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Pigment Containing Lipid Vesicles

II. Interaction of Valinomycin with Lecithin as Sensed by Chlorophyll *a*

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Summary. Valinomycin added to a suspension of chlorophyll *a* containing lecithin vesicles induces slight changes in the spectrum of chlorophyll *a*. These changes are measured as a difference spectrum between samples with and without valinomycin but of otherwise identical composition. The analysis of the experiments reveals that the effect is neither associated with the ionophoric properties of valinomycin nor due to a direct interaction of this agent with chlorophyll *a*. The molar ratio of valinomycin dissolved in the membrane to lecithin is found to be the relevant parameter, thus indicating an interaction between these two components. As a consequence, the aggregational state of the lecithin molecules is altered. Chlorophyll *a* incorporated into the membrane acts as a sensor, i.e. it reflects the alteration by a change in its spectroscopic parameters.

Valinomycin has long been known to be an effective ionophore, and a considerable body of information about its properties has been collected. Dissolved in vesicle membranes as well as in black lipid bilayers, it exhibits a strong selectivity for potassium rather than for sodium ions (Mueller & Rudin, 1967; Grell & Funck, 1973), and a detailed mechanism of ion transport through black lipid bilayers has been worked out (Stark & Benz, 1971; Stark, Ketterer, Benz & Läuger, 1971). Therefore we thought valinomycin might be a useful tool for creating well-defined diffusion potentials across the membrane of single-shelled lecithin vesicles. These membranes show rather low intrinsic permeabilities for hydrophilic ions such as sodium or chloride ions (Hauser, Oldani & Phillips, 1973), thus we simply have to establish a potassium ion gradient between the inner phase of the vesicles and the suspending medium, and to balance the osmotic difference with an opposite gradient of a salt whose cation is only little transferred through the membrane by valinomycin. When chlorophyll *a* or other pigment molecules are incorporated into the membrane, this system may serve to investigate the effect of the electric field associated

with the diffusion potential on the absorption spectrum of the pigment molecules.

Such experiments are of interest since certain changes in absorbance of isolated chloroplasts were interpreted as indicating an electric field across the inner chloroplast membrane. Chlorophyll *a* and *b* as well as carotenoids were found to contribute to the absorption changes (Witt, 1971), and thus these pigment molecules should respond to an electric field with a change of their spectroscopic parameters. Such an electrochronism was indeed demonstrated with pigment containing monolayer assemblies for chlorophyll *a* and *b* (Kleuser & Bücher, 1969; Schmidt, Reich & Witt, 1969) and later also for a carotenoid (Schmidt & Reich, 1972). We used vesicles which are bounded by a single spherical lipid bilayer containing chlorophyll *a* (Ritt & Walz, 1976). The membranes of such vesicles are rather fluid, but their bimolecular leaflet structure restricts the mobility of the membrane forming molecules to an essentially two-dimensional movement within a spherical shell (Kornberg & McConnell, 1971*a*, *b*). In this respect the vesicle membrane probably resembles more the chloroplast membrane than the dry monolayer assembly with an almost rigid array of pigment molecules.

Our experiments showed spectroscopic changes for chlorophyll *a* on addition of valinomycin to a vesicle suspension in the presence of a potassium ion gradient but, in addition, we found similar changes also in the absence of a gradient. This most unexpected phenomenon led us to the investigation of a yet unknown effect of valinomycin. The data which will be reported in this paper were all obtained without a potassium ion gradient or even without potassium at all. Hence, they cannot be related to the ionophoric properties of valinomycin. The analysis of the experimental data carried out leads us to the conclusion that valinomycin interacts with the lecithin molecules in the membrane with chlorophyll *a* acting as a sensor for this interaction.

Materials and Methods

The vesicles were prepared, the chlorophyll *a* and lecithin contents of the vesicle stock solutions were determined, and the analytical sieve chromatography was performed as described in the preceding paper (Ritt & Walz, 1976). Valinomycin (A Grade) and chlorophyll *a* were purchased from Calbiochem and Sigma, respectively, and were used without further purification. All experiments were carried out with vesicles prepared from the same batch of egg yolk lecithin and with the same supply of chlorophyll *a* (No. 53C-9510) for which high purity was found. Some experiments were repeated with chlorophyll *a* from another less pure supply (No. 103C-9570, containing traces of probably chlorophyll *a'* and xanthophyll) to check the influence of the chlorophyll *a* purity on the effects to be reported. Buffer solutions were prepared from analytical grade reagents. They contained 0.01 M 1-morpholinopropane

sulfonic acid and LiCl or NaCl with varying amounts of KCl to a total concentration of 0.2 M salt; the pH was adjusted to 7.2 with the corresponding alkali, i.e. LiOH or NaOH.

Spectra and difference spectra were run from 300 to 720 nm on an Aminco DW-2 spectrophotometer in split beam mode. The cuvettes were positioned just in front of the photomultiplier with a scattering plate inserted in between. The cuvette holder was thermostatized so that the temperature of the solutions in the cuvettes was 20 ± 0.2 °C. Samples were prepared by diluting aliquots (0.03 to 0.7 ml) of the vesicle stock solution to 7 ml with the corresponding buffer, then exactly 3 ml of the diluted suspension were pipetted into each of a pair of optically matched rectangular 1-cm quartz cuvettes. One of the cuvettes was placed in the sample holder while a third cuvette which contained buffer was inserted as reference, and the spectrum of the vesicle suspension was recorded twice. (The baseline for these spectra had been scanned before with the same cuvettes but both containing buffer.) The reference cuvette was replaced by the second cuvette containing the vesicle suspension. The absorbance differences were smoothed out with the baseline correction device of the instrument, and the baseline was recorded three times. Then usually 5 µl (in some cases 2 or 10 µl) of a 1 mM (in some cases 0.1 mM) methanolic valinomycin solution were added to the sample cuvette, the reference cuvette receiving the same volume of methanol. The resulting first difference spectrum was again recorded three times, then the same amounts of valinomycin solution and methanol were added but now to the reference and sample cuvette, respectively, thus creating the same conditions in both cuvettes. Hence, the remaining absorbances, which were also recorded three times as a second difference spectrum, should be similar to the baseline and therefore provided a check for the reproducibility of the whole system. All difference spectra were measured with a wavelength drive speed of 2 nm/sec, and with a constant 3 nm bandpass of the monochromator. Sensitivity was usually 0.01 A full scale deflection, and the response time constant of the electronics was set at 0.1 sec which was fast enough compared to wavelength speed and bandpass but decreased the noise of the detecting system considerably. The time required to scan the whole wavelength range was 3 1/2 min, with an equal time interval between each scan. After additions to the cuvettes, one scan was done without recording giving the solutions some 10 min time to reequilibrate. Hence, the total time needed to record all spectra, and including the baseline correction procedure, was about 1 1/2 hours.

Preliminary experiments had shown that no detectable absorbance changes occurred in the range of 490 to 550 nm. Advantage was taken of this fact to correct for small shifts in baseline location which were introduced by changing the electric zero point of the recorder slightly after each scan through the whole wavelength range. This gave tracings which were separated from each other and which were much easier to evaluate, especially for repeated recordings of the same difference spectrum. For the evaluation, values were read from each tracing with respect to an arbitrarily chosen zero-line at 7 nm intervals. These values were fed into a computer (Hewlett-Packard 9830 A) which first corrected the three tracings of the baseline for the above-mentioned shifts by calculating an average distance between the first and the other two tracings in the range of 490 to 550 nm. Then the three baselines were averaged to yield the true zero-line and its standard deviation. The values obtained from the tracings of the difference spectra were also first corrected for the shifts, with the first tracing of the baseline as reference. Then the values of the true zero-line were subtracted and finally the three tracings were again averaged thus yielding points and standard deviations for the corrected difference spectra at 7 nm intervals. All these data were displayed on a plotter (Hewlett-Packard 9862 A) connected to the computer, and curves were drawn through the points of the difference spectra by cubic interpolation. All plots have a break in the wavelength scale between 489 and 552 nm, i.e. the interval where the above-mentioned correcting procedure was performed. The spacing of 7 nm for the readings was chosen since it amounts to about twice the bandpass of the measuring light. Hence it is unlikely that small absorbance changes as detected by the photometric device were not observed because of a too large spacing.

Results

The spectra of the chlorophyll *a* containing vesicle suspensions resembled the spectra known for chlorophyll *a* in different solvents (Seely & Jensen, 1965) but they were not equal to one or the other of these spectra. In view of the features of solvents which give rise to different chlorophyll *a* spectra, i.e. dielectric properties, refractive index and chemical interactions (e.g. complex formation, *see* Katz & Norris, 1973), this could not be expected because a lecithin membrane is a unique solvent in this respect. The spectra were found slightly different from one vesicle preparation to another. Moreover, small changes in the Soret region occurred on aging of each preparation. But spectra obtained within about one day were usually quite similar and hence could be averaged. Fig. 1 presents a typical example; this spectrum is the average of ten individual spectra recorded with different concentrations of vesicles from the same preparation, and normalized at 668 nm which is the location of the main red peak. In the range below about 390 nm, the absorbance of the lecithin vesicles is superimposed on that of chlorophyll *a*. It was due to a scattering of the measuring light by the spherical vesicles which was not completely eliminated by the anti-scattering device of the spectrophotometer, and to the self-absorption of lecithin. No attempt was made to correct the chlorophyll *a* spectra for this contribution since such a correction would be valid only if it were based on a chlorophyll *a* free vesicle suspension with a concentration and a size distribution close to that of the corresponding chlorophyll *a* vesicles. This, however, would require rather extensive experimental efforts, especially because vesicles with and without chlorophyll *a* differ by about 10 Å in their average radius (Ritt & Walz, 1976). Part of the above-mentioned differences between spectra from different vesicle preparations and part of the changes observed on aging of the vesicles can be explained by the contribution of lecithin to the measured absorbances. The smaller the chlorophyll *a* content of the vesicles the larger was this contribution. Aging of vesicles leads to the formation of larger particles (Ritt & Walz, 1976) and hence to a concomitant increase in light scattering. The other parts of the differences observed were most probably not due to a chemical alteration of chlorophyll *a* since no degradation could be detected (Ritt & Walz, 1976). The spectroscopic parameters for chlorophyll *a* in a lecithin vesicle membrane are summarized in Table 1. They indicate chlorophyll *a* in its monomeric form (*cf.* Katz & Norris, 1973) as was also found by Tomkiewicz and Corker (1975) for a similar membrane system with

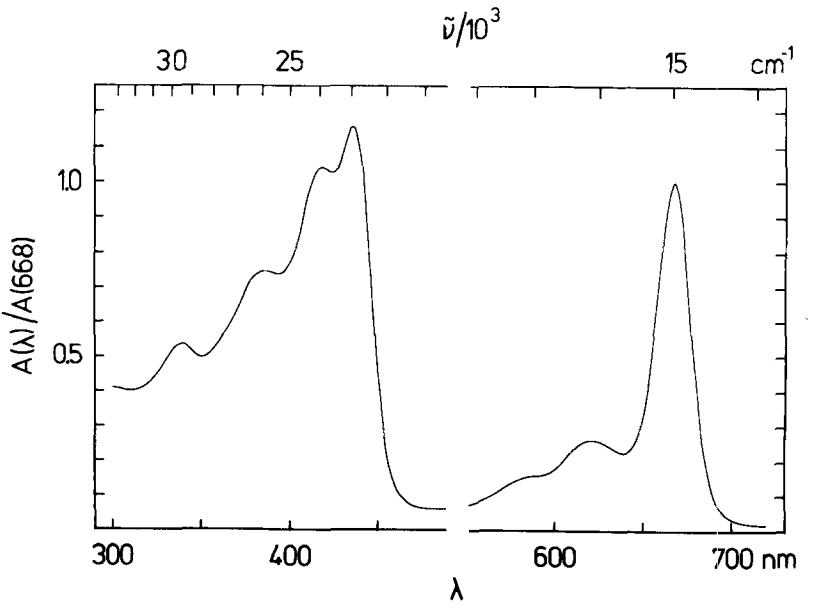

Fig. 1. Absorption spectrum of chlorophyll *a* incorporated into membranes of lecithin vesicles. The curve is an average of ten individual spectra, $A(\lambda)$, recorded with different concentrations of vesicles from the same preparation, and normalized by the absorbance at the main red peak, $A(668)$. The break in the wavelength scale between 489 and 552 nm was introduced to get similar graphs for spectra and difference spectra (*see* Fig. 2). The spectrum in this range runs almost parallel to the abscissa as a slightly wavy line. The upper scale indicates the wavenumber, $\bar{\nu}$

Table 1. Summary of spectroscopic parameters for chlorophyll *a* in the membrane of lecithin vesicles

$q \cdot 10^3$	$A(\lambda_{max})/A(668)$ for maxima located at (λ_{max} range in nm)					$\varepsilon_c(668)$ ($mM^{-1}\,cm^{-1}$)
	338–340	385–387	418–419	436–437.5	621.5–623.5	
1.6	0.72	0.84	1.07	1.24	0.27	77.7
4.3	0.65	0.84	1.15	1.23	0.27	82.8
10.3	0.58	0.79	1.08	1.20	0.27	81.0
11.3	0.55	0.76	1.03	1.18	0.26	76.8
15.4	0.53	0.73	1.02	1.15	0.26	82.3

The locations of the maxima varied slightly as indicated by the λ_{max} ranges. Mean values for the absorbance at the maxima, $A(\lambda_{max})$, relative to the absorbance at the main red peak, $A(668)$, are given for different vesicle preparations (q = molar ratio of chlorophyll *a* to lecithin). $\varepsilon_c(668)$ = extinction coefficient for the red peak whose location varied between 667.5 and 668.5 nm.

circular dichroism and electron spin resonance measurements. At the wavelength of the red peak (668 nm), Beer-Lambert's law was checked and found to be valid up to an absorbance of 1 A. With the corresponding

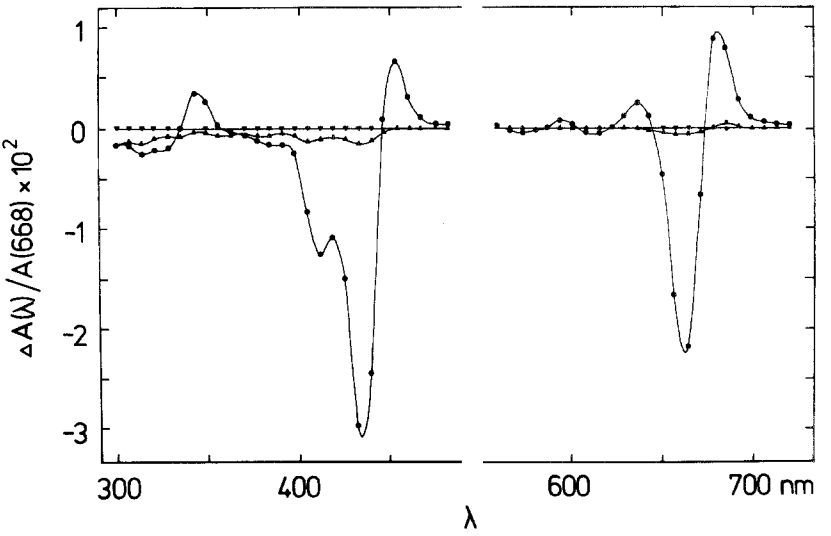

Fig. 2. Difference spectra, normalized by the chlorophyll *a* absorbance $A(668)$ (*see* Fig. 1). The absorbance changes induced by valinomycin in chlorophyll *a* containing lecithin vesicles, $\Delta A(\lambda)$, were measured and evaluated as described in Materials and Methods: ▼ zero-line, ● first and ▲ second difference spectrum. The standard deviations for all points were about twice as large as the size of the dots. Vesicles with a molar ratio of chlorophyll *a* to lecithin $q=0.0154$ were used, concentration of lipid $C_l=0.21$ mM and of valinomycin (when present) $C_v=1.67$ μM. The interior phase of the vesicles as well as the suspending medium were aqueous solutions of 0.19 M LiCl, 0.01 M KCl and 0.01 M 1-morpholinopropane sulfonic acid, pH adjusted to 7.2 with LiOH

chlorophyll *a* content of the vesicle solutions the extinction coefficient at this wavelength, $\varepsilon_c(668)$, could be calculated (*see* Table 1).

Fig. 2 shows an example of difference spectra measured and evaluated as described in Materials and Methods, and normalized by the absorbance of the vesicle suspension at 668 nm, $A(668)$, to take into account the amount of chlorophyll *a* present. Both aqueous phases, i.e. the suspending medium and that in the cavities of the vesicles, contained 10 mM KCl. Thus, no concentration gradient of potassium ions existed and no diffusion potential could arise. Nevertheless, a clear-cut difference spectrum occurred after addition of valinomycin to the sample cuvette. It has negative peaks at 412, 435 and 663 nm while positive peaks are located at 344, 453, 637 and 680 nm. The second difference spectrum recorded after adding the same amount of valinomycin to the reference cuvette was close to the baseline which indicates that the absorbance changes induced by valinomycin were reproducible as well as time independent. The remaining small differences can be explained by not exactly equal

Table 2. Interaction of valinomycin (val) with the components of the system

Component	Interaction	Effect on chlorophyll *a* spectrum	Normalized difference spectra depend on
alkali ions	val as ionophore "dissolves" ions in the membrane	electric field of the charged ion-val complex induces an electrochromic effect[a]	– ion species (selectivity of val) – ion concentration – val concentration
chlorophyll *a* (chl *a*)	formation of a val-chl *a* complex in membrane	spectrum of val-chl *a* complex different from chl *a* spectrum	x (molar ratio val-chl *a* complex to total chl *a*)
	interaction of val with chl *a*	interaction changes spectroscopic parameters of chl *a*	r_c' (molar ratio of val dissolved in the membrane to chl *a*)
lecithin (lec)	interaction of val with lec	chl *a* is sensor for the val-lec interaction	r_l' (molar ratio of val dissolved in the membrane to lec)

[a] An electrochromic effect is defined as a change of the optical absorption (or emission) spectrum of molecules in the condensed phase due to the influence of an electric field.

amounts of valinomycin in both cuvettes. Again there was a contribution of light scattering in the short-wavelength range below about 390 nm, and the deviation of the second difference spectrum from the baseline in this range most probably reflects a change in size of the vesicles during the experiment. Zero-line and second difference spectrum were averaged and the first difference spectrum was adapted to this new baseline. All data reported hereafter were obtained from such corrected difference spectra.

As far as can be judged by analytical sieve chromatography, valinomycin did not affect the integrity of the lecithin vesicles. Samples of the same vesicle preparation chromatographed on a Sepharose 4B column gave almost identical elution patterns with or without valinomycin present in the eluting buffer. The average Stokes' radii of the vesicles in the absence and presence of valinomycin differed by only 2 Å which is below the sensitivity of the method (*see* Ritt & Walz, 1976). A potential difference across the vesicle membrane was absent, hence the difference spectra could only be due to some interaction of valinomycin with one of the components of the system. Table 2 then lists the components of the system, possible interactions of them with valinomycin, and the corresponding effects which would result for the spectrum of chlorophyll *a*. Water, the main constituent of the system, was not considered because we could not think of a water-valinomycin interaction which is sensed by chlorophyll

a in the vesicle membrane. The last column of Table 2 indicates the expected dependence of the normalized difference spectra on the constituents and their concentrations or their mutual molar ratios which will enable us to discriminate between the four possibilities. (Note that the normalization with $A(668)$ eliminates the trivial dependence of the difference spectra on the amount of chlorophyll *a* present.) We therefore measured difference spectra with a) vesicles suspended in solutions containing different salts, b) vesicles with different contents of chlorophyll *a*, and c) different vesicle concentrations under conditions a) and b). In preliminary experiments with chlorophyll *a* free vesicles it was found that valinomycin concentrations up to about 2 μM could be used without oversaturating the aqueous phase considerably. Moreover, the absorption of valinomycin at this concentration in the wavelength range where the difference spectra were measured was below 2×10^{-4} and could thus be neglected. However, there was a certain amount of valinomycin adsorbed on the glass walls of the cuvette. To account for the inevitable error in valinomycin concentration due to adsorption most difference spectra were measured with the same final concentration (1.67 μM resulting from an addition of 5 μl of a 1 mM methanolic valinomycin solution to the 3 ml samples). Occasionally this concentration was varied to smaller values.

The first possibility listed in Table 2, i.e. *valinomycin acting as an ionophore*, could be dismissed since no relation between the spectroscopic changes and the ion species present in the water phase was observed. Valinomycin complexes sodium ions very little (Grell & Funck, 1973), and no complexation of lithium ions could be detected (Grell, *personal communication*). But absorbance differences of similar extent were measured when vesicles at comparable concentrations were treated with the same concentration of valinomycin in solutions containing KCl as well as in pure NaCl or LiCl solutions. Moreover, no dependence on the KCl concentration was observed if the concentrations of valinomycin and vesicles were kept constant. Thus, we do not need to distinguish between difference spectra obtained from vesicles in different salt solutions in the following considerations.

The assumption of a *complex formation between chlorophyll a and valinomycin* (*see* Table 2) is supported by the extensive investigations of Katz and co-workers (for a review *see* Katz & Norris, 1973). It was found that chlorophyll *a* when dissolved in dry apolar solvents forms dimers and oligomers by complexation of the central magnesium in one porphyrin ring with the carbonyl group of a second ring. Valinomycin could provide

such carbonyl groups as well. However, the porphyrin ring must then be oriented towards the hydrocarbon region of the membrane since traces of water interfere with complexation. The algebraic treatment given in the Appendix leads to the relation

$$\Delta E(r_c, q, C_v, \lambda) = x(r_c, q, C_v)\,\Delta\varepsilon(\lambda) \tag{1}$$

which shows that the normalized difference spectra $\Delta E(r_c, q, C_v, \lambda)$ should be equal to a product of two factors: $x(r_c, q, C_v)$, the molar ratio of complex to total chlorophyll *a*, which depends only on the concentration parameters r_c, q and C_v (for definitions *see* Appendix) and $\Delta\varepsilon(\lambda)$, the difference in extinction coefficients of the complex and the uncomplexed chlorophyll *a* divided by $\varepsilon_c(668)$, which obviously depends on the wavelength, λ, only. According to Eq. (1) all difference spectra should be similar in shape. Inspection of the results confirmed this expectation, but a more quantitative treatment was desired. We rewrite Eq. (1) for a constant wavelength, λ_0, and use this new relation to eliminate the factor x from Eq. (1):

$$\Delta\varepsilon(\lambda) = R(\lambda, \lambda_0)\,\Delta\varepsilon(\lambda_0), \tag{2}$$

where

$$R(\lambda, \lambda_0) = \Delta E(r_c, q, C_v, \lambda)/\Delta E(r_c, q, C_v, \lambda_0) \tag{3}$$

should be the same for all difference spectra. Since the chlorophyll *a* spectrum varied slightly from one vesicle preparation to another, similar variations of $R(\lambda, \lambda_0)$ determined with vesicles from different preparations were to be expected. Moreover, difference spectra with low r_c values were usually less accurate than those with large r_c values. Therefore, an averaged difference spectrum, $\langle\Delta E(\bar{r}_c, q, C_v, \lambda)\rangle$, was calculated, and a mean

$$\langle R(\lambda, \lambda_0)\rangle = \langle\Delta E(\bar{r}_c, q, C_v, \lambda)\rangle/\langle\Delta E(\bar{r}_c, q, C_v, \lambda_0)\rangle \tag{4}$$

was determined for each preparation, i.e. for each value of q, separately. Experiments with $C_v \neq 1.67\ \mu M$ were excluded for the reasons given below. Note that the averaged difference spectra depend on $\bar{r}_c$, the average of r_c values of the individual difference spectra. Fig. 3 presents $\langle R(\lambda, \lambda_0)\rangle$ for $\lambda_0 = 435$ nm, the wavelength of the largest negative peak in the Soret region, and for five values of q. The differences between the curves are most probably related to the variations of the chlorophyll *a* spectra and not to a possible dependence on q, except for the short wavelength range below about 390 nm. Here the divergence of the curves does correlate with q but this can be entirely explained by light scattering. The smaller the q the higher was the vesicle concentration and hence the contribution of scattered light which correspondingly shifted the $\langle R(\lambda, \lambda_0)\rangle$ curves to

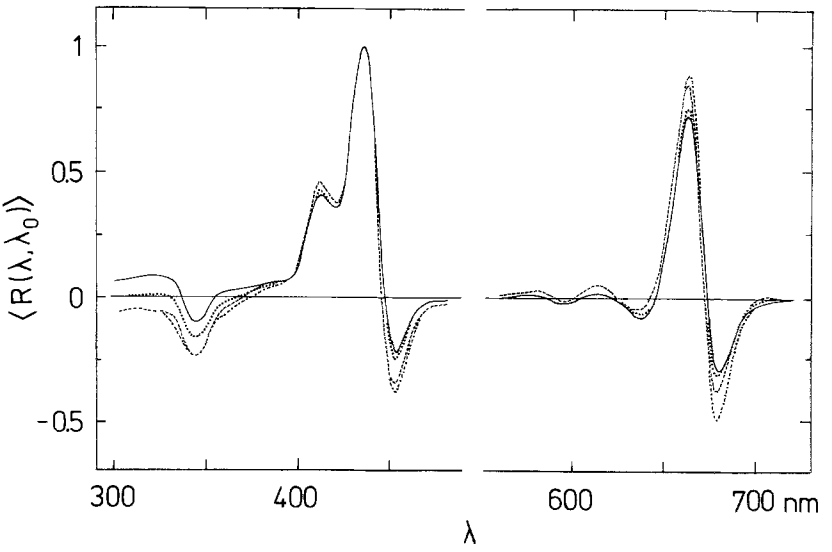

Fig. 3. Curves for $\langle R(\lambda, \lambda_0)\rangle$, calculated according to Eq. (4) from difference spectra measured with vesicles from different preparations, $\lambda_0 = 435$ nm: vesicles with $q = 0.0154$ (——), $q = 0.0113$ and 0.0103 (·····), $q = 0.0043$ (– – – –) and $q = 0.0016$ (– · – · –); q = molar ratio of chlorophyll *a* to lecithin. Similar graphs were also obtained for other values of λ_0

more negative values. Thus, $\langle R(\lambda, \lambda_0)\rangle$ is indeed independent of q. As will be shown below, a dependence on r_c seems not to exist, and C_v was constant. Therefore, we can conclude that the experiments are in agreement with Eq. (1) insofar as the splitting into two factors is concerned.

We now have to check if the experimental data are compatible with the factor x depending on the concentration parameters as predicted by the assumed complex formation. Introducing Eq. (2) into Eq. (1), we obtain after rearranging

$$\Delta E(r_c, q, C_v, \lambda)/R(\lambda, \lambda_0) = x(r_c, q, C_v)\,\Delta\varepsilon(\lambda_0). \tag{5}$$

The ratio on the left-hand side does not depend on λ and was averaged over the whole wavelength range. Using $\langle R(\lambda, \lambda_0)\rangle$ instead of the less accurate $R(\lambda, \lambda_0)$, we define a weighted mean:

$$Q(r_c, q, C_v, \lambda_0) = \sum_{\lambda} \{\text{sign}\,\langle R(\lambda, \lambda_0)\rangle\,\Delta E(r_c, q, C_v, \lambda)\}/\sum_{\lambda} |\langle R(\lambda, \lambda_0)\rangle| \tag{6}$$

where $\sum_{\lambda}$ indicates that we have summed over all the discrete values of λ at which the difference spectra were evaluated. The weight $|\langle R(\lambda, \lambda_0)\rangle|$ was introduced because otherwise even small experimental errors create large deviations for ΔE values close to zero. Combining Eqs. (5) and (6)

then gives

$$Q(r_c, q, C_v, \lambda_0) = x(r_c, q, C_v)\,\Delta\varepsilon(\lambda_0). \tag{7}$$

The dependence of x on r_c, q and C_v is rather complex [cf. Eq. (A 18)]. Therefore, we use a Taylor expansion of x with respect to r_c at the point $r_c = 0$:

$$Q(r_c, q, C_v, \lambda_0) = r_c\,\Delta\varepsilon(\lambda_0)\left[\left.\frac{\partial x}{\partial r_c}\right|_{r_c=0} + \frac{r_c}{2}\left.\frac{\partial^2 x}{\partial r_c^2}\right|_{r_c=0} + \cdots\right]. \tag{8}$$

Rearranging this equation and taking the limit for $r_c \to 0$, we find using Eq. (A 21),

$$\lim_{r_c \to 0}\left(\frac{r_c q}{Q}\right) = \frac{\alpha - 1}{\alpha K_b \rho_l \Delta\varepsilon(\lambda_0)} + \left(1 + \frac{\alpha - 1}{\alpha K_b \rho_c}\right)\frac{q}{\Delta\varepsilon(\lambda_0)} = y_0 + s q. \tag{9}$$

Although C_v was eliminated in this equation by straightforward algebra and not by an approximation, it is safer to use experiments only where $C_v = \text{const}$. This is one reason why experiments with $C_v \neq 1.67$ μM were excluded (*see above*). Complex formation thus predicts a linear dependence of $\lim_{r_c \to 0}(r_c q/Q)$ on q where the slope, s, and the intercept, y_0, determine the parameters K_b and $\Delta\varepsilon(\lambda_0)$. Values of Q were calculated according to Eq. (6), and the ratio $r_c q/Q$ was plotted versus r_c for each value of q separately. Fig. 4 presents, by way of example, such plots for $\lambda_0 = 435$ nm and for two values of q. The standard deviations marked at the points are reasonably small. The points indicate a slight but smooth dependence of $r_c q/Q$ on r_c which becomes more pronounced at larger q values. Similar plots for smaller values of q than those in Fig. 4 showed larger but still

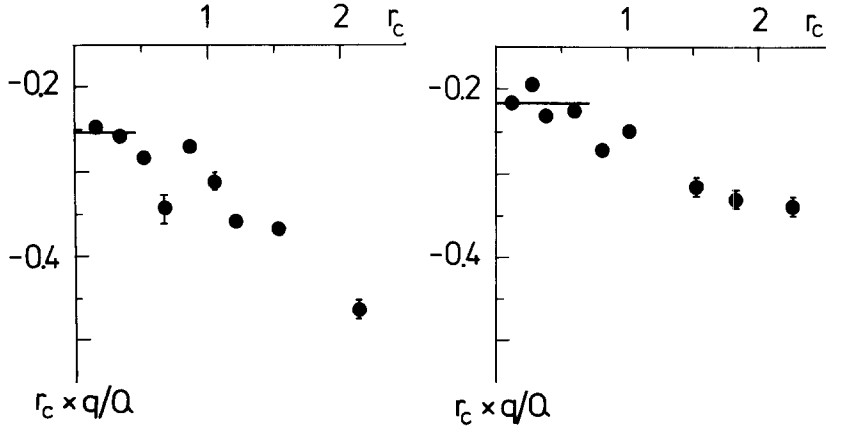

Fig. 4. Dependence of $r_c q/Q$ on r_c. Q as defined in Eq. (6), q = molar ratio of chlorophyll *a* to lecithin, r_c = molar ratio of valinomycin to chlorophyll *a*. $\lambda_0 = 435$ nm and $q = 0.0154$ (left-hand plot), $q = 0.0103$ (right-hand plot). Standard deviations are marked by bars where they were larger than the size of the dots. The horizontal lines indicate estimates for $\lim_{r_c \to 0}(r_c q/Q)$

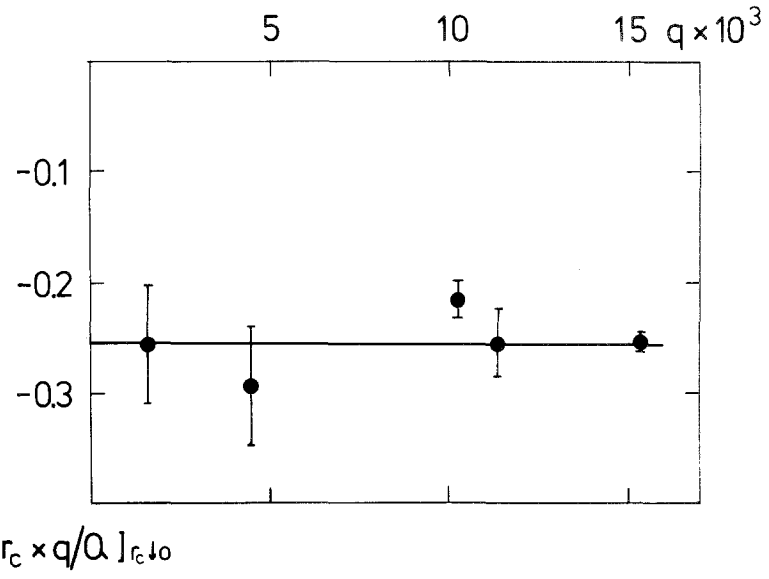

Fig. 5. Dependence of $[r_c q/Q]_{r_c \downarrow 0}$, i.e. the estimates for $\lim_{r_c \to 0}(r_c q/Q)$ (*see* Fig. 4), on q. Standard deviations are marked by bars. $\lambda_0 = 435$ nm, graphs for other values of λ_0 were similar

reasonable standard deviations due to less accurate difference spectra, and practically no dependence on r_c. The same characteristics were found for the data with other values of λ_0. All this indicates that a possible dependence of $\Delta E(r_c, q, C_v, \lambda)/\Delta E(r_c, q, C_v, \lambda_0)$ on r_c, which would have been averaged out when using Eq. (4) instead of Eq. (3), does not exist. Such a dependence would produce either large standard deviations or a scatter of the $r_c q/Q$ values.

Estimates for $\lim_{r_c \to 0}(r_c q/Q)$, denoted by $[r_c q/Q]_{r_c \downarrow 0}$, were determined from graphs like those in Fig. 4 and plotted versus q. As shown in Fig. 5 these values are essentially independent of q, hence we get from Eq. (9) with $s=0$: $K_b = -(\alpha - 1)/(\alpha \rho_c)$ and $\Delta\varepsilon(\lambda_0) = -\rho_c/(\rho_l y_0)$. But $\rho_c > 0$ and α, the distribution coefficient of valinomycin between lipid and water phase, is certainly larger than 1. We would therefore obtain a negative binding constant for the complex which is of course meaningless. Moreover, $\Delta\varepsilon(\lambda_0)$ would have the wrong sign (positive for negative peaks at λ_0, and vice versa). Attempts to find values for K_b and $\Delta\varepsilon(\lambda_0)$ with proper signs by trying several straight lines with $s \neq 0$ but still compatible with the experimental data were not successful, for one of the parameters always had the wrong sign. Hence, a complex formation between chlorophyll *a* and valinomycin can be excluded. The discussion of this possibility, however, revealed that the difference spectra can be described by a product

of two factors, one being a function of the concentration parameters and the other of the wavelength only [compare Eq. (1)]. Using $\langle R(\lambda, \lambda_0)\rangle$ defined in Eq. (4) as a factor depending on λ (and λ_0) only, we can thus write

$$\Delta E(r_c, q, C_v, \lambda) = f(r_c, q, C_v)\langle R(\lambda, \lambda_0)\rangle B(\lambda_0). \tag{10}$$

The factor f depending on the concentration parameters represents some interaction of valinomycin with one of the components of the membrane which then causes the changes in the absorption spectra of chlorophyll *a*. B denotes a constant with a value depending on the choice of λ_0.

The *interaction of valinomycin with chlorophyll a or lecithin*, listed as the last two possibilities in Table 2, are not specified enough to allow an interpretation of $\langle R(\lambda, \lambda_0)\rangle$, but they provide sufficient information to deal with $f(r_c, q, C_v)$. According to the statements in Table 2, we should find

$$f(r_c, q, C_v) = f_i(r_i') \quad \text{for } i = c \text{ and } l, \tag{11}$$

i.e. the general dependence of f on r_c, q and C_v can be expressed by functions f_c and f_l which depend only on r_c' and r_l', respectively, whereas

$$r_i' = r_i'(r_c, q, C_v) \quad \text{for } i = c \text{ and } l. \tag{12}$$

The variables r_c' and r_l' are the molar ratios of valinomycin *dissolved* in the membrane to chlorophyll *a* and to lecithin, respectively. Their dependence on r_c, q and C_v is derived in the Appendix. By means of Eq. (6) we can transform Eq. (10) into

$$Q(r_c, q, C_v, \lambda_0) = f(r_c, q, C_v)\, B(\lambda_0). \tag{13}$$

Using a Taylor expansion for $f(r_c, q, C_v)$ with respect to r_c at the point $r_c = 0$ [compare Eq. (8)] and taking into account that

$$\frac{\partial f}{\partial r_c} = \frac{\partial f_i}{\partial r_c} = \frac{\partial f_i}{\partial r_i'}\frac{\partial r_i'}{\partial r_c} \quad \text{for } i = c \text{ and } l, \tag{14}$$

we obtain in the limit $r_c \to 0$ using Eq. (A 24)

$$\lim_{r_c \to 0}\left(\frac{r_c q}{Q}\right) = \frac{\alpha - 1}{\alpha B(\lambda_0)(\partial f_c/\partial r_c')_{r_c = 0}} q \tag{15}$$

for an interaction of valinomycin with chlorophyll *a*, and

$$\lim_{r_c \to 0}\left(\frac{r_c q}{Q}\right) = \frac{\alpha - 1}{\alpha B(\lambda_0)(\partial f_l/\partial r_l')_{r_c = 0}} \tag{16}$$

for an interaction of valinomycin with lecithin, respectively. Fig. 5 clearly demonstrates that Eq. (15) has to be ruled out and thus a chlorophyll *a*-valinomycin interaction. The only remaining possibility, viz. an interaction of valinomycin with the lecithin molecules (Table 2), is strongly supported by the agreement of the experimental data with Eq. (16).

The simplest function $f_l(r_l')$ is a proportionality to r_l':

$$f(r_c, q, C_v) = k' r_l'. \tag{17}$$

To check this we do not need the full Eq. (A 23), which was derived to show the dependence of r_l' on all concentration parameters. We can make use of Eq. (A 10) for V and obtain using Eqs. (A 4), (A 7) and (A 22) as a good approximation

$$r_l' = \frac{\alpha r_e}{\alpha + r_l \rho_l / C_v - 1}. \tag{18}$$

Substituting from Eqs. (17) and (18) into Eq. (13) yields on rearranging

$$1/Q = s_1/r_l + s_2 \tag{19a}$$

or

$$r_l/Q = s_1 + s_2 r_l \tag{19b}$$

with

$$s_1 = \frac{\alpha - 1}{\alpha k' B(\lambda_0)} \quad \text{and} \quad s_2 = \frac{\rho_l}{\alpha C_v k' B(\lambda_0)}. \tag{20}$$

Note that s_2 depends on C_v, another reason why $\langle R(\lambda, \lambda_0)\rangle$ and Q were calculated only from experiments with the same C_v. Linear correlations should exist and were found (*see* Fig. 6) between $1/Q$ and $1/r_l$ as well as r_l/Q and r_l, and if Eq. (17) is valid for the whole range of r_l', s_1 and s_2 should be similar in both cases. As shown in Table 3 this is indeed true, indicating that Eqs. (17) and (18) are not oversimplifications. Moreover, the values for α calculated from s_1 and s_2 by means of Eq. (20) are independent of λ_0, as had to be expected (Table 3).

Eqs. (17) and (18) definitely establish the dependence of the difference spectra on the concentration parameters. We can therefore perform a final check with all experimental data including those with $C_v = 1.67$ μM. Values of Q [cf. Eq. (6)] were plotted versus r_l', calculated from Eq. (18) and with the mean value of α (18,600, *see* Table 3), and a proportionality was found over the whole range of r_l' (Fig. 7). The values of the proportionality constant $k' B(\lambda_0)$ are listed in Table 3, and these agree well with the values determined from s_1 and s_2. Finally, it could be shown that, for each preparation of vesicles, the product of $k' B(\lambda_0)$ and $\langle R(\lambda, \lambda_0)\rangle$

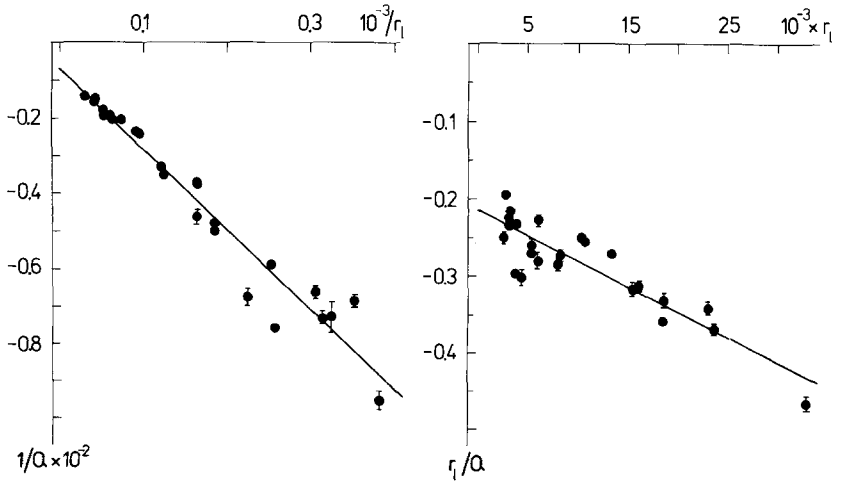

Fig. 6. Plots according to Eq. (19), i.e. $1/Q$ *vs.* $1/r_l$ on the left and r_l/Q vs. r_l on the right. Slope and intercept of the straight lines determine the parameters s_1 and s_2 (*see* Table 3). Q as defined in Eq. (6), r_l = molar ratio of valinomycin to lecithin, $\lambda_0 = 435$ nm

Table 3. Values for the parameters s_1 and s_2 in Eq. (19), together with values for α and $k' B(\lambda_0)$ calculated from these data

Parameter	Determined by method	$\lambda_0 = 412$ nm	$\lambda_0 = 435$ nm	$\lambda_0 = 663$ nm	$\lambda_0 = 680$ nm
s_1	A	− 0.500 (0.025)	−0.205 (0.010)	−0.285 (0.015)	0.640 (0.035)
	B	− 0.490 (0.020)	−0.210 (0.010)	−0.280 (0.010)	0.655 (0.030)
s_2	A	−16.5 (2.6)	−6.8 (1.1)	−9.5 (1.6)	21.5 (3.6)
	B	−16.0 (1.6)	−7.0 (0.7)	−9.3 (1.0)	21.5 (2.2)
$\alpha \cdot 10^3$	A	18.7 (3.9)	18.6 (3.9)	18.5 (4.1)	18.4 (4.1)
	B	18.9 (2.7)	18.5 (2.7)	18.6 (2.7)	18.8 (2.8)
$k' B(\lambda_0)$	A	−2.00 (0.10)	−4.90 (0.24)	−3.50 (0.18)	1.55 (0.08)
	B	−2.04 (0.08)	−4.75 (0.23)	−3.60 (0.13)	1.55 (0.07)
	C	−2.01 (0.04)	−4.75 (0.09)	−3.55 (0.07)	1.59 (0.03)

Parameters s_1 and s_2 were obtained from plots according to Eq. (19a) (method A) and Eq. (19b) (method B) (*see* Fig. 6). The distribution coefficient for valinomycin, α, and the proportionality constant $k' B(\lambda_0)$ [cf. Eqs. (10) and (17)] were calculated with the corresponding values for s_1 and s_2: from Eq. (20) $\alpha = s_1 \rho_l/(s_2 C_v) + 1$ and $k' B(\lambda_0) = 1/(s_1 + s_2 C_v/\rho_l)$; $\rho_l = 1.03$ mmole/cm^3, $C_v = 1.67$ μM. Moreover, $k' B(\lambda_0)$ was determined from the data plotted in Fig. 7 (method C).

Numbers in parentheses indicate standard deviations. The mean for α is $18{,}600 \pm 3{,}300$.

yields essentially the same curve for all λ_0. Hence within the agreement of $\langle R(\lambda, \lambda_0) \rangle$ for different preparations (cf. Fig. 3)

$$k' B(\lambda_0) \langle R(\lambda, \lambda_0) \rangle = \overline{\Delta E}(\lambda). \tag{21}$$

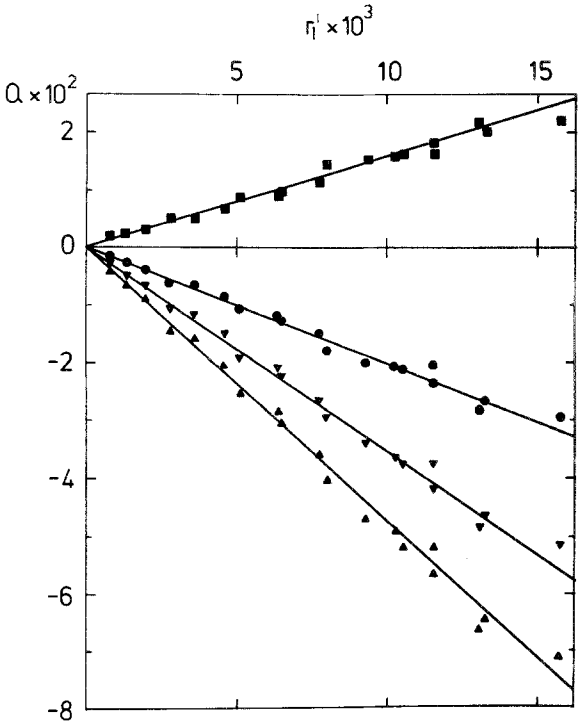

Fig. 7. Dependence of the difference spectra on the molar ratio of valinomycin dissolved in the membrane to lecithin, r_l'. According to Eqs. (6), (10) and (17): $Q = k' B(\lambda_0) r_l'$. All experimental data were used to determine the straight lines, but only some of them were plotted. Data for $\lambda_0 = 412$ nm (●), 435 nm (▲), 663 nm (▼), 680 nm (■)

From Eqs. (10), (17) and (21) it is found that

$$\Delta E(r_c, q, C_v, \lambda) = r_l' \, \overline{\Delta E}(\lambda) \tag{22}$$

where r_l' is given by Eq. (18), and all the present difference spectra are in agreement with this equation.

Discussion

The difference spectra described in this paper which were obtained by adding valinomycin to suspensions of lecithin vesicles containing chlorophyll *a* can be analyzed by Eq. (22). In terms of the experimentally accessible quantities, which are the absorbance changes, ΔA, and the concentrations of the components of the system (lecithin, chlorophyll *a*, valinomycin), C_i $(i = l, c, v)$, this equation reads [cf. Eqs. (18), (22), (A1), (A4), (A17) and (A19)]

$$\Delta A(C_l, C_c, C_v, \lambda) = F_1(C_l, C_v) \, F_2(\lambda) \, C_c \, l, \tag{23}$$

with λ and l denoting the wavelength and the optical path length, respectively. The factor F_1 depending on C_l and C_v characterizes the interaction of valinomycin and lecithin:

$$F_1(C_l, C_v) = k \frac{\alpha C_v}{\alpha C_l + \rho_l}. \tag{24}$$

α is the distribution coefficient for valinomycin between the lipid and the aqueous phase, and ρ_l denotes the molar density of lecithin in the vesicle membrane. In Eq. (24), the minor and unspecific contributions of chlorophyll *a* and valinomycin to the volume of the vesicle membrane were neglected [cf. Eq. (A 10)], and $\alpha \gg 1$ (*see* Table 3). The difference spectra are of course proportional to the chlorophyll *a* concentration, C_c, [*see* Eq. (23)] but otherwise no relevant dependence on the amount of chlorophyll *a* in the vesicle membrane exists. This means that chlorophyll *a* acts as a sensor (or a probe) for the valinomycin-lecithin interaction. The factor F_2 in Eq. (23):

$$F_2(\lambda) = \overline{\Delta E}(\lambda)\, \varepsilon_c(668)/k, \tag{25}$$

with $\varepsilon_c(668)$ denoting the extinction coefficient of the chlorophyll *a* vesicle suspension at 668 nm, expresses the characteristics of this probe (Walz, *unpublished paper*). Photochemical processes were obviously not involved, as was to be expected on account of the low intensity of the measuring light. Moreover, samples were prepared in dim light and kept in the dark until use.

Our evaluation of the experimental data is based on relatively simple schemes (*see* Table 2). More detailed schemes would produce complex mathematical expressions which, however, could be linearized in the limit of vanishing valinomycin concentration ($r_c \to 0$) and transformed into relations like those of Eqs. (15) and (16). In addition, our simple scheme seems to cover all the information contained in the present experiments, as indicated by the satisfactory fit of the difference spectra with Eq. (22), and more detailed mechanisms would need more information either from other experiments or based on a theoretical background. Moreover, the value for the distribution coefficient α obtained from our evaluation (18,600) is very reasonable. It compares well with the value of 25,000 determined by Stark *et al.* (1971) for the distribution of valinomycin between the membrane forming bulk phase of black lipid bilayers and the surrounding aqueous solution (keeping in mind that this bulk phase is essentially decane with only about 0.5 % lecithin). It should be added that α is critically dependent on the value of ρ_l which itself is determined by the

critical parameters A_l and d. Another choice of the values for these parameters will yield a different value of α but does not affect the quantity $s_1/(s_2\ C_v) = 18.1 \times 10^6$ cm^3/mole (cf. Table 3) as estimated from the experiments.

An interaction of polypeptide or depsipeptide antibiotics such as alamethicin, gramicidin A and S, enniatin B or valinomycin with different lipids (egg lecithin, dipalmitoyl- or dimyristoyl-lecithin, phosphatidylserine) of different physical state (single-shelled vesicles, unsonicated bilayers) has been observed by several authors. The experimental techniques used include nuclear magnetic resonance (Finer, Hauser & Chapman, 1969; Hsu & Chan, 1973), studies with the fluorescence probe ANS (Haynes, 1972) as well as differential scanning calorimetry, UV-spectroscopy, circular dichroism and ultrasonic absorption measurements (Grell, Funck & Eggers, 1975). Hauser, Finer and Chapman (1970) have found that the high resolution nuclear magnetic resonance signal of several protons on lecithin or phosphatidylserine molecules in the membrane of single-shelled vesicles was broadened when alamethicin was added to the aqueous phase. They interpret this result as indicating two different aggregational states of the lipids. Besides the state present in untreated vesicles there is a second state induced by alamethicin where the motional freedom of the whole phospholipid molecule is reduced. The second state appears at molar ratios of alamethicin to lipid as low as 1:1000, hence it must be regarded as an alamethicin-induced phase transition (i.e. a co-operative phenomenon), and correspondingly the data for phosphatidylserine could be described by the equilibrium scheme:

$$\mathrm{PS^I} + \mathrm{ala} \cdot \mathrm{PS^{II}_{n-1}} \rightleftarrows \mathrm{ala} \cdot \mathrm{PS^{II}_n} \quad \text{with } n = 1, 2, \ldots, 607 \tag{26}$$

where $\mathrm{PS^I}$ and $\mathrm{PS^{II}}$ denote phosphatidylserine in state I (narrow resonance line) and state II (broad line), respectively, and ala stands for alamethicin. A similar scheme for lecithin failed because the exchange rates of the equilibrium in Eq. (26) are intermediate between the proton relaxation rates of the lecithin in state I and II. It should be mentioned that alamethicin carries a negative charge, in contrast to valinomycin which is neutral. However, as pointed out by the authors, this charge is not the relevant feature for inducing a phase transition.

It is very likely that valinomycin induces a phase transition in lecithin membranes similar to that produced by alamethicin. The chlorophyll *a* molecules in the membrane are then dissolved in two different "solvents", i.e. lecithin in the state of aggregation present in untreated vesicles (state I) and lecithin in the aggregational state induced by valinomycin (state II).

If these "solvents" differ with respect to the parameters which influence the spectrum of chlorophyll *a*, the difference spectra can be explained as a "solvatochromic effect". The absorbance differences normalized by the absorbance of the vesicle suspension at 668 nm are then given by an equation identical with Eq. (A16) except for n_{vc} and ε_{vc} being replaced by n_c' and ε_c', the number of moles and the extinction coefficient of chlorophyll *a* dissolved in lecithin of state II, respectively. The ratio n_c'/N_c is equal to the ratio of volume for lecithin in state II to the total volume of the membrane, provided that the chlorophyll *a* molecules are uniformly distributed throughout the vesicle membrane, i.e. the "solubility" of chlorophyll *a* is equal in both aggregational states. With an equilibrium scheme analogous to Eq. (26) for the valinomycin-lecithin interaction:

$$\mathrm{PC^{I}} + \mathrm{val} \cdot \mathrm{PC^{II}_{m-1}} \rightleftarrows \mathrm{val} \cdot \mathrm{PC^{II}_{m}} \qquad m = 1, 2, \ldots, m_{\max} \tag{27}$$

where PC and val denote phosphatidylcholine (lecithin) and valinomycin, respectively, the volume of state II being $m\, n_v/\rho_l$ while the total volume is given by Eq. (A10). A possible difference in ρ_l for the two states was hereby neglected. At sufficiently low amounts of valinomycin, m is equal to $m_{\max}$ for all valinomycin-lecithin aggregates, hence [cf. Eq. (A7)]

$$n_c'/N_c = m_{\max}\, n_v/N_l = m_{\max}\, r_l'. \tag{28}$$

The scheme in Eq. (27) thus predicts a proportionality between the normalized difference spectra and r_l', in agreement with our present findings [cf. Eq. (22)]. A prerequisite for this conclusion is that the molecular events underlying the light absorption phenomenon are fast compared to both the exchange of lecithin molecules between the two aggregational states and the mobility of chlorophyll *a* molecules across the "boundaries" of the two states. The exchange rates can be judged by the data of Hauser *et al.* (1970) for the alamethicin-lecithin system where the time constants are comparable to the proton relaxation times and hence of the order of milliseconds (Finer, Flook & Hauser, 1972). The mobility of chlorophyll *a* molecules may be estimated from the lateral diffusion of lecithin in the membrane. Using 10 Å as minimal distance for a molecule to be moved across a phase boundary, and 10^{-8} cm^2/sec for the diffusion coefficient of lecithin (Devaux & McConnell, 1972; Träuble & Sackmann, 1972), we obtain 0.5 μsec as minimal time (Einstein's equation). Both times are indeed large compared to about 5 nsec, the average life time of excited chlorophyll *a* molecules, which governs the light absorption process (Becker, 1969).

A saturation phenomenon should be observed according to the scheme in Eq. (27): more and more domains with lecithin in state II are formed with increasing amounts of valinomycin, and finally all the membranes consist of lecithin in the valinomycin-induced aggregational state only. Therefore, the proportionality to r_l' at small r_l' values should end up in a constant for larger r_l' values with an intermediate range located around $r_l' = 1/m_{max}$. The transition region would allow for an estimation of the equilibrium constant as well as m_{max}, and by this means the constant k in Eqs. (24) and (25) would be determined. Since Fig. 7 comprises the linear part of the correlation only, supplementary experiments were carried out with r_l' values up to the limit set by the experimental conditions: with 5×10^{-4} as a lower limit for the absorbance changes in the difference spectra, $C_v < 2$ μM, $q < 0.018$ (*see* Ritt & Walz, 1976) and the other data given in the preceding section, this limit was at $r_l' \simeq 0.028$. Vesicles prepared from another less pure supply of chlorophyll *a* were used. As a consequence, the absorption spectra and $\overline{\Delta E}(\lambda)$ curves slightly differed from the previous ones but the value of α, i.e. the parameter relevant to valinomycin and lecithin, was still the same. Again, the normalized absorbance differences were proportional to r_l' over the whole range of values for this parameter, hence $m_{max} < 36$ which, however, is not surprising. Grell *et al.* (1975) have estimated that 7 to 10 lecithin molecules in vesicle membranes form one "binding site" for valinomycin, and the data for the alamethicin-lecithin system (Fig. 4 in the paper of Hauser *et al.*, 1970) suggest an m_{max} value between 2 and 10.

Based on their extensive studies with different experimental techniques, Grell *et al.* (1975) propose four types of arrangements for the valinomycin molecules within the membrane of lipid vesicles. One type (called state IV in their terminology), where four valinomycin molecules form a kind of channel through the membrane, is qualified as unlikely by the authors themselves. Another type (state I) is valinomycin adsorbed to the surface of the membrane. It is supported by the finding of Hsu and Chan (1973) that this antibiotic acts on lecithins in unsonicated bilayers predominantly in the region of the polar head groups. State II and III finally refer to the locations schematically shown in Fig. 8. At the present state of knowledge, however, we have no evidence to distinguish if valinomycin in all or only in some of these states is responsible for the postulated phase transition of lecithin.

Our experimental system represents a tool for investigating the lipid-polypeptide (or lipid-protein) interaction, independent of and thus supplementing the other experimental techniques used. Additional

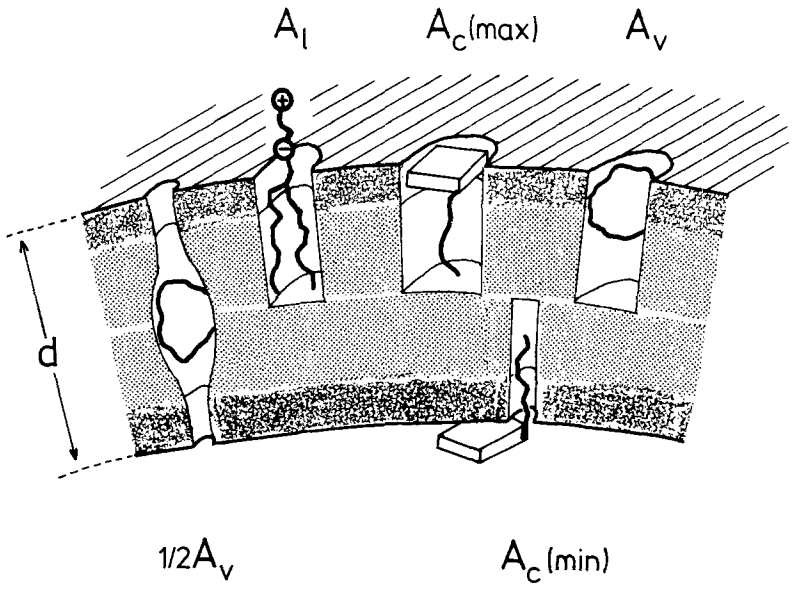

Fig. 8. Sketch of a cross-section through the vesicle membrane. The phospho-choline groups of the lecithin molecules (⊕—⊖) are drawn perpendicular to the membrane surface (Phillips, Finer & Hauser, 1972). They are strongly hydrated (Hauser, 1975) and thus belong to the aqueous phase. The lipid phase (thickness d) comprises the apolar fatty acid chains of lecithin (dotted area) and the more polar region where the glycerol moieties with the ester bonds are located (hatched area). A_l denotes the surface area occupied by one lecithin molecule. The location of chlorophyll *a* in the membrane is not yet known. Two extreme cases are shown: one where the porphyrin ring (symbolized by the square) lies parallel to the membrane surface giving a maximal surface area, A_c(max), and one where the phytyl chain only is incorporated into the lipid phase which needs a minimal surface area, A_c(min). According to Grell *et al.* (1975), the valinomycin molecules (represented by the distorted circles) are about equally distributed between the apolar and more polar region of the membrane. The two arrangements shown which correspond to state II and III in Grell's terminology are tentatively drawn to yield the same surface area, A_v (or $1/2\,A_v$ on two surfaces), for both locations

information about the characteristics of the sensor molecules such as localization or orientation in the membrane as well as data about their spectroscopic behaviour (Walz, *unpublished results*) will reveal new aspects of the aggregational states of lipids in different membranes, especially when the experiments are extended to other suitable pigment molecules as sensors. Our technique is thus comparable to the valuable measurements with fluorescence probes currently in use (*see*, e.g., Haynes, 1972). The effect of valinomycin on the aggregational state of lipids should be kept in mind when using this antibiotic as ion-selective ionophore in model membranes or biological systems. As will be discussed elsewhere[1] there are indeed

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reports in the literature that valinomycin might have effects in biological membranes not related to its ionophoric properties.

I would like to thank Prof. P. Läuger for his encouragement during the early phase of this work. I am also indebted to Prof. F. Grün and Dr. H. Hauser for many helpful discussions. The excellent technical assistance of Miss A. Kräuchi who carefully measured and evaluated most of the difference spectra is gratefully acknowledged.

Appendix

1. Definition of Parameters

The solution in a cuvette after addition of valinomycin contains, in a total volume V_0, an aqueous salt solution with N_l moles of lecithin, N_c moles of chlorophyll *a*, and N_v moles of valinomycin. The concentration of each of these components is

$$C_i = N_i/V_0, \tag{A1}$$

where the index i stands for l (lecithin), c (chlorophyll *a*) and v (valinomycin). Moreover, molar ratios are defined which may serve later as dimensionless concentration parameters. The molar ratio of chlorophyll *a* to lecithin

$$q = N_c/N_l \tag{A2}$$

is determined by the amount of chlorophyll *a* and lecithin used when preparing the vesicles, and remains constant for all vesicles of one preparation. The molar ratios of valinomycin to chlorophyll *a*,

$$r_c = N_v/N_c \tag{A3}$$

and of valinomycin to lecithin

$$r_l = N_v/N_l = q\, r_c \tag{A4}$$

depend on the vesicle concentration in the sample and the amount of valinomycin added to it. Lecithin and chlorophyll *a* together constitute the lipid phase and valinomycin, which is well soluble in lipids but only slightly so in water, distributes itself between these two phases. In the present context it is reasonable to take all vesicle membranes together and to treat them as one extended lipid phase with volume V. Similarly, we do not need to distinguish between the aqueous phases encapsulated in the cavities of the vesicles and the suspending medium, both of which had identical compositions, since valinomycin is easily permeable through the vesicle membrane. Hence we may apply the distribution law as usually

defined for extended phases,

$$\alpha = \frac{n_v/V}{n_v^*/(V_0 - V)}, \tag{A 5}$$

where α denotes the distribution coefficient of valinomycin for the vesicle-water two-phase system, while n_v and n_v^* are the number of moles of valinomycin in the lipid and in the water phase, respectively. In Eq. (A 5) no distinction was made between free valinomycin and valinomycin with a complexed alkali ion, an assumption which seems to be justified in view of the fact that no dependence of the difference spectra on the ion species was detected. Finally, we may define molar ratios similar to r_c and r_l but with the actual amount of valinomycin which is dissolved in the lipid phase:

$$r_c' = n_v/N_c \tag{A 6}$$

and

$$r_l' = n_v/N_l. \tag{A 7}$$

2. *Volume of the Lipid Phase, V*

The lecithin molecules in a bilayer structure are regularly arranged and each molecule spans about half the thickness of the membrane, d, and occupies an average area, A_l, on the surface (Fig. 8). The average volume of one lecithin molecule is thus $A_l\, d/2$, and the molar density of lecithin in the membranous aggregational state amounts to

$$\rho_l = 2/(A_l\, dN_L), \tag{A 8}$$

where N_L denotes Loschmidt's number. The precise location of chlorophyll *a* in the membrane is not yet known, but in view of the results reported by Steinemann, Stark & Läuger (1972) for black lipid membranes one can expect an arrangement with a predominant orientation. It is then meaningful to introduce a surface area, A_c (shown in Fig. 8 for two extreme cases), and a molar density, ρ_c, for chlorophyll *a* may be defined similarly to ρ_l in Eq. (A 8). When valinomycin is dissolved in the lipid phase it obviously contributes to the volume of this phase, too. To simplify matters, we treat these molecules in a similar way to lecithin and chlorophyll *a* and attribute to them a surface area, A_v (*see* Fig. 8), with a corresponding molar density, ρ_v. The volume of the lipid phase is then given by the sum of the number of moles divided by the molar density for all components which constitute the vesicle membrane:

$$V = N_l/\rho_l + N_c/\rho_c + n_v/\rho_v. \tag{A 9}$$

When estimating values for the molar densities, one has to know the thickness of the lipid phase, d, which extends between the glycerol moieties and does not comprise the phospho-choline groups (cf. Fig. 8). According to Hauser *et al.* (1973) $d=46$ Å for vesicles prepared from egg lecithin. With the generally accepted value $A_l=70$ Å^2 (Small, 1967; Johnson, Bangham, Hill & Korn, 1971; Hauser *et al.*, 1973) we thus obtain $\rho_l=1.03$ mmole/cm^3. The upper and lower limit for A_c can be estimated from the dimensions of the chlorophyll *a* molecule (Ballschmiter & Katz, 1969): $A_c(\min=45$ Å^2 and $A_c(\max)=225$ Å^2, hence 0.32 mmole/cm$^3 < \rho_c <$ 1.6 mmole/cm^3. A_v is difficult to determine, but $A_v<200$ Å^2 (cf. Mueller & Rudin, 1967; Shemyakin *et al.*, 1969; Hsu & Chan, 1973; Grell *et al.*, 1975) and therefore $\rho_v>0.36$ mmole/cm^3 seems to be reasonable. With these values for the molar densities, it was found that the contributions of chlorophyll *a* and valinomycin to the volume of the lipid phase were always less than 8 % for all experimental conditions; thus as a good approximation

$$V \simeq N_l/\rho_l. \tag{A 10}$$

3. *Complex Formation*

Chlorophyll *a* and valinomycin in the membrane associate to a complex in an equilibrium reaction: val + chl *a* $\rightleftarrows$ val · chl *a*. According to the law of mass action

$$K_b = \frac{n_{vc}/V}{n_c/V \cdot n_v/V}, \tag{A 11}$$

where K_b denotes the binding constant of the complex while n_{vc} and n_c are the number of moles of complexed and uncomplexed chlorophyll *a*, respectively. By means of Eq. (A 5) and the mass conservation equations

$$n_c + n_{vc} = N_c \tag{A 12}$$

for chlorophyll *a* and

$$n_v + n_v^* + n_{vc} = N_v \tag{A 13}$$

for valinomycin, Eq. (A 11) is transformed into

$$n_{vc}^2 - n_{vc}\{N_c + N_v + [V_0 + (\alpha - 1)\, V]/(\alpha\, K_b)\} + N_c\, N_v = 0. \tag{A 14}$$

Eq. (A 9) has to be modified to cope with the new species in the membrane, i.e. the valinomycin-chlorophyll *a* complex. Assigning a molar density,

ρ_{vc}, to this species we obtain in view of Eqs. (A 2) and (A 6):

$$V = N_l/\rho_l [1 + q(\rho_l/\rho_c + r'_c\, \rho_l/\rho_v + n_{vc}/N_c \cdot \rho_l/\Delta\rho_{vc})], \tag{A 15}$$

where $1/\Delta\rho_{vc} = 1/\rho_{vc} - 1/\rho_c$. The differences in absorbance between a vesicle suspension with and without valinomycin as a function of wavelength $\Delta A(\lambda)$ arises from different extinction coefficients for the complex, $\varepsilon_{vc}(\lambda)$, and for uncomplexed chlorophyll *a*, $\varepsilon_c(\lambda)$. Hence for the normalized difference spectra

$$\Delta A(\lambda)/A(668) = n_{vc}/N_c \cdot [\varepsilon_{vc}(\lambda) - \varepsilon_c(\lambda)]/\varepsilon_c(668) \tag{A 16}$$

with $A(668)$ denoting the absorbance of the vesicle suspension at 668 nm. When writing this equation it was assumed that Beer-Lambert's law holds true over the whole wavelength range:

$$A(\lambda) = \varepsilon_c(\lambda)\, C_c\, l, \tag{A 17}$$

l being the optical path length of the cuvette. The molar ratio of complex to total chlorophyll *a*, x, follows from Eqs. (A 14) and (A 15) together with Eqs. (A 1) to (A 3):

$$\begin{aligned} &x(r_c, q, C_v) \equiv n_{vc}/N_c = b/2a - [(b/2a)^2 - r_c/a]^{1/2} \\ &a = 1 - (\alpha - 1)/(\alpha\, K_b\, \Delta\rho_{vc}) \\ &b = 1 + r_c + r_c/(\alpha\, K_b\, C_v) + (\alpha - 1)\,[1 + q(\rho_l/\rho_c + r'_c\, \rho_l/\rho_v)]/(\alpha\, K_b\, q\, \rho_l). \end{aligned} \tag{A 18}$$

Eqs. (A 16) and (A 18), together with the abbreviations

$$\Delta A(\lambda)/A(668) \equiv \Delta E(r_c, q, C_v, \lambda) \tag{A 19}$$

and

$$[\varepsilon_{vc}(\lambda) - \varepsilon_c(\lambda)]/\varepsilon_c(668) \equiv \Delta\varepsilon(\lambda) \tag{A 20}$$

yield then Eq. (1). Note that Eq. (A 18) contains the variable r'_c depending itself on r_c, q and C_v. This makes a test against the experimental results rather difficult. Therefore, a Taylor series of x with respect to r_c was used at the point $r_c = 0$ where

$$(\partial x/\partial r_c)_{r_c = 0} = 1/b(r_c = 0) = 1/[1 + (\alpha - 1)(1 + q\, \rho_l/\rho_c)/(\alpha\, K_b\, q\, \rho_l)] \tag{A 21}$$

and r'_c is zero, too.

4. Interaction of Valinomycin with Chlorophyll a or Lecithin

By virtue of Eq. (A 5) and the mass conservation of valinomycin [Eq. (A 13) with $n_{vc} = 0$] one obtains

$$n_v = \alpha\, N_v\, V/[V_0 + (\alpha - 1)\, V]. \tag{A 22}$$

Dividing this by N_c or N_l and introducing Eq. (A9), rewritten with the help of Eqs. (A1) to (A4) and (A6), (A7) as

$$V = V_0\, C_v[1 + q(\rho_l/\rho_c + r_c'\,\rho_l/\rho_v)]/(r_c\, q\, \rho_l) = V_0\, C_v\,[1 + q\,\rho_l/\rho_c + r_l'\,\rho_l/\rho_v]/(r_c\, q\, \rho_l),$$

yields quadratic equations for r_c' and r_l' with the roots

$$r_i'(r_c, q, C_v) = -b_i/2\,a_i + [(b_i/2\,a_i)^2 + c_i/a_i]^{1/2} \qquad \text{for } i = c \text{ or } l$$

where

$$a_c = a_l\, q = (\alpha - 1)\, q\, \rho_l/\rho_v \tag{A23}$$

$$b_c = b_l = r_c\, q\, \rho_l/C_v + (\alpha - 1)(1 + q\,\rho_l/\rho_c) - \alpha\, r_c\, q\, \rho_l/\rho_v$$

$$c_c\, q = c_l = \alpha\, r_c\, q(1 + q\,\rho_l/\rho_c).$$

For a test against the experimental data we do not need all of Eq. (A23) but only the first partial derivatives of r_i' with respect to r_c at $r_c = 0$:

$$(\partial r_i'/\partial r_c)_{r_c=0} = (\partial c_i/\partial r_c)_{r_c=0}/b_i(r_c = 0) \qquad \text{for } i = c \text{ or } l$$

which are then given by

$$q(\partial r_c'/\partial r_c)_{r_c=0} = (\partial r_l'/\partial r_c)_{r_c=0} = \alpha\, q/(\alpha - 1). \tag{A24}$$

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