concentration-dependence of cation effects on chlorophyll *a* fluorescence. (A) The effect of Na-tricine concentrations in the presence and absence of 3.3 mM $CaCl_2$. (B) The effect of NaCl concentrations. (C) $CaCl_2$ induced reversal fluorescence in the presence of 10 mM Na-tricine. Spinach chloroplasts (at 5 μg chl/ml) were incubated in the presence of 100 mM sucrose and sufficient Tris-base to titrate to pH 8.0 (~ 0.12 mM) and 6.7 μM DCMU was present. Data of Gross and Hess [2].

bring about a decrease in the fluorescence to the low level although they do effect, in a competitive way, the ability of low levels of monovalent cations to reduce fluorescence [6]. Among the relatively chemically inactive species there is essentially no cation specificity for these effects, only the charge carried by the cation is important and both inorganic and organic cations can be equally effective [2–6]. The nature of the anion seems to have little or no observable effect (see for example, fig.1(A)).

3.2. *Electron-transport*

Walz, Schuldiner and Avron [7] have shown that when chloroplasts are suspended in a cation-free medium the rate of electron-transport is low. Their results are shown in fig.2. On addition of low levels of monovalent cations (2–4 mM KCl) the rate increases to a maximum level but then decreases again as the monovalent level is raised. Like fluorescence, this biphasic effect did not exhibit cation specificity, with both inorganic and organic monovalent cations being equally effective.

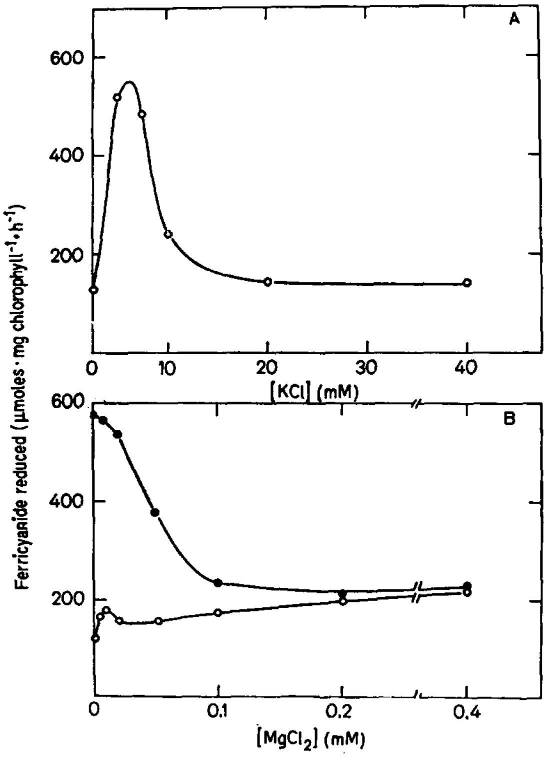


Fig.2. Dependence of the rate of ferricyanide reduction on KCl (A) and MgCl$_2$ (B) concentrations in a glycine medium. Lettuce chloroplasts (at 15 µg chl/µl) were suspended in 40 mM glycine, with 0.05 mM $K_3Fe(CN)_6$, at pH 7.4. The upper curve in (B) contained, in addition, 2 mM potassium phosphate. Data of Walz, Schuldiner and Avron [7].

As shown in fig.2(B) divalent cations did not induce a rise in the electron-transport rate but did induce a lowering of the rate when low levels of monovalent cations were present. As with the fluorescence phenomenon a much lower concentration of divalent cations was required to decrease the rate as compared with the lowering induced by monovalent cations. Walz et al. used 40 mM glycine, at pH 7.4, for their cation-free medium but the same results can be observed with 0.11 M sorbitol, brought to pH 7.5 with Tris base, and also when methyl viologen is used as an electron-acceptor (Nakatani and Barber, unpublished).

3.3. *Thylakoid stacking*

Gross and Prasher have shown [8] that when chloroplasts are suspended in a cation-free medium (0.1 M sucrose + 0.2 mM Tris base) the thylakoids are stacked. Addition of low levels of monovalent cations (about 3 mM) brings about unstacking but as the monovalent cation content of the medium is raised stacking occurs again. As with fluorescence and electron-transport, divalent cations did not induce unstacking although in the presence of low levels of monovalent cations, divalent cations are far more effective than monovalent cations at bringing about restacking. These effects seemed to manifest themselves as turbidity changes measured as variations of absorbance at 540 nm [8].

4. General conclusions from observations

Although the exact concentration-dependence is not the same (compare figs.1 and 2) there is clearly a similarity between the three phenomena presented above. These are:

(i) Antagonism between low concentrations of monovalent and divalent cations.

(ii) Antagonism between low and high levels of monovalent cations.

(iii) Essentially no specificity within a particular charge group.

These properties suggest that the three phenomena are associated with an electrical rather than a chemical effect, and eq. (12) and eq. (13) can be used to gain a better understanding of the mechanism involved. They also indicate that when chloroplasts are carefully isolated and suspended in a cation-free medium they apparently have divalent cations at their surfaces (see refs [4] and [5]).

5. Application of the Gouy-Chapman expression

5.1. *Use of equation (12)*

This quadratic can be solved for ψ_0 by assuming a value of the fixed charge density (q) and by varying C'_a and C''_a. Knowing the value of ψ_0 then allows the calculation of the surface concentration of an ion species i (C_{i0}) for any bulk concentration (C_{ia}) using the Boltzmann expression, eq. (1). This approach has already been adopted in a recent publication from our laboratory [4] but an extensive computer-analysis is shown in fig.3. Figure 3(a) and 3(b) are computer-derived curves showing how ψ_0, C'_0 and C''_0 vary under different conditions. By considering various values of C''_a it can be seen that increasing C'_a decreases ψ_0 and as expected brings about a displacement of divalent

Fig 3

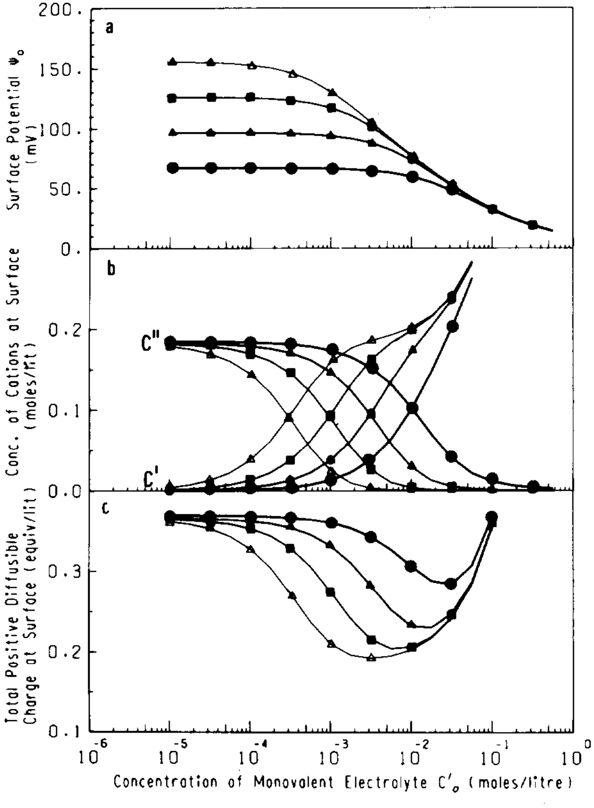
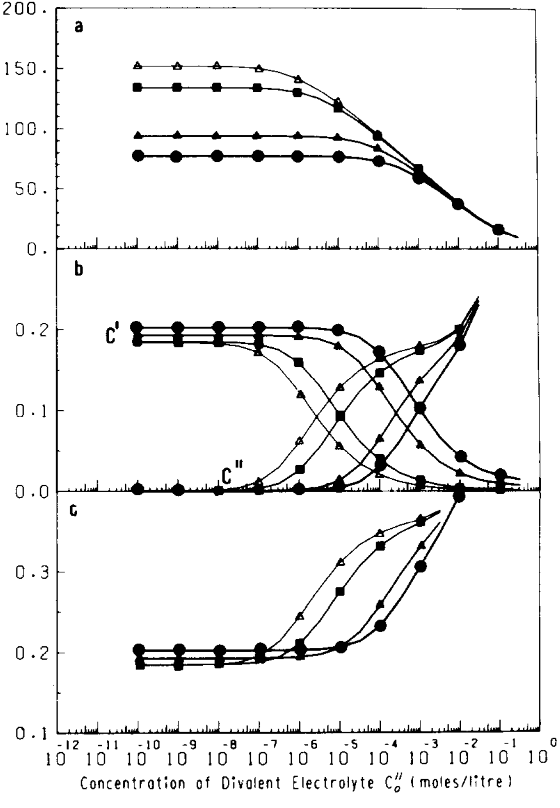


Fig 4



Figs.3 and 4. Computer-generated curves using eq. (1) and eq. (12) showing changes of (a) the surface potential ψ_0, (b) surface concentration of monovalent (C'_0) and divalent (C''_0) cations and (c) of total positive charge at the surface, for different levels of mixed electrolytes in the solution (Z^+/Z^- and Z^{2+}/Z^{2-}).

Fig.3. Effect of changing monovalent salt (10^{-5} – 3×10^{-1} M) for different starting levels of divalent salt. Open triangles 10^{-6} M, closed squares 10^{-5} M, closed triangles 10^{-4} M and closed circles 10^{-3} M.

Fig.4. Effect of changing divalent salts (10^{-10} – 3×10^{-1} M) for different levels of monovalent salts. Open triangles 5×10^{-4} M, closed squares 10^{-3} M, closed triangles 5×10^{-3} and closed circles 10^{-2} M.

cations at the surface by monovalent cations. (Note that initially C_0'' is about the same for all values of C_a''.) Neither ψ_0 nor C_0' and C_0'' reflect the biphasic effect seen in the above experiments. However, as fig.3(c) shows, if the total positive charge immediately adjacent to the membrane is plotted as a function of C_0', then when C_0'' is low, a biphasic curve is generated. The value of C_a' at which the curve is a minimum is dependent on q and the curves in fig.3 have been computed for $q = 2.5\ \mu\text{C/cm}^2$ which is equivalent to about one electronic charge per 640 Å^2. There is no reliable figure in the literature for the surface charge density of thylakoid membranes thus this value was chosen since it corresponded to the value of C_a' which brings about the lowering of fluorescence. Clearly this value is arbitrary and should be determined experimentally by particle electrophoresis. Moreover the value of q may vary for chloroplasts isolated from different material or subjected to different experimental conditions. These variations could account for the difference in the concentration ranges shown in fig.1(B) and fig.2(A). The analysis does, however, indicate that the high fluorescence/low electron-flow rate/stacking conditions were associated with a high density of diffusible charge (space charge density) at the surface of the membrane (see fig.3(c)). Decreasing the space charge density at the surface could be the reason for the lowering of fluorescence, increase in electron-flow and thylakoid unstacking. Equation (12) also predicts (see fig.3(c)) that by raising the level of C_a'' in the bathing medium results in a requirement of higher levels of C_a' to bring about the decrease in the space charge density. Such a competitive effect is observed with the monovalent cation-induced decrease in fluorescence [6]. In addition the competitive effect of different low levels of monovalent cations on the divalent cation-induced rise of fluorescence [3,5] can also be predicted by eq. (12) as shown in fig.4.

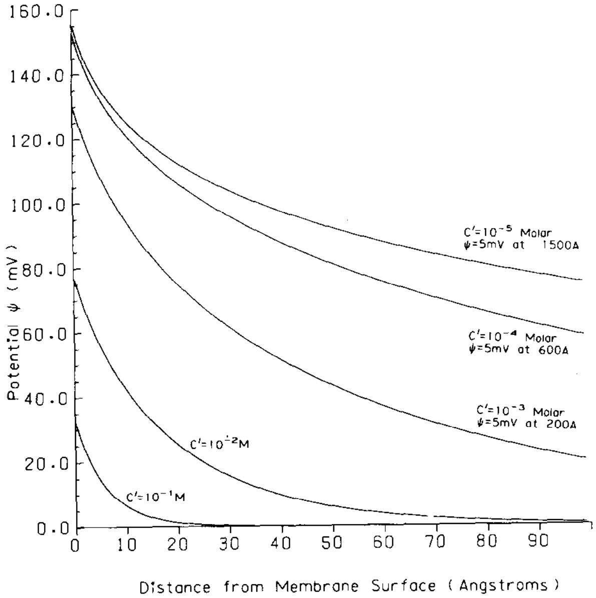


Fig.5. Computer-generated curves obtained by carrying out a numerical integration of eq. (13) showing how the electrical potential ψ varies as a function of distance (x) from the surface. The curves have been computed for a fixed divalent cation concentration in the medium (C_a'') of 10^{-6} M and for the various values of monovalent cations (C_a') as shown.

5.2. *Use of equation (13)*

A more satisfactory approach to understanding these phenomena in terms of the properties of the diffuse layer is to obtain information on changes of the electrical potential (ψ), the electrical field ($d\psi/dx$) and the space charge density ($d^2\psi/dx^2$) as a function of distance from the membrane for different mixtures of electrolytes. This is possible by integrating eq. (13) by numerical methods using a computer and assuming a q value (we have taken 2.5 μC/cm^2). This has been done for $C_a'' = 10^{-6}$ which seems a fair situation for chloroplasts suspended in a cation-free medium. As shown in fig.3(b) under this condition the surface concentration C_0'' is still high in order to counterbalance the fixed negative charges and this is expressed by the high value of ψ_0. In fig.5 the values of ψ as a function of distance from the membrane are plotted for different values of C_a' with C_a'' maintained at 10^{-6} M. As can be seen the effect of increasing C_a' is to reduce ψ_0 and at the same time, decrease the thickness of the double-layer. When the electric field ($d\psi/dx$) is plotted as a function of distance from the membrane surface (fig.6) or as a function of C_a' for different distances (fig.7), it is found to be maximum when C_a' is about 10 mM. In the case of the space charge density ($d^2\psi/dx^2$) it is found that this also shows a biphasic curve as C_a' is increased at values of x other than just $x = 0$ (as shown in fig.3(c)). However as fig.7 shows this decrease in $d^2\psi/dx^2$ is only observed up to about 10 Å from the membrane surface.

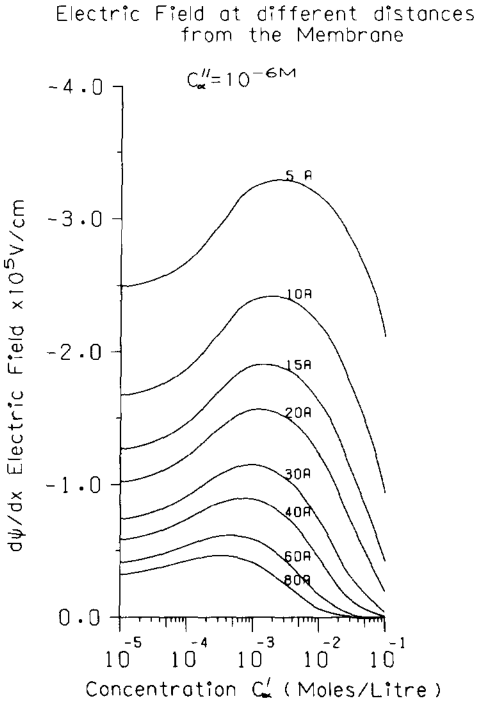


Fig.6. Computer-generated curves of the electrical field $d\psi/dx$ as a function of the bulk monovalent cation concentration (C'_a) for different values of distance (x) from the membrane surface and with $C''_a = 10^{-6}$ M (as in fig.5).

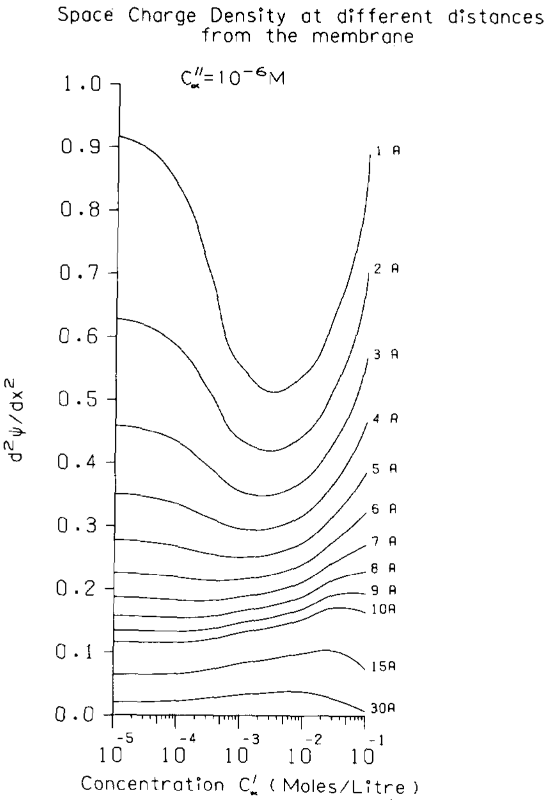


Fig.7. Computer-generated curves of $d^2\psi/dx^2$ as a function of the bulk monovalent cation concentration (C'_a) for different values of distance (x) from the membrane surface and with $C''_a = 10^{-6}$ M (as in figs 5 and 6).

6. Conclusion

It seems that the various experimental observations described above can be qualitatively explained in terms of changes in the electrical field or space charge density adjacent to the membrane surface. Whether the three phenomena are interrelated is not at present clear and exactly how the changes in the electrical properties of the thylakoid surface influence fluorescence and electron-transport still has to be established. The stacking and unstacking phenomenon is easier to understand since this effect is almost certainly due to changes in the balance between repulsion, due to the electrical diffuse layers on each membrane surface and attraction, associated with van der Waal forces. Differential (Schulze-Hardy Rule [9]) and antagonistic effects of monovalent and divalent cations on colloidal coagulation are well established [10] and have already been explained theoretically using classical double-layer theory [11]. Thus it could be that the fluorescence and electron-transport effects seen under these conditions simply result from changes in the distance between adjacent thylakoid membranes.

Overall our results emphasise the significance of the fixed negative charges on the thylakoid membranes in that they create a diffuse electrical layer and that this layer is varied greatly by the ionic composition of the suspension medium. It seems that in vivo divalent cations are the main co-ions for the fixed membrane charges.

Acknowledgements

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References

[1] Grahame, D. C. (1953) J. Chem. Phys. 21, 1054–1064.

[2] Gross, E. L. and Hess, S. C. (1973) Arch. Biochim. Biophys. 159, 832–836.

[3] Vandermeulen, D. L. and Govindjee (1974) Biochim. Biophys. Acta 368, 61–70.

[4] Barber, J. and Mills, J. (1976) FEBS Lett. 68, 288–292.

[5] Mills, J. and Barber, J. (1976) Biochim. Biophys. Acta 440, 495–505.

[6] Mills, J. (1976) Thesis, University of London, England.

[7] Walz, D., Schuldiner, S. and Avron, M. (1971) Eur. J. Biochem. 22, 439–444.

[8] Gross, E. L. and Prasher, S. H. (1974) Arch. Biochem. Biophys. 164, 460–468.

[9] Verweg, E. J. W. and Overbeak, J. (1948) Theory of the Stability of Lyophobic Colloids, Elsevier, Amsterdam.

[10] Tezak, B., Matijevic, E., Schultz, K., Kratohvil, J., Wolf, R. and Cernicki, B. (1954) J. Colloid Sci., Supplement 1, 118–127.

[11] Levine, S. and Bell, G. M. (1965) J. Colloid Sci., 20, 695–727.