Chapter 7

Taking Photosynthesis Apart. II. Photochemical Activities of Chloroplasts and Chlorophyll Solutions

CHLOROPLASTS: THE HILL REACTION[1]

We will now describe another, more direct way to separate photosynthesis into stages. From kinetic evidence (that is, rate measurements), we turn to attempts to break the cells, fractionate the cell material, and study the photochemical behavior of these fractions. This approach has long seemed hopeless. The alternative seemed to be: either live cells, capable of complete photosynthesis or dead, photochemically inert cell debris! The situation began to change thirty years ago. In 1937, an English plant biochemist, Robert Hill, following up an old observation that dry leaf powders exposed to light liberate a small amount of oxygen, discovered that this short-lived effect can be prolonged by supplying suspended leaf material with certain iron salts, such as ferric oxalate. This became known as the Hill reaction, and proved to be a very significant discovery, with which we must deal in some detail.

In Chapter 8, we will see that cells capable of photosynthesis contain,

[1] See Chapter 16 for Hill reaction studies in fractions of chloroplast material supposedly enriched in one of the two photochemical systems.

with very few exceptions, small pigmented bodies called *chloroplasts*. Microscopic observations with luminescent and motile bacteria, which are attracted by the slightest traces of oxygen, had shown long ago that chloroplasts are the sites of oxygen evolution in photosynthesis. This, and the localization of pigments in chloroplasts, suggested that they are the photosynthetic organelles of plants. When leaves are minced mechanically, for example, by means of a blender, or a pestle in a mortar, the resulting mash can be separated by means of a centrifuge into fractions, some of which contain, mostly or exclusively, whole or broken chloroplasts. These fractions prove to be the ones capable of carrying out the Hill reaction. Spinach leaves have been used more often than any others for this purpose, partly because fresh spinach is easily obtainable on the market.

Methods for preparing photochemically active suspensions from fresh, healthy leaves were improved by many investigators who rightly saw in Hill's finding a first hopeful glance into the black box of photosynthesis. It suggested that one part of photosynthesis, the liberation of oxygen in light, could be reproduced in an extracellular preparation—a suspension contaning chloroplasts or chloroplast fragments, but no cytoplasm, nuclei, or mitochondria.

It was soon found that ferric oxalate is not the only chemical to produce sustained photochemical oxygen production from chloroplast suspension. Hill himself observed that ferricyanide is even better for this purpose. Otto Warburg, in Germany, noted that quinone (orthobenzoquinone) is quite active. Subsequent studies have shown that a large number of compounds of the quinone type, as well as many organic dyes, with a similar, "quinonoid," structure, have the same capacity. All these compounds have one property in common: they are good oxidants. Ferric salts, containing the ion Fe^{3+}, can be reduced to ferrous salts, containing the ion Fe^{2+}, by the addition of an electron; quinones, Q, can be reduced to hydroquinones, QH_2, and dyes, D, to colorless leucodyes, DH_2, by addition of two hydrogen atoms:

$$Fe^{3+} + e^- \rightarrow Fe^{2+}$$
$$Q + 2H \rightarrow QH_2, \text{ or } D + 2H \rightarrow DH_2$$

It thus appeared tempting to interpret the Hill reaction as "photosynthesis with a substitute oxidant." Referring to Fig. 5.4, the CO_2-reducing enzymatic system, represented by the *upper* horizontal arrow, seems

to be lost (or damaged) in the preparation of the chloroplast suspension, so that another oxidant (hydrogen acceptor), which may be a ferric salt, a quinone, or a dye, must be substituted for the system CO_2/CH_2O.

Quantitative studies of the Hill reaction confirmed that it was in fact a photochemical oxidation of water. About the same maximum quantum efficiency and the same maximum rate of oxygen evolution (for a given amount of chlorophyll) were found in the Hill reaction as in photosynthesis.

The Hill reaction thus represents the long sought-after residual activity of a fraction of the complete photosynthetic apparatus left intact after mechanical destruction of the cells and isolation of chloroplast material.

These findings revived the old controversy as to whether the photochemical reaction of photosynthesis is concerned primarily with carbon dioxide or with water. Since the earliest investigation, it was known that H_2O and CO_2 are the two reactants involved in photosynthesis. Two attitudes had emerged: plant physiologists, botanists, and biochemists were concerned, above all, with the conversion of CO_2 into organic nutrients; photosynthesis appeared to them as "reduction of carbon dioxide to carbohydrates" in light. This attitude found its extreme expression in dividing photosynthesis into the following two steps (see Eq. 1.5):

$$CO_2 \xrightarrow{\text{light}} C + O_2;\ C + H_2O \rightarrow (CH_2O) \tag{7.1}$$

However, some chemists (among them G. Bredig in 1914) suggested that the light reaction of photosynthesis may be primarily concerned with water; for example:

$$2H_2O \xrightarrow{\text{light}} O_2 + 4H;\ 4H + CO_2 \rightarrow (CH_2O) + H_2O \tag{7.2}$$

but this view found little attention at that time.

As stated in Chapter 5, we now realize that photosynthesis is neither "decomposition of carbon dioxide" as in Eq. 7.1, nor "decomposition of water," as in Eq. 7.2, but an *oxidation-reduction reaction* between H_2O and CO_2, an uphill transfer of four hydrogen atoms from H_2O to CO_2. The two reactants are equally important, and play a symmetric role in the overall process.

However, a legitimate question remains whether, in Fig. 5.4, the photochemical, energy-storing step is closer to the removal of hydrogen from

water, or to the addition of hydrogen to carbon dioxide. It is like asking whether, on an uphill waterway, the locks are located at the lower or at the upper end. Considerations of comparative physiology, especially by C. B. van Niel (see Eq. 2.2), and the discovery of the Hill reaction, suggested that the primary photochemical reaction is more closely associated with the *dehydrogenation* of water than with the *hydrogenation* of carbon dioxide. It is, however, still incorrect to say, as it is often done, that the primary process in photosynthesis is "photolysis of water," which would suggest dissociation of H_2O into the molecules H_2 and O_2, or into the radicals OH and H.

One experimental finding, disproving the hypothesis in Eq. 7.1, is the demonstration that the source of O_2 in photosynthesis is the water molecule (as in Eq. 7.2) and not CO_2. This proof was given by Sam Ruben and Martin Kamen in Berkeley in 1941. They found that when plants were supplied ^{18}O-enriched water, the isotopic composition of the evolved oxygen was that of water and not that of CO_2.

The reducing capacity of the chloroplasts in the Hill reaction seemed at first to be insufficient to reduce CO_2 and thus to carry out complete photosynthesis; only oxidants with redox potentials more positive than 0.0 volt could be easily utilized for this reaction. More recent experiments suggested, however, that if back reactions can be avoided, even more reluctant oxidants can be reduced. In fact, it has been found possible to reduce, by means of illuminated chloroplasts, the important biological catalyst $NADP^+$ (oxidized nicotinamide adenine dinucleotide phosphate), with a potential of about —0.35 volt, if certain catalytic components (present in green plants and believed to be involved in photosynthesis) are added. Such observations were first made by W. Vishniac and S. Ochoa in New York in 1951 and confirmed by Anthony San Pietro (then at the Johns Hopkins University) and Daniel Arnon (at Berkeley). The necessary catalytic compounds include an iron-containing protein called ferredoxin and an enzyme called ferredoxin-NADP-reductase; these will be discussed in Chapter 17.

It is now widely assumed (although still not quite certain) that in true photosynthesis, the photochemical stage ends in the reduction of $NADP^+$ to NADPH (and production of a certain amount of so-called high energy phosphate, adenosine triphosphate, or ATP; see Chapters 17 and 18). Therefore, there seems to be no reason why chloroplasts, provided with catalytic supplements, should not also bring about the

reduction of CO_2. Arnon and co-workers at Berkeley found, in fact, that illuminated chloroplast suspensions, provided with a proper assortment of catalytic "co-factors," can transfer ^{14}C from labeled carbonate to organic compounds previously found to occur as intermediates in the reduction of CO_2 in live cells (see Chapter 17). This suggests that the true reducing power of illuminated isolated chloroplasts is not lower than that of the same chloroplasts in whole cells.

In Arnon's earlier experiments, the rate of CO_2 reduction (and O_2 liberation) with illuminated chloroplast preparations appeared quite low compared to that observed in photosynthesis of whole cells containing the same amount of chlorophyll. Recently, however, D. Walker in England was able to raise the rate to 10–15% of that of intact leaves; and in 1967, R. G. Jensen and J. Bassham in Berkeley obtained, *in strong light,* a rate of oxygen liberation equal to about 60% of that of intact leaves, but only in the presence of CO_2 of much higher concentration than in the air; this rate could be maintained for 6–10 minutes. The decisive factors in these experiments seem to be (1) the use of a suspension medium (developed by Norman Good of Michigan University) containing high-molecular-weight compounds, which seem to preserve intact the chloroplast membranes and prevent rapid loss of certain enzymes; and (2) quick separation of the chloroplasts from the rest of the cell material. Recently, we have found that the quantum yield of O_2 evolution by such chloroplast suspensions *in weak light* may reach 30–40% of that of intact Chlorella cells under identical conditions.

To sum up, the upper horizontal arrow in Fig. 5.4 can be reconstructed in chloroplast suspension; but as yet, such reconstructed systems have been able to operate only with reduced efficiency, and not for long.

Why live cells devote themselves to the reduction of carbon dioxide and "accept no substitutes," is one of the great wonders of photosynthesis. The rule is however, not without exceptions. For example, if *Chlorella* cells are placed in a solution of benzoquinone, they begin to act like chloroplast suspensions, that is, to liberate oxygen in light, and reduce quinone, but leave carbon dioxide alone. These cells are dead; they do not respire and cannot be revived by washing-out the quinone. Other Hill oxidants cannot be used in the same way. In many cases, they simply do not penetrate into healthy cells (although their penetration can be forced e.g., by washing the cells with glutaraldehyde.)

One well-known strong oxidant that does easily penetrate into cells

is free oxygen, and the striking property of the photosynthetic apparatus is its refusal to substitute O_2 for CO_2 as hydrogen acceptor. Substituting O_2 for CO_2 would mean photosynthesis running in a cycle:

$$H_2O + \tfrac{1}{2}O_2 \xrightarrow{\text{light}} \tfrac{1}{2}O_2 + H_2O \qquad (7.3)$$

Certain observations suggest that such a "short-circuiting" of photosynthesis can in fact occur, but only under special conditions.

Chloroplasts, freed from all adhering mitochondria, do not respire (that is, do not use up oxygen in the dark). This suggests a neat division of the two functions, photosynthesis and respiration, between the chloroplasts and the mitochondria.

Simultaneous occurrence of respiration and photosynthesis adds a difficulty to all rate measurements of photosynthesis in live cells because one process undoes what the other does. In light, one can measure only their difference, while respiration can be measured by itself in darkness. To calculate the rate of photosynthesis, one has to postulate that respiration remains the same in light. This assumption can be tested by tracer experiments in which heavy oxygen isotope is used. Such experiments, by Allan Brown and co-workers at the University of Minnesota, showed the rate of O_2 uptake in dark and in light to be not too different, at least, in chloroplast-bearing algal cells. (However, additional oxygen uptake in light, so-called *photorespiration*, does occur in many plants in certain spectral regions.)

In chloroplast-bearing plants, one can say that photosynthesis takes place in chloroplasts and respiration elsewhere in the cell. Of course, this spatial separation cannot be absolute, since the products of photosynthesis, such as the carbohydrates, sooner or later diffuse into the cytoplasm, to be used there as substrates of respiration. Inversely, not only carbon dioxide produced by respiration, but probably also certain respiration intermediates can diffuse into the chloroplasts, and interfere there with the enzymatic processes of photosynthesis. In the primitive, chloroplast-free blue-green algae, interaction between photosynthesis and respiration is much livelier than in the chloroplast-bearing higher plants; at least, Allan Brown found a marked effect of light on O_2-uptake by these algae. In the course of evolution, plants must have found it advantageous to separate respiration from photosynthesis; this may have permitted them to keep all life processes, supported by respiration, on an even keel, day or night, rain or shine.

PHOTOCHEMISTRY OF CHLOROPHYLL SOLUTIONS

The green pigment chlorophyll, (Chl *a*), is a common constituent of all photosynthesizing plant cells. In addition, these cells contain also a variable assortment of other pigments (see Chapters 8 and 9). Chlorophyll *a* could be a physical agent in photosynthesis, collecting light energy and making it somehow available for photosynthesis, or it could serve as a photocatalyst; that is, act as a light-activated chemical catalyst that takes an active, albeit reversible, part in the photosynthetic reaction. This question encourages studies of the photochemical properties of chlorophyll (and other plant pigments) outside the plant cell. Nonphotochemical oxidation-reduction catalysts, such as the iron-containing proteins called cytochromes, operate by reversible participation in the transfer of electrons (or hydrogen atoms). If the transfer of an H-atom (or an electron) from one compound (the reductant) to another compound (the oxidant) does not easily occur by itself (although it is permitted by the order of redox potentials), the provision of an intermediate system will often facilitate it. The intermediate oxidant takes the H-atoms or electrons from the reductant and gives them up to the oxidant. It thus serves as a go-between in an otherwise difficult chemical transaction. A *photocatalyst* may act similarly, except that it requires excitation by light to ply its trade. If the end result of a reaction is increase in free energy (that is, if the oxidation-reduction occurs against the gradient of redox potentials, as in photosynthesis), excitation of the catalyst is indispensable to make the reaction possible.

We are thus interested in knowing whether chlorophyll *in vitro* can serve as an oxidation-reduction photocatalyst, and whether it can mediate oxidation-reduction reactions involving storage of light energy.

Many dyes are easily reduced to colorless leucodyes and reoxidized back to colored dyes. A well-known example is methylene blue (E_0' = about 0.0 volt), which is often used in the reconstruction of biological processes; it is easily reduced to colorless leuco-methylene blue. This dye, as well as its close relative, the violet dye thionine, can bring about, in light, the oxidation of ferrous ions:

$$\text{dye} + 2\text{Fe}^{++} \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} \text{leucodye} + 2\text{Fe}^{+++} \qquad (7.4)$$

If one strongly illuminates a solution containing the dye and a ferrous salt, the blue (or violet) solution is decolorized within a few seconds. It quickly regains its color in darkness, showing that the bleached system had a higher free energy than the colored one. It is about the best imitation we know of the postulated primary photochemical process of photosynthesis. Light is utilized in this experiment, as in photosynthesis, for an uphill oxidation-reduction reaction against the gradient of electrochemical potential. In contrast to photosynthesis, however, no enzymatic agents are present to stabilize the energy-rich products, and the stored light energy is rapidly dissipated by back reaction between ferric ions and leuco thionine (or leuco-methylene blue). In our laboratory, reaction 7.4 was carried out in an emulsion of ether in water. The leucodye, formed in light, was extracted into ether, while the ferric ions stayed in water. The two solutions, the aqueous and the ethereal, could be separated in a separatory funnel. The products were thus prevented from reacting back. When the two solutions were stirred together again in the presence of alcohol, which makes them mutually soluble, the delayed back reaction took place and the color returned. (This is analogous to the products of photosynthesis, sugar and oxygen, reacting back in respiration.)

E. Rabinowitch and J. Weiss, in 1937, searched for a similar behavior of chlorophyll solutions in *methanol,* but found that these solutions can be reversibly *oxidized* (by ferric ions), rather than reversibly *reduced* (by ferrous ions).

$$\text{Chl (in methanol)} + \text{Fe}^{+++} \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} \text{Chl}^{+} + \text{Fe}^{++} \qquad (7.5)$$

In 1948 the Russian physical chemist, A. A. Krasnovsky, found that chlorophyll *a*, dissolved in *pyridine,* can be reversibly reduced in light by ascorbic acid ($E_0' = 0.0$ volt), to a pink "eosinophyll."

$$\text{chlorophyll (in pyridine)} + \text{ascorbic acid} \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} \text{eosinophyll} + \text{dehydroascorbic acid}$$

The basic nature of the solvent (pyridine) seems to be essential for the display of oxidizing, instead of reducing properties of chlorophyll. In the dark, Krasnovsky's reaction goes back, showing that it is associated with storage of free energy. Krasnovsky and co-workers went

one step further, showing that reduced chlorophyll can be reoxidized by a variety of oxidizing compounds. The net result is photocatalyzed reduction of these compounds by ascorbic acid. In many cases, this reaction leads to a net storage of chemical energy. For example, the well-known biological catalyst, riboflavin ($E_0' = -0.2$ volt) is reduced by ascorbic acid ($E_0' = 0.0$ volt) in an illuminated chlorophyll solution, overcoming an adverse potential gradient of 0.2 volt.

Krasnovsky suggested that light-excited chlorophyll molecules are reduced, in vivo, at the cost of water, as they are in vitro at the cost of ascorbic acid, and restored by reducing an appropriate intermediate, which in turn reduces carbon dioxide. However, Krasnovsky's experiments showed only reduction by ascorbic acid ($E_0' = 0.0$ volt) of compounds with redox potentials down to —0.2 volt, thus bridging one sixth of the 1.2 volt gap that has to be bridged in photosynthesis, where H_2O serves as reductant.

We will see in later chapters that photosynthesis probably involves two sets of primary photochemical reactions. One of them may involve photoreduction of an organic substrate by one pigment system, and the other, photooxidation of water by another pigment system (see Chapters 13–16). If Chl *a* is the photocatalyst, it will be oxidized in the first reaction and reduced in the second. A reaction between the oxidized form of chlorophyll *a* produced in one reaction and the reduced form produced in the second reaction is needed to close the sequence and restore the photocatalytic system to its original state. One is tempted to recall in this connection the above-mentioned tendency of chlorophyll for reversible photooxidation in alcoholic solution, and reversible photoreduction in pyridine solution. A remote, but plausible, analogy may exist between these two reactions in vitro and the two reactions of Chl *a* (probably located in different molecular environments) in photosynthesis. This is only a speculation, but it does invite systematic experiments.

Finally, we shall see in Chapter 14 that only a small fraction of all chlorophyll *a* molecules (perhaps only one per photosynthetic unit) is likely to engage in photocatalytic activity, while all others serve, similarly to accessory pigments, merely as "physical" excitation energy suppliers.