Report

# Dual-Wavelength Spectroscopy in Photosynthesis

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Photosynthetic Electron Transport, Plastidic Cytochromes, Dual-Wavelength Spectroscopy, Cross-Over Experiments; Oxidation-Reduction Potentiometry

Dual-wavelength spectroscopy allows the precise and fast measurement of small absorbance changes in highly absorbing and scattering samples ($\leqq 10^{-3}$ absorbance). The sensitivity of this method makes it possible to monitor directly redox changes of electron carriers in complex particulate biological systems. In this article, a description of the measuring principle of dual-wavelength spectroscopy is given with special reference to its application in photosynthesis.

Photosynthesis of green plants converts sunlight into chemical energy. This process takes place during photosynthetic electron transport which is located in the membranes (thylakoids) of the chloroplasts. New instrumentation and particularly advanced methods of absorption spectroscopy have tremendously pushed forward investigations on this basic process of life.

## 1. Photosynthetic Electron Transport

Fig. 1 demonstrates in some details how this process is looked upon today. Visible sunlight is adsorbed by chlorophyll and channeled into two reaction center chlorophyll molecules of the so-called photosystem I and II. Here, an electron is expelled from such a chlorophyll molecule, which is reactive by its particular albeit unknown insertion into the redox system, and taken up by a corresponding primary acceptor, thereby bridging a redox potential difference at the expenditure of light energy (heavy arrows pointing upward). The pigment then gets back an electron by an appropriate donor as indicated. According to their absorption maxima the reaction center chlorophylls are denoted P682 for photosystem II and P700 for system I, respectively.

Within the photosystem II region water is oxidized and molecular oxygen released in addition to protons and electrons. The latter ones are conducted through a "chain" of several redox carriers including photosystem I and eventually captured by the terminal acceptor $NADP^+$. NADPH is subsequently oxidized by the reduction of $CO_2$ leading to the formation of glucose. The number and nature of the redox carriers involved are not yet completely known. The primary electron acceptors at the reaction centers are the compounds Q and X, but their chemical features still have to be elucidated. Better known redox carriers are the lipophilic plastoquinone (PQ), cytochrome f (and other c-type cytochromes), cytochromes b-563 (= Cyt $b_6$) and b-559, and plastocyanin (PC), a copper containing redox protein. Furthermore, bound and soluble ferredoxins are found in the photosynthetic redox system, which are non-heme iron proteins, and a flavoprotein (Fp), the ferredoxin-NADP reductase, which catalyzes the reduction of $NADP^+$ by reduced ferredoxin. During electron transport ATP is formed (photophosphorylation) as indicated in Fig. 1 by the curved arrow. Between Q and photosystem I there is a midpoint potential difference of approximately 400 mV allowing for the formation of 1 molecule of ATP per 2 electrons transported through this part of the redox system, that is to say that at least 1 ATP is formed per 1 molecule of $NADP^+$ reduced. There is a certain degree of "coupling" between electron flow through the redox chain and ATP formation. When the absence of ADP and phosphate does not allow ATP synthesis, electron flow will decrease accordingly [1].

Our knowledge of photosynthetic electron transport derives mainly from investigations on cell-free systems, *i. e.* from functional analyses of isolated chloroplasts or thylakoids, which generally have lost $NADP^+$. Then, artificial electron (or hydrogen) acceptors can accept electrons after photosystem II or I when suspended with chloroplasts in appropriate buffers, and still evolve oxygen upon illumi-

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*Abbreviations:* DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (= dibromothymoquinone); DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Tricine-NaOH, N-tris(hydroxymethyl)-methylglycine (adjusted with NaOH).

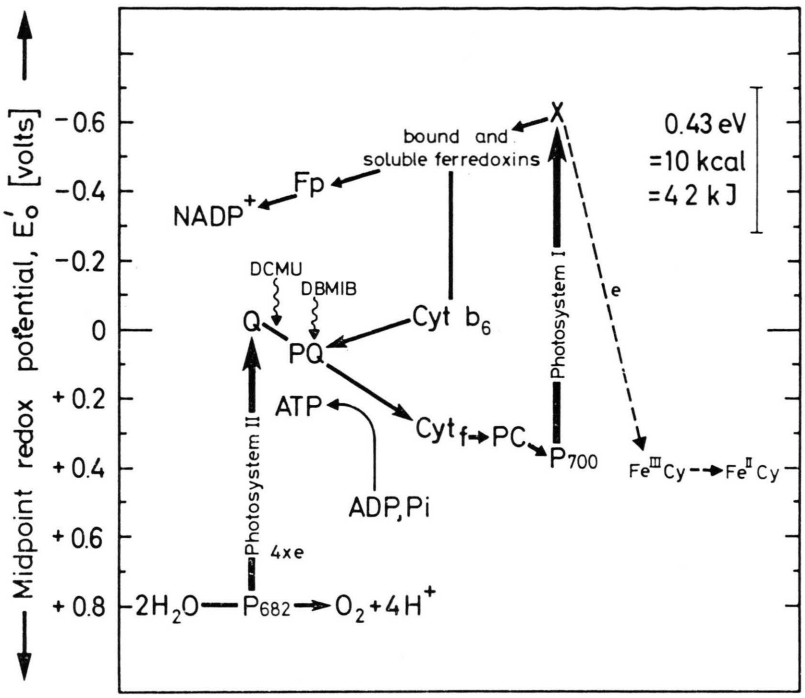


Fig. 1. Scheme of the photosynthetic electron transport. The positions of redox carriers, electron acceptors or donors indicate their midpoint potentials ($E_0'$). Cytochrome $b_6$ (= b-563) mediates cyclic electron flow from ferredoxin to plastoquinone. — See text for abbreviations.

nation (= "Hill reaction"). Examples of such chemicals are *e. g.* silicomolybdic acid, reacting at photosystem II, or potassium ferricyanide ($Fe^{III}Cy$) for system I (see Fig. 1). Accordingly, also artificial electron donors instead of water may feed in electrons to the redox chain. Furthermore, specific inhibitors are used to block certain redox carriers like DBMIB (dibromothymoquinone), a plastoquinone antagonist, or DCMU [3-(3′,4′-dichlorophenyl)-1,1-dimethylurea], which prevents electron flow between Q and PQ as indicated in Fig. 1. The ATP-forming enzyme may be inhibited by phlorizin (see section 4). These approaches have been extremely useful in studying sequential parts of the electron transport system. In addition, chemical or physical means like detergents or sonication are applied to break open the redox system and to allow studies of partial photosynthetic reaction, and separation of photosystem I from system II.

The "non-cyclic" electron transport from water to $NADP^+$ can be accompanied by a cyclic one, which diverts electrons from the photosystem I region at X and reintroduces them into the main electron transport chain at plastoquinone. This cycle most probably includes a non-heme iron protein and cytochrome $b_6$ as redox carriers. Thereby, a redox potential difference is built up by photosystem I alone and provides the energy for additional "cyclic" photophosphorylation [2, 3]. — For reviews on photosynthetic electron transport see *e. g.* [4, 5].

## 2. Instrumentation

Comprehension of a complex biological system requires the measurement of its intermediates. In electron transport systems like the photosynthetic apparatus, this involves mainly the identification of oxidation-reduction reactions of its individual components. These redox reactions can be measured by a number of physical methods such as light absorption and fluorescence spectroscopy, electron spin resonance and nuclear magnetic resonance. By combination with fast kinetic techniques (flash-photometry, stopped flow), by variation of temperature (low-temperature studies), the use of specific inhibitors and controlled oxidation-reduction poises, spectroscopy may furnish detailed information on the intermediate steps of the overall process. In the following we will deal with *dual-wavelength spectroscopy* which is a special and sensitive method of differential light-absorption spectroscopy.

Dual-wavelength spectroscopy, as introduced by Chance [6 – 8] allows the measurement of small absorbance changes (down to $2 \times 10^{-4}$ absorbance,

abbreviated by $\Delta A$ in the figures * in highly absorbing and scattering samples due to *e. g.* redox reactions of electron carriers in complex biological systems as mitochondria, chloroplasts, microsomes, etc. (see section 3). The measuring principle involves the use of a *single* cuvette, through which two predetermined monochromatic light beams of different wavelengths are alternately passed via a common light path. Being a differential technique, it compares the absorbance change at the wavelength of interest (= absorbance maximum of the measured component) with the absorbance change at a fixed reference wavelength (often an isosbestic point). Time sharing of both light beams is accomplished by a mechanical chopper, like a rotating sector mirror or a vibrating mirror. Thus, a modulated signal is produced at the cathode of a photomultiplier tube, positioned directly behind the measuring cell. The proximity of detector and sample is essential when highly scattering material such as chloroplasts is used. Before a measurement is made, the radiation intensities of the measuring and reference wavelength are optically attenuated so that the two electrical signals produced by the photomultiplier are equal although two different wavelengths with different absorption by the sample are used. This zero difference condition negates the absolute, usually high background absorption and the signal can be correspondingly amplified. A chemical or physical perturbation of the system changes the absorbance value of the measuring wavelength relative to the reference. Since both monochromatic light beams pass the same cuvette over a common optical axis, effects resulting from changes in sample density that are relatively independent of wavelength, like scattering, sedimentation or conformational changes of particles and also effects of instrumental stray light and light-source fluctuations are effectively cancelled out. To facilitate gating of sample and reference signal and to maintain the chopper speed constant a synchronization pulse is generated at the light chopper. A schematic illustration of the dual-wavelength measuring principle is shown in Fig. 2.

Usually, the *time resolution* of a dual-wavelength instrument is limited by the time sharing frequency. Chopper frequencies up to 1 kHz allow to measure kinetic reactions in the range of several milliseconds. For most photosynthetic reactions this is too slow to measure true kinetics. In this case, repetitive flash photometry (single-beam operation) has been applied together with signal averaging techniques to improve the signal-to-noise ratio [9]. Absorbance changes measured in biological material may, however, not be exactly repeatable due to baseline shifts, aging effects, etc. leading to errors

* Absorbance and optical density are used as synonyms.

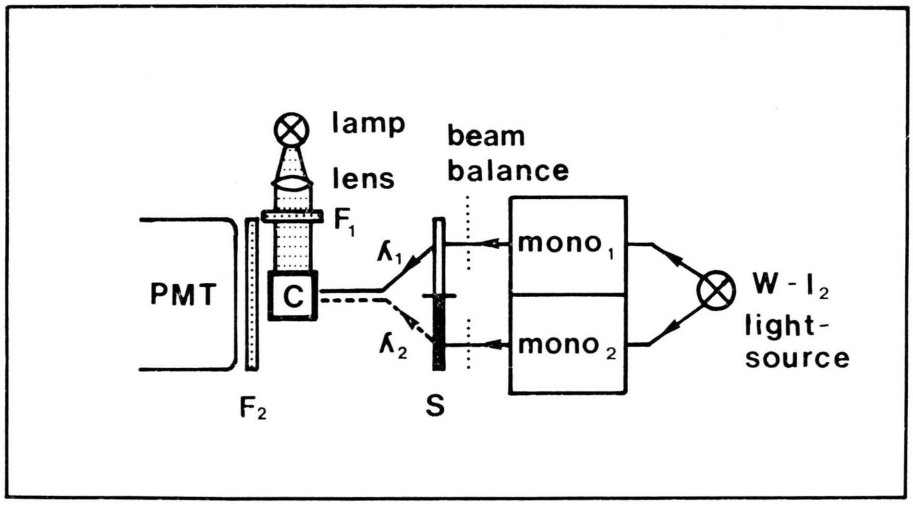


Fig. 2. Principle of dual-wavelength measurement. The dispersed radiation ($\lambda_1$, $\lambda_2$) from two gratings (Mono 1 and 2) is time-shared by a rotating sector (S) over an identical light path through a single cuvette (C). The photomultiplier (PMT) is protected against stray light from cross-illumination (which is defined by interference filter $F_1$) by a broadband interference filter ($F_2$). Instrumental details see instruction manual No. 1096A, 1972, of American Instrument Company, Silver Spring, Maryland.

by signal averaging. To improve the signal-to-noise ratios in comercially available dual-wavelength instruments, response time constants in the range from 100 $\mu$sec to several seconds are used, further limiting the possible time resolution. This, however, is not critical, since in general *steady-state* measurements are made. Only significant shifts in the redox steady state of a component induced by chemical or physical perturbations are recorded.

The *measuring wavelength* generally is determined by the absorption maximum of the component under investigation. The reference wavelength is set at a nearby isosbestic point. Although an isosbestic point makes an ideal reference, it is by no means essential. Especially in multicomponent systems other electron carriers might undergo positive or negative absorbance changes in the wavelength region of an isosbest and may give a significant contribution to the total absorbance change. Consequently, one would not expect to have true isosbestic points under all experimental conditions (see [10 – 12]). Any portion of the spectrum will suffice that does not change or where the change is small compared to the total signal. The only restriction is that the two wavelength pairs should be as close together as possible (due to Raleigh scattering); 540 nm, 561 nm and 570 nm have proven to be suitable reference wavelengths to measure spectral changes of cytochromes in chloroplasts. At any rate, the spectral changes indicative of a certain component have to be identified. This is achieved by systematic variation of the measuring wavelength with a constant reference. A point-by-point spectrum obtained in such a way should identify the component on the basis of peak maximum, band shape and bandwidth. Recently, a dual-wavelength scanning instrument operated on-line with a small computer has been described [11].

In photosynthesis, reactions induced by light are the subject of investigation. Selective excitation of the two photosystems within the electron transport chain by *cross-illumination* is achieved by providing a focusable microscope lamp (*e. g.* from Unitron) with narrow-band interference filters (half-bandwidth 1 – 10 nm, peak range between 650 nm and 720 nm). Actinic light intensity should be in the range of 100 – 1000 J/m$^2$ × sec. For certain applications high intensity monochromators may also be used. To measure absorbance changes during illumination of the sample, the photomultiplier tube is protected optically by broad-band interference filters and electronically by a phase-sensitive amplifier (lock-in amplifier) which "detects" mainly the signal produced by the measuring light which is modulated with a light-chopper.

It is advisable to have *controlled temperature* conditions (thermostated cuvette holder) during the reaction, since aside from the general temperature dependence of reactions lipid protein membrane systems might undergo phase transitions leading to different reaction pathways. On the other hand, subzero temperatures have successfully been applied for the kinetic resolution of redox reactions of cytochrome f [13].

To avoid settling of large particles such as chloroplasts during measurements in the range of minutes, the cuvette is *magnetically stirred*. This also facilitates rapid mixing of added solutions during the experiment. However, careful magnetic shielding (mu-metal sheets) of the photomultiplier tube is required.

## 3. Light-induced Absorbance Changes

In the following, we present some typical experiments performed by a dual-wavelength instrument (Aminco DW2) equipped for side-illumination and magnetic stirring. The component under investigation is cytochrome f with absorbance maxima in the visible region at 554, 523 and 420 nm in the reduced state (comp. Fig. 3 B). In higher plant and algal chloroplasts, redox reactions of cytochrome f and other c-type cytochromes are preferably measured at the $\alpha$-absorbance maximum. There are two reasons: (1) The sharp, well defined $\alpha$-band of the reduced cytochrome is replaced by a rather weak and diffuse absorption in the oxidized state, and (2) in the green part of the spectrum there is a plateau of low absorbance of chlorophylls a and b, which are the main absorbing and most abundant pigments. Although the differential extinction coefficient (reduced minus oxidized) of the cytochrome $\gamma$-band is much higher, oxidation leads to a peak shift somewhat diminished in intensity and, what is more serious, it coincides with the blue absorption maximum of chlorophyll. Apart from the high-absorbance background, redox reactions and electrochromic shifts of the chlorophyll molecules could, thereby, completely mask cytochrome absorbance changes. Cross illumination by monochromatic light as defined by an interference filter

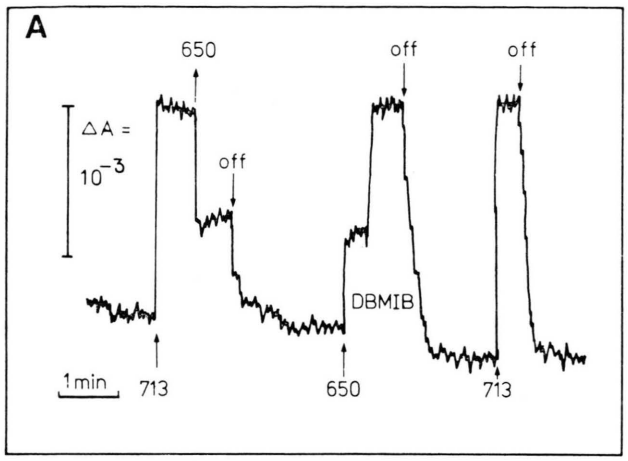


Fig. 3 A. Light-induced absorbance changes of cytochrome f of isolated spinach chloroplast thylakoids in the absence and presence of dibromothymoquinone (DBMIB). Cytochrome-f redox reactions are measured at 554 nm with 561 nm as reference; downward deflection indicates absorbance increase, *i. e.* cytochrome reduction. DBMIB (2 $\mu$M) is added during 652 nm illumination; actinic intensity for both 713 nm and 652 nm light was 100 J/m$^2$×sec. Measuring beam intensity about $10^{-3}$ J/m$^2$×sec with half-bandwidth of 2 nm; recorder time constant 1 sec. The reaction mixture (3 ml), pH 7.8, contained in mM concentration: Tricine-NaOH 50, NaCl 10, $MgCl_2$ 5 and chloroplasts with a chlorophyll concentration of 50 $\mu$g/ml.

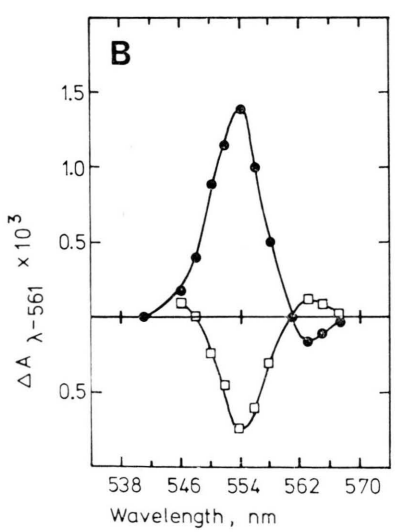


Fig. 3 B. Difference spectrum for the absorbance change induced by 713 nm (●—●) and 650 nm (□—□) light. Conditions as in Fig. 3 A.

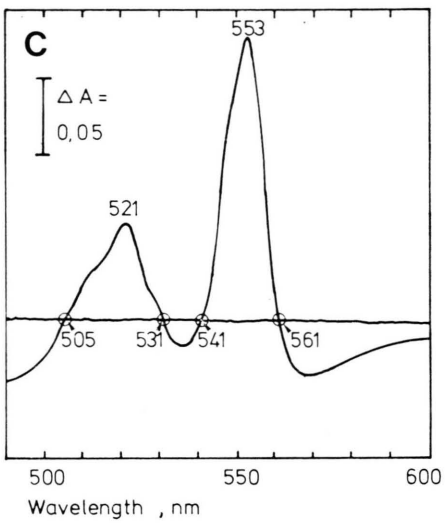


Fig. 3 C. Differential absorption spectrum of purified plastidic cytochrome c-553, reduced minus oxidized, from the alga *Bumilleriopsis filiformis* [33]; half-bandwidth: 1 nm, isosbestic points encircled.

at 650 nm will mainly excite photosystem II, whereas wavelengths above 700 nm will preferably excite photosystem I. The photomultiplier tube is protected against actinic light by a green broad-band interference filter (Balzers, DT green), when cytochrome absorbance changes are measured in the α-band region. For a recent review on photosynthetic cytochromes see Cramer and Whitmarsh [14].

The data of Fig. 3 A show that cytochrome f, which is in its reduced state in the dark, can be oxidized upon excitation of photosystem I by 713 nm light. Oxidation is measured in the dual-wavelength mode as absorbance decrease at 554 nm relative to a 561 nm reference. 650 nm light, effective to excite primarily photosystem II, leads to a more decreased oxidation-reduction level of cytochrome f, which in a subsequent dark period returns to the initial reduced state. This redox response of cytochrome f to different light qualities can be repeated many times. The experiment shows that cytochrome f should be located in the electron transport chain *between* both photosystems, showing red (650 nm)/far-red (713 nm) light antagonism ([15], compare Fig. 1). The tracing shows, however, that cytochrome f is not completely reduced by 650 nm light, suggesting a rate-limiting step in cytochrome-f photoreduction [16, 17]. The nature of this rate-limiting step will de discussed in section 4. In Fig. 3 B, the above experiment is re-

peated, varying the measuring wavelength and keeping that of the reference constant. The resulting point-by-point spectrum shows that the light-induced absorbance change measured in the region of 554 nm can be mainly attributed to redox reactions of cytochrome f. For comparison, the differential absorbance spectrum of isolated plastidic cytochrome c-554 from an alga (which has a similar absorption spectrum as the f-type cytochrome of higher plants) is shown in Fig. 3 C.

A further possibility separating photosystem-I and -II dependent redox reactions consists in the application of different fractionation procedures. As mentioned in section 1, detergent treatment of photosynthetic membranes has been used to prepare photoreactive photosystem-I and -II fragments. These experiments show that cytochromes f and $b_6$ are preferentially associated with photosystem-I fragments, whereas cytochrome b-559 is tightly bound to the photosystem-II region [18]. In reconstitution experiments it could be further demonstrated that cytochrome-f photooxidation is dependent on plastocyanin addition, whereas cytochrome-$b_6$ reduction requires ferredoxin [19]. In some algae (*e. g. Bumilleriopsis*), plastocyanin is missing and a cytochrome c-553 (Fig. 3 C) is the direct electron donor to the photosystem-I reaction center [20].

## 4. Cross-over Experiments

One of the greatest aids in spectroscopic indentification of the reaction pathways of electron carriers has been the use of specific inhibitors. Two main groups of inhibitors can be distinguished: (1) inhibitors acting on photosynthetic electron transport (such as DCMU, DBMIB, KCN, etc.) and inhibitors of the energy-conserving mechanism, such as inhibitors of the chloroplast coupling factor (= ATP synthetase) * and uncoupling agents **. (For a recent review on the mechanisms of photophosphorylation, see [21].)

* *Energy-transfer inhibitors* like phlorizin or DCCD (N,N′-dicyclohexylcarbodiimide) inhibit the ATP-synthetase reaction, thereby restricting electron transport to its "basal" rate.

** *Uncouplers* ($NH_4^+$; methylamine; FCCP, carbonyl-cyanide-*p*-trifluoromethoxy-phenylhydrazone; etc.) dissociate electron transport from phosphorylation; ATP synthesis is abolished and, generally, this leads to a stimulation of electron transport in the absence or presence of ADP and Pi. Uncouplers restore electron transport inhibited by an energy-transfer inhibitor, but not ATP formation.

The term "cross-over" has been applied to the general class of experiments where an agent causes oppositely directed changes in the oxidation state of those electron carriers surrounding the side of action of this agent [22]. As an example, the action of DBMIB, a plastoquinone antagonist [23], on cytochrome-f absorbance changes is shown. Addition of DBMIB to a chloroplast suspension, when both photosystems are excited by 650 nm light, shifts the cytochrome redox level to complete oxidation. Electron flow from photosystem II is efficiently blocked indicating that plastoquinone precedes cytochrome f on the system II side. Cytochrome-f photooxidation remains unimpaired ([24] and Fig. 3 A).

One of the unique features of electron transport systems is the coupling of electron transport and phosphorylation, *i. e.* synthesis of ATP from ADP and phosphate. No ATP is made unless electron flow occurs. Isolated chloroplasts, however, are not perfectly coupled. A "basal" electron flow occurs at one half to one fourth the rate when ADP and phosphate are present in the assay.

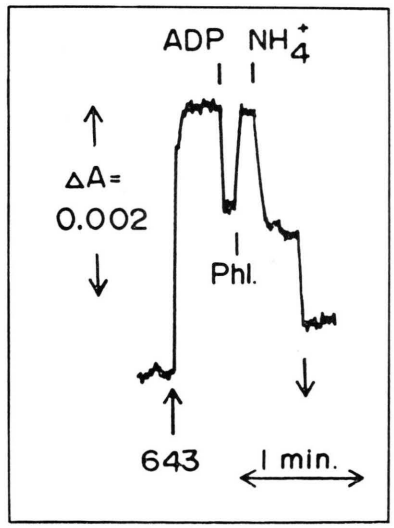


Fig. 4. Effect of ADP, phlorizin (= Phl) and $NH_4Cl$ on the light-induced absorbance changes of cytochrome f of isolated spinach chloroplasts. Additions as indicated during 643 nm illumination in mM: ADP 0.06, phlorizin 0.5, $NH_4Cl$ 2. Upward arrows: actinic light on, downward arrows: light off. Reference wavelength 540 nm. The reaction mixture, pH 7.5, contained in a final volume of 3 ml in mM: Tricine-NaOH 25, sorbitol 100, $MgCl_2$ 5, $K_2HPO_4$ 5, methylviologen (1,1′-dimethyl-4,4′-dipyridylium dichloride) 0.1, and chloroplasts with a chlorophyll concentration of 70 μg/ml [17].

Coupling means that the reaction mechanism of energy conservation limits the overall electron transport. This allows for localization of energy coupling sites, by investigating the effect of agents known to affect phosphorylation on the steady-state redox levels of electron transport carriers [22, 25]. If the rate of electron transport is limited by the absence of ADP, addition of ADP should change the steady-state redox levels of electron carriers interacting with the energy conservation site(s). Such a "cross-over" experiment is shown in Fig. 4. Preferential excitation of photosystem II in the presence of an (artificial) electron acceptor causes cytochrome-f oxidation, which is partly reversed by the addition of ADP. In the presence of the energy transfer inhibitor phlorizin, cytochrome f returns to the completely oxidized state; a subsequent addition of the uncoupler $NH_4Cl$ during illumination causes again a reductive change. Similar experiments on the steady-state redox changes of plastoquinone lead to the suggestion that energy conservation is very likely coupled to electron transfer between plastoquinone and cytochrome f [17].

## 5. Oxidation-Reduction Potentiometry

The knowledge of the basic properties of electron transport compounds involves the determination of the respective half-reduction potentials. Most of the early work related the half-reduction (midpoint) redox potential of a known compound to the unknown potential of an electron carrier [26]. However, in multicomponent systems this method is often inapplicable.

The sensitivity of the dual-wavelength method to monitor *in situ* oxidation-reduction reactions of membrane-bound electron carriers has been used extensively for redox-titration studies. The redox state, indicated by the accompanying absorbance change, is recorded as a function of redox potential of the suspension, measured simultaneously by an inert metal electrode. In order to facilitate equilibration of membrane-bound components with the electrode and also to buffer the redox potential to a certain value during the absorbance measurement, so-called mediators (redox dyes) are added to the solution (criteria for the use of mediators, see [27]).

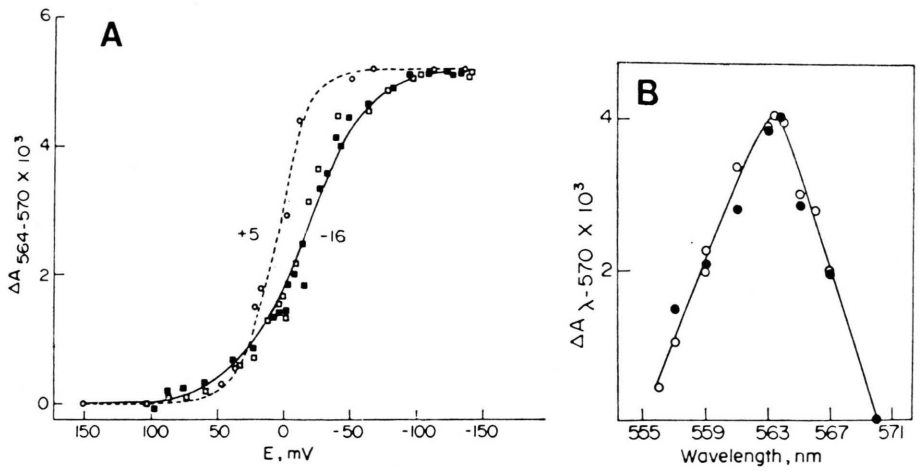


Fig. 5 A. Anaerobic dark titration of the cytochrome $b_6$ oxidation state in isolated spinach chloroplasts as a function of externally controlled oxidation-reduction potential. Oxidation changes of cytochrome $b_6$ were measured at 564 nm with 570 nm as reference. The deaerated reaction mixture, pH 8.0, contained in mM: Tricine-NaOH 25, $MgCl_2$ 5, $K_2HPO_4$ 5, and in addition the following redox dyes in $\mu$M: 1,2-naphthoquinone 10, 1,4-naphthoquinone 20, 2,5-dihydroxy-1,4-benzoquinone 10, 2-hydroxy-1,4-naphtoquinone 10. The first titration (○) was reductive; the calculated titration curve which was fitted to the data (— —) assumes a two-electron transition and a midpoint (Em) of +5 mV. Data points from subsequent oxidative (■) and reductive (□) titrations were fitted to a calculated titration curve assuming an Em of −16 mV and a one-electron transition. Chlorophyll concentration 100 $\mu$g/ml [28].

Fig. 5 B. Difference spectrum for the absorbance change titrated in Fig. 5 A. The absorbance change obtained by changing the redox potential from −90 mV to about +130 mV by addition of a single aliquot of ferricyanide (○), and from +130 mV to about −90 mV by the addition of a single aliquot of sodium dithionite (●), is plotted as a function of wavelength. The only redox buffer present was 1,4-naphthoquinone (20 $\mu$M); chlorophyll concentration 75 $\mu$g/ml. Conditions otherwise as in Fig. 5 A.

Generally the influence of oxygen must be eliminated and anaerobic techniques have to be applied (see [28] for description); this holds especially true when components in the potential region of zero volts and below are titrated.

Titrations of the absorbance change at 564 nm in the dark associated with cytochrome $b_6$, shown in Fig. 5 A, revealed a midpoint potential at approximately zero volts. This allows to place cytochrome $b_6$ on the reducing side of plastoquinone on a redox potential basis (comp. scheme of Fig. 1). The absorbance change titrated is identified as belonging to cytochrome $b_6$ by shifting the redox potential from $-90$ to $+130$ mV in dependence on wavelength (Fig. 5 B). Experiments with inhibitors have confirmed this proposal. In the presence of uncouplers a negatively directed change in potential is observed. The conclusion drawn from these kinds of experiments was that the midpoint potential of cytochrome $b_6$ depends on the energy state and/or possibly the structural integrity of the chloroplast membrane, which may be affected by the experimental procedure or chloroplast isolation techniques [28].

In a similar approach, the complete electron transport system may be "poised" at a particular potential by exogenously supplied redox agents and mediators [27–29]. Redox-linked functions, as for example light-induced absorbance changes, are consequently quenched or attenuated as a function of the redox potential of the solution. This technique was particularly useful in characterization of the primary photochemical reactions in photosynthetic bacteria and higher-plant chloroplasts. In conjunction with low temperature (light-induced changes at 77 °K) only reactions very close to primary events are measured, *i. e.* redox reactions closely connected with the excited pigments. Thermochemical reactions as the transfer of electrons through a chain of carriers would be "frozen out". Among the cytochromes investigated so far, only cytochrome b-559 photooxidation at 77 °K by photosystem II has been observed [30, 31], a reaction the meaning of which is, however, not yet understood [32].

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