THE EFFECTS OF THE DIPYRIDYL DIQUAT ON THE METABOLISM OF *CHLORELLA VULGARIS*

II.* EFFECTS OF DIQUAT IN THE LIGHT ON CHLOROPHYLL BLEACHING AND PLASTID STRUCTURE

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Summary

The effects of diquat, in the light, on chlorophyll bleaching and the fine structure of the cells of *C. vulgaris* (211–11h) have been studied. Diquat causes bleaching of chlorophyll when the cells are illuminated in water or phosphate or bicarbonate buffer. Using cultures of uniform cell size it is shown that diquat causes considerable damage to cell membranes in less than 10 hr of illumination, but that inhibition of both photosynthesis and respiration precede any damage to the plastids visible under the electron microscope.

I. Introduction

In early experiments with the herbicide diquat dibromide (1,1′-ethylene-2,2′-dipyridylium dibromide) used on higher plants, chlorophyll bleaching was reported (Funderburk and Lawrence 1964), although this was sometimes obscured by blackening of tissues (Mees 1960) or halted by rapid desiccation. In manometric experiments described in Part I of this series (Turner, Stokes, and Gilmore 1970) *Chlorella vulgaris* cells appeared fully green after a 3-hr treatment in the light by diquat, which had reduced the gas exchange to very low values. Later work showed that 10^{-3}M diquat applied to *C. vulgaris* cells at 400 f.c. caused complete bleaching of all the cells in approximately 48 hr.

In this paper the factors concerned in the bleaching of *C. vulgaris* treated with diquat in the light are examined. The effects of this treatment on the fine structure of the plastid and the cell are also reported.

II. Materials and Methods

The experimental material was *C. vulgaris* Beij, strain 211–11h Pratt, obtained from the Cambridge collection. For the work on bleaching, the alga was cultured as described by Turner, Stokes, and Gilmore (1970). For the electron-microscope studies of plastid and cell structure it proved essential to utilize cells of a more uniform size and age so as to provide a good control material. The *C. vulgaris* strain was, therefore, grown in a largely synchronous culture by a modification of the method of Morimura (1959). First a culture (A) was grown in the normal way (as in Part I) at 800 f.c. and 20°C. After 4–5 days the light intensity was reduced to 80 f.c. for a further 3–4 days. Some 20 ml of this material was used as the inoculum in 250 ml of fresh culture medium. This second culture (B) was grown under the same light and temperature regime as culture A and was then stored in darkness at 2°C. The population of cells so produced still

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contained a proportion of large cells but most of these were readily removed by an initial centrifugation at 155 *g* for 3 min. A second centrifugation at 500 *g* for 6 min provided a population of small cells of uniform size and shape. These small cells were used for the electron-microscope studies and the accompanying manometric experiments.

Methods for the measurement of gas exchange were those described in Part I. Chlorophyll determinations were carried out with a Unicam SP500 spectrophotometer by the method of Arnon (1949).

For electron microscopy the cells were removed from the Warburg vessels, centrifuged at 500 *g*, and rinsed in distilled water. After recentrifugation at 500 *g* for 5 min they were fixed for 2 hr in 6% (w/v) glutaraldehyde in 0·1M phosphate buffer (Sabatini, Bensch, and Barrnett 1963). The cells were then washed in three changes of 0·1M phosphate buffer (one wash overnight) and post-fixed for 2 hr in 2·0% (w/v) OsO_4 in veronal acetate buffer containing 0·5% (w/v) $MgCl_2$. After centrifuging at 500 *g* for 3–5 min the cells were dehydrated in a series of acetone solutions, and embedded in a mixture of Araldite–Epon (Mollenhauer 1964, No. 1 mixture). Sections were cut in a Porter–Blum microtome with glass knives and double-stained in saturated uranyl acetate in 50% ethanol (30 min) (Gibbons and Grimstone 1960) followed by the same time in lead citrate (Reynolds 1963). All sections were examined in a Siemens Elmiskop 1 electron microscope at 80 kV.

III. Results and Discussion

(*a*) *Bleaching of Chlorophyll*

C. vulgaris samples were incubated in 0·067M phosphate buffer, pH 6·7; in Warburg buffer No. 9 (modified according to Pratt 1943), pH 9·1; and in distilled water, all at 1000 f.c. and 25°C. At various times duplicate samples were removed from each treatment and the chlorophyll content of the cells determined. Detailed results are presented in Table 1. These may be briefly summarized as follows:

(1) Bleaching was much more rapid when the diquat was supplied in distilled water rather than in buffers.

(2) Bleaching was a photochemical process: the fall in chlorophyll content due to diquat treatment was arrested by darkness and resumed when light was admitted.

Reference to Part I and to Figure 1 of this paper will show that in buffered solutions of diquat (10^{-3}M) gas exchange in the light is very largely suppressed after treatment for 1 hr, whereas bleaching of chlorophyll requires treatment for more than 4 hr. We conclude that the photochemical bleaching of chlorophyll is a secondary effect of the application of diquat to *C. vulgaris* in light.

(*b*) *Effect of Diquat on Plastid Structure*

In a typical experiment, 12 replicate samples obtained from culture B of *C. vulgaris*, prepared as described under Section II, were set up as for a normal manometric experiment. At zero time another replicate sample was fixed. Diquat (10^{-3}M) was then added to six of the 12 samples and these 12 samples were then illuminated in the Warburg apparatus. At 1, 5, and 10 hr after adding diquat two vessels from each of the control and diquat treatments were removed, the duplicate samples bulked, and then fixed for electron microscopy. The results are illustrated in Figures 2–8. Experimental procedures are indicated in the legends to these figures.

Figure 2 shows the normal fine structure of a *C. vulgaris* cell taken straight from the culture medium. The cell is approximately 5 μm in diameter and has a cellulose wall approximately 50 nm thick. The cell contains a single cup-shaped chloroplast with an intact membrane and many thylakoids with well-defined lamellae. The outermost layers of the thylakoids or end granal membranes are thin, while the inner layers or partition are approximately twice as thick. Four-layered stacks of lamellae are most common (three thylakoids), but stacks of up to eight lamellae do occur. Murakami, Morimura, and Takamiya (1963) reported for *Chlorella ellipsoidea* that stacks of two to five lamellae occurred and that three-layered stacks (two thylakoids) were the most common.

TABLE 1

CHLOROPHYLL CONTENT OF *CHLORELLA VULGARIS* EXTRACTED AT VARIOUS TIMES AND EXPRESSED AS A PERCENTAGE OF THE CHLOROPHYLL CONTENT AT ZERO TIME

All percentages are corrected to the nearest whole number and all values are the mean of two samples. Light intensity 1000 f.c.; temperature 25°C

	Time in Light (hr)	Diquat Concn. 10^{-3}M	Control
Warburg buffer No. 9, pH 9·1	0	100	100
	3	85	87
	6	66	84
	7	45	—
	Intervening 16 hr dark period		
	7	42	—
	9	19	87
Phosphate buffer (Na^+ and K^+ salts), pH 6·7, 0·067M	0	100	100
	4	84	87
	7	75	88
	Intervening 16 hr dark period		
	7	66	82
	9	19	—
	11	3	82
Distilled water	0	100	100
	3	31	—
	6	2	88

As Murakami, Morimura, and Takamiya (1963) observed with *C. ellipsoidea*, there is no membrane separating the matrix of the pyrenoid from the plates of starch surrounding it, nor is there a membrane between the pyrenoid and the neighbouring lamellae. The large, oval-shaped pyrenoid, filled with a granular matrix, is traversed by a membrane, which appears to be an extension of the chloroplast lamellae.

The other organelles were those normally expected in such a cell and they included many mitochondria with poorly defined cristae scattered throughout the

cytoplasm, a single Golgi body lying close to the nucleus, a well-defined nucleolus, and a double nuclear membrane with many nuclear pores. Several vacuoles surrounded by a tonoplast are also found in the cell.

After 1 hr of illumination little change is shown in the structure of the cell (Figs. 3 and 4). In the control cell there has been an increase in the amount of starch deposited around the pyrenoid and also within the chloroplast lamellae. In the cell treated with diquat (Fig. 4) there is no such increase in starch content. This is to be expected since Figure 1 shows that after 1 hr of diquat treatment apparent photosynthesis had fallen to zero and the cells were absorbing oxygen. The evidence from the electron micrographs therefore supports that obtained from gas-exchange

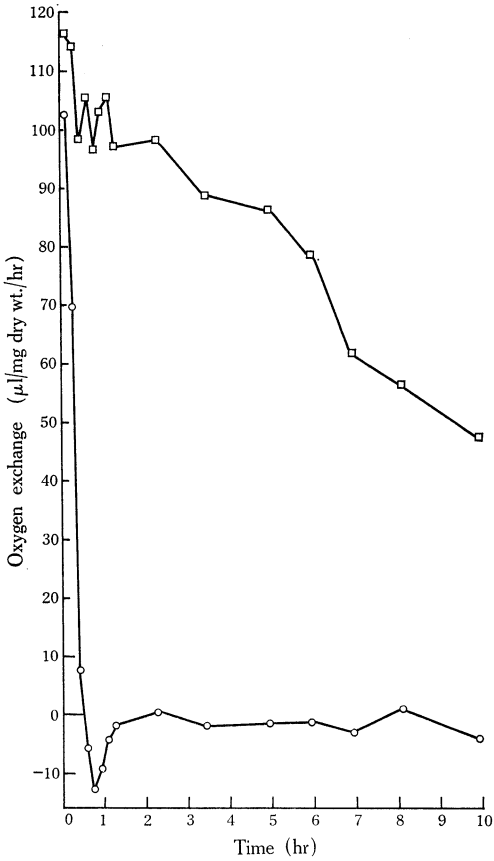


Fig. 1.—Effects of diquat on gas exchange of a synchronous culture of *C. vulgaris*. Warburg buffer No. 9, 91×10^{-6} mole/l CO_2; 25°C; 750 f.c. Each point is the mean of at least two samples. □ Control rate of apparent photosynthesis. ○ 10^{-3}M diquat. Negative values indicate oxygen influx.

measurements—that diquat inhibits photosynthesis within 1 hr and shows that this inhibition is not accompanied by any damage to the plastids visible under the electron microscope.

After 5 hr the diquat-treated cells were still absorbing oxygen at a slow rate (Fig. 1; see also Part I). There are marked differences between the control and treated cells (Figs. 5 and 6). Starch is still prominent in the control cell and the

enlarged starch grains have displaced the lamellae in the plastid. On the other hand in the diquat-treated cell there is only a small quantity of starch remaining. The lamellae show signs of buckling suggesting damage due to diquat and the pyrenoid shows signs of damage, although its traversing membrane is still present. The mitochondria and nucleus are no longer visible in the diquat-treated cell.

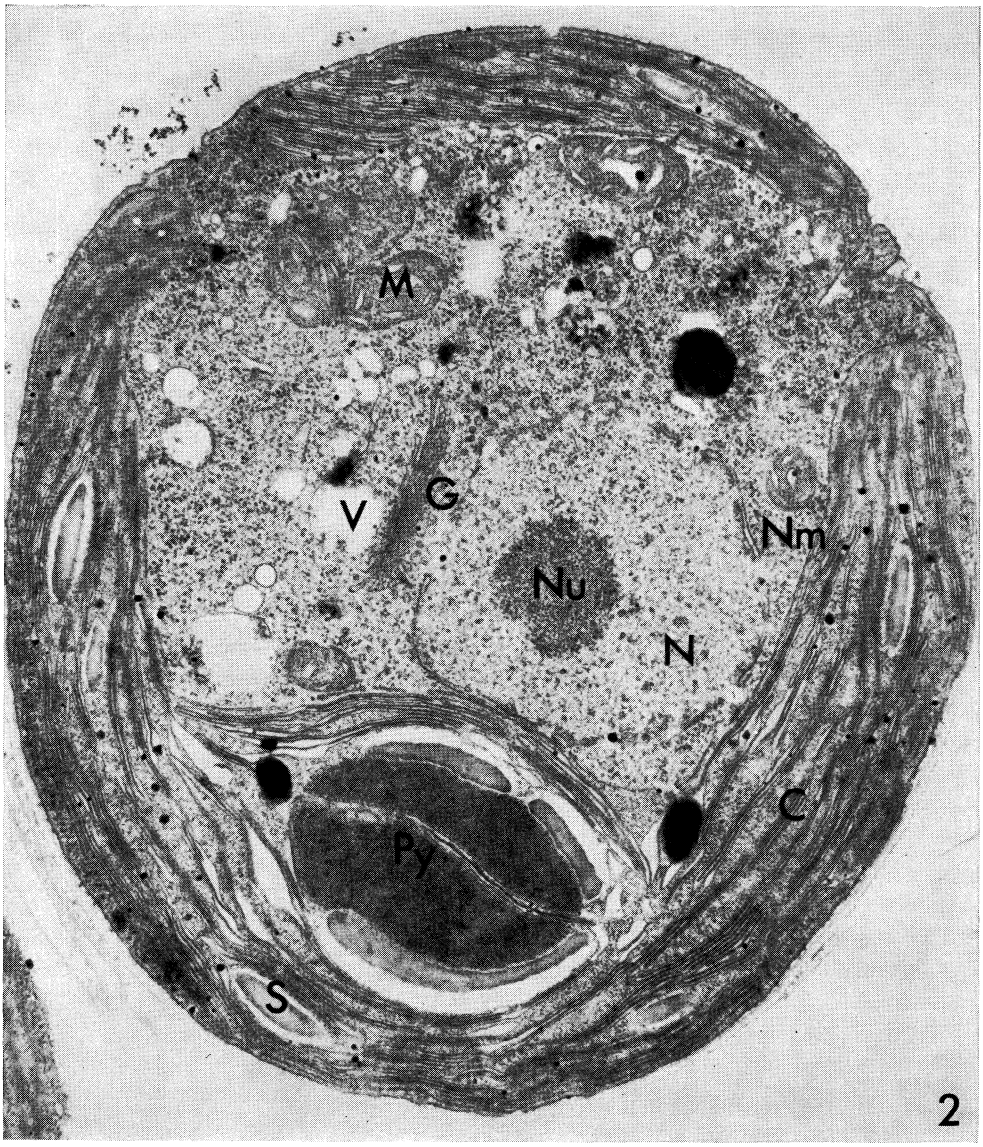
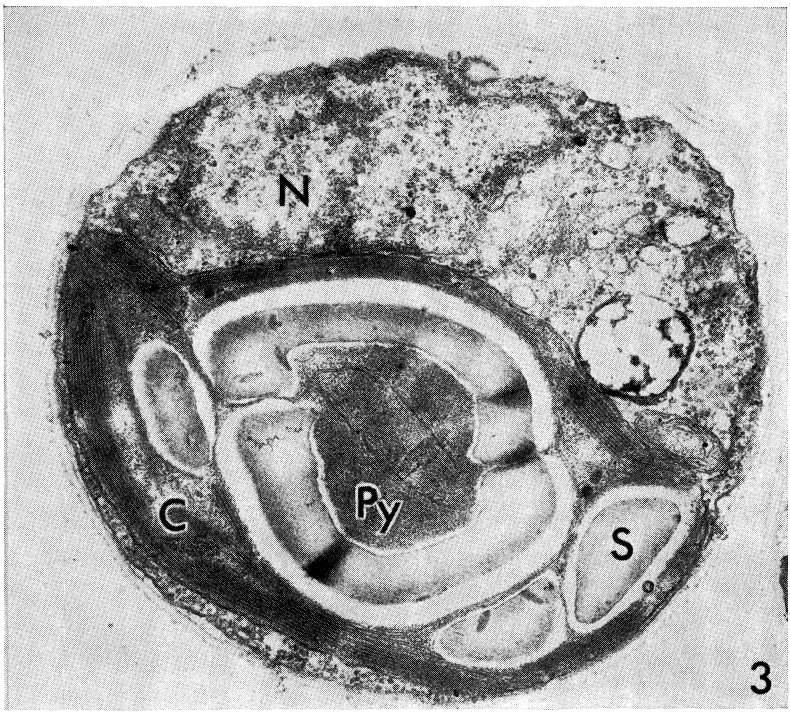
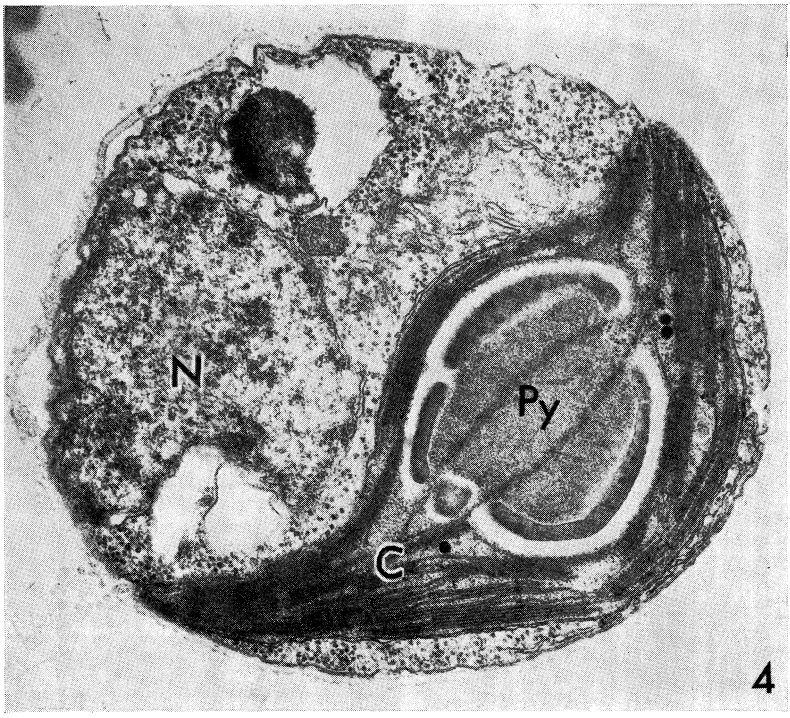
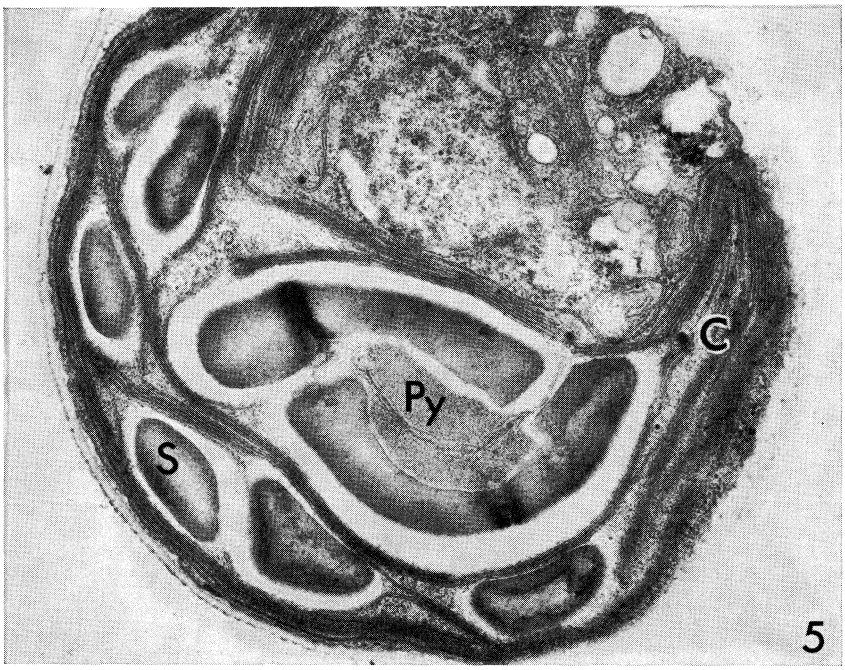


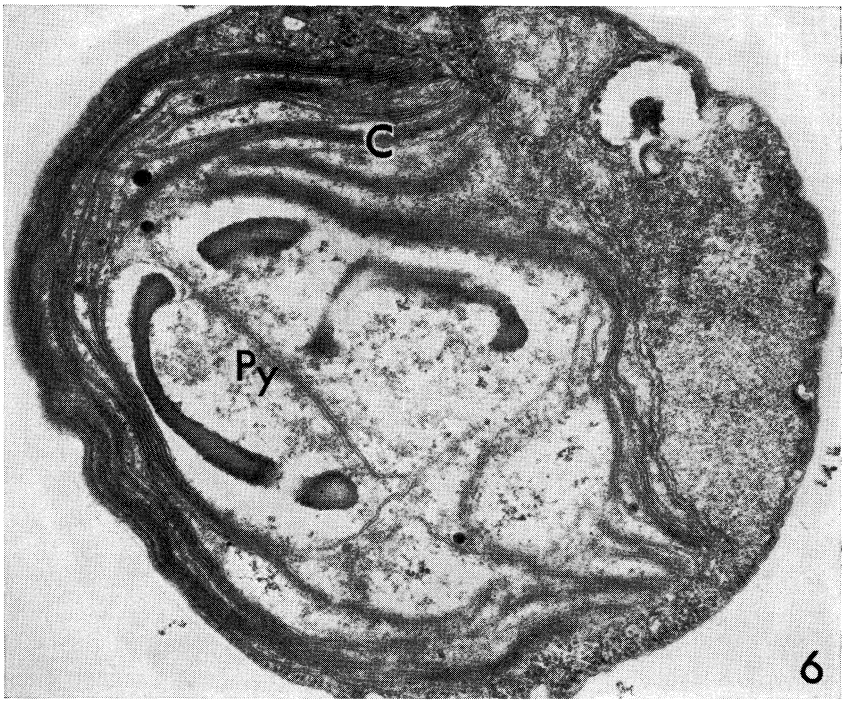
Fig. 2.—Typical *C. vulgaris* cell after dark period at start of experiment. Abbreviations used in Figures 2–8 are as follows: *N*, nucleus; *Nm*, nuclear membrane; *C*, chloroplast; *Py*, pyrenoid; *M*, mitochondria; *G*, Golgi body; *S*, starch grain; *Nu*, nucleolus; *V*, vacuole. $12K \times 2 \cdot 25 = 27{,}000$.



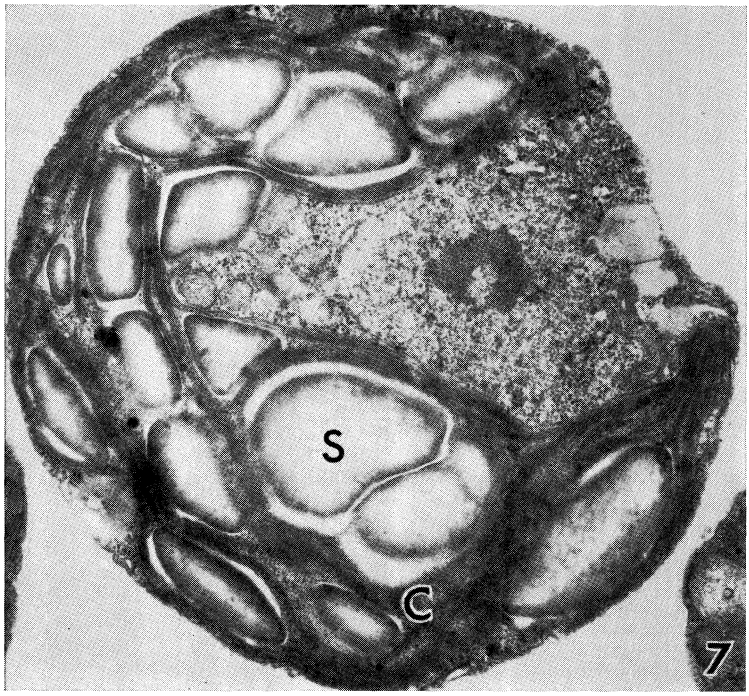


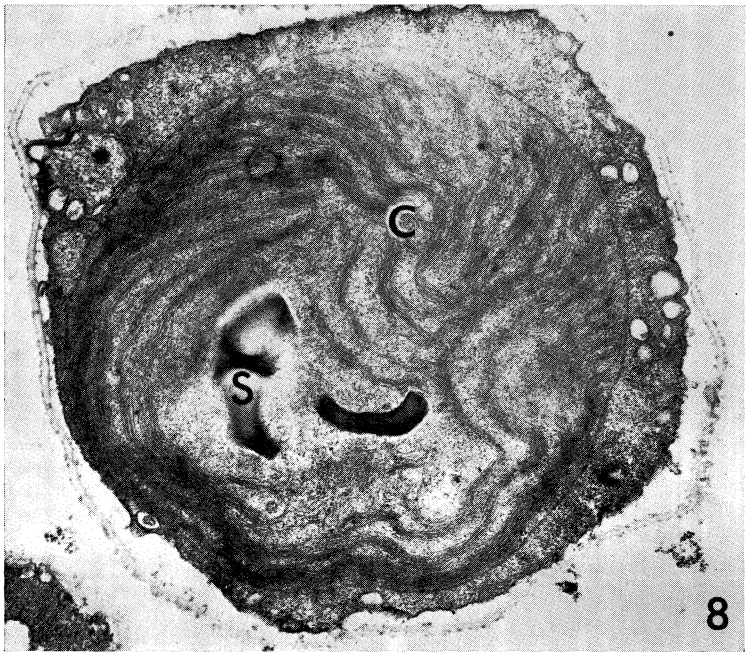
Figs. 3 and 4.—*C. vulgaris* cells illuminated for 1 hr after dark period: ***3***, control; ***4***, in presence of diquat. 15K × 2 = 30,000 for both figures.





Figs. 5 and 6.—*C. vulgaris* cells illuminated for 5 hr after dark period: **5**, control; **6**, in presence of diquat. 15K × 2 = 30,000 for both figures.





Figs. 7 and 8.—*C. vulgaris* cells illuminated for 10 hr after dark period: ***7***, control; ***8***, in presence of diquat. $15K \times 1 \cdot 5 = 22{,}500$ for both figures.

After 10 hr (Figs. 7 and 8) the differences shown at 5 hr are even more marked. Starch now fills half the control cell. In the treated cell starch is almost absent and the organization of the chloroplast lamellae has completely broken down. The limiting membrane of the plastid is still intact, but other cellular inclusions of the cytoplasm appear to be absent. At this stage the treated cells are conspicuously bleached.

IV. Conclusions

In Part I it was shown that 10^{-3}M diquat, added to *C. vulgaris* in the light, caused a complete and irreversible inhibition of photosynthesis in less than 60 min. It was concluded that this, and the subsequent inhibition of respiration, could be due to the production of a toxic substance which damaged the functional membranes of organelles. This substance could be hydrogen peroxide, produced by the oxidation of the free radical formed from diquat in the light in plastids. The electron micrographs presented in this paper show that there is no visible damage to the plastid or to mitochondria after 1 hr of treatment in the light by 10^{-3}M diquat. Damage to both types of organelle is visible after 10 hr of diquat treatment in the light.

It is now concluded that if the inhibition of photosynthesis and respiration is due to structural damage to the organelles, this must be, during the first hour, at a level not detectable by electron microscopy. The gross structural disorganization, and the bleaching of chlorophyll, observable after 5 and 10 hr of diquat treatment in the light, are secondary phenomena. They could be due to the continual production of the free radical and of the hydrogen peroxide resulting from oxidation of this radical, or more directly to photoxidation occurring in a pigmented system in which photosynthesis has been inhibited.

It is emphasized that here the effects of diquat applied in the light have been studied: in Part III of this series we shall examine the effects of diquat on the dark metabolism of *C. vulgaris*.

V. Acknowledgments

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