SUBSTRUCTURE OF CHLOROPLAST LAMELLAE

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ABSTRACT

(1) Chloroplast lamellae, like many other membranes, are composed of 90-A subunits. (2) The way in which the 90-A units associate with one another determines the quantasome structures of the membrane. There appear to be four 90-A subunits per quantasome in the most highly organized arrays. In at least some cases, the way in which the subunits bind one to another is under environmental control. (3) Extraction experiments are consistent with a model in which the membrane lipid surrounds the protein framework of the membrane.

INTRODUCTION

It is generally accepted that the quantum conversion reactions and associated transport reactions of photosynthesis reside in the internal membrane systems of chloroplasts (11, 16). The morphology of these internal membrane systems or chloroplast lamellae undergoes considerable variation not only from species to species, but within a species, depending on the physiological conditions of growth (15). In all cases, the lamellar membranes exist as closed flattened sacs termed thylakoids by Menke (6). The larger thylakoids make up the stroma lamellae of the membrane system, and the smaller thylakoids are stacked together to form the grana lamellae of the membrane system. The thylakoid itself has been shown to consist of at least two kinds of subunits. The first is the quantasome, which appears in shadowed or frozen-etched preparations of chloroplasts as viewed in the electron microscope (7, 10). The quantasome exists in a variety of arrays ranging from a random to a paracrystalline array. The quantasome as seen in a paracrystalline array is approximately 180 × 150 × 100 A in size, has a molecular weight of about 2×10^6, and contains about 230 chlorophyll molecules (10). The second kind of unit is a subunit of the quantasome itself. Individual quantasomes when viewed in shadowed preparations are seen to consist of four subunits. These subunits are spaced on approximately 90-A centers when viewed on the surface of the membrane. Cross-sections of chloroplast membranes as seen in permanganate fixed, sectioned material in the electron microscope also reveal a 90-A periodicity (8). This paper presents further evidence for the existence of 90-A subunits in the chloroplast membrane, and we propose that the ways in which these subunits associate with one another determine the quantasome structure of the membrane.

METHODS

1. CHLOROPLAST ISOLATION: Chloroplast lamellae were isolated from *Spinacia oleracea* L. as described previously by Park and Pon (11, 12). Chloroplasts were isolated from leaves of *Pharbitis nil* Chois. grown under an 8-hour photoperiod by homogenizing the leaves for 2 minutes in 0.5 M sucrose and 0.1 M $(K)PO_4$ buffer, pH 7.4. The homogenate was passed through 8 layers of cheesecloth and the chloroplasts were separated by fractional centrifugation.

2. SHADOW CASTING: The various lamellar preparations were placed on formvar-covered grids and shadowed with a nickel chromium mixture. In this technique the shadow source is a small piece of nichrome wire wrapped around a tungsten filament.

FIGURE 1 Shadowed preparation of spinach chloroplast lamellae. × 110,000.

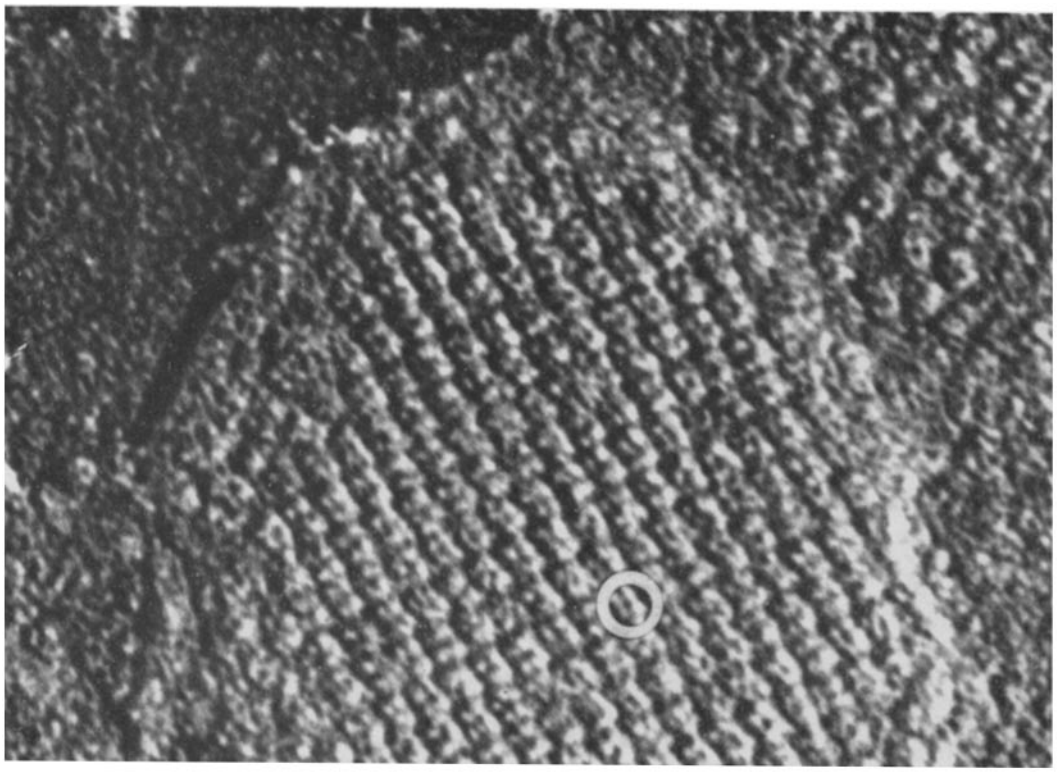

FIGURE 2 Shadowed paracrystalline quantasome array. Quantasome with contained subunits is circled. × 330,000.

After evacuation of the shadowing unit the nichrome wire is melted and approximately 90 per cent of the mass of the shadowing metal is evaporated from the filament before the remainder is used for shadow casting. This procedure fractionates the alloy so that most of the chromium is lost and a high proportion of nickel relative to chromium is used in the final shadow casting. The metal film obtained by this method does not tend to granulate under the electron beam as do films containing a higher proportion of chromium.

3. SPECTROPHOTOMETRY OF THE ELECTRON MICROSCOPE GRIDS: Carbon films cast onto freshly split mica (5) were floated onto 200-mesh copper grids. Special holders were built from carbon-impregnated epoxy resin to hold the grids, one in the reference beam and one in the sample beam of a Cary Model 14 recording spectrophotometer equipped with a scatter transmission attachment. The holders could be removed one at a time for any further required treatment and then returned to the spectrophotometer with relatively small changes in baseline. Using the 0.1 O.D. full scale slide wire, it was possible to obtain visible spectra of lamellar preparations on the grids, which could then be shadow cast and observed by electron microscopy.

4. EXTRACTION OF LAMELLAR PREPARATIONS ON ELECTRON MICROSCOPE GRIDS: The samples on electron microscope grids were extracted by removing one of the grid holders from the spectrophotometer and immersing it sequentially in 100 per cent freshly distilled acetone, 50 per cent acetone–50 per cent petroleum ether (30°–60°), and finally 100 per cent petroleum ether (30°–60°). The extractions were performed at room temperature for a duration of 1 minute in each extracting solvent. Similar extractions with lyophilized lamellar preparations in bulk show that greater than 90° of the lamellar lipid is removed by this acetone-petroleum ether extraction sequence (12).

EXPERIMENTAL RESULTS AND DISCUSSION

MEMBRANE SURFACE MORPHOLOGY: Fig. 1 is a shadow-cast preparation of spinach chloro-

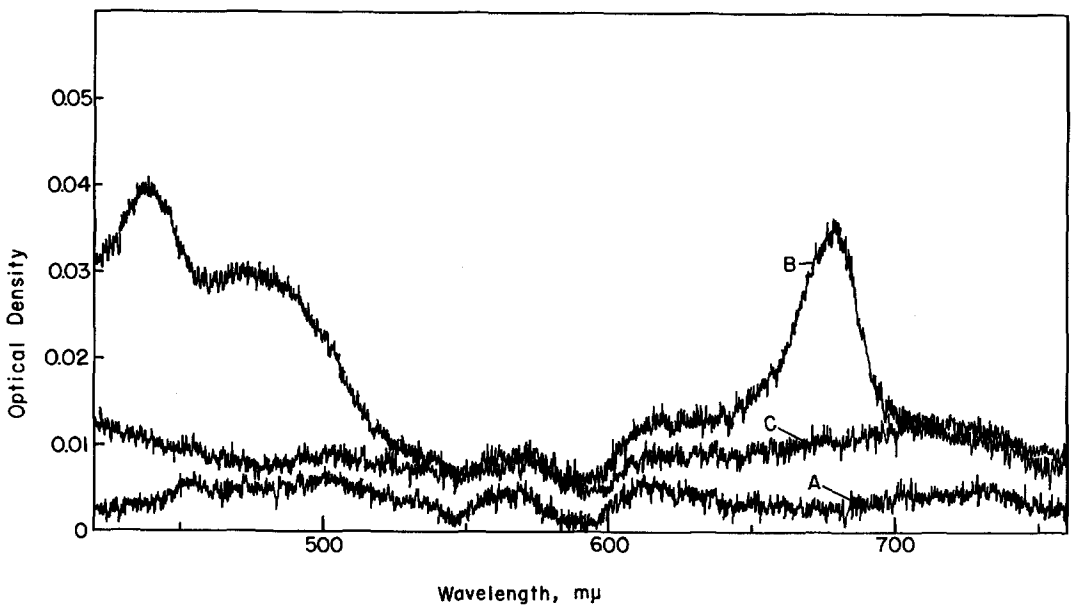

FIGURE 3 Difference spectra of carbon films supported on 200-mesh grids, *A*, before placing membranes on the sample grid, *B*, after placing membranes on sample grid, *C*, after extracting the lipids from the membranes on the sample grid. The maximum of the red peak in *B* is at 678 mμ.

plast lamellae grown under short days. Several paracrystalline quantasome arrays are evident in this preparation. The repeat distances along this array appear to be 15 per cent less than that we reported earlier (10), the repeat distance along the long axis being 160 A rather than 185 A. Fig. 2 is a shadow-cast paracrystalline quantasome array at a higher magnification. In this picture, the quantasome subunit structure is very evident. Though in some cases two of the subunits are joined together to yield only three units per quantasome; in many cases there are four subunits per quantasome. This is illustrated in the circled unit. The periodicity of the subunits (~80 A) along the membrane is particularly interesting since it is so similar to periodicities observed by Sjöstrand (14), Fernández-Morán (3), and Murakami (8) in sectioned membranes.

LIPID EXTRACTION: The lamellae in Figs. 1 and 2 are known from chemical studies to consist of approximately 50 per cent lipid and 50 per cent protein (12). In earlier studies, we had attempted to extract the lipids from aqueous suspensions of chloroplast lamellae and then to view the extracted lamellae by shadow-casting techniques. We hoped to gain by this procedure some evidence concerning the distribution of lipid and protein along the membrane surface. The initial effort was largely unsuccessful because of the water insolubility of the lamellar proteins exposed by lipid extraction. To overcome this difficulty, we developed the technique of extracting lamellae that were already adhered to carbon films (see Methods). We knew from large scale experiments with lyophilized lamellae that greater than 90 per cent of the lamellar lipids are removed by extraction with petroleum ether and acetone (12). The relatively small amounts of highly polar lipids (phospholipid and sulfolipid) in the lamellae allow fairly complete lipid extraction by acetone and petroleum ether. The large proportion of galactolipids in chloroplast lamellae allows one to avoid the use of extraction solvents such as methanol or aqueous acetone, which severely denature the lamellar proteins. Direct evidence that the extraction procedure outlined under Methods actually removes the visible absorbing lipids was obtained from spectrophotometric observations of the carbon-covered grids before and after extraction. The results of such an experiment are given in Fig. 3. Acetone and petroleum ether extraction is shown directly to remove the colored lipids and presumably, by analogy with large scale experiments, most of the colorless lipids. Electron micro-

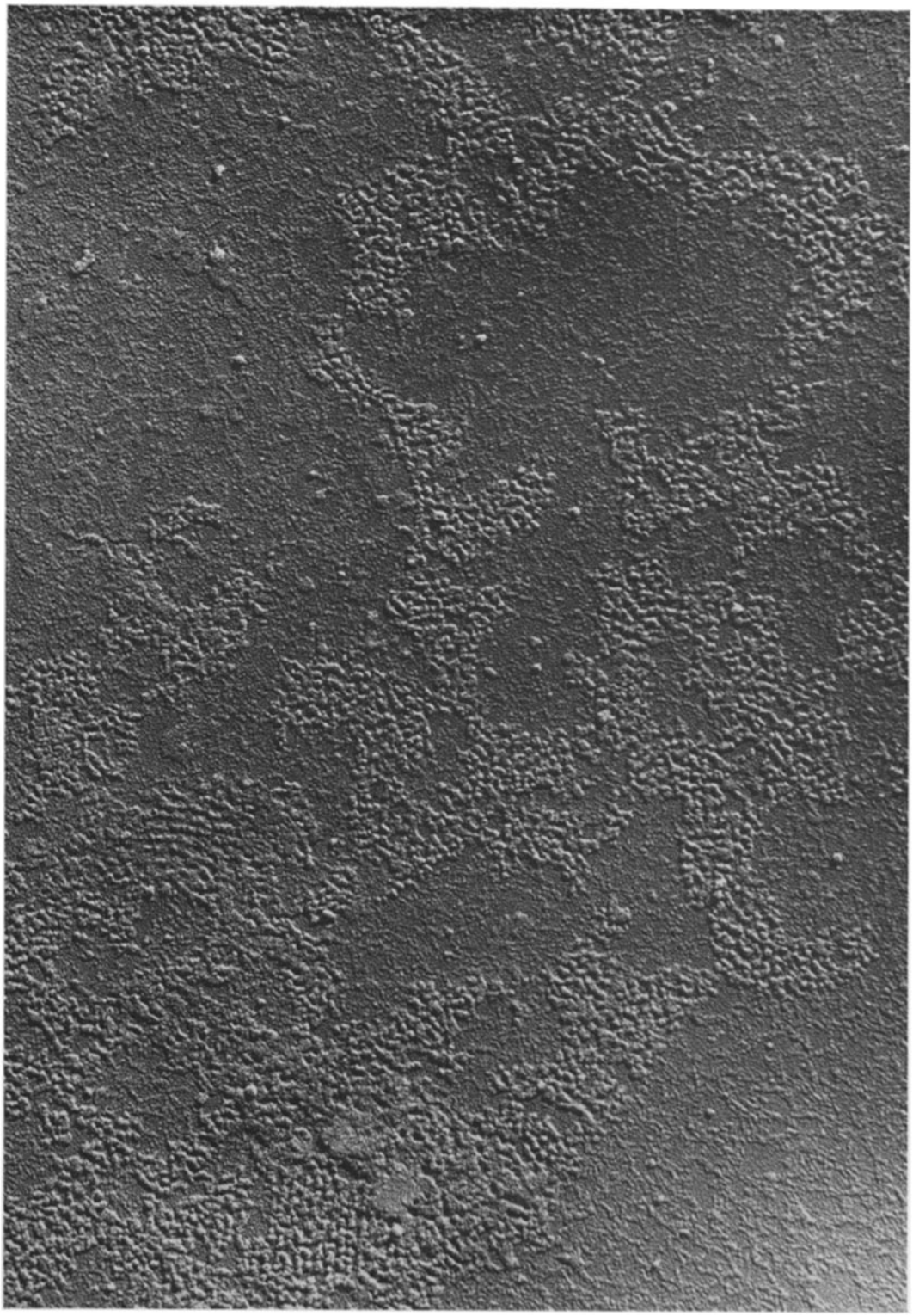

FIGURE 4 Spinach chloroplast lamellae after lipid extraction. × 100,000.

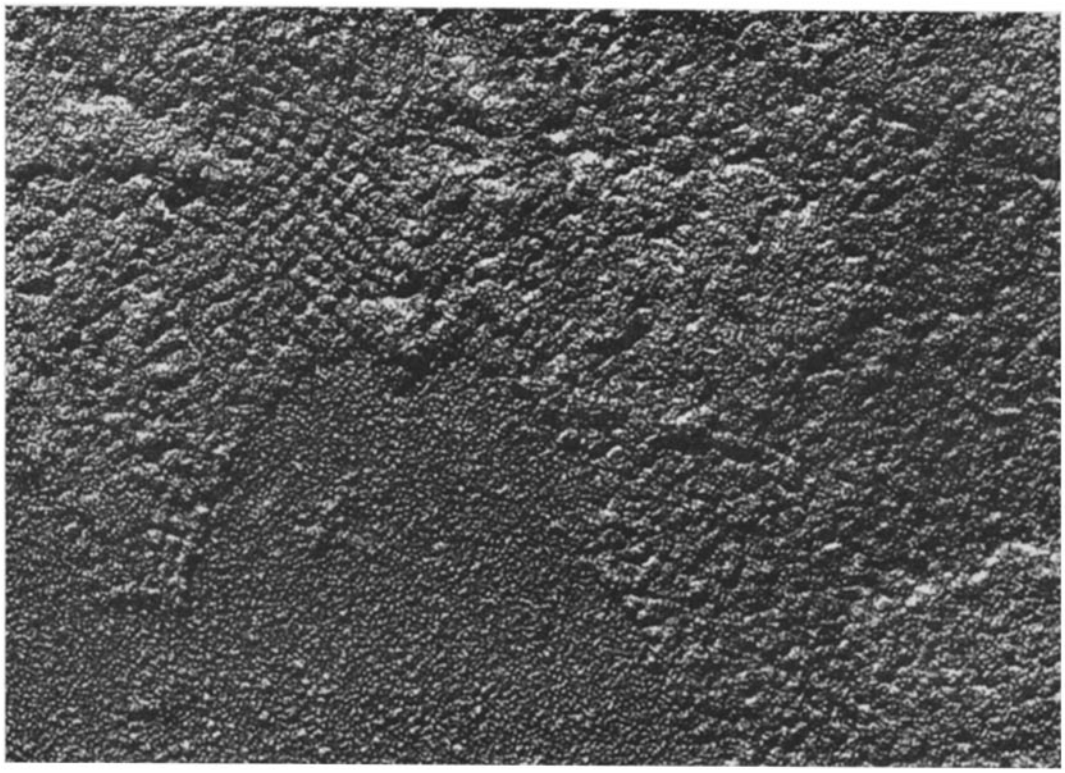

FIGURE 5 Chloroplast lamella after lipid extraction. Paracrystalline quantasome array is still evident in protein residue. × 170,000.

graphs of the extracted preparation in Fig. 3 are shown in Figs. 4 to 6. These preparations from which the lipid, representing 50 per cent of the mass, has been removed represent the protein components of the membrane. These protein components, as seen particularly in Figs. 4 and 5, still show quantasome structure. The quantasome structure in these preparations is in greater relief than in unextracted membranes. In Fig. 4 the distance between the linear quantasome arrays remains at 150 to 200 A; however, a great deal of material has been removed from between the rows. These results suggest (1) that the chloroplast lipids are wrapped around the protein framework of the membrane, and (2) that it is the degree of order in the protein framework which dictates the morphology or quantasome structure of the intact membrane.

Occasionally, as in Fig. 6, areas with an 80 to 90 A periodicity are evident in the extracted preparations. As mentioned earlier, this periodicity in the protein framework of the membrane is probably related to a similar periodicity seen in histological preparations of membranes.

QUANTASOME STRUCTURE IN PHARBITIS: The chloroplast lamellae of *Pharbitis nil*, a short-day plant, provide a useful comparison with spinach lamellae. First of all, the quantasome structure of *Pharbitis* chloroplast membranes is uniformly spread over the entire membrane system and exists on the outside of the lamellae. This is shown in Fig. 7, in which a *Pharbitis* chloroplast was osmotically ruptured while on a formvar-covered grid. The grana stacks are evident. There is also a paracrystalline area. This result immediately suggests that our observations on spinach, which seemed to indicate that the quantasome arrays are on the interior of the membrane, should be critically reinvestigated (11). *Pharbitis* also differs from spinach in the effects of sonic rupture on morphology. Fig. 8 shows a shadowed preparation of sonicated *Pharbitis* chloroplasts. Unlike the preparation in Fig. 7, approximately one-half the membrane area is covered with typical quantasome arrays, while

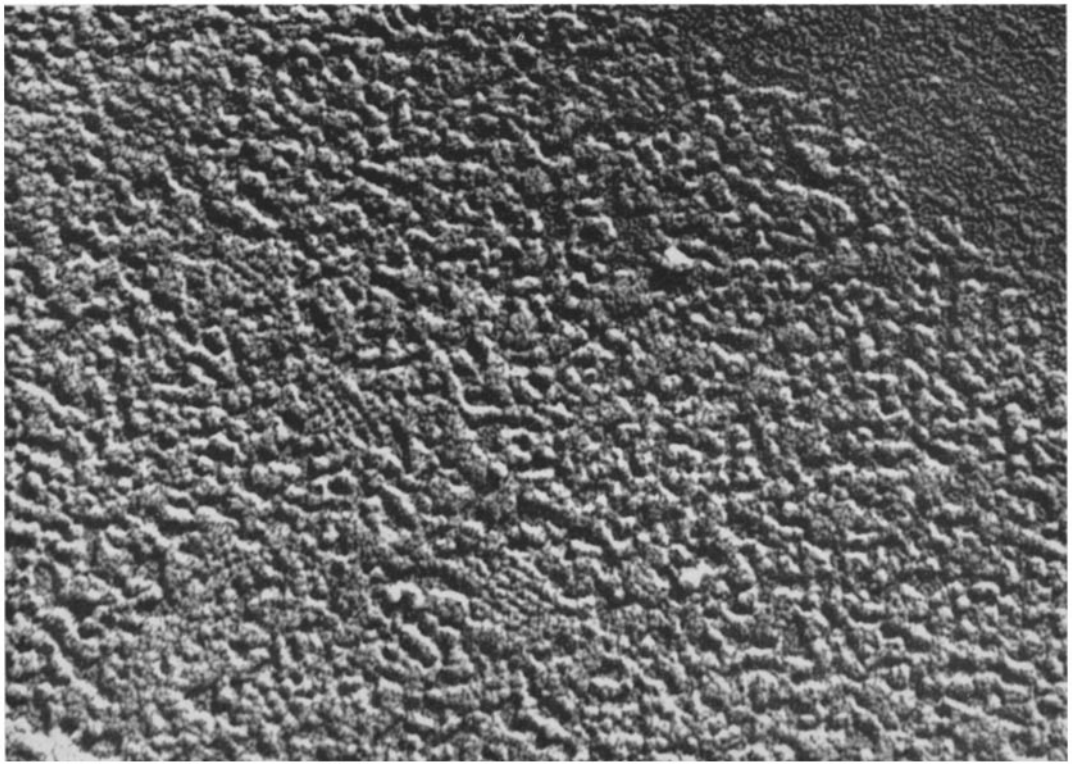

FIGURE 6 Chloroplast lamella after lipid extraction. Regions with 90-A periodicities are evident in the membrane. × 170,000.

the other half is covered with particles on 90-A centers. We interpret this result to mean that in *Pharbitis* the interior of the lamella (or thylakoid) consists of 90-A particles, while the outside demonstrates the quantasome structure. It is probable that these 90-A particles observed in shadow-cast preparations are related to those evident in sectioned material. Murakami (8) has discussed similar particles in thin sections of *Chlorella*. Negative staining of chloroplast membranes also reveals particles of this size (9) though these particles may be contaminating Fraction I protein.

DISCUSSION AND CONCLUSIONS

Studies from this and other laboratories have concentrated on three levels of organization in chloroplast membranes. The first level is the 90-A subunits which form the quantasomes; the second level is the quantasomes which form the membranes; and the third level is the characteristic thylakoid arrangement of a given chloroplast.

90-A UNITS: The 90-A units observed in shadowed and sectioned chloroplast lamellae are just one example of the existence of such membrane subunits. Sjöstrand and Fernández-Morán *et al.* demonstrated repeating structures of this size in thin sections of mitochondria (3, 14). Sjöstrand has demonstrated their presence in other cytoplasmic membranes (14). Since these various membranes have different functions, it might be inferred that the 90-A period is a basic unit of many membranes and is only secondarily related to its function. The shadow-cast preparations of 90-A particles making up the *Pharbitis* membrane are graphic support for the idea that chloroplast lamellar membranes are made up of such units. The demonstration of units of similar size as substructure in paracrystalline quantasome arrays of intact and extracted spinach membranes is further evidence that the 90-A unit is not only a basic structural unit of the membrane, but also is a reflection of the protein structure of the membrane. A single 90-A unit is much too small to be a physiological photosynthetic unit. The photosynthetic unit has to consist of at least

FIGURE 7 Shadowed preparation of *Pharbitis* grana stacks. × 66,000.

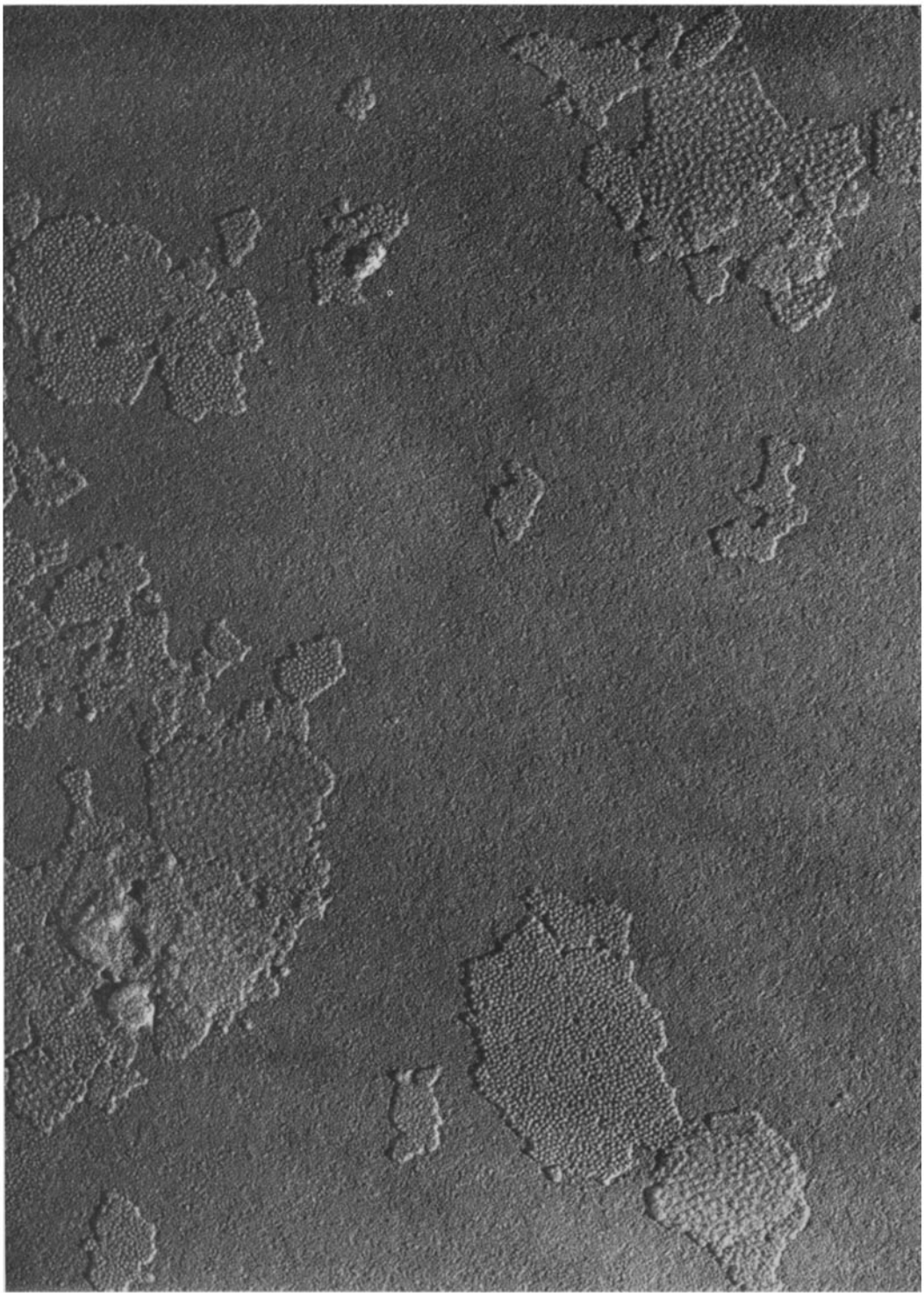

FIGURE 8 Shadowed preparation of sonicated *Pharbitis* chloroplast lamellae. × 60,000.

four such 90-A particles and their associated lipid to contain sufficient chlorophyll and cytochromes to embrace a complete electron transport pathway (10).

QUANTASOMES: The next level of organization of the membranes is found at a repeat distance of about 150 A, a particle which we have termed the quantasome. We have already noted that the quantasome is a polymorphic structure. In its most organized form it exists as a paracrystalline array. It may exist as a linear array, a random array, or become almost imperceptible (10). In spinach the types of array appear to be controlled, in part, by day length, whereas in *Pharbitis* quantasome arrays appear to be independent of photoperiod. The extraction experiments presented here support the notion that the quantasome structure is a reflection of the protein structure of the membrane. There appear to be four 90-A units per quantasome in the most orderly arrays, and the quantasome structure itself appears to depend on the way in which the 90-A units have become aggregated. It will be noticed that the *Pharbitis* quantasome, for the most part, exists in the random array as do the 90-A subunits visible on the other side of the membrane. The quantasome is calculated to contain about 230 chlorophylls and is the smallest lamellar unit qualified by chemical composition to be a photosynthetic unit (2). Good has obtained inhibitor evidence which suggests that the physiological photosynthetic unit may be larger than the quantasome (4). This problem will be most directly resolved only by isolation and characterization of the quantasome.

We noted that the quantasome arrays appear in greater relief after lipid extraction and interpreted this result to mean that the lipid is located on the exterior of the membrane. Though this interpretation must remain tentative until further experimentation, it does receive some support from other observations in the literature. In particular, Sastry and Kates (13) showed that galactose is rapidly hydrolyzed from the chloroplast galactolipids in *Phaseolus multiflorus* homogenates. Apparently, the galactose residues are at the surface of the membrane readily available for hydrolysis by soluble enzymes. Also, Branton (1) has observed that freeze-etched unit membranes in cross-sections appear as two ridges separated by a depressed trough. The trough is the center of the membrane and probably represents the area of greatest water content. Branton's observations also support our interpretation of the extraction experiments that lipid is distributed primarily on the membrane surface.

THYLAKOIDS: The thylakoids are made up of the chloroplast internal membranes which, in turn, are made up of quantasome arrays. The detailed lamellar arrangements appear almost infinite and again are under a variety of environmental controls (15). The thylakoids contain tens of thousands of chlorophyll molecules and are obviously much larger than the physiological photosynthetic unit. The final designation of a morphological photosynthetic unit corresponding to the physiological unit of Emerson and Arnold (2) awaits its isolation and characterization from lamellar material of *uniform morphology*. This last point must be stressed since it is becoming increasingly obvious that chloroplast lamellae within a given species vary greatly in both morphology and chemical composition and enzymology, depending on the conditions under which the plants are grown.

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