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CURRENT-VOLTAGE STUDIES ON THE THYLAKOID MEMBRANE IN THE PRESENCE OF IONOPHORES

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SUMMARY

The reversibility of the binding of ionophores to the thylakoid membrane is studied. While gramicidin binds practically irreversibly, valinomycin and nonactin bind reversibly, however, only a small fraction (about 1 %) of the membrane-bound valinomycin or nonactin is active in ion transport. The current-voltage relationship is evaluated under these circumstances. We have found that it is practically linear. This together with the relationship between current and ion concentration agrees qualitatively with the results reported for bimolecular lipid membranes, which contain a large fraction of negatively charged lipids. For the ionophores, valinomycin and nonactin, the binding equilibria ($K \approx 10^4$) and the turnover numbers ($\approx 3 \cdot 10^4$/s) are evaluated for their action on the thylakoid membrane. Possible reasons for the inactivity of the majority of membrane-bound ionophore molecules are discussed.

INTRODUCTION

Current-voltage studies are a useful tool for investigating the molecular mechanisms of the electric conduction of membranes. The mechanism of action of certain ion transporting antibiotics on bimolecular lipid membranes was elucidated by this approach. Evidence was provided that some of these agents act as mobile carriers [1–3], while others form ion conducting pores [4–6] or channels [7] (for a review see ref. 8).

Considering the experimental results obtained for artificial membranes and the theoretical models derived therefrom it is worthwhile studying the current-voltage behavior of biological membranes in vivo. Taking into account that more than half of the dry weight of the functional membranes of respiration and photosynthesis is non-lipid material, it is of special interest as to whether or not the electric behavior of a biological membrane can be approximated by that of the more or less homogeneous lipid matrix of a bimolecular lipid membrane.

Unfortunately the common direct measuring techniques by electrodes are restricted to macroscopic membrane systems. On submicroscopic membrane systems (photosynthesis, respiration, primary photoreception) only indirect methods are applicable. Based on the favorable properties of the functional membrane of photosynthesis we developed an indirect method for current-voltage studies on the inner membrane of chloroplasts, the thylakoid membrane [9].

A light-driven electric generator is intrinsically incorporated in the thylakoid membrane [10]. On stimulation of chloroplasts with a short flash of light, vectorial electron transport [11,12] generates an electric potential difference of the order of 100 mV in less than 20 ns [13]. Another favorable condition is the high pigmentation of the thylakoid membrane (mainly chlorophylls and carotenoids). The bulk pigments respondby electrochroic shifts of their absorption bands to the light-induced electric field in the membrane. The electrochroic nature of some of the absorption changes observed on excitation of chloroplasts with light was established by three independent lines of experimental evidence: kinetic [10], spectroscopic [14–16] and electric [17–20].

The electrochroic absorption changes represent a molecular voltmeter in the membrane which, according to evidence reviewed below, works pseudolinearly. Under the assumption that the electric capacitance of the membrane is independent of the voltage, the current density across the membrane can be calculated from the first derivative of the time course of these absorption changes.

This indirect method, however, is handicapped by the fact that the average voltage and the average current density read from absorption changes in a typical experiment result from of the order of 10^{12} photosynthetic vesicles. Although it is almost certain that the sample is homogeneous with respect to the electric potential generation after a short flash of light, the preparation procedure or other factors may cause a broad distribution in the domain of specific conductivity among the vesicles.

In this paper, experimental criteria are derived which allow for an evaluation of the non-homogeneity of the sample. For photosynthetic vesicles doped with the ion carrying antibiotics, valinomycin and nonactin, respectively, the distribution of vesicles in the domain of specific electric conductivities is determined and quantitatively taken into account in order to evaluate the current-voltage behavior of a single vesicle from the observed absorption changes. Surprisingly, only a small percentage of ionophore molecules bound to the membrane are active, while about 99 % of membrane-bound ionophores do not contribute to the electric conduction. We found a practically linear increase of the current with the voltage in the case of valinomycin and nonactin. The dependence of current on the ion concentration is linear for nonactin and exhibits saturation for valinomycin.

In our earlier studies [9] we assumed that almost all membrane-bound valinomycin molecules are active in ion transport. Under this assumption we derived a hyperbolic sine for the current-voltage relationship. The experiments reported in this paper reveal that the apparent hyperbolic sine relationship reflects a Poisson distribution of active ionophore molecules over vesicles rather than the current-voltage behavior of a single vesicle. The corrected current-voltage relationship is discussed in the light of current models for ion transport by ionophores.

MATERIALS AND METHODS

Chloroplasts

Chloroplasts of the broken type isolated according to Siggel et al. from market spinach were used [21]. The preparation method left the electric properties of the inner membranes practically unchanged, while the outer envelope was stripped off.

The chloroplasts were suspended in an aqueous volume of 15 ml (except the measurements in Fig. 6). The reaction mixture contained: tricine, 2 mM (pH 8); benzyl-viologen, 6.7 μM, as artificial electron acceptor; monovalent ions, 20 mM (NaCl and KCl, respectively as indicated). The chlorophyll concentration in the cuvette was 10 μM. Deviations from these conditions are indicated in the legends.

The ionophoric agents were dissolved in ethanol. When added to the chloroplast suspension care was taken to keep the end concentration of ethanol below 0.5 %. Nonactin was a personal gift from Dr Bickel (CIBA).

Detection of absorption changes

For the measurements of transient absorption changes at 520 nm the cuvette (optical path length 2 cm, except for Fig. 6) was placed in a rapid kinetic spectrophotometer [22]. Photosynthesis was excited with saturating flashes of light (half time of duration $\tau_{1/2} = 15\ \mu s$, wavelength interval 630–680 nm) short enough to turn over each light reaction once (single-turnover flash). To improve the signal to noise ratio the signals were induced repetitively and between 100 and 1000 transients were averaged [23]. The repetition rate was 1 cps.

Artifacts resulting from flash light scattered in the turbid chloroplast suspension were avoided by a combination of guard filters (BG 38/4 and DAL 520 (Schott)) placed on the cathode of the photomultiplier (EMI 9558 BQ, 2 inches diameter). Artifacts due to transient changes of the light scattering properties of the sample on excitation were practically eliminated by placing the cathode of the photomultiplier close to the absorption cell.

Evidence for the pseudolinear electrochroic response of carotenoids in the thylakoid membrane

Our approach to current-voltage studies by electrochroic absorption changes incorporates two possible sources of misinterpretation; besides the statistical problems which are dealt with later on in this paper, deviations from a strict proportionality between the electrochroic absorption change and the voltage across the membrane may cause error.

The linearity of the absorption changes around 520 nm in dependence on the voltage across the membrane was studied both in vivo and in vitro. The following results were obtained:

Linearity between the absorption change and the number of charges translocated. Correlation of the extent of the light-induced absorption change at 520 nm with the number of protons translocated across the functional membrane of photosynthesis yielded a 1:1 correspondence for a range of variation of six [24].

Linearity between the absorption change and the voltage across the membrane. Recently, the pseudolinear response of the electrochroic absorption change to the voltage across the membrane was experimentally confirmed by an electric induction technique. A perfect 1 : 1 correspondence of the voltage as detected by macroscopic electrodes and detected by the electrochroic absorption changes at 520 nm was observed [19]. Other evidence for a pseudolinear response of carotenoids was reported for bacterium chromatophores. The absorption changes around 523 nm (carotenoid shift) correlates with the extent of a diffusion potential across the chromatophore membrane induced artificially by a salt jump [20].

Evaluation of the voltage and the current density across the membrane from transient electrochroic absorption changes

Thus the extent of the electrochroic absorption change is linearly correlated with both the voltage and the charge separated by the membrane. This, according to Eqn 1, implies that the membrane capacitance is practically voltage independent.

$$\Delta A \sim C \cdot U = \Delta Q \qquad (1)$$

ΔA is the absorption change, U the voltage across the membrane, ΔQ the charge displacement and C the membrane capacitance.

Therefore it follows that the extent of the absorption change is proportional to the voltage across the membrane, while the electric current density ($< j >$) is proportional to the first derivative of the absorption change with respect to time (see Fig. 1).

$$< j > \sim \frac{d\Delta A}{dt} \qquad (2)$$

(For the consideration of the relation between the time course of the absorption change and the underlying electrical events see the legend of Fig. 1).

It has been argued that one should not expect linear electrochroism from symmetrical molecules like the carotenoids; which, according to ref. 14, are mainly responsible for the electrochroic absorption change around 520 nm. However, it was demonstrated for a monolayer model system that polar or dipolar headgroups of lipids create a very high constant electric field strength in the membrane. This

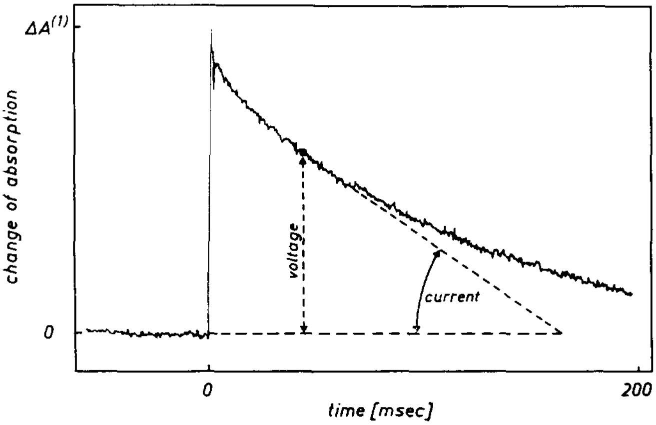

Fig. 1. Time course of the absorption change at 520 nm on excitations of photosynthesis by a short flash of light at $t = 0$. The rapid rise of absorption at this time indicates the induction of an electric potential difference across the membrane. The relaxation of the absorption change reflects the discharging of the membrane capacitance via its conductance. Evaluation of voltage and current density across the thylakoid membrane (for details see text and ref. 9).

imposes a strong bias on symmetrical molecules so that they respond pseudolinearly to the relatively smaller transient electric field across the membrane [16, 25].

Test for electric non-homogeneities in a chloroplast suspension

A complication for the evaluation of the voltage and the current density from the observed transient change of absorption ($\Delta A(t)$) arises from the fact, that it represents the sum of contributions from (of the order of) 10^{12} vesicles in the cuvette. Therefore, the extent of the absorption change is a straightforward indicator of the voltage only if the voltage transients on different vesicles are identical or, at least, obey a normal distribution around an average behavior. If not, the observed transient absorption changes do not directly reflect voltage changes on an "average vesicle".

One has to distinguish between non-homogeneity concerning on the one hand the electric potential generation and on the other hand the relaxation of the electric potential via the membrane conductance. The following arguments favor the homogeneity of a chloroplast suspension with respect to the electric potential generation. The functional unit of the electric potential is at least as big as one thylakoid [10] containing more than 10^5 chlorophyll molecules which correspond to about 400 electric generators [11]. This great number makes it reasonable to assume that the density of electric generators per membrane area will be approximately the same for all vesicles, which originated from the same batch of spinach. Since the electric potential rises with the exciting short flash of light we may assume that all vesicles start with approximately the same voltage after the termination of the flash.

Let us now ask, whether the sample is homogeneous with regard to the relaxation process, too. If not, relaxation of the electrochroic absorption change will reflect the distribution of vesicles over the domain of "specific conductances" rather than the relaxation of the voltage on an "average vesicle". The voltage at vesicles with a high conductance will drop rapidly, while it does so slowly at those with a lower one. If there were only two classes of vesicles in the suspension the decay of the electrochroic absorption change would be biphasic. The rapid phase being due to "rapid vesicles" with a high conductance, the slow phase due to "slow ones" with a low conductance.

Non-homogeneity in this sense can be tested as follows. By chemical blocking it is possible to excite only about one half of the electric generators in the thylakoid membrane [11, 26]. So either the full or approximately the halved voltage can be induced in a single-turnover flash. Thus there are two ways of generating half of the single-turnover flash value of the electrochroic absorption change: (1) by starting with half the maximal voltage at each vesicle or (2) by starting at the full voltage and waiting for relaxation to half the maximum absorption change. If the sample is non-homogeneous the ensemble which belongs to the half of the maximal absorption change obtained by these two different ways will differ, depending on the past history of each. In the first ensemble, started from half of the maximal voltage, all vesicles experience this voltage. In the other ensemble started from the full voltage, some of the vesicles (the "fast type" with a high conductance) have almost discharged to zero voltage, while others (the "slow type" with a low conductance) have practically retained the maximal value of the voltage. But together they produce half of the maximum absorption change.

Thus, the slope of the absorption change at half of the maximal extent will differ depending on the history, if the sample is non-homogeneous. On the other hand the slopes will coincide if it is homogeneous and if the current density is a unique function of the voltage.

These considerations are illustrated by a model experiment. The results are shown in Fig. 2. A non-homogeneous sample was prepared by mixing equal amounts of fresh chloroplasts and aged chloroplasts. Fresh chloroplasts alone reveal a slow decay of the electrochroic absorption change. Their membrane conductance is low. Aged chloroplasts show a faster decay, since the conductance is high. The left part of Fig. 2 shows the time course of the absorption change of the mixed sample, when all electric generators are operative. The decay is biphasic. In the right part of Fig. 2 only half of the electric generators are operative and the extent of the absorption change, as well as the voltage, is approximately halved. The slopes in both cases are quite different as expected because of the non-homogeneity.

Up to here we have argued as though there are only two kinds of vesicles present in the sample. But it is obvious, that the argument still holds if the vesicles are distributed over a spectrum of conductivities.

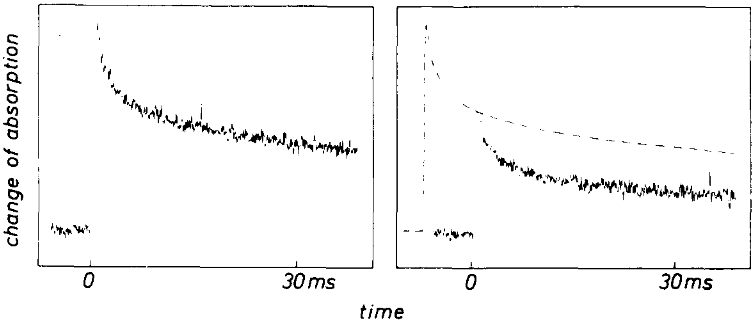

Fig. 2. Absorption change at 520 nm of a sample consisting of a mixture of aged and fresh chloroplasts. Left: All light reactions are working. $6.7 \cdot 10^{-6}$ M benzylviologen was used as the artificial electron acceptor (standard reaction medium). Right: After blocking light reaction II by $2 \cdot 10^{-6}$ M 3-(3,4-dichlorophenyl)-1,1′-dimethylurea only about one half of the light reaction was operative. As electron donor a system of $2.7 \cdot 10^{-5}$ M thymol-indophenol and 10^{-3} M ascorbate, and as electron acceptor $6.7 \cdot 10^{-6}$ M benzylviologen were used.

The alternative case to Fig. 2, a biphasic decay of a homogeneous sample, was observed for the intrinsic conductivity of the thylakoid membrane which is coupled with the ATP synthase under phosphorylating conditions [27]. The biphasic decay under these conditions vanished if the initial voltage dropped under a critical value corresponding to the bend between the rapid and the fast phase.

RESULTS AND DISCUSSION

Evidence for a non-homogeneous distribution of vesicles in the domain of specific conductivities induced by valinomycin and nonactin

We studied the conductivity of the functional membrane of photosynthesis in the presence of the ionophores, valinomycin and nonactin. Prior investigations with valinomycin were presented elsewhere [9]. The effects of valinomycin on the electrochroic absorption changes in chloroplasts were as follows. The decay was accelerated by valinomycin in the presence of K^+, however, it was practically unchanged in the presence of Na^+ as the dominating cation. This parallels the specificity of valinomycin for K^+ over Na^+ found on black lipid membranes [28]. We observed a similar effect for nonactin, although the selectivity against Na^+ was less pronounced than with valinomycin. Both agents (at moderate concentrations e.g. 20 nM for valinomycin and the half for nonactin) increased the conductance of the thylakoid membrane by orders of magnitude.

It was suggested that the vesicles behaved like a homogeneous sample in the presence of these antibiotics. At first sight one would expect homogeneity since these compounds were presented at a concentration of about 10–100 nM on 10 μM chlorophyll. Under the assumption that any ionophore molecule binds to the membrane this implies about 100 to 1000 ionophore molecules on 10^5 chlorophyll molecules, which is the "minimum" size of one vesicle. Thus the ionophore distribution over vesicles should follow a narrow normal distribution.

The outcome of the experimental tests was strikingly different from this expectation. It is obvious from an inspection of Fig. 3 that there are different slopes at the halved extent of the absorption change depending on the initial conditions. The same result was obtained for valinomycin. According to the above considerations this implies a non-homogeneous distribution of the ionophores among different vesicles. This point is difficult to understand and it was not considered in our earlier studiess which need to be modificated for this reason.

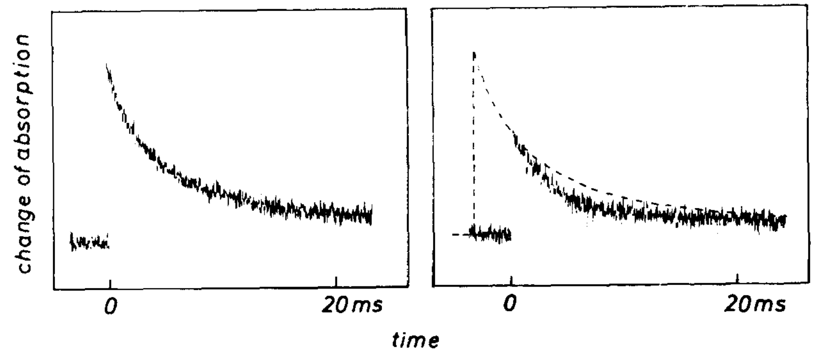

Fig. 3. Absorption change at 520 nm induced by single-turnover flashes after the addition of $1.3 \cdot 10^{-7}$ M nonactin. The average cation concentration was $2 \cdot 10^{-2}$ M (10^{-2} M KCl and 10^{-2} M NaCl). Other conditions as in Fig. 2.

Binding equilibria for valinomycin and nonactin on the thylakoid membrane

A possible explanation for the cause of the non-homogeneous distribution is that the distribution which we see is an artifact caused by the adding procedure. We added a small volume (say 10 μl) of concentrated ethanolic ionophore solution to 15 ml of a chloroplast suspension. Ionophores have a hydrophilic exterior and, therefore, they are bound very tightly by a lipophilic membrane. The vesicles which "see" the drop with the ionophores first extract most of them and bind them irreversibly. For the last vesicles to encounter the ionophore solution the probability to obtain an ionophore is low. So a rather broad distribution of ionophores over vesicles is generated.

This interpretation holds only if the ionophores do not equilibrate between the water and the membrane phase. Equilibration would cause a homogeneous distribution after an induction period. To test the above interpretation we studied the equilibration of the ionophores between the membrane and the aqueous phase. Chloroplasts were suspended in a reaction mixture, the ionophore was added and the absorption change was measured. Subsequently the chloroplasts were centrifuged, the pellet was resuspended in new reaction mixture and the absorption change was once more measured. The results are shown in Fig. 4. For chloroplasts in the presence of nonactin the decay of the electrochroic absorption change becomes slower after washing (centrifugation and resuspension in nonactin-free solution). With valinomycin the effect can also be seen, but is not so conspicuous. In the presence of gramicidin there is practically no change. In the control experiment with chloroplasts alone there is practically no change after the washing.

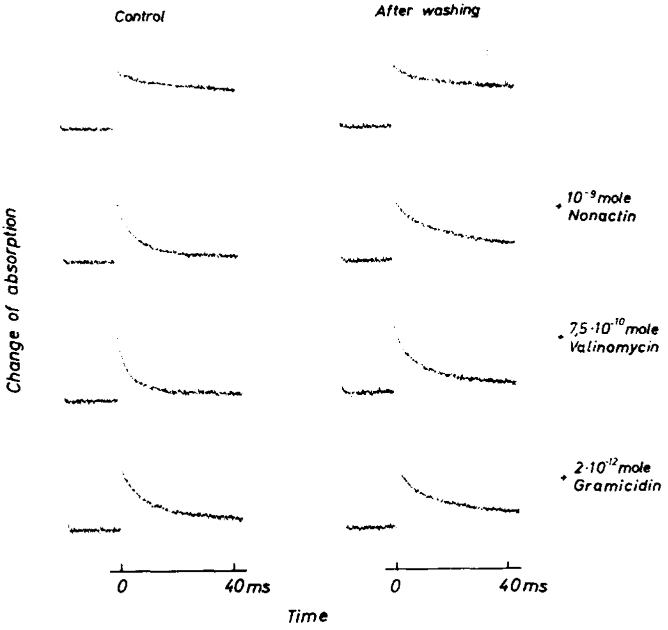

Fig. 4. Time course of the absorption change at 520 nm induced by single-turnover flashes. Left (Control) chloroplasts were suspended in the standard reaction medium plus antibiotics as indicated; Right, the chloroplasts were centrifuged (5 min at 10 000 × g) and resuspended in new standard reaction medium without the addition of antibiotics.

For valinomycin and nonactin we conclude that the amount of membrane-bound ionophores is diminished, because one part went into the newly added water phase. This proves that these ionophores bind reversibly. In contrast gramicidin binds practically irreversibly to thylakoid membranes, as is obvious from the lower traces in Fig. 4.

Thus, for valinomycin and nonactin the non-homogeneity is not caused by the adding procedure of the ionophore, but instead reflects the specific interactions of these molecules with the membrane.

The distribution of valinomycin and nonactin between the membrane and the aqueous phase

We used two different methods for these quantitative investigations. The first assay was based on the fact that the current density, j, at a given voltage, U, across the membrane mediated by the ionophore is proportional to the number, n_i, of active molecules in the membrane of a given vesicle, i.

$$j = n_i \cdot f(U) \tag{3}$$

$f(U)$ describes the current-voltage behavior of a vesicle having a single active ionophore molecule.

Since at $t = 0$ just after the exciting flash all vesicles are synchronized to the same voltage, the average current density ($< j >$) which is proportional to the total number of ionophore molecules N can be read from the slope of the absorption change at this time.

$$\frac{\mathrm{d}}{\mathrm{d}t} \Delta A \sim \frac{\mathrm{d}}{\mathrm{d}t} \sum_i U_i = \alpha f(U) \sum_i n_i = \alpha f(U) \cdot N = < j > \tag{4}$$

Where i indicates the number of the vesicle and α is a constant.

In Fig. 5 the current density determined by this method is plotted against the concentration of added nonactin (for valinomycin see ref. 1, Fig. 4). The linear relationship obtained indicates that the ionophore molecules act independently from each other.

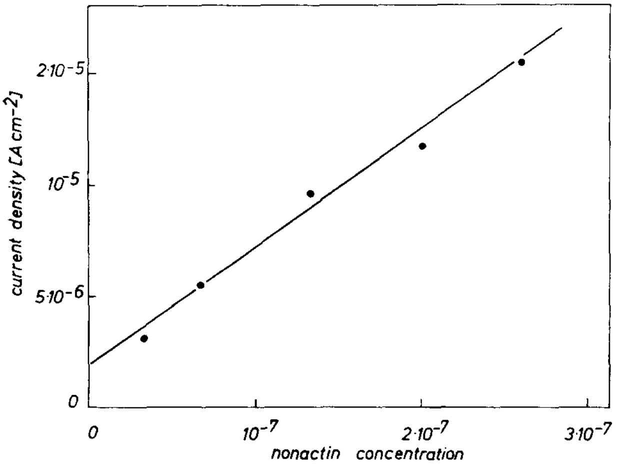

Fig. 5. Dependence of the current density at $t = 0$, corresponding to the maximal voltage induced in a single-turnover flash $U^{(\mathrm{I}+\mathrm{II})}$ on the average nonactin concentration. The segment on the ordinate indicates the basal current density due to the intrinsic conductance of the thylakoid membrane. The cation composition was 10^{-2} M KCl and 10^{-2} M NaCl.

This plot can be used to calibrate the ionophore concentration in an aqueous solution. To determine quantitatively the distribution of ionophores between the membrane and water we proceeded as follows. Chloroplasts were suspended in the reaction medium and aliquots of ionophores were added. After spinning down the chloroplasts new chloroplasts were added to the supernatant and the ionophore concentration was determined by the described assay. So we found that 30–40 % of the initially added ionophore molecules were bound to the membrane.

There exists another indirect way for the determination of the distribution. If the total amount of ionophores (concentration c_0) is kept constant, the number of bound molecules can be altered by changing the chlorophyll concentration (c_{chl}) and as consequence changing the total membrane volume (V_{M}). Assuming a thermodynamic equilibrium with a constant K for the distribution of ionophores between both phases and a linear relationship (factor of proportionality α') between the concentration of ionophores in the membrane and the current density $< j >$ the following equation holds.

$$< j > = \frac{\alpha' \cdot c_0}{1/K + \gamma \cdot c_{\mathrm{chl}}} \tag{5}$$

Where $\gamma = V_{\mathrm{M}}/(V_{\mathrm{W}} \cdot c_{\mathrm{chl}})$ with V_{W} = volume of the water phase ≈ total volume of the sample.

In Fig. 6 the reciprocal "extra current density" caused by the ionophores is plotted against the average chlorophyll concentration. The "extra current density" was calculated from the total current density read out from the slope of the transient absorption change by subtraction of the intrinsic basal conductivity in the absence

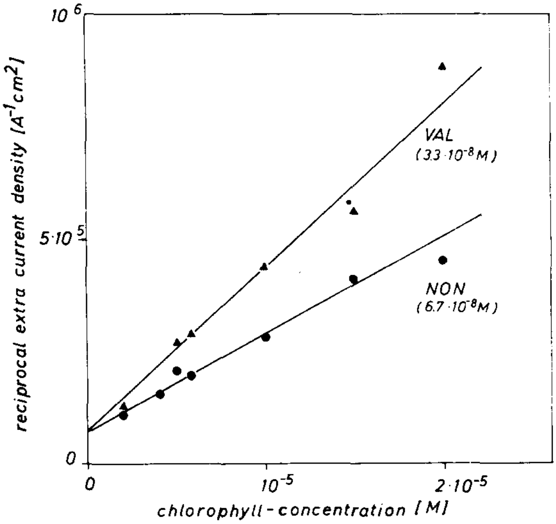

Fig. 6. Dependence of the reciprocal extra current density at the voltage $U = U^{(\mathrm{I}+\mathrm{II})}$ (at $t = 0$) on the average chlorophyll concentration. The concentration of valinomycin or nonactin was kept constant. The cation composition was $6.7 \cdot 10^{-3}$ M KCl and $1.3 \cdot 10^{-2}$ M NaCl. For these measurements the optical ground absorption of the sample was kept constant in order to optimize the signal to noise ratio. Cuvettes with different optical path lengths (1–10 cm) were used for this purpose.

of the ionophore. The observed behavior fits well with the theoretically expected linear relationship (Eqn 5) between the inverse current density and the inverse chlorophyll concentration for both valinomycin and nonactin. From the slope and the intercept on the ordinate the amount of membrane-bound molecules can be calculated according to Eqn 5. At a chlorophyll concentration of 10 μM we found that 64 % of the nonactin molecules and 76 % of the valinomycin molecules bind to the membrane.

The difference in the results of both methods may be attributed to a loss of ionophores in the vessels during the centrifugation work due to the surface active properties of these substances [29]. However, it is a reasonable basis for further argumentation that about one half of the initially added ionophore molecules are bound to the membrane under our conditions (average chlorophyll concentration, 10 μM).

From this we may calculate the order of magnitude of the distribution constant of the ionophores between the membrane and the water phase. A rough estimate of the membrane volume may be based on the following data; an area of 220 Å^2 per chlorophyll molecule in the thylakoid membrane [30]; a thickness of the hydrophobic core of the membrane of less than 70 Å. Thus the total membrane volume in a 15-ml suspension of broken chloroplasts at 10 μM chlorophyll is 1.3 μl.Taking the 1:1 (approx.) distribution of both ionophores between the membrane and the aqueous phase into account the partition equilibrium constant can be estimated; it is in the order of 10^4. This agrees with the order of magnitude reported for the partition equilibrium between artificial bimolecular lipid membranes and the aqueous phase ($5 \cdot 10^3$ for nonactin [2] and $2.5 \cdot 10^4$ for valinomycin [1]). Furthermore, from the measurements depicted in Fig. 5 it follows that the partition equilibrium constant is also higher for valinomycin than for nonactin for the thylakoid membrane.

Evidence for active and inactive binding sites for valinomycin and nonactin in the thylakoid membrane

With an ionophore concentration of 0.05 μM about 100 ionophore molecules are bound to a membrane area covered by 10^5 molecules of chlorophyll (the minimum "size" of one vesicle) as shown above. With respect to the 10^5 chlorophyll molecules we refer to, it is worth mentioning that the preparation of broken chloroplasts we used contained very little unpigmented membrane material. Thus the amount of chlorophyll is roughly proportional to the total membraneous volume in the suspension.

We have the result that more than a hundred ionophore molecules are reversibly bound to each vesicle. This gives an homogeneous value for the specific conductance that is probable for all vesicles (the relative mean square deviation is of the order of 1/10). In contrast to this expectation we find a non-homogeneous distribution. We have to conclude that only a minor fraction of the membrane-bound ionophore molecules is really active in ion transport across the membrane. As an illustration let us assume that there is a mean value of only one active molecule per vesicle besides a majority of say 99 inactive molecules. Then some of the vesicles contain no active ionophore molecules at all, some have one, some have two, and so on. The differences in the conductance between vesicles which have no active ionophore molecules (low basic conductivity) and those which have one active ionophore molecule or more, are rather large. This may cause the observed non-homogeneity of the sample.

One may argue that the membranes of the vesicles are not equally structured and so their different behavior in relation to the ionophores may be the reason for the non-homogeneous distribution. This intrinsic non-homogeneity may exist. However, it should give a narrow distribution within a homogenized chloroplast suspension.

We asked whether the observed decay curves of the electrochroic absorption change, which reveal the non-homogeneity, can be understood quantitatively on the assumption that the average load of active ionophore molecules per vesicle is rather low. For this we are concerned with the distribution of vesicles over classes with a load of zero, one, two . . . active ionophore molecules. Poisson's distribution is valid whenever the number of active binding sites per vesicle largely exceeds the number of ionophore molecules to occupy these sites. This is experimentally confirmed by the absence of any saturation of the current dependent on the ionophore concentration (Fig. 5), which we tested up to a concentration of 0.3 μM. The Poisson distribution has an important property which can be tested easily. By increasing the average number of actively bound ionophore molecules per vesicle to a number of about 10, the distribution shifts from the asymmetric Poisson to the practically symmetric Bernoulli. This makes the fraction of vesicles having no active ionophore molecules less and less important. Thus, in the observed decay curves the kink should occur at lower and lower values of the absorption change.

We calculated the time course of the absorption change with two assumptions: (1) a Poisson distribution with average loads of active ionophore molecules per vesicle from 1 to 8 (2) a linear current-voltage behavior (see below). (The quantitative treatment of the calculation is given in the Appendix.)

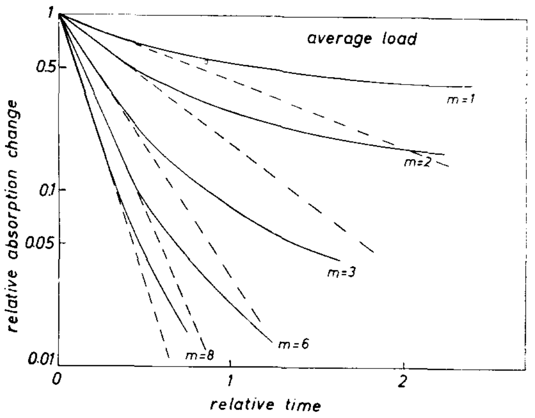

Fig. 7. Calculated time course of the logarithmic absorption change of a sample of vesicles with the following properties (see Appendix): a, all vesicles have the same voltage, at $t = 0$; b, the basic conductivity of the vesicles is zero; c, the conductivity induced by the ionophore molecules is proportional to their number; d, the current-voltage relationship of the ionophores is linear; e, the distribution of the ionophores over the vesicles obeys Poisson's distribution law; f, the observed absorption change is the sum of the absorption changes of all vesicles; g, the absorption change of a vesicle is proportional to its voltage.

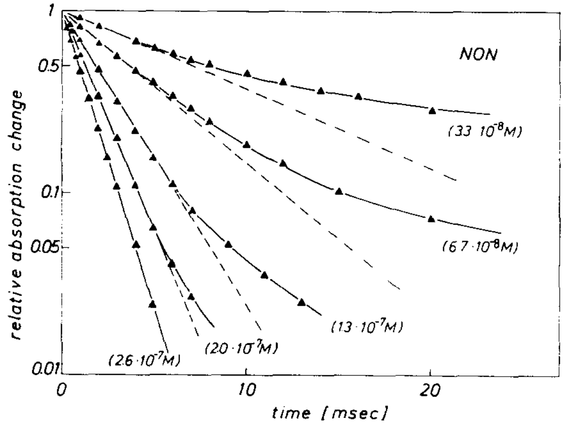

Fig. 8. Semi-logarithmic plot of the time course of the relative absorption change induced by single-turnover flashes (at $t = 0$) for increasing average nonactin concentration. Cation composition 10^{-2} M KCl and 10^{-2} M NaCl.

In Fig. 7 we plotted semi-logarithmically the calculated time course of the absorption change. With increasing average load, the time course becomes more and more exponential, as is obvious from a closer approximation to a linear semi-logarithmic plot. A similar plot of the time course of the measured electrochroic absorption changes for increasing ionophore concentrations showed good agreement with the expectation based on Poisson statistics and a linear current-voltage relationship (Fig. 8). This justifies the interpretation of the observed non-homogeneity by the Poisson distribution under the assumption that only about 1 % of the membrane-bound ionophores are active in ion transport. However, the second assumption which entered into the calculation, a linear current-voltage relationship, has still to be tested experimentally.

The current-voltage behavior of the thylakoid membrane in the presence of valinomycin and nonactin

An independent access to the current-voltage behavior which does not involve special assumptions about the kind of statistics the vesicles obey is obtained as follows. Since the electric potential difference across the membrane is generated within the short exciting flash all vesicles after termination of the flash are charged with the same voltage as discussed above. If only one light reaction (e.g. I) is active, the voltage stimulated in the flash is about one half of the voltage generated by the activity of both light reactions (I and II) [11]. Thus there are two voltages ($U^{(\mathrm{I+II})}$ and $U^{(\mathrm{I})}$) to which all vesicles can be synchronized at $t = 0$. According to Eqn 4, the average current is proportional to the slope of the absorption change on condition that all vesicles show the same voltage. The extent of the absorption change indicates pseudolinearly the voltage (constant of proportionality β). Thus Eqn 4 reads:

$$\frac{\mathrm{d}}{\mathrm{d}t}\Delta A \sim f(\beta \cdot \Delta A) \tag{4a}$$

The dependence of the first derivative of the time course of the absorption change on the extent reflects the functional relationship between the current density and the voltage at each vesicle, except for a normalization constant (β); $j \sim f(u)$.

From the two traces in Fig. 3 the ratio of the slope at the full extent and at the half (0.56) extent of the absorption change just after the flash is 7.3/3.9. Together with the slope 0 at the extent 0, these three points show that the current-voltage behavior of the inner membrane of chloroplasts in the presence of nonactin is roughly linear with a slight saturation. This agrees with the current-voltage behavior obtained from the decay of the electrochroic absorption change at higher ionophore concentration (Fig. 8). The same result was found in the case of valinomycin. Thus two independent types of experiments revealed an almost linear current-voltage behavior for valinomycin and nonactin on the thylakoid membrane.

Comparison of the results obtained for the action of valinomycin and nonactin on the thylakoid membrane with those obtained for other membranes

Conclusions as to the mechanism of ionic conduction can be inferred from the current-voltage behavior and from the dependence of the current at a fixed voltage on the concentration of the permeating ion. For nonactin this second relationship is depicted in Fig. 9, for valinomycin it is shown in ref. 9. It is striking, that the observed order of cation specificities for nonactin ($\mathrm{Na} \ll \mathrm{Cs} < \mathrm{Rb} < \mathrm{K}$) parallels the one found in two-phase extraction experiments [31], on bimolecular lipid membranes [2] and by vapor osmometry [32]. For valinomycin we found the cation specificity to be $\mathrm{Na} \ll \mathrm{K} < \mathrm{Cs} < \mathrm{Rb}$ in contrast to the sequence $\mathrm{Cs} < \mathrm{K} < \mathrm{Rb}$ found on bimolecular lipid membranes [28]. A possible clue for this discrepancy may be found in the experiments of Haynes and Pressman, who demonstrated in two-phase extraction experiments that the sequence for the cation specificity of valinomycin ($\mathrm{Cs} < \mathrm{Rb} < \mathrm{K}$) could be converted to ($\mathrm{Cs} < \mathrm{K} < \mathrm{Rb}$) when changing from nonpolar to more polar solvents [31]. Thus the microenvironnement of the valinomycin molecule in the chloroplast membrane may induce this difference in the sequence of the cation selectivity.

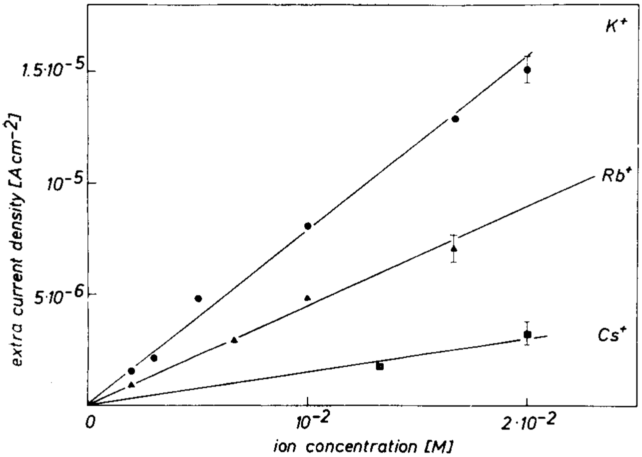

Fig. 9. Dependence of the extra current density at the voltage $U = U^{(\mathrm{I}+\mathrm{II})}$ (at $t = 0$) on the average concentration of the following permeating ions K, Rb, Cs. For constant ionic strength NaCl was added, when necessary, until a total concentration of $2 \cdot 10^{-2}$ M for the cation chlorides was achieved.

The roughly linear current-voltage relationship for valinomycin and nonactin on the thylakoid membrane is in agreement with findings on bimolecular lipid membranes [1,2]. The relationship between current and ion concentration that we found is linear for nonactin (Fig.9) and shows a pronounced saturation for valinomycin (ref. 9, Fig. 5). Stark and Benz observed a linear relationship on bimolecular lipid membranes formed from neutral lipids under conditions similar to ours (non-zero current) [1]. Only for valinomycin and a membrane formed from negatively charged lipids was a saturation observed (cation concentration higher than 10 mM) [1]. Considering the fact that the thylakoid membrane contains 13 % negatively charged lipids [33], our observations are similar to those on bimolecular lipid membranes. This suggests that the action of these ionophores on the thylakoid membrane is similar to their mode of action on the artificial system. The detailed studies revealed a carrier mechanism, the rate of which is limited either by the heterogeneous complexation rate or by the diffusion rate of the charged complex depending on the lipid composition [1,34].

Conclusions about the mechanism of ionic conduction from our pulse kinetic experiments based on a theory of the steady state behavior are justified only if an active ionophore molecule carries out very many turnovers for discharging the capacitance of the membrane. The number of turnovers of an average ionophore molecule can be calculated from our above estimates of the average load of active ionophore molecules per vesicle at a given concentration and from the decay time of the electric potential difference. Comparison of Figs 7 and 8 yields a minimum turnover time for nonactin of about $3 \cdot 10^{-5}$ s. This implies that 400 turnovers of an average nonactin molecule are required for discharging one vesicle corresponding to 10^5 chlorophyll molecules. This turnover number is of the same order of magnitude as the value estimated by Läuger under zero potential conditions [35]. It is two orders of magnitude lower compared to the value reported for mitochondria [36]. However, in the later estimate it was not considered that perhaps not all membrane-bound ionophore molecules were active in the mitochondrial membrane.

The above kinetic data give no evidence that the ionophores, valinomycin and nonactin, if active, work differently on the thylakoid membrane and on artificial black lipid membranes. It is probable that they act as mobile ion carriers. However, there is an unexpected quantitative difference since only about 1 % of membrane-bound ionophores works actively in ion transport, while the majority is inactive.

The reason for the large fraction of losses is not yet understood. One may argue that the ionophores are captured by the protein moeity of the membrane. However, we found only insignificant binding of ionophores to bovine serum albumin. We consider it improbable that the low efficiency is due to ionophores trapped by "dead" thylakoid membranes. If so, the reaction of "dead" membranes should exceed the fraction of active ones by two orders of magnitude.

A possible reason for the low efficiency of the membrane-bound ionophores may be sought in the special structure of the thylakoid membrane. The close interaction of the antenna pigments and the absence of rotational relaxation of chlorophyll a_I in photoselection experiments [37-39] gives evidence for the rigidity of large parts of the thylakoid membrane. Ionophores act as ion carriers only if the membrane is in a fluid state. They fail to act, if a fluid membrane by cooling is transformed into a solid state [40]. Thus the low proportion of active ionophores in the thylakoid membrane may reflect a relatively small fraction of fluid areas in this membrane.

APPENDIX

Calculation of the time course of the absorption change for an ensemble of vesicles doped with a number of ionophores following a Poisson distribution

Under the assumption of a linear current-voltage relationship and a zero basic conductivity for the single vesicle the extent of the absorption change contributed by a vesicle having k active and independent ionophore molecules is:

$$\Delta A_k(t) = \Delta A_k{}^0 \cdot e^{-k\rho t} \tag{A.1}$$

$\Delta A_k{}^0$ is the extent of the absorption change at $t = 0$ and ρ represents the quotient of the specific conductance and the specific capacitance of a vesicle with one ionophore molecule.

The extent of the absorption change observed on a system of M vesicles is the sum of the contributions from different vesicles.

$$\Delta A_{\mathrm{obs}}(t) = M \sum_{k=0}^{\infty} [w(k) \cdot \Delta A_k{}^0 \cdot e^{-k\rho t}] \tag{A.2}$$

The factor w (k) gives the probability for the existence of a vesicle with k active ionophore molecules. From Poisson statistics it follows that:

$$w(k) = \frac{n^k}{k!} e^{-n} \tag{A.3}$$

(where n is the mean value of the number of active ionophores per vesicle). For the observed absorption change the following equation may be obtained:

$$\Delta A_{\mathrm{obs}}(t) = \Delta A^0{}_{\mathrm{obs}} \sum_{k=0}^{\infty} \left[\frac{n^k}{k!} e^{-n} \cdot e^{-k\rho t}\right] = \Delta A^0{}_{\mathrm{obs}} \cdot e^{-n} \cdot e^{(n \cdot e^{-\rho t})} \tag{A.4}$$

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