PHOTOOXIDATION OF DIAMINOBENZIDINE (DAB) BY CHLOROPLAST LAMELLAE

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INTRODUCTION

The introduction of diaminobenzidine (DAB) into ultrastructural cytochemistry in 1966 by Graham and Karnovsky (1) for demonstrating horseradish peroxidase activity was an important milestone. Its usefulness has been extended to demonstration of mitochondrial cytochrome oxidase via cytochrome *c* by Seligman et al. (2) in 1968, and to demonstration of peroxisomes of kidney (3–5) and peroxisomes of the leaf (6). It has been shown that DAB is oxidized to a water-insoluble, lipid-insoluble, osmiophilic polymer which reliably localizes the sites of enzymatic activity (1, 2). Since DAB is a good electron donor in the mitochondrial succinoxidase system of animal tissue, it was of interest to see whether it would also act as an electron donor in some part of the chain of electron transport in photosynthesis, making it possible to study the localization of this reaction in ultrastructural preparations. The publication in 1969 of papers (7, 8) reporting that benzidine may be used as an electron donor in photoreactions supported our experiments. These have consisted of showing that DAB is oxidized by illuminated chloroplasts and that following osmication, the sites of oxidation on the membranes of the chloroplasts may be studied in the electron microscope. In the absence of light only mitochondria of the plant oxidize DAB.

MATERIALS AND METHODS

Spinach and *Elodea* leaves were used. With spinach, small segments, 1 × 2 mm, were fixed for 20 min at 0°–2°C in 1% glutaraldehyde (dissolved in 0.01 M phosphate buffer, pH 7.0). After fixation the segments were rinsed for 15 min at 0°–2°C in phosphate-sucrose buffer (0.05 M phosphate buffer, pH 7.0, containing 2.5% sucrose). The incubation medium consisted of 0.05 M phosphate buffer (pH 7.0), 2.5% sucrose, and DAB, 0.5 mg/ml. The incubation was carried out at room temperature in small vials illuminated through bottles of water by two 150 w reflector bulbs for 1 hr. With *Elodea*, pieces of stem carrying four to five leaves were fixed in 4% depolymerized paraformaldehyde at 0°–2°C for 15 min. The leaves were rinsed in tap water for 15 min, incubated in DAB (0.5 mg/ml dissolved in tap water and adjusted to pH 7.0), and illuminated at room temperature for 1 hr. The leaves were rinsed for 20 min in phosphate-sucrose buffer (spinach) or tap water (*Elodea*), then osmicated in a 2.5% solution of osmium tetroxide for 1 hr at room temperature. They were dehydrated through alcohols and embedded in Epon 812 (9). Thin sections were cut from the edge of the tissue blocks. Unstained sections were examined with an RCA EMU 4B electron microscope.

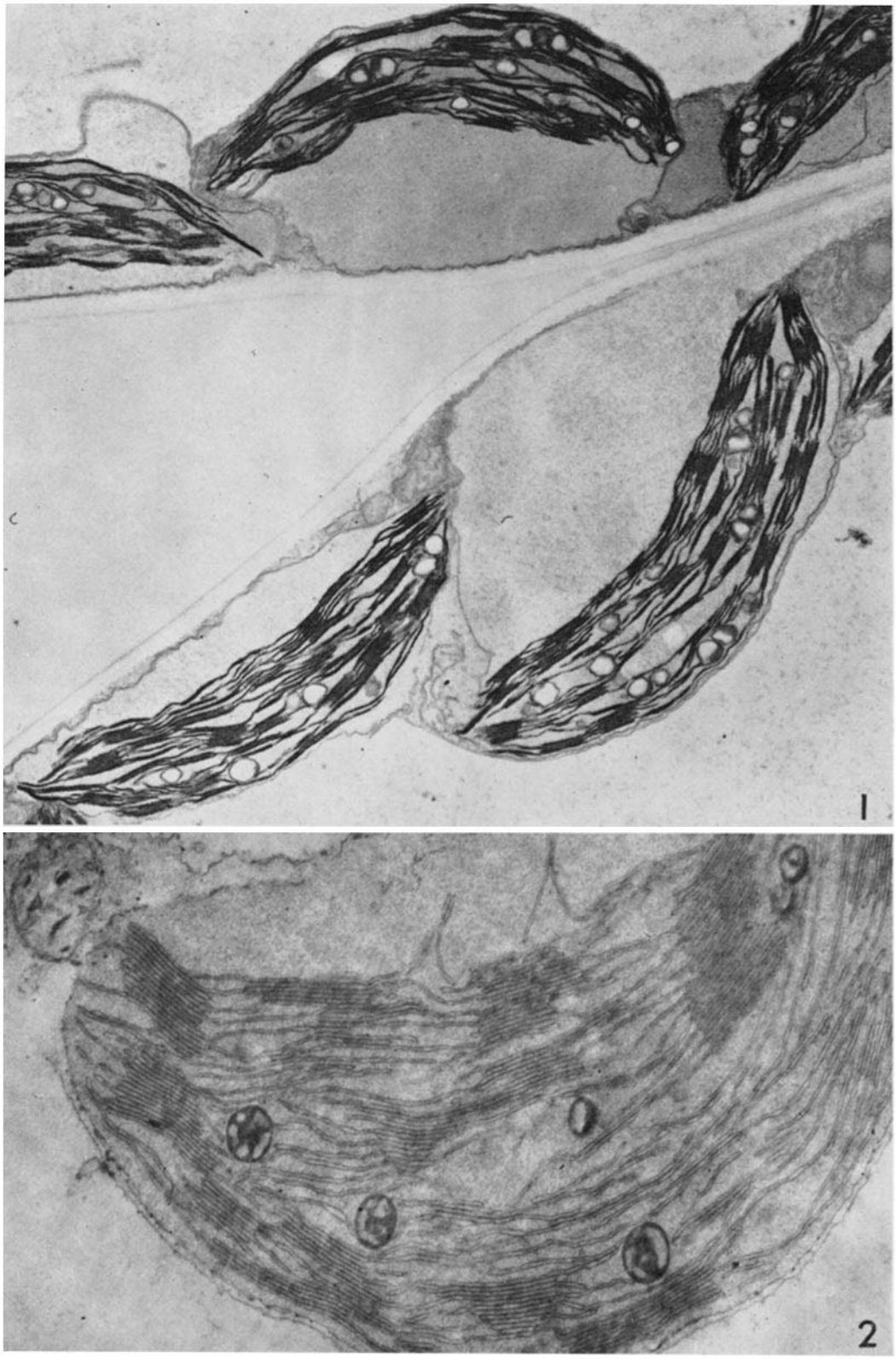

FIGURE 1 Photoreaction in spinach leaves with DAB. Note contrast due to photoreaction in the membranes of the chloroplast. These membranes include the grana and the stroma membranes, but not the outer membrane. × 12,000.

FIGURE 2 Incubation of spinach leaves with DAB in the dark. Note absence of photo-reaction in chloroplast, and note mitochondrial intracristate staining due to cytochrome oxidase activity (upper left). × 40,000.

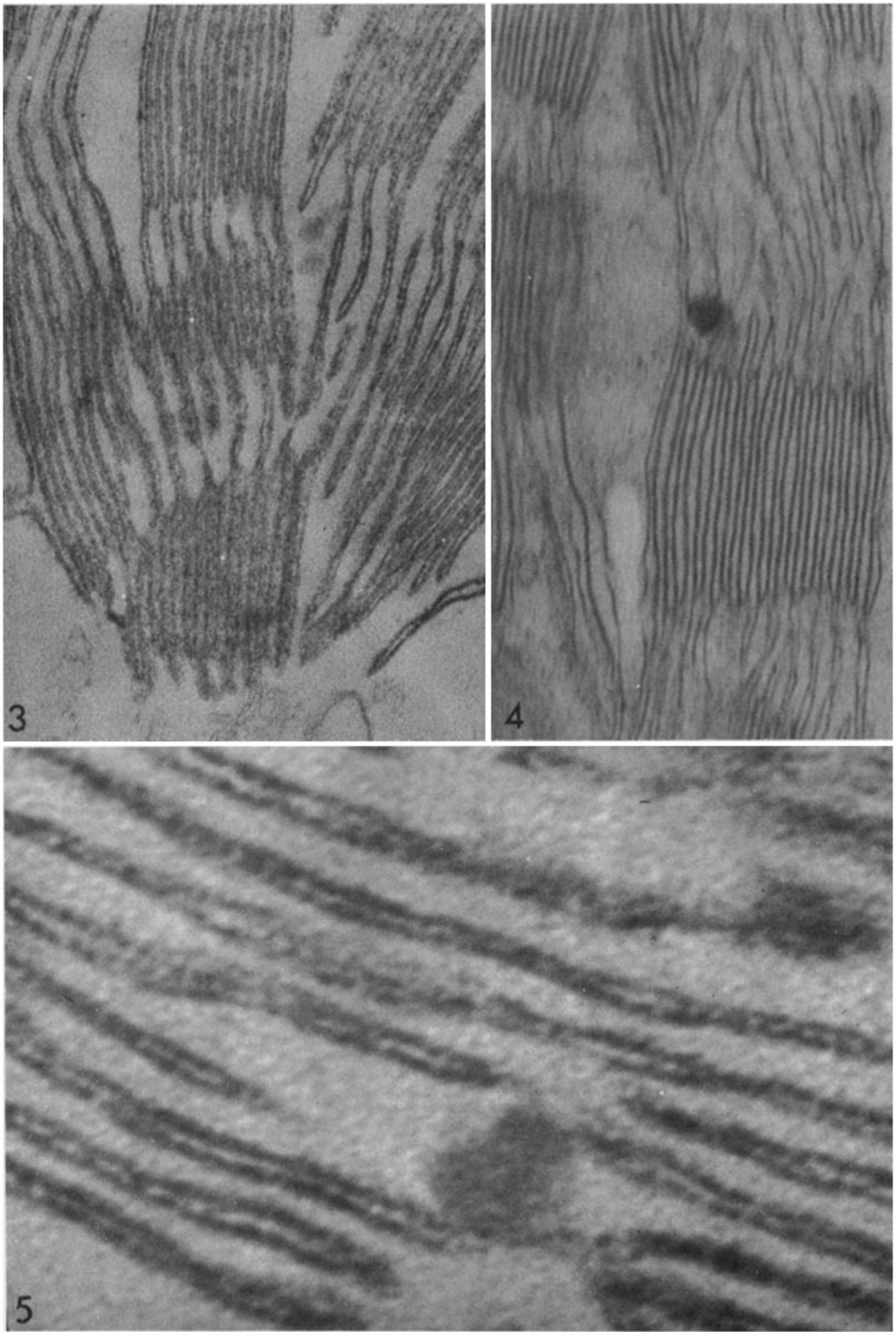

FIGURE 3 Photoreaction with DAB in *Elodea* leaf. Reaction product may be seen on the grana and stroma membranes. Compare with control in Fig. 4. × 62,000.

FIGURE 4 Incubation of *Elodea* leaf with DAB in the dark. Note absence of deposits on chloroplast membranes. × 62,000.

FIGURE 5 Same as Fig. 3. The deposits on the membranes are not uniform, but are interrupted, suggesting periodicity. × 165,000.

RESULTS AND DISCUSSION

Exposure of leaves to light in the presence of DAB caused visible darkening. After osmication further blackening occurred and the membranes of the chloroplast became very electron opaque. These membranes included the grana and the stroma membranes, but not the outer membrane (Fig. 1). When leaves were incubated with DAB in the dark, no darkening was visible; under the light microscope, chloroplasts were not stained and the membranes of the chloroplast did not show increased electron opacity (Figs. 2 and 4). Only mitochondrial intracristate staining, due to cytochrome oxidase activity, was observed (see Fig. 2 and compare with mammalian mitochondria, reference 2). In light-activated chloroplasts, the polymerization product of oxidized DAB was located on the thylakoid membrane (Figs. 3 and 5). At high magnification (Fig. 5), the deposit on the membrane was not uniform, but was interrupted, suggesting periodicity. These results support biochemical evidence about the unique relation of the photoreaction sites and the chloroplast membranes. Since some evidence has been provided (7, 8) that benzidine can supply electrons at the level of photosystem II, it could be presumed that diaminobenzidine (DAB) demonstrates the sites of this part of the photoreaction. However, benzidine does not give the visible changes noted with DAB. Furthermore, we observed that 2×10^{-4} M 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), an inhibitor of photosystem II (8, 10), did not appear to inhibit the photooxidation of DAB in light and electron microscopic preparations. Therefore, more evidence is required to determine in which photosystem the reaction with DAB occurs.

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