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STIMULATION OF MILLISECOND DELAYED LIGHT EMISSION BY KCl AND NaCl GRADIENTS AS A MEANS OF INVESTIGATING THE IONIC PERMEABILITY PROPERTIES OF THE THYLAKOID MEMBRANES

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SUMMARY

1. Transient increases in millisecond delayed light emission brought about by the establishment of KCl or NaCl gradients across the thylakoid membranes of spinach chloroplasts have been interpreted in terms of a diffusion potential model.

2. Both the magnitude and kinetics of the salt-induced signals under various conditions can be explained by assuming the intensity of emission is an exponential function of the membrane potential developed.

3. Treatment of the chloroplasts with valinomycin and gramicidin gives support to these concepts and suggests further that under the experimental conditions employed, membrane potentials of 50 to 110 mV (inside positive) can be created across the thylakoid membranes by illumination.

4. Pretreatments of the chloroplasts with various KCl concentrations were found to modify, in a predictable way, the signals due to further KCl additions.

5. Analyses of the decay of NaCl and KCl induced signals have given estimates of the rate of Cl^- transfer across the thylakoid surface under known driving conditions.

6. This analysis indicates that Cl^- entry is by a first order process and that the permeability of the thylakoid membranes to this anion may be very low.

7. It is suggested that the apparent "leakiness" of broken chloroplasts to Cl^-, and probably other monovalent ions, reflects the very large surface area to volume ratio rather that high intrinsic membrane permeabilities.

INTRODUCTION

When spinach chloroplasts are subjected to KCl gradients there is a transient increase in the intensity of one millisecond delayed light emission[1,2]. It has been suggested that these transients reflect the establisment and decay of electrical gradients resulting from the selective diffusion of the ions across the thylakoid membranes. This would indicate that a membrane potential of the correct polarity can decrease the activation energy for the return of electrons from the metastable

Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid.

states, created during the illumination, to the first singlet of chlorophyll[3]. The possibility that a membrane potential may control the intensity of millisecond delayed light emission has also been discussed by others[4,5]. Wraight and Crofts[5] have argued that the sensitivity of the millisecond emission to the high-energy state of chloroplasts[6] is partly due to the establishment of a light-induced electrical potential.

Recently I have reported a method of estimating the size of the light-induced potential[7]. The assumption was made that the intensity of luminescence (L) was related to the exponential of the membrane potential.

$$L\alpha \exp(\Delta\psi) \tag{1}$$

In the one millisecond region of the emission the electrical gradient ($\Delta\psi$) could consist of two components, a light-induced membrane potential ($\Delta\psi_L$) and a diffusion potential created artificially by a sudden salt addition to the chloroplast suspension ($\Delta\psi_s$). The size of $\Delta\psi_s$ can be estimated from the Goldman Voltage Equation[8,9]

$$\Delta\psi_s = \frac{RT}{F} \ln \frac{\Sigma_C P_C[C]_o + \Sigma_A P_A[A]_i}{\Sigma_C P_C[C]_i + \Sigma_A P_A[A]_o} \tag{2}$$

where P_C and P_A are permeability coefficients for univalent cations C and anions A and $[\,]_o$ and $[\,]_i$ are the outside and inside concentrations, respectively, and the other symbols have their usual meanings. This equation assumes that the ions move passively and independently and that a linear electrical field is developed across the membrane.

Substituting Eqn 2 into Eqn 1 gives:

$$kL_s = \frac{\Sigma_C P_C[C]_o + \Sigma_A P_A[A]_i}{\Sigma_C P_C[C]_i + \Sigma_A P_A[A]_o} \tag{3}$$

where L_s is the intensity of the salt-induced light emission and k is a proportionality constant[7,10].

In order to apply Eqn 3 it is necessary to reduce $\Delta\psi_L$ to zero and it was assumed that this could be accomplished, in the absence of light-induced ion gradients, by adding valinomycin to the suspension[7].

In this paper I want to give further support to these concepts and use them as a means of estimating ion fluxes across the thylakoid membranes.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach. Using a Polytron, about 40 g of sliced leaves were homogenised for 15 s with 100 ml of a semi-frozen solution containing 0.33 M sucrose, 1 mM $MgCl_2$ and 10 mM NaH_2PO_4 brought to pH 6.8 with KOH. The macerate was squeezed initially through two and then eight layers of muslin. The crude chloroplast suspension was rapidly centrifuged at 0 °C for 60 s at 3000 × g using an IEC refrigerated centrifuge (Model B20). The resulting pellet was subjected to osmotic shock by resuspending in the above grinding medium diluted 10-fold. After 5 min of this treatment the broken chloroplasts were centri-

fuged at 6500 × *g* for 3 min. The pellet was resuspended in ice-cold 0.33 M sucrose followed by a 3-min spin at 6500 × *g*. The pellet was then washed again with 0.33 M sucrose and finally suspended in 5 ml of a medium consisting of 0.33 M sucrose and 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) and stored at 0 °C in an ice bath. This resuspending solution contained 2 mM KOH which brought the pH to 7.0. The total chlorophyll concentration of this stock was determined by the method of Arnon[11]. For experiments an appropriate quantity of chloroplast stock was diluted with the above TES buffer to give a desired chlorophyll concentration.

The intensity of one millisecond delayed light was continuously measured with a rotating sector phosphoroscope. The illumination and viewing times were 1.5 and 0.2 ms, respectively, with four measurements per cycle. During the illumination period the chloroplasts were exposed to light transmitted through a filter combination consisting of Balzer Calflex C and a 2-mm Schott BG 38 giving an intensity of $2.7 \cdot 10^4$ ergs·cm^{-2}·s^{-1} at the cuvette as measured with a YSI-Kettering Model 65A Radiometer (Yellow Springs Instrument Company, Chicago, Ill., U.S.A.), The delayed light emitted was detected with an EMI 9659 B photomultiplier protected by a 2-mm Schott RG 665 filter. In some cases a second photomultiplier (EMI 9558) placed at right angles to the exciting light beam was used for following prompt fluorescence. This detector was shielded with a filter combination consisting of Balzer B40 695, 6-mm Schott RG 665 and the exciting light was transmitted by a Balzer Calflex C, 2-mm Schott BG 38 and 4-mm Schott BG 18 giving an intensity of $1.6 \cdot 10^4$ ergs·cm^{-2}·s^{-1}. The current pulses from the photomultipliers were passed through diode pump circuits with time constants of 0.1 s and the signals recorded on a Honeywell or Rickidenki chart recorder. Various additions to 3 ml of suspension contained in a Hellma 1-cm cuvette were made during measurement by rapidly injecting 100 μl of the appropriate stock using a syringe inserted through a light-tight rubber diaphragm. This procedure gave reproducible signals with a mixing time in the region of 200 ms.

Valinomycin was obtained from The Lilley Laboratories, Indianapolis, and gramicidin D from Sigma Chemical Company.

RESULTS

Typical signals and effect of valinomycin

Fig. 1 shows the general characteristics of the millisecond delayed light signals induced by injecting KCl or NaCl into chloroplasts which had or had not been treated with valinomycin. The membrane potential scale has been calculated by adopting exactly the same procedure to that already reported[7].

Essentially, it was assumed that since the well washed broken chloroplasts were suspended in a Cl^--free medium containing 2 mM K^+ then the value of $\Delta\psi_s$ generated by, for example, a KCl pulse would be given by a simplified form of Eqn 2:

$$\Delta\psi_s = \frac{RT}{F} \ln \frac{[K^+]_o}{2 + \beta[Cl^-]_o} \tag{4}$$

where $\beta = P_{Cl}/P_K$.

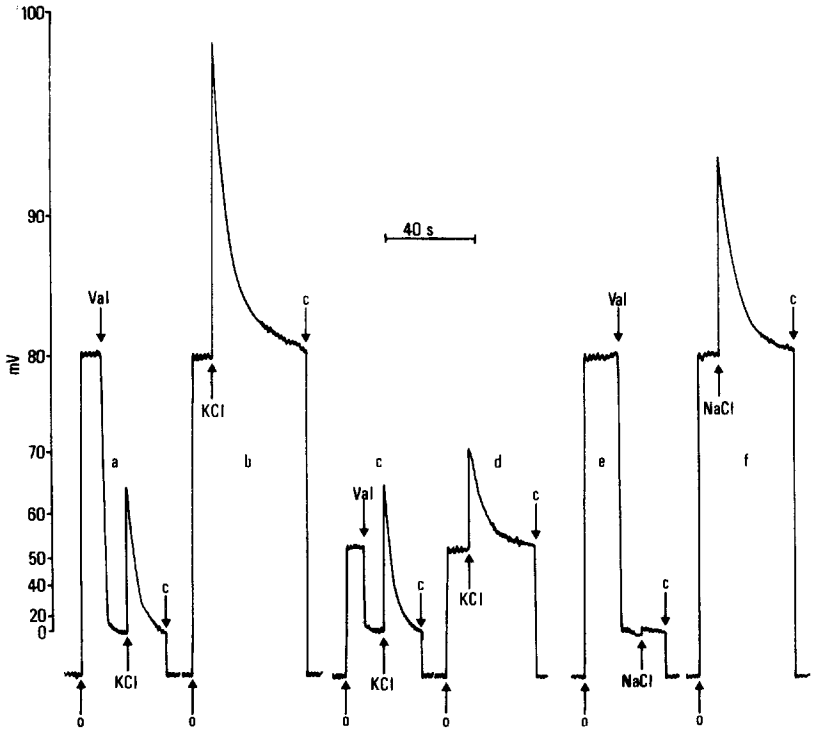

Fig. 1. The effect of valinomycin, KCl and NaCl additions on steady-state one millisecond delayed light emission. The cuvette contained 3 ml of chloroplast suspension which was illuminated 2 min before opening the shutter across the photomultiplier. The suspending medium consisted of 0.33 M sucrose, 5 mM TES buffer and 2 mM K^+ added as KOH to bring the pH to 7.0. The various additions are indicated by arrows and were made as outlined in Materials and Methods. The valinomycin concentration was 0.5 μM and KCl and NaCl additions gave a final concentration of 50 mM. Signals a, b, e and f were obtained using the same preparation while c and d were recorded on another occasion using a different batch of chloroplasts. The chlorophyll concentration in each case was 25 μg/ml. The opening and closing of the photomultiplier shutter is indicated by o and c, respectively. The membrane potential scale was calculated by using the procedure already reported[7]. Exciting light intensity was $2.7 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$s^{-1}.

By substituting Eqn 4 into Eqn 1 then:

$$kL_s = \frac{[K^+]_o}{2+\beta[Cl^-]_o} \tag{5}$$

where k is a proportionality constant. This equation assumes that no other net ion movement occurs. By measuring L_s for at least two different KCl gradients using valinomycin-treated chloroplasts, Eqn 5 can be solved for k and β and the relationship between L and $\Delta\psi$ obtained.

A notable feature of the traces shown in Fig. 1, which were obtained from different chloroplast suspensions, is the variation in the size of the valinomycin-sensitive component and how this effects the relative magnitudes of the KCl-induced transients before and after treatment with this antibiotic. When the component is large the KCl signal is greater before than after valinomycin treatment. On the other hand when the valinomycin component is small the opposite is true. It can

also be seen that the NaCl signal is very much smaller after valinomycin treatment. These results can be readily explained when taking into account the membrane potential scale which has assumed that the intensity of emission is an exponential function of this potential. It is further assumed that in the absence of ion gradients, valinomycin short circuits the light-induced membrane potential ($\Delta\psi_L$). From Trace a of Fig. 1 $\Delta\psi_L = +80$ mV inside positive (*i.e.* +80 mV), while Trace c gives $\Delta\psi_L = +52$ mV. Over the course of this work values between +50 to +110 mV have been calculated. The reason for the variation is not yet clear. As both Traces a and c show the establishment of a 50-mM KCl gradient with valinomycin-treated chloroplasts resulted in signals having an initial peak corresponding to 66 mV. With the same gradients but no valinomycin (Traces b and d) the KCl signals were quite different but it can be seen that they corresponded to about the same membrane potential (18 mV).

Kinetics of salt-induced signals and ion fluxes

If the salt-induced millisecond delayed light transients represent the establishment and fall of a membrane potential then their decay kinetics give a measure of the rate of salt entry into the interior of the thylakoids. For a KCl gradient it seems that the decay is controlled by the rate of Cl^- penetration. The method of calculating the rate of Cl– entry is shown in Fig. 2 and assumes that no net current flows. It can be seen from Fig. 2 that the electrical potential fell from 93 to 81 mV in 5 s. This corresponds, assuming only net K^+ and Cl^- movement occurs, to an increase of internal KCl from zero to 15 mM. In this way the time course of Cl^- uptake can be calculated as shown in Fig. 2. The values obtained were found to fit a first-order law. The rate constant of 0.075 s^{-1} corresponds to an initial Cl^- influx of 33 $\mu M \cdot s^{-1} \cdot \mu g^{-1}$ chlorophyll. In order to express this rate in more meaningful units it is necessary to assume surface and volume parameters of the thylakoids. I have

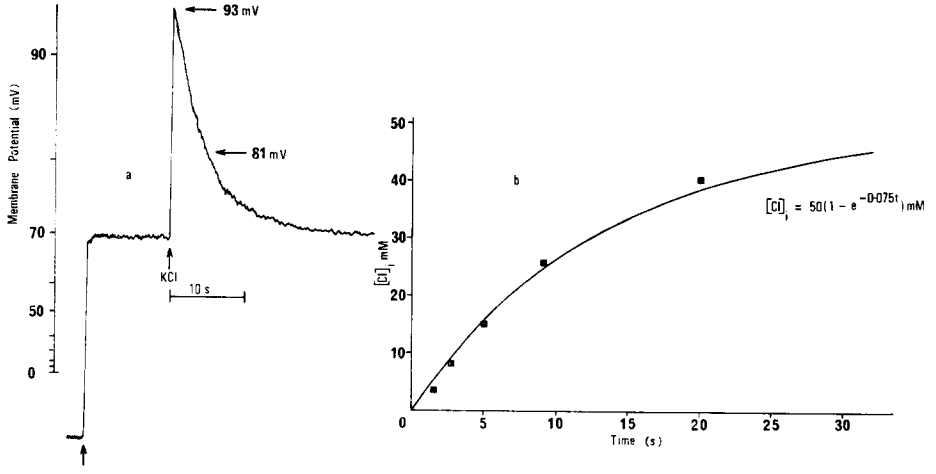

Fig. 2. (a) The kinetics of a 50 mM KCl-induced signal. Chlorophyll concn, 36 μg/ml. Other conditions are the same as given in the legend of Fig. 2. (b) The influx of Cl^- estimated from the decay of the signal shown in (a). The closed squares are experimental points and the curve has been drawn according to the first order law shown where t = time in seconds and $[Cl]_i$ is the inside Cl^- concentration in mequiv/l.

taken a single thylakoid to be a flattened disc of 4000 Å diameter and 200 Å thick. These dimensions would mean that a single thylakoid would have a surface area of $27.6 \cdot 10^{-10}$ cm^2 and a volume of $25 \cdot 10^{-16}$ cm^3. Junge and Witt[12] have also taken similar dimensions for a single thylakoid and have further assumed that 10^5 chlorophyll molecules occupy $25 \cdot 10^{-10}$ cm^2 of thylakoid membrane. This would mean that 1 μg chlorophyll is associated with 16.7 cm^2 of membrane surface and a total thylakoid volume of $15.2 \cdot 10^{-6}$. Using these values the above rate of 33 $\mu M \cdot s^{-1} \cdot \mu g^{-1}$ chlorophyll would correspond to $3 \cdot 10^{-14}$ equiv $Cl^- \cdot s^{-1} \cdot cm^{-2}$.

The rate of passive Cl^- movement across the thylakoid membranes (ϕ_{Cl}) is governed by the electrochemical potential gradient ($\partial\bar{\mu}/\partial x$) and also by its mobility (u_{Cl}) and concentration (c_{Cl}) within the membrane. This is expressed for any ion j by the general flux equation[13]:

$$\phi_j = u_j c_j \left(- \frac{\partial \bar{\mu}}{\partial x_j} \right) \tag{6}$$

The electrochemical potential can be defined as (ignoring pressure terms and activity coefficients)

$$\bar{\mu}_j = \mu_j^o + RT \ln c_j + ZF\Delta\psi \tag{7}$$

where μ_j^0 is the standard chemical potential.
Then

$$\phi_j = - u_j \frac{RT}{F} \frac{\partial c_j}{\partial x} - Z_j u_j c_j \frac{\partial \Delta\psi}{\partial x} \tag{8}$$

For the purpose of integration of Eqn 8 Goldman[8] assumed that the electrical field $\partial\Delta\psi/\partial x$ within the membrane is linear. With this assumption Eqn 9 below can be derived.

$$\phi_j = \frac{- Z_j F \Delta\psi}{RT} P_j \left[\frac{c_j^o - c_j^i \exp (Z_j F \Delta\psi / RT)}{1 - \exp (Z_j F \Delta\psi / RT)} \right] \tag{9}$$

where the permeability coefficient $P_j = u_j RT\ B/Fd$ (B is a partition coefficient and d is the thickness of the membrane) and suffix o and i mean outside and inside, respectively.

For the initial Cl^- influx induced by establishing a KCl gradient Eqn 9 can be simplified since the initial internal Cl^- concentration is zero ($c_{Cl}^i = 0$).

$$\phi_{Cl} = \frac{F\Delta\psi}{RT} P_{Cl} \frac{[Cl^-]_o}{1 - \exp (Z_j F \Delta\psi / RT)} \tag{10}$$

From Eqn 10 it can be seen that the initial influx of Cl^- into the thylakoids depends on the size of the KCl pulse, the membrane potential and the permeability constant P_{Cl}.

Fig. 3 shows the signals obtained by three different treatments. According to the voltage calibration each signal at its maximum corresponded to a similar membrane potential, in the region of 61 to 67 mV. In addition the size of the salt pulse was the same in each case (50 mM) yet the decay of the KCl signal after valinomycin treatment was quite different to those obtained on giving NaCl and KCl

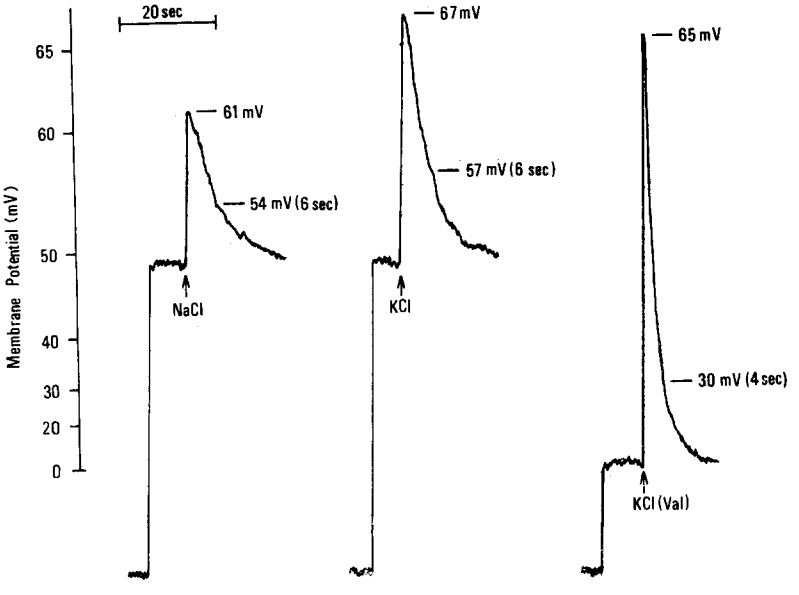

Fig. 3. The kinetics of signals induced by 50 mM KCl and NaCl and also by 50 mM KCl after treating the chloroplasts with 0.5 μM valinomycin. Chlorophyll concn, 27 μg/ml. Other details are the same as Fig. 2.

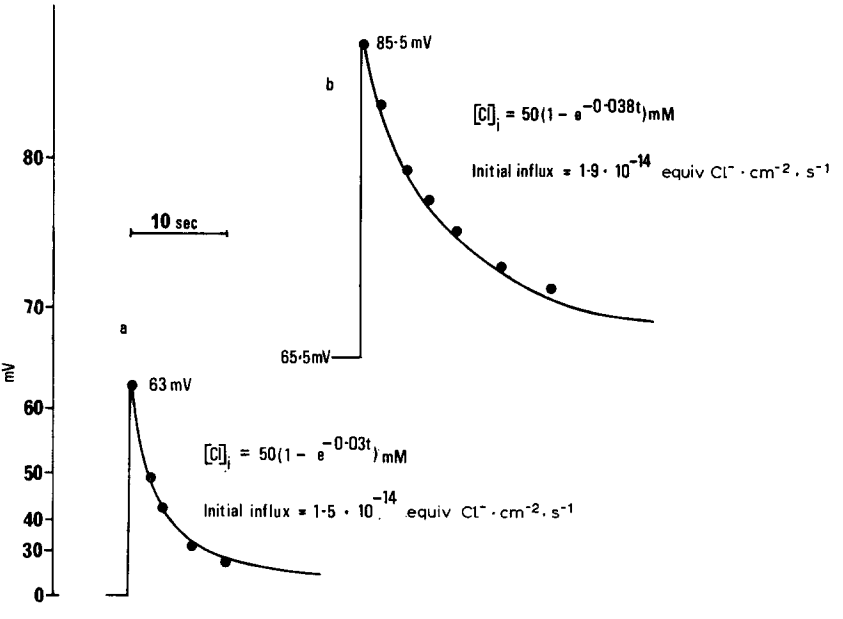

Fig. 4. Kinetic analyses of the signals induced by 50 mM KCl additions to chloroplasts which had (Signal a) or had not (Signal b) been treated with 0.5 μM valinomycin. The closed circles are points taken from the experimental signals while the curves have been theoretically constructed using the two first-order laws shown for Cl^- influx where t = time in seconds and $[Cl]_I$ is the inside Cl^- concentration in mequiv/l. Before valinomycin treatment P_{Cl}/P_K = 0.44 and after P_{Cl}/P_K = 0.03. Chlorophyll concn, 30 μg/ml. Other details of the experiment are the same as Fig. 2.

pulses to untreated chloroplasts. At first sight this would not seem to be consistent with the argument that Cl^- entry controls the decay of the signals. But calculations of the influx rates for the three signals give very similar rates for Cl^- entry, being about $4.0 \cdot 10^{-14}$ equiv $Cl^- \cdot s^{-1} \cdot cm^{-2}$. This is because there is a change in the relative permeabilities of K^+ and Cl^- after valinomycin treatment. Before valinomycin treatment it was found that $P_{Cl}/P_{Na} = 0.6$ and $P_{Cl}/P_K = 0.47$ but after the addition of the antibiotic $P_{Cl}/P_K = 0.04$. From the Goldman Voltage Equation such a change in the permeability characteristics changes the relative effect of the internal K^+ and Cl^- concentrations on the size of the diffusion potential (see Eqn 6). For example, when $P_{Cl}/P_K = 0.47$ a rise of internal KCl to 10 mM results in a decrease of the potential from about 67 to 61 mV while after valinomycin treatment a 10-mM rise of KCl inside would decrease the initial potential of 65 to about 32 mV.

In most cases, however, the size of the potential corresponding to the initial height of the salt-induced signals before and after treatment with valinomycin differ as shown in Fig. 1. In these cases, according to Eqn 10 the passive influx of Cl^- should be different. Fig. 4 shows the analysis of some experimental data in which $\Delta\psi_L = 65.5$ mV. The curves drawn correspond to net Cl^- influxes given by the first-order equations shown and are the best fit to the experimental data. The Cl^- influx (ϕ_{Cl}) corresponds to $1.9 \cdot 10^{-14}$ equiv $Cl^- \cdot s^{-1} \cdot cm^{-2}$ before valinomycin treatment and to a slightly lower influx (ϕ_{Cl}^{Val}) of $1.5 \cdot 10^{-14}$ equiv $Cl^- \cdot s^{-1} \cdot cm^{-2}$ after treatment with valinomycin. Again it can be seen that although the chloride flux is lower after valinomycin treatment the decay kinetics of the signal is faster. Using the Goldman Flux Equation (Eqn 10) it is possible to check whether the differences in the initial membrane potentials account for the differences in the influxes.

Assuming the permeability to Cl^- is not altered by valinomycin then the flux ratio for this experiment should be given by

$$\frac{\phi_{Cl}}{\phi_{Cl}^{Val}} = \frac{85.5}{63.0}\left[\frac{1 - \exp(-63F/RT)}{1 - \exp(-85.5F/RT)}\right] \tag{11}$$

This corresponds to an expected flux ratio of 1.29 which compares well with the observed ratio of 1.27.

Pretreatment with various KCl levels

As Fig. 5 shows, the size of a 50 mM KCl-induced signal is affected by pretreating the chloroplasts with various KCl levels. Qualitatively this is what would be expected from the diffusion potential model. Whether the magnitude of the inhibition observed is consistent with the theoretical approach presented above requires the application of Eqn 3.

In this experiment the chloroplasts had been treated with valinomycin and the increasing KCl pulses gave signals which corresponded to a $P_{Cl}/P_K = 0.03$ (see Fig. 6). Using this value, the size of the 50 mM KCl-induced signals after various initial KCl additions should be given by:

$$L = \frac{[K^+]_o + 0.03[Cl^-]_i}{[K^+]_i + 0.03[Cl^-]_o} \tag{12}$$

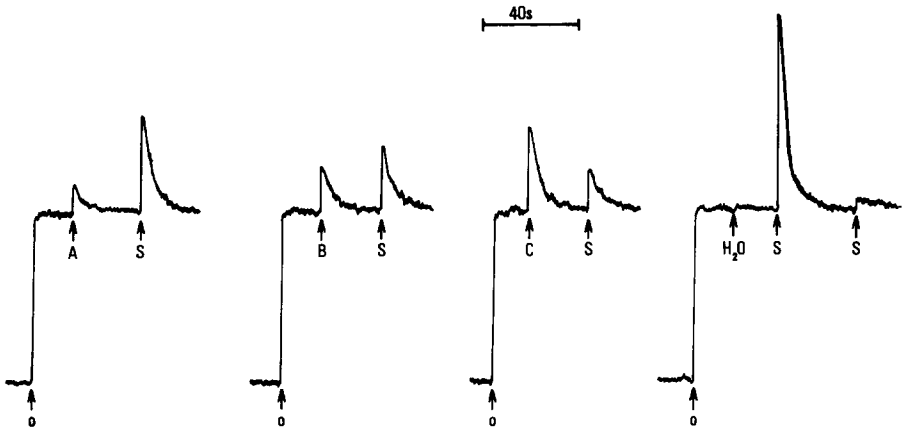

Fig. 5. The effect of preinjections of various KCl concentrations on the intensity of the 50 mM KCl-induced signals. The preinjections were: A, 3 mM KCl; B, 6 mM KCl; C, 12 mM KCl. Injections S correspond to 50 mM KCl additions. Also shown is the effect of injecting 100 μl of water. The chloroplasts had been treated with 0.5 μM valinomycin and the chlorophyll concentration was 26 μg/ml. Other details are the same as Fig. 2.

As Fig. 6 shows, good agreement between theory and experimental observation was obtained.

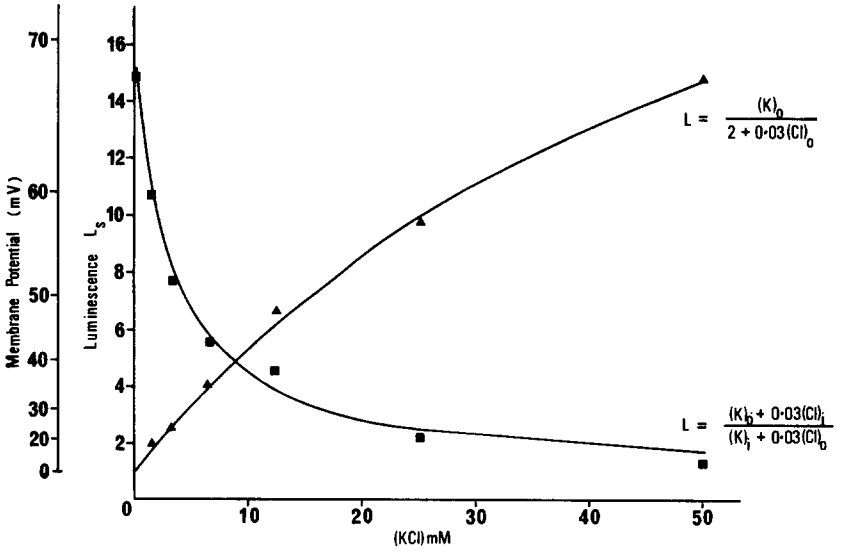

Fig. 6. A quantitative analysis of the data obtained from the experiment shown in Fig. 6. The closed squares are the initial heights of the 50 mM KCl-induced signals after the various preinjections of KCl at the concentrations indicated. The closed triangles are the initial height of the preinjection KCl signals. The curves through the experimental points have been drawn according to the equations shown and the membrane potential scale calculated in the way already reported[7].

Effect of gramicidin D

Fig. 7 shows the effect of gramicidin D on the KCl-induced signals. Unlike valinomycin treated chloroplasts it made no difference whether KCl or NaCl was used. With gramicidin concentrations of $3.3 \cdot 10^{-7}$ M and above, the intensity of emission was reduced to a value below that observed with valinomycin and there was a complete inhibition of the KCl -and NaCl-induced signals. With lower gramicidin concentrations, KCl signals could be detected and corresponded to diffusion potentials greater than those observed in the absence of this antibiotic but less than that found with valinomycin-treated chloroplasts.

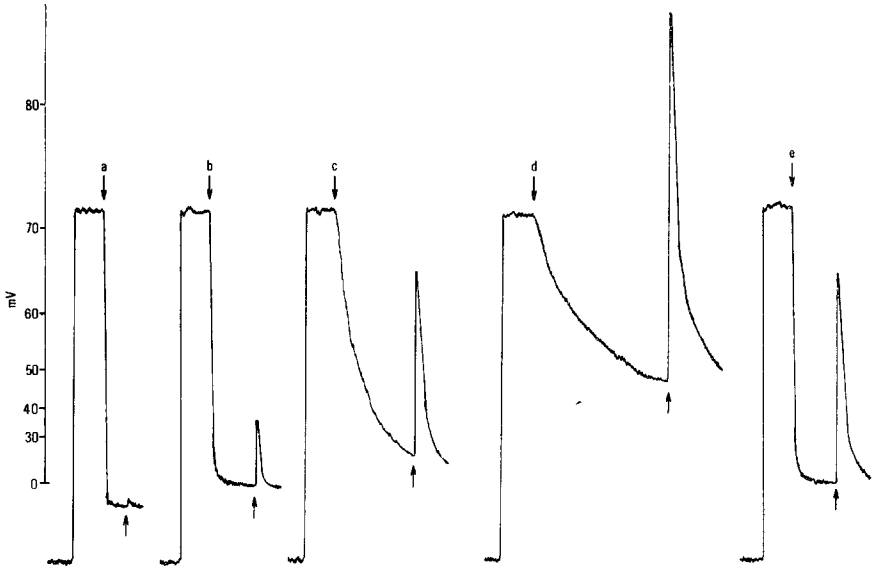

Fig. 7. The effect of gramicidin D on millisecond delayed light and the 50 mM KCl-induced signals. The upward pointing arrows indicate 50 mM KCl additions. Injections a, b, c and d correspond to $3.3 \cdot 10^{-7}$ M, $3.3 \cdot 10^{-8}$ M, $3.3 \cdot 10^{-9}$ M and $3.3 \cdot 10^{-10}$ M gramicidin additions, respectively. Injection e was 0.5 μM valinomycin. Chlorophyll concentration was 29 μg/ml and all other conditions were the same as Fig. 2.

DISCUSSION

There seems to be a good correlation between the experimental observations and theoretical concepts presented in this and earlier papers[7,10]. Under various conditions it is possible to account for both the magnitude and kinetics of the KCl and NaCl signals in terms of a diffusion potential model. From this it seems that millisecond delayed light, like the 515-nm shift[12], is an indicator of electrical gradients across the thylakoid membranes and can be used to give a measure of their size and polarity. From the analyses presented it seems that rapidly raising the external KCl or NaCl concentration and also illumination of chloroplast suspensions causes the thylakoid interiors to become electrically more positive with respect to their medium. Of course, the assumption that $\Delta\psi_L$ is reduced to zero by valinomycin can only be valid in the absence of ion gradients and may only occur if K^+

is out of equilibrium. If in fact significant light-induced ion gradients exist across the thylakoid surface then there could be a diffusional, as well as an electrogenic, component of the membrane potential. Without knowing the values of the ion gradients and the permeability properties of the membrane it would be impossible to say how valinomycin would effect such a potential. For example, suppose the major ion gradients created in the light are H^+, K^+ and Cl^-. According to the Goldman Voltage Equation[8,9] the diffusion potential ($\Delta\psi_d$) created as these ions diffuse independently and passively down their concentration gradients across an ion selective membrane would be given by:

$$\Delta\psi_d = \frac{RT}{F} \ln \frac{P_H[H^+]_o + P_K[K^+]_o + P_{Cl}[Cl^-]_i}{P_H[H^+]_i + P_K[K^+]_i + P_{Cl}[Cl^-]_o} \quad (13)$$

where P_H,P_K and P_{Cl} are the permeability coefficients and $[\,]_o$ and $[\,]_i$ mean outside and inside activities, respectively. If $P_K > P_{Cl} \gg P_H$ and $[K^+]_o > [K^+]_i$ the diffusion potential would be positive inside being mainly governed by K^+ distribution although it seems likely from the above experiments that chloride would have some influence. If on the addition of valinomycin $P_K \gg P_{Cl}$ then the potential would become more positive. On the other hand if $P_H > P_K > P_{Cl}$ and $[H^+]_i > [H^+]_o$ the diffusional potential would be inside negative. With valinomycin present there could well be a reversal in the relative permeabilities such that $P_K > P_H$. This would mean that the potential would change polarity. Clearly from these considerations there is some problem in using the analyses presented in this paper for estimating $\Delta\psi_L$ under phosphorylating conditions when ion gradients are known to exist. There are, however, reports in the literature of chloroplast particles which seem to phosphorylate in the absence of light-induced pH gradients[14,15]. Accepting Mitchell's[16] arguments this would mean that a large electrical gradient exists in these particles which presumably is derived from an electrogenic mechanism. If this is correct then the analyses of millisecond delayed light which I have presented should be of some use. If in fact pH gradients do exist in these subchloroplast particles, as suggested very recently by Rottenberg and Grunwald[17] then one is again faced with the possibility that there may be a diffusional component of the membrane potential.

By analysing the decay characteristics of the KCl and NaCl signals it is possible to estimate the Cl^- flux under known driving conditions. For a 50-mM Cl^- gradient and potentials in the region of 50 to 100 mV (inside positive) the influx of this anion corresponded to approximately 10^{-14} equiv·cm^{-2}·s^{-1}. Accepting that this value is based on rather crude estimates of thylakoid dimensions, the rate is very slow. It is usually assumed that the thylakoids are leaky to Cl^- (ref. 18). From the above analyses, however, the relatively rapid decay of the salt-induced signals is not due to a leaky membrane but almost certainly reflects the very large surface area to volume ratio which could be in the region of 10^6 (as estimated above). Using Eqn 10 it is possible to get a quantitative estimate of the permeability of the thylakoids to Cl^-. Taking the data of Fig. 2 it was found that for a Cl^- gradient of 50 mM and a membrane potential of +93 mV the influx of this anion was $4.0 \cdot 10^{-14}$ equiv $Cl^- \cdot s^{-1} \cdot cm^{-2}$. Substituting these values in Eqn 10 give $P_{Cl} = 2.1 \cdot 10^{-10}$ cm·s^{-1}.

Although this analysis seems reasonable it gives a P_{Cl} value which is at present difficult to reconcile with some of the experimental findings. It has been estimated that P_K was about 30 times greater than P_{Cl} after valinomycin treatment. This would give a P_K value in the region of 10^{-8} to 10^{-9} cm·s^{-1}. Such values would seem unacceptably low bearing in mind the reported effect of valinomycin on artificial membranes[19]. For the moment I have no clear explanation for this. It could be that the estimate of the P_{Cl}/P_K ratio after valinomycin treatment is incorrect although earlier analyses of the KCl induced 10-s delayed light using a rapid mixing system gave very similar values[1,10]. Alternatively a deviation from a Nernst Potential relationship when valinomycin is present (that is P_{Cl}/P_K ratio = 0) could be due to the net transfer of K^+ across the membrane in the absence of diffusing coions. Such a significant breakdown in the laws of electroneutrality for systems having very large surface area to volume ratios has already been discussed in some detail by Mitchell[16]. Another possibility is at the valinomycin concentrations employed there may be some loss of cation selectivity resulting in an increase of membrane conductance to protons as well as K^+.

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