Chapter 14

Difference Spectroscopy: The Roles of the Cytochromes, P700, Plastoquinone and Plastocyanin

DIFFERENCE SPECTROSCOPY

We discussed, in the preceding chapter, evidence for a two-step mechanism of photosynthesis derived from the study of the action spectra of photosynthesis, in monochromatic light and in combinations of two monochromatic beams. We will now consider relevant evidence derived from experiments of another type—*difference spectroscopy*.

What one observes in this type of study is differences between the absorption spectra of plant cells in darkness (that is, in absence of photosynthesis) and in light, when photosynthesis is going on. The sample is illuminated by a strong "actinic" beam that causes the photochemical change; the change in absorption is monitored by a weak "measuring" beam. Of course, the measuring light itself, however weak, does have a certain influence on the photosynthetic apparatus; but the presumption is that this influence is so slight that the spectrum obtained in the absence of the stronger "actinic" illumination, is characteristic of nonphotosynthesizing cells. The difference spectrum, obtained in this

way, reveals changes in the nature (or amount) of certain light-absorbing constituents in illuminated cells. One is particularly interested in changes that are reversed immediately upon return to darkness, since these are likely to originate in catalytic and photocatalytic components undergoing reversible changes during photosynthesis.

Several experimental difficulties present themselves in these studies. One is avoiding contamination of measuring light with actinic light. To minimize it, the two beams are sent into a rectangular vessel under right angles to each other (Fig. 14.1); but plant cells scatter light so strongly that some actinic light usually gets into the measuring beam. Complementary colored filters are often used to eliminate this contamination. For example, if the measuring light is blue, red actinic light can be used, and a blue filter inserted between the sample and the recording instrument. However, to plot the complete difference spectrum one must be able to vary the wavelength of the measuring beam through the whole visible spectrum; and in trying to do so, one gets into spectral regions where the wavelengths of the actinic and of the measuring light are so close that their separation by colored filters is impossible.

Two experimental tricks help. One is to send in the actinic light in the form of a strong flash, and to measure absorption changes after the flash. (The use of powerful condenser discharge flashes for this type of research was first introduced by G. Porter and R. G. W. Norrish, who received the 1967 Nobel Prize in chemistry for studies of extremely fast chemical reactions.) This procedure has the advantage of permitting to vary the time interval between illumination and measurement, and thus to obtain evidence as to the kinetics of the various absorption changes: how quickly they arise in light, and how rapidly (and following what kinetic law) they disappear in the dark.

Another trick is to *modulate* the measuring beam, for example, by means of a rotating disc, and arrange the measuring circuit (usually containing a photomultiplier, that is, a multistage photoelectric cell) in such a way that it will respond to modulated measuring light, but not to constant scattered actinic light (or the constant fluorescence excited by it).

Measurements of the difference spectra of photosynthesizing cells in constant light were first carried out by L. N. M. Duysens, and described in his doctoral dissertation (University of Utrecht, Holland, 1952). The flashing light technique was first developed by B. Kok, also in Holland

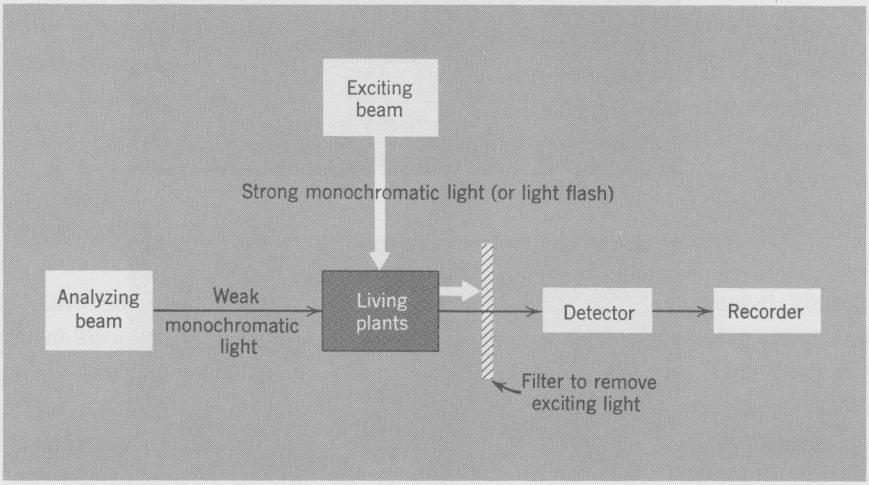

FIG. 14.1 Diagram of a difference spectrophotometer. The analyzing beam (of low intensity) is split into two beams; one goes through the sample (plant cells or living plant) and the other around the sample (not shown in the diagram). Both beams fall on the detector (a photomultiplier). The intensity of the beam that is sent around the sample is adjusted to match the intensity of the light transmitted by the sample; a zero reading is obtained on the recorder. Upon turning on the exciting beam (of high intensity), changes in absorption are observed and recorded. By varying the wavelength of the analyzing beam, a difference spectrum (that is, the difference between the absorption spectrum of cells illuminated with strong exciting light and non-illuminating cells) is obtained; it gives information concerning the pigments that undergo reversible changes during photosynthesis. By varying the wavelength of the exciting beam, an action spectrum (that is, the curve showing the effectiveness of different wavelengths of light in producing the change) is obtained; it provides information concerning the pigments that sensitize the absorption changes.

(now at Baltimore, Md.) using rotating disc flashes, and by H. T. Witt in Germany using condenser discharge flashes.

A large number of difference spectra have been obtained by the two methods, using different algae as well as chloroplast suspensions, under a variety of conditions. Figure 14.2 shows some examples.

These difference spectra reveal reversible changes in the oxidation-reduction state of several known organic compounds—three cytochromes, plastoquinone, plastocyanin, "pigment 700" and "pigment 690" (prob-

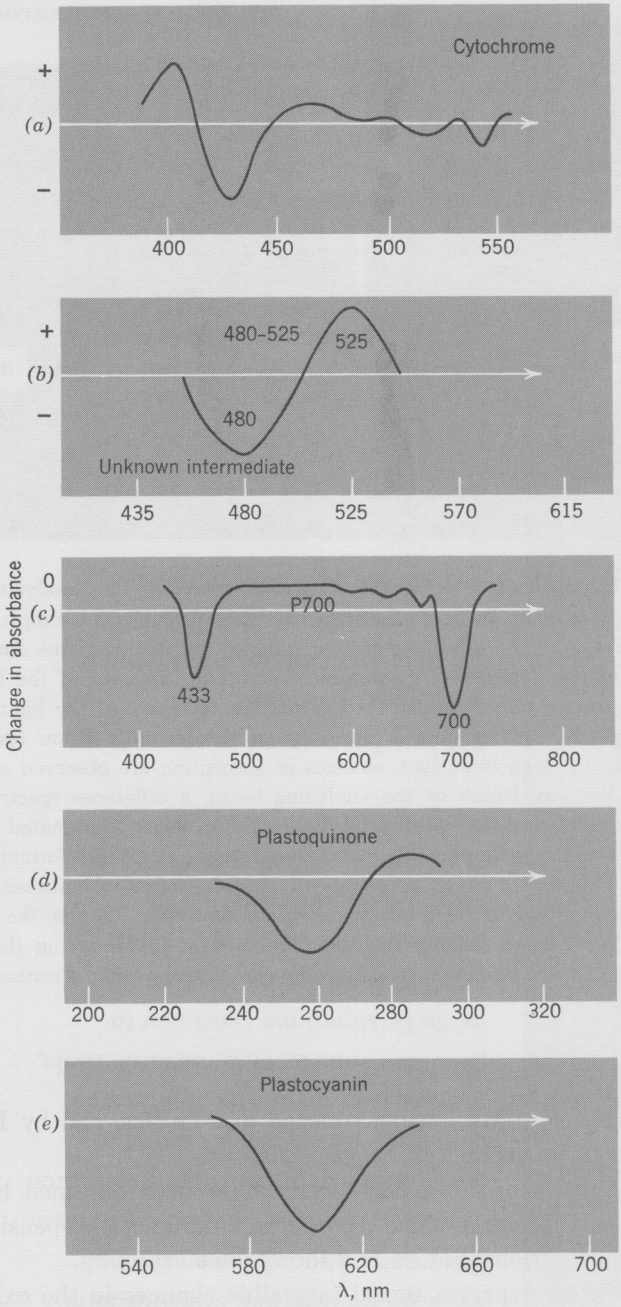

FIG. 14.2 Diagram of typical difference bands of photosynthesizing cells. Derived from the work of L. N. M. Duysens (cytochrome), B. Kok (P700), H. T. Witt, and J. Amesz (plastoquinone), and D. C. Fork and co-workers plastocyanin).

ably special forms of chlorophyll *a*)—and of some unknown ones. All these must be intermediates (or, at least, reversibly formed side products) in the reaction sequence of photosynthesis.

THE CYTOCHROMES

One hoped at first that difference spectroscopy will reveal changes in chlorophyll (or other pigments)—compounds most closely associated with the primary process in photosynthesis. Instead, Duysens' first clear-cut results (Fig. 14.2*a*) revealed, quite unexpectedly, a reversible tranformation in light of compounds whose role in photosynthesis had not been realized before—the so-called *cytochromes*. These are proteins with an attached iron-bearing prophyrin group (somewhat similar to hemoglobin). The iron in them can exist in "ferric" or "ferrous" form, and they are known to mediate, by alternating between these two forms, important oxidation-reduction steps in the respiratory chain. The cytochromes have absorption bands in the 350–600 nm range, which are generally used for their identification. These bands are different in the oxidized and the reduced state.

The two main types of cytochromes taking part in respiration are designated *b* and *c*; the former have redox potentials close to 0 volt and the latter, above +0.3 volt. When an electron is transferred from a *b*-type cytochrome to a *c*-type cytochrome, a free energy amount of about 7 Kcal/mole is liberated; two such transfers are utilized, in respiration, to produce one molecule of "high energy phosphate," ATP (Chapter 18).

In 1951, the British biochemist Robert Hill and his co-workers in Cambridge found that chloroplasts contain (at least) two cytochromes. One of them, which they called cytochrome b_6, was of "*b*-type," with a redox potential close to 0.0 volt. The other, which they called cytochrome *f*, was similar to cytochromes of the *c*-type; it had a redox potential as high as +0.42 volt. Duysens noted reversible oxidation of cytochromes (most clearly, that of cytochrome *f*) in illuminated photosynthetic cells. Since it was found that chloroplasts, freed from adhering traces of cell plasma (and thus also of mitochrondria) do not respire, that is, do not absorb oxygen in the dark, it became clear that cyto-

chromes must play a role in photosynthesis. At first, attempts were made to place them on one or the other end of the primary photochemical process, either on the "oxidation end," close to the evolution of oxygen, or on the "reduction end," close to the reduction of carbon dioxide. However, the redox potentials of the cytochromes did not fit them into these positions; and when the concept of a two-stage photochemical process arose, the hypothesis naturally offered itself that the cytochromes are intermediates *between the two stages*. (The redox potential of the couple $CO_2/(CHOH)$ is -0.4 volt; that of the couple O_2/H_2O, $+0.8$ volt; the midpoint between them is $+0.2$ volt, just where the redox potentials of the cytochromes are clustered.)

This concept received a convincing support from Duysens and co-workers' findings in 1961. Duysens' original observation, that cytochrome *f* is oxidized in light and reduced in darkness, proved incomplete; photooxidation occurred only in light absorbed in pigment system I (that is, in the red algae, on which these experiments were made, in chlorophyll *a*) while light absorbed in pigment system II (that means, in the red algae, in phycoerythrin) accelerated the return of oxidized cytochrome *f* into the reduced state (Fig. 14.3).

Robert Hill and Fay Bendall suggested, in 1960, that one of the two cytochromes found in plant cells (cytochrome b_6) could function (in its oxidized form) as primary oxidant in what we now call system II, accepting, in light, electrons originating in a water molecule, while the other (cytochrome *f*), could serve (in its reduced form) as electron donor (reductant) in what we now call system I, which transfers the electron to an organic compound such as a pyridine nucleotide. (The latter in turn uses it for enzymatic reduction of carbon dioxide; see Fig. 14.4.) Reduced cytochrome b_6 and oxidized cytochrome *f* could then react, directly or through additional intermediates, restoring the first one to its oxidized and the second one to its reduced state. About 0.4 eV of free energy would become available in this downward electron slide. By analogy with the similar steps in respiration, this energy could well be used to synthesize ATP. This is a very welcome possibility, because Melvin Calvin's studies with radiocarbon tracer have revealed a need for ATP in one (or two) steps in the enzymatic reaction sequence of photosynthesis (the "Calvin-Benson cycle," see Chapter 17). Since photosynthesis proceeds, in strong light, an order of magnitude faster than respiration, the needed ATP molecules can not be "borrowed" from cellular respira-

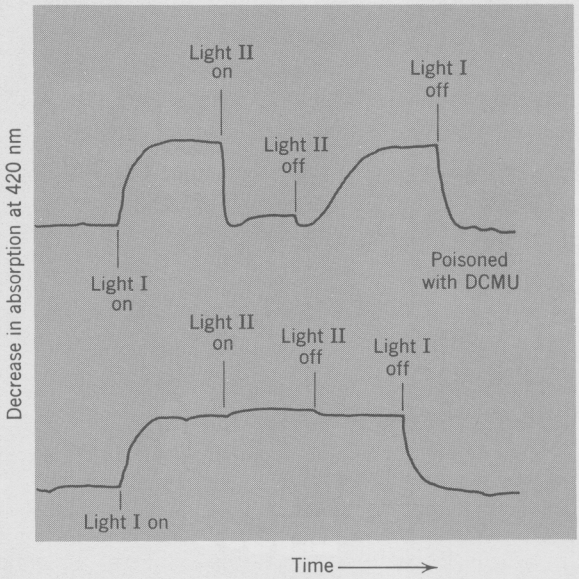

FIG. 14.3 Absorbancy changes at 420 nm owing to oxidation and reduction of cytochrome *f* in the red alga *Porphyridium cruentum*. (DCMU = 3-(3′,4′ dichlorophenyl 1,1 dimethyl urea; Light I = light absorbed mainly in pigment system I; Light II = light absorbed mainly in pigment system II.) (L. N. M. Duysens and J. Amesz, 1961.)

tion; they must be obtained as by-products of photosynthesis itself. The Hill-Bendall scheme (Fig. 14.4) provides a plausible explanation how this ATP-production can be achieved without using up additional light quanta (beyond the 8 required to move four electrons through two photochemical reactions each).

As mentioned above, Duysens' results were clearest in the case of cytochrome *f*. Hill's suggestion that a symmetric role is played by cytochrome b_6 in system II, could not be confirmed by difference spectroscopy. Also, it appeared that cytochrome b_6 may be involved in another reaction—the occasional reversal of photoreaction I accompanied by ATP-synthesis ("cyclic phosphorylation," see Chapter 18). On the other hand, recent spectroscopic evidence suggested that another *b*-type cytochrome (called cytochrome b_3) occupies the position in system II suggested by Hill and Bendall for cytochrome b_6.

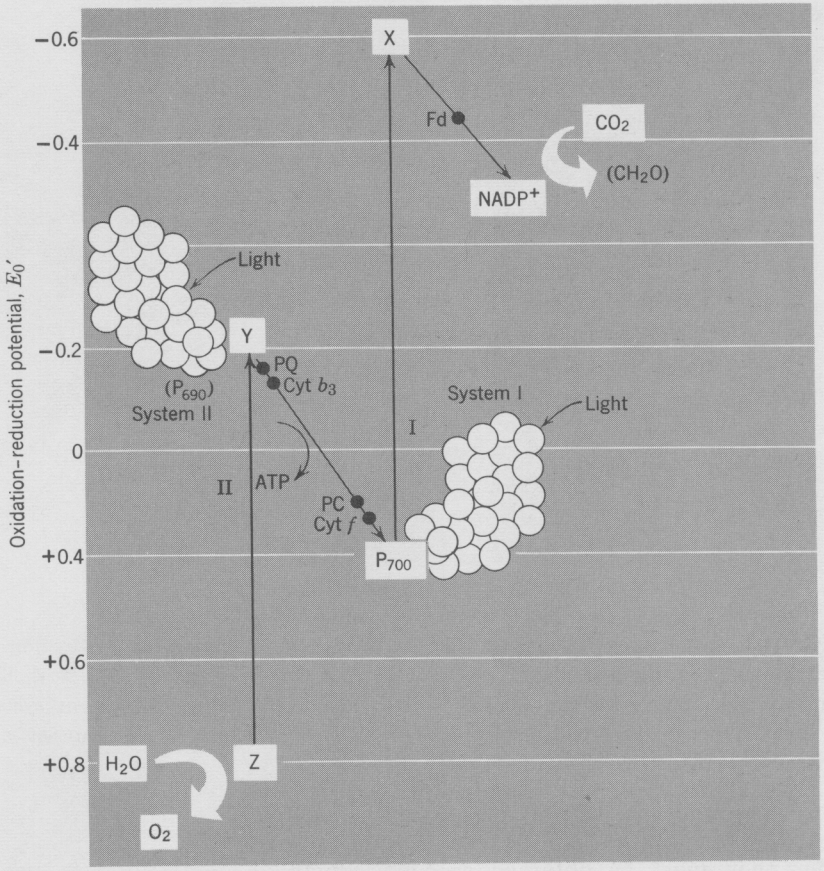

FIG. 14.4 Hill-Bendall scheme of photosynthesis in its modified form (elaboration of Fig. 13.9). *Z* and *Y* are primary electron donor and acceptor of light reaction II; P700 and *X* are primary electron donor and acceptor of light reaction I. P690, reaction center for light reaction II; P700, reaction center for light reaction I, PQ, plastoquinone; PC, plastocyanin; Cyt b_3, cytochrome b_3; Cyt *f*, cytochrome *f*, ADP, adenosine diphosphate; ATP, adenosine triphosphate; Fd, ferrodoxin; $NADP^+$, nicotinamide adenine dinucleotide phosphate.

THE DIFFERENCE BANDS AT 480 AND 520 nm

Reversible transformation of cytochromes in light explains only one part of the difference spectrum of illuminated plant cells. This part is prominent in weak light, but soon becomes light-saturated; while

other features of the difference spectrum continue to grow with increasing intensity of illumination. Some of these changes seem to be associated with reversible changes of chlorophyll (the search for which had motivated the first application of difference spectroscopy to photosynthesis); others indicated changes in certain quinone-type compounds (see below). The origin of some of the most prominent difference bands in green plants still remains uncertain. For example, in *Chlorella,* the two most striking components of the difference spectra in strong light are a decrease of absorption (a "negative difference band") at 480 nm, and an increase in absorption (a "positive difference band") at 515–520 nm (see Fig. 14.2*b*). Quantitative studies of these spectra in Witt's laboratory in Germany, and at the University of Illinois, have dealt in particular with the 520 nm band; it is not only the most prominent, but also the best reproducible of all difference bands. This band has been taken by Witt's group as an index of chlorophyll *b* changes. Yet, no final interpretation of this band exists. Observations suggest that the difference bands at 480 and 520 nm may be composite, perhaps due to superimposed changes in chlorophyll *a,* chlorophyll *b,* and the carotenoids.

PIGMENTS P700 AND P690

Bessel Kok, using the flash method, observed (in several algae and in chloroplast fragments) an interesting difference band in the far red. It was located at about 700 nm, that is, on the long-wave side of the main absorption band of Chl *a* (Fig. 14.2*c*); Kok interpreted this band as evidence of the presence, in vivo, of a minor Chl *a* component with an absorption peak of 700 nm, (and, as he later found, a Soret band at 433 nm). This component, alone in the whole pigment system, seems to be reversibly bleached in light. He called it P700, and suggested that it represents the "trap" in the pigment system in which the light energy, taken up in a photosynthetic unit, is collected, and which alone is directly involved in the primary photochemical process. Various findings indicate that it is specifically the trap in system I, engaged in the reduction of CO_2 by electrons taken from reduced cytochrome *f*.

This suggestion is supported by the oxidation-reduction potential of

P700, which Kok determined by adding redox systems of known potential and observing their effect on the P700 difference band in chloroplast suspensions. (It is difficult to make such experiments with intact cells, because the cell membrane prevents the outside redox system from affecting the potential inside the cell.) A redox potential of $E_0' \cong +0.4$ volt was estimated for P700—about right for the position suggested for it in Fig. 14.4. We can postulate that in pigment system PSI, the pigment P700 is the primary reductant (electron donor). Following its photochemical oxidation, P700 is rapidly converted back into reduced form by a dark reaction with cytochrome *f* (or plastocyanin, see below).

Another argument in favor of identifying P700 with the "trap" is the concentration of the compound. Assuming that *all* P700 is oxidized when chloroplasts are exposed to sufficiently strong light (and also that the molar absorption coefficient of P700 is the same as that of the bulk of Chl *a*), Kok calculated that about one molecule of P700 is present per 200–300 Chl *a* molecules. (If a single "trap" were present in each photosynthetic unit in system I, a ratio of one P700 per 600 Chl *a* could be anticipated.)

Kok and co-workers found that in the blue-green alga *Anacystis,* the red light, absorbed by chlorophyll *a* in PSI, oxidizes P700; while orange light, absorbed by phycocyanin (in PSII), reduces it.

It is worth mentioning that P700 cannot be identified with Chl *a* 695. The latter was estimated to form about 5%, while P700 is present only in a concentration of 0.3% of total Chl *a*.

To sum up, we can now suggest that the photosynthetic units in PSI in green plants consist of four components: (1) some chlorophyll *b*; (2) Chl *a* 670 and Chl *a* 680; (3) Chl *a* 695; and (4) a single (or a few) molecules of P700.

Long-sought-for absorbancy changes attributable to the reaction center of pigment system II have been recently identified by G. Döring, H. H. Stiehl, and H. T. Witt (1967) in whole cells of *Chlorella,* and by G. Döring, J. Bailey, W. Kreutz, and H. T. Witt (1968) in "system II particles" (see Chapter 16) obtained from spinach. Using repetitive fast flashes (of $\sim 10^{-6}$ sec duration), they were able to observe absorbancy changes decaying with a half-time of about 2×10^{-4} sec (in contrast to changes in P700 that decay with a half-time of about 2×10^{-2} sec). The peak of this change in whole cells is at 690 nm; but in separated system II particles, it appears to be shifted to about 682 nm; it is absent in

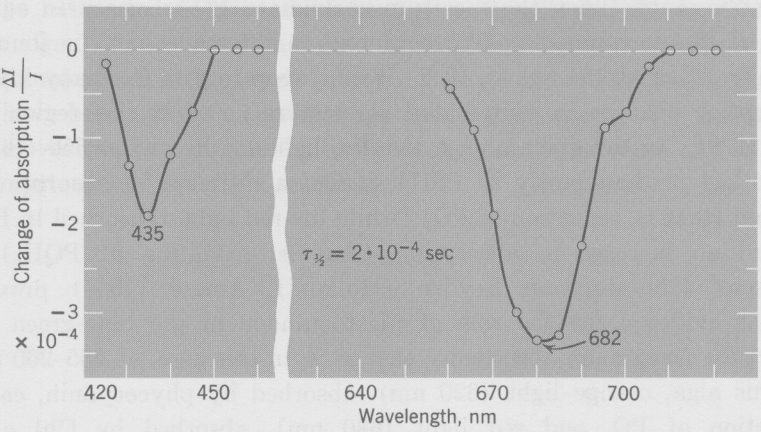

FIG. 14.5 Absorption changes with a lifetime of $\sim 2.10^{-4}$ sec in system II particles from spinach. Electron acceptor: K_3 $[Fe(CN)_6]5.10^{-5}$ M/1. Excitation: 620–710 nm (left curve) or 380–550 nm (right curve). Repetitive pulse technique has been used. For each measuring point, 4096 flashes were fired. (G. Döring, J. L. Bailey, W. Kreutz, and H. T. Witt, 1968.)

system I particles. The corresponding Soret band is observed at 435 nm (Fig. 14.5).

PLASTOQUINONE AND PLASTOCYANIN

In addition to cytochromes, P700, and P690, two other catalytic compounds were found to contribute to difference spectra of chloroplasts. One is called *plastoquinone*. It has an E_0' of about 0 volt. The other is a protein called *plastocyanin;* it is blue because it carries a copper atom. Its potential (E_0') is +0.36 volt. It seems plausible that both plastocyanin and plastoquinone act as intermediate redox catalysts in the electron path from system II to system I, plastoquinone close to cytochrome b_3, and plastocyanin close to cytochrome *f* (Fig. 14.4).

Plastoquinone (*PQ*), is one of several quinones found by fractionation of the photosynthetic system. The first suggestion that one of them is involved as intermediate in photosynthesis came from the antagonistic effect of light absorbed in pigments systems I and II on its oxidation-

reduction state. When the spectrum of oxidized PQ is measured against that of the corresponding hydroquinone, a difference band is found in the ultraviolet at 254 nm. In H. T. Witt's laboratory in Germany (1962), absorption changes in illuminated algae were noted in this region (see Fig. 19.2*d*). In the green alga *Chlorella*, light in the region 650–660 nm (absorbed predominantly in PSII) caused a decrease in absorption at 254 nm (that is, reduction of PQ); while far-red light (absorbed in PSI), caused an increase in absorption (that is, oxidation of PQH_2). In Duysens' laboratory in Leyden (Holland), Amesz (1964) provided similar evidence for the role of plastoquinone in the blue-green alga *Anacystis* (maximum absorbance change is, in this case, at 255–260 nm). In this alga, orange light (620 nm), absorbed by phycocyanin, caused reduction of PQ, and red light (680 nm), absorbed by Chl *a*, its reoxidation.

Compared to the cytochromes, plastoquinones are present in the algae in about ten times larger quantities.

Plastocyanin (*PC*) is a copper protein discovered in the green alga *Chlorella* by S. Katoh (1960) in Japan. D. C. Fork and co-workers at the Carnegie Institution at Stanford observed that light absorbed in pigment system I oxidizes plastocyanin (as shown in Fig. 14.9*c* by changes in optical density at 590 nm, the peak of the difference band between the spectra of oxidized and reduced plastocyanin). Light absorbed in pigment system II accelerated the reverse reaction—the reduction of oxidized plastocyanin. From some poisoning experiments (a method much used in enzymology), Fork concluded that plastocyanin precedes cytochrome *f* in the electron transport chain (in the green alga *Ulva*, and several other organisms). The oxidation-reduction potential of plastocyanin ($E_0' = +0.38$) is, in fact, slightly above that of cytochrome *f*. However, the exact sequence is not yet certain.

BACTERIA

Difference spectra have revealed that photosynthetic units in photosynthetic bacteria also contain "energy traps" in the form of specialized bacteriochlorophyll molecules. The maxima of the negative difference bands are at 840, 870 or 890 nm, depending on the organism. This

can be attributed to photooxidation of a "trap" (similar to P700 in green algae). Absorbancy changes attributable to bacterial cytochromes, and quinones of a certain type (ubiquinones, related to the plastoquinone) also have been noted in bacterial systems. Action spectra for the oxidation of various cytochromes in bacteria suggest the presence of two pigment systems and two light reactions.