Chapter 6

Taking Photosynthesis Apart.
# I. The Light and the Dark Stage

THE PHOTOCHEMICAL AND THE ENZYMATIC STAGE

For a long time after the establishment of the overall chemical equation of photosynthesis (Eq. 1.3) and of its energy balance (Eq. 2.1), the process remained inaccessible to further analysis. In studying many other metabolic processes, for example, respiration, fermentation, and the synthesis of many important cell components, biochemists have long since learned how to extract the cells' catalytic components, the enzymes, or enzyme-bearing structural units (subcellular particles), and to carry out the pertinent reactions outside the living cell. Substrates on which these enzymes operate also could be extracted from the cells, or prepared synthetically. Ultimately, by putting together a proper assortment of substrates and enzymes, the whole metabolic process could be reconstructed in an artificial system. In recent years, this taking apart and putting together of metabolic processes has extended into the inner sanctum of life—the self-duplication of nucleic acids, which is the basis of heredity, and the synthesis of proteins, the process by which hereditary instructions, laid down in nucleic acids, are implemented as structural and functional capacities of an organism.

Photosynthesis long resisted such taking apart and reconstruction. The photosynthesizing cell appeared as a magic box, a *camera obscura*, into

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which nature throws carbon dioxide and water, exposes it to light, and presto, out comes oxygen, and carbohydrates accumulate inside. As soon as the cell was destroyed, or substantially damaged, this magic capacity was lost. For a long time, no partial steps of photosynthesis could be reenacted with nonliving material, not even with extracts or fragments from living cells. The mechanism of photosynthesis, therefore, long remained a matter of pure speculation.

To quote one example: In 1870, a German chemist, A. von Baeyer, suggested that the transformation of carbon dioxide in photosynthesis could proceed in two consecutive steps. First, CO_2 is *reduced* to the simplest carbohydrate, formaldehyde:

$$CO_2 \xrightarrow{+4H} CH_2(OH)_2 (\rightarrow CH_2O + H_2O)$$
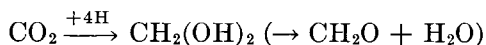
and then formaldehyde is *polymerized* to a carbohydrate, $(CH_2O)_n$. (This hypothesis was based on the capacity of formaldehyde, observed by von Baeyer, to polymerize to "formose," a sugar-like substance.) Attempts to test this hypothesis by introducing formaldehyde into plants as a substrate for sugar production failed. (Formaldehyde is a cell poison; this is why it is used as a disinfectant.) Nevertheless, until a few years ago, textbooks of plant physiology quoted von Baeyer's scheme as the best that could be said about the chemical mechanism of photosynthesis.

The reason why attempts to break photosynthesis into partial processes have proved unsuccessful is that, in contrast to metabolic processes that proceed with a *decrease* in free chemical energy, photosynthesis involves its *storage*. This makes photosynthesis as different from ordinary metabolic reactions as pumping water up into a high reservoir is different from its running down through a series of turbines and turning wheels as it runs (see Figs. 3.2 and 3.3). This pumping-up must involve formation of highly unstable intermediates, which undergo rapid enzymatic stabilization, ending in the liberation of oxygen and formation of carbohydrates. These intermediates cannot be easily extracted to reconstruct in vitro parts of the photochemical process.

Photosynthesis apparently requires certain submicroscopic structures in the cell to prevent unstable intermediates from mutual destruction and direct them into proper enzymatic reaction channels. Something similar does exist in respiration, where a certain sequence of energy releasing reactions cannot be separated from the subcellular particles

called *mitochondria.* In this case, all partial reactions occur downhill, so that intermediates could not escape by back reactions. Nevertheless, it seems important to keep them on the right track by providing a structural background that makes the reactions occur in proper order. We will deal in Chapter 8 with what is known about the structure of the subcellular particles required for photosynthesis. In the present chapter, we turn to another subject: the discovery of the division of photosynthesis into a photochemical and an enzymatic stage.

Unable to take photosynthesis apart, students of this process were in the position of mechanics wanting to interpret the operation of an automobile but not permitted to lift the hood and dismantle the engine. What could they do but resort to kinetic measurements, determining the speed with which the car runs in relation to supply and consumption of gas and air, hoping to obtain in this way some insight into the operation of the engine? Because of this situation, more numerous and precise kinetic data have been accumulated in the study of photosynthesis than in that of respiration, or of other metabolic processes more easily accessible to biochemical dismantling.

Incidentally, how does one measure the rate of photosynthesis? The earliest method was to count the oxygen bubbles rising from an illuminated submerged plant. This rough procedure was later developed into precise physical methods of measuring the amount of oxygen liberated by a suspension of unicellular algae. One such method is manometry. However, a complication appears in its use: according to Eq. 2.1, one volume O_2 is produced when one volume CO_2 is consumed. To prevent the two changes from balancing each other, the CO_2-consumption effect must be eliminated as fully as possible. This can be done by suspending the algae in a carbonate-bicarbonate mixture. Removing CO_2 from such a buffered solution merely causes the conversion of carbonate into bicarbonate, without any CO_2 being taken up from the gas above the solution. An inconvenience of this method is that it requires the use of alkaline media, which not all plants find to their liking. A more sophisticated approach permits the use of neutral or slightly acid solutions. In this method, *two* manometers are used, with a different gas volume above the suspension. The rates of exchange of CO_2 and O_2 can then be calculated separately, from the readings of two manometers, taking into account the known differences in the solubility of the two gases in water.

Other methods of measuring the rate of photosynthesis include mass spectrometry, infrared spectroscopy, calorimetry, polarimetry, and chemical analysis. The most convenient one is a variation of the polarimetric method, in which the rate of addition or subtraction of oxygen to the medium is determined by measuring the electric current flowing through it, between a platinum and a silver-silver chloride electrode. A negative potential is applied to the platinum electrode.

What organisms are most convenient for quantitative photosynthetic research? A favorite object has been the unicellular green alga *Chlorella pyrenoidosa*—somewhat like the fruit fly in the study of genetics. Another much used unicellular green alga is *Scenedesmus;* unicellular flagellates, such as *Euglena gracilis* (organisms that can live either as "animals" or as "plants") also have been used. Useful for comparative study are unicellular red algae (such as *Porphyridium cruentum*), or blue-green algae (such as *Anacystis nidulans*), and certain species of diatoms (such as *Navicula minima*).

Multicellular plants, leaves, and algal fronds, which have been the main subjects of earlier qualitative investigations, are less suitable for quantitative studies than the unicellular algae, because the latter can be suspended in an appropriate medium and stirred during the measurement to maintain uniform conditions. However, when one works with chloroplasts, the subcellular organelles in which photosynthesis occurs (see Chapters 7 and 8), higher plants can be used for preparing them. A favorite material for this purpose is spinach (*Spinacea oleracea*).

Photosynthetic *bacteria* are increasingly being used for comparative studies in photosynthesis because of the characteristic similarities and differences of their behavior compared to that of higher plants and algae. There are two main types of these bacteria—purple (such as *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*), and green (such as *Chlorobium thiosulfatophilum* and *Chloropseudomonas ethylicum*). These bacteria are abundant in stagnant, oxygen-deprived natural waters.

With recent successes in "opening the hood" of photosynthesis (see Chapters 7 and 17), interest in rate measurements has subsided. And yet, a completely satisfactory interpretation of a metabolic process would have to be quantitative, and not merely qualitative. In other words, we need to know not only the sequence of the chemical steps and the nature of enzymes catalyzing them, but also why the overall reaction

runs at the actually observed rate, and responds in a certain way to changes in external conditions. Such quantitative tests can be fatal to many a qualitatively plausible hypothesis, particularly in the case of photosynthesis, where high efficiency is an essential feature of the whole process.

Kinetic evidence of two types has led to a two-step concept of photosynthesis, involving one light-requiring step and one "dark," that is, not light-requiring step: (1) measurements of the rate of photosynthesis as function of the intensity of *steady illumination* and (2) measurements of the rate of photosynthesis in *flashing light* as function of the energy of the flashes and of the duration of dark intervals between them.

LIGHT SATURATION AND ITS IMPLICATIONS

As first noted by the German botanist J. Reinke, in 1883, the proportional increase in the rate of photosynthesis, P, with increasing intensity of illumination, I, is replaced, in sufficiently strong light, by light *saturation*, as shown in Fig. 6.1. The exact meaning of "strong" depends on whether we deal with shade-loving (umbrophile) objects, such as shade leaves or deep-water algae, or with light-loving (heliophile) ones, such as sun-exposed leaves, surface algae, and, particularly, desert and alpine plants. The plants of the first type may become light-saturated at one tenth or less of full sunlight at sea level, whereas plants of the second type may not be light-saturated even in direct sunlight at noon.

After saturation, the rate remains constant over a certain range of light intensities. In still stronger light, the rate begins to decline, particularly if the illumination is prolonged. This decline is caused by irreversible injury by light; the rate is not restored after return to lower illumination. On the other hand, the rising and the horizontal part of the light curve, $[P = f(I)]$, can be reproduced again and again by going either from lower to higher, or from higher to lower intensities.

The British plant physiologist, F. F. Blackman, was the first, in 1905, to interpret the shape of the light curves of photosynthesis as evidence of a two-step mechanism, consisting of a photochemical and a dark step. For some time, the latter has been widely called the "Blackman
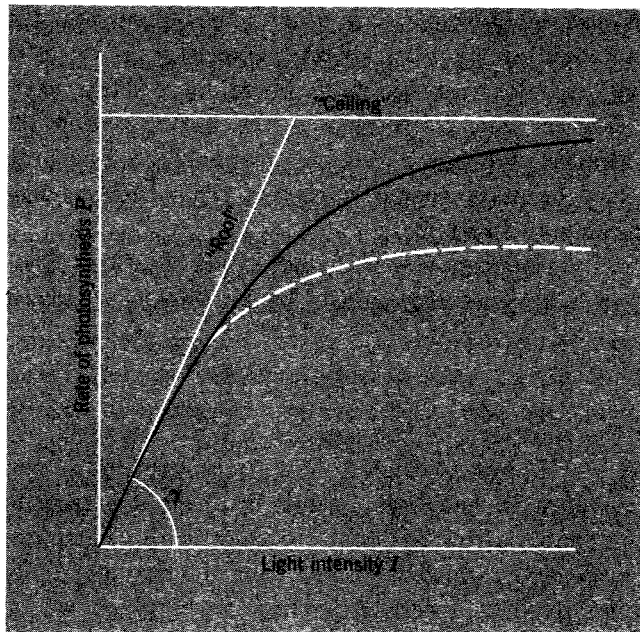
FIG 6.1   Light curve of photosynthesis $[P = f(I)]$. Dashed line shows the effect of lowering the temperature or adding a poison such as cyanide. The tangent of the angle γ measures the maximum yield of photosynthesis (provided I is the *absorbed,* not the *incident,* quantum flux).

reaction"; but this term is rarely used now, since we know that photosynthesis involves not one, but many dark enzymatic reactions.

The initial part of the light curve, in which the rate of photosynthesis increases proportionally with light intensity, corresponds, according to Blackman, to the so-called *light-limited state* of photosynthesis. In this range, as quickly as the light-produced primary photochemical products are formed, they are further transformed by dark (that is, nonphotochemical) reactions. It is the rate of supply of light that limits the overall rate under these conditions. When *light saturation* begins to manifest itself by curvature of the light curve towards the horizontal, this is evidence that the dark chemical apparatus is becoming overtaxed and incapable of taking care of all the primary light products as rapidly as they are formed.

This makes sense. However, it is worth pointing out that light satura-
tion does not occur in "ordinary" photochemistry in vitro, even if the
primary photochemical reaction *is* followed by a dark reaction. Light
saturation can, in fact, occur only if the dark reaction that follows
the photochemical step has a certain maximum "ceiling" rate. This is
not the case in ordinary chemistry, but is typical of enzyme-catalyzed
reactions in biological systems.

The general mechanism of enzyme-catalyzed reactions, first suggested
in 1913 by the German biochemist, Lenor Michaelis, consists of two
steps.

$$S + E \rightleftharpoons SE \rightarrow E + P \tag{6.1}$$

Here, S is the substrate, E the enzyme, SE a "complex" in which the
molecules S and E are associated, and P the product of the reaction.
After the complex SE had been formed by reversible association, indi-
cated by the double arrow, internal processes in this complex transform
S into the product, P, which separates from the enzyme, E. This second
transformation requires a certain average time, which we call t_e. The
inverse constant, $k_e = 1/t_e$ is called the "rate constant" of the reaction;
it is the average number of substrate molecules a single enzyme molecule
can transform in a second.

As the rate of supply of the substrate, S, increases, the enzyme mole-
cules, E, released at the end of reaction (6.1) become reloaded again
with the substrate more and more quickly. When the supply of S is
very fast, all molecules of E are kept continuously occupied. Increasing
still further the supply of S has now become useless. The enzymatic
transformation, represented by the arrow from SE to $E + P$ in Eq. 6.1,
has now become a bottleneck (in industrial parlance) or a rate-limiting
reaction (in the terminology of physical chemistry); it now limits effec-
tively the rate of the overall reaction. In photosynthesis, where S is
produced by light, this means that we have passed from the light-limited
state into the light-saturated, enzyme-limited state. It is as if soldiers
were brought to port by railroad and then shipped overseas. The ships
shuttle back and forth as fast as they can, but require certain time
for the round trip. As long as trains arrive at a leisurely rate, ships
have no difficulty in taking care of all arrivals; the whole transport
operation is "train-limited." As the rate of train arrival increases, the
whole operation passes from the "train-limited" to the "ship-limited"

state. The essential point in the case of photosynthesis is that the number of "trains" can be increased at will (by increasing the intensity of light), while the number of "ships" is limited by the finite number of enzyme molecules in the cell.

One can surmise that the rate of the photochemical production of the substrate S in Eq. 6.1 remains proportional to the intensity of illumination, even when light saturation is reached; but that excess molecules of S, not taken care of at once by the enzyme, E, crowd the too-strongly illuminated cell, like unemployed workers crowd a labor exchange when there are not enough jobs. Two things can happen in this situation. The substrate molecules S may be *stable*. Then, after their supply had been stopped (for example, by the cessation of illumination), the accumulated supply will be worked up by the enzyme. In other words, photosynthetic production will continue for awhile in darkness. Alternatively, the light-produced substrate molecules S may be unstable. In this case, they will not hang around, but disappear, like soldiers drifting home when stranded in a port without enough ships to take them overseas. Experiments show that the production of O_2 and the reduction of CO_2 do *not* continue for a significant length of time after the cessation of illumination, however strong the latter had been. This suggests that the second alternative is correct; that is, that light produces *unstable* intermediates. These have to be stabilized by an enzymatic reaction; otherwise, they are lost by sliding back, or falling aside (*i.e.*, by back reaction or side reactions).

Figure 6.1 shows that the photochemical reaction imposes on the light curves of photosynthesis, $P = f(I)$, a slanting "roof":

$$P = k_i I \tag{6.2}$$

where k_i is a proportionality constant. The dark enzymatic reaction imposes on P a horizontal "ceiling":

$$P_{\max} = \frac{k_e [E_0]}{n} \tag{6.3}$$

where $[E_0]$ is the total concentration of enzyme molecules available in the cell, and n a small whole number (see below).

So far, we did not make any assumption about the mechanism of photochemical reactions, except for the natural one that its rate is proportional to the intensity of illumination. We now make use of Einstein's

quantum theory of light, according to which light is absorbed by matter in discrete packages, the so-called energy *quanta* or *photons*. The energy content ϵ of a quantum is proportional to the frequency of the light, ν, and thus inversely proportional to its wavelength λ:

$$\epsilon = h\nu = \frac{hc}{\lambda} \tag{6.4}$$

where λ is expressed in cm; h is Planck's universal quantum constant ($h = 6.6 \times 10^{-27}$ erg sec), and c is the velocity of light, 3.0×10^{10} cm/sec).

Sixty years ago, Einstein formulated the basic law of photochemistry: *one* absorbed quantum causes the transformation of *one* molecule. This law is undoubtedly correct for the initial excitation of the absorbing molecule. But the ultimate result, measured in the number of substrate molecules transformed, or of the product molecules formed, may be quite different, depending on the efficiency of secondary reactions, which follow the primary excitation act. The number of molecules transformed by a single absorbed quantum is called the *quantum yield* of a photochemical reaction; its inverse, the number of light quanta needed to transform one substrate molecule (or to produce one product molecule), is called *quantum requirement*. If the quantum initiates a long *reaction chain*, the quantum yield may rise into the hundreds or thousands. If most primary reaction products are lost by back reactions, this yield may go down to small fractions of unity.

The proper way to measure the rate of photosynthesis in relation to light intensity is thus by the *number of molecules* transformed (that is CO_2 molecules consumed or O_2 molecules liberated) per *absorbed light quantum*. If Fig. 6.1 is drawn on this scale, with the abscissa representing the rate of absorption of quanta in einsteins/sec, and the ordinate the rate of liberation of O_2 (or consumption of CO_2) in moles/sec, the tangent to the curve (its slope) at its beginning is the *maximum quantum yield* of the process. This yield remains constant in the light-limited, linear part of the light curve, but declines as this curve bends towards the horizontal. The constant k_i in Eq. 6.2 is the maximum quantum yield of photosynthesis, as observed in the limiting case of weak light.

Equation 6.2 is based on Einstein's first law of photochemistry, whereas Eq. 6.3 is based on Guldberg and Waage's first law of reaction kinetics, the so-called *mass action law*. This law asserts that the rate

of a chemical reaction is proportional to the concentration of the mole-
cules participating in them. Thus, the rate of the second reaction in
Eq. 6.1 is proportional to the concentration of the complex $[SE]$. (Con-
centrations are often designated by square brackets, $[A]$ meaning "con-
centration of A.") The coefficient k_e is the rate constant. The maximum
possible value of $[SE]$ is $[E_0]$, the total concentration of the enzyme
in the cell. The ceiling rate in Fig. 6.1 is defined by Eq. 6.3. The small
whole number, n, is included in Eq. 6.3 because of the probability that
not one, but several (perhaps 8) products P of reaction (6.1) must be
formed for a single O_2 molecule to be liberated.

The above-suggested simple two-stage mechanism of photosynthesis
explains the fundamental fact of light saturation. Mathematical analysis
shows that it also explains the hyperbolical shape of some (although
not all!) experimental light curves $[P = f(I)]$. It explains also the way
in which *temperature* and certain *poisons* affect the rate of photosynthe-
sis (see Fig. 6.1). Lowering the temperature, or adding certain poisons
has no effect on the rate of absorption of light quanta, and thus also
on the rate of supply of the substrate S; therefore, in the light-limited
state, the rate of photosynthesis does *not* depend on these factors; but
they do affect the ceiling rate, k_eE_0. Changing the temperature changes
the rate constant, k_e, because the dark reactions generally go faster
the higher the temperature. Adding enzyme poisons diminishes the num-
ber of available enzyme molecules, E_0, and thus reduces the ceiling rate
$k_e[E_0]$. Many poisons, such as cyanide, act by combining with heavy
metal atoms (for example, iron atoms) present in an enzyme molecule,
and thus make the latter inactive. The proportion of such deactivated
enzyme molecules increases with increasing concentration of the poison,
until they are all immobilized, and the reaction rate is reduced to zero.

So far, so good; but more detailed, quantitative studies showed that
the situation is more complex. Not one, but a whole series of enzymatic
reactions, each with its own specific sensitivity to poisons and tempera-
ture changes, appear to be involved in the reaction sequence of photo-
synthesis. One of these enzymes may, under a given set of conditions,
act as *the* rate limiting one, just like the narrowest bridge may limit
the traffic-bearing capacity of a whole road. In the presence of certain
specific poisons, another enzyme may become rate-limiting, as another
bridge may become a bottleneck if it is under repair. Here is a striking
example. The maximum rate of reduction of quinone to hydroquinone

(or of ferricyanide to ferrocyanide) and of the liberation of oxygen by green cells or cell fragments in light (the so-called Hill reaction see Chapter 7) is about the same as that of photosynthesis. This suggests a common rate-limiting enzymatic reaction. And yet, upon addition of cyanide, the rate of photosynthesis goes down strongly, while that of the Hill reaction is not affected at all! This suggests that the common rate-limiting reaction is *not* sensitive to cyanide, but that the rate of some other enzymatic reaction, involved only in photosynthesis and not in the Hill reaction, is depressed by cyanide until this reaction becomes the bottleneck.

Biochemical analysis of the mechanism of CO_2-reduction in photosynthesis (see Chapter 17) led to the identification of more than a dozen enzymes involved in this process. The relation between these enzymes and the rate-limiting enzyme, whose presence had been derived many years earlier from the shape of the light curves (and other kinetic data), remains uncertain. Apparently, the main rate-limiting reaction in photosynthesis and in the Hill reaction is involved in the reaction sequence that is common to both processes, and not in the reduction of carbon dioxide, which does not occur in the Hill reaction.

PHOTOSYNTHESIS IN FLASHING LIGHT

If photosynthesis consists of a practically instantaneous primary photochemical reaction and an enzymatic dark reaction (or reactions), which require a certain average time, t_e, then the two reactions may be separated by the use of *flashing light*. Photochemical reactions offer to the experimentalist the convenient possibility of starting and stopping them as quickly and as often as desired by switching the light on and off. Also, one can easily produce light flashes, lasting only milliseconds or even microseconds, supplying a sufficient number of quanta to produce a measurable chemical change. In studying photosynthesis by means of flashing light, we want to send into a cell a practically instantaneous flash, containing enough photons to produce a measurable amount of oxygen. (An "instantaneous" flash can be defined here as one much shorter than the time, t_e, required for the completion of the rate-limiting enzymatic

reaction.) Such flash illumination can be produced *mechanically*, by placing a slit in a rotating disc in the path of a strong, steady light beam, or *electrically*, by loading up a condenser and discharging it through a vacuum tube. (Recently, the use of lasers has been initiated to produce sufficiently intense monochromatic flashes lasting only nanoseconds.)

The two methods have their advantages and disadvantages. In the rotating disc technique, if a sufficiently intense light source is available, one can vary the flash energy, E_f (the total number of quanta supplied by the flash) within very wide limits, by making the slit broader or narrower, and by rotating the disc more or less rapidly. But if one goes down to flashes shorter than a millisecond, their energy becomes small. In the condenser-discharge technique, flash duration can be made very short— a few microseconds or even less—but unless one has available an unusually powerful condenser, the total energy of the flash, E_f, is not high— while one usually would like to make it strong enough to attain flash saturation (see below). Another limitation is set by the time needed to reload the condenser before it can fire again, which usually takes $\gg 0.1$ sec.

One does not usually need to measure oxygen yield from one flash. (Certain extremely sensitive methods, for example, observation of the phosphorescence of certain phosphors, which is suppressed by the slightest traces of oxygen, do permit, however, a rough estimate of this magnitude.) Usually, it is enough to measure the oxygen volume produced by a known number of repeated flashes. In the rotating slit technique, the frequency of flashes must be sufficiently small for the dark enzymatic reaction to be completed after each flash. Experiments showed that for this purpose, the dark periods in photosynthesis must last at least 0.1 sec, so that one has to operate with not more than ten flashes per second.

The first experiments on photosynthesis in flashing light were made by Robert Emerson and William Arnold in 1932. They exposed suspensions of Chlorella cells to condenser flashes lasting about 10^{-5} sec and measured the rate of oxygen evolution in relation to the energy of the flashes and the duration of dark intervals between them. They also observed the effects of temperature and of certain poisons on the oxygen production in flashing light. These experiments gave some unexpected results.

Emerson and Arnold found that if the energy of the flashes is progressively increased, the oxygen yield *per flash,* which at first grows propor-
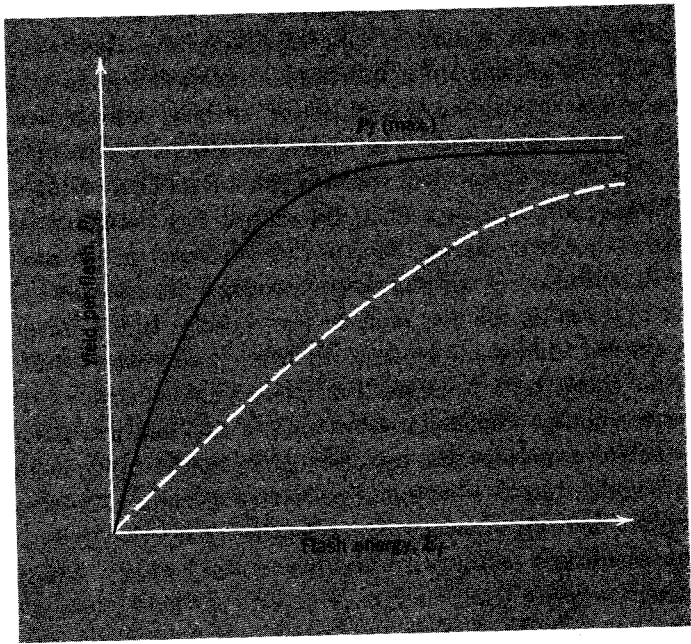
FIG. 6.2 Flash saturation of photosynthesis. Dashed line corresponds to shorter dark intervals or lower temperature. (R. Emerson and W. Arnold, 1932.)

tionately to this energy, finally shows saturation, approaching a maximum rate (Fig. 6.2)—just as the rate of photosynthesis in constant light. It must be noted that what is plotted in Fig. 6.2, in contrast to Fig. 6.1, is the *yield* P_f (per flash) and not the *rate* P (yield per unit of time exposure). The flash ceiling, $P_f{}^{max}$, is unexpectedly low. Before the relation between the enzymatic and photochemical stage in photosynthesis became clear, one did not expect saturation in flashing light to occur until each chlorophyll molecule had been given the chance to absorb a quantum of light in the flash (and thus to produce material to be worked over during the dark period). Instead, flash saturation was found to occur, in normal cells, already when only one out of 2500 chlorophyll molecules had received a quantum during the flash. We suggested above an interpretation of this observation, in terms of a limited number, $[E_0]$, of available enzyme molecules. Since enzyme molecules, and not chlorophyll molecules, have to "chew" on the photoproduct dur-

ing the dark interval, it is their number and not that of chlorophyll molecules that determines the maximum yield obtainable from a flash. To explain Emerson and Arnold's results, one could thus suggest that one molecule of the limiting enzyme is available for 2500 molecules of chlorophyll. If several molecules of the photochemical intermediate (S in reaction 6.1) are supplied to each enzyme molecule in a single flash, one of them preempts it by forming the complex (SE) and all others go to waste. This consideration led to the concept of a *photosynthetic unit* of about 2500 chlorophyll molecules, associated with a single enzyme molecule (or, we would now say, with the entrance to a single enzymatic "conveyor belt"). This important concept was introduced by H. Gaffron and K. Wohl in 1936. Some structures, observed in electron-microscope pictures of chloroplast fragments, have been tentatively identified with photosynthetic units (see Chapter 8).

One correction needs to be introduced: we measure the yield per flash by the number of oxygen molecules produced; but the production of each oxygen molecule is likely to require not one, but several primary photochemical processes, and, therefore, put the enzymatic conveyor belt to work not once, but several times. How many? We shall see that the most likely figure is *eight* (two for each hydrogen atom transferred from H_2O to CO_2). The liberation, in a flash, of one oxygen molecule per 2500 chlorophyll molecules thus may mean that one enzymatic center is present per $\frac{2500}{8}$, that is, per about 300 chlorophyll molecules. The photosynthetic unit would then consist of three hundred, rather than of two and one-half thousand chlorophyll molecules.

Like green plants, photosynthesizing bacteria also contain photosynthetic units, that is, a large number of pigment molecules present per enzymatic center. However, this number is smaller than in green plants—about 50 instead of 300.

These numbers (300 and 50) are typical of many normal, healthy cells, but may vary widely from plant to plant. For example, they are much lower for chlorophyll-deficient variegated leaves.

We have thus interpreted the maximum flash yield of photosynthesis as measure of the available amount of a rate-limiting enzyme, and estimated that the ratio between the concentrations of chlorophyll and of this enzyme is, in typical healthy plant cells, about 300 to 1.

The need for something like the postulated photosynthetic unit can

be easily seen a priori. In order to absorb enough light, a cell must contain as much as 0.1 mole chlorophyll per liter. If each chlorophyll molecule were provided with its own assortment of enzyme molecules, the latter would have to be present in the same concentration. However, enzymes are protein molecules, with a molecular weight of 10^5–10^6, while chlorophyll has a molecular weight of about 10^3. Each enzyme molecule, therefore, requires 100–1000 times the space of a single chlorophyll molecule. There is not enough space in the cell for so many enzyme molecules! Thus, many chlorophyll molecules simply *have* to share a single enzyme molecule. Fortunately, the effectiveness of enzymes is great enough for this sharing. In direct sunlight, a chlorophyll molecule will absorb photons at the rate of one to ten per second, while a good enzyme can easily transform a thousand or ten thousand substrate molecules each second; it can thus keep pace with the substrate supply from several hundred chlorophyll molecules.

The flashing light experiments can be used for another determination: that of the "working time," t_e, of the rate-limiting enzyme. For this purpose, we measure the yield of oxygen per flash as function of the dark period between flashes, t_d. (In this experiment, care must be taken to use sufficiently strong "saturating" flashes.) As the duration of the dark intervals increases, the yield per flash increases too, until the intervals reach the order of 0.1 second. Then the curve approaches saturation. This suggests that the rate-limiting enzyme requires about 0.1 sec to work up *practically all* the supply of substrate it had received during the flash. The *average* time, t_e, required to transform a substrate molecule is smaller—about 0.02 sec at room temperature.

Changes in temperature were found by Emerson to affect not the maximum yield per flash, but the dark interval needed to obtain it. This, too, agrees with the interpretation of the maximum yield as a measure of the available amount of a limiting enzyme. This conclusion is further confirmed by the effect of the addition of potassium cyanide on the flash yield; thus poison, too, does not change the maximum flash yield, but lengthens the required dark interval.

We can thus derive, from flashing light experiments, *two* independent constants:

$$[E_0] = \frac{Chl_0 n}{2500}$$

where Chl_0 is the total amount of chlorophyll; and

$$k_e = \frac{1}{t_e} = \frac{1}{0.02} = 50 \text{ sec}^{-1} \text{ (at 20°C)}$$

Now comes a very satisfying calculation: if the enzyme that limits the steady rate of photosynthesis in *constant* light is the same one that limits the yield *per flash* in *flashing* light, then the saturation rate of photosynthesis (P^{max}) in constant light (Fig. 6.1) must be equal to $k_e[E_0]/n$ (Eq. 6.3). This, in turn, should be equal to the product of the two above-determined constants, or about 0.02 Chl_0 per second. This means that the maximum rate of photosynthesis *in constant light* should be one oxygen molecule each 50 seconds per molecule of chlorophyll. And this is what it actually is! It has been known since the pioneer measurements of Richard Willstätter and Albert Stoll (1913–1918) that healthy, fully-active leaves, abundantly supplied with carbon dioxide and light, can produce one molecule of O_2 (and consume one CO_2 molecule) every 20–30 seconds per chlorophyll molecule present in them. Willstätter called this the "assimilation time" of the leaves (carbon dioxide assimilation being, we recall, another term for photosynthesis). For Chlorella cells, somewhat higher assimilation times, 40 or 50 seconds, have been found. This is close enough to the value calculated from flashing light experiments to assert that the latter actually permit factorization of the maximum rate in constant light, $k_e[E_0]$ into the two factors k_e and $[E_0]$.

However, as always in more precise study of biological phenomena, this simple relation proved to be *too* simple. Photosynthesis involves not a single one, but a number of enzymatic reactions, and more than one of them can affect the maximum rate, in flashing as well as in constant light. Subsequent flashing light experiments by James Franck and S. Weller in the United States, by Hiroshi Tamiya and co-workers in Japan, and by Bessel Kok in Holland, gave evidence of such complications. According to Tamiya, if the flash lasts several *milli*seconds (instead of *micro*seconds, as in Emerson's experiments), the maximum yield per flash rises above the saturation plateau in Fig. 6.2, and becomes dependent on temperature—despite the fact that a few milliseconds is still a short period compared to the above-calculated "working time" (about 10 milliseconds) of the "Emerson-Arnold enzyme." Kok explained this behavior by postulating a more complex mechanism, involving two

successive enzymatic steps. The maximum yield of a longer flash measures, according to Kok, the combined "reservoirs" of both enzymes $[E_0' + E_0'']$, rather than that of one enzyme only, as does the maximum yield of a shorter flash. The observed working time is, according to Kok, a function of the working times of both enzymes, more closely related to that of the second than to that of the first one.

One final cautionary remark: When there are two or more narrow bridges on a road, the maximum traffic it can bear is affected by all, and not only by the narrowest one. In the same way, if several enzymatic "bottlenecks" exist in a sequential reaction, all of them (and not only the narrowest one) affect the saturation rate of the overall process. For example, if two reactions in a series have the same maximum rate, V max, the maximum rate of the overall process will be (under certain conditions) only V max/2. The maximum rate of the overall reaction can be equated with the maximum rate of a single "limiting" step only if the limits imposed by all other steps lie far above that of the "limiting" step.

Despite these complications, it seems certain that the ratio 300 to 1 represents a significant relation between the number of pigment molecules and the number of enzymatic centers present in typical healthy green cells.

Ultimately, *kinetic* data will have to be brought into line with *biochemical* data, that is, with the amounts and action times of specific enzymes known to take part in photosynthesis (see Chapter 17). We are as yet far from achieving this aim.