Plant Physiol. (1968) 43, 1769–1780

Developmental Physiology of Bean Leaf Plastids II. Negative Contrast Electron Microscopy of Tubular Membranes in Prolamellar Bodies[1]

Albert Kahn

Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

Received March 12, 1968.

Abstract. **Proplastids and prolamellar bodies with tubular membranes were isolated from the dark grown primary leaves of bean seedlings (*Phaseolus vulgaris* L.). The combination of fluorescence microscopy and negative contrast electron microscopy provided the tentative identification of protochlorophyll holochrome as a constituent of prolamellar body membranes and new evidence for solution-filled channels within the tubular membrane systems of prolamellar bodies.**

When prolamellar bodies form in the plastids of dark-grown bean leaves, they first appear as clusters of vesicles. These vesicles then fuse into ordered groups of long tubes with few interconnections. Eventually, most internal membranes of the plastids exist as conjoined, tubular elements of paracrystalline prolamellar bodies (5, 18). The geometry of the paracrystalline stage has been described in detail from electron micrographs of thin sections (6, 7, 15, 16, 17).

The first light-elicited step in the morphological and functional transitions of tubular prolamellar body membranes into photosynthetic lamellae probably is related to protochlorophyll(ide) photoconversion to chlorophyll(ide) *a* (5, 11, 14, 18). The action spectra and energy requirements for saturating protochlorophyll(ide) conversion and potentiating the initial disordering of the tubular membranes of etiolated bean leaf prolamellar bodies are similar or identical (11, 14). It seems important, therefore, in gaining understanding of the first step in the transformation of tubular membranes into photosynthetic lamellar systems (7, 11, 14) to know at the molecular level the location and distribution of protochlorophyll(ide) in plastids with prolamellar bodies.

The association of protochlorophyll(ide) with prolamellar bodies was suggested by Boardman and Wildman (4) and Boardman and Anderson (3) through phase contrast and fluorescence microscopic observations of bean proplastids in suspensions. It seemed reasonable that the dark bodies within proplastids seen in phase contrast, which were the exclusive province of red fluorescence when irradiated with short wave blue light, were identical with the prolamellar bodies seen by electron microscopy. Klein, Bryan, and Bogorad (11), combining fluorescence and electron microscopy, obtained further evidence for the association of protochlorophyll(ide) with prolamellar bodies.

Photoreactive protochlorophyll(ide) is attached to a specific protein macromolecule, and the complex *in vitro* is called protochlorophyll holochrome (13). According to Boardman (1), the molecular weight of the complex is $6 \pm 0.5 \times 10^5$, and the diameter of an individual macromolecule is 100 to 110A. As reported here, the red fluorescence of protochlorophyll(ide) and the distinctive size of protochlorophyll holochrome permit, tentatively, the localization of the pigment-protein complex as a component of the tubular membranes of isolated prolamellar bodies by combining fluorescence microscopy and negative contrast electron microscopy.

Materials and Methods

Red kidney beans (*Phaseolus vulgaris* L.) were planted in water-saturated expanded silica and watered daily while growing in a dark chamber maintained at 26 ± 1°. All operations requiring vision were performed under a dim green safelight. After 13 to 15 days, primary, opposite leaves were harvested and weighed. From the time of grinding the leaves until the particles were fixed or observed, the preparations were maintained at 2 ± 2°.

Thirty grams of leaves were homogenized for 10 sec in 90 ml of grinding medium 0.4 M sucrose, 0.003 M $MgCl_2$, 0.1 M tricine buffer (pH 7.5) in an Omnimixer powered at 50 v. The homogenate was then stirred with a spatula and subjected to a further 10 sec of grinding at 50 v. Cheesecloth and Kleenex filtrations in succession removed leaf pieces and all cells except occasional hair cells from the homogenate. The filtrate was centrifuged at 1500

[1] This investigation was supported by NSF grant GB-2897.

rpm in two 50 ml tubes in a Sorvall SS34 rotor for 15 min, yielding Pellet I. The supernatant was discarded. The pellet contained all of the intact proplastids and some of the naked prolamellar bodies that were in the filtered leaf homogenate.

Pellet I was resuspended in 40 ml of grinding medium with the aid of a Vortex Jr. Mixer and then divided into four 10 ml samples. Two samples were maintained as dark controls, and the other 2 were irradiated for 15 min in a horizontal beam of white light at an intensity of 95 ± 10 ft-c. A 10 cm water layer was interposed between the 75W tungsten filament lamp and the suspensions, and a fan moved air over the system during the irradiation. The 4 samples were then centrifuged at 1500 rpm for 15 min to produce Pellet II. One irradiated sample and 1 dark control sample were resuspended in 2 ml each of grinding medium, and the other irradiated and dark control samples were fixed without resuspension for subsequent thin sectioning and electron microscopy.

Phase contrast and fluorescence microscopy were performed on drops of resuspended Pellet II. A system of Carl Zeiss components enabled simultaneous and independent phase contrast and fluorescence observations. For negative contrast electron microscopy, samples from the Pellet II suspensions were diluted 1:9 with 4% (w/v) sodium phosphotungstate (PTA), (pH 7.0), and processed rapidly without further precautions against light and thermal reactions. Drops of the PTA-diluted suspensions were placed on glass microscope slides for prompt phase contrast and fluorescence microscopic examination and on parlodion-coated copper mesh grids for negative contrast electron microscopy.

For thin sectioning, samples of Pellet II from both irradiated and dark control material were fixed for 1 hr at 0° in a 1:1 mixture of 1+% (v/v) glutaraldehyde and 2X grinding medium lacking $MgCl_2$. The pellets were then washed rapidly 3 times with grinding medium and fixed further at 0° for 15 min with a freshly prepared solution of 2.5% (w/v) OsO_4 made up in 0.4 M sucrose, 0.05 M tricine buffer (pH 7.5). Because OsO_4 is reduced by sucrose-tricine, the pellets were then transferred for 1 hr at 0° to 2.5% (w/v) OsO_4 made up in 0.05 M phosphate buffer (pH 7.5). From this time onward, white light was used when convenient. The final step in the fixation procedure was an overnight soak of the pellets at 4° in 1% (w/v) OsO_4 made up in 0.08 M phosphate buffer (pH 7.5). The pellets were dehydrated by passing them through a stepped series of increasing acetone concentrations. The preparations were then embedded in an Epon plastic mixture and sectioned. Sections were stained with uranyl magnesium acetate and lead citrate in succession. Electron microscopy was carried out on sectioned and negative-contrasted material with a Philips EM-200 operated at 60 kv. Magnification factors were obtained by the use of a diffraction grating replica and, where possible, from the internal standard, phytoferritin (9) with a newly determined diameter (unpublished data) of 125A.

Results

Phase Contrast and Fluorescence Microscopy. The following observations, made by light microscopy on suspensions and electron microscopy on thin sections, stem from viewing many similar preparations, including the ones described here. No photomicrographs are presented, because phase contrast pictures of prolamellar bodies are available (3), and the low intensity and fugacity of fluorescence from prolamellar bodies did not permit fluorescence photography.

Resuspended Pellet II, viewed in phase contrast, contained 3 prominent classes of proplastids, as well as naked prolamellar bodies, and smaller particles extending in size down to the limit of resolution. The majority of the proplastids were highly refractile and had smooth outlines. A second class was similar, but had jagged outlines. Neither of these 2 plastid types showed the internal dark spots characteristic of prolamellar bodies in phase contrast, but internal red spots indicating prolamellar bodies were seen by fluorescence microscopy. Both plastid types are presumed to have been intact and shrunken by hypertonicity of the suspending medium, with the difference between their internal density and that of the surrounding fluid accounting for their uniform brightness in phase contrast. The jagged profiles of the second class may have been caused by internal starch grains. Proplastids of the third class were larger, had lower refractility, and 1 or more prolamellar bodies could be discerned in each plastid. This class fitted the description of isolated proplastids given by Boardman and Wildman (4) and resembled closely Boardman and Anderson's (3) photomicrographs of proplastids.

Naked prolamellar bodies, by phase contrast and fluorescence microscopy, appeared identical with those remaining enclosed within proplastids of the third class. Some particles smaller than prolamellar bodies showed fluorescence. Few non-fluorescent particles with the same size as whole prolamellar bodies were seen.

No consistent difference between irradiated and dark control suspensions was detected, though red fluorescence sometimes seemed less in the irradiated preparations.

The dilution of the suspensions with PTA solution ruptured all of the proplastids, but at least some of the prolamellar bodies appeared intact. Equivalent tonicity reduction by dilution with water also caused lysis. Many prolamellar bodies in the PTA solution, had irregular outlines, indicating localized swelling at their peripheries. The red fluorescence of prolamellar bodies could still be stimulated, though perhaps with diminished intensity. No non-fluorescing particles in the size range of prolamellar bodies were detected in the PTA solutions.

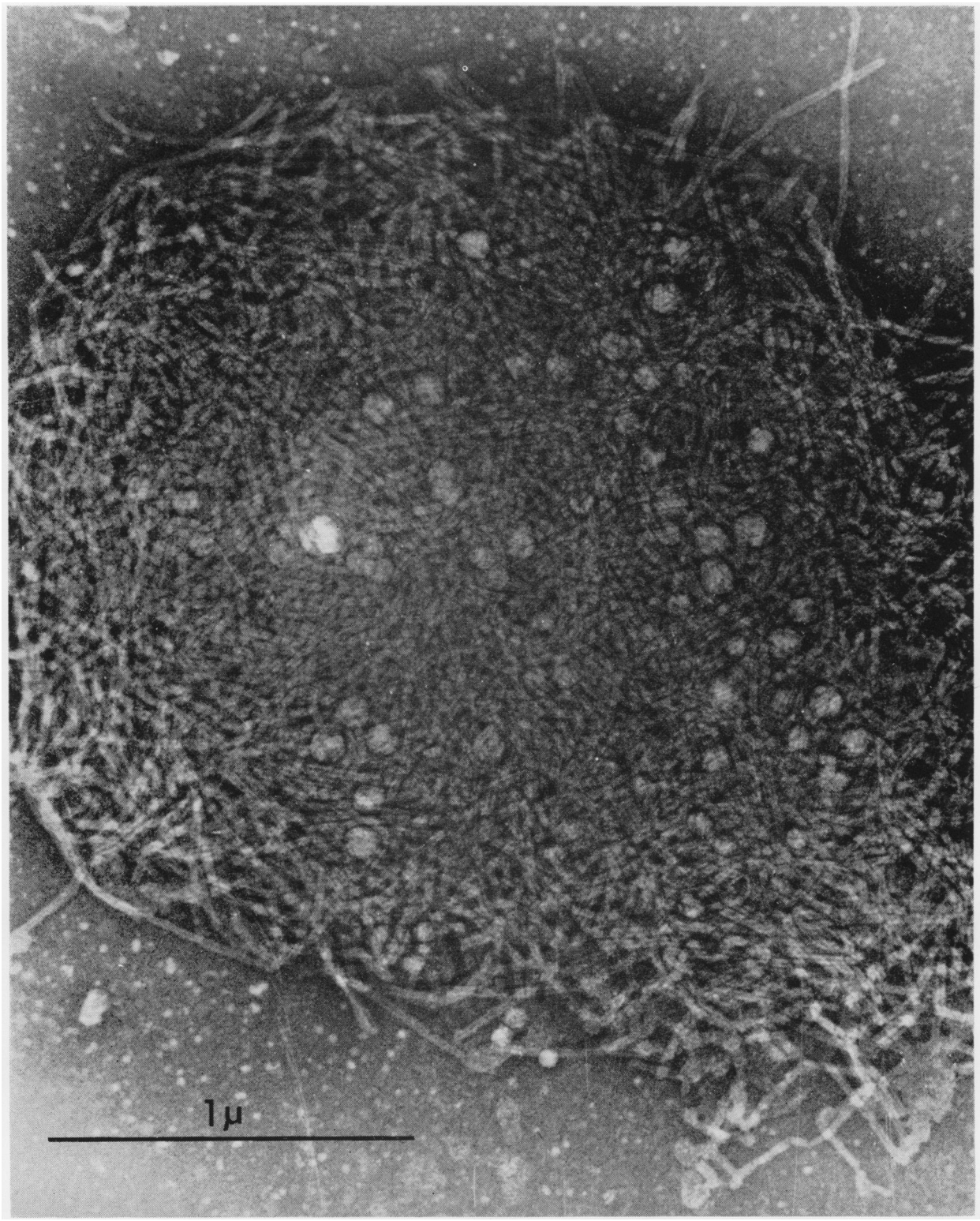

FIG. 1. Negative contrast image of the tubular membrane system of a prolamellar body. 61,000×.

Insert

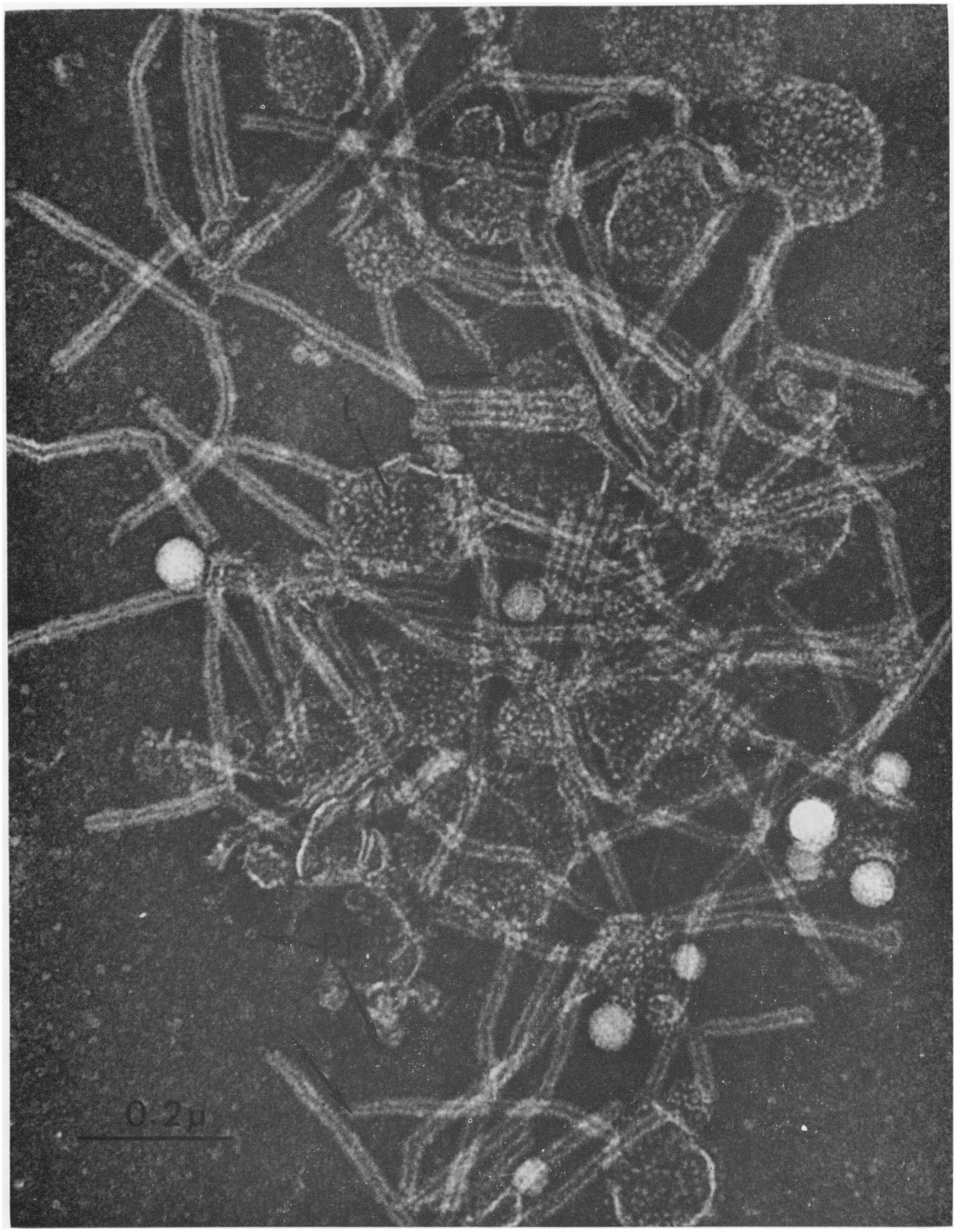

FIG. 2. Prolamellar body fragment in negative contrast showing macromolecules with a regular axial center to center spacing of 80A in the tube walls (best evident in segments denoted by 2-headed arrows) and 100A particles in non-tubular membrane segments (L). Phytoferritin (PF) provides an internal magnification standard. 130,000×.

Insert

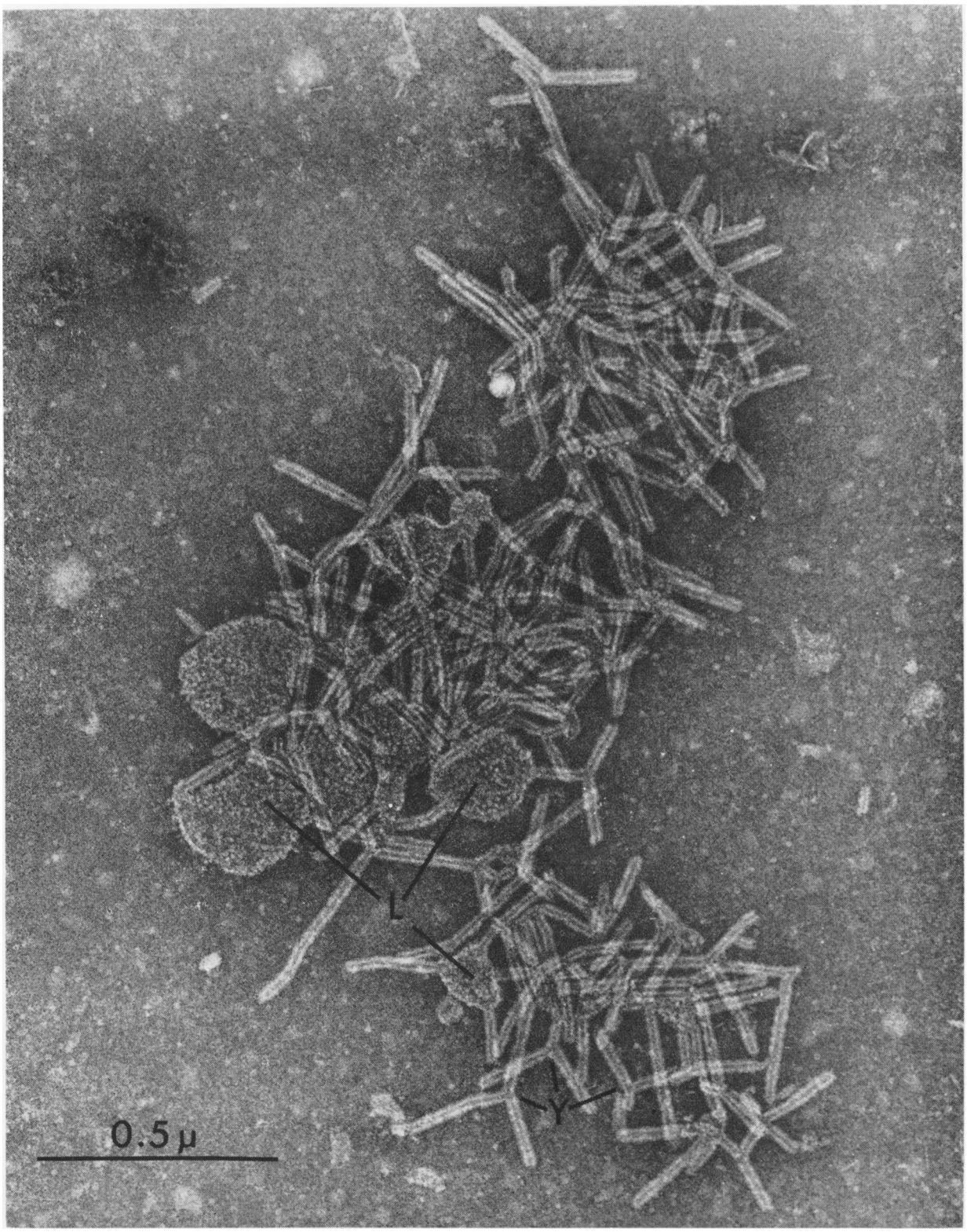

FIG. 3. Negative contrast view of a prolamellar body fragment showing several 3-membered junctions (Y) among its tubular membrane elements; 100A particles appear in the non-tubular membrane segments (L). 80,000×.

Insert

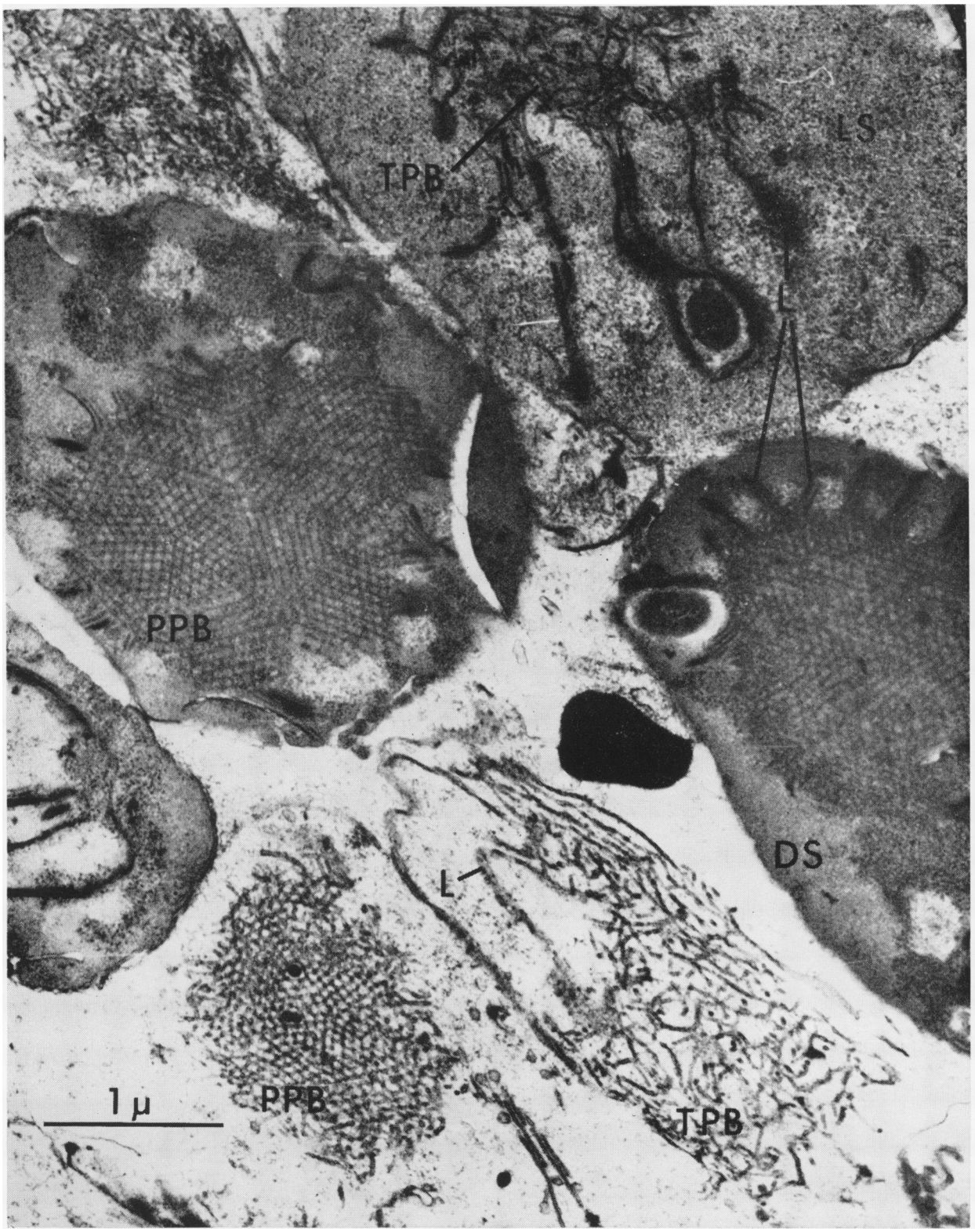

FIG. 4. Section from a pellet showing intact proplastids with stroma of high electron density (DS) and lower density (LS). Paracrystalline prolamellar bodies (PPB) and prolamellar bodies with sparsely joined tubes (TPB) are present both within and outside of proplastids. Lamellae (L) appear at the peripheries of prolamellar bodies. The images in figs 1 to 3 were gained from resuspended particles of another pellet that had been sedimented from in the same suspension as this pellet. 30,000×.

Insert

Thus, the preparations that were examined later by negative contrast electron microscopy lacked proplastids but contained prolamellar bodies and prolamellar body fragments with attached protochlorophyll(ide) or chlorophyll(ide). If any particles in the same size range as prolamellar bodies, but lacking protochlorophyll(ide) were present, they escaped detection by light microscopy.

Electron Microscopy. Isolated prolamellar bodies and fragments from the non-irradiated preparation appeared as illustrated in negative contrast in figures 1 to 3. Irradiated prolamellar bodies looked similar. The outer diameter of a tubular membrane element is about 280A. The electron dense channels of the tubes, which appear about 60 to 65A in diameter, signify penetration of PTA into the tubes. Regularly arranged macromolecules are evident in the tubular membranes (fig 2). These macromolecules seem to be close-packed with an axial center to center distance of 80A. Similar or identical macromolecules can be seen in non-tubular membrane segments which are continuous with tubular membrane (fig 2 and 3). They often are angular or elliptical in profile and have average diameters of about 100A.

Branching or junctioning among the tubular membrane elements is shown in figures 2 and 3. The Y-shaped pattern was the only mode of tube branching that was seen clearly in negative contrast. Occasionally, membrane of a junction region was non-tubular (fig 3).

For comparison with the negative contrast images, an electron micrograph of a section of Pellet II from the same experiment is presented in figure 4. The particles received no white light until after fixation with osmium tetroxide had proceeded for over 1 hr. Intact proplastids with paracrystalline prolamellar bodies are present as well as a naked prolamellar body with ordered, conjoined tubes. Some lamellae occur at the peripheries of prolamellar bodies. Because of apparent greater width when cut at low angles to their faces, such lamellae can be distinguished from tube profiles. The electron density of stroma varies as expected from the varied refractility of the proplastids when viewed by phase contrast microscopy. Prolamellar bodies at an earlier developmental stage (5, 18) also are shown. Both transverse sections and longitudinal profiles of their long tubes with sparse interconnections can be seen. In longitudinal profile the tube outer and inner diameters are approximately 255A and 60A, respectively.

No qualitative morphological difference was detected here or previously (10) between sections of unirradiated prolamellar bodies and those receiving light while in cell-free suspensions. This apparent discrepancy with an earlier report (12) will be considered separately and is not crucial here. The point is raised only to emphasize the improbability of detectable, light-dependent morphological changes in the prolamellar body membranes during light microscopy or negative staining for electron microscopy.

Discussion

The tentative identification of protochlorophyll holochrome as a constituent of the tubular membranes of prolamellar bodies rests on the following: 1) Red fluorescence was observed in isolated prolamellar bodies whose tubes contained and were surrounded by PTA solution. Thus, it is likely that the red-fluorescing protochlorophyll(ide) and its photoproduct are attached directly to the tubular membrane elements. 2) The size of the macromolecules demonstrated in prolamellar body membranes (fig 2 and 3) is consistent with the size of protochlorophyll holochrome.

Protochlorophyll holochrome has not been proved to exist as a natural complex. The possibility remains that the pigmented macromolecule characterized in solution is formed during isolation, perhaps by the binding of protochlorophyll(ide), complexed naturally with a smaller protein, to Fraction I protein which physically resembles protochlorophyll holochrome (2). The conclusion that a protochlorophyll(ide)-protein complex of some sort resides in the tubular membrane elements of isolated prolamellar bodies is not affected by this qualification. However, the tentative identification of protochlorophyll holochrome in prolamellar body membranes rests on dimensions.

The possibility of accommodating protochlorophyll holochrome in the tubular membranes can be considered using Boardman's (1) size determination of the macromolecules and by assigning 1 protochlorophyll(ide) molecule to each (2). With the tube inner diameter as 60A and the outer as 280A, the wall would have a thickness of 110A. Since the macromolecules are approximately isodiametric and have average diameters of about 100A (1), a maximum of between 5 and 6 can be fitted in a transverse profile of the tube wall. A higher number of flattened, truncated cone-shaped units could occur.

The minimum surface area of tube wall required per close-packed macromolecule would be roughly 1×10^4 A^2 in either case. Using oats, Gunning (6) and Gunning and Jagoe (7) have estimated the total surface area of tube membrane per prolamellar body (1.4 μ diam.) to be of the order of 50 μ^2 or 5×10^9 A^2. Bean prolamellar bodies and their tubular elements have dimensions that are similar to those of oat, so this estimate will be used here. Each prolamellar body could then accommodate about 5×10^5 protochlorophyll holochrome macromolecules. The etiolated bean leaves used in this study contained about 3×10^{-10} mole of protochlorophyll(ide) per leaf (unpublished data) and hence about 3×10^{-10} mole of protochlorophyll holochrome per leaf. The number of protochlorophyll holochrome macromolecules per leaf then would be about 2×10^{14}, and this many would fit in the membranes of about 4×10^8 prolamellar bodies.

Gyldenholm (8) concluded that there are 2×10^8 proplastids per etiolated bean leaf. My determina-

tions (unpublished data) using an entirely different method are in close agreement and indicate 5 to 7×10^8 proplastids per etiolated bean leaf. Proplastids can contain more than 1 prolamellar body (7), and Boardman and Wildman (4) have observed up to 4 or 5 fluorescent bodies in a single proplastid. Accordingly, the number of prolamellar bodies in a leaf is somewhat higher than the number of proplastids.

Obviously, the foregoing calculations include diverse and necessarily imprecise data. They permit, nevertheless, the suggestion that, to the nearest order of magnitude, all of the protochlorophyll holochrome that an etiolated bean leaf may contain can be accommodated spatially in the tubular membranes of prolamellar bodies.

With the assumption that all of the macromolecules demonstrated in membrane in this study have identical shape, deductions concerning their packing arrangement can be made. While many of the macromolecules show diameters of about 100A in non-tubular areas of membrane, the center to center distance of the macromolecules averages 80A in 280A diameter, undistorted tube walls (fig 2). For tubular membrane, these images are consistent with 1) helical arrangement of approximately isodiametric 100A particles in close contact or 2) annular arrangement of oriented, non-isodiametric particles. In the latter case, each particle would be oriented with its smaller diameter in the long axis of the tube membrane and its larger diameter in the transverse direction. Another orientation with respect to the membrane surface would be required for at least some particles in non-tubular membrane. The non-tubular membrane regions (fig 2 and 3) may represent distorted tubes or membrane continuous with them which swelled and collapsed during negative staining and drying. Alternatively, they may be lamellae that were already present at the peripheries of prolamellar bodies before negative staining (fig 4).

Acknowledgment

I am grateful for the skillful assistance and continued enthusiasm of Mrs. Dorothy A. Werderitsh.

Literature Cited

1. Boardman, N. K. 1962. Studies on a protochlorophyll-protein complex I. Purification and molecular-weight determination. Biochim. Biophys. Acta 62: 63–79.
2. Boardman, N. K. 1966. Protochlorophyll. In: The Chlorophylls. L. P. Vernon and G. R. Seely, eds. Academic Press, New York. p 437–79.
3. Boardman, N. K. and J. M. Anderson. 1964. Studies on the greening of dark-grown bean plants I. Formation of chloroplasts from proplastids. Australian J. Biol. Sci. 17: 86–92.
4. Boardman, N. K. and S. G. Wildman. 1962. Identification of proplastids by fluorescence microscopy and their isolation and purification. Biochim. Biophys. Acta 59: 222–24.
5. Eriksson, G., A. Kahn, B. Walles, and D. v. Wettstein. 1961. Zur makromolekularen Physiologie der Chloroplasten III. Ber. Deut. Botan. Ges. 74: 221–32.
6. Gunning, B. E. S. 1965. The greening process in plastids 1. The structure of the prolamellar body. Protoplasma 60: 111–30.
7. Gunning, B. E. S. and M. P. Jagoe. 1967. The prolamellar body. In: Biochemistry of Chloroplasts. Proc. of a NATO Adv. Study Inst., Aberystwyth, 1965. T. W. Goodwin, ed. Academic Press, New York. Vol. II. p 655–76.
8. Gyldenholm, A. O. 1967. Macromolecular physiology of plastids. V. On the nucleic acid metabolism during chloroplast development. Hereditas 59: 142–68.
9. Hyde, B. B., A. J. Hodge, A. Kahn, and M. L. Birnstiel. 1963. Studies on phytoferritin. I. Identification and localization. J. Ultrastruct. Res. 9: 248–58.
10. Kahn, A. 1966. Developmental physiology of bean leaf plastids. 1. Preliminary studies on isolated proplastids. In: Sixth Intern. Congr. Electron Microscopy, Kyoto, 1966. R. Uyeda, ed. Maruzen Co., Ltd., Tokyo. Vol. II. p 381.
11. Klein, S., G. Bryan, and L. Bogorad. 1964. Early stages in the development of plastid fine structure in red and far-red light. J. Cell Biol. 22: 433–42.
12. Klein, S. and A. Poljakoff-Mayber. 1961. Fine structure and pigment conversion in isolated etiolated proplastids. J. Biophys. Biochem. Cytol. 11: 433–40.
13. Smith, J. H. C. and D. W. Krupke. 1956. Some properties of extracted protochlorophyll holochrome. Nature 178: 751–52.
14. Virgin, H. I., A. Kahn, and D. v. Wettstein. 1963. The physiology of chlorophyll formation in relation to structural changes in chloroplasts. Photochem. Photobiol. 2: 83–91.
15. Wehrmeyer, W. 1965. Zur Kristallgitterstruktur der sogenannten Prolamellarkörper in Proplastiden etiolierter Bohnen I. Pentagondodekaeder als Mittelpunkt konzentrischer Prolamellarkörper. Z. Naturforsch. 20b: 1270–78.
16. Wehrmeyer, W. 1965. Zur Kristallgitterstruktur der sogenannten Prolamellarkörper in Proplastiden etiolierter Bohnen II. Zinkblendegitter als Muster tubulärer Anordnungen in Prolamellarkörpern. Z. Naturforsch. 20b: 1278–88.
17. Wehrmeyer, W. 1965. Zur Kristallgitterstruktur der sogenannten Prolamellarkörper in Proplastiden etiolierter Bohnen III. Wurzitgitter als Muster tubulärer Anordnungen in Prolamellarkörpern. Z. Naturforsch. 20b: 1288–96.
18. Wettstein, D. v. and A. Kahn. 1960. Macromolecular physiology of plastids. A. L. Houwink and B. J. Spit, eds. Proc. European Regional Conf. Electron Microscopy Delft. Vol. II. p 1051–54.