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LIGHT-INDUCED ABSORBANCE CHANGES AT −170 °C WITH SPINACH CHLOROPLASTS:

CHARGE SEPARATION AND FIELD EFFECT

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SUMMARY

Absorbance changes induced by illumination of spinach chloroplasts at −170° were recorded either with a single-beam kinetic spectrophotometer or with a double-beam differential spectrophotometer, in the range 500–560 nm.

1. The difference spectrum of absorbance changes measured with the kinetic apparatus indicates the reduction of C-550 (peaks at 542, 547.5 nm) and the oxidation of cytochrome b_{559} (556 nm). There is an additional peak at 518 nm. The kinetics are nearly identical at 542 and 518 nm, and about twice slower at 556 nm.

2. It is shown that C-550, cytochrome b_{559} and the 518-nm effect all belong to Photosystem II. The corresponding absorbance changes do not occur upon illumination at −170° of chloroplasts that received one flash in the presence of 3(3,4-dichlorophenyl)-1,1-dimethylurea and of hydroxylamine before being cooled. The kinetics under illumination by far-red light confirm the attribution to Photosystem II. Another type of absorbance change was identified, with a smooth difference spectrum peaking at 530 nm; it is attributed to Photosystem I.

3. In the presence of an increasing concentration of ferricyanide, a progressive and parallel decrease of the magnitude of absorbance changes is observed at 518 and 556 nm. Ferricyanide has little effect on absorbance changes at 542 nm.

4. Difference spectra (between a cuvette illuminated at −170° and a reference cuvette) recorded with the double-beam spectrophotometer are nearly identical with the Photosystem II part of the absorbance changes. The peak at 518 nm appears to be stable for at least 10 min. In the presence of ferricyanide a parallel decrease of the peaks at 518 and 556 nm is observed.

We propose that the 518 nm effect observed at −170° is due to a local field effect, that needs the reduction of the primary electron acceptor, and that is influenced by the redox state of the secondary electron donors of Photosystem II. A possible similar effect occurring in Photosystem I would be of much smaller amplitude.

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INTRODUCTION

Photochemical reactions performed at low temperature with photosynthetic structures from green plants allowed the identification of several components of the reaction center of Photosystem II (see [1] for a review). In many respects, C-550 behaves like a primary electron acceptor [2, 3]. We also found that at −170° its rate of reduction is proportional to the intensity of exciting light [4]. Cytochrome b_{559} is an electron donor in photochemical reactions; it is clearly connected in some way with C-550 [2]. However that connection is not straightforward in view of their respective kinetics of phototransformation at −170° [4] and of the influence of the state of the electron donating system on the photoreactions occurring at −50° [5]. In any case, following an illumination at a low temperature, a few species are blocked in a charged state, near the reaction center, and we can expect, due to their proximity, the existence of a strong electrical field.

Junge and Witt [6], and Emrich et al. [7] have interpreted in terms of an electrochromic effect, due to a delocalized electrical field, the light-induced absorbance increase that peaks at 515 nm in spinach chloroplasts, at room temperature and that is related to the photosynthetic phosphorylations. The fast rise-time of the 515 nm absorbance change [8] suggests that the field is directly generated by the primary charge separation. However Witt et al. [9] did not detect such an absorbance change at −160°. A possible electrochromic effect occurring at very low temperature has been reported for photosynthetic bacteria, e.g. [10, 11]. Amesz et al. [12] recently described a similar effect with spinach chloroplasts, at −40°.

In this article we report on experiments performed at −170°. We identify an absorbance change peaking at 518 nm (518-nm effect), that is connected kinetically with the reduction of C-550, but whose magnitude seems to parallel the magnitude of cytochrome b_{559} photooxidation. This effect is discussed as a possible consequence of local fields resulting from the charge separation. A Photosystem I absorbance change of small amplitude is also reported (maximum at 530 nm).

MATERIALS AND METHODS

Biological material. Chloroplasts, prepared from fresh spinach leaves [13] were resuspended in the grinding buffer (0.4 M sucrose, 0.01 M NaCl, 0.02 M Tris, at pH 7.8) plus 5 % dimethylsulfoxide. The suspension was distributed in 0.5 ml tubes and kept in liquid N_2. Each tube was thawed a few minutes before the experiment. For experiments performed in the presence of potassium ferricyanide (0.1–20 mM) or 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (20 μM) plus hydroxylamine (100 μM), the chloroplasts stayed 5 min in a tenfold diluted buffer (so as to insure the breakage of the outer envelope [14]) and were then resuspended in the normal buffer.

Differential absorption spectra. These spectra were recorded with a double-beam spectrophotometer (Perkin–Elmer, model 356), equipped with a Dewar flask in which was dipped the cell holder (path: 1 mm) (see [13] for further details). Instead of using the chart recorder, we sent the signal into a multichannel analyzer (Intertechnique, type SA 40 B). Before actinic illumination, five successive differential spectra were stored negatively; then the sample cuvette was illuminated (at −170°)

and five successive difference spectra were stored positively. That operation was reproduced about 10 times, each time with a new sample, and all the spectra were stored together so as to improve the signal/noise ratio. The photomultiplier was protected from fluorescence excited by the measuring beam by two filters (Wratten 64 and Scott BG 18-3 mm).

Kinetics of absorbance changes. The chloroplasts suspension was poured into a lucite cuvette (1 mm) which was held in a copper frame dipping in liquid N_2. The equipment has been described in detail [4]. The temperature of the sample was −170°. The signals of absorbance change were measured with a multichannel analyzer (Intertechnique, type Didac 800). The signals corresponding to several successive cuvettes were added in the memory. The kinetics and the magnitude of absorbance changes were measured under light-limited conditions. The actinic beam was opened by an electronic shutter. It was roughly monochromatic; two filter combinations were used, giving 630 nm [4] or far-red light. In the latter case the following filters were used: 2 cm of water, a Calflex (Balzers), a Schott RG 630/3 mm. a Schott RG 715/3 nm, a MTO 707 nm interference filter.

In both types of measurement (differential spectrophotometer or kinetic apparatus), the chloroplasts suspension was dark-adapted for 2 min at 20 °C before being cooled. For experiments in the presence of DCMU and/or hydroxylamine, all operations were performed in complete darkness; in some experiments one saturating flash of white light (2 μs, 0.1 J) was given at room temperature.

RESULTS

1. *Description of the absorbance changes*

Typical kinetics of evolution of absorbance changes, for chloroplast suspensions excited by 630 nm light, are presented in Figs 1 and 5 for a few selected wavelengths. The magnitude of absorbance change ΔA is plotted in Fig. 2a, as a differential spectrum. This spectrum includes well-known features due to C-550 (peaks at 542 and 547.5 nm) and to the oxidation of cytochrome b_{559} (peak at 556 nm); it includes also a previously unobserved peak at 518–520 nm.

The kinetics are dependent upon wavelength. At 542 nm (C-550) the kinetics are nearly exponential, and the rate is proportional to the intensity of actinic light, as already reported [4]. The kinetics are 2–3 times slower at 556 nm. At 518 nm the kinetics are nearly the same as the 542 nm (Fig. 1), at all intensities; however at 518

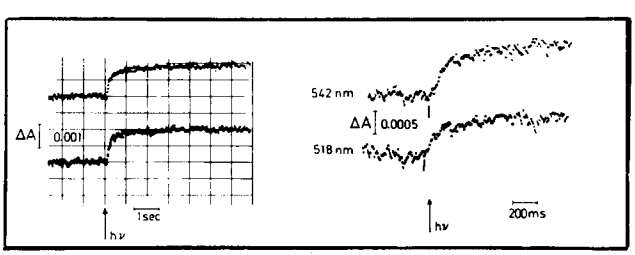

Fig. 1. Time course of absorbance changes at 542 nm (upper traces) and 518 nm (lower traces), at two scanning speeds, with the same actinic light (630 nm, 4 mW · cm^{-2}). Left traces, 1 expt; right traces, 4 expts. Chlorophyll concentration, 0.25 mg · ml^{-1}.

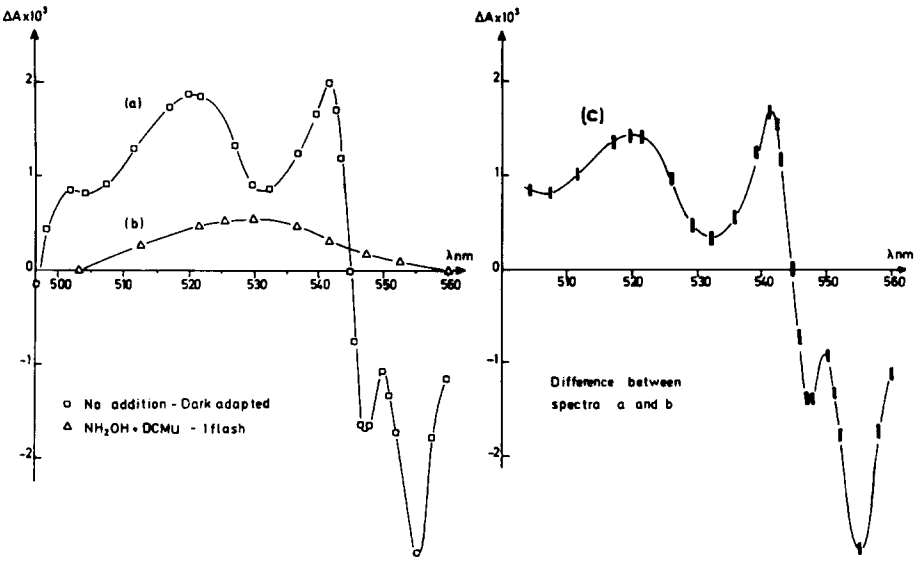

Fig. 2. Plots of absorbance changes, measured at their maximum level, with the kinetic apparatus, at −170 °. Chlorophyll concentration 0.25 mg · ml^{-1}. Curve a, control chloroplasts; curve b, addition of DCMU (20 μM) and of hydroxylamine (100 μM), (the suspension was excited by one flash before cooling); curve c, difference between curves a and b.

nm they seem to be somewhat faster (about 20 % on the average) and to show a deviation from the exponential. The curve of Fig. 5 (518 nm, excitation 630 nm) is very typical in this respect.

Difference spectra recorded with the double-beam spectrophotometer (Fig. 3a) present the same characteristics as Fig. 2a. This indicates a good stability with time (at least 10 min) for those three main features ($C\text{-}_{550}$, cytochrome b_{559}, peak at

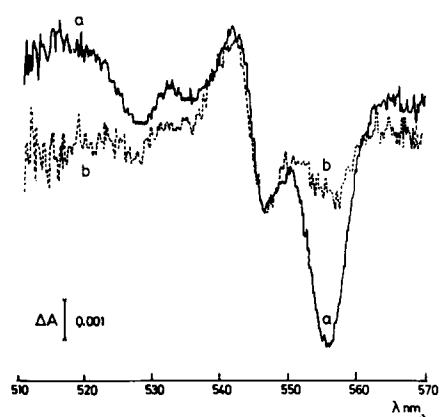

Fig. 3. Difference spectra, recorded with the double-beam spectrophotometer, for a cuvette before and after illumination at −170 °C. Chlorophyll concentration 0.4 mg · ml^{-1}. Curve a, control chloroplasts. The results for 8 samples were averaged. Curve b, addition of ferricyanide (5 mM). The results for 8 samples were averaged.

518 nm that we name 518-nm effect). A trough centered at 528 nm might be due to the β-band of cytochrome b_{559} [15]. It does not appear in Fig. 2a, probably because of the poorer resolution.

2. *Experiments in the presence of DCMU plus hydroxylamine*

It has been shown that the primary acceptor of Photosystem II is blocked in the reduced state when chloroplasts receive one flash in the presence of DCMU and hydroxylamine [16]. We used this property to separate possible contributions of Photosystem I and Photosystem II to the absorbance changes.

The kinetics of absorbance change in the presence of DCMU (20 μM) plus hydroxylamine (100 μM) (Fig. 4), or in the presence of only one of these chemicals, are identical with that of the control, provided that all operations are performed in complete darkness. If the chloroplast suspension is preilluminated by one flash at 20 °C, in the presence of both DCMU and hydroxylamine, and then rapidly cooled, the absorbance changes occurring at −170 °C are dramatically altered compared to the control (Figs 4 and 2b). There is no trace of photooxidation of cytochrome b_{559}; in the 500–550 nm wavelength range, the C-550 signal and the 518 nm effect disappear, and it remains a small absorbance increase, with a smooth difference spectrum (Fig. 2b). For a control cuvette with hydroxylamine alone, the preilluminating flash has no influence on the effect of subsequent illumination at −170 °C; with DCMU alone it induces a small decrease of the absorbance changes.

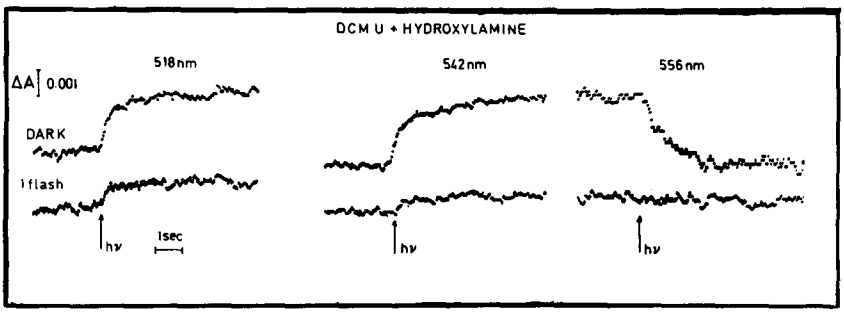

Fig. 4. Time course of absorbance changes (at −170 °) at 518, 542 and 556 nm, for chloroplasts suspensions (chlorophyll concentration: 0.25 mg · ml^{-1}) to which were added DCMU (20μM) and hydroxylamine (100 μM). Actinic light (630 nm, 4 mW · cm^{-2}) starts at upward arrow. Upper traces, no preillumination at room temperature. Lower traces, one flash at room temperature. Each trace represents the average of two samples.

The absorbance changes remaining at −170 °C, for chloroplasts preilluminated by one flash in the presence of DCMU plus hydroxylamine, are partly reversible (20–50 %), i.e. they occur at a reduced amplitude if a second step of light is given 2–5 min after the first saturating illumination. A similar reversibility, of relatively less significance, could also be observed in the 510–540 nm range with control cuvettes. Due to uncontrolled fluctuations that phenomenon has not been studied precisely; it seems to be more connected with Photosystem I reactions.

3. *Excitation with far-red light*

The signals whose difference spectrum is reported in Fig. 2b might be due to a

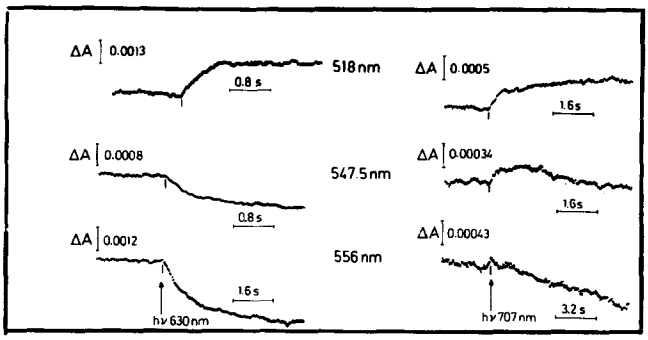

Fig. 5. Time course of absorbance changes (at −170 °), at 518, 547.5 and 556 nm, for chloroplasts suspensions (chlorophyll concentration: 0.25 mg · ml^{-1}) excited in red light (630 nm, 1 mW · cm^{-2}) or in far-red light (707 nm, 6.7 mW · cm^{-2}). Number of averaged samples: 630 nm–518 nm, 9; 547.5 nm, 6; 556 nm, 4; 707 nm–518 nm, 10; 547.5 nm, 15; 556 nm, 12.

Photosystem I reaction (it is known that P_{700} can be photooxidized at low temperature [9]) or to a previously unobserved Photosystem II reaction not blocked by preillumination in the presence of DCMU plus hydroxylamine. The second alternative is excluded by experiments performed with far-red actinic light. There are no well documented action spectra for Photosystem I and Photosystem II at low temperatures. We have made the assumption that far-red light preferentially excites Photosystem I reactions, as it does at room temperature [17].

A few kinetics of absorbance change are reported in Fig. 5, comparing excitations at 630 nm and 707 nm; they are precisely what was expected, assuming that the difference spectrum of Fig. 2b is due to Photosystem I and that the difference spectrum of Fig. 2c is due to Photosystem II. Far-red light seems to excite preferentially the Photosystem I contribution (ΔA positive at 518 and 547.5 nm, zero at 556 nm) and only very inefficiently the Photosystem II phase (ΔA positive at 518 nm, negative at 547.5 and 556 nm). In Fig. 5 (707 nm) the reactions are not saturated.

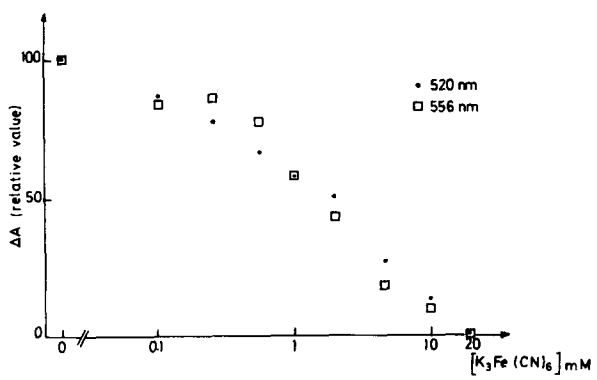

Fig. 6. A plot of absorbance changes (relative to their maximum value, at zero concentration of ferricyanide), measured at their maximum level, with the kinetic apparatus, at −170 °, for different concentrations of potassium ferricyanide. Actinic light, 630 nm, 4 mW · cm^{-2}. Two samples were used for each ferricyanide concentration. Chlorophyll concentration, 0.25 mg · ml^{-1}.

4. *Effect of ferricyanide*

Addition of potassium ferricyanide (0.1–20 mM) to chloroplast suspensions has little effect on the kinetics and magnitude of absorbance changes related to C-550. The other absorbance changes disappear progressively with increasing concentrations of ferricyanide, as seen with the double-beam spectrophotometer (Fig. 3b) and with the kinetic apparatus (Fig. 6); their kinetics are not seriously affected.

In Fig. 6, the curves corresponding to the 518-nm effect and to the photooxidation of cytochrome b_{559} are slightly different; however the difference is within our experimental uncertainty. In the experiments reported in Figs 3 and 6 a high concentration of ferricyanide was necessary for a dark oxidation of cytochrome b_{559} and for the disappearance of the 518 nm effect. A large seasonal variability was observed with regard to the amount of ferricyanide that must be added for the same effect (in [5] a nearly complete dark oxidation of cytochrome b_{559} was achieved with 0.4 mM of ferricyanide).

DISCUSSION

The different results that we report on light-induced absorbance changes at $-170°$ allow the characterization of four types of effects: reduction of C-550, oxidation of cytochrome b_{559}, a 518-nm effect, and a Photosystem I effect. Many studies have been devoted previously to the photoreduction of C-550 and to the photooxidation of cytochrome b_{559} [1]. The very low yield of these photoreactions in far-red excitation (compared to the Photosystem I effect), and their absence for chloroplasts preilluminated by one flash in the presence of DCMU plus hydroxylamine confirm that these species belong to Photosystem II and that their phototransformation cannot occur at -170 °C whenever the primary electron acceptor (as characterized at room temperature) is reduced.

The 518-nm effect is not a secondary band of C-550 for it disappears in the presence of ferricyanide when C-550 is not affected. Does it correspond to a new electron transport component or to a modification of the absorption spectrum of neighbour pigment molecules by the products of the primary photoreactions (local field)? We will provisionally prefer the second hypothesis for several reasons. First the kinetics of the 518-nm effect is practically identical with the C-550 kinetics, indicating a close relationship with the primary reaction (the small difference might result from overlap with the β-band of cytochrome b_{559}). Secondly the part of the difference spectrum that we obtain is very similar to the photoinduced 515-nm effect occurring at room temperature and attributed to a field effect [6, 7]. Moreover experiments with chloroplasts from maize mutants largely devoided of normal coloured carotenoids indicate the absence of the 518-nm effect, although the reactions involving C-550 and cytochrome b_{559} normally occur (unpublished results, in collaboration with Dr A. Faludi-Daniel).

That interpretation leads us to raise a few questions. At first it implies that the 518-nm effect is not a necessary consequence of charge separation. Indeed the Photosystem I activity results in a very different absorbance change (maximum at 530 nm), that might be due to oxidation of P_{700} [18]. Even if this absorbance change represents a light-induced field effect, its magnitude and its spectrum are very different from the Photosystem II 518-nm effect. The disappearance of that Photosystem I signal in the

presence of ferricyanide is rationalized by a chemical dark oxidation of the primary electron donor P_{700}. Moreover, in the presence of ferricyanide, the primary photoreactions of Photosystem II do occur without any detectable 518-nm effect. The results favor the hypothesis of a local field occurring at low temperature, as opposed to a delocalized field proposed by Junge and Witt [6] at room temperature, acting on pigments close to the reaction center (conformational change or electrochromism). As the 518-nm effect is stable for a few minutes, the hypothesis of an electrical field requires the additional assumption that the field does not break down, due to ions movements within that interval.

Absorbance changes similar to the 518-nm effect have been reported for photosynthetic bacteria, at low temperatures [10, 11, 19]. Also in bacteria, Case and Parson [20] found that the occurrence of pigment absorption shifts (carotenoids and bacteriochlorophyll), at room temperature, is influenced by the nature of the electron donor: a band shift occurs when cytochrome c_{555} is the electron donor, but not when cytochrome c_{552} is the donor. This result resembles our finding that the magnitude of the 518-nm effect varies with addition of ferricyanide: cytochrome b_{559} is oxidized in the dark and (as C-550 is still photoreduced with practically the same efficiency) another electron donor must be photooxidized.

We have no good rationale for the parallel disappearance of the 518-nm effect and dark oxidation of cytochrome b_{559}, with increasing concentrations of ferricyanide. We cannot decide between a specific effect of ferricyanide on low-temperature photoreactions and an action via oxidation of cytochrome b_{559}. Ferricyanide has little influence on the room-temperature 518-nm effect connected with Photosystem II activity (Vermeglio, A. and Mathis, P., unpublished; see also [21]); so we conclude that the effect of ferricyanide at low temperature is not related to a gross alteration of the photosynthetic membranes.

From the differential effect of ferricyanide on first the photoreduction of C-550 and second the photooxidation of cytochrome b_{559} and the 518-nm effect, it was hoped to get more insight into the structure of the Photosystem II reaction center. In a previous article [4] we proposed two schema that are able to account for the kinetics of reduction of C-550 and of oxidation of cytochrome b_{559}: two photoreactions working in series or in parallel. Both models will accomodate our present results provided that the chlorophyll molecule, that is supposed to be the primary electron donor to C-550, is reduced by an electron donor D whose redox potential is somewhat higher than the potential of cytochrome b_{559}. Such a donor has been proposed by Bearden and Malkin [22] in order to account for the appearance of the EPR signal of a chlorophyll cation upon illumination of spinach chloroplasts at −196 °C, at potentials higher than +475 mV. In our "in series" hypothesis, the electron donor D would be located between the chlorophyll and cytochrome b_{559}; in our "in parallel" hypothesis, D would be rapidly oxidized by Chl^+ in the photoreaction whose electron acceptor is C-550. In any hypothesis, the 518-nm effect would be linked to the photooxidation of both the hypothetical donor D and cytochrome b_{559}.

REFERENCES

1 Butler, W. L. (1973) Acc. Chem. Res. 6, 177–184
2 Erixon, K. and Butler, W. L. (1971) Biochim. Biophys. Acta 234, 381–389
3 Vermeglio, A. and Mathis, P. (1974) Bioelectrochemistry and Bioenergetics, in the press
4 Mathis, P., Michel-Villaz, M. and Vermeglio, A. (1974) Biochem. Biophys. Res. Comm. 56, 682–688
5 Vermeglio, A. and Mathis, P. (1973) Biochim. Biophys. Acta 314, 57–65
6 Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 244–254
7 Emrich, H. M., Junge, W. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1144–1146
8 Wolff, C., Buchwald, H. E., Rüppel, H., Witt, K. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1038–1041
9 Witt, H. T., Müller, A. and Rumberg, B. (1961) Nature 192, 967–969
10 Arnold, W. and Clayton, R. K. (1960) Proc. Natl. Acad. Sci. U.S., 46, 769–776
11 Dutton, P. L. (1971) Biochim. Biophys. Acta 226, 63–80
12 Amesz, J., Pulles, M. P. J. and Velthuys, B. R. (1973) Biochim. Biophys. Acta, 325, 472–482
13 Vermeglio, A. and Mathis, P. (1973) Biochim. Biophys. Acta 292, 763–771
14 Hall, D. O. (1972) Nature 235, 125–126
15 Garewal, H. S., Singh, J. and Wasserman, A. R. (1971) Biochem. Biophys. Res. Commun. 44, 1300–1305
16 Bennoun, P. (1970) Biochim. Biophys. Acta 216, 357–363
17 Vernon, L. P. and Avron, M. (1965) Annu. Rev. Biochem. 34, 269–296
18 Ke, B. (1973) Biochim. Biophys. Acta 301, 1–33
19 Kononenko, A. A., Lukashev, E. P., Rubin, A. B., Samuilov, V. D., Timofeev, K. N. and Venediktov, P. S. (1973) FEBS Lett. 30, 239–242
20 Case, G. D. and Parson, W. W. (1973) Biochim. Biophys. Acta 325, 441–453
21 Junge, W., and Ausländer, W. (1974) Biochim. Biophys. Acta 333, 59–70
22 Bearden, A. J. and Malkin, R. (1974) Biochim. Biophys. Acta 325, 266–274