

## THE SITE OF FERRICYANIDE PHOTO- REDUCTION IN THE LAMELLAE OF ISOLATED SPINACH CHLOROPLASTS: A CYTOCHEMICAL STUDY

D. O. HALL, H. EDGE AND M. KALINA

*Department of Botany, University of London, King's College,  
68 Half Moon Lane, London, S.E. 24, England, and  
Department of Cell Biology and Histology, Tel Aviv Medical School,  
University of Tel Aviv, Israel*

---

### SUMMARY

Ferricyanide was used as a Hill oxidant to localize the site of photoreduction in chloroplast lamellae. The ferrocyanide formed on illumination was complexed with copper ions to form insoluble, electron-dense precipitates of Cu ferrocyanide, which can be easily seen in unstained preparations in the electron microscope. Control experiments showed no precipitates in the dark, at zero time, in the absence of ferricyanide, or on addition of sodium ascorbate. Discrete precipitates of Cu ferrocyanide were seen on both stroma and grana lamellae. It is concluded that both stroma and grana lamellae have photosystem II activity necessary for reduction of ferricyanide.

### INTRODUCTION

In the last 2 years considerable effort has been expended in trying to establish the location of the membrane particles which catalyse photosynthetic electron transport in chloroplasts. The electron transport chain is thought to comprise photosystem II (long-wavelength system) where water is split to give oxygen and electrons, and photosystem I (short-wavelength system) where the electron's energy is raised in order to reduce NADP (see review of Avron & Vernon (1965) for the electron transport scheme and terminology used in this paper). Jacobi & Lehman (1969), and Sane, Goodchild & Park (1970) have claimed that the stroma lamellae of the chloroplast do not contain photosystem II but only photosystem I, while the grana lamellae (or thylakoids) of the granal stacks contain both photosystems I and II. If this were so we would not expect to see the reduction of ferricyanide (a Hill oxidant, i.e. electron acceptor, requiring collaboration of both photosystems I and II for reduction) in the stroma lamellae. In the present cytochemical study with isolated spinach chloroplasts we do, however, demonstrate the reduction of ferricyanide in the stroma lamellae, in addition to the grana lamellae, and conclude that photosystem II is indeed present in both stroma and grana lamellae.

In order to demonstrate the site of photoreduction in chloroplast lamellae a Hill oxidant should be used which will not migrate once it has been reduced. The reduced product must then be detectable in the electron microscope as an electron-dense

deposit. Kalina, Weavers & Pearse (1969) adapted the method of Kerpel-Fronius & Hajos (1968) in a study of the site of succinic dehydrogenase localization in mitochondria. They used ferricyanide as the terminal electron acceptor and the ferrocyanide formed was complexed with copper ions to produce an insoluble copper ferrocyanide complex at the site of reduction, with no readily detectable migration from this site. The Cu ferrocyanide particles are electron-dense (Ogawa, Saito & Mayahara, 1968; Beasley & Milligan, 1969) and can be easily seen in the electron microscope without further staining. Since ferricyanide is an excellent and universally used Hill oxidant in chloroplast studies, we have been able to use this technique to localize the site of photoreduction in the chloroplast.

There have been previous cytochemical studies of chloroplasts which used the photoreduction of tetrazolium dyes both *in vivo* and *in vitro*. Howell & Moudrianakis (1967), using the tetrazolium salt, iodo-nitro tetrazolium (INT), to demonstrate the photoreducing site in chloroplasts concluded that there was no localized reducing site, since the insoluble formazan produced was evenly spread over the membrane surface ('the entire membrane appeared to participate uniformly in the deposition of the stain'). However, further work by Shumway & Park (1969) with three different tetrazolium salts showed that tetrazolium reduction is unacceptable for the resolution of the Hill reaction site since considerable migration of the formazan occurred before precipitation. With INT the 'resulting formazan yielded filamentous strands which extended for micron distances away from the chloroplast internal membranes'. Using tetranitro-blue tetrazolium (TNBT), which was not included in Shumway and Park's investigation, Weier, Stocking & Shumway (1967) noted the deposition of the light-reduced diformazans in the middle of the partition, i.e. between adjacent lamellae in the grana stacks, of *in vivo* and isolated chloroplasts. In the experiments of Weier *et al.* (1967) it seems as if there was uniform deposition of the diformazans on the lamellae and thus one could conclude that the sites of TNBT reduction were distributed along the surface of both grana and stroma lamellae. In our work with ferricyanide we attempted to localize discrete sites of photoreduction on the chloroplast lamellae. However, due to the large size of the Cu ferrocyanide deposits and the problems of migration of product and penetration of reactants we were unable to draw any definite conclusions as to the presence or absence of discrete sites of photoreduction on individual lamellae.

#### MATERIALS AND METHODS

The photochemical reduction of ferricyanide by isolated spinach chloroplasts was followed by complexing the ferrocyanide formed with Cu; the resulting Cu ferrocyanide complex was electron-dense and could be observed in the electron microscope in unstained sections.

#### *Chloroplast preparations*

One hundred grams of freshly picked, young, greenhouse-grown, spinach leaves (variety Goliath) were homogenized for 10 s in an 800-ml MSE Atomix Blender in 200 ml of medium consisting of 0.4 M sucrose, 0.01 M sodium chloride and 0.05 M tricine (*N*-tris (hydroxymethyl) methylglycine), pH 7.5. The homogenate was passed through 8 layers of cheesecloth and the filtrate centrifuged at 1500g for 2 min in an MSE Super Minor centrifuge. The pellet was

gently resuspended in 50 ml of 0.4 M sucrose and centrifuged as before. The chloroplasts were finally resuspended in 0.4 M sucrose to give a chlorophyll concentration of 1 mg/ml (measured according to Arnon, 1949). All procedures were carried out at 0–4 °C.

### *Reaction medium*

Ferricyanide reduction was performed in a modified Kerpel-Fronius & Hajos (1968) medium containing the following final concentrations of reagents: sodium-potassium tartrate, 0.3 M; sucrose, 0.08 M; Sorensen's phosphate buffer, pH 7.6, 15 mM; copper sulphate, 20 mM; potassium ferricyanide, 2 mM. Chloroplasts equivalent to 1 mg chlorophyll were added to the reaction mixture in a final volume of 5 ml. Sodium ascorbate, 5 mM, and dichlorophenyl-dimethylurea (DCMU),  $10^{-5}$  M (added in 50  $\mu$ l methanol), were added where indicated. Tartrate was used as the chelator of Cu in the reaction mixture.

### *Incubation*

Incubation was carried out in glass test tubes. The reaction was initiated by turning on the light source, which consisted of a 150-W tungsten spot lamp to give a light intensity of approximately 9000 lx at a distance of 18 in. (45.6 cm). Incubation was carried out at 20 °C for times up to 10 min. At the end of the experiments the chloroplasts were rapidly centrifuged down in the dark at 0 °C, washed once with 10 ml of 0.4 M sucrose in 0.1 M phosphate buffer (pH 7.6), recentrifuged, and the pellet was then fixed for 2–3 h in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.6), washed in 0.4 M sucrose in 0.1 M phosphate buffer (pH 7.6), and then resuspended in 2% osmium tetroxide containing 0.33 M sucrose and 0.1 M phosphate buffer (pH 7.6) for 1 h at 0 °C. The fixed chloroplast suspension was collected by centrifugation and the pellet was resuspended in 10 ml of 0.1 M phosphate buffer (pH 7.6). Thereafter the chloroplast pellet was dehydrated in an ethanol series and embedded in Araldite.

Control experiments included (a) incubation in the dark, (b) omission of ferricyanide from the incubation medium, (c) the addition of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), and (d) the addition of sodium ascorbate to the reaction mixture after ferricyanide and chloroplasts had been added (the addition of ascorbate immediately reduced all the ferricyanide non-enzymically and the resulting ferrocyanide complexed with copper to give a heavy brown precipitate suspended in the chloroplast reaction mixture). The controls were all processed under the same conditions as the other reaction mixtures.

The series of experiments was repeated 4 times, giving similar results on each occasion.

### *Chloroplast pretreatment*

There are indications that penetration of the reactants into the chloroplast lamellae may present some problems. We have therefore tried the effects of (a) short fixation with glutaraldehyde and (b) preincubation with the reactants. The experimental details are described below; all procedures were carried out at 0 °C.

(a) The chloroplasts were resuspended in 0.4 M sucrose plus 3% glutaraldehyde for 2 min, centrifuged at 2000g for 2 min and then resuspended in 0.4 M sucrose to wash out the excess glutaraldehyde. The chloroplasts were left to stand for 1 h, recentrifuged and then resuspended in 0.4 M sucrose. Hallier & Park (1969) have shown that glutaraldehyde-fixed spinach lamellae retain both photosystem I and II activity.

(b) A portion of the chloroplast preparation was suspended in 0.4 M sucrose containing 0.3 M sodium potassium tartrate in 0.1 M phosphate buffer, pH 7.6, and 0.02 M copper sulphate for a period of approximately 15 min. These chloroplasts were then added directly to the reaction mixture in the test tubes.

### *Electron microscopy*

The Araldite blocks were sectioned on an LKB Ultramicrotome, Mark III, and viewed in an AEI EM 6B electron microscope, either stained only with OsO<sub>4</sub> or with OsO<sub>4</sub> and post-stained with lead citrate. The microscope has a double condenser system and a liquid nitrogen decontamination accessory; it was operated at 60 kV with an objective aperture of 25  $\mu$ m.

## RESULTS

A short incubation time of 30 s in the light was sufficient for the appearance of copper ferrocyanide precipitates (approximately 15.0 nm in diameter) on the chloroplast membranes (Figs. 1-4). When the incubation time was prolonged up to 10 min, more sites of Cu ferrocyanide deposition appeared on the membranes and there was an increase in the size of the electron-dense reaction products (Fig. 7 for 3-min incubation, approximately 30.0 nm diameter; Figs. 8-10 for 10-min incubation, approximately 60.0 nm diameter). At 10 min most of the grana and inter-grana (or stroma) lamellae appeared to contain the precipitates. This accumulation of Cu ferrocyanide was distinctly visible as a change in the colour of the chloroplast reaction mixture to a muddy, green-brown during the incubation.

The structural variability of the chloroplasts in the preparation, as has been noted by Weier *et al.* (1968) and Walker (1970), was reflected in the varying reactivity of the different types of chloroplasts (Figs. 1, 2). Relatively intact chloroplasts often contained little or no reaction product, whereas broken chloroplasts in the same preparation contained much more reaction. However, after prolonged incubation all of the chloroplasts, including the better-preserved ones, showed copper ferrocyanide deposits on all of their lamellae, including stroma and grana lamellae (Figs. 8-10). Similar variability in the reactivity of mitochondria has been noted in the experiments of Kerpel-Fronius & Hajos (1968) and Ogawa *et al.* (1968). Shumway & Park (1969) also noted considerable variability in chloroplast reactivity in their tetrazolium photoreduction experiments.

A relatively short incubation time of up to 3 min resulted in deposits of reaction product mainly on stroma lamellae (Figs. 2, 3, 6). Often precipitate accumulated on membranes around the periphery of relatively intact grana stacks (Fig. 11). However, the more expanded grana lamellae (Figs. 4, 6) showed more reactive sites than the closely stacked grana lamellae. Prolonged incubation resulted in an increased deposition of Cu ferrocyanide on the grana lamellae (Figs. 9, 10), probably as a result of the increasing penetration of the reactants into the grana membranes as they swelled during incubation in the reaction mixture.

The reaction products appeared initially as an electron-dense precipitate localized on the surface of individual membranes (Figs. 7, 11). Prolonging the incubation resulted in an increase in size of the copper ferrocyanide precipitate which then tended to deposit between the individual membranes, i.e. in the grana loculi (compartments) of the grana stacks or the fret channels of the stroma lamellae (for terminology see Weier & Benson, 1967). This deposition of reaction product between adjacent membranes has been seen in mitochondrial cristae by Kalina *et al.* (1969). In some experiments we noted a precipitate which consisted of elongated, 'spiky' structures appearing only in the amorphous stroma area; this non-specific precipitate appeared quite different from the specific reaction product formed on the membranes which always increased in size with longer times of incubation.

When electron micrographs of glutaraldehyde-fixed chloroplast preparations (Fig. 10) or of chloroplasts pretreated in the copper sulphate and sodium-potassium

tartrate solution were compared with those treated and incubated as described earlier, no differences were noted.

### *Control experiments*

Chloroplasts which had been incubated for up to 10 min in the dark (Figs. 5, 13) and chloroplasts placed in the reaction medium and immediately centrifuged out (zero time incubation, Fig. 12) were either completely devoid of any deposits or showed only very few sites of deposition. The few deposits sometimes found in the controls were thought to be due to the presence of different amounts of endogenous reducing reagents. Addition of ascorbate to the incubation medium, after the chloroplasts had been added, caused a heavy precipitate of copper ferrocyanide suspended in the medium, as noted previously. This non-specific precipitate was easily removed by the washing with 0.4 M sucrose. Under these circumstances no precipitate was found in the chloroplast lamellae themselves (Fig. 14), indicating that the deposition of the reaction product on the lamellae was light-dependent and that there is no non-specific binding of the reactants or product to the membranes. No reaction was observed when ferricyanide was omitted from the incubation medium (Fig. 15). DCMU at a concentration of  $10^{-5}$  M, which will completely block oxygen evolution at photosystem II and thereby inhibit ferricyanide reduction (Jagendorf & Avron, 1959), abolished copper ferricyanide deposition on the lamellae. No colour change was seen in the reaction mixture during the 10-min incubation. The chloroplast membranes became completely disoriented in the presence of this concentration of DCMU.

### DISCUSSION

The use of a cytochemical technique to decide where ferricyanide photoreduction occurs, and therefore where photosystem II is localized, is direct but has possible limitations. Although the reactants penetrate the chloroplast, and the grana and stroma lamellae, a finite time is required depending on the integrity of the chloroplast. With short incubation times the stroma lamellae react more rapidly than the grana lamellae, unless the latter have been expanded during preparation, when we find that reaction occurs as rapidly in the grana as in the stroma lamellae. The chloroplasts used in our studies were not 'intact' chloroplasts with envelopes (as discussed by Walker, 1970) but were isolated in isotonic sucrose and contained varying proportions of 'whole' and broken chloroplasts. During incubation in the reaction mixture the chloroplasts underwent further expansion and breakage. This breaking of the chloroplasts resulted in great variability of types of chloroplasts in the reaction mixture (Figs. 1, 2, 12).

In our experiments clear localization of the reaction product was observed at definite sites on the lamellae and not away from the lamellae. We therefore agree with the conclusion of Shumway & Park (1969) that the tetrazolium (INT) method as used by Howell & Moudrianakis (1967) is probably not an accurate method for localizing the site of reduction of the photochemical systems, since the formazan

molecules produced from INT were shown by Park & Shumway to migrate micron distances away from the lamellae themselves. Howell & Moudrianakis had noted an overall deposition of formazan and inferred a general distribution of reactive sites on the membranes. In the experiments of Weier *et al.* (1967), the light reduction of TNBT to the diformazan may have shown a more definite site of reduction in the partitions between adjacent lamellae because of its quite discrete localization. It is, however, possible that the diformazan produced migrates to a favourable deposition site before precipitation actually occurs on the membrane. Another attempt to show the photochemical reaction site in chloroplasts was that of Nir & Seligman (1970) who studied the photo-oxidation of diamino-benzidine (DAB). However, the site where DAB donates electrons into the electron-transport chain is unknown; moreover, since the reaction was insensitive to DCMU, the photo-oxidation of DAB cannot show the localization of photosystem II.

The large size of the Cu ferrocyanide deposits and the fact that these deposits increase in size with increasing times of illumination indicate that there may be a movement of ferrocyanide away from the initial site of reduction on the lamellae to a localized crystallization point. This may be a specific or unspecific membrane site, or an existing Cu ferrocyanide deposit. A small number of sites of deposition was observed after a short incubation of, for example, 30 s. After longer incubations, e.g. 10 min (Figs. 9, 10), many more sites were seen. This is consistent with the formation of additional points of crystallization as the concentration of Cu ferrocyanide increases within the lamellae. The failure of preincubation with tartrate and copper to increase the number of deposition sites may be due to the time required to form additional centres of crystallization for the Cu ferrocyanide and not to the problem of penetration into the lamellae. From experiments using the oxygen electrode, ferricyanide is known to penetrate rapidly into chloroplast lamellae (Baldry, Cockburn & Walker, 1968).

It is not profitable to speculate about the characteristics of the sites of deposition on the lamellae or about the distance of migration of the ferrocyanide. It is, however, unlikely that the ferrocyanide formed by reduction in the grana lamellae would migrate to the stroma lamellae before any reaction with Cu occurred, the Cu ferrocyanide only then being deposited on the stroma membrane. For example, in Fig. 2 a migration distance of 350 nm from the edge of the grana, and 500 nm from the centre of the grana, to the mid-point of the stroma fretwork would be involved.

The sites of ferricyanide reduction should correspond to the sites of photosystem II in the stroma and grana lamellae. With  $H_2O$  as the sole source of electrons (as in our experiments) the only way in which ferricyanide can be photochemically reduced in chloroplasts is via photosystem II. Ferricyanide has a potential of +0.4 V at pH 7, so that any reductant more reducing (more negative) than +0.4 V will reduce ferricyanide non-enzymically in the dark (see review of Avron & Vernon, 1965). For example, ascorbic acid, which in our controls showed no reduction on the lamellae, can reduce ferricyanide non-enzymically (Fig. 14). We conclude from our experiments on ferricyanide reduction that photosystem II operates in both stroma and grana lamellae.

M.K. thanks the Wellcome Trust for support. We thank Mr S. Reeves and Mr K. Maybury for excellent assistance. We also thank Professors C. R. Stocking, F. R. Whatley and Jean Hanson for valuable criticism of the manuscript.

## REFERENCES

- ARNON, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Pl. Physiol., Lancaster* **24**, 1-15.
- AVRON, M. & VERNON, L. P. (1965). Photosynthesis. *A. Rev. Biochem.* **34**, 269-296.
- BALDRY, C. W., COCKBURN, W. & WALKER, D. A. (1968). Inhibition, by sulphate, of oxygen evolution associated with photosynthetic carbon assimilation. *Biochim. biophys. Acta* **153**, 476-483.
- BEASLEY, M. L. & MILLIGAN, W. O. (1969). The structure and morphology of cupric ferrocyanide gels. *Trans. N.Y. Acad. Sci.* **31**, 261-279.
- HALLIER, U. W. & PARK, R. B. (1969). Photosynthetic light reactions in chemically fixed spinach thylakoids. *Pl. Physiol., Lancaster* **44**, 544-546.
- HOWELL, S. H. & MOUDRIANAKIS, E. N. (1967). Hill reaction site in chloroplast membranes: non-participation of the quantasome particle in photoreduction. *J. molec. Biol.* **27**, 323-333.
- JACOBI, G. & LEHMANN, H. (1969). Photochemical activities of chloroplast fragments. In *Progress in Photosynthesis Research*, vol. 1 (ed. H. Metzner), pp. 159-173. Tubingen, Germany: Int. Union Biol. Sci.
- JAGENDORF, A. T. & AVRON, A. (1959). Inhibitors of photosynthetic phosphorylation in relation to electron and oxygen transport pathways of chloroplasts. *Archs Biochem. Biophys.* **80**, 246-257.
- KALINA, M., WEAVERS, B. & PEARSE, A. G. E. (1969). Fine structure localization of succinic oxidase complex on mitochondrial cristae. *Nature, Lond.* **221**, 479-480.
- KERPEL-FRONIUS, S. & HAJOS, F. (1968). The use of ferricyanide for light and electron microscopic demonstration of succinic dehydrogenase activity. *Histochemie* **14**, 343-351.
- NIR, I. & SELIGMAN, A. M. (1970). Photooxidation of diaminobenzidine (DAB) by chloroplast lamellae. *J. Cell Biol.* **46**, 617-620.
- OGAWA, K., SAITO, T. & MAYAHARA, H. (1968). The site of ferricyanide reduction by reductases within mitochondria as studied by electron microscopy. *J. Histochem. Cytochem.* **16**, 49-57.
- SANE, P. V., GOODCHILD, D. J. & PARK, R. B. (1970). Characterization of chloroplast photosystems 1 and 2 separated by a non-detergent method. *Biochim. biophys. Acta* **216**, 162-178.
- SHUMWAY, L. K. & PARK, R. B. (1969). Light-dependent reduction of some tetrazolium and ditetrazolium salts by chloroplasts. *Expl Cell Res.* **56**, 29-32.
- WALKER, D. A. (1970). Three phases of chloroplast research. *Nature, Lond.* **226**, 1204-1208.
- WEIER, T. E. & BENSON, A. A. (1967). The molecular organisation of chloroplast membranes. *Am. J. Bot.* **54**, 389-402.
- WEIER, T. E., STOCKING, C. R. & SHUMWAY, L. K. (1967). The photosynthetic apparatus in chloroplasts of higher plants. *Brookhaven Symp. Biol.* **19**, 353-374.

(Received 10 March 1971)

Fig. 1. General view of chloroplasts incubated for 30 s in the light. Note the variability in the quality of preservation of the 4 chloroplasts and the electron-dense Cu ferrocyanide reaction product on the lamellae of the broken (central) chloroplast. The chloroplast on the extreme right also shows reaction product, whereas very little, or no, reaction product is seen in the 2 chloroplasts on the left. Arrows indicate Cu ferrocyanide deposits. Note oil droplets (*od*) and starch granules (*sg*).  $\times 30000$ .

Fig. 2. After 30 s incubation in the light. Variability in chloroplast integrity and the deposition of the Cu ferrocyanide mainly on the stroma lamellae in the central, broken chloroplast is noted. Arrows indicate Cu ferrocyanide deposits. (*od*, oil droplets; *sg*, starch granules.)  $\times 30000$ .

Fig. 3. After 30 s incubation in the light. Reaction product is seen mostly on the stroma lamellae, with some deposits on the grana lamellae. Arrows indicate Cu ferrocyanide deposits. (*od*, oil droplet.)  $\times 60000$ .



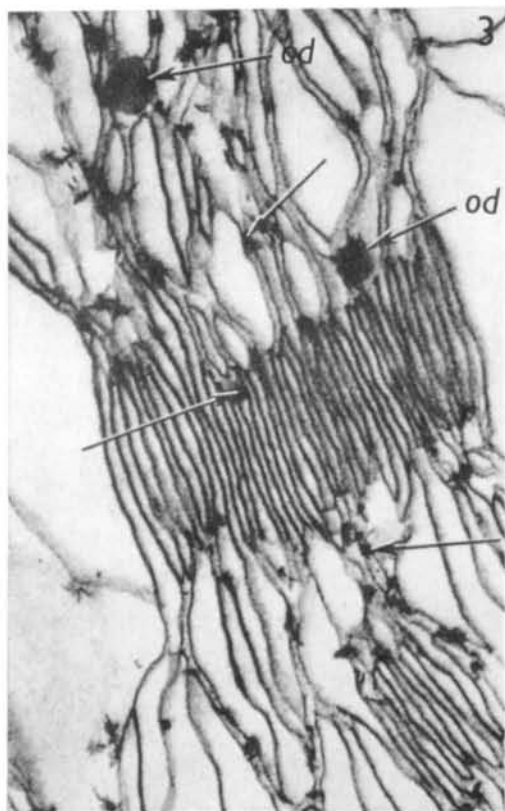
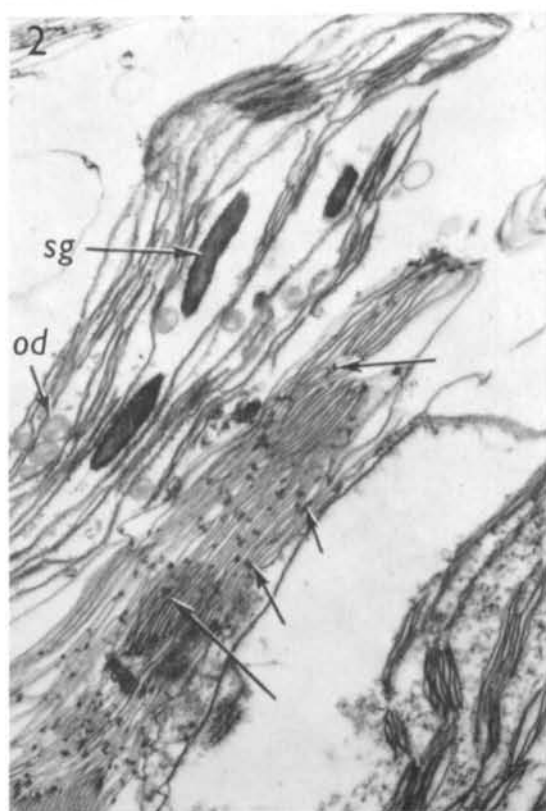
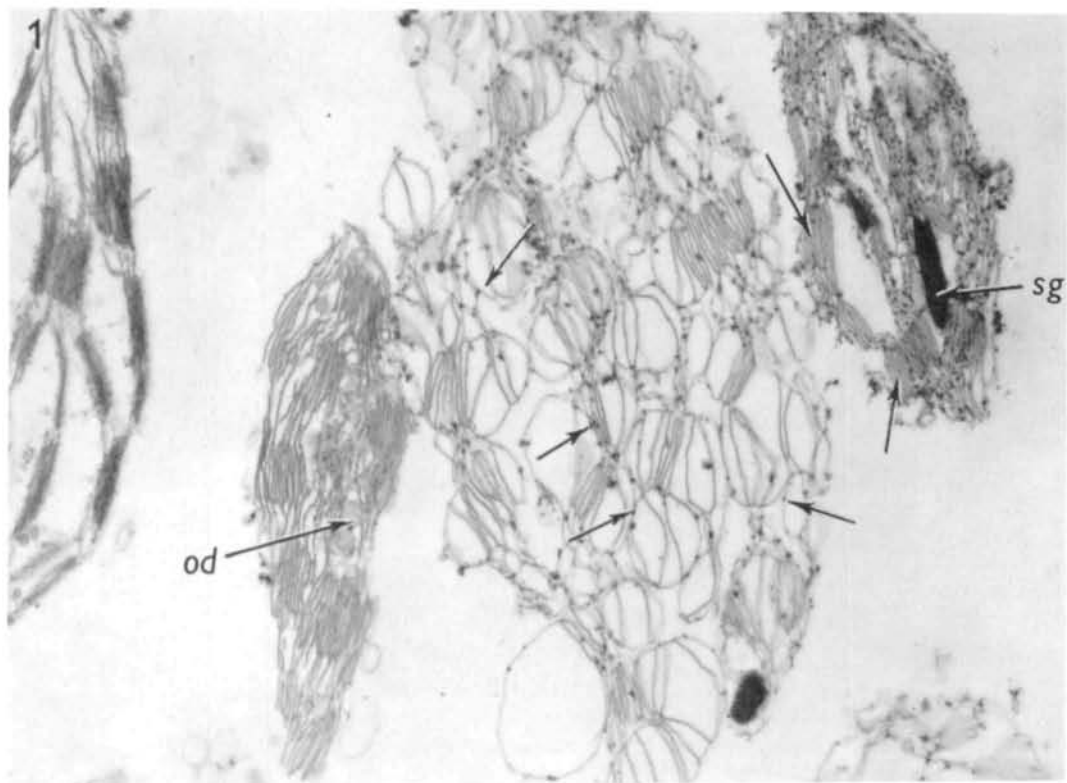


Fig. 4. Cu ferrocyanide reaction products on lamellae of a broken chloroplast after 30 s incubation in the light. Arrows indicate Cu ferrocyanide deposits. (*od*, oil droplet.)  $\times 50000$ .

Fig. 5. The absence of Cu ferrocyanide deposits in chloroplasts lamellae after 30 s incubation in the dark. This control experiment should be compared with Fig. 4. (*od*, oil droplet.)  $\times 84000$ .

Fig. 6. Higher-resolution micrograph of chloroplasts after 30 s incubation in the light. Note reaction product localized on single membranes (*a*) and the deposition within the grana loculi (*b*) and stroma frets (*c*), i.e. between adjacent membranes.  $\times 170000$ .

Fig. 7. After 3 min incubation in the light more reaction product deposits are seen. Pronounced deposition on the edges of the grana stacks is evident here, in addition to deposits in the stroma lamellae and a few isolated deposits in the grana lamellae.  $\times 50000$ .

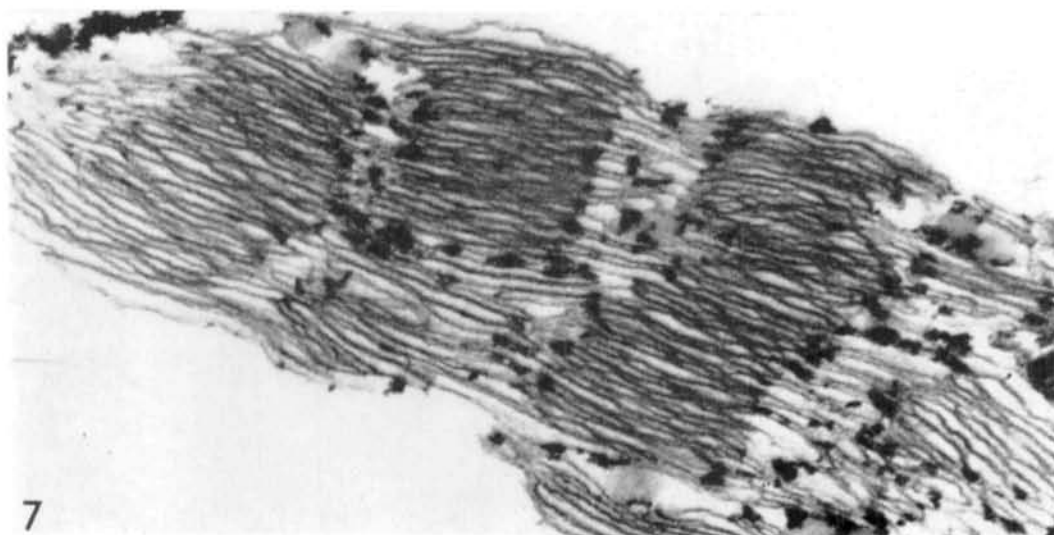
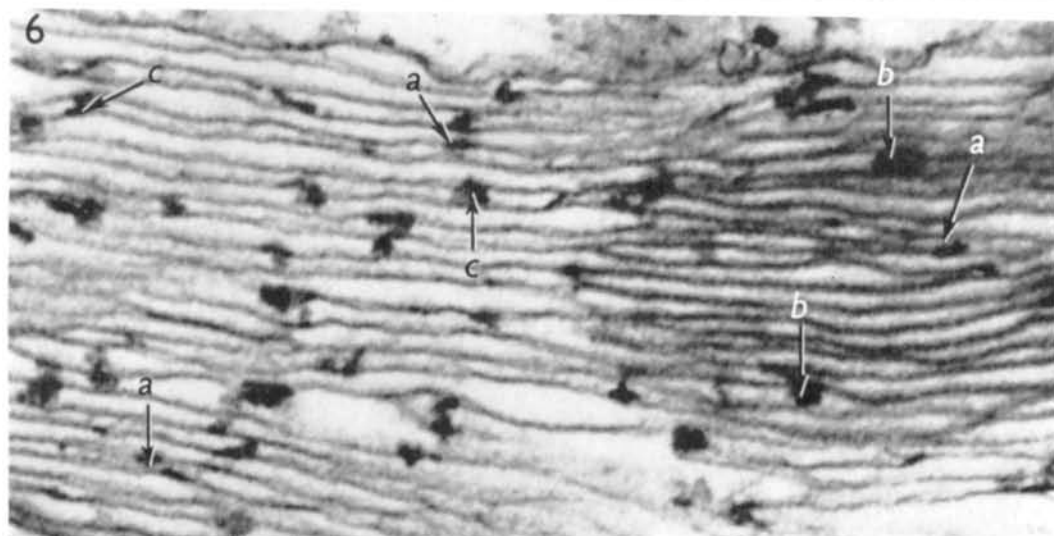
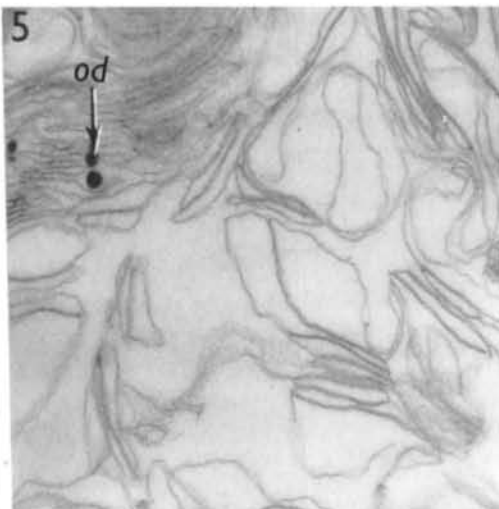
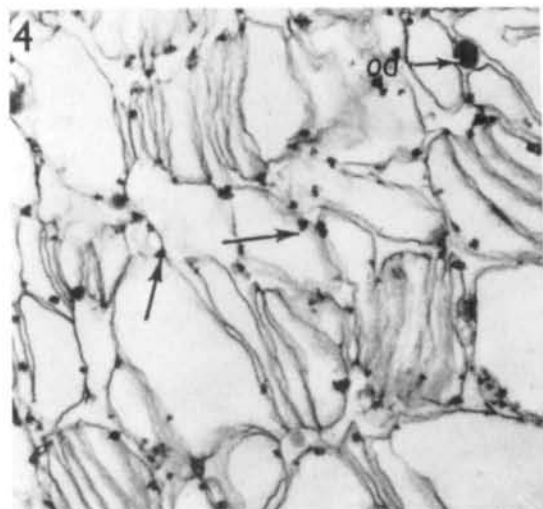


Fig. 8. General view of chloroplasts after 10 min incubation in the light. All chloroplasts contain Cu ferrocyanide deposits on nearly all their membranes.  $\times 12000$ .

Fig. 9. Portion of single chloroplast after 10 min incubation in the light. Note dense reaction deposits on grana and stroma lamellae.  $\times 60000$ .

Fig. 10. Portion of chloroplast. Conditions as in Fig. 9, except that the chloroplasts were prefixed in 3% glutaraldehyde.  $\times 30000$ .

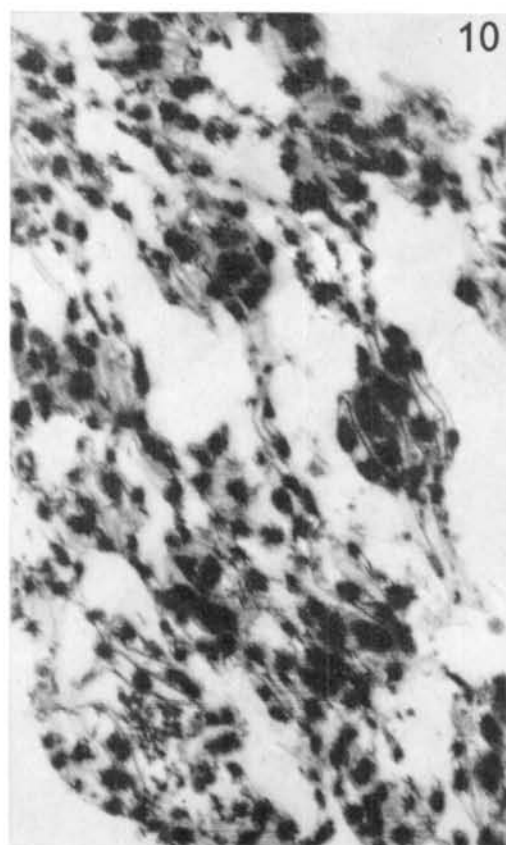
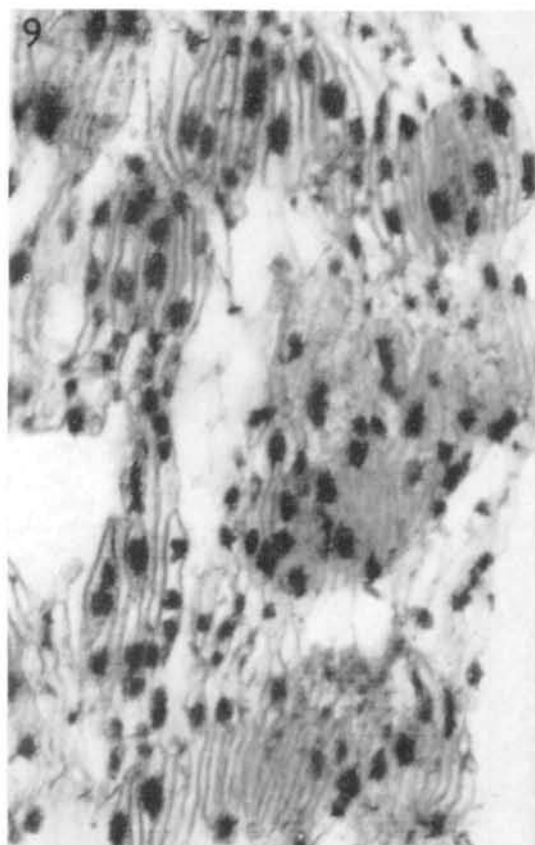
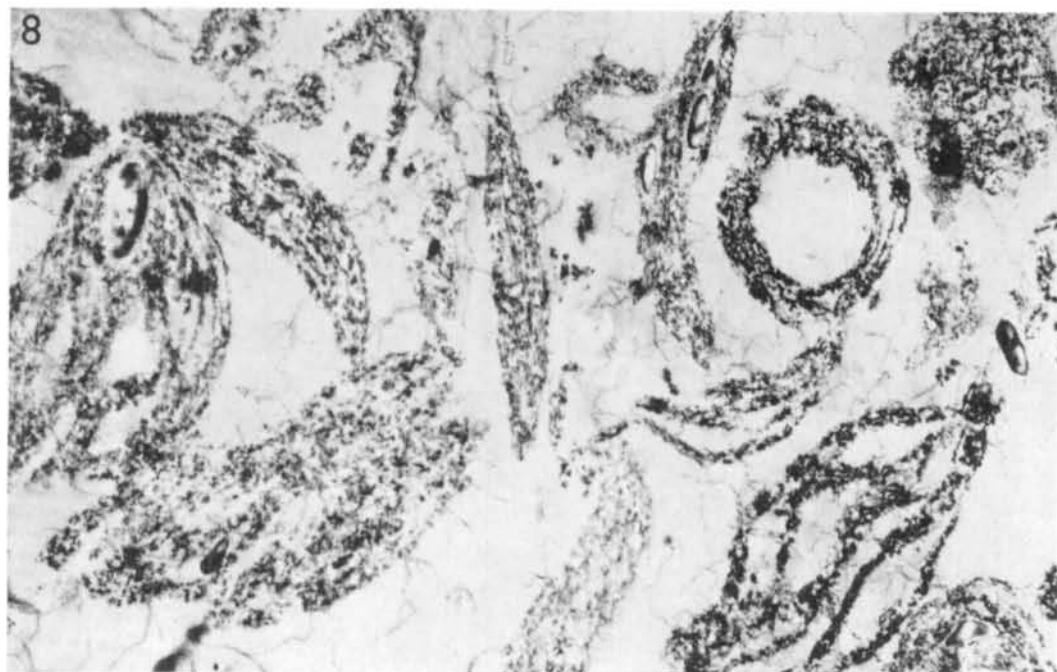


Fig. 11. High-power view of lamellae showing deposits on grana (*g*) and stroma (*s*) lamellae; 3 min incubation in the light. Note reaction product mainly in the stroma lamellae in this chloroplast even with the long incubation time. Grana stacks intact.  $\times 150000$ .

Fig. 12. Control experiment showing a general view of chloroplasts from zero time incubation. Note absence of reaction product in this and subsequent controls, i.e. Figs. 13-15. Note darkly stained oil droplets (*od*) in this figure and Figs. 13-15.  $\times 20000$ .

Fig. 13. Control experiment; 3 min incubation in the dark.  $\times 33000$ .

Fig. 14. Control experiment in which Na ascorbate was added to the reaction mixture after all other reactants had been added. For details, see Methods.  $\times 84000$ .

Fig. 15. Control experiment; 3 min incubation in the light but with ferricyanide omitted from the reaction mixture.  $\times 70000$ .

