

Addition of Lipid to the Photosynthetic Membrane: Effects on Membrane Structure and Energy Transfer

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ABSTRACT We have carried out a series of experiments in which the lipid composition of the photosynthetic membrane has been altered by the addition of lipid from a defined source under experimental conditions. Liposomes prepared by sonication are mixed with purified photosynthetic membranes obtained from spinach chloroplasts and are taken through cycles of freezing and thawing. Several lines of evidence, including gel electrophoresis and freeze-fracture electron microscopy, indicate that an actual addition of lipid has taken place. Structural analysis by freeze-fracture shows that intramembrane particles are widely separated after the addition of large amounts of lipid, with one exception: large hexagonal lattices of particles appear in some regions of the membrane. These lattices are identical in appearance with lattices formed from a single purified component of the membrane known as chlorophyll-protein complex II. The suggestion that the presence of such lattices in lipid-enriched membranes reflects a profound rearrangement of photosynthetic structures has been confirmed by analysis of the fluorescence emission spectra of natural and lipid-enriched membranes. Specifically, lipid addition in each of the cases we have studied results in the apparent detachment of chlorophyll-protein complex II from photosynthetic reaction centers. It is concluded that specific arrangements of components in the photosynthetic membrane, necessary for the normal functioning of the membrane in the light reaction of photosynthesis, can be regulated to a large extent by the lipid content of the membrane.

The photosynthetic membrane provides an ideal system for the study of the relationship between membrane structure and function. It is characterized by light-driven reactions which are easily assayed, as well as by a defined and highly ordered ultrastructure. The membranous sacs, or thylakoids, of the chloroplasts of higher plants have been extensively investigated by a number of workers (3, 4, 19). These membranes contain the machinery necessary for the light reaction of photosynthesis, involving the transport of electrons from water to appropriate acceptor molecules via a chain of electron carriers. The fundamental components of the system are two reaction centers, termed photosystem I and photosystem II. Associated with each photosystem are chlorophyll, carotenoids, and other accessory pigments.

Based on freeze-fracture electron microscopy and the use of mutants or deficient plants, a rough model of the organization of the photosynthetic membrane has been constructed. For example, tobacco plants deficient in photosystem II lack the large (140–180 Å) particles normally observed on the so-called

E face fracture face (9). It was therefore suggested that photosystem II might be related to this structure (9). Several investigators also believe that the photosystem II reaction center core is surrounded by a discrete number of units of a light-harvesting pigment-protein complex (LHC) which is responsible for absorbing much of the light energy eventually directed to the photosystem II reaction center. Both Miller et al. (10) and Armond et al. (1) have found that membranes lacking some or all of the normal complement of LHC show reductions in the sizes of particles on the E face fracture face normally associated with photosystem II.

These and other studies have led to the development of models for the organization of the photosynthetic reaction center which propose that a small core of reaction center is surrounded by several light-harvesting chlorophyll-protein complexes to form a functional photosynthetic unit (see for example references 3, 20). Up until this point, studies of the organization and biochemical composition of the photosynthetic membrane have dealt principally with protein and lipo-

protein components of the membrane. This has been the pattern of research in the field partly because of a greater interest in protein and pigment-protein components, and partly because of the availability of several methods, genetic and physical, to alter the protein composition of the membrane. It is, of course, reasonable to expect that the lipid components of the photosynthetic membrane may play a central role in thylakoid organization, but manipulating the lipid composition of the membrane in such a way that this role could be evaluated has always presented an experimental problem. Recently, however, Schneider et al. (17) have reported on a new method to add exogenous lipid to the inner mitochondrial membrane. Inspired by the successes of these workers, we have modified their techniques for use with the photosynthetic membrane.

We report here on methods to alter the lipid composition of isolated thylakoid membranes from spinach chloroplasts through the addition of exogenous lipid under experimental conditions. We have also analyzed the membranes which are produced by this procedure as to their biochemical, structural, and biophysical characteristics in comparison to the native membrane. Our experiments show that these modified membranes differ from native ones in a number of fundamental ways, and these observations suggest that specific lipids may play crucial roles in determining the structural organization of this and other biological membranes.

MATERIALS AND METHODS

Isolation of Thylakoid Membranes

After removing the central vein of each leaf, ~100 g of commercially obtained spinach leaves were washed in tap water and placed in a Waring Blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) with 150 ml of isolation buffer (0.4 M sorbitol, 0.1 M Tris base, pH 7.8). The leaves were homogenized for 20 s at high speed and the homogenate filtered through a double layer of cheesecloth. The homogenization and all isolation steps were performed at 4°C. The filtrate was centrifuged for 3 min at 300 g and the pellet discarded. The supernate was centrifuged for 10 min at 3,000 g in the SS-34 rotor of a Sorvall RC-5B centrifuge (DuPont Instruments, Wilmington, Del.). The pellet from this spin was resuspended in isolation buffer and centrifuged again for 10 min at 3,000 g. The pellet from this last spin was washed twice in 10 mM HEPES buffer, pH 6.5, and resuspended in this buffer to a final concentration of 0.5 mg chlorophyll/ml. The low ionic strength of this buffer causes thylakoid membrane unstacking, exposing a maximal amount of membrane surface area. The lower pH of this buffer increases the extent of lipid addition compared to buffers at pH 7.0 and above.

Chlorophyll Determination

Chlorophyll concentrations were determined from acetone extracts analyzed in a Cary 219 spectrophotometer using the equations of Arnon (2).

Phospholipid Determination

Phospholipid was determined by the methods of Sunderman and Sunderman (21).

Protein Determination

Protein concentrations were determined using the Buffalo Black method (16).

Preparation of Liposomes

250 mg of L-phosphatidyl choline, type II-S, obtained from Sigma Chemical Co. (St. Louis, Mo.) were placed in a test tube with 5 ml of 10 mM HEPES buffer, pH 6.5, containing 68 µg of butylated hydroxytoluene (BHT), flushed with nitrogen, and sealed with parafilm. The contents were vortexed to disperse the lipid and sonicated for 15 min in 80 kc cycle bath-type sonicator (Laboratory Supplies Co., Hicksville, N. Y.) adjusted for maximum agitation. The liposomes were used immediately after preparation.

When highly purified phospholipid was used, this procedure was modified

slightly. In this case, the contents of the vial in which the lipid had been shipped were dried under nitrogen to remove the solvent. The dried phospholipid (50 mg) was placed in a tube with 1 ml of 10 mM HEPES buffer, pH 6.5, containing BHT as described above, vortexed, and sonicated as described. The concentration of lipid used for liposome preparation was 50 mg/ml unless otherwise noted in the text. Purified phosphatidyl choline and phosphatidyl glycerol were obtained from Supelco, Inc., Bellefonte, Pa.

Freeze-Thaw Addition of Lipid to the Thylakoid Membrane

1 ml of the liposome solution and 1 ml of purified thylakoid membranes were gently mixed, giving a lipid/chlorophyll ratio of 100/1. The tube was frozen in a dry ice-acetone bath (-73.5°C), and allowed to thaw for 20 min at room temperature. For control experiments, identical mixtures were prepared and handled in an identical fashion, except for the omission of the freeze-thaw step. The mixture was layered on a discontinuous sucrose gradient (20%, 27%, 34%, and 50% sucrose in 10 mM HEPES, pH 6.5) and centrifuged for 4 h at 4°C in 200,000 g in a Beckman SW-40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

SDS PAGE

Gel electrophoresis was performed on a slab gel (a gradient of acrylamide from 7.5–15%) according to the method of Laemmli (7). The sample solution contained 0.05 M Tris HCl, pH 6.8, 5% SDS, 5% B-mercaptoethanol, and 10% glycerol. The completed gels were stained with Coomassie Blue.

Freeze-Fracture Electron Microscopy

Membrane samples were infiltrated with glycerol to a concentration of 25% (vol/vol) over the course of an hour, frozen in liquid Freon 22, and transferred to liquid nitrogen for storage. Replicas were prepared at -110°C with minimal etching according to the method of Moor and Mühlethaler (11) on a Balzers freeze-etching device. Replicas were cleaned in commercial laundry bleach and were examined in a Philips 300 electron microscope.

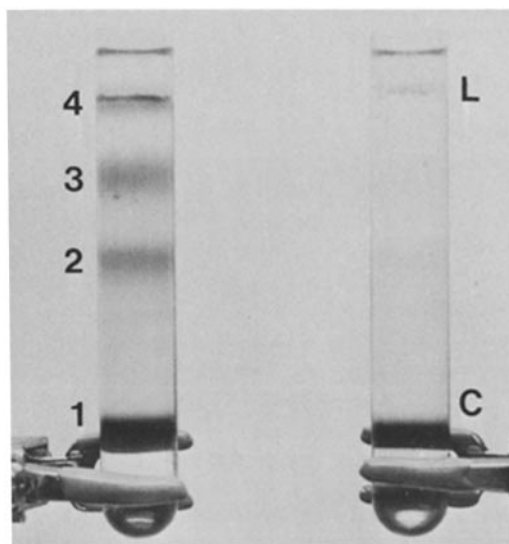


FIGURE 1 Separation of a mixture of liposomes and isolated photosynthetic membranes after freeze-thawing. Samples containing lipid and photosynthetic membranes were mixed and freeze-thawed as described in the text. Separation on a discontinuous sucrose gradient yields a band of pigmented material at each interface, as shown in the left-hand tube. The bands are labeled 1 (most dense) through 4 (least dense). The right-hand tube contains an identical mixture and has been treated in an identical manner, except that the freeze-thaw step has been omitted. The photosynthetic membranes, containing the green pigment, chlorophyll, band at the lowest interface (C), while the less dense liposomes band (L) at the uppermost interface. Results identical to those observed in the right-hand tube are obtained after separate freeze-thawing of lipid and membranes.

Assays of Photosynthetic Activity

Photosystem I activity was measured by methyl viologen-mediated oxygen consumption. The reaction was monitored on a Yellow Springs Instrument Co. (Yellow Springs, Ohio) model 53 Biological Oxygen Monitor, fitted with a #5331 oxygen probe and a #5301 standard bath assembly. In a darkened room, chloroplasts containing 10–30 μg of chlorophyll were added to a reaction mixture containing 1 mM methyl viologen, 0.5 mM dichlorophenol indophenol, 1 mM sodium ascorbate, and a sufficient amount of buffer (1 mM sodium azide, 5 mM NH_4Cl , 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mM NaCl, 1 mM Na Tricine, pH 7.0, and 0.03 M of 3-[3,4-dichlorophenyl]-1,1-dimethylurea) to give a final volume of 1.5 ml. The chamber was illuminated with a Dolan-Jenner Fiber-Lite (Dolan-Jenner Industries, Inc., Woburn, Mass.). The temperature of the circulating water bath was maintained at 24°C.

Photosystem II activity was assayed in the Cary 219 spectrophotometer (Varian Associates, Inc., Palo Alto, Calif.) at a wavelength of 620 nm. With the room darkened, the assay was performed with external illumination; the shutter switches were taped down, and 620-nm interference filters were placed over the photomultiplier ports. The reaction cuvette contained 10 mM NaCl, 50 mM NaPO_4 buffer, pH 6.8, 10–30 μg of chlorophyll, and 45 μM dichlorophenol indophenol, in a total volume of 3 ml. For saturation kinetics measurements, a series of neutral density filters kindly provided by Dr. Paul Brown of the Harvard Biological Laboratories was used to modify the intensity of the illuminating actinic beam.

Low Temperature Fluorescence

Samples for fluorescence contained 10 mM NaCl, 10 mM Trizma base, pH 7.5, 50 mM sorbitol, and 20 μl of a chloroplast suspension (containing ~10 μg of chlorophyll) in a total of 1 ml. The sample was placed in a micropipette and inserted into a transparent Dewar flask filled with liquid nitrogen. The Dewar flask was placed in a Spex Fluorolog spectrofluorimeter set at an excitation wavelength of 440 nm.

Particle Size Measurements

Particle sizes were measured on micrographs enlarged to 200,000 diameters and viewed through a $\times 7$ objective lens fitted with a micrometer scale. Between 150 and 250 particles were measured in continuous regions of the membrane on five representative micrographs.

RESULTS

Our protocol for the addition of exogenous lipid to the photosynthetic membrane involves mixing liposomes prepared by bath sonication with isolated thylakoid membranes and freezing this mixture in a dry ice-acetone bath. After thawing at room temperature, the mixture is layered on a discontinuous sucrose gradient and centrifuged for 4 h at 200,000 g. The results of this treatment are shown in Fig. 1. In the tube at the right, thylakoid membranes band at the lowest interface of the gradient, while the liposomes band at the very top of the tube. This result is not altered when liposomes and thylakoids are separately freeze-thawed. After freeze-thawing, as in the tube at the left, material containing the green pigment chlorophyll can be seen at each step of the gradient. Similar results were obtained with Sigma II-S phosphatidyl choline (10–20% pure),

TABLE I
Biochemical Analysis of Fractions Collected from the Four Gradient Steps Shown in Fig. 1

Band	Chlorophyll a/b	Chlorophyll	Protein	Phospholipid	Protein/chlorophyll	Phospholipid/protein
	mg	mg	mg	mg		
4	2.58	0.26	2.47	28.5	9.50	11.5
3	2.64	0.13	1.58	4.50	12.15	2.84
2	2.70	0.05	0.40	0.50	8.00	1.25
1	2.43	0.04	0.40	0.19	10.00	0.475

highly purified phosphatidyl choline (98% pure), and highly purified phosphatidyl glycerol (98% pure). The presence of green pigment at the upper levels of the gradient could indicate that the thylakoid membrane has been reduced in density by the addition of lipid, possibly through the actual fusion of the relatively buoyant liposomes with the more dense native membranes.

Control experiments in which the liposomes and isolated thylakoid membranes are freeze-thawed separately several times, thoroughly mixed, and then layered on the gradient, do not produce these changes in density, indicating that the freeze-

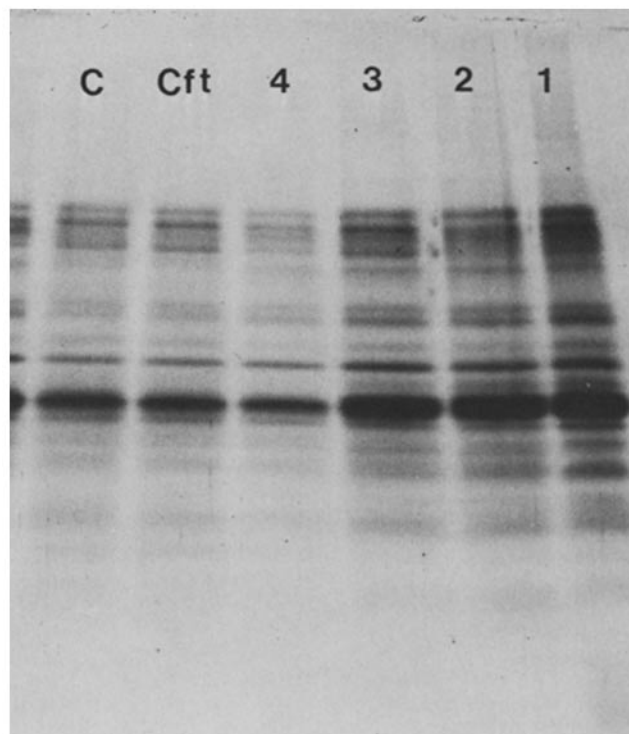


FIGURE 2 Polypeptides of the photosynthetic membrane separated by SDS PAGE. Samples include untreated membranes from the chloroplast (C), membranes subjected to freeze-thaw treatments in the absence of additional lipid (Cft), and each of the four samples taken from the separation in a gradient system similar to that shown in Fig. 1. No polypeptides seem to have been differentially extracted by the freeze-thaw procedure.

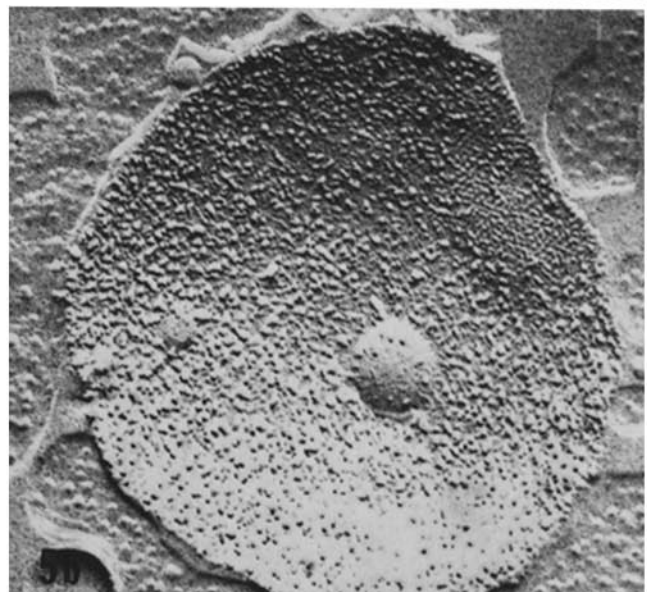
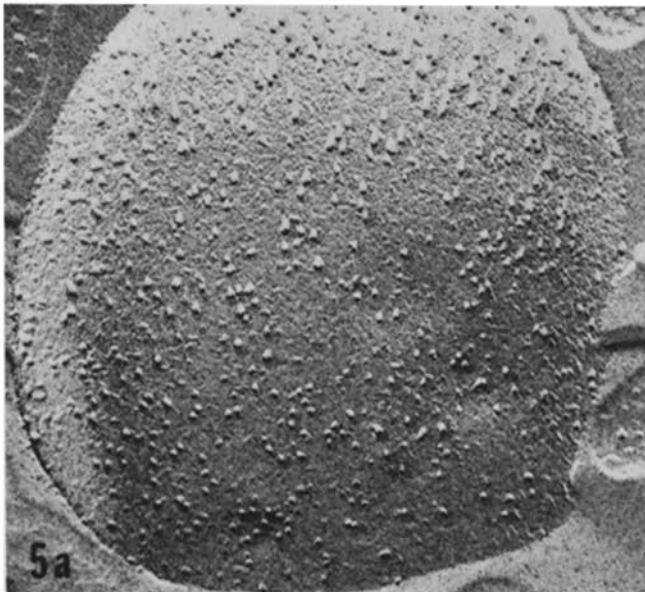
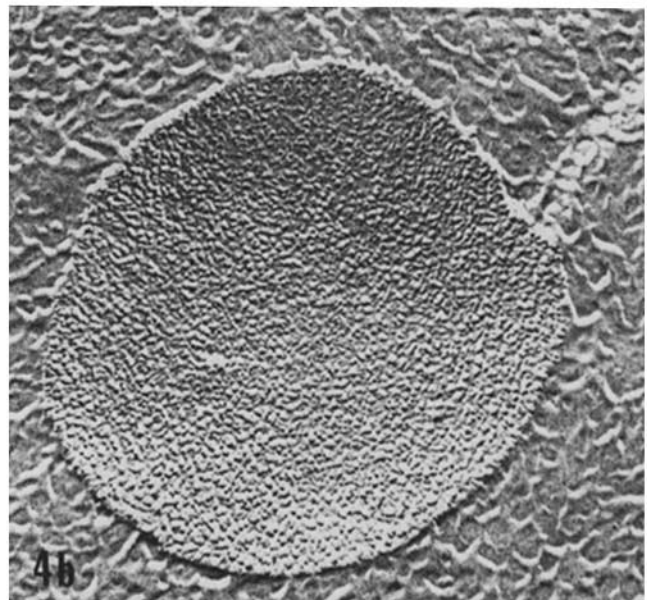
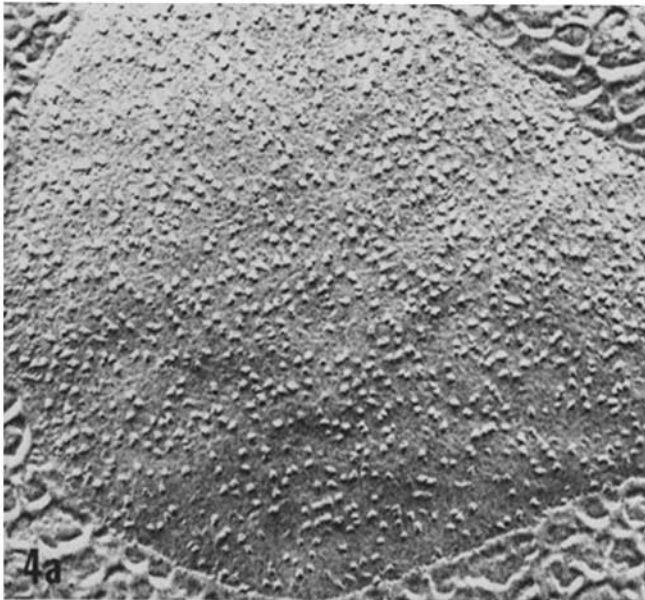
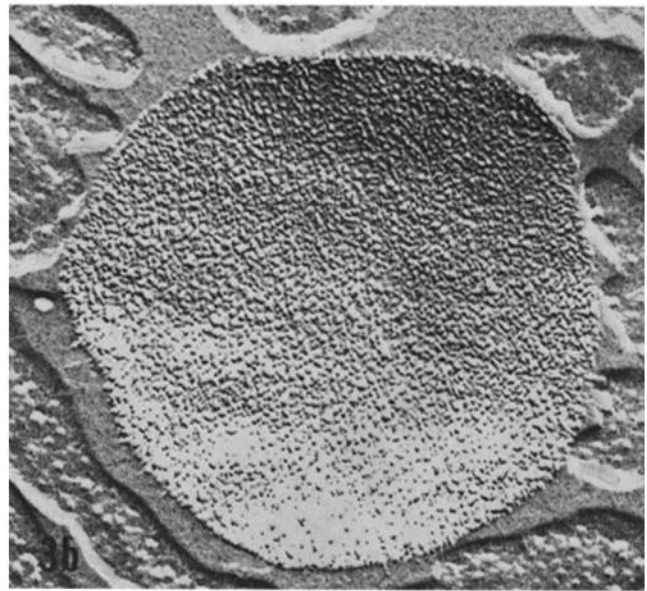
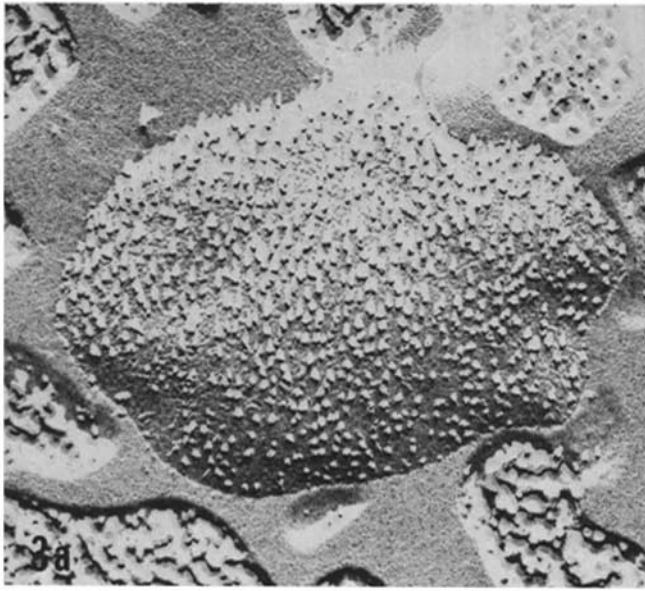
TABLE II
Effect of the Freeze-Thaw Procedure on Membrane Photosynthetic Activities

Sample	Chlorophyll a/b	Photosystem I*	Photosystem II§
Untreated thylakoid membranes	2.62	20.21	9.93
Thylakoid membranes, freeze-thaw treatment	2.60	14.08	7.64
Sucrose gradient			
Band 1	2.43	10.01	6.44
Band 2	2.70	9.74	7.83
Band 3	2.64	‡	4.77
Band 4	2.58	‡	3.07

* Nanomoles of O_2 consumed/ μg chlorophyll/min.

‡ Measurements on these bands were not possible because the large amounts of lipid present caused light-dependent O_2 consumption even in the absence of chlorophyll.

§ 10^{-10} mol of dichlorophenol indophenol/ μg of chlorophyll/min.



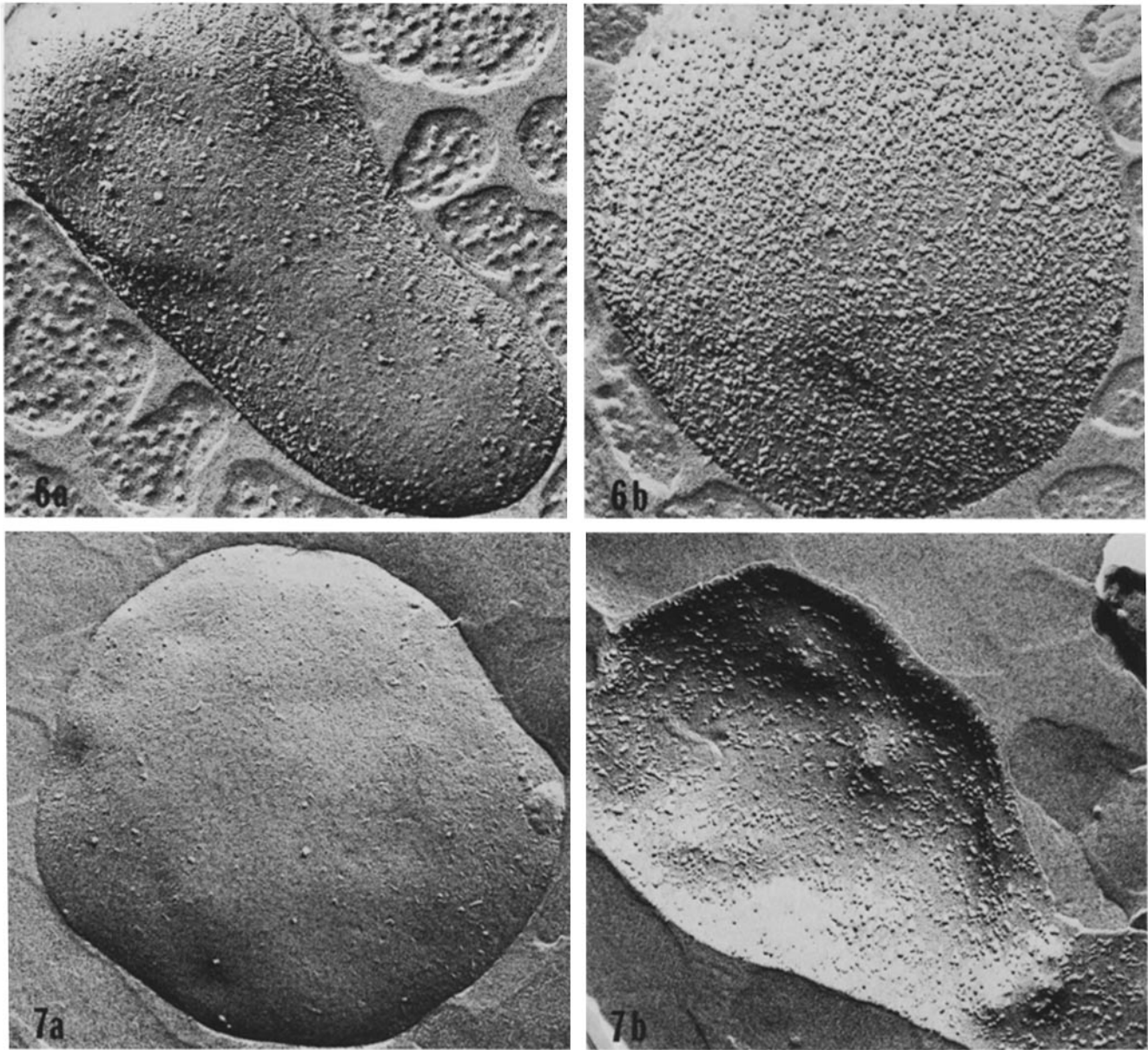


FIGURE 6 (a) E face from band 3 of the gradient system. Increased separation between E face particles is now very clear. $\times 75,000$. (b) P face from band 3. $\times 75,000$.

FIGURE 7 (a) Apparent E face from band 4 of the gradient. Separation between particles has dramatically increased. $\times 75,000$. (b) Apparent P face from band 4. Even in the densely packed P face, particle separation is pronounced. $\times 75,000$.

thaw step may have induced the association of lipid with the thylakoid membranes. Incubation of lipid and thylakoid membranes at room temperature for periods as long as 4 h also produces no change in the density of the pigmented membranes.

An analysis of the protein, chlorophyll, and phospholipid

composition of each of the bands derived from the gradient after the freeze-thaw step is presented in Table I. Although substantial variation is observed in protein/phospholipid ratios, the ratio of protein to chlorophyll, and chlorophyll *a*/chlorophyll *b* is consistent in each step of the gradient. If the decrease in density of the pigmented material were the result

FIGURE 3 (a) Fracture face E of an isolated photosynthetic membrane freeze-thawed in the absence of lipid and separated on a sucrose gradient as was band C in Fig. 1. Large ($\sim 160 \text{ \AA}$) particles are evident. $\times 75,000$. (b) P fracture face from the same preparation as a. $\times 75,000$.

FIGURE 4 (a) E fracture face from band 1 of a discontinuous gradient identical to the experimental tube in Fig. 1. $\times 75,000$. (b) P fracture face from the same sample. $\times 75,000$.

FIGURE 5 (a) E fracture face from band 2 of the gradient system. Note the increasing separation of the E face particles. $\times 75,000$. (b) P face from band 2. Note the increasing particle separation. $\times 75,000$.

of differential protein extraction, one might have expected a change in protein to chlorophyll ratios at various levels in the gradient. To further investigate the possibility of differential protein extraction, we analyzed the polypeptide composition of each gradient fraction by gel electrophoresis. The results are shown in Fig. 2. Polypeptide composition seems to be identical in each of the four bands of the sucrose gradient, in isolated unmodified membranes, and in membranes freeze-thawed in the absence of lipid. This would seem to indicate that none of the characteristic membrane polypeptides are lost during the fusion process, and that the appearance of the low density green bands in the gradient is not due merely to the presence of chlorophyll extracted from the membranes.

If these bands do indeed represent the composition of thylakoid membranes which have been reduced in density by the addition of lipid during the freeze-thaw process, it is important to establish whether or not the procedure outlined here has deleterious effects on the normal biological activity of the membrane. To answer this question, we monitored several photosynthetic parameters in our membrane samples. As can be seen in Table II, although a reduction in photosynthetic activity is observed, a substantial level of activity is preserved, even in membranes recovered from the sucrose gradient.

Isolated photosynthetic membranes display characteristic fracture faces when studied by freeze-fracture electron microscopy. These faces have been well characterized in this and

other laboratories (18, 19). More relevant to our study is that the actual addition of lipid, were it taking place, would be immediately evident with the use of this technique. If lipid were being directly incorporated into the plane of the membrane (as opposed to being merely adsorbed at the surface of the membrane), the distances between the intramembrane particles visible in freeze fracture should begin to increase. Figs. 3-7 show representative samples of membranes from the four bands of our gradient system, as well as control unmodified membranes. As can be seen in these micrographs, membrane samples taken from less dense regions of the gradient show greater distances between intramembrane particles. In addition, the results seem to show that the lipid we have added is truly incorporated into the existing membrane, mixing freely with the native lipid of the thylakoid. We infer this latter point from the absence of smooth, particle-free regions which one might expect to see were the mobility of newly added lipid molecules restricted to certain regions. We have also been unable to find any evidence of liposomes adhering to the membrane surface following the freeze-thaw procedure. Although we have chosen micrographs of membranes which are similar in shape from each of the gradient steps, it should be noted that a range of sizes is found among the membranes in each fraction. A comparison of replicas prepared from membranes frozen and thawed in the absence of lipid indicated that the fusion process does not induce membrane vesiculation.

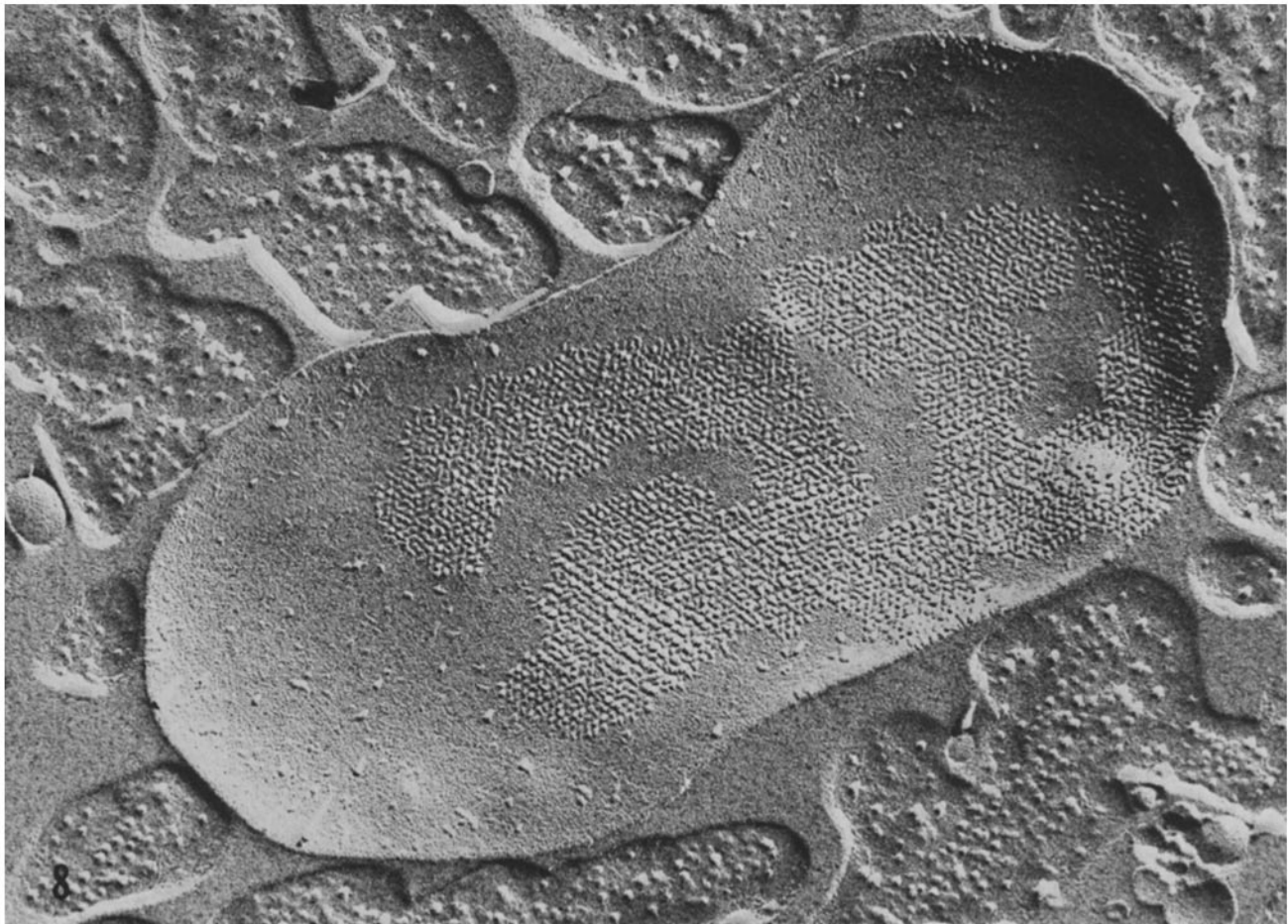


FIGURE 8 Freeze-fractured lipid-enriched membrane from the upper (least dense) band of a sucrose gradient, where extensive lipid addition has taken place. In addition to the phenomenon of increased intraparticle spacing, new structures have appeared in the membrane fracture face. These hexagonal lattices of small particles with spacings between the particle rows of 120 Å occur after addition of crude phosphatidyl choline and purified phosphatidyl glycerol (phosphatidyl choline is shown). $\times 90,000$.

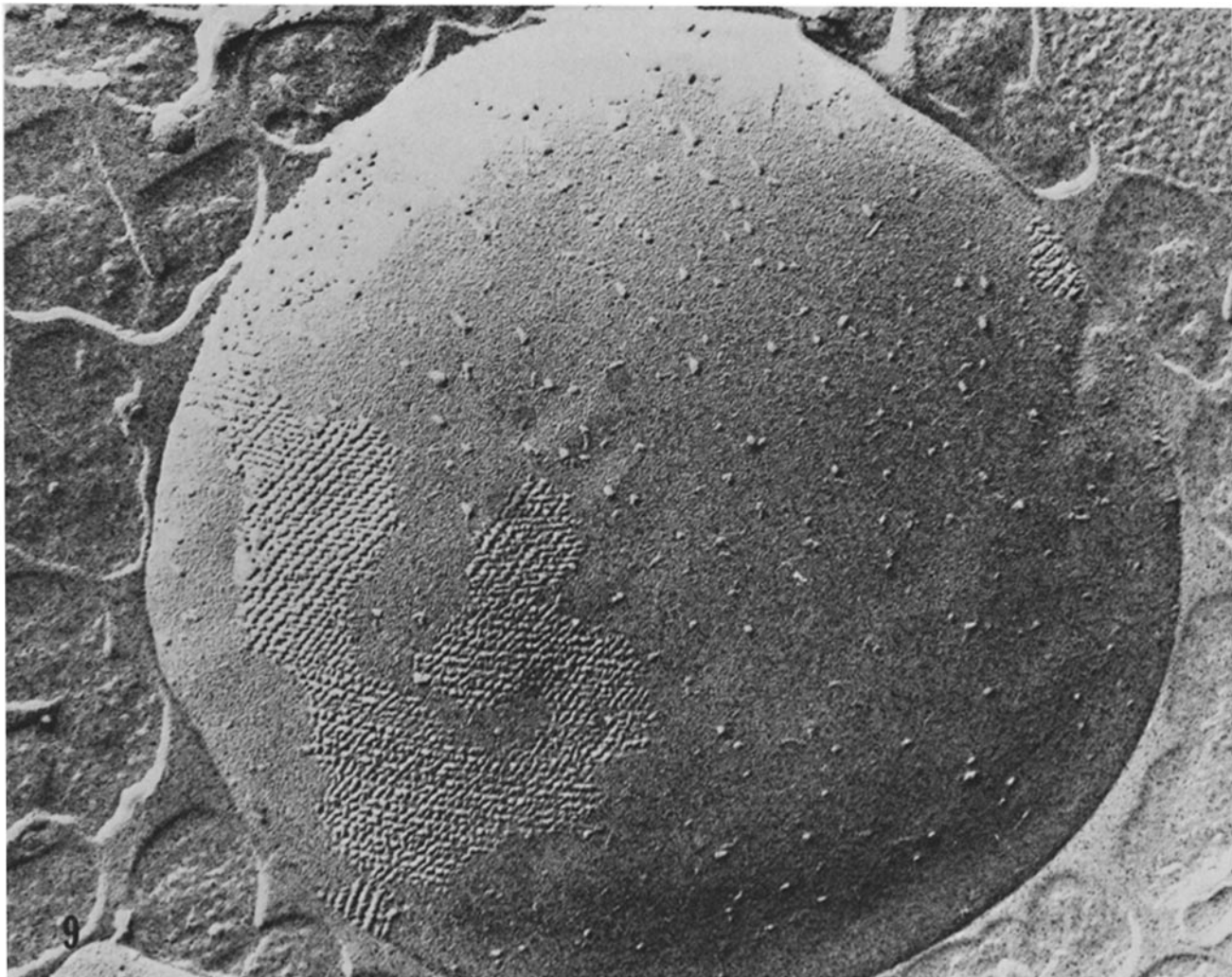


FIGURE 9 Freeze-fractured lipid-enriched membrane similar to Fig. 8, illustrating the localized nature of these lattices which appear after extensive lipid addition. Despite the decrease in particle spacing brought about by the addition of lipid during freeze-thaw, these lattices appear in certain regions of the lipid-enriched membrane. Such lattices have never been observed in membranes to which lipid has not been added, regardless of the number of times such membranes are subjected to the freeze-thaw protocol. $\times 90,000$.

Significantly, this decrease in lateral surface density of the intramembrane particles observed in freeze-fracture electron microscopy was found regardless of the type of lipid used for the experiment. This lends further support to the idea that it is the addition of lipid which has occurred during these experiments, rather than an artifact resulting from a minor contaminant in one of the lipid preparations.

Results very similar to these have been reported earlier for the mitochondrial inner membrane using the technique of Schneider et al. (17). With this study in mind, it was our expectation that the addition of lipid would result in a simple spreading out of the particles observed in freeze fracture. However, an unusual phenomenon was observed in such lipid-enriched membranes. As shown in Figs. 8 and 9, lipid-enriched membranes frequently showed the formation of regular hexagonal lattices with an interparticle spacing of $\sim 120 \text{ \AA}$. Such lattices were observed after the addition of Sigma II-S phosphatidyl choline and phosphatidyl glycerol, although they were not observed after the addition of purified phosphatidyl choline. That these lattices do not represent an artifact of the freeze-thaw protocol is shown by the fact that thylakoid mem-

branes which have been repeatedly freeze-thawed in the absence of lipid never show such structures. It is also unlikely that the formation of these lattices is related to the presence of impurities in Sigma II-S phosphatidyl choline, because identical lattices are formed after the addition of highly purified phosphatidyl choline from Supelco Laboratories (see Materials and Methods).

These patches of hexagonally arranged particles immediately suggested that a profound change in the organization of the photosynthetic membrane was taking place following the addition of lipid. Specifically, these regions of hexagonal order seemed very similar to structures formed when one of the components of the photosynthetic membrane, the so-called chlorophyll protein complex II, was purified and reconstituted into lipid vesicles (8, 20). Preparations of this pigment-protein complex made in our own laboratory (Fig. 10) made this morphological comparison especially compelling.

This pigment-protein complex is perhaps the major light-harvesting molecule in the photosynthetic apparatus of higher plants. Most investigators agree that this complex is normally associated with the photosystem II reaction complex, with from

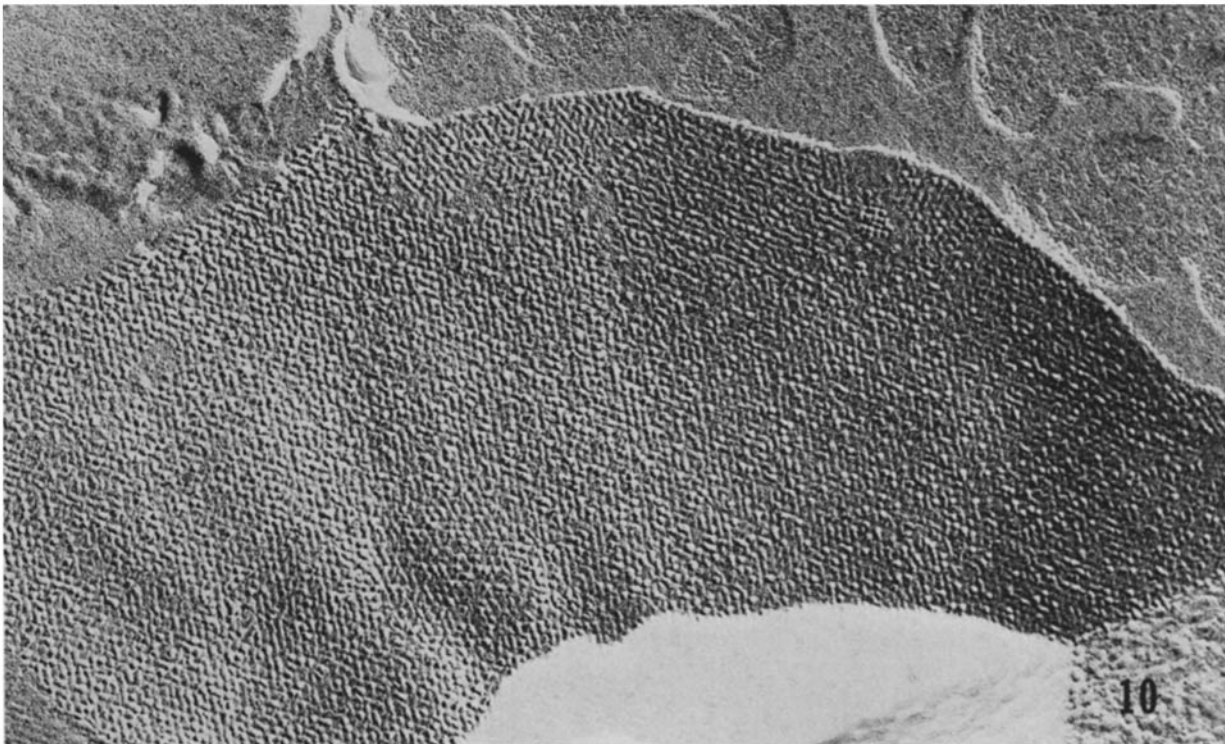


FIGURE 10 Freeze-fracture image prepared from highly purified chlorophyll-protein complex II. The spacing of particles in the regular hexagonal lattice shown in this micrograph is strikingly similar to that in the lattices observed in lipid-enriched membranes (see Figs. 8 and 9). The pigment-protein sample was kindly provided by Carol Brenner (Harvard University). $\times 90,000$.

one to four molecules of the pigment-protein complex bound to a single reaction complex (1, 10). Were the addition of lipid to cause the chlorophyll protein complex to split off from the functional photosystem II reaction complex and crystallize in hexagonal patches, profound changes in the pattern of energy distribution in the membrane might be expected to result.

Important information concerning the state of organization of the photosynthetic membrane can be obtained using the technique of low-temperature fluorescence (15). At the temperature of liquid nitrogen (77°K) all photochemistry is halted, and the excitation energy produced from the absorption of a photon is emitted as fluorescence. Fig. 11*a* illustrates the fluorescence emission spectrum of isolated unmodified thylakoid membranes. Through a series of studies (6, 12, 13), emission of fluorescence at the three characteristic peaks of 740, 695, and 685 nm has been attributed to photosystem I, photosystem II, and chlorophyll-protein complex II, respectively.

The freeze-thaw procedure itself seems to have no effect on the fluorescence emission spectrum of the membrane, nor does the mere presence of liposomes mixed among thylakoid membranes, as illustrated in Fig. 11*b*. However, the addition of lipid to the membrane by means of freeze thaw results in a spectacular change in the nature of the emission spectrum, with a vast increase in the proportion of the fluorescence now arising in the region (685 nm) associated with chlorophyll-protein complex II (see Fig. 12). This change occurs consistently with all three lipid preparations used for these experiments although the spectra are not strictly identical for the different mixtures.

These fluorescence changes, coupled with the appearance of the regular hexagonal lattices, seem to suggest that the light-harvesting complex has become dissociated from the photosystem II reaction complex. If this were the case, the individual

photosynthetic reaction centers, now stripped of their light-harvesting apparatus, should work at much lower efficiencies. This might be expected to reduce the rates of photosynthetic reactions at low levels of light, where the harvesting apparatus is most important in maintaining photosynthetic activity. As can be seen in Fig. 13, this prediction is confirmed in the lipid-enriched membranes. While unmodified membranes show an intensity-dependent saturation of photosystem II-mediated photoreduction at fairly low light intensities, the lipid-enriched membranes show far lower rates at these intensities and, in fact, could not be saturated with our experimental apparatus. This inability to saturate suggests, but does not prove, that the ultimate rate of photoreduction may be unchanged by the addition process.

To determine whether the hexagonal lattices in lipid-enriched membranes are indeed the result of a dissociation of protein complex located in the thylakoid membrane or merely represent the aggregation of a certain size class of particles, particle size measurements were carried out as described in Materials and Methods. In the calculation of these histograms, the particles of the lattices themselves were omitted. Fig. 14 shows a typical histogram prepared by pooling measurements made on the two fracture faces of unstacked native membranes in 10 mM HEPES buffer, pH 6.5. The pooling of data from both was necessary because in the lipid-enriched membranes it sometimes becomes impossible to distinguish E faces from P faces. This histogram may be compared to the data given in Fig. 15 for similar measurements on a lipid-enriched preparation. There is a decrease in average particle diameter in the lipid-enriched membranes when compared to the native membranes. The difference (102.01 \AA vs. 120.67 \AA) is significant. Using a form of the Student's *t* test (5), a value for *t* of 308.18 was obtained. Considering the total degrees of freedom, this

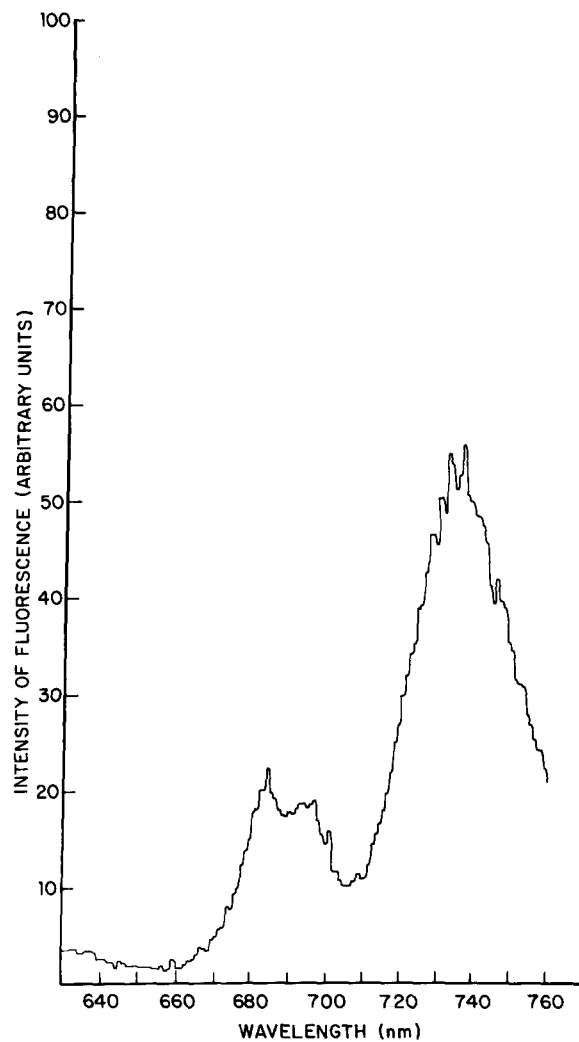
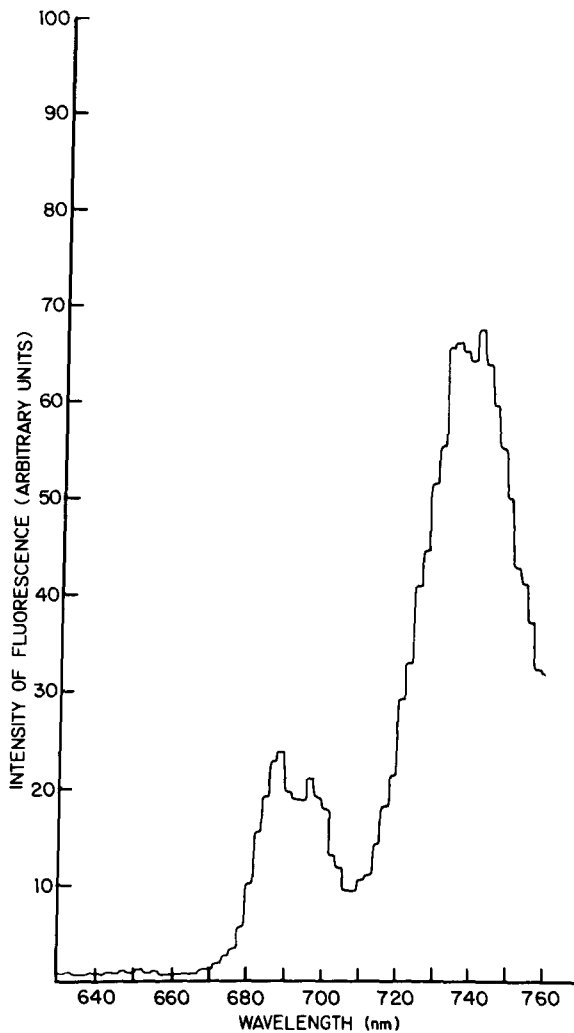


FIGURE 11 (a) Low-temperature fluorescence emission spectrum of isolated unmodified photosynthetic membranes. Emission maxima occur at 685, 695, and 740 nm. Only 20% of total fluorescence occurs between 670 and 710 nm. (b) Low-temperature fluorescence emission spectrum of isolated unmodified photosynthetic mem-

branes, thoroughly mixed with liposomes as described in the text. 24% of the total fluorescence occurs between 670 and 710 nm. Clearly, the mere physical presence of lipid has little effect on the spectrum. Freeze-thawing lipid and photosynthetic membranes separately also has no effect on low-temperature fluorescence.

corresponds to a probability $P < 0.01$ that the two populations are identical. The null hypothesis may be rejected in this case.

This decrease in the average particle size is reflected in the decrease in the number of particles of diameter $>140 \text{ \AA}$. In the native membrane, 31% of the particles are $>140 \text{ \AA}$ in diameter, while only 15% of the particles in the lipid-enriched membranes are $>140 \text{ \AA}$.

DISCUSSION

Before dealing with the implications of this work for the actual role of lipids in the organization of the photosynthetic membrane, it is necessary to consider the evidence which led us to suggest that exogenous lipids are actually incorporated into the membrane during the freeze-thaw procedure. The different densities of unmodified thylakoids and liposomes permit separation of the two via density gradient centrifugation (Fig. 1), and the presence of chlorophyll in the natural membrane provides a useful marker for its location in the gradient system. As we have noted, the presence of chlorophyll in less dense regions of the gradient following freeze-thaw suggests that the

thylakoid membranes have been dramatically reduced in density by the incorporation of lipid molecules. It is also clear that at least two other phenomena could account for the presence of pigment in this region of the gradient: the extraction of pigment molecules from the photosynthetic membrane by the lipids and the adherence of liposomes to the native membrane in such a way that the density of the aggregate is reduced relative to that of the native membrane. The first possibility may be discounted by the fact that membrane polypeptides are found in normal amounts in the upper regions of the gradient, and these less dense regions also show significant amounts of photosynthetic activity (Figs. 6-9 and Table II, respectively). Further evidence against this possibility is found in Table I, where no significant changes in chlorophyll to protein ratios are observed in the various gradient fractions. The second possibility seems to be ruled out by our failure to observe any evidence of liposome adherence to the native membrane in freeze-fracture electron microscopy, where such an occurrence should be apparent. In fact, as Figs. 3-7 show, samples taken from less dense regions of the gradient exhibit greater and greater distances between intramembrane particles.

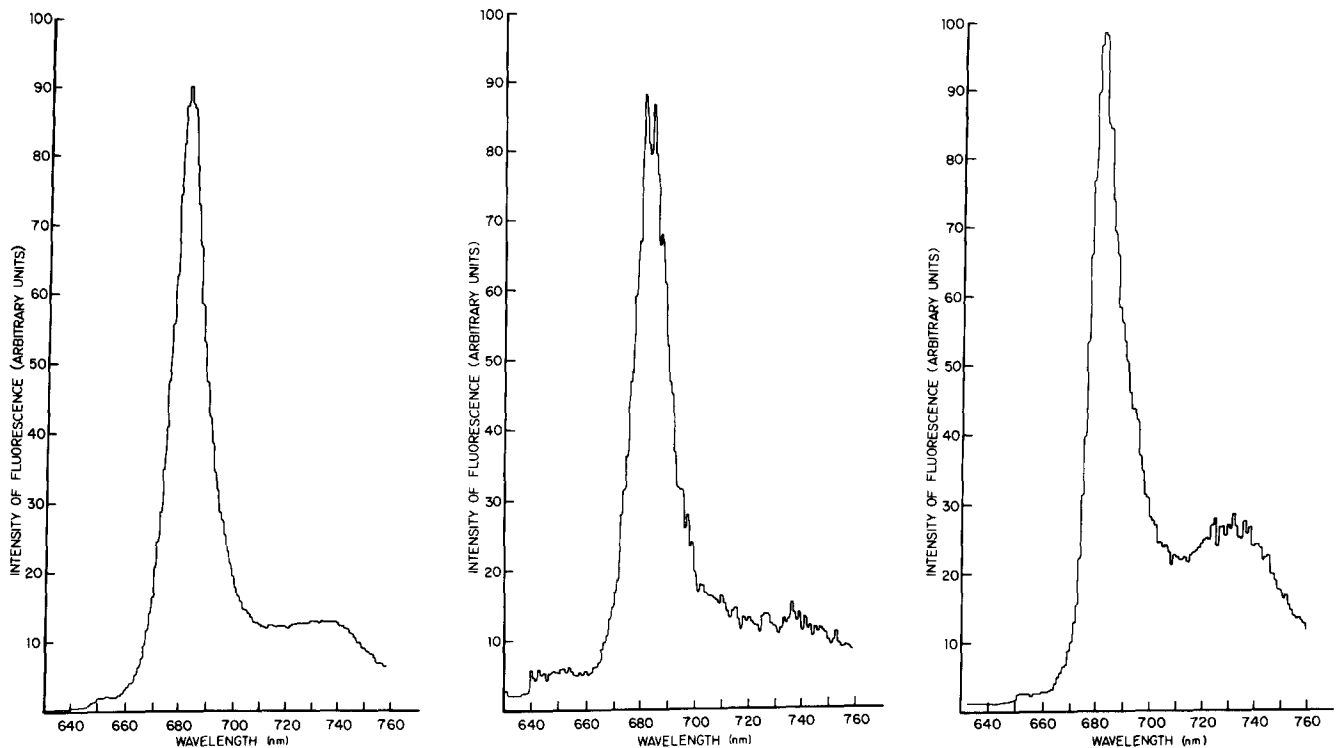
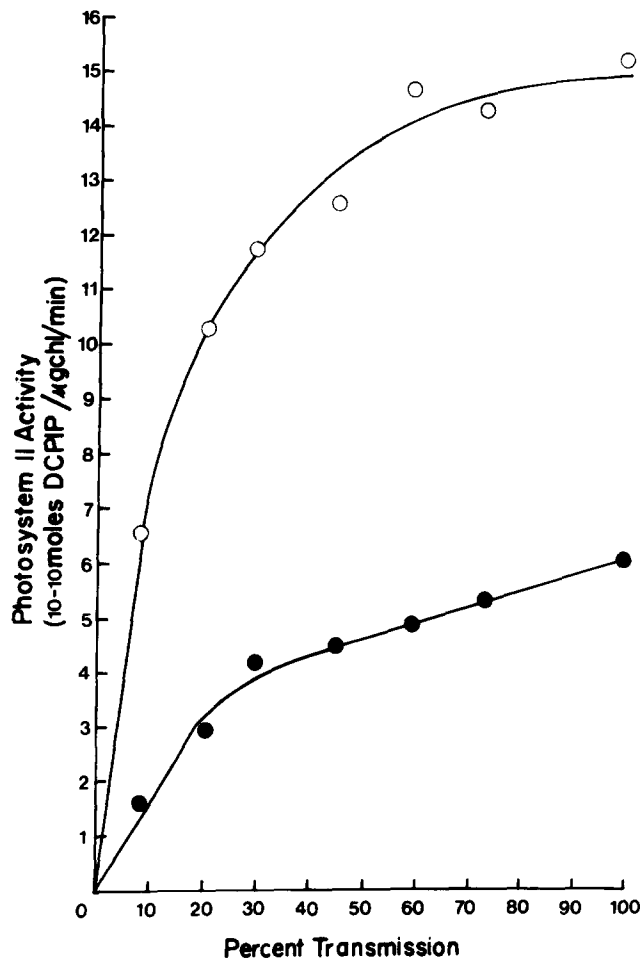


FIGURE 12 Low-temperature fluorescence emission spectra of lipid-enriched membranes formed with II-S phosphatidyl choline (a), purified phosphatidyl choline (b), and purified phosphatidyl glycerol (c). Note that in each case dramatic changes have occurred in the pattern when compared to the membranes analyzed in Fig. 11. 74% of the total fluorescence is now observed between 670 and 710 nm in a, 76% in b, and 64% in c.



That these results reflect a true addition of lipid rather than an artifact resulting from some undefined component of the lipid mixture is confirmed by the fact that identical results are obtained when either highly purified phosphatidyl choline or phosphatidyl glycerol is used. Thus, we conclude that the freeze-thaw method permits the addition of lipid of defined composition to the photosynthetic membrane.

Although these results do seem to indicate that an actual addition of lipid has taken place, it is not possible to determine, on the basis of this study, the actual mechanism of that addition. Although freeze-fracture studies failed to reveal liposomes adhering to thylakoid membranes, or liposomes internalized by thylakoid membranes, it is not possible to rule out lipid exchange as a mechanism of lipid addition. Lipid may therefore have been added to the thylakoid membrane either by direct fusion, lipid exchange, or some other mechanism.

Schneider et al. (17), the study from which our technique was derived, reported similar results in adding lipid to the mitochondrial inner membrane. However, in our experiments we have found that each of the lipid preparations that this study used significantly alters the structure and functional organization of the photosynthetic membrane. Specifically, in membranes to which lipid has been added, we observe regular hexagonal lattices with a period of $\sim 120 \text{ \AA}$. The presence of such lattices in samples to which lipid has been added, but not

FIGURE 13 Light-dependent reaction rates for photoreduction in unmodified and lipid-enriched membranes. While unmodified membranes show a saturation of photosynthetic activity at 70% of maximum intensity for the illumination system used, lipid-enriched membranes cannot be saturated with our apparatus. This result suggests a large reduction in light-harvesting efficiency has occurred in the lipid-enriched membranes.

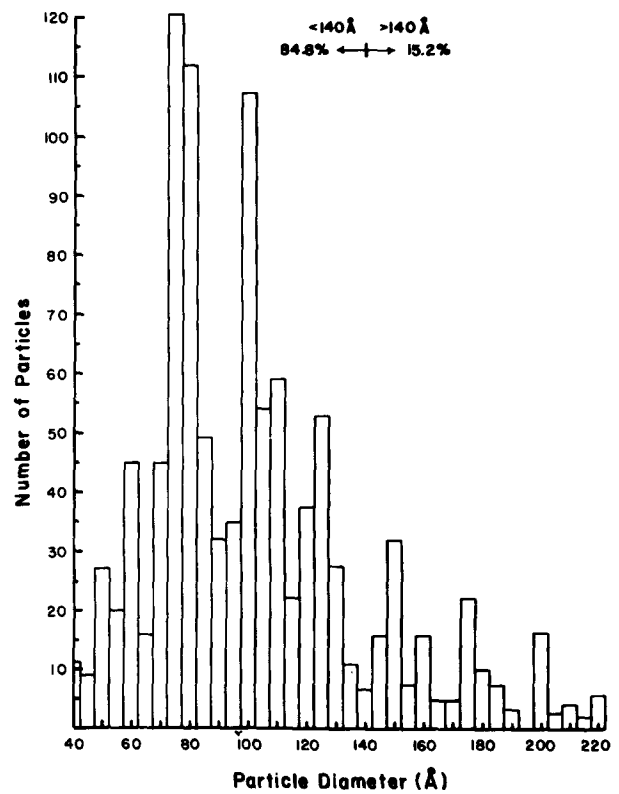
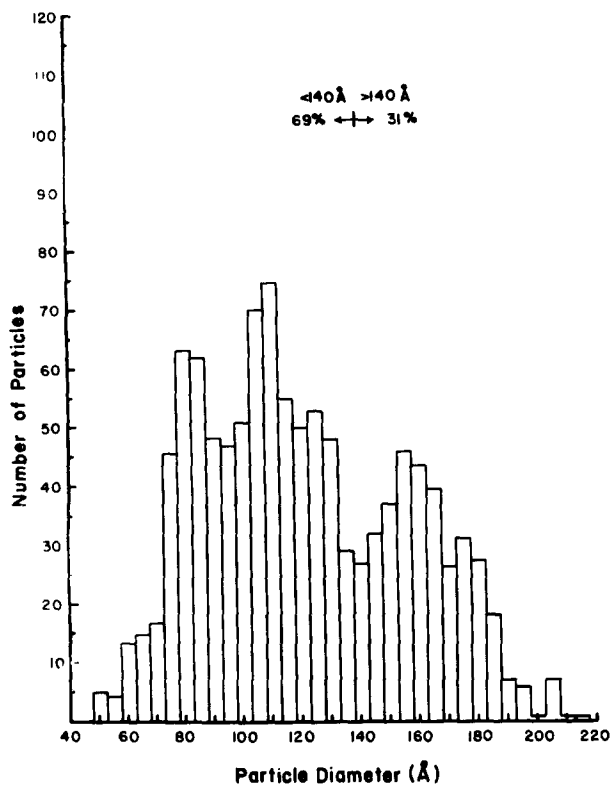


FIGURE 14 Histograms prepared from particle size measurements on native photosynthetic membranes. For the purpose of this analysis, membranes were suspended in 10 mM HEPES buffer, pH 6.5, and prepared by freeze-fracturing. The data presented here represent combined measurements on both the E and P fracture faces. $n = 1,098$; $\bar{X} = 120.6 \text{ \AA}$; and $\delta = 34.2 \text{ \AA}$.

FIGURE 15 Histograms prepared from particle size measurements on lipid-enriched membranes. All fracture faces were included in the measurements. $n = 1,062$; $\bar{X} = 102.6 \text{ \AA}$; and $\delta = 36.2 \text{ \AA}$.

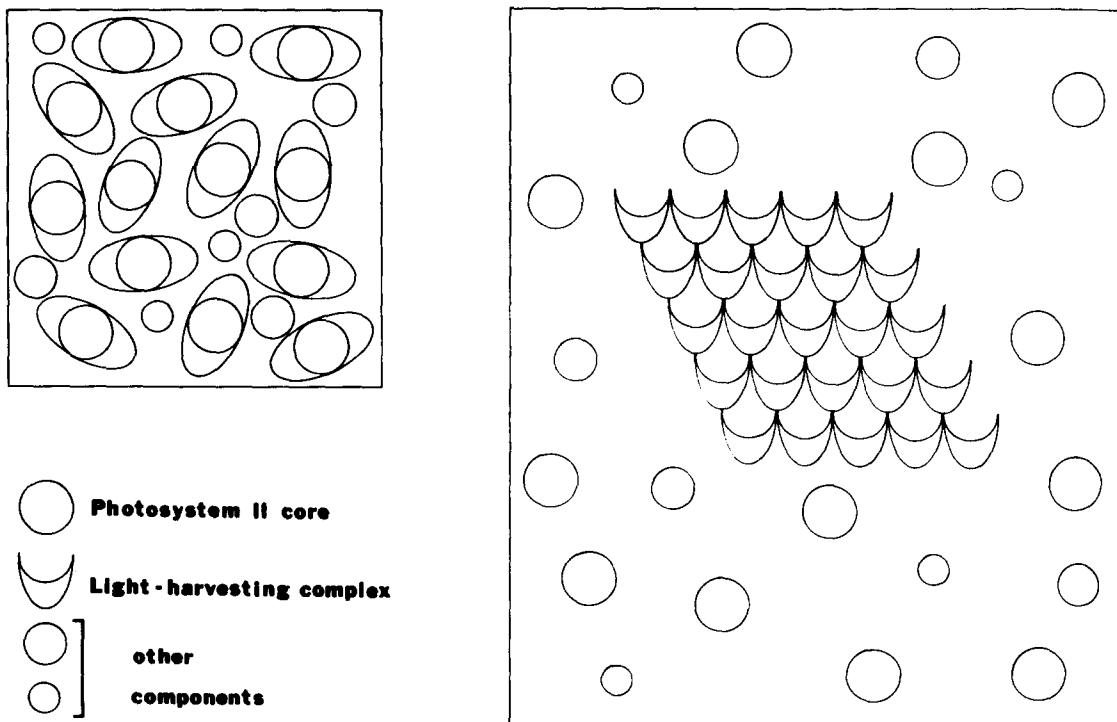


FIGURE 16 A highly schematic representation of the changes in membrane organization which accompany the addition of lipid to the photosynthetic membrane. After lipid addition, certain complexes seem to become altered in organization, and the formation of regular lattices composed of a light-harvesting complex (chlorophyll-protein complex II) seems to result.

in membranes which have merely been mixed with lipid or freeze-thawed in the absence of lipid, indicates that these structures are not artifacts of the freeze-thaw technique. These lattices are found when Sigma II-S phosphatidyl choline or purified phosphatidyl glycerol are used for the experimental procedure.

These structures bear a striking morphological similarity to the lattices observed in reconstitution experiments involving the purified chlorophyll-protein complex II. Such preparations have been made in this (Fig. 10) and other (8, 20) laboratories. This observation has led us to suggest that the addition of the particular lipids we have used for these studies results in the dissociation of the chlorophyll-protein complex from its normal site in the membrane and in its eventual crystallization into regular lattices. While no such lattices are observed after the addition of highly purified phosphatidyl choline, it is possible that this species of lipid might prevent the types of intermolecular interactions necessary for lattice formation, while still inducing the dissociation of the light-harvesting chlorophyll-protein complex from its attachment site.

Two lines of biophysical evidence support the idea that the light-harvesting chlorophyll-protein complex dissociates from the photosystem II (and possibly the photosystem I) reaction center during lipid addition. Profound changes in the pattern of low-temperature fluorescence (Figs. 11 and 12) occur with each lipid addition experiment we have carried out. Fluorescence from photosystem II (695 nm) and photosystem I (740 nm) is dramatically reduced by the lipid addition process, while direct fluorescence from the light-harvesting chlorophyll-protein complex is greatly enhanced. In our opinion, this illustrates that the light-harvesting chlorophyll-protein complex has become effectively isolated in situ following the addition of lipid. Greatly reduced efficiency of photosystem II-mediated reduction of dichlorophenol indophenol at low light intensities is also consistent with a dissociation of the light-harvesting complex from the photosystem II reaction center. There are other possibilities for thylakoid membrane organization which are also suggested by the decrease in photosystem II activity noted with increasing phospholipid content (Table II). An alternative explanation for these results might be that interactions among photosystem I, photosystem II, plastoquinone, and plastocyanin are diffusion-mediated. Therefore, increasing the lipid content of the membrane results in a decrease of the maximal rate at which electrons can move through the system. Similar diffusion-related rates of electron transport have been shown by Schneider et al. (17) for inner mitochondrial membranes. Further work would be necessary to establish this interpretation firmly, but it clearly is consistent with the results we have reported here.

Further support for the dissociation of the light-harvesting complex from other membrane components may be found in the particle size histograms of Figs. 14 and 15. The average particle size decreases after the addition of lipid with a corresponding decrease in the percentage of particles of diameter $>140 \text{ \AA}$. This decrease in the number of large E face particles, which many investigators have identified as photosystem II structures, is consistent with the idea that a dissociation of the light-harvesting complex has occurred.

These results lend support to the "core" model of photosynthetic membrane structure, which postulates that the essential components of a reaction center are surrounded by light-harvesting components which have no direct photochemical role

other than to trap light energy and funnel the excitation results from such trapping events to the functional reaction center complex. The observations reported here show that, following lipid additions to the photosynthetic membrane, changes in membrane organization occur which suggest essentially the same model. We have illustrated the nature of these changes in highly diagrammatic form in Fig. 16. If the explanation is to be accepted, what further conclusions can be drawn regarding the role of the lipid molecule in the organization of the photosynthetic apparatus?

Principally, it is clear that lipids do not play a merely supportive role in the organization of this membrane. It seems apparent that the mere ability to form a bilayer with which polypeptides can be associated is insufficient to maintain specific membrane components in their proper orientations. In these experiments, the addition of large amounts of what is essentially a foreign species of lipid seems to be directly responsible for the effects we have observed. Phosphatidyl glycerol and phosphatidyl choline have been shown to constitute 12% and 6%, respectively, of the total lipid of the higher plant photosynthetic membrane (14), and the addition of large amounts of these substances significantly alters the molecular nature of the membrane. Studies with other lipid mixtures are necessary to determine the specificity of these effects, but it seems clear that a direct dependence upon lipid composition has been shown for the proper organization of the photosynthetic membrane.

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