ELECTRON MICROSCOPY IN BIOLOGY

VOLUME 1

Edited by
Jack D. Griffith

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SERIES PREFACE

Over the past 40 years, electron microscopy in biology has changed from a frontier of cell biology to a routine tool of fields as diverse as protein chemistry and chemical carcinogenesis. In the early days of electron microscopy most studies employed thin sectioning, and a microscopist could keep abreast of the major preparative procedures and advances in the field. Now, with so many diverse applications and subspecialties, few electron microscopists have more than a reading knowledge of the preparative techniques outside their own area of study. The past few years have also seen the cost of state-of-the-art electron microscopes rise beyond the usual resources of an individual laboratory. This has forced the growth of central EM facilities serving from a few research groups to one or more departments. Those with primary interests in biological electron microscopy are being called on to supervise these facilities, and to counsel colleagues who face research problems very different from their own. Following the literature in a single field is difficult enough; scanning journals for articles of possible interest to colleagues is impossible. Furthermore, the literature of biological electron microscopy appears primarily in journals dedicated to the area of application, making it even more likely that one would miss articles outside one's normal reading.

A yearly series of invited articles can help solve this dilemma. A well-executed series can bring together articles reviewing techniques and advances in all areas of biological electron microscopy. Properly written, each article should be useful to the expert as a brief refresher and resource of reference material, and to the novice as an introduction. A collection of these volumes spanning several years should provide a valuable resource for introducing colleagues to new applications of electron microscopy and to the expert as an on-hand reference library. The series also would provide a needed format for electron microscopists to communicate among themselves in ways that the journals cannot accommodate. Most journal articles focus on the end rather than the means, and often important details are omitted, simply because of the style dictated by the format of a journal article. A

discussion of techniques can be very important in a technique-oriented field such as biological electron microscopy, and a critical review of techniques, illustrated with successful applications, makes a valuable contribution to our colleagues.

JACK D. GRIFFITH

Chapel Hill, North Carolina
March, 1981

PREFACE

This volume contains excellent articles that combine discussion of techniques and applications with critical appraisal of the interpretation of electron micrographs. The chapter by Miller on freeze-etching of chloroplast membranes provides state-of-the-art electron micrographs of chloroplasts and a valuable discussion of our growing understanding of their structure based on such data. The chapter on chromatin ultrastructure by Hamkalo and Rattner provides an overview by acknowledged experts in this field and clues into fruitful directions for future research. Moore, in Chapter 3, discusses the visualization of RNA by electron microscopy and provides examples from the work of the best laboratories in this field. The visualization and mapping of genes by the R-loop technique described by Broker and Chow is very timely. These authors have contributed greatly to the development of this very powerful technique now finding use in many laboratories. The chapter by Fotino discusses potential for biological applications of high-voltage electron microscopy in a way that is highly informative to novice and expert alike. Finally, the review by Baker of the literature of image processing serves as an excellent introduction to the field for the novice, including a complete and well-cross-referenced literature survey that will provide valuable direction for the beginner and a handbook for the expert in the field.

As editor, I thank my panel of advisors for a great deal of encouragement and counseling and for aiding with the selection of authors for this and future volumes.

JACK D. GRIFFITH

Chapel Hill, North Carolina
March, 1981

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1 FREEZE–ETCHING STUDIES OF THE PHOTOSYNTHETIC MEMBRANE

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1 Introduction
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1 INTRODUCTION

Of all the biochemical and functional adaptations of living things, none is more fundamental than photosynthesis, the ability to transduce light into chemical energy. Because of this basic importance, the chloroplast, the organelle of photosynthesis, has been intensively studied since the development of biological electron microscopy. The very first electron microscope studies (17, 19, 41) indicated the presence of an extensive membrane system in the chloroplast. These flattened membranes were later termed "thylakoids" by Menke (22). Because these structures carry out the light reaction of photosynthesis, they are also often called photosynthetic membranes. These membranes were also among the first biological structures to be studied extensively with the freeze-etching technique methodology of Moor and Mühlethaler (28).

In this review I summarize some of the recent literature relating to freeze-etching of the photosynthetic membrane, and present a comprehensive view of the organization of the thylakoid membrane as revealed by this technique. Space limitations and the nature of this volume do not permit a detailed review of the broader question of structure and function in the photosynthetic membrane. For this more general topic, the reader is referred to any number of recent reviews that treat this question from several perspectives (1, 5, 6, 35, 40).

2 GENERAL ORGANIZATION OF THE THYLAKOID MEMBRANE

Thin sections of the chloroplast illustrate at once the extensiveness of the thylakoid lamellar system (Figure 1). Thylakoid membranes are piled into large stacks called grana, and individual grana are connected by a network of

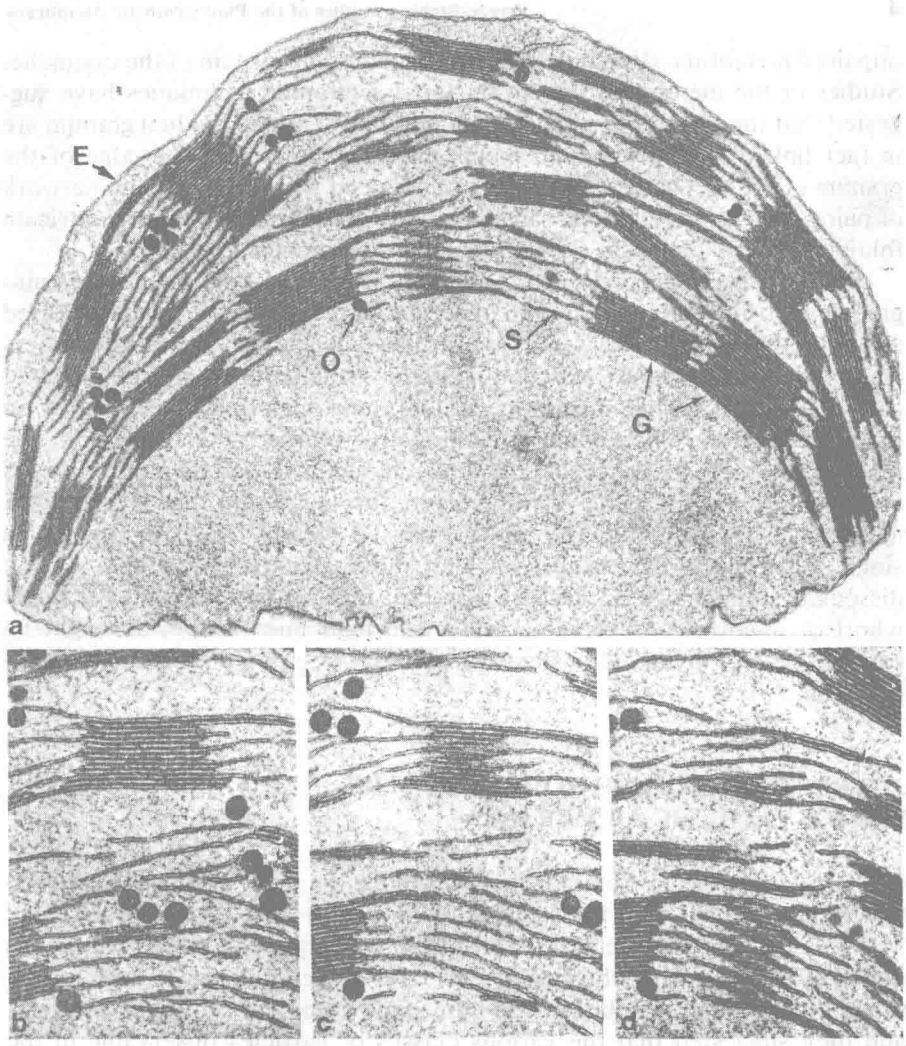

Figure 1 The structure of an intact chloroplast as visualized in thin section. (*a*) The entire organelle in an intact leaf. The chloroplast is enclosed by a pair of envelope membranes (*E*) and shows a number of dense osmiophilic granules (*O*), which probably represent lipid droplets stored in the organelle. The internal membranes of the chloroplast, which are termed thylakoids, contain the elements of the light reaction of photosynthesis and are stacked in several regions to form larger structures known as grana (*G*). (*b*), (*c*), and (*d*) A series of sequential sections taken through the central region of the chloroplast shown in (*a*) illustrates the relationship of the unstacked stromal (*S*) thylakoid membranes to the stacked membranes that comprise the grana. By following membranes across the three photographs, one can visualize the manner in which unstacked stromal membranes seem to spiral around the grana. The membranes in a single chloroplast, therefore, seem to represent a complex, interconnected network organized into two phases: stacked membranes and unstacked membranes. Magnification: (*a*): 40,000×. (*b–d*): 45,400×.

unpaired membranes that links the entire membrane system of the organelle. Studies of the membrane system by serial sectioning techniques have suggested that the apparently separate membranes of any individual granum are in fact linked by the unpaired membranes that appear at the edge of the granum (18, 32). The net result is a complicated three-dimensional network of paired and unpaired membranes that may in fact result from the intricate folding and refolding of a single thylakoid membrane sac.

It should be noted that distinctive stacking of thylakoid membranes into grana can disappear under certain experimental conditions. Izawa and Good (20) were the first to show that when chloroplasts are isolated in buffers lacking divalent cations (and containing only small amounts of monovalent cations), the thylakoid membrane unfolds and membrane stacking is no longer observed. When divalent cations are returned to the medium, the membranes begin to reassociate, and the characteristic grana morphology is once again observed. One of my reasons for suggesting that the complex membrane system of the chloroplast may be merely the elaboration of a single thylakoid is the observation that the unstacked membranes do not dissociate into small vesicles and disperse: rather, they remain as a single whorl of membrane observable under the light microscope, as might be expected if the thylakoid system were in fact composed of a single membrane sac.

3 FREEZE-FRACTURE STUDIES OF THE PHOTOSYNTHETIC MEMBRANE

Chloroplast thylakoid membranes were among the first structures to be systematically studied with the freeze-etching device developed by Moor and Mühlethaler (28), and the first report of the appearance of these membranes in freeze-fracture was published in 1965 (29). The complexity of structure in the photosynthetic membrane was immediately apparent to these workers, and they suggested that the various classes of particles observable in the replicas were manifestations of a series of enzyme-pigment complexes on the two surfaces of the thylakoid membrane.

The suggestion that particles visible in freeze-fracture replicas of the thylakoid were complexes on the surface of the membrane derived from the belief that freeze-fracturing resulted in a separation of the membrane from the surrounding ice in such a way that the membrane surface was exposed. Branton (10), in contrast, suggested that the fracturing process might actually split biological membranes, and maintained that the particles thus visualized were derived from structures embedded in the membrane, rather than exposed at its surface. A study of fracture faces in the photosynthetic membrane was reported and discussed with this interpretation in mind (13). Later reports showed that biological membranes do indeed seem to undergo splitting during the freeze-fracturing process (see reference 11 for a sum-

mary), and Branton's suggestion of membrane splitting during freeze-fracturing is now generally accepted.

However, the four fracture faces found in freeze-fractured chloroplast thylakoid membranes still presented a severe problem of interpretation, and Branton and Park (13) attempted to resolve it by suggesting that perhaps fracturing could occur at several levels within the thylakoid membrane. It then became necessary to explain why chloroplast membranes should fracture differently (i.e., at several levels) from other biological membranes, and this problem was solved in 1971 by Goodenough and Staehelin (16) in their study of fracture faces in stacked and unstacked thylakoid membranes from *Chlamydomonas*.

It now appears that the photosynthetic membrane fractures in roughly the same way as other biological membranes, and that the additional fracture faces are the result of differences in membrane internal structure between stacked and unstacked regions of the thylakoid membrane system. In the remainder of this chapter I outline our understanding of the way in which the photosynthetic membrane behaves during the fracturing process, commenting briefly about the functional organization of the membrane in the light reaction of photosynthesis.

4 PREPARATION OF MEMBRANES FOR FREEZE-FRACTURING

The membranes of the chloroplast can be examined *in situ*, whether in leaf tissue or intact algae, although this does present some disadvantages. Leaf tissue is particularly difficult to handle and fracture successfully, and algae may possess thick cell walls or other structures that tend to prevent the fracture plane from exposing the photosynthetic membrane in enough cases to provide a large sample for the microscopist. Therefore, it is quite common to isolate chloroplasts by standard techniques (43) prior to freezing. The isolated chloroplasts are infiltrated with glycerol to a concentration of 20–25% v/v for 1 hour, and frozen in liquid Freon or some suitable medium. Once frozen, the membrane samples can be stored indefinitely in liquid nitrogen until used. Replicas are prepared by standard techniques on one of several different freeze-etching devices. Freeze-fracture replicas are those that have been prepared by shadowing the frozen surface immediately following the cleavage event, or following the last pass of a fracturing knife. Such replicas show details of membrane fracture faces only.

5 FRACTURE FACES OF THE THYLAKOID MEMBRANE

Freeze-fracture replicas of stacked thylakoid membranes show four fracture faces, as illustrated in Figures 2 and 3. Two of these fracture faces are derived from membrane splitting in stacked regions of the thylakoid system, and two

Figure 2 Freeze-fractured isolated thylakoid membranes. The fracturing process reveals internal details of membrane organization, which can then be copied by the replica technique and observed in the microscope. Several different fracture faces are evident in this low-power micrograph. Magnification: 29,600×.

from the splitting of unstacked membranes. The labeling scheme now used for such fracture faces is illustrated in Figure 4. Detailed analysis of each of the four fracture faces can be carried out by careful measurement of particle sizes and distribution densities (see, e.g., references 16 and 39), and such information can be used to supplement the qualitative observation that the four fracture faces differ in terms of both the average diameters of particles present and the density with which the particles are distributed. One important piece of information gleaned from such analyses is that a wide range of particle sizes is present on any one fracture face, and it would therefore be misleading to assume a priori that the particles found on any single fracture face are biochemically or energetically homogeneous.

The effect of membrane stacking on the image of the membrane in freeze-fracture is illustrated in Figure 5. Isolated membranes that have been unstacked by washing in a buffer of low ionic strength show only two fracture faces. When such unstacked membranes are incubated with small (1–2 mm) amounts of Mg^{2+}, restacking of the membranes occurs, and four frac-

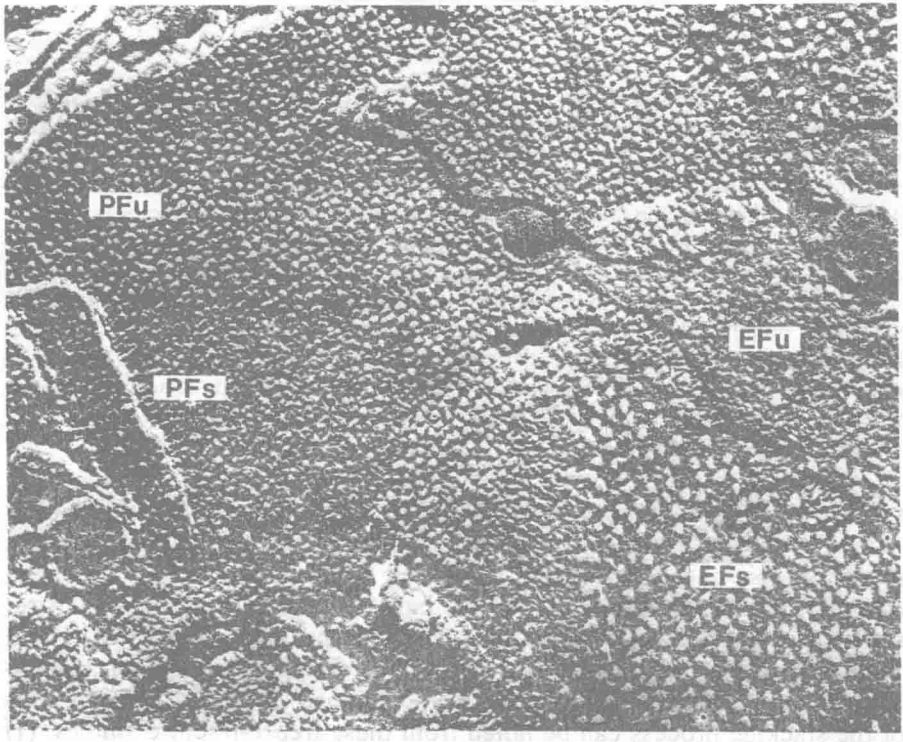

Figure 3 The four fracture faces formed by freeze-fracturing of stacked chloroplast membranes. For the details of the labeling system, see Figure 4. The particles visualized in replicas such as this represent structures revealed by membrane splitting during the fracturing process. Magnification: 103,500×.

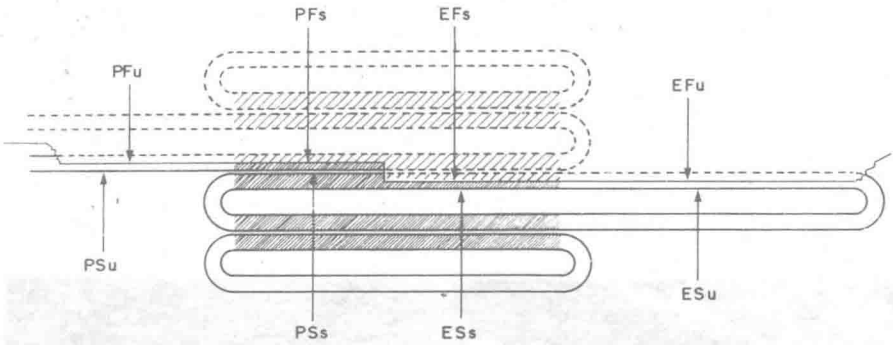

Figure 4 Four fracture faces are formed during freeze-fracturing of isolated thylakoid membranes. The membranes of a granum are shown as they might behave during freeze-fracturing. The membranes split away by the fracturing process are shown with a dotted line, while the remaining membranes are shown with solid lines. The region of membrane stacking (granum) in the center of the diagram is shaded. The fracture faces are labeled as follows: PFu, protoplasmic face, unstacked; PFs, protoplasmic face, stacked; EFs, ectoplasmic face, stacked; EFu, ectoplasmic face, unstacked. In addition, four types of membrane surface can be distinguished with this system of nomenclature, even though these surfaces cannot be observed in freeze-fractured preparations (etching, the sublimination of ice from the fractured surface, is required for that purpose): PSu, protplasmic surface, unstacked; PSs, protoplasmic surface, stacked; ESs, ectoplasmic surface, stacked; ESu, ectoplasmic surface, unstacked.

ture faces are again observable. It is of course of interest to know just what sort of molecular rearrangements this change in fracture face appearance involves. This question was first addressed by Ojakian and Satir (30), who carried out a careful study of particle behavior during unstacking and restacking in the thylakoid membrane. These authors concluded that lateral migrations of certain particle size classes were responsible for the observable changes. This finding has since been confirmed (39). Two general effects of the stacking process can be noted from these freeze-fracture studies: (1) there is a concentration of the E fracture face particle into stacked regions (see Figure 6); and (2) there is a segregation of particles on the P fracture face, with the larger particles being found in unstacked regions of the membrane and smaller particles concentrated in stacked regions (see Figure 7).

6 DEEP ETCHING OF THE PHOTOSYNTHETIC MEMBRANE

One of the principal advantages of the freeze-etching technique for the study of biological membranes is its versatility: it is possible to examine not only membrane fracture faces (in freeze-fractured samples) but also the true outer and inner surfaces of a membrane, by means of a technique known as deep etching. A sample of isolated chloroplast photosynthetic membranes is frozen in either distilled water or a very dilute (less than 10 m*M*) buffer without