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The Mechanism of Action of Valinomycin on the Thylakoid Membrane

Characterization of the Electric Current Density

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Summary. Most of the studies devoted to the mechanism by which certain antibiotics increase the ion permeability of *biological membranes* have been carried out on artificial *model systems*. Undoubtedly one of the major reasons for this was that some of the most relevant biological membrane systems are of submicroscopic dimensions and thus inaccessible to the common electrochemical measuring techniques. This holds for the inner membrane systems of chloroplasts, mitochondria, and retinal rods.

Since it is not trivial that a mechanism of action found for a model membrane works as well in a biological one with a much higher structural complexity, it seemed worthwhile to study the mechanism of action of ionophorous antibiotics on the above-mentioned biological membranes.

In this paper, a nonelectrochemical method for measuring both the voltage and the current across the inner chloroplast membrane (or thylakoid membrane) is established in extension of earlier work. This method is used to characterize the mode of action of valinomycin on the thylakoid membrane.

The mechanism of action of ionophorous antibiotics has been subject to intensive study. The special interest in these substances results mainly from the possibility that they may serve as model systems for active transport across biological membranes.

Moore and Pressman (1964) discovered the influence of valinomycin on the ion transport across the mitochondrial membrane. However, since the driving forces (of electrical or chemical nature) for the mitochondrial ion transport have not been measurable by commonly used methods, the first mechanistic evidence for the action of valinomycin has been derived from experiments with artificial model systems.

Studies by Mueller and Rudin (1967), Lev and Buzhinsky (1967), and Chappel and Crofts (1966) have revealed the ability of valinomycin to increase the electric conductance of bimolecular lipid membranes and artifi-

cial lipid vesicles. Studies on lipophilic bulk phases have induced Pressman Harris, Jagger and Johnson (1967) to propose that valinomycin and the whole class of compounds which they called "ionophores" may act as mobile ion carriers in lipid membranes. Eisenman, Ciani and Szabo (1968) found that the extra conductance which valinomycin-like macrocyclic antibiotics induce in bimolecular lipid membranes parallels their ability to extract certain ions into a lipophilic bulk phase. A quantitative treatment based on the carrier concept matched these experiments fairly well.

Liberman and Topaly (1968) have studied the dependence of the current across a valinomycin-doped bimolecular lipid membrane on the voltage and the ion concentration. A theoretical interpretation of these results based on the mobile carrier concept could be fitted to their experimental data (Markin, Pastushenko, Krishtalik, Liberman & Topaly, 1969).

Now, it is not at all trivial that the same concept of a mobile ion carrier matches the increase of conductivity induced by ionophores in a biological membrane equally well as in an artificial bimolecular lipid membrane. Thus it seems worthwhile to study the mechanism of action of valinomycin directly on a biological membrane. Unfortunately such an attempt suffers from the fact that minuscule dimensions of some of the most relevant biological membrane systems exclude the use of even miniaturized electrochemical measuring techniques. This holds for mitochondria and for the inner-membrane systems of chloroplasts and of retinal rods. Since measurement of the current-voltage behavior is necessary for the characterization of the mechanism of action of valinomycin in biological membranes, one has to strive for nonelectrochemical measuring techniques for the voltage and the current.

Recent studies have revealed that the thylakoid membrane is an appropriate subject for such an investigation. Thylakoids are disc-shaped vesicles inside of chloroplasts, in which the primary steps of photosynthesis take place.

It has been demonstrated that the thylakoid membrane (1) incorporates a light-driven electrical generator (Junge & Witt, 1968), and (2) contains pigments, which respond by electrochromic shifts of their absorption bands to the electric field across the membrane. These bandshifts have been used previously as a linear indicator for the electric potential difference across the thylakoid membrane (Junge & Witt, 1968—further papers on this subject have been summarized by Witt, Rumberg & Junge, 1968, and by Junge, Emrich & Witt, 1970). The first property of the thylakoid membrane provides an easy way for a pulse stimulation of an electric potential difference by light flashes; the second property provides something like a "molecular

voltmeter" with practically unlimited time resolution. (Wolff, Buchwald, Rüppel, Witt & Witt, 1969).

In this paper it will be demonstrated that this method of measuring the voltage across a microscopic membrane can be extended to yield also the current density across the thylakoid membrane. This will be used to characterize the current-voltage behavior of the valinomycin-doped thylakoid membrane.

Materials and Methods

We used freshly isolated spinach chloroplasts of the type characterized in a previous paper (Junge, Rumberg & Schröder, 1970). These chloroplasts were suspended in an aqueous solution to an average chlorophyll concentration of 10^{-5} M. The solution contained: 3×10^{-4} M tricine-NaOH, pH 8; 5×10^{-5} M benzylviologen (as electron acceptor); and 2×10^{-2} M NaCl. The reaction volume was 15 ml. When valinomycin was added, which had been dissolved in ethanol, care was taken to keep the end concentration of ethanol below 0.5%. This concentration does not influence photosynthesis. (The valinomycin was a generous gift of Dr. B. C. Pressman.) Temperature was kept at 21 °C.

The reaction cuvette was placed into a rapid kinetic spectrophotometer of the type which has been described by Döring, Stiehl and Witt (1967). The optical path length of the cuvette was 2 cm, the bandwidth of the measuring beam $\Delta\lambda = 5$ nm, the intensity of the measuring beam 45 erg/cm$^2 \times$ s, and the electric bandwidth of the detection system 30 kHz.

Photosynthesis was excited with saturating short flashes (duration $\tau_{1/2} = 1.5 \times 10^{-5}$ sec; wavelength interval 630 to 680 nm). The signal-to-noise ratio was improved by averaging repetitive signals (Rüppel & Witt, 1969). The repetition rate was 0.5 Hz, and the number of signals averaged about 200.

On excitation of photosynthesis by a short flash of light, an electric potential difference is induced across the thylakoid membrane. This is indicated by special absorption changes of chloroplast bulk pigments (Junge & Witt, 1968). These absorption changes have been interpreted from the fact that an absorption band undergoes a shift if the dye is exposed to a strong electric field (electrochroism). For field strength below 10^6 V/cm, these band shifts depend linearly on the electric field strength, whereas above 10^6 V/cm, second-order effects become predominant. Because the voltage induced across the thylakoid membrane by a single short flash of light lies in the order of 100 mV (Schliephake, Junge & Witt, 1968), field strength of a few 10^5 V/cm has to be expected. Thus these absorption changes can be used as a linear indicator for the voltage across the membrane. This concept has been experimentally confirmed in a series of previous papers which have been reviewed recently by Junge *et al.* (1970).

Recently this method has become extendable to systems other than the intrinsically pigmented thylakoid membrane, by the finding of an artificial field indicating dye rhodamin b which binds to lipid membranes. In the thylakoid membrane, this dye yields absorption changes whose spectrum is similar to that of its electrochromic response to an electric field *in vitro* (Emrich, Junge & Witt, 1969).

A time course of the electrochromic absorption change at 520 nm is depicted in Fig. 1. The rapid rise of absorption after excitation of photosynthesis with a short flash of light indicates the induction of an electric potential difference across the thylakoid membrane. As mentioned above, the voltage U is proportional to the change of absorption ΔA:

$$U = \alpha \cdot \Delta A . \tag{1}$$

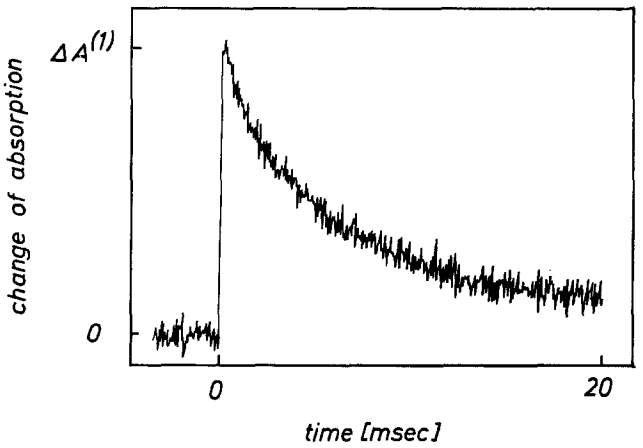

Fig. 1. Time course of the absorption change at 520 nm on excitations of photosynthes by a short flash of light at time $t=0$

The decay of absorption in the subsequent dark period indicates the discharge of th thylakoid's electric capacitance via the ion conductivity of the membrane.

It should be possible to calculate the current density across the membrane from th decay rate of the absorption. A necessary condition for this is the constancy of th thylakoid's electric capacitance. Independence of the membrane's capacitance from th voltage can be assumed in analogy to the independence which has been found for th capacitance of artificial bimolecular lipid membranes (Ohki, 1969). The independenc from time is evident for the short measuring time which is represented in Fig. 1.

When the thylakoid's electric capacitance C is constant, the voltage U is simpl proportional to the amount of charge separated by the membrane (Q):

$$Q=C\cdot U\,.$$

Any flux of charge across the membrane causes an alteration of the voltage. If the capac tance C and consequently the separated charge Q are related to one unit area of the mem brane, the current density simply becomes:

$$J=\frac{d}{dt}Q=C\frac{d}{dt}U\,. \qquad (2$$

Since the method used does not yield the voltage directly, but instead the absorptio change proportional to it, the current density becomes:

$$J=C\cdot\alpha\frac{d}{dt}\Delta A\,. \qquad (3$$

For a quantitative evaluation of the current density from the derivative of the absorptio change with respect to time, the numerical value of the product $C\cdot\alpha$ must be known. Thi can be derived from an earlier paper (Schliephake, Junge & Witt, 1968), where it has bee demonstrated that the absorption change, which is induced by a *single* saturating flas

of light $\Delta A^{(1)}$, corresponds to the translocation of two elementary charges across the capacitance of a membrane element, which is covered by one electron transport chain. The area covered by one electron transport chain is known from small-angle X-ray scattering experiments of Kreutz (1970). It amounts to about 10^5 Å^2. From these data, it follows that the proportionality constant equals:

$$C \cdot \alpha = 3.2 \cdot 10^{-8} / \Delta A^{(1)} \quad [\text{amp} \cdot \text{sec} \cdot \text{cm}^{-2}].$$

Thus the current density becomes:

$$J = 3.2 \cdot 10^{-8} \cdot \frac{d}{dt}\left(\frac{\Delta A}{\Delta A^{(1)}}\right) \quad [\text{amp} \cdot \text{cm}^{-2}] \tag{4}$$

where $\Delta A^{(1)}$ denotes the absorption change which is induced by a single, saturating short flash of light.

The procedure for calculating the current density from the decay of the absorption change is evident from Eq. (4).

We will give an example. Let us calculate the current density for the voltage corresponding to the maximum absorption change from the decay curve depicted in Fig. 1.

At $\Delta A/\Delta A^{(1)} = 1$, we differentiate the decay curve. The derivative of the relative absorption change amounts to 2.3×10^2 sec^{-1}. Making use of Eq. (4), this yields a current density of 7.4 μamp cm^{-2}.

Results and Discussion

In the preceeding section, we demonstrated how the current density across the thylakoid membrane can be calculated from decay curves of the absorption change at 520 nm, similar to those of Fig. 1. Although the current density follows from measured decay curves in absolute values, the voltage across the membrane follows only in relative ones. The proportionality constant between the voltage and the absorption change is unknown. However, this is sufficient for studying the current voltage behavior of the valinomycin-doped thylakoid membrane.

The effect of valinomycin on the decay of the absorption change at 520 nm is depicted in Fig. 2. When no valinomycin is applied to the chloroplasts, as in the experiment shown in Fig. 2A, there is a rather slow decay of absorption. This indicates the discharge of the membrane's capacitance by currents across the rather low intrinsic conductivity of the thylakoid membrane. When valinomycin is added (Fig. 2B & C), there is practically no effect if only Na^+ ions (but virtually no K^+ ions) are present, as in Fig. 2B. However, in the presence of K^+, there is a marked acceleration of the absorption change as depicted in Fig. 2C. This indicates that valinomycin increases the conductivity of the thylakoid membrane selectively for K^+ ions.

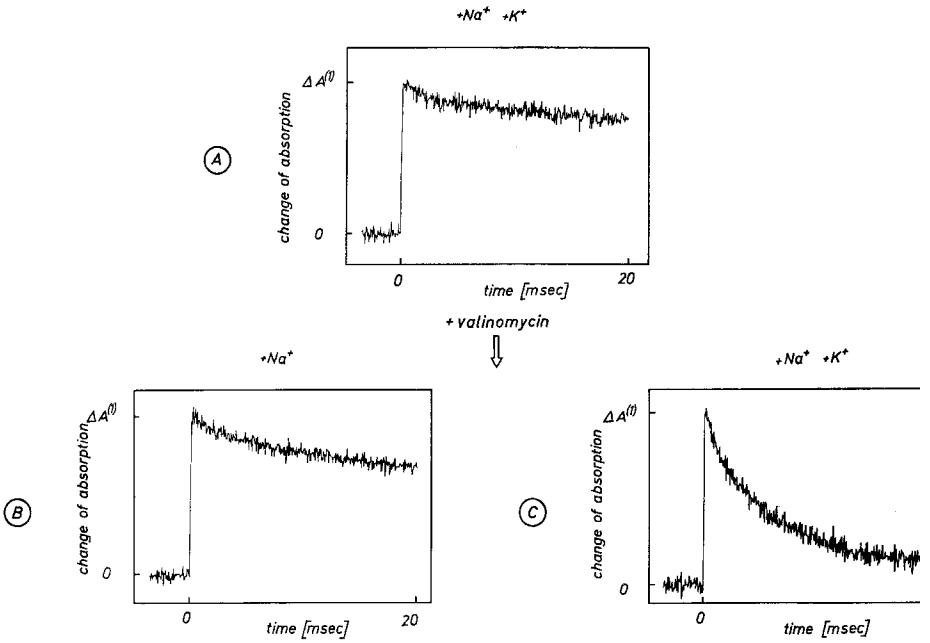

Fig. 2A–C. Time course of the absorption change at 520 nm. A. without valinomyc with Na^+ (10^{-2} M) and with K^+ (10^{-2} M); B. with valinomycin (1.1×10^{-8} M), wi Na^+ (10^{-2} M) but without K^+ ($<10^{-4}$ M); C. with valinomycin ($1.1 \cdot 10^{-8}$ M) wi Na^+ (10^{-2} M) and with K^+ (10^{-2} M)

This selectivity observed on the thylakoid membrane parallels the wel known selectivity of valinomycin on various other systems (*see*, for instanc the first observation by Moore & Pressman, 1964).

We will study the current-voltage relationship which is caused by valin mycin in the presence of K^+ ions. To do so, the current density has to b calculated from an experiment such as that in Fig. 2C for a whole set o values of $\Delta A/\Delta A^{(1)}$. However, the current density calculated from suc an experiment is not entirely due to the extra conductivity induced b valinomycin. The intrinsic conductivity of the thylakoid membrane ha to be taken into account. The current density as calculated from an exper ment with valinomycin, such as that in Fig. 2C, represents the sum of th current densities caused by the intrinsic conductivity (J_{intr}) and by the extr conductivity (J_{extra}) owing to the presence of valinomycin:

$$J_{\text{total}} = J_{\text{intr}} + J_{\text{extra}} \,. \qquad (1)$$

Since the incorporation of valinomycin into the thylakoid membran practically does not alter the membrane's intrinsic conductivity (this follow

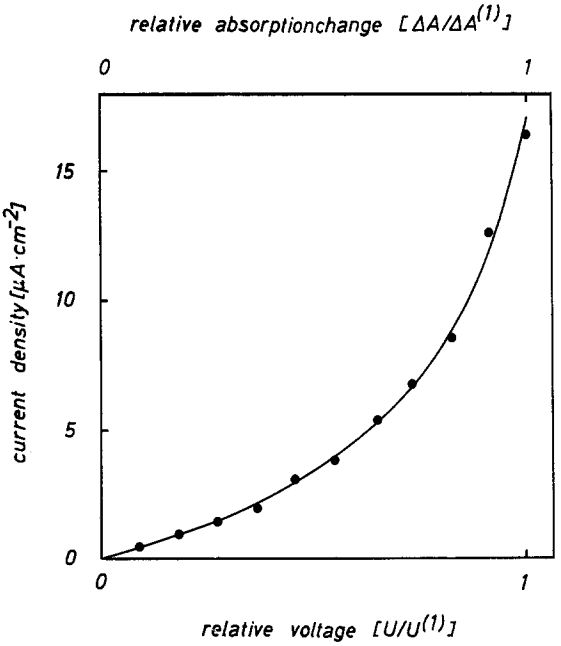

Fig. 3. Dependence of the valinomycin-mediated extra current density on the relative absorption change $\Delta A/\Delta A^{(1)}$ and on the relative voltage $U/U^{(1)}$. The points have been evaluated from the experiment (for details, *see* text). The solid curve results from the empirical Eq. (6). The average K^+ concentration was 2×10^{-2} M, and the average concentration of valinomycin 3×10^{-8} M

from a comparison of Fig. 2A and B), the intrinsic current density at a given voltage can be calculated from a decay curve without valinomycin, such as in Fig. 2A. (For a calculation of the intrinsic current density at low values of $\Delta A/\Delta A^{(1)}$, another experiment with a time span greater than in Fig. 2A is necessary.)

Thus from two experiments (one with and one without valinomycin), the extra current density caused by valinomycin can be calculated. We have done this for a set of voltages and have plotted the extra current density over the voltage. The experimental values are indicated in Fig. 3 by points. (The voltage scale factor is unknown, as mentioned above.)

Inspection of Fig. 3 reveals that the experimental points coincide with a hyperbolic sine curve which is represented by a solid line. This curve is defined by the following empirical equation:

$$J_{\text{extra}} = 1.36 \times \sinh(3.18 \times U/U^{(1)}) \quad [\mu\text{amp cm}^{-2}], \tag{6}$$

where $U^{(1)}$ denotes the voltage induced by a short saturating flash of light across the membrane.

A similar current-voltage behavior has been observed for a bimolecula lipid membrane in the presence of valinomycin (McLaughlin, Szabo & Eisenman, *personal communication*). However, these two results are at variance with the findings of Liberman and Topaly (1968), who found lower-than-first-order dependence of the current on the voltage with saturation at high voltages. Possible reasons for this variance will be discussed in a subsequent paper.

As evident from Fig. 3 and Eq. (6) respectively, the resistance of th thylakoid membrane in the presence of valinomycin and of K^+ ions i essentially non-ohmic. We will give a rigorous theoretical interpretatio of the conductivity mechanism causing this behavior in a subsequen paper. However some considerations may be presented here as they yiel an estimation of the voltage across the thylakoid membrane.

The hyperbolic sine current-voltage relationship can be understood fror a transition state approach (Eyring, 1935) under the assumption that th membrane represents one or a few energy barriers for a permeating charge species. As discussed by Ciani (1965), this approach yields a current voltage relationship of the following type:

$$J = \beta \times \sinh(U \times F/2\,\mathrm{nRT}), \qquad (7$$

where U is the voltage across the membrane, n the number of potentia barriers, β a factor depending on the concentration of the permeatin species, and F, R, and T are as usual. (Equal concentrations of the perme ating species at both boundaries of the membrane and an equidistan distribution of the energy barriers between the boundaries are assumed A comparison of the rate Eq. (7) with the empirical Eq. (6) allows a estimation of the voltage induced across the thylakoid membrane. For th voltage induced by a single, saturating short flash of light, one obtains

$$U^{(1)} = 3.18 \times \mathrm{nRT}/F \cong n \times 160\ \mathrm{mV} \quad (\text{for } T = 300\ ^\circ\mathrm{K}).$$

Since the voltage for a break through a bimolecular lipid membrane come close to this value (for a review, *see* Tien & Diana, 1968), it becomes rathe improbable by analogy that n (the number of energy barriers across th thylakoid membrane) will be greater than 1. Thus the most probable valu of the electric potential difference induced by a short flash of light becomes

$$U^{(1)} = 160\ \mathrm{mV}.$$

The voltage between one water phase and the rate-determining energ barrier, which in Eq. (7) was assumed to be in the middle of a homogenou

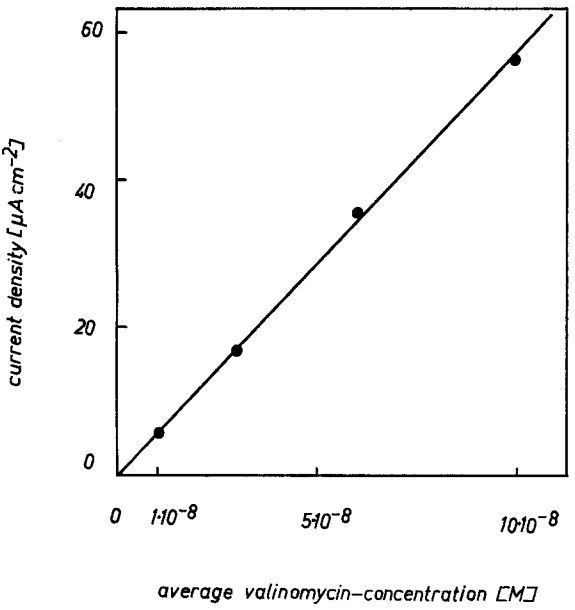

Fig. 4. Dependence of the extra current density at a voltage $U = U^{(1)}$ on the average valinomycin concentration. The average potassium concentration was 2×10^{-2} M

membrane, thus becomes one half of this value:

$$U^* = 80 \text{ mV}.$$

It is rather satisfying that this value is practically equal to the 78 mV which was estimated previously for the electric potential difference between one water phase and the rate-determining potential barrier of the intrinsic phosphorylating system in the thylakoid membrane (Junge, 1970). (It remains to be studied if the mechanism by which the active ATPase system increases the electrical conductivity of the thylakoid membrane is similar to the mechanism by which valinomycin does.)

After characterizing the current-voltage relationship, we studied the dependence of the current density at a given voltage on the concentration of valinomycin and of the permeating ion.

The dependence of the extra current density at $U = U^{(1)}$ on the valinomycin concentration is depicted in Fig. 4. The linear dependence of the current density on the overall average valinomycin concentration indicates that each molecule of the ionophore acts independently from the other ones on the membrane.

The dependence of the valinomycin-induced extra current density at $U = U^{(1)}$ on the concentration of K^+ ions is depicted in Fig. 5. Whereas

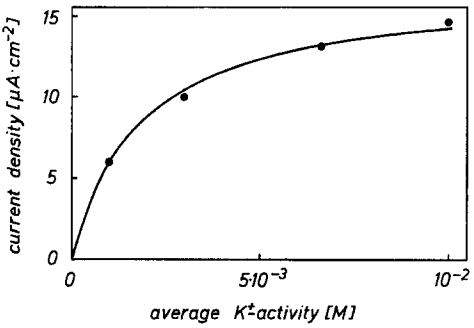

Fig. 5. Dependence of the extra current density at a voltage $U = U^{(1)}$ on the avera K^+ concentration. The average valinomycin concentration was 2.7×10^{-8} M

the increase in the extra current density is linear in the region of small K concentrations, saturation becomes obvious at higher concentrations. W will try to discover the mechanism causing this saturation.

The well-known selectivity of valinomycin which, as shown in Fig. increases the conductivity of the membrane only in the presence of K^+ ion but not in the presence of Na^+ alone, demonstrates the important ro of the valinomycin-K^+ complex for the extra current. Depending on tl kind of rate-determining step, two different dependencies of the curre on the K^+ concentration can be expected.

(1) If the movement of valinomycin-K^+ complexes is rate determinin the current density at a given voltage should be proportional to the avera concentration of these complexes.

$$J_{\text{extra}} \sim c_{\text{val-}K},$$
$$J_{\text{extra}} \sim c^0_{\text{val}} \cdot \frac{a_K}{a_K + K_D}, \quad (1$$

where c^0_{val} denotes the total valinomycin concentration in the suspensio a_K the average K^+ activity, and K_D the dissociation constant of the comple [The K^+ activity can be assumed to be equal on both sides of the thylako membrane, since the Donnan potential in the concentration range studi comes close to zero (H. Muhle, *personal communication*).]

(2) If the complexation of K^+ is rate determining, the current densi should be proportional to the average concentration of uncomplex valinomycin multiplied by the activity of the ion to be complexed (a_K

$$J_{\text{extra}} \sim c_{\text{val}} \cdot a_K,$$
$$J_{\text{extra}} \sim c^0_{\text{val}} \cdot \frac{K_D \cdot a_K}{K_D + a_K}. \quad (1$$

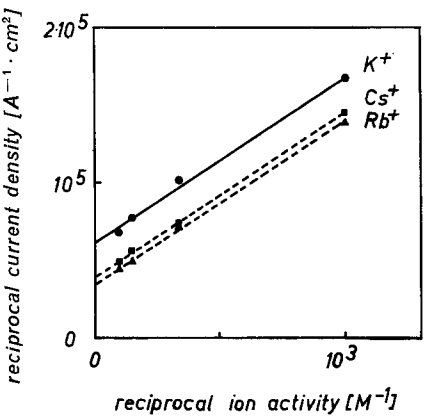

Fig. 6. Plot of the reciprocal extra current density at $U = U^{(1)}$ over the reciprocal activity of the following cations: K^+, Rb^+ and Cs^+. The average valinomycin concentration in all these experiments was 2.7×10^{-8} M

The current densities expected for these two cases differ only by a factor K_D, whereas the dependence on the K^+ activity is similar.

To test whether the experimental current saturation curve from Fig. 5 fulfills the expectation formulated in Eq. (8) or in Eq. (9), we have plotted J^{-1} as a function of a_K^{-1}. (This is similar to a Lineweaver-Burke plot.) In both cases, a straight line has to be expected:

$$J^{-1} \sim \frac{K_D}{a_K} + 1, \tag{8a}$$

$$J^{-1} \sim \frac{1}{a_K} + \frac{1}{K_D}. \tag{9a}$$

This special plot is depicted by filled circles connected by a solid line in Fig. 6. As expected, the experimental points fall on a straight line. Discrimination between the above two mechanisms is possible if K_D, the dissociation constant of the complex, is varied. We have done so by replacing K^+ by Cs^+ and Rb^+. Again, as for K^+, this plot yields straight lines for Cs^+ and Rb^+ as depicted in Fig. 6.

Since these lines are parallel to each other and since they intersect the ordinate at different points, they clearly fulfill the expectation which is formulated in Eq. (9a). In contrast to this observed behavior, Eq. (8a) predicts straight lines with different steepnesses. This suggests that the rate-determining step for the ion flux across the valinomycin-doped thylakoid membrane is the complexation of an ion by the ionophore.

This finding clearly yields evidence for the mechanism of action of valin mycin in the thylakoid membrane. However, further conclusions deser a rigorous theoretical treatment which will be presented in a subseque paper.

Making use of the proportionality in Eq. (9a), the dissociation constan of valinomycin for K^+, Cs^+, and Rb^+, can be read out from the straig lines in Fig. 6. They are: $K_D(K^+)=1.8\times10^{-3}$ M; $K_D(Cs^+)=2.8\times10^{-3}$ M and $K_D(Rb^+)=3.2\times10^{-3}$ M.

The selectivity sequence found in our experiments $-K^+>Cs^+>Rb^+$ does agree with the sequence reported by most other authors (*see*, for i stance, Pressman, 1968) as far as $K^+>Cs^+$ and $K^+>Rb^+$ are concerne Only the sequence $Cs^+>Rb^+$ is different.

However, from the structural studies of Shemyakin *et al.* (1969) becomes obvious that the selectivity of valinomycin should depend c the microscopic geometry of the medium in which it is embedded. Th it is not at all surprising that the sequence may be different for different structurized membranes.

Conclusions

It has been demonstrated that a recently developed, nonelectrochemic measuring technique can be used for an evaluation of the current-volta characteristics of a submicroscopic membrane system, which is inacessib to electrochemical measuring techniques. This method makes use of electr chromic absorption changes.

We studied the properties of the extra current across the thylakoi membrane, which was induced by valinomycin in the presence of certai alkaline ions. The results were as follows.

(1) The extra current density depends by a hyperbolic sine relationshi on the voltage across the membrane. (2) The extra current density depen linearly on the average valinomycin concentration. (3) The extra curre density is proportional to the product of the average concentration of u complexed valinomycin and of the activity of the ion to be complexe

The principle aim of this paper was the demonstration of a new metho for permeability studies on submicroscopic membrane systems; a quant tative interpretation of the experimental results will be presented in a sul sequent paper.

We wish to thank Miss Jutta Mann for her valuable technical assistance. We a very much indebted to Professors G. Eisenmann, B. Rumberg and H. T. Witt for critic comments.

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