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Pigment Containing Lipid Vesicles

I. Preparation and Characterization of Chlorophyll *a*-Lecithin Vesicles

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Summary. Vesicles obtained by sonication of chlorophyll *a*-lecithin mixtures dispersed in an aqueous medium closely resemble the well-characterized vesicles similarly prepared from pure lipids. They are bounded by one spherical lipid bilayer which contains the chlorophyll *a*. Appropriate conditions for sonication prevent substantial degradation of the membrane constituents. Up to one chlorophyll *a* molecule per 55 lecithins can be incorporated into the membranes. The average Stokes' radius of the vesicles determined by analytical sieve chromatography is 102 ± 5 Å and independent of the chlorophyll *a* content. The membrane is visible in the electron-microscope when the vesicles are treated with osmium tetroxide prior to negative staining. The osmium fixation is, however, not strong enough to allow for a preparation of the vesicles for thin sectioning (dehydration, embedding in epoxide).

Two systems have proven to be especially suited for studying lipid bilayers, the "black lipid membranes" developed by Mueller and co-workers and the "single-shelled vesicles" obtained when the multi-layered liposomes introduced by Bangham and co-workers are sonicated. Non-lipid components such as chlorophyll *a* can be incorporated into both types of bilayers, but the geometry and other properties of the two types of membranes are different.

A black lipid membrane is a planar film separating two aqueous phases which both are directly accessible. It is well suited to study photoelectric processes of the incorporated chlorophyll *a* (Tien & Verma, 1970; Trissl & Läuger, 1972), but spectroscopic measurements require special experimental techniques because of the relatively small membrane area (Cherry, Hsu & Chapman, 1971; Steinemann, Alamuti, Brodmann, Marschall & Läuger, 1971). The planar geometry facilitates dichroic measurements, and the orientation of the porphyrin ring could thus be determined (Cherry *et al.*, 1971; Steinemann, Stark & Läuger, 1972). The main disadvantage of this system is that a solvent such as *n*-decane has to be present which usually leads to an unknown and uncontrolled composition of the membrane.

A single-shelled vesicle consists of one spherical lipid bilayer with no organic solvent present. It encapsulates a small volume of an aqueous phase whose composition is known but which is not directly accessible. However, these vesicles have the advantage that the composition of their membranes can be controlled and that their physico-chemical properties are well known (Huang, 1969; Hauser, 1971; Miyamoto & Stoeckenius, 1971). In addition, the physical state of the lipids has been investigated by spin-label techniques (Kornberg & McConnell, 1971*a*, *b*) and by nuclear magnetic resonance spectroscopy (Finer, Flook & Hauser, 1972*b*; Sheetz & Chan, 1972). Stable suspensions with large numbers of vesicles can be prepared providing large overall membrane areas and thus also substantial concentrations of the membrane contained pigment. Hence, Tomkiewicz and Corker (1975) could easily detect photochemical reactions of chlorophyll *a* incorporated into the membrane of lecithin vesicles, as did Chapman and Fast (1968) and Trosper, Raveed and Ke (1970) though with the less defined system of chlorophyll *a* in multi-layered liposomes. Colbow (1973) could use the fluorescence of chlorophyll *a* in vesicles prepared from dipalmitoylphosphatidylcholine as a probe of the liquid-crystalline phase transition. We have chosen single-shelled vesicles in order to study pigment molecules dissolved in lipids by means of spectroscopic measurements. In this paper we describe, by way of example, the preparation of lecithin vesicles containing chlorophyll *a*. Chemical analysis and analytical sieve chromatography as well as examination in the electron-microscope are used to characterize the vesicles.

Materials and Methods

Lecithin was isolated from fresh egg yolks and chromatographed on alumina according to Singleton, Gray, Brown and White (1965). The purified material obtained from the column was dissolved in benzene and lyophilized. The dry material, which gave a single spot on a thin-layer chromatogram indicating pure phosphatidylcholine, was then dissolved in ethanol to yield a 5% (w/v) stock solution. Five-ml aliquots of this solution were sealed under nitrogen in ampoules which were stored at −20 °C. Chlorophyll *a* was purchased from Sigma and used without further purification. Buffer solutions were prepared from analytical grade reagents; they contained 0.01 M 1-morpholinopropane sulfonic acid and 0.2 M salt (KCl, NaCl or LiCl) and their pH was adjusted to 7.2.

Preparation of Vesicles

The preparation followed essentially the method of Huang (1969) with some modifications. All steps were carried out in darkness or in dim light to prevent a degradation of chlorophyll *a* by light. Chlorophyll *a* was dissolved in benzene and the ethanolic lecithin stock solution was added. The solvents were removed either by lyophilization or in a rotatory evaporator at 50 °C, and the remaining chlorophyll *a*-lecithin mixture was dispersed by shaking in 10 ml

of an aqueous buffer solution to yield a concentration of 1 to 2.5% (w/v). This dispersion was flushed with nitrogen for about 10 min and then sonicated with an MSE ultrasonic disintegrator (nominal frequency 20 kHz, input power 150 W) under nitrogen. The round bottom tube which contained the solution was thermostatized at 0 to 5 °C; it had a diameter of 4 cm and was filled by the 10 ml solution to a height of about 1 cm. The exponential probe with an end diameter of 1/8 inch was immersed 5 mm into the solution, and the sonicator was tuned to yield a 15 to 20 μm peak-to-peak amplitude of vibration for the probe. Sonication was discontinued after 150 min since longer time periods did not give higher yields of small vesicles. The suspension was then centrifuged at 5 °C for 60 min at 105,000 × *g* to separate undispersed lecithin and small metal pieces released from the tip of the soniprobe. The supernatant was applied to a Sepharose 4 B column (2.5 × 60 cm) and, after elution, the fraction containing the small vesicles was selected and concentrated in a Dow Hollow Fiber Beaker (type ultrafilter b/HFU-1). The vesicle suspension was circulated at a high rate through the fibers by means of a peristaltic pump in order to prevent the adsorption of lipids to the wall of the fibers. The beaker containing the fibers was filled with buffer solution and set under reduced pressure. With this device, we were able to reduce the 100 to 150 ml initial volume of the vesicle suspension to about 6 to 8 ml within 10 to 15 min. The concentrated suspension was dialyzed overnight against 1 liter of fresh buffer at 4 °C. Vesicle stock solutions thus prepared had lipid concentrations between 1 and 2.4% (w/v) and were stored in the dark at 10 °C.

Assay for Chlorophyll a and Lecithin

The chlorophyll *a* content of the vesicle stock solutions was determined by extraction of aliquots (0.05–0.4 ml) with 4 ml chloroform. After vigorous shaking, the two-phase system was separated by centrifugation, and the colorless water layer was removed by suction. The absorption spectrum of chlorophyll *a* in the remaining chloroform layer compared to that of a fresh sample of chlorophyll *a* dissolved in water-saturated chloroform showed that the two spectra were identical in the red region (above 550 nm) but different in the Soret region (below 500 nm) due to the presence of lecithin in one of the samples. Chlorophyll *a* concentrations could then be determined from the absorbance at 665 nm, the location of the main red peak. The extinction coefficient at this wavelength was determined as follows. Small aliquots of a standard chlorophyll *a* solution in chloroform were pipetted into cuvettes and either diluted to 3 ml with water-saturated chloroform, or dried by a stream of nitrogen gas followed by the addition of 3 ml dry diethylether. Absorbances were read at 665 and 662 nm for chloroform and ether solutions, respectively, both of which were proportional to the volume of the aliquots used (Beer-Lambert's law). From the extinction coefficient for chlorophyll *a* in ether at 662 nm listed on the data sheet which is supplied by Sigma with each lot, the concentration of the standard solution and by this in turn the extinction coefficient for chlorophyll *a* in chloroform at 665 nm could be calculated.

The lecithin content of the vesicle stock solutions was determined as inorganic phosphate liberated from the lipids and assayed according to Bartlett (1959).

Thin-Layer Chromatography

Lipids were separated on silica gel plates with chloroform/methanol/7 M ammonia (60:35:5, v/v) as solvents (Mangold, 1967) and chlorophylls were chromatographed on cellulose plates with petroleum ether (60–80 °C)/acetone/*n*-propanol (90:10:0.45, v/v) as solvents (Bolliger & König, 1967). Precoated plates (20 × 20 cm) purchased from Merck (Darmstadt, Germany) were used without any pretreatment. Chlorophylls did not interfere with the separation of lipids: chlorophyll *a* moved close to the solvent front as two slightly separated spots (hydrated and nonhydrated species) together with pheophytin *a*, and chloro-

phyllide *a* was about halfway between lecithin and the front. But a separation of chlorophylls on the cellulose plate was impossible in the presence of lipids since the latter were smeared over a large distance and displaced the chlorophylls (*see also* Strain, Sherma, Benton & Katz, 1965). Hence, the lipids were first removed by the following technique. A large sample was applied over a distance of 16 cm along one edge of a silical gel plate and the chromatogram was developed in two dimensions with chloroform/methanol/H_2O (65:25:4, v/v). The chlorophylls thus concentrated in spots close to the two solvent fronts were eluted from the silica gel onto a strip of filter paper and from this applied to a cellulose plate with the same solvents. Lipids were made visible on the chromatograms by exposure to iodine vapor or by spraying with ammonium molybdate-perchloric acid reagent for organic phosphates (Hanes & Isherwood, 1949). Chlorophylls were detected by the green color and the red fluorescence in UV-light.

Analytical Sieve Chromatography

Analytical sieve chromatography was performed on Sepharose 4 B (Pharmacia, Uppsala, Sweden). The column (1.5 × 30 cm) was equilibrated with buffer and then saturated with a lecithin dispersion in order to eliminate a retardation of vesicles due to an adsorption of lipids on the Sepharose gel (Huang, 1969; Hauser, Finer & Chapman, 1970). After re-equilibration with buffer, samples (1 to 1.5 ml) were applied to the column and eluted with a flow rate of 7.5 ml/hour. The optical density of the effluent was monitored at 276 and 366 nm or 254 nm with an Uvicord III (LKB Produkter AB, Bromma, Sweden). A calibration curve for the column, relating elution volume to Stokes' radius of the particles, was obtained by chromatographing horse heart cytochrome *c* (radius 15.5 Å, 0.5 mg/ml), human γ-globulin (52 Å, 1.5 mg/ml), bacteriophage ϕX-174 (170 and 125 Å with and without projections, 5×10^{11} particles/ml, donated by A. Walz, Dept. of Microbiology, Biozentrum). The void volume was determined with dextran blue 2000 (Pharmacia), DNA of phage λ (average MW 30 million, donated by A. Walz) and the large size liposomes present in each sonicated lecithin dispersion. The total volume, equal to the sum of void volume and internal volume of the column, was obtained as the elution volumes of $K_3Fe(CN)_6$, methylviologen and pyocyanine.

Electron-Microscopy

A drop of the vesicle suspension was spread on the surface of a solution of either uranyl acetate (1 or 2%, pH adjusted to 4) or phosphotungstic acid (2%, neutralized with potassium hydroxide). The vesicles were transferred onto a Formvar carbon-coated grid by touching the surface of the solution with the face of the grid. Alternatively, a drop of the vesicle suspension was placed on a grid, excess solution was drained off, and the air-dried film on the grid was stained with uranyl acetate or potassium phosphotungstate. The grids were examined immediately in a Siemens Elmiskop 101 electron-microscope operated at 80 kV. In additional experiments, the vesicle suspension was diluted 1:1 with a 2% solution of osmium tetroxide in isotonic buffer and kept at 0 °C for 30 min prior to negative staining. In order to estimate the vesicle size, a drop of the suspension was spread on a grid and, after drying in air, the vesicles were shadowed with platinum at an angle of 25°.

Results

Ultrasonic irradiation of lipid dispersions does not only cause the breakdown of the multilamellar lipid structures and the formation of single-shelled vesicles (Finer, Flook & Hauser, 1972*a*) but it can also lead

to chemical degradation of the lipids (oxidation, cleavage of covalent bonds). The oxidation of unsaturated hydrocarbon chains can be reduced to a negligible extent when working in a nitrogen atmosphere (Klein, 1970). The cleavage of covalent bonds, however, is a complex phenomenon depending on many different parameters (Hauser, 1971), and simple rules for the prevention of this side effect are not yet known. The only way to deal with this difficulty is therefore to analyze the lipid suspension for chemical degradation, and to look for an appropriate setting of the adjustable parameters by trial and error. Samples were taken from the lipid suspension at different times during the sonication and were lyophilized. The residues were dissolved in chloroform/methanol (1 : 1, *v*/*v*) and chromatographed on thin-layer plates as described in Materials and Methods. In order to estimate phosphate-containing substances quantitatively, equal areas of silica gel including the pertinent spots on the chromatogram were completely scratched off and subjected to a phosphate determination according to Bartlett (1959). It was thus found that, under the conditions for sonication described in Materials and Methods, the only detectable degradation product was lysolecithin whose amount did not exceed 1% of the total lipids. No degradation at all was observed for chlorophyll *a*.

When preparing vesicles from a two-component system such as chlorophyll *a* and lecithin, one has to think of possible phase separations. The intimate mixture of the two components prepared by lyophilization of an appropriate solution may separate into phases of different composition upon addition of an aqueous phase or during the sonication. The vesicles present after sonication and centrifugation did not indicate such a phenomenon, as judged by the elution pattern shown in Fig. 1. The absorbance at 254 nm which arises predominantly from light scattered by the particles and to a lesser extent from the absorption of lecithin and chlorophyll *a* is paralleled by the absorbance at 668 nm which is essentially due to chlorophyll *a* (Walz, 1976). Since light scattering depends on both the concentration and the size of the particles, the large vesicles eluted at volumes close to the void volume give rise to a larger absorbance than a similar concentration of smaller vesicles appearing at larger elution volumes. The curves in Fig. 1 then indicate that the molar ratio of chlorophyll *a* to lecithin was about the same for all fractions of vesicles eluted at different volumes. However, the molar ratio of chlorophyll *a* to lecithin determined for these vesicles was only 50 to 70% of that calculated from the initial composition, and the chlorophyll *a* content of the pellet obtained after centrifugation was correspondingly higher. Hence, a phase separation did occur either with the dispersion of the chlorophyll *a*-lecithin mixture

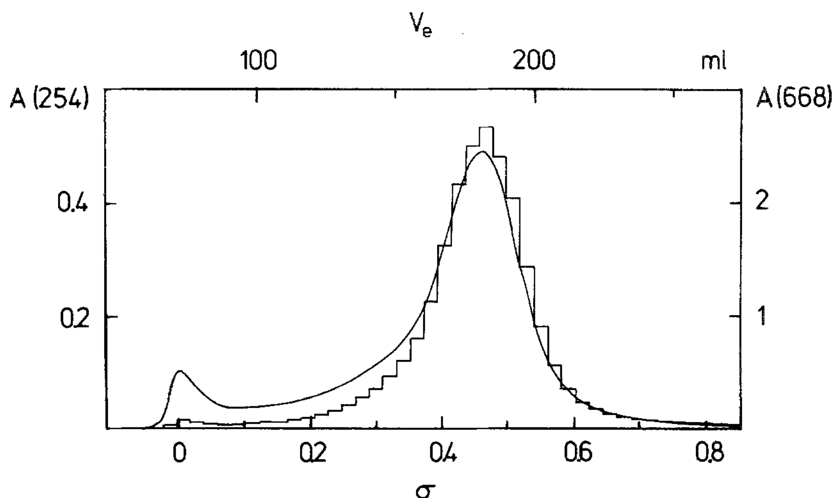

Fig. 1. Elution of chlorophyll *a* containing lecithin vesicles from a lipid-pretreated Sepharose 4B column (2.5 × 60 cm); flow rate 20 ml/hr. The curve indicates the absorbance of the effluent monitored at 254 nm in a circular flowthrough cell of 3 mm diameter (left hand scale). The histogram represents the absorbance at 668 nm measured for each fraction in a 1 cm rectangular cuvette (right hand scale). The elution volume (V_e) is given by the scale at the top; the scale on the bottom indicates the molecular sieve coefficient (σ) calculated according to Eq. (1) and with $V_0 = 71$ ml (void volume), $V_t = 310$ ml (total volume). Not shown in the Figure is a small peak eluted at volumes close to V_t; it was also present in preparations of pure lecithin vesicles and probably consisted of small micellar structures

in the aqueous phase or with the formation of the vesicles during sonication. This phase separation was very pronounced for molar ratios of the initial composition larger than 0.03 since ratios in the vesicles exceeding about 0.018 could not be obtained. This value obviously indicates a saturation limit for the solute chlorophyll *a* in the "solvent" lecithin of vesicle membranes. It should be added that a saturation value of about 0.015 was found when the vesicles were prepared from chlorophyll *a*-lecithin mixtures obtained by evaporation of the solvents (benzene and ethanol, *see* Materials and Methods) instead of lyophilization. This, by the way, was the only difference observed in the vesicles which resulted from the two techniques used to prepare the mixtures.

The vesicles eluted at volumes between about 120 and 250 ml were selected and concentrated to yield the stock solution. Our rather fast concentrating technique was completely harmless to the vesicles; a sample of the stock solution chromatographed on analytical scale as described in Materials and Methods gave an elution pattern similar to that shown in Fig. 1 except for the peak at the void volume due to the large size liposomes which was, of course, absent. According to Ackers (1967)

the molecular sieve coefficient, σ, defined as

$$\sigma = (V_e - V_0)/(V_t - V_0) \quad (1)$$

where V_e, V_0 and V_t denote the elution volume, the void volume and the total volume, respectively, is a useful parameter in order to estimate the Stokes' radii, a, of the eluted particles:

$$a = a_0 + b_0 \operatorname{erfc}^{-1} \sigma \quad (2)$$

with erfc^{-1} denoting the inverse error function complement. The parameters a_0 and b_0 are calibration constants for a given column at a constant flow rate; they were determined as described in Materials and Methods and found to be -5 Å and 206 Å, respectively. By means of the σ value at the peak of the elution curve (0.46) an average Stokes' radius of 102 ± 5 Å could be estimated for the lecithin vesicles containing chlorophyll *a*. The corresponding value for vesicles without chlorophyll *a* was similarly found to be 114 ± 7 Å in fair agreement with the data given in the literature (Huang, 1969; Hauser *et al.*, 1970; Hauser, 1971; Johnson, Bangham, Hill & Korn, 1971). In order to check whether the difference in Stokes' radii for vesicles with and without chlorophyll *a* is significant, a sample containing both types of vesicles was chromatographed. The optical density of the effluent was monitored at 276 and 366 nm, and since the ratio of the absorbances at the two wavelengths was different for vesicles with and without chlorophyll *a* but essentially independent of the size of the vesicles, the elution pattern could be resolved for the two types of vesicles. The result confirmed that lecithin vesicles with and without chlorophyll *a* represent two populations of particles whose Stokes' radii are similarly distributed but differ by about 10 Å. However, no dependence of the average radius on the chlorophyll *a* content was observed within experimental error for vesicles with molar ratios in the range of 0.002 to 0.018 (saturation point).

Vesicles suspended in *KCl or NaCl* solution and negatively stained with uranyl acetate appeared, when examined in the electron-microscope, as more or less circular structures with no further details (Fig. 2*A*). The diameters ranged between 150 and 400 Å. Negative staining with potassium phosphotungstate gave similar results, but in addition plaques of an amorphous mass were seen probably resulting from a disintegration of the vesicles in the presence of the stain. If vesicles suspended in *LiCl* solution were treated with either of the two staining solutions no intact particles could be observed, the surface of the grid was then covered with large areas of an amorphous material. The two stains used seem to be

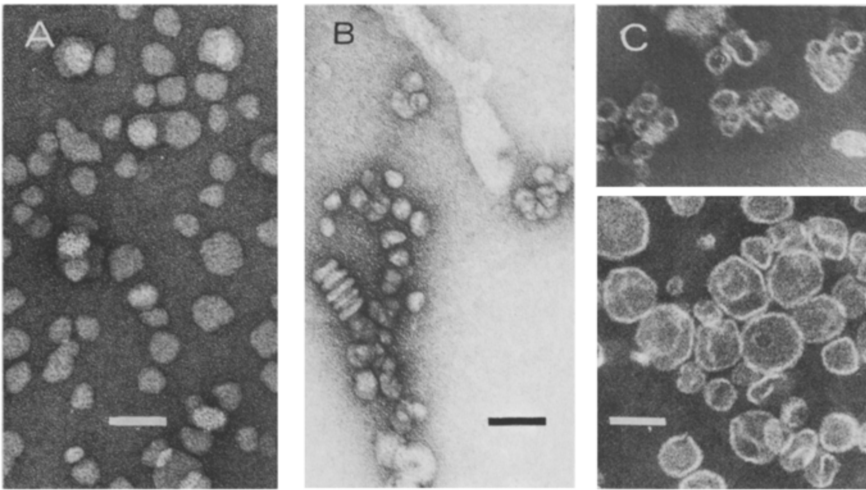

Fig. 2. Electron-micrographs of chlorophyll *a*-lecithin vesicles, suspended in 200 mM KCl solution (*A*) or 180 mM KCl, 20 mM LiCl solution (*B*), both negatively stained with uranyl acetate. (*C*): Vesicles fixed with osmium tetroxide prior to negative staining with potassium phosphotungstate: *upper part* – suspended in 180 mM KCl, 20 mM LiCl solution; *lower part* – suspended in 100 mM KCl. The interior aqueous phases of the vesicles contained 200 mM salt in all cases. The bars are 500 Å × 150,000

incompatible with Li ions and, as a consequence, the vesicles were destroyed. The effect of this incompatibility on the vesicles is demonstrated by the micrograph in Fig. 2 *B* showing vesicles suspended in KCl solution with a small amount of LiCl and negatively stained with uranyl acetate. Besides the usual circular structures, cup-like shapes and flattened particles forming stacks are found, together with small plaques of an amorphous mass.

Vesicles exposed to osmium tetroxide prior to negative staining still appeared as circular structures but were surrounded by about 40 Å thick, bright rings (*see* Fig. 2 *C*). Osmium tetroxide is soluble in apolar solvents and it is therefore likely that it permeates through the lecithin membrane. The appearance of the vesicles can then be explained by an electron dense deposit on both sides of the membrane due to an interaction of the membrane constituents with osmium tetroxide. This deposit seems to stabilize the membranous structure to a certain extent: osmium-treated vesicles were not disintegrated by potassium phosphotungstate and were less affected when negatively stained in the presence of small amounts of LiCl (compare upper part of Fig. 2 *C* with Fig. 2 *B*); they did swell (*cf.* Reeves & Dowben, 1970) but were not disrupted when subjected to an osmotic strain (*see* lower part of Fig. 2 *C*). The stabilization was not sufficient,

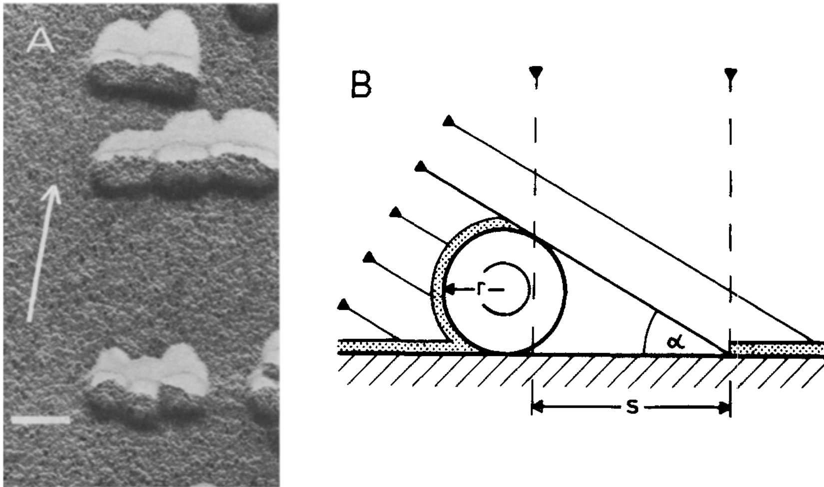

Fig. 3. (*A*): Chlorophyll *a*-lecithin vesicles shadowed with platinum in the direction indicated by the arrow at an angle of 25°. The bar is 500 Å × 150,000. (*B*): Schematic representation of the shadowing process. The cross-section shown, which is in the direction of the incident "platinum beams" (parallel solid lines) and perpendicular to the surface of the grid, cuts a vesicle through its center. The dotted areas indicate the deposits of platinum on the grid (hatched) and on the vesicle (circles). The broken lines symbolize the electron beams which project the edge of the platinum cap on the vesicle and the edge of the shadow into the plane of the micrograph

however, to allow for a thin sectioning of the vesicles; attempts in this respect using the technique of Miyamoto and Stoeckenius (1971) were not successful. In contrast to this, lecithin vesicles without chlorophyll *a* were found to be destroyed and not stabilized when exposed to osmium tetroxide. The few intact vesicles occasionally observed under this condition, however, appeared also as circular structures bounded by bright rings, similar to the chlorophyll *a* containing vesicles.

The spherical shape of the vesicles became apparent by the shadowing technique (Fig. 3*A*). The vesicles were arranged in groups on the grid probably due to the forces acting on them during evaporation of the water film. The outer radius of the vesicles, r, can be estimated by means of the distance, s, between the edge of the cap formed by the deposited platinum on the vesicle and the corresponding edge of the shadow (*cf.* Fig. 3*B*):

$$r = s \operatorname{tg} \alpha / (1 + \cos \alpha) \qquad (3)$$

where α denotes the angle between the "platinum beams" and the surface of the grid. Radii in the range of 80 to 120 Å were thus found in agreement with the average value estimated by analytical sieve chromatography.

Finally, the stability of the vesicles during storage at 10 °C in the dark was checked. Samples taken from the stock solutions at different times within two or three weeks were subjected to analytical sieve chromatography and chemical analysis. They showed the same elution patterns as immediately after preparation except for the peak eluted at the void volume which indicated a slow recurrence of large size liposomes to a small extent. The lysolecithin content occasionally increased but never exceeded 3 % after three weeks, and no degradation products of chlorophyll *a* could be detected. The only serious injury to the vesicles was due to a digestion of the lipids by bacteria which were brought into the stock solution when taking samples. This reduced the concentration of vesicles but did not affect the molar ratio of chlorophyll *a* to lecithin in the remaining vesicles. Bacterial degradation could be prevented by filtrations of the stock solution through membrane filters with pores of 0.2 µm nominal size at time intervals depending on the frequency at which samples were taken.

Discussion

Vesicles prepared from pure lipids (e.g. Huang, 1969; Saha, Papahadjopoulos & Wenner, 1970; Hauser, 1971; Sheetz & Chan, 1972) or lipid mixtures (e.g. Johnson *et al.*, 1971; Miyamoto & Stoeckenius, 1971) by sonication of aqueous lipid dispersions have been extensively investigated. The data obtained by different physico-chemical methods and by electron-microscopy are all consistent with the scheme which represents the vesicles as small cavities filled with an aqueous solution and bounded by spherical single bilayer membranes ("single-shelled vesicles"). The results which we obtained with the chlorophyll *a* containing vesicles are in line with the investigations mentioned above and thus indicate that it is possible to incorporate nonlipid molecules (such as chlorophyll *a*) into the membrane of single-shelled vesicles. Moreover, we have confirmed the findings of Hauser (1971) that chemical degradation of lipids but also of foreign molecules present in the lipid phase can be kept at a minimum when sonications are performed under carefully controlled conditions.

The Stokes' radii of vesicles prepared from different lipids were all found to be of the same order of magnitude (90 to 200 Å). Nevertheless, a slight dependence of the average radius on the type of lipids and on the lipid composition can be observed (Israelachvili & Mitchell, 1975); besides this the input of ultrasonic energy seems to have some influence (*cf.* note b to Table II in the paper of Hauser, Oldani & Phillips, 1973). The

decrease of the average radius by about 10 Å upon incorporation of chlorophyll a into lecithin vesicles prepared under identical conditions of sonication is therefore not surprising and probably indicates a certain interaction of chlorophyll a with lecithin. An influence of the interaction between lipid and nonlipid membrane components on the size of the vesicles has been found by Hauser *et al.* (1970) in the case of the polypeptide alamethicin in lecithin vesicles.

The behavior of vesicles with respect to negative staining allows some conclusions concerning stability and membrane properties of different vesicle preparations. The chlorophyll a containing vesicles may thus be regarded to be as stable as pure lecithin vesicles (compare our micrograph with Fig. 6*A* in the paper of Huang, 1969). In contrast to this, vesicles prepared from Asolectin are totally unstable (Miyamoto & Stoeckenius, 1971), and lecithin vesicles containing 4% phosphatidic acid require the addition of albumin in order not to collapse in the presence of the stain (Johnson *et al.*, 1971). On the other hand, the presence of phosphatidic acid in the membrane allows the stain to penetrate into the aqueous interior of the vesicles. Lecithin membranes with or without chlorophyll a are impermeable to the stain, and only the addition of an ionophore such as alamethicin brings about a staining of the cavities (Hauser & Irons, 1972).

It is generally accepted that osmium-fixation of biological membranes is due to a chemical reaction of osmium tetroxide with the double bonds in the fatty acid chains of the lipids (Riemersma, 1968; Schiechl, 1974). The double bonds are oxidized to vicinal diol configurations and the primary reduction product of osmium tetroxide, i.e. osmic acid, is assumed to form ester bonds with the diols thus cross-linking adjacent fatty acid chains. Egg lecithin contains up to 50 mole % unsaturated fatty acids (Singleton *et al.*, 1965) and hence the lecithin in vesicle membranes reacted with osmium tetroxide as judged by the darkening of the samples generally believed to indicate an osmium-fixation. Further evidence came from the effect of Li ions on the reaction products. We have found that a black precipitate is formed when lecithin dissolved in ethanol reacts with osmium tetroxide and, as a consequence, vesicles with and without chlorophyll a suspended in 200 mM LiCl solutions flocculated upon exposure to the fixation reagent. Nevertheless, the membranes of lecithin vesicles were not stabilized but became more fragile when treated with osmium tetroxide. The cross-linking was obviously not very effective despite the large percentage of unsaturated fatty acids present in egg lecithin. On the other hand, the formation of diols is equivalent to a conversion of unsaturated

hydrocarbon chains into saturated chains with a concomitant shift of the transition point (i.e. the phase transition from the crystalline to the liquid-crystalline state of the membrane constituents) to a higher temperature (Oldfield & Chapman, 1972). This would explain the decreased stability of the osmium-treated lecithin membranes. However, as little as 1 to 2 chlorophyll *a* molecules per 100 lecithins bring about a stabilization of the membrane. Experiments with ethanolic chlorophyll *a* solutions similar to those mentioned above with lecithin indicated that the interaction of osmium tetroxide with the porphyrin ring probably does not occur via a redox reaction as observed for the double bonds in the fatty acid chains but is rather due to a physico-chemical process, e.g. a complexation or hydrogen bonding as recently proposed by Litman and Barrnett (1972) for proteins. This could be the nucleus for a similar binding of osmium tetroxide to the polar head groups of the lipids which then proceeds over the whole surface of the membrane (*cf.* Riemersma, 1968). Asolectin, a complex mixture of phosphatides and other components, obviously contains many constituents which interact with osmium tetroxide similarly to chlorophyll *a*. Thus, the stability of the membranes of vesicles prepared from this lipid is increased so that even a dehydrating of the vesicles and an embedding in epoxide was possible (Miyamoto & Stoeckenius, 1971).

The upper limit of the molar ratio chlorophyll *a* to lecithin (1:55) determined for vesicle membranes is rather low compared to that estimated in black lipid membranes (2:10; Cherry *et al.*, 1971). It has to be kept in mind, however, that the black film which contains decane certainly has a different supramolecular structure to that in the vesicle membrane which has a strong curvature; the two systems should therefore not be compared. Tomkiewicz and Corker (1975) indicate molar ratios in chlorophyll *a*-lecithin vesicles as high as 1:10. These ratios were attained by a special technique of preparation, but the vesicle suspension was not fractionated by sieve chromatography or the actual chlorophyll *a* content estimated (*personal communication*).

In conclusion, the chlorophyll *a* containing vesicles resemble the pure lecithin vesicles; the physical state of the lipids is likely to be similar in both preparations although the pigment-lipid interaction reduces the average Stokes' radius by about 10 Å. The vesicles are homogeneous and fairly stable when stored at 10 °C. There was no detectable degradation of chlorophyll *a* since the vesicles were kept in the dark and the presence of water, which could lead to a pheophytinization or a cleavage of the ester bond between the porphyrin ring and the phytyl chain was apparently harmless. This vesicle preparation is therefore well suited to investigate

the properties of the system chlorophyll *a* in lecithin membranes (*see* Walz, 1976).

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