LIGHT-INDUCED OXIDATION OF A CHLOROPLAST b-TYPE CYTOCHROME AT −189°C

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Abstract.—The *b*-type cytochromes of chloroplasts have heretofore been viewed as photosynthetic electron carriers that probably occupy an intermediate position in a light-induced electron flow. The oxidation-reduction of such intermediate electron carriers, being removed from the primary photochemical reaction linked to photon capture by chlorophyll, would be expected to show a temperature dependence. Evidence has now been obtained that cytochrome b_{559} is photooxidized at −189°C and that this photooxidation can be induced only by "short-wavelength" monochromatic light which activates the oxygen-evolving system in chloroplasts (photosystem II). In appears, therefore, that photooxidation of cytochrome b_{559} is closely linked with photon capture by the chlorophyll pigments characteristic of photosystem II.

The cytochrome pigments are a group of conjugated proteins that function catalytically as electron carriers in cellular reactions associated with respiration and also, according to more recent evidence, with photosynthesis. The ability of cytochromes to transport electrons stems from their prosthetic group, heme, whose iron atom undergoes oxidation-reduction. Reversible shifts of cytochromes from reduced (ferrous) to oxidized (ferric) states are accompanied by well-defined spectral changes in three bands (α, β, and γ) that vary with the specific cytochrome but lie within distinct ranges: α = 550–610 mμ, β = 520–535 mμ, and γ = 415–452 mμ.

A reaction of a cytochrome component is measured by the difference in absorption spectrum between its reduced and oxidized states. In the reduced state, cytochromes exhibit characteristic absorption peaks in the α, β, and γ bands, on the basis of which they are classified as belonging to type *a*, *b*, or *c*. On oxidation, the α- and β-peaks disappear and are replaced by a weaker and more diffuse absorption; the γ-peak is somewhat diminished in intensity and displaced toward the violet end of the spectrum. When a cytochrome reacts, the diagnostically most useful absorption changes (aside from those in the γ-peak) are those at the position of the sharp α-peak; changes at the position of the β-peak may be slight and difficult to detect.

The cytochrome pigments involved in respiration are associated with mitochondria, whereas those involved in photosynthesis of green plants are localized in chloroplasts. Chloroplasts are known to contain three cytochromes. Hill and his associates demonstrated the presence of cytochrome *f*, a *c*-type cytochrome with an α absorption peak at 554 mμ,[1, 2] and cytochrome b_6, characterized by an α absorption peak at 563 mμ.[3] Spectroscopic evidence was obtained by Lundegårdh for a third cytochrome component, cytochrome b_{559}, with an α absorption peak at 559 mμ.[4] The presence of cytochrome b_{559} has since been

confirmed in several laboratories.[5-7] Cytochrome b_{559} may prove to be identical with cytochrome b_3, described by Hill and Scarisbrick[1] and known to be present in nonphotosynthetic cells.

The main feat of photosynthesis—the transformation of light energy into chemical energy—begins with the absorption of photons by chlorophyll and gives rise to electron transport that flows "uphill," against the thermodynamic gradient.[8] Coupled to this light-induced electron flow are chemical reactions in which energy is liberated and trapped in biochemically useful forms.[8, 9] Among the cellular electron carriers involved in the light-induced electron flow are cytochromes. Light-induced oxidation of cytochrome *f* has been demonstrated in intact algal cells,[10, 11] in leaves,[10, 12] in leaf homogenates,[10] and in isolated chloroplasts.[12-14]

Of special interest are the findings that a light-induced oxidation of cytochrome *f* in chloroplasts takes place at and below −150° [12, 13] (for bacterial systems, cf. ref. 15). These observations suggest that no thermochemical events intervene between the oxidation of cytochrome *f* in chloroplasts and the photon absorption act by chlorophyll.

No such photooxidation at low temperature has been reported for cytochromes of the *b*-type. However, light-induced changes of cytochrome b_6 in chloroplasts have been observed at room temperature.[7, 14, 16-18] Cramer and Butler[7] have also reported light-induced changes, at room temperature, in cytochrome b_{559}.

The lack of evidence for temperature-independent reactivity of *b*-type cytochromes has heretofore removed them from consideration as participants in the primary light reactions of photosynthesis and limited their role to electron transport in the secondary, purely chemical reactions in chloroplasts. Thus, based on the marked sensitivity of ferredoxin-catalyzed cyclic photophosphorylation to inhibition by antimycin A[19, 20]—an inhibitor known[21, 22] to impede electron transport between cytochromes *b* and *c*—cytochrome b_6 has been included as an electron carrier in this process.

As for cytochrome b_{559}, its role has remained unclear. Bendall's[23] recent report of oxidation-reduction potentials of chloroplast cytochromes, measured *in situ*, indicates that cytochrome b_{559} is more electropositive (E'_0 = +0.37 v) than cytochrome *f* (E'_0 = +0.35 v). This finding renders unlikely the suggestions[7, 24] that cytochrome b_{559} may serve as an electron donor to cytochrome *f*. By fractionating chloroplasts with digitonin, Boardman and Anderson[5] and Arnon *et al.*[6] found that cytochrome b_{559} is associated with photosystem II, but its function in that system has remained unknown. Photosystem II in chloroplasts is identified with the light-induced electron flow from water and the resultant evolution of oxygen (literature cited in ref. 25).

In an attempt to establish whether cytochrome b_{559} may be involved in the primary photochemical reactions of photosynthesis, we investigated the response of this cytochrome to illumination of chloroplasts at liquid nitrogen temperatures. We found a light-induced oxidation of cytochrome b_{559} at −189°. This finding, combined with other evidence, suggests that the oxidation of this cytochrome component represents a primary photochemical reaction of the electron transport of chloroplasts identified with photosystem II.[25]

Methods.—"Broken" chloroplasts from spinach and romaine lettuce leaves were prepared as described by Whatley and Arnon.[26] Chloroplasts from leaves of *Amaranthus edulis* Speg. were prepared according to the method of Kalberer, Buchanan, and Arnon[27] and hypotonically disrupted by placement in a 1:10 dilution of the blending medium.[27] Chlorophyll was determined as described by Arnon.[28]

Changes in cytochrome absorption spectra were measured in a dual wavelength spectrophotometer (Phoenix Precision Instrument Co.). The reference wavelengths were 538 or 568 mμ at liquid nitrogen temperature and 570 mμ at room temperature. Measurements at room temperature were made in a cuvette (1-cm light path) transparent on all four sides to allow for side illumination. The chloroplast suspension was stirred continuously with a microstirrer (Rho Scientific) placed so as not to interfere with either the actinic or the measuring light. The liquid nitrogen cuvette assembly was similar to that described by Bonner.[29] The cuvette (with Plexiglas faces) had a 2-mm light path. The remainder of the cuvette assembly, made of aluminum, was kept in thermal contact with the liquid nitrogen by an aluminum tongue. The entire cell compartment was flushed with dry nitrogen gas to prevent fogging. The temperature of the sample was measured with a platinum resistance thermometer (DigiTec model 531). The absorption changes at liquid nitrogen temperature were determined in chloroplasts kept in 50% glycerol and frozen by the single freezing procedure of Keilin and Hartree.[30]

Actinic illumination was provided by monochromatic light beams, as described previously,[31] which were introduced through a hole in the side of the spectrophotometer. For room-temperature measurements, the cuvette was illuminated directly from the side. For liquid-nitrogen-temperature measurements, the actinic beam was reflected onto the cuvette face with a 45° mirror. Balzer interference filters (approximately 10 mμ half-band width), supplemented with a Corning 2-60 filter, were used to isolate the actinic beam with an intensity of approximately 1.0×10^4 ergs/cm²/sec. To prevent the actinic beam from influencing the spectrophotometric determinations, the spectrophotometer phototube (EMI 9558 QC) was blocked with a Corning 4-96 filter and an Optics Technology 600-mμ short-pass cutoff filter.

Results and Discussion.—The spectrum of the light-induced absorbance changes in spinach chloroplasts at −189° is shown in Figure 1. The decrease in absorbance with two peaks in the α band at 548 and 552 mμ is characteristic of photooxidation of cytochrome *f* at low temperature, and the decrease in absorbance with a peak at 556 mμ is characteristic of photooxidation at low temperature of cytochrome b_{559}. Essentially the same low-temperature absorption spectra, showing the photooxidation of cytochromes *f* and b_{559}, were obtained

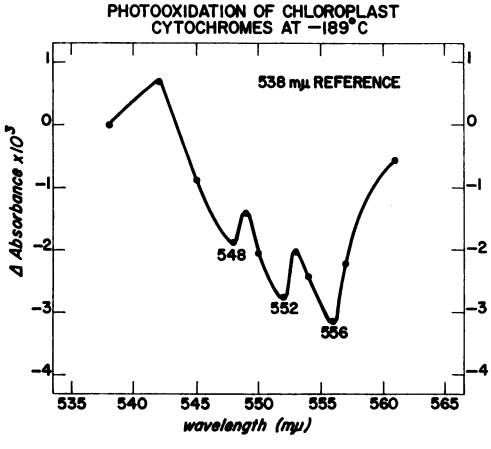

FIG. 1.—Spectrum of absorbance changes in spinach chloroplasts at −189°, induced by illumination with 664 mμ light. The reaction mixture contained (per 1.0 ml) spinach chloroplasts (equivalent to 200 μg chlorophyll), 0.5 ml glycerol, and the following (in μmoles): Tricine (*N*-Tris(hydroxymethyl) methylglycine) buffer (pH 8.2), 33.3; and sodium ascorbate, 5.

with chloroplasts isolated from romaine lettuce and *Amaranthus* leaves. At the low temperature, the light-induced spectral changes were not reversed in the dark. The absorption spectrum in Figure 1 is in good agreement with the difference spectra (oxidized minus reduced) obtained by chemical means[5, 32] at liquid nitrogen temperatures for cytochromes b_{559} and f. At low temperatures the α absorption peak of cytochrome f is split into two absorption maxima at 548 and 552 mμ and the α absorption peak of cytochrome b_{559} is shifted slightly to the violet end of the spectrum.[5, 12, 32] As expected, there were no absorption changes with a maximum at 561 mμ that is diagnostic of the α band of cytochrome b_6 at low temperature.[4] Cytochrome b_6 is auto-oxidizable[3, 5, 32] and would already be in a completely oxidized state in the isolated chloroplasts prior to illumination.

Figure 2 shows the time course of photooxidation of cytochrome b_{559} at $-189°$ in chloroplasts illuminated by 664 mμ light. There was a rapid decrease in absorbance at 557 mμ (with 568 mμ as reference)—two wavelengths at which changes in absorption due to photooxidation of cytochrome f are minimal. If the decrease in absorbance at 557 mμ is indeed due to a photooxidation of cytochrome b_{559}, then it should not occur when this cytochrome is already in an oxidized state—a condition that is readily brought about by pretreatment of chloroplasts with ferricyanide at room temperature in the dark.[5, 6] Figure 3 shows that chloroplasts treated with 5 mM potassium ferricyanide prior to freezing no longer gave the marked light-induced absorption change at 557 mμ at $-189°$.

Figure 2 also shows that illuminating chloroplasts by 714 mμ light was almost wholly ineffective in photooxidizing cytochrome b_{559}. The contrast between the effectiveness of 664 mμ and the ineffectiveness of 714 mμ illumination in photooxidizing cytochrome b_{559} might be taken as evidence that this cytochrome is involved in photosystem II, which is activated solely by the shorter wavelength illumination.[25] However, such contrasting effects of red and far-red light are not specific at low temperature. At $-189°$, far-red light was also ineffective in photooxidizing cytochrome f, although it is very effective at room temperature.[12, 14] Other evidence was therefore sought for the involvement of cytochrome b_{559} in photosystem II.

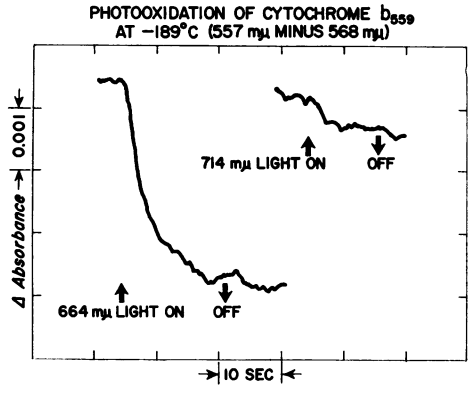

FIG. 2.—Effect of 664 and 714 mμ light on photooxidation of cytochrome b_{559} at $-189°$ in spinach chloroplasts. Experimental conditions were as described in Fig. 1.

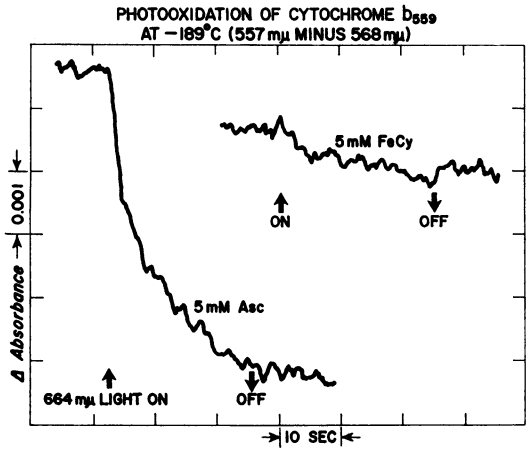

FIG. 3.—Effect of pretreatment of spinach chloroplasts with ferricyanide on photooxidation of cytochrome b_{559} at −189° by 664 mμ light. Experimental conditions were as described in Fig. 1, except that, where indicated, 5 μmoles potassium ferricyanide replaced sodium ascorbate.

In our experiments, photooxidation of cytochrome b_{559} could be observed only at low temperature (cf. ref. 7). This fact could be explained by assuming that at physiological temperatures the photooxidation of this cytochrome was immediately balanced by a reduction due to the flow of electrons from water—the electron donor in photosystem II. At room temperature, such a rapid sequence of photooxidation and reduction would escape detection with the recording technique used here. By contrast, at −189° the photooxidation of cytochrome b_{559} would become measurable, because the low temperature would probably stop the chemical reaction(s) involved in the flow of electrons from water.

To test this hypothesis, experiments were carried out at room temperature with Tris-treated chloroplasts. This treatment[33, 34] inactivates photosystem II

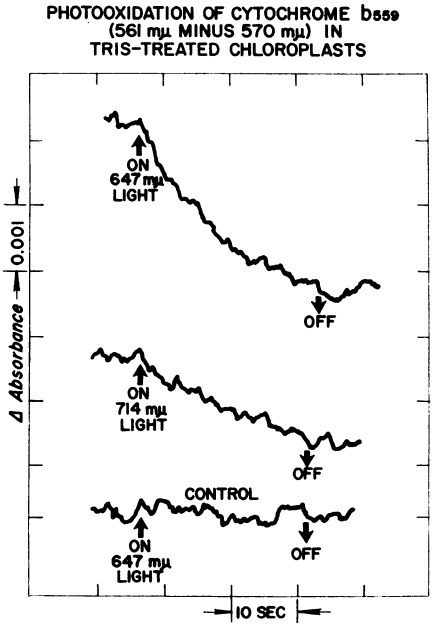

FIG. 4.—Photooxidation of cytochrome b_{559} at approximately 18° by illuminating Tris-treated chloroplasts with 647 and 714 mμ light. The reaction mixture for the two upper curves contained (per 1.0 ml) Tris-treated spinach chloroplasts (equivalent to 75 μg chlorophyll) and the following (in μmoles): Tricine buffer (pH 8.2), 33.3; and ascorbate, 1. The same reaction mixture was used for the *Control*, except that the Tris treatment of chloroplasts was omitted.

in chloroplasts to the extent that they cannot, on illumination, draw electrons from water and hence evolve oxygen. The photosystem II activity of Tris-treated chloroplasts can be restored only by supplying an artificial electron donor.[33, 34] Thus, without an artificial electron donor, photooxidation of cytochrome b_{559} in Tris-treated chloroplasts should be measurable even at room temperatures, since its subsequent reduction would be prevented.

Figure 4 shows that Tris-treated chloroplasts, prepared by a modification of the method of Yamashita and Butler,[34] gave a decrease in absorbance at 561 mμ (with 570 mμ as a reference) when illuminated with 647 mμ light at room temperature. No absorbance change was observed with the untreated chloroplasts. The wavelengths (561 and 570 mμ) were chosen to minimize any absorbance contribution from cytochrome f[2, 14] at room temperature. When a spectrum of the light-induced absorbance changes was plotted, it showed a maximum change at 560 mμ, characteristic of cytochrome b_{559}.[7] As expected, there was no indication of any oxidation of the already oxidized cytochrome b_6.[3]

Figure 4 also shows that 714 mμ actinic light was relatively ineffective in photooxidizing cytochrome b_{559} in the Tris-treated chloroplasts. In other experiments, 664 mμ light was more effective than 714 mμ light in photooxidizing cytochrome b_{559}, even when the two light beams were adjusted to give equal light absorption by the Tris-treated chloroplasts. These observations are again consistent with the view that the photooxidation of cytochrome b_{559} is closely linked with photon capture by the chlorophyll pigments of photosystem II.

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[1] Hill, R., and R. Scarisbrick, *New Phytologist*, **50**, 98 (1951).

[2] Davenport, H. E., and R. Hill, *Proc. Roy. Soc.* (*London*), **B139**, 327 (1952).

[3] Hill, R., *Nature*, **174**, 501 (1954).

[4] Lundegårdh, H., *Physiol. Plantarum*, **15**, 390 (1962).

[5] Boardman, N. K., and J. Anderson, *Biochim. Biophys. Acta*, **143**, 187 (1967).

[6] Arnon, D. I., H. Y. Tsujimoto, B. D. McSwain, and R. K. Chain, in *Comparative Biochemistry and Biophysics of Photosynthesis*, ed. K. Shibata, A. Takamiya, A. T. Jagendorf, and R. C. Fuller (Tokyo: Univ. of Tokyo Press, 1968), p. 113.

[7] Cramer, W. A., and W. L. Butler, *Biochim. Biophys. Acta*, **143**, 332 (1967).

[8] Arnon, D. I., in *Light and Life*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1961), p. 489.

[9] Arnon, D. I., *Physiol. Rev.*, **47**, 317 (1967).

[10] Lundegårdh, H., *Physiol. Plantarum*, **7**, 375 (1954).

[11] Duysens, L. M. N., J. Amesz, and B. M. Kamp, *Nature*, **190**, 510 (1961).

[12] Chance, B., and W. D. Bonner, in *Photosynthetic Mechanisms of Green Plants*, ed. B. Kok and A. T. Jagendorf (Washington, D.C.: National Academy of Sciences–National Research Council, 1963), p. 66.

[13] Witt, H. T., A. Müller, and B. Rumberg, *Nature*, **192**, 967 (1961).

[14] Avron, M., and B. Chance, in *Currents in Photosynthesis*, ed. J. B. Thomas and J. C. Goedheer (Rotterdam: Donker, 1966), p. 455.

[15] Chance, B., and M. Nishimura, these PROCEEDINGS, **46**, 19 (1960).

[16] Rumberg, B., *Biochim. Biophys. Acta*, **102**, 354 (1964).

[17] Hind, G., and J. M. Olson, in *Brookhaven Symposia in Biology*, No. 19 (1966), p. 188.

[18] Weikard, J., *Z. Naturforsch.*, **23b**, 245 (1968).

[19] Tagawa, K., H. Y. Tsujimoto, and D. I. Arnon, these PROCEEDINGS, **49**, 567 (1963).

[20] Arnon, D. I., H. Y. Tsujimoto, and B. D. McSwain, *Nature*, **214**, 562 (1967).

[21] Chance, B., and C. R. Williams, *Advan. Enzymol.*, **17**, 65 (1956).

[22] Racker, E., *Advan. Enzymol.*, **23**, 323 (1961).
[23] Bendall, D. S., *Biochem. J.*, **109**, 46P (1968).
[24] Bendall, D. S., and R. Hill, *Ann. Rev. Plant Physiol.*, **19**, 167 (1968).
[25] McSwain, B. D., and D. I. Arnon, these PROCEEDINGS, **61**, 989 (1968).
[26] Whatley, F. R., and D. I. Arnon, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1963), vol. 6, p. 308.
[27] Kalberer, P. P., B. B. Buchanan, and D. I. Arnon, these PROCEEDINGS, **57**, 1542 (1967).
[28] Arnon, D. I., *Plant Physiol.*, **24**, 1 (1949).
[29] Bonner, W. D., in *Haematin Enzymes*, ed. J. E. Falk, R. Lemberg, and R. K. Morton (Oxford: Pergamon Press, 1961), part 2, p. 485.
[30] Keilin, D., and E. F. Hartree, *Nature*, **164**, 254 (1949).
[31] Tagawa, K., H. Y. Tsujimoto, and D. I. Arnon, these PROCEEDINGS, **50**, 544 (1963).
[32] Hill, R., and W. D. Bonner, in *Light and Life*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1961), p. 424.
[33] Yamashita, T., and T. Horio, *Plant Cell Physiol.*, **9**, 268 (1968).
[34] Yamashita, T., and W. L. Butler, *Plant Physiol.*, **43**, 1978 (1968).

Editorial note: Owing to operational difficulties beyond our control, the foregoing article by Knaff and Arnon (which had been communicated to the PROCEEDINGS on March 24, 1969) was not paged up for press in the issue for June 1969 as had been scheduled. In order that the publication of the June issue would not be further delayed, Dr. Arnon agreed that the article could be rescheduled for publication in the issue for July. Accordingly, it appears herein together with the following article, which is also by Knaff and Arnon, and which had from the beginning been scheduled for this issue. (J. A. W.)