AEROBIC PHOTOBLEACHING OF VISCHERIA STELLATA

J. B. THOMAS, H. LOGTENBERG, and H. SCHOONMAN

Biophysical Research Group, Physics Institute of the State University, Utrecht, The Netherlands

SUMMARY

1. The time course of aerobic photobleaching of the chlorophyll *b*-free alga *Vischeria stellata* is of a biphasic nature. Initially the bleaching rate is about 10 times higher than under prolonged exposure to high light intensities.
2. Centrifugation considerably enhances the aerobic photobleaching rate of chloroplast fragments. Concomittantly the 685 nm component of the low-temperature fluorescence spectrum is markedly increased. The relationship between the intensity of this component and the bleaching capacity resembles an exponential one.
3. As the photobleaching proceeds, the red absorption maximum shifts to longer wavelengths. This relationship is linear.
4. Both photosystems are involved in the initial bleaching.
5. The results are discussed in terms of the spill-over model for excitation energy in photosynthesis.

1. INTRODUCTION

As reported earlier (THOMAS & BRETSCHNEIDER 1970), the aerobic photobleaching of the chlorophyll *b*-free green alga *Vischeria stellata* shows a reversed pattern of its time course as compared with that for spinach chloroplasts, whereas the type of photobleaching in spinach resembles that for *Aspidistra elatior* chloroplasts (THOMAS & NIJHUIS 1968). It means that, contrary to spinach and *Aspidistra*, *Vischeria* preparations show an initial "fast" photobleaching followed by a "slow" one.

Generally, a biphasic photobleaching of photosynthetic pigments can be explained by (1) the occurrence of two components bleaching in succession and at different rates, or by (2) the influence of a compound formed or destroyed during irradiation, or by a combination of (1) and (2). Two forms of chlorophylls, for both chlorophyll *a* and *b*, have been observed by SHLYK & NIKOLAYEVA (1963), DEROCHE-LABORIE *et al.* (1964), and DEROCHE (1969). The former authors reported that differences in rate of photobleaching occur between freshly formed and aged chlorophyll complexes. On the other hand, FRIEND & ACTON (1966) observed with isolated sugar beet chloroplasts that in light and in the presence of added linoleate the total amount of lipid peroxide increased in the first 20 minutes, whereas afterwards this peroxide was destroyed. In a personal communication Dr. Friend remarked that oxidation of unsaturated fatty acids will cause a bleaching of chlorophyll, and the oxidation coupled

with breakdown of lipid peroxide may be faster than that coupled with increase in peroxide.

Since the *Vischeria* photobleaching pattern differs from that for spinach and *Aspidistra*, and such a difference may provide some more information about the proceeding of aerobic photobleaching, this alga is used for studying both absorption and fluorescence changes during the bleaching process. The results are presented below.

2. METHODS

2.1. Preparation

Vischeria stellata was grown in Bristol's solution (STARR 1964) with the addition of 1g/l Difco proteose peptone. The culture flasks containing 100 ml medium each were placed in a Psycrotherm incubator shaker under air enriched with 5% CO_2 at 27°C. A light-dark cycle of 14 and 10 hours, respectively, was maintained throughout the growing period of 7 days.

The cells were spun down at about 2500 × *g* for 10 minutes and taken up in 0.02 M phosphate buffer, pH 7.3. The suspension was transferred to a thin-walled glass test tube provided with a rubber stopper carrying a gas inlet and outlet. It was flushed with nitrogen "extra" for 15 minutes. Next the suspension was sonicated at 0°C in a Lehfelt sonicator at a frequency of 0.8 Mcycles/sec., output 250 W, for 30 seconds. Then ice was added again to the contacting water column, and sonication was resumed after 30 seconds. This procedure was repeated 14 times. The sonication times mentioned below bear upon the sum of the actual sonication periods. The sonicated suspension was spun down at about 2500 × *g* for 25 minutes. The sediment was discarded, and, since the photobleaching in question is an oxidative one, the supernatant was flushed with air at 0°C for 15 minutes. Finally the preparation was diluted with the mentioned, ice-cold buffer until the absorbancy, 1 cm path length, was about 0.7 at the red chlorophyll maximum.

Colloidal chlorophyll was prepared by grinding *Vischeria* cells in a mortar containing carborundum "F" and a few drops of a distilled acetone water mixture, 80%. To the brei some more acetone was added, and a few drops of the intensively coloured clear supernatant were pipetted into a test tube containing 0.02 M phosphate buffer, pH 7.3. Next acetone was removed by flushing with nitrogen "extra" in the dark for several hours. Finally the concentration of the colloidal suspension was adjusted by dilution with buffer.

2.2. Absorption measurements

Absorption spectra as well as absorption difference spectra were established in a Beckman DK2 recording spectrophotometer at room temperature.

2.3. Fluorescence measurements

Emission spectra were recorded with the apparatus described by GOEDHEER (1964) at 77°K. Glycerol was added to the suspension to give a final concen-

tration of 60%. In this way no cracks occurred during cooling down to liquid nitrogen temperature. Instead of using the filter paper technique, this mixture was put into 1-cm perspex cuvettes in front of the window of the three-walled Dewar vessel according to the method of BRIL *et al.* (1969).

2.4. Irradiation

For photobleaching, 1-cm cuvettes were filled with the preparation, placed in a refrigerated box at about 4 °C in front of a 9 mm thick perspex window, and irradiated with white light from a Cimator slide projector placed outside the box. The light intensity at the front wall of the cuvette amounted to 0.06 W.cm^{-2}. In case of establishing difference spectra, the blank, protected from light, was placed in the refrigerated box as well during irradiation of the sample. In general, preparations were kept as cool as possible throughout the experiment.

3. RESULTS

3.1. Aerobic photobleaching

The time course of aerobic photobleaching of *Vischeria stellata* preparations is shown in *fig. 1*. Two phases are observed. It should be remarked that the concept of a biphasic time course rather than a logarithmic one is based on previous results (THOMAS & NIJHUIS 1968) as well as on the data of the individual experiments. An initial "high-rate" bleaching is followed by a 10 times slower one. As mentioned earlier, this sequence is reversed for spinach and *Aspidistra*.

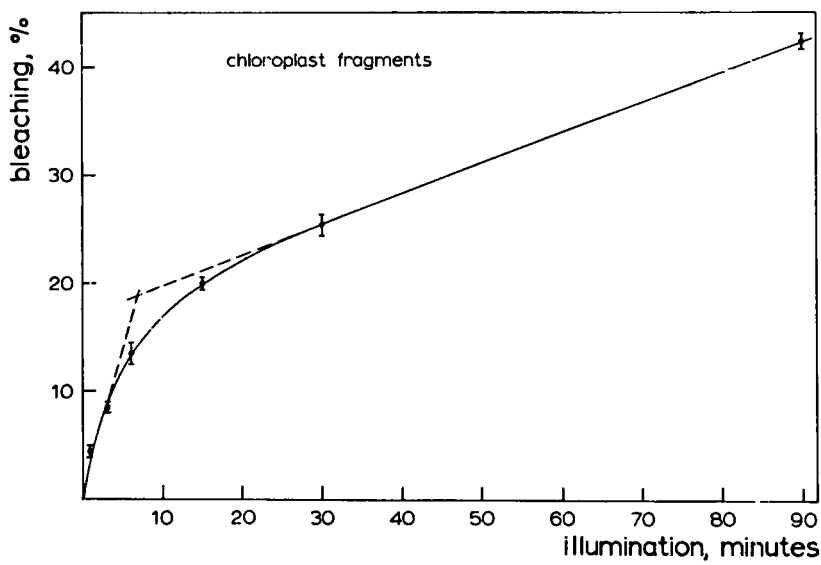

Fig. 1. Aerobic photobleaching of *Vischeria stellata* preparations. Light intensity at front wall of cuvette 0.06 W.cm^{-2}. Bleaching expressed as percentual absorption decrease. Values and standard deviations refer to 10 experiments.

It might be that part of the chlorophyll in *Vischeria* is readily freed from its carrier compound by sonication. This freed chlorophyll, either in the colloidal state or dissolved in lipids from the chloroplast fragments, might bleach at a higher rate than the intact chlorophyll-carrier complexes. Four additional experiments, not described here, demonstrated that photobleaching of *Vischeria* fragments obtained by grinding with carborundum "F" shows the same pattern as that for sonicated preparations. The possibility of occurrence of artificially freed chlorophyll is absent in intact cells. Since whole cells are considerably more resistant to photobleaching, use was made of two projectors for irradiating a 1-cm cuvette at both sides with incident intensities of 0.05 and 0.08 $W.cm^{-2}$, respectively. The light beam passed through a water layer of about 10 cm thickness. A magnetic stirrer prevented the cells suspended in the above-mentioned buffer from settling down. The cuvette was placed in a fan-driven air current. The temperature in the middle of the cuvette remained constant at 29 ± 1 °C. Four experiments were made. The results are shown in *fig. 2*. Evidently, whole cells exhibit the same type of aerobic photobleaching as do the fragments, be it at considerably increased intensity and irradiation period. The intersection of the tangents of both slopes of the curves seems to occur at lower bleaching percentages, 11, 15, and 17, than with chloroplast fragments, 19%. However, in two cases in which bleaching was measured within the first hour of irradiation

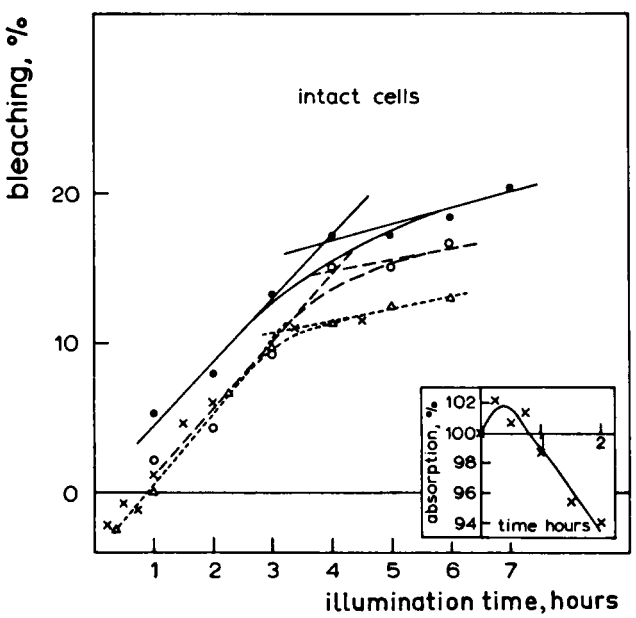

Fig. 2. Aerobic photobleaching of whole *Vischeria stellata* cells. For experimental conditions see the text. The data refer to samples from four cultures grown under identical conditions. The dotted graph serves to depict two nearly coinciding experiments (△ and ×).

as well, crosses and triangles in *fig. 2*, an initial absorption increase was observed. For one of the samples this effect is depicted in the insert. No significant changes in scattering were observed. Therefore it seems likely that, at the very early phase of irradiation, some chlorophyll is formed preponderantly to, and concomittantly with, the photobleaching process. For instance, ŠETLÍK *et al.* (1969) obtained changes in chlorophyll concentration per unit dry weight of algae when varying the light intensity. Moreover, they emphasized the difficulty of interpretation, since both adaptation and cell division may occur simultaneously. In any case, varying the light intensity was observed to induce changes in chlorophyll concentration.

If corrections are made for the initial absorption increase, shown as "negative bleaching" in *fig. 2*, the tangential intersection point shifts towards bleaching percentages about equal to that for fragment preparations. This means that free chlorophyll is not likely to occur in the latter samples. This conclusion is supported by the following observations: (1) addition of colloidal chlorophyll *a* does not noticeably change the biphasic character of the bleaching pattern at the same extinctions, (2) colloidal chlorophyll in comparable concentrations bleaches at about twice a higher rate than *Vischeria* fragments in between 15 and 90 minutes of irradiation, (3) a protective factor from spinach, *cf.* THOMAS & NIJHUIS (1968), retards the aerobic photobleaching of *Vischeria* fragments but does not affect the bleaching rate of colloidal chlorophyll *a*, and (4) addition of this nearly non-fluorescent colloidal chlorophyll does not markedly change the emission spectrum at 77 °K of *Vischeria* fragments. The colloidal chlorophyll, therefore, does not noticeably dissolve in lipids from the thylakoids.

Sub (3) mention is made of a protective factor from spinach. Such a compound could not be observed with *Vischeria*. Washing the preparations did not result in any change of the bleaching pattern and rate of the crude suspension. This result also means that the initial "high-rate" bleaching cannot be due to a washable factor that accelerates bleaching and is consumed

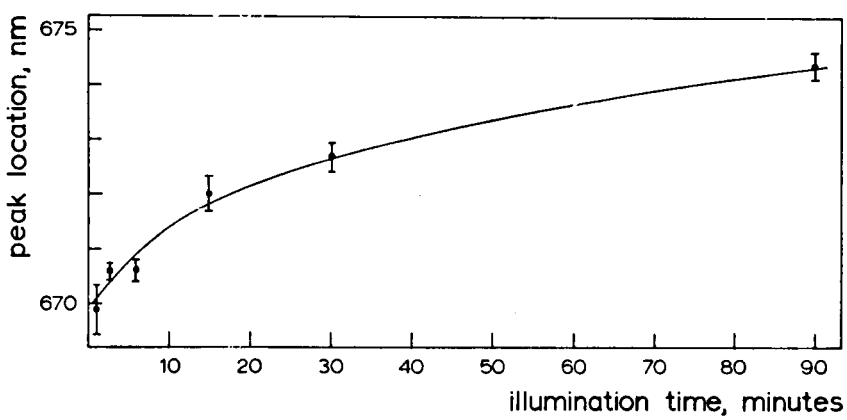

Fig. 3. Relation between duration of irradiation and location of the red absorption band. Values are means of 10 experiments.

during such a process. A biphasic time course of bleaching would equally result if a protective factor were formed during irradiation. Check experiments in which supernatants of spun-down fragments from non-irradiated and 30 minutes irradiated preparations were used as media for non-treated fragments from the same batch, showed no noticeable difference in bleaching rate and pattern for both kinds of samples. Therefore, these data suggest that the bleaching pattern is not due to changing concentrations of a washable factor affecting the bleaching rate.

During bleaching the location of the maximum of the red absorption band shifts towards longer wavelengths, *fig. 3*. This red shift indicates that, contrary to what happens in the cases studied so far, a short-wave form of chlorophyll *a* is more sensitive to irradiation than the long-wave form in *Vischeria*.

Since the contribution of scattering increases with progressive reduction of the absorption band, the measurements became less accurate with prolonged irradiation. Therefore photobleaching was not measured beyond 60%.

3.2. Effect of centrifugation

Extending the sonication period from 7 up to 12 minutes did not result in any change of the bleaching rate. It therefore seems unlikely that, under the present experimental conditions, there were any changes in the pigment molecules due to this procedure, as observed by JANUSZCZYK *et al.* (1969) in chlorophyll *a* solutions.

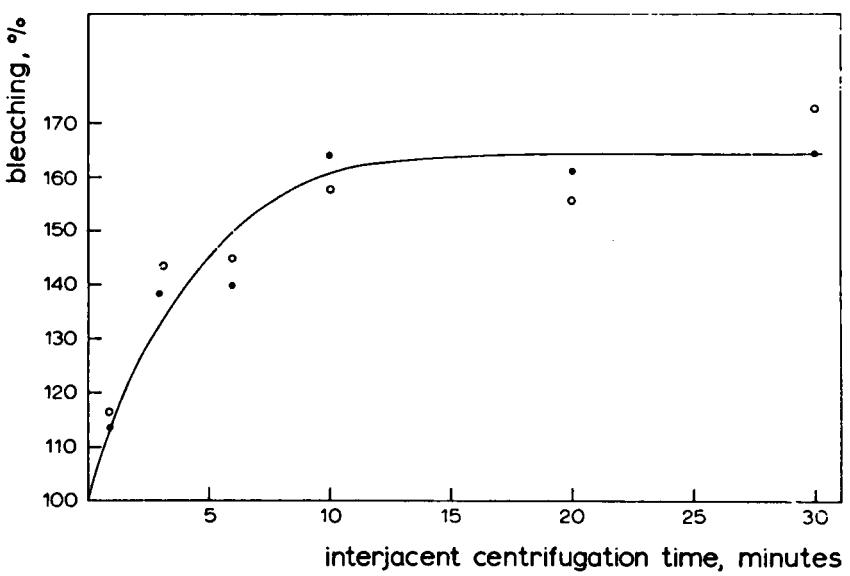

Fig. 4. An arbitrary experiment showing the effect of interjacent centrifugation on the rate of aerobic photobleaching. Irradiation times 15 (○) and 30 (●) minutes. For details see text.

If the series of alternating sonication and cooling was interrupted for a period up to 30 minutes, no detectable difference in bleaching rate with the non-interrupted one was observed. This holds for preparations kept at either 0 °C or room temperature during interruption. However, if during this period the particles were spun down and resuspended in their supernatant, the rate of photobleaching, most surprisingly, was enhanced. *Fig. 4* shows an experiment in which the suspension was centrifuged at about 2500 × *g* for varying times upon 4 minutes of sonication. After centrifugation the preparation was sonicated for 3 more minutes. The bleaching, measured after 15 and 30 minutes of irradiation, is expressed in percents of that of a blank sonicated for 7 minutes interrupted by a period of storing at 0 °C, equal to that for centrifugation of the sample. According to *fig. 4*, interjacent centrifugation results in an about 60% increase in bleaching rate. Extension of the centrifugation period beyond 10 minutes yielded no further increase in bleaching rate.

3.3. Fluorescence measurements

The shape of the emission spectrum at 77 °K was established and compared with the bleaching capacity. The fluorescence spectrum shows two major components peaking around 720 and 685 nm, termed F720 and F685, respectively. The intensity of F685 is expressed in percents of the total emission by estimating the area of the total emission as well as that of F720. The shape of the latter one was derived from preparations in which F685 was nearly annihilated by irradiation. As shown in *fig. 5*, there is a clear correlation between F685 intensity and

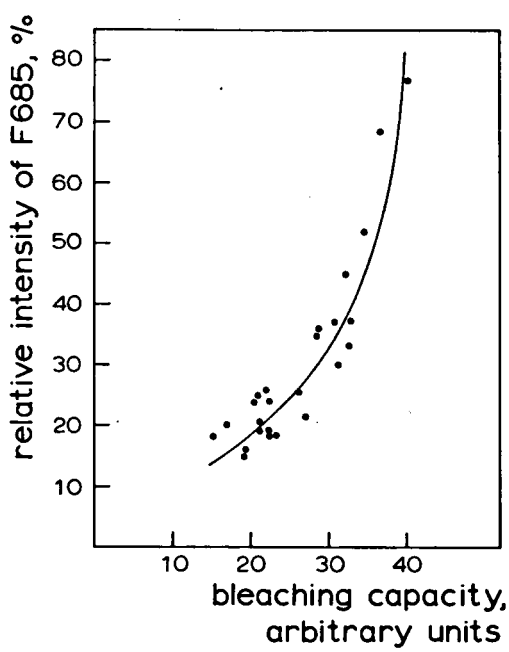

Fig. 5. Relation between bleaching capacity, measured as bleaching upon a 15 minutes' irradiation, and original, relative, F685 intensity.

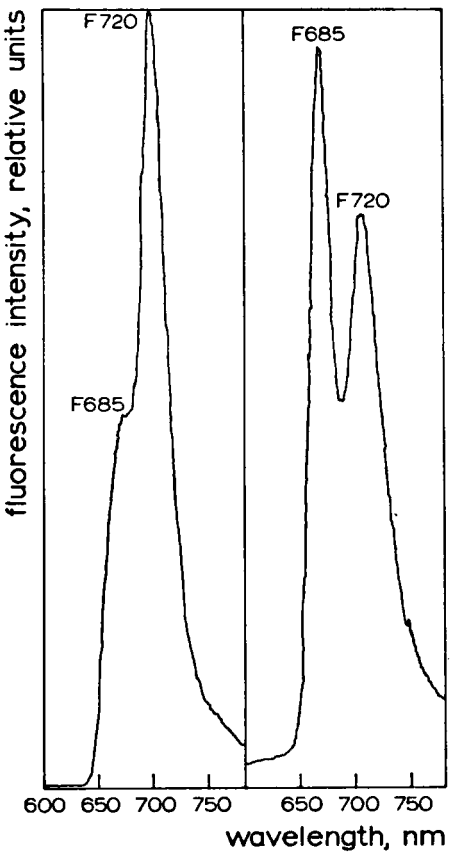

Fig. 6. Example of low-temperature emission spectra of preparations with low and high F685 contribution. For both spectra chlorophyll concentration as well as adjustment of the measuring apparatus were approximately the same.

bleaching capacity. Since centrifugation increases the bleaching rate, it enhances the F685 intensity as well. It may be added that, as the yield of chloroplast fragments is poor at short sonication times, the effect of sonication on the F685 intensity could not be measured with sufficient accuracy.

An example of emission spectra with low and high F685 contributions is given in *fig. 6*. According to various authors, see *e.g.* GOEDHEER (1968), F685 is emitted by photosystem 2, whereas F720 is mainly due to photosystem 1 emission. A high fluorescence intensity of the former system is correlated with a high photobleaching capacity of *Vischeria* preparations.

Irradiation reduces the fluorescence intensity. *Fig. 7* shows that initially this reduction is highest for F685. After about 30 minutes of irradiation only about 20% of F685 is left. Prolonged irradiation reduces this low F685 intensity only very slowly. F720, on the contrary, is affected less severely at first, but at prolonged irradiation its reduction rate is higher than that of F685. It can therefore be concluded that initially the fluorescence emitted by the short-wave system, photosystem 2, is sensitive to irradiation considerably more than the emission by the long-wave one, photosystem 1.

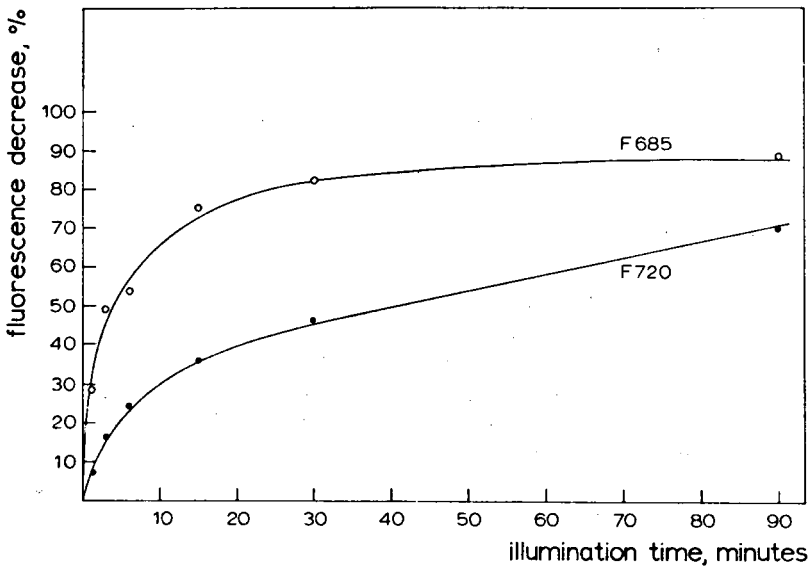

Fig. 7. Fluorescence reduction for F685 and F720 due to irradiation.

As demonstrated in *fig. 8*, photo-induced fluorescence reduction proceeds at a higher rate than does the decrease of absorption. The difference in initial rates is again larger for F685 than for F720. The fact that the relation between bleaching and fluorescence decrease for both kinds of emission is nearly linear at small reductions of absorption suggests that this decrease is not due to pro-

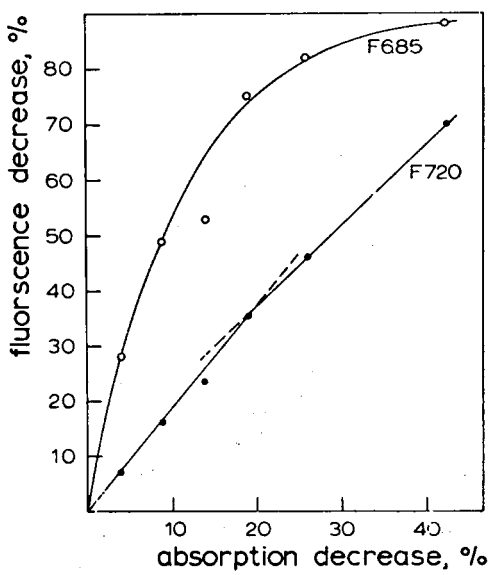

Fig. 8. Relation between photo-induced fluorescence reduction and bleaching

ducts formed during bleaching. From both *figs. 7* and *8* it is evident that fluorescence reduction for both photosystems occurs concomittantly. This statement, combined with the observation of the initial approximate linearity of the bleaching/fluorescence reduction relation in *fig. 8*, suggests that the initial "fast" bleaching refers to the sum of the bleaching of both photosystems.

4. DISCUSSION

Summarizing the results it can be stated that:

1. the biphasic character of the aerobic photobleaching in *Vischeria stellata* chloroplast fragments and whole cells is neither due to free chlorophyll nor to a washable factor affecting the bleaching rate, *figs. 1* and *2*,
2. the red shift of the absorption maximum suggests that a short-wave form of chlorophyll *a* bleaches more readily than the long-wave one, *fig. 3*,
3. the bleaching rate enhances with increasing centrifugation time at about 2500 × *g* up to 10 minutes. Prolonged centrifugation does not result in any further enhancement, *fig. 4*,
4. the relative intensity of F685 varied with various preparations, *fig. 6*, whereas an exponential-like relation exists between this intensity and the bleaching capacity, *fig. 5*, and
5. during bleaching the rates of intensity decrease for both F720 and F685 are highest in the early phase, *fig. 7*, the relation between intensity decrease and bleaching being almost linear initially, *fig. 8*, whereas a nearly linear relationship shows up at prolonged bleaching for F720 exclusively.

Considering these results, the following conclusions can be drawn.

The difference between the oxidative photobleaching of *Vischeria* fragments and those from spinach and *Aspidistra* consists not only in the earlier mentioned reversal of the bleaching pattern, but also in a reversal of the mutual sensitivities towards irradiation of both photosystems. In the alga studied chlorophyll of system 2, C_{aII} bleaches more readily than that of system 1, C_{aI}, whereas in the higher plants mentioned C_{aI} is the more sensitive pigment form. When *figs. 1* and *3* are compared, a certain similarity of the shapes of the graphs is obvious. The samples used in these experiments were prepared in an identical way, including the time of centrifugation. Plotting the data of both graphs in relation to each other yields a straight line, *fig. 9*. This linear relationship means that the ratio of the photobleached chlorophyll forms from both systems remains constant throughout the irradiation period. Consequently C_{aI} bleaching proceeds at a higher rate initially, then it levels off. Therefore it should show the pattern of C_{aII} bleaching, be it at a lower rate. That is, the time course of bleaching for photosystem 1 is of a biphasic nature as well, and the intersection point of both phases occurs at about the same bleaching percentage as in the case of photosystem 2.

When attempting to explain this phenomenon, one may think of the heterogeneity of chlorophylls as observed by SHLYK & NIKOLAYEVA (1963), DEROCHE-

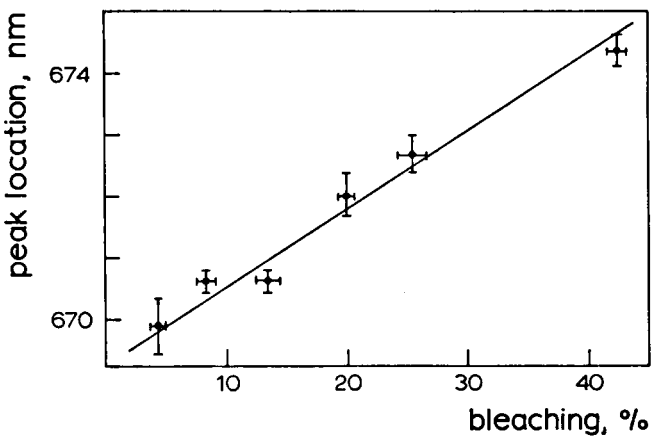

Fig. 9. Relation between absorption peak location and bleaching.

LABORIE *et al.* (1964), and DEROCHE (1969), discussed earlier. Both photosystems would then contain two kinds of chlorophyll *a* differing in bleaching rate. Since system 2 bleaches faster than system 1, the portion of the "high-rate" bleaching chlorophyll should be larger for system 2 than for system 1. Moreover, these ratios should be rather constant for various cultures, as shown by the small standard deviations in *fig. 1*. Therefore the above explanation does not seem likely, although it cannot be ruled out altogether. In this connection it is worth mentioning that MICHEL & MICHEL-WOLWERTZ (1970) obtained a single fraction exhibiting system 1 activity and two fractions with system 2 properties by disrupting spinach chloroplasts without the use of detergents and subsequent gradient centrifugation.

The linear relationship between location of the red absorption maximum and bleaching is suggestive of another possibility, namely, the dependence of the bleaching of system 1 on that of system 2. Such a dependence can be understood in terms of spill-over of excitation energy absorbed by system 2 to system 1, as originally proposed by MYERS (1963), see also, *e.g.*, MALKIN (1967). In the non-bleached state photosystem 1 receives energy by direct light absorption as well as by transfer of part of the energy absorbed by system 2. The more the latter system is bleached, the less energy it can absorb and, consequently, transfer to system 1. In this way it can be also understood why F720 intensity during the early bleaching phase decreases at a higher rate than after severe reduction of the F685 intensity, *fig. 7*. The relationship between initial F685 intensity and bleaching capacity is in line with the spill-over model, *fig. 5*. If the F685 intensity is high, this may mean that the spill-over efficiency is reduced. In that case C_{aII} retains more energy, resulting in an increased bleaching rate. Since it is found that C_{aII} bleaches faster than C_{aI}, the overall bleaching rate would be increased. In *fig. 6* it can be seen that a high intensity of F685 coincides with a low one for F720, and conversely.

As mentioned earlier, the initial rate of the biphasic bleaching, *fig. 1*, is 10 times that of the final one. Since evidence is obtained that both photosystems

start bleaching simultaneously, *figs. 7* and *8*, whereas system 2 is the more sensitive one, *fig. 3*, it means that the latter system bleaches 9 times faster than system 1 per unit chlorophyll. Considering the conclusion that any change in concentration of a possible rate-affecting factor during bleaching is unlikely, and assuming that the fluorescence decrease upon irradiation reflects bleaching of chlorophyll molecules, the data in *fig. 8* can be understood in terms of the spill-over conception. The initial reduction of fluorescence per 10% absorption decrease amounts to 53% for F685 and to 19% for F720. At a later stage, between 30% and 40% absorption decrease, these figures are 4% and 15%, respectively. For F685 this means a difference of 49% per 10% bleaching at initial and later stages. Assuming by way of rough approximation that bleaching affects fluorescence intensity and spill-over rate equally, it follows that at the later stage system 1 receives 49% less energy per 10% bleaching from system 2 than initially. Since the quantum yield for bleaching of C_{aI} is 1/9 of that forC_{aII}, it is calculated that the difference in fluorescence decrease per 10% bleaching at both stages amounts to about 5% for F720, provided the F720 intensity, based on light absorption by system 1 exclusively, is linearly related to the absorption decrease. The shape of the F720 graph, *fig. 8*, seems to be suggestive of such a relationship within the studied bleaching range. From *fig. 8* it can be read that the difference in question amounts to about 4%. This percentage is close to the computed 5%.

In conclusion, the conception of the spill-over model seems to offer a more plausible interpretation of the results than that based on the assumption of the occurrence of different proportions of slower and faster bleaching chlorophyll molecules in both photosystems.

The reason why the initial bleaching proceeds at a higher rate than at prolonged irradiation is still obscure. The effect of centrifugation, *fig. 4*, suggests that structural factors are involved. The saturation of this effect is remarkable. Its explanation, however, needs further study.

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