LOCALIZATION OF CHLOROPHYLL IN SPINACH CHLOROPLAST LAMELLAE BY FLUORESCENCE MICROSCOPY

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INTRODUCTION

Chlorophyll in green plants is thought to reside in the internal membranes or lamellae of chloroplasts (1). These membranes are observable by electron microscopy and can be roughly grouped, on the basis of size and arrangement, into grana lamellae and stroma lamellae which exist as closed, flattened sacs, or thylakoids (2). Although the chlorophyll molecule is not yet demonstrable by electron microscopy, it is readily detected by fluorescence microscopy (3–5). The deep red fluorescence of chlorophyll, observed by Sir David Brewster in 1833, was used to advantage in 1924 by Francis E. Lloyd (6) who found the chloroplasts of higher plants to be the source of the fluorescence, and again in 1962 by Spencer and Wildman (7) who further restricted the red fluorescence to the chloroplast grana.

The technique described here permits observation of the same specimen by fluorescence and electron microscopy, yielding precise information on the distribution of chlorophyll in the membranes through a direct comparison of ultrastructure and fluorescence.

MATERIALS AND METHODS

Store spinach was ground in a cold Waring blendor for 30 sec in a grinding medium composed of 0.5 M sucrose and 0.1 M $(K)PO_4$ buffer pH 7.5. This slurry was squeezed through 8 layers of cheesecloth and was centrifuged at 470 *g* for 5 min in the cold. The precipitate from this sedimentation was discarded and the supernatant was centrifuged at 900 *g* for 15 min. This precipitate was resuspended in grinding buffer and then centrifuged again at 900 *g*. Osmotic rupture was accomplished by centrifugally washing the resuspended pellet in distilled water twice at 15,000 *g*.

A drop of the chloroplast material, diluted to a pale green color with distilled water, was placed on a Formvar-covered locator grid and dried after removal of the excess liquid with an absorbent paper. The grid was then placed in a nitrogen-filled dry-box, allowed to equilibrate with the nitrogen atmosphere, and carefully placed, coated surface downwards, in a drop of deoxygenated water on a glass coverslip, which was then upturned on a microscope slide. The wet, covered grids were then quickly transferred to the fluorescence microscope and photographed.

Actinic light was provided by a Zeiss fluorescence source, using an Osram HBO 200 high pressure mercury vapor lamp. Blue and purple cut-off filters mounted in front of the source permitted only light of less than 500 mμ to reach the specimen. The microscope used was a Tiyoda trinocular research microscope equipped with an oil immersion objective and a Bessler Topcon 35-mm camera back. A red cutoff filter, passing light of greater than 630 mμ (Corning 2-58), was mounted in the tube of the microscope. In order to minimize fluorescence of the red filter itself, a second filter was mounted below the first, removing all light of less than 520 mμ. (Optical Coating Labs., Inc., Santa Rosa, California, dielectric rejection filter). The fluorescence image was recorded on Kodak High Speed Infrared Film HIR 417.

The success of the technique depends on the brilliance and duration of the fluorescence, the focus of the light microscope, and the ability of the chloroplast fragments to adhere to the Formvar film throughout the procedure. Adequate fluorescence may be insured by excluding all oxygen from the specimen, and by working quickly to keep diffusion of air under the coverslip to a minimum. Thorough drying of the grids before microscopy will prevent loss of material during manipulation. Under these conditions, exposures of between 2 and 3 sec at a magnification of 2,000 will suffice to photographically record the chlorophyll fluorescence of a single membrane thickness.

After a chosen area of the grid was photographed on the fluorescence microscope, the coverslip was carefully floated off with distilled water and the grid was removed, dried, and shadowed with chromium-nickel in a Mikros vacuum evaporator. Electron microscopy was performed in a Siemens Elmiskop I operating at 40 kv. Photographs of identical areas obtained by electron and fluorescence microscopy were then enlarged and compared.

RESULTS AND DISCUSSION

Fig. 1 is a fluorescence micrograph of chloroplast fragments spread on a Formvar film. Fig. 2 is an

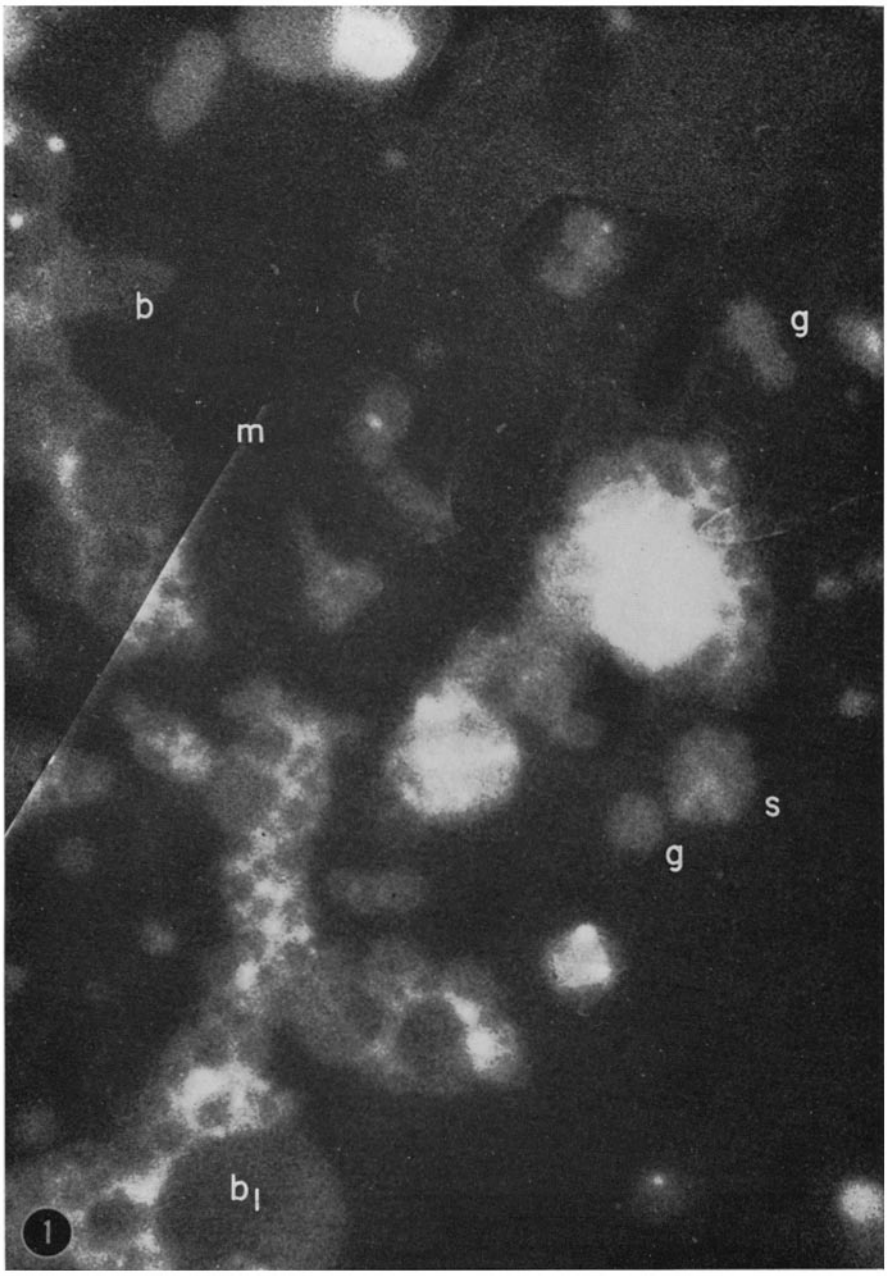

FIGURE 1 A montage of two fluorescence micrographs of chloroplast membranes on Formvar film. The line at *m* is a result of the montage. × 3,000.

electron micrograph of the same fragments after drying and shadowing. These illustrations correspond exactly and show that all the membranes visible in the electron micrograph contain approximately equal amounts of chlorophyll. Careful examination shows that the intensity of fluorescence is fairly constant and that variations can usually be correlated with variations in the number of superposed membranes. In some cases, a single thylakoid, or membrane sac, is ruptured in

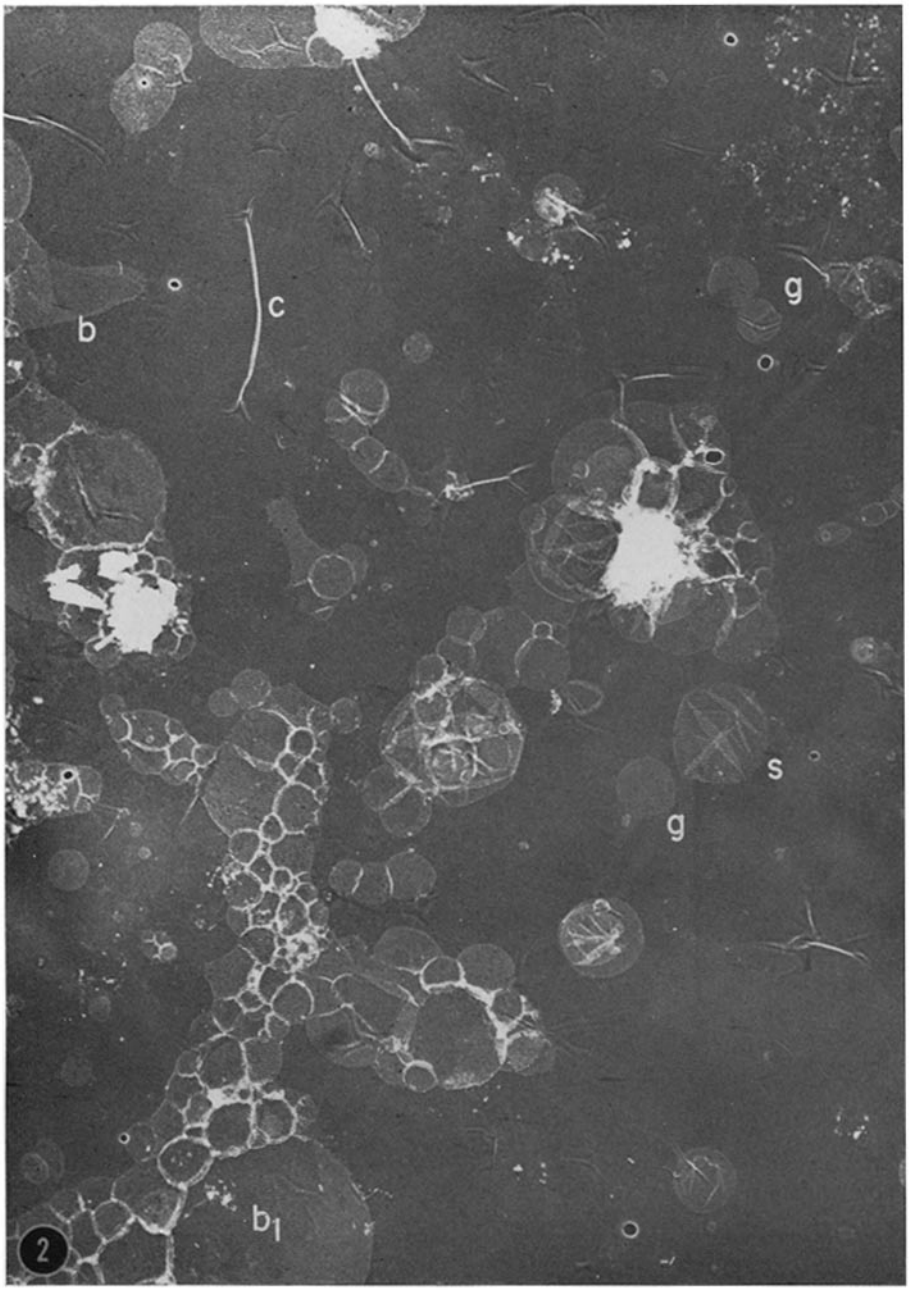

FIGURE 2 Electron micrograph of the same area of the Formvar film shown in Fig. 1. Line at *c* is an example of a fold in the Formvar. × 3,000. Folds in the membrane *s* seen here are also visible in Fig. 1. Areas labelled *b* and b_1 are only one membrane thick and show a decrease in fluoresecnce relative to adjacent areas, as explained in text. Membranes such as *s*, which measure approximately 5 μ in diameter, are probably too large to be considered grana lamellae.The membrane shown at b_1, being larger than 9 μ across, may be a limiting membrane of a chloroplast. Membranes equal to or less than "g" in size are probably grana lamellae.

such a way that only a single membrane thickness remains; these areas show a corresponding decrease in intensity of fluorescence, verifying the observation that the chlorophyll is, in fact, membrane-bound and not merely held captive within the thylakoid. Although distortion of the membranes during osmotic shock might be expected to affect the intensity of fluorescence, it seems unlikely that this distortion would relocate chlorophyll molecules from isolated sites (grana) to the completely uniform distribution we have observed.

Since the isolation procedure can be shown to isolate some whole chloroplasts with limiting membranes, and since all membranes regardless of size observed by this technique contain chlorophyll, it may be concluded that certainly the grana lamellae and stroma lamellae, and possibly the innermost limiting membrane, all contain chlorophyll.

Observation of the fluorescence of whole chloroplasts by Spencer and Wildman (7) showed strong fluorescence from the grana regions and little or none from the intergranum regions. They concluded that the chlorophyll was restricted to the grana. We feel that the technique described here has demonstrated that chlorophyll is uniformly distributed throughout the chloroplast lamellae regardless of size, and that the intense fluorescence of the grana regions of whole plastids is due to the large number of appressed membranes rather than a restriction of the chlorophyll to one membrane system.

Anticipated uses of this technique include the study of developing and senescing plastids to determine whether the distribution of chlorophyll in the lamellae is uniform throughout the life cycle of the plastids.

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REFERENCES

1. PARK, R. B., and PON, N. G., *J. Molec. Biol.*, 1961, **3,** 1.
2. MENKE, WILHELM, *Z. Naturforsch.*, 1961, B **16,** 334.
3. ANDERSON, J. M., and BOARDMAN, N. K., *Austral. J. Biol. Sc.*, 1964, **17,** 86 and 93.
4. STERN, K., *Ber. bot. Ges.*, 1920, **38.** 28.
5. EPSTEIN, H., and SCHIFF, J. A., *J. Protozool.*, 1961, **8,** 427.
6. LLOYD, F. E., *Science*, 1924, **59,** 241.
7. SPENCER, D., and WILDMAN, S. G., *Austral. J. Biol. Sc.*, 1962, **15,** 599.