BBA 76043

STRUCTURE AND REFLECTION SPECTRA OF CHLOROPHYLL–LIPID MEMBRANES

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(Received April 24th, 1972)

SUMMARY

Electrical and optical techniques have been used to investigate the structure of chlorophyll–lipid membranes. No change in either the low frequency a.c. capacitance or the optical reflectance at 632.8 nm could be detected when chlorophyll *a* was introduced into the lipid membrane. From these results, it is concluded that the chlorophyll porphyrin ring is most probably located amongst the lipid polar groups.

A preliminary investigation has been made of the application of reflection spectroscopy to lipid–pigment membranes. Reflection spectra in the range 400–550 nm have been obtained for both chlorophyll *a* and chlorophyll *b* in the membrane. These spectra are in reasonable accord with a theoretical analysis of reflection by a thin absorbing film. The analysis indicates that the reflection technique is potentially a very sensitive method for obtaining spectra of pigments in membranes.

INTRODUCTION

The isolated bimolecular lipid membrane is currently being used as a model system for investigating a variety of membrane phenomena[1]. The incorporation of chlorophyll into the lipid bilayer provides an attractive model for studying energy transfer and energy transduction in membranes[2–7]. In order to understand these processes, it is important to fully characterise the structure of the model system. For this purpose we have developed a technique for obtaining the absorption spectrum of chlorophyll in the lipid membrane. These measurements enable the chlorophyll concentration to be determined and also give information about the orientation of the porphyrin ring in the membrane[8].

Absorption measurements alone are not sufficient to identify the location of chlorophyll in the membrane. As shown in the present paper, this may be deduced by combining electrical data with the results of optical reflectance measurements. In this way it is possible to build up a fairly complete picture of the chlorophyll–lipid membrane.

We have also made a preliminary investigation of the application of reflection spectroscopy to lipid–pigment membranes. This technique is complementary to absorption spectroscopy but, as will be shown, has potentially greater sensitivity.

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METHODS

Materials

Chlorophylls *a* and *b* were extracted from pea leaves and purified by thin-layer chromatography as described previously[8]. Lecithin was extracted from fresh egg yolks and purified on an alumina column according to the method of Singleton *et al.*[9]. The lipid was stored in chloroform solution under N_2 in sealed ampoules at −20 °C until used.

The membrane-forming solution was made up by dissolving weighed quantities of lecithin and chlorophyll in *n*-decane. Normally the concentration of the solution was 1 % with respect to the dry weight of lecithin. Puriss-grade *n*-decane was obtained from Koch Light Ltd. Analar-grade reagents were used for making up the aqueous solutions. The aqueous phase was buffered at pH 7 with 50 mM tris(hydroxymethyl)-methylamine.

Electrical measurements

Electrical measurements were carried out in a cell similar to that described by Mueller and Rudin[10]. Membranes were formed by brushing the lipid solution across a 1- or 2-mm diameter hole in the side of a teflon cup. For resistance measurements, electrical connection to the aqueous phase on either side of the membrane was made *via* calomel electrodes and KCl–agar bridges. A d.c. voltage was applied to the membrane and the current measured with a Keithley 417 picoammeter. Pt electrodes were used for a.c. capacitance measurements which were carried out using a Wayne Kerr B221 Universal Bridge and Advance SG 68A Low Frequency Oscillator. Dielectric constants of solutions of chlorophyll were obtained by measuring the capacitance of a standard cell containing the solution. These measurements were made using a Wayne Kerr Autobalance Universal Bridge B642 with an internal source of 1591.5 Hz.

Optical reflectance

The method of measuring the absolute reflectance of the membrane was as previously described[11,12]. Briefly, the membrane was illuminated by light from a helium–neon laser ($\lambda = 632.8$ nm) and the reflected intensity measured with an E.M.I. 9661B photomultiplier. The incident light was at near normal incidence and polarised perpendicular to the plane of incidence. Reflectances were calibrated by replacing the membrane with a quartz plate of known reflectivity. The aqueous phase consisted of either NaCl, $CaCl_2$ or sucrose in various concentrations. The refractive index of the aqueous phase was measured on an Abbé refractometer.

Reflection spectra

In order to measure reflection spectra, the laser light source was replaced by an A.E.I. 500 W DC Type XE/D Xenon Discharge Lamp. Before being focussed on the membrane the light was first passed through a Hilger D186 monochromator fitted with a quartz prism. The reflected intensity was measured by the photomultiplier in the usual way. The spectrum was continuously scanned using a motor drive attached to the wavelength drum. Because there tended to be some drift in the lamp output, spectra were scanned in both directions and the average taken. A calibration

spectrum was obtained by measuring the output of the monochromator with the photomultiplier. The calibration curve was used to correct the membrane reflectance for variation of lamp output and photomultiplier sensitivity with wavelength.

RESULTS

Electrical measurements

Chlorophyll was found to have a marked stabilising effect on membrane conductance. We have frequently noted that the conductances of pure lecithin membranes tend to vary substantially from membrane to membrane and that the conductance of an individual membrane may drift considerably to a higher value over a period of time. In contrast, the conductances of lecithin–chlorophyll membranes were generally reproducible within a factor of 2 and were stable with time.

The capacitance of bilayers formed in 0.1 M KCl and 50 mM Tris buffer at pH 7, from a solution of lecithin–chlorophyll *a* (1:1, w/w) in *n*-decane, was found to be 0.38 ± 0.02 μF/cm^2 at 1500 Hz. This value is indistinguishable from that found for pure lecithin membranes[12,13].

Dielectric constants were determined for solutions of chlorophyll *a* in *n*-decane containing 7.5 % diethyl ether. Ether was added to ensure complete solubilization of chlorophyll at all concentrations. The variation of dielectric constant with chlorophyll concentration is shown in Fig. 1. At concentrations above 15 mg/ml and within the range of the experiment, the dielectric constant is a linear function of the concentration. The slope is 0.036 per 10 mg/ml. The limitation of available material prevented the measurements from being extended to much higher chlorophyll concentrations.

Reflectance

As shown previously[11,14], the reflectance of a lipid membrane is given by the expression:

$$\frac{\lambda R_m^{\frac{1}{2}}}{2\pi d} = \bar{n} - n_0 + \Delta \qquad (1)$$

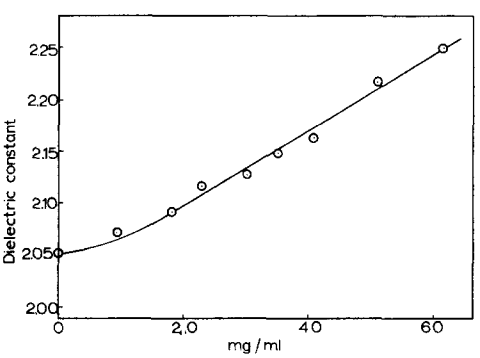


Fig. 1. Concentration dependence of dielectric constant of solutions of chlorophyll *a* in *n*-decane containing 7.5% diethyl ether.

where

$$\Delta = \frac{(\bar{n} - n_0)^2}{\bar{n} + n_0} \tag{2}$$

d is the total membrane thickness, $\bar{n}$ the mean refractive index parallel to the plane of the membrane and n_0 the refractive index of the aqueous phase. The membrane parameters may be obtained by the following procedure. First, a plot is made of $R_m^{\frac{1}{2}}$ against n_0. The intercept of the curve with the x-axis gives an approximate value of $\bar{n}$. This value is used to calculate Δ from Eqn 2 and a further plot is made of $R_m^{\frac{1}{2}}$ *versus* $(n_0 - \Delta)$. From the intercept and the slope of the second plot, values of $\bar{n}$ and d may be obtained. The data for lecithin–chlorophyll *a* membranes are plotted in Figs 2a and 2b. The results are independent of the solute used to vary n_0, since the same line fits experimental points obtained with different solutes. From Figure 2b, values of $\bar{n}$ and d for the lecithin–chlorophyll membrane are found to be: $d = 62 \pm 2$ Å and $n = 1.458 \pm 0.004$. The equivalent values for lecithin membranes[11] are $d = 62 \pm 2$ Å and $\bar{n} = 1.454 \pm 0.002$.

Reflection spectra for chlorophylls *a* and *b* in the range 400–550 nm are shown

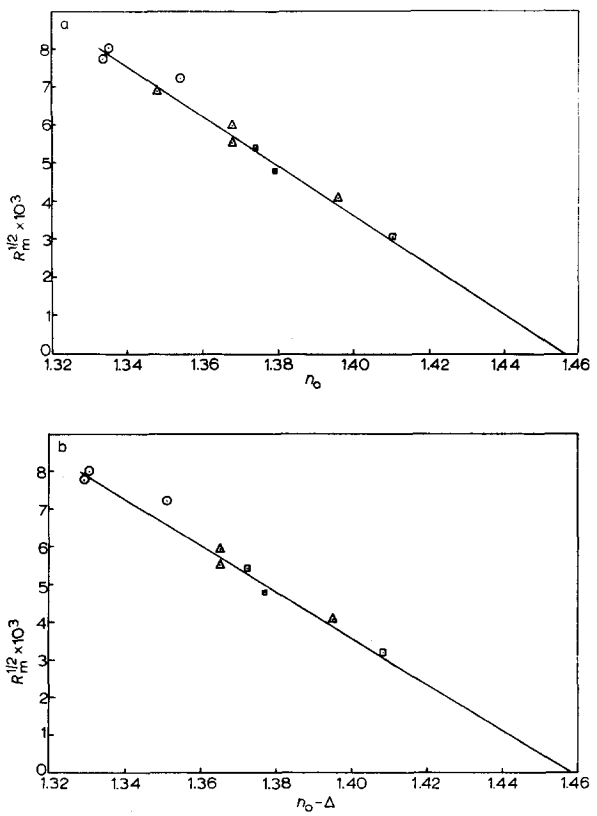
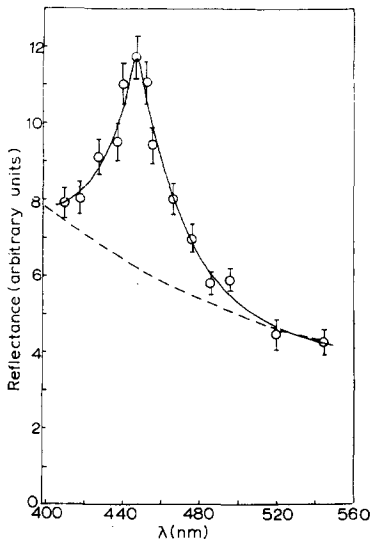


Fig. 2. Reflectance of lecithin–chlorophyll bilayers. Membrane forming solution lecithin–chlorophyll *a* (1:1, w/w) in *n*-decane. Aqueous phase: ⊙, NaCl; △, $CaCl_2$; ⊡, sucrose. (a) Variation of $R_m^{\frac{1}{2}}$ with n_0. (b) Variation of $R_m^{\frac{1}{2}}$ with $n_0 - \Delta$.



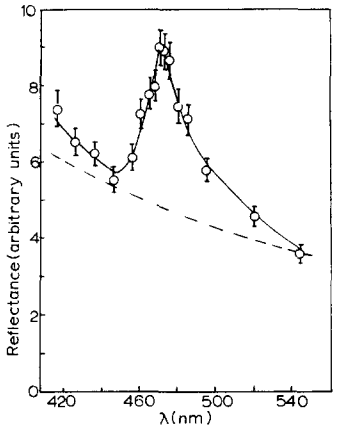


Fig. 3. Reflection spectrum of bilayer formed from lecithin–chlorophyll *a* (1:1, w/w) in *n*-decane. Aqueous phase $CaCl_2$, $n_0 = 1.396$.

Fig. 4. Reflection spectrum of bilayer formed from lecithin–chlorophyll *b* (1.5:1, w/w) in *n*-decane. Aqueous phase $CaCl_2$, $n_0 = 1.396$.

in Figs 3 and 4. The experimental arrangement did not permit measurements to be made beyond 550 nm because of the rapid fall off in the sensitivity of the photomultiplier at longer wavelengths.

DISCUSSION

Structure of the membrane

In a previous report we deduced that the porphyrin ring of chlorophyll was oriented at about 50° to the plane of the lipid bilayer[8]. The aim of the present analysis is to determine the location of the porphyrin ring within the bilayer. In this respect it is convenient to consider three distinct possibilities: (a) The porphyrin ring is located within the hydrocarbon chain region of the bilayer. (b) The porphyrin ring is located in the aqueous phase outside the lipid polar groups. (c) The porphyrin ring is located between the lipid polar groups. These possibilities are illustrated schematically in Fig. 5.

Information relevant to the first possibility may be obtained from the capacitance data. It is well established that at low frequency the capacitance of the lecithin bilayer is determined solely by the hydrocarbon region[15]. The capacitance per unit area, C, is given by the expression for a parallel plate capacitor

$$C = \frac{\varepsilon_a}{4\pi d_H} \tag{3}$$

where ε_a is the dielectric constant and d_H the thickness of the hydrocarbon region. Our experimental finding that C is unchanged by the incorporation of chlorophyll implies that ε_a and d_H are also unchanged. The alternative possibility that ε_a and d_H

both change in such a way as to leave C unaltered seem unlikely; in addition it will be shown that a thickness change is not implied by the reflectance data.

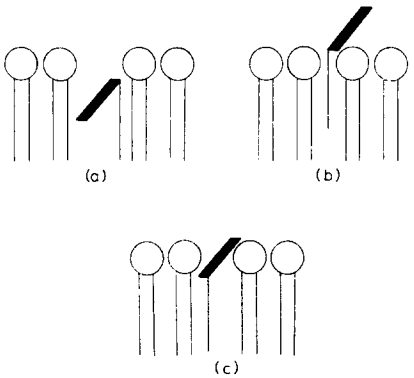


Fig. 5. Schematic diagram of one half of lipid bilayer illustrating possible locations of chlorophyll molecule.

If the porphyrin ring of chlorophyll were located in the hydrocarbon region of the bilayer it would be expected to produce some change in ε_a. The magnitude of the change may be estimated from the dielectric-constant measurements (Fig. 1), since decane may be regarded as a reasonable approximation to the fluid hydrocarbon interior of the bilayer. From Fig. 1, it may be seen that the dielectric constant has increased by about 8.5 % when the chlorophyll concentration is 60 mg/ml or 0.067 M. The chlorophyll concentration in the bilayer may be estimated from absorption data and is found to be $2 \cdot 10^{13}$–$4 \cdot 10^{13}$ molecules/cm^2 when the membrane forming solution is lecithin–chlorophyll (1:1, w/w)[7,8]. Taking the hydrocarbon thickness as 50 Å this is equivalent to a concentration of between 0.065 and 0.130 M. Thus if the porphyrin ring were in the hydrocarbon region we would expect an increase in dielectric constant, and consequently an increase in capacitance, of greater than 8.5 %. Since no change is observed, within the experimental uncertainty of $\pm$ 5 %, it is probable that the porphyrin ring is not in the hydrocarbon chain region of the bilayer.

It has previously been shown that the thickness obtained from optical reflectance measurements is the total membrane thickness[14]. The total thickness would be increased if the porphyrin rings were lying outside the lipid polar groups as in Fig. 5b. Taking the length of the porphyrin ring as 15 Å and the angle of tilt as 50°, the increase in thickness would be about 11 Å each side, or 22 Å in all. However, the optical measurements demonstrate that the thickness of lecithin and lecithin–chlorophyll membranes are the same within the experimental error of $\pm$ 2 Å. Hence we conclude that the porphyrin rings are not outside the lipid polar groups.

We have now shown that possibilities a and b in Fig. 5 are improbable and hence conclude that the porphyrin ring of chlorophyll is amongst the lipid polar groups. The present experiment gives no direct indication of the position of the phytol chain. However, in view of the hydrophobic nature of this part of the molecule, there seems little doubt that it will lie in the hydrocarbon-chain region of the bilayer. Thus we conclude that the structure of the lecithin–chlorophyll membrane is most

probably as illustrated schematically in Fig. 5c. As we have previously shown[8], the angle between the plane of the porphyrin ring and the plane of the bilayer is about 50°.

Our conclusion concerning the location of the chlorophyll porphyrin ring is at variance with Steinemann *et al.*[6] and Ting *et al.*[5] both of whom place the porphyrin ring in the aqueous phase as in Fig. 5b. The bilayers studied by Ting *et al.* were formed from an uncharacterised extract of chloroplast lipids and pigments. Their structural model is based on a thickness measurement of 105 Å obtained from optical reflectance measurements of bilayers in 0.1 M NaCl. However, we have pointed out elsewhere[14] that a reliable value of the bilayer thickness cannot be obtained from reflectance measured at a single value of n_0. The authors themselves admit that their thickness measurement might be in error by as much as 50 % owing to the use of an assumed value of refractive index. The model proposed by Steinemann *et al.* is based on the observation that chlorophyll in the bilayer may be oxidised by potassium peroxodisulfate ($K_2S_2O_8$) present in the aqueous phase. Since the polar region of the bilayer is probably accessible to small ions, this finding is not inconsistent with our conclusion that the chlorophyll porphyrin ring is amongst the lipid polar groups.

Steinemann's experiment with $K_2S_2O_8$ also demonstrates that a substantial proportion of chlorophyll molecules are not dissolved in microscopic lenses of solvent which may be present in the film. Steinemann found that approximately one half of the total chlorophyll could be oxidised by $K_2S_2O_8$ on either side of the bilayer. This is only consistent with virtually all the chlorophyll being in the surface of the membrane.

Reflection spectra

The previously developed theory of reflection by a lipid bilayer assumed that there was no absorption of the incident light[11,14]. The proximity of the wavelength of the laser output (632.8 nm) to that of the chlorophyll *a* absorption maximum in the bilayer (672 nm) raises the question of whether absorption could have any effect on the reflectance measurements. It was felt desirable to check this possibility, both theoretically and experimentally. At the same time it was possible to investigate the application of reflection spectroscopy to lipid–pigment membranes.

We first apply the theory of reflection by thin absorbing films to the lipid membrane. We assume the membrane to be a homogeneous absorbing layer of thickness d and complex refractive index given by

$$n^* = n - ik$$

An analysis of reflection by absorbing films has been given by Vašiček[16]. By adapting equation 5.9.33 of his treatment to the present case, where the film is immersed in a medium of refractive index n_0, we obtain

$$R_m = \frac{r^2(1 + e^{-2x'} - 2e^{-x'}\cos x)}{1 + r^4 - 2r^2 e^{-x'}\cos x} \tag{4}$$

where

$$r = \frac{(n - n_0)^2 + k^2}{(n + n_0)^2 + k^2} \tag{5}$$

$$x = \frac{4\pi nd}{\lambda} \tag{6}$$

$$x' = \frac{4\pi kd}{\lambda} \tag{7}$$

and the equations apply to normal incidence. At this point we note that the absorbance A of the membrane is related to the imaginary part of the refractive index by the expression

$$A = \frac{4\pi kd}{2.3\lambda} \tag{8}$$

Since the measured absorbance of membranes formed from chlorophyll *a*–lecithin (1:1, w/w) is less than 0.01 at the 439-nm absorption maximum[8], we calculate from Eqn 8 that $k < 0.13$. Assuming that the real part of the refractive index n is not very much different from the refractive index of the lecithin membrane (1.454), we deduce from Eqn 5 that $r \ll 1$. This enables a considerable simplification in Eqn 4 to be made, since we may write the denominator equal to unity and obtain

$$R_m = r^2(1 + e^{-2x'} - 2e^{-x'}\cos x) \tag{9}$$

(This simplification is equivalent to neglecting multiple reflections within the film.) By noting that for a 60-Å-thick film both $x \ll 1$ and $x' \ll 1$, Eqn 9 may be simplified further to obtain

$$R_m = 4\left[\frac{(n - n_0)^2 + k^2}{(n + n_0)^2 + k^2}\right]\sin^2\left(\frac{2\pi nd}{\lambda}\right) \tag{10}$$

For zero absorption the reflectance R_0 may be obtained from Eqn 10 by putting $k = 0$ to obtain the familiar expression[11]

$$R_0 = 4\left[\frac{(n - n_0)^2}{(n + n_0)^2}\right]\sin^2\left(\frac{2\pi nd}{\lambda}\right) \tag{11}$$

A useful alternative expression for R_m, which is valid when $k^2 \ll (n + n_0)^2$ is

$$R_m = R_0\left[1 + \frac{k^2}{(n - n_0)^2}\right] \tag{12}$$

With the aid of the above analysis we now consider the experimental data. Firstly, at 632.8 nm the absorption is below the limits of detectability in membranes formed from lecithin–chlorophyll *a* (1:1, w/w)[8]. This places an upper limit on A of 0.0005 and hence on k of 0.0095 (Eqn 8). Hence $k^2 \ll (n - n_0)^2$ and from Eqn 12, $R_m \approx R_0$ at this wavelength. Thus our use of equations derived for non-absorbing films to interpret reflectance measurements at 632.8 nm is justified.

The reflection spectra are most conveniently interpreted with reference to Eqn 12. This demonstrates that the spectrum may be divided into two components. The first of these is R_0 which is simply the reflectance in the absence of absorption. The second component is proportional to k^2 and hence is proportional to the square of the absorbance. In Figs 3 and 4 the dashed line represents R_0 calculated from the

measured parameters $n = 1.458$ and $d = 62$ Å and fitted to the experimental points at 550 nm.

The maxima of the reflection spectra occur at 445 ± 3 nm for chlorophyll *a* and at 472 ± 4 nm for chlorophyll *b* in reasonable agreement with the absorption data[8]. However, there is some discrepancy in the shapes of the bands. On the long wave-length side of their maxima, the reflection spectra decrease in intensity much less rapidly than predicted by the absorption spectra.

As a quantitative check of the theory we may use the reflection data to calculate the chlorophyll concentrations in the bilayer. At the reflection maxima we obtain values of $R_m/R_0 = 1.89$ for chlorophyll *a* and $R_m/R_0 = 1.86$ for chlorophyll *b*. Substituting in Eqn 12 we obtain values of k of 0.059 and 0.058, respectively. By combining Eqn 8 with the previously derived equation[7]

$$A = \varepsilon y M/N \tag{13}$$

where ε is the extinction coefficient, y the number of chlorophyll molecules/cm^2 in the bilayer, M the molecular weight, and N is Avagadro's number, we obtain

$$y = \frac{4\pi k d N}{2.3\varepsilon\lambda M} \tag{14}$$

Substituting the values of k and taking $d = 60$ Å and values of ε measured in ether[17], we obtain $y = 2.4 \cdot 10^{13}$ for chlorophyll *a* and $y = 1.7 \cdot 10^{13}$ for chlorophyll *b*. These values are in fairly good agreement with those obtained from absorption spectra[8].

The theory used in interpreting the reflection spectra is based on the assumption that the bilayer is a single homogeneous layer. As discussed elsewhere[14], this is undoubtedly an over-simplification of the system and a multilayer treatment is more appropriate. However, the analysis of multilayered absorbing films is highly complex and has not been attempted in the present work. For the moment it appears that the simplified theory gives a reasonable account of the reflection spectra and that the quantitative information obtained is consistent with the corresponding absorption measurements.

Reflection spectra give similar information to that obtained from absorption measurements. However, the reflection technique has in principle a considerable advantage in sensitivity over absorption. This is because the background reflectance R_0 may be reduced by increasing n_0 and becomes zero when $n_0 = n$. Thus by making measurements in solutions of high refractive index, the reflectance will be dominated by the absorption component, k, in the numerator of Eqn 10. The measurement of the spectrum is then only limited by the sensitivity of the photomultiplier. The present measurements were mainly directed towards demonstrating the feasibility of obtaining reflection spectra from pigment in lipid membranes and were carried out with a relatively simple experimental arrangement. It may be anticipated that the application of more sophisticated double-beam techniques would enable the potential sensitivity of the method to be further exploited.

ACKNOWLEDGEMENT

We thank the Science Research Council for financial support.

REFERENCES

1 P. Mueller and D. O. Rudin, *Curr. Top. Bioenerg.*, 3 (1969) 157.
2 R. J. Cherry, *Q. Rev.*, 22 (1968) 160.
3 H. T. Tien, *Nature*, 219 (1968) 272.
4 N. T. Van and H. T. Tien, *J. Phys. Chem.*, 74 (1970) 3559.
5 H. P. Ting, W. A. Huemoeller, S. Lalitha, A. L. Diana and H. T. Tien, *Biochim. Biophys. Acta*, 163 (1968) 439.
6 A. Steinemann, R. Alamati, W. Brodmann, O. Marschall and P. Läuger, *J. Membrane Biol.*, 4 (1971) 284.
7 R. J. Cherry, K. Hsu and D. Chapman, *Biochem. Biophys. Res. Commun.*, 43 (1971) 351.
8 R. J. Cherry, K. Hsu and D. Chapman, *Biochim. Biophys. Acta*, 267 (1972) 512.
9 W. S. Singleton, M. S. Gray, M. L. Brown and J. L. White, *J. Am. Oil Chem. Soc.*, 42 (1965) 53.
10 P. Mueller and D. O. Rudin, in H. Passow and R. Stampfli, *Laboratory Techniques in Membrane Biophysics*, Springer-Verlag, Berlin, 1969, p. 141.
11 R. J. Cherry and D. Chapman, *J. Mol. Biol.*, 40 (1969) 19.
12 A. W. Clowes, R. J. Cherry and D. Chapman, *Biochim. Biophys. Acta*, 249 (1971) 301.
13 T. Hanai, D. A. Haydon and J. Taylor, *Proc. R. Soc. London, Ser. A*, 281 (1964) 377.
14 R. J. Cherry and D. Chapman, *J. Theor. Biol.*, 24 (1969) 137.
15 T. Hanai, D. A. Haydon and J. Taylor, *J. Theor. Biol.*, 9 (1965) 278.
16 A. Vašiček, *Optics of Thin Films*, North Holland Publishing Co., Amsterdam, 1960.
17 F. P. Zscheile, Jr and C. L. Comar, *Botan. Gaz.*, 102 (1941) 463.