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AEROBIC PHOTOBLEACHING CAPACITY AS RELATED TO ELECTRON MICROSCOPICAL DATA ON PARTICLE SIZE DISTRIBUTION OF CHLOROPLAST FRAGMENTS FROM VISCHERIA STELLATA AND SPINACIA OLERACEA

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SUMMARY

1. The chloroplast of *Vischeria stellata* shows the general features of algal chloroplasts. The thylakoids are arranged in bands containing three of them each.
2. A correlation between increase of both aerobic photobleaching rate and relative amount of particles with dimensions ranging from 16 to 49 nm is observed for *Vischeria stellata* chloroplast fragments upon interruption, either by storage or by centrifugation, of the preparative sonication procedure.
3. With spinach chloroplast fragments no such increase in both bleaching rate and relative amount of the mentioned particles is found.
4. The time course of aerobic photobleaching for *Vischeria* chloroplast fragments is the same after grinding and sonication. For spinach, however, sonication results in a time course pattern different from that of ground preparations. The pattern of sonicated preparations resembles that of *Vischeria.*
5. The data support a previous suggestion holding that structural effects are involved in determining the aerobic photobleaching capacity.

1. INTRODUCTION

In a previous report (THOMAS *et al.* 1970) it has been suggested that the rate of aerobic photobleaching is related to structural factors in the chlorophyll *b*-free alga *Vischeria stellata.* This suggestion was based on the observation that interrupting the preparative sonication procedure by a period of centrifugation at about 2500 × *g* resulted in enhancement of the photobleaching rate. Moreover, THOMAS & BRETSCHNEIDER (1970) found that the pattern of the photobleaching time course for *Vischeria* is reversed as compared with that for spinach, which resembles that of *Aspidistra elatior* (THOMAS & NIJHUIS 1968). In relative terms, the photobleaching rate for *Vischeria* is high initially, then it declines. For both spinach and *Aspidistra* an initial low-rate bleaching is followed by a high-rate one.

In order to check the suggested relationship between structure and photobleaching characteristics, an electron microscopical study was combined with photobleaching measurements for both *Vischeria* and spinach. Since, however, the structure of spinach chloroplasts has already been studied by various authors, electron microscopy was done only on *Vischeria* cells. Subsequently, after negative staining, chloroplast fragment suspensions of both species, as used in

bleaching experiments, were analysed for differences in particle morphology and size distribution.

2. METHODS

2.1. Preparation

Vischeria stellata was grown, and chloroplast fragment suspensions were prepared from it ultrasonically, as mentioned in THOMAS *et al.* (1970). Accordingly, the sonication procedure consisted of alternately sonicating near 0 °C for 30 seconds and cooling by adding ice to the coupling water column for the same period. The sonication times mentioned below refer to the sum of the actual sonication periods. The following modification, however, was applied. In experiments on the effect of interrupting the sonication procedure, as mentioned under Results, a batch of cells, obtained by centrifuging the culture suspension, was divided into two equal parts. One of them, the reference, was sonicated for 7 minutes. Then it was stored at room temperature as long as needed for preparation of the other sample by interruption of the sonication procedure. In this way the sum of sonication and interruption periods was equal for both samples.

Spinach was obtained from the Botanic Gardens. Chloroplast fragment suspensions were prepared according to THOMAS *et al.* (1967), using a 0.02 M phosphate buffer, pH 7.3.

2.2. Absorption measurements and irradiation

The same techniques were used as described in the first-mentioned paper. The intensity of the bleaching, incandescent, light was about 0.3 $W.cm^{-2}$ at the front wall of the cuvette.

2.3. Electron microscopy

Vischeria stellata cells proved to have a thick wall apparently to such a degree impenetrable for standard fixation reagents that a special method had to be used to get pictures of cell structure showing acceptable image quality.

The method of DAMSKY *et al.* (1969) for fixation of yeast cells yielded the best results. Thick cell walls were ruptured during fixation by stirring the cells together with 0.6 mm glass beads for 1.5 to 4 minutes. The fixatives used were (1) 2% glutaraldehyde together with 1% OsO_4 at 0 °C, and (2) 2% $KMnO_4$ at 20 °C. Both solutions were buffered at pH 6.5 by 0.05 M phosphate buffer. Fixation time was 1 h.

The effect of treatment with glass beads was very clearly seen at $KMnO_4$ fixation. Damaged cells turned dark brown at once, while intact cells stayed green.

The fixed cells were dehydrated via ethanol and propyleneoxide. Small pellets were embedded in Epon 812 resin. Sections were stained with uranyl acetate and lead citrate.

Suspensions of chloroplast fragments were studied by negative staining. Equal volumes of suspension and a 4% ammonium molybdate solution were mixed

and drops were applied to Formvar-covered specimen grids. Most of the fluid was sucked off with filter paper.

3. RESULTS

3.1. Electron microscopy

The cup- or banana-shaped chloroplast of *Vischeria stellata* lies against the cell wall over a large part of its outer surface, *fig. 1*. In perpendicular section a band pattern, also found in other algae (GIBBS 1970), is revealed. Each band consists of 3 thylakoids, *fig. 2*. When cells are damaged by stirring them together with glass beads prior to fixation, the thylakoids desintegrate into vesicles and tubules, *fig. 3*. The chloroplast fragment suspension obtained by grinding or sonication shows elements of different kinds and sizes when negatively stained. They look like vesicles, discs, rods, and floccules, *fig. 4*.

The suspension particle size distribution was obtained from micrographs at 30.000× magnification by taking the largest dimension for any kind of particle. Particles with apparent size lower than 16 nm were not counted. Due to the low contrast, their size determination is rather uncertain. The structure of the dried ammonium molybdate layer itself shows contrast variations over object regions up to about 3 nm.

3.2. Aerobic photobleaching

Examples of aerobic photobleaching of both *Vischeria* and spinach chloroplast fragments are given in *fig. 5*. Curve A demonstrates the time course of bleaching for *Vischeria* chloroplast fragments prepared by sonication for 7 minutes. It agrees with the data shown in *fig. 1* of an earlier paper (THOMAS *et al.* 1970). *Fig. 5*, curve B, refers to the bleaching of a sample from the same preparation upon interruption of the sonication period after 4 minutes and for 20 minutes. The bleaching rate is clearly enhanced by this interruption during which the sample was kept at room temperature in the dark. This effect is contradictory to the opinion that interjacent storage does not affect the bleaching rate mentioned in the earlier paper. This discrepancy, most likely, is due to the modification of the preparation technique mentioned under Methods. At present it is found that storage indeed enhances the bleaching rate. This enhancement, however, is only about 40% of that due to interjacent centrifugation.

The pattern of the time course of bleaching is the same for *Vischeria* preparations obtained by either grinding or sonication (Schoonman, unpublished results). Since whole cells show this pattern as well, *fig. 2* of the earlier paper, such is not astonishing.

For spinach, however, things are quite different. In *fig. 5*, curve C, the photobleaching time course of a ground preparation is shown. If, on the contrary, this spinach preparation is sonicated, a bleaching pattern of the *Vischeria* type, curve D, results. Nevertheless, a difference between the bleaching characteristics of both sonicated species remains. A 20 minutes' interruption of the sonication period enhances the *Vischeria* bleaching, curve B, whereas it does not change

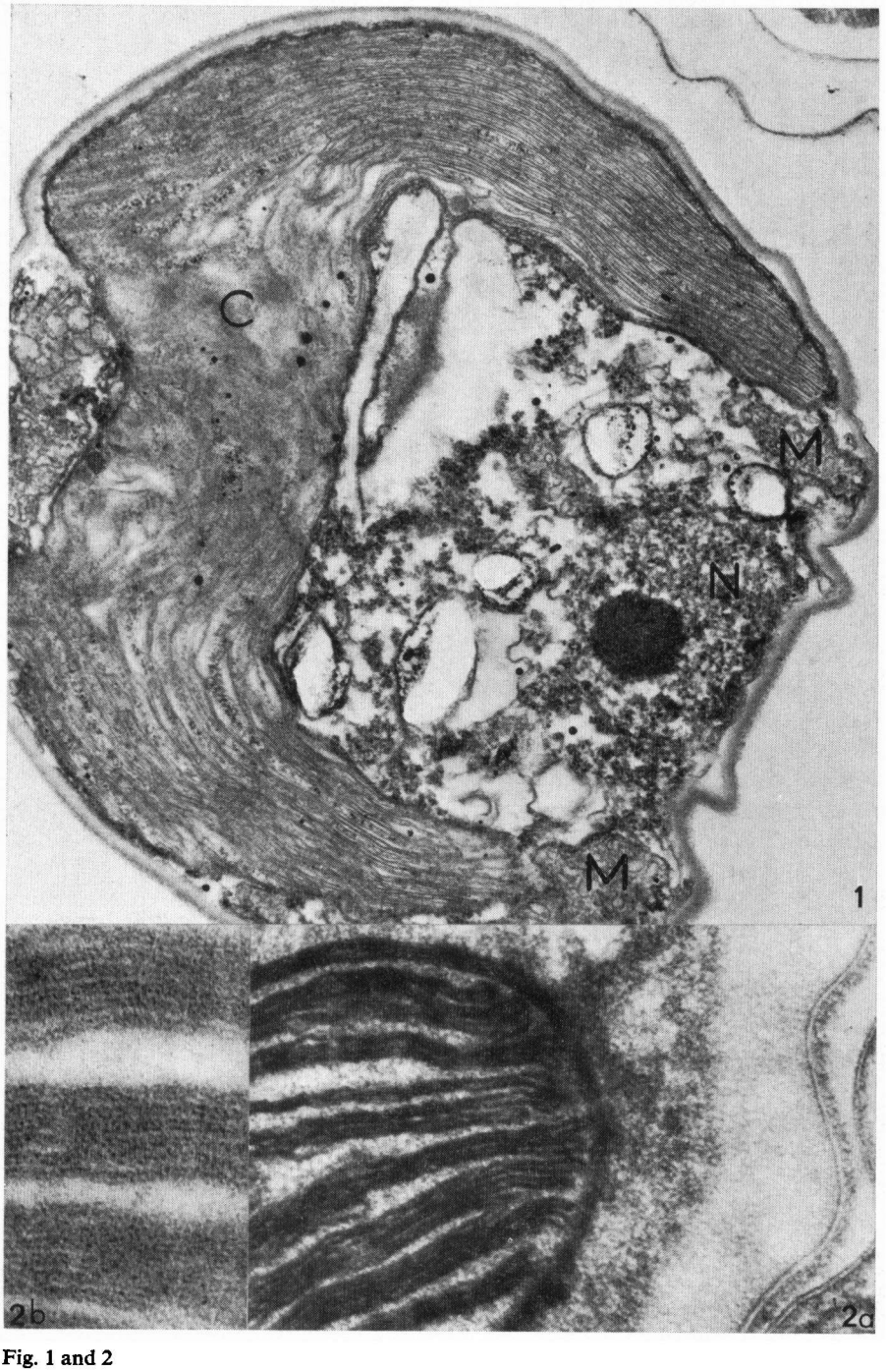

Fig. 1 and 2

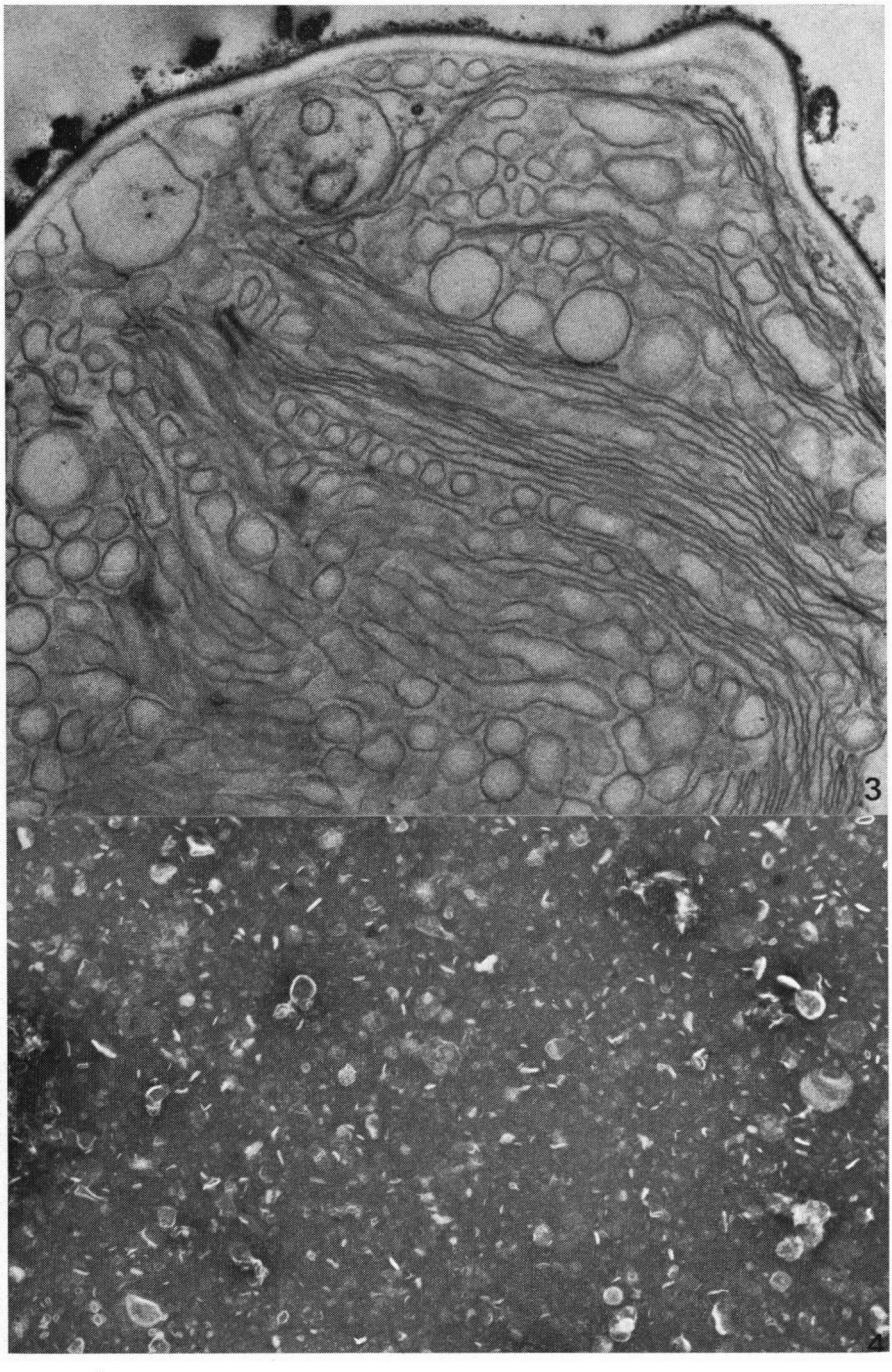

Fig. 3 and 4

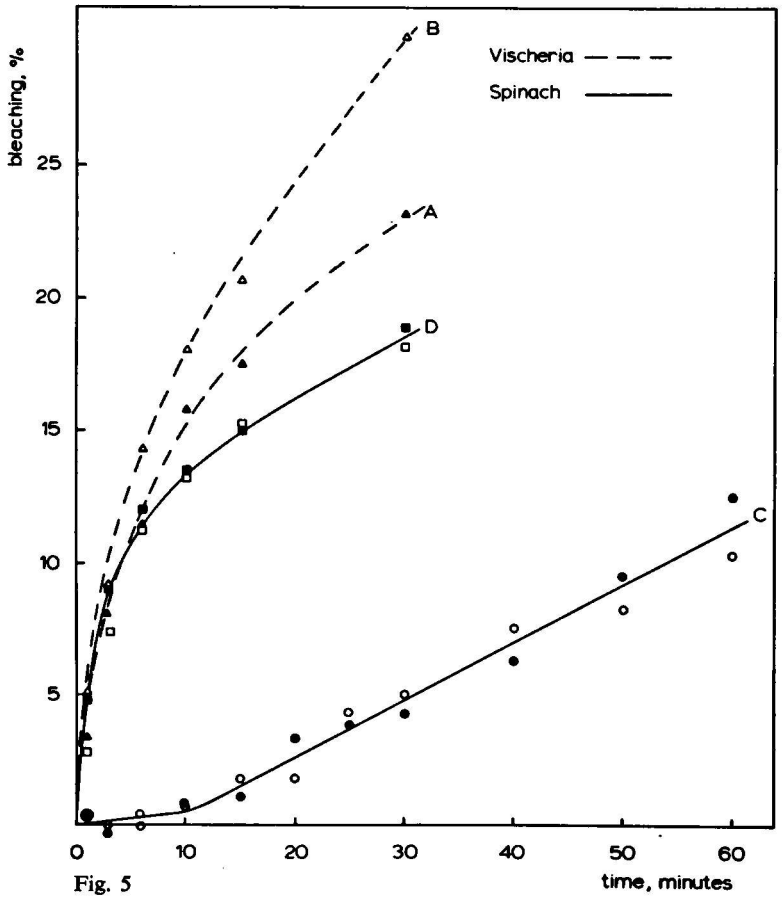
bleaching, %
Vischeria
Spinach
B
A
D
C
0
5
10
15
20
25
0
10
20
30
40
50
60
time, minutes

Fig. 5

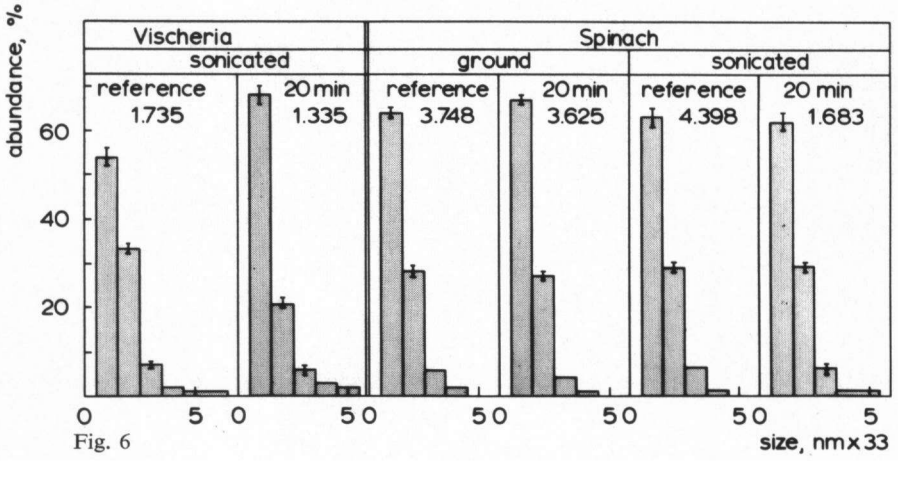
abundance, %
Vischeria
sonicated
Spinach
ground
sonicated
reference
1.735
20 min
1.335
reference
3.748
20 min
3.625
reference
4.398
20 min
1.683
60
40
20
0
5
0
50
50
50
50
5
size, nm x 33

Fig. 6

the bleaching rate for spinach preparations, curve D, open symbols. The same is true of the ground spinach samples, curve C, open circles, even if centrifuged during the interruption period.

Fig. 6 shows the particle size distribution for sonicated *Vischeria* preparations, and for ground as well as sonicated spinach samples. For *Vischeria* it is evident that a 20 minutes' interruption of sonication results in an increased amount of 16–49 nm particles at the expense of 49–82 nm ones. However, for spinach no such distinct change in size distribution of the counted particles is observed. This invariability holds for both ground and sonicated preparations.

4. DISCUSSION

A most striking phenomenon, *fig. 5*, curves C and D, consists of a change of the time course pattern for aerobic photobleaching of spinach chloroplast fragment suspensions upon sonication. The sequence 'low-rate' bleaching followed by 'high-rate' bleaching is reversed by this treatment, and, thus, the latter pattern resembles that for *Vischeria* preparations. However, interruption of the sonication procedure does not affect spinach bleaching, whereas it enhances that of *Vischeria*. From this result one may conclude that the spinach material is likely to be more stable than the algal particles are. This conclusion is supported by the fact that such an interruption period results in an increased relative amount of 16–49 nm fragments at the expense of 49–82 nm ones, whereas such an increase is not observed for spinach preparations, *fig. 6*. It should be emphasized that in the electron micrographs, it is not possible to discriminate between chlorophyll-carrying and chlorophyll-free fragments. The particle size distributions, therefore, are only indicative of the relative sturdiness of the suspended material as a whole.

LIST OF FIGURES

Fig. 1. Cell of *Vischeria stellata*. Fixation: glutaraldehyde together with OsO_4. C; chloroplast, N; nucleus, M; mitochondrion.

Fig. 2. Band pattern of *Vischeria* chloroplast. Fixation: $KMnO_4$. Each band consists of 3 thylakoids, see insert.

Fig. 3. Disintegration of thylakoids due to glass bead treatment prior to fixation. Subsequent fixation: glutaraldehyde-OsO_4.

Fig. 4. Fragments of *Vischeria* chloroplasts, negatively stained with ammonium molybdate.

Fig. 5. Time course of aerobic photobleaching of *Vischeria* and spinach chloroplast fragment suspensions. Triangles and squares: closed: 7 min. sonication, open: 4+3 min. sonication + 20 min. interruption; circles: closed: ground, open: ground + 20 min. centrifugation.

Fig. 6. Particle size distributions for *Vischeria* and spinach chloroplast fragment preparations. For sonicated samples "reference" indicates a non-interrupted 7 minutes' sonication treatment, "20 min." refers to a 20 minutes' interruption of this procedure. For ground samples "reference" means grinding without, and "20 min." grinding with a 20 minutes' interruption. Counts were made by the first two authors independently, and at different regions of the pictures. The vertical bars indicate the difference between both counts. Their mean is chosen for the height of the columns. Absence of bars means coincidence of counts. Numbers refer to the number of particles counted.

The change of the spinach bleaching pattern upon anaerobic sonification suggests that this treatment creates a situation typical of *Vischeria*. Since sonication does not markedly change the measured particle size distribution in spinach preparations, the alteration of the bleaching pattern possibly is due to fragments smaller than 16 nm. In such a case the dimensions of these particles are equal to, or smaller than, those of the photosynthetic unit (BRANTON & PARK 1967). As a result of sonication, a number of these units, or fragments thereof, may become devoid of some protection procured by the original condition. Such a situation can be understood if, e.g., these particles are freed from their original embedding in the thylakoid membrane, or at least a loosening occurs of the membrane structure in their direct environment. The fact that the slopes of the bleaching curves for ground and sonicated spinach samples are equal beyond 10–15 minutes of irradiation is in line with this suggestion.

Three additional experiments showed that during bleaching the red chlorophyll absorption maximum of sonicated spinach samples does not shift to the short-wave side, as is the case for this peak of ground preparations. This result indicates that the bleaching of photosystem 2 is preferentially enhanced by sonication. According to THOMAS *et al.* (1970), a preferential bleaching of system 2 chlorophyll occurs in *Vischeria*. These observations support the suggestions that sonication of spinach chloroplasts changes their normal condition into one reminiscent of that of *Vischeria* chloroplasts. Comparison of both kinds of preparations, therefore, possibly is a useful tool for studies of thylakoid functions in photosynthesis.

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